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Wpływ wybranych ksenoestrogenów i hiperglikemii na aktywność inhibitorów aromatazy w badaniach modelowych *in vitro*

Effect of metalloestrogens and hyperglycemia on the effectiveness of aromatase inhibitors in a hormone-dependent breast cancer cell model

Rozprawa doktorska
w dziedzinie nauk medycznych i nauk o zdrowiu
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STRESZCZENIE

Rak piersi jest najczęściej diagnozowanym nowotworem złośliwym u kobiet, w 2020 roku zdiagnozowano go u ponad 2,3 miliona pacjentek na świecie i szacuje się, że liczba ta wzrośnie w 2040 roku do ponad 3 milionów. Nawet jedna trzecia pacjentek z rakiem piersi, choruje również na cukrzycę. Cukrzyca i hiperglikemia nie tylko zwiększają ryzyko rozwoju raka piersi, ale także wiążą się z wyższym o 40% ryzykiem zgonu w porównaniu do pacjentek bez cukrzycy. Według Międzynarodowej Agencji Badań nad Rakiem, dotychczas uznane czynniki ryzyka zachorowania na raka piersi, m.in. genetyczne, hormonalne, ekspozycja na promieniowanie jonizujące, czy wspomniana już cukrzyca, odpowiadają za niecałe 37% przypadków nowotworów piersi, co skłania do dalszych badań nad rolą innych czynników, np. zanieczyszczeń środowiska, czy ksenoestrogenów w jego patogenezie. Ksenoestrogeny to substancje egzogenne, które mogą naśladować działanie estrogenów. W leczeniu hormonozależnego raka piersi, który jest najczęściej diagnozowanym podtypem, podstawą leczenia jest hormonoterapia mająca na celu wyeliminowanie pobudzającego działania estrogenów na komórki nowotworowe. Jednocześnie pacjentki stosujące terapię endokrynną (np. inhibitorami aromatazy) narażone są na występujące powszechnie w codziennym środowisku życia ksenoestrogeny, których wpływ na skuteczność leków stosowanych w hormonoterapii w dużej mierze pozostaje nieznany.

Celem niniejszej pracy doktorskiej była ocena wpływu wybranych ksenoestrogenów – jonów metali Cr^{3+} i Al^{3+}, obecnych m.in. w suplementach diety (chrom), czy antyperspirantach (glin) na skuteczność inhibitorów aromatazy (letrozolu i eksemestanu), podstawowej grupy leków stosowanych w hormonoterapii raka piersi u pacjentek po menopauzie. Dodatkowo oceniono, czy ewentualna interakcja pomiędzy lekami a metaloestrogenami różni się w zależności od panujących w modelu komórkowym warunków, odzwierciedlających normo- i hiperglikemię. Poddano również ocenie profil bezpieczeństwa inhibitorów aromatazy, ze szczególnym uwzględnieniem występowania zaburzeń gospodarki węglowodanowej (np. hiperglikemii) oraz ze strony układu sercowo-naczyniowego.

W ramach pracy wykonano badania na modelu komórkowym hormonozależnego raka piersi - liniach komórkowych MCF-7 oraz MCF-7/DOX (linia oporna na doksorubicynę), eksponowanych na wybrane metaloestrogeny, leki oraz ich kombinacje w warunkach normo- i hiperglikemii. Skuteczność inhibitorów aromatazy stosowanych pojedynczo, bądź łącznie z metaloestrogenami, oceniono wykonując test żywotności komórek, cytometryczną ocenę apoptozy, nekrozy, analizę cyklu komórkowego oraz ilościowy pomiar stężeń białek zaangażowanych w proces apoptozy (Bcl-2, BAX) i angiogenezy (VEGF-A). Profil

bezpieczeństwa inhibitorów aromatazy oceniono wykonując przegląd systematyczny i metaanalizę działań niepożądanych raportowanych w badaniach klinicznych.

Przeprowadzone badania wykazały, że w warunkach odzwierciedlających normoglikemię, kombinacja inhibitorów aromatazy z metaloestrogenami, w obu liniach komórkowych, może prowadzić do zmniejszenia aktywności leków, co obserwowano jako zwiększenie żywotności komórek nowotworowych, zmniejszenie liczby komórek ulegających apoptozie i nekrozie, wzrost stężenia białka antyapoptotycznego Bcl-2 (co skutkowało wzrostem współczynnika Bcl-2/BAX), a także wzrost stężenia VEGF-A. Wykazano ponadto, że zmniejszenie aktywności inhibitorów aromatazy pod wpływem metaloestrogenów, nie było związane z ich wpływem na cykl komórkowy. W warunkach hiperglikemii, w obu liniach komórkowych, jednoczesna ekspozycja na metaloestrogeny i inhibitory aromatazy nie zmniejszała istotnie działania leków (nie zwiększała żywotności komórek nowotworowych, nie zmniejszała odsetka komórek ulegających apoptozie/nekrozie, nie zwiększała współczynnika Bcl-2/BAX). Jednocześnie obserwowano znacznie mniejszą aktywność leków przy wysokim stężeniu glukozy panującym w modelu komórkowym. Bezpośrednie porównanie wyników otrzymanych w warunkach normo- i hiperglikemii wskazuje, iż na efektywność inhibitorów aromatazy większy wpływ ma wysokie stężenie glukozy obecne w modelu komórkowym, niż ich kombinacja z metaloestrogenami. Wyniki wykonanego przeglądu systematycznego i metaanalizy dostarczyły natomiast dowodów na zwiększone ryzyko występowania zdarzeń sercowo-naczyniowych, a także na możliwe występowanie zaburzeń gospodarki węglowodanowej (hiperglikemii, cukrzycy) w czasie leczenia inhibitorami aromatazy.

Uzyskane wyniki wskazują, że obecne w codziennym środowisku życia człowieka metaloestrogeny, mogą w warunkach normoglikemii negatywnie wpływać na skuteczność leków stosowanych w hormonoterapii. Potrzebne są dalsze badania, aby w pełni wyjaśnić wykazane interakcje i móc im skutecznie zapobiegać. Otrzymane wyniki wskazują również na kluczową rolę hiperglikemii w obniżaniu efektywności inhibitorów aromatazy. Stale rosnąca liczba chorych na cukrzycę, a co za tym idzie także wzrost liczby pacjentek jednocześnie zmagających się z cukrzycą i nowotworem piersi sprawia, że leczenie raka piersi u takich pacjentek staje się sporym wyzwaniem klinicznym. Biorąc pod uwagę fakt, że główną grupą pacjentek stosującą inhibitory aromatazy są pacjentki po menopauzie oraz uzyskane w badaniach wyniki, wydaje się, że systematyczne monitorowanie glikemii w trakcie hormonoterapii może być jednym ze sposobów na kontrolowanie jej skuteczności.

SUMMARY

Breast cancer is the most common cancer diagnosed among women, in 2020 it was diagnosed in over 2.3 million patients worldwide and it is estimated that this number will increase to over 3 million in 2040. Up to one-third of breast cancer patients also have diabetes. Diabetes and hyperglycemia not only increase the risk of developing breast cancer, but are also associated with a 40% higher risk of death compared to patients without diabetes. According to the International Agency for Research on Cancer, previously recognized risk factors for breast cancer, including genetic, hormonal, exposure to ionizing radiation, or the aforementioned diabetes, are responsible for less than 37% of breast cancer cases, which prompts further research into the role of other factors, such as environmental pollution or xenoestrogens, in its pathogenesis. Xenoestrogens are exogenous substances that can mimic the action of estrogens. In the treatment of hormone-dependent breast cancer, which is the most frequently diagnosed subtype of breast cancer, the basis of treatment is hormone therapy the purpose of which is to eliminate the stimulating effect of estrogens on cancer cells. At the same time, patients using endocrine therapy (e.g. aromatase inhibitors) are exposed to xenoestrogens, commonly found in the everyday environment, whose impact on the effectiveness of drugs used in hormone therapy remains largely unknown.

The aim of this doctoral dissertation was to assess the impact of selected xenoestrogens - metal ions Cr^{3+} and Al^{3+}, present, among others, in dietary supplements (chromium) or antiperspirants (aluminum) on the effectiveness of aromatase inhibitors (letrozole and exemestane), the basic group of drugs used in hormone therapy of breast cancer in postmenopausal patients. In addition, it was assessed whether the possible interaction between drugs and metalloestrogens differs depending on the conditions prevailing in the cell model, reflecting normo- and hyperglycemia. The safety profile of aromatase inhibitors was also assessed, with particular emphasis on the occurrence of carbohydrate metabolism disorders (e.g. hyperglycemia) and cardiovascular system disorders.

Studies were performed on a cellular model of hormone-dependent breast cancer - MCF-7 and MCF-7/DOX cell lines (doxorubicin-resistant line), exposed to selected metalloestrogens, drugs and their combinations in conditions of normo- and hyperglycemia. The effectiveness of aromatase inhibitors used alone or in combination with metalloestrogens was assessed by performing a cell viability test, cytometric assessment of apoptosis, necrosis, cell cycle analysis and quantitative measurement of the concentration of proteins involved in the process

of apoptosis (Bcl-2, BAX) and angiogenesis (VEGF-A). The safety profile of aromatase inhibitors was assessed by performing a systematic review and meta-analysis of adverse events reported in clinical trials.

The conducted studies have shown that under conditions reflecting normoglycemia, the combination of aromatase inhibitors with metalloestrogens in both cell lines may lead to a decrease in drug activity, which was observed as an increase in the viability of cancer cells, a decrease in the number of cells undergoing apoptosis and necrosis, an increase in the concentration of the anti-apoptotic protein Bcl- 2 (resulting in an increase in the Bcl-2/BAX ratio), as well as an increase in VEGF-A concentration. It was also shown that the decrease in the activity of aromatase inhibitors under the influence of metalloestrogens was not related to their effect on the cell cycle. Under hyperglycemic conditions, in both cell lines, simultaneous exposure to metalloestrogens and aromatase inhibitors did not significantly reduce the effect of drugs (it did not increase the viability of cancer cells, did not reduce the percentage of cells undergoing apoptosis/necrosis, did not increase the Bcl-2/BAX ratio). At the same time, significantly lower drug activity was observed at high glucose concentrations prevailing in the cellular model. A direct comparison of the results obtained in the conditions of normo- and hyperglycemia indicates that the effectiveness of aromatase inhibitors is more influenced by the high concentration of glucose present in the cellular model than their combination with metalloestrogens. The results of the systematic review and meta-analysis provided evidence of an increased risk of cardiovascular events, as well as the possible occurrence of carbohydrate metabolism disorders (hyperglycemia, diabetes) during treatment with aromatase inhibitors.

The obtained results indicate that metalloestrogens present in the everyday environment of human life may negatively affect the effectiveness of drugs used in hormone therapy in conditions of normoglycemia. Further research is needed to fully explain the interactions shown and to be able to effectively prevent them. The obtained results also indicate the crucial role of hyperglycemia in reducing the effectiveness of aromatase inhibitors. The constantly growing number of patients with diabetes, and thus also the increase in the number of patients suffering from diabetes and breast cancer at the same time, makes the treatment of breast cancer in such patients a clinical challenge. Taking into account the fact that the main group of patients using aromatase inhibitors are post-menopausal patients and the results obtained in the studies, it seems that systematic monitoring of glycaemia during hormonal therapy may be one of the ways to control its effectiveness.

1. WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Monotematyczny cykl publikacji, będący podstawą ubiegania się o stopień doktora nauk farmaceutycznych stanowią łącznie cztery artykuły, publikowane w czasopismach naukowych z listy A, o zasięgu międzynarodowym, posiadające współczynnik wpływu IF. Na cykl składają się trzy prace oryginalne oraz jeden artykuł przeglądowy. We wszystkich publikacjach jestem pierwszym autorem oraz autorem korespondencyjnym.

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2. **Boszkiewicz K,** Piwowar A, Petryszyn P. Aromatase Inhibitors and Risk of Metabolic and Cardiovascular Adverse Effects in Breast Cancer Patients-A Systematic Review and Meta-Analysis. J Clin Med. 2022 May 31;11(11):3133. doi: 10.3390/jcm11113133.

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   IF: 0,555 Punkty MEiN: 100

Sumaryczny współczynnik IF za cykl publikacji: 13,571

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2. WPROWADZENIE

2.1. Rak piersi – informacje ogólne.

Rak piersi jest najczęściej diagnozowanym nowotworem złośliwym na świecie u kobiet, w 2020 roku zdiagnozowano go u ponad 2,3 miliona pacjentek i szacuje się, że liczba ta wzrośnie w 2040 roku do ponad 3 milionów [1]. Według statystyk Amerykańskiego Towarzystwa Onkologii Klinicznej (ASCO, *ang. American Society of Clinical Oncology*), 1 na 8 kobiet zachoruje na inwazyjnego raka piersi w swoim życiu, a dla 1 na 39 z nich, rak piersi stanie się przyczyną śmierci [2]. W Polsce w 2020 roku odnotowano 17 511 nowych przypadków raka piersi, co stanowiło aż 23,8% wszystkich zachorowań na nowotwory złośliwe u kobiet. Niemal 80% przypadków (78,6%) dotyczyło kobiet w wieku ≥ 50 lat. Rak piersi, choć rzadko, występuje również u mężczyzn, stanowiąc 0,5-1% wszystkich przypadków [3-5]. W Polsce nowotwory złośliwe są u obu płci drugą co do częstości przyczyną śmierci (pierwszą od wielu lat pozostają choroby układu sercowo-naczyniowego), zaś pod względem umieralności u kobiet rak piersi ustępuje jedynie rakowi płuca, który odpowiada za 17,6% wszystkich zgonów z powodu nowotworów złośliwych (rak piersi – 15,3%) [3].

W większości przypadków nie udaje się ustalić przyczyny zachorowania na raka piersi, którego etiologia najczęściej jest wieloczynnikowa. Do najbardziej istotnych czynników ryzyka należą czynniki demograficzne - płeć oraz wiek, ponadto wymieniane są czynniki genetyczne (nosicielstwo mutacji genowej, głównie *BRCA1* i *BRCA2,* rodzinne występowanie raka piersi, zwłaszcza w młodym wieku), czynniki związane z reprodukcją (wczesna pierwsza miesiączka, późna menopauza, późny wiek pierwszego porodu), czynniki hormonalne (np. stosowanie hormonalnej terapii zastępczej), występowanie łagodnych zmian rozrostowych w piersiach, ekspozycja na promieniowanie jonizujące, nadwaga i otyłość (zwłaszcza w okresie pomenopauzalnym), a także nadmierna konsumpcja alkoholu, palenie tytoniu oraz cukrzyca [4,6]. Według Międzynarodowej Agencji Badań nad Rakiem (IARC, *ang. International Agency for Research on Cancer*), dotychczas uznane i wymienione wyżej czynniki ryzyka zachorowania na raka piersi, odpowiadają jedynie za około 36,8% przypadków, co skłania do dalszych badań nad rolą innych czynników, np. zanieczyszczeń środowiska, czy ksenoestrogenów w patogenezie nowotworu piersi [7].

Nowotwór piersi nie jest chorobą homogenną. W zależności od ekspresji kluczowych receptorów na komórkach nowotworowych – receptorów steroidowych (estrogenowych – ERs, *ang. estrogen receptors* i progesteronowych – PgRs, *ang. progesterone receptors*) oraz

receptora ludzkiego naskórkowego czynnika wzrostu typu 2 - HER2 (*ang. human epidermal growth factor receptor 2*), wyróżnia się trzy główne podtypy raka piersi, które różnią się między sobą nie tylko rodzajem stosowanego leczenia systemowego, ale i rokowaniem. Najczęściej występującym podtypem nowotworu piersi jest rak hormonozależny (luminalny), który występuje u 70% zdiagnozowanych pacjentek. W przypadku kobiet po menopauzie, podtyp ten występuje jeszcze częściej – nawet w 75% przypadków. Luminalny rak piersi charakteryzuje się obecnością co najmniej 1% komórek nowotworowych wykazujących ekspresję receptorów steroidowych oraz najlepszym rokowaniem – wskaźnik przeżycia 5-letniego dla wczesnych stadiów wynosi w USA ≥ 99%, zaś średni czas przeżycia w stadium zaawansowanym to 4-5 lat. W leczeniu systemowym podtypu hormonozależnego stosuje się hormonoterapię u wszystkich pacjentek oraz chemioterapię, która stosowana jest u niektórych chorych, w zależności od występowania innych czynników, zwiększających ryzyko nawrotu, np. stopień złośliwości, współczynnik proliferacji komórek nowotworowych (Ki67), zajęcie węzłów chłonnych. Kolejnym podtypem jest rak piersi HER2 dodatni, który wykrywa się w 15-20% wszystkich zachorowań. Podtyp ten charakteryzuje się nadmierną ekspresją receptora HER2 na komórkach nowotworowych oraz nadmierną amplifikacją genu *HER2*. Uważa się, że w około 50% przypadków nowotworu HER2 dodatniego, równocześnie występuje ekspresja receptorów steroidowych. Przeżycie 5-letnie we wczesnym stadium w podtypie HER2 dodatnim szacuje się na podstawie danych amerykańskich na ≥ 94%, zaś średnie przeżycie w stadium zaawansowanym – na około 5 lat. Terapia systemowa obejmuje chemioterapię skojarzoną z leczeniem celowanym anty-HER2 (przeciwciała monoklonalne – trastuzumab, pertuzumab), a także z hormonoterapią w przypadku obecności receptorów ERs/PgRs). Najgorzej rokującym podtypem raka piersi, który diagnozowany jest w około 15% przypadków, jest rak piersi potrójnie ujemny (*ang. triple-negative breast cancer* - TNBC), który nie wykazuje ekspresji receptorów steroidowych, ani nadmiernej ekspresji receptora HER2. W leczeniu systemowym TNBC zastosowanie znajduje chemioterapia. Przeżycie 5-letnie we wczesnym stadium wynosi w USA ≥ 85%, zaś średnie przeżycie w zaawansowanym stadium waha się od 10 do 13 miesięcy [8-11].

Ocenę stopnia zaawansowania nowotworu piersi określa się na podstawie klasyfikacji TNM (*ang. tumor-node-metastasis*), bieżąco aktualizowanej przez Amerykański Wspólny Komitet ds. Raka (AJCC, *ang. American Joint Committee on Cancer*). Obecnie, od stycznia 2018 roku, obowiązuje jej ósma edycja. Klasyfikacja TNM opiera się na ocenie guza pierwotnego (*ang. tumor* – T), regionalnych węzłów chłonnych (*ang. node* – N) oraz

przerzutów odległych (*ang. metastases* – M). Biorąc pod uwagę poszczególne cechy, klasyfikacja pozwala na wyróżnienie stopnia zaawansowania od 0 do IV, który determinuje rokowanie, a także sposób postępowania. Stopień 0 oznacza bardzo wczesne stadium nowotworu, jest to tzw. rak nieinwazyjny. Stadium I oraz II oznacza inwazyjnego, ale nadal wczesnego raka piersi, w którym występowanie komórek nowotworowych ogranicza się do piersi lub najbliższych węzłów chłonnych. W stopniu III występują przerzuty do regionalnych węzłów chłonnych i/lub okolicznych tkanek, stąd stadium to jest określane jako miejscowo zaawansowane. Rak piersi w stopniu IV, nazywany również nowotworem piersi uogólnionym/rozsianym, to najbardziej zaawansowana postać raka piersi, gdzie stwierdza się obecność przerzutów odległych, najczęściej w kościach, wątrobie, płucach, mózgu. Prawdopodobieństwo przeżycia 5 lat od chwili diagnozy w zależności od stadium nowotworu piersi zmniejsza się wraz ze wzrostem stopnia zaawansowania - od 100% w stadium 0 i I do 22% w stadium IV [9,12].

