Uniwersytet Przyrodniczy w Poznaniu
Wydział Nauk o Żywności i Żywieniu

mgr inż. Agata Gołąbek-Grenda

Bioaktywność resweratrolu i jego naturalnych pochodnych w regulacji procesów zaangażowanych w rozwój endometriozy – badania na modelach komórkowych *in vitro*

Bioactivity of resveratrol and its natural derivatives in the regulation of processes involved in the development of endometriosis – *in vitro* cell model studies

Rozprawa doktorska w dziedzinie nauk rolniczych
w dyscyplinie technologia żywności i żywienia
Doctoral dissertation in the field of agricultural sciences
in the discipline of nutrition and food technology

Promotor:
Prof. UPP dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu

Poznań, 2026

Serdeczne podziękowania składam:

Promotor pracy doktorskiej,
Pani Prof. UPP dr hab. Annie Olejnik,
za okazaną pomoc merytoryczną, wskazówki i wyrozumiałość,
a także nieustanną motywację i inspirację do dalszego rozwoju naukowego.

Panu Prof. UPP dr hab. Wojciechowi Juzwie oraz
Panu Prof. dr hab. n. med. Mariuszowi Kaczmarkowi,
za wkład w powstanie niniejszej pracy, poświęcony czas
i cenne rady podczas analiz cytometrycznych.

Kierownikowi Katedry Biotechnologii i Mikrobiologii Żywności,
Panu Prof. UPP dr hab. Wojciechowi Białasowi,
za życzliwość oraz stworzenie możliwości realizacji prac badawczych.

Wszystkim pracownikom i doktorantom Katedry Biotechnologii i Mikrobiologii Żywności,
za wszelką pomoc, chęć dzielenia się wiedzą
i przyjazną atmosferę pracy.

Moim Najbliższym, w szczególności Mężowi i Rodzicom,
za obecność, wiarę oraz codzienne wsparcie.

Niniejszą pracę dedykuję
Synowi Jerzemu oraz śp. Babci Cecylii.

Wykaz publikacji naukowych wchodzących w skład rozprawy doktorskiej

Publikacja 1

Gołąbek, **A**., Kowalska, K., i Olejnik, A. (2021). Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives. *Nutrients*, *13*(4), 1347. https://doi.org/10.3390/nu13041347

IF_{2021} = 6,706; punkty wg MNiSW = 140

Publikacja 2

Gołąbek-Grenda, **A**., i Olejnik, A. (2022). In vitro modeling of endometriosis and endometriotic microenvironment—Challenges and recent advances. *Cellular Signalling*, 97, 110375. https://doi.org/10.1016/j.cellsig.2022.110375

IF_{2022} = 4,8; punkty wg MNiSW = 100

Publikacja 3

Gołąbek-Grenda, **A**., Kaczmarek, M., Juzwa, W., i Olejnik, A. (2023). Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways. *Scientific Reports*, *13*(1), 11468. https://doi.org/10.1038/s41598-023-38692-8

IF_{2023} = 3,8; punkty wg MNiSW = 140

Publikacja 4

Gołąbek-Grenda, **A**., Juzwa, W., Kaczmarek, M., i Olejnik, A. (2024). Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages. *Nutrients*, *16*(20), 3483. https://doi.org/10.3390/nu16203483

IF_{2024} = 5,0; punkty wg MNiSW = 140

Publikacja 5

Gołąbek-Grenda, **A**., i Olejnik, A. (2026). Stilbene-based strategies for the management of endometriosis: Targeting cell adhesion, migration, and ECM-driven invasion. *Journal of Functional Foods*, *140*, 107277. https://doi.org/10.1016/j.jff.2026.107277

IF_{2024}= 4,0; punkty wg MNiSW = 100

Charakterystyka bibliometryczna publikacji wchodzących w skład rozprawy doktorskiej:

- Sumaryczny wskaźnik *Impact factor* (IF) według *Journal Citation Reports* = 24,307
- Całkowita liczba punktów według listy MNiSW obowiązującej w roku opublikowania prac = 620
- Liczba cytowań według *Web of Science* = 120, *Scopus* = 127

Spis treści

Wykaz skrótów

α-SMA	alfa-aktyna mięśni gładkich
17β-HSD2	dehydrogenaza 17β-hydroksysteroidowa typu 2
AIF	czynnik indukujący apoptozę (*apoptosis inducing factor*)
BAX	gen kodujący białko BAX (*BCL2-associated X protein)*
BCL2	gen kodujący białko BCL-2 (*B-cell lymphoma 2*)
BCL2L1	gen kodujący białko BCL-XL (*B-cell lymphoma-extra large protein*)
CASP	geny kodujące białka kaspaz
CAT	gen kodujący katalazę
CCL2	gen kodujący białko chemotaktyczne monocytów MCP-1
COX-2	cyklooksygenaza 2
CRP	białko C-reaktywne
CXCL10	gen kodujący białko IP-10 (*interferon gamma-induced protein 10*)
DAPI	4′,6-diamidyno-2-fenyloindol
E2	estradiol
ECM	macierz zewnątrzkomórkowa (*extracellular matrix*)
EMT	przejście epitelialno-mezenchymalne
ER-α,-β	receptory estrogenowe α i β
FAS	gen kodujący białko Fas (*FS-7-associated surface antygen*)
FasL	ligand Fas
FDA	Agencja Żywności i Leków
FITC	izotiocyjanianem fluoresceiny
FN1	gen kodujący białko fibronektynę
GAPDH	dehydrogenaza aldehydu 3-fosfoglicerynowego
GnRH	hormon uwalniający gonadotropiny
GPX	gen kodujący białko GPx peroksydazy glutationowej
HIF-1α/2α	czynniki indukowane hipoksją 1α/2α
IFN-γ	interferon gamma
JNK	kinaza N-końcowa c-Jun
LPS	lipopolisacharyd
M1	makrofagi typu M1(prozapalne)
M2	makrofagi typu M2 (przeciwzapalne)

MTT	bromek 3-(4,5-dimetylotiazol-2yl)-2,5-difenylotetrazolowy
NAC	N-acetylocysteina
NFKB1	gen kodujący białko NF-Kb (*nuclear factor-kappa B*)
NLPZ	niesteroidowe leki przeciwzapalne
Nrf2	jądrowy czynnik erytroidalny 2 związany z czynnikiem 2
PGE2	prostaglandyna E2
PI	jodek propidyny
PI3K/AKT/mTOR	szlak kinazy 3-fosfatydyloinozytolu/kinazy białkowej B /mechanistycznego celu rapamycyny
PMA	octan mirystynianu forbolu
PR	receptor progesteronowy
PTGS2	gen kodujący białko syntazy prostaglandyny-endoperoksydowej 2
ROS	reaktywne formy tlenu
SIRT1	deacetylaza sirtuinowa 1 zależna od NAD+
SOD	gen kodujący białko dysmutazy ponadtlenkowej
TdT	terminalna deoksynukleotydylotransferaza
TGF-β	transformujący czynnik wzrostu β
TIMP	tkankowy inhibitor metaloproteinaz
TNF-α	czynnik martwicy nowotworów α
TUNEL	test *terminal deoxynucleotidyl transferase dUTP nick end labeling*
UDP	urydyno-5′-difosforan
VEGF	naczyniowo-śródbłonkowy czynnik wzrostu
FITC	izotiocyjanian fluoresceiny

Streszczenie w języku polskim

Endometrioza jest definiowana jako obecność i rozrost tkanki endometrialnej poza jej fizjologicznym usytuowaniem, głównie w obrębie jajników i otrzewnej, ale również w odległych narządach. Chorobie towarzyszy przewlekły stan zapalny, liczne dolegliwości bólowe oraz zaburzenia funkcji rozrodczych wynikające z mechanizmów zaangażowanych w jej rozwój. Etiologia endometriozy nie została dotychczas w pełni poznania, jednak podkreśla się istotną rolę czynników hormonalnych, zapalnych, genetycznych i środowiskowych. Obecnie standardowym postępowaniem terapeutycznym jest leczenie objawowe – przeciwbólowe i hormonalne – które często wiąże się z występowaniem działań niepożądanych. W obrębie zmian endometrialnych dochodzi do deregulacji wielu procesów komórkowych i szlaków sygnalizacji molekularnej odpowiedzialnych za proliferację, apoptozę, adhezję, przebudowę macierzy zewnątrzkomórkowej (ECM) oraz angiogenezę, co czyni je potencjalnym celem działania naturalnych związków bioaktywnych.

Celem głównym przedstawionej pracy było określenie potencjału biologicznego resweratrolu i jego naturalnych pochodnych (pterostilbenu, piceatannolu i polidatyny) w prewencji i terapii endometriozy. W badaniach wykorzystano modele komórkowe *in vitro* do oceny działania antyproliferacyjnego i proapoptotycznego na komórki endometriotyczne linii 12Z, aktywności antyoksydacyjnej i przeciwzapalnej w modelu ko-hodowli komórek 12Z i makrofagów, a także zdolności badanych związków do hamowania adhezji, migracji i inwazji komórek 12Z. Resweratrol i jego pochodne wykazały selektywne działanie antyproliferacyjne i proapoptotyczne wobec komórek 12Z, przy jednocześnie niższej cytotoksyczności względem komórek endometrialnych linii T HESC. Najsilniejszy efekt obserwowano dla pterostilbenu i resweratrolu, które skutecznie indukowały apoptozę, czego potwierdzeniem była aktywacja kaspaz efektorowych, wzrost ekspresji białek proapoptotycznych oraz nasilenie fragmentacji DNA. Dominujące i szerokie działanie przeciwzapalne resweratrolu wynikało z obniżenia ekspresji i wydzielania kluczowych mediatorów stanu zapalnego. Z kolei aktywność pterostilbenu była związana przede wszystkim ze stymulacją mechanizmów obrony antyoksydacyjnej, czego efektem było istotne ograniczenie populacji makrofagów akumulujących ROS. Wszystkie badane związki skutecznie hamowały migrację i inwazję komórek 12Z, głównie poprzez ograniczenie degradacji ECM, związane z modulacją ekspresji metaloproteinazy MMP-2 i jej tkankowego inhibitora TIMP-2. Pterostilben niemal całkowicie znosił zdolność komórek 12Z do inwazji w modelu Matrigel^{TM}. Uzyskane wyniki wskazują na szereg bioaktywnych, dotychczas nieopisanych właściwości stilbenów wobec endometriozy, które mogą stanowić podstawę do dalszych zaawansowanych badań przedklinicznych oraz rozwoju strategii dietoterapeutycznych wspomagających jej leczenie.

Słowa kluczowe: modele endometriozy *in vitro*, stilbeny, apoptoza, stan zapalny, inwazja

Streszczenie w języku angielskim

Endometriosis is defined as the presence and growth of endometrial tissue in areas outside its physiological location, primarily within the ovaries and peritoneum, but also in distant organs. The disease is accompanied by chronic inflammation, numerous pain symptoms, and reproductive dysfunction resulting from the mechanisms involved in its development. The etiology of endometriosis is not fully understood, but the significant role of hormonal, inflammatory, genetic, and environmental factors is emphasized. Currently, the standard therapeutic approach is symptomatic treatment – analgesics and hormonal care – which are often associated with adverse reactions. Within endometriotic lesions, numerous cellular processes and molecular signaling pathways responsible for proliferation, apoptosis, adhesion, extracellular matrix (ECM) remodeling, and angiogenesis are dysregulated, making these processes potential targets for natural bioactive compounds.

The main aim of this study was to determine the biological potential of resveratrol and its natural derivatives (pterostilbene, piceatannol, and polydatin) in the prevention and treatment of endometriosis. *In vitro* cell models were used to assess the antiproliferative and proapoptotic effects on 12Z endometriotic cells, antioxidant, and anti-inflammatory activity in a co-culture model of 12Z cells and macrophages, and the potential of the investigated compounds to inhibit adhesion, migration, and invasion of 12Z cells. Resveratrol and its derivatives demonstrated selective antiproliferative and proapoptotic effects on 12Z cells, while exerting lower cytotoxicity towards the endometrial T HESC cell line. The strongest effects were observed with pterostilbene and resveratrol, which effectively induced apoptosis, as demonstrated by the activation of effector caspases, increased expression of proapoptotic proteins, and increased DNA fragmentation. The broad, dominant anti-inflammatory effects of resveratrol were mediated by reduced expression and secretion of key inflammatory mediators. Pterostilbene's activity, in turn, was especially associated with stimulation of antioxidant defense mechanisms, resulting in a significant reduction in the population of ROS-accumulating macrophages. All tested agents effectively inhibited 12Z cell migration and invasion, mainly by limiting ECM degradation, which was associated with modulation of MMP-2 and its tissue inhibitor TIMP-2 expression. Pterostilbene almost completely abolished 12Z cells' ability to invade the Matrigel™ model. The results indicate a series of bioactive, previously undescribed properties of stilbenes against endometriosis, which may lay the foundation for further advanced preclinical studies and the development of dietary therapeutic strategies to support its treatment.

Keywords: *in vitro* models of endometriosis, stilbenes, apoptosis, inflammation, invasion

1. Wstęp

1.1 Endometrioza jako problem medyczny, społeczny i ekonomiczny

Endometrioza jest chorobą ginekologiczną charakteryzująca się szerokim spektrum objawów klinicznych oraz zróżnicowaniem podtypów. W 2021 roku opracowano jej ujednoliconą definicję oraz zestaw powiązanych terminów, co miało na celu standaryzację klasyfikacji i badań nad chorobą. Zgodnie z przyjętą definicją, endometrioza polega na obecności tkanek przypominających endometrium poza jego fizjologiczną lokalizacją, zwykle z towarzyszącym stanem zapalnym (Tomassetti i in., 2021). Ogniska choroby występują najczęściej w obrębie miednicy mniejszej i dzielą się na trzy główne typy: zmiany powierzchowne, torbiele endometrialne jajnika oraz endometriozę głęboko naciekającą, choć mogą także pojawiać się w narządach odległych (Kędzia i in., 2024). Klasyfikacja Amerykańskiego Towarzystwa Medycyny Reprodukcyjnej uwzględnia charakter i rozległość zmian w skali I–IV, jednak słabo koreluje z nasileniem objawów (Griffiths i in., 2024).

Zmiany endometrialne wykazują wrażliwość na działanie hormonów płciowych, co prowadzi do cyklicznych krwawień, rozwoju stanu zapalnego oraz dolegliwości bólowych (Ferrero i in., 2015). Do najczęstszych objawów klinicznych należą bolesne miesiączki, dyspareunia, ból podczas oddawania moczu lub defekacji oraz przewlekły ból miednicy. Ponadto pacjentki mogą doświadczać wzdęć, zaburzeń jelitowych, przewlekłego zmęczenia czy nudności (Gete i in., 2023; Kędzia i in., 2024). Choroba może prowadzić do niepłodności poprzez zaburzenia procesów rozrodczych (Elizur i in., 2025), a także negatywnie wpływać na jakość życia, funkcjonowanie społeczne i zdrowie psychiczne, zwiększając ryzyko wystąpienia depresji i zaburzeń lękowych (Gete i in., 2023; van Barneveld i in., 2022). Należy jednak podkreślić, że endometrioza może przebiegać bezobjawowo nawet u 25% pacjentek (Moradi i in., 2021).

Złożoność i niespecyficzność objawów endometriozy prowadzą do istotnych opóźnień diagnostycznych sięgających średnio 7 lat. Wynikają one z niespecyficzności symptomów, braku swoistych biomarkerów, niskiej świadomości społecznej oraz niedostatecznej oceny objawów przez personel medyczny (Zondervan i in., 2020). Szacuje się, że endometrioza dotyczy 6–13% kobiet na świecie, przy czym częściej występuje u kobiet niepłodnych oraz przewlekłym bólem miednicy (Zondervan i in., 2020; Rahmioglu i Zondervan, 2024; Li i in., 2025). Choroba najczęściej dotyczy kobiet w wieku rozrodczym, pomiędzy 25. a 45. rokiem życia (Smolarz i in., 2021), i wiąże się z istotnym obciążeniem ekonomicznym, porównywalnym z innymi chorobami przewlekłymi (Ellis i in., 2022). Współcześnie

endometrioza postrzegana jest jako choroba wieloukładowa o złożonej etiologii, zależnej od czynników hormonalnych, zapalnych, genetycznych i środowiskowych, co czyni ją przedmiotem wielu intensywnych badań multidyscyplinarnych (Giudice i in., 2023; Griffiths i in., 2024).

1.2 Mechanizmy patogenezy endometriozy oraz czynniki ryzyka rozwoju choroby

Komórki, według ewolucyjnej zasady organizacji tkanek, są zdolne do naprawy DNA, dzielą się po otrzymaniu odpowiednich sygnałów od komórek sąsiednich oraz pozostają w swojej fizjologicznie wyznaczonej lokalizacji. W przypadku endometriozy ektopowo zlokalizowana (z gr. *ect* – poza, *topos* – miejsce) tkanka endometrialna nie spełnia trzeciej reguły, co stanowi jedną z najbardziej niewyjaśnionych cech tej choroby. Istnieje kilka teorii wyjaśniających pochodzenie endometriozy oraz mechanizmy rozprzestrzeniania się tkanki endometrialnej, jednak najbardziej akceptowaną pozostaje teoria wstecznej menstruacji zaproponowana przez. Sampsona w 1927 roku. Następne badania wykazały, że różnica między częstością występowania refluksu menstruacyjnego a rzeczywista zapadalnością na endometriozę wynika z udziału dodatkowych mechanizmów warunkujących rozwój choroby, obejmujących m.in. czynniki immunologiczne, genetyczne i epigenetyczne (Laganà i in., 2019; Y. Wang i in., 2020). Do klinicznie istotnych czynników ryzyka endometriozy zalicza się m.in. czynniki demograficzne, takie jak wyższa częstość występowania choroby w populacji kobiet pochodzenia azjatyckiego (Williams i in., 2019); czynniki związane z cyklem menstruacyjnym, w tym wczesna pierwsza miesiączka i krótkie cykle menstruacyjne oraz brak przebytych porodów (Missmer i in., 2004); czynniki genetyczne i konstytucjonalne, takie jak rodzinne występowanie endometriozy oraz zwiększona zgodność zachorowań u bliźniąt jednojajowych (Kim i in., 2021), a także niski lub prawidłowy wskaźnik masy ciała. Wśród czynników środowiskowych związanych ze stylem życia wymienia się codzienne spożywanie alkoholu (Missmer i in., 2004) oraz ekspozycję na substancje chemiczne zaburzające gospodarkę hormonalną, w tym polichlorowane bifenyle i dioksyny (Shafrir i in., 2018).

1.3 Zaburzenia komórkowe i molekularne w endometriozie

Rozwój endometriozy jest wynikiem licznych zaburzeń komórkowych i nieprawidłowej sygnalizacji molekularnej. Kluczową rolę w progresji choroby odgrywają zaburzenia hormonalne, immunologiczne oraz nieprawidłowa regulacja procesów proliferacji, apoptozy, adhezji komórkowej, przebudowy macierzy zewnątrzkomórkowej (ECM, *extracellular matrix*)

i angiogenezy. Endometrioza rozwija się w warunkach zwiększonego poziomu estrogenów wynikającego z nadmiernej aktywności aromatazy cytochromu P450 oraz zmniejszenia aktywności dehydrogenazy 17β-hydroksysteroidowej typu 2 (17β-HSD2, *17β-hydroxysteroid dehydrogenase type 2*) (Smolarz i in., 2020). Odpowiedź komórek na estrogen jest zwiększona wskutek dominacji receptora estrogenowego β (ER-β) przy jednoczesnym obniżeniu ekspresji receptora ER-α, co związane jest z nieprawidłową metylacją promotorów genów kodujących te receptory. Jednocześnie obserwuje się zjawisko oporności na progesteron, wynikające ze zmniejszenia liczby receptorów progesteronowych (PR), szczególnie izoformy PR-B, co prowadzi do utraty hamującego wpływu progesteronu na proliferację komórek i stan zapalny. Mechanizmy te tworzą pętlę dodatniego sprzężenia zwrotnego – obniżony PR zmniejsza aktywność 17β-HSD2, prowadząc do zwiększenia stężenia estradiolu (E2) i wzmocnienia sygnalizacji zależnej od ER-β, który ostatecznie obniża ekspresję PR poprzez wiązanie się z promotorem jego genu. U pacjentek z endometriozą obserwuje się również zaburzenia stężenia gonadotropin oraz deregulację osi podwzgórze-przysadka-nadnercza (Bulun i in., 2010; Qi i in., 2025; Y. Wang i in., 2020).

Zmiany endometriotyczne, płyn otrzewnowy oraz eutopowe endometrium kobiet z endometriozą charakteryzują się nadmierną produkcją mediatorów stanu zapalnego, w tym cyklooksygenazy-2 (COX-2, *cyclooxygenase-2*), interleukin IL-1β, IL-6, IL-8, czynnika martwicy nowotworów (TNF-α, *tumor necrosis factor αlpha*), prostaglandny E2 (PGE2) i białka chemotaktycznego monocytów 1 (MCP-1, *monocyte chemoattractant protein-1*) (Lousse i in., 2012; Smolarz i in., 2021). Do ognisk endometriozy rekrutowane są liczne komórki układu odpornościowego, w tym makrofagi, limfocyty B i T, komórki dendrytyczne CD1a+, komórki NK (*natural killer*), neurofile oraz mieloidalne komórki supresorowe (T. Zhang, He, i in., 2023; Qi i in., 2025). Obecność zwiększonej liczby aktywowanych makrofagów o obniżonej zdolności fagocytarnej sprzyja utrzymywaniu przewlekłego stanu zapalnego. Kwestia polaryzacji makrofagów w kierunku fenotypu M1/M2 w przebiegu endometriozy pozostaje przedmiotem dyskusji, jednak ich istotna rola w progresji choroby oraz bólu neuropatycznym została jednoznacznie potwierdzona (Ding i in., 2021). Komórki endometriotyczne, podobnie jak komórki nowotworowe, wykazują zwiększoną zdolność do proliferacji oraz oporność na apoptozę. Komórki obecne w zmianach endometrialnych charakteryzują się podwyższonym poziomem cyklin stymulujących proliferację (A2, B1, B2 i D1) oraz obniżoną ekspresją inhibitorów cyklu komórkowego (p21, p27, p16, p18 i p19) (Szymański i in., 2024). Proliferacji sprzyjają również wysokie stężenie E2 i obniżona aktywność czynników regulowanych przez progesteron (np. FOXO1, *forkhead box protein*

O1), a także nadekspresja białek związanych z proliferacją, takich jak marker proliferacji Ki-67, kinaza tymidynowa 1 oraz cyklina E1 (P. Zhang i Wang, 2023). Na upośledzenie szlaku apoptozy wpływa również obniżona ekspresja receptora Fas (*FS-7-associated surface antigen*) (Sbracia i in., 2016), podwyższone stężenie ligandu FasL w płynie otrzewnowym (Garcia-Velasco i in., 2002), zwiększona ekspresja białek anty-apoptotycznych BCL-2 (*B cell lymphoma gene-2*) i BCL-XL (*B-cell lymphoma-extra large*), obniżona ekspresja białka BAX (*BCL2-associated X protein*) (Agic i in., 2009) oraz zredukowana aktywność kaspaz-3 (Delbandi i in., 2020), -8 i -9 (Cho i in., 2018). Duże znaczenie w promowaniu adhezji komórek endometrialnych w miejscach ektopowych ma zaburzona ekspresja integryn i glikoprotein transbłonowych zaangażowanych w przyleganie komórek do ECM oraz zdolności migracyjne komórek i ich inwazyjność (Lessey i Kim, 2017; J. Zhang i in., 2022). Wykazano, że zmiany endometrialne posiadają cechy przejścia epitelialno-mezenchymalnego (EMT, *epithelial mesenchymal transition*), na co wskazuje wzrost ekspresji markerów mezenchymalnych, przy jednoczesnym obniżeniu poziomu nabłonkowego markera E-kadheryny (Jin i in., 2025). Odkrycie produktów degradacji ECM w płynie otrzewnowym pacjentek z endometriozą zwróciło uwagę na znaczenie przebudowy ECM w patogenezie choroby (Spuijbroek i in., 1992). Kluczową rolę w tym procesie odgrywa podwyższona aktywność licznych metaloproteinaz macierzy (MMP, *matrix metalloproteinases*), w tym MMP-1, -2, -3, -7, -9, 10, -11, -12, -23, -26, obserwowana w tkankach, surowicy i płynie otrzewnowym pacjentek z endometriozą, przy jednoczesnym obniżeniu poziomu ich tkankowych inhibitorów – TIMP-1 i TIMP-2 (*tissue inhibitors of metalloproteinases*) (Ke i in., 2021; Madjid i in., 2020). W zmianach endometrialnych dochodzi do miejscowej neoangiogenezy, która jest wzmacniana przez czynniki wzrostu, czynniki proangiogenne, hormony steroidowe i cytokiny zapalne obecne w płynie otrzewnowym (Rocha i in., 2013). Podwyższona ekspresja oraz stężenie czynników indukowanych hipoksją HIF-1α/2α (*hypoxia-inducible factors* 1α/2α), a także czynników wzrostu śródbłonka naczyniowego VEGF-A i VEGF-C (*vascular endothelial growth factors*) oraz zwiększona aktywność ich receptorów, prowadzą do aktywacji MMPs, co w konsekwencji umożliwia efektywna degradację ECM i rozrost naczyń krwionośnych w obrębie tkanki (Bo i Wang, 2024; Chung i Han, 2022). W badaniach *in vitro* oraz próbach klinicznych wykazano również zaburzenia stężeń białek regulujących integralność i dojrzewanie naczyń, takich jak angiopoetyny ANG1 i ANG2 oraz receptor kinazy tyrozynowej TIE-2 (Sabolová i in., 2024; Malik i in., 2025). Pomimo ostatniego postępu badań naukowych, wiele fundamentalnych kwestii związanych z zaburzeniami występującymi w zmianach endometrialnych na poziomie komórkowym i molekularnym nadal pozostaje

niewyjaśnionych. Jednym z kluczowych kierunków badań może być identyfikacja nieopisanych dotąd komórek progenitorowych lub macierzystych obecnych we krwi, które mogą odpowiadać za regenerację komórek w ogniskach endometriozy (Filby i in., 2020). Ponadto badania genetyczne stanowią obecnie ważny element wyjaśniania patogenezy choroby i obejmują m.in. analizy genów kandydujących, badania sprzężeń i powiązań genetycznych oraz badania asocjacyjne całego genomu. Coraz częściej podkreśla się również znaczenie mechanizmów epigenetycznych, w tym regulacji zależnej od mikroRNA, które wpływają na przebieg procesów komórkowych oraz ekspresję genów w endometriozie (Laganà i in., 2019).

1.4 Modele eksperymentalne endometriozy

Opracowanie modeli *in vitro* i *in vivo* odzwierciedlających główne cechy endometriozy stanowi duże wyzwanie badawcze, ponieważ wymaga uwzględnienia wieloczynnikowej etiologii choroby, licznych procesów patofizjologicznych, a także jej heterogenności i złożonego charakteru. W publikacji wchodzącej w skład niniejszej rozprawy doktorskiej (publikacja 2) przedstawiono najnowsze osiągniecia z zakresu modelowania endometriozy w warunkach *in vitro*. Podkreślono złożoną strukturę mikrośrodowiska zmian endometrialnych, obejmującą komórki nabłonkowe, zrębowe, immunologiczne i śródbłonkowe, a także liczne czynniki zapalne, hormonalne i angiogenne wpływające na fenotypowe i czynnościowe przeprogramowanie komponentu komórkowego. Artykuł stanowi syntetyczne opracowanie aktualnego stanu wiedzy dotyczącego modeli komórkowych i tkankowych naśladujących endometriozę, wskazując jednocześnie istniejące luki badawcze i aspekty modelowania wymagające dalszego doskonalenia. Zwrócono uwagę na konieczność dokładnej charakterystyki materiału biologicznego izolowanego od pacjentek, rozwijania standardowych modeli komórkowych 2D opartych na pierwotnych i unieśmiertelnionych liniach komórkowych, stosowania ko-hodowli komórek endometriotycznych z innymi typami komórek oraz wykorzystywania trójwymiarowych systemów hodowli. W artykule scharakteryzowano dostępne komercyjnie linie komórkowe, wskazując ich specyficzne markery, obszary zastosowań oraz kluczowe cechy funkcjonalne. Pomimo ich przydatności istotnym ograniczeniem endometrialnych i endometriotycznych linii komórkowych pozostaje brak specyficznej organizacji tkankowej, co utrudnia odwzorowanie sygnalizacji międzykomórkowej oraz interakcji komórek ze składnikami ECM. W pracy omówiono również badania wykorzystujące ko-hodowlę komórek endometriozy z makrofagami, komórkami mezotelium otrzewnej oraz komórkami śródbłonka naczyń. W publikacji 2 przedstawiono również najnowsze technologie umożliwiające zwiększenie fizjologicznej relewancji modeli *in*

vitro, w tym trójwymiarowe organoidy, sferoidy, modele bioprintingowe oraz mikroprzepływowe systemy typu „*organ-on-a-chip*". Dostępne dane literaturowe wskazują, że innowacyjne rozwiązania technologiczne pozwalają na wierniejsze odwzorowanie właściwości ECM, przestrzennej komunikacji komórkowej, fizykochemicznego mikrośrodowiska zmian endometrialnych oraz gradientów biochemicznych. Podkreśla się, że dalszy rozwój inżynierii tkankowej oraz zaawansowanych mikroprzepływowych modeli 3D może w przyszłości umożliwić dokładniejszą identyfikację celów terapeutycznych, opracowywanie biomarkerów diagnostycznych oraz bardziej precyzyjne testowanie nowych substancji leczniczych (Gołąbek-Grenda i Olejnik, 2022).

Obok modeli komórkowych i tkankowych duże znaczenie poznawcze mają modele gryzoni, które dostarczyły istotnych informacji na temat modulacji stanu zapalnego, stresu oksydacyjnego oraz płodności w patofizjologii endometriozy. Modele te mają jednak poważne ograniczenia wynikające z braku naturalnego występowania cyklu menstruacyjnego i konieczności chirurgicznego przeszczepienia tkanki macicy w celu indukcji choroby (Edwards i in., 2013). W ostatnich latach podjęto również próby opracowania modelu mysiego modyfikowanego genetycznie do modelowania endometriozy *de novo* bez interwencji chirurgicznej (Burns i in., 2022). W wielu badaniach nad endometriozą wykorzystano ssaki z rzędu *Primates,* głównie makaki rezus i pawiany, ze względu na zbliżoną fizjologię rozrodu i spontaniczną endometriozę. Większość badań na naczelnych innych niż człowiek, koncentrowała się na testowaniu leków i potwierdzeniu skuteczności ich działania, na przykład inhibitorów aromatazy oraz antagonistów progesteronu (Malvezzi i in., 2020). Prowadzenie eksperymentów interwencyjnych na dużych zwierzętach wiąże się z wysokimi kosztami, a także jest ograniczone względami etycznymi oraz rosnącym sprzeciwem opinii publicznej, dlatego rekomendacje dotyczące badań z wykorzystaniem naczelnych stają się coraz bardziej rygorystyczne (Malvezzi i in., 2020). Alternatywy dla badań *in vivo* pozostają przedmiotem intensywnej dyskusji w społeczności naukowej, przemyśle oraz wśród agencji regulacyjnych, szczególnie od momentu wdrożenia do prawa europejskiego Dyrektywy 2010/63/UE w sprawie ochrony zwierząt wykorzystywanych do celów naukowych oraz odpowiadającej jej polskiej Ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych. Regulacje te podkreśliły znaczenie badań naukowych prowadzonych z wykorzystaniem metod alternatywnych, umożliwiających ograniczenie zakresu badań na zwierzętach oraz ich realizację zgodnie z koncepcją etyczną 3R (*Replacement, Reduction, Refinement*). Ponadto w 2020 roku amerykańska Agencja Żywności i Leków (FDA) uznała wartość modeli 3D *in vitro* w ocenie leków, promując ich stosowanie w badaniach

przedklinicznych (H. Wang i in., 2021). W ostatnich latach osiągnięto znaczący postęp w rozwoju technologii modelowania *in vitro*, które stanowią obiecującą platformę do poznawania patofizjologii chorób, takich jak endometrioza. Należy jednak podkreślić, że nawet najbardziej zaawansowane modele *in vitro* wymagają dalszego doskonalenia oraz intensywnych prac nad optymalizacją i planowaniem doświadczeń, a także nad właściwą interpretacją i ekstrapolacją uzyskiwanych danych.

1.5 Strategie leczenia i dostępne terapie endometriozy

Do chwili obecnej nie opracowano terapii umożliwiającej całkowite wyeliminowanie wszystkich objawów endometriozy. Zgodnie z wytycznymi Europejskiego Towarzystwa Rozrodu Człowieka i Embriologii z 2022 roku, cele leczenia obejmują redukcję bólu, poprawę jakości życia, zachowanie płodności oraz ograniczenie nawrotów choroby. Zalecane postępowanie powinno mieć charakter zindywidualizowany i uwzględniać obraz kliniczny, choroby współistniejące oraz plany prokreacyjne pacjentki (Becker i in., 2022). W farmakoterapii rekomenduje się stosowanie niesteroidowych leków przeciwzapalnych (NLPZ) oraz terapii hormonalnej. NLPZ, w tym inhibitory cyklooksygenazy COX-2, hamują produkcję prostaglandyn, natomiast dwuskładnikowe hormonalne środki antykoncepcyjne oraz progestageny blokują owulację, obniżają poziom estrogenów i ograniczają proliferację ognisk endometriozy, sprzyjając ich regresji (Kędzia i in., 2024; Zondervan i in., 2020). Do najczęściej obserwowanych działań niepożądanych terapii należą zaburzenia ze strony przewodu pokarmowego i układu nerwowego, a także nieregularne krwawienia, wahania nastroju i przyrost masy ciała (J. Brown i in., 2017; Mitchell i in., 2022). Leki drugiego rzutu, w tym agoniści i antagoniści gonadoliberyny (GnRH, *gonadotropin-releasing hormone*) oraz inhibitory aromatazy, umożliwiają indukcję kontrolowanego hipoestrogenizmu przy zmniejszonym ryzyku działań ubocznych (Bulun i in., 2019; Zondervan i in., 2020). Testowane obecnie syntetyczne modulatory receptora progesteronowego wykazują zdolność do selektywnego hamowania proliferacji endometrium bez wywoływania istotnego niedoboru estrogenów (Reis i in., 2020). Leczenie chirurgiczne, zwykle laparoskopowe, zaleca się w przypadkach nieskuteczności, złej tolerancji lub przeciwwskazań do farmakoterapii, szczególnie u kobiet planujących ciążę. Pomimo całkowitego usunięcia zmian endometrialnych, nawroty choroby występują dość często i dotyczą około 22% pacjentek po 2 latach i 40–50% po 5 latach od zabiegu, co podkreśla istotną rolę pooperacyjnego leczenia hormonalnego (Allaire i in., 2023). Ostatnie rekomendacje Polskiego Towarzystwa Ginekologów i Położników wskazują również na znaczenie terapii wspomagających,

obejmujących dietoterapię, fizjoterapię oraz opiekę psychologiczną, które mogą wzmacniać efekty farmakoterapii i stanowić ważny element przygotowania pacjentki do leczenia operacyjnego (Kędzia i in., 2024).

1.6 Czynniki żywieniowe w profilaktyce i leczeniu endometriozy

W ostatnich latach odnotowano wzrost liczby badań obserwacyjnych, randomizowanych badań kontrolowanych oraz systematycznych przeglądów literatury dotyczących zależności między sposobem żywienia a ryzykiem rozwoju endometriozy oraz skutecznością terapii dietetycznych. Zmiany w diecie uzyskały wysokie oceny w badaniach opartych na samoocenie kobiet dotkniętych chorobą pod względem zdolności do zmniejszenia bólu miednicy oraz łagodzenia objawów żołądkowo-jelitowych (Abulughod i in., 2024). W prospektywnym badaniu obejmującym lata 1991–2015 wykazano, że wzorzec żywienia zgodny z AHEI-2010 (*Alternate Healthy Eating Index 2010*), bogaty w owoce i warzywa, a ubogi w czerwone mięso i tłuszcze trans, wiązał się z niższym o 13% ryzykiem rozpoznania endometriozy (Dougan i in., 2024). Najnowsze badania wskazują, że składniki diety mogą modulować stan zapalny, stres oksydacyjny oraz metabolizm estrogenów, wpływając tym samym na przebieg i objawy choroby (L. Zhou, Liu, i in., 2025).

Na podstawie dostępnych danych wyróżniono kilka modeli żywieniowych, które mogą mieć wpływ na przebieg endometriozy. Należą do nich: dieta śródziemnomorska, przyczyniająca się do zmniejszenia wskaźników zapalnych oraz łagodzenia objawów bólowych (Cirillo i in., 2023); dieta bogata w błonnik, która poprzez obniżenie poziomu estrogenów, poprawę mikrobiomu i redukcję stresu oksydacyjnego może ograniczać ryzyko endometriozy (Parazzini i in., 2004; Harris i in., 2018); dieta bezglutenowa, w przypadku której u około 75% pacjentek odnotowano istotne zmniejszenie dolegliwości bólowych (Marziali i in., 2012); dieta FODMAP, redukująca objawy żołądkowo-jelitowe u pacjentek z zespołem jelita drażliwego współistniejącym z endometriozą (Moore i in., 2017; van Haaps i in., 2023); dieta niskoniklowa, która w 3-miesięcznym badaniu pilotażowym zmniejszyła objawy żołądkowo-jelitowe, ginekologiczne i ogólnoustrojowe (Borghini i in., 2020), a także dieta przeciwzapalna, której skuteczność oceniano na podstawie biomarkerów zapalnych, takich jak IL-6, białko C-reaktywne (CRP) i TNF-α (P. Liu i in., 2023). W dużym badaniu prospektywnym wykazano, że spożywanie tłuszczów trans oraz produktów mięsnych wiązało się ze zwiększonym o 48% ryzykiem rozpoznania endometriozy (Missmer i in., 2010). Stwierdzono również, że spożywanie czerwonego mięsa może sprzyjać zwiększonej produkcji estradiolu oraz nasilać ekspresję markerów stanu zapalnego ściśle związanych z progresją choroby (Papier i in., 2021;

L. Zhou, Liu, i in., 2025). Z kolei badania żywieniowe wskazują na potencjalnie korzystny wpływ produktów mlecznych, prawdopodobnie związany z zawartością wapnia i witaminy D (Nodler i in., 2020). Pomimo identyfikacji schematów żywieniowych potencjalnie korzystnych w profilaktyce endometriozy oraz łagodzeniu jej objawów, molekularne mechanizmy działania poszczególnych składników odżywczych nadal pozostają nie w pełni poznane. Wyniki interwencji żywieniowych są często niejednoznaczne ze względu na brak jednorodności metodologicznej, obejmującej m.in. zróżnicowane schematy i protokoły badań, niewielkie grupy uczestniczek oraz odmiennie punkty końcowe analiz. Opracowanie precyzyjnych zaleceń żywieniowych wymaga zatem dokładniejszego poznania wpływu diety na poziom estrogenów, skład mikrobiomu jelitowego oraz procesy zapalne związane z patofizjologią choroby.

1.7 Potencjał naturalnych związków bioaktywnych w terapii endometriozy

Bioaktywne działanie naturalnych związków zawartych w żywności oraz ich znaczenie w terapii endometriozy stanowią obecnie obszar intensywnych badań naukowych. Coraz więcej dowodów wskazuje, że żywność, zwłaszcza pochodzenia roślinnego, jest cennym źródłem związków o wysokim potencjale prewencyjnym i terapeutycznym w odniesieniu do endometriozy. Składniki odżywcze oraz związki bioaktywne, poprzez łagodzenie stanu zapalnego i stresu oksydacyjnego, mogą przyczyniać się do zapobiegania rozwojowi endometriozy oraz modyfikowania jej przebiegu. Do grupy związków o istotnym potencjale prozdrowotnym w endometriozie zaliczono witaminy C i E, które dzięki wysokiej aktywność przeciwutleniającej przyczyniają się do obniżenia stresu oksydacyjnego oraz zmniejszenia dolegliwości bólowych u pacjentek (Amini i in., 2021). Wykazano również, że suplementacja witaminy D prowadzi do znacznej poprawy w zakresie bólu miednicy, obniżenia stężenia białka CRP oraz zwiększenia całkowitej zdolności antyoksydacyjnej organizmu (Mehdizadehkashi i in., 2021). N-acetylocysteina (NAC), będąca prekursorem glutationu, wykazuje działanie przeciwutleniające poprzez aktywacje licznych mechanizmów obrony antyoksydacyjnej; jej stosowanie wiązało się ze zmniejszeniem wielkość torbieli endometrialnych jajnika oraz ograniczeniem dolegliwości bólowych, w tym bolesnych miesiączek, dyspareunii i przewlekłego bólu miednicy (Porpora i in., 2013). Wysokie spożycie długołańcuchowych kwasów tłuszczowych omega-3, zwłaszcza kwasu eikozapentaenowego (EPA), wiązało się z obniżeniem ryzyka rozwoju endometriozy (Hopeman i in., 2015), a także ze zmniejszeniem objawów bólowych oraz obniżeniem poziomu markerów zapalnych, takich jak PGE2 i CA-125 (Signorile i in., 2018). EPA hamuje przekształcanie kwasu arachidonowego w prozapalne

molekuły, takie jak PGE2 i leukotrien B4, które odgrywają kluczową rolę w generowaniu bólu w endometriozie (Sienko i in., 2024).

Ważną grupę związków bioaktywnych w dietoterapii endometriozy stanowią polifenole, których potencjał przedstawiono w artykule przeglądowym (publikacja 1), wchodzącym w skład niniejszej rozprawy doktorskiej. W artykule szczegółowo omówiono związki należące do grupy flawonoli, flawonów, izoflawonoidów, flawanonów, chalkonów i flawanoli. Szczególną uwagę poświęcono aktywności biologicznej resweratrolu, udokumentowanej w licznych badaniach *in vitro* i *in vivo* oraz w próbach klinicznych, które wskazują na jego zdolność do modulowania kluczowych szlaków patofizjologii endometriozy. Dotychczasowe badania eksperymentalne i analizy kliniczne jednoznacznie pokazują, że związki takie jak resweratrol, galusan epigallokatechiny, kwercetyna czy apigenina mogą zmniejszać rozmiar ognisk endometrialnych, hamować ich wzrost poprzez indukcję apoptozy i inhibicję proliferacji oraz ograniczać angiogenezę. Polifenole wykazują działanie wielokierunkowe, modulując kluczowe markery oraz ścieżki sygnałowe w różnych modelach biologicznych choroby. Pomimo obiecujących wyników badań przedklinicznych, wdrożenie polifenoli do praktyki klinicznej wymaga jednak przeprowadzenia dalszych, dobrze zaprojektowanych badań klinicznych, które pozwolą określić optymalne dawki oraz warunki bezpiecznej i skutecznej terapii, szczególnie w kontekście leczenia długoterminowego. Biorąc pod uwagę udokumentowany w badaniach naukowych potencjał biologiczny naturalnych związków polifenolowych, można przypuszczać, że mogą one stanowić wartościowe wsparcie terapii endometriozy i przyczyniać się do łagodzenia objawów tej choroby (Gołąbek i in., 2021).

1.8 Resweratrol i jego pochodne jako związki aktywne biologicznie w endometriozie

Resweratrol, przedstawiciel stilbenów należących do polifenoli, zbudowany jest z 14-węglowego szkieletu i dwóch pierścieni fenylowych połączonych podwójnym wiązaniem etylenowym; jego masa cząsteczkowa wynosi 228,247 g/mol. Obecność podwójnego wiązania umożliwia występowanie w dwóch formach stereoizomerycznych – cis- i trans-, z których forma trans dominuje pod względem aktywności biologicznej i zastosowań farmaceutycznych (Salla i in., 2024). Stilbeny powstają w roślinach w procesie biosyntezy fitoaleksyn, związków chroniących je przed stresem, takim jak infekcje grzybicze, promieniowanie UV, uszkodzenia mechaniczne (Teka i in., 2022).

Resweratrol został po raz pierwszy wyizolowany w 1940 roku z korzenia ciemiężycy wielokwiatowej (*Veratum gradiflorum*), a następnie z korzenia rdestowca ostrokończystego

(*Polygonum cuspidatum),* który należy do najbogatszych naturalnych źródeł tego związku (296–377 µg/g) (Khattar i in., 2022). Od lat 90., kiedy zidentyfikowano go jako składnik czerwonego wina, resweratrol stał się przedmiotem intensywnych badań naukowych (Nunes i in., 2018). Występuje głównie w skórkach winogron (50–100 µg/g owoców), natomiast jego stężenie w winie mieści się w zakresie 0,352–1,990 µg/mL (Khattar i in., 2022). Obecność resweratrolu w winie powiązano z tzw. „paradoksem francuskim", czyli niską częstością chorób wieńcowych w populacji stosującej dietę wysokotłuszczową spożywającą znaczne ilości czerwonego wina (Renaud i de Lorgeril, 1992). Związek ten występuje również w niższych stężeniach w korzeniach, łodygach, liściach, kwiatach, owocach i nasionach wielu gatunków roślin, m.in. sosny, orzeszków ziemnych, borówki i żurawiny, morwy, eukaliptusa, chlebowców, gniotowców oraz lilii (Tian i Liu, 2020; Khattar i in., 2022), a także w ziołach, takich jak melisa lekarska, mięta pieprzowa, rumianek pospolity, dziurawiec zwyczajny czy bazylia słodka (Mekinić i in., 2016).

Właściwości przeciwnowotworowe resweratrolu zostały po raz pierwszy opisane w 1997 roku przez zespół Janga, który wykazał, że związek ten może oddziaływać na każdy etap rozwoju nowotworu – od inicjacji, przez promocję, aż po progresję (Jang i in., 1997). Szacuje się, że od czasu przełomowej publikacji Janga ukazało się ponad 13 000 prac naukowych zawierających resweratrol w tytule. Resweratrol był badany w kontekście szerokiego spektrum potencjalnych korzyści zdrowotnych, obejmujących działanie kardioprotekcyjne, neuroprotekcyjne, przeciwnowotworowe, przeciwcukrzycowe, przeciwbakteryjne oraz przeciwstarzeniowe. Wiele z tych efektów wynika ze zdolności resweratrolu do modulowania stresu oksydacyjnego, śmierci apoptotycznej komórek, procesów zapalnych, metabolizmu glukozy oraz przemian estrogenu. Mimo obiecujących wyników potwierdzających potencjał przeciwnowotworowy resweratrolu uzyskanych w badaniach przedklinicznych, badań klinicznych w tym obszarze jest stosunkowo niewiele, co może wynikać m.in. z braku odpowiednich biomarkerów do monitorowania skuteczności leczenia. Natomiast, liczba badań klinicznych oceniających wpływ resweratrolu na płodność, endometriozę, zespół policystycznych jajników oraz inne schorzenia ginekologiczne znacznie przewyższa odsetek badań dotyczących jego działania przeciwnowotworowego (K. Brown i in., 2024). Mechanizmy regulowane przez resweratrol odgrywają kluczową rolę w progresji endometriozy, co wskazuje na jego potencjalne właściwości ochronne w prewencji i przebiegu tej choroby.

Resweratrol indukuje apoptozę zarówno poprzez tzw. szlak zewnętrzny zależny od receptorów śmierci, jak i wewnętrzną (mitochondrialną) ścieżkę sygnalizacyjną. W komórkach

raka jelita grubego HT29 po ekspozycji na resweratrol wykazano redystrybucję receptorów śmierci (CD95, DR4, DR5), białka adaptorowego FADD (*Fas-associated protein with death domain*) oraz prokaspazy-8, co prowadziło do aktywacji kompleksu DISC inicjującego apoptozę (Delmas i in., 2004). Wykazano również, że resweratrol hamuje anty-apoptotyczne białka rodziny BCL-2 oraz aktywuje białka pro-apoptotyczne, takie jak BAD, BAK i BAX (Aziz i in., 2006; Bhardwaj i in., 2007). Istotną rolę odgrywa także regulacja osi białka supresorowego nowotworów i deacetylazy sirtuiowej 1 (p53/SIRT1) oraz hamowanie szlaku kinazy 3-fosfatydyloinozytolu/kinazy białkowej B/mechanistycznego celu rapamycyny (PI3K/Akt/mTOR), co potwierdzono w komórkach raka jelita grubego, jajnika, piersi, macicy, prostaty i szpiczaka mnogiego (Brockmueller i in., 2023; J.-H. Ko i in., 2017). Resweratrol wywołuje apoptozę komórek nowotworowych również poprzez modulację elementów szlaku kinaz białkowych aktywowanych mitogenami (MAPK) tj., obejmującego kinazy regulowane sygnałem pozakomórkowym (ERK1/2, ERK3/4, ERK5, ERK7/8), kinazy N-końcowej Jun (JNK1/2/3) oraz białko p38 (Hankittichai i in., 2023). Według innych danych związek ten moduluje sygnalizację sfingolipidów, zwiększając poziom ceramidów i zmniejszając poziom sfingozyno-1-fosforanu, co sprzyja apoptozie i ograniczeniu proliferacji komórek raka jelita grubego, piersi i prostaty (Gaggini i in., 2023). Ponadto, resweratrol wpływa na cykl komórkowy poprzez regulację cyklin oraz kinaz zależnych od cyklin (Gatouillat i in., 2010). Nowsze badania dotyczące jego działania proapoptotycznego i antyproliferacyjnego koncentrują się również na regulacji miRNA oraz lncRNA *(long non-coding RNA*) (H. Zhang, Liu, i in., 2023; H. Zhang, Wang, i in., 2023).

Przewlekły stan zapalny i stres oksydacyjny stanowią kluczowe elementy patofizjologii endometriozy. Resweratrol wykazywał działanie przeciwzapalne w różnych modelach chorób poprzez hamowanie aktywności czynników transkrypcyjnych AP-1 i NF-κB, co prowadziło do zmniejszenia transkrypcji genów enzymów syntetyzujących eikozanoidy (np. COX-2) oraz obniżenia produkcji PGE2 (Das i Das, 2007). W makrofagach resweratrol działał poprzez szlaki TLR4/NF-κB/MAPK, hamując fosforylację kaskady białek sygnałowych (IκBα, p38, JNK, ERK1/2) i ograniczając ekspresję markerów zapalnych, takich jak TNF-α, IL-6 i IL-8 (Tong i in., 2020; Monmai i Baek, 2024). Aktywując SIRT1, która deacetyluje podjednostkę p65 NF-κB, resweratrol zmniejszał jego aktywność transkrypcyjną i odpowiedź zapalną (Sun i in., 2024). Dowiedziono również, że resweratrol działa jako silny antyoksydant – neutralizuje reaktywne formy tlenu (ROS, *reactive oxigen species*), chroni DNA i lipidy przed peroksydacją oraz zwiększa aktywność enzymów antyoksydacyjnych. Poprzez aktywację jądrowego czynnika 2 związanego z czynnikiem erytroidowym 2 (Nrf2, *nuclear factor erythroid 2-related*

factor 2) indukuje ekspresję enzymów fazy II detoksykacji, takich jak NQO w komórkach białaczki (Hsieh i in., 2006) oraz QR w komórkach raka piersi (Bianco i in., 2005). W modelach *in vitro* i *in vivo* raka jajnika wykazano wzrost aktywności enzymów dysmutazy ponadtlenkowej (SOD, *superoxide dismutase*), peroksydazy glutationowej (GPx, *glutathione peroxidase*) i katalazy (CAT, *catalase*) oraz obniżenie markerów stresu oksydacyjnego (Ibrahim i in., 2021; J. Wang i in., 2022).

Nowsze kierunki badań dotyczą wpływu resweratrolu na mikrośrodowisko immunologiczne, w tym przeprogramowanie makrofagów i limfocytów T CD8+ (W. Zhang i in., 2022) oraz na wykorzystaniu biomarkerów (profile miRNA, metabolomika) do monitorowania efektów terapii (Y. Lee i Im, 2021; Z.-D. Zhang, Tao, i in., 2023). Ze względu na strukturalne podobieństwo do 17β-estradiolu resweratrol może wiązać się z receptorami ER, wykazując zarówno działanie estrogenowe, jak i antyestrogenowe, Analizy dynamiki molekularnej sugerują, że resweratrol preferencyjnie indukuje antagonistyczną konformację ER-α, wpływa na procesy genomowe i niegenomowe zależne od ER oraz na ekspresję i degradację proteasomalną ER. Działa również pośrednio, modulując poziomy estrogenów i steroidów w organizmie (Qasem, 2020). Badania przedkliniczne dotyczące estrogenowego działania resweratrolu obejmowały głównie linie komórkowe raka piersi (Pozo-Guisado i in., 2005), jajnika (Bowers i in., 2000) oraz endometrium (Bhat i Pezzuto, 2001), a także modele uterotropowe *in vivo* (Freyberger i in., 2001). W badaniach klinicznych wpływ resweratrolu na sygnalizację estrogenową oceniano w przypadku m.in. zespołu policystycznych jajników PCOS (Banaszewska i in., 2016), otyłości (Chow i in., 2014) oraz endometriozie (Maia i in., 2012).

Wielokierunkowe działanie resweratrolu, ograniczające poszczególne etapy progresji nowotworzenia, a mające znaczenie również w endometriozie, obejmuje przede wszystkim hamowanie ekspresji MMPs, inaktywację przejścia EMT, redukcję włóknienia poprzez blokadę szlaku sygnałowego transformującego czynnika wzrostu β/białek Smad (TGF-β/Smad) oraz blokowanie angiogenezy zależnej od VEGF (J.-H. Ko i in., 2017). Hamowanie ekspresji MMPs, zwłaszcza MMP-9, przekładało się na zmniejszenie zdolności migracyjnych i inwazyjnych komórek, co potwierdzono m.in. w komórkach raka piersi MDA-MB231 (M.-F. Lee i in., 2011) oraz raka jajnika (Kadry, 2024). Wykazano również, że resweratrol obniża ekspresję MMP-2 i MMP-9 w tkance i płynie endometrium oraz surowicy (Kodarahmian i in., 2019). W tej samej grupie badanych zaobserwowano spadek ekspresji markerów angiogenezy i stanu zapalnego (VEGF i TNF-α) w endometrium (Khodarahmian i in., 2021). Resweratrol

hamował również EMT indukowane przez TGF-β w wielu modelach nowotworowych (J.-H. Ko i in., 2017).

Związek ten może także przeciwdziałać włóknieniu, co ma znaczenie zarówno w kontekście nowotworowym, jak i w powstawaniu zrostów w endometriozie. Wykazano, że resweratrol inaktywuje oś TGF-β/Smad oraz obniża ekspresję α-SMA (*alpha-smooth muscle actin*) i kolagenu w modelach uszkodzenia nerek i serca oraz w fibroblastach związanych z nowotworem (Dana i in., 2022; J. Wang i in., 2018; Y. Zhang i in., 2018). Ponadto resweratrol ogranicza angiogenezę – kluczowy proces również dla unaczynienia zmian endometrialnych – m.in. poprzez blokadę szlaku STAT3/HIF-1α/VEGF, co prowadziło do zmniejszenia ekspresji VEGF oraz markerów unaczynienia, takich jak CD31, w modelach *in vitro* i *in vivo* (Savio i in., 2022; H. Wang i in., 2020).

Resweratrol może wpływać również na redukcję markerów neurozapalnych i dolegliwości bólowych, co ma istotne znaczenie w endometriozie, gdzie przewlekły ból wiąże się z rozbudową unerwienia zmian i postępującym neurozapaleniem. W modelu zwierzęcym bólu neuropatycznego resweratrol hamował ekspresję cytokin prozapalnych (TNF-α, IL-1β, IL-6), zwiększał poziom przeciwzapalnej IL-10, obniżał markery pobudzenia neuronów (CGRP, c-Fos) oraz ograniczał aktywność astrocytów i mikrogleju – komórek kluczowych dla regulacji neurozapalenia (Y. Yang i in., 2016). Obiecującym kierunkiem działania resweratrolu w kontekście leczenia bólu neuropatycznego oraz nadmiernego unerwienia tkanek w endometriozie jest jego zdolność do aktywacji semaforyny 3A (Sema3A), białka pełniącego funkcję inhibitora wzrostu włókien nerwowych (Tsuji i in., 2024). Ma to szczególne znaczenie w świetle najnowszych doniesień dotyczących roli hipoksji i sygnalizacji HIF-1α w modulacji ekspresji Sema3A w endometriozie (R. Yang i in., 2024).

Resweratrol, mimo licznych doniesień o jego właściwościach prozdrowotnych, ma ograniczone zastosowanie kliniczne, co wynika głównie z jego szybkiego metabolizmu oraz niskiej biodostępności. Brak wystarczających i jednoznacznych danych klinicznych dotyczących bezpieczeństwa oraz biodostępności sprawia, że związek ten nie spełnia rygorystycznych wymagań agencji regulacyjnych, takich jak FDA, co utrudnia jego rejestrację jako produktu leczniczego (Salla i in., 2024). Resweratrol wyróżnia się spośród polifenoli stosunkowo wysokim stopniem wchłaniania w przewodzie pokarmowym po podaniu doustnym, sięgającym około 70–75%. Transport resweratrolu zachodzi na drodze biernej dyfuzji przez nabłonek lub poprzez tworzenie kompleksów z transporterami błonowymi, takimi jak integryny czy transportery ABC (*ATP-binding cassette*). W enterocytach jelita cienkiego oraz hepatocytach wątroby resweratrol ulega szybkiej biotransformacji do rozpuszczalnych

w wodzie metabolitów glukuronidowych i siarczanowych, co obniża poziom wolnej formy resweratrolu we krwi i ułatwia jego wydalanie z moczem. Intensywne reakcje sprzęgania powodują, że biodostępność niezmienionej cząsteczki w krążeniu ogólnym jest bardzo niska i wynosi poniżej 1% (Smoliga i Blanchard, 2014; Walle i in., 2004). Ograniczona dostępność resweratrolu do wchłaniania w przewodzie pokarmowym wynika także z jego niskiej rozpuszczalności w wodzie. Istotną rolę w dystrybucji tkankowej resweratrolu odgrywa wiązanie jego wolnej formy z albuminą i lipoproteinami, co umożliwia dysocjację w błonach komórek posiadających receptory dla albuminy i lipoprotein o niskiej gęstości (Gambini i in., 2015). Dodatkowym czynnikiem wpływającym na jego przemiany jest metabolizm przez mikrobiotę jelitową. Dihydroresweratrol syntetyzowany przez bakterie jelitowe oraz wolny resweratrol wykrywano w tkankach głównie po długotrwałym podawaniu, natomiast po jednorazowej dawce dominują metabolity glukuronidowe i siarczanowe. Biodostępność resweratrolu i jego metabolitów zależy zatem zarówno od czasu, jak i dawki podawania (Chaplin i in., 2018). Zaobserwowano również znaczną zmienność międzyosobniczą w biodostępności resweratrolu, co może prowadzić do różnic w odpowiedzi fizjologicznej organizmu (Smoliga i Blanchard, 2014).

Dotychczas analizowano wiele strategii mających na celu poprawę biodostępności resweratrolu, w tym jego podawanie z różnymi produktami spożywczymi, stosowanie proszków mikronizowanych, łączenie z innymi substancjami fitochemicznymi (np. kwercytyną) lub standardowymi chemioterapeutykami, a także wykorzystanie systemów kontrolowanego uwalniania i nanoformulacji (Robertson i in., 2022; Smoliga i Blanchard, 2014). Innym podejściem jest zastosowanie pochodnych resweratrolu otrzymywanych poprzez m.in. hydroksylację, aminację, amidację, imidację, metoksylację, prenylację, halogenację, glikozylację oraz oligomeryzację. Ze względu na liczbę jednostek 1,2-difenyloetylenowych stilbeny dzieli się na dwa główne typy: monomeryczne i oligomeryczne. Monomery różnią się rodzajem podstawników chemicznych, natomiast oligomery mogą występować jako dimery, trimery, tetramery lub wyższe oligomery. Modyfikacje strukturalne wpływają na rozpuszczalność, absorpcję, biodostępność, stabilność metaboliczną oraz aktywność biologiczną tych związków (Mendonça i in., 2024; L. Zhou, Cai, i in., 2025).

Przykładem metylowanej pochodnej resweratrolu jest pterostilben (3,5-dimetoksy-4′-hydroksystilben), w którym dwie grupy –OH podstawione są grupami $–OCH_3$. Pterostilben występuje naturalnie w owocach jadalnych, takich jak borówki, winogrona, żurawina czy orzeszki ziemne, gdzie powstaje w wyniku *O*-metylacji resweratrolu (Nagarajan i in., 2022; Valletta i in., 2021). Silne działanie przeciwutleniające i przeciwzapalne pterostilbenu stanowi

podstawę szerokiego spektrum właściwości biologicznych, obejmujących m.in. efekty hipoglikemiczne, hipolipemizujące, przeciwgrzybicze, przeciwwirusowe oraz neuroprotekcyjne (Y. Liu i in., 2020; Mendonça i in., 2024). W badaniach *in vitro* pterostilben skutecznie hamował proliferację wielu typów komórek nowotworowych, m.in. raka żołądka, płuca, wątroby, jamy ustnej, trzustki, układu limfatycznego, jelita grubego, prostaty, piersi, czerniaka, białaczki i szpiczaka mnogiego. W modelach *in vivo* ograniczał rozwój guzów oraz procesy powstawania przerzutów. Jego działanie przeciwnowotworowe jest związane z hamowaniem proliferacji poprzez regulację szlaków AKT/mTOR, ERK1/2, JAK/STAT3, indukcją apoptozy poprzez AKT/mTOR/p70S6K i ERS, blokowaniem inwazji i przerzutowania poprzez modulację szlaków Src/FAK oraz Rac1/WAVE/Arp2/3, a także ograniczaniem populacji nowotworowych komórek macierzystych poprzez szlak GRP78 (Mendonça i in., 2024). Lipofilowy charakter pterostilbenu ułatwia jego przenikanie przez barierę krew-mózg, co może przynosić korzyści dla ośrodkowego układu nerwowego (P. Liu i in., 2024). Pterostilben charakteryzuje się również wyższą biodostępnością po podaniu doustnym (80–95%), co przypisuje się zwiększonej lipofilowości (obecność dwóch grup metoksylowych) oraz stabilności metabolicznej (jedna grupa hydroksylowa), skutkującej wolniejszą glukuronidacją i dłuższym okresem półtrwania w porównaniu z resweratrolem (Brás i in., 2025). Pterostilben charakteryzuje się korzystnym profilem bezpieczeństwa, co wykazano zarówno w badaniach przedklinicznych, jak i klinicznych. W modelach zwierzęcych był dobrze tolerowany nawet przy bardzo wysokich dawkach sięgających 3000 mg/kg mc./dobę. Natomiast badania kliniczne wskazują na dobrą tolerancję u ludzi w dawkach terapeutycznych wynoszących do 250 mg/dobę. Niestety wciąż brakuje danych potwierdzających bezpieczeństwo długoterminowego przyjmowania pterostilbenu, co jest istotne w świetle doniesień sygnalizujących potencjalną hepatotoksyczność w przypadku zastosowania wysokich dawek. Autorzy prac podkreślają konieczność prowadzenia dalszych badań nad optymalnym dawkowaniem i bezpieczeństwem w przypadku chronicznego narażenia (Lin i in., 2020; P. Liu i in., 2024).

Do naturalnych, najczęściej występujących hydroksylowanych pochodnych resweratrolu należą m.in. oksyresweratrol, piceatannol, dihydroksystilbeny oraz heksahydroksystilbeny. Piceatannol (3,5,3′,4′-tetrahydroksy-trans-stilben), który był przedmiotem badań w niniejszej pracy doktorskiej, jest najlepiej zbadaną hydroksylowaną pochodną resweratrolu zawierającą dodatkową grupę hydroksylową w pozycji 3′ drugiego pierścienia aromatycznego. Piceatannol występuje naturalnie w borówkach, granacie, pestkach marakui, białych i czerwonych winogronach, orzeszkach ziemnych, rdeście ostrokończystym, białej herbacie i roślinach

strączkowych. Może być syntetyzowany z pochodnych estru CoA lub powstawać w wyniku metabolicznej hydroksylacji resweratrolu. Związek ten wykazuje szerokie spektrum właściwości biologicznych, obejmujących działanie antyoksydacyjne, przeciwzapalne, przeciwnowotworowe, antyproliferacyjne, neuroprotekcyjne, a także korzystne efekty metaboliczne, przeciwmiażdżycowe, kardioprotekcyjne oraz aktywność przeciwbakteryjną i przeciwwirusową (Piotrowska i in., 2012; Al-Jaber i in., 2024). Jego właściwości cytotoksyczne oceniono wobec wielu linii komórkowych, w tym raka jelita grubego, różnych typów białaczek, czerniaka, raka wątroby oraz raka jajnika. (Rajan i in., 2024). Do kluczowych mechanizmów molekularnych regulowanych przez piceatannol należy selektywna inhibicja kinaz tyrozynowych, istotnych w regulacji odpowiedzi immunologicznej oraz procesów związanych kancerogenezą (Kwon i in., 2012; Piotrowska i in., 2012). Efekty antyoksydacyjne i cytoprotekcyjne wywołane przez piceatannol mogą być związane z regulacją szlaków sygnałowych kontrolowanych przez Nrf2 (Mendonça i in., 2024). Ponadto liczne dane wskazują na hamujący wpływ piceatannolu na NF-κB oraz szlaki JAK2/STAT3, PI3K/Akt/mTOR, EGFR i inne ścieżki sygnałowe związane ze stanem zapalnym i proliferacją (Al-Jaber i in., 2024). Obecność dwóch grup hydroksylowych w pozycjach 3′ i 4′ (układ katecholowy w pierścieniu B) ma kluczowe znaczenie dla zdolności piceatannolu do neutralizowania wolnych rodników i wychwytywania niesparowanych elektronów, co prowadzi do powstania stabilnego rodnika semichinonowego i skuteczniejszej ochrony antyoksydacyjnej w porównaniu z resweratrolem. Podobnie jak zwiazek macierzysty, piceatannol charakteryzuje się ograniczoną biodostępnością, a jego metabolizm w wątrobie zachodzi głównie przez glukuronidację, sulfatację i metylację. Najnowsze analizy *in silico* wykazały, że obecność układu katecholowego zwiększa jednak stabilność metaboliczną związku, a powstające metabolity cechują się niską cytotoksycznością i dobrą przenikalnością przez tkanki przewodu pokarmowego (Rajan i in., 2024).

Kolejną pochodną resweratrolu, która była przedmiotem badań realizowanych w niniejszej pracy doktorskiej, była polidatyna (piceid) – 3,4′,5-trihydroksystilbeno-3-O-β-D-glukopiranozyd. Rośliny szczególnie bogate w polidatynę to rdestowiec japoński, w korzeniach którego stwierdza się około 16 mg polidatyny i 1,8 mg resweratrolu na gram suchej masy, a także różne gatunki winorośli, pistacje i chmiel. W roślinach polidatyna pełni funkcję magazynowej, lepiej rozpuszczalnej formy resweratrolu (Kozłowska i Czekała, 2017). Jej biosynteza zachodzi z udziałem urydynodifosfo-(UDP)-glukoronylotransferaz, wykorzystujących UDP-glukozę jako donor reszty cukrowej (T. Liu i in., 2021). Polidatyna wykazuje w badaniach *in vitro* i *in vivo* wielotorowe działanie biologiczne, obejmujące

modulację szlaków oksydacyjno-zapalnych, poprawę profilu lipidowego oraz regulację metabolizmu energetycznego. Jej potencjał przeciwnowotworowy oceniono w modelach raka krtani, nosogardła, jajnika, płuc, szyjki macicy, glejaka wielopostaciowego, szpiczaka mnogiego i kostniakomięsaka. Działanie polidatyny było związane z hamowaniem proliferacji komórek i indukcją apoptozy poprzez modulację szlaku PI3K/Akt/mTOR, aktywację kaspaz, obniżenie stosunku BCL-2/BAX oraz ograniczanie migracji i inwazji (↓MMP-2, ↓MMP-9). W modelach metabolicznych polidatyna wykazywała działanie przeciwcukrzycowe poprzez aktywację AMPK i regulację szlaku IRS-1/PI3K/AKT, a także zmniejszała niealkoholowe stłuszczenie wątroby. Jej działanie kardioprotekcyjne polegało na aktywacji eNOS/NO i osi PI3K/AKT, czego efektem była ochrona antyoksydacyjna i przeciwzapalna (Karami i in., 2022; Luo i in., 2022). Potencjał terapeutyczny polidatyny w endometriozie oceniano w badaniach przedklinicznych i próbach klinicznych, które wykazały, że związek ten w połączeniu z palmitoiloetanoloamidą (PEA) zmniejsza stan zapalny i liczbę komórek tucznych, ogranicza rozmiar i rozwój zmian endometrialnych oraz łagodzi objawy bólowe, poprawiając jednocześnie jakość życia i stan psychiczny pacjentek (Di Paola i in., 2016; Stochino Loi i in., 2019). Polidatyna jest wchłaniana w żołądku i jelitach zarówno przez dyfuzję bierną, jak i transport aktywny z udziałem zależnego od sodu transportera glukozy SGLT1 (Luo i in., 2022). Niektóre dane wskazują, że transport przeznabłonkowy polidatyny przebiega szybciej niż w przypadku formy aglikonowej resweratrolu. Polidatyna ulega jednak hydrolizie przez β-glikozydazy wytwarzane przez mikrobiotę jelitową, co sprzyja wchłanianiu aglikonu i prowadzi do powstania metabolitów koniugowanych podobnych jak w przypadku resweratrolu (Nunes i in., 2018). Metabolizm polidatyny zależny od mikrobioty jelitowej i biokonwersja przez bakteryjne β-glukozydazy są dobrze udokumentowane i wskazują na indywidualną farmakokinetykę tej pochodnej (Zhao i in., 2022).

Nowe strategie poprawy właściwości farmakokinetycznych i dostarczania pochodnych resweratrolu obejmują przede wszystkim rozwój zaawansowanych systemów dostarczania leków, takich jak stałe nanocząsteczeki lipidowe funkcjonalizowane bioaktywnymi ligandami, w tym np. laktoferryną i siarczanem chondroityny (Aly i in., 2023), liposomy, micele, nanokapsułki polimerowe, kropki kwantowe i polimerowe nanokapsułki (Karami i in., 2022), układy samoemulgujące (Bachmaier i in., 2022), biomimetyczne systemy dostarczania nanocząstek (M. Zhou, Niu, i in., 2025), amorficzne dyspersje stałe (Rosiak i in., 2023) oraz kokryształy (Bofill i in., 2021). Formulacje te poprawiają rozpuszczalność, stabilność i biodostępność związków, a także umożliwiają selektywne ich dostarczanie do tkanek docelowych, w tym przenikanie przez barierę krew–mózg. Resweratrol i jego naturalne

pochodne są związkami wykazującymi wielokierunkowe działanie biologiczne, obejmujące wpływ na procesy komórkowe i molekularne, kluczowe dla patogenezy endometriozy. Udokumentowane w licznych badaniach ich silne właściwości przeciwzapalne oraz zdolność do neutralizacji ROS mogą mieć szczególne znaczenie w endometriozie, w której przewlekły stan zapalny i stres oksydacyjny stanowią główne czynniki promujące rozwój zmian ektopowych. Ponadto inne dobrze poznane efekty biologiczne resweratrolu, takie jak zdolność do hamowania proliferacji komórek, indukcji apoptozy oraz ograniczania angiogenezy, migracji i przerzutowania, a także włóknienia, przemawiają za wyborem tego związku jako potencjalnego czynnika terapeutycznego w endometriozie.

Włączenie do badań, prowadzonych w ramach pracy doktorskiej, różnych pochodnych resweratrolu było związane z ich korzystniejszym profilem farmakokinetycznym oraz potencjalnie wyższą aktywnością biologiczną po podaniu doustnym. Modyfikacje strukturalne stilbenów wpływają bowiem na ich stabilność metaboliczną, lipofilowość, biodostępność oraz zdolność przenikania do komórek. Potencjał antyendometriotyczny wybranych stilbenów był analizowany z zastosowaniem modeli *in vitro* endometriozy, które stanowią cenne narzędzie badawcze umożliwiające precyzyjną analizę dynamiki procesów komórkowych i molekularnych w ocenie terapeutycznego działania związków bioaktywnych. Badania wykorzystujące różne komórkowe układy modelowe imitujące *in vitro* zjawiska istotne dla patogenezy endometriozy mogą dostarczyć nowych danych na temat potencjalnej aktywności resweratrolu oraz jego analogów, które mogą być podstawą rekomendacji tych związków do dalszych, szeroko zakrojonych badań przedklinicznych i klinicznych.

2. Hipotezy badawcze i cel pracy

Analiza danych literaturowych oraz przyjęta koncepcja badawcza stanowiły podstawę do sformułowania następujących hipotez badawczych:

H1. Resweratrol i jego naturalne pochodne wykazują selektywne działanie ukierunkowane na komórki endometriozy.

H2. Analizowane stilbeny hamują proliferację i indukują apoptozę w komórkach endometriotycznych.

H3. Resweratrol i jego naturalne pochodne działają antyoksydacyjnie i przeciwzapalnie w mikrośrodowisku zmian endometriotycznych poprzez hamowanie ekspresji i produkcji kluczowych mediatorów stanu zapalnego.

H4. Analizowane stilbeny ograniczają adhezję komórek endometriotycznych do składników ECM oraz zmniejszają ich migrację i inwazję.

W celu weryfikacji postawionych hipotez badawczych zastosowano układy doświadczalne umożliwiające ocenę aktywności biologicznej resweratrolu i jego naturalnych pochodnych w zakresie działania antyproliferacyjnego i proapoptotycznego na komórki endometriotyczne, aktywności antyoksydacyjnej i przeciwzapalnej w mikrośrodowisku endometriozy oraz zdolności do hamowania adhezji, migracji i inwazji komórek endometriotycznych.

Cel główny, którym była **analiza potencjału antyendometriotycznego resweratrolu i jego pochodnych – pterostilbenu, piceatannolu i polidatyny – z wykorzystaniem modeli komórkowych odzwierciedlających kluczowe procesy zaangażowane w rozwój endometriozy**, realizowano w oparciu o następujące cele szczegółowe**:**

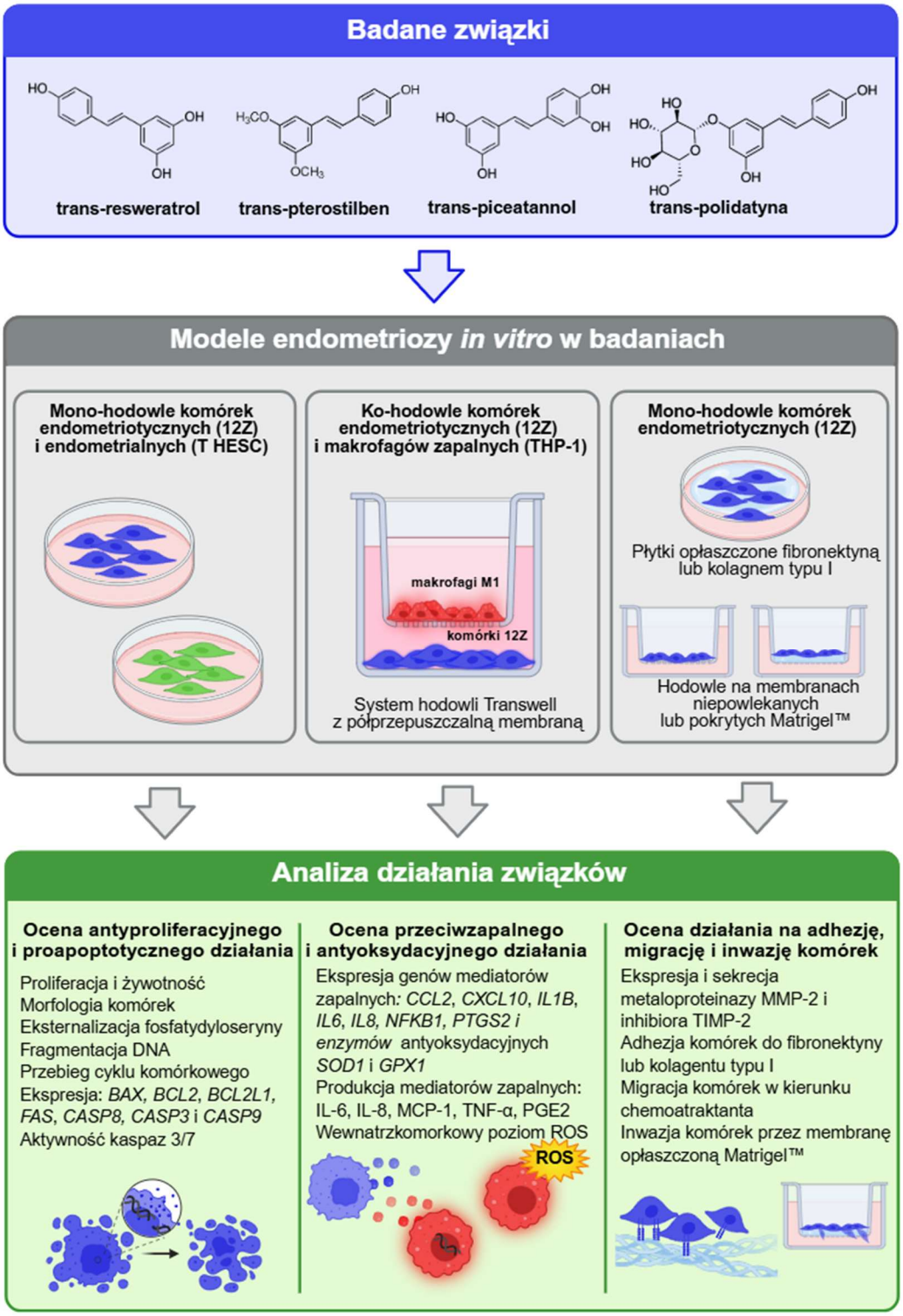
C1: Ocena cytotoksycznego działania resweratrolu i jego pochodnych wobec komórek endometrialnych i endometriotycznych.

C2: Analiza antyproliferacyjnych i proapoptotycznych mechanizmów działania resweratrolu i jego pochodnych w komórkowym modelu endometriozy *in vitro*.

C3: Ocena aktywności antyoksydacyjnej i przeciwzapalnej analizowanych stilbenów w modelu stanu zapalnego endometriozy.

C4: Ocena potencjału resweratrolu i jego pochodnych do hamowania procesów adhezji, migracji i inwazji komórek endometriozy *in vitro*.

Prace badawcze prowadzono zgodnie ze schematem badań przedstawionym na Rycinie 1.



Rycina 1. Schemat badań. Rycinę przygotowano w programie https://BioRender.com.

3. Materiały i metody badań

3.1 Analizowane związki

W pracy analizowano aktywność biologiczną trans-resweratrolu, trans-pterotilbenu, trans-piceatannolu i trans-polidatyny, które pozyskano z firmy Merck KGaA (Darmstadt, Niemcy). Związki rozpuszczano w 96% alkoholu etylowym i dodawano do pożywki hodowlanej zachowując końcowe stężenie alkoholu na poziomie 0,5%.

3.2 Linie komórkowe

W badaniach zastosowano następujące linie komórkowe:

- Ludzkie komórki endometriotycze linii 12Z (T0764) pozyskane z kolekcji Applied Biological Materials, Inc. (Richmond, British Columbia, Kanada).
- Ludzkie komórki podścieliska endometrium linii T HESC (CRL-4003™).
- Ludzkie komórki ostrej białaczki monocytowej linii THP-1 (TIB-202™).

Linie komórkowe T HESC i THP-1 pozyskano z kolekcji ATCC (American Type Culture Collection, Manassas, VA, USA).

3.3 Warunki hodowli komórek

Hodowle komórek prowadzono zgodnie z procedurami rekomendowanymi przez kolekcje, z których zostały pozyskane. Komórki endometriotyczne linii 12Z hodowano w pożywce DMEM/F12 (*Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12*) (Sigma-Aldrich Co., St. Louis, MO, USA/Merck Group, Darmstadt, Niemcy) z dodatkiem 10% surowicy bydlęcej (FBS, *fetal bovine serum*) (Gibco, Grand Island, NY, USA). Hodowle komórek zrębu endometrium linii T HESC prowadzono z użyciem pożywki DMEM/F12 bez czerwieni fenolowej (Sigma-Aldrich), suplementowanej 10% FBS filtrowaną węglem aktywnym (Sigma-Aldrich), 1% mieszaniną insuliny, transferryny i selenu (roztwór ITS+1) (Sigma-Aldrich). Do hodowli monocytów linii THP-1 stosowano pożywkę RPMI 1640 (Sigma-Aldrich) z dodatkiem 10% FBS oraz 0,05 mM 2-merkaptoetanolu (Sigma-Aldrich). Do standardowych hodowli komórkowych dodawano gentamycynę (Sigma-Aldrich) w stężeniu 50 mg/l.

Wszystkie komórki hodowano w temperaturze 37°C, w atmosferze gazowej zawierającej 95% powietrza i 5% CO_2. Do pasażowania adherentnych komórek linii 12Z i T HESC stosowano roztwór trypsyno-wersenu (Sigma-Aldrich), który po uwolnieniu komórek od

powierzchni był inaktywowany pożywką z dodatkiem FBS. Podczas prowadzenia hodowli rutynowo wykonywano testy na obecność mykoplazm w celu wykluczenia infekcji.

3.4 Modele komórkowe

3.4.1 Modele komórek endometriotycznych i endometrialnych zastosowane w badaniach nad antyproliferacyjnym i proapoptotycznym potencjałem stilbenów

W testach cytotoksyczności zastosowano 24-godzinne hodowle komórek endometriotycznych linii 12Z i komórek endometrialnych linii T HESC, które zakładano przy początkowej ilości komórek wynoszącej 0,15 × 10^5/cm². Komórki eksponowano na analizowane stilbeny w stężeniach 6,25; 12,5; 25; 50; 100; 200; 300; 400 μM przez 48 godzin. Każda seria doświadczalna obejmowała również hodowle kontrolne, w których komórki poddawano działaniu alkoholu etylowego w stężeniu 0,5%, stosowanego do rozpuszczenia związków. Stężenie alkoholu w hodowlach kontrolnych odpowiadało jego zawartości w hodowlach z dodatkiem analizowanego związku.

W badaniach nad aktywnością antyproliferacyjną i proapoptotyczną resweratrolu i jego pochodnych zastosowano 24-godzinne hodowle komórek endometriotycznych linii 12Z, które przygotowywano przy początkowej ilości komórek wynoszącej 0,15 × 10^5/cm². Komórki traktowano resweratrolem (10, 50, 100 μM), pterostilbenem (10, 50, 100 μM), piceatannolem (50, 100, 200 μM) i polidatyną (50, 100, 200 μM) przez 48 godzin w standardowych warunkach hodowli. Jako związek referencyjny indukujący apoptozę zastosowano kamptotecynę w stężeniach 5 μM i 50 μM.

3.4.2 Model komórkowy stanu zapalnego w endometriozie zastosowany w badaniach nad przeciwzapalnym potencjałem stilbenów

Do badań nad aktywnością przeciwzapalną i antyoksydacyjną resweratrolu i jego pochodnych w odniesieniu do mikrośrodowiska endometriozy opracowano model komórkowy obejmujący ko-hodowlę komórek endometriotycznych 12Z na płytkach 6-dołkowych i makrofagów typu M1 uzyskanych poprzez różnicowanie ludzkich monocytów linii THP-1 rosnących na półprzepuszczalnych membranach. Monocyty THP-1 wysiewano na membrany w ilości 2 × 10^5/cm^2 i traktowano octanem mirystynianu forbolu (PMA, 10 ng/ml) przez 24 godziny w celu indukcji ich różnicowania do makrofagów. Następnie przy użyciu pożywki indukującej – RPMI 1640 z 10% FBS z dodatkiem 50 ng/ml IFN-γ i 10 pg/ml lipopoliacharydów (LPS) z *E. coli* – przeprowadzano 24-godzinną polaryzację makrofagów do prozapalnego fenotypu M1. Równolegle w płytce 6-dołkowej zakładano hodowlę komórek 12Z

przy inokulum 0,3 × 10^5/cm^2 w pożywce DMEM/F12 z 10% dodatkiem FBS. Po 24 godzinach wymieniano pożywkę na RPMI 1640 z 2% dodatkiem FBS. W celu uzyskania komórkowego układu doświadczalnego tworzono ko-hodowlę makrofagów M1 i komórek 12Z utrzymywaną w pożywce RPMI 1640 z 2% dodatkiem FBS. Ko-hodowle traktowano resweratrolem i jego pochodnymi przez 24 godziny.

Procedury różnicowania i polaryzacji komórek opracowano poprzez dobór odpowiednich warunków eksperymentalnych. Optymalizacji poddawano początkową liczbę monocytów THP-1, czas ekspozycji na czynniki różnicujące i polaryzujące oraz stężenia induktorów, korzystając z wcześniej opublikowanych danych (Genin i in., 2015; Smith i in., 2015). Analizy cytotoksyczności badanych związków przeprowadzono w mono-hodowlach makrofagów THP-1 i komórek 12Z. Komórki 12Z i komórki THP-1 po 24-godzinnej stymulacji PMA traktowano przez 24 godziny stilbenami (1 μM–50 μM) rozpuszczonymi w podłożu RPMI 1640 z 2% dodatkiem FBS.

3.4.3 Model komórkowy zastosowany w badaniach nad wpływem stilbenów na adhezję, migrację i inwazję komórek endometriozy

W badaniach nad wpływem stilbenów na procesy adhezji, migracji i inwazji komórek endometriozy zastosowano mono-hodowlę komórek endometriotycznych linii 12Z, którą prowadzono na powierzchni opłaszczonej fibronektyną lub kolagenem typu I (Millicoat™ Human Fibronectin/Collagen Type I Coated Strips, Millipore #ECM101,104) oraz na półprzepuszczalnych membranach (BD Falcon FluoroBlok Insert System i BD Biocoat FluoroBlok Invasion System, BD Bioscience, Bedford, MA, USA). Przed analizami przeprowadzono testy cytotoksyczności badanych związków. Hodowle zakładano na płytkach pokrytych kolagenem typu I (5 μg/cm^2) przy początkowej ilości komórek 2,5 × 10^4 /cm^2 i inkubowano przez 24 godziny. Następnie komórki poddano działaniu stilbenów (5, 10, 25, 50 μM) przez kolejne 24 godziny w standardowych warunkach hodowli.

3.5 Metody analityczne

3.5.1 Testy cytotoksyczności

Analizę cytotoksyczności resweratrolu i jego pochodnych przeprowadzono przy pomocy dwóch testów: MTT oraz Alamar blue.

Test MTT opiera się na przekształceniu żółtej rozpuszczalnej w wodzie soli tetrazolowej (bromek 3-(4,5-dimetylotiazol-2yl)-2,5-difenylotetrazolowy) do zredukowanej formy nierozpuszczalnego w wodzie formazanu o barwie fioletowej. Formazan powstający w wyniku

aktywności dehydrogenaz mitochondrialnych, gromadzi się w postaci kryształów w komórkach aktywnych metabolicznie. Po ekspozycji komórek na badane związki do hodowli dodano roztwór MTT (0,5 mg/ml) i prowadzono inkubację w temperaturze 37°C przez 3 godziny. Powstały formazan ekstrahowano z komórek przy użyciu izopropanolu; ekstrakcję prowadzono w temperaturze pokojowej na wytrząsarce przez 20 minut. Absorbancję mierzono przy długości fali λ = 570 nm (pomiar właściwy) i λ = 690 nm (pomiar referencyjny) z wykorzystaniem czytnika mikropłytek Tecan Infinite M200 (Tecan Group Ltd., Männedorf, Szwajcaria).

Do oceny cytotoksyczności badanych związków wykorzystano odczynnik alamarBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA). Test Alamar Blue opiera się na konwersji niebieskiego, niefluorescencyjnego barwnika – resazuryny, przenikającego przez błony komórkowe – do silnie fluorescencyjnej, różowej rezorufiny. Przekształcenie resazuryny w rezorufinę zachodzi wyłącznie w komórkach żywych, aktywnie metabolicznych. Po 4 godzinach inkubacji z odczynnikiem alamarBlue™ mierzono fluorescencję przy użyciu czytnika mikropłytek TECAN Infinite M200, przy długości fali wzbudzenia λ = 560 nm i emisji λ = 600 nm.

Na podstawie wyników testów MTT i Alamar Blue wyznaczono wartości stężeń cytotoksycznych IC (*inhibitory concentration*), w tym: IC10 i IC50, odpowiadające stężeniom stilbenów powodującym odpowiednio 10% i 50% zmniejszenie żywotności i aktywności metabolicznej komórek.

3.5.2 Mikroskopowa ocena morfologii komórek

Morfologię komórek endometriotycznych 12Z oraz komórek linii THP-1 poddanych różnicowaniu i polaryzacji oceniano z użyciem odwróconego mikroskopu świetlnego Axiovert 40C z kontrastem fazowym (Carl Zeiss AG, Oberkochen, Niemcy).

Za pomocą mikroskopu fluorescencyjnego Zeiss Axiovert 200 (Carl Zeiss AG, Oberkochen, Niemcy) obserwowano i identyfikowano morfologiczne symptomy śmierci apoptotycznej w komórkach 12Z poddanych działaniu resweratrolu i jego pochodnych. Komórki hodowano na szkiełkach Millicell® EZ Slide (Merck KGaA Darmstadt, Niemcy) i traktowano badanymi związkami zgodnie ze standardowym protokołem. Po ekspozycji komórki 12Z utrwalano 4% paraformaldehydem, przemywano PBS, a następnie permeabilizowano błony komórkowe za pomocą 0,1% Tritonu X-100 i barwiono 1 µg/ml DAPI. W preparatach obserwowano morfologiczne cechy apoptozy, takie jak zmniejszenie objętości komórki, fragmentacja komórki, powstawanie ciałek apoptotycznych, kondensacja chromatyny.

3.5.3 Oznaczenie adhezji komórek 12Z do składników ECM

Do analizy adhezji komórek 12Z zastosowano naczynia hodowlane powlekane ludzką fibronektyną lub kolagenem typu I (Millicoat™ Human Fibronectin/Collagen Type I Coated Strips, Millipore, Merck KGaA). Test adhezji przeprowadzano zgodnie z protokołem zalecanym przez producenta. Komórki 12Z wysiewano w ilości 2,5 × 10^4/cm² i traktowano stilbenami w stężeniach 5, 10, 25 i 50 µM. Następnie komórki delikatnie odklejano od podłoża z użyciem roztworu Non-Enzymatic Cell Dissociation Solution i przemywano buforem PBS. Uwolnione komórki wysiewano ponownie w zoptymalizowanej gęstości 2,9 × 10^5/cm^2 i inkubowano przez 1 godzinę w celu umożliwienia ich adhezji do powierzchni pokrytych fibronektyną i kolagenem typu I. Po inkubacji komórki przemywano buforem PBS zawierającym Ca^{2+}/Mg^{2+}, a następnie barwiono 0,2% roztworem fioletu krystalicznego przez 5 minut. Po usunięciu barwnika komórki trzykrotnie przemywano buforem PBS. Związany z komórkami barwnik rozpuszczano w roztworze do ekstrakcji, który stanowiła mieszanina (1:1) złożona z 0,1 M NaH_2PO_4 (pH 4,5) i 50% etanolu. Hodowle inkubowano z delikatnym mieszaniem przez 5 minut w temperaturze pokojowej. Po ekstrakcji barwnika mierzono absorbancję przy długości fali λ = 540 nm za pomocą czytnika mikropłytek Tecan Infinite M200. Dołki pokryte albuminą surowicy bydlęcej wykorzystano jako kontrolę negatywną. Po barwieniu wyniki wizualizowano również przy użyciu odwróconego mikroskopu świetlnego z kontrastem fazowym (Zeiss Axiovert 40C).

3.5.4 Oznaczenie migracji i inwazji komórek 12Z

Do oceny migracji i inwazji komórek 12Z poddanych działaniu stilbenów wykorzystano systemy Corning® FluoroBlok™ Inserts (Corining, Inc., Corning, NY, USA) oraz BD Biocoat™ FluoroBlok Invasion System (BD Bioscience, Bedford, MA, USA). Układ składał się z dwóch kompartmentów; w części górnej układu umieszczono wkład z membraną PET FluoroBlok™ o porowatości 8 µm. Membrany pokryte matrycą BD Matrigel™ stosowano w testach inwazji, natomiast w testach migracji wykorzystano membrany nieopłaszczone. Wkłady z membranami umieszczano w dołkach płytek 24-dołkowych.

Komórki 12Z wysiewano na membrany w optymalnej gęstości 2 × 10^4 komórek i inkubowano w pożywce hodowlanej w obecności analizowanych związków. W celu wywołania procesu migracji i inwazji do dolnych kompartmentów dodawano pożywkę DMEM/F12 zawierającą 10% FBS, pełniącą rolę chemoatraktanta. Hodowle inkubowano w standardowych warunkach przez 22 godziny. Po inkubacji usuwano pożywkę znad komórek, a następnie wkłady z membranami przenoszono do nowych płytek 24-dołkowych

zawierających barwnik – acetoksymetylowy ester kalceiny (4 μg/ml) w buforowanym roztworze soli Hanksa. Hodowle inkubowano przez 60 minut. Migrację i inwazję komórek 12Z oznaczano za pomocą czytnika mikropłytek Tecan Infinite M200, mierząc fluorescencję przy długości fali wzbudzenia λ = 485 nm i emisji λ = 530 nm. Potencjał migracyjny i inwazyjny określano jako wartości względne w odniesieniu do kontroli.
Indeks inwazji obliczano według wzoru:

$$I_{indeks} = \frac{\text{względna inwazja komórek (\%)}}{\text{względna migracja komórek (\%)}}$$

Wyniki testów migracji i inwazji potwierdzano dodatkowo obserwacjami mikroskopowymi z wykorzystaniem mikroskopu fluorescencyjnego Zeiss Axiovert 200.

3.5.5 Analiza procesu apoptozy w komórkach endometriotycznych

3.5.5.1 Oznaczenie eksternalizacji fosfatydyloseryny

Eksternalizacja błonowego fosfolipidu – fosfatydyloseryny – z wewnętrznej do zewnętrznej warstwy błony komórkowej, stanowiąca wczesny marker apoptozy, była oznaczana za pomocą barwienia dwoma barwnikami: (1) białkową aneksyną V, zależną od jonów wapnia, skoniugowaną z izotiocyjanianem fluoresceiny (FITC), wykazującą wysokie powinowactwo do fosfatydyloseryny zlokalizowanej na powierzchni komórek apoptotycznych, oraz (2) jodkiem propidyny (PI), który wiąże się z DNA w komórkach o zaburzonej integralności błony komórkowej, charakterystycznej dla później fazy apoptozy. Komórki 12Z po ekspozycji na analizowane związki uwalniano z powierzchni płytek wielodołkowych, a następnie zawieszano w buforze do uzyskania stężenia 1×10^6/ml i barwiono przy użyciu zestawu BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). Komórki analizowano na cytometrze przepływowym z bioobrazowaniem Amnis™ FlowSight™ (Luminex Corp., Austin, TX, USA). Do opracowania uzyskanych danych zastosowano oprogramowanie IDEAS® (wersja 6.2.187, Luminex Corp.). Na postawie analizy wyróżniano cztery subpopulacje komórek: (1) komórki żywe (aneksyna V^-/PI^-), (2) komórki wczesnoapoptotyczne (aneksyna V^+/PI^-), (3) komórki późnoapoptotyczne (aneksyna V^+/PI^+) oraz (4) komórki nekrotycznne (aneksyna V^-/PI^+).

3.5.5.2 Detekcja fragmentacji DNA

Ocenę fragmentacji DNA w komórkach linii 12Z przeprowadzono przy użyciu testu TUNEL (*terminal deoxynucleotidyl transferase dUTP nick end labeling*), z zastosowaniem

zestawu In Situ Cell Death Detection Kit (Roche Diagnostics International Ltd., Rotkreuz, Szwajcaria). Metoda ta umożliwia detekcję pęknięć nici DNA poprzez przyłączenie fluorescencyjnie znakowanych nukleotydów dUTP katalizowanych przez terminalną deoksynukleotydylotransferazę (TdT).

Komórki 12Z eksponowane na resweratrol i jego pochodne utrwalano w 4% paraformaldehydzie, a następnie permeabilizowano błony komórkowe roztworem 0,1% Triton X-100 w 0,1% cytrynianie sodu. Po przemyciu buforem PBS komórki inkubowano z mieszaniną reakcyjną zawierającą dUTPs znakowane fluoresceiną oraz enzym TdT. Akwizycję danych wykonano przy użyciu cytometru przepływowego z funkcją obrazowania Amnis™ FlowSight™.

3.5.5.3 Oznaczenie aktywności kaspaz-3/7

Do oznaczenia aktywności enzymów charakterystycznych dla wykonawczej fazy apoptozy wykorzystano zestaw Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Corp., Madison, WI, USA). Zasada działania testu opiera się na sekwencyjnym proteolitycznym rozszczepieniu profluorescencyjnego substratu bisamidowego Z-DEVD-R110 do peptydów DEVD (Asp–Glu–Val–Asp), co prowadzi do uwolnienia rodaminy 110 emitującej silne światło fluorescencji. W celu wykonania oznaczenia aktywności kaspaz-3/7 hodowlę komórek 12Z prowadzono na białych płytkach 96-dołkowych z transparentnym dnem. Hodowle komórkowe przygotowywano analogicznie jak w analizach pozostałych wskaźników apoptozy, uwzględniając początkową ilość komórek, dawki związków i czas ekspozycji. Jako kontrolę pozytywną zastosowano hodowle komórek traktowane kamptotecyną w stężeniach 5 μM i 50 μM. Analizę przeprowadzono zgodnie z instrukcją producenta. Fluorescencję mierzono przy użyciu czytnika mikropłytek Tecan Infinite M200, przy długości fali wzbudzenia λ = 499 nm i emisji λ = 521 nm. Uzyskane wyniki znormalizowano względem zawartości białka komórkowego.

Wpływ stilbenów na ekspresję kaspaz analizowano również na poziomie molekularnym z zastosowaniem metody Real-Time PCR, którą opisano w rozdziale 3.5.11.

3.5.6 Analiza cyklu komórkowego

Analiza cyklu komórkowego z wykorzystaniem barwienia PI umożliwia ilościową ocenę zawartości DNA. Uzyskane dane pozwalają na określenie odsetka komórek znajdujących się w poszczególnych fazach cyklu, tj. w fazach G0/G1, S oraz G2/M. Metoda ta umożliwia również detekcję populacji komórek o obniżonej zawartości DNA, charakterystycznej m.in. dla komórek apoptotycznych, która na histogramach pojawia się jako populacja sub-G0/G1

(Darzynkiewicz i in., 2001). Komórki endometriotyczne linii 12Z po ekspozycji na badane związki uwalniano od powierzchni hodowlanej, przemywano PBS, utrwalano w 70% etanolu i inkubowano w temperaturze 4°C przez 30 min w celu zwiększenia przepuszczalności błon komórkowych. Po przepłukaniu komórek w PBS osad komórkowy zawieszano w PBS zawierającym 50 µg/ml PI i 100 µg/ml rybonukleazy A. Po 30-minutowej inkubacji w 37°C komórki analizowano na cytometrze przepływowym FACSCanto (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Analizę danych przeprowadzano przy użyciu oprogramowania FACS Diva (Becton, Dickinson and Company).

3.5.7 Oznaczenie wewnątrzkomórkowych reaktywnych form tlenu

Oznaczenia ROS produkowanych przez makrofagi hodowane w ko-hodowli z komórkami 12Z po ekspozycji na stilbeny przeprowadzono z wykorzystaniem zestawu CellROX™ Deep Red Flow Cytometry Assay Kit (Life Technologies, Carlsbad, CA, USA). Odczynnik CellROX™ Deep Red Reagent w formie zredukowanej nie emituje fluorescencji i łatwo przenika do wnętrza komórek. Po utlenieniu generuje silny sygnał fluorescencji, którego intensywność jest proporcjonalna do poziomu ROS w żywych komórkach. W analizie uwzględniono również identyfikację komórek martwych z zastosowaniem barwnika Sytox™ Green Dead Cell Stain (Life Technologies, Carlsbad, CA, USA). Makrofagi po ekspozycji na stilbeny w układzie ko-hodowli z komórkami endometriotycznymi uwalniano od powierzchni hodowlanej i zawieszano w pożywce RPMI 1640 bez czerwieni fenolowej. Do zawiesiny komórek dodawano odczynnik CellROX™ Deep Red do stężenia końcowego 500 nM, po czym prowadzono inkubację przez 60 minut w temperaturze 37°C w ciemności. Podczas ostatnich 15 minut barwienia wprowadzano odczynnik Sytox™ Green Dead Cell Stain w stężeniu 45 nM. Pomiar fluorescencji oraz obrazowanie komórek przeprowadzono z wykorzystaniem cytometru przepływowego Amnis™ FlowSight™. Na podstawie różnic w intensywności sygnału fluorescencji wyodrębniano trzy subpopulacje komórek: (1) komórki żywe z nadprodukcją ROS, (2) komórki żywe niewykazujące oznak stresu oksydacyjnego oraz (3) komórki martwe ze szczątkowym sygnałem ROS.

3.5.8 Ilościowe oznaczenie cytokin z zastosowaniem cytometrycznych macierzy kulkowych

Do oznaczeń wykorzystano pożywkę z ko-hodowli makrofagów i komórek 12Z, pobraną po 24-godzinnej ekspozycji na stilbeny. Przed analizą pożywkę wirowano w celu usunięcia zanieczyszczeń komórkowych. Oznaczenia cytokin wykonano przy użyciu zestawu Human

Inflammatory Cytokine Cytometric Bead Array (CBA) (BD Biosciences, Franklin Lakes, NJ, USA), zgodnie z protokołem producenta. W pierwszym etapie do analizowanych próbek dodawano polimerowe kulki opłaszczone odpowiednimi przeciwciałami i prowadzono inkubację przez 1 godzinę w temperaturze pokojowej, w ciemności. Następnie do mieszaniny reakcyjnej dodawano przeciwciała sprzężone z barwnikiem – fikoerytryną. Po 2 godzinach inkubacji i przepłukaniu osad zawieszano w buforze analitycznym. Cytokiny oznaczano na cytometrze przepływowym FACSAriaII (BD Biosciences) z zastosowaniem oprogramowania FACSDiva v6.1.2 (BD Biosciences), a uzyskane wyniki analizowano przy użyciu programu FCAP Array™ Software (BD Biosciences). Dla każdej oznaczanej cytokiny sporządzono krzywą kalibracyjną obejmującą zakres wykrywalności od 1,0 do 5000 pg/ml.

3.5.9 Oznaczenie białka komórkowego

Po ekspozycji komórek na analizowane związki, komórki endometriotyczne 12Z i makrofagi THP-1 dwukrotnie płukano zimnym buforem PBS, a następnie zawieszano w buforze ekstrakcyjnym RIPA zawierającym inhibitory proteaz i inkubowano na lodzie przez 5 minut. Następnie próby wirowano (14 000 rpm, 5 min, 4°C) w celu oddzielenia pozostałości komórkowych. Supernatant zawierający białka komórkowe przechowywano w temperaturze – 80°C do momentu analizy. Zawartość całkowitego białka komórkowego oznaczono z wykorzystaniem zestawu Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) według protokołu producenta.

3.5.10 Oznaczenie stężenia czynników prozapalnych i metaloproteinazy MMP-2 metodą ELISA

Oznaczenia czynników prozapalnych (MCP-1, TNF-α i PGE2) w pożywce z ko-hodowli komórek endometriotycznych i makrofagów oraz metaloproteinazy MMP-2 w pożywce z hodowli komórek 12Z eksponowanych na analizowane stilbeny wykonano z zastosowaniem zestawów do analizy immunoenzymatycznej (ELISA), produkowanych przez firmy R&D Systems, Inc. (Minneapolis, MIN, USA) oraz Cayman Chemical (Ann Arbor, MI, USA). Oznaczenia przeprowadzano zgodnie z protokołami zalecanymi przez producentów. Stężenia analizowanych wskaźników określano na podstawie krzywych kalibracyjnych.

3.5.11 Analiza ekspresji genów metodą Real-Time PCR

Komórki 12Z i makrofagi THP-1 poddawano działaniu odczynnika TRI-Reagent (Sigma-Aldrich) w celu izolacji całkowitego RNA komórkowego. Stężenie i czystość wyizolowanego RNA oznaczano za pomocą spektrofotometru Nano Drop-1000 (Thermo Fisher Scientific,

Waltham, MA, USA). Syntezę komplementarnego DNA (cDNA) na matrycy 1 µg całkowitego RNA prowadzono z wykorzystaniem zestawu Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics International Ltd., Rotkreuz, Szwajcaria), zgodnie z instrukcją producenta. Otrzymane cDNA wykorzystano jako matrycę w reakcji łańcuchowej polimerazy z analizą w czasie rzeczywistym (Real-Time PCR), prowadzonej w termocyklerze Applied Biosystems 7500 (Life Technologies, Carlsbad, CA, USA). Reakcje przygotowywano z użyciem zestawu SYBR™Select Master Mix (Life Technologies, Carlsbad, CA, USA), zgodnie z instrukcją producenta. Zastosowane startery, których sekwencje przedstawiono w tabeli 2 (publikacja 2) i tabeli 1 (publikacja 4) zaprojektowano przy użyciu narzędzia BLAST (*Basic Local Alignment Search Tool*). Mieszanina reakcyjna o objętości końcowej 25 µl zawierała: 1 µl matrycy cDNA, startery specyficzne forward i reverse (1 µM), 12,5 µl SYBR™ Select Master Mix oraz wodę do uzupełnienia objętości końcowej 25 µl. Program reakcji PCR obejmował wstępną denaturację w temperaturze 94°C przez 10 minut, a następnie 40 cykli PCR: denaturację w 95°C przez 40 s, przyłączanie starterów w 59°C przez 30 s i elongację w 72°C przez 30 s. Po zakończeniu reakcji przeprowadzono analizę krzywej topnienia w celu potwierdzenia specyficzności amplifikacji. Poziomy transkryptów normalizowano względem ekspresji genów referencyjnych: β-aktyny w komórkach endometriotycznych 12Z oraz dehydrogenazy aldehydu 3-fosfoglicerynowego (GAPDH, *glyceraldehyde 3-phosphate dehydrogenase*) w makrofagach THP-1. Zmiany poziomu ekspresji genów wyrażono jako krotność względem komórek kontrolnych nieeksponowanych na analizowane związki. Względną ekspresję genów obliczano metodą $2^{-\Delta\Delta Ct}$ (Livak i Schmittgen, 2001).

3.6 Analiza statystyczna

Wszystkie eksperymenty biologiczne oraz analizy przeprowadzono w co najmniej dwóch powtórzeniach. Dane przedstawiono jako średnie arytmetyczne ± odchylenie standardowe (SD). Normalność rozkładu danych weryfikowano testem Shapiro-Wilka, a jednorodność wariancji testem Levene'a. Do porównania dwóch grup zastosowano test t-Studenta. W przypadku porównania trzech lub większej liczby grup przeprowadzano jednokierunkową analizę wariancji (ANOVA), a następnie test *post hoc* Tukeya. Za istotne statystycznie uznawano wartości $p \leq 0{,}05$. Analizy statystyczne wykonano przy użyciu oprogramowania STATISTICA w wersji 13.3 (Statsoft, Inc., Tulsa, OK, USA).

4. Wyniki

4.1 Ocena antyproliferacyjnego i proapoptotycznego działania resweratrolu i jego pochodnych w komórkach endometriotycznych (publikacja 3)

W ramach pracy doktorskiej analizowano potencjał proapoptotyczny resweratrolu i jego naturalnych analogów wobec nabłonkowych komórek endometriotycznych. Oceniono wpływ tych związków na żywotność i morfologię komórek, markery biochemiczne apoptozy, fragmentację DNA, regulację ekspresji genów pro- i antyapoptotycznych oraz aktywność kaspaz. Porównano również odpowiedź komórek endometriotycznych linii 12Z i komórek endometrialnych linii T HESC na działanie analizowanych stilbenów. Wyniki badań przestawiono w publikacji pt. „*Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways*" (publikacja 3) (Gołąbek-Grenda i in., 2023).