2.2. Rak piersi – leczenie ze szczególnym uwzględnieniem hormonoterapii.

Cele leczenia raka piersi różnią się w zależności od stopnia jego zaawansowania. W przypadku nowotworu w stadium 0, a więc w raku przedinwazyjnym, u większości chorych zalecana jest obserwacja, a gdy występują dodatkowe czynniki ryzyka (np. dodatni wywiad rodzinny, występowanie mutacji *BRCA1/2*), możliwa jest profilaktyczna amputacja piersi z rekonstrukcją. Celem jest więc zabezpieczenie pacjentki przed progresją nowotworu do stadiów inwazyjnych. W stopniach I-III kluczowe jest usunięcie komórek nowotworowych guza pierwotnego oraz zajętych węzłów chłonnych, aby zapobiec powstaniu przerzutów odległych. W tym celu wykorzystuje się zarówno leczenie miejscowe (chirurgia, radioterapia), jak i leczenie systemowe (w zależności od podtypu nowotworu – hormonoterapię, chemioterapię, leczenie celowane). W leczeniu zaawansowanego raka piersi (stadium IV) stosuje się leczenie paliatywne, które ma na celu wydłużenie czasu przeżycia pacjentki oraz łagodzenie objawów towarzyszących chorobie, a nie wyleczenie [9,12].

We wszystkich przypadkach hormonozależnego raka piersi, niezależnie od wieku pacjentki oraz dodatkowych wskazań do zastosowania chemioterapii, w leczeniu systemowym stosowana jest hormonoterapia. Leczenie hormonalne może zostać wdrożone zarówno jako leczenie przedoperacyjne (neoadjuwantowe), jak i pooperacyjne - jako terapia uzupełniająca. W obu przypadkach hormonoterapia ma za zadanie wyeliminować działanie endogennych estrogenów, pobudzających proliferację komórek nowotworowych [8,9]. W zależności od stanu menopauzalnego pacjentki, stosuje się więc inne podejście. Zgodnie z obowiązującymi

rekomendacjami leczenia hormonozależnego raka piersi, przedstawionymi przez Europejskie Towarzystwo Onkologii Klinicznej (ESMO, *ang. European Society for Medical Oncology*) oraz Polskie Towarzystwo Onkologii Klinicznej, hormonoterapia stosowana we wczesnym stadium raka piersi (stadium I-III wg klasyfikacji TNM) u pacjentek przed menopauzą obejmuje stosowanie tamoksyfenu przez 5-10 lat. U chorych, u których w tym czasie doszło do biochemicznie potwierdzonej menopauzy, można zamienić tamoksyfen na inhibitor aromatazy. U pacjentek przed menopauzą, z grupy wysokiego ryzyka nawrotu można zastosować inhibitor aromatazy zamiast tamoksyfenu, ale jedynie w połączeniu z farmakologiczną supresją jajników przy użyciu analogów gonadoliberyny (aGnRH, *ang. gonadotropin-releasing hormone analogs*). U pacjentek po menopauzie w hormonoterapii stosuje się tamoksyfen, inhibitory aromatazy lub ich sekwencję przez łączny czas 5-10 lat. W leczeniu zaawansowanego raka piersi (stadium IV wg klasyfikacji TNM), niezależnie od stanu menopauzalnego pacjentki, w hormonoterapii zastosowanie znajduje podobny zestaw leków, który w przypadku kobiet przed menopauzą uzupełniony jest o supresję/ablację jajników. W pierwszej linii leczenia stosowane są:

- tamoksyfen,
- inhibitory aromatazy (AIs, *ang. aromatase inhibitors*),
- fulwestrant w wysokich dawkach (tj. 500 mg *i.m.*),
- połączenie inhibitora aromatazy/fulwestrantu z inhibitorem zależnej od cyklin kinazy 4 i 6 (CDK4/6 inhibitor, *ang. cyclin-dependent kinase 4 and 6 inhibitor*).

W kolejnych liniach leczenia, w zależności od wcześniej zastosowanych leków, stosuje się fulwestrant lub inhibitor aromatazy w skojarzeniu z inhibitorem CDK4/6, eksemestan w połączeniu z ewerolimusem, octan megestrolu, a u chorych z mutacją genu *PIK3CA* (najczęstsza mutacja w hormonozależnym raku piersi) – kombinację inhibitora kinazy fosfatydyloinozytolu-3 (PI3K) - alpelisibu z fulwestrantem [9,13,14].

Hormonoterapia w raku piersi ma na celu wyeliminowanie stymulacji komórek nowotworowych przez estrogeny. Efekt taki można osiągnąć poprzez:

- hamowanie wytwarzania steroidowych hormonów płciowych w jajnikach (aGnRH),
- hamowanie obwodowej konwersji androgenów w estrogeny (inhibitory aromatazy),
- blokowanie wiązania się estrogenów z receptorami estrogenowymi w komórkach nowotworowych (selektywne modulatory receptora estrogenowego - SERMs, *ang. selective estrogen receptor modulators*),

- częściową degradację receptorów estrogenowych w komórkach nowotworowych (selektywni antagoniści receptora estrogenowego - SERDs, *ang. selective estrogen receptor down-regulators*) [4,15].

Lekami najczęściej stosowanymi w leczeniu hormonalnym nowotworu piersi są tamoksyfen (grupa SERM) oraz letrozol, anastrozol i eksemestan (inhibitory aromatazy) [15]. Tamoksyfen przez wiele lat uznawany był za złoty standard hormonoterapii, stosowany zarówno u kobiet przed, jak i po menopauzie. Mechanizm działania tamoksyfenu polega na kompetencyjnym wiązaniu się z receptorami estrogenowymi na komórkach nowotworowych, dzięki czemu blokowane jest działanie estrogenów, stymulujące ich proliferację. W niektórych tkankach tamoksyfen wykazuje aktywność agonistyczną, która może być korzystna (np. działanie agonistyczne w tkance kostnej, dzięki czemu u pacjentek po menopauzie nie dochodzi do demineralizacji kości), jak również odpowiada za najistotniejsze klinicznie działania niepożądane tego leku (powikłania zakrzepowo-zatorowe, przerost błony śluzowej macicy, wzrost ryzyka raka endometrium) [9,15,16].

Inhibitory aromatazy, dzięki swej wysokiej skuteczności klinicznej, stały się na przestrzeni ostatnich lat lekami stosowanymi jako pierwsza linia leczenia u pacjentek po menopauzie z hormonozależnym rakiem piersi. U kobiet po menopauzie estrogeny powstają w wyniku konwersji androgenów do estrogenów, która zachodzi w tkankach obwodowych (np. w tkance tłuszczowej, skórze, mięśniach, wątrobie). W procesie tym niezbędna jest aktywność enzymu – aromatazy, a więc inhibitory aromatazy hamując jej aktywność, eliminują powstawanie estrogenów w organizmie. Ze względu na budowę chemiczną, wyróżnia się steroidowe (eksemestan) i niesteroidowe (letrozol, anastrozol) inhibitory aromatazy. Należy podkreślić, że u kobiet przed menopauzą zastosowanie inhibitorów aromatazy jest możliwe jedynie w połączeniu z dodatkową supresją czynności jajników. Mechanizm działania inhibitorów aromatazy skutkuje typowymi działaniami niepożądanymi, związanymi z niedoborem estrogenów. Są to m.in. bóle stawów i mięśni, obniżenie gęstości mineralnej kości, zaburzenia gospodarki lipidowej. Niektóre badania wskazują również na zwiększone ryzyko rozwoju insulinooporności oraz cukrzycy [17-22].

2.3. Metaloestrogeny.

Z uwagi na stale utrzymującą się tendencję wzrostową zapadalności na raka piersi w populacji ogólnej, analiza oddziaływania różnych czynników, w tym środowiskowych, w patogenezie raka piersi jest przedmiotem wielu badań [23,24]. W ostatnich latach sporo

uwagi poświęca się ksenoestrogenom, zaliczanym do szerokiej grupy związków endokrynnie czynnych (EDC, *ang. endocrine disrupting compounds*), które jak wskazują dane z piśmiennictwa, mogą oddziaływać z układem hormonalnym człowieka [25]. Liczne publikacje potwierdzają, iż ksenoestrogeny mogą pobudzać receptory estrogenowe i tym samym konkurować lub naśladować działanie endogennych estrogenów, m.in. wpływać na proliferację komórek nowotworowych [23,26,27]. Zdecydowanie mniej wiadomo na temat wpływu ksenoestrogenów na skuteczność leczenia najczęstszego podtypu raka piersi – nowotworu hormonozależnego, w którego terapii wykorzystuje się leczenie farmakologiczne mające na celu eliminację działania endogennych estrogenów.

Metaloestrogeny to istotna ze względów toksykologicznych grupa ksenoestrogenów, którą stanowią jony metali, np. kadmu, glinu, chromu, miedzi, ołowiu, niklu, rtęci. Choć większość z nich jest uwalniana do środowiska ze źródeł przemysłowych (górnictwo, hutnictwo, galwanizacja) i dostają się do organizmu człowieka w ramach ekspozycji zawodowej, to część z nich jest powszechna w codziennym środowisku życia człowieka i często nie jest kojarzona z negatywnym wpływem na zdrowie. Przykładem mogą być sole glinu, będące jednym z głównych składników dezodorantów i antyperspirantów, czy sole chromu [III], znajdujące się w suplementach diety witaminowo-mineralnych, „na odchudzanie", a także regulujących stężenie glukozy we krwi [24,28].

Choć człowiek może być narażony na związki glinu z wielu różnych źródeł, w tym z diety, czy poprzez stosowanie środków zobojętniających sok żołądkowy, to uważa się, iż to właśnie regularne stosowanie dezodorantów i antyperspirantów zawierających w składzie sole glinu (najczęściej chlorek glinu [III] lub chlorowodorek glinu [III]) odpowiada za największą ekspozycję na jony tego metalu. Produkty te stosuje się bezpośrednio w sąsiedztwie piersi, pod pachą, często również na skórę podrażnioną w wyniku golenia, co dodatkowo zwiększa jego przenikanie do organizmu [29]. Badania na komórkach nowotworowych piersi - MCF-7 wykazały, że glin działa estrogenopodobnie - zakłóca wiązanie się estradiolu z receptorem estrogenowym i naśladuje jego działanie. Co więcej, odkryto, że długotrwałe narażenie na glin w stężeniach 10-300 μM (stężenia niższe niż w kosmetykach) może prowadzić do nadmiernej i nieprawidłowej proliferacji komórek nabłonka gruczołu piersiowego, a także zwiększać migrację i inwazyjność komórek raka piersi. Działanie to było poprzedzone m.in. wzrostem syntezy DNA oraz podwójnymi pęknięciami nici DNA, co sugeruje, że glin wykazuje działanie podobne do onkogenów [30,31]. Z kolei chrom w środowisku występuje na dwóch głównych stopniach utlenienia jako chrom sześciowartościowy - Cr [VI] oraz trójwartościowy - Cr [III].

Chrom heksawalentny został uznany przez Międzynarodową Agencję Badań nad Rakiem za związek kancerogenny. Narażenie na chrom sześciowartościowy jest związane najczęściej z ekspozycją zawodową i dotyczy wielu gałęzi przemysłu, np. farbiarskiego, metalurgicznego, garbarskiego. Z kolei źródłem jonów Cr^{3+} są najczęściej wspomniane już suplementy diety. W literaturze dostępne są sprzeczne dane dotyczące cytotoksyczności oraz genotoksyczności chromu triwalentnego. Niektóre wskazują na to, że sole i związki chromu triwalentnego mogą indukować uszkodzenia DNA, wymianę chromatyd siostrzanych, stres oksydacyjny oraz prowadzić do powstania adduktów Cr-DNA. Ponadto w badaniach *in vitro*, podobnie jak w przypadku glinu, wykazano, że jony Cr^{3+} wykazują aktywność estrogenopodobną [28,32,33]. Pomimo powszechnej ekspozycji na wyżej opisane metaloestrogeny, nie ma w dostępnym piśmiennictwie danych dotyczących wpływu tych związków na skuteczność leków stosowanych w hormonoterapii raka piersi. Dodatkowo, jak wcześniej wspomniano, nie są one zazwyczaj kojarzone z negatywnym oddziaływaniem na organizm człowieka, co sprawiło, że spośród metaloestrogenów wybrano jony Cr^{3+} i Al^{3+} do dalszych badań.

2.4. Hiperglikemia a rak piersi.

Szacuje się, że nawet jedna trzecia pacjentek z rakiem piersi, choruje również na cukrzycę [34]. Cukrzyca nazywana jest często epidemią XXI wieku, w 2021 roku na cukrzycę chorowało 537 milionów ludzi. Biorąc pod uwagę szacunki Międzynarodowej Federacji Diabetologicznej (IDF, *ang. International Diabetes Federation*) prognozujące stały wzrost zachorowań na cukrzycę – do 643 milionów do 2030 roku oraz do 783 milionów do 2045 roku, trudno nie zgodzić się z tym określeniem [35]. Cukrzyca jest przewlekłą chorobą metaboliczną, charakteryzującą się hiperglikemią, wynikającą z zaburzenia wydzielania i/lub działania insuliny na komórki i tkanki. Utrzymująca się hiperglikemia może prowadzić do uszkodzenia wielu narządów, zwłaszcza nerek, serca, naczyń krwionośnych, czy nerwów [36]. Prócz tego, pacjenci z cukrzycą mają zwiększone ryzyko zachorowania na wiele typów nowotworów, w tym nowotwór piersi [37-41].

Komórki nowotworowe zużywają znacznie więcej glukozy niż komórki prawidłowe. Ten wzmożony metabolizm glukozy określany jest jako efekt Warburga i polega na pozyskiwaniu energii na drodze glikolizy z wytworzeniem mleczanu, pomimo dostępności tlenu. Takie przełączenie metaboliczne ma za zadanie zaspokoić zwiększone zapotrzebowanie energetyczne komórek nowotworowych [42,43]. Hiperglikemia działa w inny sposób na komórki prawidłowe i nowotworowe – w prawidłowych indukuje apoptozę, zaś w nowotworowych chroni przed apoptozą i dodatkowo nasila ich migrację, co sprawia, że

u pacjentów nowotworowych z hiperglikemią częściej dochodzi do powstawania przerzutów [44].

Cukrzyca i hiperglikemia nie tylko zwiększają ryzyko rozwoju raka piersi, ale także wiążą się z 40% wyższym ryzykiem zgonu w porównaniu do pacjentek bez cukrzycy [34]. Jednym z możliwych wyjaśnień zwiększonej śmiertelności z powodu tego nowotworu u pacjentek z cukrzycą jest mniejsza skuteczność stosowanych leków w warunkach hiperglikemii. W przeglądzie systematycznym, do którego włączono badania przedkliniczne, Gerards i wsp. [44] analizowali wpływ hiperglikemii na skuteczność chemioterapii. W większości badań panująca w modelu badawczym hiperglikemia, skutkowała osłabieniem skuteczności chemioterapii, co obserwowano jako zmniejszenie aktywności antyproliferacyjnej i/lub zakłócenie sygnalizacji apoptotycznej [44]. Hiperglikemia indukuje w komórkach stres oksydacyjny i generuje powstanie reaktywnych form tlenu (ROS, *ang. reactive oxygen species*). Nadmierne wytwarzanie ROS aktywuje jądrowy czynnik transkrypcyjny NF kappa B (NF- κB, *ang. nuclear factor kappa B*), który odgrywa kluczową rolę w procesie onkogenezy i progresji nowotworu. Dane z piśmiennictwa wskazują, że nadmierna ekspresja NF- κB może zwiększać proliferację komórek nowotworowych, nasilać angiogenezę, hamować apoptozę, a także przyczyniać się do wystąpienia oporności na terapię endokrynną [45-47].

3. CELE PRACY

Celem niniejszej rozprawy doktorskiej był:

1. Przegląd, analiza i podsumowanie aktualnego stanu wiedzy na temat wpływu ksenoestrogenów na skuteczność leków stosowanych w terapii hormonozależnego raka piersi.
2. Ocena profilu bezpieczeństwa inhibitorów aromatazy, stosowanych w leczeniu hormonozależnego raka piersi u pacjentek po menopauzie, ze szczególnym uwzględnieniem zaburzeń gospodarki węglowodanowej (np. hiperglikemii).
3. Ocena wpływu wybranych ksenoestrogenów – jonów metali Cr^{3+} i Al^{3+} na efektywność steroidowego (eksemestan) i niesteroidowego (letrozol) inhibitora aromatazy w modelu komórkowym hormonozależnego raka piersi.
4. Ocena, czy interakcja pomiędzy metaloestrogenami – jonami metali Cr^{3+} i Al^{3+} a inhibitorami aromatazy różni się w zależności od panujących w modelu komórkowym warunków, odzwierciedlających warunki normo- i hiperglikemii.

Wzajemne interakcje metaloestrogenów, inhibitorów aromatazy oraz hiperglikemii nie były dotychczas badane i analizowane, co sprawia, iż zagadnienia te mają znaczący aspekt poznawczy. Zidentyfikowanie możliwych interakcji pomiędzy AIs a obecnymi w środowisku metaloestrogenami, może wpłynąć pozytywnie na skuteczność i bezpieczeństwo terapii hormonozależnego raka piersi, dzięki wdrożeniu odpowiednich działań prewencyjnych.

4. MATERIAŁ I METODY

Metody stosowane w trakcie badań zostały szczegółowo opisane w artykułach oryginalnych wchodzących w skład cyklu publikacji, stanowiących podstawę niniejszej rozprawy doktorskiej. Poniżej znajduje się ogólny opis zastosowanych metod badawczych.

4.1. Przegląd systematyczny i metaanaliza (publikacja 2).

Przegląd systematyczny oraz metaanalizę wykonano zgodnie z zaleceniami PRISMA (*ang. Preferred reporting items for systematic reviews and meta-analyses*) [48], a protokół przeglądu systematycznego zarejestrowano w Międzynarodowym Prospektywnym Rejestrze Przeglądów Systematycznych – PROSPERO. Przeszukano bazy PubMed (Medline), EMBASE oraz Cochrane Central Register of Controlled Trials, bez stosowania kryteriów językowych, zgodnie z postawionym pytaniem klinicznym w modelu PICO(S):

- P – ang. *patient,* pacjent – pacjentki po menopauzie z hormonozależnym rakiem piersi,
- I – ang. *intervention,* interwencja, grupa badana – inhibitory aromatazy III generacji (anastrozol, eksemestan, letrozol), stosowane w terapii neoadjuwantowej, adjuwantowej lub przedłużonej terapii adjuwantowej,
- C – ang. *comparison,* porównanie, grupa kontrolna – tamoksyfen, placebo lub brak leczenia,
- O – ang. *outcome*, punkt końcowy – częstość występowania działań niepożądanych ze strony układu sercowo-naczyniowego (nadciśnienie tętnicze, zdarzenia sercowo-naczyniowe, takie jak zaburzenia rytmu serca, choroba niedokrwienna serca, zawał mięśnia sercowego, niewydolność serca, migotanie przedsionków) i metabolicznych (hiperglikemia, zwiększenie masy ciała, dyslipidemia),
- S – ang. *study design*, typ badań - kontrolowane, randomizowane badania kliniczne z udziałem ludzi (faza II i III).

Selekcja badań włączonych do przeglądu systematycznego i metaanalizy była prowadzona niezależnie przez dwóch autorów, zaś wszelkie rozbieżności w interpretacji były omawiane i wyjaśniane z udziałem wszystkich autorów. Ostatecznie do przeglądu systematycznego zakwalifikowano 21 publikacji, zaś do metaanalizy 18 publikacji. Każda z publikacji została poddana ocenie jakości przy pomocy narzędzia Cochrane Collaboration [49]. Wykonano 4 oddzielne metaanalizy (metaanaliza przy użyciu modelu losowego) w zależności od projektu badań klinicznych. Ilorazy szans (OR) oraz 95% przedziały ufności

(95% CI) obliczono i zilustrowano za pomocą wykresów typu *forest plot*. Niejednorodność wyników badań (*ang. heterogeneity of results*) oszacowano przy pomocy testu statystycznego I^2. Wszystkie analizy wykonano przy użyciu pakietu R-„meta" wersja 4.19.0, a wartości $p < 0,05$ uznano za istotne statystycznie.

4.2. Metody eksperymentalne.

4.2.1. Hodowla komórkowa.

W badaniach wykorzystano linię komórkową estrogenozależnego raka piersi – MCF-7, którą zakupiono w CLS Cell Lines Service GmbH (Eppelheim, Niemcy) i następnie hodowano w kompletnym medium wzrostowym DMEM (*ang. Dulbecco's modified eagle's medium*). Aby zasymulować warunki normoglikemii i hiperglikemii, hodowlę prowadzono w dwóch wariantach, wykorzystując w tym celu media hodowlane z różnym stężeniem glukozy – DMEM high-glucose o stężeniu glukozy 25 mM (warunki hiperglikemii) oraz DMEM low-glucose o stężeniu glukozy 5,5 mM (warunki normoglikemii) (Biological Industries, Izrael). Jest to metoda dobrze opisana w istniejącej literaturze naukowej [50-53]. Medium wzrostowe wzbogacano bydlęcą surowicą płodową (Biological Industries, Izrael) – 10% v/v, dodatkiem antybiotyków (streptomycyna 10,000 U/mL, penicylina 10 mg/mL, Biological Industries, Izrael), 2 mM L-glutaminy (Gibco, USA) oraz 10^{-9}M testosteronu (Sigma Aldrich, USA). Drugą badaną linię komórkową – MCF-7/DOX (linia oporna na doksorubicynę, z nadekspresją P-gp - glikoproteiny P) otrzymano z linii MCF-7 poprzez trzymiesięczną hodowlę w obecności niskich stężeń doksorubicyny, którą następnie hodowano analogicznie do linii MCF-7. Hodowla komórkowa prowadzona była w warunkach jałowych, w sterylnych, jednorazowych naczyniach hodowlanych, w warunkach standardowych, tzn. w inkubatorze w 5% CO_2 i temperaturze 37°C.

W czasie badań prowadzonych w ramach niniejszej pracy doktorskiej, linie komórkowe były eksponowane na działanie inhibitorów aromatazy (letrozolu lub eksemestanu) oraz metaloestrogenów (jony metali Cr^{3+} lub Al^{3+}) lub ich kombinacje.

4.2.2. Roztwory podstawowe i testowe AIs oraz metaloestrogenów.

Przed przystąpieniem do eksperymentów sporządzono roztwory podstawowe inhibitorów aromatazy oraz metaloestrogenów:

- 100 mM roztwór letrozolu [$C_{17}H_{11}N_5$] w DMSO,
- 100 mM roztwór eksemestanu [$C_{20}H_{24}O_2$] w DMSO,

- 1 mM roztwór chlorku glinu [$AlCl_3$] w wodzie dejonizowanej,
- 1 mM roztwór heksahydratu chlorku chromu (III) [$CrCl_3$ x $6H_2O$] w wodzie dejonizowanej.

Wszystkie odczynniki pochodziły z firmy Sigma Aldrich, USA. Roztwory podstawowe przechowywane były w temperaturze -20°C. Roztwory robocze przygotowywane były bezpośrednio przed każdym eksperymentem poprzez rozcieńczenie w odpowiedniej ilości medium hodowlanego. Stężenia roztworów roboczych zostały dobrane w oparciu o dane dostępne w piśmiennictwie oraz wstępne badania własne (dane niepublikowane).

4.2.3. Test żywotności komórek – XTT.

Do wykonania testu żywotności komórek użyto 96-dołkowych płytek, na które osadzano $1x10^4$ komórek na dołek. Następnie komórki inkubowano z odpowiednimi stężeniami badanych związków przez 72 godziny. Po tym czasie wykonywano test XTT (Roche Diagnostics, Niemcy) zgodnie z instrukcją producenta. Test XTT jest testem kolorymetrycznym, bazującym na reakcji redukcji soli tetrazolowej (XTT, sól sodowa 2,3-bis[2-metoksy-4-nitro-5-sulfofenylo]-2H-tetrazolio-5-karboksyanilid) do formazanu. Reakcja ta zachodzi przy udziale enzymów mitochondrialnych, absorbancja jest więc proporcjonalna do ilości żywych, aktywnych metabolicznie komórek. Absorbancję mierzono w aparacie Synergy HTX Multi-Mode Microplate Reader (BioTek, USA) przy długości fali λ=450 nm i przy długości fali referencyjnej λ=650 nm.

4.2.4. Analizy z wykorzystaniem cytometru przepływowego.

Badania przeprowadzono z wykorzystaniem cytometru przepływowego CyFlow®SPACE (Sysmex, Japonia), zaś ich wyniki analizowano przy użyciu oprogramowania FCS Express 7 Cytometry (De Novo Software, USA). Do pomiaru fluorescencji aneksyny V skoniugowanej z tioizocyjanianem fluoresceiny (Annexin V-FITC) zastosowano wzbudzenie laserowe 488 nm (50 mW) i filtr 536/40 (BP). Fluorescencję jodku propidyny (PI) mierzono za pomocą wzbudzenia laserowego 488 nm (50 mW) i filtra 675/20 (BP).

4.2.4.1. Ocena apoptozy i nekrozy przy pomocy cytometrii przepływowej.

Do oceny liczby komórek ulegających apoptozie oraz nekrozie po 72-godzinnej ekspozycji na badane związki i ich kombinacje, wykorzystano zestaw Annexin V-FITC Apoptosis Kit (Invitrogen, USA), który zawiera Annexin V-FITC oraz PI. Annexin V-FITC wiąże się specyficznie z fosfatydyloseryną (przemieszczanie się fosfofadyloseryny na zewnętrzną stronę błony komórkowej jest jednym z pierwszych etapów programowanej śmierci

komórkowej), zaś jodek propidyny wnika do wnętrza komórki i wiąże się z kwasami nukleinowymi. Na podstawie tego testu możliwe jest zróżnicowanie populacji komórek na cztery grupy: komórki żywe, nie barwiące się żadnym odczynnikiem [Annexin V-FITC-, PI-], komórki apoptotyczne we wczesnym stadium apoptozy [Annexin V-FITC+, PI-], komórki w późnym stadium apoptozy i/lub nekrozy [Annexin V-FITC+, PI+] oraz komórki nekrotyczne [Annexin V-FITC-, PI+]. Szczegółowa procedura testu została opisana w załączonej publikacji 3 oraz 4.

4.2.4.2. Analiza cyklu komórkowego.

Wykorzystując jodek propidyny, który wiąże się stechiometrycznie do DNA komórki, możliwe jest również określenie zawartości DNA w komórkach (ilość PI związanego z komórką jest proporcjonalna do ilości DNA). Ilość DNA w komórce zależna jest od fazy cyklu życiowego, w której znajduje się komórka, tak więc analiza cyklu komórkowego dostarcza informacji na temat rozkładu procentowego komórek w poszczególnych jego fazach – fazie G0/G1, fazie S oraz fazie G2/M. W badaniach użyto komercyjnego roztworu barwiącego – FxCycle^{TM}PI/RNase Staining Solution (Invitrogen, USA). Szczegółowa procedura testu została opisana w załączonej publikacji 3.

4.2.5. Testy immunoenzymatyczne ELISA.

Testy immunoenzymatyczne ELISA wykorzystano do oceny ilościowej stężenia białek zaangażowanych w proces apoptozy – Bcl-2 (białko antyapoptotyczne), BAX (białko proapoptotyczne), oraz białka VEGF-A (ang. *Vascular Endothelial Growth Factor*). Oznaczenia były wykonywane w lizatach komórkowych, które uzyskiwano z komórek poddanych 48-godzinnej inkubacji z badanymi związkami lub ich kombinacjami. Przed wykonaniem testów ELISA, w lizatach komórkowych oznaczano całkowite stężenie białka przy pomocy testu Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Testy ELISA - Nori Human Apoptosis Regulator BAX ELISA Kit, Nori Human Bcl-2 ELISA Kit oraz Nori Human VEGF-A ELISA Kit (Genorise Scientific, USA), zostały wykonane zgodnie z instrukcją producenta. Absorbancje przy długości fali λ=570 nm zmierzono przy pomocy Synergy HTX Multi-Mode Microplate Reader (BioTek, USA). Do obliczenia stężeń Bcl-2, BAX oraz VEGF-A użyto 4-parametrowej krzywej logistycznej (4PL, ang. *four parameter logistic curve*), wygenerowanej ze strony internetowej Arigo Biolaboratories (https://www.arigobio.com/elisa-analysis). Stężenia białek przeliczono na 100 µl białka całkowitego w próbce. Obliczono również współczynnik Bcl-2/BAX (Bcl-2/BAX ratio). Wszystkie powyższe procedury opisano szczegółowo w publikacji 3 oraz 4.

4.2.6. Analiza statystyczna.

Analizę statystyczną uzyskanych wyników wykonano za pomocą programu GraphPadPrism 9 (GraphPad Software, USA). Każdy eksperyment został powtórzony trzykrotnie, a wynik został przedstawiony jako średnia arytmetyczna oraz odchylenie standardowe SD (*ang.* s*tandard deviation*). Normalność rozkładu sprawdzono testem Shapiro-Wilka. Do analizy danych uzyskanych w eksperymentach prowadzonych w warunkach normo- i hiperglikemii zastosowano jednoczynnikową analizę wariancji (*ang*. *one-way ANOVA*) oraz testy porównań wielokrotnych (test Tukeya, a w przypadku testu żywotności komórek dla pojedynczych związków – test Dunnetta). Porównując wyniki uzyskane w warunkach normoglikemii vs. hiperglikemii, zastosowano dwuczynnikową analizę wariancji (*ang. two-way ANOVA*) oraz testy porównań wielokrotnych (test Tukeya). We wszystkich analizach za wartość istotną statystycznie przyjęto $p<0,05$.

5. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY

5.1. Publikacja 1.

Pierwsza publikacja pt.: *The impact of xenoestrogens on effectiveness of treatment for hormone-dependent breast cancer - current state of knowledge and perspectives for research* jest pracą przeglądową, opublikowaną w 2020 roku w czasopiśmie Annals of Agricultural and Environmental Medicine. Miała ona na celu przegląd, analizę i podsumowanie aktualnego stanu wiedzy na temat interakcji ksenoestrogenów z lekami stosowanymi w terapii hormonozależnego raka piersi oraz wskazanie obszarów wymagających dalszych badań.

Ksenoestrogeny to substancje egzogenne, zakłócające funkcjonowanie układu hormonalnego. Poprzez oddziaływanie z receptorami estrogenowymi, ksenoestrogeny mogą działać jako ich antagoniści lub agoniści. Mogą również zaburzać syntezę i metabolizm endogennych estrogenów, jak i wpływać na ekspresję receptorów estrogenowych [54]. Wyróżnia się kilka najważniejszych klas ksenoestrogenów – fitoestrogeny (np. genisteina, apigenina), mykoestrogeny (np. zearalenon), metaloestrogeny (jony metali np. kadmu, glinu, chromu), pestycydy (np. dichlorodifenylotrichloroetan - DDT) oraz chemikalia przemysłowe (np. bisfenole, parabeny) [55].

Przegląd dostępnej literatury wykazał, że fitoestrogeny, w szczególności flawonoid genisteina, są najlepiej zbadaną grupą ksenoestrogenów pod kątem interakcji z lekami stosowanymi w terapii hormonozależnego raka piersi. Ich źródłem mogą być zarówno leki oraz suplementy diety przeznaczone do łagodzenia objawów menopauzy (większość z nich zawiera kilka fitoestrogenów w składzie), jak i pożywienie (m.in. soja, siemię lniane, koniczyna). W badaniach *in vitro* oraz *in vivo* wykazano, że genisteina może zmniejszać skuteczność działania tamoksyfenu, inhibitora aromatazy - letrozolu, a także kombinacji leków letrozol + palbocyklib (AI + inhibitor CDK4/6) [56-59]. Przeanalizowano również pracę, w której badano wpływ czterech, dostępnych na rynku wieloskładnikowych suplementów diety na skuteczność leków stosowanych w hormonoterapii raka piersi. Wykazano, że wszystkie testowane suplementy aktywują zależny od receptora estrogenowego wzrost proliferacji komórek MCF-7, który nie został zahamowany ani przez 4-hydroksytamoksyfen (4-OH-TAM, aktywny metabolit tamoksyfenu), ani przez letrozol [60]. Spośród pozostałych ksenoestrogenów, nie będących pochodzenia naturalnego, odnaleziono doniesienia jedynie dla bisfenolu A (BPA) oraz metylparabenu. Badania *in vitro* wskazują, że jednoczesna ekspozycja na 4-OH-TAM i bisfenol A zmniejsza efekt terapeutyczny leku, a efekt ten jest tym słabszy im

wyższe jest stężenie BPA [61]. Z kolei metylparaben może przyczyniać się do wystąpienia chemooporności na leki stosowane w leczeniu raka piersi (tamoksyfen, fulwestrant) [62].

Na podstawie wykonanego przeglądu piśmiennictwa, została postawiona teza, iż obszarem wymagającym dalszych badań jest analiza oddziaływania ksenoestrogenów innych niż fitoestrogeny, np. metaloestrogenów, na skuteczność leków stosowanych w leczeniu hormonozależnego raka piersi. W dostępnej literaturze brakuje tego typu doniesień. Biorąc pod uwagę, że ekspozycja na metaloestrogeny jest powszechna, a w przypadku wielu z nich, np. jonów Al^{3+} oraz Cr^{3+} często nie jest kojarzona z negatywnym wpływem na zdrowie, stało się to inspiracją do dalszych badań realizowanych w ramach niniejszej rozprawy doktorskiej.

5.2. Publikacja 2.

Druga wchodząca w skład cyklu publikacji praca pt.: *Aromatase Inhibitors and Risk of Metabolic and Cardiovascular Adverse Effects in Breast Cancer Patients-A Systematic Review and Meta-Analysis* została opublikowana w 2022 roku w czasopiśmie Journal of Clinical Medicine. Jest to przegląd systematyczny oraz metaanaliza, które przez sporą część wysokopunktowanych czasopism naukowych klasyfikowane są jako prace oryginalne [63]. Metaanaliza wykorzystuje metody statystyczne do ilościowej syntezy danych z wielu badań klinicznych. W hierarchii danych naukowych, zgodnie z zasadami EBM (*ang. evidence based medicine*), przeglądy systematyczne i metaanalizy znajdują się na jej szczycie [64].

Celem niniejszej publikacji była ocena profilu bezpieczeństwa inhibitorów aromatazy, stosowanych w leczeniu hormonozależnego raka piersi u pacjentek po menopauzie, ze szczególnym uwzględnieniem działań niepożądanych ze strony układu sercowo-naczyniowego oraz metabolicznych, takich jak hiperglikemia, zwiększenie masy ciała, czy dyslipidemia.

Inhibitory aromatazy to obecnie najczęściej stosowana grupa leków w hormonoterapii raka piersi. W zależności od sytuacji klinicznej, terapia inhibitorami aromatazy trwa od 2 (leczenie sekwencyjne z tamoksyfenem) do 10 lat (przedłużona hormonoterapia), co sprawia iż działania niepożądane związane z ich stosowaniem, mogą mieć istotny wpływ na przebieg leczenia i dobrostan pacjentek [65,66]. Najczęstszymi działaniami niepożądanymi zgłaszanymi podczas terapii inhibitorami aromatazy są bóle mięśni i stawów, obniżenie gęstości mineralnej kości oraz inne dolegliwości związane ze spadkiem stężenia estrogenów w organizmie (np. suchość pochwy, uderzenia gorąca, nocne poty). Pacjentki leczone tą grupą leków są również bardziej podatne na rozwój hiperlipidemii, hipercholesterolemii i nadciśnienia tętniczego, które są uznanymi czynnikami ryzyka rozwoju chorób sercowo-naczyniowych [19]. Niektóre badania wskazują również na zwiększone ryzyko rozwoju insulinooporności oraz cukrzycy u takich osób [21,22,67]. Biorąc pod uwagę fakt, że inhibitory aromatazy są stosowane głównie u kobiet po menopauzie, a okres pomenopauzalny sam w sobie związany jest ze zwiększonym ryzykiem wystąpienia zdarzeń sercowo-naczyniowych, zaburzeń gospodarki lipidowej i węglowodanowej, ocena czy ryzyko rozwoju tych zaburzeń dodatkowo zwiększa się przy stosowaniu farmakoterapii AI, jest istotnym problemem badawczym [68].

Model badawczy.

Przegląd systematyczny oraz metaanalizę wykonano zgodnie z zaleceniami PRISMA (ang. *Preferred reporting items for systematic reviews and meta-analyses*) [48], a protokół

przeglądu systematycznego zarejestrowano w Międzynarodowym Prospektywnym Rejestrze Przeglądów Systematycznych – PROSPERO. Sformułowanie pytania klinicznego w modelu PICO(S) pozwoliło na zawężenie poszukiwań badań pierwotnych, co ostatecznie zaowocowało włączeniem 21 publikacji do przeglądu systematycznego oraz 18 z nich do metaanalizy. Ze względu na zróżnicowane schematy terapeutyczne stosowane w analizowanych badaniach klinicznych, zdecydowano się wykonać cztery oddzielne analizy w zależności od stosowanego w grupie badanej i kontrolnej leczenia:

1. pacjentki stosujące w monoterapii inhibitor aromatazy vs. tamoksyfen,
2. pacjentki stosujące monoterapię inhibitorem aromatazy lub terapię sekwencyjną tamoksyfen → inhibitor aromatazy vs. tamoksyfen,
3. pacjentki stosujące w monoterapii inhibitor aromatazy vs. pacjentki stosujące monoterapię tamoksyfenem lub terapię sekwencyjną tamoksyfen → inhibitor aromatazy,
4. pacjentki stosujące monoterapię inhibitorem aromatazy vs. pacjentki otrzymujące placebo.