4.1.1 Wpływ resweratrolu i jego pochodnych na żywotność komórek endometrialnych i endometriotycznych

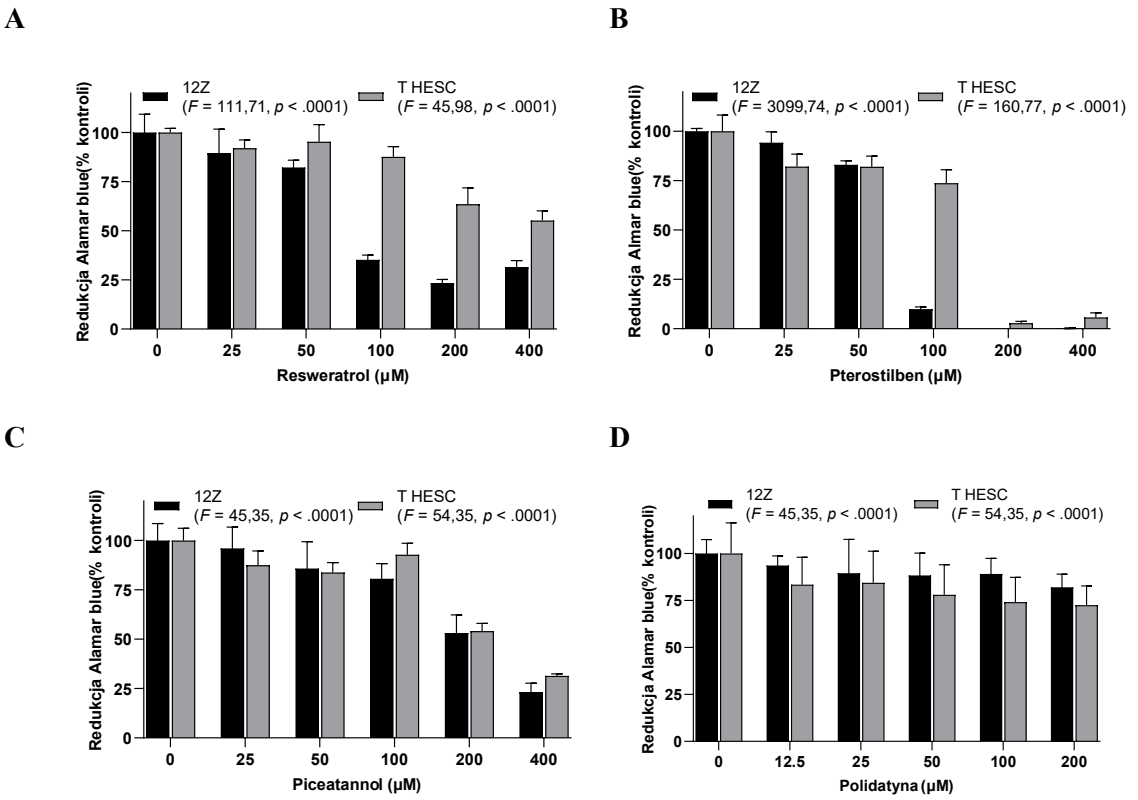
Ocenę żywotności i aktywności metabolicznej komórek poddanych działaniu resweratrolu i jego pochodnych przeprowadzono z użyciem testów MTT i Alamar Blue. Wykazano, że komórki endometrialne linii T HESC i endometriotyczne linii 12Z różnią się wrażliwością na testowane stilbeny, na co wskazują wartości dawek IC_{10} i IC_{50} wyznaczone w oparciu o wyniki uzyskane w teście MTT, które zostały przedstawione w Tabeli 1. Analiza wartości IC_{50} wykazała, że cytotoksyczność resweratrolu i pterostilbenu jest odpowiednio 5- i 2-krotnie niższa wobec prawidłowych komórek endometrium niż wobec komórek endometriotycznych. Podobnie, piceatannol i polidatyna wywołały znacznie słabsze efekty cytotoksyczne w hodowli prawidłowych komórek T HESC (Tabela 1). Wyniki uzyskane w teście Alamar Blue potwierdziły charakter działania badanych związków na komórki 12Z i T HESC (Rycina 2). Resweratrol i pterostilben wprowadzone do hodowli komórek 12Z indukowały najsilniejsze efekty cytotoksyczne, natomiast pozostałe dwie pochodne – piceatannol i polidatyna – wykazywały istotnie słabsze działanie ograniczające wzrost komórek 12Z w porównaniu ze związkiem macierzystym. Polidatyna charakteryzowała się najniższą cytotoksycznością spośród badanych związków; w komórkach T HESC nie indukowała efektów cytotoksycznych w stężeniach ≤ 400 μM, natomiast w komórkach 12Z pierwsze efekty cytotoksyczne obserwowano przy dawce 86,76 μM.

Tabela 1. Dawki cytotoksyczne IC_{10} i IC_{50} resweratrolu, pterostilbenu, piceatannolu i polidatyny wyznaczone w hodowli komórek endometriotycznych (12Z) i endometrialnych (T HESC) za pomocą testu MTT. Dane stanowią średnie uzyskane z trzech niezależnych eksperymentów (n=3) ± SD.
(Gołąbek-Grenda i in., 2023)

Analizowane związki	komórki 12Z		komórki T HESC	
	IC_{10}	**IC_{50}**	**IC_{10}**	**IC_{50}**
Resweratrol (μM)	8.99 ± 4.29	49.32 ± 11.4	160.35 ± 7.05$^{\#\#}$	250.99 ± 1.58$^{\#\#}$
Pterostilben (μM)	29.34 ± 4.46	73.88 ± 6.0	113.79 ± 5.11*, $^{\#\#}$	145.12 ± 7.71**, $^{\#\#}$
Piceatannol (μM)	167.50 ± 11.36**	223.76 ± 6.47**	222.68 ± 14.49**, $^{\#}$	363.72 ± 16.98**, $^{\#\#}$
Polidatyna (μM)	86.76 ± 10.01**	242.85 ± 22.51**	> 400	> 400

*p ≤0,05, **p ≤ 0,001 poziom istotności różnic pomiędzy analogami resweratrolu a resweratrolem.

#p ≤ 0,05, ##p ≤0,001 poziom istotności różnic pomiędzy działaniem analizowanych stilbenów na komórki 12Z i T HESC.

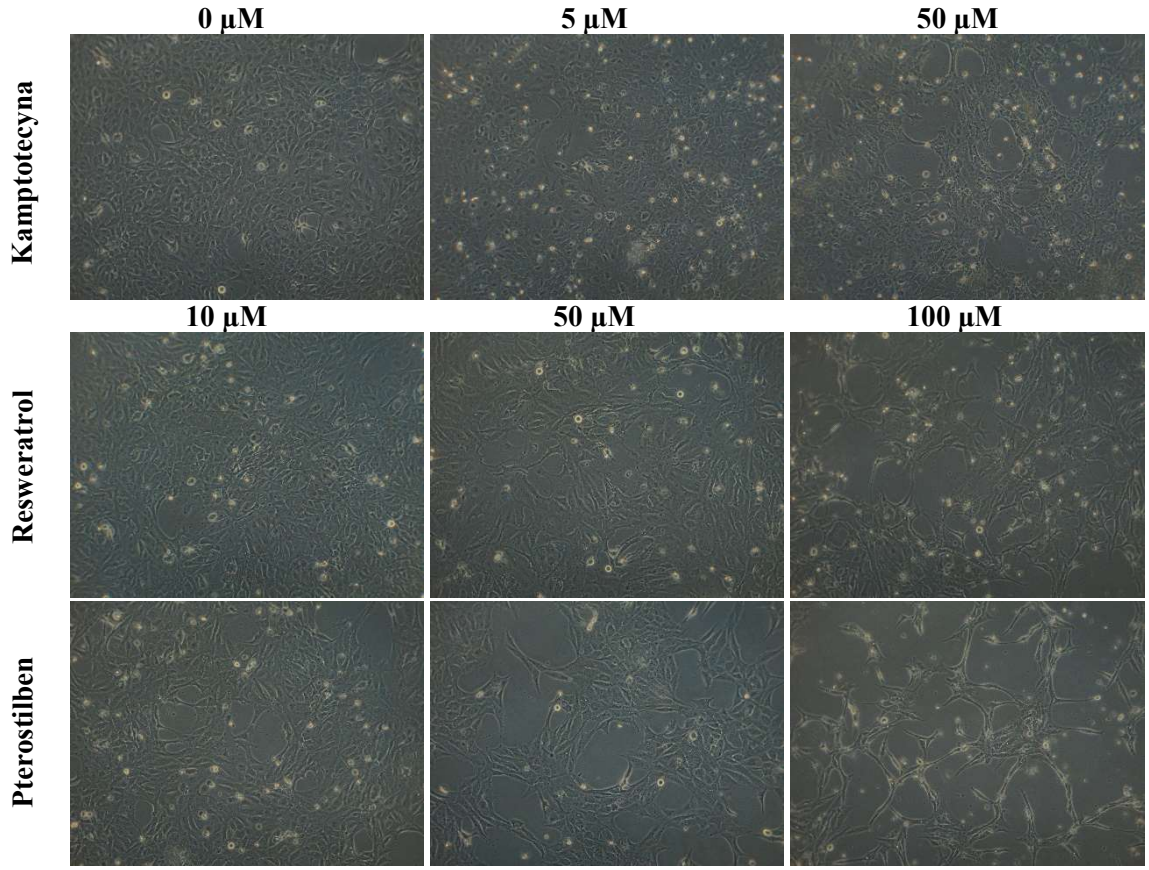


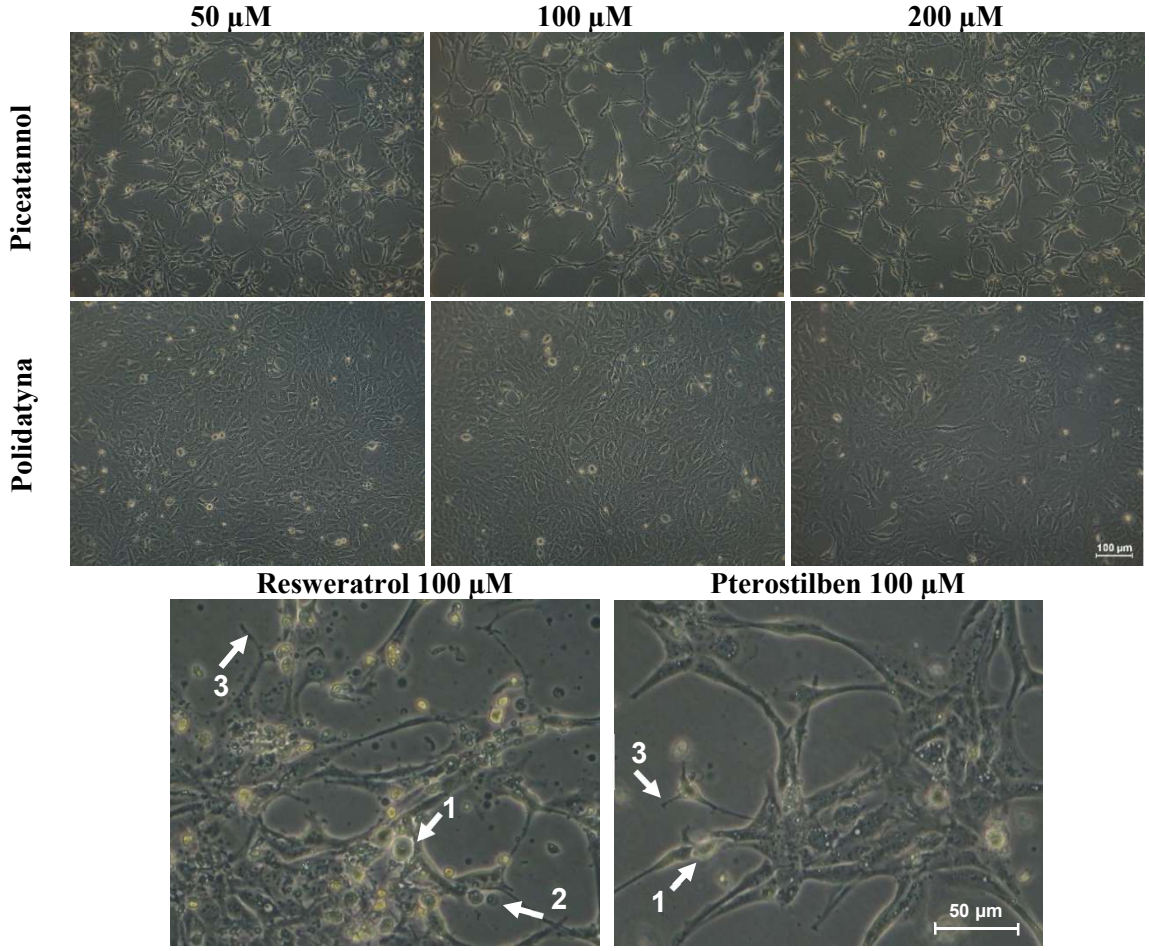
Rycina 2. Wpływ resweratrolu (A), pterostilbenu (B), piceatannolu (C) i polidatyny (D) na żywotność komórek endometriotycznych 12Z i endometrialnych T HESC, oznaczoną za pomocą testu Alamar blue. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). Istotność statystyczną oceniono z wykorzystaniem analizy wariancji (ANOVA) (*p, F*).
(Gołąbek-Grenda i in., 2023)

4.1.2 Zmiany morfologiczne komórek endometriotycznych po ekspozycji na resweratrol i jego pochodne

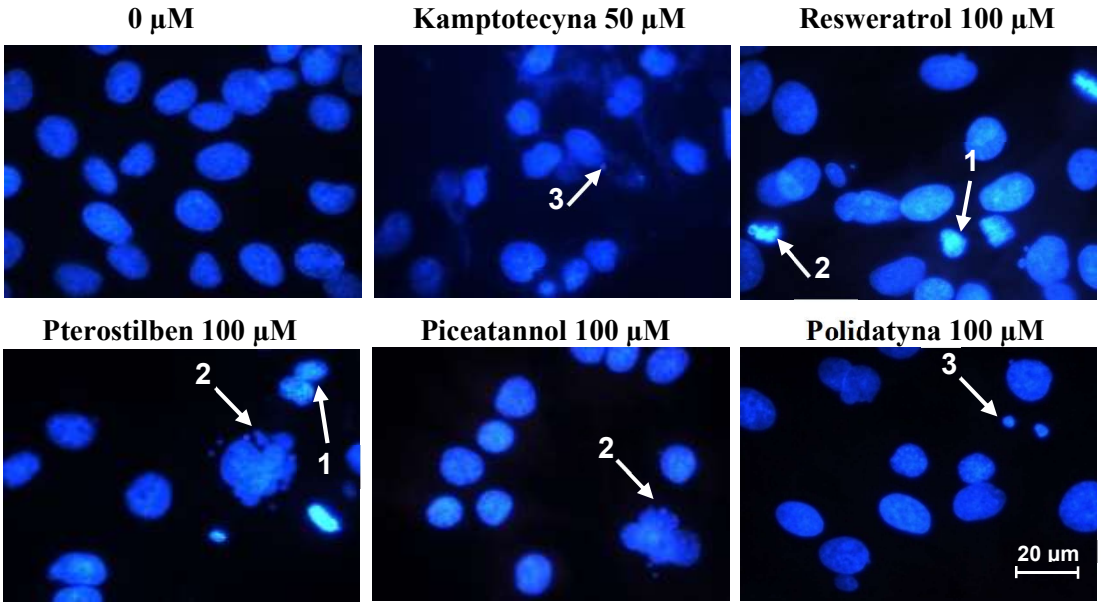
Na podstawie mikroskopowej analizy komórek endometriotycznych eksponowanych na stilbeny stwierdzono zależne od dawki hamowanie wzrostu komórek, naruszenie integralności monowarstwy komórkowej oraz utratę kontaktu pomiędzy sąsiadującymi komórkami. Obserwowano obkurczenie komórek, zmiany ich kształtu i wielkości, zagęszczenie cytoplazmy, tworzenie pęcherzykowatych uwypukleń błony komórkowej oraz formowanie kolczastych wypustek cytoplazmatycznych (Rycina 3A). Zmiany morfologiczne komórek endometriotycznych obserwowane po ekspozycji na analizowane związki uznano za charakterystyczne dla procesu apoptozy. W mikroskopowej ocenie procesu apoptozy zastosowano metodę barwienia fluorescencyjnego z wykorzystaniem DAPI, który wiąże się z chromatyną jądrową. Analiza obrazów mikroskopowych komórek traktowanych stilbenami wykazała obecność zmian typowych dla śmierci apoptotycznej, takich jak zwiększona kondensacja chromatyny jądrowej oraz tworzenie ciałek apoptotycznych (Rycina 3B). Efekty wywoływane przez stilbeny porównywano z działaniem kamptotecyny (5 μM i 50 μM), zastosowanej jako związek referencyjny.

A





B

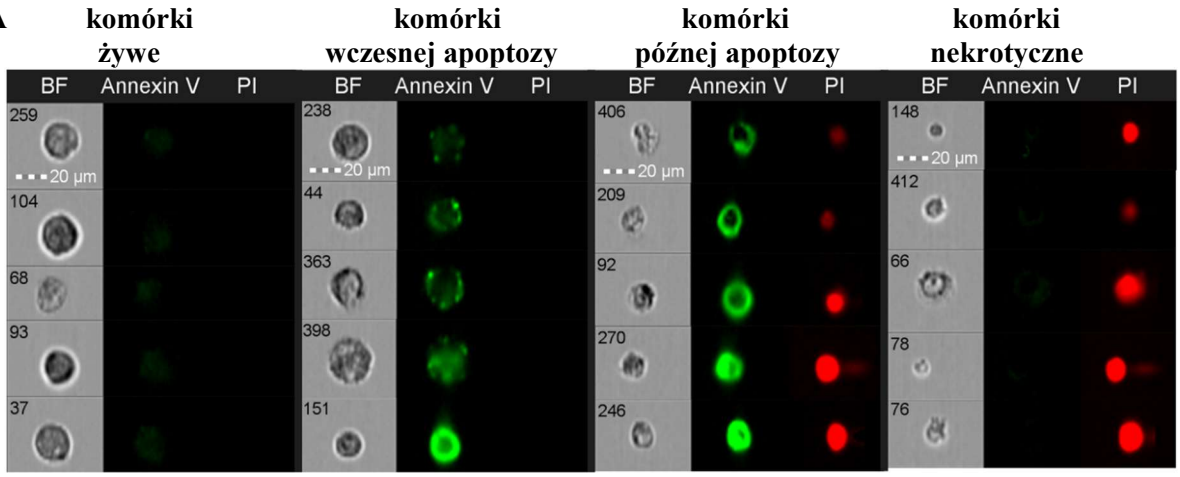


Rycina 3. Morfologiczne cechy apoptozy komórek 12Z po traktowaniu resweratrolem i jego analogami widoczne pod mikroskopem świetlnym odwróconym z kontrastem fazowym, zaokrąglenie komórek (1), pęcherzykowate uwypuklenia błony komórkowej (2) oraz kolczaste wypustki cytoplazmatyczne (3) panel A; oraz pod mikroskopem fluorescencyjnym po barwieniu komórek DAPI, kondensacja chromatyny (1), fragmentacja jądra komórkowego (2) oraz obecność ciałek apoptotycznych (3) panel B. (Gołąbek-Grenda i in., 2023)

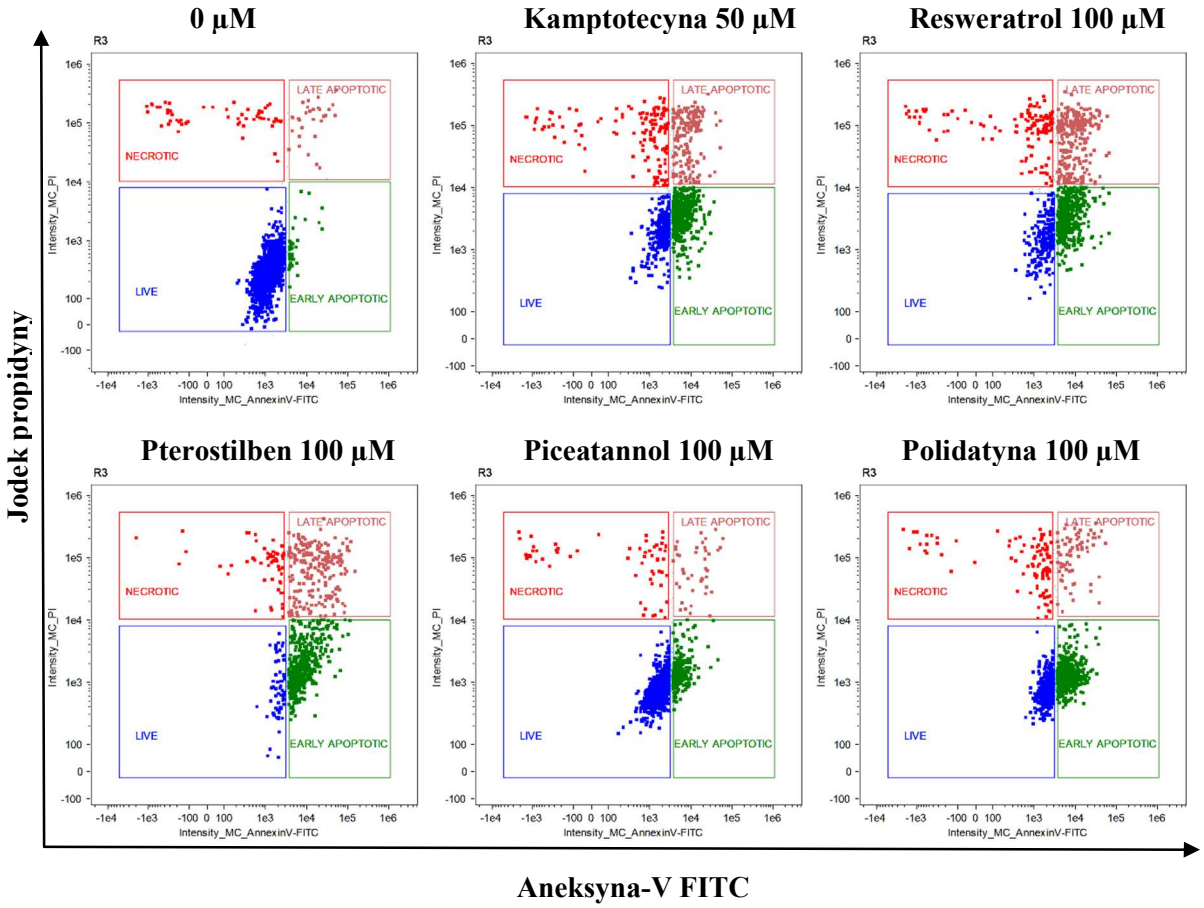
4.1.3 Cytometryczna analiza proapoptotycznego działania stilbenów w komórkach endometriotycznych

Jednym z objawów pojawiających się we wczesnej fazie apoptozy jest eksternalizacja fosfatydyloseryny na zewnętrzną powierzchnię błony komórkowej. Zaburzenia asymetrii błony plazmatycznej komórek 12Z eksponowanych na stilbeny analizowano metodą podwójnego barwienia aneksyną V i PI przy zastosowaniu cytometrii przepływowej połączonej z bioobrazowaniem. Z analizowanej populacji komórek eksponowanych na stilbeny wyodrębniono cztery subpopulacje: komórki żywe (Aneksyna V^-/PI^-), komórki we wczesnej fazie apoptozy (Aneksyna V^+/PI^-), komórki w późnej fazie apoptozy (Aneksyna V^+/PI^+) oraz komórki nekrotyczne (Aneksyna V^-/ PI^+). Wymienione subpopulacje przedstawiono na przykładowych grafikach uzyskanych metodą bioobrazowania pojedynczych komórek (Rycina 4A) oraz na wykresach kropkowych opracowanych na podstawie pomiaru intensywności fluorescencji Aneksyny V i PI (Rycina 4B).

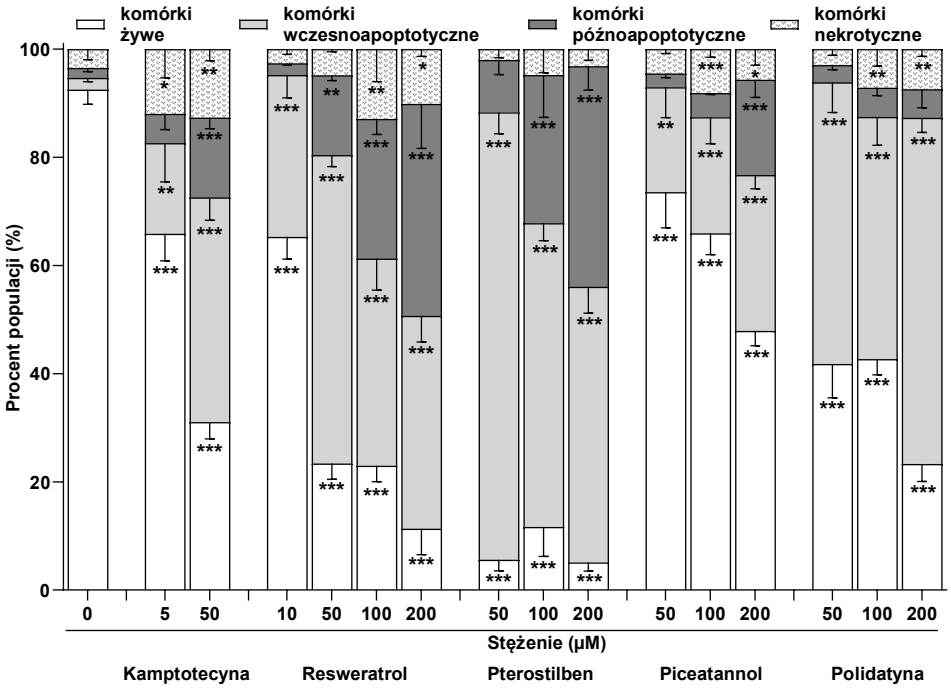
Na podstawie wyników analizy cytometrycznej stwierdzono, że resweratrol i jego pochodne, w sposób zależny od zastosowanej dawki, zmniejszają subpopulację komórek żywych, jednocześnie zwiększając subpopulacje komórek we wczesnej i późnej fazie apoptozy, przy stosunkowo niewielkiej subpopulacji komórek nekrotycznych (Rycina 4C). W komórkach 12Z traktowanych resweratrolem, pterostilbenem i piceatannolem w stężeniach 50, 100 i 200 µM odnotowano zależne od dawki zwiększenie liczby komórek w późnej fazie apoptozy. Wykazano, że najsilniejszym induktorem apoptozy jest pterostilben; po ekspozycji komórek na ten związek w stężeniu 200 µM aż 94% komórek wykazywało cechy charakterystyczne dla apoptozy. W przeciwieństwie do pterostilbenu, polidatyna powodowała głównie wzrost odsetka komórek znajdujących się we wczesnej fazie apoptozy (Rycina 4C).



B



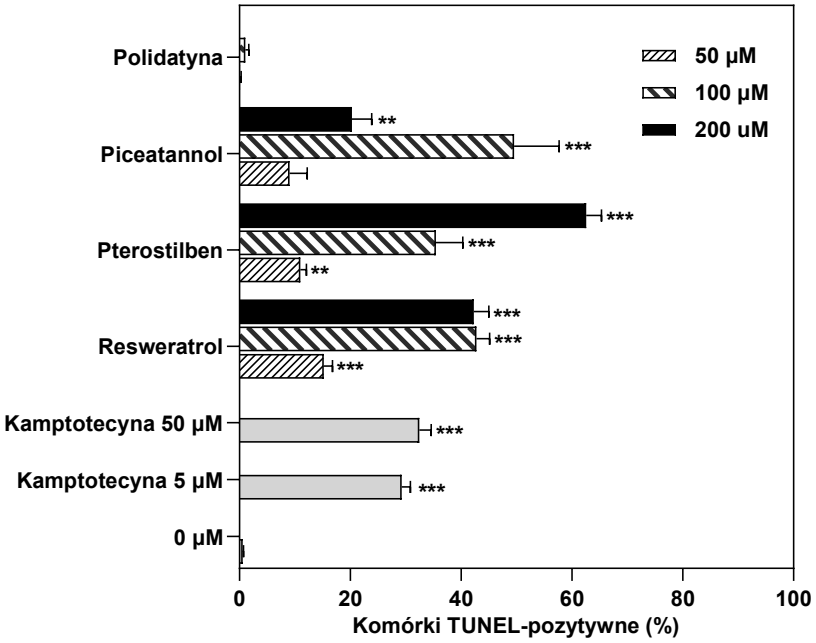
C



Rycina 4. Analiza cytometryczna komórek endometriotycznych 12Z eksponowanych przez 48 godzin na resweratrol, pterostilben, piceatannol i polidatynę oraz barwionych aneksyną V-FITC i jodkiem propidyny (PI). Przykładowe obrazy komórek, obejmujące obraz w jasnym polu (BF - brightfield) oraz obraz po wybarwieniu aneksyną V-FITC i PI (n = 4; podziałka = 20 µm) przedstawiono w panelu A.

Panel B przedstawia przykładowe wykresy punktowe ilustrujące podział analizowanych komórek na cztery subpopulacje. W panelu C zaprezentowano udział komórek żywych, wczesnoapoptotycznych i poźnoapoptotycznych oraz komórek nekrotycznych w populacji komórek 12Z. Kontrolę negatywną (0) stanowił 0,5% etanol zastosowany jako rozpuszczalnik stilbenów, natomiast kontrolę pozytywną stanowiła kamptotecyna. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001 w odniesieniu do komórek kontrolnych. (Gołąbek-Grenda i in., 2023)

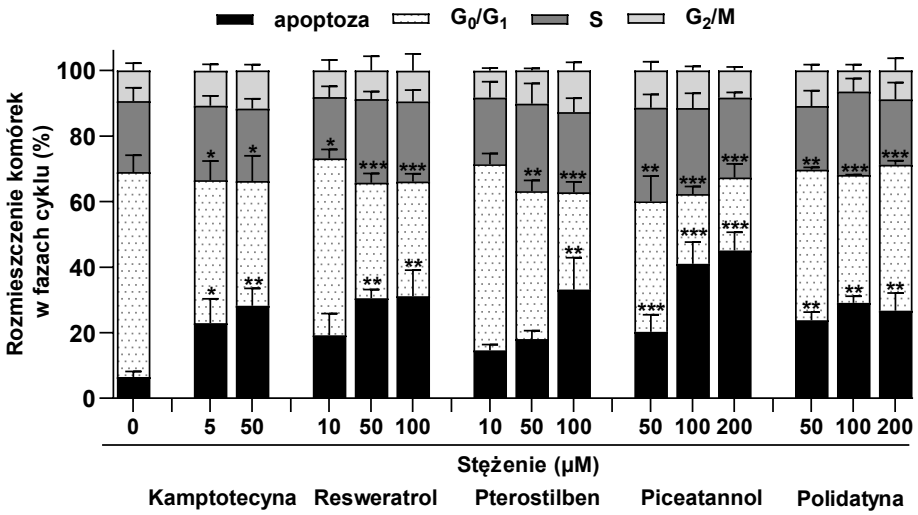
Kolejnym wskaźnikiem procesu apoptozy analizowanym w badaniach była degradacja nici DNA, oceniana z wykorzystaniem testu TUNEL. Najbardziej nasilona fragmentacja DNA występowała w komórkach 12Z poddanych działaniu resweratrolu (42,8%) i piceatannolu (49,6%) w stężeniu 100 µM oraz pterostilbenu w stężeniu 200 µM (62,6%). W przypadku ekspozycji komórek endometriotycznych na polidatynę nie stwierdzono istotnej degradacji DNA (1,08%). Kamptotecyna zastosowana jako kontrola pozytywna, w stężeniu 50 µM, indukowała uszkodzenia DNA w 32,5% populacji komórek linii 12Z (Rycina 5).



Rycina 5. Fragmentacja DNA w komórkach 12Z po ekspozycji na resweratrol i jego analogi, oznaczona metodą TUNEL. Kontrolę negatywną (0) stanowił 0,5% etanol zastosowany jako rozpuszczalnik stilbenów, natomiast kontrolę pozytywną stanowiła kamptotecyna (5 i 50 µM). Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001 w odniesieniu do nietraktowanych komórek kontrolnych. (Gołąbek-Grenda i in., 2023)

W ramach pracy analizowano również wpływ resweratrolu i jego pochodnych na przebieg cyklu komórkowego w komórkach endometriotycznych. Analiza cytometryczna komórek barwionych PI umożliwiła określenie rozmieszczenia komórek w poszczególnych fazach cyklu komórkowego. Otrzymane wyniki wykazały, że resweratrol, pterostilben i piceatannol istotnie zwiększały udział subpopulacji komórek apoptotycznych o obniżonej

zawartości DNA, jednocześnie zmniejszając udział subpopulacji komórek znajdujących się w fazie G1/G0 (Rycina 6). Najwyższy odsetek komórek apoptotycznych obserwowano po zastosowaniu resweratrolu (31,2 ± 8,0%) i pterostilbenu (33,1 ± 9,7%) w stężeniu 100 µM oraz piceatannolu w stężeniu 200 µM (45,1 ± 5,6%). Efekty proapoptotycznego działania badanych związków były porównywalne lub silniejsze niż działanie kamptotecyny zastosowanej w stężeniu 50 µM (Rycina 6).



Rycina 6. Wpływ resweratrolu i jego analogów na przebieg cyklu komórkowego w komórkach endometriotycznych linii 12Z. Kamptotecynę zastosowano jako kontrolę pozytywną. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001 w odniesieniu do nietraktowanych komórek kontrolnych.
(Gołąbek-Grenda i in., 2023)

4.1.4 Wpływ resweratrolu i jego pochodnych na ekspresję genów regulujących przebieg apoptozy

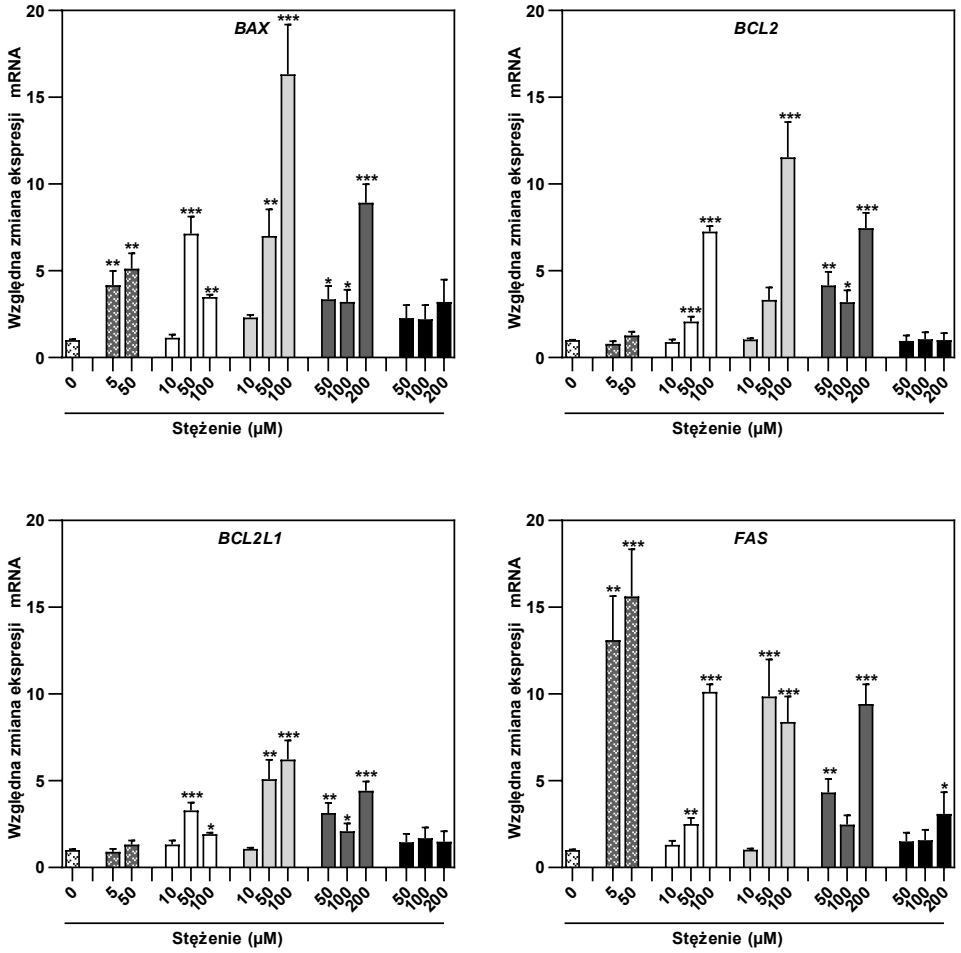
Proapoptotyczne działanie stilbenów na komórki endometriotyczne 12Z oceniano również na poziomie molekularnym poprzez analizę ekspresji genów kodujących białka regulujące zewnętrzny, receptorowy szlak apoptozy związany z błoną komórkową oraz wewnętrzny, mitochondrialny szlak apoptozy. Analiza molekularna wykazała, że resweratrol i jego analogi wpływają na ekspresję wybranych genów pro-apoptotycznych oraz genów kodujących białka anty-apoptotyczne (Rycina 7).

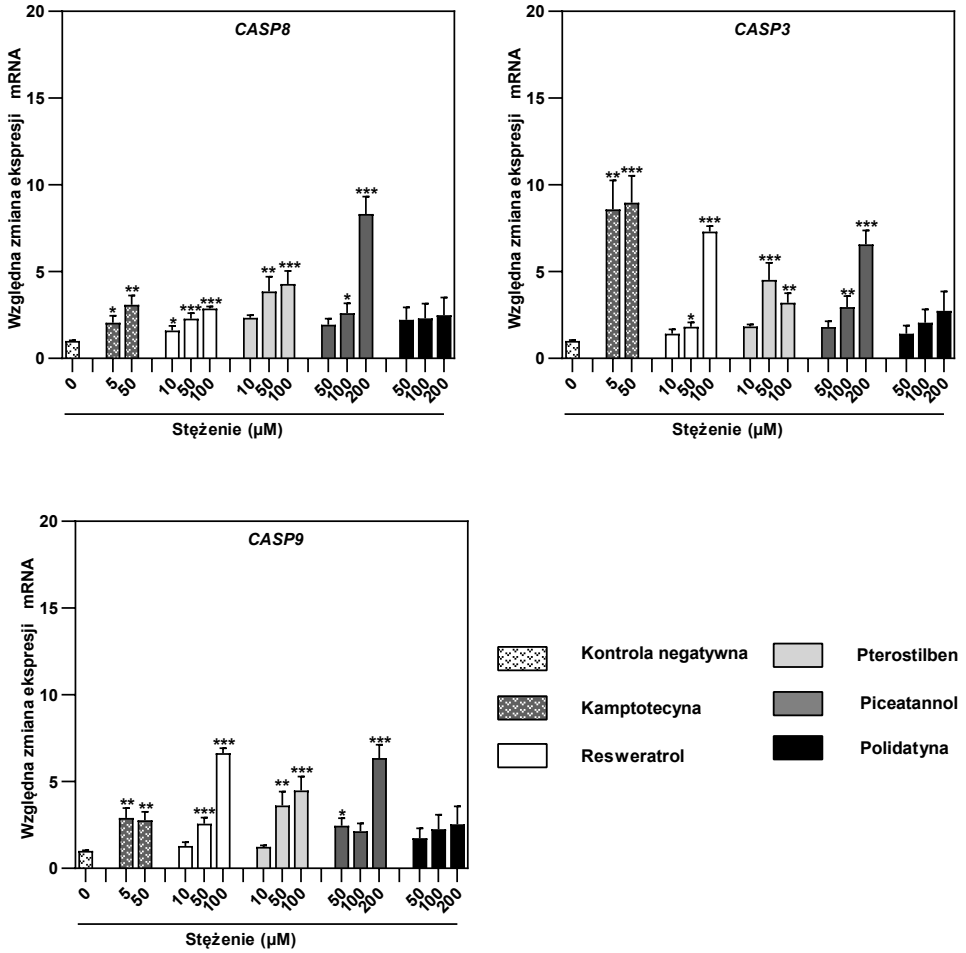
Badania z użyciem resweratrolu (50 µM i 100 µM), pterostilbenu (50 µM i 100 µM) i piceatannolu (50 µM, 100 µM i 200 µM) dowiodły, że związki te zwiększają poziom transkryptów mRNA genu *BAX*, kodującego wielodomenowe białko pro-apoptotyczne odpowiedzialne za uwalnianie cytochromu c wskutek zwiększenia przepuszczalności błony mitochondrialnej. Istotny wzrost ekspresji genu *BAX* zaobserwowano w komórkach 12Z

traktowanych resweratrolem w dawce 50 µM (7-krotny wzrost) i pterostilbenu w stężeniu 100 µM (16-krotny wzrost) (Rycina 7).

Resweratrol, pterostilben i piceatannol, w przeciwieństwie do kamptotecyny, wpływały również na ekspresję genów antyapoptotycznych *BCL2* i *BCL2L1*, których produkty stabilizują błonę mitochondrialną i zapobiegają propagacji sygnału proapoptotycznego. Uzyskane wyniki wskazują, że analizowane związki mogą inicjować apoptozę poprzez modulację równowagi pomiędzy cząsteczkami pro- i anty-apoptotycznymi. Wszystkie badane stilbeny, zwłaszcza w wyższych dawkach, powodowały ok. 10-krotny wzrost ekspresji mRNA genu *FAS* kodującego receptor śmierci należący do nadrodziny receptorów czynnika martwicy nowotworów TNF, inicjujący zewnętrzny szlak apoptozy (Rycina 7).

Wykazano również statystycznie istotny wpływ resweratrolu, pterostilbenu i piceatannolu na wzrost ekspresji genów kodujących kaspazy inicjatorowe szlaku receptorowego i mitochondrialnego – *CASP8* i *CASP9* – oraz kaspazę wykonawczą *CASP3*. Natomiast polidatyna nie powodowała istotnych zmian w profilu ekspresji analizowanych genów, z wyjątkiem genu *FAS* (Rycina 7).





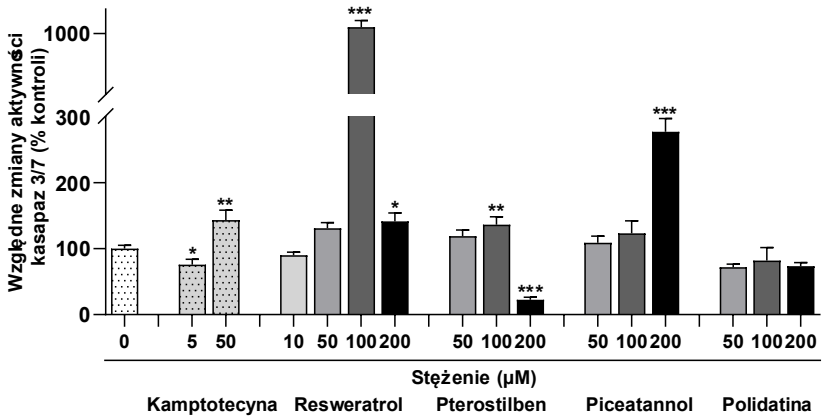
Rycina 7. Wpływ resweratrolu i jego analogów na ekspresję mRNA genów *BAX, BCL2*, *BCL2L1, FAS*, *CASP8, CASP3* i *CASP9* w komórkach endometriotycznych 12Z. Ekspresję genów analizowano metodą qRT-PCR, a uzyskane dane znormalizowano względem ekspresji genu β-aktyny i wyrażono jako względny poziom ekspresji mRNA w odniesieniu do kontroli. Kamptotecynę zastosowano jako kontrolę pozytywną. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001 w odniesieniu do nietraktowanych komórek kontrolnych. (Gołąbek-Grenda i in., 2023)

4.1.5 Wpływ resweratrolu i jego pochodnych na aktywność kaspazy 3 i 7

Promocję apoptozy w komórkach endometriotycznych przez stilbeny analizowano również poprzez oznaczenie aktywności efektorowych kaspaz 3 i 7 (Rycina 8). Wykazano istotny, zależny od dawki wzrost aktywności kaspazy 3/7 pod wpływem resweratrolu ($F = 2467$; $p \leq 0,001$), osiągając najwyższy, ponad 10-krotny wzrost przy stężeniu 100 μM, co pozostawało zgodne ze zwiększoną ekspresją genu *CASP3* (Rycina 7).

Pterostilben w stężeniu 100 μM powodował umiarkowaną aktywację kaspaz 3/7 (1,4-krotny wzrost aktywności), natomiast w stężeniu 200 μM prowadził do zahamowania ich aktywności. Piceatannol zastosowany w stężeniu 200 μM zwiększał aktywność kaspazy 3/7 2,8-krotnie, co korelowało ze wzrostem ekspresji genu *CASP3*. Nie zaobserwowano natomiast

zwiększenia aktywności badanych enzymów po ekspozycji komórek 12Z na polidatynę, niezależnie od zastosowanego stężenia (Rycina 8).



Rycina 8. Wpływ resweratrolu i jego analogów na aktywność kaspaz 3/7 w komórkach endometriotycznych linii 12Z. Kamptotecynę zastosowano jako kontrolę pozytywną. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001 w odniesieniu do nietraktowanych komórek kontrolnych.
(Gołąbek-Grenda i in., 2023)

4.2 Ocena aktywności przeciwzapalnej resweratrolu i jego pochodnych w mikrośrodowisku endometriozy (publikacja 4)

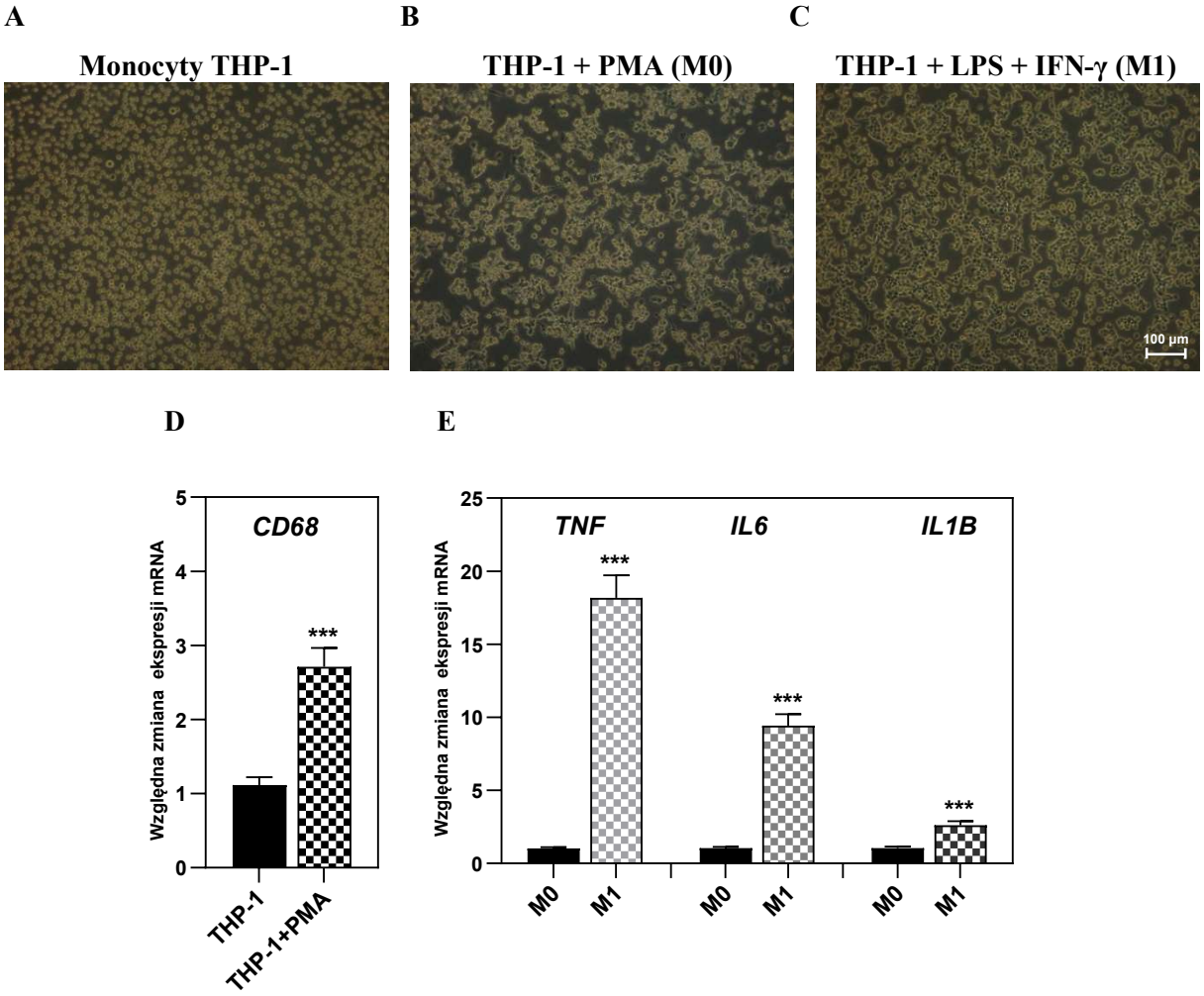
W ramach pracy doktorskiej analizowano wpływ resweratrolu i jego pochodnych na zaburzenia immunologiczne oraz stres oksydacyjny występujące w mikrośrodowisku endometriozy. W badaniach zastosowano model endometriozy z indukowanym stanem zapalnym, oparty na ko-hodowli makrofagów prozapalnych typu M1 z komórkami endometriotycznymi linii 12Z. W opracowanym układzie modelowym oceniano wpływ stilbenów na ekspresję mediatorów zapalnych oraz produkcję ROS. Wyniki badań przedstawiono w publikacji pt. „*Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages*" (publikacja 4) (Gołąbek-Grenda i in., 2024).

4.2.1 Opracowanie modelu komórkowego imitującego stan zapalny w mikrośrodowisku endometriozy

W pierwszym etapie prac nad modelowaniem układu doświadczalnego opracowano model ludzkich makrofagów o fenotypie M1. W badaniach wykorzystano ludzkie monocyty linii THP-1, które w warunkach standardowej hodowli wzrastają w zawiesinie i charakteryzują się kulistą morfologią (Rycina 9A). W celu uzyskania makrofagów typu M0 monocyty THP-1 różnicowano za pomocą PMA. W wyniku różnicowania obserwowano przyleganie komórek do

powierzchni, zmiany ich morfologii i wielkości (Rycina 9B), a także zwiększenie ekspresji mRNA genu *CD68*, stanowiącego główny marker makrofagów (Rycina 9D).

Dalszą polaryzację makrofagów indukowano za pomocą LPS i IFN-γ. Makrofagi stymulowane LPS i IFN-γ charakteryzowały się spłaszczoną i rozgałęzioną morfologią (Rycina 9C) oraz zwiększoną ekspresją genów *TNF*, *IL6*, *IL1B,* kodujących kluczowe białka prozapalne. Uzyskane komórki wykazywały cechy typowe dla makrofagów stanu zapalnego typu M1 (Rycina 9E).



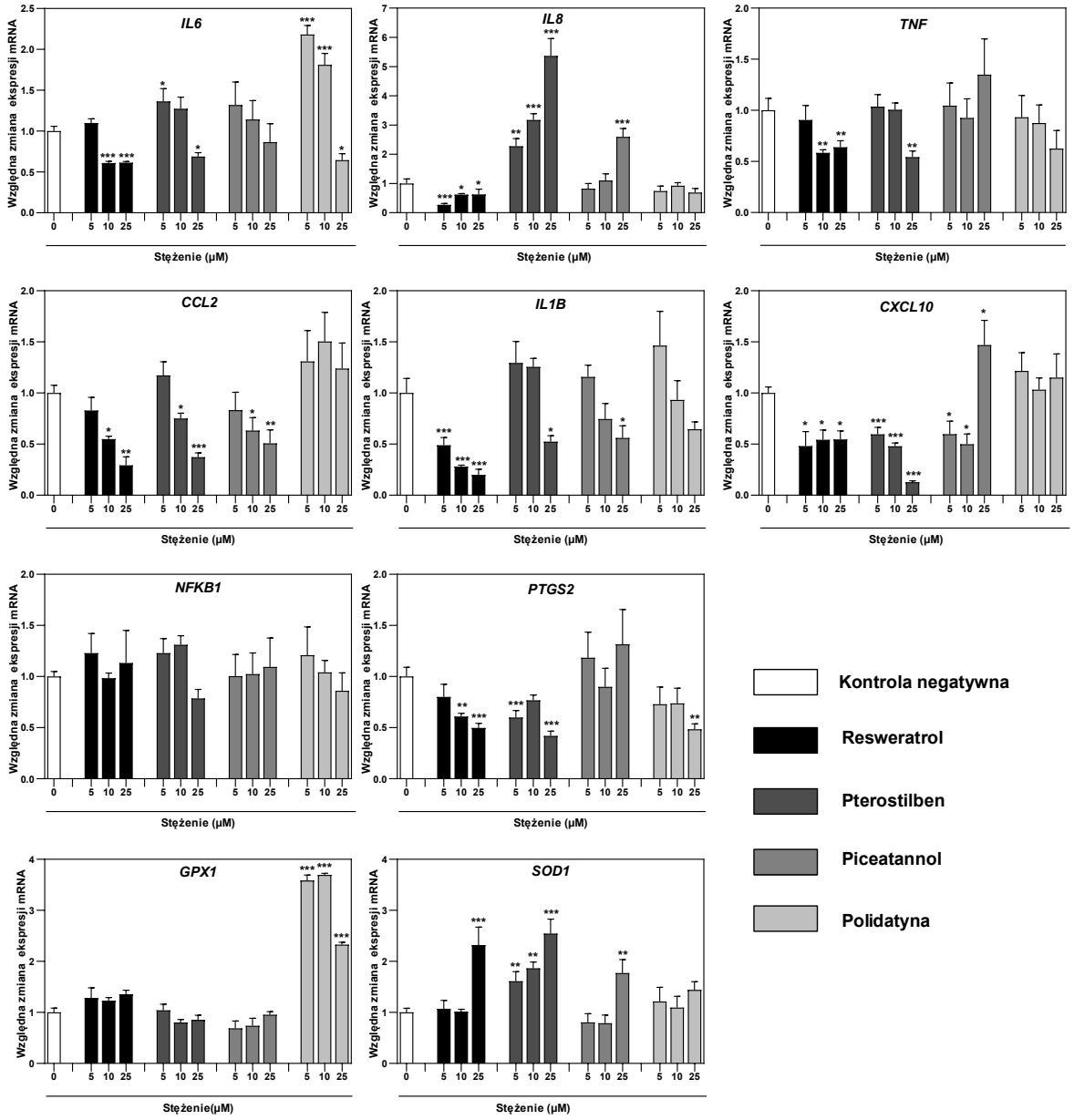
Rycina 9. Morfologia komórek THP-1 przed indukcją różnicowania w kierunku makrofagów (A), po stymulacji PMA (B) oraz po polaryzacji z użyciem LPS i IFN-γ (C). Zdjęcia wykonano przy powiększeniu 100×. Ekspresję markera *CD68* w makrofagach THP1 M0 po indukcji PMA przedstawiono na wykresie D. Wykres E przedstawia ekspresję genów prozapalnych w makrofagach spolaryzowanych do fenotypu M1. Dane znormalizowano względem ekspresji genu *GAPDH* i wyrażono jako względny poziom ekspresji mRNA (krotność kontroli). Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do kontroli negatywnej. (Gołąbek-Grenda i in., 2024)

W badaniach wstępnych oceniono poziom cytotoksyczności analizowanych stilbenów w hodowli komórek 12Z i makrofagów uzyskanych z monocytów THP-1. Wyniki wykazały, że resweratrol i jego pochodne charakteryzują się zbliżoną cytotoksycznością wobec komórek

endometriotycznych 12Z i makrofagów THP-1 w modelowych warunkach stanu zapalnego endometriozy. Szczegółowe dane dotyczące wpływu resweratrolu na hodowlę komórek 12Z i makrofagów THP-1 przedstawiono w publikacji 4 na rycinie 4. Resweratrol i jego analogi w stężeniach ≤ 25 μM nie powodowały obniżenia żywotności komórek 12Z i THP-1. Natomiast po zastosowaniu związków w dawce 50 μM obserwowano istotne zmniejszenie żywotności komórek endometriotycznych. Z tego względu badania w układzie ko-hodowli komórek 12Z i makrofagów THP-1 prowadzono z wykorzystaniem niecytotoksycznych stężeń stilbenów wynoszących 5, 10 i 25 μM.

4.2.2 Wpływ resweratrolu i jego pochodnych na ekspresję mediatorów zapalnych w makrofagach hodowanych z komórkami endometriotycznymi

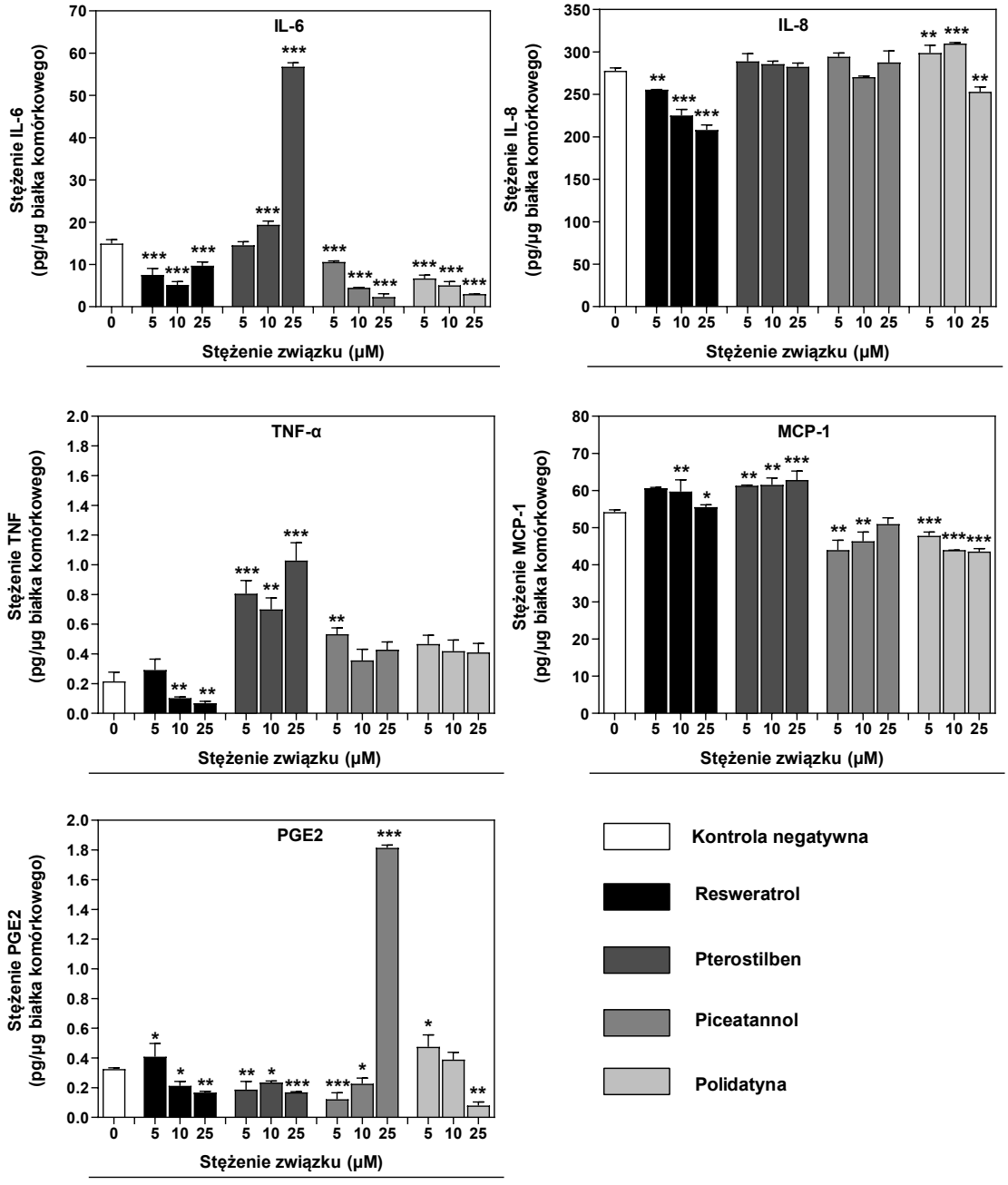
Poziom ekspresji genów kodujących czynniki zaangażowane w proces zapalny (*CCL2*, *CXCL10*, *IL1B*, *IL6*, *IL8, NFKB1* i *PTGS2*) oraz endogenne enzymy antyoksydacyjne (*SOD1* i *GPX1*) w makrofagach hodowanych wspólnie z komórkami 12Z przedstawiono na Rycinie 10. W układzie ko-hodowli eksponowanym na resweratrol zaobserwowano obniżenie ekspresji mRNA genów kodujących mediatory stanu zapalnego, takich jak *IL6*, *IL8*, *IL1B*, *TNF*, *CCL2*, *CXCL10* i *PTGS2*, przy czym najsilniejsze efekty odnotowano po zastosowaniu resweratrolu w stężeniach 10 μM i 25 μM. Największy spadek ekspresji mRNA dotyczył genów *CCL2* (3,5-krotny) i *IL1B* (5-krotny) w komórkach eksponowanych na resweratrol w dawce 25 μM. Pterostilben i piceatannol również obniżały ekspresję markerów prozapalnych, takich jak *IL6*, *IL8*, *IL1B* i *CCL2*. Pterostilben istotnie redukował także ekspresję genu *CXCL10*; po zastosowaniu najwyższego stężenia (25 μM) poziom ekspresji tego genu był 7,7-krotnie niższy niż w hodowli kontrolnej. W przypadku polidatyny hamowanie ekspresji większości analizowanych markerów zapalnych nie było istotne statystycznie. Działanie supresyjne tego związku dotyczyło jedynie ekspresji genów *IL6* i *PTGS2* i obserwowano je wyłącznie po zastosowaniu najwyższej dawki (Rycina 10).



Rycina 10. Wpływ resweratrolu i jego analogów na ekspresję mRNA genów *IL6*, *IL8, TNF, CCL2*, *IL1B*, *CXCL10*, *NFKB1*, *PTGS2*, *GPX1 i SOD1* w makrofagach hodowanych wspólnie z komórkami endometriotycznymi. Analizy przeprowadzono metodą qRT-PCR, a uzyskane wyniki znormalizowano względem ekspresji genu *GAPDH* i wyrażono jako względny poziom ekspresji mRNA (krotność kontroli). Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do nietraktowanych komórek układu kontrolnego. (Gołąbek-Grenda i in., 2024)

W ramach pracy wykonano również ilościowe oznaczenia cytokin i chemokin wydzielanych w modelu stanu zapalnego endometriozy po ekspozycji na resweratrol i jego pochodne. Analizy przeprowadzono z wykorzystaniem testów CBA i ELISA, a uzyskane wyniki przedstawiono na Rycinie 11. Na podstawie otrzymanych danych stwierdzono, że resweratrol i jego analogi w zróżnicowany sposób wpływają na produkcję mediatorów stanu zapalnego. Resweratrol stosowany w dawkach 10 μM i 25 μM istotnie obniżał nadprodukcję

IL-6, IL-8, TNF-α i PGE2 przez makrofagi. Piceatannol i polidatyna wyraźnie hamowały wydzielanie IL-6 i MCP-1. Polidatyna i pterostilben wykazywały działanie ograniczające syntezę PGE2. Pterostilben, w przeciwieństwie do pozostałych stilbenów, wykazywał odmienną aktywność, powodując zwiększenie produkcji IL-6, MCP-1 i TNF-α (Rycina 11).



Rycina 11. Wpływ resweratrolu i jego analogów na uwalnianie cytokin/chemokin w modelu ko-hodowli makrofagów i komórek endometriozy. Oznaczenia ilościowe wykonano za pomocą testów CBA i ELISA. Dane podano w przeliczeniu na zawartość białka komórkowego w analizowanej próbie. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do komórek hodowanych w układzie kontrolnym nietraktowanym stilbenami. (Gołąbek-Grenda i in., 2024)

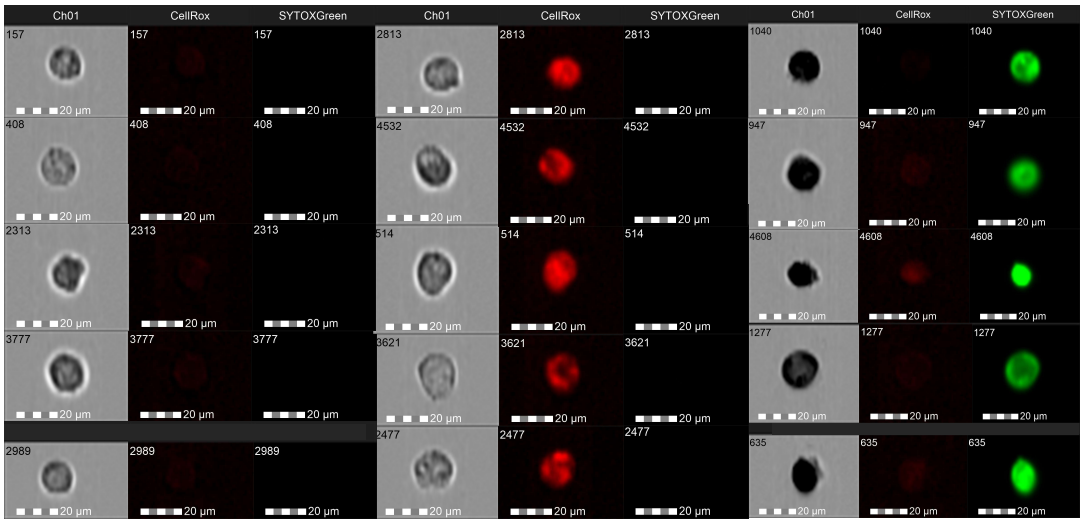
4.2.3 Wpływ resweratrolu i jego pochodnych na poziom reaktywnych form tlenu w makrofagach hodowanych z komórkami endometriotycznymi

Wewnątrzkomórkową produkcję ROS w makrofagach hodowanych wspólnie z komórkami endometriozy oznaczano metodą podwójnego barwienia z wykorzystaniem odczynników Sytox® Green Dead Stain oraz CellROX® Deep Red. Znakowane komórki analizowano za pomocą cytometrii przepływowej w celu określenia dwóch parametrów: poziomu stresu oksydacyjnego oraz żywotności komórek. ROS identyfikowano w makrofagach na wewnętrznej powierzchni błony komórkowej oraz w cytozolu. Przykładowe obrazy komórek przedstawiono na Rycinie 12A.

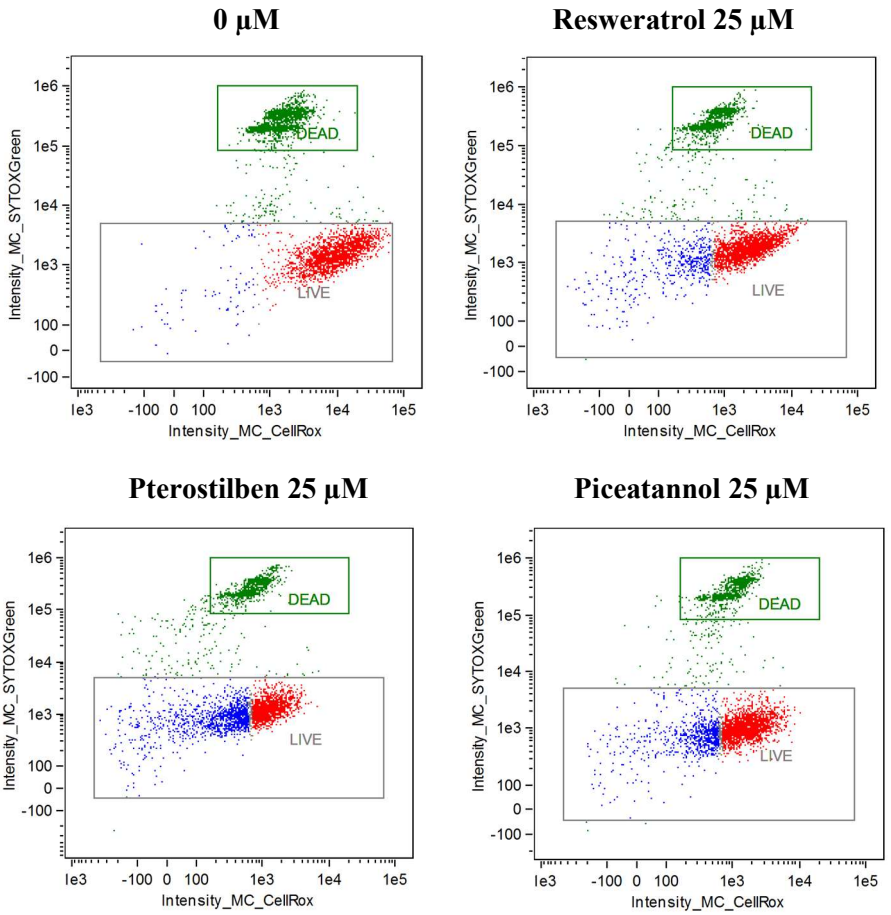
Zastosowanie multipleksowej metody barwienia umożliwiło rozróżnienie żywych komórek z podwyższonym poziomem ROS od komórek martwych, co pozwoliło na wyodrębnienie trzech subpopulacji: (1) żywych komórek bez oznak stresu oksydacyjnego, (2) żywych komórek z nadprodukcją ROS oraz (3) komórek martwych wykazujących szczątkowy sygnał ROS. Przykładowe wykresy kropkowe przedstawiono na Rycinie 12B. Dane pokazane na Rycinie 12C wskazują, że resweratrol i jego analogi, w sposób zależny od dawki, zwiększają udział populacji żywych komórek bez nadprodukcji ROS oraz zmniejszały subpopulację makrofagów akumulujących ROS. Efekty antyoksydacyjne obserwowano już przy zastosowaniu stilbenów w najniższym stężeniu wynoszącym 5 μM. Najsilniejsze działanie wykazywał pterostilben, który najefektywniej redukował odsetek komórek ROS-dodatnich. Zaobserwowany efekt związany z ograniczeniem wewnątrzkomórkowej akumulacji ROS potwierdzono również w ilościowej analizie sygnału fluorescencji barwnika CellROX® Deep Red przedstawionej na Rycinie 12D. Pterostilben i piceatannol, stosowane w największych dawkach (25 μM), okazały się najskuteczniejsze w ograniczaniu nadprodukcji ROS w makrofagach.

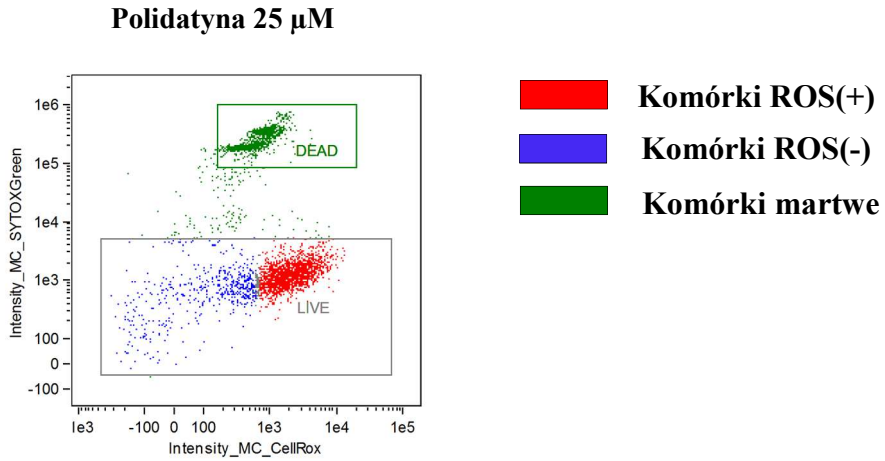
W ramach pracy analizowano również wpływ stilbenów na ekspresję mRNA genów *SOD1* i *GPX1* kodujących enzymy antyoksydacyjne. Wyniki badań wykazały, że resweratrol, pterostilben i piceatannol, stosowane w największej dawce 25 μM, wzmacniają ekspresję genu *SOD1*, natomiast nie wpływają istotnie na poziom mRNA *GPX1*. Wzrost ilości transkryptu mRNA genu *GPX1* obserwowano jedynie po zastosowaniu polidatyny, która we wszystkich badanych stężeniach powodowała istotną stymulację ekspresji tego genu (Rycina 10).

A

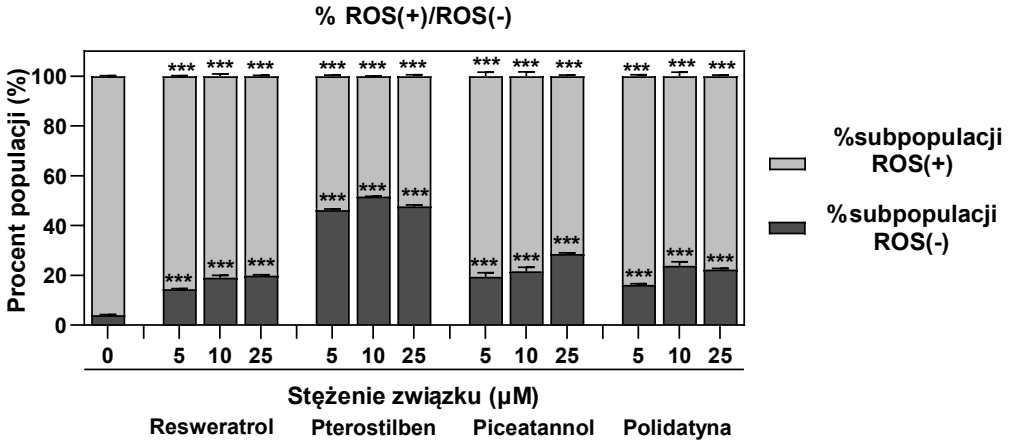

Komórki żywe ROS(-)
Komórki żywe ROS(+)
Komórki martwe
Ch01
CellRox
SYTOXGreen
20 µm

B

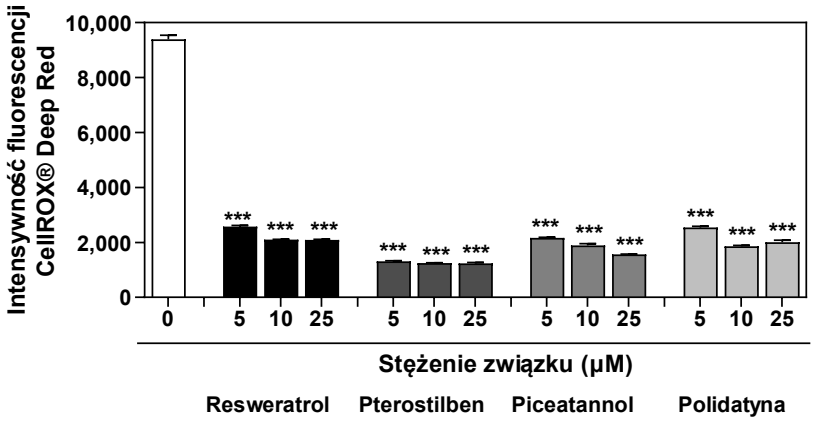

0 µM
Resweratrol 25 µM
Pterostilben 25 µM
Piceatannol 25 µM
DEAD
LIVE
Intensity_MC_SYTOXGreen
Intensity_MC_CellRox
1e6
1e5
1e4
1e3
100
0
-100
1e3
-100
0
100
1e3
1e4
1e5



C



D



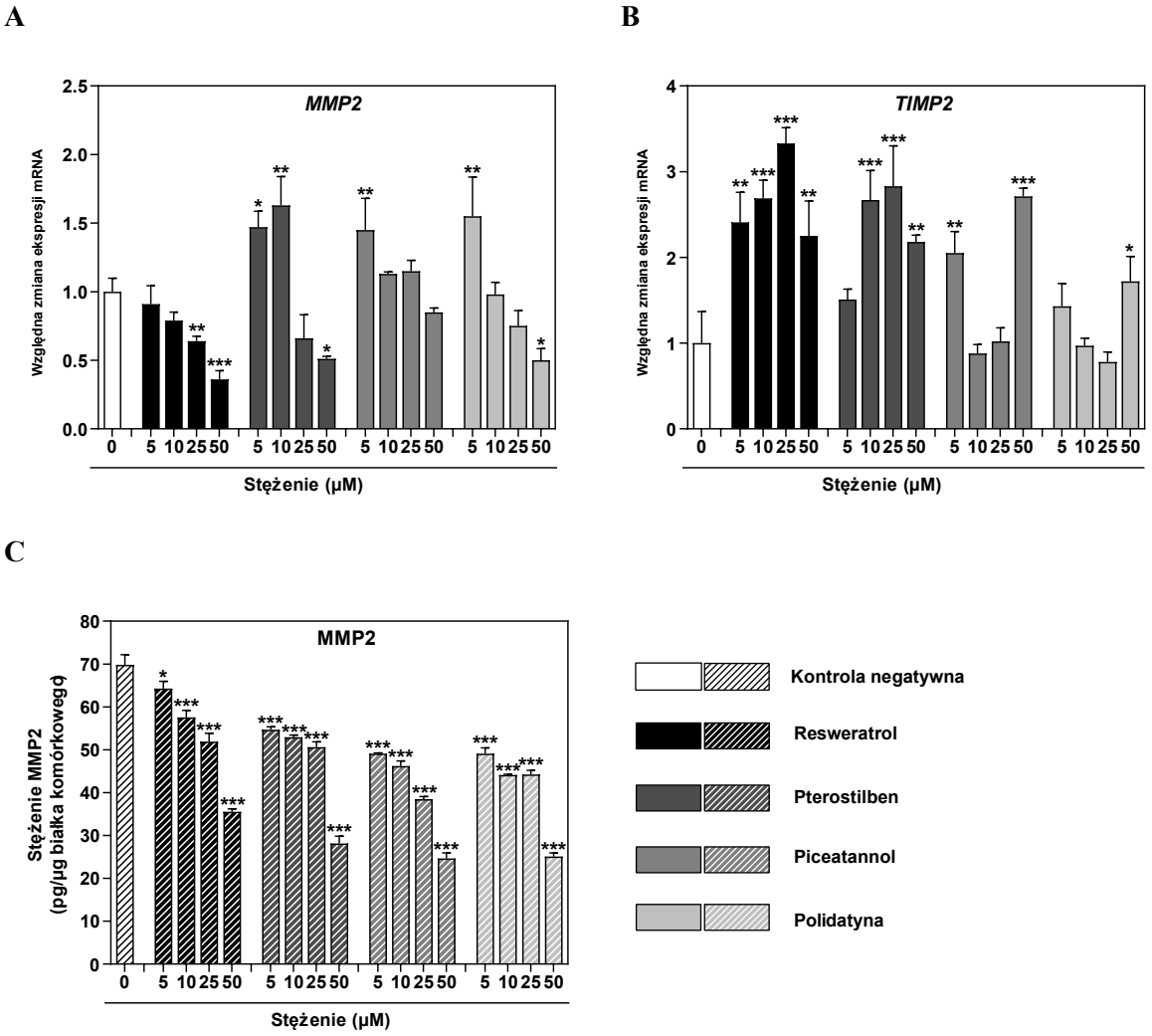
Rycina 12. Wpływ resweratrolu i jego analogów na status oksydacyjny makrofagów hodowanych z komórkami endometriotycznymi 12Z. Przykładowe obrazy komórek, w tym obraz w jasnym polu (BF; *brightfield*) oraz obrazy po wybarwieniu CellROX® Deep Red i Sytox® Green Dead Stain przedstawiono w panelu A (n = 4; podziałka = 20 µm). Panel B przedstawia przykładowe wykresy punktowe ilustrujące podział analizowanych komórek na trzy subpopulacje. Na wykresie C zaprezentowano procentowy rozkład subpopulacji żywych makrofagów ROS-pozytywnych i ROS-negatywnych barwionych odczynnikami CellROX® Deep Red i Sytox® Green Dead, analizowanych metodą cytometrii przepływowej. Wykres D przedstawia ilościową analizę intensywności fluorescencji odczynnika CellROX® Deep Red. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do makrofagów kontrolnych nietraktowanych stilbenami. (Gołąbek-Grenda i in., 2024)

4.3 Ocena wpływu resweratrolu i jego pochodnych na adhezję, migrację i inwazję komórek endometriotycznych (publikacja 5)

W ramach pracy doktorskiej analizowano potencjalną aktywność terapeutyczną resweratrolu i jego pochodnych w kontekście hamowania migracji i inwazji komórek endometriotycznych oraz ograniczania ich adhezji do składników ECM. Oceniano również wpływ analizowanych związków na ekspresję i sekrecję kluczowych mediatorów proteolizy ECM, takich jak MMP-2 i TIMP-2. Wyniki badań przestawiono w publikacji pt. „*Stilbene-based strategies for the management of endometriosis: targeting cell adhesion, migration, and ECM-driven invasion*" (publikacja 5) (Gołąbek-Grenda i Olejnik, 2026).

4.3.1 Wpływ resweratrolu i jego pochodnych na ekspresję i sekrecję MMP-2 i TIMP2 w komórkach endometriotycznych

Wpływ resweratrolu i jego analogów na ekspresję kluczowych enzymów uczestniczących w proteolizie ECM w komórkach endometriotycznych 12Z przedstawiono na Rycinie 13. Uzyskane wyniki wskazują na istotne zmniejszenie liczby kopii transkryptu *MMP2* w komórkach eksponowanych na resweratrol (↓64%), pterostilben (↓46%) i polidatynę (↓55%) zastosowane w najwyższej testowanej dawce 50 μM. Efekt hamowania ekspresji mRNA genu *MMP2* obserwowano również po zastosowaniu resweratrolu w niższym stężeniu wynoszącym 25 μM (↓46%) (Rycina 13A). Resweratrol i jego pochodne, w sposób zależny od dawki, obniżały także sekrecję MMP-2 przez komórki endometriotyczne 12Z, zarówno w formie aktywnej jak i nieaktywnej (pro-MMP). Najsilniejszy efekt inhibicyjny obserwowano po zastosowaniu stilbenów w dawce 50 μM, które powodowały ok. 2,0–2,8-krotne zmniejszenie stężenia MMP-2 (Rycina 13C). Efektem działania stilbenów na komórki 12Z było istotne wzmocnienie ekspresji inhibitora MMP-2 – *TIMP2*. Najwyższy poziom ekspresji obserwowano po zastosowaniu resweratrolu i pterostilbenu w stężeniu 25 μM, które zwiększały liczbę kopii transkryptu *TIMP2* odpowiednio 3,3- i 2,8-krotnie. Piceatannol i polidatyna, stosowane w najwyższym stężeniu 50 μM, wykazywały podobne działanie stymulujące ekspresję *TIMP2* (Rycina 13B).



Rycina 13. Wpływ resweratrolu i jego analogów na ekspresję mRNA genów *MMP2* (A) i *TIMP2* (B), analizowaną metodą qRT-PCR, oraz na wydzielanie MMP-2 (C), oznaczane metodą ELISA. Uzyskane dane znormalizowano względem ekspresji genu *GAPDH* i wyrażono jako względny poziom ekspresji mRNA (krotność kontroli). Stężenie MMP-2 przeliczono względem zawartości białka całkowitego w analizowanej próbie. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do komórek hodowanych w układzie kontrolnym nietraktowanym stilbenami. (Gołąbek-Grenda i Olejnik, 2026)

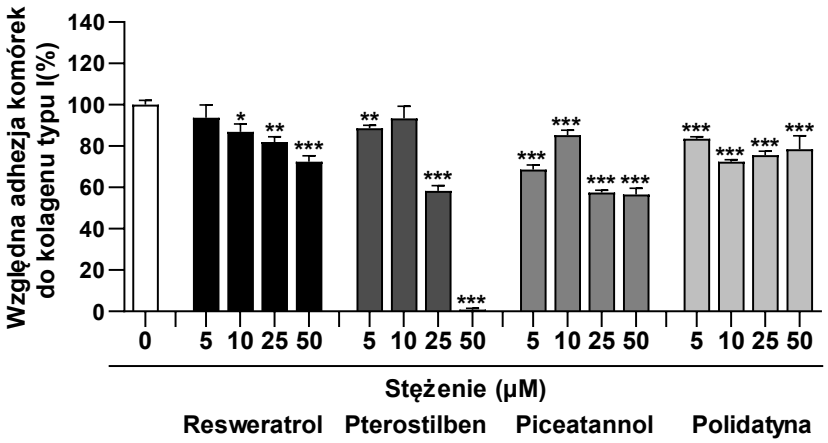
4.3.2 Wpływ resweratrolu i jego pochodnych na adhezję komórek endometriotycznych do składników ECM

Wpływ stilbenów na adhezję komórek endometriotycznych do składników ECM analizowano po naniesieniu komórek 12Z na powierzchnie pokryte kolagenem typu I lub fibronektyną, a następnie po ich wybarwieniu fioletem krystalicznym. Uzyskane wyniki przedstawiono na Rycinie 14A i 14B.

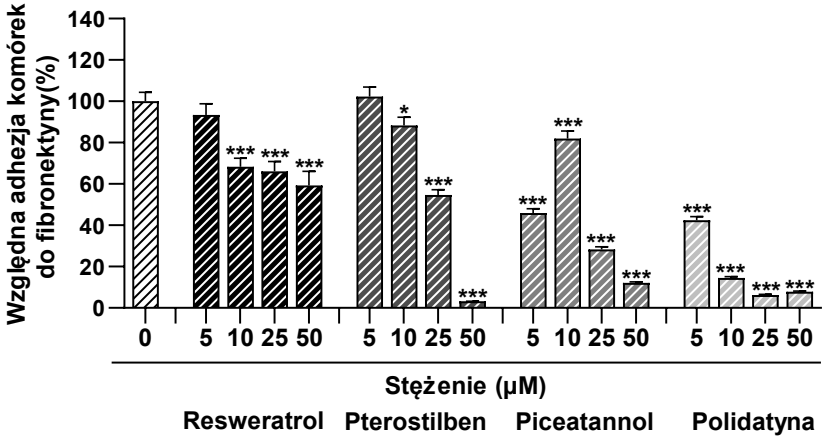
Na podstawie przeprowadzonych analiz stwierdzono, że wszystkie badane stilbeny osłabiały adhezję komórek do składników ECM. Resweratrol w dawkach ≥10 μM ograniczał przyczepność komórek 12Z zarówno do kolagenu typu I, jak i fibronektyny. Pterostilben,

stosowany w stężeniach 25–50 µM, również silnie hamował adhezję komórek. W największej dawce redukował liczbę komórek adherujących do kolagenu o 99,2%, a do fibronektyny o 97%, wykazując najsilniejsze działanie spośród analizowanych związków. Zmniejszenie adhezji komórek 12Z obserwowano również po ekspozycji na piceatannol, który wykazywał aktywność we wszystkich stosowanych stężeniach; w dawce 50 µM ograniczał adhezję do kolagenu o 43%, a do fibronektyny o 88%. Porównywalne efekty uzyskano również po zastosowaniu polidatyny, która silne działanie hamujące adhezję komórek do fibronektyny wykazywała już w dawce 5 µM. Wyniki badań potwierdzono analizą mikroskopową, a reprezentatywne obrazy komórek barwionych fioletem krystalicznym przedstawiono na Rycinie 14C.

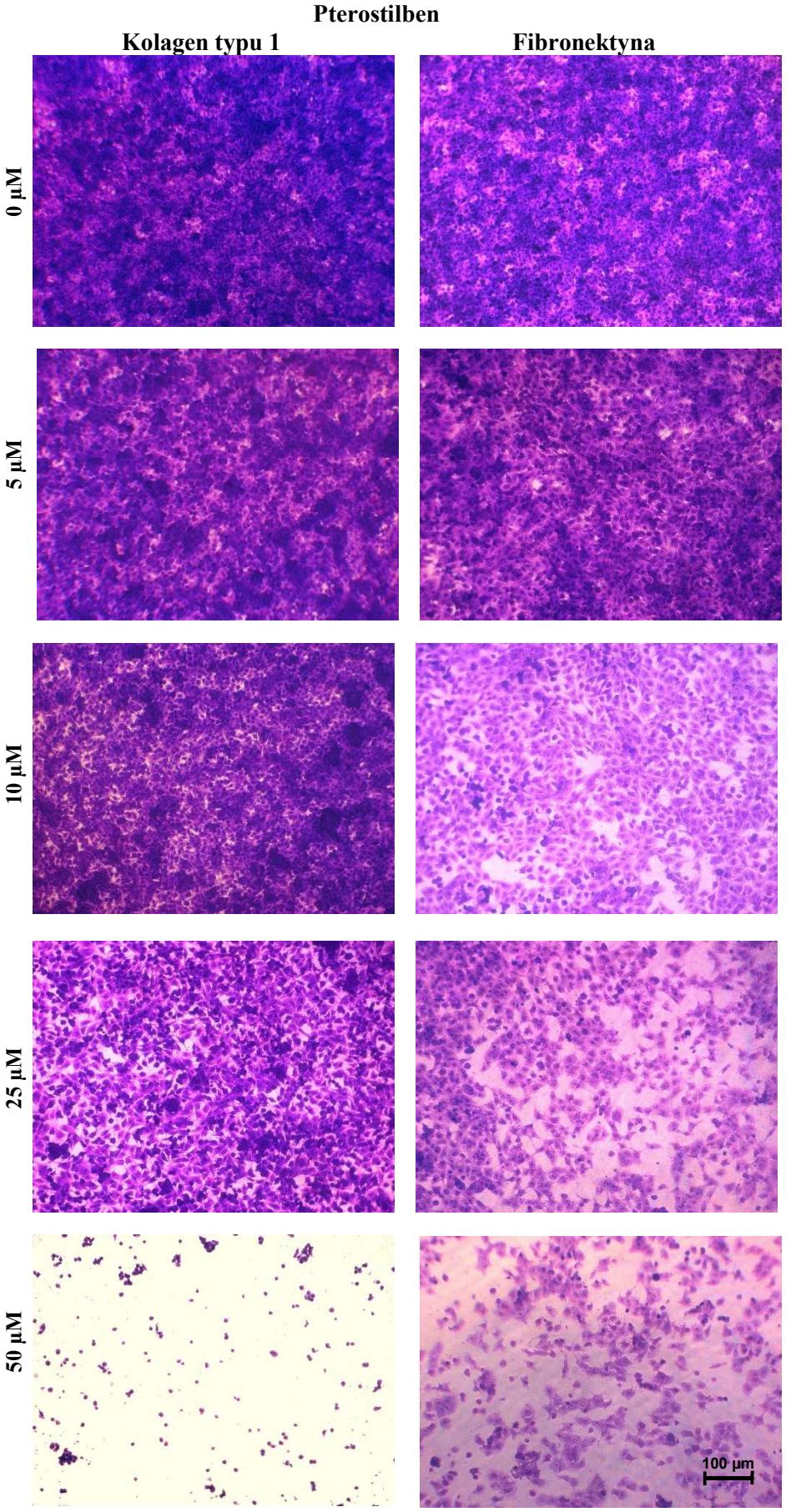
A



B



C


Pterostilben
Kolagen typu 1
Fibronektyna
0 µM
5 µM
10 µM
25 µM
50 µM
100 µm

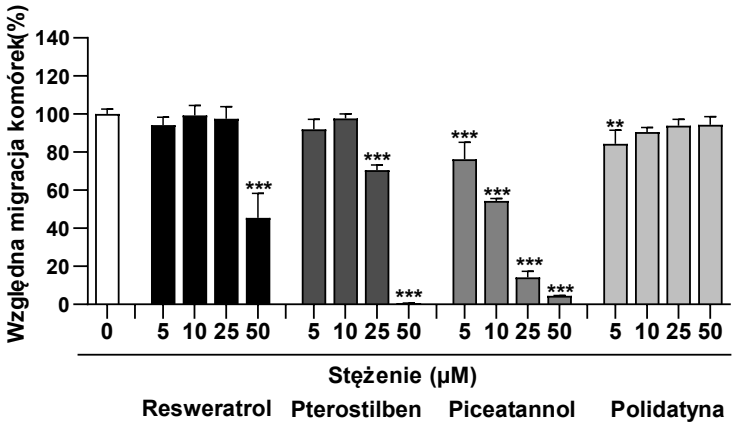
Rycina 14. Wpływ resweratrolu i jego analogów na adhezję komórek endometriotycznych linii 12Z. Komórki wybarwiano fioletem krystalicznym. Wyniki przedstawiono jako względną adhezję komórek 12Z do kolagenu typu I (A) i fibronektyny (B). W panelu C pokazano przykładowe obrazy komórek traktowanych pterostilbenem, wybarwionych po adhezji do kolagenu typu I lub fibronektyny. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do komórek hodowanych w układzie kontrolnym nietraktowanym stilbenami. (Gołąbek-Grenda i Olejnik, 2026)

4.3.3 Wpływ resweratrolu i jego pochodnych na migrację i inwazję komórek endometriotycznych

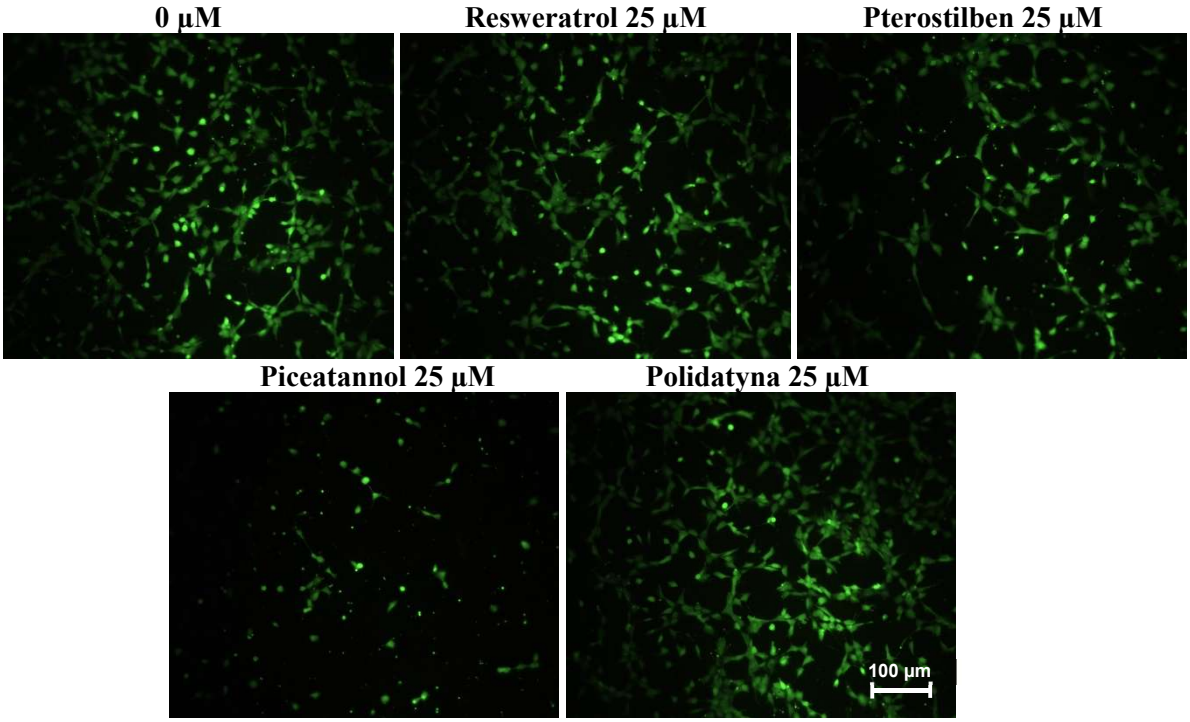
Wpływ stilbenów na procesy umożliwiające rozprzestrzenianie się komórek endometriotycznych analizowano w układach dwukomorowych rozdzielonych półprzepuszczalną membraną. Układy zawierające niepowlekane membrany zastosowano do oceny migracji komórek, natomiast do analizy inwazji wykorzystano membrany pokryte substancją BD Matrigel™ imitującą ECM. Wyniki badań przedstawiono na Rycinie 15A i 15B.

Najsilniejszy wpływ na migrację komórek 12Z przez niepowlekaną membranę w kierunku FBS, stosowanej jako chemoatraktant, wykazywał pterostilben (25 μM i 50 μM), który w dawce 50 μM niemal całkowicie hamował przemieszczanie komórek przez membranę (↓99%). Pterostilben w stężeniu 50 μM silnie ograniczał również inwazję komórek 12Z (↓91%). Procesy migracji i inwazji były także zaburzane przez piceatannol, którego działanie miało charakter zależny od dawki. Ekspozycja komórek na ten związek w najmniejszym badanym stężeniu 5 μM powodowała redukcję liczby komórek inwazyjnych o 60%. Polidatyna nie wpływała istotnie na zdolność migracji komórek endometriozy, natomiast wykazywała umiarkowane działanie antyinwazyjne. Podobnie resweratrol, w sposób zależny od dawki, ograniczał inwazję komórek 12Z, podczas gdy jego aktywność antymigracyjna była widoczna jedynie po zastosowaniu najwyższego stężenia 50 μM (Rycina 15A). Obrazy komórek endometriotycznych po migracji i inwazji przedstawiono na Rycinie 15B. Działanie prowadzące do ograniczenia inwazyjności komórek, które było obserwowane głównie po zastosowaniu resweratrolu i polidatyny, zostały potwierdzone przez wartości wskaźnika inwazyjności, które określają zależność pomiędzy potencjałem migracyjnym i inwazyjnym komórek endometriozy. Wartości wskaźnika inwazyjności dla komórek 12Z eksponowanych na pterostilben i piceatannol były istotnie niższe niż wartości uzyskane w hodowli kontrolnej. Natomiast po zastosowaniu pterostibenu i piceatannolu w wysokiej dawce 50 μM odnotowano wyraźny wzrost wskaźnika inwazyjności (Rycina 15C i 15E).

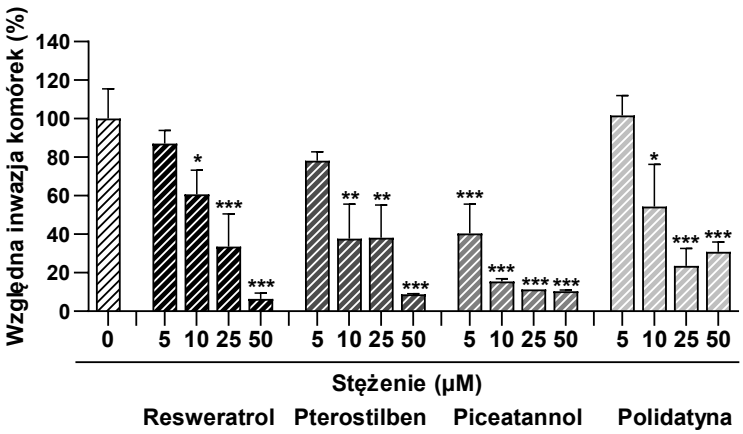
A


Względna migracja komórek(%)
140
120
100
80
60
40
20
0
0 5 10 25 50 5 10 25 50 5 10 25 50 5 10 25 50
Stężenie (µM)
Resweratrol Pterostilben Piceatannol Polidatyna

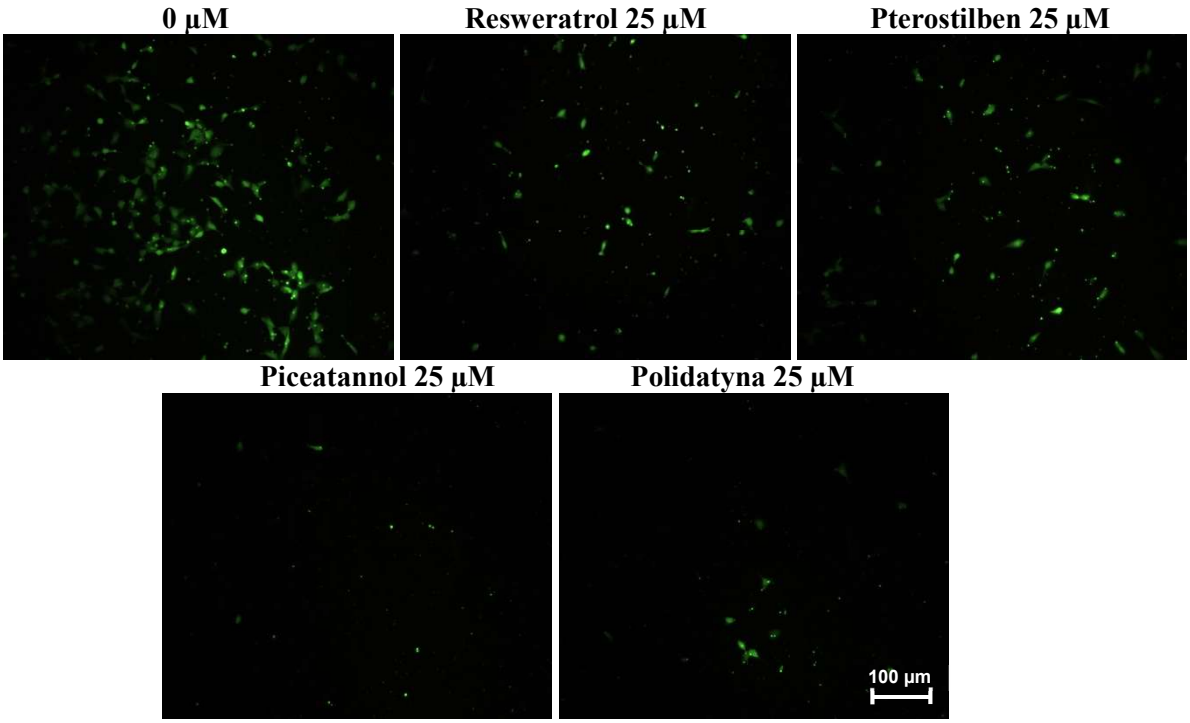
B


0 µM
Resweratrol 25 µM
Pterostilben 25 µM
Piceatannol 25 µM
Polidatyna 25 µM
100 µm

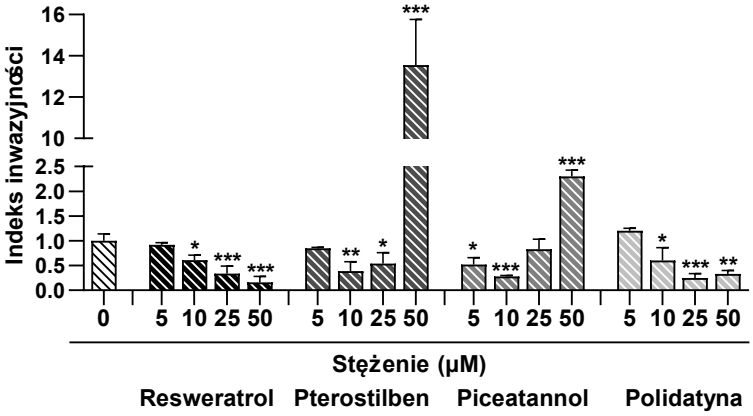
C


Względna inwazja komórek (%)
140
120
100
80
60
40
20
0
0 5 10 25 50 5 10 25 50 5 10 25 50 5 10 25 50
Stężenie (µM)
Resweratrol Pterostilben Piceatannol Polidatyna

D



E



Rycina 15. Wpływ resweratrolu i jego analogów na migrację i inwazję komórek endometriotycznych linii 12Z. Komórki wysiewano na membrany pokryte Matrigel™ w celu oznaczenia inwazji oraz na membrany niepowlekane w celu oceny migracji, a następnie poddawano działaniu resweratrolu i jego analogów przez 24 godziny. Komórki, które uległy migracji lub inwazji wybarwiano barwnikiem fluorescencyjnym kalceiną AM. Wyniki przedstawiono jako względną migrację (A) i inwazję (C) komórek 12Z po ekspozycji na analizowane stilbeny. W panelach B i D przedstawiono przykładowe obrazy komórek migrujących przez membranę niepowlekaną (B) lub membranę pokrytą Matrigel™ (D). Wykres E przedstawia wartości wskaźnika inwazyjności. Dane przedstawiono jako średnie arytmetyczne ± SD z trzech niezależnych eksperymentów (n = 3). *p ≤ 0,05, **p ≤ 0,01, ***p ≤ 0,001 w odniesieniu do komórek hodowanych w układzie kontrolnym nietraktowanym stilbenami.
(Gołąbek-Grenda i Olejnik, 2026)

5. Dyskusja

W pierwszej pracy eksperymentalnej (publikacja 3), otwierającej cykl badań nad potencjałem terapeutycznym resweratrolu i jego pochodnych w leczeniu endometriozy, przedstawiono wyniki dotyczące wpływu analizowanych związków na szlaki przeżycia komórek endometriotycznych oraz regulację ich śmierci, w tym sygnalizację apoptozy. Istotną przesłanką do opracowania nowych strategii terapeutycznych endometriozy, opartych na modulowaniu programowanej śmierci komórek poprzez mobilizację stymulatorów apoptozy, są doniesienia wskazujące na obniżoną podatność eutopowego i ektopowego endometrium na apoptozę u pacjentek z endometriozą w porównaniu z endometrium kobiet bez tej choroby (Delbandi i in., 2020). We wstępnych eksperymentach oceniających cytotoksyczność analizowanych stilbenów zastosowano ludzkie komórki endometriozy linii 12Z oraz kontrolne, nieendometriotyczne komórki T HESC pochodzące z podścieliska endometrium. Umożliwiło to wykazanie różnic w odpowiedzi komórkowej pomiędzy tkanką endometrialną a endometriotyczną, co może mieć istotne znaczenie w ocenie aktywności potencjalnych czynników terapeutycznych. Dowiedziono, że komórki endometrium T HESC są bardziej oporne na cytotoksyczne działanie stilbenów niż komórki endometriozy 12Z. Na podstawie wartości IC_{50} stwierdzono, że cytotoksyczność resweratrolu wobec komórek endometriozy 12Z jest około 5-krotnie wyższa niż wobec komórek endometrium T HESC. Analogi resweratrolu wykazywały zróżnicowaną aktywność cytotoksyczną zarówno w odniesieniu do komórek pochodzących z endometrium, jak i zmian endometrialnych.

Pterostilben, dimetylowy analog resweratrolu charakteryzujący się lepszym wchłanianiem jelitowym oraz większą stabilnością metaboliczną w wątrobie (McCormack i McFadden, 2013), nie był dotychczas badany jako potencjalny czynnik terapeutyczny w endometriozie. Jego działanie cytotoksyczne na komórki endometriozy 12Z było słabsze niż działanie resweratrolu, natomiast hamowanie proliferacji komórek endometrium T HESC było silniejsze niż w przypadku związku macierzystego. W literaturze pterostilben opisywany jest jako jeden z najsilniej cytotoksycznych stilbenów, co wiąże się przede wszystkim z obecnością grup metoksylowych w jego strukturze. W najnowszych przeglądach literatury wskazuje się, że pterostilben wywiera efekty cytotoksyczne w różnych modelach komórkowych w zakresie stężeń 25–100 μM (Ma i in., 2019; Medrano-Padial i in., 2021).

Pozostałe dwie pochodne resweratrolu – piceatannol i polidatyna – w znacznie mniejszym stopniu ograniczały proliferację zarówno komórek 12Z, jak i T HESC, przy czym najsłabsze działanie wykazała polidatyna. Chociaż część doniesień sugeruje, że hydroksylacja

resweratrolu w pozycjach 3’ i 4’ prowadzi do zwiększenia cytotoksyczności piceatannolu, inne badania, zwłaszcza prowadzone na liniach komórek białaczki i czerniaka, wykazały brak istotnego działania cytotoksycznego tego związku nawet w wysokich stężeniach 100–400 μM (Medrano-Padial i in., 2021). Podobne zależności dotyczące aktywności stilbenów zaobserwowano w badaniach przeprowadzonych na komórkach raka piersi. Po 48-godzinnej ekspozycji komórek raka piersi linii MCF-7 na stilbeny w zakresie stężeń 2,5–100 μM resweratrol najsilniej hamował proliferację komórek, piceatannol wykazywał słabsze działanie, natomiast piceid nie wykazywał istotnej cytotoksyczności (Komorowska i in., 2021).

Dokonując oceny podatności komórek zrębu endometrium linii T HESC oraz nabłonkowych komórek endometriozy linii 12Z na cytotoksyczne działanie analizowanych związków, należy podkreślić, że zastosowane linie komórkowe pochodzą z różnych typów tkanki endometrium, co stanowi istotne ograniczenie w bezpośredniej interpretacji i porównaniu uzyskanych wyników. Wykorzystanie linii podścieliska T HESC wynikało z braku dostępnych reprezentatywnych linii komórek nabłonkowych endometrium. Podobny model komórkowy zastosowano w pracy Madanes i in. (2022), w której wykazano, że resweratrol w dawkach 50 i 100 μM po 48 godzinach ekspozycji zmniejszał żywotność i potencjał proliferacyjny komórek endometriozy 12Z i komórek zrębu endometrium St-T1b w porównywalnym stopniu. Ponadto związek ten w podobnym zakresie hamował proliferację komórek nabłonkowych izolowanych zarówno od pacjentek z endometriozą, jak i z grupy kontrolnej. Z kolei inne badania wykazały, że resweratrol w stężeniach ≤100 μM po 48 godzinach ekspozycji nie wpływał istotnie na żywotność ektopowych komórek podścieliska endometrium izolowanych od pacjentek z endometriozą. Dopiero zastosowanie resweratrolu w stężeniu 200 μM prowadziło do obniżenia żywotności komórek o około 50% (Kolahdouz Mohammadi i in., 2020; Taguchi i in., 2016). W badaniach nad proapoptotycznym potencjałem resweratrolu i jego analogów zaobserwowano, że obecność tych związków w hodowli komórek endometriozy w stężeniu 50 μM zwiększa odsetek komórek charakteryzujących się eksternalizacją fosfatydyloseryny, stanowiącą wczesny marker procesu apoptozy. Należy zauważyć, że pterostilben istotnie zwiększał populację komórek zarówno we wczesnej, jak i później fazie apoptozy. Podobnie w literaturze opisano silniejsze proapoptotyczne działanie pterostilbenu w porównaniu z resweratrolem, m.in. w komórkach raka szyjki macicy (Chatterjee i in., 2018; Shin i in., 2020). Jego wyższy potencjał proapoptotyczny wiązano z aktywacją szlaku apoptozy zależnego od p53 oraz modulacją szlaku Nrf-2 poprzez indukcję stresu siateczki śródplazmatycznej w komórkach raka szyjki macicy (Chatterjee i in., 2018; B. Zhang i in., 2014).

Po ekspozycji komórek endometriozy na wszystkie analizowane stilbeny w stężeniu 50 μM stwierdzono oligonukleosomalną fragmentację DNA oraz pojawienie się subpopulacji komórek o zmniejszonej zawartości DNA, widocznej przed frakcją komórek w fazie G_0/G_1 na histogramach obrazujących przebieg cyklu komórkowego. Pęknięcia nici DNA stanowią charakterystyczną cechę późnych etapów apoptozy i wynikają z aktywności endonukleaz, w tym deoksyrybonukleazy aktywowanej kaspazą (CAD, *caspase-activated DNase*), która ulega aktywacji po rozszczepieniu inhibitora ICAD przez kaspazę-3 (Schindler i in., 2006). Analiza aktywności kaspaz na poziomie molekularnym i komórkowym wykazała, że resweratrol istotnie zwiększa ekspresję mRNA kaspazy-3 (ok. 7-krotnie) oraz jej aktywność proteolityczną (ok. 10-krotnie). Natomiast pterostilben, będący najsilniejszym induktorem pęknięć DNA, znacznie słabiej aktywował kaspazę-3. Dostępne dane literaturowe sugerują ponadto, że apoptoza indukowana przez pterostilben może przebiegać częściowo niezależnie od kaspaz, w przeciwieństwie do resweratrolu i jego pozostałych analogów (Tolomeo i in., 2005). Ścieżki aktywacji śmierci komórki mają złożony charakter i często nakładają się na siebie. Przykładowo klasyczny szlak apoptozy zależny od białek z rodziny BCL i kaspaz może uruchamiać dodatkowe mechanizmy związane z uwalnianiem z mitochondriów czynnika indukującego apoptozę (AIF, *apoptosis inducing factor*), który bezpośrednio indukuje fragmentację jądrowego DNA (Cregan i in., 2004). Wykazano, że uwalnianie AIF może być inicjowane przez nadekspresję *BAX* w komórkach HeLa i 293T (Arnoult i in., 2003), co koresponduje z podwyższoną ekspresją *BAX* wywołaną przez pterostilben w komórkach endometriozy, obserwowaną w badaniach przedstawionych w pracy doktorskiej. Zróżnicowany potencjał apoptotyczny resweratrolu i pterostilbenu wynika prawdopodobnie z zależności pomiędzy strukturą chemiczną a aktywnością biologiczną związku. W badaniach innych autorów stwierdzono, że obecność motywu 3,5-dimetoksylowego sprzyja działaniu proapoptotycznemu, nawet w komórkach wykazujących fenotyp oporności wielolekowej lub ekspresję onkogenu antyapoptotycznego (Tolomeo i in., 2005).

W przeprowadzonych eksperymentach zaobserwowano również hamujący wpływ piceatannolu na komórki endometriozy. Wykazano, że indukcja apoptozy przez piceatannol może być związana z rozległą fragmentacją jądrowego DNA w komórkach 12Z, pomimo stosunkowo niskiej cytotoksyczności tego związku, określonej wartością IC_{50} wynoszącą 223,76 μM, znacząco wyższą niż wartości wyznaczone dla resweratrolu (49,32 μM) i pterostilbenu (73,88 μM). Stwierdzono, że piceatannol w stężeniu 100 μM powodował niemal 50% fragmentację DNA w komórkach 12Z oraz istotny wzrost ekspresji mRNA kaspazy-8, -9 i -3. Podobne wyniki uzyskano w badaniach prowadzonych na komórkach raka

wątrobowokomórkowego HepG2, w których piceatannol w stężeniach 100 μM i 200 μM, mimo niskiej cytotoksyczności, indukował charakterystyczną dla procesu apoptozy fragmentację DNA oznaczoną testem TUNEL (Morales i Haza, 2012). Zaskakującym zjawiskiem obserwowanym w komórkach endometriozy było zwiększenie ekspresji genu kodującego antyapoptotyczne białko BCL-2 po ekspozycji na resweratrol, pterostilben i piceatannol w wyższych dawkach, przy czym najsilniejszy efekt wzmacniający ekspresję mRNA *BCL2* wywoływał pterostilben. Białko BCL-2 uczestniczy w regulacji przepuszczalności błony mitochondrialnej, z której uwalniany jest m.in. cytochrom c, odpowiedzialny za kaskadową aktywację kaspaz (Westphal i in., 2014). Badania innych autorów wykazały, że pterostilben zwiększa ekspresję *BCL2* w ludzkich komórkach nerwowych poprzez aktywację receptora ER-α, prowadzącą do genomowej regulacji ekspresji *BCL2*, dzięki czemu może wykazywać działanie neuroprotekcyjne (Z. Song i in., 2015). Rozmieszczenie grup hydroksylowych w pierścieniach aromatycznych stilbenów sprawia, że związki te wykazują podobieństwo strukturalne do endogennych i syntetycznych estrogenów. Jednym z biologicznych celów działania resweratrolu jest receptor estrogenowy, który reguluje ekspresję genów związanych z proliferacją i przeżyciem komórek, takich jak *BCL2*. Regulacja ta może przebiegać zarówno poprzez klasyczny szlak zależny od wiązania receptora do specyficznych sekwencji DNA w promotorach genów docelowych, określanych jako ERE (*estrogen-responsive elements*), jak i poprzez mechanizmy nieklasyczne, polegające na pośrednim oddziaływaniu receptora z DNA za pośrednictwem różnych czynników transkrypcyjnych. Ponadto możliwa jest również szybka, niegenomowa aktywacja szlaków sygnalizacyjnych zachodząca w wyniku bezpośrednich interakcji z receptorami kinaz tyrozynowych lub kinazami białkowymi (Kawiak i Kostecka, 2022). Związki estrogenowe, w tym niektóre stilbeny, mogą działać jako selektywne modulatory receptora estrogenowego, wywołując zróżnicowane efekty agonistyczne lub antagonistyczne, zależnie od typu komórek i rodzaju genu docelowego (Oseni i in., 2008). Wiadomo również, że komórki endometriozy 12Z oraz komórki zrębu endometrium T HESC wykorzystane w pracy doktorskiej są ER-dodatnie, co stwarza możliwość prowadzenia dalszych badań nad modulacją sygnalizacji ER jako potencjalnym kierunkiem terapeutycznym w endometriozie (Banu i in., 2008; Logan i in., 2013).

Przedmiotem badań prezentowanych w rozprawie była również polidatyna, będąca naturalnym prekursorem resweratrolu, w którym jedna z grup hydroksylowych została zastąpiona resztą glukopiranozydową. Modyfikacja ta umożliwia przenikanie związku przez błony komórkowe z wykorzystaniem naturalnych mechanizmów transportu glukozy oraz specyficznych transporterów błonowych. Dotychczasowe badania eksperymentalne sugerują,

że terapeutyczne działanie polidatyny może być związane z jej aktywnością przeciwzapalną (Lanzilli i in., 2012), antyoksydacyjną (Bröhan i in., 2011) oraz przeciwnowotworową (Shah i in., 2022). Wyniki badań prowadzonych w ramach niniejszej pracy doktorskiej wskazują, że wpływ polidatyny na indukcję apoptozy w komórkach endometriozy jest ograniczony, a jej aktywność biologiczna pozostaje niższa w porównaniu z pozostałymi analizowanymi stilbenami. Pomimo indukcji wczesnej fazy apoptozy, potwierdzonej eksternalizacją fosfatydyloseryny, polidatyna nie prowadziła do aktywacji późnych etapów tego procesu; nie obserwowano zwiększenia ekspresji kaspaz ani białek proapoptotycznych. Na podstawie badań prowadzonych na komórkach nowotworowych sugeruje się, że niższa aktywność biologiczna polidatyny w porównaniu z resweratrolem może wynikać z ograniczonego wychwytu komórkowego, zależnego od specyficznych transporterów błonowych lub mechanizmów endocytozy związanych z tratwami lipidowymi (Su i in., 2013).

Podsumowując badania nad proapoptotycznym działaniem resweratrolu i jego naturalnych pochodnych można stwierdzić, że analizowane związki, poprzez specyficzną indukcję apoptozy z udziałem różnych szlaków sygnalizacji śmierci komórek, mogą mieć potencjalne znaczenie w profilaktyce i leczeniu endometriozy. Na szczególną uwagę zasługuje pterostilben, który w porównaniu z resweratrolem, wykazywał wyższą aktywność proapoptotyczną. Potencjał biologiczny tego związku wobec komórek endometriozy został, zgodnie z dostępną literaturą, przedstawiony po raz pierwszy.

Kolejnym aspektem oceny potencjału biologicznego stilbenów, przedstawionym w publikacji 4, było ich działanie przeciwzapalne w zapalnym mikrośrodowisku endometriozy, imitowanym w modelowym układzie ko-hodowli makrofagów THP-1 z komórkami endometriozy 12Z. Podejście to jest w pełni uzasadnione, ponieważ przewlekły stan zapalny towarzyszący endometriozie, podtrzymywany między innymi przez aktywowane makrofagi, stanowi jeden z głównych czynników patogenetycznych choroby. Odtworzenie złożoności reakcji zapalnych oraz interakcji pomiędzy różnymi typami komórek zaangażowanych w rozwój stanu zapalnego towarzyszącego endometriozie jest jednak bardzo trudne, a w warunkach *in vitro* praktycznie niemożliwe. Z tego względu w badaniach zastosowano uproszczony model komórkowy oparty na makrofagach o fenotypie prozapalnym, uzyskanych w wyniku różnicowania i polaryzacji monocytów THP-1. W układzie ko-hodowli makrofagów THP-1 z komórkami endometriozy 12Z przeprowadzono badania nad wpływem analizowanych stilbenów na ekspresję kluczowych markerów stanu zapalnego oraz stresu oksydacyjnego w aktywowanych makrofagach związanych z endometriozą.

W celu opracowania odpowiedniego układu doświadczalnego przeprowadzono szereg prac eksperymentalnych związanych z optymalizacją protokołu różnicowania i polaryzacji makrofagów, z uwzględnieniem zapewnienia odpowiednich warunków hodowli zarówno dla makrofagów, jak i komórek endometriozy oraz skutecznej indukcji stanu zapalnego. W procesie optymalizacji modelu uwzględniono również zalecenia literaturowe dotyczące ko-hodowli komórek endometriotycznych z makrofagami pochodzącymi z monocytów THP-1 (Smith i in., 2015). W badaniach wstępnych wykazano, że indukcja różnicowania monocytów THP-1 oraz aktywacja stanu zapalnego w makrofagach prowadziła do zwiększenia ekspresji genów kodujących kluczowe markery tych procesów, takich jak *CD68*, *IL6*, *IL1β* oraz *TNF*. Dotychczas opracowano jedynie nieliczne modele doświadczane uwzględniające udział układu odpornościowego w patogenezie endometriozy. W części badań makrofagi związane z endometriozą uzyskiwano poprzez zastosowanie pożywki kondycjonowanej pochodzącej z hodowli ludzkich komórek endometriozy (11Z, 12Z, 22B) (Woo i in., 2017). W innych pracach komórki endometriozy 12Z traktowano medium kondycjonowanym uzyskanym z hodowli zróżnicowanych i spolaryzowanych makrofagów M0, M1 i M2 (Sekulovski i in., 2019). Zaproponowany w niniejszej pracy doktorskiej model ko-hodowli prozapalnych makrofagów i komórek endometriotycznych umożliwiał ich wzajemne oddziaływania międzykomórkowe za pośrednictwem specyficznych czynników wydzielanych do wspólnego środowiska hodowlanego. W konsekwencji uzyskano układ lepiej odzwierciedlający zapalne mikrośrodowisko endometriozy, co umożliwiło uzyskanie danych o większej istotności fizjologicznej.

Badania przeprowadzone w komórkowym modelu stanu zapalnego w endometriozie, zaprezentowane w publikacji 4, wykazały, że resweratrol i jego analogi hamują ekspresję transkryptów kluczowych mediatorów prozapalnych. Należy jednak podkreślić, że analizowane stilbeny różniły się profilem działania oraz regulacją ekspresji poszczególnych genów. Resweratrol, pterostilben i polidatyna w stężeniu 25 μM istotnie obniżały ekspresję *IL6* na poziomie mRNA oraz syntezę IL-6 na poziomie białka. Makrofagi związane z endometriozą stanowią główne źródło IL-6, która uczestniczy w rekrutacji monocytów do ognisk choroby oraz stymuluje proliferację komórek podścieliska endometrium (Ramírez-Pavez i in., 2021). Po wprowadzeniu pterostilbenu do układu ko-hodowli zaobserwowano jednak odmienną odpowiedź, związaną ze zwiększoną produkcją IL-6 przez makrofagi. Zjawisko to może wynikać ze stymulacji komórek endometriozy 12Z do nasilonego wydzielania czynników indukujących produkcję tej interleukiny przez makrofagi. Podobną zależność opisano również w modelu opartym na zastosowaniu pożywek kondycjonowanych (Woo i in., 2017).

Po ekspozycji ko-hodowli na resweratrol odnotowano istotne obniżenie ekspresji mRNA *IL1B* w makrofagach. IL-1β odgrywa wielokierunkową rolę w patogenezie endometriozy – stymuluje makrofagi do syntezy IL-6 i nasila produkcję PGE2 oraz uczestniczy w syntezie estradiolu poprzez wiązanie czynnika steroidogennego 1 z promotorami genów steroidogennych i aromatazy (Chen i in., 2023). Resweratrol, pterostilben i piceatannol powodowały również obniżenie ekspresji transkryptu genu *CCL2* kodującego białko MCP-1, które jest dobrze poznaną cytokiną chemotaktyczną dla monocytów i makrofagów, działającą zarówno parakrynnie, jak i autokrynnie, której zwiększone stężenie wykrywane jest w płynie otrzewnowym u kobiet z endometriozą (Ramírez-Pavez i in., 2021). Doniesienia literaturowe wskazują, że źródłem MCP-1 mogą być również komórki nabłonkowe endometriozy, które poprzez wytwarzanie tej cytokiny indukują kierunkową migrację makrofagów (Woo i in., 2017).

Badania prowadzone w ramach pracy doktorskiej wykazały, że spośród wszystkich analizowanych stilbenów jedynie resweratrol obniżał ekspresję i syntezę IL-8, będącej ważnym chemoatraktantem w endometriozie. Uzyskane wyniki wskazują na silne działanie przeciwzapalne resweratrolu oraz odmienne właściwości immunomodulujące jego naturalnych pochodnych. Przeprowadzone badania wykazały, że resweratrol, pterostilben i polidatyna istotnie obniżają ekspresję mRNA *PTGS2* (COX-2) w makrofagach hodowanych z komórkami endometriozy, co w konsekwencji prowadziło do ograniczenia syntezy PGE2. COX-2 odgrywa istotną rolę zarówno w fizjologii, jak i patologii tkanek, uczestnicząc w regulacji procesów zapalnych, proliferacyjnych i angiogennych. Ze względu na liczne działania niepożądane NLPZ, analizowane stilbeny mogą stanowić dla nich potencjalną alternatywę terapeutyczną dzięki ukierunkowanemu działaniu na COX-2 (Connolly, 2003; Tassinari i in., 2023).

Kolejnym ważnym czynnikiem zaangażowanym w rozwój stanu zapalnego, analizowanym w modelu *in vitro* endometriozy, jest TNF-α. Według dostępnych danych poziom TNF-α w płynie otrzewnowym koreluje ze stopniem zaawansowania endometriozy, co wskazuje na istotną rolę tej cytokiny w modulacji odpowiedzi immunologicznej przez związki o działaniu przeciwzapalnym (Ziętek i in., 2015). Ekspresja TNF-α w aktywowanych makrofagach pozostających w ko-hodowli z komórkami endometriozy była modulowana przez resweratrol i pterostilben, jednak ograniczenie produkcji tej cytokiny obserwowano wyłącznie po ekspozycji na resweratrol. Wbrew oczekiwaniom nie zaobserwowano wpływu resweratrolu i jego pochodnych na ekspresję czynnika transkrypcyjnego NF-κB, będącego centralnym regulatorem odpowiedzi komórkowej na LPS, cytokiny oraz stres w wielu typach komórek, w tym w makrofagach. LPS jest silnym i szybko działającym induktorem wiązania NF-κB

z DNA, co stanowi kluczowy element wczesnej odpowiedzi zapalnej makrofagów. Badania przeprowadzone na komórkach THP-1 wykazały, że po stymulacji LPS wiązanie NF-κB z DNA utrzymuje się przez około 12 godzin, natomiast po 24 godzinach ulega osłabieniu, co stanowi istotny etap regulacji ekspresji genów umożliwiający wygaszenie odpowiedzi zapalnej (Sharif i in., 2007). Z uwagi na dynamiczny charakter aktywacji NF-κB po indukcji LPS, wpływ analizowanych związków prawdopodobnie nie został uchwycony w wybranym punkcie czasowym eksperymentu. Badania *in vitro* opisywane w literaturze wykazały, że resweratrol i pterostilben silnie modulują odpowiedź zapalną monocytów i makrofagów w monohodowlach, obniżając ekspresję oraz wydzielanie kluczowych mediatorów zapalenia, takich jak PGE2, IL-6, TNF-α i PTGS2, przy czym efekty były często wyraźniejsze na poziomie transkrypcji niż sekrecji białek. Działanie tych związków badano w różnych punktach czasowych – ekspresję genów w komórkach THP-1 oznaczano po 4 godzinach aktywacji, natomiast sekrecję białek po 24 godzinach hodowli. Działanie stilbenów analizowano w stężeniach 5–25 μM, zbliżonych do stosowanych w ramach niniejszej pracy doktorskiej. Jednakże, ze względu na odmienne schematy eksperymentalne oraz różnice w stężeniach induktorów, bezpośrednie porównanie wyników uzyskanych w różnych badaniach nie jest możliwe (Feng i in., 2019; Schwager i in., 2017). Analizy właściwości przeciwzapalnych resweratrolu prowadzono również w monohodowlach komórek endometriozy. Kolahdouz Mohammadi i in. (2020) jako pierwsi opisali hamujący wpływ resweratrolu na ekspresję MCP-1, IL-6, IL-8 i RANTES w ektopowych komórkach podścieliska endometrium pochodzących od kobiet z endometriozą. Podobne badania z wykorzystaniem komórek zrębu endometrium wykazały, że resweratrol poprzez endogenną aktywację SIRT1 istotnie ograniczał produkcję IL-8 indukowaną przez TNF-α (Taguchi i in., 2016). Zastosowanie komórek endometriotycznych wraz z makrofagami w złożonym modelu *in vitro* umożliwiło częściowe odtworzenie interakcji międzykomórkowych w mikrośrodowisku endometriozy. Uzyskane wyniki stanowią nowość naukową i poszerzają wiedzę na temat działania stilbenów na procesy zapalne towarzyszące endometriozie.

Kluczowym zaburzeniem leżącym u podstaw endometriozy jest stres oksydacyjny oraz podwyższony poziom ROS, które napędzają przewlekły stan zapalny. Stan zapalny i produkcja ROS stanowią wzajemnie nasilające się mechanizmy – proces zapalny indukuje wytwarzanie ROS, natomiast stres oksydacyjny wywołany ich nadmierną produkcją podtrzymuje przewlekłą odpowiedź zapalną. W badaniach klinicznych u pacjentek z endometriozą stwierdzono zwiększony poziom ROS oraz obniżoną całkowitą zdolność antyoksydacyjną organizmu (J. Lee i in., 2025). Z tego względu redukcja ROS jest obecnie postrzegana jako istotny cel

terapeutyczny w endometriozie. W badaniach będących przedmiotem rozprawy doktorskiej analizowano wpływ resweratrolu i jego analogów na poziom ROS w aktywowanych makrofagach hodowanych z komórkami endometriozy. Wyniki doświadczeń wykazały, że analizowane stilbeny wprowadzone do modelu stanu zapalnego w endometriozie istotnie ograniczały akumulację ROS w makrofagach. Ekspozycja na stilbeny już w niskich stężeniach (5 μM) prowadziła do zmniejszenia frakcji komórek ROS-dodatnich. Najsilniejsze działanie obserwowano po zastosowaniu pterostilbenu, który redukował frakcję makrofagów nadprodukujących ROS o około 50%. Ograniczenie wewnątrzkomórkowej akumulacji ROS mogło być związane zarówno z wysokim potencjałem antyoksydacyjnym analizowanych stilbenów, jak i z ich zdolnością do aktywacji komórkowych systemów antyoksydacyjnych, co zostało potwierdzone w niniejszej pracy. Analizy na poziomie molekularnym wykazały, że resweratrol i jego pochodne powodowały zwiększenie ekspresji mRNA enzymu antyoksydacyjnego SOD1 w makrofagach. Stymulujący wpływ stilbenów na aktywność endogennych enzymów antyoksydacyjnych stanowi istotny aspekt ich działania przeciwoksydacyjnego i przeciwzapalnego w endometriozie, zwłaszcza biorąc pod uwagę fakt, że w płynie otrzewnowym oraz surowicy pacjentek z endometriozą często obserwuje się obniżoną aktywność enzymów obrony antyoksydacyjnej, takich jak SOD, CAT oraz GPx. Dane literaturowe wskazują, że zaburzenia aktywności endogennych enzymów antyoksydacyjnych, w tym SOD – stanowiącej pierwszą linię obrony przed ROS – oraz GPx, kluczowego enzymu odpowiedzialnego za usuwanie nadtlenków, mogą przyczyniać się do rozwoju uszkodzeń oksydacyjnych w endometriozie a jednocześnie stanowić potencjalne biomarkery tej choroby (Ekarattanawong i in., 2017; Mulgund i in., 2015).

Źródłem ROS w endometriozie są między innymi makrofagi, erytrocyty i apoptotyczne komórki endometriozy. ROS i czynniki prozapalne pozostają ze sobą w ścisłej zależności, przyczyniając się do nasilenia bólu oraz zaburzeń detoksykacji produktów peroksydacji lipidów w warunkach stresu oksydacyjnego. Cytokiny prozapalne i chemotaktyczne odgrywają istotną rolę w rekrutacji i aktywacji komórek fagocytujących, będących głównymi producentami ROS (Didziokaite i in., 2023). Narastający stres oksydacyjny w jamie otrzewnej prowadzi z kolei do uszkodzeń tkanek oraz utrzymywania się przewlekłego stanu zapalnego, co sprzyja proliferacji i włóknieniu ektopowych ognisk endometrium (Chen i in., 2023). Kumulatywny i progresywny charakter tych procesów podkreśla ich istotne znaczenie w progresji endometriozy. Z tego względu ograniczanie stanu zapalnego i łagodzenie stresu oksydacyjnego stanowią ważny cel terapeutyczny w endometriozie. Dlatego modele eksperymentalne umożliwiające ocenę

procesów zapalnych i stresu oksydacyjnego w mikrośrodowisku endometriozy są istotnym narzędziem w badaniach przedklinicznych.

Analizując wyniki przeprowadzonych badań, należy uwzględnić pewne ograniczenia wynikające z zastosowanego modelu komórkowego endometriozy. Opracowany układ doświadczalny obejmował jedynie dwa typy komórek – komórki nabłonkowe endometriozy oraz aktywowane makrofagi – co może być niewystarczające w kontekście złożonego i dynamicznego mikrośrodowiska zmian endometrialnych (Gołąbek-Grenda i Olejnik, 2022). Ponadto w badaniach wykorzystano wyłącznie jeden fenotyp makrofagów (M1), co nie odzwierciedla w pełni złożonej natury i funkcji tej populacji komórek w endometriozie, szczególnie w świetle trwającej dyskusji dotyczącej kryteriów fenotypowej klasyfikacji makrofagów M1 i M2. Niektóre doniesienia wskazują, że populacja makrofagów prozapalnych występuje głównie we wczesnych stadiach choroby, natomiast w bardziej zaawansowanych etapach dochodzi do polaryzacji w kierunku fenotypu M2, wykazującego właściwości przeciwzapalne i naprawcze oraz aktywność profibrotyczną (Kobayashi i Imanaka, 2022; Laganà i in., 2020).

Kolejne ograniczenie badań związane jest z biodostępnością analizowanych stilbenów, ich przemianami metabolicznymi zachodzącymi podczas trawienia żołądkowo-jelitowego oraz procesami wchłaniania jelitowego, które nie zostały uwzględnione w planie badawczym pracy doktorskiej. Aby zniwelować wpływ metabolizmu i biodostępności na ocenę biofunkcjonalności stilbenów efekty immunomodulujące i antyoksydacyjne analizowano przy możliwie niskich, fizjologicznie osiągalnych stężeniach związków. Podejście to uwzględniało doniesienia Feng i in. (2019) oraz T. T. Y. Wang i in. (2008), którzy wykazali w warunkach *in vitro* (komórki THP-1) i *in vivo* (model gryzoni), że resweratrol moduluje odpowiedź immunologiczną zarówno w dawkach uznawanych za fizjologiczne (5 μM), jak i farmakologiczne (>10 μM).

W kolejnej pracy naukowej, stanowiącej kontynuację badań nad potencjałem terapeutycznym resweratrolu i jego analogów, oceniono wpływ analizowanych stilbenów na adhezję komórek endometriotycznych do składników ECM oraz ich migrację i inwazję (publikacja 5). Odtworzenie podstawowych procesów komórkowych związanych z progresją endomteriozy w warunkach *in vitro* wymaga uwzględnienia zaproponowanego współcześnie trójetapowego, sekwencyjnego modelu rozwoju choroby, obejmującego adhezję komórek do mezotelium jamy otrzewnej, remodelowanie błony podstawnej i macierzy zewnątrzkomórkowej i ostateczną implantację komórek wraz z neowaskularyzacją w miejscu ektopowym (H. Liu i Lang, 2011; C. Zhang i in., 2024). Komórki endometrialne ulegają

implantacji poprzez pokonywanie nienaruszonych barier strukturalnych, złożonych z komórek nabłonkowych, błon podstawnych i włókien kolagenowych, lub poprzez wnikanie do wnętrza ECM otrzewnej w miejscach wcześniejszego uszkodzenia tkanek, po czym dochodzi do ich lokalnej ekspansji (Nisolle i in., 2000). Adhezja komórek endometriotycznych do składników ECM, takich jak kolagen, laminina czy fibronektyna, stanowi kluczowy etap inicjacji i rozwoju ognisk endometriozy. Proces ten umożliwia komórkom pochodzącym z endometrium przytwierdzenie się do mezotelium jamy otrzewnej oraz innych struktur ektopowych. Adhezja komórek regulowana jest głównie przez integryny oraz inne cząsteczki adhezyjne, których ekspresja może być modulowana przez czynniki zapalne i hormonalne. Zaburzenia regulacji tych mechanizmów sprzyjają trwałemu zakotwiczeniu komórek oraz ich przeżyciu poza środowiskiem macicy (Pollock i in., 2014).

Wyniki analiz przeprowadzonych w ramach pracy doktorskiej jednoznacznie wskazały na antyadhezyjny potencjał testowanych stilbenów. Wykazano, że analizowane związki skutecznie ograniczają adhezję komórek 12Z do składników ECM, przy czym bardziej preferencyjnie hamują adhezję komórek do fibronektyny niż do kolagenu typu I. Wyższy potencjał antyadhezyjny wobec fibronektyny może wynikać ze słabszego wiązania się komórek 12Z z tym białkiem, co obserwowano również w badaniach prowadzonych przez innych autorów (Koks i in., 2000). Działanie antyadhezyjne resweratrolu udowodniono także w modelach *in vitro* raka jajnika, w których związek ten hamował przyleganie komórek A2780, OVCAR-3 i SKOV3 do mezotelium otrzewnej (Mikuła-Pietrasik i in., 2014). Podobnie analogi resweratrolu, w tym pterostilben, piceatannol i polidatyna, ograniczały adhezję komórek do kolagenu typu I i fibronektyny oraz wykazywały działanie przeciwzwłóknieniowe poprzez redukcję syntezy tych białek (Lan i in., 2020; Marchisio i in., 2025; Pan i in., 2019).

W endometrium ektopowym i eutopowym identyfikowane są różne typy kolagenu, jednak w obrębie zmian endometrialnych, szczególnie głęboko naciekających, dominującym składnikiem ECM pozostaje kolagen typu I. Białko to sprzyja migracji i inwazyjności komórek oraz wspiera procesy włóknienia (Stejskalová i in., 2021; Vissers i in., 2024). Oprócz kolagenu istotnym składnikiem ECM jest również fibronektyna, która pełni ważną funkcję w procesie adhezji komórek endometriozy, dla której wykazano korelację pomiędzy ekspresją genu fibronektyny (FN1) a umiarkowaną i ciężką postacią choroby (Sapkota i in., 2017; Singh i in., 2010). W świetle dostępnych danych, zwiększona ekspresja kolagenu typu I, fibronektyny oraz białek fibrynotycznych, takich jak α-SMA i CTGF, w tkance ektopowej wskazuje na ich istotne zaangażowanie w proces inwazji komórek endometriotycznych (Warzecha i in., 2022; Z. Zhang i in., 2019).

Po adhezji migracja komórek endometriotycznych stanowi kolejny kluczowy etap progresji choroby, umożliwiający rozprzestrzenianie się ognisk endometrialnych w obrębie jamy otrzewnej. Proces ten zależy od reorganizacji cytoszkieletu oraz dynamicznych interakcji pomiędzy komórką a ECM. Współczesne badania wskazują, że składniki ECM mogą bezpośrednio regulować migrację komórek poprzez wpływ na organizację filamentów aktynowych oraz aktywację szlaków sygnałowych związanych z ruchem komórkowym (C. Zhang i in., 2024). Najbardziej zaawansowanym etapem progresji endometriozy jest inwazja komórek endometriotycznych oparta na ich zdolności do degradacji błony podstawnej oraz penetracji otaczających tkanek. Mechanizm ten jest ściśle związany z aktywnością MMPs oraz zaburzeniem równowagi pomiędzy MMPs a ich inhibitorami TIMPs. Najnowsze dane wskazują również na istotne znaczenie procesu EMT, który zwiększa właściwości migracyjne i inwazyjne komórek endometriotycznych (Wei i in., 2025).

Wyniki badań przeprowadzonych w ramach pracy doktorskiej wykazały, że analizowane stilbeny ograniczały inwazję komórek 12Z przez membranę opłaszczoną macierzą Matrigel™, imitującą ECM. Uzyskane dane sugerują, że działanie stilbenów może być związane przede wszystkim z hamowaniem degradacji ECM, a w mniejszym stopniu z regulacją chemotaksji komórek. Należy jednak podkreślić, że resweratrol w najwyższym testowanym stężeniu 50 μM, wykazującym działanie cytotoksyczne, istotnie ograniczał również przemieszczanie się komórek 12Z w kierunku chemoatraktanta. Wynik ten pozostaje zgodny z doniesieniami Madanes i in. (2022), którzy wykazali, że resweratrol w dawkach 50 μM i 100 μM hamuje migrację komórek 12Z w teście gojenia ran. Badania prowadzone przez innych autorów wykazały ponadto, że resweratrol w stężeniach 25 μM i 50 μM istotnie hamuje proliferację, migrację i inwazję ektopowych komórek podścieliska endometrium izolowanych od pacjentek z endometriozą (Kong i in., 2020).

Na podstawie badań prezentowanych w niniejszej rozprawie stwierdzono, że silnym działaniem hamującym migrację i inwazję komórek endometriozy charakteryzuje się pterostilben, który w dawce 50 μM niemal całkowicie eliminował zdolność komórek 12Z do inwazji. Przeciwinwazyjne właściwości pterostilbenu zostały wcześniej wykazane w badaniach prowadzonych na komórkach raka piersi linii MDA-MB-231 oraz raka szyjki macicy linii HeLa (H. S. Ko i in., 2014; Shin i in., 2020). Kolejną pochodną resweratrolu wykazującą hamujący wpływ na migrację i inwazję komórek endometrizoy był piceatannol. Jego aktywność przeciwinwazyjna została opisana w odniesieniu do komórek raka prostaty DU145, w których prowadził do zmniejszenia aktywności MMP-9 i uPA (*urokinase-type plasminogen activator*), ograniczenia wydzielania VEGF oraz obniżenia poziomu IL-6 poprzez modulację szlaku

sygnałowego IL-6/STAT3 (Kwon i in., 2012). W badaniach dotyczących przeciwzapalnej aktywności stilbenów przedstawionych w publikacji 4, związkiem o najwyższym potencjale hamującym produkcję IL-6 w komórkach 12Z był właśnie piceatannol, co może sugerować mechanizm działania zbliżony do obserwowanego w komórkach raka prostaty DU145.

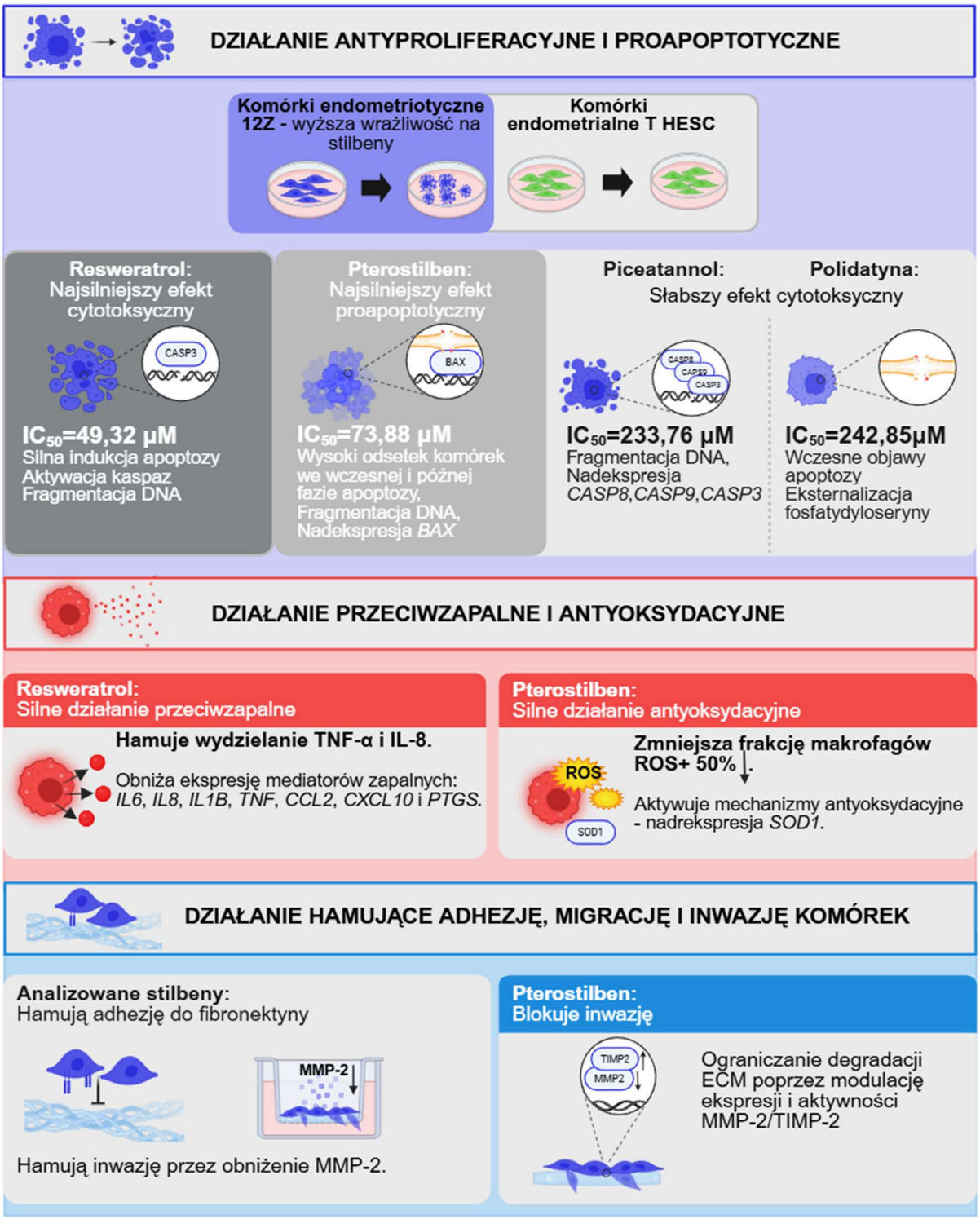
Działanie analizowanych związków prowadzące do ograniczenia inwazji komórek 12Z było prawdopodobnie związane z obniżeniem ekspresji i aktywności enzymu proteolitycznego MMP-2 oraz zwiększeniem ekspresji jego inhibitora TIMP-2. Badane stilbeny wywierały zależny od dawki efekt hamujący zarówno na aktywną formę MMP-2, jak i jej nieaktywny prekursor pro-MMP-2. Aktywność enzymów proteolitycznych wytwarzanych przez komórki endometrialne stanowi kluczowy element procesu inwazji i rozwoju endometriozy otrzewnowej. Enzymy proteolityczne umożliwiają ektopowym komórkom endometrium adhezję, przetrwanie oraz penetrację tkanek. Potwierdzają to między innymi obserwacje dotyczące podwyższonego poziomu MMP-2 w płynie otrzewnowym, zwiększona ekspresja MMP-2 w tkance endometrialnej oraz korelacje pomiędzy wariantami i haplotypami genów *MMP2* i *TIMP2* a ryzykiem rozwoju zaawansowanych stadiów choroby (Cho i in., 2013; Huang i in., 2004; Jana i in., 2016). Badania prowadzone przez innych autorów wykazały również zdolność resweratrolu stosowanego w stężeniach 50–100 μM do modulowania stosunku MMP-2/TIMP-1 w komórkach endometriozy linii 12Z i St-T1b (Madanes i in., 2022). Ponadto, działanie antyinwazyjne resweratrolu obserwowane w różnych typach nowotworów, w tym raka żołądka, jamy ustnej i nerek, wiązano również z hamowaniem ekspresji MMP-2 i MMP-9. Dokładne mechanizmy molekularne, za pośrednictwem których resweratrol ogranicza proces przerzutowania nowotworów, pozostają nadal nie w pełni wyjaśnione, jednak coraz częściej wskazuje się na udział w modulacji szlaku sygnałowego NF-κB, będącego jednym z głównych regulatorów unikania programowanej śmierci komórki (B. Song i in., 2023).

Na podstawie analiz przeprowadzonych w ramach rozprawy doktorskiej stwierdzono, że badane stilbeny wywierały zbliżony wpływ na poziom ekspresji metaloproteinaz. Można zatem przypuszczać, że potencjalnym celem działania tych związków mogą być również inne cząsteczki zaangażowane w regulację migracji i inwazji komórek endometrialnych, w szczególności markery nabłonkowe i mezenchymalne uczestniczące w procesach przejścia EMT. Projekt eksperymentalny przedstawiony w publikacji 5 nie obejmował analizy całego profilu składników ECM ani wszystkich kluczowych metaloproteinaz i receptorów integrynowych pośredniczących w adhezji komórek do białek ECM. Sygnalizacja zależna od komórkowych cząsteczek adhezyjnych może stanowić istotny czynnik determinujący adhezję komórek endometriotycznych oraz rozprzestrzenianie się zmian chorobowych. Zagadnienie to

wydaje się szczególnie interesującym kierunkiem dalszych badań nad potencjałem prewencyjnym i terapeutycznym stilbenów w endometriozie. Wyniki badań dotyczących potencjału antyinwazyjnego stilbenów, zaprezentowane w publikacji 5, pozwalają wnioskować, że resweratrol i jego naturalne analogi obniżają migracyjny i inwazyjny potencjał komórek endometriozy poprzez modulację aktywności transkrypcyjnej oraz redukcję poziomu białek zaangażowanych w przebudowę ECM, a także poprzez wpływ na interakcje pomiędzy komórką a ECM. Zgromadzone dane wskazują na istotny antyendometriotyczny potencjał pterostilbenu i piceatannolu, które oddziałują poprzez regulację procesów związanych z ruchliwością komórek oraz degradacją ECM. Pełne wyjaśnienie mechanizmów ich działania wymaga jednak dalszych, bardziej szczegółowych badań. Dodatkowym ograniczeniem interpretacji uzyskanych wyników jest niewielka liczba dostępnych doniesień literaturowych dotyczących aktywności tych związków w modelach endometriozy.

Niniejsza praca wykazała wpływ badanych stilbenów na kluczowe procesy związane z rozwojem endometriozy, obejmujące zaburzenia proliferacji i apoptozy, nasilony stres oksydacyjny oraz przewlekły stan zapalny, a także adhezję, migrację i inwazję komórek endometriozy oraz przebudowę ECM. Określono szereg bioaktywnych właściwości analizowanych związków wobec endometriozy oraz przedstawiono sugerowane mechanizmy ich działania, potwierdzając tym samym postawione w pracy doktorskiej hipotezy badawcze oraz realizację celu głównego i celów szczegółowych.

Uzyskane dane, prezentujące dotychczas nieopisane działanie analizowanych stilbenów, a także identyfikacja związków o najwyższej aktywności biologicznej, mogą stanowić dobrą podstawę do dalszych badań nad ich potencjalnym zastosowaniem we wspomaganiu terapii endometriozy. Dodatkowo otrzymane wyniki podkreślają znaczenie zaawansowanych modeli *in vitro* w badaniach właściwości potencjalnych środków terapeutycznych, umożliwiających uwzględnienie złożoności komórkowych, humoralnych i pozakomórkowych komponentów mikrośrodowiska choroby. Grafika zamieszczona na Rycinie 16 podsumowuje wielokierunkowy charakter działania resweratrolu i jego naturalnych analogów, wskazując na ich potencjalne znaczenie w modulacji procesów odpowiedzialnych za rozwój i utrzymywanie się zmian endometrialnych.



Rycina 16. Działanie resweratrolu i jego naturalnych pochodnych w modelach endometriozy *in vitro* ze wskazaniem najważniejszych aktywności związków. Rycinę przygotowano w programie https://BioRender.com.

6. Wnioski

Na podstawie przeprowadzonych badań sformułowano następujące wnioski:

I. Resweratrol i jego pochodne wykazują wyższy potencjał cytotoksyczny wobec komórek endometriotycznych linii 12Z niż wobec komórek endometrialnych linii T HESC, co wskazuje na ich działanie ukierunkowane na komórki patologiczne.

II. Najsilniejsze działanie cytotoksyczne na komórki 12Z wykazują resweratrol i pterostilben, natomiast najsłabszy efekt hamowania proliferacji obserwuje się w przypadku polidatyny.

III. Pterostilben charakteryzuje się wysokim potencjałem proapoptotycznym, powoduje zwiększenie populacji komórek we wczesnej i późnej fazie apoptozy, nasila fragmentację DNA oraz wzmacnia ekspresję proapoptotycznego genu *BAX*, przy jednocześnie umiarkowanej aktywacji kaspaz.

IV. Silne działanie proapoptotyczne resweratrolu wobec komórek endometriozy 12Z jest związane głównie z aktywacją kaspazo-zależnego szlaku apoptozy, co potwierdza istotny wzrost ekspresji i aktywności kaspazy-3 oraz nasilona fragmentacja DNA.

V. Piceatannol indukuje proces apoptozy poprzez zwiększenie ekspresji kaspazy-8, -9 i -3, czemu towarzyszy nasilona fragmentacja DNA w komórkach endometriotycznych.

VI. Polidatyna wykazuje najsłabszy efekt proapoptotyczny, ograniczając się do indukcji wczesnych etapów apoptozy związanych z eksternalizacją fosfatydyloseryny, bez aktywacji późniejszych cech charakterystycznych dla komórek apoptotycznych.

VII. Resweratrol wykazuje najsilniejsze działanie przeciwzapalne w modelu ko-hodowli komórek 12Z i makrofagów obniżając ekspresję kluczowych mediatorów stanu zapalnego (*IL6*, *IL8*, *IL1B*, *TNF*, *CCL2*, *CXCL10* oraz *PTGS*) oraz produkcję TNF-α i IL-8.

VIII. Pochodne resweratrolu różnią się zdolnością modulowania odpowiedzi immunologicznej, zwłaszcza w zakresie wpływu na wydzielanie IL-6 i MCP-1.

IX. Analizowane stilbeny istotnie ograniczają akumulację ROS w makrofagach. Pterostilben najsilniej redukuje frakcję komórek nadprodukujących ROS, co wiąże się ze zwiększoną ekspresją dysmutazy ponadtlenkowej *SOD1*.

X. Resweratrol i jego pochodne hamują adhezję komórek endometriotycznych 12Z, ograniczając ich przyleganie do składników ECM, szczególnie do fibronektyny.

XI. Badane związki ograniczają migrację i inwazję komórek 12Z, głównie poprzez zmniejszenie degradacji ECM, wynikającej z modulowania ekspresji i aktywności metaloproteinazy MMP-2 oraz jej inhibitora TIMP-2.

XII. Najwyższą aktywność przeciwinwazyjną wykazuje pterostilben, który w stężeniu 50 μM niemal całkowicie znosi zdolność komórek 12Z do inwazji w modelu Matrigel™.

7. Bibliografia

Abulughod, N., Valakas, S., i El-Assaad, F. (2024). Dietary and Nutritional Interventions for the Management of Endometriosis. *Nutrients*, *16*(23), 3988. https://doi.org/10.3390/nu16233988

Agic, A., Djalali, S., Diedrich, K., i Hornung, D. (2009). Apoptosis in Endometriosis. *Gynecologic and Obstetric Investigation*, *68*(4), 4. https://doi.org/10.1159/000235871

Al-Jaber, H. I., Shakya, A. K., Al-Qudah, M. A., Barhoumi, L. M., Abu-Sal, H. E., Hasan, H. S., Al-Bataineh, N., Abu-Orabi, S., i Mubarak, M. S. (2024). Piceatannol, a comprehensive review of health perspectives and pharmacological aspects. *Arabian Journal of Chemistry*, *17*(9), 105939. https://doi.org/10.1016/j.arabjc.2024.105939

Allaire, C., Bedaiwy, M. A., i Yong, P. J. (2023). Diagnosis and management of endometriosis. *CMAJ : Canadian Medical Association Journal*, *195*(10), E363–E371. https://doi.org/10.1503/cmaj.220637

Aly, S., El-Kamel, A. H., Sheta, E., i El-Habashy, S. E. (2023). Chondroitin/Lactoferrin-dual functionalized pterostilbene-solid lipid nanoparticles as targeted breast cancer therapy. *International Journal of Pharmaceutics*, *642*, 123163. https://doi.org/10.1016/j.ijpharm.2023.123163

Amini, L., Chekini, R., Nateghi, M. R., Haghani, H., Jamialahmadi, T., Sathyapalan, T., i Sahebkar, A. (2021). The Effect of Combined Vitamin C and Vitamin E Supplementation on Oxidative Stress Markers in Women with Endometriosis: A Randomized, Triple-Blind Placebo-Controlled Clinical Trial. *Pain Research & Management*, *2021*, 5529741. https://doi.org/10.1155/2021/5529741

Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J. C., Cecconi, F., i Youle, R. J. (2003). Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *The EMBO Journal*, *22*(17), 4385–4399. https://doi.org/10.1093/emboj/cdg423

Aziz, M. H., Nihal, M., Fu, V. X., Jarrard, D. F., i Ahmad, N. (2006). Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3'-kinase/Akt pathway and Bcl-2 family proteins. *Molecular Cancer Therapeutics*, *5*(5), 1335–1341. https://doi.org/10.1158/1535-7163.MCT-05-0526

Bachmaier, K., Stuart, A., Singh, A., Mukhopadhyay, A., Chakraborty, S., Hong, Z., Wang, L., Tsukasaki, Y., Maienschein-Cline, M., Ganesh, B. B., Kanteti, P., Rehman, J., i Malik, A. B. (2022). Albumin Nanoparticle Endocytosing Subset of Neutrophils for Precision Therapeutic Targeting of Inflammatory Tissue Injury. *ACS Nano*, *16*(3), 4084–4101. https://doi.org/10.1021/acsnano.1c09762

Banaszewska, B., Wrotyńska-Barczyńska, J., Spaczynski, R. Z., Pawelczyk, L., i Duleba, A. J. (2016). Effects of Resveratrol on Polycystic Ovary Syndrome: A Double-blind, Randomized, Placebo-controlled Trial. *The Journal of Clinical Endocrinology and Metabolism*, *101*(11), 4322–4328. https://doi.org/10.1210/jc.2016-1858

Banu, S. K., Lee, J., Starzinski-Powitz, A., i Arosh, J. A. (2008). Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells. *Fertility and Sterility*, *90*(4), 972–987. https://doi.org/10.1016/j.fertnstert.2007.07.1358

Becker, C. M., Bokor, A., Heikinheimo, O., Horne, A., Jansen, F., Kiesel, L., King, K., Kvaskoff, M., Nap, A., Petersen, K., Saridogan, E., Tomassetti, C., van Hanegem, N., Vulliemoz, N., Vermeulen, N., i ESHRE Endometriosis Guideline Group. (2022). ESHRE guideline: Endometriosis. *Human Reproduction Open*, *2022*(2), hoac009. https://doi.org/10.1093/hropen/hoac009

Bhardwaj, A., Sethi, G., Vadhan-Raj, S., Bueso-Ramos, C., Takada, Y., Gaur, U., Nair, A. S., Shishodia, S., i Aggarwal, B. B. (2007). Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. *Blood*, *109*(6), 2293–2302. https://doi.org/10.1182/blood-2006-02-003988

Bhat, K. P., i Pezzuto, J. M. (2001). Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. *Cancer Research*, *61*(16), 6137–6144.

Bianco, N. R., Chaplin, L. J., i Montano, M. M. (2005). Differential induction of quinone reductase by phytoestrogens and protection against oestrogen-induced DNA damage. *Biochemical Journal*, *385*(Pt 1), 279–287. https://doi.org/10.1042/BJ20040959

Bo, C., i Wang, Y. (2024). Angiogenesis signaling in endometriosis: Molecules, diagnosis and treatment (Review). *Molecular Medicine Reports*, *29*(3), 1–14. https://doi.org/10.3892/mmr.2024.13167

Bofill, L., Barbas, R., de Sande, D., Font-Bardia, M., Ràfols, C., Albertí, J., i Prohens, R. (2021). A Novel, Extremely Bioavailable Cocrystal of Pterostilbene. *Crystal Growth & Design*, *21*(4), 2315–2323. https://doi.org/10.1021/acs.cgd.0c01716

Borghini, R., Porpora, M. G., Casale, R., Marino, M., Palmieri, E., Greco, N., Donato, G., i Picarelli, A. (2020). Irritable Bowel Syndrome-Like Disorders in Endometriosis: Prevalence of Nickel Sensitivity and Effects

of a Low-Nickel Diet. An Open-Label Pilot Study. *Nutrients*, *12*(2), 341. https://doi.org/10.3390/nu12020341

Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., i Klinge, C. M. (2000). Resveratrol Acts as a Mixed Agonist/Antagonist for Estrogen Receptors α and β. *Endocrinology*, *141*(10), 3657–3667. https://doi.org/10.1210/endo.141.10.7721

Brás, L. P., Luís, Â., Chatel, G., Socorro, S., i Duarte, A. P. (2025). Stilbenes from Vine Extracts: Therapeutic Potential and Mechanisms. *International Journal of Molecular Sciences*, *26*(17), 8269. https://doi.org/10.3390/ijms26178269

Brockmueller, A., Buhrmann, C., Shayan, P., i Shakibaei, M. (2023). Resveratrol induces apoptosis by modulating the reciprocal crosstalk between p53 and Sirt-1 in the CRC tumor microenvironment. *Frontiers in Immunology*, *14*. https://doi.org/10.3389/fimmu.2023.1225530

Bröhan, M., Jerkovic, V., i Collin, S. (2011). Potentiality of Red Sorghum for Producing Stilbenoid-Enriched Beers with High Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, *59*(8), 4088–4094. https://doi.org/10.1021/jf1047755

Brown, J., Crawford, T. J., Allen, C., Hopewell, S., i Prentice, A. (2017). Nonsteroidal anti-inflammatory drugs for pain in women with endometriosis. *The Cochrane Database of Systematic Reviews*, *2017*(1), CD004753. https://doi.org/10.1002/14651858.CD004753.pub4

Brown, K., Theofanous, D., Britton, R. G., Aburido, G., Pepper, C., Sri Undru, S., i Howells, L. (2024). Resveratrol for the Management of Human Health: How Far Have We Come? A Systematic Review of Resveratrol Clinical Trials to Highlight Gaps and Opportunities. *International Journal of Molecular Sciences*, *25*(2), 747. https://doi.org/10.3390/ijms25020747

Bulun, S. E., Cheng, Y.-H., Pavone, M. E., Xue, Q., Attar, E., Trukhacheva, E., Tokunaga, H., Utsunomiya, H., Yin, P., Luo, X., Lin, Z., Imir, G., Thung, S., Su, E. J., i Kim, J. J. (2010). Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. *Seminars in Reproductive Medicine*, *28*(1), 36–43. https://doi.org/10.1055/s-0029-1242991

Bulun, S. E., Yilmaz, B. D., Sison, C., Miyazaki, K., Bernardi, L., Liu, S., Kohlmeier, A., Yin, P., Milad, M., i Wei, J. (2019). Endometriosis. *Endocrine Reviews*, *40*(4), 1048–1079. https://doi.org/10.1210/er.2018-00242

Burns, K. A., Pearson, A. M., Slack, J. L., Por, E. D., Scribner, A. N., Eti, N. A., i Burney, R. O. (2022). Endometriosis in the Mouse: Challenges and Progress Toward a 'Best Fit' Murine Model. *Frontiers in Physiology*, *12*. https://doi.org/10.3389/fphys.2021.806574

Chaplin, A., Carpéné, C., i Mercader, J. (2018). Resveratrol, Metabolic Syndrome, and Gut Microbiota. *Nutrients*, *10*(11), 1651. https://doi.org/10.3390/nu10111651

Chatterjee, K., AlSharif, D., Mazza, C., Syar, P., Al Sharif, M., i Fata, J. E. (2018). Resveratrol and Pterostilbene Exhibit Anticancer Properties Involving the Downregulation of HPV Oncoprotein E6 in Cervical Cancer Cells. *Nutrients*, *10*(2), 243. https://doi.org/10.3390/nu10020243

Chen, S., Liu, Y., Zhong, Z., Wei, C., Liu, Y., i Zhu, X. (2023). Peritoneal immune microenvironment of endometriosis: Role and therapeutic perspectives. *Frontiers in Immunology*, *14,* 1134663. https://doi.org/10.3389/fimmu.2023.1134663

Cho, Y. J., Kim, N. H., Jeong, K.-A., Lee, J. Y., Moon, H.-S., Kim, H.-L., i Chung, H. W. (2013). Association between MMP-2 and TIMP-2 gene polymorphisms and advanced-stage endometriosis in Korean women. *American Journal of Reproductive Immunology*, *69*(1), 73–84. https://doi.org/10.1111/aji.12020

Cho, Y. J., Lee, S. H., Park, J. W., Han, M., Park, M. J., i Han, S. J. (2018). Dysfunctional signaling underlying endometriosis: Current state of knowledge. *Journal of molecular endocrinology*, 60(3), R97–R113. https://doi.org/10.1530/JME-17-0227

Chow, H.-H. S., Garland, L. L., Heckman-Stoddard, B. M., Hsu, C.-H., Butler, V. D., Cordova, C. A., Chew, W. M., i Cornelison, T. L. (2014). A pilot clinical study of resveratrol in postmenopausal women with high body mass index: Effects on systemic sex steroid hormones. *Journal of Translational Medicine*, *12*, 223. https://doi.org/10.1186/s12967-014-0223-0

Chung, M. S., i Han, S. J. (2022). Endometriosis-Associated Angiogenesis and Anti-angiogenic Therapy for Endometriosis. *Frontiers in Global Women's Health*, *3*, 856316. https://doi.org/10.3389/fgwh.2022.856316

Cirillo, M., Argento, F. R., Becatti, M., Fiorillo, C., Coccia, M. E., i Fatini, C. (2023). Mediterranean Diet and Oxidative Stress: A Relationship with Pain Perception in Endometriosis. *International Journal of Molecular Sciences*, *24*(19), 14601. https://doi.org/10.3390/ijms241914601

Connolly, T. P. (2003). Cyclooxygenase-2 Inhibitors in Gynecologic Practice. *Clinical Medicine and Research*, *1*(2), 105–110. https://doi.org/10.3121/cmr.1.2.105

Cregan, S. P., Dawson, V. L., i Slack, R. S. (2004). Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene*, *23*(16), 2785–2796. https://doi.org/10.1038/sj.onc.1207517

Dana, P., Thumrongsiri, N., Tanyapanyachon, P., Chonniyom, W., Punnakitikashem, P., i Saengkrit, N. (2022). Resveratrol Loaded Liposomes Disrupt Cancer Associated Fibroblast Communications within the Tumor Microenvironment to Inhibit Colorectal Cancer Aggressiveness. *Nanomaterials*, *13*(1), 107. https://doi.org/10.3390/nano13010107

Darzynkiewicz, Z., Juan, G., i Bedner, E. (2001). Determining cell cycle stages by flow cytometry. *Current Protocols in Cell Biology*, *Chapter 8*, Unit 8.4. https://doi.org/10.1002/0471143030.cb0804s01

Das, S., i Das, D. K. (2007). Anti-inflammatory responses of resveratrol. *Inflammation & Allergy Drug Targets*, *6*(3), 168–173. https://doi.org/10.2174/187152807781696464

Delbandi, A.-A., Mahmoudi, M., Shervin, A., Heidari, S., Kolahdouz-Mohammadi, R., i Zarnani, A.-H. (2020). Evaluation of apoptosis and angiogenesis in ectopic and eutopic stromal cells of patients with endometriosis compared to non-endometriotic controls. *BMC Women's Health*, *20*(1), 3. https://doi.org/10.1186/s12905-019-0865-4

Delmas, D., Rébé, C., Micheau, O., Athias, A., Gambert, P., Grazide, S., Laurent, G., Latruffe, N., i Solary, E. (2004). Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. *Oncogene*, *23*(55), 8979–8986. https://doi.org/10.1038/sj.onc.1208086

Di Paola, R., Fusco, R., Gugliandolo, E., Crupi, R., Evangelista, M., Granese, R., i Cuzzocrea, S. (2016). Co-micronized Palmitoylethanolamide/Polydatin Treatment Causes Endometriotic Lesion Regression in a Rodent Model of Surgically Induced Endometriosis. *Frontiers in Pharmacology*, *7*, 382. https://doi.org/10.3389/fphar.2016.00382

Didziokaite, G., Biliute, G., Gudaite, J., i Kvedariene, V. (2023). Oxidative Stress as a Potential Underlying Cause of Minimal and Mild Endometriosis-Related Infertility. *International Journal of Molecular Sciences*, *24*(4), 3809. https://doi.org/10.3390/ijms24043809

Ding, S., Guo, X., Zhu, L., Wang, J., Li, T., Yu, Q., i Zhang, X. (2021). Macrophage-derived netrin-1 contributes to endometriosis-associated pain. *Annals of Translational Medicine*, *9*(1), 29. https://doi.org/10.21037/atm-20-2161

Dougan, M. M., Fest, S., Cushing-Haugen, K., Farland, L. V., Chavarro, J., Harris, H. R., i Missmer, S. A. (2024). A prospective study of dietary patterns and the incidence of endometriosis diagnosis. *American Journal of Obstetrics and Gynecology*, *231*(4), 443.e1-443.e10. https://doi.org/10.1016/j.ajog.2024.04.030

Edwards, A. K., Nakamura, D. S., Virani, S., Wessels, J. M., i Tayade, C. (2013). Animal models for anti-angiogenic therapy in endometriosis. *Journal of Reproductive Immunology, 97*(1), 85–94. https://doi.org/10.1016/j.jri.2012.10.012

Ekarattanawong, S., Tanprasertkul, C., Somprasit, C., Chamod, P., Tiengtip, R., Bhamarapravatana, K., i Suwannarurk, K. (2017). Possibility of using superoxide dismutase and glutathione peroxidase as endometriosis biomarkers. *International Journal of Women's Health*, *9*, 711–716. https://doi.org/10.2147/IJWH.S141021

Elizur, S. E., Mostafa, J., Berkowitz, E., i Orvieto, R. (2025). Endometriosis and infertility: Pathophysiology, treatment strategies, and reproductive outcomes. *Archives of Gynecology and Obstetrics*. https://doi.org/10.1007/s00404-025-08124-1

Ellis, K., Munro, D., i Clarke, J. (2022). Endometriosis Is Undervalued: A Call to Action. *Frontiers in Global Women's Health*, *3*, 902371. https://doi.org/10.3389/fgwh.2022.902371

Feng, L., Yasmeen, R., Schoene, N. W., Lei, K. Y., i Wang, T. T. Y. (2019). Resveratrol differentially modulates immune responses in human THP-1 monocytes and macrophages. *Nutrition Research*, *72*, 57–69. https://doi.org/10.1016/j.nutres.2019.10.003

Ferrero, S., Alessandri, F., Racca, A., i Maggiore, U. L. R. (2015). Treatment of pain associated with deep endometriosis: Alternatives and evidence. *Fertility and Sterility*, *104*(4), 771–792. https://doi.org/10.1016/j.fertnstert.2015.08.031

Filby, C. E., Rombauts, L., Montgomery, G. W., Giudice, L. C., i Gargett, C. E. (2020). Cellular Origins of Endometriosis: Towards Novel Diagnostics and Therapeutics. *Seminars in Reproductive Medicine*, *38*(2–03), 201–215. https://doi.org/10.1055/s-0040-1713429

Freyberger, A., Hartmann, E., Hildebrand, H., i Krötlinger, F. (2001). Differential response of immature rat uterine tissue to ethinylestradiol and the red wine constituent resveratrol. *Archives of Toxicology*, *74*(11), 709–715. https://doi.org/10.1007/s002040000186

Gaggini, M., Fenizia, S., i Vassalle, C. (2023). Sphingolipid Levels and Signaling via Resveratrol and Antioxidant Actions in Cardiometabolic Risk and Disease. *Antioxidants*, *12*(5), 1102. https://doi.org/10.3390/antiox12051102

Gambini, J., Inglés, M., Olaso, G., Lopez-Grueso, R., Bonet-Costa, V., Gimeno-Mallench, L., Mas-Bargues, C., Abdelaziz, K. M., Gomez-Cabrera, M. C., Vina, J., i Borras, C. (2015). Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and

Humans. *Oxidative Medicine and Cellular Longevity*, *2015*, 837042. https://doi.org/10.1155/2015/837042

Garcia-Velasco, J. A., Mulayim, N., Kayisli, U. A., i Arici, A. (2002). Elevated soluble Fas ligand levels may suggest a role for apoptosis in women with endometriosis. *Fertility and Sterility*, *78*(4), 855–859. https://doi.org/10.1016/s0015-0282(02)03320-4

Gatouillat, G., Balasse, E., Joseph-Pietras, D., Morjani, H., i Madoulet, C. (2010). Resveratrol induces cell-cycle disruption and apoptosis in chemoresistant B16 melanoma. *Journal of Cellular Biochemistry*, *110*(4), 893–902. https://doi.org/10.1002/jcb.22601

Genin, M., Clement, F., Fattaccioli, A., Raes, M., i Michiels, C. (2015). M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer*, *15*, 577. https://doi.org/10.1186/s12885-015-1546-9

Gete, D. G., Doust, J., Mortlock, S., Montgomery, G., i Mishra, G. D. (2023). Associations between endometriosis and common symptoms: Findings from the Australian Longitudinal Study on Women's Health. *American Journal of Obstetrics and Gynecology*, *229*(5), 536.e1-536.e20. https://doi.org/10.1016/j.ajog.2023.07.033

Giudice, L. C., Horne, A. W., i Missmer, S. A. (2023). Time for global health policy and research leaders to prioritize endometriosis. *Nature Communications*, *14*(1), 8028. https://doi.org/10.1038/s41467-023-43913-9

Gołąbek, A., Kowalska, K., i Olejnik, A. (2021). Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives. *Nutrients*, *13*(4), 1347. https://doi.org/10.3390/nu13041347

Gołąbek-Grenda, A., Juzwa, W., Kaczmarek, M., i Olejnik, A. (2024). Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages. *Nutrients*, *16*(20), 3483. https://doi.org/10.3390/nu16203483

Gołąbek-Grenda, A., Kaczmarek, M., Juzwa, W., i Olejnik, A. (2023). Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways. *Scientific Reports*, *13*(1), 11468. https://doi.org/10.1038/s41598-023-38692-8

Gołąbek-Grenda, A., i Olejnik, A. (2022). In vitro modeling of endometriosis and endometriotic microenvironment—Challenges and recent advances. *Cellular Signalling*, *97*, 110375. https://doi.org/10.1016/j.cellsig.2022.110375

Gołąbek-Grenda, A., i Olejnik, A. (2026). Stilbene-based strategies for the management of endometriosis: Targeting cell adhesion, migration, and ECM-driven invasion. *Journal of Functional Foods*, *140*, 107277. https://doi.org/10.1016/j.jff.2026.107277

Griffiths, M. J., Horne, A. W., Gibson, D. A., Roberts, N., i Saunders, P. T. K. (2024). Endometriosis: Recent advances that could accelerate diagnosis and improve care. *Trends in Molecular Medicine*, *30*(9), 875–889. https://doi.org/10.1016/j.molmed.2024.06.008

Hankittichai, P., Thaklaewphan, P., Wikan, N., Ruttanapattanakul, J., Potikanond, S., Smith, D. R., i Nimlamool, W. (2023). Resveratrol Enhances Cytotoxic Effects of Cisplatin by Inducing Cell Cycle Arrest and Apoptosis in Ovarian Adenocarcinoma SKOV-3 Cells through Activating the p38 MAPK and Suppressing AKT. *Pharmaceuticals*, *16*(5), 755. https://doi.org/10.3390/ph16050755

Harris, H. R., Eke, A. C., Chavarro, J. E., i Missmer, S. A. (2018). Fruit and vegetable consumption and risk of endometriosis. *Human Reproduction (Oxford, England)*, *33*(4), 715–727. https://doi.org/10.1093/humrep/dey014

Hopeman, M. M., Riley, J. K., Frolova, A. I., Jiang, H., i Jungheim, E. S. (2015). Serum Polyunsaturated Fatty Acids and Endometriosis. *Reproductive Sciences*, *22*(9), 1083–1087. https://doi.org/10.1177/1933719114565030

Hsieh, T., Lu, X., Wang, Z., i Wu, J. M. (2006). Induction of quinone reductase NQO1 by resveratrol in human K562 cells involves the antioxidant response element ARE and is accompanied by nuclear translocation of transcription factor Nrf2. *Medicinal Chemistry*, *2*(3), 275–285. https://doi.org/10.2174/157340606776930709

Huang, H.-F., Hong, L.-H., Tan, Y., i Sheng, J.-Z. (2004). Matrix metalloproteinase 2 is associated with changes in steroid hormones in the sera and peritoneal fluid of patients with endometriosis. *Fertility and Sterility*, *81*(5), 1235–1239. https://doi.org/10.1016/j.fertnstert.2003.10.027

Ibrahim, M. A., Albahlol, I. A., Wani, F. A., Abd-Eltawab Tammam, A., Kelleni, M. T., Sayeed, M. U., Abd El-Fadeal, N. M., i Mohamed, A. A. (2021). Resveratrol protects against cisplatin-induced ovarian and uterine toxicity in female rats by attenuating oxidative stress, inflammation and apoptosis. *Chemico-Biological Interactions*, *338*, 109402. https://doi.org/10.1016/j.cbi.2021.109402

Jana, S., Chatterjee, K., Ray, A. K., DasMahapatra, P., i Swarnakar, S. (2016). Regulation of Matrix Metalloproteinase-2 Activity by COX-2-PGE2-pAKT Axis Promotes Angiogenesis in Endometriosis. *PLOS ONE*, *11*(10), e0163540. https://doi.org/10.1371/journal.pone.0163540

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., i Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, *275*(5297), 218–220. https://doi.org/10.1126/science.275.5297.218

Jin, P., Cai, J., Chen, N., Liu, Y., Zhao, H., Wang, Y., Chen, J., Li, M., Xiao, T., Shan, C., Yu, M., i Zhang, J. V. (2025). TGF-β/snail-mediated epithelial-to-mesenchymal transition disrupts estradiol metabolism through suppressing the HSD17B2 expression in endometriotic epithelial cells. *Biochemical and Biophysical Research Communications*, *771*, 151964. https://doi.org/10.1016/j.bbrc.2025.151964

Kadry, M. O. (2024). Resveratrol-based nano-formulations as an emerging therapeutic strategy for ovarian carcinoma: Autophagy stimulation and SIRT-1/Beclin/MMP-9/P53/AKT signaling. *Cancer Nanotechnology*, *15*(1), 36. https://doi.org/10.1186/s12645-024-00274-2

Karami, A., Fakhri, S., Kooshki, L., i Khan, H. (2022). Polydatin: Pharmacological Mechanisms, Therapeutic Targets, Biological Activities, and Health Benefits. *Molecules*, *27*(19), 6474. https://doi.org/10.3390/molecules27196474

Kawiak, A., i Kostecka, A. (2022). Regulation of Bcl-2 Family Proteins in Estrogen Receptor-Positive Breast Cancer and Their Implications in Endocrine Therapy. *Cancers*, *14*(2), 279. https://doi.org/10.3390/cancers14020279

Ke, J., Ye, J., Li, M., i Zhu, Z. (2021). The Role of Matrix Metalloproteinases in Endometriosis: A Potential Target. *Biomolecules*, *11*(11), 1739. https://doi.org/10.3390/biom11111739

Kędzia, M., Basta, P., Czajkowski, K., Gogacz, M., Spaczyński, R., Mroczkowska, B., Stojko, R., Szaflik, T., Szubert, M., Szyłło, K., Zaborowski, M., i Sieroszewski, P. (2024). Rekomendacje Polskiego Towarzystwa Ginekologów i Położników dotyczące postępowania u kobiet z endometriozą. *Ginekologia i Perinatologia Praktyczna*, *9*(2), 89–123.

Khattar, S., Khan, S. A., Zaidi, S. A. A., Darvishikolour, M., Farooq, U., Naseef, P. P., Kurunian, M. S., Khan, M. Z., Shamim, A., Khan, M. M. U., Iqbal, Z., i Mirza, Mohd. A. (2022). Resveratrol from Dietary Supplement to a Drug Candidate: An Assessment of Potential. *Pharmaceuticals*, *15*(8), 957. https://doi.org/10.3390/ph15080957

Khodarahmian, M., Amidi, F., Moini, A., Kashani, L., Salahi, E., Danaii-Mehrabad, S., Nashtaei, M. S., Mojtahedi, M. F., Esfandyari, S., i Sobhani, A. (2021). A randomized exploratory trial to assess the effects of resveratrol on VEGF and TNF-α 2 expression in endometriosis women. *Journal of Reproductive Immunology*, *143*, 103248. https://doi.org/10.1016/j.jri.2020.103248

Kim, H. J., Lee, H.-S., Kazmi, S. Z., Hann, H. J., Kang, T., Cha, J., Choi, S., Swan, H., Kim, H., Lee, Y. S., i Ahn, H. S. (2021). Familial risk for endometriosis and its interaction with smoking, age at menarche and body mass index: A population-based cohort study among siblings. *BJOG: An International Journal of Obstetrics and Gynaecology*, *128*(12), 1938–1948. https://doi.org/10.1111/1471-0528.16769

Ko, H. S., Kim, J. S., Cho, S. M., Lee, H.-J., Ahn, K. S., Kim, S.-H., i Lee, E.-O. (2014). Urokinase-type plasminogen activator expression and Rac1/WAVE-2/Arp2/3 pathway are blocked by pterostilbene to suppress cell migration and invasion in MDA-MB-231 cells. *Bioorganic & Medicinal Chemistry Letters*, *24*(4), 1176–1179. https://doi.org/10.1016/j.bmcl.2013.12.115

Ko, J.-H., Sethi, G., Um, J.-Y., Shanmugam, M. K., Arfuso, F., Kumar, A. P., Bishayee, A., i Ahn, K. S. (2017). The Role of Resveratrol in Cancer Therapy. *International Journal of Molecular Sciences*, *18*(12), 2589. https://doi.org/10.3390/ijms18122589

Kobayashi, H., i Imanaka, S. (2022). Understanding the molecular mechanisms of macrophage polarization and metabolic reprogramming in endometriosis: A narrative review. *Reproductive Medicine and Biology*, *21*(1), e12488. https://doi.org/10.1002/rmb2.12488

Kodarahmian, M., Amidi, F., Moini, A., Kashani, L., Shabani Nashtaei, M., Pazhohan, A., Bahramrezai, M., Berenjian, S., i Sobhani, A. (2019). The modulating effects of Resveratrol on the expression of MMP-2 and MMP-9 in endometriosis women: A randomized exploratory trial. *Gynecological Endocrinology,35*(8), 719–726. https://doi.org/10.1080/09513590.2019.1576612

Koks, C. A., Groothuis, P. G., Dunselman, G. A., de Goeij, A. F., i Evers, J. L. (2000). Adhesion of menstrual endometrium to extracellular matrix: The possible role of integrin alpha(6)beta(1) and laminin interaction. *Molecular Human Reproduction*, *6*(2), 170–177. https://doi.org/10.1093/molehr/6.2.170

Kolahdouz Mohammadi, R., Delbandi, A.-A., Khodaverdi, S., Arefi, S., Arablou, T., i Shidfar, F. (2020). The Effects of Resveratrol Treatment on Bcl-2 and Bax Gene Expression in Endometriotic Compared with Non-Endometriotic Stromal Cells. *Iranian Journal of Public Health*, *49*(8), 1546–1554. https://doi.org/10.18502/ijph.v49i8.3900

Komorowska, D., Gajewska, A., Hikisz, P., Bartosz, G., i Rodacka, A. (2021). Comparison of the Effects of Resveratrol and Its Derivatives on the Radiation Response of MCF-7 Breast Cancer Cells. *International Journal of Molecular Sciences*, *22*(17), 9511. https://doi.org/10.3390/ijms22179511

Kong, X., Xu, X., Zhou, L., Zhu, M., Yao, S., Ding, Y., Liu, T., Wang, Y., Zhang, Y., Li, R., Tang, X., Ling, J., Wu, J., Zhu, X., Gu, Y., i Zhou, H. (2020). MTA1, a Target of Resveratrol, Promotes Epithelial-Mesenchymal Transition of Endometriosis via ZEB2. *Molecular Therapy Methods & Clinical Development*, *19*, 295–306. https://doi.org/10.1016/j.omtm.2020.09.013

Kozłowska, M., i Czekała, Ł. (2017). Stilbenes and their role in disease resistance. *Progress in Plant Protection*, *57*(1), 27–35. https://doi.org/10.14199/ppp-2017-004

Kwon, J. Y., Seo, S. G., Heo, Y.-S., Yue, S., Cheng, J.-X., Lee, K. W., i Kim, K.-H. (2012). Piceatannol, Natural Polyphenolic Stilbene, Inhibits Adipogenesis via Modulation of Mitotic Clonal Expansion and Insulin Receptor-dependent Insulin Signaling in Early Phase of Differentiation *. *Journal of Biological Chemistry*, *287*(14), 11566–11578. https://doi.org/10.1074/jbc.M111.259721

Laganà, A. S., Garzon, S., Götte, M., Viganò, P., Franchi, M., Ghezzi, F., i Martin, D. C. (2019). The Pathogenesis of Endometriosis: Molecular and Cell Biology Insights. *International Journal of Molecular Sciences*, *20*(22), 5615. https://doi.org/10.3390/ijms20225615

Laganà, A. S., Salmeri, F. M., Ban Frangež, H., Ghezzi, F., Vrtačnik-Bokal, E., i Granese, R. (2020). Evaluation of M1 and M2 macrophages in ovarian endometriomas from women affected by endometriosis at different stages of the disease. *Gynecological Endocrinology*, *36*(5), 441–444. https://doi.org/10.1080/09513590.2019.1683821

Lan, T., Zhuang, L., Li, S., Yang, G., Xuan, Y., i Guo, J. (2020). Polydatin attenuates hepatic stellate cell proliferation and liver fibrosis by suppressing sphingosine kinase 1. *Biomedicine & Pharmacotherapy*, *130*, 110586. https://doi.org/10.1016/j.biopha.2020.110586

Lanzilli, G., Cottarelli, A., Nicotera, G., Guida, S., Ravagnan, G., i Fuggetta, M. P. (2012). Anti-inflammatory Effect of Resveratrol and Polydatin by In Vitro IL-17 Modulation. *Inflammation*, *35*(1), 240–248. https://doi.org/10.1007/s10753-011-9310-z

Lee, J., Yeo, S. G., Lee, J. M., Kim, S. S., Lee, J.-W., Chung, N., i Park, D. C. (2025). Expression of Free Radicals and Reactive Oxygen Species in Endometriosis: Current Knowledge and Its Implications. *Antioxidants*, *14*(7), 877. https://doi.org/10.3390/antiox14070877

Lee, M.-F., Pan, M.-H., Chiou, Y.-S., Cheng, A.-C., i Huang, H. (2011). Resveratrol modulates MED28 (Magicin/EG-1) expression and inhibits epidermal growth factor (EGF)-induced migration in MDA-MB-231 human breast cancer cells. *Journal of Agricultural and Food Chemistry*, *59*(21), 11853–11861. https://doi.org/10.1021/jf202426k

Lee, Y., i Im, E. (2021). Regulation of miRNAs by Natural Antioxidants in Cardiovascular Diseases: Focus on SIRT1 and eNOS. *Antioxidants*, *10*(3), 377. https://doi.org/10.3390/antiox10030377

Lessey, B. A., i Kim, J. J. (2017). Endometrial Receptivity in Eutopic Endometrium of Women with Endometriosis It is affected, let me show you why. *Fertility and sterility*, *108*(1), 19–27. https://doi.org/10.1016/j.fertnstert.2017.05.031

Li, R., Zhang, L., i Liu, Y. (2025). Global and regional trends in the burden of surgically confirmed endometriosis from 1990 to 2021. *Reproductive Biology and Endocrinology*, *23*(1), 88. https://doi.org/10.1186/s12958-025-01421-z

Lin, W.-S., Leland, J. V., Ho, C.-T., i Pan, M.-H. (2020). Occurrence, Bioavailability, Anti-inflammatory, and Anticancer Effects of Pterostilbene. *Journal of Agricultural and Food Chemistry*, *68*(46), 12788–12799. https://doi.org/10.1021/acs.jafc.9b07860

Liu, H., i Lang, J. H. (2011). Is abnormal eutopic endometrium the cause of endometriosis? The role of eutopic endometrium in pathogenesis of endometriosis. *Medical Science Monitor*, *17*(4), RA92–RA99. https://doi.org/10.12659/MSM.881707

Liu, P., Maharjan, R., Wang, Y., Zhang, Y., Zhang, Y., Xu, C., Geng, Y., i Miao, J. (2023). Association between dietary inflammatory index and risk of endometriosis: A population-based analysis. *Frontiers in Nutrition*, *10*, 1077915. https://doi.org/10.3389/fnut.2023.1077915

Liu, P., Tang, W., Xiang, K., i Li, G. (2024). Pterostilbene in the treatment of inflammatory and oncological diseases. *Frontiers in Pharmacology*, *14*, 1323377. https://doi.org/10.3389/fphar.2023.1323377

Liu, T., Liu, Y., Li, L., Liu, X., Guo, Z., Cheng, J., Zhu, X., Lu, L., Zhang, J., Fan, G., Xie, N., Lu, J., i Jiang, H. (2021). De Novo Biosynthesis of Polydatin in Saccharomyces cerevisiae. *Journal of Agricultural and Food Chemistry*, *69*(21), 5917–5925. https://doi.org/10.1021/acs.jafc.1c01557

Liu, Y., You, Y., Lu, J., Chen, X., i Yang, Z. (2020). Recent Advances in Synthesis, Bioactivity, and Pharmacokinetics of Pterostilbene, an Important Analog of Resveratrol. *Molecules*, *25*(21), 5166. https://doi.org/10.3390/molecules25215166

Livak, K. J., i Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Logan, P. C., Ponnampalam, A. P., Steiner, M., i Mitchell, M. D. (2013). Effect of cyclic AMP and estrogen/progesterone on the transcription of DNA methyltransferases during the decidualization of

human endometrial stromal cells. *Molecular Human Reproduction*, *19*(5), 302–312. https://doi.org/10.1093/molehr/gas062

Lousse, J.-C., Van Langendonckt, A., Defrere, S., Ramos, R. G., Colette, S., i Donnez, J. (2012). Peritoneal endometriosis is an inflammatory disease. *Frontiers in Bioscience*, *4*, 23–40. https://doi.org/10.2741/358

Luo, J., Chen, S., Wang, L., Zhao, X., i Piao, C. (2022). Pharmacological effects of polydatin in the treatment of metabolic diseases: A review. *Phytomedicine*, *102*, 154161. https://doi.org/10.1016/j.phymed.2022.154161

Ma, Z., Zhang, X., Xu, L., Liu, D., Di, S., Li, W., Zhang, J., Zhang, H., Li, X., Han, J., i Yan, X. (2019). Pterostilbene: Mechanisms of its action as oncostatic agent in cell models and *in vivo* studies. *Pharmacological Research*, *145*, 104265. https://doi.org/10.1016/j.phrs.2019.104265

Madanes, D., Meresman, G., Valla, S. A., Hassan, N., Kiesel, L., Greve, B., Barañao, R. I., Götte, M., & Ricci, A. G. (2022). Resveratrol impairs cellular mechanisms associated with the pathogenesis of endometriosis. *Reproductive biomedicine online*, *44*(6), 976–990. https://doi.org/10.1016/j.rbmo.2022.02.008

Madjid, T. H., Ardiansyah, D. F., Permadi, W., i Hernowo, B. (2020). Expression of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 in Endometriosis Menstrual Blood. *Diagnostics*, *10*(6), 364. https://doi.org/10.3390/diagnostics10060364

Maia, H., Haddad, C., Pinheiro, N., i Casoy, J. (2012). Advantages of the association of resveratrol with oral contraceptives for management of endometriosis-related pain. *International Journal of Women's Health*, *4*, 543–549. https://doi.org/10.2147/IJWH.S36825

Malik, H., Zamouri, S., Akkawi, S., Mehra, S., Mouaki, R., Sathyapalan, T., Nandakumar, M., Butler, A. E., i Atkin, S. L. (2025). Endothelial Protein Changes Indicative of Endometriosis in Unexplained Infertility, an Exploratory Study. *International Journal of Molecular Sciences*, *26*(13), 6485. https://doi.org/10.3390/ijms26136485

Malvezzi, H., Marengo, E. B., Podgaec, S., i Piccinato, C. de A. (2020). Endometriosis: Current challenges in modeling a multifactorial disease of unknown etiology. *Journal of Translational Medicine*, *18*(1), 311. https://doi.org/10.1186/s12967-020-02471-0

Marchisio, L., Gaudillat, Q., Muller, J., Zedet, A., Tissot, M., Harakat, D., Sénéjoux, F., Rolin, G., Cardey, B., Girard, C., i Pudlo, M. (2025). Synthesis and evaluation of piceatannol derivatives as novel arginase inhibitors with radical scavenging activity and their potential for collagen reduction in dermal fibroblasts. *European Journal of Medicinal Chemistry*, *287*, 117376. https://doi.org/10.1016/j.ejmech.2025.117376

Marziali, M., Venza, M., Lazzaro, S., Lazzaro, A., Micossi, C., i Stolfi, V. M. (2012). Gluten-free diet: A new strategy for management of painful endometriosis related symptoms? *Minerva Chirurgica*, *67*(6), 499–504.

McCormack, D., i McFadden, D. (2013). A review of pterostilbene antioxidant activity and disease modification. *Oxidative Medicine and Cellular Longevity*, *2013*, 575482. https://doi.org/10.1155/2013/575482

Medrano-Padial, C., Prieto, A. I., Puerto, M., i Pichardo, S. (2021). Toxicological Evaluation of Piceatannol, Pterostilbene, and ε-Viniferin for Their Potential Use in the Food Industry: A Review. *Foods*, *10*(3), 592. https://doi.org/10.3390/foods10030592

Mehdizadehkashi, A., Rokhgireh, S., Tahermanesh, K., Eslahi, N., Minaeian, S., i Samimi, M. (2021). The effect of vitamin D supplementation on clinical symptoms and metabolic profiles in patients with endometriosis. *Gynecological Endocrinology*, *37*(7), 640–645. https://doi.org/10.1080/09513590.2021.1878138

Mekinić, I. G., Skroza, D., Ljubenkov, I., i Katalinić, V. (2016). Insight into the Presence of Stilbenes in Medicinal Plants Traditionally Used in Croatian Folk Medicine. *Natural Product Communications*, *11*(6), 833–835.

Mendonça, E. L. S. S., Xavier, J. A., Fragoso, M. B. T., Silva, M. O., Escodro, P. B., Oliveira, A. C. M., Tucci, P., Saso, L., i Goulart, M. O. F. (2024). E-Stilbenes: General Chemical and Biological Aspects, Potential Pharmacological Activity Based on the Nrf2 Pathway. *Pharmaceuticals*, *17*(2), 232. https://doi.org/10.3390/ph17020232

Mikuła-Pietrasik, J., Sosińska, P., i Książek, K. (2014). Resveratrol inhibits ovarian cancer cell adhesion to peritoneal mesothelium in vitro by modulating the production of α5β1 integrins and hyaluronic acid. *Gynecologic Oncology*, *134*(3), 624–630. https://doi.org/10.1016/j.ygyno.2014.06.022

Missmer, S. A., Chavarro, J. E., Malspeis, S., Bertone-Johnson, E. R., Hornstein, M. D., Spiegelman, D., Barbieri, R. L., Willett, W. C., i Hankinson, S. E. (2010). A prospective study of dietary fat consumption and endometriosis risk. *Human Reproduction*, *25*(6), 1528–1535. https://doi.org/10.1093/humrep/deq044

Missmer, S. A., Hankinson, S. E., Spiegelman, D., Barbieri, R. L., Malspeis, S., Willett, W. C., i Hunter, D. J. (2004). Reproductive history and endometriosis among premenopausal women. *Obstetrics and Gynecology*, *104*(5 Pt 1), 965–974. https://doi.org/10.1097/01.AOG.0000142714.54857.f8

Missmer, S. A., Hankinson, S. E., Spiegelman, D., Barbieri, R. L., Marshall, L. M., i Hunter, D. J. (2004). Incidence of laparoscopically confirmed endometriosis by demographic, anthropometric, and lifestyle factors. *American Journal of Epidemiology*, *160*(8), 784–796. https://doi.org/10.1093/aje/kwh275

Mitchell, J.-B., Chetty, S., i Kathrada, F. (2022). Progestins in the symptomatic management of endometriosis: A meta-analysis on their effectiveness and safety. *BMC Women's Health*, *22*, 526. https://doi.org/10.1186/s12905-022-02122-0

Monmai, C., i Baek, S.-H. (2024). Anti-Inflammatory Effects of the Combined Treatment of Resveratrol- and Protopanaxadiol-Enriched Rice Seed Extract on Lipopolysaccharide-Stimulated RAW264.7 Cells. *Molecules*, *29*(18), 4343. https://doi.org/10.3390/molecules29184343

Moore, J. S., Gibson, P. R., Perry, R. E., i Burgell, R. E. (2017). Endometriosis in patients with irritable bowel syndrome: Specific symptomatic and demographic profile, and response to the low FODMAP diet. *The Australian & New Zealand Journal of Obstetrics & Gynaecology*, *57*(2), 201–205. https://doi.org/10.1111/ajo.12594

Moradi, Y., Shams-Beyranvand, M., Khateri, S., Gharahjeh, S., Tehrani, S., Varse, F., Tiyuri, A., i Najmi, Z. (2021). A systematic review on the prevalence of endometriosis in women. *The Indian Journal of Medical Research*, *154*(3), 446–454. https://doi.org/10.4103/ijmr.IJMR_817_18

Morales, P., i Haza, A. I. (2012). Selective apoptotic effects of piceatannol and myricetin in human cancer cells. *Journal of Applied Toxicology: JAT*, *32*(12), 986–993. https://doi.org/10.1002/jat.1725

Mulgund, A., Doshi, S., i Agarwal, A. (2015). Chapter 25—The Role of Oxidative Stress in Endometriosis. W R. R. Watson (Red.), *Handbook of Fertility* (s. 273–281). Academic Press. https://doi.org/10.1016/B978-0-12-800872-0.00025-1

Nagarajan, S., Mohandas, S., Ganesan, K., Xu, B., i Ramkumar, K. M. (2022). New Insights into Dietary Pterostilbene: Sources, Metabolism, and Health Promotion Effects. *Molecules*, *27*(19), 6316. https://doi.org/10.3390/molecules27196316

Nisolle, M., Casanas-Roux, F., i Donnez, J. (2000). Early-stage endometriosis: Adhesion and growth of human menstrual endometrium in nude mice. *Fertility and Sterility*, *74*(2), 306–312. https://doi.org/10.1016/s0015-0282(00)00601-4

Nodler, J. L., Harris, H. R., Chavarro, J. E., Frazier, A. L., i Missmer, S. A. (2020). Dairy consumption during adolescence and endometriosis risk. *American journal of obstetrics and gynecology*, *222*(3), 257.e1-257.e16. https://doi.org/10.1016/j.ajog.2019.09.010

Nunes, S., Danesi, F., Rio, D. D., i Silva, P. (2018). Resveratrol and inflammatory bowel disease: The evidence so far. *Nutrition Research Reviews*, *31*(1), 85–97. https://doi.org/10.1017/S095442241700021X

Oseni, T., Patel, R., Pyle, J., i Jordan, V. C. (2008). Selective Estrogen Receptor Modulators and Phytoestrogens. *Planta medica*, *74*(13), 1656–1665. https://doi.org/10.1055/s-0028-1088304

Pan, J., Shi, M., Li, L., Liu, J., Guo, F., Feng, Y., Ma, L., i Fu, P. (2019). Pterostilbene, a bioactive component of blueberries, alleviates renal fibrosis in a severe mouse model of hyperuricemic nephropathy. *Biomedicine & Pharmacotherapy*, *109*, 1802–1808. https://doi.org/10.1016/j.biopha.2018.11.022

Papier, K., Hartman, L., Tong, T. Y. N., Key, T. J., i Knuppel, A. (2021). Higher Meat Intake Is Associated with Higher Inflammatory Markers, Mostly Due to Adiposity: Results from UK Biobank. *The Journal of Nutrition*, *152*(1), 183–189. https://doi.org/10.1093/jn/nxab314

Parazzini, F., Chiaffarino, F., Surace, M., Chatenoud, L., Cipriani, S., Chiantera, V., Benzi, G., i Fedele, L. (2004). Selected food intake and risk of endometriosis. *Human Reproduction*, *19*(8), 1755–1759. https://doi.org/10.1093/humrep/deh395

Piotrowska, H., Kucinska, M., i Murias, M. (2012). Biological activity of piceatannol: Leaving the shadow of resveratrol. *Mutation Research*, *750*(1), 60–82. https://doi.org/10.1016/j.mrrev.2011.11.001

Pollock, K., Jaraczewski, T. J., Carroll, M. J., Lebovic, D. I., i Kreeger, P. K. (2014). Endometriotic Epithelial Cell Response to Macrophage-Secreted Factors is Dependent on Extracellular Matrix Context. *Cellular and molecular bioengineering*, *7*(3), 409–420. https://doi.org/10.1007/s12195-014-0339-6

Porpora, M. G., Brunelli, R., Costa, G., Imperiale, L., Krasnowska, E. K., Lundeberg, T., Nofroni, I., Piccioni, M. G., Pittaluga, E., Ticino, A., i Parasassi, T. (2013). A Promise in the Treatment of Endometriosis: An Observational Cohort Study on Ovarian Endometrioma Reduction by N-Acetylcysteine. *Evidence-based Complementary and Alternative Medicine : eCAM*, *2013*, 240702. https://doi.org/10.1155/2013/240702

Pozo-Guisado, E., Merino, J. M., Mulero-Navarro, S., Lorenzo-Benayas, M. J., Centeno, F., Alvarez-Barrientos, A., i Fernandez-Salguero, P. M. (2005). Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kappaB. *International Journal of Cancer*, *115*(1), 74–84. https://doi.org/10.1002/ijc.20856

Qasem, R. J. (2020). The estrogenic activity of resveratrol: A comprehensive review of in vitro and in vivo evidence and the potential for endocrine disruption. *Critical Reviews in Toxicology*, *50*(5), 439–462. https://doi.org/10.1080/10408444.2020.1762538

Qi, Q., Li, Y., Chen, Z., Luo, Z., Zhou, T., Zhou, J., Zhang, Y., Chen, S., i Wang, L. (2025). Update on the pathogenesis of endometriosis-related infertility based on contemporary evidence. *Frontiers in Endocrinology*, *16,* 1558271. https://doi.org/10.3389/fendo.2025.1558271

Rahmioglu, N., i Zondervan, K. T. (2024). Endometriosis: Disease mechanisms and health disparities. *Bulletin of the World Health Organization*, *102*(12), 919–921. https://doi.org/10.2471/BLT.24.292660

Rajan, R. K., Engels, M., i Ramanathan, M. (2024). Predicting phase-I metabolism of piceatannol: An in silico study. *In Silico Pharmacology*, *12*(1), 52. https://doi.org/10.1007/s40203-024-00228-x

Ramírez-Pavez, T. N., Martínez-Esparza, M., Ruiz-Alcaraz, A. J., Marín-Sánchez, P., Machado-Linde, F., i García-Peñarrubia, P. (2021). The Role of Peritoneal Macrophages in Endometriosis. *International Journal of Molecular Sciences*, *22*(19), 10792. https://doi.org/10.3390/ijms221910792

Reis, F. M., Coutinho, L. M., Vannuccini, S., Batteux, F., Chapron, C., i Petraglia, F. (2020). Progesterone receptor ligands for the treatment of endometriosis: The mechanisms behind therapeutic success and failure. *Human Reproduction Update*, *26*(4), 565–585. https://doi.org/10.1093/humupd/dmaa009

Renaud, S., i de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet*, *339*(8808), 1523–1526. https://doi.org/10.1016/0140-6736(92)91277-f

Robertson, I., Wai Hau, T., Sami, F., Sajid Ali, M., Badgujar, V., Murtuja, S., Saquib Hasnain, M., Khan, A., Majeed, S., i Tahir Ansari, M. (2022). The science of resveratrol, formulation, pharmacokinetic barriers and its chemotherapeutic potential. *International Journal of Pharmaceutics*, *618*, 121605. https://doi.org/10.1016/j.ijpharm.2022.121605

Rocha, A. L. L., Reis, F. M., i Taylor, R. N. (2013). Angiogenesis and Endometriosis. *Obstetrics and Gynecology International*, *2013*, 859619. https://doi.org/10.1155/2013/859619

Rosiak, N., Tykarska, E., i Cielecka-Piontek, J. (2023). Amorphous Pterostilbene Delivery Systems Preparation—Innovative Approach to Preparation Optimization. *Pharmaceutics*, *15*(4), 1231. https://doi.org/10.3390/pharmaceutics15041231

Sabolová, G., Špaková, I., Artimovič, P., Bohuš, P., Rabajdová, M., i Mareková, M. (2024). The Pivotal Role of the Key Angiogenic Factors in the Development of Endometrioid Pathologies of the Uterus and Ovary. *Cancers*, *16*(16), 2772. https://doi.org/10.3390/cancers16162772

Salla, M., Karaki, N., El Kaderi, B., Ayoub, A. J., Younes, S., Abou Chahla, M. N., Baksh, S., i El Khatib, S. (2024). Enhancing the Bioavailability of Resveratrol: Combine It, Derivatize It, or Encapsulate It? *Pharmaceutics*, *16*(4), 569. https://doi.org/10.3390/pharmaceutics16040569

Sapkota, Y., Steinthorsdottir, V., Morris, A. P., Fassbender, A., Rahmioglu, N., De Vivo, I., Buring, J. E., Zhang, F., Edwards, T. L., Jones, S., O, D., Peterse, D., Rexrode, K. M., Ridker, P. M., Schork, A. J., MacGregor, S., Martin, N. G., Becker, C. M., Adachi, S., … Nyholt, D. R. (2017). Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. *Nature Communications*, *8*, 15539. https://doi.org/10.1038/ncomms15539

Savio, M., Ferraresi, A., Corpina, C., Vandenberghe, S., Scarlata, C., Sottile, V., Morini, L., Garavaglia, B., Isidoro, C., i Stivala, L. A. (2022). Resveratrol and Its Analogue 4,4′-Dihydroxy-trans-stilbene Inhibit Lewis Lung Carcinoma Growth In Vivo through Apoptosis, Autophagy and Modulation of the Tumour Microenvironment in a Murine Model. *Biomedicines*, *10*(8), 1784. https://doi.org/10.3390/biomedicines10081784

Sbracia, M., Valeri, C., Antonini, G., Biagiotti, G., Pacchiarotti, A., i Pacchiarotti, A. (2016). Fas and Fas-Ligand in Eutopic and Ectopic Endometrium of Women With Endometriosis: The Possible Immune Privilege of Ectopic Endometrium. *Reproductive Sciences*, *23*(1), 81–86. https://doi.org/10.1177/1933719115594019

Schindler, C. K., Pearson, E. G., Bonner, H. P., So, N. K., Simon, R. P., Prehn, J. H., i Henshall, D. C. (2006). Caspase-3 Cleavage and Nuclear Localization of Caspase-Activated DNase in Human Temporal Lobe Epilepsy. *Journal of Cerebral Blood Flow & Metabolism*, *26*(4), 583–589. https://doi.org/10.1038/sj.jcbfm.9600219

Schwager, J., Richard, N., Widmer, F., i Raederstorff, D. (2017). Resveratrol distinctively modulates the inflammatory profiles of immune and endothelial cells. *BMC Complementary and Alternative Medicine*, *17*, 309. https://doi.org/10.1186/s12906-017-1823-z

Sekulovski, N., Whorton, A. E., Shi, M., MacLean, J. A., i Hayashi, K. (2019). Endometriotic inflammatory microenvironment induced by macrophages can be targeted by niclosamide†. *Biology of Reproduction*, *100*(2), 398–408. https://doi.org/10.1093/biolre/ioy222

Shafrir, A. L., Farland, L. V., Shah, D. K., Harris, H. R., Kvaskoff, M., Zondervan, K., i Missmer, S. A. (2018). Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, *51*, 1–15. https://doi.org/10.1016/j.bpobgyn.2018.06.001

Shah, M. A., Hamid, A., Faheem, H. I., Rasul, A., Baokbah, T. A. S., Haris, M., Yousaf, R., Saleem, U., Iqbal, S., Alves, M. S., Khan, Z., Hussain, G., Alsharfi, I., Khan, H., i Jeandet, P. (2022). Uncovering the Anticancer Potential of Polydatin: A Mechanistic Insight. *Molecules*, *27*(21), 7175. https://doi.org/10.3390/molecules27217175

Sharif, O., Bolshakov, V. N., Raines, S., Newham, P., i Perkins, N.D.(2007). Transcriptional profiling of the LPS induced NF-κB response in macrophages.*BMC Immunology*, *8*,1. https://doi.org/10.1186/1471-2172-8-1

Shin, H. J., Han, J. M., Choi, Y. S., i Jung, H. J. (2020). Pterostilbene Suppresses both Cancer Cells and Cancer Stem-Like Cells in Cervical Cancer with Superior Bioavailability to Resveratrol. *Molecules*, *25*(1), 228. https://doi.org/10.3390/molecules25010228

Sienko, A., Cichosz, A., Urban, A., Smolarczyk, R., Czajkowski, K., i Sienko, J. (2024). The effect of two anti-inflammatory dietary components, omega-3 and resveratrol, on endometriosis. *Ginekologia Polska*, *95*(7), 573–583. https://doi.org/10.5603/gpl.97573

Signorile, P. G., Viceconte, R., i Baldi, A. (2018). Novel dietary supplement association reduces symptoms in endometriosis patients. *Journal of Cellular Physiology*, *233*(8), 5920–5925. https://doi.org/10.1002/jcp.26401

Singh, P., Carraher, C., i Schwarzbauer, J. E. (2010). Assembly of fibronectin extracellular matrix. *Annual Review of Cell and Developmental Biology*, *26*, 397–419. https://doi.org/10.1146/annurev-cellbio-100109-104020

Smith, M. P., Young, H., Hurlstone, A., i Wellbrock, C. (2015). Differentiation of THP1 Cells into Macrophages for Transwell Co-culture Assay with Melanoma Cells. *Bio-protocol*, *5*(21), e1638. https://doi.org/10.21769/bioprotoc.1638

Smolarz, B., Szyłło, K., i Romanowicz, H. (2020). The Genetic Background of Endometriosis: Can ESR2 and CYP19A1 Genes Be a Potential Risk Factor for Its Development? *International Journal of Molecular Sciences*, *21*(21). https://doi.org/10.3390/ijms21218235

Smolarz, B., Szyłło, K., i Romanowicz, H. (2021). Endometriosis: Epidemiology, Classification, Pathogenesis, Treatment and Genetics (Review of Literature). *International Journal of Molecular Sciences*, *22*(19), 10554. https://doi.org/10.3390/ijms221910554

Smoliga, J. M., i Blanchard, O. (2014). Enhancing the Delivery of Resveratrol in Humans: If Low Bioavailability is the Problem, What is the Solution? *Molecules*, *19*(11), 17154–17172. https://doi.org/10.3390/molecules191117154

Song, B., Wang, W., Tang, X., Goh, R. M. W.-J., Thuya, W. L., Ho, P. C. L., Chen, L., i Wang, L. (2023). Inhibitory Potential of Resveratrol in Cancer Metastasis: From Biology to Therapy. *Cancers*, *15*(10), 2758. https://doi.org/10.3390/cancers15102758

Song, Z., Han, S., Pan, X., Gong, Y., i Wang, M. (2015). Pterostilbene mediates neuroprotection against oxidative toxicity via oestrogen receptor α signalling pathways. *The Journal of Pharmacy and Pharmacology*, *67*(5), 720–730. https://doi.org/10.1111/jphp.12360

Spuijbroek, M. D., Dunselman, G. A., Menheere, P. P., i Evers, J. L. (1992). Early endometriosis invades the extracellular matrix. *Fertility and Sterility*, *58*(5), 929–933. https://doi.org/10.1016/s0015-0282(16)55437-5

Stejskalová, A., Fincke, V., Nowak, M., Schmidt, Y., Borrmann, K., von Wahlde, M.-K., Schäfer, S. D., Kiesel, L., Greve, B., i Götte, M. (2021). Collagen I triggers directional migration, invasion and matrix remodeling of stroma cells in a 3D spheroid model of endometriosis. *Scientific Reports*, *11*(1), 4115. https://doi.org/10.1038/s41598-021-83645-8

Stochino Loi, E., Pontis, A., Cofelice, V., Pirarba, S., Fais, M. F., Daniilidis, A., Melis, I., Paoletti, A. M., i Angioni, S. (2019). Effect of ultramicronized-palmitoylethanolamide and co-micronized palmitoylethanolamide/polydatin on chronic pelvic pain and quality of life in endometriosis patients: An open-label pilot study. *International Journal of Women's Health*, *11*, 443–449. https://doi.org/10.2147/IJWH.S204275

Su, D., Cheng, Y., Liu, M., Liu, D., Cui, H., Zhang, B., Zhou, S., Yang, T., i Mei, Q. (2013). Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro. *PLOS ONE*, *8*(1), e54505. https://doi.org/10.1371/journal.pone.0054505

Sun, H., Li, D., Wei, C., Liu, L., Xin, Z., Gao, H., i Gao, R. (2024). The relationship between SIRT1 and inflammation: A systematic review and meta-analysis. *Frontiers in Immunology*, *15*, 1465849. https://doi.org/10.3389/fimmu.2024.1465849

Szymański, M., Bonowicz, K., Antosik, P., Jerka, D., Głowacka, M., Soroka, M., Steinbrink, K., Kleszczyński, K., i Gagat, M. (2024). Role of Cyclins and Cytoskeletal Proteins in Endometriosis: Insights into Pathophysiology. *Cancers*, *16*(4), 836. https://doi.org/10.3390/cancers16040836

Taguchi, A., Koga, K., Kawana, K., Makabe, T., Sue, F., Miyashita, M., Yoshida, M., Urata, Y., Izumi, G., Tkamura, M., Harada, M., Hirata, T., Hirota, Y., Wada-Hiraike, O., Fujii, T., i Osuga, Y. (2016). Resveratrol Enhances Apoptosis in Endometriotic Stromal Cells. *American Journal of Reproductive Immunology*, *75*(4), 486–492. https://doi.org/10.1111/aji.12489

Tassinari, V., Smeriglio, A., Stillittano, V., Trombetta, D., Zilli, R., Tassinari, R., Maranghi, F., Frank, G., Marcoccia, D., i Di Renzo, L. (2023). Endometriosis Treatment: Role of Natural Polyphenols as Anti-Inflammatory Agents. *Nutrients*, *15*(13), 2967. https://doi.org/10.3390/nu15132967

Teka, T., Zhang, L., Ge, X., Li, Y., Han, L., i Yan, X. (2022). Stilbenes: Source plants, chemistry, biosynthesis, pharmacology, application and problems related to their clinical Application-A comprehensive review. *Phytochemistry*, *197*, 113128. https://doi.org/10.1016/j.phytochem.2022.113128

Tian, B., i Liu, J. (2020). Resveratrol: A review of plant sources, synthesis, stability, modification and food application. *Journal of the Science of Food and Agriculture*, *100*(4), 1392–1404. https://doi.org/10.1002/jsfa.10152

Tolomeo, M., Grimaudo, S., Cristina, A. D., Roberti, M., Pizzirani, D., Meli, M., Dusonchet, L., Gebbia, N., Abbadessa, V., Crosta, L., Barucchello, R., Grisolia, G., Invidiata, F., i Simoni, D. (2005). Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells. *The International Journal of Biochemistry & Cell Biology*, *37*(8), 1709–1726. https://doi.org/10.1016/j.biocel.2005.03.004

Tomassetti, C., Johnson, N. P., Petrozza, J., Abrao, M. S., Einarsson, J. I., Horne, A. W., Lee, T. T. M., Missmer, S., Vermeulen, N., Zondervan, K. T., Grimbizis, G., i De Wilde, R. L. (2021). An international terminology for endometriosis, 2021†,‡. *Human Reproduction Open*, *2021*(4), hoab029. https://doi.org/10.1093/hropen/hoab029

Tong, W., Chen, X., Song, X., Chen, Y., Jia, R., Zou, Y., Li, L., Yin, L., He, C., Liang, X., Ye, G., Lv, C., Lin, J., i Yin, Z. (2020). Resveratrol inhibits LPS-induced inflammation through suppressing the signaling cascades of TLR4-NF-κB/MAPKs/IRF3. *Experimental and Therapeutic Medicine*, *19*(3), 1824–1834. https://doi.org/10.3892/etm.2019.8396

Tsuji, G., Yumine, A., Kawamura, K., Takemura, M., i Nakahara, T. (2024). Induction of Semaphorin 3A by Resveratrol and Pinostilbene via Activation of the AHR-NRF2 Axis in Human Keratinocytes. *Antioxidants*, *13*(6), 732. https://doi.org/10.3390/antiox13060732

Valletta, A., Iozia, L. M., i Leonelli, F. (2021). Impact of Environmental Factors on Stilbene Biosynthesis. *Plants*, *10*(1), 90. https://doi.org/10.3390/plants10010090

van Barneveld, E., Manders, J., van Osch, F. H. M., van Poll, M., Visser, L., van Hanegem, N., Lim, A. C., Bongers, M. Y., i Leue, C. (2022). Depression, Anxiety, and Correlating Factors in Endometriosis: A Systematic Review and Meta-Analysis. *Journal of Women's Health*, *31*(2), 219–230. https://doi.org/10.1089/jwh.2021.0021

van Haaps, A. P., Wijbers, J. V., Schreurs, A. M. F., Vlek, S., Tuynman, J., De Bie, B., de Vogel, A. L., van Wely, M., i Mijatovic, V. (2023). The effect of dietary interventions on pain and quality of life in women diagnosed with endometriosis: A prospective study with control group. *Human Reproduction*, *38*(12), 2433–2446. https://doi.org/10.1093/humrep/dead214

Vissers, G., Giacomozzi, M., Verdurmen, W., Peek, R., i Nap, A. (2024). The role of fibrosis in endometriosis: A systematic review. *Human Reproduction Update*, *30*(6), 706–750. https://doi.org/10.1093/humupd/dmae023

Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., i Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *32*(12), 1377–1382. https://doi.org/10.1124/dmd.104.000885

Wang, H., Brown, P. C., Chow, E. C. Y., Ewart, L., Ferguson, S. S., Fitzpatrick, S., Freedman, B. S., Guo, G. L., Hedrich, W., Heyward, S., Hickman, J., Isoherranen, N., Li, A. P., Liu, Q., Mumenthaler, S. M., Polli, J., Proctor, W. R., Ribeiro, A., Wang, J., … Huang, S. (2021). 3D cell culture models: Drug pharmacokinetics, safety assessment, and regulatory consideration. *Clinical and Translational Science*, *14*(5), 1659–1680. https://doi.org/10.1111/cts.13066

Wang, H., Jia, R., Lv, T., Wang, M., He, S., i Zhang, X. (2020). Resveratrol Suppresses Tumor Progression via Inhibiting STAT3/HIF-1α/VEGF Pathway in an Orthotopic Rat Model of Non-Small-Cell Lung Cancer (NSCLC). *OncoTargets and therapy*, *13*, 7057–7063. https://doi.org/10.2147/OTT.S259016

Wang, J., He, F., Chen, L., Li, Q., Jin, S., Zheng, H., Lin, J., Zhang, H., Ma, S., Mei, J., i Yu, J. (2018). Resveratrol inhibits pulmonary fibrosis by regulating miR-21 through MAPK/AP-1 pathways. *Biomedicine & Pharmacotherapy*, *105*, 37–44. https://doi.org/10.1016/j.biopha.2018.05.104

Wang, J., Jia, R., Celi, P., Zhuo, Y., Ding, X., Zeng, Q., Bai, S., Xu, S., Yin, H., Lv, L., i Zhang, K. (2022). Resveratrol Alleviating the Ovarian Function Under Oxidative Stress by Alternating Microbiota Related Tryptophan-Kynurenine Pathway. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.911381

Wang, T. T. Y., Hudson, T. S., Wang, T.-C., Remsberg, C. M., Davies, N. M., Takahashi, Y., Kim, Y. S., Seifried, H., Vinyard, B. T., Perkins, S. N., i Hursting, S. D. (2008). Differential effects of resveratrol on androgen-responsive LNCaP human prostate cancer cells in vitro and in vivo. *Carcinogenesis*, *29*(10), 2001–2010. https://doi.org/10.1093/carcin/bgn131

Wang, Y., Nicholes, K., i Shih, I.-M. (2020). The Origin and Pathogenesis of Endometriosis. *Annual Review of Pathology*, *15*, 71–95. https://doi.org/10.1146/annurev-pathmechdis-012419-032654

Warzecha, D., Załęcka, J., Mańka, G., Kiecka, M., Lipa, M., Spaczyński, R., Piekarski, P., Banaszewska, B., Jakimiuk, A., Issat, T., Rokita, W., Młodawski, J., Szubert, M., Sieroszewski, P., Raba, G., Szczupak, K., Kluz, T., Kluza, M., Wielgoś, M., … Laudański, P. (2022). Plasma and Peritoneal Fluid Fibronectin and Collagen IV Levels as Potential Biomarkers of Endometriosis. *International Journal of Molecular Sciences*, *23*(24), 15669. https://doi.org/10.3390/ijms232415669

Wei, N., Guan, H., Zhang, Y., Shi, J., Ma, J., Shi, R., Qi, X., Dong, Z., i Zhao, R. (2025). Molecular mechanisms underlying the inhibition of cell migration and invasion in endometriosis: Advances in pharmacological research (Review). *Biomedical Reports*, *23*(3), 152. https://doi.org/10.3892/br.2025.2030

Westphal, D., Dewson, G., Menard, M., Frederick, P., Iyer, S., Bartolo, R., Gibson, L., Czabotar, P. E., Smith, B. J., Adams, J. M., i Kluck, R. M. (2014). Apoptotic pore formation is associated with in-plane insertion of Bak or Bax central helices into the mitochondrial outer membrane. *Proceedings of the National Academy of Sciences*, *111*(39), E4076–E4085. https://doi.org/10.1073/pnas.1415142111

Williams, C., Long, A. J., Noga, H., Allaire, C., Bedaiwy, M. A., Lisonkova, S., i Yong, P. J. (2019). East and South East Asian Ethnicity and Moderate-to-Severe Endometriosis. *Journal of Minimally Invasive Gynecology*, *26*(3), 507–515. https://doi.org/10.1016/j.jmig.2018.06.009

Woo, J.-H., Yang, Y.-I., Ahn, J.-H., Choi, Y. S., i Choi, J.-H. (2017). Interleukin 6 secretion from alternatively activated macrophages promotes the migration of endometriotic epithelial cells. *Biology of Reproduction*, *97*(5), 660–670. https://doi.org/10.1093/biolre/iox118

Yang, R., Yang, F., Wei, Y., Huang, B., Cao, T., Tan, H., Liu, D., Zou, Q., Wen, J., Wen, L., Lu, X., Yu, C., Cai, H., Xie, X., Jiang, S., Yao, S., i Liang, Y. (2024). Hypoxia-induced Semaphorin 3A promotes the development of endometriosis through regulating macrophage polarization. *International Immunopharmacology*, *138*, 112559. https://doi.org/10.1016/j.intimp.2024.112559

Yang, Y., Hu, L., Xia, Y., Jiang, C., Miao, C., Yang, C., Yuan, M., i Wang, L. (2016). Resveratrol suppresses glial activation and alleviates trigeminal neuralgia via activation of AMPK. *Journal of Neuroinflammation*, *13*(1), 84. https://doi.org/10.1186/s12974-016-0550-6

Zhang, B., Wang, X.-Q., Chen, H.-Y., i Liu, B.-H. (2014). Involvement of the Nrf2 pathway in the regulation of pterostilbene-induced apoptosis in HeLa cells via ER stress. *Journal of Pharmacological Sciences*, *126*(3), 216–229. https://doi.org/10.1254/jphs.14028fp

Zhang, C., Cheng, H., Ye, X., Cui, H., Li, Y., Zhu, H., i Chang, X. (2024). ECM1 promotes migration and invasion in endometriosis. *Reproductive Biology*, *24*(1), 100826. https://doi.org/10.1016/j.repbio.2023.100826

Zhang, H., Liu, Y., Han, Z., Xu, Q., Zhang, N., Wang, J., Zheng, X., Ding, Y., Yin, Z., i Zhang, X. (2023). Integrated analysis of lncRNA and mRNA for the apoptosis of porcine ovarian granulosa cells after polyphenol resveratrol treatment. *Frontiers in Veterinary Science*, *9*, 1065001. https://doi.org/10.3389/fvets.2022.1065001

Zhang, H., Wang, J., Xie, F., Liu, Y., Qiu, M., Han, Z., Ding, Y., Zheng, X., Yin, Z., i Zhang, X. (2023). Identification of microRNAs implicated in modulating resveratrol-induced apoptosis in porcine granulosa cells. *Frontiers in Cell and Developmental Biology*, *11*, 1169745. https://doi.org/10.3389/fcell.2023.1169745

Zhang, J., Wang, L., Li, C., Zhang, H., Li, R., i Li, M. (2022). Letrozole promotes the expression of integrin αvβ3 and HOXA10 in endometrium of endometriosis. *Systems Biology in Reproductive Medicine*, *68*(2), 121–128. https://doi.org/10.1080/19396368.2021.2013577

Zhang, P., i Wang, G. (2023). Progesterone Resistance in Endometriosis: Current Evidence and Putative Mechanisms. *International Journal of Molecular Sciences*, *24*(8), 6992. https://doi.org/10.3390/ijms24086992

Zhang, T., He, Y., Man, G. C. W., Ding, Y., Wang, C. C., i Chung, J. P. W. (2023). Myeloid-derived suppressor cells: A new emerging player in endometriosis. *International Review of Cell and Molecular Biology*, *375*, 191–220. https://doi.org/10.1016/bs.ircmb.2022.11.004

Zhang, W., Zhang, R., Chang, Z., i Wang, X. (2022). Resveratrol activates CD8+ T cells through IL-18 bystander activation in lung adenocarcinoma. *Frontiers in Pharmacology*, *13*. https://doi.org/10.3389/fphar.2022.1031438

Zhang, Y., Lu, Y., Ong'achwa, M. J., Ge, L., Qian, Y., Chen, L., Hu, X., Li, F., Wei, H., Zhang, C., Li, C., i Wang, Z. (2018). Resveratrol Inhibits the TGF-β1-Induced Proliferation of Cardiac Fibroblasts and Collagen Secretion by Downregulating miR-17 in Rat. *BioMed Research International*, *2018*, 8730593. https://doi.org/10.1155/2018/8730593

Zhang, Z., Suo, L., Chen, Y., Zhu, L., Wan, G., i Han, X. (2019). Endometriotic Peritoneal Fluid Promotes Myofibroblast Differentiation of Endometrial Mesenchymal Stem Cells. *Stem Cells International*, *2019*, 6183796. https://doi.org/10.1155/2019/6183796

Zhang, Z.-D., Tao, Q., Bai, L.-X., Qin, Z., Liu, X.-W., Li, S.-H., Yang, Y.-J., Ge, W.-B., i Li, J.-Y. (2023). The Transport and Uptake of Resveratrol Mediated via Glucose Transporter 1 and Its Antioxidant Effect in Caco-2 Cells. *Molecules*, *28*(12), 4569. https://doi.org/10.3390/molecules28124569

Zhao, G., Yang, L., Zhong, W., Hu, Y., Tan, Y., Ren, Z., Ban, Q., Yang, C. S., Wang, Y., i Wang, Z. (2022). Polydatin, A Glycoside of Resveratrol, Is Better Than Resveratrol in Alleviating Non-alcoholic Fatty Liver Disease in Mice Fed a High-Fructose Diet. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.857879

Zhou, L., Cai, X., Wang, Y., Yang, J., Wang, Y., Deng, J., Ye, D., Zhang, L., Liu, Y., i Ma, S. (2025). Chemistry and biology of natural stilbenes: An update. *Natural Product Reports*, *42*(2), 359–405. https://doi.org/10.1039/D4NP00033A

Zhou, L., Liu, B., Jian, X., Jiang, L., i Liu, K. (2025). Effect of dietary patterns and nutritional supplementation in the management of endometriosis: A review. *Frontiers in Nutrition*, *12,* 1539665. https://doi.org/10.3389/fnut.2025.1539665

Zhou, M., Niu, H., Huang, G., Zhou, M., Cui, D., Li, H., Wen, H., Zhang, H., Liang, F., i Chen, R. (2025). Biomimetic Nano-delivery of Small-Molecule Piceatannol Modulates Tumor Stemness and Suppresses Colorectal Cancer Metastasis via Hippo/YAP1/SOX9 Signaling. *Small (Weinheim an Der Bergstrasse, Germany)*, *21*(2), 2407191. https://doi.org/10.1002/smll.202407191

Ziętek, A., Futyma, K., Nowakowski, Ł., Gogacz, M., i Rechberger, T. (2015). Progress on macrophage's proinflammatory products as markers of acute endometriosis. *Journal of Acute Disease*, *4*(3), 169–172. https://doi.org/10.1016/j.joad.2015.04.001

Zondervan, K. T., Becker, C. M., i Missmer, S. A. (2020). Endometriosis. *The New England Journal of Medicine*, *382*(13), 1244–1256. https://doi.org/10.1056/NEJMra1810764

Spis rycin

Opublikowane artykuły naukowe wchodzące w skład rozprawy doktorskiej

nutrients

MDPI

Review

Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives

Agata Gołąbek, Katarzyna Kowalska and Anna Olejnik *

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego St., 60-627 Poznan, Poland; agata.golabek@up.poznan.pl (A.G.); katarzyna.kowalska@up.poznan.pl (K.K.)
* Correspondence: anna.olejnik@up.poznan.pl

Abstract: Endometriosis represents an often painful, estrogen-dependent gynecological disorder, defined by the existence of endometrial glands and stroma exterior to the uterine cavity. The disease provides a wide range of symptoms and affects women's quality of life and reproductive functions. Despite research efforts and extensive investigations, this disease's pathogenesis and molecular basis remain unclear. Conventional endometriosis treatment implies surgical resection, hormonal therapies, and treatment with nonsteroidal anti-inflammatory drugs, but their efficacy is currently limited due to many side effects. Therefore, exploring complementary and alternative therapy strategies, minimizing the current treatments' adverse effects, is needed. Plants are sources of bioactive compounds that demonstrate broad-spectrum health-promoting effects and interact with molecular targets associated with endometriosis, such as cell proliferation, apoptosis, invasiveness, inflammation, oxidative stress, and angiogenesis. Anti-endometriotic properties are exhibited mainly by polyphenols, which can exert a potent phytoestrogen effect, modulating estrogen activity. The available evidence derived from preclinical research and several clinical studies indicates that natural biologically active compounds represent promising candidates for developing novel strategies in endometriosis management. The purpose of this review is to provide a comprehensive overview of polyphenols and their properties valuable for natural treatment strategy by interacting with different cellular and molecular targets involved in endometriosis progression.