Najistotniejsze wyniki i ich omówienie.

Dostępne w badaniach pierwotnych dane pozwoliły na wykonanie metaanalizy dla czterech punktów końcowych – zdarzenia sercowo-naczyniowe (definiowane jako choroba wieńcowa serca, zawał serca, niewydolność serca, migotanie przedsionków, arytmia), nadciśnienie tętnicze, przyrost masy ciała oraz dyslipidemia (definiowana jako wszelkie zaburzenia lipidowe, np. hiperlipidemia, hipercholesterolemia, hipertriglicerydemia). Wykazano, że stosowanie inhibitorów aromatazy w monoterapii lub w terapii sekwencyjnej z tamoksyfenem związane jest z 16% wzrostem ryzyka wystąpienia zdarzeń sercowo-naczyniowych w porównaniu do stosowania tamoksyfenu w monoterapii (OR = 1.16; 95% CI 1.04-1.30) oraz z 24% wzrostem ryzyka, gdy porównywano grupę otrzymującą AI w monoterapii vs. grupa otrzymująca tamoksyfen w monoterapii lub w sekwencji z AI (OR = 1.24; 95% CI 1.11-1.38). Brak istotności statystycznej przy porównaniu grup stosujących AI vs. placebo (OR = 1.08; 95% CI 0.88-1.33), może wskazywać, że wzrost ryzyka zdarzeń sercowo-naczyniowych jest związane raczej z kardioprotekcyjnym działaniem tamoksyfenu, aniżeli kardiotoksycznością AI. Niezależnie od konstrukcji analizy, nie wykazano statystycznie istotnego wzrostu ryzyka wystąpienia nadciśnienia tętniczego, przyrostu masy ciała oraz dyslipidemii u pacjentek stosujących AI. Warto jednak zaznaczyć, że w przypadku nadciśnienia

tętniczego oraz dyslipidemii badania wykazywały znaczną niejednorodność wyników, co utrudnia jednoznaczną interpretację.

Dane w badaniach pierwotnych dotyczące związanej ze stosowaniem AI hiperglikemii były niewystarczające, aby wykonać metaanalizę. Hiperglikemia raportowana była jedynie w 5 badaniach klinicznych o różnej konstrukcji, zaś zgodnie z przyjętymi założeniami, wykonanie metaanalizy było możliwe przy występowaniu danego działania niepożądanego co najmniej w 3 badaniach o takiej samej konstrukcji. Mimo to wydaje się, że występowanie zaburzeń gospodarki węglowodanowej u pacjentek leczonych AI jest klinicznie istotne. W badaniu Iwata i wsp. [69], w którym porównywano skuteczność i bezpieczeństwo dwóch AI – anastrozolu i eksemestanu w pierwszej linii leczenia zaawansowanego raka piersi, hiperglikemia raportowana była odpowiednio u 47,7% i 51,4% pacjentek. Inni autorzy wskazują, że leczenie AI może wiązać się z rozwojem insulinooporności oraz cukrzycy. Badania przeprowadzone z udziałem pacjentek, które zakończyły leczenie raka piersi, wykazały, że stosowanie hormonoterapii wiązało się z ryzykiem rozwoju cukrzycy i było znacząco wyższe dla AI (HR=4.27; 95% CI 1.42-12.84; p=0.010) niż dla tamoksyfenu (HR=2.25; 95% CI 1.19-4.26; p=0.013) [21,22].

Podsumowując, wykonany przegląd systematyczny i metaanaliza stanowią cenne uzupełnienie wiedzy na temat działań niepożądanych inhibitorów aromatazy, które są mniej kojarzone z tą grupą leków. Wyniki wskazują na zwiększone ryzyko występowania zdarzeń sercowo-naczyniowych w czasie stosowania AI zamiast tamoksyfenu, jest to jednak spowodowane w większej mierze kardioprotekcyjnym działaniem tamoksyfenu niż kardiotoksycznością AI. Biorąc pod uwagę fakt, że główną grupą pacjentek stosującą AI są pacjentki po menopauzie, warto również monitorować bezpieczeństwo farmakoterapii pod kątem jej wpływu na gospodarkę węglowodanową oraz lipidową.

5.3. Publikacja 3.

Trzecia publikacja pt.: *The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model,* to publikacja oryginalna, opublikowana w 2023 roku w czasopiśmie Cancers. Opisane w niej badania miały na celu ocenę wpływu wybranych metaloestrogenów - jonów metali Cr^{3+} i Al^{3+} na efektywność steroidowego (eksemestan) i niesteroidowego (letrozol) inhibitora aromatazy w modelu komórkowym hormonozależnego raka piersi w warunkach normoglikemii.

Model badawczy.

Badania eksperymentalne, których wyniki znajdują się w publikacji 3 oraz 4, zostały wykonane na dwóch liniach komórkowych estrogenozależnego raka piersi – linii MCF-7 oraz MCF-7/DOX (linii opornej na doksorubicynę, z nadekspresją P-gp). Linia MCF-7/DOX została włączona do badań, ponieważ część pacjentek z hormonozależnym rakiem piersi, u których występują dodatkowe czynniki ryzyka nawrotu (np. masywne zajęcie węzłów chłonnych ≥4, rozmiar guza >5 cm, wysokie ryzyko na podstawie profilu molekularnego), otrzymuje przed- lub pooperacyjną chemioterapię, polegającą na sekwencyjnym stosowaniu wielolekowych schematów opartych głównie na antracyklinach (np. doksorubicyna) i taksoidach (np. docetaksel, paklitaksel) [9]. Może to prowadzić do selekcji komórek opornych na doksorubicynę - pod wpływem prowadzonej chemioterapii, w komórkach nowotworowych piersi może rozwinąć się wtórna oporność wielolekowa (MDR, *ang. multidrug resistance*), wynikająca m.in. z nadmiernej ekspresji białka transportowego P-gp, należącego do rodziny białek ABC, odpowiedzialnego za wyrzut chemioterapeutyków z komórki. Dane z piśmiennictwa wskazują, że nadmierna ekspresja P-gp występuje u 15,5% pacjentek z inwazyjnym rakiem piersi z przerzutami do węzłów chłonnych [70-73]. Obie linie komórkowe były eksponowane na działanie pojedynczych związków (inhibitorów aromatazy – letrozolu lub eksemestanu, metaloestrogenów – jonów Al^{3+} lub Cr^{3+}) oraz ich kombinacji.

Najistotniejsze wyniki i ich omówienie.

Przeprowadzone badania potwierdziły, zgodnie z wcześniejszymi przypuszczeniami, że linie komórkowe MCF-7 oraz MCF-7/DOX reagują inaczej na badane leki i metaloestrogeny. Komórki MCF-7/DOX były mniej wrażliwe na cytotoksyczne działanie obu inhibitorów aromatazy oraz na apoptozę. Towarzyszyło temu, m.in. niższe, wykrywane w lizatach komórkowych, stężenie białka BAX (białko o aktywności proapoptotycznej) oraz wyższy współczynnik Bcl-2/BAX, który wskazuje na zmniejszoną wrażliwość na apoptozę komórek

MCF-7/DOX [74]. Jednocześnie komórki MCF-7/DOX były mniej wrażliwe na stymulujące proliferację działanie metaloestrogenów. Warto podkreślić słabsze działanie letrozolu w porównaniu do eksemestanu w obu liniach komórkowych, we wszystkich badanych układach, które manifestowało się mniej nasilonym działaniem cytotoksycznym i proapoptotycznym.

W badaniu obserwowano statystycznie istotne zmniejszenie aktywności inhibitorów aromatazy w kombinacji z metaloestrogenami w obu badanych liniach komórkowych. W linii MCF-7 kombinacja eksemestanu w niższym stężeniu (100 μM) z Al^{3+} lub Cr^{3+} zwiększała żywotność komórek nowotworowych, a więc zmniejszała cytotoksyczne działanie leku. Kombinacja z metaloestrogenami zmniejszała również skuteczność letrozolu (w stężeniu 100 μM), co podobnie, było obserwowane jako zwiększenie żywotności komórek nowotworowych. Te same zależności wykazano w linii MCF-7/DOX, ale przy kombinacji metaloestrogenów z wyższym stężeniem eksemestanu (200 μM) i obu stężeń letrozolu.

Apoptoza, czyli programowana śmierć komórki, zachodzi w odpowiedzi na różne bodźce i może być indukowana przez wewnętrzny (mitochondrialny) i zewnętrzny szlak apoptozy. Szlak mitochondrialny jest regulowany przez rodzinę białek Bcl-2 – równowagę pomiędzy białkami proapoptotycznymi, np. BAX, a antyapoptotycznymi, np. Bcl-2 [75]. Działanie inhibitorów aromatazy, tak jak wielu innych leków stosowanych w leczeniu nowotworów, prowadzi do śmierci komórek poprzez inicjację apoptozy lub dysregulację cyklu komórkowego. Letrozol (niesteroidowy AI) hamuje proliferację komórek nowotworowych poprzez zatrzymanie cyklu komórkowego (w fazie G0/G1) oraz aktywację mitochondrialnego szlaku apoptozy. Eksemestan (steroidowy AI) zmniejsza proliferację komórek MCF-7 poprzez zatrzymanie cyklu komórkowego w fazie G0/G1 oraz G2/M, podobnie jak letrozol aktywuje apoptozę poprzez szlak wewnętrzny oraz dodatkowo nasila autofagię [76-79]. W wykonanych badaniach zaobserwowano, że połączenie eksemestanu (w stężeniu 100 μM) z Al^{3+} lub Cr^{3+} prowadzi do istotnej statystycznie redukcji liczby komórek MCF-7 oraz MCF-7/DOX ulegających apoptozie, co współistnieje ze wzrostem współczynnika Bcl-2/BAX. W przypadku letrozolu istotną statystycznie redukcję odsetka komórek ulegających apoptozie zaobserwowano jedynie w komórkach MCF-7 przy kombinacji wyższego stężenia letrozolu (100 μM) z metaloestrogenami. Nie towarzyszyła temu jednak istotna statystycznie zmiana współczynnika Bcl-2/BAX.

Wykazano ponadto, że zmniejszenie aktywności inhibitorów aromatazy pod wpływem metaloestrogenów, nie jest związane z wpływem na cykl komórkowy. Estrogeny zwiększają proliferację komórek m.in. poprzez stymulację przejścia przez fazę G0/G1 cyklu komórkowego. AIs, zarówno steroidowe, jak i niesteroidowe, blokując działanie estrogenów, zatrzymują cykl komórkowy w fazie G0/G1 (eksemestan również w fazie G2/M), co potwierdziliśmy w naszych badaniach [77,79]. Jednoczesna ekspozycja komórek MCF-7 oraz MCF-7/DOX na AIs oraz metaloestrogeny nie skutkowała statystycznie istotną zmianą dystrybucji komórek w poszczególnych fazach cyklu komórkowego.

Podsumowując, publikacja 3 dowodzi, że badane metaloestrogeny – jony Cr^{3+} i Al^{3+} mogą w warunkach normoglikemii zmniejszać efektywność inhibitorów aromatazy. Obniżenie skuteczności działania leków było obserwowane jako zwiększenie żywotności komórek nowotworowych oraz zmniejszenie ich wrażliwości na apoptozę. Ze względu na powszechne narażenie na badane metaloestrogeny, potrzebne są dalsze badania, które w pełni wyjaśnią wykazane interakcje, aby móc skutecznie im przeciwdziałać w trakcie terapii pacjentek z hormonozależnym rakiem piersi.

5.4. Publikacja 4.

Czwarta w cyklu publikacja pt.: *High glucose reduces anti-tumor activity of aromatase inhibitors in a hormone-dependent breast cancer cell model,* jest kolejną publikacją oryginalną, która została przyjęta do publikacji 14.06.2023 przez czasopismo Acta Poloniae Pharmaceutica - Drug Research (w chwili wydruku rozprawy doktorskiej – manuskrypt, po otrzymaniu pozytywnych recenzji, został przyjęty do publikacji; decyzja czasopisma została zamieszczona w załączniku 4). Celem pracy była ocena, czy metaloestrogeny – jony metali Cr^{3+} i Al^{3+} zmniejszają skuteczność inhibitorów aromatazy w modelu hormonozależnego raka piersi w warunkach hiperglikemii.

Model badawczy.

Badania wykonano w tym samym modelu badawczym, podobnie jak w publikacji 3, na dwóch liniach komórkowych – MCF-7 oraz MCF-7/DOX. Różnicą było zastosowanie medium hodowlanego z wysokim stężeniem glukozy – 25 mM, które odzwierciedla warunki hiperglikemii (DMEM high-glucose, Biological Industries, Izrael) [50-53]. Model uwzględnia więc dodatkowy czynnik – hiperglikemię, co ma swoje uzasadnienie merytoryczne. Dane z piśmiennictwa wskazują, iż hiperglikemia może nasilać proliferację komórek nowotworowych, hamować ich apoptozę oraz indukować powstawania oporności na stosowane leczenie [45-47]. Biorąc pod uwagę powyższe, a także wcześniej przedstawione informacje (publikacja 2), dotyczące zwiększonego ryzyka występowania insulinooporności oraz cukrzycy u pacjentek stosujących hormonoterapię, a także fakt, że główną grupą pacjentek stosujących AIs są pacjentki po menopauzie, u których sam stan menopauzalny sprzyja występowaniu zaburzeń gospodarki węglowodanowej, uznano, że uwzględnienie w badaniach dodatkowego czynnika – hiperglikemii, może dostarczyć cennych informacji odnośnie potencjalnych interakcji AIs z metaloestrogenami. Wzajemne interakcje ksenoestrogenów, inhibitorów aromatazy oraz hiperglikemii nie były dotychczas analizowane i brakuje takich danych w piśmiennictwie, co sprawia, że wyniki zaprezentowane w publikacji 4 mają dużą wartość poznawczą.

Najistotniejsze wyniki i ich omówienie.

W publikacji 4 zawarto wyniki eksperymentów wykonanych w warunkach hiperglikemii oraz w przypadku oznaczenia stężenia białka VEGF-A porównanie wyników uzyskanych w warunkach normoglikemii vs. hiperglikemii, gdyż wyniki oznaczenia VEGF-A nie były wcześniej publikowane. Dane z piśmiennictwa wskazują, że wysokie stężenia glukozy,

odzwierciedlające hiperglikemię, mogą nie tylko zwiększać proliferację komórek nowotworowych, ale również hamować ich apoptozę (m.in. poprzez ↑ Bcl-2) oraz nasilać angiogenezę (poprzez ↑ VEGF) [45-47], co potwierdzono w wykonanych badaniach. Co warte podkreślenia, poziom ekspresji VEGF-A może korelować z wysokim ryzykiem nawrotu choroby, a także odpowiadać za niepowodzenie stosowanych terapii przeciwnowotworowych [80,81].

W warunkach hiperglikemii, w obu liniach komórkowych jednoczesna ekspozycja na metaloestrogeny i inhibitory aromatazy nie zmniejszała istotnie działania leków (nie zwiększała żywotności komórek nowotworowych, nie zmniejszała odsetka komórek ulegających apoptozie/nekrozie, nie zwiększała współczynnika Bcl-2/BAX). Jednocześnie obserwowano znacznie mniejszą aktywność leków przy wysokim stężeniu glukozy panującym w modelu komórkowym niż w warunkach normoglikemii (mniejsza cytotoksyczność, mniejszy odsetek komórek ulegających apoptozie/nekrozie). Podobnie jak w publikacji 3, komórki linii MCF-7/DOX były mniej wrażliwe na działanie leków, a letrozol wykazywał słabszą aktywność niż eksemestan. W warunkach hiperglikemii, w linii MCF-7/DOX aktywność cytotoksyczna letrozolu była zbliżona do kontroli, niezależnie od kombinacji z metaloestrogenem, co wskazuje na znaczące obniżenie skuteczności działania leku przez wysokie stężenie glukozy.

Wykazano ponadto, że w obu liniach komórkowych w warunkach hiperglikemii stężenia VEGF-A były wyższe, a dodatkowo linia MCF-7/DOX (niezależnie od stężenia glukozy) charakteryzowała się wyższymi stężeniami VEGF-A. Zaobserwowano również, że stężenie VEGF-A obniżyło się pod wpływem obu inhibitorów aromatazy w obu liniach komórkowych, przy czym spadek stężenia był większy w warunkach normoglikemicznych. W warunkach normoglikemii obserwowano również interakcje między inhibitorami aromatazy a metaloestrogenami, których efektem był statystycznie istotny wzrost stężenia VEGF-A po dodaniu metaloestrogenu do inhibitora aromatazy w środowisku hodowli (np. w linii MCF-7 – eksemestan 100 μM VEGF-A = 148.5 pg/ml vs. eksemestan 100 μM + Al^{3+} VEGF-A = 331.5 pg/mL; $p<0.0001$/ vs. eksemestan 100 μM + Cr^{3+} VEGF-A = 245.5 pg /mL; $p<0.0001$), czego nie obserwowano w warunkach hiperglikemii, gdzie stężenie VEGF-A było na podobnym poziomie niezależnie od obecności metaloestrogenów.

Podsumowując, moje wcześniejsze badania (w warunkach normoglikemii) wykazały, że skuteczność inhibitorów aromatazy może zostać zmniejszona w wyniku interakcji z metaloestrogenami, czego nie zaobserwowano w warunkach hiperglikemii. Warto podkreślić,

że aktywność inhibitorów aromatazy w obecności wysokich stężeń glukozy była znacznie niższa, niezależnie od kombinacji z metaloestrogenami, co wskazuje na kluczową rolę hiperglikemii w osłabianiu ich aktywności. Stale rosnąca liczba chorych na cukrzycę, a co za tym idzie także wzrost liczby pacjentów jednocześnie zmagających się z cukrzycą i nowotworem piersi sprawia, że leczenie raka piersi u takich pacjentek staje się sporym wyzwaniem klinicznym. Systematyczne kontrolowanie glikemii może mieć istotne implikacje terapeutyczne.