Keywords: endometriosis; diet therapy; polyphenols; molecular targets; apoptosis; invasion; angiogenesis; inflammation; oxidative stress

Citation: Gołąbek, A.; Kowalska, K.; Olejnik, A. Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives. *Nutrients* **2021**, *13*, 1347. https://doi.org/10.3390/nu13041347

Academic Editor: Susan S. Percival

Received: 1 April 2021
Accepted: 16 April 2021
Published: 18 April 2021

1. Introduction

Endometriosis is a chronic gynecological disorder, defined by the implantation of endometrial glands and stroma outside the uterine cavity, mainly in the pelvic peritoneum and ovaries. It affects approximately 10–15% of women of reproductive age, which extrapolates to around 190 million women worldwide [1,2]. Most affected patients often suffer from chronic pelvic pain, dyspareunia, dysmenorrhea, abnormal uterine bleeding, and infertility [3,4]. All these symptoms provide an impact on a patient's quality of life, resulting in depression, anxiety, and impaired social function caused by the severity of pain [5]. The successful diagnosis of endometriosis requires surgical exploration or laparoscopy with histological confirmation [6]; therefore, the prevalence of the disease, its symptoms, associated disorders, and risk factors are limited data, only to the group of patients with definitive diagnosis [7].

Various hypotheses have been proposed to explain endometriosis pathology and tissue scattering throughout the abdominal cavity [8]. Distinct biological and clinical features indicate different types of endometriosis. Endometriosis can occur in several forms, according to histopathology and its anatomical localization in the pelvis: as the ovarian endometriotic cysts, deep infiltrating endometriosis, and superficial peritoneal lesions of varying colors [9]. Although scientists devoted significant effort to explain pathogenesis

and endometriosis-associated pain, the basics of many biological processes remain enigmatic. Despite multiple theories explaining endometriosis pathogenesis, every concept suggests that endometriosis is a complex multifactorial and heterogeneous disease of uncertain etiology; its development and progression are influenced by genetic, immunological, hormonal, and environmental factors [10]. Recently, Lagana et al. (2019) summarized existing theories and divided them into two main categories: the transplantation and in situ [8]. Transplantation theory assumes that endometriosis develops due to eutopic endometrium metastasis to distinct sites by the hematogenous or lymphatic spread [8,11]. In contrast, in situ theory refers to endometriosis tissue existence caused by coelomic metaplasia, peritoneal mesothelium transformation into glandular endometrium or embryologic origin [8]. A separate theory does not explain the different clinical presentations and pathological forms in endometriosis. The formation and persistence of endometriotic tissues at ectopic sites essentially depend on the fundamental biological process as adhesion, proliferation, cell invasion, local inflammation, immune dysregulation, and neoangiogenesis [2]. Another increasingly reported important hallmark in endometriosis's pathogenesis is the growth of new nerve fibers in endometriotic lesions, stimulated by inflammatory mediators and responsible for endometriosis-associated chronic pain conditions [12].

2. Current Treatment

Endometriosis therapy depends on the patient's predominant symptoms, such as age, side-effect profile, the extent and location of endometriotic lesions, and previous treatment [13]. The main therapeutic approach includes surgery, pharmacotherapy, and long-term comprehensive individual treatment [14,15]. Surgical intervention represents a primary treatment aimed at destroying or removing ectopic endometriotic lesions. Indeed, surgery generally increases pain relief in some but not all women. Although the surgical resection option completely removes all visible ectopic endometriotic lesions, high recurrence rates of up to 50% within five years of surgery are reported [16].

The most common pharmacological approach for endometriosis therapy is nonsteroidal anti-inflammatory drugs for managing pain symptoms, combined with oral contraceptives [17,18]. Hormone therapy aims to induce hypoestrogenemia, inhibition of tissue proliferation, and inflammation [19]. Second-line treatments that suppress systemic estrogen levels include progestin monotherapy and gonadotrophin-releasing hormone agonists (GnRH) [20]. The efficacy of conventional medical treatments is limited or intermittent in most patients. It brings a plethora of side effects like perimenopausal stage symptoms, osteoporosis, breakthrough bleeding, lipid profile changes, and liver dysfunction, resulting from the hypo-estrogenic state induced by this medical approach [21,22]. Consequently, exploring additional and alternative options is needed to improve treatment outcomes for patients with endometriosis. Strategies complement conventional drug therapy may include non-pharmaceutical options: acupuncture, diet changes, supplementations, and phytotherapy [23–25]. Natural therapies constitute an option of paramount importance that can accelerate the healing process and help minimize the current treatment's adverse effects. The search for new alternative therapies should start with demonstrating their benefits and safety, evidence-based core outcomes obtained using endometriotic models [26].

3. Nutritional and Dietary Aspects of Endometriosis

Because of the unsuccessful treatment and chronic character of endometriosis, many women affected by endometriosis use additional management strategies to control this disease themselves [27]. According to the last Australian national online survey, as many as 76% of endometriosis women use non-pharmacological practices and lifestyle choices like relaxation techniques, movement, and nutrition. Almost half the women managed dietary supporting, and the diet's effectiveness had high self-reported improvement scores [28]. In recent years, an increasing number of endometriosis patients have focused on health-promoting and therapy-supporting dietary factors [29].

Plants are promising sources of bioactive compounds, potentially improving therapeutic strategies [30]. Different pathways involved in the physiological and pathological processes associated with endometriosis, including altered inflammatory microenvironment, the attachment and invasion mechanisms, angiogenesis, estrogen activity, menstrual cyclicity, organochlorine burden, and prostaglandin metabolism, can be a target of food bioactive compounds [15,29,31]. Dietary treatment of endometriosis may be based on the estrogen dependency of endometriosis, and estrogen-lowering diet components may be used to treat or regress endometriosis [29,32]. Changing dietary patterns for endometriosis may help reduce inflammatory markers, shown to be increased in endometriosis [33]. The diet to treat endometriosis can moderate prostaglandins' effects responsible for the pain during endometriosis progression [34]. Different active compounds offering various therapeutic properties such as antiproliferative, anti-inflammatory, antioxidant, and analgesic properties are considered a complex group of molecular compounds effective in endometriosis [35].

There are many scientific studies on the effects of nutrition on endometriosis. Most papers have reported case-control studies evaluating the role of diet on endometriosis risk, while the diet effectiveness and diet compound potential in endometriosis treatment have investigated less frequently. In our opinion, a scientific basis for the action of bioactive compounds in the management of endometriosis should be considered, and their possible curative effect described to explain their contribution to the healing process and impact on the control of severe endometriotic symptoms.

4. Molecular Targets in Endometriosis Dietary Management

Plants are sources of natural bioactive compounds that demonstrate broad-spectrum health-promoting effects and interact with molecular targets associated with endometriosis, such as cell proliferation and apoptosis, cell adhesion, invasiveness, inflammation, oxidative stress, and angiogenesis. The anti-endometriotic potential of nutraceuticals can also concern a potent phytoestrogenic effect modulating estrogen activity. Dysregulated physiological processes related to endometriosis, contributing potential molecular targets for bioactive polyphenol compounds, are schematically presented in Figure 1.

4.1. Cell Survival and Apoptosis

The proliferation of endometrial cells is primarily controlled by interactions between sex steroids and their corresponding receptors. Endometriotic tissue's proliferative potential is significantly higher in the eutopic endometrium of women with endometriosis than in the endometrium of disease-free patients [36]. The complex microenvironment consists of proinflammatory and endocrine mediators in surrounding endometriotic lesions, promoting endometriotic cells' proliferation [37]. Ectopic endometriotic tissues are characterized by reported markedly higher levels of estrogen receptor beta (ERβ) compared with eutopic endometrial tissues and cells. ERβ plays a critical role in anti-apoptosis signaling and is responsible for a mechanism of evasion from endogenous immune surveillance for cell survival through inactivation of TNFα-induced apoptosis complex I and II and the apoptosome. ERβ also activates the cytoplasmic inflammasome components, resulting in increasing interleukin 1β (IL-1β), enhancing adhesion and proliferation of endometrial cells. [38]. Furthermore, proliferation is enhanced by high levels of estrogen in the microenvironment of endometriotic lesions provided by locally increased expression of aromatase and decreased 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2 in endometriotic implants [9].

Nuclear factor kappa B (NF-κB), a pleiotropic transcription factor, also plays a critical role in developing endometriosis by protecting cells from apoptosis via activating anti-apoptotic genes and inducing the proliferation of the endometriotic cells [39]. Under normal conditions, endometrial cells from healthy women during menstruation do not survive in ectopic location because cell turnover is regulated by apoptosis, avoiding cell dissemination and attachment. Endometrial tissue's impaired susceptibility to apoptosis

leads to increased invasiveness and the abnormal survival of endometrial cells in the peritoneum [40]. Different studies suggest that dysregulated expression of specific proteins associated with apoptosis, including B-cell lymphoma 2 (BCL-2) protein family, B-cell lymphoma-extra large (BCL-XL) protein, BCL-2 associated X (BAX) protein, FAS/FASL system, cysteine-aspartic proteases (caspases), and survivin represent possible factors involving in apoptosis resistance in endometriotic cells [41–43]. Some studies have evaluated the effect of different bioactive food compounds on mechanisms regulating endometriotic cells proliferation and apoptosis, suggesting their therapeutic potential.

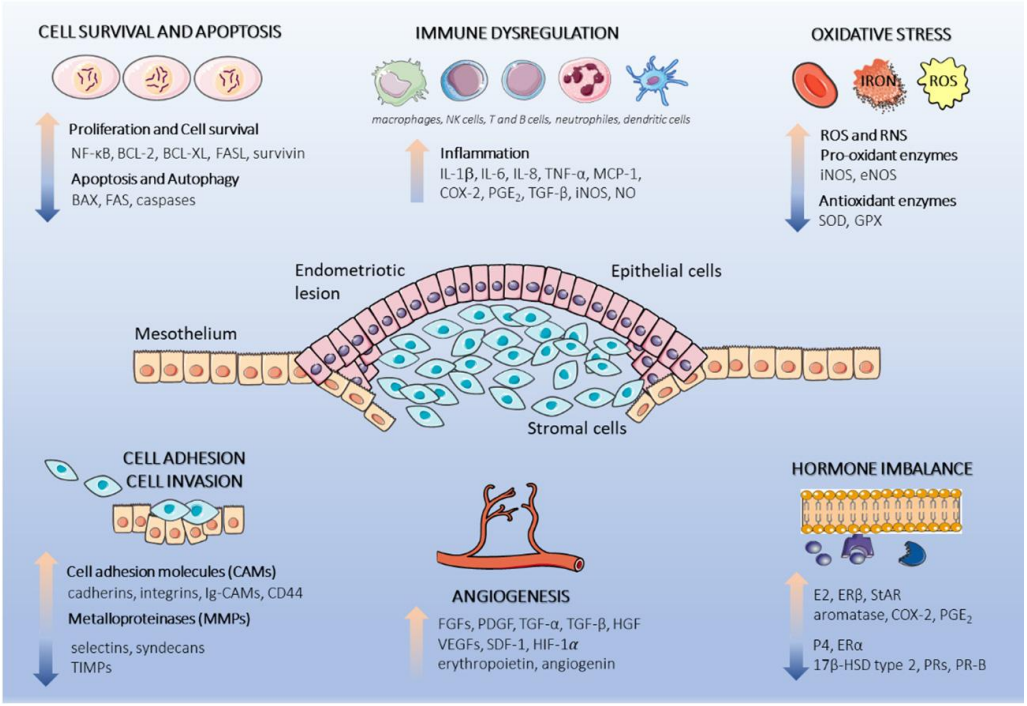


Figure 1. Schematic representation of dysregulated physiological processes in the endometriotic lesion. Endometriotic lesions consist of epithelial and stromal endometrial cells originating form retrograde menstruation. The endometrial cells subsequently attach to the underlying peritoneal mesothelium, proliferate, and initiate pro-endometriotic microenvironment formation. Cell proliferation and resistance to apoptosis, cell adhesion, inflammation, oxidative stress, and hormone signaling are augmented by the interactions of multiple cells and secretion of cytokines, chemokines, and hormones, which facilitate endometriotic lesion progression. This figure was made using graphic components obtained from the website: www.servier.com/powerpoint-imagebank (15 March 2021). Abbreviations used in graphics: 17β-HSD type 2, 17β-hydroxysteroid dehydrogenase type 2; BAX, BCL-2 associated X protein; BCL-XL, B-cell lymphoma-extra large; BCL-2, B-cell lymphoma 2; COX-2, cyclooxygenase-2; eNOS, endothelial nitric oxide synthase; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; E2, estradiol; FAS, tumor necrosis factor receptor superfamily member 6; FASL tumor necrosis factor ligand superfamily member; FGFs, fibroblast growth factors; GPX, glutathione peroxidase; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible-factor 1-alpha; IL-1β, interleukin 1β, IL-6, interleukin 6; IL-8, interleukin 8; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein 1; NF-κB, nuclear factor kappa B; NO, nitric oxide; PGE_2, prostaglandin E_2; PR-B, progesterone receptor isoform B; P4, progesterone; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDF-1, stromal-cell-derived-factor 1; StAR, steroidogenic acute regulatory protein; SOD, superoxide dismutase; TGF-α, transforming growth factor alpha; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase-1; VEGF, vascular endothelial growth factor.

4.2. Cell Attachment and Invasion

The initial attachment of refluxed endometrial tissue fragments to the pelvic peritoneum is the principal of Sampson's retrograde theory of endometriosis origin [44]. Subperitoneal endometriotic lesions' establishment requires remodeling of the extracellular matrix of peritoneal mesothelium and invasion in their surrounding environment [45]. Menstrual effluent and morphological alterations can easily damage the intact mesothelium—a protective barrier against the implantation; the own adhesion site can also be created to implant regurgitated endometrial cells [46–48]. The adhesion of endometrium fragments to the peritoneum in women with endometriosis is enhanced by the overproduction of cellular adhesion molecules that facilitate intercellular binding and cellular attachments with the extracellular matrix; including CD44 transmembrane glycoprotein, cell adhesion molecules (CAM) such as integrins, cadherins, selectins, the immunoglobulin superfamily (Ig-CAM), and transmembrane-anchored proteoglycans like syndecans [49,50].

The progression of the invasion of adjacent tissues requires extracellular matrix degradation. The breakdown and remodeling of extracellular matrix thought to be mainly modulated by matrix metalloproteinases (MMP), especially MMP-1, 2, 3, 9, and 11 and their inhibitors (tissue inhibitors of metalloproteinases, TIMP) [51]. MMPs are the initial mediators of maintenance and survival of endometriotic lesions, mainly that their expression enhances significantly in endometriotic implants [52]. Different hormones, inflammatory cytokines, including IL-6, IL-1, and growth factors, regulate MMPs. The primary inhibitor is progesterone, which might indirectly regulate MMP expression through the plasminogen activator pathway, increasing the levels of plasminogen activator inhibitor 1 (PAI-1) and reducing the activation of latent MMP-proenzyme by plasmin [53].

4.3. Angiogenesis

The formation of new blood vessels is fundamental to creating and maintaining endometriotic lesions, especially in the peritoneal microenvironment, indicating the importance of new blood supply in endometriosis development. The local neovascularization is augmented by the complex mixture of growth factors, proangiogenic factors, steroid hormones, inflammatory cytokines present in peritoneal fluid [54]. Many pro-angiogenic factors, including fibroblast growth factors (FGF), platelet-derived endothelial cell growth factor (PDGF), transforming growth factor alpha and beta (TGF-α and TGF-β), hepatocyte growth factor (HGF), erythropoietin, angiogenin, tumor necrosis factor alpha (TNF-α), and IL-8 have been detected at increased concentrations in peritoneal fluid of women with endometriosis [54]. Vascular endothelial growth factor (VEGF), produced in high amounts by activated peritoneal macrophages, neutrophils, lymphocytes, and endometrial stromal cells, is considered the most crucial angiogenic agent to induce proliferation and migration of endothelial cells, that can also increase vascular permeability [55,56]. VEGF is exerted in response to physiological activators, such as inflammation and hypoxia. Bone-marrow-derived endothelial progenitor cells detected in early-stage of endometriotic lesions are recruited by hypoxia-inducible-factor1-alpha (HIF-1α) and stromal-cell-derived-factor 1 (SDF-1) [57,58]. New agents, like antiangiogenic factors, could constitute a new and promising approach to treating this disease [59].

4.4. Immune Dysregulation

Inflammation has a vital role in the progression of endometriosis and is associated with altering immune cell profile in the peritoneal cavity [33]. The endometrial tissue is a significant source of inflammatory mediators like cytokines, chemokines, and prostaglandins, which attract macrophages, neutrophils, monocytes, eosinophils, and T cells, that can affect processes in the abdominal cavity [60]. The peritoneal fluid of endometriosis is characterized by the enhanced concentration of inflammatory mediators like IL-1, IL-6, IL-8, TGF-β, TNF-α, VEGF, cyclooxygenase-2 (COX-2), and monocyte chemoattractant protein 1 (MCP-1) [61,62]. Changes in inflammatory mediators result from the aberrant function of almost all types of immune cells in women with endometriosis.

Peritoneal macrophages are the most prevalent immune cells, found in the highest quantity in the peritoneal fluids. The peritoneal environment is regulated by the activated macrophages, which can scavenge cellular debris, and remove red blood cells and damaged tissue fragments. Lesion resident macrophages can induce tissue repair, inflammation, and angiogenesis through the secretion of soluble mediators like cytokines, prostaglandins, and enzymes. However, in the peritoneum of endometriosis-affected women, macrophages have a decreased phagocytic activity, and the amount of regurgitated endometrial cells in the peritoneal cavity may be higher. The imbalance in M1 and M2 macrophages was reported in endometriosis with an upregulation of the M2 type. M2 macrophages are supposed to have a crucial role in endometriosis development by modulating adaptive immune response and, consequently, promoting the implantation and proliferation of endometrial cells [33]. Macrophage-derived factors such as prostaglandins and cytokines produced in the peritoneal cavity, may also modulate natural killer cells activity [63]. The cytotoxic function of NK cells against endometrial cells reaching the peritoneal cavity is decreased and is inversely related to the advanced stages of the disease [64]. Moreover, reduced T cells' cytotoxic activity, modulation of pro-inflammatory cytokine secretion and altering Th1 cytokine profile prevalent in the early stages of the disease, to a Th2 cytokine profile in late stages and autoantibody production by B lymphocytes were observed [8,65]. The presence of this altered inflammatory niche could favor the implantation and development of endometriotic lesions from refluxed endometrial tissue. There is substantial evidence that the immune system plays a crucial role in this disease. Novel treatment strategies targeting immune pathways are urgently needed [13].

4.5. Oxidative Stress

Oxidative stress occurs due to an imbalance between reactive oxygen/nitrogen species (ROS/RNS) production and the organism's ability to scavenge and detoxify ROS/RNS harmful effects [66]. Essential molecules in the body, such as membrane lipids, nucleic acids and proteins, are ROS targets. Oxidative stress is involved in the pathogenesis of many chronic diseases, such as cancer, neurodegenerative and cardiovascular diseases, and ageing [67]. Endometriosis may involve ROS production because macrophages, erythrocytes, and apoptotic endometrial tissue, transplanted into the peritoneal cavity through retrograde menstruation, are found to be inducers of oxidative stress. The accumulation of iron in the different components of the peritoneal cavity of endometriosis patients has reported. Peritoneal iron overload may be a consequence of hemoglobin breakdown or bleeding of peritoneal lesions. The macrophages and lymphocytes are recruited and activated by releasing the proinflammatory heme products and the oxidative stress signals. Enhanced activity of peritoneal macrophages and lymphocytes, in turn, strengthen the oxidation stress in the endometriotic peritoneum [68]. Moreover, ROS activates NF-κB, which leads to the expression of multiple genes engaged in cell growth, angiogenesis, and inflammation mechanisms regulation in the endometriosis [69]. A subclass of nitrogen-containing compounds can play detrimental effects in endometriosis pathology. In women with endometriosis, the increased concentration of endothelial and inducible forms of nitric oxide synthase (eNOS and iNOS), the enzymes that ultimately produce nitric oxide (NO), has been found in the peritoneal fluid. In endometriosis condition, the elevated levels of NOS and NO are correlated with impaired reproductive biological processes, e.g., ovulation, fertilization, implantation, and embryonic development. Moreover, the increased endometriosis-related eNOS activity has shown a positive correlation between estrogen and progesterone levels [70]. Additionally, aberrant change in the potential of endogenous antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPX) can contribute to the oxidative damage that occurs in endometriosis [70]. Estrogen participates in the SOD induction to overcome the excessive oxidative stress; however, overexpression of this antioxidant enzyme has adverse effects and contributes to promoting endometriotic cell survival and protecting them from apoptosis. Superoxide anion produced in large amounts mediates several signaling pathways and contributes

to endometriosis-related pain through activation of nociceptive neurons [71]. Oxidative stress has been suggested as an etiological factor of chronic pelvic pain, and antioxidant supplementation has a proven impact on reducing pain in endometriosis patients [72]. These findings highlight the importance of developing therapeutic approaches targeting oxidative imbalance: reducing the oxidative stress status may exert protective effects against endometriosis.

4.6. Hormonal Imbalance

Hormonal imbalance in endometriosis is the master regulator of the alternations of multiple cellular functions, such as proliferation, invasion, angiogenesis, inflammation, and neurogenesis and pain generation [55]. Endometrial tissues are controlled by steroid hormones like 17β-estradiol and progesterone, which change the expression of hundreds of genes during different phases of the menstrual cycle. Eutopic endometrial tissues and endometriotic tissues in ectopic locations contain the immunoreactive estrogen receptors and progesterone receptors (PR); therefore, they respond to 17β-estradiol and progesterone with apparently similar histological changes [73]. Local estrogen production in both the ectopic and eutopic endometrium is considered to stimulate tissue's growth and play a crucial role in regulating the immunological mechanisms responsible for controlling the development of endometriosis. The enzyme aromatase, a member of the cytochrome P450 superfamily, is responsible for the last step in the synthesis of estradiol through the aromatization of androgens (androstenedione and testosterone) into estrogens (estrone and estradiol, respectively) [74]. In premenopausal women with endometriosis, estradiol arises from three major tissue sites that express aromatase. These are ovaries, which primarily convert cholesterol to estradiol and secrete it periodically into the circulation, peripheral tissues such as the adipose tissue and skin, that convert androstenedione to estrone in relatively small quantities, and endometriotic tissue, synthesizing *de novo* high amounts of estradiol from cholesterol via aromatase and steroidogenic acute regulatory protein (StAR) [73]. Contrary to endometriotic lesions, normal endometrium cannot synthesize estrogen due to these enzymes' absence [75]. In endometriosis, local estradiol levels increase due to upregulation of estradiol-producing aromatase expression and reduction in 17β-HSD type 2 activity, implicated in the inactivation of estradiol through the oxidation to less active estrone [76]. In normal conditions, the expression of 17β-HSD type 2 in epithelial cells is activated by paracrine signaling triggering by the progesterone via PRs on stromal cells. There are two isoforms of PRs: PR-A and PR-B; PR-B likely plays a more crucial biological function in the endometrium. Progesterone resistance in endometriotic tissue is due to the total reduction in PRs and a drastic downregulation of PR-B in endometriotic stromal cells [77]. In the case of estrogen receptors (ER), a significant reduction in isotype ERα and an increase in ERβ is observed. The elevated expression of ERβ is associated with its promoter hypomethylation, while the decrease in ERα is due to its promoter hypermethylation and direct inhibition by ERβ [78]. Increasing the E2/ERβ ratio is considered to enhance lesion survival and inflammation. It is linked with the positive feedback loop by stimulation of COX-2 activation, which is involved in producing hormones, such as prostaglandin E_2 (PGE_2), with significance in inflammation and pain. In turn, PGE_2 influences steroidogenic genes, mainly overexpressing aromatase, and supports the sustained production of estradiol [78,79]. Novel alternative treatment to regulate estradiol biosynthesis and modulate its coactivators without suppressing ovulation, instead of conventional pharmacotherapy, could be designed to more beneficially control this disorder. The substances that bind competitively to ERs, such as phytoestrogens, are recommended for future research assessment to demonstrate their effect on the levels of hormones and inflammatory markers in endometriosis.

5. Potential of Polyphenol Compounds in Endometriosis Management

There are increasing investigations about the beneficial effect of bioactive compounds in preventing chronic non-communicable diseases, including cardiovascular diseases and

cancers. Several studies have evaluated the relationship between polyphenols' administration, such as flavonoids and phytoestrogens, and female cancer risk. Grosso et al. (2016) observed that regular consumption of diets rich in flavonoids is associated with decreased breast and ovarian cancer risk [80]. In the meta-analysis, Micek et al. (2020) have found that higher dietary intake of phytoestrogens (i.e., isoflavones) is inversely correlated with the risk of breast cancer mortality and recurrence [81]. Endometriosis and breast cancer are estrogen-dependent conditions and share similar cellular processes like proliferation, invasion, neo-angiogenesis, metastases, and reproduction-associated risk factors. Significant similarities between endometriosis and cancer encourage investigating the protective effect of natural bioactive molecules against this disease [82].

Moreover, endometriosis has been linked to chronic systemic inflammation, oxidative stress, and an atherogenic lipid profile, leading to initiating and maintaining cardiovascular diseases [83]. The meta-analysis showed the linear relationship between increased dietary intake of total flavonoids and lower risk of cardiovascular disease [84]. Similar dietary patterns rich in bioactive food compounds may exert beneficial effects toward endometriosis.

Specific plant-derived bioactive compounds, exhibiting anti-proliferative, anti-oxidant, anti-inflammatory, anti-angiogenic, pro-apoptotic, immunomodulatory, and estrogen modulating activities, can affect different pathways involved in endometriosis's etiopathogenesis and provide new strategies for treating or regressing endometriosis [85,86]. Numerous preclinical research and clinical trials in endometriosis studies have attributed to phytochemicals a broad range of beneficial biological activities and described molecular mechanisms and pathways through which they may effectively influence disease progression [68,87]. The studies on potential therapeutic phytochemicals had mostly focused on polyphenolic compounds.

Polyphenols comprise a large group of bioactive compounds synthesized by plants that are an integral part of the human diet, widely recognized for their multiple functional health-promoting benefits, mainly anti-oxidant and anti-inflammatory properties, with importance in endometriosis [88]. Phenol-Explorer, the first comprehensive database on polyphenol content in foods (http://phenol-explorer.eu/) (accessed on 12 April 2021), classifies polyphenols into six primary groups: flavonoids, lignans, non-phenolic metabolites, other polyphenols, phenolic acids, and stilbenes. Flavonoids constituting the largest group of dietary polyphenols have been divided into nine subgroups, including anthocyanins, chalcones, dihydrochalcones, dihydroflavonols, flavanols, flavanones, flavones, flavonols, and isoflavonoids (http://phenol-explorer.eu/) (accessed on 12 April 2021). The above classification has been applied to organize structurally different polyphenol compounds as promising candidates for developing novel strategies in treating endometriosis. This article describes polyphenols' molecular action profiles, which target fundamental disease processes' complex pathophysiology. Table 1 summarizes polyphenols' mechanisms of action and molecular targets in endometriosis management.

Table 1. Natural polyphenol compounds and their mechanisms of action and molecular targets analyzed in endometriosis preclinical and clinical studies.

Mechanisms of Action	Molecular Targets	Disease Model	Ref.
Quercetin			
↓Cell proliferation ↑Apoptosis	↓Mitochondrial membrane potential, ↓ERK1/2, ↓p38 MAPK, ↓AKT, DNA fragmentation	Endometriosis cell lines	[89]
↓Cell proliferation	↓*CCND1*, ↓Cyclin D1	Murine endometriosis model	[89]
↓Cell proliferation, ↑Autophagy ↓Endometriotic lesion size ↓Oxidative stress	↓Serum E2, ↓Serum TNF-α ↑NQO1 enzyme activity, mTOR inhibition ↑ *NRF2*, ↑*BECN1*, ↑*ATG5*	Rat endometriosis model	[90]
↓Inflammation ↓Endometriosis-related pain ↓Endometriotic lesion size	↓Serum PGE_2 ↓Serum CA-125	30 patients with IV endometriosis stage treated for 3 months with 200 mg quercetin	[91]
Apigenin			
↓Cell proliferation ↑Apoptosis	ERK1/2 and JNK inhibition, ↑BAX, ↑Cyt- c, ↑ROS, ↑ER stress, ↑Calcium ions in cytosol ↓Mitochondrial membrane potential	Endometriosis cell lines	[92]
↓Cell proliferation ↓Inflammation	↓COX-2, ↓PGE_2, ↓IL-8 NF-κB inhibition	Primary endometriotic stromal cells from ovarian endometrioma	[93]
↓Angiogenesis, ↓Inflammation ↓Endometriotic implant volume	↓Peritoneal VEGF, ↓Peritoneal TNF-α ↓Peritoneal IL-6	Rat endometriosis model	[94]
Baicalein			
Cell cycle arrest, ↓Cell viability ↑Apoptosis	NF-κB inhibition, ↑Cells in the G1 phase ↓Cells in the S and G2/M phases, ↓BCL-2	Endometrial stromal cells from patients with ovarian endometriosis	[95]
↓Cell invasiveness	↓MMP-9, ↓MMP-2, ↓MT1-MMP, ↓TGFB1, ↓FURIN	Ectopic endometrial stromal cells	[96]
↓Cell invasiveness ↓Endometriotic lesion growth	↓MT1-MMP, ↓FURIN, ↓TGFB1	Murine endometriosis model	[96]
Wogonin			
↓Cell proliferation Cell cycle arrest	↑Cells in the G2/M phase, ↓ERα	Endometrial stromal T-HESC cells Primary endometriotic stromal cells	[97]
↓Endometriotic implant size ↓Cell proliferation, ↑Apoptosis	↓Proliferating cells ↑Apoptotic cells	Murine endometriosis model	[97]

Table 1. *Cont.*

Mechanisms of Action	Molecular Targets	Disease Model	Ref.
Rosmarinic acid			
↓Cell proliferation, ↓Oxidation	Cell cycle arrest in the G2/M phase ↓ROS	Endometrial stromal T-HESC cell line Primary endometriotic stromal cells	[97]
↓Endometriotic tissue volume	↓PCNA positive cells, ↑Apoptotic cells in lesions	Murine endometriosis model	[97]
Genistein and daidzein			
↓Cell proliferation ↓Inflammation ↓Estrogen biosynthesis	NF-κB inhibition, ↓*IL6*, ↓*IL8*, ↓*COX2*, ↓PGE_2 Higher affinity toward ERβ than ERα ↓*CYP19A1* ↓Aromatase activity ↓Glucocorticoid-regulated kinase in serum	Primary endometriotic stromal cells from ovarian endometrioma	[98]
↓Endometriotic lesions growth	↓Ki-67-positive cells	Murine endometriosis model	[98]
Genistein			
↓Endometriotic implant size	Antagonistic estrogen activity	Rat endometriosis model	[99]
Puerarin			
↓Cell invasion ↓Angiogenesis	↓MMP-9 ↑TIMP-1 ↓ICAM-1 ↓VEGF	Primary endometriotic stromal cells Chick chorioallantoic membrane model	[100]
↓Cell proliferation ↑Antiestrogen activity	↓*CCND1*, ↓Cyclin D1, ↓*CDC25A*, ↓Cdc25A ↑ERα corepressors (SMRT, NCoR) ↓ERα coactivators (SRC-1, SRC-3)	Endometriotic stromal cells	[101]
↑Antiestrogen activity	↓*CYP19A1*, ↓aromatase activity, ↓*HSD17B1*, ↑*HSD17B2*, ↓COX-2, ↓*COX2*, ↓PGE_2, ↓ERβ, ↓E2	Rat endometriotic model	[102]
Naringenin			
↓Cell proliferation ↑Apoptosis	PI3K and MAPK pathway activation ↓Mitochondrial membrane potential, ↓ROS	Endometriosis cell lines	[103]
↓Endometriotic lesions growth ↓Angiogenesis, ↓Inflammation ↑Apoptosis ↓Endometriosis prognostic markers	↓MMP-2, ↓MMP-9, ↓TNF-α, ↓NO, ↑ROS ↓Mitochondrial membrane potential, ↓VEGF ↓BCL-2, ↓PCNA, ↑Caspase-3, ↑Cyt-C ↓Nrf2/HO1/NQO1 signaling, ↓TAK1, ↓PAK1	Rat endometriosis model	[104]

Table 1. *Cont.*

Mechanisms of Action	Molecular Targets	Disease Model	Ref.
Xanthohumol			
↓Endometriotic lesions growth ↓Angiogenesis	↓PI3K ↓Microvessel density	Murine endometriosis model	[105]
Epigallocatechin gallate			
↓Cell proliferation, ↓Cell migration ↓Cell invasion, ↓Fibrogenesis	↓MAPK signaling, ↓Smad signaling ↓*αSMA*, ↓*Col-I*, ↓*CTGF*, ↓*FN*	Endometriotic and endometrial stromal cells from patients	[106]
↓Endometriotic implant size ↓Angiogenesis	↓Microvessel number and size ↓*VEGFC/VEGFR2* expression and signalling	Murine endometriosis model	[107]
↓Endometriotic lesions growth ↑Apoptosis, ↓Angiogenesis	↓Lesion size and weight, ↓Serum VEGF ↑Apoptotic cells in lesions, ↓αSMA, ↓CD31	Murine endometriosis model	[108]
Curcumin			
↑Cell adhesion ↓Inflammation	↓*ICAM1*, ↓ICAM-1, ↓*VCAM1*, ↓VCAM-1 ↓IL-6, ↓IL-8, ↓MCP-1, NF-κB inhibition	Primary endometriotic stromal cells	[109]
↓Cell proliferation	↓E2 production	Primary endometriotic stromal cells	[110]
Cell cycle arrest ↑Apoptosis	↑Cells in the G1 phase, ↓Cells in the S phase ↓VEGF	Primary endometriotic and endometrial stromal cells	[111]
↓Inflammation ↓Angiogenesis	↓IL-6, ↓IL-8, ↓IP-10, ↓G-CSF, ↓MCP-1 ↓RANTES	Primary endometriotic stromal cells derived from eutopic endometrium	[112]
↑Apoptosis ↓Endometriotic lesions growth	Cytochrome c-mediated mitochondrial pathway modulation, p53-dependent and -independent pathway modulation, NF-κB inhibition, ↓MMP-3	Murine endometriosis model	[113]
↓Inflammation, ↓Oxidation	↓MMP-9, ↓TNF-α, ↓Lipid and protein oxidation	Murine endometriosis model	[114]
↓Endometriotic tissues weight and volume	↓VEGF	Rat endometriosis model	[115]
↓Angiogenesis ↓Endometriotic lesions growth	↓Microvessel density	Rat endometriosis model	[116]

Table 1. *Cont.*

Mechanisms of Action	Molecular Targets	Disease Model	Ref.
Resveratrol			
↓Cell invasion	↓Cell invasion in Matrigel	Primary endometriotic stromal cells	[117]
Estrogenic activity	Dose-dependent agonistic and antagonistic activity	Endometrial Ishikawa cell line	[118]
↓Cell proliferation, ↑Apoptosis	↓Cell number, DNA fragmentation	Primary endometrial epithelial cells	[119]
↓Endometriotic lesion number and volume, ↓Cell proliferation ↑Apoptosis, ↓Angiogenesis	↓PCNA, ↓CD34, ↓Peritoneal VEGF ↓Vascular density area, DNA fragmentation	Murine endometriosis model	[119]
↓Cell proliferation, ↑Apoptosis	↓MKI67, ↑PCNA, DNA fragmentation	Murine endometriosis model	[117]
↓Cell proliferation	↓ERα, ↓Ki-67	Immunocompromised mouse endometriosis model (RAG-2 knockout)	[118]
↓Cell proliferation	↓IGF-1, ↓*HGF*, ↓HGF	Primary eutopic and ectopic endometrial stromal cells from endometriosis patients	[120]
↓Cell proliferation, ↑Apoptosis ↓Angiogenesis	↑*P53*, ↑*BAX*, ↑*CASP3*, ↑*SIRT1*, ↓NO	3D primary endometriotic and endometrial cell cultures	[121]
↓Inflammation	↓*MCP1*, ↓*IL6*, ↓*IL8*, ↓MCP-1, ↓IL-6, ↓IL-8, ↓RANTES	Primary ectopic endometrial stromal cells	[122]
↓Endometriotic lesion size ↓Angiogenesis, ↓Inflammation	↓VEGF in peritoneal fluid, plasma and tissue ↓MCP-1 in peritoneal fluid and plasma	Rat endometriosis model	[123]
↓Endometriotic lesion size ↓Angiogenesis, ↓Inflammation	↓VEGF in peritoneal fluid and endometriotic tissue. ↓MCP-1 in peritoneal fluid	Rat endometriosis model	[124]
↓Endometriotic lesion size ↓Oxidation	↑SOD activity in serum and tissue ↑GPX activity in serum and tissue	Rat endometriosis model	[125]
↓Cell proliferation, ↓Cell migration ↓Cell invasion	EMT process suppression ↓MTA1, ↓ZEB2	Primary endometrial stromal cells Murine endometriosis model	[126]
↓Endometriosis-related pain	↓aromatase activity, ↓COX-2	Patients treated for 2 months with 30 mg resveratrol to the hormone therapy	[127]
↓Cell invasion	↓*MMP2*, ↓*MMP9*, ↓MMP-2, ↓MMP-9 in endometrial tissue, fluid and serum	34 patients with endometriotic infertility treated with 400 mg resveratrol twice daily for 12–14 weeks with contraceptives	[128]

Table 1. *Cont.*

Mechanisms of Action	Molecular Targets	Disease Model	Ref.
↓ Angiogenesis, ↓Inflammation	↓*VEGF*, ↓*TNF*, ↓VEGF, ↓TNF-α in the eutopic endometrial tissue	34 patients with endometriosis within the implantation window treated with 400 mg resveratrol for 12–14 weeks	[129]

↑ Increase; ↓ Decrease; the gene name is italicized, protein name is not italicized. Abbreviations used in Table 1: HSD17B1, HSD17B2, 17β-hydroxysteroid dehydrogenase type 1 and 2; AKT, protein kinase B (PKB); ATG5, autophagy protein 5; BAX, BCL-2 associated X protein; BCL-2, B-cell lymphoma 2; BECN1, Beclin 1; CA-125, mucin-16; CASP-3, caspase-3; CCND1, G1/S-specific cyclin-D1; CD31, platelet endothelial cell adhesion molecule; CDC25A, M-phase inducer phosphatase 1; Col- I, collagen type1; COX-2, cyclooxygenase-2; CTGF, connective tissue growth factor; CYP19A1, aromatase gene; Cyt-C, cytochrome c; E2, estradiol; ER stress, endoplasmic reticulum stress; ERK1/2, extracellular signal-regulated kinase 1/2; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; FN, fibronectin; FURIN, oaired basic amino acid cleaving enzyme (proprotein convertase of MMPs); G-CSF, granulocyte colony-stimulating factor; GPX, glutathione peroxidase; HGF, hepatocyte growth factor; HO1, heme oxygenase; ICAM-1, intercellular adhesion molecule 1; IGF-1, insulin-like growth factor-1; IL-6, interleukin 6; IL-8, interleukin 8; IP-10, interferon gamma-induced protein 10; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; MKI67, marker of proliferation Ki-67; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; MTA1, metastasis-associated protein 1; mTOR, mammalian target of rapamycin; MT1-MMP, membrane type 1-matrix metalloproteinase; NCoR, nuclear receptor corepressor 1; NF-κB, nuclear factor kappa B; NO, nitric oxide; NQO1, NAD(P)H quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; P53, cellular tumor antigen p53; PAK1, p21-activated kinase 1; PCNA, proliferating cell nuclear antigen; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SIRT1, sirtuin 1; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SOD, superoxide dismutase; SRC-1, steroid receptor coactivator 1; SRC-3, steroid receptor coactivator 3; TAK1, transforming growth factor beta-activated kinase 1; TGFB1, transforming growth factor beta-1 proprotein; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor; VEGFC, vascular endothelial growth factor C; VEGFR2, vascular endothelial growth factor receptor 2; ZEB2, zinc finger E-box-binding homeobox 2; αSMA, α-Smooth muscle actin.

5.1. Flavonoids

5.1.1. Flavonols

Quercetin

Quercetin is a naturally occurring flavonol found in various fruits and vegetables such as onions, cauliflower, apples, berries, and chili peppers [89]. Quercetin exerts the anticancer effects by reducing cancer cell viability and enhancing apoptosis and autophagy through the modulation of Akt, mammalian target of rapamycin (mTOR), and HIF-1α signaling pathways [130]. An in vitro study demonstrated that quercetin inhibits cell proliferation and induces cell cycle arrest and apoptosis in endocervical cell lines VK2/E6E7 and End1/E6E7. Cell apoptosis symptoms included DNA fragmentation, loss of mitochondrial membrane potential and downregulation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK), and AKT signaling molecules [89].

Antiproliferative and anti-inflammatory effects of intraperitoneally injected quercetin were observed in an auto-implanted mouse model of endometriosis. A decrease in cyclin D1 expression in the luminal and glandular epithelium was observed in response to quercetin injection [89]. In rats with induced endometriosis, quercetin administration significantly decreased endometrial implants' size and serum E2 and TNF-α level compared to the related control group. The results indicated that quercetin treatment significantly increased antioxidant NAD(P)H enzyme quinone oxidoreductase 1 (NQO1) activity and its major transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) expression. The study depicted that quercetin, unlike conventional treatments of endometriosis, does not undesirably affect estrogen and progesterone production, emphasizing an advantage of this compound over the currently used medications. Furthermore, regression of endometrial lesions was found as an effect of enhanced autophagy through mTOR inhibition and overexpression autophagy-related markers, including Beclin-1 and Atg5, after the combined treatment with quercetin and metformin [90].

Favorable effects of natural active ingredients affecting main pathophysiological pathways of endometriosis demonstrated in preclinical models require proof in clinical trials. Signorile et al. (2018) investigated 30 patients defined as stage IV of endometriosis treated for three months with a dietary supplement containing, e.g., 200 mg quercetin. Clinicians assessed the reduction in the inflammatory response and endometriosis's symptoms and its harmful effects on affected organs in patients with endometriosis. Experimental data obtained proved a significant effect in reducing the pain and other disabling disease symptoms in the patients treated with a dietary supplement. The substantial decrease in serum PGE_2 levels observed after treatment indicated supplement anti-inflammatory potential. Moreover, in treated patients, the endometriosis foci' size was reduced due to the decreased serum CA-125 concentration, as the authors believe. The authors suggest that further studies of dietary supplement combined with standard therapies are necessary to establish potentially practical new endometriosis treatment strategies [91].

5.1.2. Flavones

Apigenin

Scientists suggest that apigenin (4′,5,6-trihydroxyflavone), present in significant amounts in various fruits (apples, grapes, oranges) and vegetables (onion, parsley, celery), is suitable for the prevention and treatment of diverse diseases such as diabetes, metabolic disorders, cardiovascular and neuronal diseases, and cancers [131]. Apigenin prevents or inhibits disease progression by the involvement in cell cycle arrest, apoptosis induction, anti-inflammatory, and free radical scavenging mechanisms; thus, it is also proposed as a potential therapeutic agent in reproductive disorders [92]. In this context, apigenin reduced the proliferation of two human endometriotic cell lines in a dose-dependent manner and induced apoptosis by inhibition of phosphorylation of ERK1/2 and c-Jun N-terminal kinase (JNK) proteins and increasing pro-apoptotic proteins, including BAX and cytochrome c. Additionally, apigenin treated cells accumulated excessive ROS, experienced lipid peroxi-

dation and endoplasmic reticulum stress and lost mitochondrial membrane potential with an increase in calcium ions in the cytosol [92]. In another study, apigenin reduced mitogenic activity, inflammatory response, and attenuated TNF-α–induced cell proliferation and COX-2/PGE_2 synthesis via the inactivation of the NF-κB pathway in endometriotic stromal cells [93]. The biological potential of apigenin derivatives-rich extracts obtained from *A. austriaca* flowers has been shown in a rat endometriosis model. The most active apigenin-rich fraction remarkably decreased adhesion and endometriotic implant volumes and TNF-α, VEGF, and IL-6 levels [94]. Consistent with apigenin's biological activity results, this flavone is suggested as a potential novel therapeutic agent to overcome current limitations in preventing or treating endometriosis. However, further preclinical basic research and clinical trials, in particular, are strongly required.

Baicalein

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from *Scutellaria baicalensis* Georgi's root, a traditional herb in Chinese herbal medicine [132]. Baicalein's pharmacological properties, such as anti-tumor, anti-bacterial, anti-viral, anti-allergic, anti-oxidant, and cytoprotective effects, have been reviewed recently [95]. Anti-cancer effects, target mechanisms and signaling pathways of baicalein are of increasing interest from scientists. Baicalein has been studied in many types of malignancy, including bladder, melanoma, prostate, and breast cancer, as well as in gynecologic tumors such as cervical and ovarian cancer [133]. Baicalein strongly inhibited the proliferation of breast cancer MCF-7 cells and induced apoptosis via suppressing 17β-estradiol-induced transactivation [134]. Other in vivo studies revealed the inhibitory effect of baicalein against cervical cancer [135]. Molecular mechanisms described so far include regulation of cell cycle by inhibition several cyclins or cyclin-dependent kinases (CDK), cell proliferation arresting through the Wnt/β-catenin signaling pathway, suppression of PD-L1 expression to promote anti-tumor immunity, as well as induction of apoptosis and autophagy via the ERK/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway and caspase activation [136–138]. Due to the reported anti-tumor properties, Jin et al. (2017) and Ke et al. (2021) evaluated baicalein anti-endometriotic potential using human endometrial stromal cells in vitro. The results indicated that baicalein reduces ectopic endometrial stromal cell viability and induces apoptosis through the NF-κB signaling pathway. Following baicalein treatment, the level of anti-apoptotic protein BCL-2 decreased significantly. The number of cells in the G1 phase increased with a simultaneous decline in the number of cells in the S and G2/M phases compared with non-treated control cells [95]. More recent findings indicate that baicalein reduces ectopic endometrial stromal cell invasiveness, as demonstrated by analysis of invasion-related proteins, including MMP-9, MMP-2 and membrane type 1-matrix metalloproteinase (MT1-MMP) [96]. In a mouse model of endometriosis, intraperitoneal administration of baicalein inhibited ectopic lesions' growth, reduced MT1-MMP, proprotein convertase of MMPs (FURIN) and TGFB1 expression. Thus, baicalein with a potent pro-apoptotic and anti-invasive activity may provide a novel treatment strategy for endometriosis, as the authors suggested [96]. Promising preclinical research results indicate that undertaking clinical studies on endometriosis treatment with baicalein is warranted and recommended to obtain strong evidence for therapeutic baicalein potential.

Wogonin

Wogonin (*O*-methylated flavone), another flavonoid compound found in *Scutellaria baicalensis* Georgi root extract, has been reported in preclinical studies as an anti-tumor agent [97]. An anti-proliferative effect of wogonin has been analyzed in human endometrial stromal T-HESC cell line and primary stromal cell cultures established from endometrial biopsies from women with endometriosis. In these in vitro models, wogonin significantly inhibited primary and T-HESC cell proliferation causing cell cycle arrest at the G2/M phase and attenuated the ERα expression. In a murine model of endometriosis, wogonin reduced

lesion size and decreased the number of cells in the proliferation state, simultaneously increasing the number of apoptotic cells [97].

5.1.3. Isoflavonoids

Daidzein and Genistein

Isoflavones, a subgroup of the flavonoids found in soybeans, share a similar structure with estradiol but reportedly display antagonistic estrogen properties [22]. Structural similarities due to their heterocyclic phenolic structure facilitate isoflavones bind to estrogen receptors ERα and ERβ and mimic estrogenic actions [139]. Soy isoflavones are found mainly as glycosides with daidzein and genistein are the major representative isoflavone aglycones abundant in soybeans, which are converted by β-glycosidase derived from bacteria that colonize the small and large intestine [140,141].

A few in vitro and in vivo studies have investigated the effect of isoflavones on endometriosis. Daidzein-rich isoflavone aglycones (DRIA) inhibited cell proliferation of the endometriotic stromal cells at clinically relevant concentrations. DRIAs led to reduced expression of IL-6, IL-8, COX-2, and limited aromatase activity, as well as decreased levels of glucocorticoid-regulated kinase and PGE_2 in serum. DRIAs also reduced the number, weight and Ki-67 proliferative activity of endometriosis-like lesions in the murine endometriotic model [142]. Another study showed that genistein causes regression of an endometriotic implant in a rat model. In the presence of estrogen, genistein has demonstrated antagonistic estrogen activity on endometriotic implants [98]. The results from animal studies indicate DRIAs as a potential therapeutic agent for improving treatment strategies in endometriosis.

Clinical research showed the reduced risk of endometriosis development following soy isoflavones' ingestion. A significant correlation between higher urinary isoflavone levels (genistein and daidzein) and decreased risk of advanced endometriosis (stage III and IV), but not early endometriosis (stage I and II) occurrence, was observed within a case-control study involving Japanese women [99]. Future clinical studies and meta-analyses focusing on the benefits of phytoestrogen-rich product consumption by endometriosis patients are strongly required.

Puerarin

Puerarin is the primary bioactive ingredient isolated from the root of the *Pueraria lobata*. It has proven potential in managing cardiovascular diseases, cerebrovascular disorders, cancer, Parkinson's disease, Alzheimer's disease, diabetes, and diabetic complications [143]. Puerarin exerted a vasodilatory effect by activating large conductance voltage and calcium-activated potassium channels in rat models. Puerarin could significantly inhibit diabetic vascular complications by decreasing P-selectin, low-density lipoproteins, and cholesterol in the blood and downregulating mRNA expression of aorta vascular cell adhesion molecule in diabetic rats. The preventive activity of puerarin against oxidative stress-induced neurodegeneration has been attributed to blocking ROS overproduction and lipid peroxidation, inducing nuclear Nrf2 protein expression, and activating catalase and GSH peroxidase and the transcription and translation levels of heme oxygenase 1 [143].

Puerarin is commonly regarded as a phytoestrogen because it binds to estrogen receptors and competes with estradiol to exhibit an anti-estrogenic effect [144]. Its role in treating endometriosis, an estrogen-dependent disorder, has been well elucidated in several studies. Puerarin suppressed invasion and vascularization of endometriosis tissue activated by E2 through reducing the accumulation of MMP-9, ICAM-1, and VEGF and increasing the level of tissue inhibitor of metalloproteinase 1 (TIMP-1) in primary endometriotic stromal cells and in chick chorioallantoic membrane in vivo model [100]. Puerarin can suppress estrogen-stimulated proliferation of endometriotic stromal cells and downregulate the transcription and protein level of cyclin D1 and cdc25A by promoting ERα corepressors' recruitment and limiting the recruitment of coactivators. The investigation suggests that a puerarin-dependent interaction between ERα and corepressor complexes may be pivotal

for puerarin's anti-estrogenic effect, indicating that this isoflavone could be a potential therapeutic compound for endometriosis treatment [101]. In vivo investigations showed that puerarin inhibits aromatase and COX-2 in ectopic endometrium, thereby reducing E2 and PGE_2 and consequently prevents the growth of ectopic endometriotic cells [102]. Clinical studies on the potential of puerarin in the treatment of endometriosis have not been conducted to date.

5.1.4. Flavanones

Naringenin

Naringenin, mainly found in citrus fruits, is known for having anti-oxidant, anti-proliferative, anti-inflammatory, and anti-angiogenic properties in chronic and metabolic diseases [145,146]. In cancers, as preclinical studies reported, naringenin induced ROS-mediated cell death and inhibited cell migration and invasion [146,147]. Naringenin being a phytoestrogen, impairs ERα signaling by interfering with MAPK, PI3K, and ERK1/2 signaling pathways [148]. Considering the biological potential, scientists undertook investigations to evaluate the ameliorative role of naringenin in endometriosis.

In vitro studies showed that naringenin suppresses proliferation and induces apoptosis in endometriosis cells by decreasing mitochondrial membrane potential and ROS generation. The apoptotic effect of naringenin involved activation of PI3K and MAPK cell signaling pathways [103]. An animal study revealed naringenin potential against endometriosis by reducing expression of various endometriosis prognostic markers (TAK1, PAK1, VEGF, PCNA, MMP2, and MMP-9) in endometriotic lesions developed in rats. Naringenin prevented endometriosis propagation via anti-inflammatory mechanisms by modulating the serum TNF-α, NO level, and proapoptotic effects as ROS overproduction and the loss of mitochondrial membrane potential. Further, naringenin provoked depletion of Nrf2, a transcriptional factor controlling transcription of endogenous antioxidant enzymes, which protect cells against oxidative damage and consequent down-regulation of cytoprotective genes, thereby inhibiting endometriotic cell proliferation [104]. The main direction for future studies indicated by scientists concerns the continuation of research on naringenin potential in endometriosis treatment by applying endometrium samples from a larger population of endometriotic patients [104].

5.1.5. Chalcones

Xanthohumol

Xanthohumol, a prenylated flavonoid, originates from naringenin chalcone and found in hop's female inflorescences (*Humulus lupulus* L.) [149]. Numerous studies have demonstrated its pleiotropic chemopreventive activity for cancer due to anti-proliferative, anti-inflammatory, and anti-angiogenic properties [149]. Xanthohumol effectively inhibited endometriotic lesions' development and reduced the level of PI3K protein in a BALB/c mouse model of endometriosis. Moreover, xanthohumol decreased the microvessels' density and did not adversely affect the reproductive tract organs, suggesting its potential for the selective treatment of endometriosis [105].

5.1.6. Flavanols

Epigallocatechin Gallate

Epigallocatechin gallate (EGCG) is the main flavanol found in black and white tea, especially in green tea [150]. This compound has received enormous pharmacological attention because of its putative beneficial health effects for treating different cancer types, based on its anti-oxidant, anti-angiogenic, and anti-proliferative properties [151,152].

Ex vivo studies showed that EGCG suppressed cell proliferation and induced cell death in endometrial epithelial cells from human biopsies [119]. Interestingly, EGCG more effectively inhibited cell proliferation of endometriotic stromal cells than their normal counterparts, such as endometrial stromal cells [152]. Studies performed by Matsuzaki and Darcha (2014) revealed that treatment with EGCG significantly inhibited proliferation,

migration, and invasion of endometrial and endometriotic stromal cells via inhibition of MAPK and Smad signaling pathways. EGCG also attenuated cell-mediated collagen gel contraction according to both endometriotic and endometrial cells. EGCG treatment substantially decreased the expression of markers αSMA, collagen-I, fibronectin (FN), and connective tissue growth factor (CTGF) known to be involved in fibrogenesis in endometriotic stromal cells [106]. Another study demonstrated that treatment with EGCG for two weeks reduced endometriotic lesions by downregulating angiogenic VEGFA mRNA expression and upregulating NF-κB and MAPK 1 mRNA expression [153]. The subsequent experiments showed that EGCG exerted an inhibitory effect on endometriosis-associated angiogenesis by decreasing the number and size of microvessels and VEGF-C/VEGF receptor 2 (VEGFR-2) expression and signaling [107]. A pro-drug of EGCG (pro-EGCG, EGCG octaacetate) with improved stability and bioavailability exhibited higher antioxidant, antiangiogenic, and inhibitory activity than EGCG in the mice model. Treatment with the pro-EGCG resulted in poor lesion neovascularization and decreased plasma VEGF concentration [108]. EGCG, which exerts potent anticancer effects, could represent an anti-angiogenic agent for endometriosis.

Currently, the Chinese University of Hong Kong has started a clinical trial to evaluate green tea's efficacy and safety in endometriosis. In the study, 185 patients with endometrioma will randomize into either experimental group, which will receive high-purity EGCG (400 mg of SUNPHENON EGCG) twice per day or a placebo group. Supplement administration will take three months before a planned surgery. Change in endometriotic lesion size will be a primary measured outcome of the study. Further, monitoring pain, quality of life, endometriotic lesions collected as biopsies, the number and density of neovasculature and severe adverse events and side effects are scheduled as the secondary outcomes (Clinical Trials.gov ID: NCT02832271).

5.2. Other Polyphenols

Curcumin

Curcumin is a polyphenolic monomer extracted from turmeric of *Curcuma longa* L. Scientific research suggests that curcumin represents some potential therapeutic roles as an anti-inflammatory, anti-cancer, and anti-aging agent [154]. Several in vitro and in vivo studies have proved its pharmacological activities in endometriosis management.

In human endometriotic stromal cells, curcumin attenuated TNF-α-stimulated expression of ICAM-1, VCAM-1, and secretion of IL-6, IL-8, and MCP-1 by inhibiting activation of the transcription factor NF-κB [109]. Additionally, curcumin reduced endometrial cell proliferation by decreasing estrogen level [110]. Moreover, treatment with curcumin altered human endometriotic and endometrial stromal cell morphology, and interfered with cell proliferation and cell division. Curcumin arrested the cell cycle in the G1 phase and induced apoptosis via downregulation of VEGF expression in human endometriotic and endometrial stromal cells [111]. A recent study has shown that curcumin is a potent inhibitor of secretion of pro-inflammatory and pro-angiogenic chemokines and cytokines by stromal cells derived from eutopic endometrium in endometriosis and by normal endometrial stromal cells [112].

In the murine model, curcumin administration was associated with the reduction in endometriosis progression and apoptosis activation. Curcumin caused the regression of endometriosis predominantly via the cytochrome c-mediated mitochondrial pathway of apoptosis, and apoptotic responses include both p53-dependent and independent path. Curcumin acted as an inhibitor of NF-κB translocation and downregulated MMP-3, thus mediating endometriosis regression [113]. Besides, curcumin therapy in endometriosis mice demonstrated a protective role against lipid peroxidation and protein oxidation [114]. In experimental rat models, curcumin decreased the weight and volume of endometriotic tissues in a dose-dependent manner via downregulating VEGF expression [115]. Curcumin inhibited endometriotic foci development by interfering with microvessels' density, showing potent antiangiogenic activity [116]. In summary, curcumin targets multiple molecular

and cellular mechanisms in the pathogenesis of endometriosis, such as inflammation, attachment and angiogenesis, that might considerably improve and relieve endometriosis management.

Interestingly, the Medical University of Vienna recruited endometriosis patients for a randomized interventional clinical trial of a curcumin dietary supplement called Flexofytol, administered in two capsules containing 42 mg of curcumin twice a day for four months. Clinicians assumed that the average pain score from baseline to four months after treatment begins would be a primary outcome measured. As the secondary outcomes, they defined a decrease in the number of days with pain, alleviation of dyspareunia, dysuria, dyschezia, and the change in the quality of life and sexual function (Clinical Trials.gov ID: NCT04150406).

5.3. Phenolic Acids

Rosmarinic Acid

Rosmarinic acid, found in several plants such as *Rosmarinus officinalis*, *Salvia officinalis*, and *Thymus* sp., is a phenolic compound with various health-promoting and therapeutic properties [155,156]. Its antioxidant, anti-inflammatory, anti-tumor, and anti-angiogenic functions may be relevant for endometriosis treatment. Ferella's team (2018) assessed the anti-endometriosis potential of rosmarinic acid and carnosic acid (phenolic diterpene), which occur together in rosemary (*Rosmarinus officinalis*) and common sage (*Salvia officinalis*). They applied human endometrial stromal cell culture and a BALB/c model with induced endometriotic-like lesions. In the study, both compounds suppressed cell proliferation and reduced the size of endometriotic lesions in mice. Furthermore, rosmarinic acid promoted apoptosis in endometriotic tissue and reduced intracellular ROS accumulation in human endometrial stromal cells [97].

5.4. Stilbenes

Resveratrol

Resveratrol is a phytoalexin polyphenol, which various plants naturally produce in response to ultraviolet radiation and fungal infections [157]. Resveratrol naturally occurs in many plant species, including peanuts, berries, legumes, and grasses; however, resveratrol's richest sources are grapes and red wine. The first scientific reports about the resveratrol phenomenon presented in 1992 indicated resveratrol as a cardioprotective agent addressed to the French paradox and initiated extensive research activity on this compound [158]. Besides its cardioprotective effects, resveratrol has become well known due to its anti-cancer potential, first demonstrated a few years later, as appeared by its ability to suppress all carcinogenesis stages [159]. Ever since, numerous studies suggest that resveratrol has various health beneficial properties, including anti-cancer, anti-inflammatory, anti-oxidant, anti-microbial, and anti-angiogenic functions [160]. It can also protect against many age-related diseases, such as cardiovascular disease, diabetes, arthritis, and neurodegenerative disorders [161].

Several possible molecular targets for the protective effects of resveratrol have been proposed. COX-2, a key regulator of tumorigenesis development and circulatory homeostasis, has been inhibited by resveratrol in the in vitro and in vivo models of cancers. Cardiovascular benefits of resveratrol refer to its property to facilitate endothelial NO production, preventing thrombogenic and atherogenic processes by relaxing vascular smooth muscle cells and upregulating blood flow [161]. Resveratrol's capacity to extend lifespan underlies activation of silent mating type information regulation homolog 1 (SIRT1), a class III histone deacetylase responsible for controlling gene expression, DNA repair, metabolism, response to oxidative stress, mitochondrial function, and biogenesis. SIRT1 protects cells from oncogenic transformation and acts on specific targets in defined cellular signaling pathways in tumors. Resveratrol has been reported to possess neuroprotective, anti-tumorigenic functions via SIRT1 activation [162].

Resveratrol is widely considered an innovative natural agent in the alternative and complementary therapy of severe conditions by affecting multiple pivotal transduction pathways involved in disease progression. Resveratrol anti-inflammatory potential, including prostaglandin synthesis inhibition, has been suggested to probably contribute to the prevention and treatment of endometriosis [163]. The therapeutic effect of resveratrol on endometriosis was first evaluated by Bruner-Tran et al. (2011). They reported that resveratrol could reduce the number and size of endometriotic lesions in a nude mouse model. The resveratrol effect correlated with decreased endometrial cell proliferation and increased apoptotic cell death in lesions. In an in vitro study, resveratrol induced a concentration-dependent reduction in endometrial stromal cell invasiveness [117]. Since that report, interest in resveratrol has grown significantly, including resveratrol's protective mechanisms in endometriosis.

In the next research, resveratrol reduced human endometrial epithelial cell proliferation and increased apoptosis in primary cultures [119]. Compared to estrogen, resveratrol acted as both an agonist at low concentrations, whereas it played an antagonistic role at high concentrations. In the immunocompromised mouse model of endometriosis, reduced cell proliferation in the lesion has been observed with concomitantly decreasing epithelial ERα level [118]. Moreover, a recent study revealed that resveratrol treatment reduced expression of IGF-1 and HGF, pivotal molecules in endometriotic lesion growth and angiogenesis, in eutopic and ectopic endometrial stromal cells derived from endometriosis patients [120]. Another recent study looked at the effect of resveratrol at different concentrations on tissue growth, angiogenesis and NO secretion, and expression of apoptosis-related genes in human endometriotic and endometrial tissue in 3D culture. The mean growth of endometriotic and endometrial tissue showed significant dose-dependent inhibition during the treatment with resveratrol. Furthermore, resveratrol dose- and time-dependently reduced NO concentration in endometriotic and endometrial tissues. Experimental data also demonstrated that the expression of proapoptotic genes P53, BAX, CASP3, and SIRT1 increased significantly in the endometriotic and endometrial tissues after treatment with various resveratrol doses [121]. In vitro study showed the anti-inflammatory effect of resveratrol reflected in the suppression of MCP-1, IL-6, IL-8, and RANTES in ectopic endometrial stromal cells of endometriotic women [122].

Several promising consistent in vivo studies reported the beneficial effects of resveratrol on endometriotic lesions in animal models. Namely, resveratrol attenuated the inflammatory response in the peritoneal environment and decreased endometriosis development through its anti-angiogenic and anti-inflammatory properties. Resveratrol inhibitory effect was evaluated using an experimentally induced endometriosis rat model. Results showed a significant reduction in the implant size, a decrease in VEGF and MCP-1 in the peritoneal fluid and VEGF in plasma, and highly suppressed of VEGF expression in the endometriotic tissue within considerable histological changes in the endometriotic foci following 14-day treatment with resveratrol at a dose of 10 mg/kg [123]. In a similar rat model with induced endometriosis, resveratrol's effectiveness was evaluated based on its ability to inhibit angiogenesis and inflammation. After a 21-day administration, resveratrol in a 60 mg/kg dose reduced the implants' areas and lowered VEGF and MCP-1 levels. Interestingly, resveratrol's therapeutic potential was comparable with leuprolide acetate, used as a drug in conventional endometriosis treatment [124]. In another study in the rat endometriosis model, resveratrol showed ameliorative effects on endometriotic implants due to its potent antioxidant properties. After treatment, scientists observed a dose-dependent reduction in endometriotic implant volumes and increased SOD and GPX activities in rats' sera and tissues [125]. In a more recent study, Kong et al. (2020) presented the role of epithelial-mesenchymal transition in endometriosis's pathogenesis and resveratrol suppressive potential within metastasis-associated pathways in endometriosis. Resveratrol inhibited the proliferation, migration, and invasion of endometrial stromal cells in in vitro cultures, and suppressed ectopic endometrium growth in the mouse endometriosis model. Kong's team observed that resveratrol suppressed the epithelial-mesenchymal

transition process in endometriosis mice and endometrial cells. In these experimental models, resveratrol downregulated the expression of metastasis-associated protein 1 (MTA1) and transcription factor zinc finger E-box-binding homeobox 2 (ZEB2), essential components that contribute to metastasis by promoting the transformation of epithelial cells into mesenchymal cells [126].

Clinicians also investigated the therapeutic potential of resveratrol in endometriosis in several clinical trials. Most clinical studies generally presupposed resveratrol's capacity to potentiate the actions of oral contraceptives in the treatment of endometriosis through its hypo-estrogenic action [164]. Maia et al. (2012) assessed the advantages of the association of resveratrol with oral contraceptives for the management of endometriosis-related pain. They monitored the resveratrol effect in 12 patients who failed to obtain pain relief after treatment with an oral contraceptive containing drospirenone with ethinylestradiol. After two months of using 30 mg of resveratrol to the standard hormone therapy, 82% of patients reported complete resolution of dysmenorrhea and a significant reduction in pain occurrence. In a separate experiment, the same authors investigated resveratrol's potential for regulating COX-2 and aromatase expression, as reducing these enzymes is significant in overcoming chronic pelvic pain. Clinicians analyzed the aromatase and COX-2 expression in the endometrial tissue of 42 patients, of which 26 patients administered the combination of resveratrol with an oral contraceptive. The results showed that the combined therapy successfully reduces the aromatase and COX-2 expression compared with hormone therapy alone [127].

Another clinical trial investigating the use of resveratrol as an adjuvant treatment of pain assumed a randomized, double-blind, controlled comparison of resveratrol versus placebo to treat endometriosis-related pain. The study involved 44 eligible women with a laparoscopic diagnosis of endometriosis. It investigated whether 40 mg/day of resveratrol with a monophasic contraceptive pill could attenuate pain scores after 42 days of administration. The result presented no difference between treatments when comparing pain scores between groups after treatment with resveratrol plus contraceptive and contraceptive with placebo. The secondary outcomes of this research measured the CA-125 and prolactin level. The obtained data documented the reduction in CA-125 plasma level in both the placebo and resveratrol groups but did not find changes in prolactin level [165].

More recently, researchers at the Tehran University of Medical Sciences carried out an exploratory clinical trial, which included 34 patients with endometriosis-associated infertility. Participants of the study, randomly and equally divided into control and treatment groups, took 400 mg resveratrol twice daily for 12–14 weeks along with oral contraceptives in the last three weeks. The clinical trial showed that resveratrol could modify the inflammation process in the endometrium of women with endometriosis. The results obtained revealed downregulation of the mRNA and protein expression of MMP-2 and MMP-9 in endometrium tissue, endometrium fluid, and serum following resveratrol treatment. In conclusion, the authors emphasized the need for further research on the effect of resveratrol on MMP-2 and MMP-9 expression in women with endometriosis [128].

The same group of scientists examined the effect of resveratrol on VEGF and TNF-α expression in the eutopic endometrium of infertile patients with endometriosis within the implantation window in a randomized exploratory trial. As previously, the study involved 34 patients, divided into a placebo-controlled group and treatment group of 17 patients administrated 400 mg of resveratrol for 12 to 14 weeks. The obtained results indicated that resveratrol decreased VEGF and TNF-α expression as markers of angiogenesis and inflammation. According to the authors, further clinical trials are necessary to reveal resveratrol involvement in different pathways like angiogenesis, inflammation, and oxidative stress to confirm resveratrol potential as a therapeutic approach in endometriosis treatment [129].

6. Conclusions and Future Perspectives

The pathophysiologic mechanisms involved in developing an extremely heterogeneous disease, like endometriosis, have not yet been fully elucidated, and available treat-

ment interventions are currently limited. The discovery of new therapeutic agents and improvements in existing treatment strategies seems to be necessary. Natural polyphenols exhibit a pleiotropic action profile and could exert anti-endometriotic effects via comprehensive interactions with multiple molecular targets associated with endometriosis, such as cell proliferation, apoptosis, inflammation, oxidative stress, angiogenesis, and invasiveness. Moreover, some of these phytochemicals can exert a potent phytoestrogenic effect modulating the estrogen networks without inducing severe side effects contrary to the conventional anti-estrogenic therapy against endometriosis. The available results of some studies reveal that natural bioactive compounds do not affect fertility, reproductive organs, and the development of offspring [105,108]. Additionally, an alternative endometriosis therapy approach with therapeutic potential similar to conventional management is inexpensive and suitable for long-term and safe endometriosis patients' treatment.

However, existing evidence suggesting an attractiveness of naturally occurring substances for treating endometriosis has been provided mainly by pre-clinical in vitro research and animal studies. Despite several clinical trials performed, there is still insufficient proofs to recommend implementing natural treatment strategies in daily clinical applications. Besides, detailed pharmacokinetic and pharmacodynamic data on the administration of many polyphenols are still unavailable; thus, further clinical studies are necessary to confirm the efficacy of these compounds in the treatment of endometriosis. Another significant limitation is the lack of physiological relevance of high polyphenol doses not achievable at routine food consumption, usually applied in the experimental endometriosis in vitro models. Moreover, most in vitro studies showed the therapeutic potential of naturally occurring polyphenols without considering their gastrointestinal digestion and metabolism by gut microorganisms. The extensive gastrointestinal metabolism of polyphenol compounds influences their bioavailability and intestinal absorption and, consequently, their therapeutic efficacy in tissues and organs. Furthermore, in combining natural agents with conventional pharmacotherapy, studies on bioactive compound-drug interactions are mandatory. Knowledge of the precise mechanisms of action of the natural compounds in the endometriosis's network of several cell signaling pathways is required, and further studies on this topic are needed.

It is worth emphasizing that polyphenols' overconsumption, especially as highly concentrated supplements isolated from the food matrix, may cause harmful health effects [166]. Reduced thiamin and folic acid transport have been reported due to polyphenols' pre-absorptive interactions during digestion in the gastrointestinal tract. Similarly, significant changes in some drugs' bioavailability can be observed due to polyphenols' interaction with drug transporters or digestive enzymes [167]. Besides, polyphenols can exert an iron-chelating effect and reduce iron absorption, which leads to deterioration of iron status. Previously published research hypothesized that soy-derived isoflavones adversely affect patients with endometriosis and estrogen-sensitive breast and endometrial cancer due to estrogen-like biologic activities and hormone-interfering properties [168,169]. However, most recent clinical trials have not confirmed the adverse effects of isoflavones and other phytoestrogens on women with risk and active estrogen-dependent cancers [170,171]. According to the latest reports, isoflavone-rich food and supplements are safe dietary factors for peri- and post-menopausal women and endometriosis patients [99,172].

In our opinion, natural-based endometriosis therapies are unlikely to serve as an exclusive therapy strategy and exert a potent curative effect, but they can overcome endometriosis-related symptoms. Combined with precision medical methods individually tailored to adjust patients' needs, the natural compounds can constitute an integral part and a central direction of developing future endometriosis therapeutic concepts.

Funding: This publication was co-financed within the framework of the Polish Ministry of Science and Higher Education's program: "Regional Initiative Excellence" in the years 2019–2022 (No. 005/RID/2018/19)", financing amount 12 000 000 PLN.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zondervan, K.T.; Becker, C.M.; Koga, K.; Missmer, S.A.; Taylor, R.N.; Viganò, P. Endometriosis. *Nat. Rev. Dis. Primers* **2018**, *4*, 9. [CrossRef] [PubMed]
2. Zondervan, K.T.; Becker, C.M.; Missmer, S.A. Endometriosis. *N. Engl. J. Med.* **2020**, *382*, 1244–1256. [CrossRef] [PubMed]
3. Dawson, A.; Fernandez, M.L.; Anglesio, M.; Yong, P.J.; Carey, M.S. Endometriosis and endometriosis-associated cancers: New insights into the molecular mechanisms of ovarian cancer development. *Ecancermedicalscience* **2018**, *12*. [CrossRef] [PubMed]
4. Murakami, K.; Kotani, Y.; Nakai, H.; Matsumura, N. Endometriosis-Associated Ovarian Cancer: The Origin and Targeted Therapy. *Cancers* **2020**, *12*, 1676. [CrossRef] [PubMed]
5. Missmer, S.; Tu, F.F.; Agarwal, S.K.; Chapron, C.; Soliman, A.M.; Chiuve, S.; Eichner, S.; Flores-Caldera, I.; Horne, A.W.; Kimball, A.B.; et al. Impact of Endometriosis on Life-Course Potential: A Narrative Review. *Int. J. Gen. Med.* **2021**, *ume 14*, 9–25. [CrossRef]
6. Giudice, L.C.; Kao, L.C. Endometriosis. *Lancet* **2004**, *364*, 1789–1799. [CrossRef]
7. Parasar, P.; Ozcan, P.; Terry, K.L. Endometriosis: Epidemiology, Diagnosis and Clinical Management. *Curr. Obstet. Gynecol. Rep.* **2017**, *6*, 34–41. [CrossRef]
8. Laganà, A.S.; Garzon, S.; Götte, M.; Viganò, P.; Franchi, M.; Ghezzi, F.; Martin, D.C. The Pathogenesis of Endometriosis: Molecular and Cell Biology Insights. *Int. J. Mol. Sci.* **2019**, *20*, 5615. [CrossRef]
9. Wang, Y.; Nicholes, K.; Shih, I.-M. The Origin and Pathogenesis of Endometriosis. *Annu. Rev. Pathol. Mech. Dis.* **2020**, *15*, 71–95. [CrossRef]
10. Zheng, W.; Cao, L.; Xu, Z.; Ma, Y.; Liang, X. Anti-Angiogenic Alternative and Complementary Medicines for the Treatment of Endometriosis: A Review of Potential Molecular Mechanisms. *Evidence-Based Complement. Altern. Med.* **2018**, *2018*, 1–28. [CrossRef]
11. Li, F.; Alderman, M.H.; Tal, A.; Mamillapalli, R.; Coolidge, A.; Hufnagel, D.; Wang, Z.; Neisani, E.; Gidicsin, S.; Krikun, G.; et al. Hematogenous Dissemination of Mesenchymal Stem Cells from Endometriosis. *Stem Cells* **2018**, *36*, 881–890. [CrossRef]
12. Medina, M.G.; Lebovic, D.I. Endometriosis-associated nerve fibers and pain. *Acta Obstet. Gynecol. Scand.* **2009**, *88*, 968–975. [CrossRef]
13. Lin, Y.-H.; Chen, Y.-H.; Chang, H.-Y.; Au, H.-K.; Tzeng, C.-R.; Huang, Y.-H. Chronic Niche Inflammation in Endometriosis-Associated Infertility: Current Understanding and Future Therapeutic Strategies. *Int. J. Mol. Sci.* **2018**, *19*, 2385. [CrossRef] [PubMed]
14. Mehedintu, C.; Plotogea, M.N.; Ionescu, S.; Antonovici, M. Endometriosis still a challenge. *J. Med. Life* **2014**, *7*, 349–357.
15. Della Corte, L.; Noventa, M.; Ciebiera, M.; Magliarditi, M.; Sleiman, Z.; Karaman, E.; Catena, U.; Salvaggio, C.; Falzone, G.; Garzon, S. Phytotherapy in endometriosis: An up-to-date review. *J. Complement. Integr. Med.* **2020**, *17*. [CrossRef]
16. Guo, S.-W. Recurrence of endometriosis and its control. *Hum. Reprod. Updat.* **2009**, *15*, 441–461. [CrossRef]
17. Allen, C.; Hopewell, S.; Prentice, A.; Gregory, D. Nonsteroidal anti-inflammatory drugs for pain in women with endometriosis. *Cochrane Database Syst. Rev.* **2009**, *2*, CD004753. [CrossRef]
18. Dunselman, G.A.J.; Vermeulen, N.; Becker, C.; Calhaz-Jorge, C.; D'Hooghe, T.; De Bie, B.; Heikinheimo, O.; Horne, A.W.; Kiesel, L.; Nap, A.; et al. ESHRE guideline: Management of women with endometriosis. *Hum. Reprod.* **2014**, *29*, 400–412. [CrossRef] [PubMed]
19. Greene, A.D.; Lang, S.A.; Kendziorski, J.A.; Sroga-Rios, J.M.; Herzog, T.J.; Burns, K.A. Endometriosis: Where are we and where are we going? *Reproduction* **2016**, *152*, R63–R78. [CrossRef]
20. Gheorghisan-Galateanu, A.; Gheorghiu, M.L.; Gheorghisan-Galateanu, A. Hormonal Therapy in Women of Reproductive Age with Endometriosis: An Update. *Acta Endocrinol. (Bucharest)* **2019**, *15*, 276–281. [CrossRef]
21. E Schindler, A. Dienogest in long-term treatment of endometriosis. *Int. J. Women's Health* **2011**, *3*, 175–184. [CrossRef]
22. Soares, S.R.; Martínez-Varea, A.; Hidalgo-Mora, J.J.; Pellicer, A. Pharmacologic therapies in endometriosis: A systematic review. *Fertil. Steril.* **2012**, *98*, 529–555. [CrossRef] [PubMed]
23. Jurkiewicz-Przondziono, J.; Lemm, M.; Kwiatkowska-Pamuła, A.; Ziółko, E.; Wójtowicz, M.K. Influence of diet on the risk of developing endometriosis. *Ginekol. Polska* **2017**, *88*, 96–102. [CrossRef]
24. Xu, Y.; Zhao, W.; Li, T.; Zhao, Y.; Bu, H.; Song, S. Effects of acupuncture for the treatment of endometriosis-related pain: A systematic review and meta-analysis. *PLoS ONE* **2017**, *12*, e0186616. [CrossRef] [PubMed]
25. Wattier, J.-M. Conventional analgesics and non-pharmacological multidisciplinary therapeutic treatment in endometriosis: CNGOF-HAS Endometriosis Guidelines. *Gynecol. Obstet. Fertil. Senol.* **2018**, *46*, 248–255. [CrossRef] [PubMed]
26. Taylor, H.S.; Osteen, K.G.; Bruner-Tran, K.L.; Lockwood, C.J.; Krikun, G.; Sokalska, A.; Duleba, A.J. Novel Therapies Targeting Endometriosis. *Reprod. Sci.* **2011**, *18*, 814–823. [CrossRef]
27. O'Hara, R.; Rowe, H.; Fisher, J. Self-management in condition-specific health: A systematic review of the evidence among women diagnosed with endometriosis. *BMC Women's Health* **2019**, *19*, 1–19. [CrossRef]
28. Armour, M.; Sinclair, J.; Chalmers, K.J.; Smith, C.A. Self-management strategies amongst Australian women with endometriosis: A national online survey. *BMC Complement. Altern. Med.* **2019**, *19*, 1–8. [CrossRef]
29. Missmer, S.A.; Chavarro, J.E.; Malspeis, S.; Bertone-Johnson, E.R.; Hornstein, M.D.; Spiegelman, D.; Barbieri, R.L.; Willett, W.C.; Hankinson, S.E. A prospective study of dietary fat consumption and endometriosis risk. *Hum. Reprod.* **2010**, *25*, 1528–1535. [CrossRef]

30. Kasi, P.D.; Tamilselvam, R.; Skalicka-Woźniak, K.; Nabavi, S.M.; Daglia, M.; Bishayee, A.; Pazoki-Toroudi, H.; Nabavi, S. Molecular targets of curcumin for cancer therapy: An updated review. *Tumor Biol.* **2016**, *37*, 13017–13028. [CrossRef]
31. Kong, S.; Zhang, Y.-H.; Liu, C.-F.; Tsui, I.; Guo, Y.; Ai, B.-B.; Han, F.-J. The Complementary and Alternative Medicine for Endometriosis: A Review of Utilization and Mechanism. *Evidence-Based Complement. Altern. Med.* **2014**, *2014*, 1–16. [CrossRef]
32. Trabert, B.; Peters, U.; De Roos, A.J.; Scholes, D.; Holt, V.L. Diet and risk of endometriosis in a population-based case–control study. *Br. J. Nutr.* **2010**, *105*, 459–467. [CrossRef] [PubMed]
33. Riccio, L.D.G.C.; Santulli, P.; Marcellin, L.; Abrão, M.S.; Batteux, F.; Chapron, C. Immunology of endometriosis. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *50*, 39–49. [CrossRef] [PubMed]
34. Parazzini, F.; Vigano, P.; Candiani, M.; Fedele, L. Diet and endometriosis risk: A literature review. *Reprod. Biomed. Online* **2013**, *26*, 323–336. [CrossRef]
35. Mohammadi, R.K.; Arablou, T. Resveratrol and endometriosis: In vitro and animal studies and underlying mechanisms (Review). *Biomed. Pharmacother.* **2017**, *91*, 220–228. [CrossRef]
36. Jiang, Q.-Y.; Wu, R.-J. Growth mechanisms of endometriotic cells in implanted places: A review. *Gynecol. Endocrinol.* **2012**, *28*, 562–567. [CrossRef]
37. Laux-Biehlmann, A.; D'Hooghe, T.; Zollner, T.M. Menstruation pulls the trigger for inflammation and pain in endometriosis. *Trends Pharmacol. Sci.* **2015**, *36*, 270–276. [CrossRef]
38. Han, S.J.; Jung, S.Y.; Wu, S.-P.; Hawkins, S.M.; Park, M.J.; Kyo, S.; Qin, J.; Lydon, J.P.; Tsai, S.Y.; Tsai, M.-J.; et al. Estrogen Receptor β Modulates Apoptosis Complexes and the Inflammasome to Drive the Pathogenesis of Endometriosis. *Cell* **2015**, *163*, 960–974. [CrossRef] [PubMed]
39. Azuma, Y.; Taniguchi, F.; Nakamura, K.; Nagira, K.; Khine, Y.M.; Kiyama, T.; Uegaki, T.; Izawa, M.; Harada, T. Lipopolysaccharide promotes the development of murine endometriosis-like lesions via the nuclear factor-kappa B pathway. *Am. J. Reprod. Immunol.* **2017**, *77*, e12631. [CrossRef] [PubMed]
40. Ballester, M.; Gonin, J.; Rodenas, A.; Bernaudin, J.-F.; Rouzier, R.; Coutant, C.; Daraï, E. Eutopic endometrium and peritoneal, ovarian and colorectal endometriotic tissues express a different profile of Nectin-1, -3, -4 and nectin-like molecule 2. *Hum. Reprod.* **2012**, *27*, 3179–3186. [CrossRef]
41. Agic, A.; Djalali, S.; Diedrich, K.; Hornung, D. Apoptosis in Endometriosis. *Gynecol. Obstet. Investig.* **2009**, *68*, 217–223. [CrossRef] [PubMed]
42. Taniguchi, F.; Kaponis, A.; Izawa, M.; Kiyama, T.; Deura, I.; Ito, M.; Iwabe, T.; Adonakis, G.; Terakawa, N.; Harada, T. Apoptosis and endometriosis. *Front. Biosci.* **2011**, *3*, 648–662. [CrossRef] [PubMed]
43. Sbracia, M.; Valeri, C.; Antonini, G.; Biagiotti, G.; Pacchiarotti, A.; Pacchiarotti, A. Fas and Fas-Ligand in Eutopic and Ectopic Endometrium of Women With Endometriosis: The Possible Immune Privilege of Ectopic Endometrium. *Reprod. Sci.* **2016**, *23*, 81–86. [CrossRef] [PubMed]
44. Yovich, J.L.; Rowlands, P.K.; Lingham, S.; Sillender, M.; Srinivasan, S. Pathogenesis of endometriosis: Look no further than John Sampson. *Reprod. Biomed. Online* **2020**, *40*, 7–11. [CrossRef] [PubMed]
45. Paul, S.; Bhattacharya, P.; Das Mahapatra, P.; Swarnakar, S. Melatonin protects against endometriosis via regulation of matrix metalloproteinase-3 and an apoptotic pathway. *J. Pineal Res.* **2010**, *49*, 156–168. [CrossRef]
46. Weusten, A.Y.D.; Groothuis, P.G.; Dunselman, G.A.; De Goeij, A.F.; Arends, J.W.; Evers, J.L. Morphological changes in mesothelial cells induced by shed menstrual endometrium in vitro are not primarily due to apoptosis or necrosis. *Hum. Reprod.* **2000**, *15*, 1462–1468. [CrossRef]
47. Kyama, C.M.; Overbergh, L.; Mihalyi, A.; Meuleman, C.; Mwenda, J.M.; Mathieu, C.; D'Hooghe, T.M. Endometrial and peritoneal expression of aromatase, cytokines, and adhesion factors in women with endometriosis. *Fertil. Steril.* **2008**, *89*, 301–310. [CrossRef]
48. Canis, M.; Bourdel, N.; Houlle, C.; Gremeau, A.-S.; Botchorishvili, R.; Matsuzaki, S. Trauma and endometriosis. A review. May we explain surgical phenotypes and natural history of the disease? *J. Gynecol. Obstet. Hum. Reprod.* **2017**, *46*, 219–227. [CrossRef]
49. Carr, B.R.; Arici, A.; Witz, C.A. Cell Adhesion Molecules and Endometriosis. *Semin. Reprod. Med.* **2003**, *21*, 173–182. [CrossRef]
50. Rutherford, E.J.; Hill, A.D.K.; Hopkins, A.M. Adhesion in Physiological, Benign and Malignant Proliferative States of the Endometrium: Microenvironment and the Clinical Big Picture. *Cells* **2018**, *7*, 43. [CrossRef]
51. Bałkowiec, M.; Maksym, R.B.; Włodarski, P.K. The bimodal role of matrix metalloproteinases and their inhibitors in etiology and pathogenesis of endometriosis (Review). *Mol. Med. Rep.* **2018**, *18*, 3123–3136. [CrossRef]
52. Weigel, M.T.; Krämer, J.; Schem, C.; Wenners, A.; Alkatout, I.; Jonat, W.; Maass, N.; Mundhenke, C. Differential expression of MMP-2, MMP-9 and PCNA in endometriosis and endometrial carcinoma. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2012**, *160*, 74–78. [CrossRef]
53. Bruner, K.L.; Eisenberg, E.; Gorstein, F.; Osteen, K.G. Progesterone and transforming growth factor-β coordinately regulate suppression of endometrial matrix metalloproteinases in a model of experimental endometriosis. *Steroids* **1999**, *64*, 648–653. [CrossRef]
54. Rocha, A.L.L.; Reis, F.M.; Taylor, R.N. Angiogenesis and Endometriosis. *Obstet. Gynecol. Int.* **2013**, *2013*, 1–8. [CrossRef] [PubMed]
55. Burney, R.O.; Giudice, L.C. Pathogenesis and pathophysiology of endometriosis. *Fertil. Steril.* **2012**, *98*, 511–519. [CrossRef]
56. Kianpour, M.; Nematbakhsh, M.; Ahmadi, S.M.; Jafarzadeh, M.; Hajjarian, M.; Pezeshki, Z.; Safari, T.; Eshraghi-Jazi, F. Se-rum and Peritoneal Fluid Levels of Vascular Endothelial Growth Factor in Women with Endometriosis. *Int. J. Fertil. Steril.* **2013**, *7*, 96–99. [PubMed]

57. Laschke, M.; Elitzsch, A.; Vollmar, B.; Vajkoczy, P.; Menger, M. Combined inhibition of vascular endothelial growth factor (VEGF), fibroblast growth factor and platelet-derived growth factor, but not inhibition of VEGF alone, effectively suppresses angiogenesis and vessel maturation in endometriotic lesions. *Hum. Reprod.* **2005**, *21*, 262–268. [CrossRef] [PubMed]
58. Becker, C.M.; Rohwer, N.; Funakoshi, T.; Cramer, T.; Bernhardt, W.; Birsner, A.; Folkman, J.; D'Amato, R.J. 2-Methoxyestradiol Inhibits Hypoxia-Inducible Factor-1α and Suppresses Growth of Lesions in a Mouse Model of Endometriosis. *Am. J. Pathol.* **2008**, *172*, 534–544. [CrossRef] [PubMed]
59. Machado, D.E.; Abrao, M.S.; Berardo, P.T.; Takiya, C.M.; Nasciutti, L.E. Vascular density and distribution of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Flk-1) are significantly higher in patients with deeply infiltrating endometriosis affecting the rectum. *Fertil. Steril.* **2008**, *90*, 148–155. [CrossRef] [PubMed]
60. Rizner, T.L. Diagnostic potential of peritoneal fluid biomarkers of endometriosis. *Expert Rev. Mol. Diagn.* **2015**, *15*, 557–580. [CrossRef]
61. Agic, A.; Xu, H.; Finas, D.; Banz, C.; Diedrich, K.; Hornung, D. Is Endometriosis Associated with Systemic Subclinical Inflammation? *Gynecol. Obstet. Investig.* **2006**, *62*, 139–147. [CrossRef] [PubMed]
62. Sikora, J.; Mielczarek-Palacz, A.; Kondera-Anasz, Z. Association of the Precursor of Interleukin-1β and Peritoneal Inflammation-Role in Pathogenesis of Endometriosis. *J. Clin. Lab. Anal.* **2016**, *30*, 831–837. [CrossRef]
63. Oosterlynck, D.J.; Meuleman, C.; Waer, M.; Koninckx, P.R.; Vandeputte, M. Immunosuppressive activity of peritoneal fluid in women with endometriosis. *Obstet. Gynecol.* **1993**, *82*, 206–212.
64. Laganà, A.S.; Triolo, O.; Salmeri, F.M.; Granese, R.; Palmara, V.I.; Frangez, H.B.; Bokal, E.V.; Sofo, V. Natural Killer T cell subsets in eutopic and ectopic endometrium: A fresh look to a busy corner. *Arch. Gynecol. Obstet.* **2016**, *293*, 941–949. [CrossRef]
65. Osuga, Y.; Koga, K.; Hirota, Y.; Hirata, T.; Yoshino, O.; Taketani, Y. Lymphocytes in Endometriosis. *Am. J. Reprod. Immunol.* **2010**, *65*, 1–10. [CrossRef]
66. Gupta, S.; Agarwal, A.; Krajcir, N.; Alvarez, J.G. Role of oxidative stress in endometriosis. *Reprod. Biomed. Online* **2006**, *13*, 126–134. [CrossRef]
67. Pisoschi, A.M.; Pop, A. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem.* **2015**, *97*, 55–74. [CrossRef]
68. Harlev, A.; Gupta, S.; Agarwal, A. Targeting oxidative stress to treat endometriosis. *Expert Opin. Ther. Targets* **2015**, *19*, 1447–1464. [CrossRef]
69. Lousse, J.-C.; Van Langendonckt, A.; Defrere, S.; Ramos, R.G.; Colette, S.; Donnez, J. Peritoneal endometriosis is an inflammatory disease. *Front. Biosci.* **2012**, *E4*, 23–40. [CrossRef]
70. Mulgund, A.; Doshi, S.; Agarwal, A. Chapter 25—The Role of Oxidative Stress in Endometriosis. In *Handbook of Fertility*; Watson, R.R., Ed.; Academic Press: San Diego, CA, USA, 2015; pp. 273–281. ISBN 978-0-12-800872-0.
71. Nagayasu, M.; Imanaka, S.; Kimura, M.; Maruyama, S.; Kobayashi, H. Nonhormonal Treatment for Endometriosis Focusing on Redox Imbalance. *Gynecol. Obstet. Investig.* **2021**, 1–12. [CrossRef]
72. Santanam, N.; Kavtaradze, N.; Murphy, A.; Dominguez, C.; Parthasarathy, S. Antioxidant supplementation reduces endometriosis-related pelvic pain in humans. *Transl. Res.* **2013**, *161*, 189–195. [CrossRef]
73. Bulun, S.E.; Monsavais, D.; Pavone, M.E.; Dyson, M.; Xue, Q.; Attar, E.; Tokunaga, H.; Su, E.J. Role of Estrogen Receptor-β in Endometriosis. *Nat. Med.* **2012**, *18*, 1016–1018. [CrossRef] [PubMed]
74. Mori, T.; Ito, F.; Koshiba, A.; Kataoka, H.; Tanaka, Y.; Okimura, H.; Khan, K.N.; Kitawaki, J. Aromatase as a target for treating endometriosis. *J. Obstet. Gynaecol. Res.* **2018**, *44*, 1673–1681. [CrossRef]
75. Chantalat, E.; Valera, M.-C.; Vaysse, C.; Noirrit, E.; Rusidze, M.; Weyl, A.; Vergriete, K.; Buscail, E.; Lluel, P.; Fontaine, C.; et al. Estrogen Receptors and Endometriosis. *Int. J. Mol. Sci.* **2020**, *21*, 2815. [CrossRef] [PubMed]
76. Smolarz, B.; Szyłło, K.; Romanowicz, H. The Genetic Background of Endometriosis: Can ESR2 and CYP19A1 Genes Be a Potential Risk Factor for Its Development? *Int. J. Mol. Sci.* **2020**, *21*, 8235. [CrossRef]
77. Bulun, S.E.; Cheng, Y.-H.; Pavone, M.E.; Yin, P.; Imir, G.; Utsunomiya, H.; Thung, S.; Xue, Q.; Marsh, E.E.; Tokunaga, H.; et al. 17β-Hydroxysteroid Dehydrogenase-2 Deficiency and Progesterone Resistance in Endometriosis. *Semin. Reprod. Med.* **2010**, *28*, 044–050. [CrossRef]
78. García-Gómez, E.; Vázquez-Martínez, E.R.; Reyes-Mayoral, C.; Cruz-Orozco, O.P.; Camacho-Arroyo, I.; Cerbón, M. Regulation of Inflammation Pathways and Inflammasome by Sex Steroid Hormones in Endometriosis. *Front. Endocrinol.* **2020**, *10*, 935. [CrossRef]
79. Marquardt, R.M.; Kim, T.H.; Shin, J.-H.; Jeong, J.-W. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? *Int. J. Mol. Sci.* **2019**, *20*, 3822. [CrossRef] [PubMed]
80. Grosso, G.; Godos, J.; Lamuela-Raventos, R.; Ray, S.; Micek, A.; Pajak, A.; Sciacca, S.; D'Orazio, N.; Del Rio, D.; Galvano, F. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Mol. Nutr. Food Res.* **2016**, *61*. [CrossRef] [PubMed]
81. Micek, A.; Godos, J.; Brzostek, T.; Gniadek, A.; Favari, C.; Mena, P.; Libra, M.; Del Rio, D.; Galvano, F.; Grosso, G. Dietary phytoestrogens and biomarkers of their intake in relation to cancer survival and recurrence: A comprehensive systematic review with meta-analysis. *Nutr. Rev.* **2021**, *79*, 42–65. [CrossRef]
82. Bhyan, S.B.; Zhao, L.; Wee, Y.; Liu, Y.; Zhao, M. Genetic links between endometriosis and cancers in women. *PeerJ* **2019**, *7*, e8135. [CrossRef] [PubMed]

83. Taskin, O.; Rikhraj, K.; Tan, J.; Sedlak, T.; Rowe, T.C.; Bedaiwy, M.A. Link between Endometriosis, Atherosclerotic Cardiovascular Disease, and the Health of Women Midlife. *J. Minim. Invasive Gynecol.* **2019**, *26*, 781–784. [CrossRef] [PubMed]
84. Micek, A.; Godos, J.; Del Rio, D.; Galvano, F.; Grosso, G. Dietary Flavonoids and Cardiovascular Disease: A Comprehensive Dose–Response Meta-Analysis. *Mol. Nutr. Food Res.* **2021**, *65*, 2001019. [CrossRef] [PubMed]
85. Wieser, F.; Cohen, M.; Gaeddert, A.; Yu, J.; Burks-Wicks, C.; Berga, S.L.; Taylor, R.N. Evolution of medical treatment for endometriosis: Back to the roots? *Hum. Reprod. Updat.* **2007**, *13*, 487–499. [CrossRef] [PubMed]
86. Flower, A.; Liu, J.P.; Chen, S.; Lewith, G.; Little, P. Chinese herbal medicine for endometriosis. *Cochrane Database Syst. Rev.* **2009**, *3*, CD006568. [CrossRef]
87. Sesti, F.; Capozzolo, T.; Pietropolli, A.; Collalti, M.; Bollea, M.R.; Piccione, E. Dietary therapy: A new strategy for management of chronic pelvic pain. *Nutr. Res. Rev.* **2010**, *24*, 31–38. [CrossRef]
88. Koes, R.; Verweij, W.; Quattrocchio, F. Flavonoids: A colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* **2005**, *10*, 236–242. [CrossRef]
89. Park, S.; Lim, W.; Bazer, F.W.; Whang, K.-Y.; Song, G. Quercetin inhibits proliferation of endometriosis regulating cyclin D1 and its target microRNAs in vitro and in vivo. *J. Nutr. Biochem.* **2019**, *63*, 87–100. [CrossRef]
90. Jamali, N.; Zal, F.; Mostafavi-Pour, Z.; Samare-Najaf, M.; Poordast, T.; Dehghanian, A. Ameliorative Effects of Quercetin and Metformin and Their Combination Against Experimental Endometriosis in Rats. *Reprod. Sci.* **2021**, *28*, 683–692. [CrossRef]
91. Signorile, P.G.; Viceconte, R.; Baldi, A. Novel dietary supplement association reduces symptoms in endometriosis patients. *J. Cell. Physiol.* **2018**, *233*, 5920–5925. [CrossRef]
92. Park, S.; Lim, W.; Bazer, F.W.; Song, G. Apigenin induces ROS-dependent apoptosis and ER stress in human endometriosis cells. *J. Cell. Physiol.* **2018**, *233*, 3055–3065. [CrossRef]
93. Suou, K.; Taniguchi, F.; Tagashira, Y.; Kiyama, T.; Terakawa, N.; Harada, T. Apigenin inhibits tumor necrosis factor α–induced cell proliferation and prostaglandin E2 synthesis by inactivating NFκB in endometriotic stromal cells. *Fertil. Steril.* **2011**, *95*, 1518–1521. [CrossRef]
94. Ilhan, M.; Ali, Z.; Khan, I.A.; Taştan, H.; Akkol, E.K. Promising activity of Anthemis austriaca Jacq. on the endometriosis rat model and isolation of its active constituents. *Saudi Pharm. J.* **2019**, *27*, 889–899. [CrossRef]
95. Jin, Z.; Huang, J.; Zhu, Z. Baicalein reduces endometriosis by suppressing the viability of human endometrial stromal cells through the nuclear factor-κB pathway in vitro. *Exp. Ther. Med.* **2017**, *14*, 2992–2998. [CrossRef]
96. Ke, J.; Yang, J.; Li, J.; Xu, Z.; Li, M.; Zhu, Z. Baicalein inhibits FURIN-MT1-MMP-mediated invasion of ectopic endometrial stromal cells in endometriosis possibly by reducing the secretion of TGFB1. *Am. J. Reprod. Immunol.* **2021**, *85*, 13344. [CrossRef]
97. Ferella, L.; Bastón, J.I.; Bilotas, M.A.; Singla, J.J.; González, A.M.; Olivares, C.N.; Meresman, G.F. Active compounds present in Rosmarinus officinalis leaves and Scutellaria baicalensis root evaluated as new therapeutic agents for endometriosis. *Reprod. Biomed. Online* **2018**, *37*, 769–782. [CrossRef] [PubMed]
98. Yavuz, E.; Oktem, M.; Esinler, I.; Toru, S.A.; Zeyneloglu, H.B. Genistein causes regression of endometriotic implants in the rat model. *Fertil. Steril.* **2007**, *88*, 1129–1134. [CrossRef]
99. Tsuchiya, M.; Miura, T.; Hanaoka, T.; Iwasaki, M.; Sasaki, H.; Tanaka, T.; Nakao, H.; Katoh, T.; Ikenoue, T.; Kabuto, M.; et al. Effect of Soy Isoflavones on Endometriosis: Interaction with Estrogen Receptor 2 Gene Polymorphism. *Epidemiology* **2007**, *18*, 402–408. [CrossRef] [PubMed]
100. Wang, D.; Liu, Y.; Han, J.; Zai, D.; Ji, M.; Cheng, W.; Xu, L.; Yang, L.; He, M.; Ni, J.; et al. Puerarin Suppresses Invasion and Vascularization of Endometriosis Tissue Stimulated by 17β-Estradiol. *PLoS ONE* **2011**, *6*, e25011. [CrossRef] [PubMed]
101. Ji, M.; Liu, Y.; Yang, S.; Zhai, D.; Zhang, D.; Bai, L.; Wang, Z.; Yu, J.; Yu, C.; Cai, Z. Puerarin suppresses proliferation of endometriotic stromal cells in part via differential recruitment of nuclear receptor coregulators to estrogen receptor-α. *J. Steroid Biochem. Mol. Biol.* **2013**, *138*, 421–426. [CrossRef]
102. Yu, J.; Zhao, L.; Zhang, D.; Zhai, D.; Shen, W.; Bai, L.; Liu, Y.; Cai, Z.; Li, J.; Yu, C. The Effects and Possible Mechanisms of Puerarin to Treat Endometriosis Model Rats. *Evidence-Based Complement. Altern. Med.* **2015**, *2015*, 1–11. [CrossRef] [PubMed]
103. Park, S.; Lim, W.; Bazer, F.W.; Song, G. Naringenin induces mitochondria-mediated apoptosis and endoplasmic reticulum stress by regulating MAPK and AKT signal transduction pathways in endometriosis cells. *Mol. Hum. Reprod.* **2017**, *23*, 842–854. [CrossRef] [PubMed]
104. Kapoor, R.; Sirohi, V.K.; Gupta, K.; Dwivedi, A. Naringenin ameliorates progression of endometriosis by modulating Nrf2/Keap1/HO1 axis and inducing apoptosis in rats. *J. Nutr. Biochem.* **2019**, *70*, 215–226. [CrossRef]
105. Rudzitis-Auth, J.; Körbel, C.; Scheuer, C.; Menger, M.D.; Laschke, M.W. Xanthohumol inhibits growth and vascularization of developing endometriotic lesions. *Hum. Reprod.* **2012**, *27*, 1735–1744. [CrossRef] [PubMed]
106. Matsuzaki, S.; Darcha, C. Antifibrotic properties of epigallocatechin-3-gallate in endometriosis. *Hum. Reprod.* **2014**, *29*, 1677–1687. [CrossRef] [PubMed]
107. Xu, H.; Becker, C.M.; Lui, W.T.; Chu, C.Y.; Davis, T.N.; Kung, A.L.; Birsner, A.E.; D'Amato, R.J.; Man, G.C.W.; Wang, C.C. Green tea epigallocatechin-3-gallate inhibits angiogenesis and suppresses vascular endothelial growth factor C/vascular endothelial growth factor receptor 2 expression and signaling in experimental endometriosis in vivo. *Fertil. Steril.* **2011**, *96*, 1021.e1–1028.e1. [CrossRef]

108. Wang, C.C.; Xu, H.; Man, G.C.W.; Zhang, T.; Chu, K.O.; Chu, C.Y.; Cheng, J.T.Y.; Li, G.; He, Y.X.; Qin, L.; et al. Prodrug of green tea epigallocatechin-3-gallate (Pro-EGCG) as a potent anti-angiogenesis agent for endometriosis in mice. *Angiogenesis* **2012**, *16*, 59–69. [CrossRef]
109. Kim, K.-H.; Na Lee, E.; Park, J.K.; Lee, J.-R.; Kim, J.-H.; Choi, H.-J.; Kim, B.-S.; Lee, H.-W.; Lee, K.-S.; Yoon, S. Curcumin Attenuates TNF-α-induced Expression of Intercellular Adhesion Molecule-1, Vascular Cell Adhesion Molecule-1 and Proinflammatory Cytokines in Human Endometriotic Stromal Cells. *Phytotherapy Res.* **2012**, *26*, 1037–1047. [CrossRef]
110. Zhang, Y.; Cao, H.; Yu, Z.; Peng, H.-Y.; Zhang, C.-J. Curcumin Inhibits Endometriosis Endometrial Cells by Reducing Estra-diol Production. *Iran. J. Reprod. Med.* **2013**, *11*, 415–422.
111. Cao, H.; Wei, Y.-X.; Zhou, Q.; Zhang, Y.; Guo, X.-P.; Zhang, J. Inhibitory effect of curcumin in human endometriosis endometrial cells via downregulation of vascular endothelial growth factor. *Mol. Med. Rep.* **2017**, *16*, 5611–5617. [CrossRef]
112. Chowdhury, I.; Banerjee, S.; Driss, A.; Xu, W.; Mehrabi, S.; Nezhat, C.; Sidell, N.; Taylor, R.N.; Thompson, W.E. Curcumin attenuates proangiogenic and proinflammatory factors in human eutopic endometrial stromal cells through the NF-κB signaling pathway. *J. Cell. Physiol.* **2019**, *234*, 6298–6312. [CrossRef]
113. Jana, S.; Paul, S.; Swarnakar, S. Curcumin as anti-endometriotic agent: Implication of MMP-3 and intrinsic apoptotic pathway. *Biochem. Pharmacol.* **2012**, *83*, 797–804. [CrossRef]
114. Swarnakar, S.; Paul, S. Curcumin arrests endometriosis by downregulation of matrix metalloproteinase-9 activity. *Indian J. Biochem. Biophys.* **2009**, *46*, 59–65.
115. Zhang, C.-J.; Hu, Y.-Y.; Zhang, Y.; Cao, H.; Wang, H. Inhibitory effect of curcumin on angiogenesis in ectopic endometrium of rats with experimental endometriosis. *Int. J. Mol. Med.* **2010**, *27*, 87–94. [CrossRef]
116. Bin, L. Estrogen-Independent Inhibition of Curcumin on Formation of Endometriotic Foci in Rats with Experimental Endometriosis. *Med. J. West China* **2012**, 5.
117. Bruner-Tran, K.L.; Osteen, K.G.; Taylor, H.S.; Sokalska, A.; Haines, K.; Duleba, A.J. Resveratrol Inhibits Development of Experimental Endometriosis In Vivo and Reduces Endometrial Stromal Cell Invasiveness In Vitro1. *Biol. Reprod.* **2011**, *84*, 106–112. [CrossRef] [PubMed]
118. Amaya, S.C.; Savaris, R.F.; Filipovic, C.J.; Wise, J.D.; Hestermann, E.; Young, S.L.; Lessey, B.A. Resveratrol and Endometrium: A Closer Look at an Active Ingredient of Red Wine Using in Vivo and in Vitro Models. *Reprod. Sci.* **2014**, *21*, 1362–1369. [CrossRef]
119. Ricci, A.; Olivares, C.; Bilotas, M.; Bastón, J.; Singla, J.; Meresman, G.; Barañao, R. Natural therapies assessment for the treatment of endometriosis. *Hum. Reprod.* **2013**, *28*, 178–188. [CrossRef] [PubMed]
120. Arablou, T.; Delbandi, A.; Khodaverdi, S.; Arefi, S.; Kolahdouz-Mohammadi, R.; Heidari, S.; Mohammadi, T.; Aryaeian, N. Resveratrol reduces the expression of insulin-like growth factor-1 and hepatocyte growth factor in stromal cells of women with endometriosis compared with nonendometriotic women. *Phytother. Res.* **2019**, *33*, 1044–1054. [CrossRef] [PubMed]
121. Khazaei, M.R.; Rashidi, Z.; Chobsaz, F.; Niromand, E.; Khazaei, M. Inhibitory effect of resveratrol on the growth and angiogenesis of human endometrial tissue in an In Vitro three-dimensional model of endometriosis. *Reprod. Biol.* **2020**, *20*, 484–490. [CrossRef] [PubMed]
122. Kolahdouz-Mohammadi, R.; Shidfar, F.; Khodaverdi, S.; Arablou, T.; Heidari, S.; Rashidi, N.; Delbandi, A. Resveratrol treatment reduces expression of MCP-1, IL-6, IL-8 and RANTES in endometriotic stromal cells. *J. Cell. Mol. Med.* **2021**, *25*, 1116–1127. [CrossRef] [PubMed]
123. Ergenoğlu, A.M.; Yeniel, A. Özgür; Erbaş, O.; Aktuğ, H.; Yildirim, N.; Ulukuş, M.; Taskiran, D. Regression of Endometrial Implants by Resveratrol in an Experimentally Induced Endometriosis Model in Rats. *Reprod. Sci.* **2013**, *20*, 1230–1236. [CrossRef]
124. Cenksoy, P.O.; Oktem, M.; Erdem, O.; Karakaya, C.; Cenksoy, C.; Erdem, A.; Guner, H.; Karabacak, O. A potential novel treatment strategy: Inhibition of angiogenesis and inflammation by resveratrol for regression of endometriosis in an experimental rat model. *Gynecol. Endocrinol.* **2015**, *31*, 219–224. [CrossRef] [PubMed]
125. Yavuz, S.; E Aydin, N.; Celik, O.; Yilmaz, E.; Ozerol, E.; Tanbek, K. Resveratrol successfully treats experimental endometriosis through modulation of oxidative stress and lipid peroxidation. *J. Cancer Res. Ther.* **2014**, *10*, 324–329. [CrossRef] [PubMed]
126. Kong, X.; Xu, X.; Zhou, L.; Zhu, M.; Yao, S.; Ding, Y.; Liu, T.; Wang, Y.; Zhang, Y.; Li, R.; et al. MTA1, a Target of Resveratrol, Promotes Epithelial-Mesenchymal Transition of Endometriosis via ZEB2. *Mol. Ther. Methods Clin. Dev.* **2020**, *19*, 295–306. [CrossRef]
127. Maia, H.; Haddad, C.; Pinheiro, N.; Casoy, J. Advantages of the association of resveratrol with oral contraceptives for management of endometriosis-related pain. *Int. J. Women's Health* **2012**, *4*, 543–549. [CrossRef] [PubMed]
128. Kodarahmian, M.; Amidi, F.; Moini, A.; Kashani, L.; Nashtaei, M.S.; Pazhohan, A.; Bahramrezai, M.; Berenjian, S.; Sobhani, A. The modulating effects of Resveratrol on the expression of MMP-2 and MMP-9 in endometriosis women: A randomized exploratory trial. *Gynecol. Endocrinol.* **2019**, *35*, 719–726. [CrossRef] [PubMed]
129. Khodarahmian, M.; Amidi, F.; Moini, A.; Kashani, L.; Salahi, E.; Danaii-Mehrabad, S.; Nashtaei, M.S.; Mojtahedi, M.F.; Esfandyari, S.; Sobhani, A. A randomized exploratory trial to assess the effects of resveratrol on VEGF and TNF-α 2 expression in endometriosis women. *J. Reprod. Immunol.* **2021**, *143*, 103248. [CrossRef] [PubMed]
130. Meresman, G.F.; Götte, M.; Laschke, M.W. Plants as source of new therapies for endometriosis: A review of preclinical and clinical studies. *Hum. Reprod. Updat.* **2021**, *27*, 367–392. [CrossRef] [PubMed]
131. Salehi, B.; Venditti, A.; Sharifi-Rad, M.; Kręgiel, D.; Sharifi-Rad, J.; Durazzo, A.; Lucarini, M.; Santini, A.; Souto, E.B.; Novellino, E.; et al. The Therapeutic Potential of Apigenin. *Int. J. Mol. Sci.* **2019**, *20*, 1305. [CrossRef] [PubMed]

132. Kim, Y.O.; Leem, K.; Park, J.; Lee, P.; Ahn, D.-K.; Lee, B.C.; Park, H.K.; Suk, K.; Kim, S.Y.; Kim, H. Cytoprotective effect of Scutellaria baicalensis in CA1 hippocampal neurons of rats after global cerebral ischemia. *J. Ethnopharmacol.* **2001**, *77*, 183–188. [CrossRef]
133. Liu, H.; Dong, Y.; Gao, Y.; Du, Z.; Wang, Y.; Cheng, P.; Chen, A.; Huang, H. The Fascinating Effects of Baicalein on Cancer: A Review. *Int. J. Mol. Sci.* **2016**, *17*, 1681. [CrossRef]
134. Po, L.S.; Chen, Z.-Y.; Tsang, D.S.C.; Leung, L.K. Baicalein and genistein display differential actions on estrogen receptor (ER) transactivation and apoptosis in MCF-7 cells. *Cancer Lett.* **2002**, *187*, 33–40. [CrossRef]
135. Peng, Y.; Li, Q.; Li, K.; Zhao, H.; Han, Z.; Li, F.; Sun, M.; Zhang, Y. Antitumor activity of baicalein on the mice bearing U14 cervical cancer. *Afr. J. Biotechnol.* **2011**, *10*, 14169–14176. [CrossRef]
136. Waisundara, V.Y.; Siu, S.Y.; Hsu, A.; Huang, D.; Tan, B.K. Baicalin upregulates the genetic expression of antioxidant enzymes in Type-2 diabetic Goto-Kakizaki rats. *Life Sci.* **2011**, *88*, 1016–1025. [CrossRef] [PubMed]
137. Ke, M.; Zhang, Z.; Xu, B.; Zhao, S.; Ding, Y.; Wu, X.; Wu, R.; Lv, Y.; Dong, J. Baicalein and baicalin promote antitumor immunity by suppressing PD-L1 expression in hepatocellular carcinoma cells. *Int. Immunopharmacol.* **2019**, *75*, 105824. [CrossRef] [PubMed]
138. Xia, X.; Xia, J.; Yang, H.; Li, Y.; Liu, S.; Cao, Y.; Tang, L.; Yu, X. Baicalein blocked cervical carcinoma cell proliferation by targeting CCND1 via Wnt/β-catenin signaling pathway. *Artif. Cells Nanomed. Biotechnol.* **2019**, *47*, 2729–2736. [CrossRef] [PubMed]
139. Limer, J.L.; Speirs, V. Phyto-oestrogens and breast cancer chemoprevention. *Breast Cancer Res.* **2004**, *6*, 119. [CrossRef]
140. Wang, H.; Murphy, P.A. Isoflavone Content in Commercial Soybean Foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673. [CrossRef]
141. Wagner, J.D.; Anthony, M.S.; Cline, J.M. Soy Phytoestrogens: Research on Benefits and Risks. *Clin. Obstet. Gynecol.* **2001**, *44*, 843–852. [CrossRef]
142. Takaoka, O.; Mori, T.; Ito, F.; Okimura, H.; Kataoka, H.; Tanaka, Y.; Koshiba, A.; Kusuki, I.; Shigehiro, S.; Amami, T.; et al. Daidzein-rich isoflavone aglycones inhibit cell growth and inflammation in endometriosis. *J. Steroid Biochem. Mol. Biol.* **2018**, *181*, 125–132. [CrossRef]
143. Zhou, Y.-X.; Zhang, H.; Peng, C. Puerarin: A Review of Pharmacological Effects. *Phytotherapy Res.* **2014**, *28*, 961–975. [CrossRef]
144. Hwang, Y.P.; Jeong, H.G. Mechanism of phytoestrogen puerarin-mediated cytoprotection following oxidative injury: Estrogen receptor-dependent up-regulation of PI3K/Akt and HO-1. *Toxicol. Appl. Pharmacol.* **2008**, *233*, 371–381. [CrossRef] [PubMed]
145. Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. HL-60 differentiating activity and flavonoid content of the readily extractable fraction prepared from citrus juices. *J. Agric. Food Chem.* **1998**, *47*, 128–135. [CrossRef]
146. Bao, L.; Liu, F.; Guo, H.-B.; Li, Y.; Tan, B.-B.; Zhang, W.-X.; Peng, Y.-H. Naringenin inhibits proliferation, migration, and invasion as well as induces apoptosis of gastric cancer SGC7901 cell line by downregulation of AKT pathway. *Tumor Biol.* **2016**, *37*, 11365–11374. [CrossRef]
147. Lim, W.; Park, S.; Bazer, F.W.; Song, G. Naringenin-Induced Apoptotic Cell Death in Prostate Cancer Cells Is Mediated via the PI3K/AKT and MAPK Signaling Pathways. *J. Cell. Biochem.* **2017**, *118*, 1118–1131. [CrossRef] [PubMed]
148. Galluzzo, P.; Ascenzi, P.; Bulzomi, P.; Marino, M. The Nutritional Flavanone Naringenin Triggers Antiestrogenic Effects by Regulating Estrogen Receptor α-Palmitoylation. *Endocrinology* **2008**, *149*, 2567–2575. [CrossRef] [PubMed]
149. Jiang, C.-H.; Sun, T.-L.; Xiang, D.-X.; Wei, S.-S.; Li, W.-Q. Anticancer Activity and Mechanism of Xanthohumol: A Prenylated Flavonoid From Hops (Humulus lupulus L.). *Front. Pharmacol.* **2018**, *9*, 530. [CrossRef] [PubMed]
150. Farzaei, M.H.; Bahramsoltani, R.; Rahimi, R. Phytochemicals as Adjunctive with Conventional Anticancer Therapies. *Curr. Pharm. Des.* **2016**, *22*, 4201–4218. [CrossRef] [PubMed]
151. Singh, A.K.; Chakravarty, B.; Chaudhury, K. Nanoparticle-Assisted Combinatorial Therapy for Effective Treatment of Endometriosis. *J. Biomed. Nanotechnol.* **2015**, *11*, 789–804. [CrossRef]
152. Chen, D.; Wan, S.B.; Yang, H.; Yuan, J.; Chan, T.H.; Dou, Q.P. EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. *Advances in Clinical Chemistry* **2011**, *53*, 155–177. [CrossRef]
153. Xu, H.; Lui, W.; Chu, C.; Ng, P.; Wang, C.; Rogers, M. Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model. *Hum. Reprod.* **2008**, *24*, 608–618. [CrossRef]
154. Kotha, R.R.; Luthria, D.L. Curcumin: Biological, Pharmaceutical, Nutraceutical, and Analytical Aspects. *Molecules* **2019**, *24*, 2930. [CrossRef] [PubMed]
155. Adomako-Bonsu, A.G.; Chan, S.L.; Pratten, M.; Fry, J.R. Antioxidant activity of rosmarinic acid and its principal metabolites in chemical and cellular systems: Importance of physico-chemical characteristics. *Toxicol. Vitr.* **2017**, *40*, 248–255. [CrossRef]
156. Alagawany, M.; El-Hack, M.E.A.; Farag, M.R.; Gopi, M.; Karthik, K.; Malik, Y.S.; Dhama, K. Rosmarinic acid: Modes of action, medicinal values and health benefits. *Anim. Health Res. Rev.* **2017**, *18*, 167–176. [CrossRef] [PubMed]
157. Rauf, A.; Imran, M.; Butt, M.S.; Nadeem, M.; Peters, D.G.; Mubarak, M.S. Resveratrol as an anti-cancer agent: A review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 1428–1447. [CrossRef] [PubMed]
158. Siemann, E.H.; Creasy, L.L. Concentration of the Phytoalexin Resveratrol in Wine. *Am. J. Enol. Vitic.* **1992**, *43*, 49–52.
159. Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.W.; Fong, H.H.S.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; et al. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. *Science* **1997**, *275*, 218–220. [CrossRef]
160. Frémont, L. Biological effects of resveratrol. *Life Sci.* **2000**, *66*, 663–673. [CrossRef]
161. Nakata, R.; Takahashi, S.; Inoue, H. Recent Advances in the Study on Resveratrol. *Biol. Pharm. Bull.* **2012**, *35*, 273–279. [CrossRef]

162. Chao, S.-C.; Chen, Y.-J.; Huang, K.-Y.; Kuo, K.-L.; Yang, T.-H.; Wang, C.-C.; Tang, C.-H.; Yang, R.-S.; Liu, S.-H. Induction of sirtuin-1 signaling by resveratrol induces human chondrosarcoma cell apoptosis and exhibits antitumor activity. *Sci. Rep.* **2017**, *7*, 1–11. [CrossRef]
163. Dull, A.-M.; Moga, M.A.; Dimienescu, O.G.; Sechel, G.; Burtea, V.; Anastasiu, C.V. Therapeutic Approaches of Resveratrol on Endometriosis via Anti-Inflammatory and Anti-Angiogenic Pathways. *Molecules* **2019**, *24*, 667. [CrossRef] [PubMed]
164. Ilhan, M.; Dereli, F.T.G.; Akkol, E.K.; Gürağaç, F.T. Novel Drug Targets with Traditional Herbal Medicines for Overcoming Endometriosis. *Curr. Drug Deliv.* **2019**, *16*, 386–399. [CrossRef]
165. da Silva, D.M.; Gross, L.A.; Neto, E.D.P.G.; Lessey, B.A.; Savaris, R.F. The Use of Resveratrol as an Adjuvant Treatment of Pain in Endometriosis: A Randomized Clinical Trial. *J. Endocr. Soc.* **2017**, *1*, 359–369. [CrossRef] [PubMed]
166. Cory, H.; Passarelli, S.; Szeto, J.; Tamez, M.; Mattei, J. The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Front. Nutr.* **2018**, *5*, 87. [CrossRef] [PubMed]
167. Zhang, H.; Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **2016**, *8*, 33–42. [CrossRef]
168. Yang, X.; Belosay, A.; Hartman, J.A.; Song, H.; Zhang, Y.; Wang, W.; Doerge, D.R.; Helferich, W.G. Dietary soy isoflavones increase metastasis to lungs in an experimental model of breast cancer with bone micro-tumors. *Clin. Exp. Metastasis* **2015**, *32*, 323–333. [CrossRef]
169. Carbonel, A.A.F.; Calió, M.L.; Santos, M.A.; Bertoncini, C.R.A.; Sasso, G.D.S.; Simões, R.S.; Simões, M.J.; Soares, J.M. Soybean isoflavones attenuate the expression of genes related to endometrial cancer risk. *Climacteric* **2014**, *18*, 389–398. [CrossRef]
170. Zhong, X.-S.; Ge, J.; Chen, S.-W.; Xiong, Y.-Q.; Ma, S.-J.; Chen, Q. Association between Dietary Isoflavones in Soy and Legumes and Endometrial Cancer: A Systematic Review and Meta-Analysis. *J. Acad. Nutr. Diet.* **2018**, *118*, 637–651. [CrossRef]
171. Dong, J.-Y.; Qin, L.-Q. Soy isoflavones consumption and risk of breast cancer incidence or recurrence: A meta-analysis of prospective studies. *Breast Cancer Res. Treat.* **2010**, *125*, 315–323. [CrossRef]
172. EFSA Panel on Food Additives and Nutrient Sources Added to Food. Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones. *EFSA J.* **2015**, *13*, 4246. [CrossRef]

Cellular Signalling 97 (2022) 110375

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Review

In vitro modeling of endometriosis and endometriotic microenvironment – Challenges and recent advances

Agata Gołąbek-Grenda, Anna Olejnik *

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego St., 60-627 Poznan, Poland

ARTICLE INFO

Keywords:
endometriotic niche
2D and 3D endometriosis models
spheroids
organoids
microfluidics
bioprinting

ABSTRACT

Endometriosis is a chronic condition with high prevalence in reproductive age women, defined as the growth of endometrial tissue outside the uterine cavity, most commonly on the pelvic peritoneum. The ectopic endometrial lesions exist in a unique microenvironment created by the interaction of epithelial, stromal, endothelial, glandular, and immune cell components, dominated by inflammatory, angiogenic, and endocrine signals. Current research is directed at understanding the complex microenvironment of the lesions and its relationship with different endometriosis stages, phenotypes, and disease symptoms and at the development of novel diagnostic and therapeutic concepts that minimalize the undesirable side effects of current medical management. Recreating pathophysiological cellular and molecular mechanisms and identifying clinically relevant metrics to assess drug efficacy is a great challenge for the experimental disease models.

This review summarizes the complete range of available *in vitro* experimental systems used in endometriotic studies, which reflect the multifactorial nature of the endometriotic lesion. The article discusses the simplistic *in vitro* models such as primary endometrial cells and endometriotic cell lines to heterogeneous 2D co-cultures, and recently more common, 3D systems based on self-organization and controlled assembly, both in microfluidic or bioprinting methodologies.

Basic research models allow studying fundamental pathological mechanisms by which menstrual endometrium adheres, invades, and establishes lesions in ectopic sites. The advanced endometriosis experimental models address the critical challenges and unsolved problems and provide an approach to drug screening and medicine discovery by mimicking the complicated behaviors of the endometriotic lesion.

1. Introduction

Studies during the last decades have revealed that endometriosis is a prevalent estrogen-dependent gynecological disease characterized by the appearance of ectopic endometrium-like tissue outside the uterine cavity [1]. The endometrial deposits appear as implants on the surface of the peritoneum and pelvic organs. To establish an endometriotic lesion, endometrial stromal and epithelial cells must initially attach to pelvic

Abbreviations: AKT, protein kinase B; CAM, chorioallantoic membrane; CA-125, cancer antigen 125; CCL25, thymus-expressed chemokine; CCR9, chemokine C receptor 9; CDH1, cadherin-1; CK7, cytokeratin-7; CK107a, cell surface marker for cytotoxicity; COX-2, cyclooxygenase-2; CYP19A1, cytochrome P450 family 19 subfamily A member 1; DJ-1, Parkinson disease protein 7; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; E2, estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ESC, endometrial stromal cells; FOXO1, FOXO3, forkhead box protein O1, -O3; Frag-1, fibroblast growth factor activating gene 1; HGF, hepatocyte growth factor; HOX, homeobox genes; HSD17β2, 17β-hydroxysteroid dehydrogenase 2; hTERT, human telomerase reverse transcriptase; IGFBP-1, insulin-like growth factor-binding protein 1; KIT, receptor protein-tyrosine kinase; LGR6, leucine rich repeat containing G protein-coupled receptor 6; MCP-1/CCL2, monocyte chemoattractant protein-1; mIL-6R, membrane-bound IL-6 receptor; MR, mannose receptor; NF-κB, nuclear factor-κB; NKG2A, natural killer cell protein group 2-A; NKp46, natural killer cell p46-related protein; NOTCH-1, neurogenic locus notch homolog protein 1; NRP, neuropilin-1; PAX2, paired box protein; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2 – catalytic α polypeptide; PPAR-γ, proliferator activator receptor-gamma; PR, progesterone receptor; PRL, prolactin; p120ctn, catenin delta-1; RA, retinoic acid; RAC1, Ras-related C3 botulinum toxin substrate 1; RANTES, regulated upon activation, normal T cell expressed and secreted; Sema3A, semaphorin 3A; sIL-6R, soluble IL-6 receptor; SOX9, SRY-box transcription factor 9; SPs, side population cells; SSEA-1, stage-specific embryonic antigen-1; StAR, steroidogenic acute regulatory protein; STAT3, signal transducer and activator of transcription 3; STING, stimulator of interferon gene; SUSD2, sushi domain containing-2; SV40, Simian virus 40.

* Corresponding author.
E-mail address: anna.olejnik@up.poznan.pl (A. Olejnik).

https://doi.org/10.1016/j.cellsig.2022.110375
Received 8 May 2022; Received in revised form 2 June 2022; Accepted 3 June 2022
Available online 9 June 2022

and intra-abdominal surfaces and then invade the extracellular matrix of the mesothelial cell layer. The breaks in the mesothelium facilitate the implantation and proliferation of endometrial tissue fragments and may be essential for forming peritoneal endometriosis, the most common type of endometriosis [2]. The endometriotic lesions can also occur in the ovaries developing ovary cysts or in adjacent organs (deep infiltrating endometriosis) and into the tissue between the vagina and rectum (rectovaginal endometriosis) [3]. Ectopic endometrial implants impair the reproductive system and cause condition-defining symptoms. The extensive lesions and their adhesions can cause the emergence of fibrotic tissue, leading to distortion of the pelvic organs, which may contribute to fertility problems and chronic debilitating pelvic pain [4].

Current treatment choices contain analgesics, hormonal intervention and surgical management and focus on treating each patient's symptoms rather than curing the disease completely. All therapeutic concepts are usually aimed at pain control, regulating the menstrual cycle and achieving pregnancy [5]. There is a substantial need to unravel the complex pathophysiology of the condition as a basis for identifying new methods of diagnosis and treatment. Several theories on the origin of endometriosis have been offered, like Sampson's retrograde menstruation theory, coelomic metaplasia and lymphovascular metastasis concepts. Endometriosis pathophysiology is also strongly influenced by genetic predisposition, immune response and environmental agents [1,6]. Research from the last decade suggests that endometriosis is due to immunological surveillance disturbances based on multiple lines of defective immune responses [7]. Understanding the causation and consequences of local immune dysregulation at ectopic sides is fundamental to developing new treatment options for this disease, as inflammation and subsequent fibrosis account for the major clinical symptoms, including severe pelvic pain, urinary problems, and infertility. Inflammation is probably exerted by hemorrhage and tissue injury due to blood accumulation within endometriotic lesions. Then, reactive oxygen species (ROS) initiate cell death, elicit inflammatory response and facilitate the implantation and survival of endometriotic lesions on the peritoneum surface. The presence of hemosiderin-laden macrophages at the location of the endometriotic lesion confirms the described process. The inflammatory response is sustained due to the effort of eliminate the abnormal tissue at the aberrant site. Both immune and endometrial cells secrete cytokines and growth factors, which prime the endometriotic lesion progression [8,9]. Hence, it is highly challenging to construct clinically relevant models to identify fundamental mechanisms of menstrual endometrium adhesion, invasion, and establishment in ectopic sites and to develop new therapeutical approaches. This review reports experimental tools for endometriosis research and describes how they mimic cellular and molecular mechanisms involved in endometriosis pathogenesis and development.

2. Endometriotic niche

Endometriotic lesions exist in a complex and dynamic environment created by interactions of multiple cells and inflammatory, angiogenic, and endocrine signals. The epithelial, stromal, immune, endothelial, and glandular cell populations are heterogenous in the endometriotic lesions and represent inflammation-associated alternations. Endometrial implants refluxed into the peritoneal cavity create a feed-forward loop and stimulate the infiltration of immune cells, which generate an immunological imbalance. It is also worth emphasizing that the endometrial implants possess an immune microenvironment comparable to a tumor-like inflammatory system [10]. Investigating the main pathways involved in the pathogenesis of endometriosis requires detailed insight into the endometriotic's lesion cellular and non-cellular composition and its relationship with different types of disease, stages, and symptoms. Fig. 1 presents the endometriotic lesion microenvironment and the factors expressed by the cells that can contribute to the development and progression of peritoneal endometriosis.

2.1. Endometriotic epithelial and stromal cells

The endometrium undergoes monthly cycles of proliferation, differentiation, breakdown, shedding, and regeneration under the influence of circulating ovarian steroids: estrogen and progesterone [11]. The endometrial layer comprises luminal and glandular epithelia that

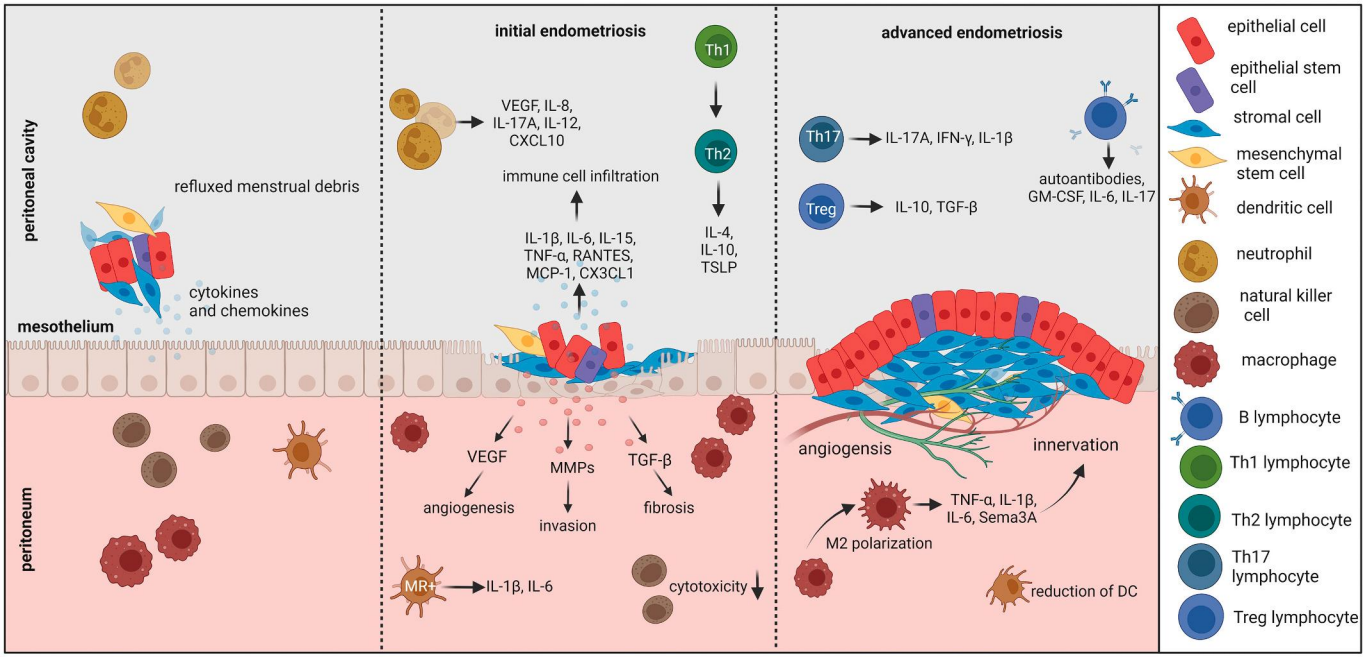


Fig. 1. Schematic presentation of endometriotic lesion microenvironment and factors expressed by neighboring cells contributing to peritoneal endometriosis development and progression.
Abbreviations: DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; MR, mannose receptor;Sema3A, semaphorin 3A; TSLP, thymic stromal lymphopoietin.

invaginate into the stroma after birth through tubulogenesis [12]. Endometrial epithelial cells are characterized by columnar morphology, apical-basal polarity, and lateral junctions that tightly connect the cells and are the first uterine point of contact for blastocysts. The glandular epithelium secretes mucins, nourishes, protects the uterus from infection, and increases receptivity for embryo apposition and implantation [12,13]. Stromal cells differentiate into larger, rounder decidual cells and undergo a mesenchymal-epithelial transition (MET) under the influence of ovarian-derived progesterone during the secretory phase of each menstrual cycle to prepare for pregnancy [14]. Decidual cells regulate trophoblast invasion, induce the spiral-artery network remodeling, assist the maternal immunotolerance and protect conceptus against allorecognition. In the absence of a pregnancy, the corpus luteum demises, circulating progesterone concentrations fall, and endometrial stromal cells (ESC) upregulate intracellular inflammatory signaling, which contributes to menstrual cascade propagation and tissue breakdown [15]. Although endometrial stromal and epithelial cells have separate important roles in the endometrium, cell–cell communication between them is crucial for normal endometrial function and maintenance of pregnancy [16]. Sampson's theory defining endometriosis posits that menstrual tissues fragments reflux through the fallopian tubes into the peritoneal cavity [17]. Most women experience retrograde menstruation, but only 10%-20% of them develop endometriosis; thus, in the function of tissue fragments and/or immune dysregulation, molecular alterations must be involved in the implantation and survival of the displaced endometrial cells [17].

Epithelial and stromal cells are the two major structural cell types in endometriotic implants. Most endometriotic lesions consist primarily of stromal cells and fewer epithelial components with morphological variations distinguishing from typical glands observed in the eutopic endometrium [18,19]. The endometriotic stromal cells express steroidogenic genes and enzymes, including steroidogenic acute regulatory protein (StAR) and aromatase, which are involved in the local estradiol synthesis from cholesterol. In contrast to endometriotic lesions, normal endometrial cells cannot synthesize estrogen due to the lack of these enzymes [20]. StAR initializes the first step of estrogenic formation by facilitating the transport of cytosolic cholesterol into the mitochondrion. Aromatase is responsible for the final stage of E2 formation, the aromatization of androstenedione and testosterone (androgens), originated primarily from adrenal and/or ovary, respectively, to estrone and E2 (estrogens) [21]. E2 directly induces cyclooxygenase-2 (COX-2) to form prostaglandin E2 (PGE2), responsible for evoking severe menstrual cramps and chronic pelvic pain. PGE2, in turn, is the most potent known stimulator of StAR and aromatase in endometriotic stromal cells [22].

Among estrogen receptors in endometriotic stromal cells, estrogen receptor beta (ERβ) level is abnormally higher, and estrogen receptor alpha (ERα) level is lower than in normal endometrium because of altered DNA methylation. The high ratio of ERβ/ERα in endometriotic stromal cells leads to increased ERβ binding to the progesterone receptor (PR) promoter, which may be the reason for observed low PR expression in endometriosis [23]. Production locally large amounts of progesterone by endometriotic stromal cells and PR deficiency confer a progesterone resistance, linked with their reduced capacity for decidualization [24]. In the healthy endometrium, progesterone exerts an antiestrogenic effect by inducing 17β-hydroxysteroid dehydrogenase 2 (HSD17β2), catalyzing the conversion of biologically active estradiol to less estrogenic estrone. Progesterone acts by PR and increases the formation of retinoic acid (RA) in ESC, which in turn induces HSD17β2 expression in neighboring endometrial epithelial cells by paracrine signaling. In contrast, endometriotic stromal cells are failed to respond to progesterone and do not produce RA. Lack of RA leads to a deficiency of HSD17β2 in epithelial cells, thus giving rise to E2. Estradiol production owing to aberrant aromatase enzymatic activity combined with HSD17β2 deficiency as a consequence of progesterone resistance contribute to the unnormal high levels of estradiol in endometriotic tissue [25,26].

Endometriotic stromal cells also secrete large amounts of immune mediators such as IL-1β, IL-6, TNF-α, RANTES (regulated upon activation, normal T cell expressed and secreted), and MCP-1 (monocyte chemoattractant protein-1) [1]. Thus, autoregulatory positive-feedback mechanisms between inflammation and estrogen production ensure chronic overexpression of genes encoding the aromatase and COX-2 enzymes and sustained formation of their products, estradiol and PGE2, in endometriotic lesions [1,27]. Proliferation and invasiveness of endometriotic stromal cells are supported by different chemokines produced in the endometriotic microenvironment: RANTES, also called CCL5, CCL2, CXCL8, and thymus-expressed chemokine, known as CCL25. The elevated level of CCL25 and its receptor chemokine C receptor 9 (CCR9) in peritoneal fluid of endometriosis patients indicates its role in the pathophysiology. Activation of CCL25/CCR9 signaling mediates the invasiveness of stromal cells. Treatment of the stromal cells with 17β-estradiol (E2) and 2,3,7,8- tetrachlorodibenzo-p-dioxin resulted in higher expression of CCR9 and invasiveness initiation by expression of MMP-2 and MMP-9 [28]. Further studies indicate that CCL25 secreted by endometriotic stromal cells and macrophages facilitate Tregs differentiation, the IL-10 and TGF-β production, and secretion by native T cells by activating the AKT/signal transducer and activator of transcription 3 (STAT3) signaling pathway. CCL25 upregulation inhibits Treg apoptosis, which stimulates stromal cell growth and invasion through IL-10, TGF-β and CD73 functional molecules, leading to an increase in MMP2 and COX-2 expression in stromal cells [29].

Recently, the contribution of endometriotic epithelial cells to the development of the inflammatory environment surrounding the lesions has been reported [30]. Epithelium in endometriosis and adenomyosis up-regulates stimulator of interferon gene (STING), which is involved in the enrichment of host's innate immunity by inducing type I interferon in response to cyclic dinucleotides [31]. There are several potential activators of the STING pathway in endometriosis, like immune factors resulting from the autoinflammatory nature of the disease, epithelial cell injuries or rapid cell proliferation associated with cancer-driver gene mutations. STING expression in the glandular epithelial cells from endometriosis correlates with the density of inflammatory cell infiltration within the epithelium in both lesions and eutopic endometrium [30]. Following retrograde menstruation theory, immune dysfunction has been hypothesized to facilitate successful lesion development after displacement of refluxed endometrial debris into pelvic locations. Dysregulated innate and adaptive immune systems cannot initiate the mechanisms responsible for menstrual debris cleavage, immune cell infiltration, and tissue repair [32].

2.2. Macrophages

Many studies have demonstrated the abnormal distribution of macrophages in patients with endometriosis, with an increased macrophage population in the peritoneal cavity and eutopic endometrium [10]. Peritoneal macrophages in endometriosis women display enhanced activation of the proinflammatory cytokine transcription factor – nuclear factor-κB (NF-κB), along with high secretion levels of the proinflammatory cytokines such as TNF-α, IL-6, and IL-1β [33]. Furthermore, macrophages have been found to down-regulate the expression of IL-24 and its receptor in endometriotic stromal cells. The suppression of IL-24 leads to increased viability and invasiveness of endometriotic stromal cells by enhancing the expression of relative proliferation gene Ki-67, proliferating cell nuclear antigen (PCNA), and COX-2, and by decreasing the CD82 level. Additionally, abnormally low levels of IL-24 may contribute to macrophages' recruitment through CD82/CCL2 signaling and the impaired phagocytosis of macrophages by the COX-2/PGE2 axis, finally improving survival, growth, and implantation of ectopic endometrium [34].

The M1 and M2 dichotomy of macrophage phenotype in endometriosis has been broadly described. The elucidation of the mechanism of the macrophage recruitment into the endometriotic lesion can provide a

better comprehension of the physiopathology of this disease. Recent findings have indicated that abnormal endometriotic cells can induce anti-inflammatory M2 polarization of macrophages located in the peritoneal fluid. Macrophages can be educated by chemokines produced in the endometriotic milieu. Numerous molecules with aberrant expression in the endometriotic lesion have been identified as responsible for the recruitment of macrophages. MCP-1 seems to activate macrophages via paracrine and autocrine signals, as reported by Mei et al. (2019), where MCP-1 and IL-22 derived from primary endometriotic stromal cells stimulated by natural killers contributed to the recruitment of macrophages [35]. A high level of RANTES from the endometriotic cells may also attract macrophages and induce macrophage tolerance [36]. Other chemokines like IL-8, which is involved in macrophages recruitment in cancer, are also engaged in creating the inflammatory and immunosuppressed environment. Fractalkine, a specific factor that chemoattracts and activates monocytes and macrophages, derived from endometriotic stromal cells, recruits more macrophages into the ectopic foci and induces M2 polarization of macrophages [37]. Data on the phenotypic profile of macrophages present in the peritoneal cavity of women with endometriosis indicates a decrease in the percentage of M1 (CD86+) and an increase in the ratio of CD163+/CD86+ M2 macrophages [38]. Peritoneal macrophages in endometriosis cannot eliminate the endometrial tissues due to defective phagocytic capacity, resulting from up-regulation of signal regulatory protein-α (SIRP-α) and its receptor CD47, while decreasing the corresponding expression of the CD36 scavenger receptor. The expression of CD200 receptor (CD200R) and CD200 (CD200R ligand) was increased in the ectopic endometrial tissues. It is an estrogen-induced molecule that attenuates macrophage phagocytosis and may contribute to the immune escape of endometriotic cells [39]. The presence of nerve fibers is associated with the induction of pain in endometriosis patients. Recent observations suggest that macrophages and nerve fibers attract directly, reinforced by colony-stimulating factor 1 (CSF-1) and CCL-2 secreted from nerves.

Semaphorin 3A (Sema3A), a member of the semaphoring family, is a neuronal chemorepellent acting as a crucial axonal guidance molecule and transducing signal through binding to its specific receptors neuropilin (NRP) and PlexinA1, which are located on the surface of target neurons. Expression of Sema3A and its receptors have been identified on monocyte-derived macrophages. Sema3A/NRP-1 signaling guides macrophages to the hypoxic microenvironment, accompanied by reprogramming of macrophages toward the M2 phenotype [40]. Sema3A and its receptors mediate the tumor cell migration by activating the vascular endothelial growth factor receptor (VEGFR) and similar signaling is probably involved in the macrophages' reprogramming [41]. In endometriosis controlled by estradiol, macrophage-nerve cross-talk has been reported [42].

CD68+ macrophages were reported to co-localize with nerve fibers within peritoneal endometriotic implants. Further analysis distinguished at least two subsets by assessing the expression of canonical markers. Therefore, the frequency of $CD14^{high}$ of peritoneal macrophages did not significantly correlate with pain scores, while $CD14^{low}$ showed a significant positive relationship with pain scores in endometriosis patients [43].

Elevated levels of several cytokines in the peritoneal cavity can enhance the dysregulated state of macrophages and other immune cells. Immune cells, after infiltration of endometrial ectopic sites, secrete many cytokines, including IL-6, the most studied pro-inflammatory cytokine in endometriosis [44]. IL-6 is a multifunctional cytokine secreted mainly by activated macrophages and exerts multiple activities via its receptor (IL-6R), which exists in two forms: membrane-binding receptor (mIL-6R) and soluble receptor (sIL-6R). A recent study revealed that activated macrophages and mIL-6R were consistently increased in peritoneal fluid in endometriosis patients. It was found that endometriotic macrophages are both significant producers of IL-6 and the main target cells of IL-6 [45].

IL-6 was also reported as inducer of NOTCH-1 expression by mediating the function of E-proteins. The NOTCH family transmembrane receptors are responsible for transduction of extracellular signals and controlling various cellular processes such as proliferation, survival, and immune modulation. The aberrant expression of NOTCH-1 was reported in ectopic endometrial tissue. In turn, IL-6, after binding with its receptor, stimulates p38MAPK phosphorylation and stabilizes E-proteins, encoding a class of basic helix-loop-helix transcription factors that regulate NOTCH-1 expression.

IL-6 induces NOTCH-1 expression in human endometriotic epithelial 12Z cells and increases the number of endometriotic lesions in the mouse model of endometriosis. Hence, increased NOTCH-1 through IL-6 activation in the epithelium of endometriotic lesions could enhance glandular cell proliferation and further accelerate lesion development [46]. In this sense, endometriosis in the initial phase leads to macrophage polarization towards a phenotype similar to M2 and M1 in advanced stages, demonstrating impaired phagocytic and antigen-presenting abilities. Elevated levels of various cytokines in the peritoneal cavity maintain a chronic inflammatory environment by promoting a dysregulated state of macrophages and other immune cells.

2.3. Neutrophils

In the context of endometriosis, an increased number of neutrophils in the peritoneal fluid is observed, especially in the advanced stages. The neutrophil infiltration is likely a consequence of the elevated plasma and peritoneal fluid concentration of potent neutrophil chemoattractants such as IL-8, growth-regulated gene α, neutrophil-activating peptide 78, and human neutrophil peptides 1, 2, and 3 [47,48].

Neutrophils produce and accumulate pro-inflammatory cytokines, such as vascular endothelial growth factor (VEGF), IL-8, IL-12, IL-17A, CXCL10, which support disease progression and induce chronic inflammation characteristic of endometriosis [48]. Some studies propose that the neutrophil-to-lymphocyte ratio is indicative of disease progression and valuable as a diagnostic tool, but further studies are warranted to clarify the neutrophils' involvement in endometriosis pathogenesis [49].

2.4. Natural killer cells

NK cells in the peritoneal fluid of endometriosis women have reduced cytotoxicity due to decreased natural cytotoxicity receptor (NKp46) and a cell surface marker for cytotoxicity (CK107a) and the increased inhibitory receptor CD94/NKG2A [32]. Consequently, NK cells lose the function of apoptosis stimulation, which contributes to the immune escape of endometrial fragments in the peritoneal cavity. IL-6, IL-15, and TGF-β released by endometriotic cells down-regulate the killing activity of NK cells, which subsequently promotes the survival of endometrial tissue for advanced implantation [48].

2.5. Dendritic cells

Dendritic cells are heterogeneous monocyte lineage specialized in antigen presentation, determining immune activation or anergy by high expression of co-stimulatory or co-inhibitory mediators [50]. In terms of the frequency of dendritic cells in the lesion or peritoneal cavity, significant reduction of dendritic cells is consistent with larger endometriosis-like lesions and markedly reduced activation of T lymphocytes [51]. Clinicians recently documented that endometriosis patient peritoneal fluids contain a high proportion of mannose receptor (MR) positive BDCA1+ dendritic cells. Mannose receptors are responsible for recognizing antigens derived from dead endometrial cells and their phagocytosis [51]. Further, MR+ dendritic cells can contribute distinctly to the escape of immune surveillance through secretion of IL-6 and IL-1β.

2.6. T and B lymphocytes

T lymphocytes are one of the crucial components of the adaptive immune system implicated in the pathogenesis of endometriosis. It is assumed that the balance of Th1 and Th2 helper cells shifted towards Th2 causes disease progression [52]. Indeed, studies of cytokines in peritoneal fluid demonstrated the increased levels of anti-inflammatory Th2 cytokines, such as IL-4, IL-10, and thymic stromal lymphopoietin (TSLP) and reduced levels of IFN-γ [53,54].

The higher frequency of the immunosuppressive Th17 subset in the peritoneal fluid is associated with the increased severity of the disease. The Th17-derived proinflammatory cytokines, such as IL-17A, IFN-γ, and IL-1β, are abundant in the peritoneal cavity [55]. IL-17A, the primary cytokine product of Th17, stimulates endometriosis stromal cell proliferation, IL-8 and COX-2 expression; therefore, the Th17 pathway contributes an attractive therapeutic target as in similar chronic inflammatory conditions [56,57].

Treg is another subset of helper T cells with specialized anti-inflammatory activity, thereby maintaining the immune system tolerance to self-antigens. Extensive studies have reported many suppressive Treg cells in the peritoneal fluid of women with endometriosis, suggesting a decreased elimination of ectopic endometrial tissue fragments [58]. CCL25 produced by endometriotic stromal cells and macrophages can initiate local immune tolerance by up-regulating the quantity and functionality of Treg cells. In turn, Tregs can also enhance ESC proliferation and invasion through the production of IL-10 and TGF-β [29].

In the pathogenesis of endometriosis, B lymphocytes have been suggested to play a role by secreting autoantibodies [59]. Anti-endometrial antibodies bounded to the glandular component of ectopic and eutopic endometrium were detected with increased immunoreactivity within the progress of endometriosis. The presence of autoantibodies should be considered with others coexisting with endometriosis immunological and autoimmune-mediated diseases. The anti-endometrial antibodies might also bind embryos and sperms, thus, in turn, contributing to endometriosis-related infertility [52].

An additional function of B lymphocytes is the production of cytokines such as IL-6, granulocyte-macrophage colony-stimulating factor, and IL-17, which have been shown to modulate the activity of immune cells such as CD4+ T cells and exacerbate the inflammatory microenvironment [60]. The synergistic effect of many cell types, including macrophages, endometrial cells, lymphocytes, and Tregs and soluble mediators such as cytokines and chemokines, facilitates this invasive, pro-angiogenic, proliferative endometriotic niche, allowing survival and implantation of shed endometrial cells. Further studies are therefore warranted to fully understand the interaction of immune cells within the endometriotic lesion microenvironment.

2.7. Mesothelial cells

The peritoneal mesothelium is an epithelial-like monolayer that covers the surface of the peritoneal membrane and maintains serosal homeostasis. The mesothelial lining is continuous with cells joined tightly by junctions and acts as a semipermeable membrane regulating the transport of ions, water, and other solutes, involved in the activation of peritoneal inflammation and the recruitment of neutrophils and macrophages [61]. The basement membrane and connective tissue constitute the peritoneal membrane's extracellular matrix (ECM), composed mainly of mesothelial-originated adhesive molecules such as type I and IV collagen, laminin and fibronectin [62].

The adhesion mechanism of the primary endometrial cells to the peritoneal surface remains unresolved. Potentially, the intact mesothelium prevents adhesion and invasion of shed endometrial tissue, which binds preferably to the ECM components [63]. However, more common observations suggest that menstrual effluent can adhere directly to the peritoneal mesothelium and create its invasion site by damaging the mesothelium, thus exposing the ECM. The spread and invasion of endometrial epithelial and stromal cells may be facilitated by loss of mesothelial cell adhesion and increased expression of matrix metalloproteases (MMPs), both in the peritoneal cells of the lesion and ectopic endometrial cells [64,65].

Another important contributor to the formation of the lesions is the change of mesothelial cell morphology from cuboidal to columnar cell types under the influence of inflammatory stimulus [63]. Human peritoneal mesothelial cells have been found to undergo an epithelial–mesenchymal transition (EMT) in response to menstrual effluent [66]. EMT is a process of losing epithelial cell morphology by decreasing E-cadherin expression, disintegrating tight junctions and cytoskeleton reorganization, and gaining mesenchymal cell characteristics by expressing N-cadherin, vimentin, smooth muscle actin, and acquiring migration and invasion of mesenchymal cells [67]. Metaplastic change cells of the visceral and parietal peritoneum into endometriotic lesions support Meyer's theory that postulates coelomic metaplasia as an endometriosis origin [68]. These changes constitute prerequisites for establishing endometriotic lesions because both in peritoneal and ovarian endometriotic lesions, the mesenchymal markers are up-regulated, while epithelial markers are downregulated [69]. Many authors concluded that the peritoneum of women with endometriosis is biologically changed due to up-regulation of cytokines and growth factors like TNF-α, HGF, TGF-β, IL-1β, IL-6, IL-8, and MCP-1. These immune mediators are produced in response to adjacent endometrial epithelial cells and may contribute to altered peritoneal immune state and enhanced invasion of ectopic cells [70,71]. A powerful preclinical study recently showed that pelvic peritoneal mesothelial cells of women with endometriosis exhibit a switch from normal mitochondrial respiration to a glycolytic phenotype. In the tumor microenvironment, lactate facilitates metastasis, angiogenesis, and immune suppression, while the same processes are involved in the formation and survival of endometriotic lesions. This finding provides new insight into the therapeutic concept by targeting metabolic processes with a non-contraceptive treatment for women with endometriosis [72].

2.8. Stem cells

Endometrial stem cells' ability to regenerate the entire endometrium makes them ideal suspects in endometriosis. The endometrium is a remarkable tissue with vast regenerative capacity composed of two main zones: the outer functional layer - dense glandular tissue and a loose connective stroma and the inner basal layer, which primarily contains the stroma, basal gland region, and supporting leukocytes and vascular system [73]. The functional layer and a small portion of the basal layer are shed during each menstrual cycle. Therefore every cyclic regeneration requires replenishment of all cellular compartments repeatedly, which is ensured by the resident stem/progenitor cell population [68].

Endometrial stem/progenitor cells are clonogenic, heterogeneous, and include all cell lineages of the endometrium. It has been suggested that endometrial stem/progenitor cells are transported in retrograde menstrual efflux, which, through retrograde menstruation, gain access to the peritoneal cavity, where they start the lesion development *via* their clonogenic activity [74,75]. Endometrial stem/progenitor cell population is generally categorized into: endometrial mesenchymal progenitor/stem cells (eMSCs), endometrial epithelial stem/progenitor cells, side population cells (SPs). CD140b+ CD146+ eMSCs are located perivascularly in both the functional and basal compartment. The second population of eMSCs is represented by the sushi domain containing-2 (SUSD2+) perivascular cells. Epithelial progenitor cells are a subset of stage-specific embryonic antigen-1 (SSEA-1+) cells located at the basal glandular epithelium and may form individual colonies. Clonogenic epithelial cells are enriched for N-cadherin, enhancing self-renewal by serial cloning. An epithelial cell hierarchy in the human endometrium based on the N-cadherin and SSEA-1 has been proposed. N-cadherin+ epithelial cells is the most primitive population observed in endometrial gland bases in the basalis, followed by SSEA-1+ cells and dual-negative

cells in the functionalis glands and some of the luminal epithelium [76]. Endometrial SPs are considered heterogeneous populations containing endothelial cell markers (CD31), hematopoietic cell markers (CD34 and CD45), the epithelial cell marker EMA, and mesenchymal stem cell markers (CD90, CD105, and CD146). eMSC in ectopic lesions have a higher proliferative potential and migration and angiogenesis capacity than eutopic eMSCs from healthy controls. Moreover, the expression of SSEA-1 in ectopic epithelial cells is similar to that in eutopic basalis epithelium, which supports the theory of a stem cell origin of endometriosis [77]. N-cadherin+ epithelial cells were observed in 50% of peritoneal and deep infiltrating lesions. Under the peritoneal microenvironment, endogenous stem/progenitor cells have altered molecular properties. On the other hand, the estrogenic environment in the endometriotic implant probably may not be directly involved in the regulation of stem/progenitor cells due to the lack of ERα in the SUSD2+, eMSCs, and SPs [76]. The expression of other adult stem cell markers like Musashi-1, OCT4, SOX15, SOX2, KIT, NOTCH-1, Numb, and the corneal epithelial progenitor cell marker importin 13 was also reported in the endometriotic milieu. An elevated level of cytokine avidin A in peritoneal fluid increased the expression of connective tissue growth factor (CTGF) in SUSD2+ eMSCs, which may induce myofibroblast differentiation in SUSD2+ eMSCs through Smad2/3 activity [78]. Moreover, the endometrial stem cell abnormal expression profiles of stemness- and cancer-associated genes, such as KIT, HIF2α, and E-cadherin, were demonstrated in endometriosis patients [79].

Alternatively, stem cells contributing to endometriotic lesions may arise from a different extrauterine source. This hypothesis is appealing as bone marrow-derived cells (BMDSCs) can seed the endometrium and differentiate into mature endometrial cell types [80]. The chemoattraction of BMDSCs was linked with high E2 and chemokine CXCL12 secretion in the endometriotic tissue. After delivery of circulating BMDSCs, they exhibit properties of stromal and epithelial cells. Cytokine secretion by the implanted BMDSCs promotes the proliferation of ectopic endometrial cells [81].

Endometrial stem cells likely play an important role in the pathology of the reproductive tract, such as endometriosis. Future research can use stem cell systems to characterize better the molecular pathways regulating endometrial stem/progenitor cells in this gynecological disorder. There is a growing body of evidence that endometrial stem/progenitor cells are a promising therapeutic target for endometriosis but require a cautious therapeutic approach to target endometriotic lesion establishment without altering the normal endometrium.

3. *In vitro* modeling of endometriosis

Developing *in vitro* models that resemble the main characteristics of endometriosis is a challenging task for several reasons, ranging from not fully elucidated causes of how the disease begins and persists. Multiple theories of disease origin (i.e. retrograde menstruation, lymphovascular metastasis, metaplasia) have been proposed along with various factors contributing to pathogenesis and progression. Further, endometriosis manifests as diverse disease forms such as superficial peritoneal lesions, endometrioma, and deeply infiltrating lesions. Currently, the main challenge to designing the experimental models of endometriosis goes beyond reconstructing factors affecting this disease and identifying potential therapeutic targets. Tools for endometriosis research should also incorporate relevant disease characteristics, follow the cellular and molecular mediators' network and mimic clinical behavior observed in patients. Although *in vitro* models cannot fully contemplate all endometriosis-related biological mechanisms, they enable the determination of critical cellular and molecular parameters in disease and the screening of therapeutic compounds, with easier access to research material, lower ethical issues, and costs. To make important insights into endometriosis pathophysiology, scientists construct experimental models based on the patient-derived tissues, fluids and cells, primary cell cultures, and cell lines, combining them in two-dimensional (2D) cocultures or three-dimensional (3D) models utilized in the research more recently.

3.1. Patient-derived tissue, fluids, and cells

Many centers worldwide collect tissue samples of ectopic and eutopic endometrium and fluids from women with and without endometriosis undergoing laparoscopy for various research purposes. However, protocols variability used for collecting, processing, and storing specimens probably limits the compilation and reproducibility of data obtained in different research laboratories and practical worldwide cooperation. The World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonization Project (EPHect) focused on overcoming limitations through the adoption of well-described standard operating procedures (SOPs) about gathering samples and standardization of surgical patients data [82].

Biopsies can be isolated from the peritoneal lining, ovarian cysts, or deep infiltrating nodules. Lesion biopsies may be contaminated with surrounding peritoneal tissue; therefore, including peritoneum and endometrium control tissues is recommended in the examination. The menstruation cycle phase and intake of exogenous hormones should also be considered as these factors can modulate the composition and concentration of peritoneal fluid [83]. Healthy patients generally do not undergo laparoscopy and endometrium biopsy; thus, access to control samples is significantly limited.

Identification of potential diagnostic biomarkers and novel drug targets for the disease is possible through molecular profile analysis based on changes in DNA, RNA, protein, and metabolite levels detected in various bodily fluids. The collection of blood, urine, saliva, endometrial fluid, peritoneal fluid, and menstrual effluent in endometriosis research according to the SOPs envisaged by the WERF EPHect allows for significant advances to be made in the detection of endometriosis biomarker relationships through multi-center collaborative studies.

3.2. Human primary endometrial epithelial and stromal cells

Primary endometriosis cell cultures are generally prepared from surgical biopsies from various lesions, including ovarian cysts, peritoneal implants, and deep infiltrating endometriosis, with cell isolation performed according to standardized protocols [84,85]. Briefly, tissue fragments are rinsed, minced, and digested with collagenase at standard culture conditions. Epithelial glands are separated from stromal cells, blood cells, and debris by serial filtration using narrow gage sieves and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. Separately, stromal cells derived from collagenase digestion of endometriotic tissues are plated to the culture flasks and maintained under the same culture conditions [86].

Endometrial epithelial and stromal cells isolated directly from endometrial biopsies and ectopic endometriotic tissues exhibit the individual *in vivo* phenotypic and functional markers [87]. Two types of cells could be distinguished: one displaying stromal cell features (cytokeratin/E-cadherin-negative), the other epithelial-like (cytokeratin-positive/E-cadherin-negative). However, analysis of the cellular composition of endometriotic lesions revealed two types of cytokeratin-expressing epithelial cells. E-cadherin-negative epithelial cells less frequent in the peritoneal endometriosis biopsies were invasive *in vitro*, whereas noninvasive endometrial epithelial cells were E-cadherin positive. The invasive phenotype of endometriotic cells is reminiscent of carcinoma cells and might play an important role in developing and invading endometriosis [88,89]. Despite the successful separation of the stromal and epithelial cells derived from endometriotic samples, cultures of stromal-like cells displayed variability in morphology indistinguishable from the endometrial stroma [90]. The degree of similarity between the two tissues is of particular interest because endometrial tissue is regarded as a likely precursor tissue for endometriosis. Primary endometrial cells isolated from the eutopic endometrium have

significant application in endometriosis research. Recently, a quality control pipeline to assure sufficient quality of *in vitro* research in the field of endometriosis was proposed. The construction of primary cell cultures from ectopic tissues should confirm the absence of mesothelial, endothelial, immune, and vascular cells and subsequently detect endometrial specific markers such as paired box 2 (PAX2), expressed highly in both ectopic and eutopic stromal and epithelial cells, with further characterization regarding the expression of steroid hormone receptors and epithelial and stromal markers [91].

Epithelial progenitor and mesenchymal stem cells derived from the basal layer of the human endometrium are likely responsible for its immense regenerative capacity; they may also be implicated in the development of endometriosis due to their occurrence together with menstrual debris in the peritoneum [92]. The importance of endometrial stem cells in the establishment and progression of endometriotic lesions have been identified using endometrial stem cells isolated from the ectopic lesions of endometriosis patients and eutopic endometrium of the control group [93].

Primary cells can better shed light on the onset of endometriosis; however, in *in vitro* conditions, they have a limited life span. Nevertheless, establishing endometriosis cell lines requires the prerequisite isolation and culture of primary cells.

3.3. Endometrial and endometriotic cell lines

As culture time and passaging of primary endometrial cells are limited in standard culture conditions, it is necessary to establish immortalized endometrial cell lines as suitable to the construction of experimental *in vitro* models. Cell lines are obtained from primary cells isolated from the endometrium or endometriotic lesions, which survive in culture following the first passage. Established immortalized cell lines have the ability to unlimited proliferation; they can be repeatedly passaged and reliably recovered from biobank after cryopreservation. Continuous cell lines constitute a viable tool for functional studies on endometriosis. Their main characteristics are summarized in Table 1 and described in the following chapters.

3.3.1. Endometrial stromal cell lines

3.3.1.1. St-T1b cell line. Samalecos's group established an endometrial stromal St-T1b cell line from primary cells derived from proliferative endometrium of patients with tubal disease awaiting *in vitro* fertilization treatment [94]. St-T1b cell line can be maintained in continuous culture owing to immortalization by introducing the human telomerase reverse transcriptase (hTERT) into primary cells. Cells display typical morphology of proliferative endometrial fibroblasts and express appropriate markers like vimentin and CD90, with lack an epithelioid marker cytokeratin-7 (CK7) expression. The expression of progesterone receptor (PR) and ERα is detectable in St-T1b cells, whereas ERβ transcripts are below the detection limit. Despite PR transcripts expression, the combination of progestin with E2 fails to induce decidualization-related morphologic changes in St-T1b cells. On the other hand, upon cAMP stimulation, St-T1b cells respond to progestin and express typical marker genes of decidualization, namely those coding for decidual PRL (dPRL), IGFBP-1 and FOXO1. cAMP admittedly acts as a decidualization stimulus but is insufficient to sustain the decidual phenotype over an extended period. Interestingly, long-term maintenance of decidualization can be achieved by adding P4. It has been found that St-T1b cells can constitute a valid substitute for primary endometrial cells, mainly in the studies on the mechanisms of decidualization [94].

The St-T1b cell line has been utilized in invasion studies, which have shown that down-regulation of miR200b in the eutopic endometrium can promote cell release from the surrounding tissue and facilitate invasive growth at ectopic sites [95]. In such research, normal ESC may constitute a reference control for endometriotic cells to evaluate the invasive process involved in endometriosis and reveal the mechanisms modulating the stromal cell invasiveness [96].

3.3.1.2. SHT290 cell line. Cell line SHT290 was obtained by introducing the hTERT into human normal ESC [97]. SHT290 cells exhibit uniform bipolar fibroblast-like morphology and weak cytokeratin 18 and 13 expression. They strongly express vimentin, typical of differentiated stromal cells of mesenchymal origin, and decidualization markers prolactin and plasminogen inhibitor type-1. Also, they have progesterone receptor and estrogen receptors functional in the decidualization response. Endometrial stromal SHT290 cells may differentiate and regulate the growth of epithelial cells in response to ovarian steroid hormones, which expand their application in the studies on paracrine factors and the reciprocal stromal-epithelial interactions in the endometrium [97]. Rai and Shivaji (2011) applied the SHT290 cell line to investigate the role of human protein deglycase (DJ-1) in the pathogenesis of endometriosis [98]. Table 1 presents this research briefly.

3.3.1.3. T-HESC cell line. T-HESC line (telomerase-transformed human endometrial stromal cells) display morphological changes and biochemical endpoints of decidualization after exposure to a progestational milieu [99]. This cell line, karyotypically and phenotypically similar to the primary parent cells, responding to hormone stimulation as cultured primary stromal cells, has become a widely used invaluable tool for studying physiological functions of uterine endometrium and its pathological changes.

The T-HESC line has been applied to study TGF-β signaling pathways in endometrial and endometriotic cells [100] identify the role of chronic NOTCH-1 signaling activation underlying progesterone resistance in endometriosis [101]. Recently, Holdsworth-Carson and co-workers found that manipulating the long intergenic non-coding RNA 339 (LINC00339) expression in HESC cells impacts immune defense pathways, which are fundamental biological pathways that stimulate endometriosis development [102].

3.3.1.4. KC02-44D cell line. The KC02-44D cell line is a human endometrial stromal cell line established by infection with lentivirus pLenti6-hTERT vector to study endometrial function and decidualization mechanisms induced *in vitro* [103]. This cell line responds to IL-1β through inducing COX-2 expression and PGE2 production, similarly to primary cells. KC02-44D decidualization is caused by progestin and cAMP and the regulation of PR expression by E2. Cells exposed to ovarian steroids show stromal cell biochemical properties and markers such as vimentin, CD10, or CD13, but they do not display cytokeratin, a marker of epithelial cells. The KC02-44D cell line has been considered a representative cell line and a valuable tool for studying normal and pathological processes in human endometrium [103].

3.3.2. Endometrial epithelial cell lines

3.3.2.1. Ishikawa cells. Ishikawa cells were isolated from a well-differentiated human endometrial epithelial adenocarcinoma [104]. The cells maintain functional steroid receptors: estrogen and progesterone receptors (ER and PR). Although this cell line does not represent endometrial or endometriotic tissue, it retains characteristics of endometrial glandular epithelium. According to scientists, the Ishikawa cell line is a valuable model for studying normal endometrium function [105]. The PR in these cells is immunologically and biochemically similar to PR in normal endometrium; it is induced by estrogen and suppressed by progesterone [106]. Moreover, Ishikawa cells express many structural proteins and enzymes specific to normal endometrium [107,108]; therefore, they have been used in several studies on endometriosis as a representative cell line for human endometrium (Table 1).

Table 1
Cell lines, their characteristics and applications in endometriosis research.

Cell line (origin)	Immortality	Specific markers	Cell culture system	Research area	Main findings	References
Endometrial stromal cells (ESCs)						
St-T1b (endometrium)	Yes	ERα (+), ERβ (–), PR (+), Vim (+), CD90 (+), CK7 (–)	Monoculture	Endometrium decidualization	Reduction of decidualization markers (dPRL, IGFBP-1, FOXO1) upon progestin treatment.	[94]
			Monoculture	Migration, invasion	Increase in miR-200b, decrease in EMT and invasiveness.	[95]
			Spheroids (ESCs, 12Z cells)	Migration, invasion	Model of collapsed endometrium placed on a Matrigel or type I collagen. Directional migration followed by matrix remodeling was observed due to increased MMP2 and MMP14.	[96]
SHT290 (endometrium)	Yes	ERα (+), ERβ (–), PR (+), Vim (+), prolactin (+), PAI-1 (+)	Co-culture (ESCs, Ishikawa cells)	Paracrine interactions, hormone response	The inhibited proliferation of Ishikawa cells treated with estrogen or progesterone.	[97]
			Monoculture	DJ-1 effect on cell proliferation, migration, invasion	Enhanced DJ-1 expression, increased cell invasion.	[98]
T-HESC (endometrium)	Yes	Vim (+)	Monoculture	Endometrium decidualization	Increased decidualization markers (IGFBP-1, FN, dPRL, TF, PAI-1, FasL) after treatment with E2/MPA.	[94]
				TGF-β signalling, cell adhesion	Increased TGF-β1 secretion and PAI-1 expression, decreased cell adhesion.	[100]
				Progesterone resistance, NOTCH-1 regulation	in progestogenic environment: ↓ NOTCH-1: reduced cell number, ↑ PGR expression; ↑ NOTCH-1: increased cell number, ↓ PGR expression.	[101]
				Non-coding RNA 339 in endometriosis	LINC00339 impacts immune defence pathways in T-HESC cells.	[102]
KC02-44D (endometrium)	Yes	Vim (+), CD10 (+), CD13 (+), CK (–)	Monoculture	Endometrium decidualization	Increased decidualization by progestin and cAMP. Induced PR by E2. Decreased decidualization by IL-1β.	[103]
Ishikawa cells (endometrial adenocarcinoma)	Yes	ERα (+), ERβ (+), PR (+)	Monoculture	Cell line establishment	Estrogen independency.	[104]
				Effects of hormone treatment on cell growth and PR expression	Enhanced proliferation by 4-OH tamoxifen (anti-oestrogen), decreased proliferation by 4-OH tamoxifen + ICI 164,384 (anti-oestrogen) ↑ PR after treatment with 4-OH tamoxifen, ↓PR after treatment with 4-OH tamoxifen + ICI 164,384	[105]
				Migration	miRNA (let-7f) modulation by aromatase inhibitor, decreased cell migration	[121]
			Monoculture, low-invasive control	Migration, EMT process	Impaired claudins localization, EMT observed in endometriotic cells.	[122]
Endometriotic stromal cells						
Hs 832(C).T (ovarian endometriosis)	Yes	Vim (+), CK (−), E-cad (−)	Monoculture	Cell line establishment	Characteristic of cell line (ultrastructural features, chromosome number, doubling time).	[109]
				Apoptosis induction by propofol	↑ FOXO1, ↑ FOXO3, ↑ Bim, ↑ pro-caspase-3, ↑ active caspase-3, ↑ p53, ↑ p21 expression.	[111]
				Migration	↑ E-cadherin, ↑ Claudin-1, ↑ Claudin-3 ↓Vimentin, ↓ SNAIL, ↓ TWIST1, ↓ ZEB1 expression under LINC00261 (lncRNA) regulation.	[112]
				Invasion	Inhibited autophagy, ↓ exogenous fascin-1 expression, enhanced endometriotic cell invasiveness.	[113]
22B (peritoneal endometriosis)	Yes	Vim (+), CK (−), E-cad (−), ERα, β (+), PR (±), P450 (+)	Monoculture	Proliferation, apoptosis, invasion, angiogenesis, inflammation	↑ EGF, ↑ EGFR1, ↑ EGFR2, ↑ EGFR3 ↑ MMP2, ↑ MMP9, ↑ VEGF, ↑ VEGFR-1, -2, -3 ↑ IL-1β, ↑ TNF-α expression.	[89,117]
Endometriotic epithelial cells						
EEC145T (peritoneal endometriosis)	No	Vim (+), CK (+), E-cad (−), ERα, β (+), PR (+)	Monoculture	Invasion	Increased invasion by peritoneal fluid CA-125.	[115]
				Adhesion	Decreased adhesion by CA-125.	[116]
10Z (peritoneal endometriosis)	Yes	Vim (+), CK (+), E-cad (−), ERα, β (+), (+), PR (+), P450 (+)	Monoculture	Invasion	The 10Z cell line in non-invasive.	[89]
12Z (peritoneal endometriosis)	Yes		Monoculture	Proliferation, apoptosis, invasion, angiogenesis, inflammation	↑ EGF, ↑ EGFR1, ↑ EGFR2, ↑ EGFR3 ↑ MMP2, ↑ MMP9 ↑ VEGF, ↑ VEGFR-1, -2, -3 ↑ TNF-α expression.	[89,117]
11Z, 49Z, 108Z (peritoneal endometriosis)	No					
12Z (peritoneal endometriosis)	Yes		Monoculture	Proliferation	Reduced proliferation under treatment with GHRH antagonist.	[123]
				Inflammation	↓ CXCR4 expression.	[124]

(*continued on next page*)

Table 1 (*continued*)

Cell line (origin)	Immortality	Specific markers	Cell culture system	Research area	Main findings	References
				NOTCH signalling Association between PR and EMT process	↑ NOTCH-1 expression under IL-6 regulation. EMT induction, ↓ PR expression. ↑ mesenchymal marker (CDH2) expression.	[46] [125]
EEC16-TERT (ovarian endometriosis)	Yes	Vim (+), CK (−), E-cad (−), ERα (–), PR (–)	Monoculture	Cell line establishment, transcriptome analysis	Cell phenotype transformation is lower for EEC16-TERT cells than for 12Z cells. Increased cell migration and invasiveness. ↑ HAS1, KRT19, CDH20, ALDH1A2 and ↓ HOXC11, HOXC12, SOD3, CALCR expression compared to normal ovarian epithelial cells.	[118]
			monoculture 3D spheroid	Neoplastic transformation	Activation of Src and Wnt signalling.	[119]

↑ Increase; ↓ Decrease. Abbreviations used in Table 1: ALDH1A2, aldehyde dehydrogenase 1 family member A2; BIM, Bcl2-interacting mediator of cell death; CALCR, calcitonin receptor; CDH2, cadherin 2; CDH20, cadherin 20; CD90, cluster of differentiation 90; CK, cytokeratin; CXCR4, C-X-C chemokine receptor type 4; E-cad, E-cadherin; FasL, Fas Ligand; FN, fibronectin; GHRH, growth hormone–releasing hormone; HAS1, hyaluronan synthase 1; HOXC11, homeobox C11; HOXC12, homeobox C12; ICI 164,384, antiestrogen – undecanamide; KRT19, keratin 19; LINC00261, long intergenic non-protein coding RNA 261; LINC00339, intergenic non-protein coding RNA 339; MPA, medroxyprogesterone acetate; NOTCH-1, neurogenic locus notch homolog protein 1; PAI1, plasminogen activator inhibitor-1; p21, cyclin-dependent kinase inhibitor 1A; P450, cytochrome P450 aromatase; p53, tumor protein 53; SNAIL, snail 1 zinc finger protein; SOD3, superoxide dismutase 3; TF, tissue factor; TWIST1, twist-related protein 1; VEGFR, vascular endothelial growth factor receptor; ZEB1, zinc finger E-box-binding homeobox 1.

3.3.3. *Endometriotic cell lines*

3.3.3.1. Hs 832(C).T cell line. Endometriotic stromal The Hs 832(C).T cell line was isolated from the inner and outer surfaces of the benign ovarian cyst from an endometriosis patient [109]. The cells have been partially characterized as stromal (vimentin-positive, cytokeratin-negative, E-cadherin-negative) but not well described for molecular markers for endometrial epithelium and stroma, despite being authenticated by short tandem repeat profiling [110]. This cell line was widely used as a representative of stromal ectopic endometriotic fraction in studies on endometriosis development in terms of cell proliferation and apoptosis [111], migration [112] and invasion [113]. Nevertheless, the commercial availability of this cell line was discontinued due to its slow growth rate [110].

3.3.3.2. 10Z, 11Z, 12Z, 49Z, 108Z, EEC145T, 22B cell lines. Zeitvogel et al. (2001) established a set of endometriotic cell lines derived from peritoneal endometriotic biopsies [89]. Scientists immortalized two cell types representing stromal (cytokeratin-negative, vimentin-positive) and epithelial (cytokeratin-positive, vimentin-positive) cell features with SV40 T-antigen. Both cell types were E-cadherin-negative. They obtained 11 cytokeratin-positive epithelial-like cell lines and 13 cytokeratin-negative fibroblastoid cell lines with spindle-shaped morphology. Although these established cell lines are not neoplastic, they share some features with carcinoma cells, like invasive capacity. N-cadherin-dependent motility has been identified in the endometriotic cell lines, which may be relevant for their invasiveness and migration similar to cancerous cells. Furthermore, endometriotic cells expressed catenins like β-catenin and p120ctn in the cell membrane, which are implicated in the transformed phenotype [89]. Only 10Z and 12Z cell lines became immortal; other cell lines had a limited life span or died. EEC145T cell line retained its initial characteristic until 25 passages and then began to lose the cytokeratin expression, invasive capacity and expression of fibroblast growth factor activating gene 1 (Frag-1), but maintained its proliferative potential [114]. The EEC145T cell line was used to study the effect of CA-125 on cell invasiveness and adhesion [115,116]. Almost all established endometriotic cell lines expressed aromatase cytochrome P-450, responsible for the local production of estradiol in the endometriotic lesions. In contrast, they showed low expression of steroids markers like ERα, ERβ, and PR [89]. Further, many genes and proteins expression patterns, identified in women with endometriosis, have been detected in established human endometrial epithelial cells (12Z, 49Z, 108Z, and 11Z) and stromal cells (22B) [117]. The cell cycle regulation and cell survival gene profiles reveal mitogenic and survival characteristics of endometriotic cell lines.

Established human endometriotic epithelial (12Z, 49Z, 108Z, and 11Z) and stromal (22B) cell lines have become valuable tools to investigate the mechanisms of action behind endometriosis due to representing genes expression patterns observed in women with endometriosis. Currently, they constitute a stable, available material in preclinical laboratory practice. 12Z cell line has been widely employed as a research tool to construct endometriosis *in vitro* models. In the last 5 years, more than 50 studies utilized this endometriotic epithelial cell line together with endometriotic stromal cell line or normal endometrial cells as a research model to explore endometriosis development and treatment. Table 1 presents the most relevant studies using endometriotic stromal and epithelial cell lines, which provided important information on the pathological mechanisms in endometriosis and unraveled novel biomarkers and therapeutic approaches.

3.3.3.3. EEC16 cell line. EEC16 cell line was established from a superficial endometriosis lesion on the ovary. Cells exhibit an epithelial morphology with mesenchymal markers like cytokeratin and vimentin and do not express ERα, P- and N-Cadherin [118]. The phenotype of the EEC16 cells was compared with normal ovarian surface epithelial cells (OSECs) and 12Z cells. Results showed that EEC16 cells represent a less transformed phenotype than 12Z cells but have more migratory and invasive capacity than normal OSECs. Comparative transcriptome analysis of primary EEC16 and OSEC lines indicated that 1780 genes are expressed significantly differentially, especially genes associated with adhesion, vasculature development, migration and cell contractility, inflammatory responses, and responses to hypoxia [118].

The limited lifespan of EEC16 cells was increased following transduction with lentiviral-TERT (EEC16-TERT-L) or retroviral-TERT (EEC16-TERT-R) human telomerase [119]. TERT-transduced cells were continuously passaged for over 200 days, in contrast to the non-transduced cells that underwent replicative senescence after 60–70 days. TERT expressing cells retained the normal karyotype of the primary cells; there is no evidence of their tumorigenicity after injection to mice. Although the primary EEC16 and TERT-expressing EEC16 cells share similar morphologies, they differentially express epithelial and mesenchymal markers. Primary cells represent epithelial features with cytokeratin and mesenchymal features with vimentin and without E-cadherin expression, whereas EEC16-TERT cells are cytokeratin-negative but maintain vimentin expression, which strongly suggests the involvement of EMT through immortalization. In previous experiments, the TERT-immortalized OSECs did not undergo EMT; thus, mechanisms facilitating the EMT process during immortalization are dedicated to endometriotic cell lines [120]. Furthermore, a reduction in expression of key tumor suppressor genes predisposing the occurrence of

endometriosis-associated ovarian cancers in EEC16-TERT lines was observed in comparison with primary EEC16 cells.

Neoplastic transformation of EEC16-TERT was dependent on active Src and Wnt signaling. Src activation may promote the survival of ectopic endometrial cells in the peritoneal cavity. Src activation in endometrial tissues of women may therefore predispose to endometriosis and have potential clinical relevance in the diagnosis of endometriosis in the endometrial biopsy specimens. If, in turn, activated Src signaling commonly occurs in cancer, it may raise the intriguing hypothesis that Src activation can constitute a prerequisite of endometriosis-associated ovarian cancer. There are possibilities to detect the Src signature noninvasively in eutopic endometrium of women at the highest risk of developing endometriosis-associated ovarian cancer [119]. The established TERT-immortalized models of ovarian endometriosis may have diverse applications for profoundly understanding the biology of endometriosis and the neoplastic transformation of endometriosis into ovarian cancer, therefore identifying novel therapeutic targets for the disease.

Much of the preclinical research in endometriosis was conducted with the *in vitro* tools, namely primary cell cultures and cell lines. Primary endometriotic cells isolated directly from fresh tissues of donor patients have been poorly investigated, mainly due to the limited endometriotic tissue availability and the small number of cells. The primary cell cultures obtained from the peritoneal lesion and chocolate cyst wall consist of mostly heterogeneous cell populations variable in size and morphology. Primary cell cultures provide more physiologically relevant data than those generated using cell lines; therefore, they are often combined with advanced technologies such as 3D cell culture models. However, cell lines isolated from primary cultures that acquired the ability to unlimited proliferation ensure the stability and reproducibility of the experiments. Cell lines provide pure homogeneous populations of cells cultured in precisely controlled culture conditions. Characteristics of cell lines should be closely monitored due to possible loss of tissue-specific cell properties and genetic instability during multiple serial passages and long-term cultivation. The major limitation of the cell line is that it relies on the cell population, which has been transformed and represents only a limited subset of disease phenotypes, usually exhibiting features of undifferentiated cells, and does not accurately reflect the *in vivo* environment. 2D cell cultures are usually applied to preliminary analyses of cellular structures, biochemical pathways, cell metabolism, and gene and protein expression in controlled culture environments. They have severe limitations, including the lack of specific cell-to-cell and cell-to-ECM signaling due to the isolation of cells from the 3D tissue geometry.

4. Heterogeneous cell culture models of endometriosis

4.1. Co-cultures of endometriotic cells with macrophages

Endometriotic cells' communication with macrophages is still poorly understood. The mechanisms of macrophage actions and reciprocal interactions with endometriotic cells may be imitated in the heterogeneous cell cultures utilizing conditioned media and various cell types grown in the co-cultures. Woo et al. (2017) suggested that human endometriotic epithelial cells can increase macrophage migration by locally producing chemotactic MCP-1. They performed a transwell cell migration assay with human endometriotic epithelial 11Z and 12Z cells that significantly increased THP-1 macrophage migration compared to endometrial epithelial cells. In contrast, human endometriotic stromal 22B cells did not mediate the macrophage migration. Macrophages stimulated by IL-13 and IL-4 secreted by endometriotic epithelial cells underwent M2 polarization, promoting migration of endometriotic cells through IL-6 overproduction [126]. A similar study indicated that 12Z cells secrete cytokines and chemokines upon stimulation by factors derived from THP-1 macrophages. In the 12Z/THP-1 co-culture model, niclosamide - a well-known essential drug, was found to strongly suppress the production of most cytokines and chemokines secreted by 12Z cells [127]. Also, macrophages derived from healthy women were incubated in the contact co-culture with ESC of eutopic endometrium from endometriosis women. This cell culture system was utilized to clarify the role of IL-24 and its receptors in the regulation of endometrial stromal cell proliferation and invasiveness during endometriosis [34]. In another study, human monocyte U937 cell line, human peritoneal mesothelial HMrSV5 cell line, and eutopic ESC isolated from patients with endometriosis were applied for constructing the combined, contact and transwell co-culture models. Scientists proved that secretion of signaling molecules with significant importance for endometriosis progression and endometriotic foci development is possible to induce at the higher level in the co-culture model consisting of three cell types. They confirmed that cross-talk between the different cells in the inflamed peritoneum might play a crucial role in the pathogenesis of endometriosis [128]. Table 2 and Fig. 2 show endometriosis experiments in the described co-culture system.

4.2. Co-cultures of endometriotic cells with peritoneal cells

Once placed in the peritoneal cavity, endometrial cells create their invasion site by directly damaging the mesothelium (Fig. 1); thus, it is justified to include mesothelial cells in modeling endometriosis. Human peritoneal cells were used to establish co-culture with endometrial or endometriotic cells. The human pleural cavity mesothelial MeT-5A cell line was co-cultured with T-HESC, Ishikawa, and 12Z cell lines to study the effect of endometriosis-related cytokines on endometrial cell adhesion. The study demonstrated the sialylation effect on endometrial cells' adhesion and the impact of TGF-β1 on the endometrium attachment to the peritoneum [129].

The attachment of endometrial epithelial and stromal cells to the peritoneal mesothelium has been investigated in several studies using the LP9 mesothelial cell line. Studies performed by Lucidi et al. (2005) showed similar endometrial cell binding to LP9 cells as to mesothelial cells obtained from parietal peritoneum and normal ovarian surface epithelium. The results suggest that the LP9 cell line is an appropriate *in vitro* surrogate for patient-derived peritoneal mesothelial cells. ESCs labelled with the calcein-AM were plated over the confluent peritoneal mesothelial cells (PMCs) from different sources and co-cultured simultaneously to perform an attachment assay [130].

Adhesion experiments were also performed using the co-culture of endometrial epithelioid cell line (EM42) and LP9 cells. Moreover, in the invasion assay, LP9 cells and PMCs were grown to confluence on Matrigel^{TM} coated chambers and the labelled EM42 cells were placed over LP9 covered membranes. The study provided evidence of the potential suppression of the endometriosis lesion arrangement among peritoneal mesothelium by proliferator activator receptor-gamma (PPAR-γ) agonist treatment [131].

In a similar study, peritoneal mesothelial cells increased invasion of ESC and EM42 cells through invasion chambers coated with Matrigel; this culture system served as a modelled peritoneum. Other noteworthy research indicated that co-culture of endometrial cells and peritoneal mesothelial cells increases transcription of genes implicated in invasion and metastasis and persistence of endometriosis [2].

Human peritoneal mesothelial cells (HPMCs) and immortalized human endometrial stromal cells (SHT290) were co-cultured in the transwell system to treat of HPMCs exhibiting glycolytic metabolic phenotype with a non-hormonal compound dichloroacetate (DCA). SHT290 was plated in the upper compartment of cell culture inserts, while HPMCs treated with DCA were incubated in basal chamber. DCA diminished the glycolytic phenotype of HPMCs and decreased stromal cell proliferation [72]. The abnormal metabolic reprogramming was corrected by treating with DCA. The findings are now translating to women, by using DCA in an exploratory-phase clinical trial (ClinicalTrials.gov identifier NCT04046081) [132].

Research findings obtained in co-culture models of endometriotic

Table 2
Cell co-culture systems in endometriosis research.

Co-culture model	Cell types	Cell culture system	Research area	Main findings	Reference
Endometriotic cells and macrophages	11Z or 12Z cells, THP-1 macrophages	Transwell co-culture	Migration	Increased macrophage migration by MCP-1 produced by endometriotic cells. Macrophage M2 polarization stimulated with IL-13 and IL-4 secreted by endometriotic epithelial cells. Enhanced endometriotic cell migration by IL-6 secreted by THP-1 cells.	[126]
	22B cells, THP-1 macrophages	Transwell co-culture	Migration	22B cells do not mediate macrophage migration.	[126]
	12Z cells, THP-1 macrophages	12Z cells cultured in conditioned media from THP-1 cells	Inflammation	THP-1 macrophages stimulated CCL5, CXCL12, MDK production in 12Z cells. Niclosamide suppressed cytokine and chemokine production in 12Z cells.	[127]
	Macrophages from healthy women, ESCs of eutopic and ectopic endometrium from endometriosis women	Contact co-culture	Viability, proliferation, invasion	↑ IL-24, IL-20R1, IL-20R2 expression in endometrial cells. ↓ IL-24, IL-20R1 in ESCs under co-culture with macrophages. Macrophages reduced the inhibitory effect of IL-24 on viability, invasion, expression of Ki-67, PCNA and COX-2 genes, and the stimulatory effect on the tumor metastasis suppressor gene CD82 in ESCs.	[34]
	U937 monocytes, HMrSV5 peritoneal mesothelial cells, eutopic ESCs from endometriosis patients	Contact and transwell co-cultures	Inflammation	↑ RANTES and MIP-1α expression in the co-culture of U937 and HMrSV5 cells. The highest RANTES and MIP-1α secretion in the co-culture of three cell types. Increase in MMP-9 and MMP-2 activity in co-culture after E2 and dioxin treatment.	[128]
Endometriotic cells and peritoneal cells	T-HESCs or Ishikawa or 12Z cells, MeT-5A human pleural cavity mesothelial cells	Contact co-culture	Adhesion	TGF-β1 increased endometrial cell adhesion to peritoneal mesothelium. ↑ ST6Gal1 and ST6Gal2 expression in T-HESCs, Ishikawa and 12Z cells treated with TGF-β1. Activation of TGF-βRI/SMAD2/3 signaling in endometrial cells. Terminal sialic acid glycan epitopes of endometrial cells interact with receptors in Met-5A cells.	[129]
	Primary ESCs of healthy endometrium, PMCs from the anterior abdominal wall and normal ovarian epithelium, LP9 mesothelial cells	Contact co-culture	Adhesion	Endometrial cells bound to LP9 cells as to mesothelial cells from parietal peritoneum and ovarian surface epithelium. Cells derived from the endometrium, rather than peritoneal mesothelium, has the highest effect on ESC binding to PMCs.	[130]
	EM42 endometrial epithelioid cells, LP9 mesothelial cells	Contact co-culture	Adhesion, invasion	Reduced binding of EM42 cells to LP9 cells by CTZ treatment.	[131]
	Primary ESCs of healthy endometrium, EM42 endometrial epithelioid cells, LP9 mesothelial cells	Contact co-culture	Invasion, metastasis	Increased invasion of ESCs and EM42 cells. ↑ CD44, ERK, CSF-1, c-fms, c-Met expression in ESCs, EM42, and LP9 cells.	[2]
	Primary HPMCs of endometriotic pelvic peritoneum, human endometrial stromal SHT290 cell line	Transwell co-culture, HPMCs incubated with DCA, TGF-β1, or DCA+TGF-β1	HPMCs glycolysis, SHT290 proliferation	DCA decreased basal and TGF-β1–stimulated HPMCs lactate secretion and proliferation of SHT290 cells.	[72]
Endometriotic cells and endothelial cells	Ad-MSC, ESCendo, ESCcyst, HUVECs	Transwell co-culture, HUVECs cultured in conditioned media from the Ad-MSC /ESCendo and Ad-MSC/ESCcyst co-cultures	Angiogenesis	Increased tube formation by HUVECs under conditioned media from Ad-MSC/ESCendo and Ad-MSC/ESCcyst co-cultures.	[135]
	E-MSCs from eutopic and ectopic endometrium of ovarian endometriosis, HUVECs	Direct and transwell co-cultures	Angiogenesis	↑ CD31 expression in E-MSCs and acquired endothelial phenotype. E-MSCs integrated into tube-like structures in contact with HUVECs.	[136]

↑ Increase; ↓ Decrease; Abbreviations used in Table 2: ESCs, endometrial stromal cells; PMCs, peritoneal mesothelial cells; Ad-MSC, mesenchymal stromal cells from allogeneic adipose tissue; ESCendo, stromal cells from endometrium; ESCcyst, endometriotic ovarian cysts; HPMCs, human primary peritoneal mesothelial cells; HUVECs, human umbilical vein endothelial cells; E-MSCs, endometrial mesenchymal stromal cells; CD31, platelet endothelial cell adhesion molecule; CD44, cluster of differentiation 44; CD82, cluster of differentiation 82; c-fms, macrophage colony stimulating factor receptor 1; c-Met, receptor for hepatocyte growth factor; CSF-1, colony stimulating factor 1; ERK, extracellular signal-regulated kinase; MDK, midkine; SMAD2, mothers against decapentaplegic homolog 2; ST6GAL1, beta-galactoside alpha-2,6-sialyltransferase 1; ST6Gal2, beta-galactoside alpha-2,6-sialyltransferase 2; TGF-BRI, TGF-β receptor inhibitor; DCA, dichloroacetate.

cells with peritoneal cells summarizes Table 2.

4.3. Co-culture of endometriotic cells with endothelial cells and/or mesenchymal stromal cells

Endometriotic lesions are characterized by intense vascularization with the significantly enhanced secretion of VEGF in peritoneal fluids [133,134]. An increasing number of studies suggests that multiple mechanisms contribute to the vascularization of endometriotic lesions. Altered autologous mesenchymal cells in ectopic lesions are likely involved in several cellular mechanisms contributing to the engraftment of endometriotic lesions. The effects of mesenchymal stromal cells from allogeneic adipose tissue (Ad-MSC) on stromal cells from endometrium (ESCendo) and endometriotic ovarian cysts (ESCcyst) were analyzed using tube formation assay (*in vitro* angiogenesis assay). Different co-culture systems were employed in this study to explore the potential

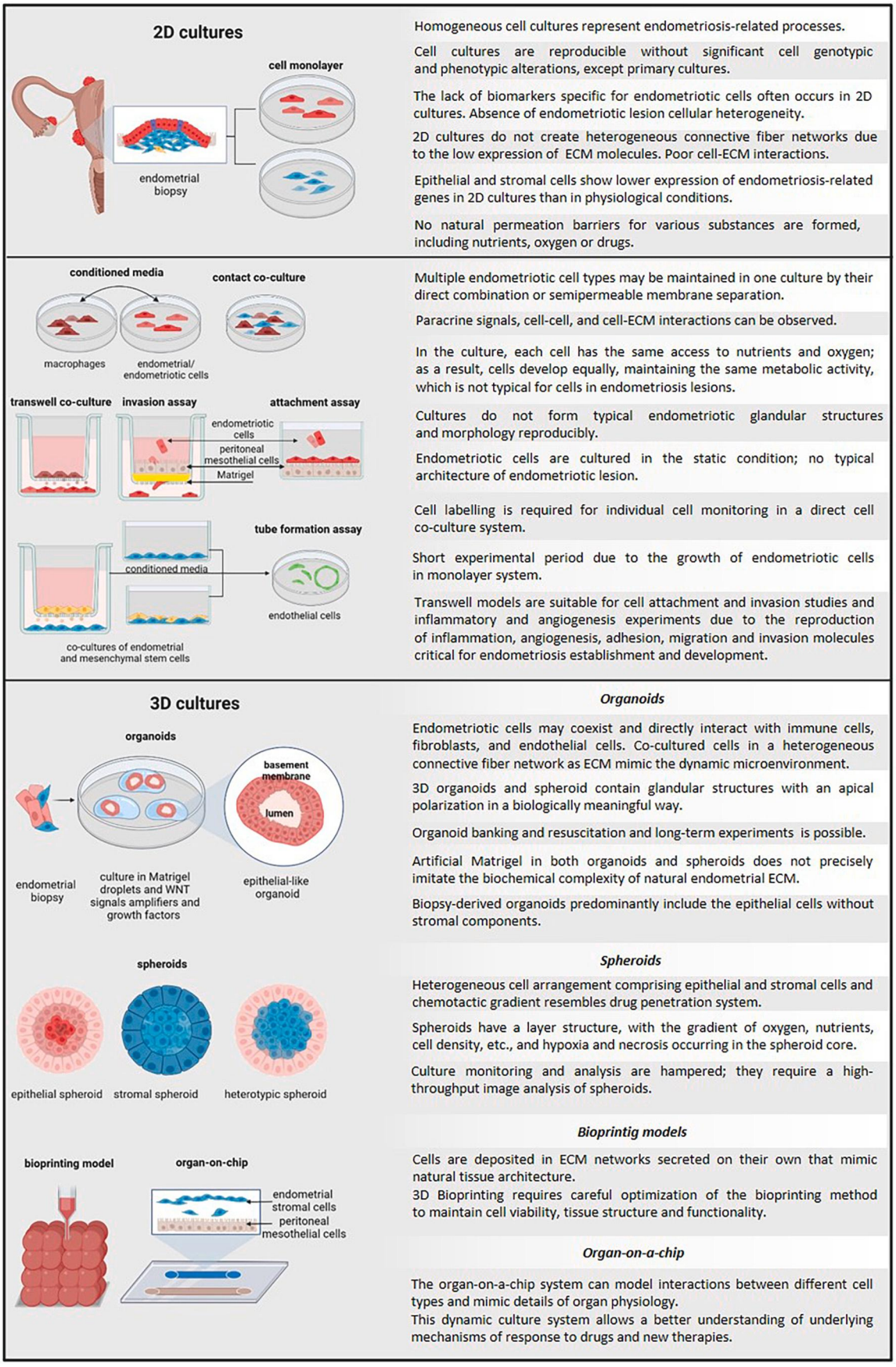


Fig. 2. A spectrum of experimental models mimicking the endometriosis microenvironment.
The figure shows the advantages and limitations associated with each model category.

effect everted by mesenchymal stromal cells. ESCendo and ESCcyst cells were cultured alone as negative controls or with Ad-MSC using inserts in a transwell system. ESCendo and ESCcyst cells were cultured in the conditioned media derived from Ad-MSC and directly in the co-culture with Ad-MSC and ESCendo or ESCcyst. The conditioned medium collected from the Ad-MSC/ESCendo and Ad-MSC/ESCcyst co-cultures on human umbilical vein endothelial cells (HUVEC) tube formation was investigated. In this study, all conditioned media from co-cultures increased tube formation of HUVEC on Matrigel, showing support activity of mesenchymal stromal cells in the tube formation in endometriosis [135].

In the studies on the angiogenic potential of endometrial

Table 3
3D models in endometriosis research.

3D model	Cell types	Research area	Main findings	Reference
Organoids	Epithelial-like cells from endometrium biopsies	Reproduction of tissue epithelium, hormonal response	Glandular-type organization and expression of specific markers. Respond to the core regulatory hormones, estrogen and progesterone. Mucus production.	[13,138]
	Epithelial-like cells from superficial endometriosis, deep peritoneal lesions and eutopic endometrium of endometriosis patient	Inflammation Invasion	Altered signaling of integrin, PI3K−AKT and Wnt pathways (organoids from endometriosis). ↑ IL-1β and IL-8 expression (ectopic organoids from I-II stages of endometriosis). ↑ SOX9, LGR6, MMP2 expression (ectopic organoids from II-IV stages of endometriosis).	[139]
	Epithelial cells from endometriosis, endometrium and ovarian endometriosis	Epigenetic mechanisms	Disturbances in methylation patterns for the HOX genes and their cofactors.	[140]
	primary endometrial epithelial and SCs from healthy patients	Proliferation	Increased proliferation under elevated androgen levels characteristic of PCOS. Identification of disease markers in neoplasms of the endometrium and urogenital tract in organoids cultured in excess of testosterone.	[141]
	Primary endometriotic tissues from biopsies	Drug screening (metabolites of ellagic acid)	Inhibition of endometriosis organoid outgrowth by natural compounds. Sensitivity of all organoids lines resulting in decreasing cell viability.	[142]
Chicken chorioallantoic membranes (cams)	Endometrial tissues from biopsies	Invasion	Increased invasion of endometrial glands and stromal cells into the CAM mesenchymal layer.	[147]
	Endometrial and endometriotic tissues from biopsies	Invasion	↑ MMPs expression in the endometriotic and endometrial fragments on CAMs.	[144,148]
	Endometrial tissue from biopsies	Angiogenesis	Reduced vascular development and impaired endometriotic-like lesions formations on CAMs under treatment with angiostatic agents.	[149]
	Human endometriosis cyst	Aromatase signaling	Decreased endometriotic lesion size under treatment with aromatase inhibitors.	[150]
Amniotic membrane	Endometrial tissue from biopsies	Adhesion	Increased attachment of endometrial fragments to damaged amnionic tissue. ↓ ß1 integrin expression; ↓ E- and P-cadherin expression in endometrial cells treated with collagenase.	[152]
Spheroids	EEC16 cells, 12Z cells	Inflammation, modulation, hormonal signaling	Higher IL-6, IL-8, CXCR1, CXCL12, CXCR4, PTGS2, CYP19A1, HGF, MMP2 expression in 3D spheroid than in 2D culture.	[118]
	Primary endometriotic SCs from ovarian endometriosis	Drug screening (dienogest)	High level of aromatase, COX-2, NF-kB p50, ERα, ERβ, PR expression.	[157]
	St-T1b cells, primary endometriotic SCs, 12Z cells, St-T1b/12Z co-culture	Invasion Migration	↓ RAC1, ↑ MMP2 and MMP14, ↑ CDH1 expression of St-T1b cells. Higher directional migration with significant collagen I remodeling of St-T1b cells in 3D compared to 2D condition. No directional migration following matrix remodeling of 12Z cells. Increased migration of 12Z cells on Matrigel under treatment with miR-145 and decreased migration under treatment with miR-200b. No directional migration following matrix remodeling in St-T1b/12Z co-culture. The increased spheroid area.	[96]
	Primary stromal cells and 12Z cells co-culture	Drug screening (metabolites of ellagic acid)	Decreased spheroid area and affected spheroid integrity after the treatment.	[142]
Ioprinting models	12Z spheroids, HEYA8 spheroids, T-HESC spheroids	Inflammation, estrogen signaling	↑ MCP-1 expression in 12Z spheroids under TNF-α stimulation Higher CYP19A1, HSD17β1, ESR1 expression in 12Z spheroids than in monolayer. Core (T-HESC) and shell (12Z) pattern of the larger construct.	[161]
Organ-on-a-chip	Primary endometrial and endometriotic SCs and human PMCs (from endometriosis and healthy patients)	Invasion	Reduced resistance of human PMCs from endometriotic lesions to endometrial and endometriotic SCs invasion compared to normal PMCs.	[167]
	Mouse ovaries and follicles tissue, human fallopian tube, uterine endometrium and ectocervical tissues	Female reproductive tract simulation	Ovarian steroid hormone production by follicles. Follicle maturation and differentiation. Ectocervix tissues maintained fully differentiated squamous architecture. Liver microtissues bound steroid hormones produced during microfluidic cultures.	[166]
	Primary endometrial SCs from healthy patients, HUVECs	Decidualization vascularization	Differentiation of stroma into functional decidual cells. Development of cytoskeleton alignment and tight junction formation by endothelial cells. Sensitivity of the model to steroids.	[168]

↑ Increase; ↓ Decrease; Abbreviations used in Table 3: HUVECs, human umbilical vein endothelial cells; HSD17β1, 17β-hydroxysteroid dehydrogenase 1; PCOS, polycystic ovary syndrome; PMCs, peritoneal mesothelial cells; SCs, stromal cells.

mesenchymal stromal cells (E-MSCs), direct and transwell co-culture models were constructed with HUVECs and mesenchymal stromal cells isolated from eutopic and ectopic tissues of patients affected by ovarian endometriosis. In the tubulogenic assay, eutopic and ectopic E-MSCs were plated with HUVEC on growth factor-reduced Matrigel dedicated to form capillary-like structures *in vitro*. In this study, E-MSCs acquired an endothelial phenotype. They participated in vessel organization together with HUVECs, which appeared probably as a consequence of cell-to-cell interactions involving adhesion-related mechanisms. These data reveal that a fraction of E-MSCs in the endometriotic lesion microenvironment takes part in the formation of endothelium not only by the capacity of recruitment of perivascular cells but also by the acquirement of the endothelial phenotype and differentiate into endothelial cells [136].

5. Endometriosis 3D models

The limitation of existing *in vitro* models of endometriosis is culturing the endometrial stromal and epithelial cells as monolayers in 2D conditions. In the *in vivo* endometriotic lesions, endometriotic cells coexist and interact with immune cells, fibroblasts, and endothelial cells and create a dynamic 3D microenvironment in the heterogeneous connective fiber network as ECM. Several studies have reported improved *in vitro* models of endometriosis at which target cells are maintained in the 3D arrangements that mimic endometriotic foci more closely than the same cells cultured in 2D conditions (Fig. 2). Table 3 presents the most relevant conclusions concerning the constructed 3D *in vitro* models of endometriosis.

5.1. Organoids

Organoids are genetically stable and self-organizing 3D systems containing progenitor/stem and differentiated cells that resemble the functionality and biology of the original tissue [137]. Turco et al. (2017) and Boretto et al. (2017) created the first highly expandable 3D organoids from the human endometrium. These organoids were built from endometrium biopsies dissociated and embedded in Matrigel under Wnt-activating conditions to form organoid structures. The culture conditions ensured the reproduction of tissue epithelium physiology and allowed long-term expansion with the maintenance of genetic and phenotypic stability [13]. Genomic analysis with RNA sequencing showed that endometrial organoids reveal more closely the epithelium of the tissue than stroma, including glandular-type organization and expression of specific markers. Moreover, the organoids respond to the core regulatory hormones, estrogen and progesterone, and reproduce functional behavior such as mucus production [138].

Boretto et al. (2019) conducted further research on the characteristics of organoids to mimic many endometrial diseases. They created endometriotic organoids from various endometriosis types (including superficial and deep peritoneal lesions) at different clinical stages together with eutopic-endometrium organoids from endometriosis patients. Culture media require active WNT and EGF to grow organoids from these lesions efficiently. Organoids embedded in the Matrigel imitating a basement membrane organize into structures with an apical polarization that contain microvilli and ciliated and non-ciliated secretory cells with secretory vacuoles facing the lumen. Organoids from endometriosis exhibit disease-associated traits with altered signaling pathways such as integrin, PI3K−AKT, and Wnt compared to healthy endometrium-derived organoids [139]. In organoids from ectopic sites of endometriosis, upregulated inflammatory cytokines IL-1β and IL-8 were observed, predominantly in the I-II stages. Whilst endometrium stem cell markers (SOX9, LGR6) and metalloproteinase (MMP2) were significantly increased in stages II and IV in line with luminal invasion, as found commonly in the endometriosis [139].

In a novel study, Esfandiari et al. (2021) investigated epigenetic mechanisms that underlie endometriosis in epithelial organoids from the endometrium and cystic ovarian endometriosis. The results confirmed the differences in the expression of four human homeobox (HOX) gene clusters and their regulator proteins or cofactors in the endometrium of women with endometriosis, concluding that endometriosis organoids maintain epigenetic changes [140].

Recently, Wiwatpanit et al. (2020) has generated a heterogenous organoid with both epithelial and stromal cells of the endometrium. The structure of the new multicellular, scaffold-free endometrial organoid system had the self-organization ability of epithelial cells on the outer surface and center stromal compartment. Authors hypothesized that the stromal population would provide the supportive layer for the epithelial lining, resembling the native tissue. Organoids were treated with physiologic levels of hormones to mimic a normal follicular phase or PCOS hormone profiles. Organoids were treated with physiologic levels of hormones to mimic a normal follicular phase or PCOS hormone profiles. They can be applied to study other endometrial processes and diseases, where epithelial and stromal cells interactions play a significant role [141].

The organoids developed by Boretto et al. (2019), constructed using tissues from endometriotic biopsies, were utilized to screen ellagic acid metabolites urolithin A and B activity. Natural antioxidants significantly inhibited organoid outgrowth and decreased cell viability. The IC_{50} dose of each compound was in concordance with that determined in the 2D monoculture of St-T1b and 12Z cells [142].

Endometriosis organoids provide unique human-derived experimental models from different clinical stages, including paired organoids from ectopic and eutopic endometrium, recapitulate endometriosis heterogeneity, summarize critical features of the primary tissue, and faithfully reproduce the disease genotype. The organoids exhibit robust expandability *in vitro*; thus, the number of primary biopsies is not limited [139]. Nevertheless, the specific origin of endometriotic organoids shows only the patient-specific and type lesion-specific features. A significant limitation of the organoids comes from the reduced nutrient exchange during *in vitro* culture; therefore, maintaining cultures in spinning bioreactors or co-culturing with endothelial cells may improve the perfusion [143]. Tissue-derived organoids predominantly include the epithelial compartment without stromal and immune components; thus, they do not fully reflect the cellular complexity of the native endometriotic lesion. This inconvenience partly reduces the potential application of this model in clinical medicine [139].

5.2. Chicken chorioallantoic membranes (CAMs) and amniotic membranes cultures

To analyze the processes of adhesion, invasion, and angiogenesis in the peritoneum, biological membranes, such as the chicken chorioallantoic membrane (CAM) of fertilized chicken eggs, can be cultured with endometrial cells in laboratory culture conditions [144]. This extraembryonic membrane serves as a gas exchange surface. Its function is supported by a dense capillary network created after fusing adjacent mesodermal layers of the chorion and the allantois at day 3 of incubation and grows until day 10 [145]. The CAM assay has been commonly used to perform angiogenesis, tumor cell invasion and metastasis studies through multi-species transplantation without risk of rejection because the early chicken embryo lacks a complete immune system [146]. Human endometrial fragments create endometriosis-like lesions with endometrial glands and stromal cells, which invade into the mesenchymal layer of the CAM [147]. The host's immature immune system allows endometrial fragments of all cycle stages to invade the CAM's epithelium [144]. After induction of endometriotic lesions on CAMs, expression of MMPs is upregulated in the endometriotic and endometrial fragments, which probably have a significant role in the development of the early endometriosis [144,148].