6. PORÓWNANIE WYNIKÓW UZYSKANYCH W WARUNKACH NORMO- I HIPERGLIKEMII

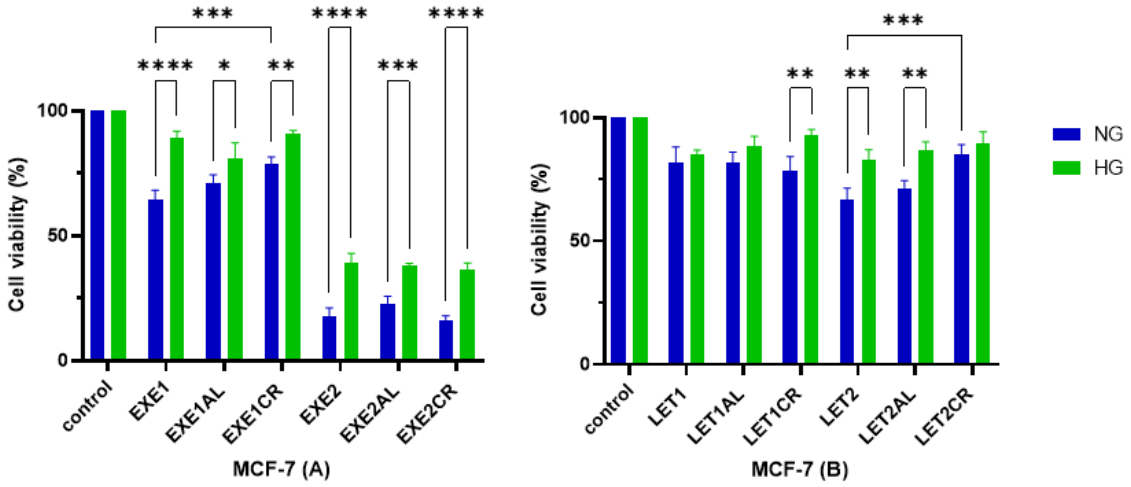
Eksperymenty w warunkach normo- i hiperglikemii były prowadzone w tym samym czasie, z wykorzystaniem tych samych linii komórkowych oraz metod, co pozwala na ich porównanie. Opublikowane prace zawierały zestawienie wyników otrzymanych w określonych warunkach – normo- lub hiperglikemii (z wyjątkiem oznaczenia stężenia VEGF-A w publikacji 4), dlatego poniżej zostało zamieszczone dodatkowe, bezpośrednie porównanie pozostałych wyników. Aby ułatwić interpretację wyników, posłużono się następującymi skrótami: NG – warunki normoglikemii; HG – warunki hiperglikemii; EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 = 100 μM; AL = 100 μM; CR = 100 μM; EXE1AL = kombinacja 100 μM eksemestanu z 100 μM glinem etc. Porównując wyniki uzyskane w warunkach normoglikemii vs. hiperglikemii, zastosowano dwuczynnikową analizę wariancji (ang. *two-way* ANOVA) oraz testy porównań wielokrotnych (test Tukeya). We wszystkich analizach za wartość istotną statystycznie przyjęto $p<0,05$.

Wyniki porównania poszczególnych eksperymentów, prowadzonych w warunkach normo- i hiperglikemii zamieszczono poniżej na rycinach S1-S6 (S, *ang. supplementary*).

6.1. Test żywotności komórek (*ang. cell viability*).

Linia MCF-7.

Wyniki dla linii komórkowej MCF-7 przedstawiono na rycinie S1. W warunkach hiperglikemii eksemestan w obu stężeniach wykazywał niższą cytotoksyczność (EXE1 NG – 65% vs. EXE1 HG – 89%, p<0,0001; EXE2 NG – 18% vs. EXE2 HG – 39%, p<0,0001). Kombinacja eksemestanu (100 µM) i Cr^{3+} zwiększała żywotność komórek MCF-7 w warunkach normoglikemii, czego nie obserwowano w hiperglikemii. Letrozol, niezależnie od warunków glikemii, wykazywał słabsze działanie cytotoksyczne niż eksemestan. Podobnie jak eksemestan, w warunkach hiperglikemii letrozol charakteryzował się słabszym działaniem, ale dopiero w wyższym stężeniu (100 µM) różnica ta jest istotna statystycznie (LET2 NG - 67% vs. LET2 HG - 83%, p=0,0014). W warunkach normoglikemii połączenie letrozolu w wyższych stężeniach (100 µM) z Cr^{3+} i Al^{3+} zwiększało żywotność komórek (LET2 – 67%; LET2CR – odpowiednio 85%; LET2AL – 70%; różnica pomiędzy LET2 a LET2CR była istotna statystycznie p = 0,0028) i podobnie jak w przypadku eksemestanu interakcja ta nie była obserwowana w warunkach hiperglikemii.

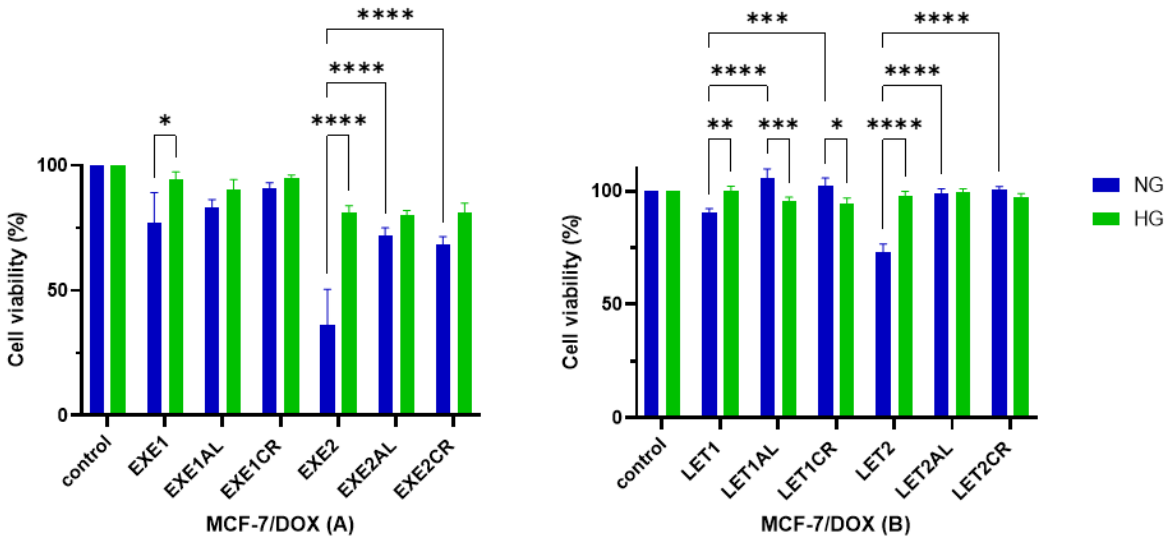


Rycina S1. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na żywotność komórek linii komórkowej MCF-7 w warunkach normoglikemii (NG) i hiperglikemii (HG). Żywotność komórek MCF-7 eksponowanych na eksemestan (A) i letrozol (B), pojedynczo lub w kombinacji z Cr^{3+} lub Al^{3+}. EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; p<0,05; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

Linia MCF-7/DOX.

Wyniki dla linii komórkowej MCF-7/DOX przedstawiono na rycinie S2. Oba inhibitory aromatazy były mniej cytotoksyczne dla komórek MCF-7/DOX niż dla komórek MCF-7.

Eksemestan w obu stężeniach charakteryzował się słabszą cytotoksycznością wobec komórek MCF-7/DOX w warunkach hiperglikemii w porównaniu do normoglikemii (EXE1 NG – 77% vs. EXE1 HG – 94%, p=0,0351; EXE2 NG – 36% vs. EXE2 HG – 81%, p < 0,0001). W warunkach prawidłowej glikemii połączenie eksemestanu (200 µM) z Cr^{3+} lub Al^{3+} znacznie zmniejszało skuteczność leku, ale takiej interakcji nie obserwowano w warunkach hiperglikemii. Inkubacja komórek z kombinacją letrozolu (w obu stężeniach) i metaloestrogenów w warunkach normoglikemicznych zmniejszała jego skuteczność, czego efektem było zwiększenie żywotności komórek MCF-7/DOX. W warunkach wysokiego stężenia glukozy wykazano statystycznie istotne zmniejszenie skuteczności letrozolu (skuteczność nie różniła się znacząco od kontroli), niezależnie od kombinacji z metaloestrogenami (LET1 NG – 91% vs. LET1 HG – 100%, p=0,0017; LET2 NG – 73% vs. LET2 HG – 98 %, p<0,0001).



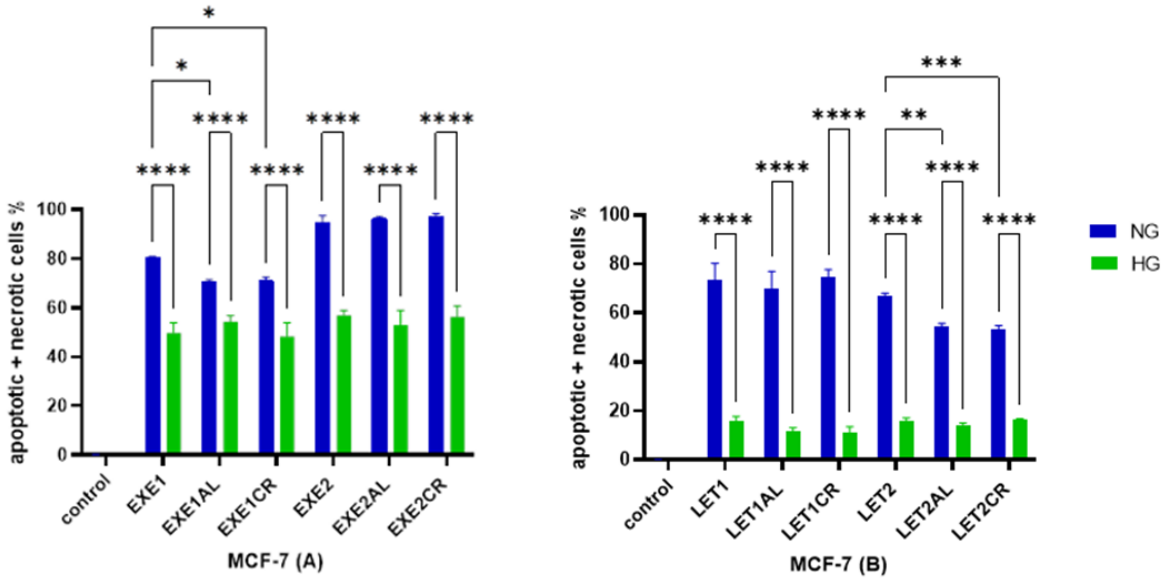
Rycina S2. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na żywotność komórek linii komórkowej MCF-7/DOX w warunkach normoglikemii (NG) i hiperglikemii (HG). Żywotność komórek MCF-7/DOX eksponowanych na eksemestan (A) i letrozol (B), pojedynczo lub w kombinacji z Cr^{3+} lub Al^{3+}. EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; p<0,05; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

6.2. Ocena apoptozy i nekrozy.

Linia MCF-7.

Wyniki dla linii komórkowej MCF-7 przedstawiono na rycinie S3. Aktywność inhibitorów aromatazy w warunkach hiperglikemii była zdecydowanie niższa niż w warunkach normoglikemii, na co wskazuje mniejszy odsetek komórek MCF-7 ulegających apoptozie oraz

nekrozie. Różnice były istotne statystycznie we wszystkich badanych próbkach (oprócz kontroli, gdzie odsetek wynosił dla NG – 0,14% i HG – 0,04%), zaś dla prób badanych: EXE1 NG - 80% vs. EXE1 HG - 50%, p<0,0001; EXE2 NG - 95% vs. EXE2 HG - 57%, p<0,0001; LET1 NG – 73% vs. LET1 HG – 16%, p<0,0001; LET2 NG - 67% vs. LET2 HG - 16%, p<0,0001. W warunkach normoglikemii, kombinacja eksemestanu (100 µM) lub letrozolu (100 µM) z metaloestrogenami skutkowała statystycznie istotnym zmniejszeniem ich skuteczności, podczas gdy w warunkach hiperglikemii aktywność inhibitorów aromatazy była istotnie mniejsza, niezależnie od kombinacji z metaloestrogenami.

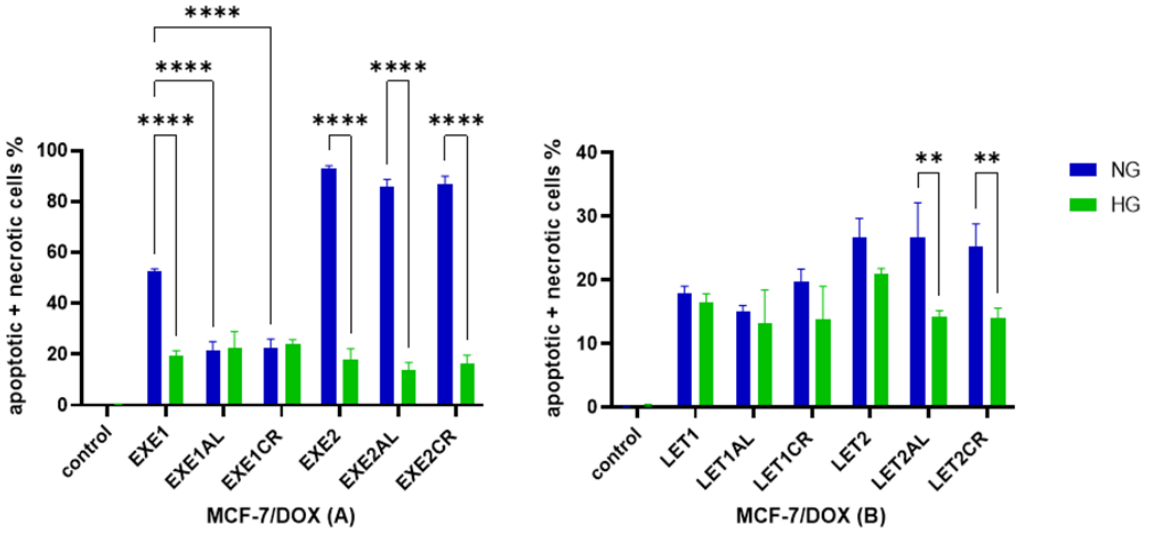


Rycina S3. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na odsetek komórek MCF-7 ulegających apoptozie i nekrozie w warunkach normoglikemii (NG) i hiperglikemii (HG). Odsetek komórek apoptotycznych (wczesna i późna apoptoza) i nekrotycznych po ekspozycji na eksemestan (A) i letrozol (B), pojedynczo lub w kombinacji z Cr^{3+} lub Al^{3+}. EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; p<0,05; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

Linia MCF-7/DOX.

Wyniki dla linii komórkowej MCF-7/DOX przedstawiono na rycinie S4. W linii komórkowej MCF-7/DOX istotne różnice w aktywności inhibitorów aromatazy w zależności od stężenia glukozy w modelu komórkowym zaobserwowano dla eksemestanu - EXE1 NG - 53% vs. EXE1 HG - 19%, p<0,0001; EXE2 NG – 93% vs. EXE2 HG – 18%, p<0,0001 (wyniki uzyskane dla letrozolu nie różniły się istotnie statystycznie). Połączenie eksemestanu (100 µM) i metaloestrogenów w warunkach normoglikemii skutkowało zmniejszeniem skuteczności leku, czego nie obserwowano w warunkach hiperglikemii, gdzie aktywność leku była niska

niezależnie od inkubacji komórek w kombinacji z metaloestrogenem (mniej niż 20% komórek uległo apoptozie i nekrozie).

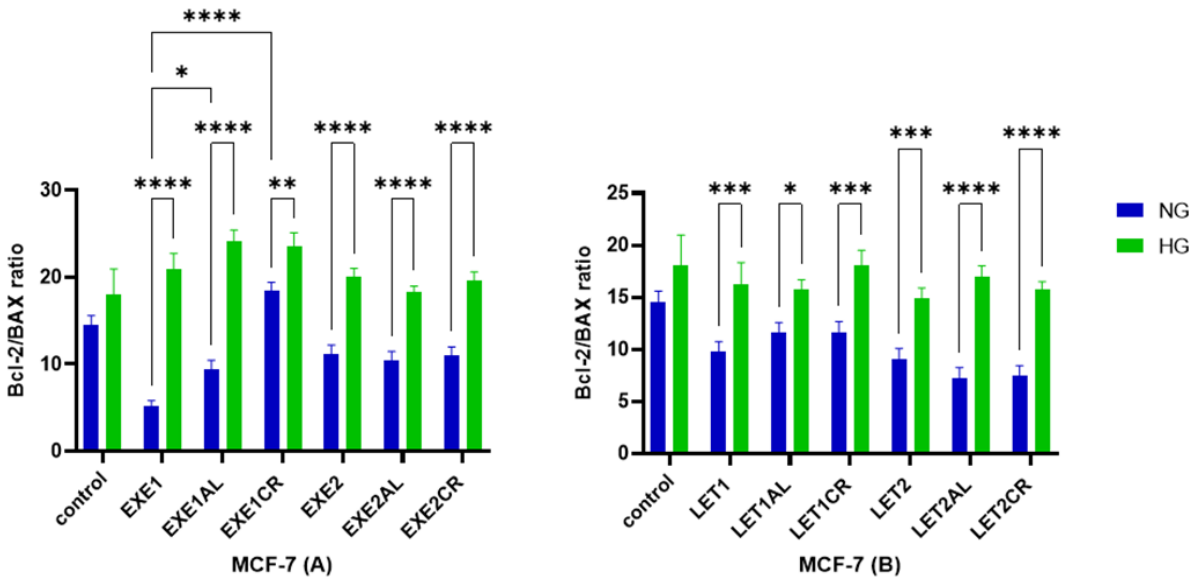


Rycina S4. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na odsetek komórek MCF-7/DOX ulegających apoptozie i nekrozie w warunkach normoglikemii (NG) i hiperglikemii (HG). Odsetek komórek apoptotycznych (wczesna i późna apoptoza) i nekrotycznych po ekspozycji na eksemestan (A) i letrozol (B), pojedynczo lub w kombinacji z Cr^{3+} lub Al^{3+}. EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; $p<0,05$; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

6.3. Współczynnik Bcl-2/BAX.

Linia MCF-7.