CAM model has been widely applied to investigate angiogenesis mechanisms involved in endometriosis establishment. The decreased vascular development and impaired endometriotic-like lesion formation

were observed after treatment of endometrium transplanted onto CAMs with angiostatic agents [149]. A significant decrease in endometriotic lesion size derived from the human endometriosis cyst biopsies was observed after treatment with aromatase inhibitors. However, inhibition of aromatization was blunted by CAM embryo gender and local estrogen production [150].

Human amniotic membranes have been used in endometrial tissue adhesion studies. Endometrial fragments did not adhere to the integral side of epithelium, but they attached to the damaged epithelium or stromal side of the amnionic tissue. Therefore, the amniotic membrane has been found to represent a suitable tool to study the interactions between endometrial tissue and ECM [151]. The pivotal role of adhesion molecules in endometrial-ECM interactions was confirmed in studies showing that collagenase-digested endometrial cells cannot adhere to stripped amniotic membranes and lost expression of β1 integrins and E- and P-cadherin [152]. Grafting endometriotic tissues into CAMs may be an appropriate assay to study angiogenesis and invasion of endometriotic implants. However, this model is not suitable for analyzing every aspect of endometriotic lesion development due to the undefined expression of adhesion molecules, limited time for experiments, and different growth factor profiles resulting from the embryological origin of the tissue. Furthermore, the detailed quantification of the vascularization process is limited to high-resolution epi-illumination microscopy [145]. The amniotic membrane consists of principal ECM components, like fibronectin, collagen type IV, laminin, and heparan sulfate proteoglycan, and can serve to study the interaction between endometrial tissue and ECM. However, the use of native material poses significant challenges due to reported low biomechanical consistency and rapid biodegradation and requires careful storage methods. The main drawbacks are the inconvenient forms and differences in the content of growth factors and regulators responsible for ECM modulation [153].

5.3. Spheroids

Different types of cells can spontaneously assemble into spheroids under culture *in vitro* conditions, at which cell–cell and cell–ECM interactions over standard cell–substrate interactions are facilitated [154]. The predominantly hemophilic cadherin–cadherin binding between neighboring cells results in forming a compacted 3D structure that closely reproduces the native organization of studied tissues [155]. Spheroids exhibit spherical geometry with a layered structure of proliferating cells in the external zone, quiescent in the middle area and necrotic cells inhabiting the spheroid centre. The formation of heterogeneous cell arrangements attributes to the limited nutrient diffusion and oxygen transport through cellular barriers in the spheroid [156].

Brueggmann et al. (2014) developed a spheroid model of endometriosis composed of ovarian endometriosis epithelial EEC16 cells and peritoneal endometriosis epithelial 12Z cells. EEC cells grown in 3D cultures histologically resembled *in vivo* endometriosis lesions more closely than 2D cultures. After 7 days of culture, EEC16 and 12Z cells formed dense, smooth, and symmetrical spheroids, and their histology was comparable to endometriotic lesions in the uterosacral ligament and peritoneum. Analysis of the expression of endometriosis relevant genes in EEC16 cell cultures indicated the upregulation of several chemokines, interleukins and their receptors under 3D compared to 2D conditions. Higher expression of genes involved in specific endometriosis-related pathways like an immune response (IL-6, IL-8, PTGS2, CXCR1, CXCL12, CXCR4), microenvironmental modulation (HGF, MMP2) and hormonal signaling (CYP19A1) was observed in the 3D cultures. Scientists, therefore, concluded that the constructed endometriosis 3D models are more representative of human endometriotic lesions than 2D cultures and may enhance the identification of novel targeted therapeutic strategies of the disease [118]. Spheroid cultures were also constructed using primary ESCs isolated from ovarian endometrioma specimens. ESCs in spheroids highly expressed proinflammatory genes, proteolytic enzymes, and growth factors, including aromatase, COX-2, NF-κB p50 nuclear localization, and steroid receptors [157].

Spheroids of endometrial stromal St-T1b cells, primary endometriotic stromal cells, epithelial endometriotic 12Z cells and cell co-culture (St-T1b and12Z cells) were constructed using the hanging drop method. The cell type influenced the size of the spheroid. It was found that epithelial 12Z cells and their co-culture with stromal St-T1b cells generate a larger spheroid area than stromal cells alone. Further, a subset of genes related to the endometriotic cell invasion was altered in 3D spheroid culture compared to 2D monoculture. St-T1b spheroids exhibited downregulation of Ras-related C3 botulinum toxin substrate 1 (RAC1) expression and expression upregulation of proteolytic genes (MMP2 and MMP14*)* and epithelial marker cadherin-1 (CDH1). In contrast, none of these markers was significantly altered in 12Z spheroids compared to 2D monoculture. The invasive behavior of constructed spheroids was assessed using two ECM-derived hydrogels: Matrigel and type I collagen. Generally, a higher spheroid area occurred on collagen than on Matrigel due to MMPs proteolytic activity. Still, no directional migration followed by matrix remodeling was observed in the co-culture or monoculture of 12Z cells. Additionally, the impact of microRNA signaling on the endometrial phenotype has been evaluated. Treatment with miR-145 reduced the migrated area of 12Z spheroids on collagen type I compared to controls, whilst microRNA miR-200b reduced 12Z cell sprouting on Matrigel [96].

Recently, the patient's primary ectopic endometriotic stromal cells and the immortalized 12Z cells were co-cultured in the 3D spheroid. Spheroids constructed with the same ratio of 12Z and primary endometriotic stroma were generated using the hanging drop method described by Stejskalova et al. (2021). The effects of urolithin A and B were verified by assessing several different morphological characteristics of spheroids. The treatment with antioxidant compounds affected the spheroid circularity and integrity and significantly decreased cell viability within the spheroids [142].

Endometrial spheroids resemble the endometrial tissue structure offering a multicellular 3D *in vitro* system that can capture *in vivo* endometriotic lesion heterogeneity. Several studies indicated that spheroids could be organized using co-culture of endometrial/endometriotic stromal and epithelial cells with specific endometriotic lesion's core and shell pattern. Moreover, the self-organization and growth of cells in the 3D spheroid conditions allow *de novo* secretion of ECM. Using the endometriotic cell lines in the construction of spheroids provides easy access and ensures the stability of biological material and the reproducibility of experiments. Facilitated genetic modifications of cells, for example, to express fluorescent proteins, enable efficient separation of co-cultured cells and the detection of their reciprocal interactions. A 3D spheroid cell culture allows following various aspects of endometriosis cell biology like hormone signaling, cell–cell adhesion, growth factors' and neovascularization signaling. Spheroids generally exhibit a drug resistance profile congenial to that found in solid tumors. They represent structurally similar conditions to the *in vivo* environment, including changes in oxygen and growth factor gradients, cell–cell and cell–ECM interactions, and dense cellular structure limiting drug diffusion. Thus they are more clinically relevant than 2D cell cultures and could meet a critical clinical need to determine novel treatments targeting endometriosis [158]. A significant disadvantage of spheroid culture as a disease model is that the cell lines represent only a limited subset of disease phenotypes [96]. Furthermore, the cellular phenotype may be influenced by the 3D structure and geometry and cell–cell and cell–ECM interactions, and comparing the effect with 2D culture is hampered [118]. Monitoring the 3D spheroid culture requires advanced microscopy imaging with the appropriate histological characterization of cells located inside and on the outside lining of the spheroid. Overall, 3D spheroids of endometriosis are superior to existing monolayer cultures and constitute a powerful *in vitro* platform for identifying and translating novel targeted therapeutic strategies for endometriosis.

5.4. Bioprinting models

The bioprinting method involves printing and patterning the cells on a substrate or tissue using an automated dispensing system [159]. Generally, bioprinting techniques can be divided into scaffold-based or scaffold-free methods. The 3D construct resembles the native ECM environment in a hydrogel, nanofibers, or films facilitating cell growth and proliferation. Scaffold-free bioprinting involves the embedding of cell or tissue aggregates in the form of a spheroid, honeycomb, cylinder into the confined space of the 3D print mould. In such a system, cells can secrete the ECM on their own and, most importantly, reflect actual tissue functionality when the 3D supported material is removed [160].

The Kenzan biofabrication method was recently used to produce 12Z cell spheroids, epithelial ovarian cancer HEYA8 cell spheroids, and heterotypic spheroids with T-HESC cells. In this bioprinting method, a metal microneedle array holds cell spheroids. The Kenzan biofabricated spheroids were fused into a larger tissue construct; after that, the Kenzan mould was removed, leaving scaffold-free construct of spheroids, which secreted ECM themselves. The performed experiments confirmed that 12Z cell spheroids might be a good recapitulation of disease-associated dysregulations by increased releasing of inflammatory factors and elevated expression of estrogen-related genes compared to monolayer. The Kenzan method of biofabrication was applied to improve both the size and complexity of endometriotic models *in vitro*. Furthermore, the mixture of T-HESC and 12Z cell heterotypic spheroids were biofabricated into larger constructs. T-HESC cells are condensed in the center and surrounded by a 12Z cell layer in the spheroids. After biofabrication into larger constructs, 12Z cells maintained the same growth pattern [161]. Scientists found biofabricated constructs containing heterotypic spheroids to represent innovative and valuable tissue-like scaffold-free 3D models of endometriosis for creating a disease microenvironment *in vitro*.

Bioprinting of *in vitro* models enables shape and size modification and mimics the microenvironment and cellular interactions. The scaffold-free model developed by Wendel et al. (2020) was a larger construct using spheroids as building blocks, generated for a short time (72 h) with maintaining characteristic core and shell patterns of common stromal and epithelial cell co-culture. Additionally, spheroids were biofabricated into multiple shapes and sizes, including cubes and hollow tubes. However, biofabrication of the endometriosis model met several limitations, i.e., destructive pressure during bioprinting; thus, optimization to minimize the cellular damage was necessary [161]. Although 3D bioprinitig made significant progress by summarizing tissue complexity, it is noteworthy that they are usually labor- and time-consuming in the optimization process. Numerous experimental aspects need to be considered to design effective models, including applying an appropriate printing technique and biomaterial, cell population, bio-microenvironment, microarchitecture, and tissue functionality.

5.5. Organ-on-a-chip

A micro-nano technology enables *in vitro* modeling of cell–cell interactions in dynamic microenvironments [162]. Organ-on-a-chip models have been applied in the studies of specific functions of the female reproductive tract such as the placenta [163], uterus [164] and ovary [165], which are composed of several multi-tissues interconnected through complex endocrine pathways and communication mechanisms. Recently developed microfluidic systems simulate the female reproductive tract and endocrine loops between modules imitating the ovary, fallopian tube, uterus, cervix, and liver, with a continuous flow between tissues. A microfluidic platform with the reproductive tract tissues and peripheral organs has been reported as a powerful tool that mimics the menstrual cycle and pregnancy-like endocrine loops with great potential for drug discovery, toxicology studies, and probably endometriosis research [166].

Chen et al. (2012) developed a microfluidic system with ESC and peritoneal mesothelial cells obtained from endometriotic patients and healthy control individuals. First, the cells were patterned in separate areas to stimulate their growth *in vitro* and then co-cultured in the same compartment to enhance their mutual interactions. Observation of direct interactions revealed that endometriotic peritoneal mesothelial cells lost contact between cells and was invaded by the surrounding endometriotic ESC. Findings obtained in this microfluidic system indicated the pivotal role of peritoneal physiology in endometriosis development [167]. In newer models, endometriotic organoids with lesion-derived stromal cells and immune and fibromuscular cells integrated into microfluidic platforms can imitate a microvascularized and innervated environment resembling endometriotic lesions [168,169].

The organ-on-a-chip concept is the close resemblance of the tissue microenvironment that replicates with predictable fluid dynamics. The advantages of microfluidics include low cost due to small sample volume, high resolution, sensitivity, and short analysis [170]. Reproductive organ-on-a-chip models may capture the tissue complexity and measure, characterize, and validate the secretome quantitatively, which may enable the identification of novel biomarkers associated with reproductive disease for clinical and pharmaceutical applications. Since the dimensions of the fluids are small, the device surface area of the device plays a significant role, and cell growth should be closely monitored. Outcomes repetitiveness requires precise parameters of the experiment and a qualified researcher.

6. Conclusions and future perspectives

Endometriosis is considered a complex multifactorial and heterogeneous disease of unknown etiology that is difficult in diagnosis, monitoring, and therapeutic approaches. Basic research into a variety of signaling pathways crucial for implantation, proliferation, invasion, and angiogenesis is important in understanding the mechanisms leading to the initiation, progression and establishment of endometriosis [171]. A substantial need to unravel molecular and cellular mechanisms underlying endometriosis is constantly pointed out as a basis for developing novel diagnostic and therapeutic methods.

In recent years, scientists developed various *in vitro* models of varying complexity that constitute valuable tools to elucidate mechanisms and pathways involved in the pathogenesis of endometriosis and endometriosis-related symptoms. Most cell culture systems are maintained in 2D conditions; however, more advanced 3D models are increasingly being applied to mimic better the unique endometriosis microenvironment and endometriotic lesions. 3D models may imitate cell-to-cell and cell-to-ECM interactions, cellular physicochemical microenvironment, and tissue-specific architecture. They offer the opportunity to study endometriotic cell communication with neighboring cells and analyze specific paracrine cross-talks between cells [172]. Various 3D geometry cell culture models have been developed in two decades. Noteworthy to mention endometriotic organoids and spheroids that differ in structures, cellular elements, properties, and applications in endometriosis research. However, the most promising 3D systems appear to be the microfabrication technologies that enable reconstitution of the organ architecture on a chip. The microfluidic system can individually compartmentalize each cell type in independent chambers and reproduce cellular functions essential to test therapeutic effects and toxicity [173]. Microfluidic platforms with integrated endometriotic organoids, endometriosis lesion-derived stromal cells and immune and fibromuscular cells have been recently developed to reconstitute a microvascularized and innervated environment resembling endometriotic lesions [168,169]. It is believed that the endometriosis models formed by the combination of patient-derived endometriotic cells, tissue engineering, and innovative microfluidic 3D models will be a powerful tool for therapeutic target identification, development of diagnostic biomarkers and screening of novel drugs. However, scientists should be deeply aware that even the best *in vitro* systems are only simplified

research models, and pharmacological *in vivo* studies are necessary to confirm *in vitro* evidence and translate promising data to clinical applications [174].

3D *in vitro* endometriosis models exhibit numerous merits and potential applications in preclinical studies. However, most of them cannot mimic the whole complexity of the endometriosis microenvironment with all cell types, mediators, pathologies, and systemic stimuli from the nervous, endocrine, or blood systems involved in endometriosis development constituting potential targets in endometriosis management. Therefore, in advanced *in vitro* research, more than one endometriosis model should be applied to reflect the endometriosis niche better and monitor significant endometriosis-related signaling pathways at cellular and molecular levels.

Advances in modeling *in vitro* technologies could potentially revolutionize the understanding of endometriosis pathophysiology and allow the identification of new targets to develop practical therapeutic approaches. The desired goal of modeling endometriosis is obtaining reproducible and robust research data, which provide evidence for alternative diagnostic and therapeutic strategies. To compare and evaluate the suitability of *in vitro* models developed for endometriosis studies, they should be validated using reference drugs with an appropriate selection system depending on the target and biological mechanism of interest. The scientific community recognizes the need for extensive endometriosis research starting from preclinical studies based on *in vitro* models - suffering-free systems that represent a serious alternative to animal testing.

Author contributions

All authors were involved in drafting and revising the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

References

[1] S.E. Bulun, B.D. Yilmaz, C. Sison, K. Miyazaki, L. Bernardi, S. Liu, A. Kohlmeier, P. Yin, M. Milad, J. Wei, Endometriosis, Endocr. Rev. 40 (2019) 1048–1079, https://doi.org/10.1210/er.2018-00242.

[2] A.S. Nair, H.B. Nair, R.S. Lucidi, A.J. Kirchner, R.S. Schenken, R.R. Tekmal, C. A. Witz, Modeling the early endometriotic lesion: mesothelium-endometrial cell co-culture increases endometrial invasion and alters mesothelial and endometrial gene transcription, Fertil. Steril. 90 (2008) 1487–1495, https://doi.org/10.1016/j.fertnstert.2007.09.047.

[3] W.-W. Wee-Stekly, C.C.Y. Kew, B.S.M. Chern, Endometriosis: A review of the diagnosis and pain management, Gynecol. Minim. Invasive Therapy 4 (2015) 106–109, https://doi.org/10.1016/j.gmit.2015.06.005.

[4] P. Viganò, J. Ottolina, L. Bartiromo, G. Bonavina, M. Schimberni, R. Villanacci, M. Candiani, Cellular components contributing to fibrosis in endometriosis: a literature review, J. Minim. Invasive Gynecol. 27 (2020) 287–295, https://doi.org/10.1016/j.jmig.2019.11.011.

[5] L. Kuznetsov, K. Dworzynski, M. Davies, C. Overton, Guideline Committee, Diagnosis and management of endometriosis: summary of NICE guidance, BMJ 358 (2017), j3935, https://doi.org/10.1136/bmj.j3935.

[6] P. Bellelis, S. Podgaec, M.S. Abrão, Environmental factors and endometriosis, Rev. Assoc. Med. Bras. 57 (2011) (1992) 448–452, https://doi.org/10.1590/s0104-42302011000400022.

[7] P.T.K. Saunders, A.W. Horne, Endometriosis: etiology, pathobiology, and therapeutic prospects, Cell 184 (2021) 2807–2824, https://doi.org/10.1016/j.cell.2021.04.041.

[8] Y. Wang, K. Nicholes, I.-M. Shih, The origin and pathogenesis of endometriosis, Annu. Rev. Pathol. 15 (2020) 71–95, https://doi.org/10.1146/annurev-pathmechdis-012419-032654.

[9] R.B. Maksym, M. Hoffmann-Młodzianowska, M. Skibińska, M. Rabijewski, A. Mackiewicz, C. Kieda, Immunology and immunotherapy of endometriosis, J. Clin. Med. 10 (2021) 5879, https://doi.org/10.3390/jcm10245879.

[10] L.K. Symons, J.E. Miller, V.R. Kay, R.M. Marks, K. Liblik, M. Koti, C. Tayade, The immunopathophysiology of endometriosis, Trends Mol. Med. 24 (2018) 748–762, https://doi.org/10.1016/j.molmed.2018.07.004.

[11] L. Deligdisch-Schor, A. Mareş Miceli, Hormonal biophysiology of the uterus, Adv. Exp. Med. Biol. 1242 (2020) 1–12, https://doi.org/10.1007/978-3-030-38474-6_1.

[12] E.W. Lopez, Z. Vue, R.R. Broaddus, R.R. Behringer, A.B. Gladden, The ERM family member Merlin is required for endometrial gland morphogenesis, Dev. Biol. 442 (2018) 301–314, https://doi.org/10.1016/j.ydbio.2018.08.006.

[13] M.Y. Turco, L. Gardner, J. Hughes, T. Cindrova-Davies, M.J. Gomez, L. Farrell, M. Hollinshead, S.G.E. Marsh, J.J. Brosens, H.O. Critchley, B.D. Simons, M. Hemberger, B.-K. Koo, A. Moffett, G.J. Burton, Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium, Nat. Cell Biol. 19 (2017) 568–577, https://doi.org/10.1038/ncb3516.

[14] D. Croxatto, P. Vacca, F. Canegallo, R. Conte, P.L. Venturini, L. Moretta, M. C. Mingari, Stromal cells from human decidua exert a strong inhibitory effect on NK cell function and dendritic cell differentiation, PLoS ONE 9 (2014), e89006, https://doi.org/10.1371/journal.pone.0089006.

[15] R. Garry, R. Hart, K.A. Karthigasu, C. Burke, A re-appraisal of the morphological changes within the endometrium during menstruation: a hysteroscopic, histological and scanning electron microscopic study, Hum. Reprod. 24 (2009) 1393–1401, https://doi.org/10.1093/humrep/dep036.

[16] S. Pawar, A.M. Hantak, I.C. Bagchi, M.K. Bagchi, Minireview: steroid-regulated paracrine mechanisms controlling implantation, Mol. Endocrinol. 28 (2014) 1408–1422, https://doi.org/10.1210/me.2014-1074.

[17] R. Kolahdouz-Mohammadi, F. Shidfar, S. Khodaverdi, T. Arablou, S. Heidari, N. Rashidi, A. Delbandi, Resveratrol treatment reduces expression of MCP-1, IL-6, IL-8 and RANTES in endometriotic stromal cells, J. Cell. Mol. Med. 25 (2021) 1116–1127, https://doi.org/10.1111/jcmm.16178.

[18] S.E. Bulun, Endometriosis (2009), https://doi.org/10.1056/NEJMra0804690.

[19] M.A.S. Fonseca, K.N. Wright, X. Lin, F. Abbasi, M. Haro, J. Sun, L. Hernandez, N. L. Orr, J. Hong, Y. Choi-Kuaea, H.M. Maluf, B.L. Balzer, I. Cass, M. Truong, Y. Wang, M.D. Pisarska, H. Dinh, A. EL-Naggar, D. Huntsman, M.S. Anglesio, M. T. Goodman, F. Medeiros, M. Siedhoff, K. Lawrenson, A cellular and molecular portrait of endometriosis subtypes, 2021, https://doi.org/10.1101/2021.05.20.445037.

[20] T. Mori, F. Ito, A. Koshiba, H. Kataoka, O. Takaoka, H. Okimura, K.N. Khan, J. Kitawaki, Local estrogen formation and its regulation in endometriosis, Reprod. Med. Biol. 18 (2019) 305–311, https://doi.org/10.1002/rmb2.12285.

[21] S.E. Bulun, Z. Lin, G. Imir, S. Amin, M. Demura, B. Yilmaz, R. Martin, H. Utsunomiya, S. Thung, B. Gurates, M. Tamura, D. Langoi, S. Deb, Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment, Pharmacol. Rev. 57 (2005) 359–383, https://doi.org/10.1124/pr.57.3.6.

[22] E. Attar, H. Tokunaga, G. Imir, M.B. Yilmaz, D. Redwine, M. Putman, B. Gurates, R. Attar, N. Yaegashi, D.B. Hales, S.E. Bulun, Prostaglandin E2 via steroidogenic factor-1 coordinately regulates transcription of steroidogenic genes necessary for estrogen synthesis in endometriosis, J. Clin. Endocrinol. Metab. 94 (2009) 623–631, https://doi.org/10.1210/jc.2008-1180.

[23] Q. Xue, Z. Lin, Y.-H. Cheng, C.-C. Huang, E. Marsh, P. Yin, M.P. Milad, E. Confino, S. Reierstad, J. Innes, S.E. Bulun, Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis, Biol. Reprod. 77 (2007) 681–687, https://doi.org/10.1095/biolreprod.107.061804.

[24] L. Aghajanova, K. Tatsumi, J.A. Horcajadas, A.M. Zamah, F.J. Esteban, C. N. Herndon, M. Conti, L.C. Giudice, Unique transcriptome, pathways, and networks in the human endometrial fibroblast response to progesterone in endometriosis, Biol. Reprod. 84 (2011) 801–815, https://doi.org/10.1095/biolreprod.110.086181.

[25] Y.-H. Cheng, A. Imir, V. Fenkci, M.B. Yilmaz, S.E. Bulun, Stromal cells of endometriosis fail to produce paracrine factors that induce epithelial 17beta-hydroxysteroid dehydrogenase type 2 gene and its transcriptional regulator Sp1: a mechanism for defective estradiol metabolism, Am. J. Obstet. Gynecol. 196 (2007), https://doi.org/10.1016/j.ajog.2006.12.014, 391.e1–7; discussion 391.e7-8.

[26] Y.-H. Cheng, P. Yin, Q. Xue, B. Yilmaz, M.I. Dawson, S.E. Bulun, Retinoic Acid (RA) Regulates 17β-Hydroxysteroid dehydrogenase type 2 expression in endometrium: interaction of RA receptors with Specificity Protein (SP) 1/SP3 for estradiol metabolism, J. Clin. Endocrinol. Metab. 93 (2008) 1915–1923, https://doi.org/10.1210/jc.2007-1536.

[27] H.S. Sun, K.-Y. Hsiao, C.-C. Hsu, M.-H. Wu, S.-J. Tsai, Transactivation of steroidogenic acute regulatory protein in human endometriotic stromalcells is mediated by the prostaglandin EP2 receptor, Endocrinology 144 (2003) 3934–3942, https://doi.org/10.1210/en.2003-0289.

[28] Y. Wang, J. Yu, X. Luo, X. Wang, M. Li, L. Wang, D. Li, Abnormal regulation of chemokine TECK and its receptor CCR9 in the endometriotic milieu is involved in pathogenesis of endometriosis by way of enhancing invasiveness of endometrial stromal cells, Cell. Mol. Immunol. 7 (2010) 51–60, https://doi.org/10.1038/cmi.2009.102.

[29] M.-Q. Li, Y. Wang, K.-K. Chang, Y.-H. Meng, L.-B. Liu, J. Mei, Y. Wang, X.-Q. Wang, L.-P. Jin, D.-J. Li, CD4+Foxp3+ regulatory T cell differentiation mediated by endometrial stromal cell-derived TECK promotes the growth and invasion of endometriotic lesions, Cell Death Dis. 5 (2014) e1436, https://doi.org/10.1038/cddis.2014.414.

[30] H. Qu, L. Li, T.-L. Wang, T. Seckin, J. Segars, I.-M. Shih, Epithelial cells in endometriosis and adenomyosis upregulate STING expression, Reprod. Sci. 27 (2020) 1276–1284, https://doi.org/10.1007/s43032-019-00127-z.

[31] H. Ishikawa, Z. Ma, G.N. Barber, STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity, Nature 461 (2009) 788–792, https://doi.org/10.1038/nature08476.

[32] Y. Liang, J. Wu, W. Wang, H. Xie, S. Yao, Pro-endometriotic niche in endometriosis, Reprod. BioMed. Online 38 (2019) 549–559, https://doi.org/10.1016/j.rbmo.2018.12.025.

[33] J.-C. Lousse, A. Van Langendonckt, S. Defrere, R.G. Ramos, S. Colette, J. Donnez, Peritoneal endometriosis is an inflammatory disease, Front. Biosci. (Elite Ed). 4 (2012) 23–40, https://doi.org/10.2741/358.

[34] J. Shao, B. Zhang, J.-J. Yu, C.-Y. Wei, W.-J. Zhou, K.-K. Chang, H.-L. Yang, L.-P. Jin, X.-Y. Zhu, M.-Q. Li, Macrophages promote the growth and invasion of endometrial stromal cells by downregulating IL-24 in endometriosis, Reproduction 152 (2016) 673–682, https://doi.org/10.1530/REP-16-0278.

[35] J. Mei, W.-J. Zhou, S.-Y. Li, M.-Q. Li, H.-X. Sun, Interleukin-22 secreted by ectopic endometrial stromal cells and natural killer cells promotes the recruitment of macrophages through promoting CCL2 secretion, Am. J. Reprod. Immunol. 82 (2019), e13166, https://doi.org/10.1111/aji.13166.

[36] X.-Q. Wang, J. Yu, X.-Z. Luo, Y.-L. Shi, Y. Wang, L. Wang, D.-J. Li, The high level of RANTES in the ectopic milieu recruits macrophages and induces their tolerance in progression of endometriosis, J. Mol. Endocrinol. 45 (2010) 291–299, https://doi.org/10.1677/JME-09-0177.

[37] Y. Wang, Y. Fu, S. Xue, A. Ai, H. Chen, Q. Lyu, Y. Kuang, The M2 polarization of macrophage induced by fractalkine in the endometriotic milieu enhances invasiveness of endometrial stromal cells, Int. J. Clin. Exp. Pathol. 7 (2013) 194–203.

[38] M.-F. Nie, Q. Xie, Y.-H. Wu, H. He, L.-J. Zou, X.-L. She, X.-Q. Wu, Serum and ectopic endometrium from women with endometriosis modulate macrophage M1/M2 polarization via the Smad2/Smad3 pathway, J. Immunol. Res. 2018 (2018) 6285813, https://doi.org/10.1155/2018/6285813.

[39] L.-C. Weng, S.-H. Hou, S.-T. Lei, H.-Y. Peng, M.-Q. Li, D. Zhao, Estrogen-regulated CD200 inhibits macrophage phagocytosis in endometriosis, J. Reprod. Immunol. 138 (2020), 103090, https://doi.org/10.1016/j.jri.2020.103090.

[40] J. Wu, H. Xie, S. Yao, Y. Liang, Macrophage and nerve interaction in endometriosis, J. Neuroinflammation 14 (2017) 53, https://doi.org/10.1186/s12974-017-0828-3.

[41] A. Casazza, D. Laoui, M. Wenes, S. Rizzolio, N. Bassani, M. Mambretti, S. Deschoemaeker, J.A. Van Ginderachter, L. Tamagnone, M. Mazzone, Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity, Cancer Cell 24 (2013) 695–709, https://doi.org/10.1016/j.ccr.2013.11.007.

[42] E. Greaves, J. Temp, A. Esnal-Zufiurre, S. Mechsner, A.W. Horne, P.T.K. Saunders, Estradiol is a critical mediator of macrophage-nerve cross talk in peritoneal endometriosis, Am. J. Pathol. 185 (2015) 2286–2297, https://doi.org/10.1016/j.ajpath.2015.04.012.

[43] D.A. Gibson, F. Collins, B. De Leo, A.W. Horne, P.T.K. Saunders, Pelvic pain correlates with peritoneal macrophage abundance not endometriosis, Reprod. Fertil. 2 (2021) 47–57, https://doi.org/10.1530/RAF-20-0072.

[44] J. Jiang, Z. Jiang, M. Xue, Serum and peritoneal fluid levels of interleukin-6 and interleukin-37 as biomarkers for endometriosis, Gynecol. Endocrinol. 35 (2019) 571–575, https://doi.org/10.1080/09513590.2018.1554034.

[45] S. Li, X. Fu, T. Wu, L. Yang, C. Hu, R. Wu, Role of interleukin-6 and its receptor in endometriosis, medical science monitor: international medical, J. Exp. Clin. Res. 23 (2017) 3801, https://doi.org/10.12659/MSM.905226.

[46] Y. Song, R.-W. Su, N.R. Joshi, T.H. Kim, B.A. Lessey, J.-W. Jeong, A.T. Fazleabas, Interleukin-6 (IL-6) activates the NOTCH1 signaling pathway through E-proteins in endometriotic lesions, J. Clin. Endocrinol. Metab. 105 (2020) 1316–1326, https://doi.org/10.1210/clinem/dgaa096.

[47] S.P. Monsanto, A.K. Edwards, J. Zhou, P. Nagarkatti, M. Nagarkatti, S.L. Young, B.A. Lessey, C. Tayade, Surgical removal of endometriotic lesions alters local and systemic proinflammatory cytokines in endometriosis patients, Fertil. Steril. 105 (2016) 968–977.e5, https://doi.org/10.1016/j.fertnstert.2015.11.047.

[48] G. Izumi, K. Koga, M. Takamura, T. Makabe, E. Satake, A. Takeuchi, A. Taguchi, Y. Urata, T. Fujii, Y. Osuga, Involvement of immune cells in the pathogenesis of endometriosis, J. Obstet. Gynaecol. Res. 44 (2018) 191–198, https://doi.org/10.1111/jog.13559.

[49] A. Tokmak, G. Yildirim, E. Öztaş, S. Akar, K. Erkenekli, P. Gülşen, N. Yilmaz, M. Uğur, Use of neutrophil-to-lymphocyte ratio combined with CA-125 to distinguish endometriomas from other benign ovarian cysts, Reprod. Sci. 23 (2016) 795–802, https://doi.org/10.1177/1933719115620494.

[50] R.M. Steinman, Decisions about dendritic cells: past, present, and future, Annu. Rev. Immunol. 30 (2012) 1–22, https://doi.org/10.1146/annurev-immunol-100311-102839.

[51] G. Izumi, K. Koga, M. Takamura, T. Makabe, M. Nagai, Y. Urata, M. Harada, T. Hirata, Y. Hirota, T. Fujii, Y. Osuga, Mannose receptor is highly expressed by peritoneal dendritic cells in endometriosis, Fertil. Steril. 107 (2017) 167–173.e2, https://doi.org/10.1016/j.fertnstert.2016.09.036.

[52] Y. Osuga, K. Koga, Y. Hirota, T. Hirata, O. Yoshino, Y. Taketani, Lymphocytes in endometriosis, Am. J. Reprod. Immunol. 65 (2011) 1–10, https://doi.org/10.1111/j.1600-0897.2010.00887.x.

[53] K. Szyllo, H. Tchorzewski, M. Banasik, E. Glowacka, P. Lewkowicz, A. Kamer-Bartosinska, The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis, Mediat. Inflamm. 12 (2003) 131–138, https://doi.org/10.1080/0962935031000134842.

[54] Y. Urata, Y. Osuga, G. Izumi, M. Takamura, K. Koga, M. Nagai, M. Harada, T. Hirata, Y. Hirota, O. Yoshino, Y. Taketani, Interleukin-1β stimulates the secretion of thymic stromal lymphopoietin (TSLP) from endometrioma stromal cells: possible involvement of TSLP in endometriosis, Hum. Reprod. 27 (2012) 3028–3035, https://doi.org/10.1093/humrep/des291.

[55] K.-K. Chang, L.-B. Liu, L.-P. Jin, B. Zhang, J. Mei, H. Li, C.-Y. Wei, W.-J. Zhou, X.-Y. Zhu, J. Shao, D.-J. Li, M.-Q. Li, IL-27 triggers IL-10 production in Th17 cells via a c-Maf/RORγt/Blimp-1 signal to promote the progression of endometriosis, Cell Death Dis. 8 (2017), e2666, https://doi.org/10.1038/cddis.2017.95.

[56] T. Hirata, Y. Osuga, K. Hamasaki, O. Yoshino, M. Ito, A. Hasegawa, Y. Takemura, Y. Hirota, E. Nose, C. Morimoto, M. Harada, K. Koga, T. Tajima, S. Saito, T. Yano, Y. Taketani, Interleukin (IL)-17A stimulates IL-8 secretion, cyclooxygensase-2 expression, and cell proliferation of endometriotic stromal cells, Endocrinology. 149 (2008) 1260–1267, https://doi.org/10.1210/en.2007-0749.

[57] P. Miossec, J.K. Kolls, Targeting IL-17 and TH17 cells in chronic inflammation, Nat. Rev. Drug Discov. 11 (2012) 763–776, https://doi.org/10.1038/nrd3794.

[58] J. Olkowska-Truchanowicz, K. Bocian, R.B. Maksym, A. Białoszewska, D. Włodarczyk, W. Baranowski, J. Ząbek, G. Korczak-Kowalska, J. Malejczyk, CD4^{+} CD25^{+} FOXP3^{+} regulatory T cells in peripheral blood and peritoneal fluid of patients with endometriosis, Hum. Reprod. 28 (2013) 119–124, https://doi.org/10.1093/humrep/des346.

[59] G.W. Randall, P.A. Gantt, R.L. Poe-Zeigler, C.A. Bergmann, M.E. Noel, W. R. Strawbridge, B. Richardson-Cox, J.R. Hereford, R.H. Reiff, Serum antiendometrial antibodies and diagnosis of endometriosis, Am. J. Reprod. Immunol. 58 (2007) 374–382, https://doi.org/10.1111/j.1600-0897.2007.00523.x.

[60] P. Shen, S. Fillatreau, Antibody-independent functions of B cells: a focus on cytokines, Nat. Rev. Immunol. 15 (2015) 441–451, https://doi.org/10.1038/nri3857.

[61] S.E. Mutsaers, C.M.-A. Prêle, S. Pengelly, S.E. Herrick, Mesothelial cells and peritoneal homeostasis, Fertil. Steril. 106 (2016) 1018–1024, https://doi.org/10.1016/j.fertnstert.2016.09.005.

[62] C.A. Witz, I.A. Montoya-Rodriguez, S. Cho, V.E. Centonze, L.F. Bonewald, R. S. Schenken, Composition of the extracellular matrix of the peritoneum, J. Soc. Gynecol. Investig. 8 (2001) 299–304, https://doi.org/10.1016/s1071-5576(01)00122-8.

[63] G.A.J. Dunselman, P.G. Groothuis, A.F.P.M. de Goeij, J.L.H. Evers, The Mesothelium, Teflon or Velcro?: Mesothelium in endometriosis pathogenesis, Hum. Reprod. 16 (2001) 605–607, https://doi.org/10.1093/humrep/16.4.605.

[64] C. Ma, R.W. Tarnuzzer, N. Chegini, Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in mesothelial cells and their regulation by transforming growth factor-beta1, Wound Repair Regen. 7 (1999) 477–485, https://doi.org/10.1046/j.1524-475x.1999.00477.x.

[65] N.Y. Sotnikova, Y.S. Antsiferova, L.V. Posiseeva, D.N. Shishkov, D.V. Posiseev, E. S. Filippova, Mechanisms regulating invasiveness and growth of endometriosis lesions in rat experimental model and in humans, Fertil. Steril. 93 (2010) 2701–2705, https://doi.org/10.1016/j.fertnstert.2009.11.024.

[66] A.Y. Demir, P.G. Groothuis, A.W. Nap, C. Punyadeera, A.F.P.M. de Goeij, J.L. H. Evers, G.A.J. Dunselman, Menstrual effluent induces epithelial–mesenchymal transitions in mesothelial cells, Hum. Reprod. 19 (2004) 21–29, https://doi.org/10.1093/humrep/deh042.

[67] M. Zeisberg, E.G. Neilson, Biomarkers for epithelial-mesenchymal transitions, J. Clin. Invest. 119 (2009) 1429–1437, https://doi.org/10.1172/JCI36183.

[68] A.S. Dhesi, S.S. Morelli, Endometriosis: a role for stem cells, Women's Health (Lond. Engl.) 11 (2015) 35–49, https://doi.org/10.2217/WHE.14.57.

[69] J. Bartley, A. Jülicher, B. Hotz, S. Mechsner, H. Hotz, Epithelial to mesenchymal transition (EMT) seems to be regulated differently in endometriosis and the endometrium, Arch. Gynecol. Obstet. 289 (2014) 871–881, https://doi.org/10.1007/s00404-013-3040-4.

[70] M. Song, S.A. Karabina, N. Kavtaradze, A.A. Murphy, S. Parthasarathy, Presence of endometrial epithelial cells in the peritoneal cavity and the mesothelial inflammatory response, Fertil. Steril. 79 (Suppl. 1) (2003) 789–794, https://doi.org/10.1016/s0015-0282(02)04836-7.

[71] V.J. Young, J.K. Brown, P.T.K. Saunders, A.W. Horne, The role of the peritoneum in the pathogenesis of endometriosis, Hum. Reprod. Update 19 (2013) 558–569, https://doi.org/10.1093/humupd/dmt024.

[72] A.W. Horne, S.F. Ahmad, R. Carter, I. Simitsidellis, E. Greaves, C. Hogg, N. M. Morton, P.T.K. Saunders, Repurposing dichloroacetate for the treatment of women with endometriosis, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 25389–25391, https://doi.org/10.1073/pnas.1916144116.

[73] P.G.M. Figueira, M.S. Abrão, G. Krikun, H. Taylor, Stem cells in endometrium and their role in the pathogenesis of endometriosis, Ann. N. Y. Acad. Sci. 1221 (2011) 10–17, https://doi.org/10.1111/j.1749-6632.2011.05969.x.

[74] I.E. Sasson, H.S. Taylor, Stem cells and the pathogenesis of endometriosis, Ann. N. Y. Acad. Sci. 1127 (2008) 106–115, https://doi.org/10.1196/annals.1434.014.

[75] C.E. Filby, L. Rombauts, G.W. Montgomery, L.C. Giudice, C.E. Gargett, Cellular origins of endometriosis: towards novel diagnostics and therapeutics, Semin. Reprod. Med. 38 (2020) 201–215, https://doi.org/10.1055/s-0040-1713429.

[76] F.L. Cousins, F.O. Dorien, C.E. Gargett, Endometrial stem/progenitor cells and their role in the pathogenesis of endometriosis, Best Pract Res Clin Obstet Gynaecol. 50 (2018) 27–38, https://doi.org/10.1016/j.bpobgyn.2018.01.011.

[77] Y. Kong, Y. Shao, C. Ren, G. Yang, Endometrial stem/progenitor cells and their roles in immunity, clinical application, and endometriosis, Stem Cell Res Ther 12 (2021) 474, https://doi.org/10.1186/s13287-021-02526-z.

[78] K. Kasai, T. Kato, Y. Kadota, O. Erdenebayar, K. Keyama, T. Kawakita, K. Yoshida, A. Kuwahara, T. Matsuzaki, M. Irahara, Intraperitoneal administration of activin A promotes development of endometriotic lesions in a mouse model of endometriosis, J. Med. Investig. 66 (2019) 123–127, https://doi.org/10.2152/jmi.66.123.

[79] S. Ponandai-Srinivasan, K.L. Andersson, M. Nister, M. Saare, H.A. Hassan, S. J. Varghese, M. Peters, A. Salumets, K. Gemzell-Danielsson, P.G.L. Lalitkumar, Aberrant expression of genes associated with stemness and cancer in endometria and endometrioma in a subset of women with endometriosis, Hum. Reprod. 33 (2018) 1924–1938, https://doi.org/10.1093/humrep/dey241.

[80] H. Du, H.S. Taylor, Contribution of bone marrow-derived stem cells to endometrium and endometriosis, Stem Cells 25 (2007) 2082–2086, https://doi.org/10.1634/stemcells.2006-0828.
[81] P. Chen, R. Mamillapalli, S. Habata, H.S. Taylor, endometriosis cell proliferation induced by bone marrow mesenchymal stem cells, Reprod. Sci. 28 (2021) 426–434, https://doi.org/10.1007/s43032-020-00294-4.
[82] A. Fassbender, N. Rahmioglu, A.F. Vitonis, P. Viganò, L.C. Giudice, T. M. D'Hooghe, L. Hummelshoj, G.D. Adamson, C.M. Becker, S.A. Missmer, K. T. Zondervan, G.D. Adamson, C. Allaire, R. Anchan, C.M. Becker, M.A. Bedaiwy, G.M. Buck Louis, C. Calhaz-Jorge, K. Chwalisz, T.M. D'Hooghe, A. Fassbender, T. Faustmann, A.T. Fazleabas, I. Flores, A. Forman, I. Fraser, L.C. Giudice, M. Gotte, P. Gregersen, S.-W. Guo, T. Harada, D. Hartwell, A.W. Horne, M.L. Hull, L. Hummelshoj, M.G. Ibrahim, L. Kiesel, M.R. Laufer, K. Machens, S. Mechsner, S. A. Missmer, G.W. Montgomery, A. Nap, M. Nyegaard, K.G. Osteen, C.A. Petta, N. Rahmioglu, S.P. Renner, J. Riedlinger, S. Roehrich, P.A. Rogers, L. Rombauts, A. Salumets, E. Saridogan, T. Seckin, P. Stratton, K.L. Sharpe-Timms, S. Tworoger, P. Vigano, K. Vincent, A.F. Vitonis, U.-H. Wienhues-Thelen, P.P. Yeung, P. Yong, K.T. Zondervan, World endometriosis research foundation endometriosis phenome and biobanking harmonisation project: IV. Tissue collection, processing, and storage in endometriosis research, Fertil. Steril. 102 (2014) 1244–1253, https://doi.org/10.1016/j.fertnstert.2014.07.1209.
[83] R.h.f. Hunter, E. Cicinelli, N. Einer-Jensen, Peritoneal fluid as an unrecognised vector between female reproductive tissues, Acta Obstet. Gynecol. Scand. 86 (2007) 260–265, https://doi.org/10.1080/00016340601155098.
[84] A.-P. Kao, K.-H. Wang, C.-C. Chang, J.-N. Lee, C.-Y. Long, H.-S. Chen, C.-F. Tsai, T.-H. Hsieh, E.-M. Tsai, Comparative study of human eutopic and ectopic endometrial mesenchymal stem cells and the development of an in vivo endometriotic invasion model, Fertil. Steril. 95 (2011) 1308–1315.e1, https://doi.org/10.1016/j.fertnstert.2010.09.064.
[85] S.P. Renner, P.L. Strissel, M.W. Beckmann, J. Lermann, S. Burghaus, J. Hackl, P. A. Fasching, R. Strick, Inhibition of adhesion, proliferation, and invasion of primary endometriosis and endometrial stromal and ovarian carcinoma cells by a nonhyaluronan adhesion barrier gel, Biomed. Res. Int. 2015 (2015), 450468, https://doi.org/10.1155/2015/450468.
[86] J. de Joliniere, P. Validire, M. Canis, M. Doussau, M. Levardon, J. Gogusev, Human endometriosis-derived permanent cell line (FbEM-1): establishment and characterization, Hum. Reprod. Update 3 (1997) 117–123, https://doi.org/10.1093/humupd/3.2.117.
[87] I.P. Ryan, E.D. Schriock, R.N. Taylor, Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro, J. Clin. Endocrinol. Metab. 78 (1994) 642–649, https://doi.org/10.1210/jcem.78.3.8126136.
[88] R. Gaetje, S. Kotzian, G. Herrmann, R. Baumann, A. Starzinski-Powitz, Nonmalignant epithelial cells, potentially invasive in human endometriosis, lack the tumor suppressor molecule E-cadherin, Am. J. Pathol. 150 (1997) 461–467.
[89] A. Zeitvogel, R. Baumann, A. Starzinski-Powitz, Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model, Am. J. Pathol. 159 (2001) 1839–1852, https://doi.org/10.1016/S0002-9440(10)63030-1.
[90] C.J. Matthews, C.P. Redfern, B.H. Hirst, E.J. Thomas, Characterization of human purified epithelial and stromal cells from endometrium and endometriosis in tissue culture, Fertil. Steril. 57 (1992) 990–997, https://doi.org/10.1016/s0015-0282(16)55014-6.
[91] A. Romano, S. Xanthoulea, E. Giacomini, B. Delvoux, E. Alleva, P. Vigano, Endometriotic cell culture contamination and authenticity: a source of bias in in vitro research? Hum. Reprod. 35 (2020) 364–376, https://doi.org/10.1093/humrep/dez266.
[92] C.E. Gargett, K.E. Schwab, R.M. Zillwood, H.P.T. Nguyen, D. Wu, Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium, Biol. Reprod. 80 (2009) 1136–1145, https://doi.org/10.1095/biolreprod.108.075226.
[93] Y. Liu, S. Liang, F. Yang, Y. Sun, L. Niu, Y. Ren, H. Wang, Y. He, J. Du, J. Yang, J. Lin, Biological characteristics of endometriotic mesenchymal stem cells isolated from ectopic lesions of patients with endometriosis, Stem Cell Res Ther 11 (2020) 346, https://doi.org/10.1186/s13287-020-01856-8.
[94] A. Samalecos, K. Reimann, S. Wittmann, H.M. Schulte, J.J. Brosens, A.-M. Bamberger, B. Gellersen, Characterization of a novel telomerase-immortalized human endometrial stromal cell line, St-T1b, Reprod. Biol. Endocrinol. 7 (2009) 76, https://doi.org/10.1186/1477-7827-7-76.
[95] J.C. Eggers, V. Martino, R. Reinbold, S.D. Schäfer, L. Kiesel, A. Starzinski-Powitz, A.N. Schüring, B. Kemper, B. Greve, M. Götte, microRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4, Reprod. BioMed. Online 32 (2016) 434–445, https://doi.org/10.1016/j.rbmo.2015.12.013.
[96] A. Stejskalová, V. Fincke, M. Nowak, Y. Schmidt, K. Borrmann, M.-K. von Wahlde, S.D. Schäfer, L. Kiesel, B. Greve, M. Götte, Collagen I triggers directional migration, invasion and matrix remodeling of stroma cells in a 3D spheroid model of endometriosis, Sci. Rep. 11 (2021) 4115, https://doi.org/10.1038/s41598-021-83645-8.
[97] C.S. Barbier, K.A. Becker, M.A. Troester, D.G. Kaufman, Expression of exogenous human telomerase in cultures of endometrial stromal cells does not alter their hormone responsiveness, Biol. Reprod. 73 (2005) 106–114, https://doi.org/10.1095/biolreprod.104.035063.
[98] P. Rai, S. Shivaji, The role of DJ-1 in the pathogenesis of endometriosis, PLoS ONE 6 (2011), e18074, https://doi.org/10.1371/journal.pone.0018074.
[99] G. Krikun, G. Mor, A. Alvero, S. Guller, F. Schatz, E. Sapi, M. Rahman, R. Caze, M. Qumsiyeh, C.J. Lockwood, A novel immortalized human endometrial stromal cell line with normal progestational response, Endocrinology 145 (2004) 2291–2296, https://doi.org/10.1210/en.2003-1606.
[100] C. Sui, E. Mecha, C.O. Omwandho, A. Starzinski-Powitz, A. Stammler, H.-R. Tinneberg, L. Konrad, PAI-1 secretion of endometrial and endometriotic cells is Smad2/3- and ERK1/2-dependent and influences cell adhesion, Am. J. Transl. Res. 8 (2016) 2394–2402.
[101] D.M. Brown, H.-C. Lee, S. Liu, C.M. Quick, L.M. Fernandes, F.A. Simmen, S.-J. Tsai, R.C.M. Simmen, Notch-1 signaling activation and progesterone receptor expression in ectopic lesions of women with endometriosis, J. Endocr. Soc. 2 (2018) 765–778, https://doi.org/10.1210/js.2018-00007.
[102] S.J. Holdsworth-Carson, M. Churchill, J.F. Donoghue, S. Mortlock, J.N. Fung, C. Sloggett, J. Chung, L. Cann, W.T. Teh, K.-R. Campbell, R. Luwor, M. Healey, G. Montgomery, J.E. Girling, P.A.W. Rogers, Elucidating the role of long intergenic non-coding RNA 339 in human endometrium and endometriosis, Mol. Hum. Reprod. 27 (2021), https://doi.org/10.1093/molehr/gaab010.
[103] M. Yuhki, T. Kajitani, T. Mizuno, Y. Aoki, T. Maruyama, Establishment of an immortalized human endometrial stromal cell line with functional responses to ovarian stimuli, Reprod. Biol. Endocrinol. 9 (2011) 104, https://doi.org/10.1186/1477-7827-9-104.
[104] M. Nishida, K. Kasahara, M. Kaneko, H. Iwasaki, K. Hayashi, Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors, Nihon Sanka Fujinka Gakkai Zasshi 37 (1985) 1103–1111.
[105] A. Jamil, J.D. Croxtall, J.O. White, The effect of anti-oestrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells (Ishikawa), J. Mol. Endocrinol. 6 (1991) 215–221, https://doi.org/10.1677/jme.0.0060215.
[106] B.A. Lessey, A.O. Ilesanmi, A.J. Castelbaum, L. Yuan, S.G. Somkuti, K. Chwalisz, P.G. Satyaswaroop, Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin, J. Steroid Biochem. Mol. Biol. 59 (1996) 31–39, https://doi.org/10.1016/s0960-0760(96)00103-3.
[107] G. Emons, B. Schröder, O. Ortmann, S. Westphalen, K.D. Schulz, A.V. Schally, High affinity binding and direct antiproliferative effects of luteinizing hormone-releasing hormone analogs in human endometrial cancer cell lines, J. Clin. Endocrinol. Metab. 77 (1993) 1458–1464, https://doi.org/10.1210/jcem.77.6.8263128.
[108] B.A. Littlefield, E. Gurpide, L. Markiewicz, B. McKinley, R.B. Hochberg, A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids, Endocrinology 127 (1990) 2757–2762, https://doi.org/10.1210/endo-127-6-2757.
[109] R.B. Owens, H.S. Smith, W.A. Nelson-Rees, E.L. Springer, Epithelial cell cultures from normal and cancerous human tissues, J. Natl. Cancer Inst. 56 (1976) 843–849, https://doi.org/10.1093/jnci/56.4.843.
[110] X. Wang, L. Parodi, S.M. Hawkins, Translational applications of linear and circular long noncoding RNAs in endometriosis, Int. J. Mol. Sci. 22 (2021) 10626, https://doi.org/10.3390/ijms221910626.
[111] S. Feng, Y. Sun, Protective role of propofol in endometriosis and its mechanism, Exp. Ther. Med. 16 (2018) 3646–3650, https://doi.org/10.3892/etm.2018.6648.
[112] L. Sha, L. Huang, X. Luo, J. Bao, L. Gao, Q. Pan, M. Guo, F. Zheng, H. Wang, Long non-coding RNA LINC00261 inhibits cell growth and migration in endometriosis, J. Obstet. Gynaecol. Res. 43 (2017) 1563–1569, https://doi.org/10.1111/jog.13427.
[113] X. Luo, W. Cheng, S. Wang, Z. Chen, J. Tan, Autophagy suppresses invasiveness of endometrial cells through reduction of fascin-1, Biomed. Res. Int. 2018 (2018), e8615435, https://doi.org/10.1155/2018/8615435.
[114] A. Starzinski-Powitz, R. Gaetje, A. Zeitvogel, S. Kotzian, H. Handrow-Metzmacher, G. Herrmann, E. Fanning, R. Baumann, Tracing cellular and molecular mechanisms involved in endometriosis, Hum. Reprod. Update 4 (1998) 724–729, https://doi.org/10.1093/humupd/4.5.724.
[115] R. Gaetje, D.W. Winnekendonk, A. Scharl, M. Kaufmann, Ovarian cancer antigen CA 125 enhances the invasiveness of the endometriotic cell line EEC 145, J. Soc. Gynecol. Investig. 6 (1999) 278–281, https://doi.org/10.1016/s1071-5576(99)00027-1.
[116] R. Gaetje, D.W. Winnekendonk, A. Ahr, M. Kaufmann, Ovarian cancer antigen CA 125 influences adhesion of human and mammalian cell lines in vitro, Clin. Exp. Obstet. Gynecol. 29 (2002) 34–36.
[117] S.K. Banu, J. Lee, A. Starzinski-Powitz, J.A. Arosh, Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells, Fertil. Steril. 90 (2008) 972–987, https://doi.org/10.1016/j.fertnstert.2007.07.1358.
[118] D. Brueggmann, C. Templeman, A. Starzinski-Powitz, N.P. Rao, S.A. Gayther, K. Lawrenson, Novel three-dimensional in vitro models of ovarian endometriosis, J. Ovarian Res. 7 (2014) 17, https://doi.org/10.1186/1757-2215-7-17.
[119] K. Lawrenson, N. Lee, H.A.M. Torres, J.M. Lee, D. Brueggmann, P.N. Rao, H. Noushmehr, S.A. Gayther, Src as a novel therapeutic target for endometriosis, Gynecol. Oncol. 135 (2014) 100–107, https://doi.org/10.1016/j.ygyno.2014.06.016.
[120] K. Lawrenson, B. Grun, E. Benjamin, I.J. Jacobs, D. Dafou, S.A. Gayther, Senescent fibroblasts promote neoplastic transformation of partially transformed ovarian epithelial cells in a three-dimensional model of early stage ovarian cancer, Neoplasia. 12 (2010) 317–325.
[121] S. Cho, L. Mutlu, Y. Zhou, H.S. Taylor, Aromatase inhibitor regulates let-7 expression and let-7f induced cell migration in endometrial cells from women

with endometriosis, Fertil. Steril. 106 (2016) 673–680, https://doi.org/10.1016/j.fertnstert.2016.05.020.
[122] F. Horné, R. Dietze, E. Berkes, F. Oehmke, H.-R. Tinneberg, I. Meinhold-Heerlein, L. Konrad, Impaired localization of claudin-11 in endometriotic epithelial cells compared to endometrial cells, Reprod. Sci. 26 (2019) 1181–1192, https://doi.org/10.1177/1933719118811643.
[123] F. Köster, L. Jin, Y. Shen, A.V. Schally, R.-Z. Cai, N.L. Block, D. Hornung, G. Marschner, A. Rody, J.B. Engel, D. Finas, Effects of an antagonistic analog of growth hormone-releasing hormone on endometriosis in a mouse model and in vitro, Reprod. Sci. 24 (2017) 1503–1511, https://doi.org/10.1177/1933719117691140.
[124] A. Ruiz, L. Ruiz, M. Colón-Caraballo, B.J. Torres-Collazo, J.B. Monteiro, M. Bayona, A.T. Fazleabas, I. Flores, Pharmacological blockage of the CXCR4-CXCL12 axis in endometriosis leads to contrasting effects in proliferation, migration, and invasion, Biol. Reprod. 98 (2018) 4–14, https://doi.org/10.1093/biolre/iox152.
[125] L. Ma, T. Andrieu, B. McKinnon, L. Duempelmann, R.-W. Peng, C. Wotzkow, C. Müller, M.D. Mueller, Epithelial-to-mesenchymal transition contributes to the downregulation of progesterone receptor expression in endometriosis lesions, J. Steroid Biochem. Mol. Biol. 212 (2021), 105943, https://doi.org/10.1016/j.jsbmb.2021.105943.
[126] J.-H. Woo, Y.-I. Yang, J.-H. Ahn, Y.S. Choi, J.-H. Choi, Interleukin 6 secretion from alternatively activated macrophages promotes the migration of endometriotic epithelial cells, Biol. Reprod. 97 (2017) 660–670, https://doi.org/10.1093/biolre/iox118.
[127] N. Sekulovski, A.E. Whorton, M. Shi, J.A. MacLean, K. Hayashi, Endometriotic inflammatory microenvironment induced by macrophages can be targeted by niclosamide†, Biol. Reprod. 100 (2019) 398–408, https://doi.org/10.1093/biolre/ioy222.
[128] J. Yu, Y. Wang, W.-H. Zhou, L. Wang, Y.-Y. He, D.-J. Li, Combination of estrogen and dioxin is involved in the pathogenesis of endometriosis by promoting chemokine secretion and invasion of endometrial stromal cells, Hum. Reprod. 23 (2008) 1614–1626, https://doi.org/10.1093/humrep/den125.
[129] H.-J. Choi, T.-W. Chung, H.-J. Choi, J.H. Han, J.-H. Choi, C.-H. Kim, K.-T. Ha, Increased α2-6 sialylation of endometrial cells contributes to the development of endometriosis, Exp. Mol. Med. 50 (2018) 164, https://doi.org/10.1038/s12276-018-0167-1.
[130] R.S. Lucidi, C.A. Witz, M. Chrisco, P.A. Binkley, S.A. Shain, R.S. Schenken, A novel in vitro model of the early endometriotic lesion demonstrates that attachment of endometrial cells to mesothelial cells is dependent on the source of endometrial cells, Fertil. Steril. 84 (2005) 16–21, https://doi.org/10.1016/j.fertnstert.2004.10.058.
[131] S.K. Kavoussi, C.A. Witz, P.A. Binkley, A.S. Nair, D.I. Lebovic, Peroxisome-proliferator activator receptor-gamma activation decreases attachment of endometrial cells to peritoneal mesothelial cells in an in vitro model of the early endometriotic lesion, Mol. Hum. Reprod. 15 (2009) 687–692, https://doi.org/10.1093/molehr/gap061.
[132] H.W. Leow, M. Koscielniak, L. Williams, P.T.K. Saunders, J. Daniels, A.M. Doust, M.-C. Jones, G.D. Ferguson, Y. Bagger, A.W. Horne, L.H.R. Whitaker, Dichloroacetate as a possible treatment for endometriosis-associated pain: a single-arm open-label exploratory clinical trial (EPiC), Pilot Feasibility Stud. 7 (2021) 67, https://doi.org/10.1186/s40814-021-00797-0.
[133] D.J. Oosterlynck, C. Meuleman, H. Sobis, M. Vandeputte, P.R. Koninckx, Angiogenic activity of peritoneal fluid from women with endometriosis, Fertil. Steril. 59 (1993) 778–782, https://doi.org/10.1016/s0015-0282(16)55859-2.
[134] M. Samimi, M.H. Pourhanifeh, A. Mehdizadehkashi, T. Eftekhar, Z. Asemi, The role of inflammation, oxidative stress, angiogenesis, and apoptosis in the pathophysiology of endometriosis: Basic science and new insights based on gene expression, J. Cell. Physiol. 234 (2019) 19384–19392, https://doi.org/10.1002/jcp.28666.
[135] F. Abomaray, S. Gidlöf, B. Bezubik, M. Engman, C. Götherström, Mesenchymal stromal cells support endometriotic stromal cells in vitro, Stem Cells Int. 2018 (2018), e7318513, https://doi.org/10.1155/2018/7318513.
[136] S. Canosa, A. Moggio, A. Brossa, G. Pittatore, G.L. Marchino, S. Leoncini, C. Benedetto, A. Revelli, B. Bussolati, Angiogenic properties of endometrial mesenchymal stromal cells in endothelial co-culture: an in vitro model of endometriosis, Mol. Hum. Reprod. 23 (2017) 187–198, https://doi.org/10.1093/molehr/gax006.
[137] A.L. Bredenoord, H. Clevers, J.A. Knoblich, Human tissues in a dish: the research and ethical implications of organoid technology, Science. 355 (2017) eaaf9414, https://doi.org/10.1126/science.aaf9414.
[138] M. Boretto, B. Cox, M. Noben, N. Hendriks, A. Fassbender, H. Roose, F. Amant, D. Timmerman, C. Tomassetti, A. Vanhie, C. Meuleman, M. Ferrante, H. Vankelecom, Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability, Development. 144 (2017) 1775–1786, https://doi.org/10.1242/dev.148478.
[139] M. Boretto, N. Maenhoudt, X. Luo, A. Hennes, B. Boeckx, B. Bui, R. Heremans, L. Perneel, H. Kobayashi, I. Van Zundert, H. Brems, B. Cox, M. Ferrante, H. Uji-i, K.P. Koh, T. D'Hooghe, A. Vanhie, I. Vergote, C. Meuleman, C. Tomassetti, D. Lambrechts, J. Vriens, D. Timmerman, H. Vankelecom, Patient-derived organoids from endometrial disease capture clinical heterogeneity and are amenable to drug screening, Nat. Cell Biol. 21 (2019) 1041–1051, https://doi.org/10.1038/s41556-019-0360-z.
[140] F. Esfandiari, R. Favaedi, H. Heidari-Khoei, F. Chitsazian, S. Yari, A. Piryaei, F. Ghafari, H. Baharvand, M. Shahhoseini, Insight into epigenetics of human endometriosis organoids: DNA methylation analysis of HOX genes and their cofactors, Fertil. Steril. 115 (2021) 125–137, https://doi.org/10.1016/j.fertnstert.2020.08.1398.
[141] T. Wiwatpanit, A.R. Murphy, Z. Lu, M. Urbanek, J.E. Burdette, T.K. Woodruff, J. J. Kim, Scaffold-free endometrial organoids respond to excess androgens associated with polycystic ovarian syndrome, J. Clin. Endocrinol. Metab. 105 (2020) 769–780, https://doi.org/10.1210/clinem/dgz100.
[142] B. Mc Cormack, N. Maenhoudt, V. Fincke, A. Stejskalova, B. Greve, L. Kiesel, G. F. Meresman, H. Vankelecom, M. Götte, R.I. Barañao, The ellagic acid metabolites urolithin A and B differentially affect growth, adhesion, motility, and invasion of endometriotic cells in vitro, Hum. Reprod. 36 (2021) 1501–1519, https://doi.org/10.1093/humrep/deab053.
[143] F. Esfandiari, N. Mansouri, M. Shahhoseini, H.H. Khoei, G. Mikaeeli, H. Vankelecom, H. Baharvand, Endometriosis organoids: prospects and challenges, Reprod. BioMed. Online (2022), https://doi.org/10.1016/j.rbmo.2022.03.016.
[144] A.W. Nap, G.A.J. Dunselman, A.F.P.M. de Goeij, J.L.H. Evers, P.G. Groothuis, Inhibiting MMP activity prevents the development of endometriosis in the chicken chorioallantoic membrane model, Hum. Reprod. 19 (2004) 2180–2187, https://doi.org/10.1093/humrep/deh408.
[145] M.W. Laschke, M.D. Menger, In vitro and in vivo approaches to study angiogenesis in the pathophysiology and therapy of endometriosis, Hum. Reprod. Update 13 (2007) 331–342, https://doi.org/10.1093/humupd/dmm006.
[146] I. Moreno-Jiménez, G. Hulsart-Billstrom, S.A. Lanham, A.A. Janeczek, N. Kontouli, J.M. Kanczler, N.D. Evans, R.O. Oreffo, The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering, Sci. Rep. 6 (2016) 32168, https://doi.org/10.1038/srep32168.
[147] J.W. Maas, P.G. Groothuis, G.A. Dunselman, A.F. de Goeij, H.A. Struijker-Boudier, J.L. Evers, Development of endometriosis-like lesions after transplantation of human endometrial fragments onto the chick embryo chorioallantoic membrane, Hum. Reprod. 16 (2001) 627–631, https://doi.org/10.1093/humrep/16.4.627.
[148] I. Juhasz-Böss, A. Hofele, C. Lattrich, S. Buchholz, O. Ortmann, E. Malik, Matrix metalloproteinase messenger RNA expression in human endometriosis grafts cultured on a chicken chorioallantoic membrane, Fertil. Steril. 94 (2010) 40–45, https://doi.org/10.1016/j.fertnstert.2009.02.052.
[149] A.W. Nap, G.A.J. Dunselman, A.W. Griffioen, K.H. Mayo, J.L.H. Evers, P. G. Groothuis, Angiostatic agents prevent the development of endometriosis-like lesions in the chicken chorioallantoic membrane, Fertil. Steril. 83 (2005) 793–795, https://doi.org/10.1016/j.fertnstert.2004.06.080.
[150] N. Pluchino, G. Poppi, L. Yart, R. Marci, J.-M. Wenger, J.-C. Tille, M. Cohen, Effect of local aromatase inhibition in endometriosis using a new chick embryo chorioallantoic membrane model, J. Cell. Mol. Med. 23 (2019) 5808–5812, https://doi.org/10.1111/jcmm.14372.
[151] P.J. van der Linden, A.F. de Goeij, G.A. Dunselman, H.W. Erkens, J.L. Evers, Endometrial cell adhesion in an in vitro model using intact amniotic membranes, Fertil. Steril. 65 (1996) 76–80.
[152] P.J. van der Linden, A.F. de Goeij, G.A. Dunselman, H.W. Erkens, J.L. Evers, Amniotic membrane as an in vitro model for endometrium-extracellular matrix interactions, Gynecol. Obstet. Investig. 45 (1998) 7–11, https://doi.org/10.1159/000009914.
[153] S. Leal-Marin, T. Kern, N. Hofmann, O. Pogozhykh, C. Framme, M. Börgel, C. Figueiredo, B. Glasmacher, O. Gryshkov, Human amniotic membrane: a review on tissue engineering, application, and storage, J. Biomed. Mater. Res. B Appl. Biomater. 109 (2021) 1198–1215, https://doi.org/10.1002/jbm.b.34782.
[154] K. Białkowska, P. Komorowski, M. Bryszewska, K. Miłowska, Spheroids as a type of three-dimensional cell cultures—examples of methods of preparation and the most important application, Int. J. Mol. Sci. 21 (2020) 6225, https://doi.org/10.3390/ijms21176225.
[155] R.-Z. Lin, R.-Z. Lin, H.-Y. Chang, Recent advances in three-dimensional multicellular spheroid culture for biomedical research, Biotechnol. J. 3 (2008) 1172–1184, https://doi.org/10.1002/biot.200700228.
[156] B. Pinto, A.C. Henriques, P.M.A. Silva, H. Bousbaa, Three-dimensional spheroids as in vitro preclinical models for cancer research, Pharmaceutics. 12 (2020) 1186, https://doi.org/10.3390/pharmaceutics12121186.
[157] K. Yamanaka, B. Xu, I. Suganuma, I. Kusuki, S. Mita, Y. Shimizu, K. Mizuguchi, J. Kitawaki, Dienogest inhibits aromatase and cyclooxygenase-2 expression and prostaglandin E^2 production in human endometriotic stromal cells in spheroid culture, Fertil. Steril. 97 (2012) 477–482, https://doi.org/10.1016/j.fertnstert.2011.11.005.
[158] M. Nowacka, K. Sterzynska, M. Andrzejewska, M. Nowicki, R. Januchowski, Drug resistance evaluation in novel 3D in vitro model, Biomed. Pharmacother. 138 (2021), 111536, https://doi.org/10.1016/j.biopha.2021.111536.
[159] S. Vanaei, M.S. Parizi, S. Vanaei, F. Salemizadehparizi, H.R. Vanaei, An overview on materials and techniques in 3D bioprinting toward biomedical application, Eng. Reg. 2 (2021) 1–18, https://doi.org/10.1016/j.engreg.2020.12.001.
[160] S. Agarwal, S. Saha, V.K. Balla, A. Pal, A. Barui, S. Bodhak, Current developments in 3D bioprinting for tissue and organ regeneration–a review, Front. Mech. Eng. 6 (2020) 90, https://doi.org/10.3389/fmech.2020.589171.
[161] J.R.H. Wendel, X. Wang, L.J. Smith, S.M. Hawkins, Three-dimensional biofabrication models of endometriosis and the endometriotic microenvironment, Biomedicines. 8 (2020) E525, https://doi.org/10.3390/biomedicines8110525.
[162] V. Mancini, V. Pensabene, Organs-on-chip models of the female reproductive system, Bioengineering (Basel) 6 (2019) 103, https://doi.org/10.3390/bioengineering6040103.
[163] J.S. Lee, R. Romero, Y.M. Han, H.C. Kim, C.J. Kim, J.-S. Hong, D. Huh, Placenta-on-a-chip: a novel platform to study the biology of the human placenta, J. Matern.

Fetal Neonatal Med. 29 (2016) 1046–1054, https://doi.org/10.3109/14767058.2015.1038518.
[164] W.-X. Li, G.-T. Liang, W. Yan, Q. Zhang, W. Wang, X.-M. Zhou, D.-Y. Liu, Artificial uterus on a microfluidic chip, Chin. J. Anal. Chem. 41 (2013) 467–472, https://doi.org/10.1016/S1872-2040(13)60639-8.
[165] J.B. Nagashima, R. El Assal, N. Songsasen, U. Demirci, Evaluation of an ovary-on-a-chip in large mammalian models: species specificity and influence of follicle isolation status, J. Tissue Eng. Regen. Med. 12 (2018) e1926–e1935, https://doi.org/10.1002/term.2623.
[166] S. Xiao, J.R. Coppeta, H.B. Rogers, B.C. Isenberg, J. Zhu, S.A. Olalekan, K. E. McKinnon, D. Dokic, A.S. Rashedi, D.J. Haisenleder, S.S. Malpani, C.A. Arnold-Murray, K. Chen, M. Jiang, L. Bai, C.T. Nguyen, J. Zhang, M.M. Laronda, T. J. Hope, K.P. Maniar, M.E. Pavone, M.J. Avram, E.C. Sefton, S. Getsios, J. E. Burdette, J.J. Kim, J.T. Borenstein, T.K. Woodruff, A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle, Nat. Commun. 8 (2017) 14584, https://doi.org/10.1038/ncomms14584.
[167] Z. Chen, Y. Dai, Z. Dong, M. Li, X. Mu, R. Zhang, Z. Wang, W. Zhang, J. Lang, J. Leng, X. Jiang, Co-cultured endometrial stromal cells and peritoneal mesothelial cells for an in vitro model of endometriosis, Integr. Biol. (Camb). 4 (2012) 1090–1095, https://doi.org/10.1039/c2ib00172a.
[168] J.S. Gnecco, V. Pensabene, D.J. Li, T. Ding, E.E. Hui, K.L. Bruner-Tran, K. G. Osteen, Compartmentalized culture of perivascular stroma and endothelial cells in a microfluidic model of the human endometrium, Ann. Biomed. Eng. 45 (2017) 1758–1769, https://doi.org/10.1007/s10439-017-1797-5.
[169] R.E. Young, D.D. Huh, Organ-on-a-chip technology for the study of the female reproductive system, Adv. Drug Deliv. Rev. 173 (2021) 461–478, https://doi.org/10.1016/j.addr.2021.03.010.
[170] A.E. Danku, E.-H. Dulf, C. Braicu, A. Jurj, I. Berindan-Neagoe, Organ-on-a-chip: a survey of technical results and problems, Front. Bioeng. Biotechnol. 10 (2022) https://www.frontiersin.org/article/10.3389/fbioe.2022.840674 (accessed May 30, 2022).
[171] R. Kapoor, C.A. Stratopoulou, M.-M. Dolmans, Pathogenesis of endometriosis: new insights into prospective therapies, Int. J. Mol. Sci. 22 (2021) 11700, https://doi.org/10.3390/ijms222111700.
[172] D. Antoni, H. Burckel, E. Josset, G. Noel, Three-dimensional cell culture: a breakthrough in vivo, Int. J. Mol. Sci. 16 (2015) 5517–5527, https://doi.org/10.3390/ijms16035517.
[173] M. Baker, Tissue models: a living system on a chip, Nature 471 (2011) 661–665, https://doi.org/10.1038/471661a.
[174] P. Groothuis, Model systems in endometriosis research: translation, translation, translation!, Front. Reprod. Health 3 (2022). https://www.frontiersin.org/article/10.3389/frph.2021.809366 (accessed February 18, 2022).

scientific reports

Check for updates

OPEN

Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways

Agata Gołąbek-Grenda[1], Mariusz Kaczmarek[2,3], Wojciech Juzwa[1] & Anna Olejnik[1]✉

The specific characteristics of endometriotic cells are their ability to evade the apoptotic machinery and abnormal response to apoptotic stimuli. Natural-originated compounds may constitute a beneficial strategy in apoptosis modulation in endometriosis. We investigated and compared the potency of natural resveratrol analogs, including piceatannol, polydatin, and pterostilbene, in targeting cell death pathways, including apoptosis-related morphologic and biochemical processes, alongside the modulation of the critical genes expression. Upon resveratrol and pterostilbene treatment, a significant reduction of endometriotic cell viability and an increased apoptotic proportion of cells were noted. The lower antiproliferative potential was found for piceatannol and polydatin. Endometrial stromal T HESC cells were significantly more resistant than endometriotic epithelial 12Z cells to the cytotoxic activity of all analyzed compounds. They differentially affected endometriotic cell viability, cell cycle, anti- and proapoptotic genes regulation, caspases expression and enzymatic activity, and DNA fragmentation. Pterostilbene-mediated endometriotic cell apoptosis modulation was confirmed to be most effective but without evident caspase 3 upregulation. Our study provides valuable insight into the apoptogenic activity of resveratrol and its natural analogs in endometriotic cells. Data obtained revealed the highest therapeutic potential of pterostilbene by effectively targeting cell death determinants in endometriosis, strengthening its optimization in further extensive research.

Abbreviations

AIF	Apoptosis-inducing factor
BAX	BCL2 associated X protein
BCL-2	B-cell lymphoma 2
BCL2L1	BCL-2-like protein 1
BID	BH3-interacting domain death agonist
BOK	BCL-2-related ovarian killer protein
CAD	Caspase-activated DNase
CASP	Caspase
CDKs	Cyclin-dependent kinases
CYB1B1	Cytochrome P450 family 1 subfamily B member 1
ER	Estrogen receptor
ERE	Estrogen response elements
E2	17β-Estradiol
FAS	Tumor necrosis factor receptor superfamily member 6
FASL	FAS ligand
MAPK/ERK	Mitogen activated protein kinase extracellular-signal-regulated kinase
PI3K/AKT	Phosphatidylinositol 3-kinase/protein kinase
Nrf-2	Nuclear erythroid 2-related factor 2
TNF-α	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand

[1]Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego St., 60-627 Poznan, Poland. [2]Department of Cancer Immunology, Chair of Medical Biotechnology, Poznan University of Medical Sciences, 61-866 Poznan, Poland. [3]Department of Cancer Diagnostics and Immunology, Greater Poland Cancer Centre, 61-866 Poznan, Poland. ✉email: anna.olejnik@up.poznan.pl

Endometriosis is an idiopathic condition characterized by ectopic growth and function of endometrial tissue outside the uterine cavity in line with a recent the International Statistical Classification of Diseases and Related Health Problems definition published latterly by WHO[1]. According to Sampson's concept, menstrual effluents, refluxed into the peritoneal cavity, contain viable endometrial cells that can implant and initiate the formation of endometriotic lesions[2]. The retrograde dissemination of the endometrial cells is a common phenomenon and appears in all women of reproductive age, regardless of whether endometriosis is present[3]. In healthy women, the misplaced endometrial cells are eliminated by the programmed cell death required to maintain cellular homeostasis effectively. The reduced endometrial tissue sensitivity to apoptosis probably causes active ectopic endometriotic lesions development[4].

Novel therapies focus on various molecular targets, and apoptosis-inducing agents may be a promising therapeutic strategy for endometriosis. Natural plant-derived compounds, including resveratrol is emerging as a promising anticancer agent due to apoptosis induction and cell cycle arrest in various cancer cell types[5,6]. This compound was reported to trigger the molecular proapoptotic cancer-related mechanisms via the mitochondrial-apoptosome-mediated intrinsic pathway and the death receptor-induced extrinsic pathway in different cancers[7]. Anticancer and apoptogenic mechanisms of resveratrol were widely investigated in many in vitro and in vivo studies, which were discussed in several review articles[8,9]. Although the abnormal tissue that grows from endometriosis is not cancerous, it shares several characteristics in terms of the metastasis-like behavior of cells, attachment to other tissues, and invasion of distant body sites[10]. The evidence of shared similar pathogenetic mechanisms of endometriosis and cancers in women may be an avenue for identifying potential targets for future disease management and treatment.

Recently, more attention has been paid to increasing resveratrol bioavailability. The metabolized fraction of resveratrol that reaches the bloodstream and is distributed into the target tissues to exert biological activity is low for its rapid and extensive phase II metabolism by glucuronidation and sulfation on the hydroxyl groups[11]. Scientific interest is emerging in some methoxylated and hydroxylated derivatives of resveratrol to enhance its therapeutic versatility. Due to additional methoxy groups, pterostilbene, a dimethylether analog of resveratrol (3,5-dimethoxy-4′-hydroxystilbene), enhances structural stability and ensures better lipophilicity, absorption, cellular uptake, and bioavailability compared with the maternal compound[12]. Moreover, several studies have focused on the activity of piceatannol (3,3′,4,5′-tetrahydroxystilbene) with higher stability than resveratrol during metabolism[13]. Also, piceatannol has been reported to reduce the high incidence of cardiovascular diseases, exert therapeutic effects in different cancers, and prevent arrhythmia, hypercholesterolemia, angiogenesis, and atherosclerosis[14,15]. Polydatin (3,4′,5-trihydroxystilbene-3-β-D-glucoside), known as piceid, is occupied by a glucopyranoside ring in the C-3 hydroxyl moiety. Glycosylated forms of resveratrol presented greater bioavailability and displayed potent anti-inflammatory and antioxidative activities[16,17].

In our opinion, extensive studies on the modulation of endometriotic and endometrial cell apoptosis are needed to assess molecular mechanisms underlying the antiproliferative effect and regulation of cell cycle arrest by the natural compound in endometriosis. To our knowledge, we present the first study evaluating the therapeutic potential of natural resveratrol derivatives in endometriosis. We aimed to examine resveratrol, pterostilbene, piceatannol, and polydatin effects on proliferation, cell cycle distribution, and symptomatic apoptosis in the endometriotic 12Z cell line. We also investigated the potential of resveratrol and its derivatives to regulate the gene expression of proapoptotic and antiapoptotic molecules.

Results

Effect of resveratrol and its derivatives on endometriotic and endometrial cell viability. The effect of resveratrol and its natural analogs on the viability of endometriotic epithelial 12Z cells and endometrial stromal T HESC cells was assessed using the MTT assay. Table 1 presents the IC_{10} and IC_{50} values (the inhibitor concentration required for 10% and 50% reduction in enzyme activity) obtained after 48-h incubation with the tested compounds. In 12Z cell culture, the IC_{10} and IC_{50} values of piceatannol and polydatin were significantly higher than those of resveratrol. The cytotoxicity of resveratrol and pterostilbene to 12Z cells was not markedly different. T HESC and 12Z cells varied in susceptibility to the treatment with stilbenes tested, as evidenced by the cytotoxic doses shown in Table 1. Pterostilbene revealed the highest cytotoxic potential for T HESC cells, significantly higher than the parent compound. However, comparing IC_{50} values, pterostilbene cytotoxicity in T HESC cell culture was almost twofold lower than in 12Z cell culture. Similarly, resveratrol evoked much weaker cytotoxic effects in T HESC cells than in 12Z cells. Polydatin at concentrations up to 400 µM was not cytotoxic

Tested compounds	Endometriotic cells—12Z		Endometrial cells—T HESC	
	IC_{10} (µM)	IC_{50} (µM)	IC_{10} (µM)	IC_{50} (µM)
Resveratrol	8.99 ± 4.29	49.32 ± 11.4	160.35 ± 7.05$^{\#\#}$	250.99 ± 1.58$^{\#\#}$
Pterostilbene	29.34 ± 4.46	73.88 ± 6.0	113.79 ± 5.11*, $^{\#\#}$	145.12 ± 7.71**, $^{\#\#}$
Piceatannol	167.50 ± 11.36**	223.76 ± 6.47**	222.68 ± 14.49**, $^{\#}$	363.72 ± 16.98**, $^{\#\#}$
Polydatin	86.76 ± 10.01**	242.85 ± 22.51**	> 400	> 400

Table 1. Effect of resveratrol, pterostilbene, piceatannol, and polydatin on endometriotic epithelial (12Z) and endometrial stromal (T HESC) cell viability, determined by the MTT reduction assay. *$p < 0.05$, **$p < 0.001$ significance of differences between cell treatment with resveratrol analogs compared to resveratrol. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ significance of differences between cytotoxic doses of stilbenes to 12Z and T HESC cells.

to the T HESC cells, while at a dose of 86.76 µM, it induced the first cytotoxic symptoms in 12Z cells. It is worth noting the Alamar Blue assay proved a similar cytotoxic effect of analyzed compounds in 12Z and T HESC cells, although it was less sensitive than the MTT assay. The increased susceptibility of endometriotic 12Z cells than endometrial T HESC cells to resveratrol and its natural analogs was consistent in both assays (Fig. 1). To summarize, resveratrol, pterostilbene, and piceatannol had a more apparent toxic effect on endometriotic cells than endometrial cells.

Effects of resveratrol and its analogs on the endometriotic cell monolayer morphology. After the treatment of resveratrol and its analogs, morphological changes in the endometriotic cell monolayer were observed under the inverted phase contrast microscope (Fig. 2). As the concentration of the analyzed compound increased, the cell culture became sparsely populated and defective. The most significant disturbances in the development of the endometriotic cell monolayer were detected following treatment with resveratrol and pterostilbene. Inhibition of adherent cell growth, losing contact with adjacent cells, and typical apoptotic features, such as rounding and membrane blebbing, were recorded dependently on the dose of stilbene tested. The arrested proliferation and echinoid spikes on the apoptotic cell surface and rampant death of endometriotic cells were observed, especially after exposition to high resveratrol and pterostilbene dosage. However, the first disturbances in 12Z cell monolayer development were noted after cell treatment with cytotoxic doses as low as 10 µM (Fig. 2).

Microscopic detection of endometriotic cell apoptosis. The apoptogenic properties of the tested compounds were monitored using fluorescence microscopy analysis of treated endometriotic 12Z cells after staining with DAPI (Fig. 3). The cells displayed common characteristics typical for apoptosis, such as chromatin condensation and cleavage, formation of pyknotic bodies of condensed chromatin, and cell disintegration into apoptotic bodies and microvesicles. The results indicated evident changes typical for apoptotic cells, such as increased nuclear condensation, formation of apoptotic bodies, and fragmentation in multiple segregated body features.

Effect of resveratrol and natural analogs on endometriotic cell death induction. After treating endometriotic 12Z cells with stilbenes, the phosphatidylserine externalization was measured by Annexin V and PI double staining (Fig. 4A). Examples of the single-cell images from sub-populations: live cells (Annexin V/

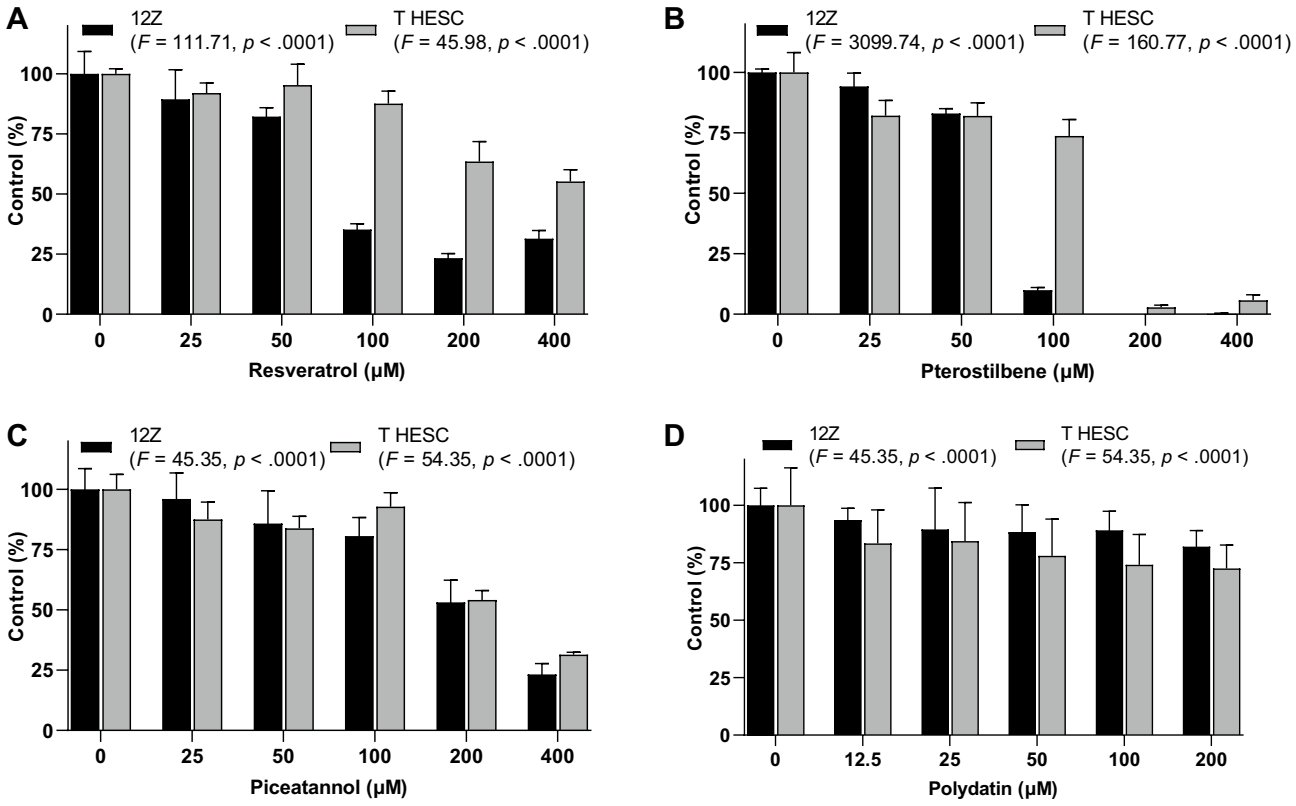


Figure 1. Effect of resveratrol (**A**), pterostilbene (**B**), piceatannol (**C**), and polydatin (**D**) on endometriotic epithelial (12Z) and endometrial stromal (T HESC) cell viability, determined by Alamar Blue reduction assay. The values represent the means (n = 3) ± SD. The significance of the main effects was determined by ANOVA (*p*, *F*).

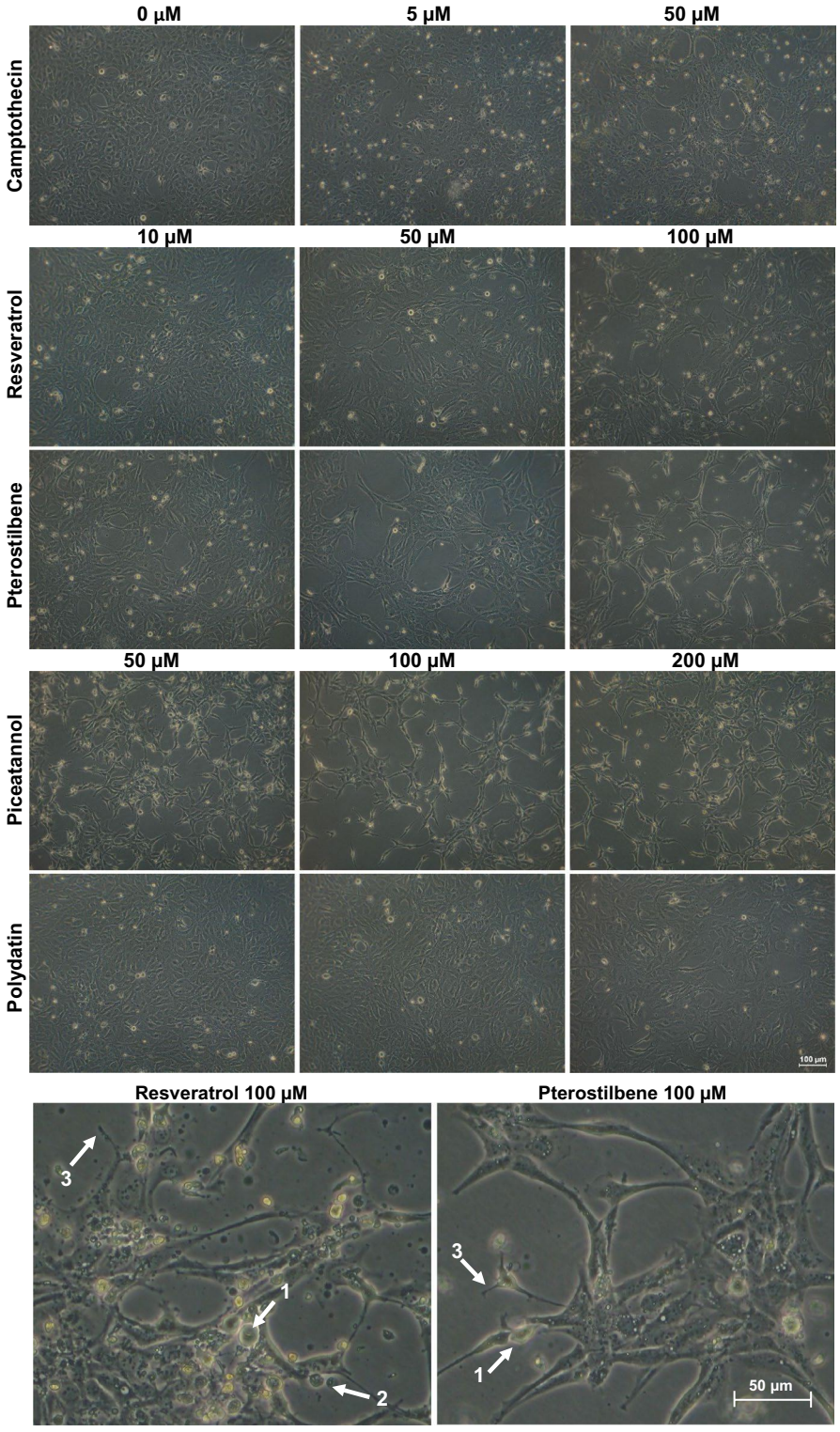


Figure 2. Endometriotic 12Z cell monolayer morphology after 48-h treatment with resveratrol and its analogs. Disturbances in 12Z cell culture developed under treatment with stilbenes were compared with 12Z cell cultures non-treated (negative control) and treated with camptothecin as a reference compound (positive control). Photos were taken at a magnification of 100×. Arrows indicate cell rounding (1), membrane blebbing (2), and echinoid spikes (3) in endometriotic cell culture under treatment with 100 µM of resveratrol and pterostilbene.

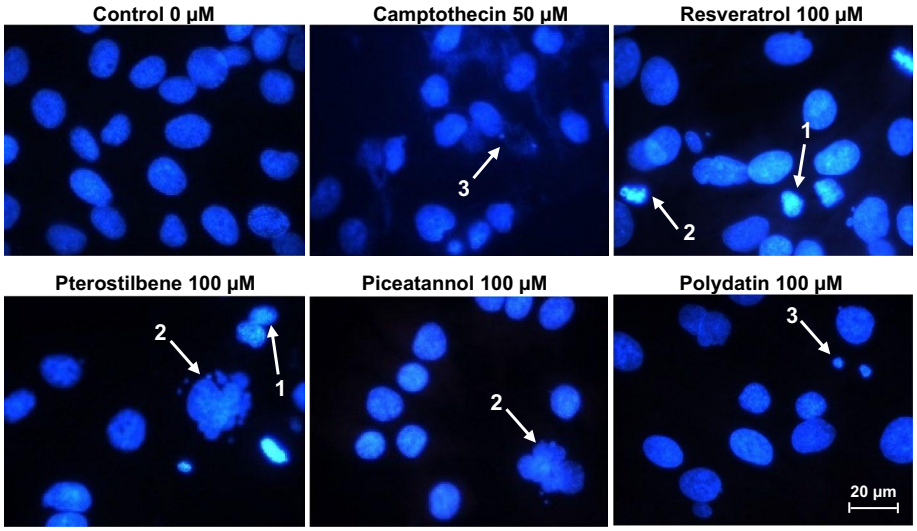


Figure 3. Morphological apoptosis of 12Z cells after treatment with resveratrol and its analogs and staining with DAPI. Control cells have intact nuclei, whereas treated cells depict pyknosis-condensed chromatin (1), nucleus fragmentation (2), and apoptotic bodies (3). White arrows indicate typical features of apoptosis. Photos were taken at a magnification of 400×.

PI-double negative), early apoptotic cells (Annexin V-positive/PI-negative), late apoptotic cells (Annexin V/PI-double positive), necrotic cells (Annexin V-negative/PI-positive) are presented in Fig. 4B.

Flow cytometry analysis of the stained cells can distinguish apoptotic cells from necrotic cells. The results in Fig. 4C show that resveratrol and its derivatives dose-dependently decreased the population of live cells and simultaneously increased the population of early and late apoptotic cells with the limited population of mechanical necrotic cells. Under treatment with resveratrol, pterostilbene, and piceatannol at concentrations of 50 µM, 100 µM, and 200 µM, a significant dose-dependent increase in the population of late apoptotic cells was observed in treated 12Z cell cultures. Pterostilbene was the most efficient apoptosis inducer based on the overall apoptotic rate parameter. In the 12Z cell culture exposed to pterostilbene at a dose of 200 µM for 48 h, approximately 94% of treated cells were classified as apoptotic cells. In contrast, 12Z cells treated with polydatin underwent apoptosis with an increased population of early apoptosis cells without induction of late apoptosis (Fig. 4C).

Effect of resveratrol and natural analogs on DNA fragmentation. Cell death via apoptosis in 12Z cells after exposure to resveratrol and its natural derivatives was determined by detecting DNA fragmentation (Fig. 5). DNA degradation was correlated in a dose-dependent manner. TUNEL assay showed increased DNA fragmentation in endometriotic 12Z cells treated for 48 h with resveratrol, pterostilbene, and piceatannol. The most significant DNA strand breaks were observed in the cells exposed to resveratrol and piceatannol at the concentration of 100 µM (42.8% and 49.6%, respectively) and 200 µM pterostilbene (62.6%). In contrast, polydatin did not induce DNA damage in treated 12Z cells (1.08%). Tunel-positive cell population in the 12Z cell culture treated with camptothecin was estimated at 32.5%, compared to 0.6% in the vehicle-treated control culture.

Effect of resveratrol and its analogs on cell cycle progression. To investigate whether resveratrol and its analogs have interfered with cell cycle progression in endometriotic 12Z cells, we analyzed the cell cycle distribution by flow cytometry. The obtained data showed that treatment with resveratrol and its analogs caused a significant increase in an apoptotic cell population with lower DNA content and simultaneously a decrease in cell population in G_1/G_0 cycle phase depending on the compound concentration, except for polydatin, which did not increase the number of apoptotic cells in a dose-dependent manner (Fig. 6). The largest subpopulation of apoptotic cells was found in the cell cultures treated with resveratrol and pterostilbene at the concentration of 100 µM (31.2 ± 8.0% and 33.1 ± 9.7%, respectively) and piceatannol at a concentration of 200 µM (45.1 ± 5. 6%).

Effect of resveratrol and its analogs on the expression of genes involved in apoptosis. The effect of resveratrol and its natural analogs on the expression of pro- or antiapoptotic regulators, was examined by real-time PCR. The analysis of mRNA expression showed that the analyzed compounds affect the expression of several proapoptotic genes and genes of two pro-survival proteins. The enhanced expression of pro-survival B-cell lymphoma 2 (*BCL-2*) and BCL2-like 1 (*BCL2L1*) genes was observed in 12Z cells treated with resveratrol (50 µM and 100 µM), pterostilbene (50 µM and 100 µM), and piceatannol (50 µM, 100 µM, and 200 µM) (Fig. 7). On the other hand, these stilbenes significantly increased the mRNA expression of the *BAX* gene, which promotes apoptosis. The highest *BAX* mRNA expression was observed in 12Z cells treated with pterostilbene at

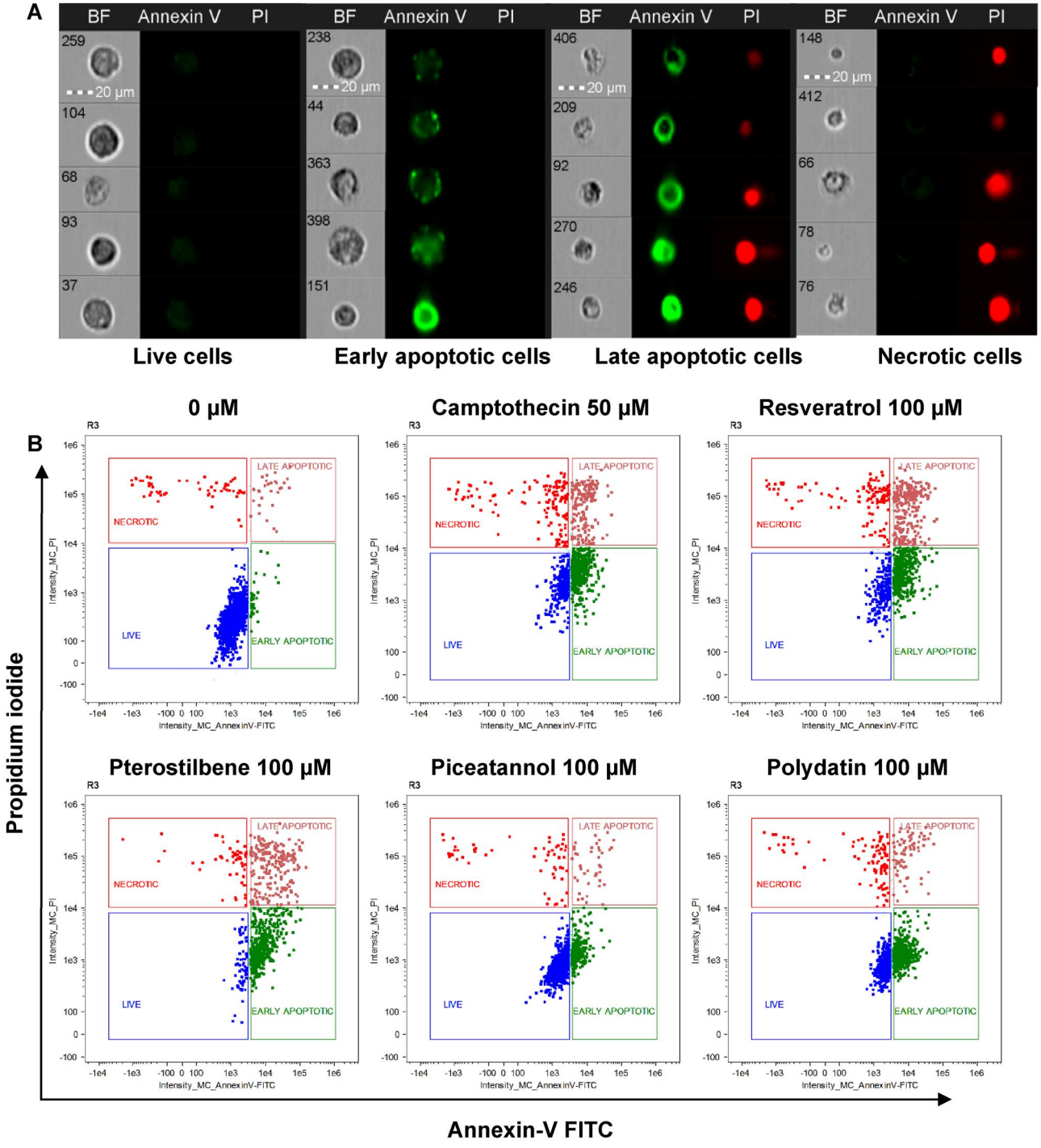


Figure 4. Flow cytometry analysis of endometriotic 12Z cells stained with PI and FITC-Annexin V. Cells were treated with resveratrol, pterostilbene, piceatannol, polydatin and their vehicle (0.5% ethanol) for 48 h. Camptothecin was used as a positive control. (**A**) Representative images of each distinct cellular subset. n = 4; scale bars = 20 µm. (**B**) Representative flow cytometry dot plots. (**C**) Summary of FITC-Annexin V and PI stained populations in 12Z cell cultures. Data show the percentage distribution of four fractions: PI-negative/FITC-Annexin V-negative, PI-positive/FITC-Annexin V-negative, PI-negative/FITC-Annexin V-positive, and PI-positive/FITC-Annexin V-positive. The results are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

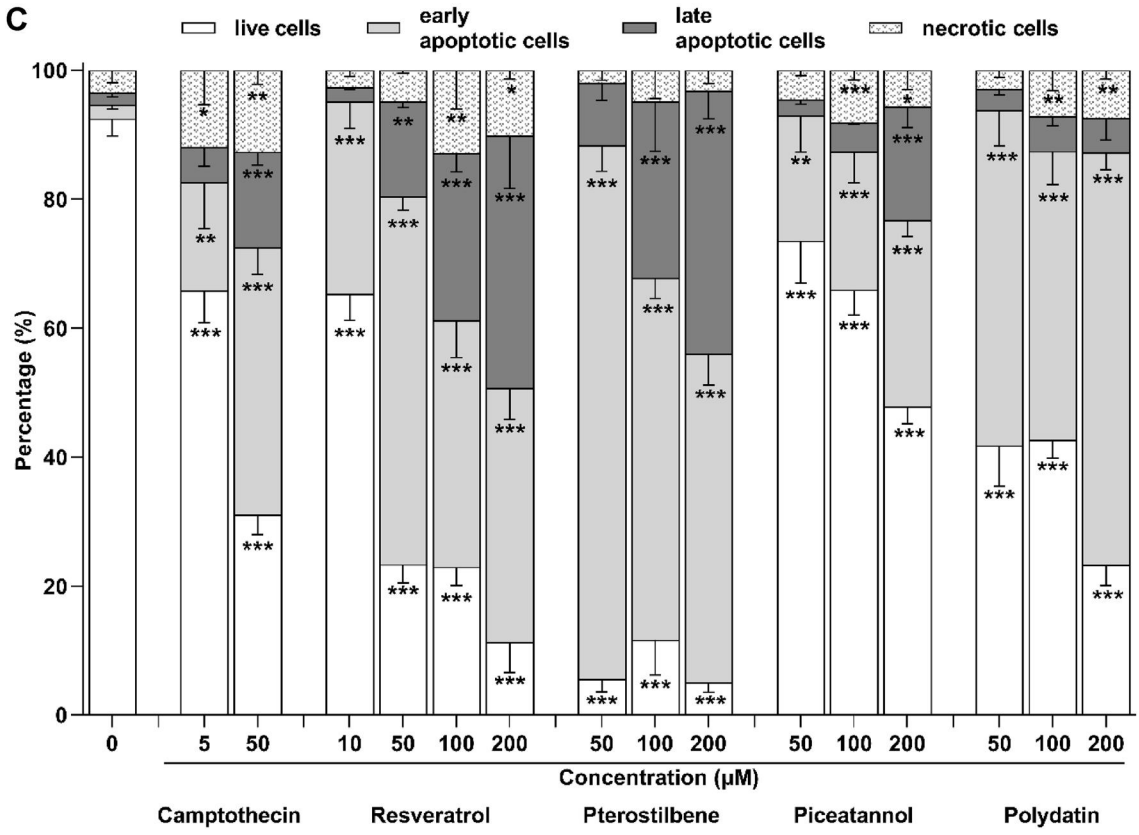


Figure 4. (continued)

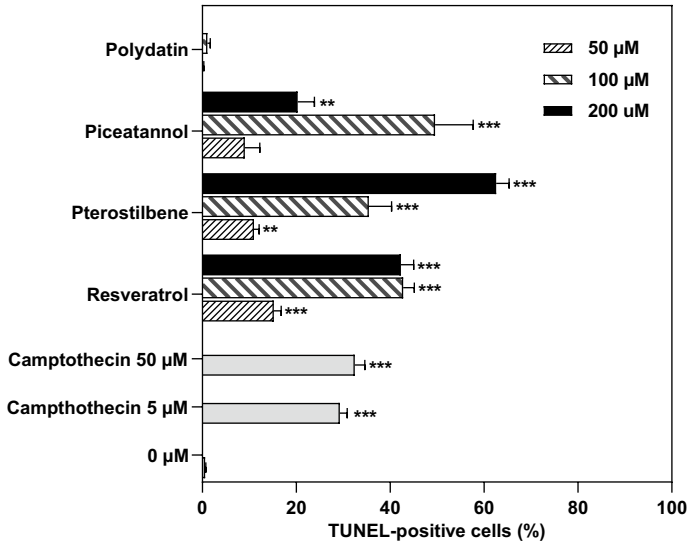


Figure 5. DNA fragmentation in 12Z cells after treatment with resveratrol and its analogs determined using TUNEL flow cytometry analysis. Results of experiments performed in triplicates are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

a concentration of 100 μM; in this treatment, the *BAX* expression level increased approximately 16-fold. *BAX* overexpression was also documented in 12Z cells exposed to resveratrol. This compound at a dose of 50 μM revealed a similar capacity to enhance *BAX* mRNA expression, increasing *BAX* transcripts by approximately sevenfold. The results indicate that the analyzed compounds can initiate cell death by varying the balance between *BAX* and *BCL-2*.

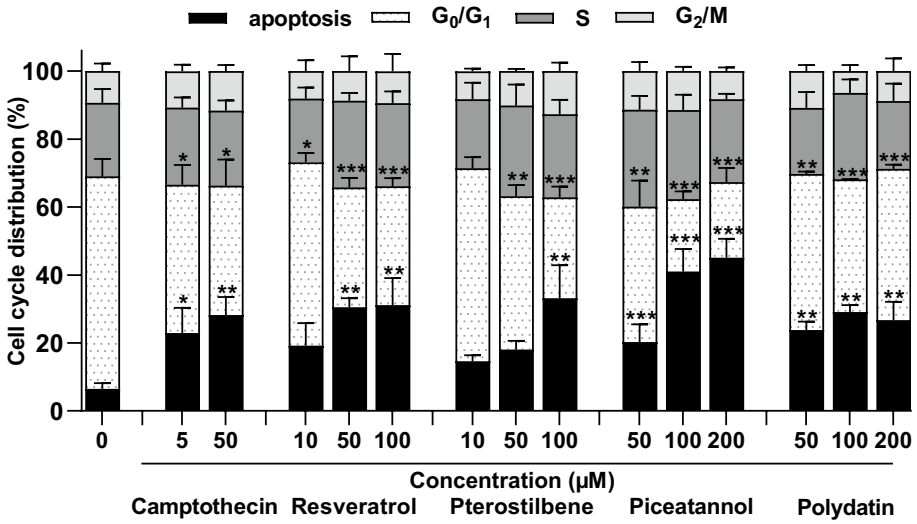


Figure 6. Effect of resveratrol and its analogs on cell cycle progression in endometriotic 12Z cells. Camptothecin was used as a reference apoptosis inducer. The values represent the means (n = 3) ± SD. Significant differences $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to vehicle control.

Moreover, after 12Z cell treatments with resveratrol and pterostilbene (50 μM and 100 μM) and piceatannol (200 μM), the mRNA expression of death receptor FAS was significantly increased. The *FAS* overexpression depended on the concentration of stilbenes, reaching a relative expression up to tenfold higher in treated endometriotic cells. The role of proteases in resveratrol-, pterostilbene-, and piceatannol-induced apoptosis was also indicated by the upregulation of well-known initiator caspases of the mitochondrial and death receptor apoptotic pathways such as caspases 8, 9 and executioner caspase 3 (Fig. 7). Polydatin did not significantly increase mRNA transcripts of the analyzed *BAX*, *FAS*, *CASP3*, *CASP8*, and *CASP9* genes.

Effect of resveratrol and its analogs on the activity of caspases-3/7. The effect of resveratrol and its analogs on the effector caspases-3/7 activity in endometriotic cells is shown in Fig. 8. Resveratrol evoked a dose-dependent caspase-3/7 activation in 12Z cells ($F = 2467.29$, $p < 0.0001$). The most potent up-regulating effect was observed under treatment with resveratrol at a concentration of 100 μM, which caused a 10.3-fold increase in caspase-3/7 activity. It was consistent with the results of *CASP3* gene expression activation. As shown in Fig. 8, pterostilbene at a concentration of 100 μM modestly activated caspases 3/7, although it inhibited their activity at 200 μM. There was a 2.8-fold activation of caspase 3/7 enzymes following treatment with piceatannol at a 200 μM dose, which complies with the *CASP3* mRNA expression level. Caspase 3/7 activity was not significantly changed in the 12Z cells by their exposure to polydatin.

Discussion

An important clue for developing new therapeutic strategies in endometriosis is that the eutopic and ectopic endometrium of endometriotic patients shows less susceptibility to apoptosis than the endometrium of non-endometriotic patients[18]. For this reason, manipulating programmed cell death processes by enhancing apoptotic stimulants of endometriotic cells could be a promising approach to treating endometriosis. This study was designed to evaluate the effects of natural analogs of resveratrol on the inhibition of endometriotic cell survival pathways and upregulation of cell death pathways, including apoptosis signaling.

The present study determined the cytotoxic effect of the analyzed stilbenes on human endometrial stromal cells (T HESC) and human endometriotic epithelial cells (12Z). The 12Z cell line retains characteristics of the active phase of endometriosis and is considered suitable for studying the cellular and molecular context of endometriosis[19]. In contrast, the T HESC line represents a distinct stromal compartment of the endometrium and resembles typical endometrial features; therefore, it has been widely applied in endometriosis studies[20,21]. However, endometriotic stromal cells were not employed in the experiments due to the lack of an established immortalized stromal cell line. Recently, the 22B cell line derived from the stromal compartment of endometriotic tissue was no longer available for current research because of the cessation of cell growth during culture[22]. A more recent study by Madanes et al. described the effect of resveratrol on human endometrial stromal St-T1b and epithelial 12Z cells[23]. Resveratrol at a concentration of 100 μM after 48-h treatment was found to reduce cell viability by 45.4% and 49.2% in 12Z and St-T1b cell cultures, respectively. The effects of resveratrol were analyzed on primary cultures of human endometrial epithelial cells obtained from eutopic endometrial biopsies of patients with endometriosis. After exposure to 50 μM and 100 μM resveratrol, cells from control women and endometriosis patients showed a significantly lower proliferative potential[24]. In other studies, resveratrol at concentrations up to 100 μM did not influence the viability of endometrial stromal cells derived from patients with endometriosis after 48-h treatment. Increasing the dose of resveratrol to 200 μM reduced endometriotic stromal cell viability to approximately 50%[25,26]. Resveratrol by itself did not induce apoptosis in the ectopic

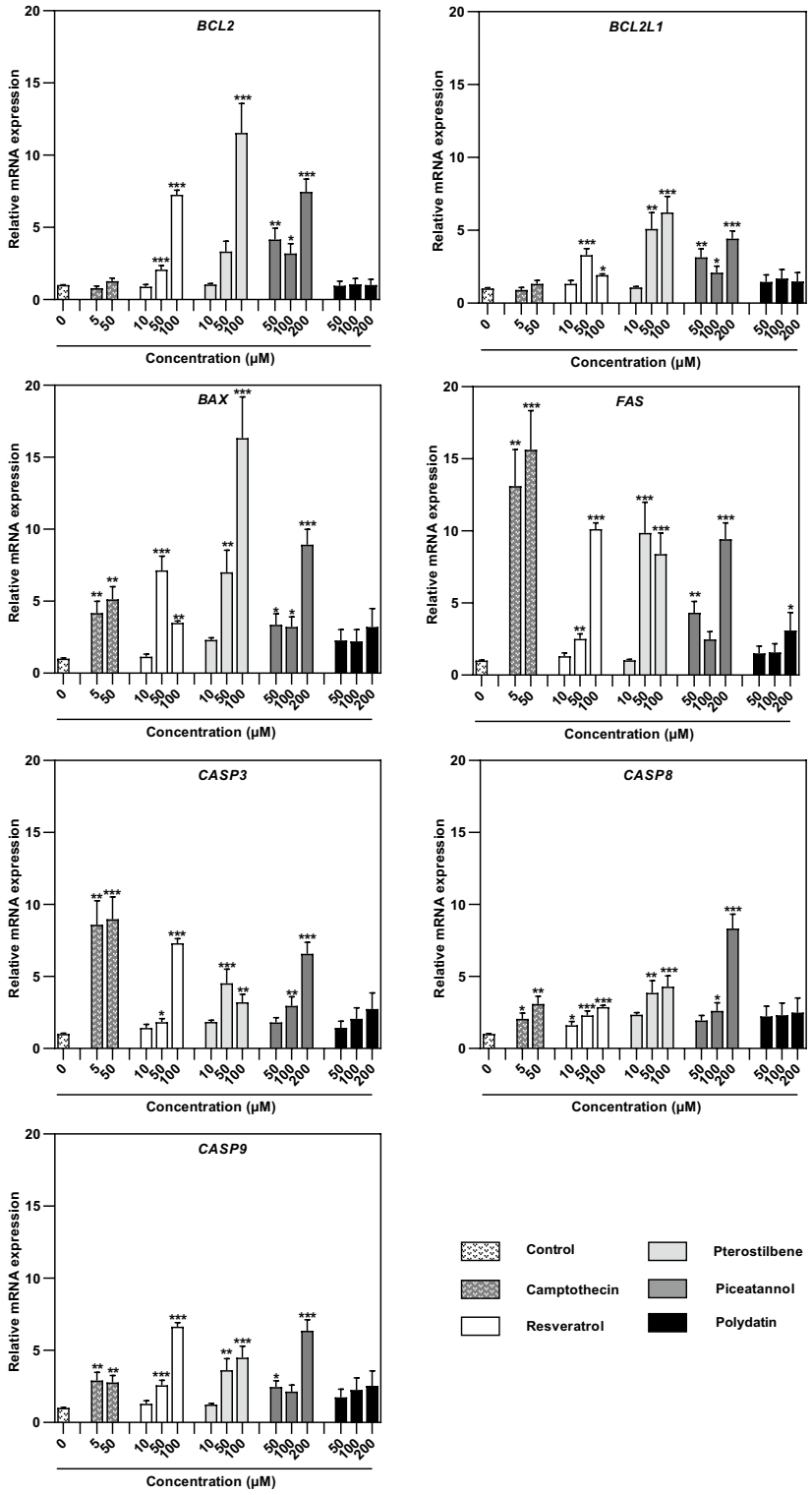


Figure 7. Effect of resveratrol and its analogs on the mRNA expression of *BCL2*, *BCL2L1*, *BAX*, *FAS*, *CASP3*, *CASP8*, and *CASP9* genes in endometriotic 12Z cells, assessed by real-time PCR. Data were normalized against *β-actin* and expressed as relative mRNA expression (-fold of control). The values represent the means (n = 3) ± SD. Significant differences $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to vehicle control.

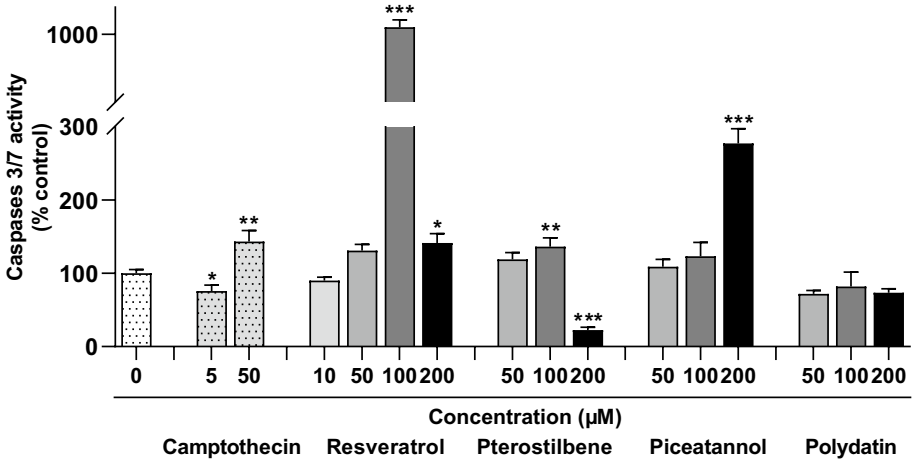


Figure 8. Effect of resveratrol and its analogs on caspases-3/7 activation. The values represent the means (n = 3) ± SD. Significant differences $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle control.

stromal cells; however, it enhanced the effect of TNF-α-related-apoptosis-inducing ligand (TRAIL) and acted by silencing survivin expression[26]. The control non-endometriotic T HESC cells derived from human endometrium were also applied in our cytotoxicity experiments since the distinction in the cellular response between endometrial and endometriotic tissue is an important issue in evaluating the activity of the therapeutic agent. Endometrial T HESC cells were more resistant to their cytotoxic activity than endometriotic 12Z cells. Based on the IC_{50} values, the cytotoxicity of resveratrol was fivefold higher for the endometriotic 12Z cells than for the endometrial T HESC cells. Resveratrol treatment seems to have a different effect on endometrial and endometriotic cells, with a harmful impact directed to endometriotic cells. Nonetheless, diverse cell population compartment representativeness must be accentuated in assessing the susceptibility of endometriotic and endometrial cells.

The results presented in this work indicated that natural resveratrol analogs are cytotoxic to cells derived from the endometrium and endometriotic lesions with different degrees of potency. Pterostilbene, a dimethyl ether analog of resveratrol with better intestinal absorption and elevated hepatic stability, has been reported to regulate pathogenic pathways associated with carcinogenesis, hematologic diseases, neurological, and age-related disorders[27]. Pterostilbene inhibited the growth of highly malignant melanoma cells at a bioavailable concentration by interfering with the molecular signaling during cell division. Moreover, pterostilbene demonstrated activity in lymphoma cell lines with a mutation of the Fas gene, resistant to apoptosis induced by resveratrol and piceatannol[28]. Compared to resveratrol, fewer hydroxyl groups result in less susceptibility to conjugation metabolism; therefore, a longer half-life is predicted for pterostilbene[29]. However, until now, pterostilbene has not been investigated as an anti-endometriotic agent. Its cytotoxic effect on endometriotic 12Z cells was lower than resveratrol. On the other hand, pterostilbene inhibited the proliferation of endometrial T HESC cells more potently than the parent compound.

Plant-derived piceatannol has been identified as an antileukemic compound and a potent inhibitor of the proliferation and migration of vascular endothelial cells. As a result, piceatannol can limit the development of tumors by inhibiting neovascularization. Polydatin, the glycosylated form of resveratrol, can increase the quantity of trans-resveratrol due to the hydrolysis by β-glucosidase secreted by bacteria in the colon. In recent years, several studies have suggested that polydatin possesses a higher capacity than resveratrol to alleviate oxidative stress and has a more potent anti-inflammatory effect[30]. It was hypothesized that the additional hydroxyl group enhances the piceatannol reactivity and makes it a more potent free radical scavenger than resveratrol[28]. However, interference in various cellular targets and proven antitumorigenic effects[14,31], piceatannol and polydatin biological activity are not yet determined in endometriosis. The potentially bioactive nature of the resveratrol analogs strongly justifies the research undertaking on their preventive and therapeutic effects on endometriosis. However, comprehensive and extensive research is needed to clarify molecular and cellular regulations of endometriotic cell growth and expansion treated with the proposed new alternative compounds.

We observed that under treatment with resveratrol and its analogs, the percentage of positive cells for Annexin V, an early sign of apoptosis, was increased significantly. Still, the most efficient increase in the population of apoptotic cells was caused by pterostilbene. Cumulative evidence has pointed out that pterostilbene shows stronger antiproliferative and apoptotic effects than resveratrol in human cervical cancer cells[32,33]. The experiments with pterostilbene ascertain it acts as a robust agent capable of regulating the p53-dependent apoptotic pathway and targeting the Nrf-2 pathway by developing endoplasmic reticulum-mediated stress in cervical cancer cells[32,34]. The superior inhibitory effects of pterostilbene compared to resveratrol were associated with the induction of p53 and p21, which contribute to cell cycle arrest at S and G_2/M phases and subsequent reduction of cyclin E1 and cyclin B1[33].

Our findings indicate that the first signs of an increase in the incidence of apoptosis were observed at 50 μM concentration of all analyzed compounds, identified by oligonucleosomal DNA fragmentation and accumulation

on the histogram as subG1 apoptosis fraction. The DNA strand breaks represent a hallmark of the later stages of apoptosis, which are mediated by several endonucleases. Caspase-activated DNase (CAD) is expressed as a heterodimer with its inhibitor ICAD, cleaved by caspase-3 leading to CAD activation, degrades the nuclear material[35].

We analyzed the activation of caspases at the molecular and cellular levels. Resveratrol upregulated caspase-3 mRNA expression and proteolytic activity by 7.3- and 10.3-fold, respectively. Caspase 3 expression in pterostilbene-induced apoptosis in endometriotic cells was much weaker, even though this compound generated the most significant number of DNA strand breaks. Data on the apoptotic activity of resveratrol and its natural analogs for human T lymphoma cells revealed that the pan-caspase-inhibitor Z-VAD-fmk could inhibit apoptosis induced by trans-resveratrol, piceatannol, and 3′-hydroxypterostilbene but not apoptosis induced by pterostilbene, suggesting that it could activate apoptosis through a caspase-independent mechanism[36]. It has now been recognized that in response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effectors such as apoptosis-inducing factor (AIF) and endonuclease G. Following release and translocation to the nucleus, AIF becomes an active executioner of the cell death by the initiation of large-scale DNA fragmentation[37]. It is essential to realize that apoptosis evolved complex death pathways, which overlap with each other. The traditional pathway involving BCL family proteins and caspases may recruit an additional response mechanism, such as caspase-independent AIF release, to facilitate the completion of apoptosis[37]. Overexpression of BAX was shown to trigger the release of AIF in 293 T and HeLa cells, suggesting that it can participate in multiple death paradigms[38]. According to this statement, in our study, pterostilbene was found to enhance *BAX* expression significantly. Different proapoptotic potentials between resveratrol and pterostilbene presented in this work are probably due to the structure–activity relationships. It has been found that the 3,5-dimethoxy motif facilitated the apoptogenic activity of the compound, even in cells expressing the multidrug-resistant phenotype or the antiapoptotic oncogene[36].

An interesting inhibitory effect of piceatannol on the endometriotic cells was observed in our study. The experimental data suggest that apoptosis induction by piceatannol could be related to the extensive fragmentation of nuclear DNA in the treated 12Z cells, despite a relatively high IC_{50} value (223.76 μM) indicating lower cytotoxic potency than resveratrol and pterostilbene. The only difference between the resveratrol and piceatannol is the presence of an additional hydroxy group in one of the piceatannol's aromatic rings. Piceatannol was found to exhibit potent tyrosine kinase inhibitory activity and inhibit a variety of tyrosine kinases involved in cell proliferation and overexpressed in different cancer cells[39]. Data obtained show that piceatannol at 100 μM led to almost 50% internucleosomal DNA fragmentation in 12Z cells with a significant increase in caspase-8, -9, and -3 mRNA expression. Similar observations concerning a minor piceatannol cytotoxic effect were made in hepatoma HepG2 cells at piceatannol doses ranging from 0 to 200 μM after 24, 48, and 72 h of treatment. Likewise, a significant percentage of apoptotic cells was detected in HepG2 cells treated with piceatannol at 100 μM and 200 μM, which also confirmed the TUNEL assay[40]. Our findings indicate that resveratrol, pterostilbene, and piceatannol at higher concentrations up-regulated the expression of the antiapoptotic *BCL2* gene, and pterostilbene evoked the most substantial effect. Protein BCL-2 resides in the outer mitochondrial wall and inhibits cytochrome *c* release[41]. Pterostilbene was previously found to increase the expression of antiapoptotic BCL-2 and activate MAPK/ERK and PI3K/AKT signal pathways in human neuronal SH-SY5Y cells in neuroprotective experiments. Pterostilbene, which exhibits estrogenic activity, mediated neuroprotective potential via mainly ER-α receptor, which activated the BCL-2 expression presumably through genomic estrogen signaling[42]. The distribution of hydroxyl groups in aromatic rings in the stilbenes makes them similar in chemical structure to endogenous and synthetic estrogens such as 17β-estradiol (E2) or diethylstilbestrol[43]. One early identified biological target of resveratrol was the estrogen receptor (ER), and the compound was classified as a phytoestrogen. After dimerization of ER receptors and translocation to the nucleus, they bind to estrogen response elements (ERE) within target genes. The transcription of genes by ER can also be mediated through the non-classical genomic pathway by its direct binding to DNA with different transcription factors (e.g., AP-1, SP-1, or NF-κB) without binding to ERE. Genes involved in proliferation and survival or apoptosis, such as cyclin D1 and *BCL2*, are regulated by ER binding to the SP-1[44]. Many estrogenic compounds display varying degrees of agonism/antagonism depending on the cell type and target gene, and they are referred to as selective estrogen receptor modulators (SERMs). Resveratrol was thought to affect breast cancer cells through ER-dependent signaling pathways, some studies demonstrated that resveratrol analogs decreased triple-negative breast cancer cell viability in a dose-dependent manner, indicating the existence of an alternative, ER-independent mechanism[45]. The well-characterized endometriotic epithelial 12Z cells used in our experiments are ER-positive (ER-α, ER-β)[46]. Endometrial stromal T HESC cells were also found to express ER and were applied in analyzing the regulation of steroid receptors upon treatment with their antagonists and induction of decidualization[47,48]. Affecting mediators down-regulating ER signaling constitutes an essential direction for therapeutic purposes because estradiol at strikingly high quantities is the master regulator of endometriotic tissue pathology[49].

Polydatin represents a natural precursor of resveratrol, in which the hydroxyl is replaced and occupied by a glucopyranoside ring, enabling much better bioavailability than resveratrol, benefiting from the way entering cells via glucose carriers[50]. A number of studies have suggested that polydatin therapeutic effect originates from its anti-inflammatory[51], antioxidant[52], and anticancer[53] activities. It remains unclear whether polydatin, like resveratrol, has antioxidation and antitumor properties or constitutes a clinically advantageous approach. In our studies, the effect of polydatin on endometriotic cell apoptosis was relatively minor. All analyzed compounds showed higher inhibition capacity in cell viability than polydatin did. Even though polydatin induced the early apoptosis stage under all measured concentrations (50 μM, 100 μM, 200 μM), cells did not go into the late apoptotic phase as under treatment with other stilbenes. Dose-independently, polydatin unaffected neither caspase activation nor expression of proapoptotic proteins. The reason that polydatin shows lower biological activity than resveratrol at the same concentration was investigated in the different tumor cells. The results

indicated that polydatin was harder uptaken by the cells than resveratrol, and probably specific transporters in cells or lipid rafts endocytosis may determine its bioactivity[54].

Active compounds from natural products hold, in general, multiple molecular targets and can affect different signaling pathways involved in proliferation, apoptosis, invasion, migration, inflammation, reactive oxidative stress, and angiogenesis in the complex disease such as endometriosis[6]. New developed therapeutics for endometriosis will be based on the prevention of off-target toxicities like side effects on the eutopic endometrium or function of the reproductive tract. Therefore, the long-term safety of natural products, target pathway selectivity, and prevent recurrence will be assessed in future studies. Moreover, challenges should be addressed to improve bioavailability by implementing prodrugs, solid dispersions, and lipid-based formulation technologies[55]. Recently, some locally administered platforms were proposed to deliver drugs for endometriosis treatment, utilizing delivery technologies already explored in cancers. A polymeric drug delivery system to control the prolonged release of curcumin from nanofibers in the peritoneum and pelvic cavity was developed in a mouse model of endometriosis. Besides, in the rat endometriosis model, polymer/nucleic acid conjugates reduced the ectopic endometrial lesions in ectopic locations. The small-molecule drugs or siRNA can be targeted to the ectopic endometriotic tissue by the micelle systems and polymeric or peptide conjugates. Different nanoparticle structures (metal-oxide nanoparticles, silver nanoparticles) can be designed to recognize specific receptors in the lesions' microenvironment and target drug delivery[56]. Endometriosis drug delivery technologies are a relatively new approach but are essential for clinically translatable therapeutics for women's health applications.

As discussed above, the study results indicate that resveratrol and its natural analogs may offer substantial preventive and therapeutic potential for treating endometriosis by specific induction of apoptosis via death-signaling pathways. It is worth emphasizing that non-endometriotic T HESC cells were significantly less affected by resveratrol and its key phytoestrogenic analogs. The significant effects of pterostilbene on the proapoptotic events contributed to considerable inhibition of endometriotic cell proliferation, which was more preferential than resveratrol. The anti-endometriotic potency of pterostilbene has been unveiled and described for the first time in the presented work. Given the previous reports and our insights, we assumed more attention should be paid to the estrogenic activity caused by natural phytoestrogens in evaluating their bioactivity. We conclude that the properties of resveratrol and pterostilbene, involving the selective suppression of endometriotic cell proliferation and induction of apoptosis, support their preventive and therapeutic activity against endometriosis.

Materials and methods

Unless specified otherwise, all chemicals were purchased from Merck KGaA (Darmstadt, Germany).

Endometriotic and endometrial cell cultures and treatment. The immortalized human endometriotic epithelial cells (12Z), derived from peritoneal endometriosis lesions, were obtained from Applied Biological Materials Inc. (Richmond, Canada). The 12Z cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), containing 10% fetal bovine serum (FBS) and 50 mg/L gentamycin. American Type Culture Collection (ATCC, Manassas, VA, USA) was the source for human endometrial stromal cells immortalized by telomerase (T HESC, CRL-4003). The cells were maintained routinely in phenol red-free DMEM/F12 supplemented with 10% charcoal/dextran treated FBS, 1% ITS + 1, and 50 mg/L gentamycin, at 37 °C and 5% CO_2. Both cell lines were negative for mycoplasma contamination; mycoplasma-free cells were confirmed repeatedly during the study.

Cells were seeded at a density of 0.15×10^5 cells/cm^2 in 6-well plates. The 24-h cell cultures were treated with resveratrol and its derivatives for 48 h under standard culture conditions. Camptothecin was applied as a reference apoptosis inducer; its final concentrations were set to 5 μM and 50 μM.

Cell viability assay. Cell viability and metabolic activity were determined using the MTT colorimetric assay, according to the protocol described in previous studies[57]. Based on the obtained data, the dose–response curves were plotted, and inhibitory concentrations (IC) were calculated. In addition, cell proliferation was measured using the alamarBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Fluorescence was measured using a Tecan M200 Infinite microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Microscopic detection of cell apoptosis. After treatment, cell monolayer morphology was assessed under an inverted phase contrast microscope Axiovert 40C (Zeiss, Germany). Moreover, 12Z cells cultured on Millicell EZ SLIDE glass (Millipore, Merck KGaA) were subjected to the standard treatment protocol. Following exposure, cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100, and stained with 1 μg/ml DAPI. The cell nuclear morphology was assessed by fluorescence microscopic inspection (Axiovert 200, Zeiss, Germany).

Annexin-V/PI staining assay. Apoptosis was determined by Annexin V and propidium iodide (PI) double staining with the FITC Annexin V Apoptosis Detection kit (BD Pharmingen, Heidelberg, Germany) by flow cytometry according to the manufacturer's instructions. Briefly, cells were harvested and washed with PBS; then, they were suspended in the binding buffer to obtain 1×10^6 cells/ml concentration and stained with FITC Annexin V and PI. The cells were analyzed with an Amnis™ FlowSight™ flow cytometer (Luminex Corporation, TX, USA). Cell populations were categorized into four subpopulations: live cells (Annexin V/PI-double negative), early apoptotic cells (Annexin V-positive/PI-negative), late apoptotic cells (Annexin V/PI-double positive), necrotic cells (Annexin V-negative/PI-positive).

Gene	Accession	No. Sequence (5′–3′)	Amplicon (bp)
Hs *BCL2*	NM-000633.3	F: ATC GCC CTG TGG ATG ACT GAG T R: GCC AGG AGA AAT CAA ACA GAG GC	127
Hs *BCL2L1*	NM-138578.3	F: GCC ACT TAC CTG AAT GAC CAC C R: AAC CAG CGG TTG AAG CGT TCC T	131
Hs *BAX*	NM-001291428.2	F: TCA GGA TGC GTC CAC CAA GAA G R: TGT GTC CAC GGC GGC AAT CAT C	103
Hs *FAS*	NM-152872.4	F: GGA CCCA GAA TAC CAA GTG CAG R: GTT GCT GGT GAG TGT GCA TTC C	125
Hs *CASP3*	NM-004346.4	F: GGA AGC GAA TCA ATG GAC TCT GG R: GCA TCG ACA TCT GTA CCA GAC C	146
Hs *CASP8*	NM-001372051.1	F: AGA AGA GGG TCA TCC TGG GAG A R: TCA GGA CTT CCT TCA AGG CTG C	142
Hs *CASP9*	NM-001278054.2	F: GTT TGA GGA CCT TCG ACC AGC T R: CAA CGT ACC AGG AGC CAC TCT T	129

Table 2. The primers sequence used for real-time PCR.

TUNEL assay. The detection of DNA fragmentation in 12Z cells was performed using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay with In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA). The test detects DNA breakage that is identified by terminal deoxynucleotidyl transferase. After treatment, the harvested cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. After washing in PBS, cells were subjected to TUNEL labeling with fluorescein-labeled dUTP and terminal deoxynucleotidyl transferase mixture. Cells were analyzed by flow cytometry using an Amnis™ FlowSight™ flow cytometer.

Cell cycle analysis. Cell cycle progression was analyzed by flow cytometry using PI staining. After treatment, 12Z cells were collected, washed with PBS, and fixed in 70% ethanol. The cells were then washed, resuspended in PBS, and stained with 50 µg/ml PI in the presence of 100 µg/ml RNase A. Cell cycle distribution was analyzed using a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA, USA). Data analysis was performed with the FACS Diva software (Becton Dickinson).

Caspase-3/7 activity. To quantitate the proteolytic activity of caspase 3 and 7, we performed an assay based on the proteolytic cleavage of the C-terminal side of the aspartate residue in the DEVD peptide substrate by caspase 3/7 and into the fluorescent rhodamine 110. Caspase 3/7 activity was determined using the APO-One Homogenous Caspase-3/7 Assay (Promega Corporation, Wisconsin, USA) following the manufacturer's instructions. The obtained data were normalized to cellular protein content. The total protein was quantified by BCA assay (Pierce® BCA Protein Assay Kit, Thermo Scientific Inc., USA) according to the manufacturer's protocol.

RNA extraction and real-time PCR. Gene expression analysis was carried out according to the previously described protocol[58]. TRI reagent was applied for total RNA isolation, synthesis cDNA Transcriptor First-Strand kit (Roche Diagnostics GmbH, Mannheim, Germany) for first-strand cDNA synthesis, and SYBR® Select Master Mix (Life Technologies, Carlsbad, CA, USA) for real-time PCR. The primers used for the amplification of cDNAs are listed in Table 2. The levels of transcripts were normalized using β-actin as an internal control, which was previously applied in 12Z cells[59]. Relative mRNA expression was shown as fold change calculated using the $2^{-\Delta\Delta Ct}$ method compared with control cells.

Statistical analysis. All data are presented as means ± SD from three independent replications. Statistical analysis was performed using STATISTICA version 13.3 software (Statsoft, Inc., Tulsa, OK, USA). The Shapiro–Wilk test was used to assess distributional assumptions, and the equality of variances hypothesis was verified with Levene's test. A Student's *t*-test was used to compare two groups of data. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to determine the differences between the mean values of multiple groups. $p \leq 0.05$ was the cut-off point for a significant difference.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 19 February 2023; Accepted: 12 July 2023
Published online: 15 July 2023

References

1. ICD-11. International Classification of Diseases and Related Health Problems (ICD-11). International Classification of Diseases and Related Health Problems tool (Version 11); 2020. https://icd.who.int. Accessed 14 January 2023. (2020).

2. Sampson, J. Benign and malignant endometrial implants in peritoneal cavity, and their relation to certain ovarian tumors. *Surg. Gynecol. Obstet.* **38**, 287–311 (1924).
3. Halme, J., Hammond, M. G., Hulka, J. F., Raj, S. G. & Talbert, L. M. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet. Gynecol.* **64**, 151–154 (1984).
4. Nasu, K. *et al.* Aberrant expression of apoptosis-related molecules in endometriosis: A possible mechanism underlying the pathogenesis of endometriosis. *Reprod. Sci.* **18**, 206–218 (2011).
5. Heo, J.-R., Kim, S.-M., Hwang, K.-A., Kang, J.-H. & Choi, K.-C. Resveratrol induced reactive oxygen species and endoplasmic reticulum stress-mediated apoptosis, and cell cycle arrest in the A375SM malignant melanoma cell line. *Int. J. Mol. Med.* **42**, 1427–1435 (2018).
6. Gołąbek, A., Kowalska, K. & Olejnik, A. Polyphenols as a diet therapy concept for endometriosis-current opinion and future perspectives. *Nutrients* **13**, 1347 (2021).
7. Ko, J.-H. *et al.* The role of resveratrol in cancer therapy. *Int. J. Mol. Sci.* **18**, 2589 (2017).
8. Rauf, A. *et al.* Resveratrol as an anti-cancer agent: A review. *Crit. Rev. Food Sci. Nutr.* **58**, 1428–1447 (2018).
9. Zhang, L.-X. *et al.* Resveratrol (RV): A pharmacological review and call for further research. *Biomed. Pharmacother.* **143**, 112164 (2021).
10. Kapoor, R., Stratopoulou, C. A. & Dolmans, M.-M. Pathogenesis of endometriosis: New insights into prospective therapies. *Int. J. Mol. Sci.* **22**, 11700 (2021).
11. Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E. & Walle, U. K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* **32**, 1377–1382 (2004).
12. Nawaz, W. *et al.* Therapeutic versatility of resveratrol derivatives. *Nutrients* **9**, 1188 (2017).
13. Setoguchi, Y. *et al.* Absorption and metabolism of piceatannol in rats. *J. Agric. Food Chem.* **62**, 2541–2548 (2014).
14. Seyed, M. A., Jantan, I., Bukhari, S. N. A. & Vijayaraghavan, K. A Comprehensive review on the chemotherapeutic potential of piceatannol for cancer treatment, with mechanistic insights. *J. Agric. Food Chem.* **64**, 725–737 (2016).
15. Banik, K. *et al.* Piceatannol: A natural stilbene for the prevention and treatment of cancer. *Pharmacol. Res.* **153**, 104635 (2020).
16. Ravagnan, G. *et al.* Polydatin, a natural precursor of resveratrol, induces β-defensin production and reduces inflammatory response. *Inflammation* **36**, 26–34 (2013).
17. Potdar, S., Parmar, M. S., Ray, S. D. & Cavanaugh, J. E. Protective effects of the resveratrol analog piceid in dopaminergic SH-SY5Y cells. *Arch. Toxicol.* **92**, 669–677 (2018).
18. Delbandi, A.-A. *et al.* Evaluation of apoptosis and angiogenesis in ectopic and eutopic stromal cells of patients with endometriosis compared to non-endometriotic controls. *BMC Women's Health* **20**, 3 (2020).
19. Gołąbek-Grenda, A. & Olejnik, A. In vitro modeling of endometriosis and endometriotic microenvironment—challenges and recent advances. *Cell. Sig.* https://doi.org/10.1016/j.cellsig.2022.110375 (2022).
20. Sui, C. *et al.* PAI-1 secretion of endometrial and endometriotic cells is Smad2/3- and ERK1/2-dependent and influences cell adhesion. *Am. J. Transl. Res.* **8**, 2394–2402 (2016).
21. Horné, F. *et al.* Impaired localization of claudin-11 in endometriotic epithelial cells compared to endometrial cells. *Reprod. Sci.* **26**, 1181–1192 (2019).
22. Samartzis, E. P., Fink, D., Stucki, M. & Imesch, P. Doxycycline reduces MMP-2 activity and inhibits invasion of 12Z epithelial endometriotic cells as well as MMP-2 and -9 activity in primary endometriotic stromal cells in vitro. *Reprod. Biol. Endocrinol.* **17**, 38 (2019).
23. Madanes, D. *et al.* Resveratrol impairs cellular mechanisms associated with the pathogenesis of endometriosis. *Reprod. Biomed. Online* **44**, 976–990 (2022).
24. Ricci, A. G. *et al.* Natural therapies assessment for the treatment of endometriosis. *Hum. Reprod.* **28**, 178–188 (2013).
25. Kolahdouz Mohammadi, R. *et al.* The effects of resveratrol treatment on Bcl-2 and bax gene expression in endometriotic compared with non-endometriotic stromal cells. *Iran J. Public Health* **49**, 1546–1554 (2020).
26. Taguchi, A. *et al.* Resveratrol enhances apoptosis in endometriotic stromal cells. *Am. J. Reprod. Immunol.* **75**, 486–492 (2016).
27. McCormack, D. & McFadden, D. A review of pterostilbene antioxidant activity and disease modification. *Oxid. Med. Cell. Longev.* **2013**, 575482 (2013).
28. Roupe, K. A., Remsberg, C. M., Yáñez, J. A. & Davies, N. M. Pharmacometrics of stilbenes: Seguing towards the clinic. *Curr. Clin. Pharmacol.* **1**, 81–101 (2006).
29. Dellinger, R. W., Gomez Garcia, A. M. & Meyskens, F. L. Differences in the glucuronidation of resveratrol and pterostilbene: altered enzyme specificity and potential gender differences. *Drug Metab. Pharmacokinet.* **29**, 112–119 (2014).
30. Zhao, G. *et al.* Polydatin, a glycoside of resveratrol, is better than resveratrol in alleviating non-alcoholic fatty liver disease in mice fed a high-fructose diet. *Front. Nutr.* **9**, 857879 (2022).
31. Jiao, Y., Wu, Y. & Du, D. Polydatin inhibits cell proliferation, invasion and migration, and induces cell apoptosis in hepatocellular carcinoma. *Braz. J. Med. Biol. Res.* **51**, e6867 (2018).
32. Chatterjee, K. *et al.* Resveratrol and pterostilbene exhibit anticancer properties involving the downregulation of HPV oncoprotein E6 in cervical cancer cells. *Nutrients* **10**, 243 (2018).
33. Shin, H. J., Han, J. M., Choi, Y. S. & Jung, H. J. Pterostilbene suppresses both cancer cells and cancer stem-like cells in cervical cancer with superior bioavailability to resveratrol. *Molecules* **25**, 228 (2020).
34. Zhang, B., Wang, X.-Q., Chen, H.-Y. & Liu, B.-H. Involvement of the Nrf2 pathway in the regulation of pterostilbene-induced apoptosis in HeLa cells via ER stress. *J. Pharmacol. Sci.* **126**, 216–229 (2014).
35. Schindler, C. K. *et al.* Caspase-3 cleavage and nuclear localization of caspase-activated DNase in human temporal lobe epilepsy. *J. Cereb. Blood Flow Metab.* **26**, 583–589 (2006).
36. Tolomeo, M. *et al.* Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells. *Int. J. Biochem. Cell Biol.* **37**, 1709–1726 (2005).
37. Cregan, S. P., Dawson, V. L. & Slack, R. S. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23**, 2785–2796 (2004).
38. Arnoult, D. *et al.* Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J.* **22**, 4385–4399 (2003).
39. Potter, G. A. *et al.* The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. *Br. J. Cancer* **86**, 774–778 (2002).
40. Morales, P. & Haza, A. I. Selective apoptotic effects of piceatannol and myricetin in human cancer cells. *J. Appl. Toxicol.* **32**, 986–993 (2012).
41. Westphal, D. *et al.* Apoptotic pore formation is associated with in-plane insertion of Bak or Bax central helices into the mitochondrial outer membrane. *PNAS* **111**, E4076–E4085 (2014).
42. Song, Z., Han, S., Pan, X., Gong, Y. & Wang, M. Pterostilbene mediates neuroprotection against oxidative toxicity via oestrogen receptor α signalling pathways. *J. Pharm. Pharmacol.* **67**, 720–730 (2015).
43. Kobylka, P. *et al.* Resveratrol analogues as selective estrogen signaling pathway modulators: structure-activity relationship. *Molecules* **27**, 6973 (2022).
44. Kawiak, A. & Kostecka, A. Regulation of Bcl-2 family proteins in estrogen receptor-positive breast cancer and their implications in endocrine therapy. *Cancers* **14**, 279 (2022).

45. Horgan, X. J., Tatum, H., Brannan, E., Paull, D. H. & Rhodes, L. V. Resveratrol analogues surprisingly effective against triple-negative breast cancer, independent of ERα. *Oncol. Rep.* **41**, 3517–3526 (2019).
46. Banu, S. K., Lee, J., Starzinski-Powitz, A. & Arosh, J. A. Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells. *Fertil. Steril.* **90**, 972–987 (2008).
47. Logan, P. C., Ponnampalam, A. P., Steiner, M. & Mitchell, M. D. Effect of cyclic AMP and estrogen/progesterone on the transcription of DNA methyltransferases during the decidualization of human endometrial stromal cells. *Mol. Hum. Reprod.* **19**, 302–312 (2013).
48. Plaza-Parrochia, F. *et al.* Molecular mechanisms of androstenediol in the regulation of the proliferative process of human endometrial cells. *Reprod. Sci.* **24**, 1079–1087 (2017).
49. Bulun, S. E. *et al.* Role of estrogen receptor-β in endometriosis. *Semin. Reprod. Med.* **30**, 39–45 (2012).
50. Jiang, Q. *et al.* Protective effects of polydatin on lipopolysaccharide-induced acute lung injury through TLR4-MyD88-NF-κB pathway. *Int. Immunopharmacol.* **29**, 370–376 (2015).
51. Lanzilli, G. *et al.* Anti-inflammatory effect of resveratrol and polydatin by in vitro IL-17 modulation. *Inflammation* **35**, 240–248 (2012).
52. Bröhan, M., Jerkovic, V. & Collin, S. Potentiality of red sorghum for producing stilbenoid-enriched beers with high antioxidant activity. *J. Agric. Food Chem.* **59**, 4088–4094 (2011).
53. Shah, M. A. *et al.* Uncovering the anticancer potential of polydatin: A mechanistic insight. *Molecules* **27**, 7175 (2022).
54. Su, D. *et al.* Comparision of piceid and resveratrol in antioxidation and antiproliferation activities in vitro. *PLoS One* **8**, e54505 (2013).
55. Hung, S. W. *et al.* Pharmaceuticals targeting signaling pathways of endometriosis as potential new medical treatment: A review. *Med. Res. Rev.* **41**, 2489–2564 (2021).
56. Swingle, K. L., Ricciardi, A. S., Peranteau, W. H. & Mitchell, M. J. Delivery technologies for women's health applications. *Nat. Rev. Bioeng.* https://doi.org/10.1038/s44222-023-00040-w (2023).
57. Olejnik, A. *et al.* Antioxidant effects of gastrointestinal digested purple carrot extract on the human cells of colonic mucosa. *Food Chem.* **190**, 1069–1077 (2016).
58. Kowalska, K., Dembczyński, R., Gołąbek, A., Olkowicz, M. & Olejnik, A. ROS modulating effects of lingonberry (*Vaccinium vitis-idaea* L.) polyphenols on obese adipocyte hypertrophy and vascular endothelial dysfunction. *Nutrients* **13**, 885 (2021).
59. Wendel, J. R. H., Wang, X., Smith, L. J. & Hawkins, S. M. Three-dimensional biofabrication models of endometriosis and the endometriotic microenvironment. *Biomedicines* **8**, E525 (2020).

Author contributions

G.-G.A. Conceptualization, Methodology, Investigation, Data Curation, Writing—Original Draft, Visualization, Project administration. K. M. and J. W. Methodology, Investigation. O. A. Conceptualization, Methodology, Formal analysis, Writing—Review & Editing.

Funding

The Ministry of Education and Science (Poland) partially financed the study within the funding for young scientists at Poznan University of Life Sciences.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to A.O.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

nutrients

MDPI

Article

Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages

Agata Gołąbek-Grenda [1], Wojciech Juzwa [1], Mariusz Kaczmarek [2,3] and Anna Olejnik [1,*]

[1] Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego St., 60-627 Poznan, Poland; agata.golabek@up.poznan.pl (A.G.-G.); wojciech.juzwa@up.poznan.pl (W.J.)
[2] Department of Cancer Immunology, Poznan University of Medical Sciences, Garbary 15 St., 61-866 Poznan, Poland; markacz@ump.edu.pl
[3] Gene Therapy Laboratory, Department of Cancer Diagnostics and Immunology, Greater Poland Cancer Centre, Garbary 15 St., 61-866 Poznan, Poland
* Correspondence: anna.olejnik@up.poznan.pl

Abstract: Background: Inflammation and immune cell dysfunction are critical facilitators of endometriosis pathophysiology. Macrophages are renowned for stimulating lesion growth, vascularization, innervation, and pain generation. By combining macrophages and endometriotic cells, we determined if resveratrol and its natural analogs can target the immune dysregulation and oxidative imbalance in endometriosis. **Methods:** After treatment with compounds (5, 10, 25 µM), we evaluated the expression of key inflammatory and oxidative stress markers, cytokines release, and ROS production by applying q-PCR, ELISA, Cytometric Beads Array, and multiplexed fluorogenic staining and flow cytometry analysis with bioimaging. **Results:** The results showed that endometriosis-related macrophages treated with stilbenes have impaired expression of pro-inflammatory markers (*IL6*, *IL8*, *IL1B*, *TNF*, *CCL2*, *CXCL10*, *PTGS2*). The effect of resveratrol, pterostilbene, and piceatannol was observed, especially in reducing *IL1B*, *CCL2*, and *CXCL10* genes up to 3.5-, 5-, and 7.7-fold at 25 µM, respectively. Also, with piceatannol or polydatin exposure, the IL-6 decrease was noticeable. This study reported an antioxidant effect by reducing ROS-positive cells from 96% to 48% by pterostilbene. Results from flow cytometry correlated with the transcript activation of detoxification enzymes (*SOD*, *GPX*). **Conclusions:** Prospects for potential therapy based on regulating the immune microenvironment and reducing the accumulation of free radicals with stilbenes application were described in the article.

Keywords: endometriosis; inflammation; macrophages; co-culture; transwell system; oxidative stress; resveratrol

Citation: Gołąbek-Grenda, A.; Juzwa, W.; Kaczmarek, M.; Olejnik, A. Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages. *Nutrients* **2024**, *16*, 3483. https://doi.org/10.3390/nu16203483

Academic Editor: Antoni Sureda

Received: 21 August 2024
Revised: 11 October 2024
Accepted: 12 October 2024
Published: 14 October 2024

1. Introduction

Endometrial macrophages play key roles in mediating the menstrual cycle through the initiation of the breakdown of endometrium during menstruation [1] or contribution to tissue remodeling during the establishment of pregnancy [2]. Macrophages exhibit extensive plasticity, and their phenotype and function are highly specific to the local tissue milieu. Dysregulation of their activity occurs in abnormalities and pathologies of the uterus, including endometriosis. It is evident that macrophages are abundant in endometrial lesions and are critical for their establishment and growth. Their enhanced ability to produce pro-inflammatory cytokines contributes to a microenvironment that favors pelvic inflammation and promotes other immune cells' recruitment, endometriotic cells' proliferation, angiogenesis, and innervation, as well as leads to the generation of

endometriosis-associated pain [3,4]. Therefore, the reciprocal communication between macrophages and endometriotic cells appears, and their intercellular dialogue sustains the formation of endometriosis lesions. As macrophages are the predominant cellular component in endometriotic tissue, the targeting of macrophage alternation could be critical in developing potential therapeutic candidates.

Resveratrol is a phenomenal natural compound commonly occurring in many plant species, including coarse cereals, potatoes, beans, fruits, and nuts [5]. It became well known due to its anti-cancer potential and promising anti-inflammatory, anti-oxidant, and anti-microbial properties investigated in extensive research [6]. Recently, it was reported that grapes do not serve as the richest source of resveratrol, but rather that the richest sources are tangerine, peach, walnut, and sweet potato. Obtaining resveratrol in the daily diet from everyday food items is worth encouraging, as red wine should not be the primary source of resveratrol due to the harmful impact of alcohol on human health. Recent studies have indicated that the current daily resveratrol intake, estimated at 0.7 mg/day, is insufficient to achieve the health-beneficial effects [5]. Therefore, there is a definite need to increase resveratrol intake to 30–150 mg/day and improve its bioavailability through the development of breeding techniques, consuming foods enriched in resveratrol as well as improvement of extraction methods, and utilization of innovative drug-delivery methods in nanoparticle structures [5].

In searching for potential health-beneficial agents with increased bioavailability, it is necessary to investigate the biological mechanisms for which resveratrol analogs and metabolites can reach the target tissues and exert biological activity. Experiments utilizing analogs demonstrated that resveratrol hydroxylation to piceatannol (3,5,3′,4′-tetrahydroxy-trans-stilbene) attributed the compound's antioxidant activity and growth-inhibitory effects [7]. This plant-derived stilbene is found in peanuts, teas, berries, and passion fruit. Passion fruit is a rich piceatannol source in its seed, detected at a 4.8 mg/g concentration. Although the benefits of piceatannol have not been studied as extensively as resveratrol, studies have shown a range of health-promoting biological properties with an emphasis on antileukemic, antiangiogenic, and cardioprotective potential, as well as promoting fat metabolism [8]. A recent randomized, double-blind, placebo-controlled crossover comparison study found that consuming 10 mg of piceatannol daily from passion fruit seeds for 7 days increases fat burning in healthy subjects [9]. Pterostilbene (3,5-dimethoxy-4′-hydroxystilbene), with two extra methoxy groups in its structure, shows greater lipophilicity than the parent compound and is, therefore, characterized by higher intestinal permeability, cellular absorption, and increased stability [10]. Pterostilbene is mainly found in blueberries and *Vaccinium* berries up to 500 ng/g. The amount of pterostilbene in dietary sources appears insufficient to exert health benefits. Therefore, formulating pure compound supplements or employing strategies like transgenic alteration or metabolic engineering offers a method to provide nutritional levels. Currently, several clinical trials for pterostilbene are being undertaken, e.g., in treating acute kidney injury and improving vascular endothelial functions and cardiovascular health. In clinical studies, dietary blueberry supplements or combining pterostilbene with other drugs, mainly at 25–125 mg doses, are applied [11]. Polydatin (3,4′,5-trihydroxystilbene-3-β-D-glucoside), in its glycoside resveratrol form, possesses different biological characteristics, such as greater bioavailability [12,13]. The reported therapeutic targets of polydatin include many that have beneficial biological activities against cancers, infections, cardiovascular diseases, diabetes, and gastric and hepatic failure [14]. Grapes, mulberries, cocoa products, peanuts, and hop flowers (*Humulus lupulus*) are prominent dietary sources of polydatin. However, polydatin was primarily isolated from the rhizome *Polygonum cuspidatum* at amounts of up to 14.43 mg/g. Polydatin has been evaluated in different clinical trials involving women's diseases and gastrointestinal and liver diseases. The effectiveness was achieved in a 40–600 mg dose range per day [14,15]. A combination of polydatin and palmitoylethanolamide impaired inflammatory factors and exhibited analgesic effects on endometriosis in several in vivo and clinical studies [14].

Natural bioactive compounds can target pathological processes involved in endometriosis, like altered inflammatory microenvironment and oxidative stress [6]. Numerous studies suggest that resveratrol demonstrated an immunomodulatory role in immunologic disorders, like cancers, autoimmune, neurodegenerative, metabolic, cardiovascular, and infectious diseases [16,17]. Regarding immune cells, it has been found that resveratrol affects the anti-inflammatory profile in macrophages, exhibits inhibitory function on T cells, reduces CD4+CD25+ regulatory T cells' suppressive function, and stimulates the killing activity of NK cells. Studies on macrophages revealed that resveratrol regulates TLR-mediated inflammatory responses, nuclear factor kappa B (NF-κB) activation, and cyclooxygenase-2 (COX-2) expression. It attenuated TLR4-TRAF6, mitogen-activated protein kinase (MAPK), and AKT pathways in lipopolysaccharide (LPS)-stimulated macrophages. Resveratrol reduced granulocyte-macrophage colony-stimulating factor (GM-CSF) production, decreased oxidative LPS effect, and modulated immune response linked to prostaglandin E2 (PGE2) and Sirtuin 1 (SIRT1) level [16,18]. Since endometriosis is an inflammatory pathology, this study aims to investigate the comparative anti-inflammatory effects of resveratrol, pterostilbene, piceatannol, and polydatin on endometriosis. It is suggested that macrophage influence on endometriotic cells may be fundamentally important for endometriosis development; however, the cell interactions have not been fully understood. Therefore, this study investigated the effect of stilbenes in the co-culture model of macrophages and endometriotic cells. We analyzed the expression of key inflammatory and oxidative stress markers, production of cytokines, and radical scavenging activity in the activated endometriosis-related macrophages. We also discussed whether the co-culture system could imitate the inflammatory niche in endometriosis.

2. Materials and Methods

2.1. Chemicals

Unless stated otherwise, all chemicals were purchased from Merck KGaA (Darmstadt, Germany). Resveratrol and its analogs were also supplied by Merck KGaA as a solid form and with a high purity (resveratrol $\geq$ 99%; pterostilbene $\geq$ 97%; piceatannol $\geq$ 98%; polydatin $\geq$ 98.0%). Trans isomers of compounds were used in this study (Figure 1). The stock solution of resveratrol, pterostilbene, piceatannol (80 mM), and polydatin (40 mM) was prepared by dissolving in 96% alcohol and stored at −80 °C. Working solutions (5 mM, 2 mM, 1 mM) were prepared immediately before experiments by diluting the stock solution in 96% alcohol. Then, they were added to the cell culture medium, maintaining 0.5% alcohol concentration in each culture.

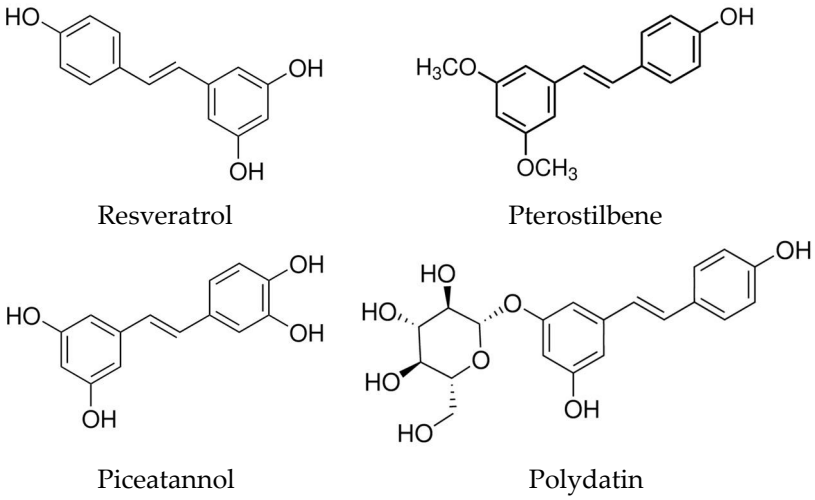


Figure 1. Chemical structures of resveratrol, pterostilbene, piceatannol, and polydatin.

2.2. Cell Cultures

Immortalized human endometriotic epithelial cells (12Z) were obtained from Applied Biological Materials Inc. (Richmond, BC, Canada). They were cultured in Dulbecco's Modified Eagle Medium combined with Nutrient Mixture F-12 (DMEM/F12) supplemented with fetal bovine serum (FBS, 10%) and gentamycin (50 mg/L). Human monocyte THP-1 cells (ATCC TIB-202) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen, Waltham, MA, USA) containing FBS (10%), gentamycin (50 mg/L), and 2-mercaptoethanol (0.05 mM). THP-1 cells were maintained at a minimum density of 2×10^5 cells/mL and subcultured when their concentration reached 8×10^5 cells/mL. They were passaged every 48 h by adding a fresh medium or complete medium replacement until the culture reached appropriate cell density. Both cell lines were grown at 37 °C under an air (95%)/CO_2 (5%) atmosphere. During the experiments, they were regularly tested regularly for mycoplasma contamination.

2.3. Macrophages Differentiation and Co-Culture Setup

Monocytes were stimulated with 10 ng/mL PMA (phorbol-12 myristate 13-acetate) for 24 h, followed by washing with RPMI 1640 to eliminate the effect of PMA, as described by Smith et al. [19]. Morphological changes in cell size, pseudopodia formation, and cell adhesion were observed to assess the acquisition of a macrophage-like phenotype. The co-culture system was prepared in 6-well hanging cell culture inserts (0.4 µm pore size; Merck KGaA, Darmstadt, Germany). THP-1 monocytes were seeded (2×10^5 cells/cm^2) into the upper compartment of the transwell system in 2 mL of RPMI 1640 medium supplemented with 10% FBS and placed into a 6-well plate containing 3 mL of medium. After seeding, THP-1 cells were treated immediately with PMA (10 ng/mL) for 24 h to stimulate differentiation. A new 6-well plate was prepared the same day with 12Z cells, seeded at 0.3×10^5 cells/cm^2 in DMEM/F12 with 10% FBS. PMA-activated THP-1 cells (in the insert) and 12Z cells (in the 6-well plate) were incubated separately for 24 h for their attachment. The next day, the differentiated THP-1 cells were washed with the medium. Macrophages were polarized to M1 macrophages with 50 ng/mL IFN-γ (PeproTech, Cranbury, NJ, USA) and 10 pg/mL LPS (PeproTech, Cranbury, NJ, USA) in RPMI 1640 medium supplemented with 10% FBS according to the method described by Smith et al. and Genin et al. [19,20]. Simultaneously, the medium in the 6-well plate with 12Z cells was changed to RPMI 1640 with 2% FBS. After 24 h, the chambers containing THP-1-derived macrophages were hung on the 6-well plates with the 12Z cells to obtain a co-culture cell system. Macrophages and 12Z cells were co-cultured in RPMI 1640 medium with 2% FBS and treated with resveratrol and its derivatives for 24 h. Figure 2 presents a schematic experimental design that includes macrophage differentiation, macrophage polarization, a co-culture setup, treatment with analyzed compounds, and further analysis.

2.4. Cytotoxicity Assay

THP-1 cells were cultured in 24-well plates at an initial density of 1×10^5/cm^2 and stimulated by 24 h incubation with 10 ng/mL PMA. The cultures were then treated with resveratrol at 1 µM to 50 µM and incubated for 24 h under standard culture conditions in RPMI 1640 medium and 2% FBS. The 12Z cells were grown in 24-well plates at an initial density of 0.25×10^5/cm^2 and treated with resveratrol at the same concentration range and under the same culture conditions. Cell viability and metabolic potential in both cultures were determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following the method described previously [21]. Absorbance was measured with a Tecan M200 Infinite microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

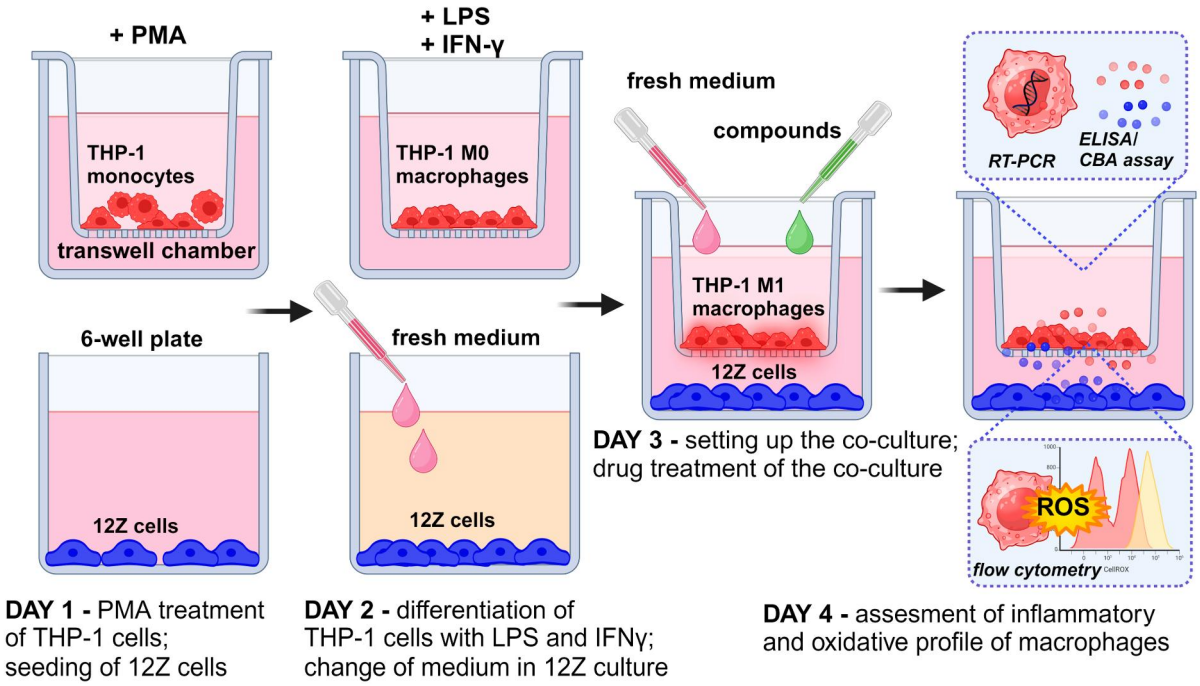


Figure 2. Schematic representation of research design workflow.

2.5. RNA Extraction and Real-Time PCR

Total RNA from THP-1-derived macrophages co-cultured with 12Z cells was extracted using Trizol reagent (Invitrogen, Waltham, MA, USA) according to the previously described protocol [22]. Complementary deoxyribonucleic acids (cDNAs) were synthesized using the cDNA Transcriptor First-Strand kit (Roche Diagnostics GmbH, Penzberg, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR® Select Master Mix (Life Technologies, Carlsbad, CA, USA). The PCR reaction included 1 µL cDNA, 1 µL of specific forward and reverse primers (5 µM), 12.5 µL SYBR® Select Master Mix, and nuclease-free water in the amount to a final volume of 25 µL. The PCR reaction mixture was denatured at 94 °C for 10 min, followed by 40 cycles of 95 °C for 40 s, 59 °C for 30 s, and 72 °C for 30 s. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct represents the threshold cycle. GAPDH was used as the internal control. The primers applied for cDNA amplification are shown in Table 1.

Table 1. Primer sequences used in real-time PCR.

Gene	Accession	No. Sequence (5′-3′)	Amplicon (bp)
CCL2	NM_002982.4	F: AGAATCACCAGCAGCAAGTGTCC R: TCCTGAACCCACTTCTGCTTGG	98
CXCL10	NM_001565.4	F: GAACTGTACGCTGTACCTGCA R: TTGATGGCCTTCGATTCTGGA	172
GAPDH	NM_002046.7	F: GTCTCCTCTGACTTCAACAGCG R:ACCACCCTGTTGCTGTAGCCAA	131
GPX1	NM_000581.4	F: GTGCTCGGCTTCCCGTGCAAC R: CTCGAAGAGCATGAAGTTGGGC	123
IL1B	NM_000576.3	F: CCACAGACCTTCCAGGAGAATG R: GTGCAGTTCAGTGATCGTACAGG	131
IL6	NM_000600.5	F: AGACAGCCACTCACCTCTTCAG R: TTCTGCCAGTGCCTCTTTGCTG	132
IL8	NM_001354840.3	F: GAGAGTGATTGAGAGTGGACCAC R: CACAACCCTCTGCACCCAGTTT	112
NFKB1	NM_003998.4	F: GCAGCACTACTTCTTGACCACC R: TCTGCTCCTGAGCATTGACGTC	130

Table 1. *Cont.*

Gene	Accession	No. Sequence (5′-3′)	Amplicon (bp)
PTGS2	NM_000963.4	F: CGGTGAAACTCTGGCTAGACAG R: GCAAACCGTAGATGCTCAGGGA	156
SOD1	NM_000454.5	F: CTCACTCTCAGGAGACCATTGC R: CCACAAGCCAAACGACTTCCAG	129
TNF	NM_000594.4	F: CTCTTCTGCCTGCTGCACTTTG R: ATGGGCTACAGGCTTGTCACTC	135

2.6. Quantitation of Cytokines by Cytometric Bead Array

The conditioned media from macrophages and 12Z cells co-culture were collected 24 h after treatment with compounds, centrifuged to remove cellular debris, and applied for subsequent experiments or stored at −80 °C until use. The concentrations of Interleukin-6 (IL-6) and Interleukin 8 (IL-8) in the conditioned media were determined with the Human Inflammatory Cytokine Cytometric Bead Array (CBA) (BD Biosciences, Franklin Lakes, NJ, USA), as previously described [23]. For each kit, a standard calibration curve with maximum and minimum detection limits (1.0–5000 pg/mL) was plotted. Samples were analyzed using a FACSAriaII flow cytometer (BD Biosciences) and FACSDiva v6.1.2 software (BD Biosciences).

2.7. Quantitation of Cytokines by ELISA

The monocyte chemoattractant protein-1 (MCP-1) (Biorbyt, Cambridge, UK; sensitivity: 1 pg/mL; specificity: natural and recombinant human CCL2) and tumor necrosis factor-alpha (TNF-α) (R&D Systems, Minneapolis, MN, USA; sensitivity: 0.5–5.5 pg/mL; specificity: natural and recombinant human TNF-α) levels were determined using enzyme-linked immunosorbent assay (ELISA) according to the suppliers' standard protocols. The prostaglandin E2 (PGE2) concentration was measured using respective ELISA kits (Cayman Chemical, Ann Arbor, MI, USA; sensitivity: 40 pg/mL; specificity: natural PGE2) following the manufacturer's instructions. The absorbance of reaction mixtures was measured using a Tecan M200 Infnite microplate reader. Results are expressed in pg of cytokine normalized per µg of proteins quantified using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Assessment of Intracellular ROS by Flow Cytometry

Reactive oxygen species (ROS) generation was determined using a CellROX® Deep Red Flow Cytometry Assay Kit (Life Technologies, Carlsbad, CA, USA). In a reduced state, the cell-permeable non-fluorescent CellROX Deep Reagent exhibits a strong fluorogenic signal upon oxidation. The fluorescence intensity of CellROX® Deep Red reflects the ROS levels in live cells. Dead cells were measured using the Sytox® Green Dead Cell Stain (Life Technologies, Carlsbad, CA, USA).

Macrophages were co-cultured with 12Z cells according to standard protocol. After treatment of the co-cultures with resveratrol or its derivatives, macrophages were gently detached from the surface of the flask, rinsed, and centrifuged. Cells were resuspended in RPMI 1640 medium without phenol red, and CellROX® Deep Red reagent was added to the samples at a final concentration of 500 nM and incubated for 60 min at 37 °C in the dark. During the final 15 min of staining, Sytox Green Dead Stain was added in an optimized concentration of 45 nM. Cells were subjected to imaging Amnis™ FlowSight™ flow cytometer (Luminex Corp., Austin, TX, USA) equipped with three lasers for excitation (405, 488, and 642 nm), five fluorescence channels (acquisition by a multi-channel CCD camera), and a side scatter detector. Post-acquisition data analysis was performed using ImageStream Data Exploration and Analysis Software (IDEAS® version 6.2.187, Luminex Corp., Austin, TX, USA). Approximately 0.5×10^4 cells were analyzed in each sample. Single-color compensation controls for CellROX® Deep Reagent and Sytox® Green Dead Stain were also acquired using the integrated software INSPIRE® (version 201.1.0.765;

Luminex Corp., Austin, Austin, TX, USA) for data acquisition. The analysis detected oxidatively stressed (live cells), non-stressed cells (live cells), and dead cells representing distinct subpopulations.

2.9. Statistical Analysis

The obtained results were expressed as means ± SD from three independent replications. The hypothesis of equality of variances was verified with Levene's test. Student's *t*-test was used to compare two groups of data. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to determine the differences between the mean values of multiple groups. A statistically significant value was considered at $p < 0.05$; asterisks indicated the level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical analysis was performed using STATISTICA version 13.3 software (Statsoft, Inc., Tulsa, OK, USA).

3. Results

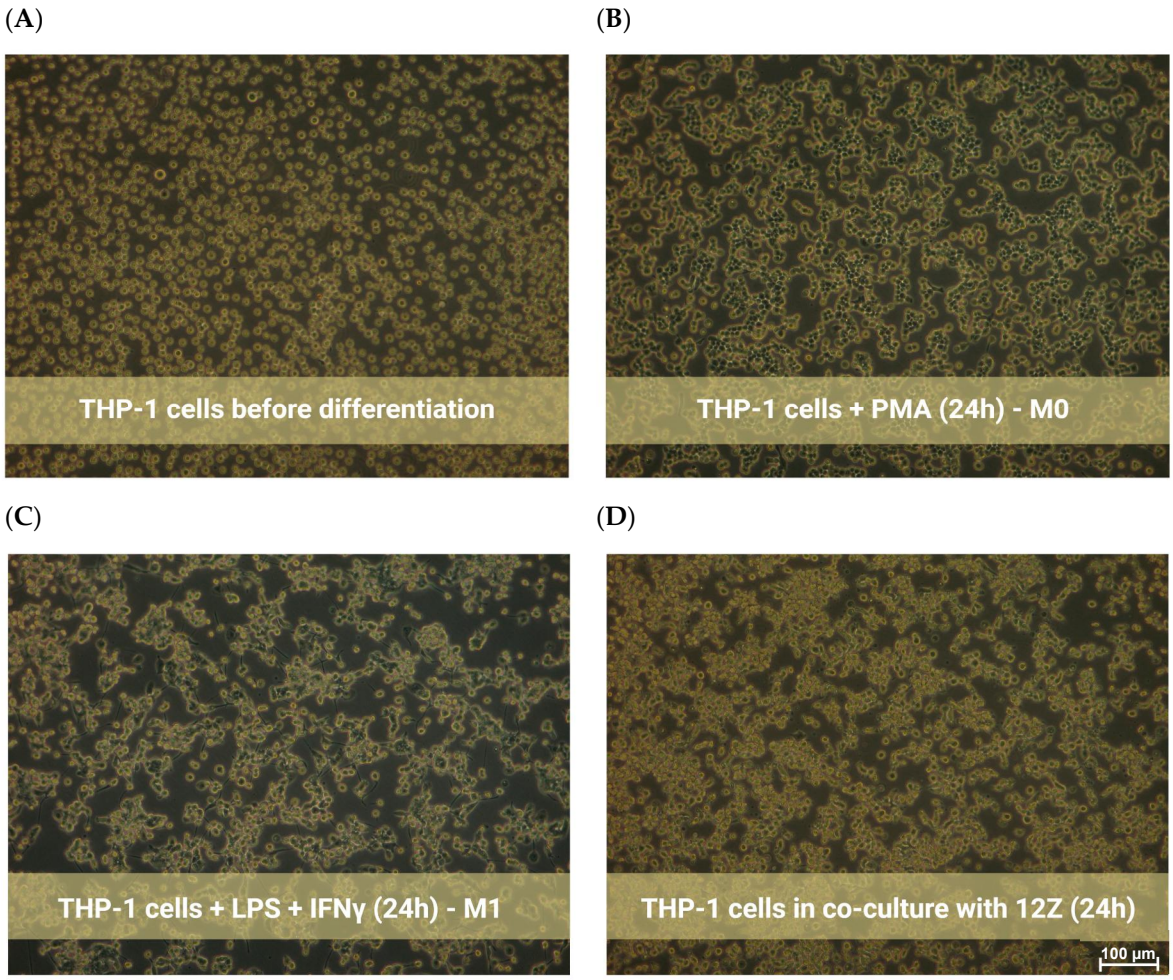
3.1. Differentiation, Polarization of Macrophages, and a Co-Culture of Endometriotic 12Z Cells and M1-Polarized THP-1 Cells

Human THP-1 monocytes were seeded into the upper compartment of the transwell vessel and subjected to differentiation into macrophages using PMA. As shown in Figure 3A, THP-1 monocytes displayed a suspended culture before induction and were characterized as small, round, and transparent. THP-1 cells exposed to PMA became adherent and larger, a characteristic of M0 cells (Figure 3B). The increase in recognized macrophage marker *CD68* (cluster of differentiation 68), revealed by the qRT-PCR assay, also illustrated the macrophage-like THP-1 (M0 THP-1) phenotype (Figure 3E). Cells acquired a flatter, more spread, and branching morphology M1 phenotype after exposure to LPS and IFN- γ (Figure 3C). As expected, the mRNA expression of *IL1B*, *TNF*, and *IL6* (M1 macrophage markers) was preferentially upregulated (Figure 3E). These results suggest that THP-1 cells were efficiently polarized into M1-like macrophages after PMA and LPS plus IFN-γ induction.

To explore the effects of resveratrol and its analog on the co-culture of polarized THP-1 macrophages and endometriotic 12Z cells, each population was cultured in indirect contact using transwell inserts. Monocytes were seeded onto 0.4 μm pore-size membranes in the inserts, which allowed the exchange of soluble factors and avoided cell transmigration. The monocyte differentiation and then polarization were launched as optimized for monoculture. Polarized THP-1 macrophages were co-cultured with 12Z endometriotic cells in the presence of resveratrol and its analog at different concentrations. At the end of incubation, RNA was extracted from macrophages to measure M1 marker expression, a conditioned medium was collected to quantify the secreted factors, and cells were harvested to measure ROS generation. Macrophages after co-culture with 12Z were presented in Figure 3D.

3.2. Effect of Resveratrol and Its Derivatives on 12Z Cell and THP-1-Derived Macrophage Proliferation

The effect of resveratrol and its natural analogs on the proliferation of endometriotic epithelial 12Z cells and macrophages differentiated from THP-1 monocytes was assessed using the MTT assay. Increasing resveratrol concentration in the endometriotic and macrophage cultures did not cause a suppression of cell proliferation. The compound dosage, responsible for a 20% reduction in viability of 12Z cells, was estimated at 50 μM of compound (Figure 4). The analyzed resveratrol analogs exhibited cytotoxic effects on 12Z cells and THP-1 macrophages comparable to the parent compound. Consequently, we have chosen three dosages: 5, 10, and 25 μM for further studies in the co-culture system.



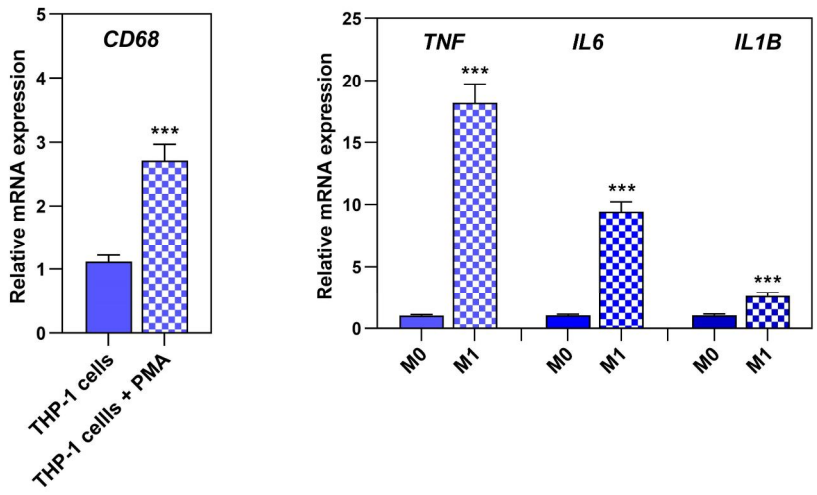


Figure 3. Morphological comparison of THP-1 cells before (**A**) and after PMA induction (**B**) and after M1 polarization (**C**) and co-culture with 12Z (**D**) using a light microscope (magnification × 100). Expression of markers typical for macrophages after differentiation (THP-1 cells + PMA) and macrophages after polarization to M1 phenotype (**E**). Data were normalized against GAPDH and expressed as relative mRNA expression (-fold of control). Statistical analysis assessed differences between exposed cells and negative control with significant differences *** $p \leq 0.001$.

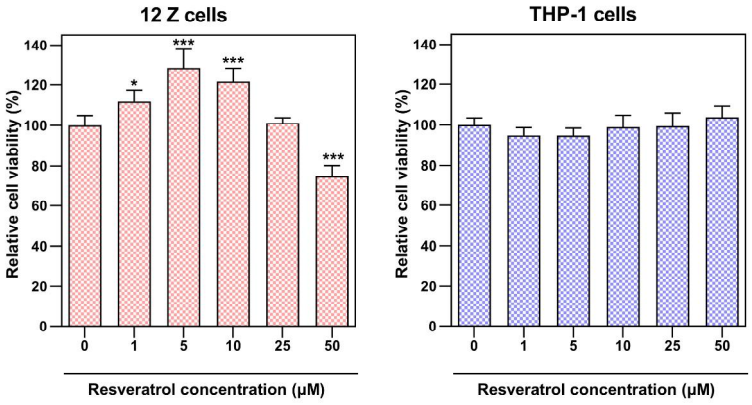


Figure 4. The effect of resveratrol on endometriotic epithelial cell (12Z) and differentiated macrophage (THP-1) proliferation analyzed by MTT reduction assay. The values represent the means (n = 6) ± SD. The post hoc Tukey test determined the significance of the effects of resveratrol concentration (* $p \leq 0.05$; *** $p \leq 0.001$).

3.3. Effect of Resveratrol and Its Derivatives on the Expression of Genes Related to the Inflammatory and Oxidative Profile of Macrophages Co-Cultured with Endometriotic Cells

To examine the effect of resveratrol and its derivatives on the macrophages co-cultured with endometriotic cells, qRT-PCR assays were used to analyze the RNA levels of proinflammatory markers *CCL2*, *CXCL10*, *IL1B*, *IL6*, *IL8*, *NFKB1*, and *PTGS2* and endogenous antioxidant enzymes *SOD1* and *GPX1* (Figure 5). Compared to the non-treated, inflamed co-culture model of macrophages and endometriotic cells, in response to resveratrol, we observed the decreased expression of inflammatory markers, such as *IL6*, *IL8*, *IL1B*, *TNF*, *CCL2*, *CXCL10*, and *PTGS2*, mostly at 10 μM and 25 μM concentrations. Especially *CCL2* and *IL1B* showed a significant dose-dependent decrease, reaching 3.5-fold and 5-fold levels at 25 μM concentration, respectively. Pterostilbene and piceatannol revealed a similar capacity to modify the expression of pro-inflammatory markers such as *IL6*, *IL8*, *IL1B*, and *CCL2*. The expression of *CCL2* and *IL1B* also followed a dose-dependent decrease after treatment with pterostilbene and piceatannol. The observed effect of pterostilbene on the down-expression of *CXCL10* was notably pronounced, with the maximum dose reaching 7.7-fold. *IL8* did not follow the pattern of a concentration-dependent decrease of expression after treatment with pterostilbene and piceatannol, whereas only resveratrol induced a significant transcript reduction (up to 3.7-fold). Changes in inflammatory genes related to the treatment under polydatin were not evident, with a reduction only for *IL6* and *PTGS2* at the maximum dose. In the case of the oxidative stress response, the genes encoding *SOD1* showed significant up-regulation at the highest dose of resveratrol, pterostilbene, and piceatannol. The macrophages in the co-culture model treated with the same compounds showed *GPX1* being slightly induced throughout. However, polydatin displayed a high activation pattern and the highest induction (up to 3.7-fold).

3.4. Effect of Resveratrol and Its Derivatives on the Cytokine/Chemokine Secretion in an Inflamed Co-Culture Model of Macrophages and Endometriotic Cells

In this experiment, CBA analysis and ELISA assay were used to quantify inflammatory cytokines and chemokines to verify the effect of resveratrol and its analogs on the inflamed co-culture model of macrophages and endometriotic cells. The results for soluble factor quantities calculated per μg of the cell protein are summarized in Figure 6. Compared with the non-treated co-culture model, resveratrol significantly decreased protein levels of IL-6, IL-8, TNF-α, and PGE2 at 10 μM and 25 μM. We detected increased released soluble factors in co-culture supernatants in response to pterostilbene. The increase in IL-6 to 56.8 pg/ug of cell protein was most striking. Only the generation of PGE2 under pterostilbene was significantly reduced at all doses. When piceatannol or polydatin was included in the exposure, the level of IL-6 was markedly decreased. The release of MCP-1 was reduced

marginally but significantly in response to piceatannol or polydatin. A noticeable elevated level of PGE2 was detected in the 25 µM piceatannol-exposed co-culture. The exposure to polydatin induced, in turn, a significant reduction in PGE2 quantity in the co-culture supernatant at the highest dose.

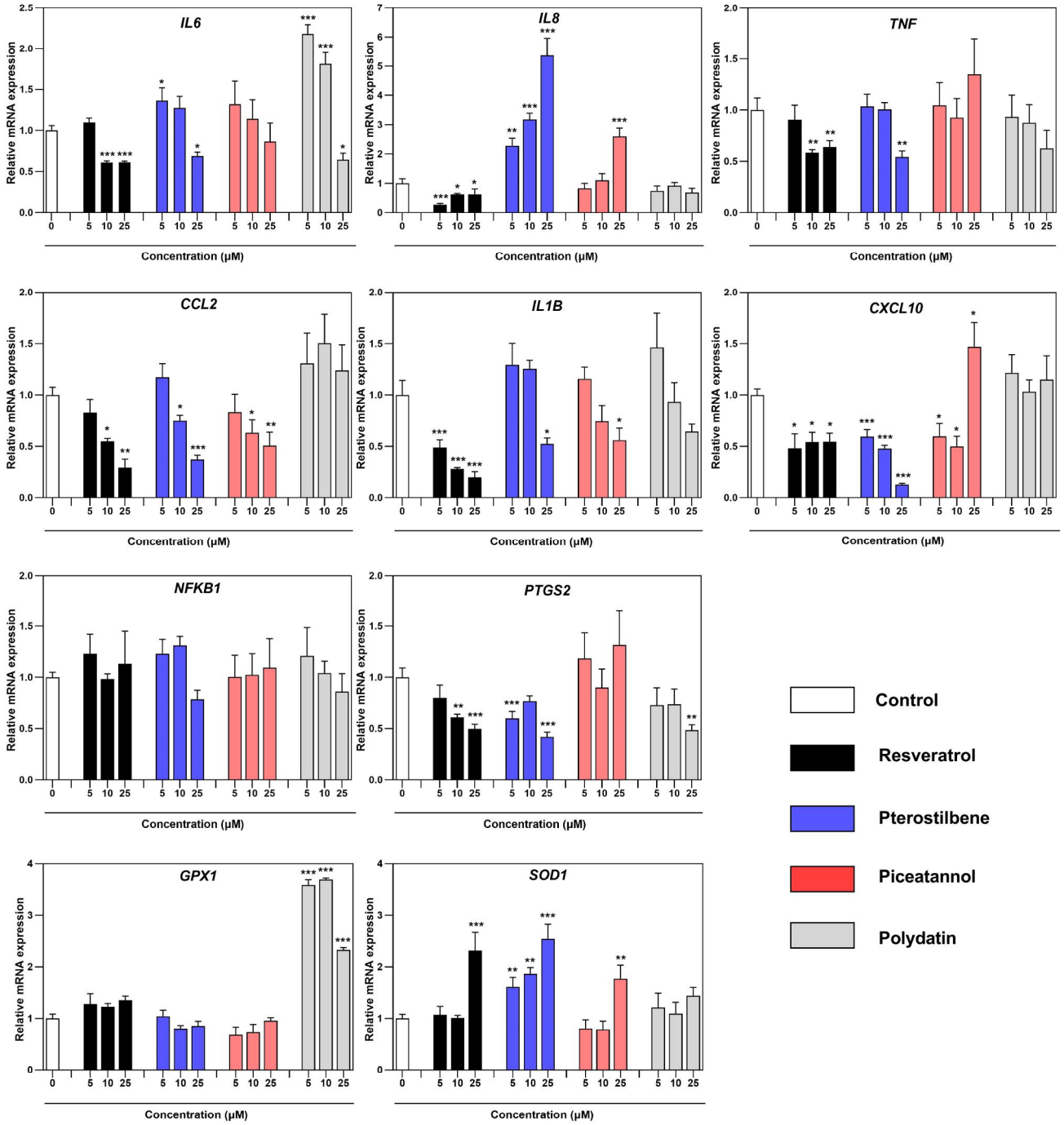


Figure 5. Effect of resveratrol and its analogs on the mRNA expression of *CCL2*, *CXCL10*, *GPX1*, *IL1B*, *IL6*, *IL8*, *NFKB1*, *PTGS2*, *SOD1*, and *TNF* genes in macrophages co-cultured with endometriotic cells, assessed by real-time PCR. Data were normalized against *GAPDH* and expressed as relative mRNA expression (-fold of control). The means ± SD of three independent experiments performed in triplicates are displayed. Statistical analysis assessed differences between the exposed cells and the negative control with significant differences * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

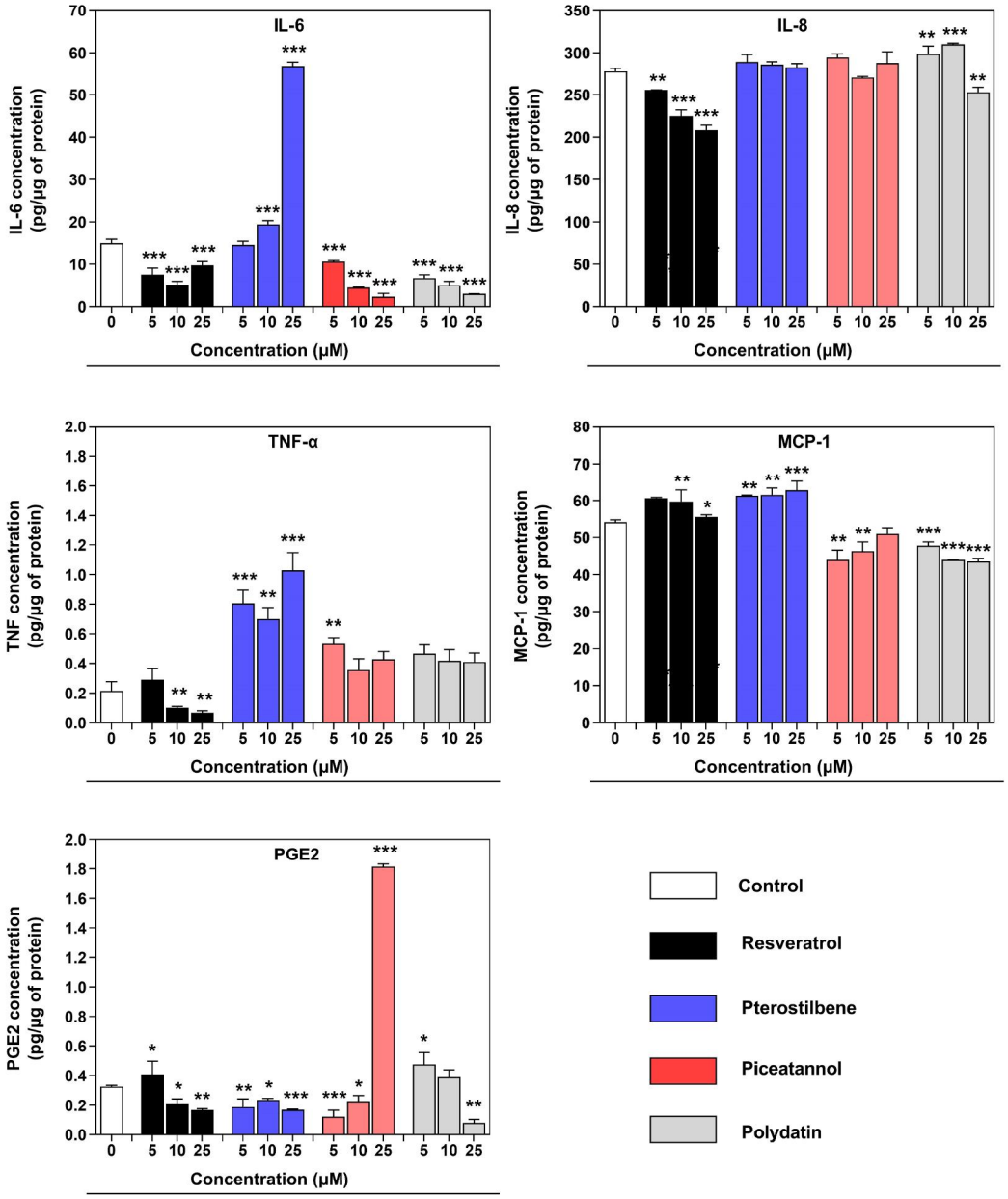


Figure 6. Effect of resveratrol and its analogs on the cytokine/chemokine release in a co-culture model of macrophages and endometriotic cells, assessed by Human Inflammatory Cytokine CBA and ELISA. Data were normalized per µg of proteins in each cell sample. The means ± SD of three independent experiments performed in triplicates are displayed. Statistical analysis assessed differences between the exposed cells and the negative control with significant differences * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.5. Effect of Resveratrol and Its Derivatives on the Intracellular ROS Generation in an Inflamed Co-Culture Model of Macrophages and Endometriotic Cells

Intracellular ROS production by macrophages co-cultured with endometriotic cells was examined using CellROX® Deep Red Reagent, a non-fluorescent dye that becomes fluorescent upon oxidation by ROS. Cell viability was monitored using Sytox® Green Dead Stain, which is impermeable to living cells. Double-stained cells were analyzed by flow cytometry to detect two specified parameters: oxidative stress and cell viability. ROS-dependent oxidation and viability of cells were followed in real-time during quantifying by flow cytometry (Figure 7). The CellROX® signal was detected on the internal cell membrane and in the cytosol. The ROS signal was distributed in clusters across different

cell parts, eventually merging until the cytosol was fully occupied. Using multiplexed reagents, we differentiated live stressed cells from dead cells; therefore, three distinct sub-populations of cells were specified: live non-stressed cells, live oxidatively stressed cells, and dead cells. Examples of the single-cell images from these sub-populations are presented in Figure 7A. The results in Figure 7B,C show that resveratrol and its analogs dose-dependently increased the population of non-stressed live cells and decreased the sub-population of macrophages producing ROS. Even while exposed to the lowest concentration (5 μM) of all analyzed compounds, a significant rise in the non-stressed population was observed. Pterostilbene was the most effective in reducing the ROS-positive fraction of cells. We assessed ROS levels by determining the fluorescence intensity of CellROX® Deep Red using flow cytometry to investigate the effect of resveratrol and its analogs on ROS generation. Using fluorescence intensity as a read-out of radical production, the highest dose of piceatannol and pterostilbene was found to most effectively prevent ROS production by macrophages by 6- and 7.6-fold, respectively (Figure 7D). Quantification of the fluorescence signal confirmed that all analyzed compounds significantly diminished the accumulation of free radicals.

(**A**)

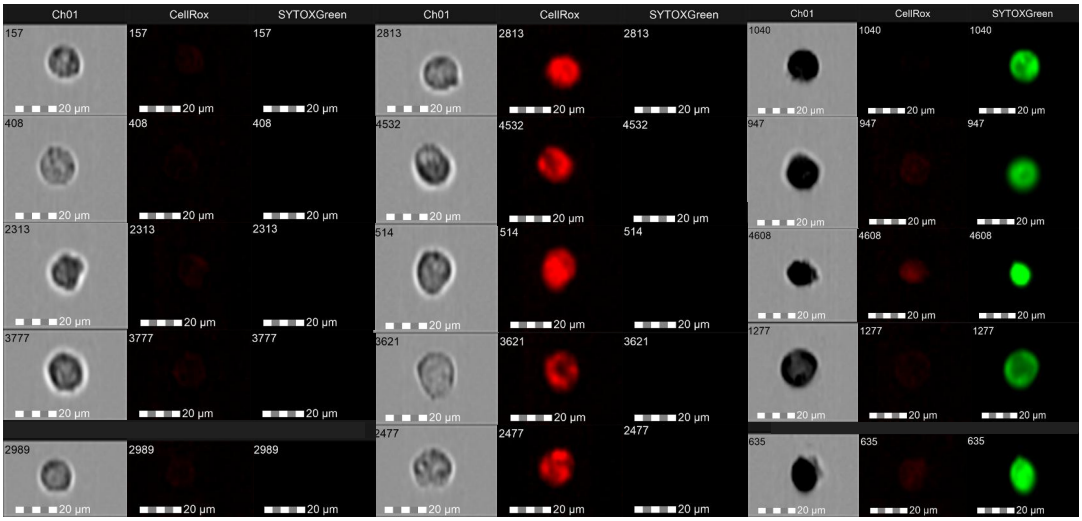


Figure 7. *Cont.*

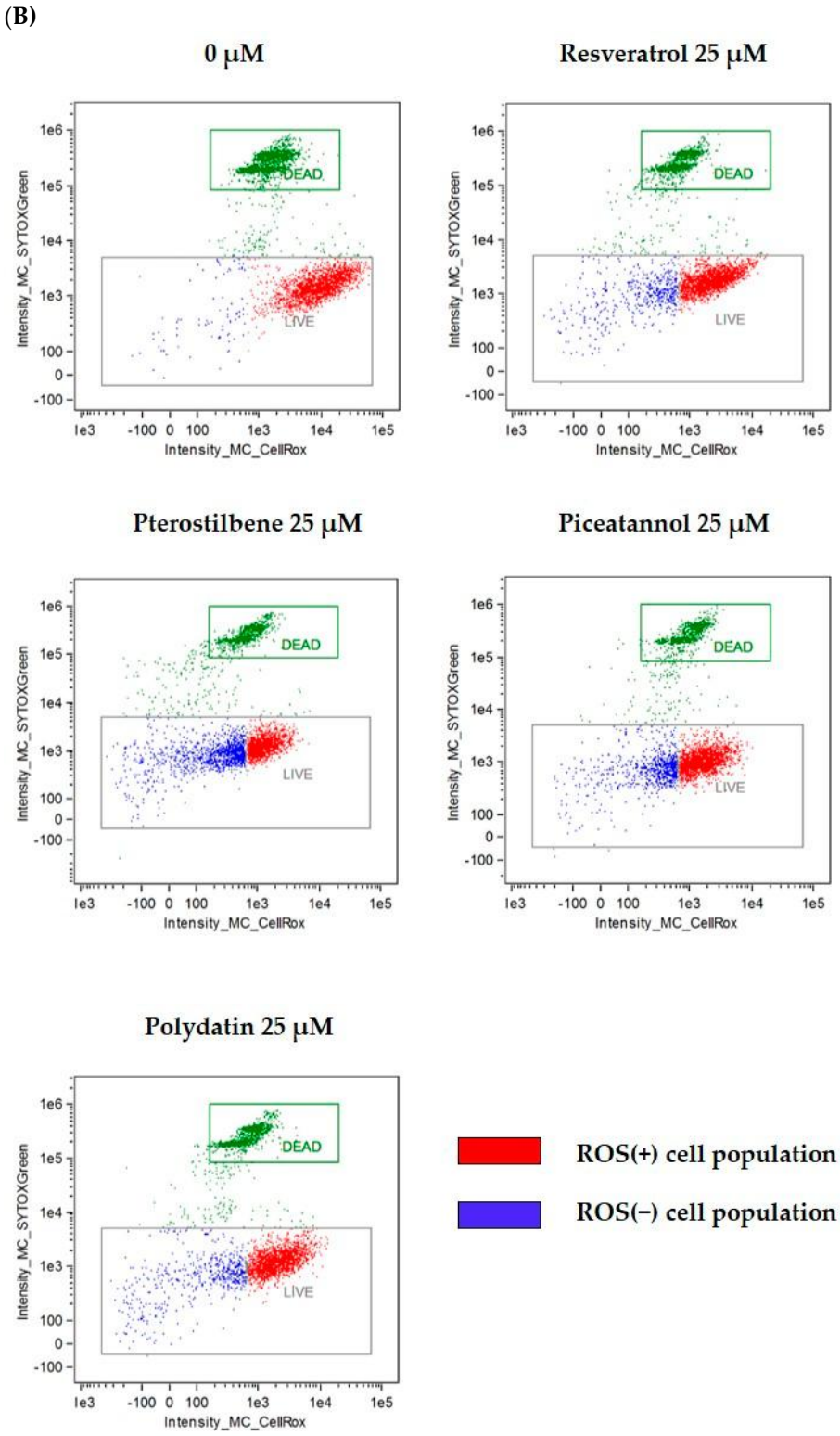


Figure 7. *Cont.*

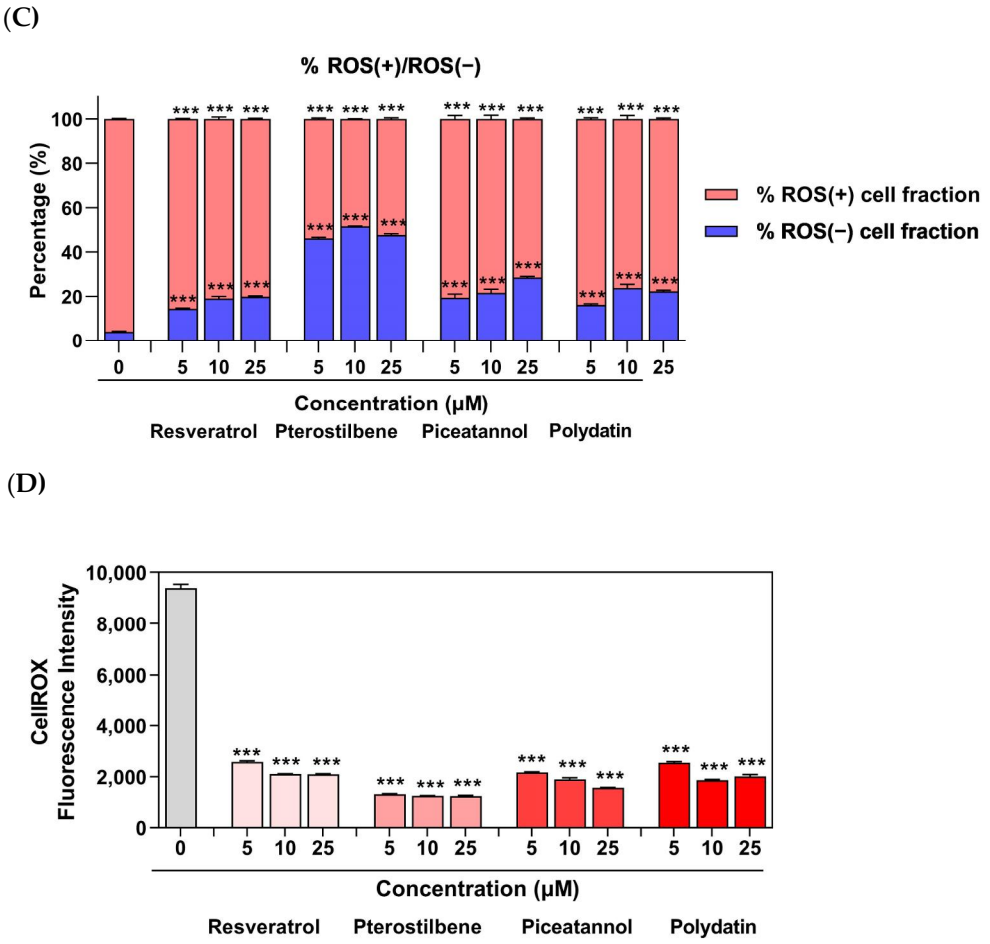


Figure 7. Flow cytometry analysis of macrophages cocultured with 12Z cells stained with CellROX® Deep Red Reagent and Sytox® Green Dead stain. Cells were treated with resveratrol, pterostilbene, piceatannol, polydatin, and their vehicle (0.5% ethanol) for 24 h. (**A**) Representative images of each distinct cellular subset. $n = 4$; scale bars = 20 μm. (**B**) Representative flow cytometry dot plots. (**C**) Summary of CellROX® Deep Red Reagent and Sytox® Green Dead stained populations in macrophages. Data show the percentage distribution of two fractions: live non-stressed cells and live oxidatively stressed cells. (**D**) Quantitative analysis of fluorescence intensity of CellROX® Deep Red. Statistical analysis assessed differences between the exposed cells and the vehicle control with significant differences *** $p \leq 0.001$.

4. Discussion

Endometriosis clinical presentation is often identified with chronic inflammation in the pelvic cavity [24]. Increased monocytes and macrophages in the peritoneal fluid of endometriosis may release a wide range of components, including prostaglandins, cytokines, growth factors, iron, hormones, and enzymes, which are presumed to be the essence of disease initiation and progression [25]. Endometriosis and cancer share a common inflammatory pathogenesis involving TNF-α, IL-1, IL-6, IL-8, and PGE2. These are related to macrophage activation, retrograde menstruation, iron overload, and triggering aberrant inflammatory signaling [26]. In actuality, the dualistic concept of macrophage polarization has been discussed because it may not reflect the dynamic immune environment in vivo [27].

In the peritoneum lesions, there are mixtures of recruited resident and differentiated macrophages; thus, the classification of M1/M2 macrophages may be oversimplified. As it is challenging to replicate the complexity of macrophages using an in vitro cell-based tool, the present study applied proinflammatory phenotypes of monocyte-derived macrophages.

To mimic the condition of the inflamed state, a co-culture of endometriotic 12Z cells and macrophages derived from THP-1 was established.

So far, only several in vitro co-culture models have been developed to imitate endometriosis with the immune system represented. To establish immuno-active co-cultures, human endometrial epithelial and stromal cells (HES, HESC), human endometriotic epithelial and stromal cells (12Z, 22B), and THP-1 cells differentiated into macrophages were used. The endometriosis-associated macrophages were macrophages exposed to the conditioned medium obtained from human endometriotic cell cultures [28]. In the studies on the interactions between macrophages and endometriotic epithelial cells, THP-1 cells were differentiated and polarized, and then, 12Z cells were treated with the conditioned media taken from M0, M1, and M2 macrophage cultures [29]. The experimental protocol based on the co-culture of proinflammatory macrophages and endometriotic cells, which was used in this work, allowed for the mutual activation of macrophages and endometriotic cells via soluble factors and enabled the obtaining of more physiologically relevant findings regarding the response of endometriotic microenvironment to anti-inflammatory substances.

The differentiation and polarization protocol for macrophages was optimized in this study, and favorable growth conditions were selected to establish their co-culture with endometriotic cells. The co-culture setup was chosen depending on the doubling rate of the cell line used and the planned length of the experiment, according to the recommendations previously reported [19]. As highlighted, several factors can impact the outcomes of co-cultures with macrophages between research groups, like PMA concentration and differentiation period, LPS and IFN-γ concentration, polarization time, cells' seeding density, and time of simultaneous co-culture [20,30]. These variabilities might explain different results in the co-culture establishment and systemic response to the treatment.

Our objective in this study was to assess potential differences between resveratrol and its analogs regarding the anti-inflammatory curative potential and resolution of oxidative stress. In studies on tumors, resveratrol has been proven to downregulate the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, and mitigating the inflammatory milieu within the tumor microenvironment hinders the development of angiogenesis, tissue invasion, and metastasis processes [31]. In addition, resveratrol promotes antioxidant defense pathways in several cancer cell lines by eliminating free radicals, enhancing the activities of SOD, CAT, and GPX [17]. As endometriosis behaves malignantly, our study was undertaken to unravel the effect of resveratrol and its analogs in the inflamed model of this disease. The results shown in this article demonstrate that resveratrol and its analogs can suppress the expression of transcript copies of key pro-inflammatory regulators. As already mentioned, not all induced genes followed the same pattern of downregulation depending on the analyzed compound. However, some genes, such as *IL6*, *IL1B*, and *CCL2*, displayed particular responsiveness to resveratrol, pterostilbene, and piceatannol. Resveratrol, pterostilbene, and polydatin, at 25 μM, led to a significant reduction in *IL6* expression. Macrophages in endometriosis produce IL-6, which is implicated in monocyte recruitment and, together with its receptor (IL-6R), regulates endometrial stromal cell growth in vitro [32]. The inhibitory effect on the IL-6 concentration in the co-culture supernatant was observed in our studies after treatment with resveratrol, piceatannol, and polydatin. The outstanding result of the IL-6 concentration after treatment with pterostilbene may be associated with the stimulation effect of endometriotic cells to produce high amounts of IL-6 by 12Z-associated macrophages described in the similar model based on the conditioned media experiments [28]. The immunological role of IL-1β is to stimulate macrophages for the synthesis of IL-6 and activation of COX-2 and enhancement of PGE2 levels, contributing to the synthesis of estradiol by binding of steroidogenic factor 1 (SF-1) to promoters of steroidogenic and aromatase genes [33]. However, the content of this cytokine in the peritoneal fluid of women with endometriosis is still under discussion [32]. The exposure of co-culture to the resveratrol has a prominent effect on reducing *IL1B* expression. The general response of *CCL2* expression remained the same for the resveratrol, pterostilbene, and piceatannol at 10 μM and 25 μM concentrations. Meanwhile, piceatannol

and polydatin noticeably reduced their expressions. MCP-1, a well-known chemoattractant cytokine for monocytes/macrophages, is elevated in the peritoneal fluid in women with endometriosis and can act in a paracrine and autocrine on macrophages [32]. The increased level of MCP-1 noted in the co-culture supernatant was probably related to MCP-1 secretion by endometriotic 12Z cells. It was previously reported that MCP-1 secreted from endometriotic epithelial cells recruits macrophages in endometriotic lesions [28]. Another key chemoattractant in endometriosis is IL-8; in our study, *IL8* gene expression and IL-8 protein secretion into co-culture supernatant were reduced only after incubation with resveratrol at all analyzed concentrations.

COX-2, a rate-limiting enzyme in PGE2 production, is overexpressed in endometriotic tissues inducible by inflammation and other pathogenic stimuli. COX-2 is an essential therapeutic target for anti-inflammatory drugs used in treating endometriosis-affected women, which evoke severe side effects such as gastrointestinal, renal, disorders, and cardiovascular toxicity [34]. In light of this, polyphenols may provide alternative nonsteroidal anti-inflammatory drugs because they often are recognized as COX-2 selective [34,35]. Our studies demonstrate that resveratrol, pterostilbene, and polydatin notably reduced COX-2 (*PTGS2*) expression in macrophages co-cultured with endometriotic cells. A qualitatively similar response was observed for the PGE2 concentration in the co-culture supernatant, and the highest concentration of polydatin evoked the most substantial effect.

TNF-α, a product of macrophages, was transcriptionally decreased by the treatment with resveratrol and pterostilbene in our co-culture model. However, the release of this cytokine into the culture medium was blocked only by a maternity compound –resveratrol. Even though TNF-α is a physiological cytokine in the proliferative and secretory-phase endometrium, scientific data indicate a correlation between the concentration of TNF-α in peritoneal fluid and the stage of endometriosis [36].

Resveratrol is a known regulator of inflammatory mediator production through SIRT1 activation. SIRT1, an anti-inflammatory NAD+-dependent deacetylating enzyme, directly deacetylates the RelA/p65 of NF-κB on lysine 310, a crucial site for NF-κB transcriptional activity. Resveratrol potentially enhances the SIRT1 expression and facilitates its attachment to the RelA/p65 substrate. Another important observation revealed that resveratrol blocked NF-κB-regulated cIAP-2 transcription and sensitized cells to TNF-α-induced apoptosis [37]. In several studies, resveratrol treatment blocked nuclear translocation of the p65 subunit of NF-κB in myeloid U-937 cells [38], in TNF-induced prostate cancer PC-3 cells [39], and in human CD34+ cells [40]. Moreover, resveratrol targets include AMP-activated protein kinase (AMPK), whereas AMPK activation increases the NAD+/NADH ratio and triggers its downstream pathways, such as SIRT1 activity [41]. AMPK, a negative regulator of NF-κB, has been reported to be implicated in the inflammatory response in macrophages [42]. SIRT1 plays a direct regulatory role in macrophage functions during inflammation, both in the secretion of cytokines and the expression of cell adhesion molecules as intracellular cell adhesion molecules 1 (ICAM-1) [16]. Several in vitro studies on macrophages have confirmed that resveratrol and its analogs, e.g., piceatannol, upregulate *SIRT1* expression via nuclear translocation of NF-κB [43,44]. In this study, we observed no effect of resveratrol and its derivatives on the expression of the NF-κB transcription factor, a central regulator of the LPS, cytokine, and stress responses in multiple cell types, including macrophages. Studies on the THP-1 cell line have indicated that LPS rapidly induces NF-κB DNA-binding, persists until 12 h of incubation, and is lost entirely after 24 h [45]; therefore, the effect of analyzed compounds in our experiments was not noticeable. Future studies on the NF-κB mechanisms in endometriosis-associated macrophages under treatment with resveratrol and its analogs should clarify the regulation of signaling upstream molecules and their effect on the particular cytokine pathway. Regarding the limitations of in vitro observations, different time checkpoints and cell lines can be included to provide essential insights into the molecular mechanism by which resveratrol suppresses the NF-κB signaling pathway.

Several previous studies have elucidated the impact of resveratrol on the immune response of monocytes and macrophages cultured in vitro. Schwager et al. investigated

the resveratrol effect in peripheral blood leukocytes, human umbilical vein endothelial cells, and murine RAW 264.7 and human THP-1 macrophages. Their research presented that resveratrol at a concentration of 25 μM impaired NO production in LPS-activated RAW 264.7 macrophages and exerted significant inhibitory effects on PGE2. Moreover, resveratrol at 25–50 μM markedly reduced the production of IL-6, IL-12(p70), TNF-α, and GM-CSF. At doses as low as 6.25 μM, the effects evoked by resveratrol were more pronounced at the transcription level than protein secretion. The consistent regulation of inflammatory processes was noticed with THP-1 cells, which indicated no species-specific relationships. At 25 μM, resveratrol almost entirely dampened the PGE2 production in LPS-activated THP-1 cells. Furthermore, IL-1β, IL-6, and *CXCL10*/IP-10 were significantly reduced. Regarding gene expression regulation, *IL1B*, *IL6*, *TNF*, and chemokines, including *CCL2* and *CXCL10*, were clearly down-regulated. Interestingly, similarly to our studies, the expression level of *CXCL8* was not decreased by resveratrol treatment. It should be noted that, in the research reported, RNA was extracted from THP-1 cells after 4 h of activation, and cell culture supernatants were recovered after 24 h [46]. Likewise, in another study, resveratrol (5–25 μM) pretreatments markedly reduced *IL6* and *PTGS2* mRNA levels in LPS-stimulated cells but did not influence *IL1B* and *CXCL8* transcripts. The increased IL-6 protein secretion by LPS-treated differentiated THP-1 cells was also lowered by treatment with resveratrol at concentrations ranging from 10 to 25 μM. Moreover, the inhibitory effect of pterostilbene was documented in the differentiated LPS-induced THP-1 macrophages, in which IL-6 was down-regulated at the mRNA and protein levels [47].

Several promising and consistent in vitro studies have shown that resveratrol has beneficial effects on the mono-culture of endometriotic cells regarding its anti-inflammatory properties. Kolahdouz-Mohammadi et al. were the first to describe the inhibitory effect of resveratrol therapy on MCP-1, IL-6, IL-8, and RANTES expression in ectopic endometrial stromal cells of endometriotic women [48]. Similar studies with endometriotic stromal cells demonstrated that resveratrol treatment significantly suppressed TNF-α-induced IL-8 release through endogenous SIRT1 activation [49]. However, the significant effects of resveratrol and its analogs on the inflammatory pathology in endometriosis have been described for the first time in the present work. The use of endometriotic cells together with macrophages underlined the importance of establishing a complex in vitro model, which addresses interactions between cells in the endometriotic microenvironment. The obtained results expand current knowledge about the anti-inflammatory properties of the stilbenes, examined on the mono-culture of endometriotic cells or immune cells.

Macrophages, erythrocytes, and apoptotic endometriotic cells are found to induce oxidative stress in endometriosis. ROS and pro-inflammatory factors are connected, contributing to pain and the failure to detoxify lipid peroxidase products under oxidative stress. Pro-inflammatory and chemotactic cytokines play a significant role in the recruitment and activation of phagocytic cells, which are the leading producers of ROS [50]. With the deepening of oxidative stress in the pelvic cavity, harmful damage, like tissue injury and chronic inflammation, is beginning to appear, resulting in the proliferation and fibrosis of ectopic endometrial lesions [33]. Our findings indicate that all analyzed stilbenes significantly reduced ROS accumulation in macrophages co-cultured with 12Z cells. A reduced fraction of ROS-positive cells was detected even with the treatment of the lowest concentration of the stilbenes (5 μM). It is worth emphasizing that the reduction in cells excessively producing ROS was observed under pterostilbene treatment by about 50%. These findings are correlated with the increasing expression of the antioxidant enzyme SOD1 in macrophages. These results are consistent with evidence suggesting that aberrant changes in endogenous antioxidant enzymes SOD as the first line of defense against oxygen free radicals and GPX—a major peroxide-scavenging enzyme—can contribute to the oxidative damage in endometriosis and be proposed as a biomarker for endometriosis [51,52].

Like other model in vitro experiments, our studies have some limitations that should be pointed out when discussing the findings. First, in the experimental system, we included two types of cell lines. However, the endometriosis niche is known to exist in a complex

environment with a dynamic population of epithelial, stromal, immune, endothelial, and glandular cells [53]. On the other hand, validating the specific pathology, like altered inflammatory processes in the disease, mostly requires using cells, which are the most committed. Secondly, only one phenotype of macrophages was used to form co-culture with endometriotic cells. To validate the present results more significantly, further studies involving naive (M0) and alternatively activated (M2) macrophage populations are needed to represent a more comprehensive immunodeficiency manifestation of the disease. Additionally, which phenotype of macrophages is responsible for disease progression is still debated. Some analyses highlight that the pro-inflammatory macrophage population mainly occurs in the early stages of the disease, whereas the switch to M2-polarization appears in advanced stages together with pro-fibrotic activity [54,55]. The point that is often emphasized is that many physiological or pathological macrophages do not show a clear M1 or M2 phenotype, and using the terms M1 and M2 with a specific phenotypic scoring criterion is confusing. Moreover, in that type of study, the verification of properties of natural compounds should be addressed to improve the bioaccessibility of the drug by considering their gastrointestinal digestion and metabolism by gut microorganisms [6]. Therefore, in our experiments, we investigated the immunomodulatory effects of resveratrol and its derivatives at low physiologically attainable concentrations, based on the last work of Feng et al., who observed in vitro and in vivo physiological (5 μM) and pharmacological (>10 μM) effects of resveratrol on THP-1 cells and rodent model [47,56].

5. Conclusions

Summarizing our findings and considering the conclusions from the reported studies on inflamed co-culture of pro-inflammatory macrophages and endometriotic cells, we can state that resveratrol and its analogs can target immune dysregulation and oxidative imbalance in endometriosis. Most of the observed changes in gene expression, cytokine/chemokine concertation, and real-time production of ROS support this hypothesis, with some notable exceptions. Differences in the anti-inflammatory and antioxidant efficiency of the compounds confirmed that each stilbene can act preferentially on specific pathways in the cell. Our findings may open the way to evaluating alternative therapy and dietary support in endometriosis treatment and alleviating endometriosis-related disorders. Optimally designed functional food products enriched with stilbenes could be a valuable source of these compounds in the daily diet of women with endometriosis. However, further extended pre-clinical studies are necessary to provide strong evidence of resveratrol and its analogs' therapeutic potential. The research should be programmed to develop optimal stilbene delivery methods and improve their bioavailability, increasing effectivity and decreasing efficient administration doses [11,14]. In vitro studies on human cells provide data on the potential activity and predicted effectivity of natural compounds that may enhance their relevance and applicability in further extensive research. Despite promising results indicating that resveratrol and its analogs attenuate immunopathology and oxidative stress in endometriosis [57], these data cannot be directly translated into clinical effects. In addition, several recent clinical trials revealed the positive use of resveratrol as an adjuvant treatment against, e.g., endometriotic chronic pelvic pain [58,59]. Therefore, well-controlled further clinical trials are absolutely required to evaluate the therapeutic potential of stilbenes administered as functional food products, dietary supplements, or drug formulations.