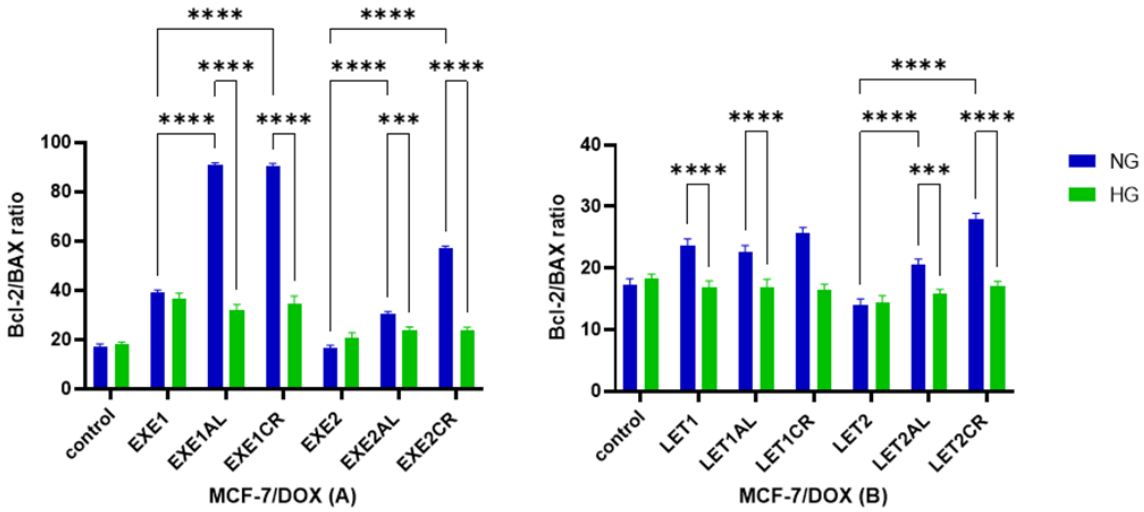
Wyniki dla linii komórkowej MCF-7 przedstawiono na rycinie S5. W warunkach hiperglikemii zaobserwowano wzrost stężenia Bcl-2 (białka antyapoptotycznego), co skutkowało wyższymi stosunkami Bcl-2/BAX i większą opornością na apoptozę. W warunkach normoglikemii obserwowano statystycznie istotny wzrost stosunku Bcl-2/BAX po dodaniu metaloestrogenów do środowiska hodowli zawierającego eksemestan (100 µM), co wskazywało na zmniejszenie proapoptotycznego działania leku. W warunkach hiperglikemii, kombinacja z metaloestrogenami nie miała istotnego wpływu na wartości współczynnika Bcl-2/BAX: EXE1 HG Bcl-2/BAX 20,89 vs. EXE1AL HG 24,21; p=0,1673; EXE1 HG Bcl-2/BAX 20,89 vs. EXE1CR HG 23,58; p=0,4433. W przypadku letrozolu nie zaobserwowano interakcji z metaloestrogenami, charakteryzującej się zwiększaniem współczynnika Bcl-2/BAX niezależnie od warunków glikemii, w jakich prowadzono doświadczenie.



Rycina S5. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na współczynnik Bcl-2/BAX w linii komórkowej MCF-7 w warunkach normoglikemii (NG) i hiperglikemii (HG). EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; p<0,05; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

Linia MCF-7/DOX.

Wyniki dla linii komórkowej MCF-7/DOX przedstawiono na rycinie S6. W komórkach MCF-7/DOX występują mniejsze różnice w wielkości stosunku Bcl-2/BAX w zależności od warunków glikemii panujących w eksperymencie. Co ciekawe, w badanych kombinacjach zaobserwowano wyższe wartości stosunku Bcl-2/BAX w warunkach odzwierciedlających normoglikemię, co wskazuje, że w linii komórkowej MCF-7/DOX, białka Bcl-2 i BAX w mniejszym stopniu niż w MCF-7 zaangażowane są w mechanizm śmierci komórkowej. Jednak podobnie jak w przypadku linii komórkowej MCF-7, obserwowanych interakcji pomiędzy inhibitorem aromatazy a metaloestrogenem w warunkach normoglikemii, nie obserwuje się przy wysokich stężeniach glukozy.



Rycina S6. Wpływ metaloestrogenów, inhibitorów aromatazy i ich kombinacji na współczynnik Bcl-2/BAX w linii komórkowej MCF-7/DOX w warunkach normoglikemii (NG) i hiperglikemii (HG). EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; Cr^{3+}/Al^{3+} = 100 µM. Wyniki przedstawiono jako średnią ± SD, n=3; $p<0,05$; *p=0,0332; **p=0,0021; ***p=0,0002; ****p<0,0001. Na wykresie zaznaczono jedynie różnice istotne statystycznie.

Podsumowując, porównanie wyników otrzymanych w warunkach normo- i hiperglikemii wskazuje, iż na efektywność inhibitorów aromatazy większy wpływ ma stężenie glukozy, odzwierciedlające warunki hiperglikemii, obecne w modelu komórkowym, niż ich kombinacja z metaloestrogenami. W warunkach hiperglikemii inhibitory aromatazy, niezależnie od połączenia z metaloestrogenem, działają mniej skutecznie, co obserwowano jako zwiększenie żywotności komórek nowotworowych, a także mniejszy odsetek komórek ulegających apoptozie i nekrozie. Otrzymane wyniki wskazują więc na kluczową rolę hiperglikemii w obniżaniu efektywności inhibitorów aromatazy, co zwraca uwagę na konieczność monitorowania glikemii w trakcie hormonoterapii.

7. PODSUMOWANIE I WNIOSKI

1. Artykuł przeglądowy dostarczył informacji na temat negatywnego wpływu ksenoestrogenów (głównie fitoestrogenów) na skuteczność leków stosowanych w terapii endokrynnej raka piersi. Analiza piśmiennictwa wykazała, że obszarem wymagającym dalszych badań jest analiza interakcji szeroko rozpowszechnionych w środowisku życia człowieka ksenoestrogenów należących do pozostałych grup, np. metaloestrogenów, z lekami najczęściej stosowanymi w hormonoterapii raka piersi (inhibitorami aromatazy), czego dotyczyły badania wykonane w ramach niniejszej pracy doktorskiej.

2. Wybrane do badań eksperymentalnych metaloestrogeny - jony Cr^{3+} i Al^{3+} mogą w warunkach normoglikemii znacząco zmniejszać efektywność inhibitorów aromatazy (steroidowego AI – eksemestanu oraz niesteroidowego AI – letrozolu) w modelu komórkowym hormonozależnego raka piersi. Obniżenie skuteczności działania leków było obserwowane jako zwiększenie żywotności komórek nowotworowych oraz zmniejszenie ich wrażliwości na apoptozę i nekrozę. Potrzebne są dalsze badania, które w pełni wyjaśnią zaobserwowane interakcje, aby móc skutecznie im przeciwdziałać w trakcie terapii pacjentek z hormonozależnym rakiem piersi.

3. W warunkach hiperglikemii nie wykazano osłabienia działania inhibitorów aromatazy pod wpływem metaloestrogenów. Natomiast hiperglikemia, niezależnie od obecności metaloestrogenów, statystycznie istotnie (w porównaniu do wyników uzyskanych w warunkach normoglikemii) zmniejszała skuteczność inhibitorów aromatazy w modelu komórkowym hormonozależnego raka piersi.

4. Stosowanie inhibitorów aromatazy, prócz charakterystycznych dla tej grupy leków działań niepożądanych, takich jak bóle mięśni i stawów, obniżenie gęstości mineralnej kości, może również zwiększać ryzyko występowania zdarzeń sercowo-naczyniowych, insulinooporności oraz cukrzycy.

5. Ze względu na stosowanie inhibitorów aromatazy głównie u kobiet po menopauzie, u których występuje zwiększone ryzyko wystąpienia zaburzeń gospodarki węglowodanowej, a także w oparciu o uzyskane wyniki badań eksperymentalnych wskazujących na kluczową rolę hiperglikemii w zmniejszaniu efektywności hormonoterapii, ważnym wydaje się regularne monitorowanie glikemii u pacjentek w trakcie leczenia inhibitorami aromatazy.

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- projektu dla Młodych Naukowców pt. „Wpływ wybranych metaloestrogenów na aktywność leków stosowanych w terapii raka piersi w badaniach modelowych *in vitro*”, ujętym w systemie SIMPLE Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu pod nr STM.D150.20.048,

- projektu konkursowego pt. „Wpływ metaloestrogenów i hiperglikemii na efektywność inhibitorów aromatazy w modelu hormonozależnego raka piersi”, realizowanego w ramach subwencji na utrzymanie i rozwój potencjału badawczego Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu, ujętego w systemie SIMPLE pod nr SUBK.D150.22.039.

10. WYKAZ STOSOWANYCH SKRÓTÓW

4PL - *ang. four parameter logistic curve* - 4-parametrowa krzywa logistyczna

aGnRH - *ang. gonadotropin-releasing hormone analogs* - analogi gonadoliberyny

AIs - *ang. aromatase inhibitors* - inhibitory aromatazy

AJCC - *ang. American Joint Committee on Cancer* - Amerykański Wspólny Komitet ds. Raka

ASCO - *ang. American Society of Clinical Oncology* - Amerykańskie Towarzystwo Onkologii Klinicznej

BAX - *ang. Bcl-2-associated X protein* - białko proapoptotyczne

Bcl-2 – *ang. B-cell lymphoma 2 protein* - białko antyapoptotyczne

CDK4/6 inhibitor - *ang. cyclin-dependent kinase 4 and 6 inhibitor* - inhibitorem zależnej od cyklin kinazy 4 i 6

EBM - *ang. evidence based medicine* – medycyna oparta na dowodach

EDCs - *ang. endocrine disrupting compounds* - związki endokrynnie czynne

ERs - *ang. estrogen receptors* – receptory estrogenowe

ESMO - *ang. European Society for Medical Oncology* - Europejskie Towarzystwo Onkologii Klinicznej

HER2 - *ang. human epidermal growth factor receptor 2* - receptor ludzkiego naskórkowego czynnika wzrostu typu 2

IDF - *ang. International Diabetes Federation* – Międzynarodowa Federacja Diabetologiczna

IARC - *ang. International Agency for Research on Cancer* - Międzynarodowa Agencja Badań nad Rakiem

MDR - *ang. multidrug resistance* - oporność wielolekowa

NF- κB - *ang. nuclear factor kappa B* - jądrowy czynnik transkrypcyjny NF kappa B

PgRs - *ang. progesterone receptors* – receptory progesteronowe

PRISMA - *ang. Preferred reporting items for systematic reviews and meta-analyses* - Preferowane pozycje sprawozdawcze dla przeglądów systematycznych i metaanaliz

ROS - *ang. reactive oxygen species* – reaktywne formy tlenu

SD - *ang.* s*tandard deviation* - odchylenie standardowe

SERDs - *ang. selective estrogen receptor down-regulators* - selektywni antagoniści receptora estrogenowego

SERMs - *ang. selective estrogen receptor modulators* - selektywne modulatory receptora estrogenowego

TNBC - *ang. triple-negative breast cancer* - rak piersi potrójnie ujemny

TNM - *ang. tumor-node-metastasis* – klasyfikacja stopnia zaawansowania nowotworu, polegająca na ocenie guza pierwotnego, regionalnych węzłów chłonnych oraz przerzutów odległych

VEGF-A - *ang. Vascular Endothelial Growth Factor* – czynnik wzrostu śródbłonka naczyniowego A

11. SPIS ZAŁĄCZNIKÓW

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REVIEW ARTICLE

The impact of xenoestrogens on effectiveness of treatment for hormone-dependent breast cancer – current state of knowledge and perspectives for research

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Abstract

Introduction. Breast cancer is the most common cancer occurring in women and causing the highest number of deaths among them. The role of xenoestrogens has been the subject of many studies in the pathogenesis of breast cancer. Less is known about the impact of xenoestrogens on the effectiveness of hormone therapy used to treat breast cancer, and thus possible drug-xenostrogen interactions.

Objective. The aim of this review is to summarize the current state of knowledge and present perspectives for further research on the impact of xenoestrogens on the effectiveness of drugs used in the treatment of hormone-dependent breast cancer.

Current state of knowledge. Phytoestrogens, in particular flavonoid genistein, are the best studied group of xenoestrogens in terms of interaction with drugs used in the treatment of breast cancer, due to their frequent use, including their use in alleviating the adverse effects of hormone therapy. Analyzing the current state of knowledge, it seems that phytoestrogens intake should be avoided during conventional anti-cancer treatment. Of the other xenoestrogens, bisphenol A (BPA) is one of the best-tested compounds for interactions with drugs used to treat breast cancer. It has been shown that bisphenol A could reduced therapeutic effect of active tamoxifen metabolite and cytostatics used in breast cancer treatment.

Conclusion. Confirmation in clinical trials of the results obtained *in vitro* and *in vivo* tests, would enable the creation of specific recommendations for patients undergoing breast cancer treatment, especially hormone therapy. An area requiring further research is the analysis of the effects of xenoestrogens other than phytoestrogens, e.g. metalloestrogens, on the effects of drugs used in the treatment of breast cancer.

Key words

breast cancer, estrogens, xenoestrogens, drug-xenoestrogen interaction

INTRODUCTION

Breast cancer is the most common cancer among women, affecting 2.1 million women annually, worldwide, and causing the highest number of cancer-related deaths among women [1]. In the USA in 2019, almost 267 thousand new cases of breast cancer were diagnosed, which accounted for 30% of all cancers among women [2]. In Poland in 2016, breast cancer was diagnosed in almost 19 thousand women, which constituted 23% of all diagnosed cancers in women [3]. The etiopathogenesis of breast cancer is complex and multifactorial, depending on genetic, environmental and hormonal conditions. The most frequently mentioned risk factors for breast cancer are age, obesity, genetic predisposition, previous breast cancer or non-cancerous diseases, and the activities of endogenous and exogenous estrogens, e.g. xenoestrogens found in the environment or food, which may interfere with the functioning of the endocrine system [4, 5]. It is estimated that nearly 70% of malign tumours are caused by environmental factors, whereas in breast cancer this percentage reaches 90–95% [6]. In addition, differences in the incidence of breast cancer are observed depending on the place of residence – women living in the countryside are about 1.5 times less often diagnosed with breast cancer than women living in cities [4]. It is also indicated that the higher breast cancer incidence in urban areas could be due to higher exposure to carcinogens, and may also be due to changes in lifestyle factors, including sedentary lifestyle. Thus, higher socio-economic status is associated with a higher incidence of breast cancer [7].

Estrogens (estradiol, estrone and estriol) play a key role in maintaining the hormonal homeostasis of the body through proper tissue development and the regulation of many physiological functions, including the menstrual cycle and reproduction in women [8]. In addition to their regulatory action, it is indicated that endogenous estrogens may play a role in the pathogenesis of breast cancer. Estrogens can induce the growth of cancer cells via estrogen receptors (ERs), as well as increase the rate of cell mutation (genotoxic effect) [8, 9]. Data from the literature indicate that the altered expression and function of estrogen receptors is crucial for the process of initiation and progression of hormone-dependent cancers such as breast cancer [10]. There are two types of estrogen receptors – estrogen receptor alpha (ERα,

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estrogen receptor alpha) and estrogen receptor beta (ERβ, *estrogen receptor beta*), both of which belong to the group of intracellular nuclear receptors and act as ligand-activated transcription factors. ERα and ERβ receptors may exert an opposite effect on the proliferation of mammary gland cells – ERβ receptors are indicated as proapoptotic agents, and ERα receptors as those with procancerogenic effects. According to existing knowledge, ERα plays a major role in the pathogenesis of breast cancer, while the role of ERβ in carcinogenesis is not yet fully explained [11, 12].

According to the expression of estrogen and progesterone receptors (PR, *progesterone receptor*), as well as the receptor for human epidermal growth factor type 2 (ERBB2, *erb-b2 receptor tyrosine kinase 2*; formerly HER2, *human epidermal growth factor receptor 2*), three main subtypes of breast cancer may be distinguished: ER/PR+/ERBB2-, occurring most frequently, in about 70% of cancer cases, ER/PR+ or ER/PR-/ERBB2+ (15–20% of cancers) and triple-negative cancer, without expression of the above receptors, which accounts for about 15% of cases [13].

In most patients with breast cancer, one of the basic elements of systemic treatment is preoperative or postoperative chemotherapy based on the sequential use of multi-drug regimens based on cytostatics – anthracyclines (e.g. doxorubicin, epirubicin) and taxoids (e.g. docetaxel, paclitaxel). Chemotherapy is used in patients with breast cancer – with or without steroid receptor expression. In the treatment of breast tumours with steroid receptor expression (hormone-dependent breast cancer), the basis of treatment is hormone therapy, eliminating the stimulatory effect of estrogens on the proliferation of cancer cells [14].

The role of xenoestrogens, which can mimic the action of endogenous estrogens, has been the subject of many studies in the pathogenesis of breast cancer. Definitely less is known about the impact of xenoestrogens on the effectiveness of hormone therapy used to treat breast cancer, and thus possible drug-xenostrogen interactions. It seems that these interactions may have a significant impact on the effectiveness of the therapies. Due to the widespread nature of exposure to xenoestrogens, this is a clinically important issue.

OBJECTIVE

The aim of this literature review is to summarize the current state of knowledge and present perspectives for further research on the impact of xenoestrogens on the effectiveness of drugs used in the treatment of hormone-dependent breast cancer.

MATERIALS AND METHOD

The presented review is based on a literature research performed in January 2020, using electronic medical databases PubMed, Embase and Scopus to identify studies relating to xenoestrogens in the context of interactions with drugs used in breast cancer hormone therapy. Search terms comprised of the following words: breast cancer, xenoestrogens, phytoestrogen, interaction, tamoxifen, aromatase inhibitor and their combination. Seventeen original research articles on animal and cell models published between 2002–2019, which seemed to be the most fitting in relation to this issue, were selected for review.

Drugs used in the treatment of hormone-dependent breast cancer. The main aim of hormone therapy used in patients with hormone-dependent breast cancer is to eliminate the stimulating effect of estrogens on cancer cells [13]. This effect can be achieved by using several groups of drugs: selective estrogen receptor modulators (SERMs, *selective estrogen receptor modulators*), selective estrogen receptor antagonists (SERDs, *selective estrogen receptor down-regulators*), aromatase inhibitors (AIs, *aromatase inhibitors*), or gonadoliberin analogues (aGnRH, *gonadotropin-releasing hormone analogues*).

For many years, tamoxifen has been considered the gold standard in the treatment of hormone-dependent breast cancer in both pre- and postmenopausal women. This is a selective estrogen receptor modulator which, by binding to estrogen ER on cancer cells, blocks the possibility of their stimulation by estrogens and inhibits their proliferation [15, 16]. Tamoxifen in some tissues, e.g. in the skeleton, by binding to estrogen receptors, has an agonist effect that mimics the effect of estrogens [16]. Another mechanism of action is characterized by fulvestrant, a selective estrogen receptor antagonist which, unlike tamoxifen, does not show any agonist effect. Fulvestrant leads to degradation of the estrogen receptor and enables the complete elimination of the effect of estrogens on the cell [17].

Reducing the effect of estrogens on cancer cells can also be achieved by using drugs that suppress estrogen production in the body. Gonadoliberin analogues are used in premenopausal patients whose estrogens are mainly produced in the ovaries. They block the pituitary gonadotropin secretion and inhibiting estrogen production in the ovaries [18]. In postmenopausal women, in whom peripheral tissues (adipose tissue, liver, skin) become the main source of estrogens, aromatase inhibitors – both steroidal (exemestane) and non-steroidal (letrozole, anastrozole), block the activity of aromatase and inhibit the conversion of androgens to estrogens [15].

In accordance with the recommendations of the Polish Society of Clinical Oncology and the recommendations of an international group of experts participating in the St. Gallen conference in 2019, the pharmacological treatment of hormone-dependent early breast cancer (stages I-III according to TNM classification [*tumour, node, metastasis*]), in premenopausal patients mainly includes the use of tamoxifen (routinely used at a dose of 20 mg/day) for 5–10 years. In postmenopausal women, tamoxifen, aromatase inhibitors or their sequences are used, also for a total of 5–10 years. In the case of advanced breast cancer (stage IV according to TNM classification), tamoxifen, high dose fulvestrant (500 mg *i.m.*) or combination therapy – aromatase inhibitor or fulvestrant + cyclin-dependent kinase inhibitor CDK4/6 (*cyclin-dependent kinase*), e.g. palbocyclib, are used [14, 19].