Author Contributions: Conceptualization, A.G.-G. and A.O.; methodology, A.G.-G. and A.O.; validation, A.G.-G., W.J. and M.K.; formal analysis, A.O.; investigation, A.G.-G., W.J. and M.K.; data curation, A.G.-G.; writing—original draft preparation, A.G.-G.; writing—review and editing, A.O.; visualization, A.G.-G.; supervision, A.O.; project administration, A.G.-G.; funding acquisition, A.G.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially financed by the Ministry of Education and Science (Poland) within the project for young scientists at Poznan University of Life Sciences.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of this study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Critchley, H.O.D.; Babayev, E.; Bulun, S.E.; Clark, S.; Garcia-Grau, I.; Gregersen, P.K.; Kilcoyne, A.; Kim, J.-Y.J.; Lavender, M.; Marsh, E.E.; et al. Menstruation: Science and Society. *Am. J. Obstet. Gynecol.* **2020**, *223*, 624–664. [CrossRef] [PubMed]
2. Chambers, M.; Rees, A.; Cronin, J.G.; Nair, M.; Jones, N.; Thornton, C.A. Macrophage Plasticity in Reproduction and Environmental Influences on Their Function. *Front. Immunol.* **2021**, *11*, 607328. [CrossRef] [PubMed]
3. Greaves, E.; Temp, J.; Esnal-Zufiurre, A.; Mechsner, S.; Horne, A.W.; Saunders, P.T.K. Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis. *Am. J. Pathol.* **2015**, *185*, 2286–2297. [CrossRef]
4. Yang, H.-L.; Zhou, W.-J.; Chang, K.-K.; Mei, J.; Huang, L.-Q.; Wang, M.-Y.; Meng, Y.; Ha, S.-Y.; Li, D.-J.; Li, M.-Q. The Crosstalk between Endometrial Stromal Cells and Macrophages Impairs Cytotoxicity of NK Cells in Endometriosis by Secreting IL-10 and TGF-β. *Reproduction* **2017**, *154*, 815–825. [CrossRef]
5. Gołąbek, A.; Kowalska, K.; Olejnik, A. Polyphenols as a Diet Therapy Concept for Endometriosis-Current Opinion and Future Perspectives. *Nutrients* **2021**, *13*, 1347. [CrossRef]
6. Xu, Y.; Fang, M.; Li, X.; Wang, D.; Yu, L.; Ma, F.; Jiang, J.; Zhang, L.; Li, P. Contributions of Common Foods to Resveratrol Intake in the Chinese Diet. *Foods* **2024**, *13*, 1267. [CrossRef]
7. Murias, M.; Jäger, W.; Handler, N.; Erker, T.; Horvath, Z.; Szekeres, T.; Nohl, H.; Gille, L. Antioxidant, Prooxidant and Cytotoxic Activity of Hydroxylated Resveratrol Analogues: Structure–Activity Relationship. *Biochem. Pharmacol.* **2005**, *69*, 903–912. [CrossRef]
8. Krambeck, K.; Oliveira, A.; Santos, D.; Pintado, M.M.; Baptista Silva, J.; Sousa Lobo, J.M.; Amaral, M.H. Identification and Quantification of Stilbenes (Piceatannol and Resveratrol) in Passiflora Edulis By-Products. *Pharmaceuticals* **2020**, *13*, 73. [CrossRef]
9. Tanzil, A.D.; Al, E. Effects of Oral Intake of Piceatannol on Fat Burning—A Randomized, Double—Blind, Placebo—Controlled Crossover Comparison Study—. *Jpn. Pharmacol. Ther.* **2020**, *48*, 1235–1240.
10. Yan, W.; Ren, D.; Feng, X.; Huang, J.; Wang, D.; Li, T.; Zhang, D. Neuroprotective and Anti-Inflammatory Effect of Pterostilbene Against Cerebral Ischemia/Reperfusion Injury via Suppression of COX-2. *Front. Pharmacol.* **2021**, *12*, 770329. [CrossRef]
11. Nagarajan, S.; Mohandas, S.; Ganesan, K.; Xu, B.; Ramkumar, K.M. New Insights into Dietary Pterostilbene: Sources, Metabolism, and Health Promotion Effects. *Molecules* **2022**, *27*, 6316. [CrossRef] [PubMed]
12. Ravagnan, G.; De Filippis, A.; Cartenì, M.; De Maria, S.; Cozza, V.; Petrazzuolo, M.; Tufano, M.A.; Donnarumma, G. Polydatin, A Natural Precursor of Resveratrol, Induces β-Defensin Production and Reduces Inflammatory Response. *Inflammation* **2013**, *36*, 26–34. [CrossRef] [PubMed]
13. Potdar, S.; Parmar, M.S.; Ray, S.D.; Cavanaugh, J.E. Protective Effects of the Resveratrol Analog Piceid in Dopaminergic SH-SY5Y Cells. *Arch. Toxicol.* **2018**, *92*, 669–677. [CrossRef] [PubMed]
14. Karami, A.; Fakhri, S.; Kooshki, L.; Khan, H. Polydatin: Pharmacological Mechanisms, Therapeutic Targets, Biological Activities, and Health Benefits. *Molecules* **2022**, *27*, 6474. [CrossRef] [PubMed]
15. Tang, D.; Zhang, Q.; Duan, H.; Ye, X.; Liu, J.; Peng, W.; Wu, C. Polydatin: A Critical Promising Natural Agent for Liver Protection via Antioxidative Stress. *Oxid. Med. Cell Longev.* **2022**, *2022*, 9218738. [CrossRef] [PubMed]
16. Malaguarnera, L. Influence of Resveratrol on the Immune Response. *Nutrients* **2019**, *11*, 946. [CrossRef] [PubMed]
17. Meng, T.; Xiao, D.; Muhammed, A.; Deng, J.; Chen, L.; He, J. Anti-Inflammatory Action and Mechanisms of Resveratrol. *Molecules* **2021**, *26*, 229. [CrossRef]
18. Donnez, J.; Binda, M.M.; Donnez, O.; Dolmans, M.-M. Oxidative Stress in the Pelvic Cavity and Its Role in the Pathogenesis of Endometriosis. *Fertil. Steril.* **2016**, *106*, 1011–1017. [CrossRef] [PubMed]
19. Smith, M.P.; Young, H.; Hurlstone, A.; Wellbrock, C. Differentiation of THP1 Cells into Macrophages for Transwell Co-Culture Assay with Melanoma Cells. *Bio Protoc.* **2015**, *5*, e1638. [CrossRef]
20. Genin, M.; Clement, F.; Fattaccioli, A.; Raes, M.; Michiels, C. M1 and M2 Macrophages Derived from THP-1 Cells Differentially Modulate the Response of Cancer Cells to Etoposide. *BMC Cancer* **2015**, *15*, 577. [CrossRef]
21. Olejnik, A.; Rychlik, J.; Kidoń, M.; Czapski, J.; Kowalska, K.; Juzwa, W.; Olkowicz, M.; Dembczyński, R.; Moyer, M.P. Antioxidant Effects of Gastrointestinal Digested Purple Carrot Extract on the Human Cells of Colonic Mucosa. *Food Chem.* **2016**, *190*, 1069–1077. [CrossRef] [PubMed]
22. Kowalska, K.; Dembczyński, R.; Gołąbek, A.; Olkowicz, M.; Olejnik, A. ROS Modulating Effects of Lingonberry (*Vaccinium Vitis-Idaea* L.) Polyphenols on Obese Adipocyte Hypertrophy and Vascular Endothelial Dysfunction. *Nutrients* **2021**, *13*, 885. [CrossRef] [PubMed]

23. Golabek, A.; Kaczmarek, M.; Dondajewska, E.; Sakrajda, K.; Mackiewicz, A.; Dams-kozlowska, H. Application of a Three-dimensional (3D) Breast Cancer Model to Study Macrophage Polarization. *Exp. Ther. Med.* **2021**, *21*, 482. [CrossRef] [PubMed]
24. Hogg, C.; Horne, A.W.; Greaves, E. Endometriosis-Associated Macrophages: Origin, Phenotype, and Function. *Front. Endocrinol.* **2020**, *11*, 7. [CrossRef]
25. Scutiero, G.; Iannone, P.; Bernardi, G.; Bonaccorsi, G.; Spadaro, S.; Volta, C.A.; Greco, P.; Nappi, L. Oxidative Stress and Endometriosis: A Systematic Review of the Literature. *Oxid. Med. Cell Longev.* **2017**, *2017*, 7265238. [CrossRef]
26. Oală, I.E.; Mitranovici, M.-I.; Chiorean, D.M.; Irimia, T.; Crișan, A.I.; Melinte, I.M.; Cotruș, T.; Tudorache, V.; Moraru, L.; Moraru, R.; et al. Endometriosis and the Role of Pro-Inflammatory and Anti-Inflammatory Cytokines in Pathophysiology: A Narrative Review of the Literature. *Diagnostics* **2024**, *14*, 312. [CrossRef]
27. Ding, S.; Guo, X.; Zhu, L.; Wang, J.; Li, T.; Yu, Q.; Zhang, X. Macrophage-Derived Netrin-1 Contributes to Endometriosis-Associated Pain. *Ann. Transl. Med.* **2021**, *9*, 29. [CrossRef]
28. Woo, J.-H.; Yang, Y.-I.; Ahn, J.-H.; Choi, Y.S.; Choi, J.-H. Interleukin 6 Secretion from Alternatively Activated Macrophages Promotes the Migration of Endometriotic Epithelial Cells. *Biol. Reprod.* **2017**, *97*, 660–670. [CrossRef]
29. Sekulovski, N.; Whorton, A.E.; Shi, M.; MacLean, J.A.; Hayashi, K. Endometriotic Inflammatory Microenvironment Induced by Macrophages Can Be Targeted by Niclosamide. *Biol. Reprod.* **2019**, *100*, 398–408. [CrossRef]
30. Kämpfer, A.A.M.; Urbán, P.; Gioria, S.; Kanase, N.; Stone, V.; Kinsner-Ovaskainen, A. Development of an in Vitro Co-Culture Model to Mimic the Human Intestine in Healthy and Diseased State. *Toxicol. Vitr.* **2017**, *45*, 31–43. [CrossRef]
31. Talib, W.H.; Alsayed, A.R.; Farhan, F.; Al Kury, L.T. Resveratrol and Tumor Microenvironment: Mechanistic Basis and Therapeutic Targets. *Molecules* **2020**, *25*, 4282. [CrossRef] [PubMed]
32. Ramírez-Pavez, T.N.; Martínez-Esparza, M.; Ruiz-Alcaraz, A.J.; Marín-Sánchez, P.; Machado-Linde, F.; García-Peñarrubia, P. The Role of Peritoneal Macrophages in Endometriosis. *Int. J. Mol. Sci.* **2021**, *22*, 10792. [CrossRef] [PubMed]
33. Chen, S.; Liu, Y.; Zhong, Z.; Wei, C.; Liu, Y.; Zhu, X. Peritoneal Immune Microenvironment of Endometriosis: Role and Therapeutic Perspectives. *Front. Immunol.* **2023**, *14*, 1134663. [CrossRef]
34. Connolly, T.P. Cyclooxygenase-2 Inhibitors in Gynecologic Practice. *Clin. Med. Res.* **2003**, *1*, 105–110. [CrossRef]
35. Tassinari, V.; Smeriglio, A.; Stillittano, V.; Trombetta, D.; Zilli, R.; Tassinari, R.; Maranghi, F.; Frank, G.; Marcoccia, D.; Di Renzo, L. Endometriosis Treatment: Role of Natural Polyphenols as Anti-Inflammatory Agents. *Nutrients* **2023**, *15*, 2967. [CrossRef] [PubMed]
36. Ziętek, A.; Futyma, K.; Nowakowski, Ł.; Gogacz, M.; Rechberger, T. Progress on Macrophage's Proinflammatory Products as Markers of Acute Endometriosis. *J. Acute Dis.* **2015**, *4*, 169–172. [CrossRef]
37. Yeung, F.; Hoberg, J.E.; Ramsey, C.S.; Keller, M.D.; Jones, D.R.; Frye, R.A.; Mayo, M.W. Modulation of NF-κB-Dependent Transcription and Cell Survival by the SIRT1 Deacetylase. *EMBO J.* **2004**, *23*, 2369–2380. [CrossRef]
38. Manna, S.K.; Mukhopadhyay, A.; Aggarwal, B.B. Resveratrol Suppresses TNF-Induced Activation of Nuclear Transcription Factors NF-Kappa B, Activator Protein-1, and Apoptosis: Potential Role of Reactive Oxygen Intermediates and Lipid Peroxidation. *J. Immunol.* **2000**, *164*, 6509–6519. [CrossRef]
39. Benitez, D.A.; Hermoso, M.A.; Pozo-Guisado, E.; Fernández-Salguero, P.M.; Castellón, E.A. Regulation of Cell Survival by Resveratrol Involves Inhibition of NF Kappa B-Regulated Gene Expression in Prostate Cancer Cells. *Prostate* **2009**, *69*, 1045–1054. [CrossRef] [PubMed]
40. Jeong, J.-Y.; Silver, M.; Parnes, A.; Nikiforow, S.; Berliner, N.; Vanasse, G.J. Resveratrol Ameliorates TNFα-Mediated Suppression of Erythropoiesis in Human $CD34^+$ Cells via Modulation of NF-κB Signalling. *Br. J. Haematol.* **2011**, *155*, 93–101. [CrossRef]
41. Xu, L.; Botchway, B.O.A.; Zhang, S.; Zhou, J.; Liu, X. Inhibition of NF-κB Signaling Pathway by Resveratrol Improves Spinal Cord Injury. *Front. Neurosci.* **2018**, *12*, 690. [CrossRef] [PubMed]
42. Liu, X.; Wang, N.; Zhu, Y.; Yang, Y.; Chen, X.; Fan, S.; Chen, Q.; Zhou, H.; Zheng, J. Inhibition of Extracellular Calcium Influx Results in Enhanced IL-12 Production in LPS-Treated Murine Macrophages by Downregulation of the CaMKKβ-AMPK-SIRT1 Signaling Pathway. *Mediators Inflamm.* **2016**, *2016*, 6152713. [CrossRef] [PubMed]
43. Kawakami, S.; Kinoshita, Y.; Maruki-Uchida, H.; Yanae, K.; Sai, M.; Ito, T. Piceatannol and Its Metabolite, Isorhapontigenin, Induce SIRT1 Expression in THP-1 Human Monocytic Cell Line. *Nutrients* **2014**, *6*, 4794–4804. [CrossRef]
44. Yamamoto, T.; Li, Y.; Hanafusa, Y.; Yeh, Y.; Maruki-Uchida, H.; Kawakami, S.; Sai, M.; Goto, T.; Ito, T.; Kawada, T. Piceatannol Exhibits Anti-inflammatory Effects on Macrophages Interacting with Adipocytes. *Food Sci. Nutr.* **2016**, *5*, 76–85. [CrossRef] [PubMed]
45. Sharif, O.; Bolshakov, V.N.; Raines, S.; Newham, P.; Perkins, N.D. Transcriptional Profiling of the LPS Induced NF-κB Response in Macrophages. *BMC Immunol.* **2007**, *8*, 1. [CrossRef] [PubMed]
46. Schwager, J.; Richard, N.; Widmer, F.; Raederstorff, D. Resveratrol Distinctively Modulates the Inflammatory Profiles of Immune and Endothelial Cells. *BMC Complement. Altern. Med.* **2017**, *17*, 309. [CrossRef]
47. Feng, L.; Yasmeen, R.; Schoene, N.W.; Lei, K.Y.; Wang, T.T.Y. Resveratrol Differentially Modulates Immune Responses in Human THP-1 Monocytes and Macrophages. *Nutr. Res.* **2019**, *72*, 57–69. [CrossRef]
48. Kolahdouz-Mohammadi, R.; Shidfar, F.; Khodaverdi, S.; Arablou, T.; Heidari, S.; Rashidi, N.; Delbandi, A. Resveratrol Treatment Reduces Expression of MCP-1, IL-6, IL-8 and RANTES in Endometriotic Stromal Cells. *J. Cell. Mol. Med.* **2021**, *25*, 1116–1127. [CrossRef]

49. Taguchi, A.; Wada-Hiraike, O.; Kawana, K.; Koga, K.; Yamashita, A.; Shirane, A.; Urata, Y.; Kozuma, S.; Osuga, Y.; Fujii, T. Resveratrol Suppresses Inflammatory Responses in Endometrial Stromal Cells Derived from Endometriosis: A Possible Role of the Sirtuin 1 Pathway. *J. Obstet. Gynaecol. Res.* **2014**, *40*, 770–778. [CrossRef]
50. Didziokaite, G.; Biliute, G.; Gudaite, J.; Kvedariene, V. Oxidative Stress as a Potential Underlying Cause of Minimal and Mild Endometriosis-Related Infertility. *Int. J. Mol. Sci.* **2023**, *24*, 3809. [CrossRef] [PubMed]
51. Mulgund, A.; Doshi, S.; Agarwal, A. Chapter 25—The Role of Oxidative Stress in Endometriosis. In *Handbook of Fertility*; Watson, R.R., Ed.; Academic Press: San Diego, CA, USA, 2015; pp. 273–281, ISBN 978-0-12-800872-0.
52. Ekarattanawong, S.; Tanprasertkul, C.; Somprasit, C.; Chamod, P.; Tiengtip, R.; Bhamarapravatana, K.; Suwannarurk, K. Possibility of Using Superoxide Dismutase and Glutathione Peroxidase as Endometriosis Biomarkers. *Int. J. Womens Health* **2017**, *9*, 711–716. [CrossRef] [PubMed]
53. Gołąbek-Grenda, A.; Olejnik, A. In Vitro Modeling of Endometriosis and Endometriotic Microenvironment—Challenges and Recent Advances. *Cell. Signal.* **2022**, *97*, 110375. [CrossRef] [PubMed]
54. Laganà, A.S.; Salmeri, F.M.; Ban Frangež, H.; Ghezzi, F.; Vrtačnik-Bokal, E.; Granese, R. Evaluation of M1 and M2 Macrophages in Ovarian Endometriomas from Women Affected by Endometriosis at Different Stages of the Disease. *Gynecol. Endocrinol.* **2020**, *36*, 441–444. [CrossRef] [PubMed]
55. Kobayashi, H.; Imanaka, S. Understanding the Molecular Mechanisms of Macrophage Polarization and Metabolic Reprogramming in Endometriosis: A Narrative Review. *Reprod. Med. Biol.* **2022**, *21*, e12488. [CrossRef]
56. Wang, T.T.Y.; Hudson, T.S.; Wang, T.-C.; Remsberg, C.M.; Davies, N.M.; Takahashi, Y.; Kim, Y.S.; Seifried, H.; Vinyard, B.T.; Perkins, S.N.; et al. Differential Effects of Resveratrol on Androgen-Responsive LNCaP Human Prostate Cancer Cells in Vitro and in Vivo. *Carcinogenesis* **2008**, *29*, 2001–2010. [CrossRef]
57. Sienko, A.; Cichosz, A.; Urban, A.; Smolarczyk, R.; Czajkowski, K.; Sienko, J. The Effect of Two Anti-Inflammatory Dietary Components, Omega-3 and Resveratrol, on Endometriosis. *Ginekol. Pol.* **2024**, *95*, 573–583. [CrossRef]
58. Indraccolo, U.; Indraccolo, S.R.; Mignini, F. Micronized Palmitoylethanolamide/Trans-Polydatin Treatment of Endometriosis-Related Pain: A Meta-Analysis. *Ann. Ist. Super. Sanita* **2017**, *53*, 125–134. [CrossRef]
59. Mendes da Silva, D.; Gross, L.A.; de Neto, E.P.G.; Lessey, B.A.; Savaris, R.F. The Use of Resveratrol as an Adjuvant Treatment of Pain in Endometriosis: A Randomized Clinical Trial. *J. Endocr. Soc.* **2017**, *1*, 359–369. [CrossRef]

Journal of Functional Foods 140 (2026) 107277

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Stilbene-based strategies for the management of endometriosis: targeting cell adhesion, migration, and ECM-driven invasion

Agata Gołąbek-Grenda, Anna Olejnik *

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego St., 60-627 Poznan, Poland

ARTICLE INFO

Keywords:
Stilbenes
Resveratrol analogs
Endometriosis
12Z endometriotic cells
ECM proteolysis
Metalloproteinases

ABSTRACT

Endometriosis develops when endometrial fragments implant in the peritoneal cavity, overcome structural barriers, or enter through existing tissue injury, leading to the formation of ectopic endometrium-like tissue. This study aimed to investigate the therapeutic potential of resveratrol and its natural analogs in modulating migratory, invasive, and adhesive interactions of endometriotic cells. Adhesion to ECM components, such as collagen I and fibronectin, was assessed by crystal violet staining and showed that stilbenes reduced cell attachment more pronouncedly on fibronectin-coated surfaces. Migration and invasion through Matrigel-coated chambers were dose-dependently suppressed by all compounds, with pterostilbene and piceatannol exerting the most potent inhibitory effects. Expression and secretion of key ECM proteolysis mediators, MMP-2 and TIMP-2, analyzed by qRT-PCR and ELISA, were modulated by stilbene treatment. These findings highlight the potential of resveratrol and its analogs as promising agents for preventing local growth, migration, and invasion of endometriotic cells.

1. Introduction

The presence and growth of endometrial-like epithelial and stromal tissue in extrauterine locations, such as the abdominal–pelvic peritoneum and visceral organs, is termed endometriosis (Mason et al., 2020). It is prevalent mainly in reproductive-age women; up to 10–15% and increases dramatically up to 25–50% in women with infertility (Macer & Taylor, 2012; Malvezzi et al., 2020). Patients with endometriosis can experience chronic or cyclic pelvic pain, dysmenorrhea, dysuria, dyspareunia, symptoms of the digestive tract, and related fertility problems (Ek et al., 2015; Macer & Taylor, 2012). Endometriotic and normal eutopic endometrial tissue share some histological features; however, distinct anomalies have also been reported in cell proliferation, tissue structure, immune response, vascularization, and cytokine and steroid production (Sharpe-Timms, 2001). It is emphasized that endometriosis exhibits several characteristics of malignant neoplasms, including increased cell survival, cell invasion, migration, inflammation, and angiogenesis (Dawson et al., 2018).

The typical theory of disease establishment posits that depleted menstrual endometrial cells translocate through the fallopian tubes and adhere to sites of ectopic implantation on peritoneal surfaces or other distant tissues, where they subsequently proliferate and invade (Nisolle et al., 2000). The specific mechanisms underlying the adhesion and invasion of endometrial cells within the peritoneal lining are still a matter of debate. Indeed, it remains unresolved whether endometrial stromal or epithelial cells can attach to the peritoneum, or whether the mesothelium prevents the adhesion of shed endometrial tissue (Griffith et al., 2010; Wang et al., 2011; Witz et al., 1999). The extracellular matrix (ECM) of the peritoneum is a highly dynamic network of proteins and soluble factors as membrane fibrous proteins (collagens I, III, IV, and elastin), proteoglycans, and adhesive proteins (laminin, fibronectin, vitronectin, and tenascin) redistributed at different proportions at their base membrane's surface and deeper dense connective tissue (Adachi et al., 2011; Klemmt et al., 2007; Witz et al., 2001). ECM degradation, the initial event in the onset of endometriosis, is primarily facilitated by zinc-dependent endopeptidases – metalloproteinases (MMPs). Excess MMPs is found in both eutopic and ectopic endometriotic tissue (Luddi et al., 2020) as well as in the serum and peritoneal fluid of endometriosis

Abbreviations: α-SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor; ECM, extracellular matrix; MMPs, metalloproteinases; MTA1, metastasis-associated protein 1; NF-κB, nuclear factor-κB; NuRD, nucleosome remodeling and deacetylase; Rac1/WAVE/Arp2/3, Ras-related C3 botulinum toxin substrate 1/WASP-family verprolin-homologous protein/Actin-related protein 2/3 complex pathway; STAT3, signal transducer and activator of transcription 3; TIMPs, tissue inhibitors of metalloproteinases; VEGF, vascular endothelial growth factor.

* Corresponding author.
E-mail addresses: agata.golabek@up.poznan.pl (A. Gołąbek-Grenda), anna.olejnik@up.poznan.pl (A. Olejnik).

https://doi.org/10.1016/j.jff.2026.107277
Received 18 December 2025; Received in revised form 18 March 2026; Accepted 30 March 2026
Available online 3 April 2026

patients (Huang et al., 2004). There is growing evidence indicating that elevated levels of specific MMPs (MMP-1; -2; -3; -7; -9; -10; -11; -12; -23; -26) are responsible for ECM remodeling in the peritoneal environment of endometriosis, regulating key processes such as invasion, angiogenesis, and fibrosis (Ke et al., 2021). The imbalance caused by excessive MMP activity leads to unregulated matrix degradation, which is exacerbated by reduced tissue inhibitors of metalloproteinases (TIMPs), acting as enzyme inhibitors (Gottschalk et al., 2000).

Since endometrial cell attachment to pelvic intra-abdominal surfaces and invasion of the mesothelial ECM are crucial steps in the development of endometriotic lesions, they represent important targets for novel therapeutic strategies. Alternative therapeutic approaches leverage natural, edible bioactive compounds that can improve the clinical management of patients with endometriosis, especially given the escalating reports of unacceptable side effects and high recurrence rates of current medical treatments and interventions (Becker et al., 2017). In several preclinical and clinical studies, plant compounds derived from food have emerged as promising candidates for targeting dysregulated physiological processes, including increased cell proliferation, enhanced inflammatory response, neovascularization, apoptosis resistance, and disrupted steroid signaling. Additionally, a growing number of patients with endometriosis have highlighted the importance of dietary factors that can promote health and support their therapy (Gołąbek et al., 2021; Meresman et al., 2020; Ricci et al., 2013). The group of natural polyphenolic compounds confirmed for their bioactivity in endometriosis pre-clinical and clinical studies includes quercetin, apigenin, baicalein, wogonin, rosmarinic acid, genistein, daidzein, puerarin, naringenin, xanthohumol, epigallocatechin gallate, curcumin, and resveratrol (Gołąbek et al., 2021).

Resveratrol and its naturally occurring analogs have garnered significant attention for their potential therapeutic benefits in managing inflammatory and oncological conditions. However, their effects on treating endometriosis remain underexplored. The health-promoting properties of resveratrol are notably limited due to its relatively low bioavailability and unfavorable pharmacokinetic profile. As a result, several natural derivatives of resveratrol have been investigated for their potentially enhanced functional benefits. One such derivative, pterostilbene, is created by replacing two hydroxyl groups with methoxy groups. This modification increases its lipophilicity and contributes to its much higher oral bioavailability (80–95%), greater metabolic stability, slower glucuronidation, and longer half-life compared to resveratrol (P. Liu et al., 2024). Pterostilbene, presented in various edible fruits such as blueberries, grapes, cranberries, peanuts, has shown potential benefits in hindering and treating multiple cancers by controlling cell proliferation and inducing cell death, and limiting cancerous cells spread (P. Liu et al., 2024). Polyhydroxylated derivatives of resveratrol, such as piceatannol, exhibit improved water solubility, which results in quicker absorption, higher bioavailability, and increased metabolic stability compared to the parent molecule (Salla et al., 2024). Piceatannol is mainly found in blueberries, pomegranates, and passion fruit. It has garnered attention for its broad antioxidant and anti-inflammatory properties as well as for some beneficial metabolic, anti-atherosclerotic, and cardioprotective effects (Al-Jaber et al., 2024). Glycosylation, plays a crucial role in the accumulation of resveratrol within plant tissues. By attaching a sugar residue to the stilbene backbone, this process enhances the compound's hydrophilicity, bioactivity, and, most importantly, stability, which is particularly advantageous for delivering prodrug forms of such compounds from the food sources (Gachon et al., 2005). Polydatin, a glycosylated derivative of resveratrol that is abundantly found in Japanese knotweed, has also shown a wide range of biological activities in both in vitro and in vivo studies. These properties involve various mechanisms, including modulation of oxidative and inflammatory pathways, inhibition of apoptosis, improvement of lipid profiles, and regulation of energy metabolism in different diseases (Karami et al., 2022).

In previous studies, we examined the bioactivity of resveratrol and its natural food-derived analogs in their ability to prevent or reduce the pathological processes involved in endometriosis development. We investigated their effects on endometriotic cell proliferation and observed an increased incidence of apoptosis following treatment (Gołąbek-Grenda et al., 2023). Additionally, we analyzed changes in the expression of inflammatory mediators and oxidative stress markers, cytokine secretion, and reactive oxygen species generation in activated endometriosis-related macrophages after exposure to resveratrol and its analogs (Gołąbek-Grenda et al., 2024). As part of our research on the anti-endometriotic effects of resveratrol, pterostilbene, piceatannol, and polydatin, this study aimed to investigate their therapeutic potential to modulate the migratory, invasive, and adhesive interactions of endometriotic cells with ECM components.

2. Materials and methods

2.1. Chemicals

The high-purity compounds: resveratrol (>99%), pterostilbene (>97%), piceatannol (>98%), and polydatin (>98%), analyzed in this study, were supplied by Merck KGaA (Darmstadt, Germany). All other chemicals used in the experiments, unless stated otherwise, were also obtained from Merck KGaA.

2.2. Cell culture

Human immortalized endometriotic epithelial cells (12Z cells) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). These cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS) and 50 mg/L gentamycin. The cells were passaged when the culture reached 80–85% confluency by dissociating the cell monolayer with 0.05% trypsin-EDTA solution. The cells were grown at 37 °C in an air environment with 5% CO_2, and they were regularly checked for mycoplasma contamination. The 12Z cell line is a well-established model for endometriosis, derived from endometriotic tissue positive for N-Cadherin, a known marker of invasiveness (Zeitvogel et al., 2001).

2.3. Cytotoxicity assay

12Z cells were cultured in 96-well plates coated with rat tail collagen type I (0.05 mg/mL); the coating was performed according to the manufacturer's protocol. Briefly, a collagen solution was applied to the wells (5 μg/cm^2), incubated at 37 °C for 2 h, and then rinsed twice with PBS. Coated tissue culture dishes were used for cell seeding at an initial density of 2.5×10^4 cells/cm^2 (8.5×10^3 cells/well). The 24-h cell cultures were treated with stilbenes at concentrations of 5 μM, 10 μM, 25 μM, and 50 μM and incubated for 24 h under standard culture conditions. Working solutions of analyzed compounds were prepared in 96% ethanol. We added them to the culture medium, maintaining 0.5% alcohol concentration. The cytotoxicity of stilbenes was estimated using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay according to the previously described protocol with minor modifications (Gołąbek-Grenda et al., 2024; Olejnik et al., 2016). On the day of analysis, MTT solution (5 mg/mL) was added to the cell cultures, and they were incubated at 37 °C for 3 h. After incubation, the MTT-containing medium was removed, and the formed formazan was solubilized in DMSO for 30 min. The absorbance was measured at 540 nm using a Tecan M200 Infinite microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Collagen-coated wells containing medium without cells served as the blank control during the absorbance readings.

2.4. Adhesion assay

Millicoat™ Human Fibronectin/Collagen Type I Coated Strips (Millipore #ECM101104) were used for the adhesion assay, which was performed according to the manufacturer's protocol, previously used by other investigators (Hannan & Salamonsen, 2008; Ngamkitidechakul et al., 2003). Briefly, the strips were rehydrated with PBS for at least 15 min at room temperature (RT). The 12Z cells were seeded at a density of 2.5×10^4 cells/cm^2 (2.4×10^5 cells/well) and treated with stilbenes at concentrations of 5, 10, 25, and 50 μM. Then, the cells were gently detached with Non-enzymatic Cell Dissociation Solution and washed with PBS. After treatment, cells were plated at an optimal density of 2.9×10^5 cells/cm^2 (1×10^5 cells/well) and allowed to attach to fibronectin- and collagen type I-coated wells for 1 h. Following washing with PBS containing Ca^{2+}/Mg^{2+}, the adhesive cells were stained with 0.2% crystal violet for 5 min at RT. The stain was removed from the wells, followed by three washes with PBS. To enable visualization, the cell-bound stain was dissolved using Solubilization Buffer (a 1:1 mixture of 0.1 M NaH_2PO_4, pH 4.5 and 50% ethanol). The wells were then incubated with gentle rotation for 5 min at RT. Dye release was determined by the colorimetric method; absorbance was measured at 540 nm using a Tecan M200 Infinite microplate reader. The wells coated with bovine serum albumin served as negative assay controls for each treatment. The experiments were repeated three times with three replicates per experiment. After staining, the results were visualized under an inverted phase-contrast microscope (Axiovert 40C, Zeiss, Oberkochen, Germany).

2.5. Cell migration and invasion assays

The BD Falcon FluoroBlok Insert System and BD Biocoat FluoroBlok Invasion System (BD Bioscience, Bedford, MA, USA) were used to assess the migration and invasion potential of 12Z cells treated with stilbenes, as described in a previously published procedure (Olejnik et al., 2018; Partridge & Flaherty, 2009). The insert consists of FluoroBlok™ PET membranes (8 μm pore size), coated with a uniform layer of BD Matrigel™ matrix for invasion testing and uncoated for migration testing. After rehydration of the invasive chambers, cells were seeded at an optimal density of 2×10^4/well into the apical chambers and incubated in culture medium supplemented with the analyzed compounds at concentrations of 5 μM, 10 μM, 25 μM, and 50 μM. The serum gradient (DMEM/F12 medium with 10% FBS) was used to facilitate migration and invasion into the basal chambers. The plates were incubated at 37 °C in an air atmosphere with 5% CO_2 for 22 h.

Following incubation, the medium was removed, and the inserts were transferred into new 24-well plates containing Calcein AM fluorescent dye (BD Bioscience) at a concentration of 4 μg/mL in Hanks' buffered salt solution. The plates were then incubated for 1 h under standard culture conditions. Invasive cells were detected using a Tecan Infinite microplate reader at wavelengths of 485/530 nm, with data reported as relative fluorescence units. The migration and invasion potentials of 12Z cells treated with stilbenes were quantified as relative migration and invasion compared to the untreated vehicle control. The invasion index was calculated using the formula:

$$I_{index} = A/B \quad (1)$$

where A denotes the relative cell invasion (%) and B relative cell migration (%).

All experiments were conducted in triplicate and repeated twice. The endpoints of cell migration and invasion assays were verified using fluorescent microscopy (Axiovert 200, Zeiss, Oberkochen, Germany).

2.6. RNA extraction and real-time PCR

Total RNA was extracted from endometriotic cells using Trizol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions and a previously described protocol (Kowalska et al., 2021). The extracted total RNA was then reverse-transcribed into first-strand cDNA using the cDNA Transcriptor First-Strand kit (Roche Diagnostics GmbH; Penzberg; Germany) according to the supplier's procedure. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR® Select Master Mix (Life Technologies; Carlsbad; CA; USA). The following primers were used: β-actin forward primer: 5′-CCTTGCACATGCCG GAG-3′; β-actin reverse primer: 5′-GCACAGAGCCTCGCCTT-3′; MMP-2 forward primer: 5′-AGCGAGTGGATGCCGCCTTTAA-3′; MMP2 reverse primer: 5′-CATTCCAGGCATCTGCGATGAG-3′; TIMP-2 forward primer: 5′-ACCCTCTGTGACTTCATCGTGC-3′; TIMP-2 reverse primer: 5′-GGAGATGTAGCACGGGATCATG-3′. The mRNA levels of MMP-2 and TIMP-2 were normalized to the control gene β-actin, which is routinely applied in 12Z cells (Wendel et al., 2020). The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.7. Quantitation of MMP2 by ELISA

The concentration of matrix metalloproteinase 2 (MMP-2) in the cell culture supernatant was determined using an enzyme-linked immunosorbent assay (ELISA) - Total MMP-2 Quantikine ELISA (#MMP200) from R&D Systems (Minneapolis, MN, USA), with an assay range of 0.5–32 ng/mL, according to the supplier's standard procedure. The results were normalized to μg of cellular protein content, which was quantified using the Pierce® BCA Protein Assay Kit from Thermo Fisher Scientific (Waltham, MA, USA) according to the manufacturer's protocol.

2.8. Statistical analysis

The statistical analysis was performed using STATISTICA version 13.3 software (Statsoft, Inc., Tulsa, OK, USA). All data were assessed for normality and homoscedasticity. A one-way analysis of variance was conducted, followed by Tukey's post hoc test to examine differences among multiple groups. *P*-values <0.05, <0.01, and <0.001 were considered significant, with asterisks indicating the significance level. The data are presented as means ± standard deviation (SD).

3. Results

3.1. Effect of resveratrol and its analogs on the viability of endometriotic cells

The effects of resveratrol and its natural analogs on endometriotic cells viability was assessed using an MTT-based assay. 12Z cells were cultured for 24 h with either a vehicle or the analyzed compounds at concentrations of 5 μM, 10 μM, 25 μM, and 50 μM. Treatment with stilbenes at 5 μM, 10 μM, and 25 μM did not result in significant changes in cell viability (Fig. 1). However, resveratrol and polydatin at the highest concentration tested (50 μM) reduced cell viability by 20–30%.

3.2. Effect of resveratrol and its analogs on the MMP-2 and TIMP2 expression in endometriotic cells

The effects of resveratrol and its analogs on MMP-2 mRNA expression and protein secretion was measured in epithelial endometriotic 12Z cell cultures treated with stilbenes for 24 h. Treatment of 12Z cells with the highest tested dose (50 μM) of resveratrol (↓64%), pterostilbene (↓46%), and polydatin (↓55%) significantly reduced the number of MMP-2 transcript copies (Fig. 2A). Additionally, resveratrol at a lower concentration of 25 μM also inhibited MMP-2 mRNA expression (↓46%). In contrast, when the 12Z cells were treated with the lowest concentration (5 μM) of resveratrol derivatives, a slight increase in MMP-2

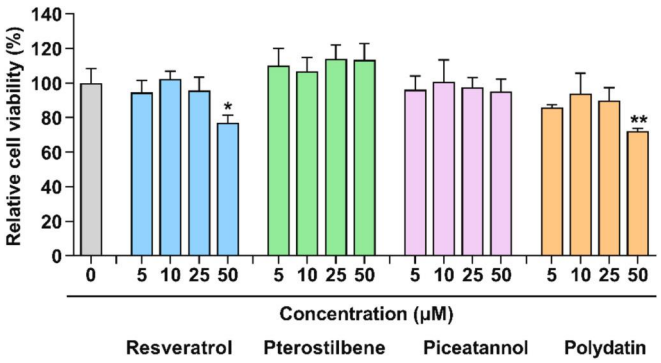


Fig. 1. The effect of resveratrol and its analogs on the viability of human endometriotic 12Z cells. The 12Z cells were treated for 24 h with either a vehicle (0.5% ethanol) or stilbenes at concentrations ranging from 5 to 50 μM. An MTT assay was conducted to determine cell viability. Results were expressed as relative cell viability, calculated relative to vehicle (control) ± SD. The significance of the effects of different stilbenes was evaluated using the post hoc Tukey test (* $p < 0.05$; ** $p < 0.01$).

mRNA expression was observed (Fig. 2A). Resveratrol and its derivatives significantly affected MMP-2 secretion, including both active MMPs and pro-MMPs, in the endometriotic cell cultures (Fig. 2C). After exposing 12Z cells to stilbenes, a dose-dependent decrease in MMP-2 concentration was observed. The most significant reduction in MMP-2 production occurred at 50 μM for each of the compounds tested. Notably, the inhibitory effects of stilbenes on MMP-2 secretion by 12Z cells were comparable across the different compounds. Compared with untreated cells, the MMP-2 concentration in cell cultures exposed to the highest stilbene dose decreased by approximately 2.0–2.8-fold (Fig. 2C).

In the study, the effects of stilbenes on TIMP2 mRNA expression were examined in 12Z cells (Fig. 2B). Treatment with resveratrol and pterostilbene significantly enhanced expression of TIMP2. Specifically, resveratrol and pterostilbene at 25 μM increased TIMP2 mRNA levels by 3.3- and 2.8-fold, respectively. A similar increase in TIMP2 expression was observed with piceatannol at 50 μM. However, treatment with polydatin, except at the highest dosage, showed no significant effect (Fig. 2B).

3.3. *Effect of resveratrol and its analogs on endometriotic cell adhesion to collagen and fibronectin*

To better understand the effects of resveratrol, pterostilbene, and piceatannol on the cellular events involved in migration and invasion, we investigated their impact on the endometriotic cell adhesion to ECM proteins. We incubated cells with varying concentrations of resveratrol and its analogs, then dissociated them and plated them on collagen- or fibronectin-coated surfaces. The results showed that all analyzed stilbenes impaired cellular adhesion to ECM components. Resveratrol similarly affected cell adhesion to both collagen and fibronectin (Fig. 3A and B).

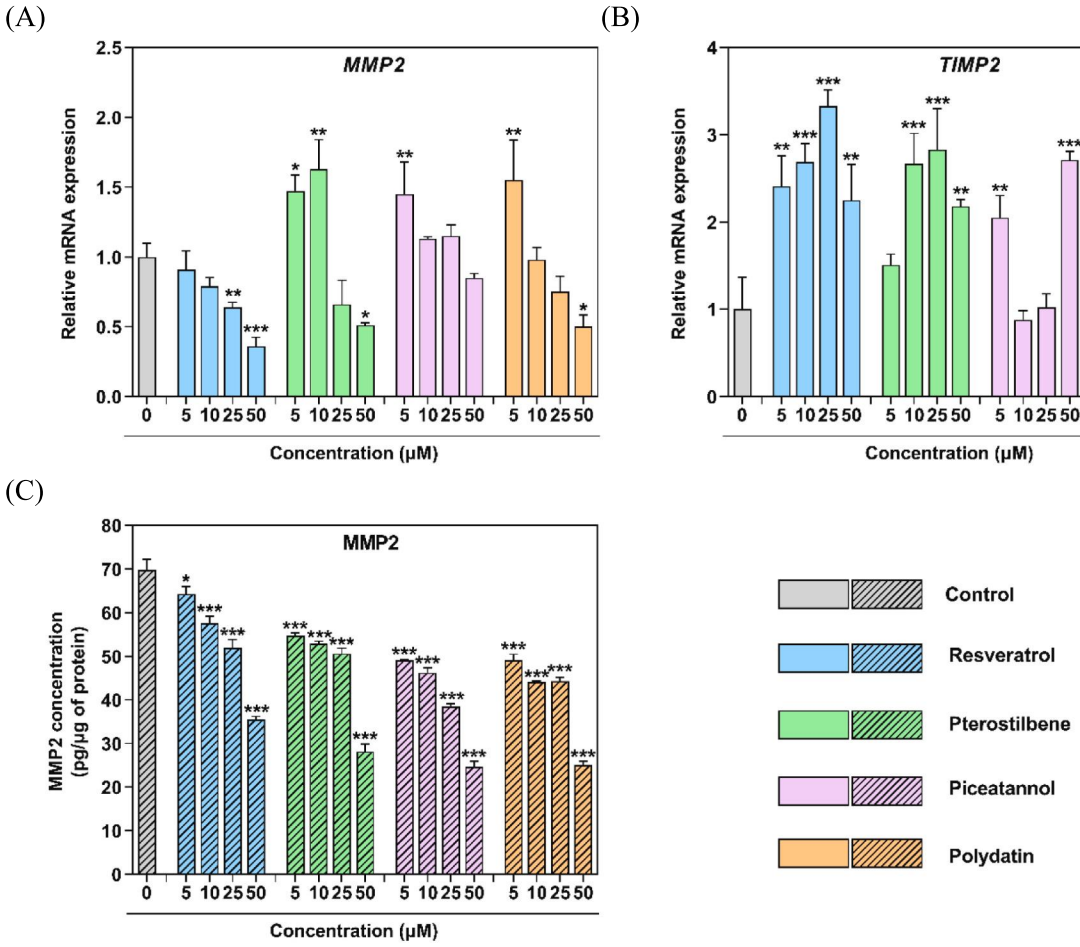


Fig. 2. Effect of resveratrol and its analogs on MMP2 (A) and TIMP2 (B) expression analyzed by qRT-PCR and MMP-2 secretion (C), analyzed by ELISA. The endometriotic 12Z cells were treated for 24 h with a vehicle or different concentrations of resveratrol and its analogs. (A, B) Data were normalized against β-actin and expressed as relative mRNA expression (−fold of control). (C) MMP-2 concentration was normalized per μg of proteins in each cell sample. Each bar represents the mean ± SD of three independent triplicated experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle control.

The adhesion of cells treated with resveratrol decreased dose-dependently, starting at 10 μM concentration. At the highest dose of 50 μM, resveratrol reduced adhesion to collagen- and fibronectin-coated surfaces to 73% and 59%, respectively. Pterostilbene, at both 25 μM and 50 μM, also significantly impacted cell adhesion to collagen and fibronectin. Among all the compounds analyzed, pterostilbene at the highest dose was the most effective in reducing cell adhesion to ECM proteins, leading to reductions of up to 0.8% to collagen and 3% to fibronectin. Piceatannol inhibited endometriotic cell adhesion at all tested concentrations, with a more pronounced effect on the fibronectin molecule. Exposure to 50 μM piceatannol decreased cell adhesion to collagen and fibronectin, resulting in reductions to 57% and 12%, respectively (Fig. 3A and B). Similarly, polydatin treatment caused comparable inhibition of cell adhesion to collagen. The ability of polydatin to decrease endometriotic cell adhesion to fibronectin was dose-dependent, with adhesion reduced to 42% at a concentration of 5 μM. Light microscopy of representative samples, stained with crystal violet, confirmed these results (Fig. 3C).

3.4. Effect of resveratrol and its analogs on endometriotic cell migration and invasion

To assess the anti-metastatic effects, we examined the motility of endometriotic cells treated with resveratrol and its analogs in the invasion and migration systems. The migration of 12Z cells through an uncoated membrane toward FBS, used as a chemoattractant, was not significantly affected by resveratrol treatment, except at the highest concentration tested (50 μM) (Fig. 4A). However, in the Matrigel invasion assay, resveratrol inhibited cell invasion in a dose-dependent manner. Resveratrol at concentrations of 10 μM, 25 μM, and 50 μM decreased cell invasion to approximately 60%, 34%, and 6%, respectively, compared with the vehicle control (Fig. 4C). Pterostilbene also inhibited cell migration at concentrations of 25 μM and 50 μM. At the highest dose, it nearly eliminated cell migration, reducing it to less than 1% (Fig. 4A). We also observed significant effects of pterostilbene on 12Z cell invasion, leading to a substantial reduction in invasiveness to 9% at 50 μM (Fig. 4C). Both cell migration and invasion were notably influenced by piceatannol across all tested concentrations in a dose-dependent manner (Fig. 4A and C). For example, even the lowest dose of piceatannol (5 μM) reduced the invading cell population by 60% (Fig. 4C). In our study, polydatin did not significantly impair migratory

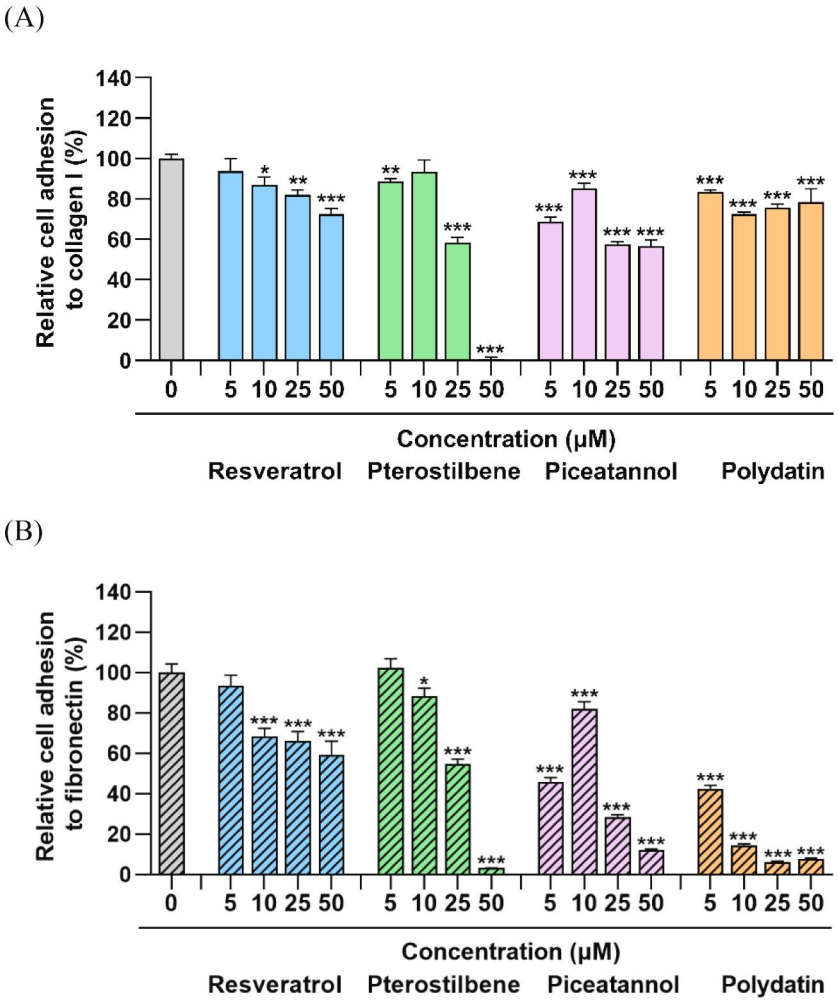


Fig. 3. Effect of resveratrol and its analogs on adhesion of endometriotic 12Z cells to collagen type I and fibronectin. The cells were pre-treated for 24 h with a vehicle or resveratrol and its analogs and then seeded on surfaces coated with EMC proteins. The adhered cells were stained with crystal violet. Results were expressed as relative adhesion of 12Z cells pre-treated with analyzed stilbenes to (A) collagen type I and (B) fibronectin. (C) The representative light microscope visualization of cells pre-treated with pterostilbene adhered to collagen or fibronectin (scale bar 100 μm). Data represent the mean ± SD of three independent experiments performed in triplicate. Significant differences were indicated at *p*-values $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to vehicle control (ANOVA, Tukey's post hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

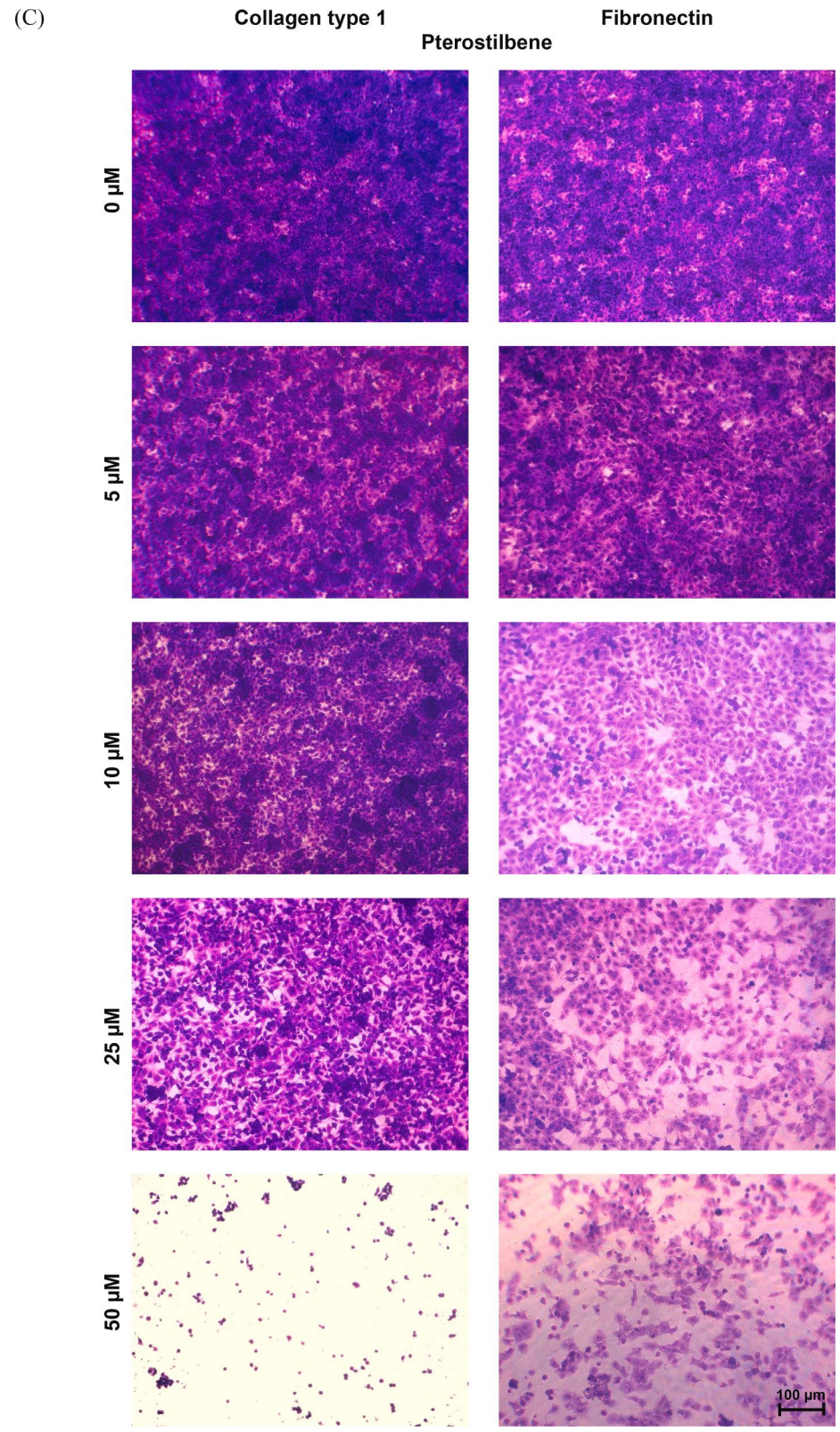


Fig. 3. (*continued*).

potential, except at the 5 µM concentration, where relative cell migration decreased by 16% (Fig. 4A). Conversely, the anti-invasion effects of polydatin were observed at doses of 10 µM (↓54%), 25 µM (↓24%), and 50 µM (↓31%) (Fig. 4C). Representative micrographs showing migrating and invasive cells treated with stilbenes at a concentration of 25 µM are presented below (Fig. 4B and D).

The treatment of 12Z cells with resveratrol and polydatin at concentrations of 10 µM, 25 µM, and 50 µM significantly reduced the invasion index, which measures the relationship between the migratory and invasive potentials of endometriotic cells (Fig. 4E). Specifically, the invasion index decreased to 0.16 and 0.25 with the application of 50 µM resveratrol and 25 µM polydatin, respectively. In contrast, the effects of

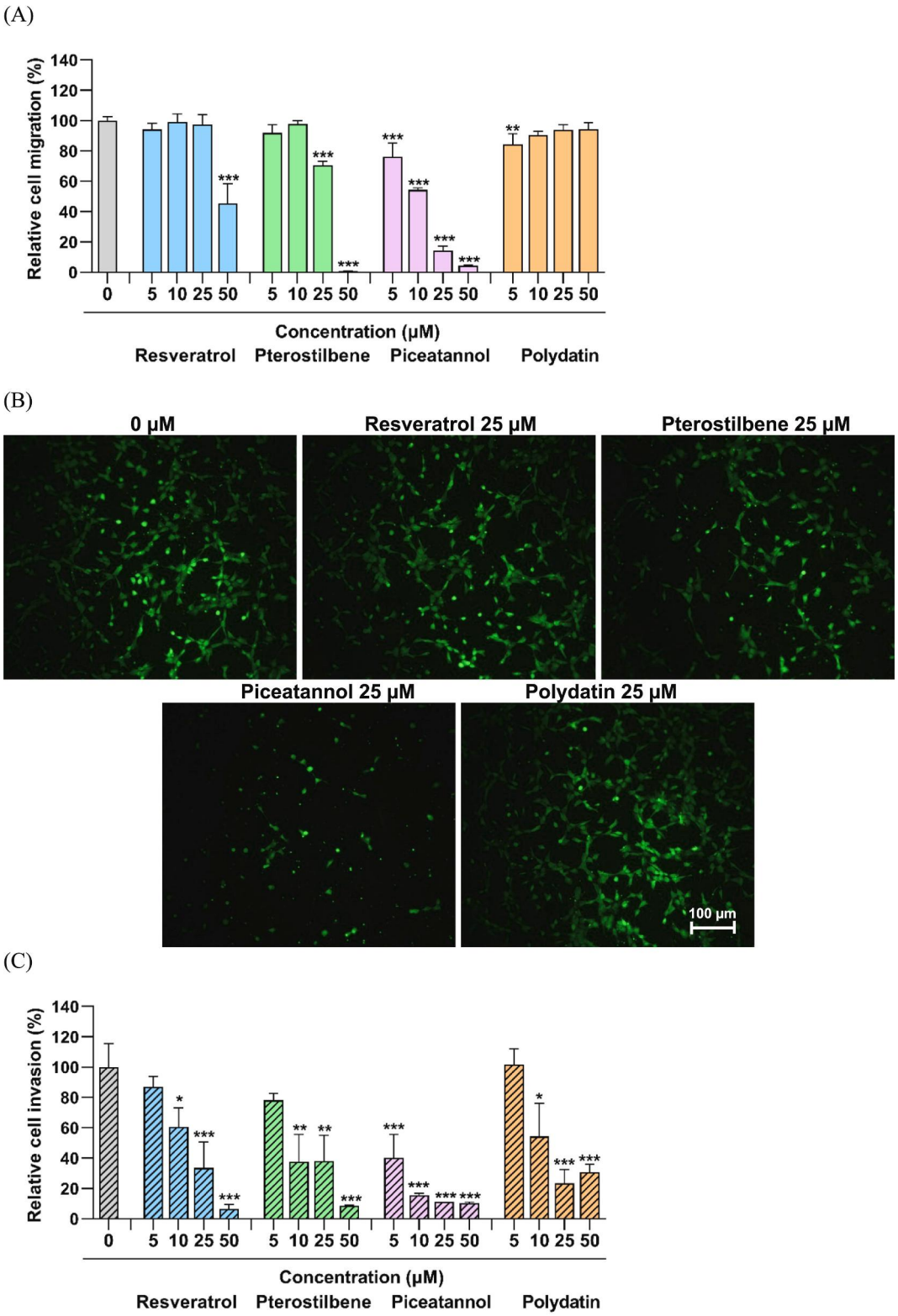


Fig. 4. Effect of resveratrol and its analogs on the migration and invasion of endometriotic 12Z cells. The cells were plated on Matrigel™-coated surfaces for the invasion assay and on uncoated membranes for the migration assay. The cells were then treated for 24 h with a vehicle control or resveratrol and its analogs. The migrated and invaded cells were stained using Calcein AM fluorescent dye. Data are presented as (A) relative cell migration and (C) invasion of 12Z cells treated with the analyzed stilbenes, along with representative images of cells migrating through (B) the uncoated membrane and through (D) the Matrigel™ matrix membrane. (E) The invasive index values were calculated for both non-treated (control) and stilbene-treated cells. Significant differences are indicated with *p*-values: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared to the vehicle control (ANOVA, Tukey's post hoc test).

pterostilbene and piceatannol on the invasion index were strongly dose-dependent. The invasive index values estimated for 12Z cells treated

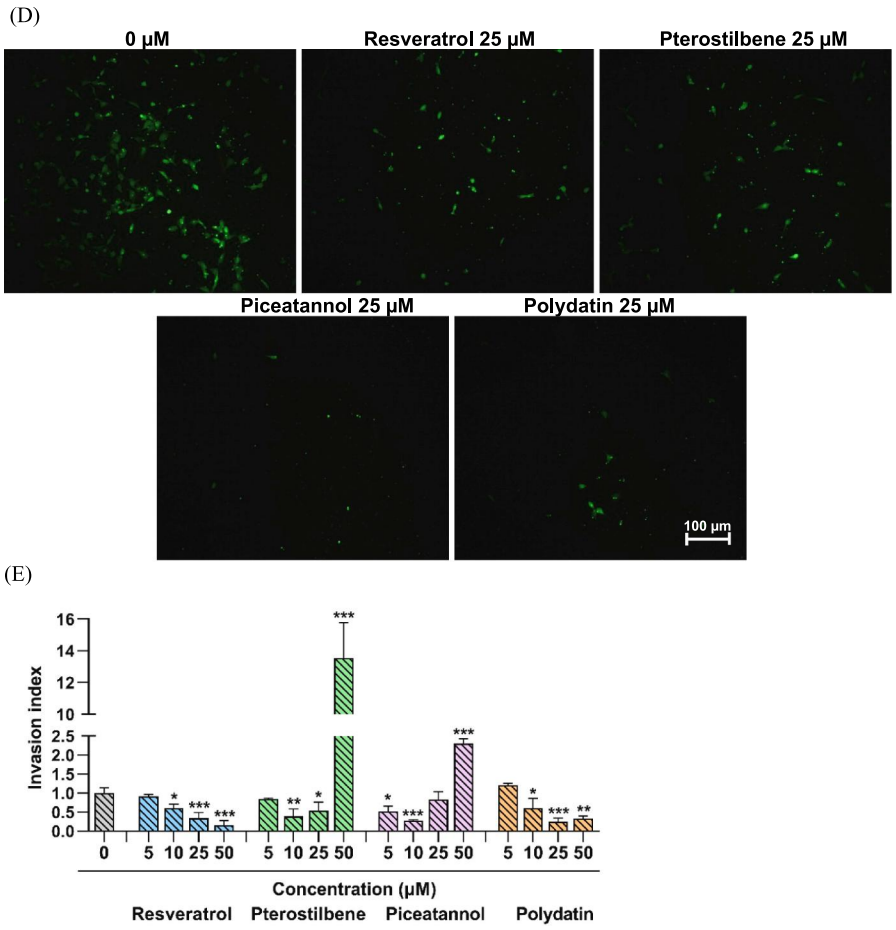


Fig. 4. (*continued*).

with 10 μM and 25 μM pterostilbene, and 5 μM and 10 μM piceatannol, were significantly lower than those observed in non-treated cell cultures. However, when cells were exposed to a high dose (50 μM) of these stilbenes, the invasion index increased markedly (Fig. 4E).

4. Discussion

The dynamic relationship between endometriotic tissue and the peritoneum is crucial for the progression and development of the disease. According to the recently proposed 3 A model of events, attachment, aggression, and angiogenesis evolve sequentially to form endometriotic foci (Liu & Lang, 2011; Zhang et al., 2024). Endometrial fragments establish implantation by first overcoming either intact structural barriers composed of epithelial cells, basement membranes, and collagen, or gaining entry via pre-existing tissue injury, followed by local expansion (Nisolle et al., 2000). To replicate the fundamental adhesion and invasion processes associated with endometriosis, several in vitro models utilizing tissue fragments and isolated cells have been developed. Cell culture systems that study the migration and invasion of endometrial and endometriotic cells have employed commercially available basement membrane matrices, such as Matrigel (Adammek et al., 2013), fibronectin, and collagen (Fan, 2020; Yotova et al., 2012). In another approach, endometrial cells were combined with chorioallantoic (Nap et al., 2004) or amniotic membrane (van der Linden et al., 1996). Some studies emulated the ability of endometrial cells to damage the peritoneum by constructing co-culture models incorporating peritoneal mesothelial cells (Lucidi et al., 2005; Nair et al., 2008). In the present study, we investigated the inhibitory effect of resveratrol and its natural analogs on the migration, invasiveness, and adhesive capacities of endometriotic cells to ECM proteins. We utilized the immortalized endometriotic cell line 12Z, known for its invasive phenotype and derived from N-Cadherin–positive endometriotic tissue (Zeitvogel et al., 2001). Several research groups have reported the motility and invasiveness of this cell line (Eggers et al., 2016; Samartzis et al., 2019; Wilson et al., 2020). However, most published findings refer to endometrial cells in invasion and migration assays (Koks et al., 2000; van der Linden et al., 1994), therefore, the different characteristics of these cells should be addressed.

In our study, we analyzed the adhesion of endometriotic cells to collagen type I and fibronectin, the primary ECM components that play a critical role in cell adhesion and invasion at endometriotic sites. Different types of collagen (I, III, and IV) have been identified in both ectopic and eutopic endometrium, however, collagen type I predominates in the vicinity of lesions, especially around deep endometrial implants (Stovall et al., 1992; Vissers et al., 2024). A comprehensive understanding of the role of collagen in endometriosis, as part of the fibrosis process, is presented in a recently published systematic review (Vissers et al., 2024). Collagen type I is a component of the ECM in endometriotic lesions, and its accumulation in ectopic endometrial implants indicates fibrotic remodeling (Talebloo et al., 2023). Moreover, collagen type I is believed to facilitate the migratory and invasive behavior of endometriotic cells, thereby contributing to lesion progression (Stejskalová et al., 2021). In a recent studies, collagen type I served as the exposed stroma model to evaluate the invasive behavior of endometrial stromal and epithelial endometriotic cell lines, as well as their co-cultures and spheroids (Stejskalová et al., 2021). In addition to collagen, fibronectin,; another key ECM component contributing significantly to hemostasis and tissue repair, may serve as a potential

adhesion target for endometriotic cells (Adachi et al., 2011). Besides interacting with various biomolecules, fibronectin proteins can polymerize into extensive fibrillar networks, that, under mechanical tension,; modulate both molecular activities and cellular behavior (Singh et al., 2010). Regarding endometriosis, a comprehensive meta-analysis integrating data from 11 genome-wide association studies revealed a correlation between the FN1 gene, which encodes fibronectin, and moderate-to-severe endometriosis (Sapkota et al., 2017). In the recent study, collagen type I and fibronectin, along with other fibrotic proteins such as alpha-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF), were markedly increased in ectopic endometrial tissue compared to both eutopic endometrium from the same endometriosis patients and normal endometrial tissue from individuals without the disease (Zhang et al., 2019). Moreover, one recent study on potential diagnostic biomarkers for endometriosis demonstrated significantly elevated fibronectin levels in both peritoneal fluid and plasma of affected women, whereas collagen type IV levels remained unchanged compared to controls (Warzecha et al., 2022). In light of these findings, the involvement of collagen type IV in the invasiveness of endometrial cells into the peritoneal wall appears to be questionable. Therefore, in our analysis, we included fibronectin and collagen type I as the dominant parts of the ECM in the endometriotic invasion site.

Our experiments showed that stilbenes can effectively modify endometriotic cells' anchorage to ECM by reducing attachment to the collagen type I and fibronectin-coated surfaces. Generally, we observed that the analyzed compounds more preferentially inhibited cell adhesion to fibronectin than to collagen type I. The inhibitory effect was more pronounced after treatment with piceatannol and polydatin. Data reported in the literature indicate that endometrial cells adhere less to fibronectin than to collagen I and IV (Koks et al., 2000), which may explain the higher anti-adhesive potential of the analyzed stilbenes to fibronectin compared to collagen. Inhibitory activity toward both target proteins was observed with resveratrol at a concentration as low as 10 μM. The promising anti-adhesive potential of this compound was also shown in in vitro ovarian cancer models. It was found that resveratrol prevents the attachment of ovarian cancer cells (A2780; OVCAR-3; SKOV3) to human peritoneal mesothelial cells and impedes the initial phase of peritoneal cancer spread. In the reported studies, cancer cells were exposed to resveratrol for a longer time (72 h) and at higher concentrations (10 μM, 50 μM, 100 μM) compared to our experiments, however, the resveratrol's inhibitory effect was not associated with apoptosis or necrosis (Mikuła-Pietrasik et al., 2014). In our previous research, we demonstrated that 12Z cells are more sensitive to resveratrol than analyzed analogs, as indicated by an IC_{10} value of 8.99 μM after 48 h of exposure (Gołąbek-Grenda et al., 2023). Research has indicated that certain resveratrol analogs can help prevent adhesion to collagen type I and fibronectin. Similar protective effects have also been observed for pterostilbene (Pan et al., 2019), piceatannol (Marchisio et al., 2025), and polydatin (Lan et al., 2020). These compounds have been shown to alleviate fibrosis by decreasing the production of collagen and fibronectin in experimental disease models. The obtained results indicate that all investigated stilbenes effectively reduce the invasiveness of 12Z cells in a dose-dependent manner. However, not all examined compounds, particularly resveratrol and polydatin, had a significant impact on 12Z cell migration. This suggests that their mechanism of action is more closely related to ECM degradation rather than the regulation of cellular motility. The results confirmed the compounds' preferential effect and were further supported by their ability to activate ECM proteolysis. However, it is noteworthy that resveratrol,; at the highest tested concentration of 50 μM with identified cytotoxicity, significantly reduced cell migration. This aligns with the findings reported by Madanes et al. (Madanes et al., 2022), which demonstrated that resveratrol at doses of 50 μM and 100 μM inhibits 12Z cell migration in a wound healing (scratch) assay. Kong et al. also observed the effects of resveratrol on ectopic endometrial stromal cells isolated from patients with endometriosis. In their study, they used resveratrol at concentrations of 25 μM and 50 μM in both wound healing and transwell invasion assays. The results indicated that resveratrol significantly inhibits the proliferation, migration, and invasion of endometriotic cells. These findings suggest that the therapeutic effects of resveratrol are mediated by the modulation of metastasis-associated protein 1 (MTA1), which is a key component of the nucleosome remodeling and deacetylase (NuRD) complex (Kong et al., 2020). In our studies, pterostilbene effectively reduced the migration and invasion of endometriotic cells, almost completely eliminating their ability to invade at a 50 μM dose. The anti-invasive mechanisms of pterostilbene were also revealed in human breast cancer MDA-MB-231 cells. Pterostilbene effectively inhibited serum-induced migration and invasion of breast cancer cells without affecting their viability, by blocking NF-κB-mediated uPa (urokinase-type plasminogen activator) expression and the Rac1/WAVE/Arp2/3 (Ras-related C3 botulinum toxin substrate 1/WASP-family verprolin-homologous protein/Actin-related protein 2/3) signaling pathway (Ko et al., 2014). The anti-invasive effects of pterostilbene were also demonstrated in the HeLa cervical cancer model, where it showed a superior migration-inhibitory potential (Shin et al., 2020). Similar to pterostilbene, piceatannol significantly reduced endometriotic cell migration in our experiments. This compound was found to suppress the migration and invasion of androgen-independent DU145 prostate cancer cells, likely by inhibiting the enzymatic activities of MMP-9 and uPA; as well as by reducing VEGF secretion. Additionally, piceatannol has been shown to decrease cellular IL-6 production, which in turn enhances uPA and VEGF secretion, promotes STAT3 phosphorylation, and increases the migration of DU145 cells (Kwon et al., 2012). Interestingly, in our recent study, piceatannol significantly decreased IL-6 secretion in 12Z cells, making it the most effective among the tested stilbenes (Gołąbek-Grenda et al., 2024). The shared epithelial characteristics and metastatic potential of these cells suggest that piceatannol may exert its effects through a similar mechanism involving IL-6/STAT3 signaling.

Consistent with the results obtained from migration and invasion assays, resveratrol and its analogs were found to downregulate MMP-2 and upregulate TIMP-2 expression. The data indicate that these compounds exert a dose-dependent inhibitory effect on both active MMP-2 and pro-MMP-2 levels. There is compelling evidence that the proteolytic activity of MMPs plays a significant role in the invasion of the peritoneum in endometriosis. Specifically, elevated levels of MMP-2 activity have been observed in the peritoneal fluid of women with endometriosis compared to healthy controls (Huang et al., 2004). Additionally, increased MMP-2 expression has been detected in endometriotic tissue (Jana et al., 2016), whereas the individual variations and haplotypes of MMP-2 and MMP-2 forms endogenous inhibitor, TIMP-2, were found to be correlated with an elevated risk of advanced-stage of endometriosis (Cho et al., 2013). Comparable studies conducted by Madanes et al. showed that resveratrol at concentrations of 50 μM and 100 μM significantly reduced the MMP-2/TIMP-1 ratio in endometriotic 12Z cells and the endometrial stromal cell line St-T1b (Madanes et al., 2022). TIMP-1 demonstrates a stronger affinity for MMP-9, but it also inhibits the inactive forms of MMP-1, MMP-2, and MMP-3. It has been found to be decreased in exfoliated endometrial cells and is associated with an increased risk of endometriosis (Grzechocińska et al., 2017; Madjid et al., 2020). These findings regarding endometriosis are similar to observations made in cancer research, particularly concerning the well-documented anti-metastatic effects of resveratrol in various types of cancer. Researchers noted that resveratrol treatment significantly inhibited the invasion of gastric, oral, and renal cancer cells, accompanied by concomitant suppression of MMP-2 and MMP-9 expression. Despite extensive research, the precise molecular mechanisms through which resveratrol inhibits the invasion and migration of cancer cells remain incompletely understood. Studies indicate that resveratrol can suppress cancer metastasis by modulating the NF-κB signaling pathway. NF-κB is a key transcriptional regulator that allows malignant and premalignant cells to evade the apoptosis-driven

surveillance mechanisms that typically prevent tumor growth (Song et al., 2023).

Furthermore, resveratrol has been shown to affect the epithelial–mesenchymal transition (EMT) process by modulating the expression of key marker proteins such as N-cadherin, E-cadherin, vimentin, Snail, and Twist (Song et al., 2023). In our research, we found that the metalloproteinase downregulation did not significantly differ among the compounds studied. The findings suggest that other molecules; specifically epithelial or mesenchymal markers, may also play a role in driving the invasion–metastasis cascade and may be influenced by stilbenes. This area presents a potential direction for future research. Given the current understanding of resveratrol's molecular effects on cancer cells, as well as the well-established phenotypic and mechanistic similarities between endometriotic and neoplastic cells, it seems plausible and scientifically valid to explore the regulatory pathways governing invasion and migration in response to resveratrol. Although our study provides interesting new data, it has a significant limitation due to the use of an endometriotic cell line that represent a narrow spectrum of disease phenotypes. To gain a deeper understanding of our findings, future research should utilize a wider variety of cell types, such as endothelial, mesothelial, or primary cell populations. Moreover, our experimental design did not incorporate a comprehensive range of ECM components or matrix metalloproteinases. In addition; we did not address integrins, which are a widely expressed class of cell adhesion receptors that play a crucial role in mediating cell attachment to ECM proteins like collagen, laminin, fibronectin, and vitronectin. When integrins bind to their ligands, they activate intracellular signaling pathways that regulate key cellular processes, including differentiation, motility, adhesion, and intercellular interactions (Adachi et al., 2011). Given their role in mediating adhesive and migratory behavior, integrin signaling may be a critical factor in determining the adhesion of endometriotic cells and their contribution to the lesion dissemination. Our future research will consider these factors to better understand the impact of stilbenes on the invasive potential of endometriotic cells.

In conclusion, our findings demonstrate that resveratrol and its naturally occurring analogs suppress the migratory and invasive abilities of endometriotic cells. They achieve this by modulating the transcriptional activity and protein levels of molecules implicated in ECM remodeling and by influencing cell–ECM interactions. To gain a deeper understanding of the basic mechanisms through which these compounds act, further comprehensive investigations are needed, especially to clarify the regulatory dynamics of the specific signaling pathways responsible for the observed therapeutic effects. Both our previous and current studies have demonstrated the enhanced anti-endometriotic potential of pterostilbene and piceatannol, although this aspect remains underreported in the existing literature. Based on our current findings and earlier reports, it can be inferred that these natural compounds may contribute to the management of endometriosis by modulating key pathological processes involved in the formation of endometriotic lesions. This insight offers potential for the development of improved supportive treatment regimens.

CRediT authorship contribution statement

Agata Gołąbek-Grenda: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Olejnik:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Ethics statement

No animal or human experiments were conducted in this work. All research was performed in compliance with ethical regulations and guidelines.

Funding

This research was funded by the National Science Centre, Poland (grant number 2025/57/N/NZ9/00709). The publication was financed by the Polish Minister of Science and Higher Education as part of the Strategy of the Poznan University of Life Sciences for 2024–2026 in the field of improving scientific research and development work in priority research areas.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The original data presented in this article will be made available upon request.

References

Adachi, M., Nasu, K., Tsuno, A., Yuge, A., Kawano, Y., & Narahara, H. (2011). Attachment to extracellular matrices is enhanced in human endometriotic stromal cells: A possible mechanism underlying the pathogenesis of endometriosis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 155*(1), 85–88. https://doi.org/10.1016/j.ejogrb.2010.10.026

Adammek, M., Greve, B., Kässens, N., Schneider, C., Brüggemann, K., Schüring, A. N., … Götte, M. (2013). MicroRNA miR-145 inhibits proliferation, invasiveness, and stem cell phenotype of an in vitro endometriosis model by targeting multiple cytoskeletal elements and pluripotency factors. *Fertility and Sterility, 99*(5), 1346–1355.e5. https://doi.org/10.1016/j.fertnstert.2012.11.055

Al-Jaber, H. I., Shakya, A. K., Al-Qudah, M. A., Barhoumi, L. M., Abu-Sal, H. E., Hasan, H. S., … Mubarak, M. S. (2024). Piceatannol, a comprehensive review of health perspectives and pharmacological aspects. *Arabian Journal of Chemistry, 17*(9), Article 105939. https://doi.org/10.1016/j.arabjc.2024.105939

Becker, C. M., Gattrell, W. T., Gude, K., & Singh, S. S. (2017). Reevaluating response and failure of medical treatment of endometriosis: A systematic review. *Fertility and Sterility, 108*(1), 125–136. https://doi.org/10.1016/j.fertnstert.2017.05.004

Cho, Y. J., Kim, N. H., Jeong, K.-A., Lee, J. Y., Moon, H.-S., Kim, H.-L., & Chung, H. W. (2013). Association between MMP-2 and TIMP-2 gene polymorphisms and advanced-stage endometriosis in Korean women. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 69*(1), 73–84. https://doi.org/10.1111/aji.12020

Dawson, A., Fernandez, M. L., Anglesio, M., Yong, P. J., & Carey, M. S. (2018). Endometriosis and endometriosis-associated cancers: New insights into the molecular mechanisms of ovarian cancer development. *Ecancermedicalscience, 12*. https://doi.org/10.3332/ecancer.2018.803

Eggers, J. C., Martino, V., Reinbold, R., Schäfer, S. D., Kiesel, L., Starzinski-Powitz, A., … Götte, M. (2016). MicroRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4. *Reproductive Biomedicine Online, 32*(4), 434–445. https://doi.org/10.1016/j.rbmo.2015.12.013

Ek, M., Roth, B., Ekström, P., Valentin, L., Bengtsson, M., & Ohlsson, B. (2015). Gastrointestinal symptoms among endometriosis patients—A case-cohort study. *BMC Women's Health, 15*, 59. https://doi.org/10.1186/s12905-015-0213-2

Fan, H. (2020). In-vitro models of human endometriosis. *Experimental and Therapeutic Medicine, 19*(3), 1617–1625. https://doi.org/10.3892/etm.2019.8363

Gachon, C. M. M., Langlois-Meurinne, M., & Saindrenan, P. (2005). Plant secondary metabolism glycosyltransferases: The emerging functional analysis. *Trends in Plant Science, 10*(11), 542–549. https://doi.org/10.1016/j.tplants.2005.09.007

Gołąbek, A., Kowalska, K., & Olejnik, A. (2021). Polyphenols as a diet therapy concept for endometriosis-current opinion and future perspectives. *Nutrients, 13*(4). https://doi.org/10.3390/nu13041347

Gołąbek-Grenda, A., Juzwa, W., Kaczmarek, M., & Olejnik, A. (2024). Resveratrol and its natural analogs mitigate immune dysregulation and oxidative imbalance in the endometriosis niche simulated in a co-culture system of endometriotic cells and macrophages. *Nutrients, 16*(20), Article 20. https://doi.org/10.3390/nu16203483

Gołąbek-Grenda, A., Kaczmarek, M., Juzwa, W., & Olejnik, A. (2023). Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways. *Scientific Reports, 13*(1), 11468. https://doi.org/10.1038/s41598-023-38692-8

Gottschalk, C., Malberg, K., Arndt, M., Schmitt, J., Roessner, A., Schultze, D., Kleinstein, J., & Ansorge, S. (2000). Matrix metalloproteinases and TACE play a role in the pathogenesis of endometriosis. *Advances in Experimental Medicine and Biology, 477*, 483–486. https://doi.org/10.1007/0-306-46826-3_49

Griffith, J. S., Rodgers, A. K., & Schenken, R. S. (2010). Reviews: In vitro models to study the pathogenesis of endometriosis. *Reproductive Sciences (Thousand Oaks, Calif.), 17*(1), 5–12. https://doi.org/10.1177/1933719109338221

Grzechocińska, B., Dąbrowski, F., Cyganek, A., Panek, G., & Wielgoś, M. (2017). The role of metalloproteinases in endometrial remodelling during menstrual cycle. *Ginekologia Polska, 88*(6), 337–342. https://doi.org/10.5603/GP.a2017.0063

Hannan, N. J., & Salamonsen, L. A. (2008). CX3CL1 and CCL14 regulate extracellular matrix and adhesion molecules in the trophoblast: Potential roles in human embryo implantation. *Biology of Reproduction, 79*(1), 58–65. https://doi.org/10.1095/biolreprod.107.066480

Huang, H.-F., Hong, L.-H., Tan, Y., & Sheng, J.-Z. (2004). Matrix metalloproteinase 2 is associated with changes in steroid hormones in the sera and peritoneal fluid of patients with endometriosis. *Fertility and Sterility, 81*(5), 1235–1239. https://doi.org/10.1016/j.fertnstert.2003.10.027

Jana, S., Chatterjee, K., Ray, A. K., DasMahapatra, P., & Swarnakar, S. (2016). Regulation of matrix metalloproteinase-2 activity by COX-2-PGE2-pAKT axis promotes angiogenesis in endometriosis. *PLoS ONE, 11*(10), Article e0163540. https://doi.org/10.1371/journal.pone.0163540

Karami, A., Fakhri, S., Kooshki, L., & Khan, H. (2022). Polydatin: Pharmacological mechanisms, therapeutic targets, biological activities, and health benefits. *Molecules, 27*(19), 6474. https://doi.org/10.3390/molecules27196474

Ke, J., Ye, J., Li, M., & Zhu, Z. (2021). The role of matrix metalloproteinases in endometriosis: A potential target. *Biomolecules, 11*(11), 1739. https://doi.org/10.3390/biom11111739

Klemmt, P. A. B., Carver, J. G., Koninckx, P., McVeigh, E. J., & Mardon, H. J. (2007). Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: Towards a mechanistic model for endometriosis progression. *Human Reproduction, 22*(12), 3139–3147. https://doi.org/10.1093/humrep/dem262

Ko, H. S., Kim, J. S., Cho, S. M., Lee, H.-J., Ahn, K. S., Kim, S.-H., & Lee, E.-O. (2014). Urokinase-type plasminogen activator expression and Rac1/WAVE-2/Arp2/3 pathway are blocked by pterostilbene to suppress cell migration and invasion in MDA-MB-231 cells. *Bioorganic & Medicinal Chemistry Letters, 24*(4), 1176–1179. https://doi.org/10.1016/j.bmcl.2013.12.115

Koks, C. A., Groothuis, P. G., Dunselman, G. A., de Goeij, A. F., & Evers, J. L. (2000). Adhesion of menstrual endometrium to extracellular matrix: The possible role of integrin alpha(6)beta(1) and laminin interaction. *Molecular Human Reproduction, 6*(2), 170–177. https://doi.org/10.1093/molehr/6.2.170

Kong, X., Xu, X., Zhou, L., Zhu, M., Yao, S., Ding, Y., Liu, T., Wang, Y., Zhang, Y., Li, R., Tang, X., Ling, J., Wu, J., Zhu, X., Gu, Y., & Zhou, H. (2020). MTA1, a target of resveratrol, promotes epithelial-mesenchymal transition of endometriosis via ZEB2. *Molecular Therapy - Methods & Clinical Development, 19*, 295–306. https://doi.org/10.1016/j.omtm.2020.09.013

Kowalska, K., Dembczyński, R., Gołąbek, A., Olkowicz, M., & Olejnik, A. (2021). ROS modulating effects of lingonberry (vaccinium vitis-idaea l.) polyphenols on obese adipocyte hypertrophy and vascular endothelial dysfunction. *Nutrients, 13*(3), 885. https://doi.org/10.3390/nu13030885

Kwon, G. T., Jung, J. I., Song, H. R., Woo, E. Y., Jun, J.-G., Kim, J.-K., … Park, J. H. Y. (2012). Piceatannol inhibits migration and invasion of prostate cancer cells: Possible mediation by decreased interleukin-6 signaling. *The Journal of Nutritional Biochemistry, 23*(3), 228–238. https://doi.org/10.1016/j.jnutbio.2010.11.019

Lan, T., Zhuang, L., Li, S., Yang, G., Xuan, Y., & Guo, J. (2020). Polydatin attenuates hepatic stellate cell proliferation and liver fibrosis by suppressing sphingosine kinase 1. *Biomedicine & Pharmacotherapy, 130*, Article 110586. https://doi.org/10.1016/j.biopha.2020.110586

van der Linden, P. J., de Goeij, A. F., Dunselman, G. A., Erkens, H. W., & Evers, J. L. (1996). Endometrial cell adhesion in an in vitro model using intact amniotic membranes. *Fertility and Sterility, 65*(1), 76–80.

van der Linden, P. J., de Goeij, A. F., Dunselman, G. A., van der Linden, E. P., Ramaekers, F. C., & Evers, J. L. (1994). Expression of integrins and e-cadherin in cells from menstrual effluent, endometrium, peritoneal fluid, peritoneum, and endometriosis. *Fertility and Sterility, 61*(1), 85–90. https://doi.org/10.1016/s0015-0282(16)56457-7

Liu, H., & Lang, J. H. (2011). Is abnormal eutopic endometrium the cause of endometriosis? The role of eutopic endometrium in pathogenesis of endometriosis. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 17*(4), RA92–RA99. https://doi.org/10.12659/MSM.881707

Liu, P., Tang, W., Xiang, K., & Li, G. (2024). Pterostilbene in the treatment of inflammatory and oncological diseases. *Frontiers in Pharmacology, 14*, Article 1323377. https://doi.org/10.3389/fphar.2023.1323377

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta delta C(T)) method. *Methods (San Diego, Calif.), 25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Lucidi, R. S., Witz, C. A., Chrisco, M., Binkley, P. A., Shain, S. A., & Schenken, R. S. (2005). A novel in vitro model of the early endometriotic lesion demonstrates that attachment of endometrial cells to mesothelial cells is dependent on the source of endometrial cells. *Fertility and Sterility, 84*(1), 16–21. https://doi.org/10.1016/j.fertnstert.2004.10.058

Luddi, A., Marrocco, C., Governini, L., Semplici, B., Pavone, V., Luisi, S., Petraglia, F., & Piomboni, P. (2020). Expression of matrix metalloproteinases and their inhibitors in endometrium: High levels in endometriotic lesions. *International Journal of Molecular Sciences, 21*(8), 2840. https://doi.org/10.3390/ijms21082840

Macer, M. L., & Taylor, H. S. (2012). Endometriosis and infertility: A review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstetrics and Gynecology Clinics of North America, 39*(4), 535–549. https://doi.org/10.1016/j.ogc.2012.10.002

Madanes, D., Meresman, G., Valla, S. A., Hassan, N., Kiesel, L., Greve, B., … Ricci, A. G. (2022). Resveratrol impairs cellular mechanisms associated with the pathogenesis of endometriosis. *Reproductive BioMedicine Online*. https://doi.org/10.1016/j.rbmo.2022.02.008

Madjid, T. H., Ardiansyah, D. F., Permadi, W., & Hernowo, B. (2020). Expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in endometriosis menstrual blood. *Diagnostics (Basel), 10*(6), Article 6. https://doi.org/10.3390/diagnostics10060364

Malvezzi, H., Marengo, E. B., Podgaec, S., & Piccinato, C. A. (2020). Endometriosis: Current challenges in modeling a multifactorial disease of unknown etiology. *Journal of Translational Medicine, 18*, Article 311. https://doi.org/10.1186/s12967-020-02471-0

Marchisio, L., Gaudillat, Q., Muller, J., Zedet, A., Tissot, M., Harakat, D., Sénéjoux, F., Rolin, G., Cardey, B., Girard, C., & Pudlo, M. (2025). Synthesis and evaluation of piceatannol derivatives as novel arginase inhibitors with radical scavenging activity and their potential for collagen reduction in dermal fibroblasts. *European Journal of Medicinal Chemistry, 287*, Article 117376. https://doi.org/10.1016/j.ejmech.2025.117376

Mason, B. R., Chatterjee, D., Menias, C. O., Thaker, P. H., Siegel, C. L., & Yano, M. (2020). Encyclopedia of endometriosis: A pictorial rad-path review. *Abdominal Radiology (New York), 45*(6), 1587–1607. https://doi.org/10.1007/s00261-019-02381-w

Meresman, G. F., Götte, M., & Laschke, M. W. (2020). Plants as source of new therapies for endometriosis: A review of preclinical and clinical studies. *Human Reproduction Update*. https://doi.org/10.1093/humupd/dmaa039

Mikuła-Pietrasik, J., Sosińska, P., & Książek, K. (2014). Resveratrol inhibits ovarian cancer cell adhesion to peritoneal mesothelium in vitro by modulating the production of α5β1 integrins and hyaluronic acid. *Gynecologic Oncology, 134*(3), 624–630. https://doi.org/10.1016/j.ygyno.2014.06.022

Nair, A. S., Nair, H. B., Lucidi, R. S., Kirchner, A. J., Schenken, R. S., Tekmal, R. R., & Witz, C. A. (2008). Modeling the early endometriotic lesion: Mesothelium-endometrial cell co-culture increases endometrial invasion and alters mesothelial and endometrial gene transcription. *Fertility and Sterility, 90*(4 Suppl), 1487–1495. https://doi.org/10.1016/j.fertnstert.2007.09.047

Nap, A. W., Dunselman, G. A. J., de Goeij, A. F. P. M., Evers, J. L. H., & Groothuis, P. G. (2004). Inhibiting MMP activity prevents the development of endometriosis in the chicken chorioallantoic membrane model. *Human Reproduction, 19*(10), 2180–2187. https://doi.org/10.1093/humrep/deh408

Ngamkitidechakul, C., Warejcka, D. J., Burke, J. M., O'Brien, W. J., & Twining, S. S. (2003). Sufficiency of the reactive site loop of maspin for induction of cell-matrix adhesion and inhibition of cell invasion: CONVERSION OF OVALBUMIN TO a MASPIN-LIKE MOLECULE *. *Journal of Biological Chemistry, 278*(34), 31796–31806. https://doi.org/10.1074/jbc.M302408200

Nisolle, M., Casanas-Roux, F., & Donnez, J. (2000). Early-stage endometriosis: Adhesion and growth of human menstrual endometrium in nude mice. *Fertility and Sterility, 74*(2), 306–312. https://doi.org/10.1016/s0015-0282(00)00601-4

Olejnik, A., Kaczmarek, M., Olkowicz, M., Kowalska, K., Juzwa, W., & Dembczyński, R. (2018). ROS-modulating anticancer effects of gastrointestinally digested ribes nigrum l. fruit extract in human colon cancer cells. *Journal of Functional Foods, 42*, 224–236. https://doi.org/10.1016/j.jff.2018.01.011

Olejnik, A., Rychlik, J., Kidoń, M., Czapski, J., Kowalska, K., Juzwa, W., … Moyer, M. P. (2016). Antioxidant effects of gastrointestinal digested purple carrot extract on the human cells of colonic mucosa. *Food Chemistry, 190*, 1069–1077. https://doi.org/10.1016/j.foodchem.2015.06.080

Pan, J., Shi, M., Li, L., Liu, J., Guo, F., Feng, Y., Ma, L., & Fu, P. (2019). Pterostilbene, a bioactive component of blueberries, alleviates renal fibrosis in a severe mouse model of hyperuricemic nephropathy. *Biomedicine & Pharmacotherapy, 109*, 1802–1808. https://doi.org/10.1016/j.biopha.2018.11.022

Partridge, J., & Flaherty, P. (2009). An in vitro FluoroBlok tumor invasion assay. *Journal of Visualized Experiments: JoVE, 29*, 1475. https://doi.org/10.3791/1475

Ricci, A. G., Olivares, C. N., Bilotas, M. A., Bastón, J. I., Singla, J. J., Meresman, G. F., & Barañao, R. I. (2013). Natural therapies assessment for the treatment of endometriosis. *Human Reproduction (Oxford, England), 28*(1), 178–188. https://doi.org/10.1093/humrep/des369

Salla, M., Karaki, N., El Kaderi, B., Ayoub, A. J., Younes, S., Abou Chahla, M. N., … El Khatib, S. (2024). Enhancing the bioavailability of resveratrol: Combine it, derivatize it, or encapsulate it? *Pharmaceutics, 16*(4), 569. https://doi.org/10.3390/pharmaceutics16040569

Samartzis, E. P., Fink, D., Stucki, M., & Imesch, P. (2019). Doxycycline reduces MMP-2 activity and inhibits invasion of 12Z epithelial endometriotic cells as well as MMP-2 and -9 activity in primary endometriotic stromal cells in vitro. *Reproductive Biology and Endocrinology, 17*(1), 38. https://doi.org/10.1186/s12958-019-0481-z

Sapkota, Y., Steinthorsdottir, V., Morris, A. P., Fassbender, A., Rahmioglu, N., De Vivo, I., … Nyholt, D. R. (2017). Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. *Nature Communications, 8*, Article 15539. https://doi.org/10.1038/ncomms15539

Sharpe-Timms, K. L. (2001). Endometrial anomalies in women with endometriosis. *Annals of the New York Academy of Sciences, 943*, 131–147. https://doi.org/10.1111/j.1749-6632.2001.tb03797.x

Shin, H. J., Han, J. M., Choi, Y. S., & Jung, H. J. (2020). Pterostilbene suppresses both cancer cells and cancer stem-like cells in cervical cancer with superior bioavailability to resveratrol. *Molecules, 25*(1), 228. https://doi.org/10.3390/molecules25010228

Singh, P., Carraher, C., & Schwarzbauer, J. E. (2010). Assembly of fibronectin extracellular matrix. *Annual Review of Cell and Developmental Biology, 26*, 397–419. https://doi.org/10.1146/annurev-cellbio-100109-104020

Song, B., Wang, W., Tang, X., Goh, R. M. W.-J., Thuya, W. L., Ho, P. C. L., … Wang, L. (2023). Inhibitory potential of resveratrol in cancer metastasis: From biology to therapy. *Cancers, 15*(10), Article 10. https://doi.org/10.3390/cancers15102758

Stejskalová, A., Fincke, V., Nowak, M., Schmidt, Y., Borrmann, K., von Wahlde, M.-K., … Götte, M. (2021). Collagen i triggers directional migration, invasion and matrix remodeling of stroma cells in a 3D spheroid model of endometriosis. *Scientific Reports, 11*(1), 4115. https://doi.org/10.1038/s41598-021-83645-8

Stovall, D. W., Anners, J. A., & Halme, J. (1992). Immunohistochemical detection of type i, III, and IV collagen in endometriosis implants*†. *Fertility and Sterility, 57*(5), 984–989. https://doi.org/10.1016/S0015-0282(16)55013-4

Talebloo, N., Bernal, M. A. O., Kenyon, E., Mallett, C. L., Fazleabas, A., & Moore, A. (2023). Detection of endometriosis lesions using Gd-based collagen i targeting probe in murine models of endometriosis. *Molecular Imaging and Biology, 25*(5), 833–843. https://doi.org/10.1007/s11307-023-01833-6

Vissers, G., Giacomozzi, M., Verdurmen, W., Peek, R., & Nap, A. (2024). The role of fibrosis in endometriosis: A systematic review. *Human Reproduction Update, 30*(6), 706–750. https://doi.org/10.1093/humupd/dmae023

Wang, D., Liu, Y., Han, J., Zai, D., Ji, M., Cheng, W., Xu, L., Yang, L., He, M., Ni, J., Cai, Z., & Yu, C. (2011). Puerarin suppresses invasion and vascularization of endometriosis tissue stimulated by 17β-Estradiol. *PLoS ONE, 6*(9), Article e25011. https://doi.org/10.1371/journal.pone.0025011

Warzecha, D., Załęcka, J., Mańka, G., Kiecka, M., Lipa, M., Spaczyński, R., Piekarski, P., Banaszewska, B., Jakimiuk, A., Issat, T., Rokita, W., Młodawski, J., Szubert, M., Sieroszewski, P., Raba, G., Szczupak, K., Kluz, T., Kluza, M., Wielgoś, M., … Laudański, P. (2022). Plasma and peritoneal fluid fibronectin and collagen IV levels as potential biomarkers of endometriosis. *International Journal of Molecular Sciences, 23*(24), 24. https://doi.org/10.3390/ijms232415669

Wendel, J. R. H., Wang, X., Smith, L. J., & Hawkins, S. M. (2020). Three-dimensional biofabrication models of endometriosis and the endometriotic microenvironment. *Biomedicines, 8*(11), E525. https://doi.org/10.3390/biomedicines8110525

Wilson, M. R., Reske, J. J., Holladay, J., Neupane, S., Ngo, J., Cuthrell, N., … Chandler, R. L. (2020). ARID1A mutations promote P300-dependent endometrial invasion through super-enhancer hyperacetylation. *Cell Reports, 33*(6), Article 108366. https://doi.org/10.1016/j.celrep.2020.108366

Witz, C. A., Monotoya-Rodriguez, I. A., & Schenken, R. S. (1999). Whole explants of peritoneum and endometrium: A novel model of the early endometriosis lesion. *Fertility and Sterility, 71*(1), 56–60. https://doi.org/10.1016/S0015-0282(98)00400-2

Witz, C. A., Montoya-Rodriguez, I. A., Cho, S., Centonze, V. E., Bonewald, L. F., & Schenken, R. S. (2001). Composition of the extracellular matrix of the peritoneum. *Journal of the Society for Gynecologic Investigation, 8*(5), 299–304. https://doi.org/10.1016/s1071-5576(01)00122-8

Yotova, I., Quan, P., Gaba, A., Leditznig, N., Pateisky, P., Kurz, C., & Tschugguel, W. (2012). Raf-1 levels determine the migration rate of primary endometrial stromal cells of patients with endometriosis. *Journal of Cellular and Molecular Medicine, 16*(9), 2127–2139. https://doi.org/10.1111/j.1582-4934.2011.01520.x

Zeitvogel, A., Baumann, R., & Starzinski-Powitz, A. (2001). Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. *The American Journal of Pathology, 159*(5), 1839–1852. https://doi.org/10.1016/S0002-9440(10)63030-1

Zhang, C., Cheng, H., Ye, X., Cui, H., Li, Y., Zhu, H., & Chang, X. (2024). ECM1 promotes migration and invasion in endometriosis. *Reproductive Biology, 24*(1), Article 100826. https://doi.org/10.1016/j.repbio.2023.100826

Zhang, Z., Suo, L., Chen, Y., Zhu, L., Wan, G., & Han, X. (2019). Endometriotic peritoneal fluid promotes myofibroblast differentiation of endometrial mesenchymal stem cells. *Stem Cells International, 2019*, Article 6183796. https://doi.org/10.1155/2019/6183796

Oświadczenia

Anna Olejnik

imię i nazwisko promotora

Oświadczenie promotora rozprawy doktorskiej

Oświadczam, że niniejsza rozprawa doktorska pt.: *Bioaktywność resweratrolu i jego naturalnych pochodnych w regulacji procesów zaangażowanych w rozwój endometriozy – badania na modelach komórkowych in vitro* została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego.

Data 25.05.2026 Podpis promotora rozprawy

Anna Olejnik

Agata Gołąbek-Grenda

imię i nazwisko doktoranta

Oświadczenie autora rozprawy doktorskiej

Niniejszym oświadczam, że przedłożoną rozprawę doktorską pt.: *Bioaktywność resweratrolu i jego naturalnych pochodnych w regulacji procesów zaangażowanych w rozwój endometriozy – badania na modelach komórkowych in vitro* została napisałem samodzielnie, tj.:

- nie zleciłem opracowania rozprawy lub jej części innym osobom,
- nie przepisałem rozprawy lub jej części z innych opracowań i prac związanych tematycznie z moją pracą,
- korzystałem jedynie z niezbędnych konsultacji,
- wszystkie elementy rozprawy, które zostały wykorzystane do jej realizacji (cytaty, ryciny, tabele, programy itp.), a nie będące mojego autorstwa, zostały odpowiednio zaznaczone wraz z podaniem źródła ich pochodzenia,
- rozprawa nie była wcześniej przedmiotem procedur związanych z uzyskaniem stopnia naukowego.

Oświadczam ponadto, że niniejsza wersja rozprawy jest identyczna z załączoną wersją elektroniczną.
Mam świadomość, że złożenie nieprawdziwego oświadczenia skutkować będzie niedopuszczeniem do dalszych czynności postępowania w sprawie nadania stopnia doktora lub cofnięciem decyzji o nadaniu mi stopnia doktora oraz wszczęciem postępowania dyscyplinarnego.

Data 25.05.2026

Agata Gołąbek-Grenda

podpis autora rozprawy

Mgr inż. Agata Gołąbek-Grenda
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Katarzyna Kowalska, Anna Olejnik, 2021, Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives, Nutrients, 13(4), 1347* mój indywidualny udział w jej powstaniu polegał na opracowaniu krytycznego przeglądu literatury, napisaniu części manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 70%.

Data 25.05.2026

Podpis

Agata Gołąbek-Grenda

Prof. UPP, dr hab. Katarzyna Kowalska
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Katarzyna Kowalska, Anna Olejnik, 2021, Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives. Nutrients, 13(4), 1347* mój indywidualny udział w jej powstaniu polegał na napisaniu części manuskryptu oraz korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 10%.

Data 25.05.2026

Podpis

Kowalska Katarzyna

Prof. UPP, dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Katarzyna Kowalska, Anna Olejnik, 2021, Polyphenols as a Diet Therapy Concept for Endometriosis—Current Opinion and Future Perspectives, Nutrients, 13(4), 1347* mój indywidualny udział w jej powstaniu polegał na napisaniu części manuskryptu oraz recenzowaniu i korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 20%.

Data 25/05/2026

Podpis

Anna Olejnik

Mgr inż. Agata Gołąbek-Grenda
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Anna Olejnik, 2022, In vitro modeling of endometriosis and endometriotic microenvironment – Challenges and recent advances. Cellular Signalling, 97, 110375* mój indywidualny udział w jej powstaniu polegał na opracowaniu krytycznego przeglądu literatury i napisaniu części manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 75%.

Data 25.05.2026

Podpis

Agata Gołąbek-Grenda

Prof. UPP, dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Anna Olejnik, 2022, In vitro modeling of endometriosis and endometriotic microenvironment – Challenges and recent advances, Cellular Signalling, 97, 110375* mój indywidualny udział w jej powstaniu polegał na napisaniu części manuskryptu oraz recenzowaniu i korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 25%.

Data 25/05/2026

Podpis

Anna Olejnik

Mgr inż. Agata Gołąbek-Grenda
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Mariusz Kaczmarek, Wojciech Juzwa, Anna Olejnik, 2023, Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways, Scientific Reports, 13, 11468* mój indywidualny udział w jej powstaniu polegał na prowadzeniu hodowli komórek, przygotowaniu prób do analiz cytometrycznych dotyczących detekcji fragmentacji DNA (test TUNEL), oznaczenia eksternalizacji fosfatydyloseryny i analiz przebiegu cyklu komórkowego, wykonaniu testów cytotoksyczności, analiz mikroskopowych, analiz Real-Time PCR oraz analiz aktywności kaspaz-3/7. Zakres prac obejmował opracowanie koncepcji, metodologii, przeprowadzenie analiz, analizę wyników, interpretację wyników, sformułowanie wniosków, opracowanie grafik oraz napisanie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 65%.

Data 25.05.2026

Podpis

Agata Gołąbek-Grenda

Prof. dr hab. n. med. Mariusz Kaczmarek
Katedra Biotechnologii Medycznej
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu
ul. Rokietnicka 8, 61-806 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Mariusz Kaczmarek, Wojciech Juzwa, Anna Olejnik, 2023, Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways, Scientific Reports, 13, 11468* mój indywidualny udział w jej powstaniu polegał na wykonaniu analiz cyklu komórkowego na cytometrze przepływowym. Zakres prac obejmował opracowanie metodologii i przeprowadzenie analiz oraz opracowanie danych do publikacji.
Mój całkowity udział w powstaniu tej publikacji wynosi 10%.

Data 18.05.2026 r.

Podpis

.....

UNIWERSYTET MEDYCZNY
im. Karola Marcinkowskiego w Poznaniu
Katedra Biotechnologii Medycznej
Zakład Immunologii Nowotworów
ul. Rokietnicka 8
60-806 Poznań

Prof. UPP, dr hab. inż. Wojciech Juzwa
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Mariusz Kaczmarek, Wojciech Juzwa, Anna Olejnik, 2023, Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways, Scientific Reports, 13, 11468* mój indywidualny udział w jej powstaniu polegał na wykonaniu analiz cytometrycznych dotyczących detekcji fragmentacji DNA (test TUNEL) i oznaczenia eksternalizacji fosfatydyloseryny. Zakres prac obejmował opracowanie metodologii i przeprowadzenie analiz oraz opracowanie danych do publikacji. Mój całkowity udział w powstaniu tej publikacji wynosi 10%.

Data 25.05.2026 r.

Podpis .....

Prof. UPP, dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Mariusz Kaczmarek, Wojciech Juzwa, Anna Olejnik, 2023, Natural resveratrol analogs differentially target endometriotic cells into apoptosis pathways, Scientific Reports, 13, 11468* mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji i metodologii badań, wykonaniu analizy statystycznej wyników oraz recenzowaniu i korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 15%.

Data 25/05/2026 Podpis

Anna Olejnik

Mgr inż. Agata Gołąbek-Grenda
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Wojciech Juzwa, Mariusz Kaczmarek, Anna Olejnik, 2024, Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages, Nutrients, 16(20), 3483* mój indywidualny udział w jej powstaniu polegał na prowadzeniu hodowli komórek, przygotowaniu prób do ilościowego oznaczeniu cytokin i oznaczenia wewnątrzkomórkowych reaktywnych form tlenu metodami cytometrycznymi, wykonaniu analiz testem ELISA, analiz Real-Time PCR, oznaczeniu białka komórkowego. Zakres prac obejmował opracowanie koncepcji, metodologii, przeprowadzenie analiz, analizę wyników, interpretację wyników, sformułowanie wniosków, opracowanie grafik oraz napisanie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 65%.

Data 25.05.2026 Podpis

Agata Gołąbek-Grenda

Prof. UPP, dr hab. inż. Wojciech Juzwa
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Wojciech Juzwa, Mariusz Kaczmarek, Anna Olejnik, 2024, Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages, Nutrients, 16(20), 3483* mój indywidualny udział w jej powstaniu polegał na wykonaniu analiz cytometrycznych, dotyczących oznaczenia wewnątrzkomórkowych reaktywnych form tlenu. Zakres prac obejmował opracowanie metodologii i przeprowadzenie analiz oraz opracowanie danych do publikacji.
Mój całkowity udział w powstaniu tej publikacji wynosi 10%.

Data 25.05.2026 r.

Podpis .....

Prof. dr hab. n. med. Mariusz Kaczmarek
Katedra Biotechnologii Medycznej,
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu
ul. Rokietnicka 8, 61-806 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Wojciech Juzwa, Mariusz Kaczmarek, Anna Olejnik, 2024, Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages, Nutrients, 16(20), 3483* mój indywidualny udział w jej powstaniu polegał na ilościowym oznaczeniu cytokin metodą cytometryczną. Zakres prac obejmował opracowanie metodologii i przeprowadzenie analiz oraz opracowanie danych do publikacji.
Mój całkowity udział w powstaniu tej publikacji wynosi 10%.

Data 18.05.2026 r.

Podpis

UNIWERSYTET MEDYCZNY
im. Karola Marcinkowskiego w Poznaniu
Katedra Biotechnologii Medycznej
Zakład Immunologii Nowotworów
ul. Rokietnicka 8
60-806 Poznań

Prof. UPP, dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Wojciech Juzwa, Mariusz Kaczmarek, Anna Olejnik, 2024, Resveratrol and Its Natural Analogs Mitigate Immune Dysregulation and Oxidative Imbalance in the Endometriosis Niche Simulated in a Co-Culture System of Endometriotic Cells and Macrophages, Nutrients, 16(20), 3483* mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji i metodologii badań, wykonaniu analizy statystycznej wyników oraz recenzowaniu i korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 15%.

Data 25/05/2026 Podpis

Anna Olejnik

Mgr inż. Agata Gołąbek-Grenda
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Anna Olejnik, 2026, Stilbene-based strategies for the management of endometriosis: Targeting cell adhesion, migration, and ECM-driven invasion, Journal of Functional Foods, 140, 107277* mój indywidualny udział w jej powstaniu polegał na prowadzeniu hodowli komórek, wykonaniu analiz adhezji komórek, analiz migracji i inwazji komórkowej, analiz testem ELISA, analiz Real-Time PCR, oznaczeniu białka komórkowego. Zakres prac obejmował opracowanie koncepcji, metodologii, przeprowadzenie analiz, analizę wyników, interpretację wyników, sformułowanie wniosków, opracowanie grafik oraz napisanie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 80%.

Data 25.05.2026

Podpis

Agata Gołąbek-Grenda

Prof. UPP, dr hab. Anna Olejnik
Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 48, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Agata Gołąbek-Grenda, Anna Olejnik, 2026, Stilbene-based strategies for the management of endometriosis: Targeting cell adhesion, migration, and ECM-driven invasion, Journal of Functional Foods, 140, 107277* mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji i metodologii badań wykonaniu analizy statystycznej wyników eksperymentów oraz recenzji i korekcie manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wynosi 20%.

Data 25/05/2026

Podpis

Anna Olejnik