The schematic mechanism of the action of drugs used in the treatment of breast cancer is presented in Figure 1.

Xenoestrogens. Xenoestrogens are exogenous substances that interfere with the functioning of the endocrine system. As defined by the European Commission, 'it is an exogenous substance or mixture that changes the functions of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations' [20]. By interacting with estrogen receptors, xenoestrogens can act as their antagonists or agonists. They can also interfere with

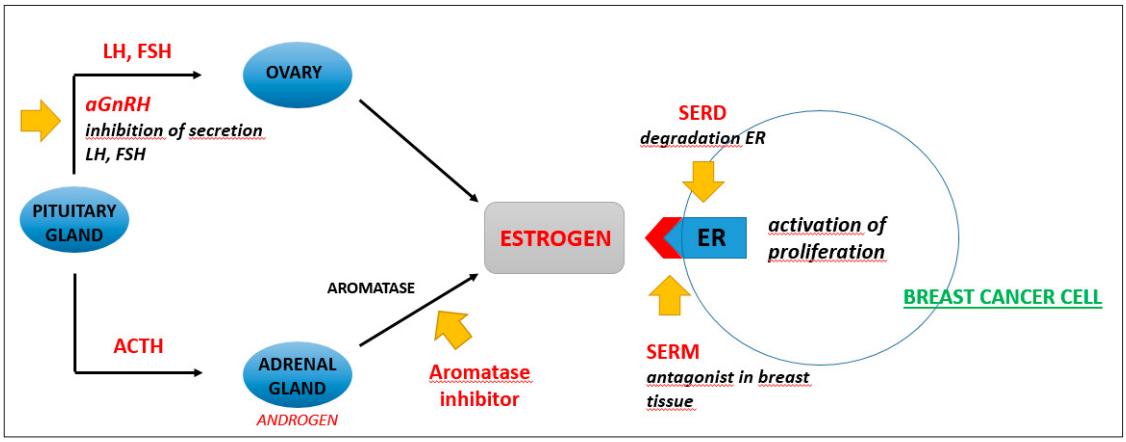

Figure 1. Mechanism of the action of drugs used in hormone therapy for breast cancer. Aromatase inhibitors and gonadoliberin analogues inhibit estrogen production in the body. SERD causes estrogen receptor degradation, while SERM in breast tissue antagonizes endogenous estrogens [13–16].
ER – estrogen receptor; FSH – follicle stimulating hormone; LH – luteinizing hormone; ACTH – adrenocorticotropic hormone; aGnRH – Gonadoliberin analogues; SERM – selective estrogen receptor modulator; SERD – a selective estrogen receptor antagonist

the synthesis and metabolism of endogenous estrogens, as well as affect the synthesis of ER. Such numerous and diverse mechanisms of xenoestrogen action are the result of the diversity of substances belonging to this group.

There are several major classes of xenoestrogens, which are show in Figure 2. This classification is based on elements of the chemical structure, which are characteristic for a group [21]. Among them, the best known group of xenoestrogens are phytoestrogens, which are a heterogeneous group of chemical compounds, currently including over 100 different substances. Based on their chemical structure, phytoestrogens are divided into isoflavones, coumestans or lignans. Their source is most often food (e.g. soy-rich foods) and dietary supplements, or over-the-counter medications used to relieve menopausal symptoms. Another group of xenoestrogens are mycoestrogens, produced by some yeasts and fungi, which are impurities in cereal products. Xenoestrogens also include metalloestrogens, including some metals – cadmium (Cd), mercury (Hg), copper (Cu), aluminum (Al), cobalt (Co), nickel (Ni), chromium (Cr) or lead (Pb). Most of them are released into the environment from industrial sources (mining, metallurgy, electroplating). Significant amounts of some metals, however, are also found in everyday life, e.g. cadmium is found in tobacco smoke, aluminum in deodorants and antiperspirants, and chromium (III) in dietary supplements used to lower blood glucose. Xenoestrogens also include

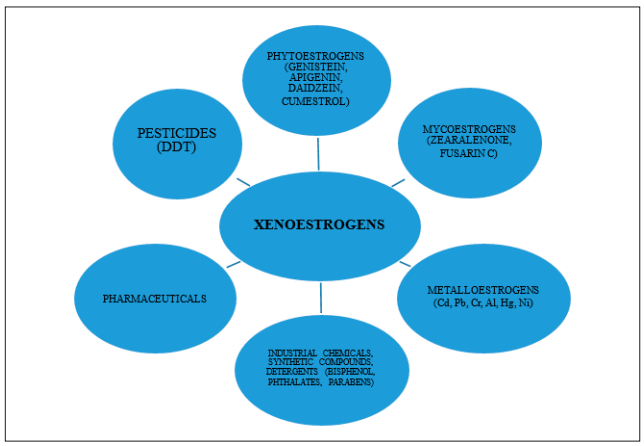

Figure 2. Classes of xenoestrogens [based on 19]

pesticides, pharmaceuticals and industrial chemicals, synthetic compounds and detergents [20, 21].

The source of xenoestrogens are various types of chemicals from industry, agriculture and technological processes, and therefore exposure to xenoestrogens may vary significantly depending on the living environment or the nature of the work performed. For example, the DDT pesticide (dichlorodiphenyltrichloroethane), commonly used in agriculture and forestry, despite being banned in the 1970s, still circulates in food chains in areas where it was previously used [22]. Exposure to pesticides occurs especially in agriculture and the chemical industry, although all persons working or living on farms may also be exposed. Occupational exposure to xenoestrogens occurs primarily in people working in the production of plastics (bisphenol A, phthalates), epoxy resins, or in the metallurgy and metal processing sectors, and industries that require welding and soldering (especially metalloestrogens – cadmium, mercury, arsenic, iron) [23].

Increasing evidence from epidemiological studies, as well as an increasingly better understanding of the mechanisms that connect toxic substances with the development of breast cancer, indicate that exposure to some environmental xenobiotics, many of which are common, in everyday products as well as their degradation products, can lead to increased risk of developing breast cancer. It is indicated that exposure to chemical compounds and radiation in the environment, both in single and combined actions or in interactions, contribute to the increasing incidence of breast cancer [24]. To better understand these relationship, in 2003 there began the BCERP (Breast Cancer and the Environment Research Programme) – a transdisciplinary study of the effects of environmental exposures on mammary development and breast cancer risk development, not only in adults, but also during the other periods of susceptibility, such as puberty [25].

The role of xenoestrogens in the pathogenesis of breast cancer has been the subject of many studies. Numerous publications confirm that many compounds commonly found in the environment can bind to estrogen receptors and thus compete or mimic the action of endogenous estrogens (e.g. stimulate the proliferation of cancer cells). This has been confirmed in *in vitro* and *in vivo* tests for

bisphenol A [26, 27, 28, 29], phthalates [30], parabens [31] and metalloestrogens [32, 33, 34], as well as mycoestrogens [35, 36]. Data on phytoestrogens, often used as a prophylaxis for breast cancer, are inconclusive [37]. In the case of isoflavone-genistein, attention is paid to its differentiated effects on MCF-7 breast cancer cells depending on the concentration used – low concentrations of genistein (10nM – 1μM) stimulate cell proliferation, while higher concentrations (> 10μM) have antiproliferative effects, which makes it difficult to unequivocally assess the appropriateness of its use in women [38].

DESCRIPTION OF THE STATE OF KNOWLEDGE

Phytoestrogens and mycoestrogens. Treatment with tamoxifen or aromatase inhibitors, due to a significant decrease in estrogen levels in the body, are often associated with uncomfortable menopause symptoms in patients, such as hot flashes, mood disorders or weight gain. One of the most common ways to deal with these symptoms is the use of dietary supplements containing plant extracts, which are a rich source of phytoestrogens, e.g. from red clover, black bedbug, hops or soybeans. Due to their vegetable, natural origin and availability without a prescription, these preparations are widely considered safe, which may result in their use by a patient without the knowledge and control of a doctor [39, 40, 41]. Phytoestrogens, in particular flavonoid genistein, are the best studied group of xenoestrogens in terms of interaction with drugs used in the treatment of hormone-dependent breast cancer. Their source can be both the above-mentioned drugs and dietary supplements intended to alleviate menopausal symptoms, as well as food (including soy, linseed, sesame, oats, clover, coffee) [42]. There are also several reports about the mycoestrogen zearalenone, which is a mycotoxin, produced by fungi of the genus *Fusarium* and occurring as a contaminant in bread and various types of cereals – corn, barley, wheat and rice [42].

One of the first reports describing the interaction between flavonoid-genistein and tamoxifen was the study by Jones et al. [43] performed on a postmenopausal breast cancer model using the T47D human breast cancer cell line. The addition of tamoxifen to a culture medium was shown to inhibit the proliferation of T47D breast cancer cells and stop the cell cycle in the G1 phase, which confirms the antitumour efficacy of the drug. The addition of low levels of genistein (<10 mM) reversed the therapeutic effect of tamoxifen [43]. Similar results were obtained by Ju et al. [44] conducting a study on ovariectomized mice implanted with estrogen-dependent breast cancer cells (MCF-7). The main parameter studied was the tumour surface at week 32 of the experiment. The mice were assigned to one of 6 groups depending on the substances used or their combination: 1) control; 2) 0.25 mg estradiol; 3) 0.25 mg estradiol + 2.5 mg tamoxifen; 4) 0.25 mg estradiol + 5 mg tamoxifen; 5) 0.25 mg estradiol + 2.5 mg tamoxifen + 1,000 ppm genistein; and 6) 0.25 mg estradiol + 5 mg tamoxifen + 1,000 ppm genistein. Estradiol and tamoxifen were administered as subcutaneous implants, while genistein (1,000 ppm per day) was added to food. It was shown that the use of tamoxifen at both doses inhibited estradiol stimulated tumour growth in mice, while this effect was inhibited in the presence of phytoestrogen, which indicates that genistein antagonizes the effect of tamoxifen [44]. Similar observations were made by Du et al. [45] examining the effect of 3 doses of genistein (250 ppm, 500 ppm and 1,000 ppm of genistein) on the effectiveness of tamoxifen in ovariectomized mice implanted with MCF-7 cells. It was shown that low doses of genistein (250 ppm and 500 ppm) significantly reduced the effect of tamoxifen, while higher doses (1,000 ppm genistein) did not affect the activity of the drug [45].

Liu et al. [46], in a mouse model, studied the effect of a diet containing high and low doses of isoflavones (genistein and daidzein) on the effectiveness of tamoxifen used in the prevention of breast cancer. The researchers showed that mice receiving a diet containing low doses of isoflavones (~211 μg/g) and tamoxifen (5 mg as a subcutaneous implant) had a significantly faster tumour growth. In addition, *in vitro* studies performed on MCF-7 cells confirmed that low doses of phytoestrogen administered together with tamoxifen promote cell proliferation. In turn, enriching the diet of mice with high doses of isoflavones (~500 μg/g), as well as adding them to the culture medium in *in vitro* tests, inhibits tumour growth in mice and MCF-7 cells in culture. The authors emphasize that the demonstrated interaction between low doses of genistein and tamoxifen may be important for patients using this drug in the treatment or prevention of breast cancer [46].

Literature data indicate that the ability of genistein to promote the proliferation of breast cancer cells is associated with its high affinity for the ERα. Seo et al. [47] evaluated the effect of genistein and apigenin (also a phytoestrogen from the flavonoid group) on breast cancer cell lines with steroid receptor expression – MCF-7 and T47D and without expression – MDA-MB-435. Both phytoestrogens have been shown to stimulate the proliferation of cells expressing estrogen receptors, which was not seen with the MDA-MB-435 cell line. Various concentrations of phytoestrogens were tested in the range from 10 nM – 10 mM, both in the presence and absence of 0.1 mM 4-hydroxy tamoxifen (4-OH-Tam, *4-hydroxytamoxifen*), the active metabolite of tamoxifen. Genistein, in addition to stimulating cell proliferation of the MCF-7 and T47D lines, additionally inhibited the antiproliferative effect of 0.1 μM 4-OH-Tam (at a concentration of 10 μM in MCF-7 cells and at concentrations from 10 nM – 10 μM in T47D cells). Apigenin only slightly inhibited 4-OH-Tam, which is probably due to its lower affinity for ERα than genistein. The authors of the study point out that the concentration of phytoestrogens achieved in the human body (from diet and/or additional supplementation) are usually in the range of 1 – 18.5 μM. Thus, these are the concentrations that can stimulate the growth of ER-positive breast cancer cells, as well as block the effects of tamoxifen. Therefore, phytoestrogens, and in particular genistein, used in the form of dietary supplements, among others for fighting hot flashes in women using tamoxifen, may be unfavourable for them, as they may reduce the effectiveness of hormone therapy [47].

Important information about the interaction of tamoxifen and phytoestrogens has been provided by a study conducted by Constantinou et al. [48] on female Sprague-Dawley rats, whose diet, depending on the study group, was enriched with tamoxifen (0.125 mg/kg diet), genistein (140 mg/kg diet), daidzein (105 mg/kg diet) or a combination of tamoxifen with each of the phytoestrogens at the aforementioned doses. Observations were made with respect to

the group in which no additional dietary modifications were introduced. After a week of using the above-mentioned diet modifications, all animals initiated a tumour process with dimethylbenzanthracene (DMBA). It was shown that in the group of females fed with tamoxifen and daidzein, the smallest incidence of cancer occurred, whereas in the group receiving tamoxifen and genistein, the incidence of cancer was higher than in the group receiving only tamoxifen (60% TAM + DAI group vs 95% control group DMBA vs 79% group TAM + GEN vs 73% group TAM; data represent the percentage of animals in which a tumour developed). It is therefore clear that some phytoestrogens, in this case genistein, can negatively affect the action of tamoxifen, while others, such as daidzein, have a synergistic effect [48]. Emodine, which is an anthraquinone classified as a phytoestrogen, also inhibits tamoxifen. In a study conducted on MCF-7 and ZR 75–1 (ER-positive and HER2 positive breast cancer cell line), the simultaneous use of emodine and endoxifen (the active metabolite of tamoxifen) weakened its therapeutic effect by increasing the expression of cyclin D1, which is a key protein that stimulates the growth of cancer tissue [49].

Considering that most of the supplements used to relieve menopausal symptoms are multi-component preparations containing a combination of several phytoestrogens, the effect of which may accumulate, van Duursen et al. [39] studied the effects of both genistein and 8-prenylnarygenin alone and 4 multi-component dietary supplements on the effectiveness of 4-hydroxy tamoxifen and letrozole. Studies have been conducted on the coculture of the MCF-7 and H295R (*human adrenal cortex cell line*) cell lines assessing tumour cell proliferation, aromatase activity and steroidogenesis, and on the BG1Luc4E2 line (*estrogen-responsive recombinant human ovarian*) to assess ERα activation. Genistein, 8-prenylnarygenin, as well as all the tested supplements containing various combinations of phytoestrogens, have been shown to activate an estrogen-dependent increase in MCF-7 cell proliferation that was not inhibited by either 4-hydroxy tamoxifen (co-administered with genistein or 8-prenylnarygenin) or by letrozole (in each combination tested – with genistein, 8-prenylnarygenin, and with each of the 4 others commercially available). It was also shown that all tested supplements and genistein increased aromatase activity, while 8-prenylnarygenin strongly inhibited its activity. Based on the results obtained, the authors recommend the avoidance of the use of dietary supplements containing phytoestrogens by patients undergoing breast cancer treatment [39].

Similarly to the studies with tamoxifen, Ju et al. [50] assessed the effect of genistein on aromatase inhibitor therapy – letrozole. In this study, ovariectomized mice were implanted with estrogen-dependent breast cancer cells (MCF-7) and randomly assigned to one of 10 groups depending on the compounds given to the mice or a combination thereof: 1) control; 2) 250 ppm genistein; 3) 500 ppm of genistein; 4) 1,000 ppm genistein; 5) 5 mg androstenedione (aromatase substrate); 6) 1 mg letrozole; 7) 5 mg androstenedione + 1 mg letrozole; 8) 5 mg androstenedione + 1 mg letrozole + 250 ppm genistein; 9) 5 mg androstenedione + 1 mg letrozole + 500 ppm genistein; 10) 5 mg androstenedione + 1 mg letrozole + 1,000 ppm genistein. Androstenedione and tamoxifen were administered as subcutaneous implants, while genistein was added to food at appropriate daily doses. The main parameter studied was the tumour area, evaluated in the 19th week of the experiment. Letrozole was shown to be effective in inhibiting tumour growth in mice; however, this effect was inhibited by the presence of genistein (at each concentration tested). Concentration dependence, however, was observed: the higher the dose of genistein used, the greater the tumour growth [50].

Different results, i.e. lack of interaction between formestane, a second generation steroid aromatase inhibitor (currently no longer used in the treatment of breast cancer) and phytoestrogens derived from the root bug extract, were shown in the animal model by Nißlein et al. [51]. The experiment was conducted on female Sprague-Dawley rats in which the tumour process was initiated by administration of DMBA. The rats were then assigned to one of 4 groups receiving: 1) 3.5 mg of formestane + black cohosh root extract; 2) 5 mg of formestane + black cohosh root extract; 3) 5 mg formestane; 4) control group not receiving additional compounds. It was shown that the use of formestane in each group was associated with significant inhibition of tumour growth, and the use of phytoestrogen did not affect its activity [51].

In 2015, the American Food and Drug Administration (FDA) approved the use of combined therapy with letrozole + palbociclib in the treatment of advanced breast cancer in postmenopausal women. The results of the PALOMA-1 clinical trial conducted in 2009–2012 on 165 patients, indicated that the use of combination therapy compared to the use of standard therapy with letrozole alone, improves progression-free survival rates by 10 months (group using combination therapy – average survival without disease progression – 20.2 months; letrozole treatment – 10.2 months). This was a significant advance in the treatment of advanced breast cancer in postmenopausal women. Warth et al. [52] examined the effect of xenoestrogens present in the diet (genistein – phytoestrogen, zearalenone – mycoestrogen) on the effectiveness of letrozole and palbociclib treatment. An *in vitro* study using the MCF-7 and T47D breast cancer cell lines showed that the combination of letrozole and palbociclib effectively inhibited tumour cell proliferation, while the addition of both genistein (1 μM) and zearalenone (100 nM) counteracted this effect. At the same time, using the Western-blot method, the combination of letrozole and palbociclib has been shown to inhibit the activity of the intracellular mTOR kinase pathway (responsible, among others, for the occurrence of treatment resistance), which is also reversed when genistein or zearalenone are added. The results obtained show how significant an effect the xenoestrogens have on the effectiveness of breast cancer treatment. The authors indicate that confirmation of the results obtained in an animal model, as well as in clinical trials, will enable the creation of specific nutritional recommendations for patients who undergo breast cancer hormone therapy [52].

Gallo et al. [53] studied the effect of soy extract, standardized on the content of genistein and daidzein, on the action of fulvestrant, which inhibited the formation of breast cancer in ovariectomized mice. Low doses of phytoestrogens – 50 mg soy extract/kg bw/day, have been shown to slightly increase the inhibitory effect of fulvestrant, while higher doses – 100 mg soy extract/kg bw/day significantly reduced the antitumour activity of the drug [53]. In contrast, the lack of interaction between fulvestrant and genistein was indicated by the results of experiments by Dess et al. [54], who showed that low doses of genistein (1μM) and zearalenone (1nM), similar to the pesticide DDT (dichlorodiphenyltrichloroethane

known for breast cancer proliferation promoting properties), increase the activity of cyclin-dependent kinase 2 (Cdk2), synthesis of cyclin D1 and hyperphosphorylation of pRbl05 (retinoblastoma protein) in MCF-7 cells. This indicates that both genistein and zearalenone stimulate proliferation, stimulating MCF-7 breast cancer cells to enter the cell cycle. The use of the anti-estrogen – fulvestrant (at a concentration of 100 nM), inhibiting the activation of Cdk2, enabled a complete reversal of this effect [54].

Multidrug resistance (MDR) is one of the main reasons for the failure of systemic cancer therapy. Various mechanisms attributed to MDR includes increased expression of drug efflux transporters, changes in tumour microenvironment and cancer stem cell regulation, increased epigenetic microRNA regulations, drug target modification, altered apoptotic signalling pathway and increased DNA repair mechanism [55]. One of the mechanisms responsible for MDR in the aspects of breast cancer development is the over-expression of proteins from the ABC (ATP-binding cassette) family of membrane transporters, which limits intracellular accumulation of cytostatic drugs and their effectiveness. ABC over-expression can be intrinsic or acquired through induction for example, by exposure to therapeutic drugs, environmental toxicants and micronutrients present in the diet. If such an induction occurs during chemotherapy, lower therapeutic response and a worse disease outcome are expected [56].

The most important transporters in breast cancer therapy are P-glycoprotein (P-gp/ABCB1), multi-drug resistance protein (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2), which are involved in the transport of, e.g. doxorubicin, epirubicin or paclitaxel. Clinical evidence points to an association between transporter expression and cancer disease prognosis. It is indicated that expression levels of BCRP predict survival after neoadjuvant chemotherapy for breast cancer, while Pgp and MRP1expression have little predictive value [57]. Rigalli et al. [58] studied the effect of genistein (at 3 concentrations – 0.1 μM, 1 μM and 10 μM) on the expression and function of ABC proteins on MCF-7 and MDA-MB-231 cell lines. Increased expression of ABCC1 (+ 121%) and ABCG2 (+ 281%) proteins in MCF-7 cells was demonstrated in the presence of 10 μM genistein, while no increase in their expression was observed at lower concentrations. In addition, in MCF-7 cells in the presence of 10 μM genistein, an increased efflux of doxorubicin (+ 55%) and mitoxantrone (+ 136%) was observed, which is associated with an increase in resistance to cytostatistics. Genistein had no effect on increasing ABCB1 protein expression, increasing the efflux of doxorubicin and mitoxantrone in MDA-MB-231 cells, while at 0.1 μM and 1 μM, it increased ABCC1 protein expression by 70% and 74%, respectively. The results indicate a risk of interaction between genistein and cytostatics used in the treatment of breast cancer, which could significantly reduce the effectiveness of chemotherapy. If genistein exerted a similar activity *in vivo*, a detrimental effect on both efficacy of chemotherapeutic drugs and on disease prognosis could be expected. These data reinforce the necessity of avoiding genistein consumption during treatment [58].

Other xenoestrogens. Of the other xenoestrogens which are not of natural origin, bisphenol A (BPA) is one of the best-tested compounds for interactions with drugs used to treat breast cancer. Bisphenol A is a synthetic diphenyl compound, with a structural similarity to a strong estrogen receptor agonist – diethylstilbestrol, which may mimic the effects of estrogen in the body. BPA is a compound widely distributed in the environment as it is a chemical raw material used in the production of plastics and epoxy resins [59]. Hence, the main source of BPA exposure are plastic containers for food and drinks, dental materials, protective coatings, and thermal paper. BPA is a compound with the ability to accumulate in tissues, and its presence has been detected in healthy people, including in urine and blood serum samples [59].

Goodson et al. [60] showed that the exposure of mammary epithelial cells (HRBEC – high-risk donor breast epithelial cell) obtained from donors (at high risk of developing breast cancer) to detectable levels of bisphenol A in human blood 100 pM – 100 nM BPA), results in a change in the expression of genes associated with the activation of the mTOR pathway, which increases the survival of cancer cells [60]. Another issue explored by this group of researchers was the effect of bisphenol A on the therapeutic effectiveness of one of the main active tamoxifen metabolites – 4-hydroxy tamoxifen. It was shown that with the simultaneous use of 4-hydroxy tamoxifen (10 μM) and bisphenol A (100 nM), the therapeutic effect of 4-hydroxy tamoxifen is reduced, measured as the percentage of cells undergoing apoptosis – both breast cancer cell lines (T47D, SKBR3) and HRBEC. This effect was even greater the higher the BPA concentration – only 50% of the cells underwent apoptosis at a BPA concentration of 100 nM [61].

LaPensee et al. [62] assessed whether bisphenol A affects the action of commonly used cytostatics – doxorubicin, cisplatin and vinblastine, and the mechanism of possible interactions. To this end, *in vitro* tests were performed on both estrogen-sensitive breast cancer cells (T47D) and estrogen-insensitive breast cancer cells (MDA-MB-468). Cytotoxicity of 3 different concentrations of doxorubicin (5 ng/mL, 25 ng/mL, 124 ng/mL), cisplatin (100 ng mL, 200 ng/mL, 400 ng/mL) and vinblastine (1 ng/mL, 5 ng/mL, 25 ng/mL) were assessed in the absence and presence of bisphenol A (at 1 nM). All cytostatics at each concentration were shown to be cytotoxic in the absence of BPA on both cancer cell lines. The addition of BPA antagonized this effect. It was also shown that this effect is not associated with BPA via estrogen receptors, but is the result of the increased expression of antiapoptotic proteins (Bcl-2 and Bcl-xL). Thus, the authors confirmed that bisphenol A at nanomolar doses occurring in humans as a result of environmental exposure, may reduce the effectiveness of chemotherapy, which should be taken into account when using anti-cancer therapy [62].

Research by Osuna et al. [63] published in 2017, suggests that another synthetic xenoestrogen – methylparaben, contributes to the occurrence of chemo-resistance to drugs used in the treatment of breast cancer (tamoxifen, fulvestrant). This may be due to the direct stimulating effect of methylparaben on tumour-initiating cells (TICs), as well as by modulating the activity of stem cells that remain resistant to antiestrogens, by increasing the expression of the NANOG protein, which promotes stem cell differentiation. In a study performed on the MCF-7 cell line, it was shown that neither tamoxifen nor fulvestrant block the effects of methylparaben [63].

Table 1 summarizes the interaction studies of xenoestrogens with substances used in the treatment of hormone-dependent breast cancers in *in vivo* and *in vitro* tests.

Table 1. Summary of interaction studies of xenoestrogens with substances used in the treatment of hormone-dependent breast cancer in *in vivo* and *in vitro* tests

Author/ Lear	Cell line/Animal	Xenoestrogen	Drug	Results
SERM – tamoxifen or active metabolite of tamoxifen (4-hydroxytamoxifen or endoxifen).				
Jones et al., 2002 [37]	T47D	GEN <10μM	TAM	GEN reversed the inhibitory effects of tamoxifen on both proliferation and G1 arrest.
Ju et al., 2002 [38]	MCF-7 implanted in ovariectomized athymic mice	GEN 1,000 ppm	TAM	GEN negated the inhibitory effect of TAM; increased expression of estrogen-responsive genes (e.g. pS2, PR and cyclin D1).
Liu et al., 2005 [40]	MCF-7; mouse mammary tumour cell line	diet with low doses of isoflavones (~ 211 μg/g); diet with high doses of isoflavones (~ 500 μg/g)	TAM	Low doses of GEN, co-administered with TAM, promote cell proliferation; in contrast TAM with high doses of GEN that are growth inhibitory.
Constantinou et al., 2005 [42]	Sprague-Dawley rats given DMBA	GEN (140 mg/kg diet), DAI (105 mg/kg diet) as a diet component	TAM	GEN and TAM combination had increased tumour multiplicity, compared with TAM alone; DAI with TAM had reduced tumour multiplicity.
Seo et al., 2006 [41]	MCF-7, T47D, MDA-MB-435	GEN, API in concentration 10nM -10μM	4-OH-TAM	GEN antagonizes the anti-proliferative effect of 4-OH-TAM; API even at 10μM appeared to only have a moderate effect on blocking TAM effect.
Goodson et al., 2011 [51]	HRBEC, MCF-7, T47D, SKBR3	BPA (100pM to 100nM), mePB (10nM to 1μM)	4-OH-TAM	HRBECs pretreated with BPA, or mePB surmounted antiestrogenic effects of tamoxifen showing dose-dependent apoptosis evasion and induction of cell cycling.
Du et al., 2012 [39]	MCF-7 implanted in ovariectomized athymic mice	GEN 250 ppm, 500 ppm, 1000 ppm	TAM	Inhibitory effect of TAM was significantly negated by the low doses of GEN (250 and 500 ppm), whereas the 1,000 ppm. GEN did not have the same effect.
Kim et al., 2019 [43]	MCF-7, T47D, ZR-75–1, BT474	Emodin 15, 30, 60 μM	Endoxifen	Combination emodin and endoxifen attenuated treatment effect via cyclin D1 and pERK up-regulation in ER(+) breast cancer cell lines.
Aromatase inhibitors – steroidal (formestane) and non-steroidal (letrozole). Combination AI with CDK4/6 inhibitor – palbociclib.				
Niβlein et al., 2007 [45]	Sprague-Dawley rats given DMBA	iCR – 60 mg herbal substances per kg body weight	Formestane	iCR did not antagonize or diminish the antitumoural effects of formestane
Ju et al, 2008 [44]	MCF-7 implanted in ovariectomized athymic mice	GEN 250 ppm, 500 ppm, 1000 ppm	LET	GEN reversed the inhibitory effect of LET in a dose-dependent manner.
van Duursen et al., 2013 [33]	MCF-7/H295R, BG1Luc4E2	GEN, 8PN, 4 commercially available menopausal suplements	4-OH-TAM, LET	GEN, 8PN and 4 supplements all induced ER-dependent tumour cell proliferation, which could not be prevented by LET and 4OH-TAM
Warth et al., 2018 [46]	MCF-7, T47D	GEN 1μM, ZEA 100 nM	PAL+LET	GEN and ZEA reversed the inhibitory effect of PAL+LET combination on cancer cells proliferation.
SERD – fulvestrant				
Dees et al., 1997 [48]	MCF-7	GEN 0,1μM, ZEA 10 nM	FUL (ICI 182 780)	FUL suppressed dietary estrogen-mediated activation of Cdk2.
Gallo et al., 2007 [47]	MCF-7 implanted in ovariectomized athymic mice	SSE containing GEN, DAI at 50 or 100 mg/kg per day	FUL (ICI 182 780)	Concomitant administration of 50 mg/kg per day SSE slightly potentiated the inhibitory activity of FUL, while at 100 mg/kg per day, SSE partially negated FUL activity.
Osuna et al., 2017 [54]	MCF-7, MDA-MB-231	mePB 10 nM	TAM, FUL	mePB increases breast cancer tumour proliferation through enhanced TIC activity and regulates stem cell genes (inc. NANOG); TAM and FUL do not block these effects.
Cytostatic agents e.g. doxorubicin, cisplatin				
LaPensee et al., 2009 [53]	T47D, MDA-MB-468	BPA 1nM	DOX, CIS, VIN	BPA antagonizes the cytotoxicity of chemotherapeutic drugs in both ER-positive and ER-negative breast cancer cells
Rigalli et al., 2016 [49]	MCF-7, MDA-MB-231	GEN 0,1μM, 1μM, 10μM	DOX, MXR	In MCF-7 cells, GNT (10 μM, increased DOX efflux (+55%) and MXR efflux (+136%).

T47D, MCF-7 – ER-positive breast cancer cell lines; MDA-MB-435 – ER-negative breast cancer cell lines; H295R – human adrenal cortex cell line; BG1Luc4E2 – estrogen-responsive recombinant human ovarian cell line; HRBEC – high-risk donor breast epithelial cells; DMBA – dimethylbenzanthracene; pERK – phosphorylated extracellular signal-regulated kinase; TIC – tumour initiating cells; GEN – genistein; API – apigenin; ZEA – zearalenone; DAI – daidzein; 8-PN – 8-prenylnaringenin; iCR – isopropanolic extract of black cohosh; SSE – standarized soy extract; mePB – methylparaben; BPA – bisphenol A; TAM – tamoxifen; 4-OH-TAM – 4-hydroxytamoxifen; LET – letrozole; PAL – palbociclib; FUL – fulvestrant; endoxifen – active metabolite of TAM; DOX – doxorubicin, MXR – mitoxantrone, CIS – cisplatin, VIN – vinblastine

CONCLUSIONS

Given our widespread exposure to xenoestrogens, as well as the steady increase in the incidence of breast cancer, examination of the impact of these endocrine active compounds found in the human environment on the effectiveness of therapies used to treat hormone-dependent breast cancer is becoming an important clinical and social issue. As shown in this literature review, most research has focused on phytoestrogens, due to their frequent use, including their use in alleviating the adverse effects of hormone therapy. Analyzing the current state of knowledge, it seems that their intake should be avoided during conventional anti-cancer treatment, due to the possibility of reducing the effectiveness of therapy and thus increasing the risk of disease progression. Confirmation in clinical trials of the results obtained *in vitro* and *in vivo* tests, would enable the creation of specific nutritional recommendations for patients undergoing breast cancer hormone therapy, which may improve the effectiveness of treatment.

An area requiring further research is analysis of the effects of xenoestrogens other than phytoestrogens, e.g. metalloestrogens, on the effects of drugs used in the treatment of breast cancer. Exposure to metalloestrogens is common (e.g. cadmium – smoking, chromium (III) – dietary supplements, or aluminum – the use of deodorants and antiperspirants), and their carcinogenic potential has been proven in many *in vitro* tests, which suggests that, like phytoestrogens, they may affect the effectiveness of the therapies used in the treatment of hormone-dependent breast cancer.

Another interesting and important aspect for conducting further research is to examine the potential relationship between the exposure to environmental xenoestrogens with confirmed carcinogenic potential, e.g. pesticides, industrial chemicals or metalloestrogens, and the effectiveness of treatment of hormone-dependent breast cancer. Due to the differences in the incidence of breast cancer depending on the place of residence, as well as the varied exposure to environmental xenoestrogens depending on the living environment, this seems to be a clinically relevant issue.

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Review

Aromatase Inhibitors and Risk of Metabolic and Cardiovascular Adverse Effects in Breast Cancer Patients—A Systematic Review and Meta-Analysis

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Abstract: Aromatase inhibitors (AIs) have been considered first-line therapy for patients with hormone-dependent breast cancer due to their high efficacy and good tolerability. However, AIs are not free of adverse events, and studies show that therapy with AIs is associated with an increased risk of cardiovascular events and the development of insulin resistance and diabetes. We searched the Cochrane Central Register of Controlled Trials, PubMed and EMBASE up to 27 October 2020 for the prevalence of cardiovascular and/or metabolic adverse effects during treatment with AIs in postmenopausal women with breast cancer. A meta-analysis was performed using a random effects model. Odds ratios and 95% confidence intervals were calculated and illustrated using forest plot charts. We performed separate analyses depending on trial design. Twenty two studies met the inclusion criteria. AIs were associated with a higher risk of cardiovascular events, especially when we compared study arms in which AIs were used (alone or in sequence with TAM) with the arms in which TAM was used alone (OR = 1.16; 95%CI 1.04–1.30) or when comparing patients taking AIs alone to patients taking TAM alone or in sequence with AIs (OR = 1.24; 95%CI 1.11–1.38). A pooled analysis of five trials comparing adjuvant AIs to TAM showed the odds for arterial hypertension being 1.31 times higher for patients taking AIs; however, this did not reach statistical significance (OR = 1.31; 95%CI 0.47–3.65). We have not shown an increased risk of dyslipidemia or weight gain with the use of AIs. Our results suggest that postmenopausal women with breast cancer treated with AIs have an increased risk of cardiovascular events in comparison with TAM, potentially due more to a cardioprotective effect of the latter than the cardiotoxicity of AIs. We were unable to prove a similar association for hypertension, dyslipidemia, hyperglycemia or weight gain. Further high-quality RCTs and post-marketing safety observational studies are needed to definitively evaluate the impact of AIs on metabolic disorders in breast cancer patients.

Keywords: breast cancer; aromatase inhibitors; adverse effects

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1. Introduction

Breast cancer is the most common cancer in women, affecting 2.1 million women worldwide each year and causing the largest number of cancer-related deaths in women [1]. According to the data providing by American Cancer Society, 13% of women will develop breast cancer in their lifetime, and 3% of them will die from it [2]. Breast cancer is a heterogeneous disease; the most common subtype of breast cancer, occurring in about 70% of cancer cases (75% in postmenopausal patients), is hormone-dependent breast cancer [3]. The pharmacological treatment of hormone-dependent early breast cancer in pre-menopausal patients mainly includes the use of tamoxifen for 5–10 years or AIs combined with ovarian suppression. In postmenopausal women, tamoxifen, aromatase inhibitors or their sequences are used, also for a total of 5–10 years. In the case of advanced

breast cancer, a combination therapy–aromatase inhibitor or fulvestrant + cyclin-dependent kinase inhibitor CDK4/6, e.g., palbocyclib or tamoxifen/aromatase inhibitor/high dose fulvestrant, is used. In premenopausal women, simultaneous ovarian suppression is necessary [3,4]. Over the past decade, aromatase inhibitors (AIs) became a first-line therapy for patients with hormone-dependent breast cancer because of their greater clinical efficacy and prolonged disease-free survival compared to tamoxifen (TAM) [3]. AIs inhibit the action of the enzyme aromatase. Aromatase (estrogen synthetase) is a member of the cytochrome P450 superfamily of monooxygenases and catalyzes the demethylation of androgens' carbon 19, producing phenolic 18-carbon estrogens [5]. In postmenopausal women, the major source of estrogen is the conversion of androgens to estrogens in skin, muscle and adipose tissue. Aromatase inhibitors block this pathway, reducing estrogen-mediated cancer cell proliferation in hormone receptor-positive breast cancer [5]. Based on their chemical structure, there are steroidal (exemestane) and non-steroidal (letrozole, anastrozole) aromatase inhibitors [3].

Although AIs have a more favorable risk–benefit profile compared to tamoxifen, such as lower incidence of life-threatening adverse events, for instance thromboembolic episodes and the occurrence of endometrial cancer, they are not free of side effects [5,6]. The most common adverse events during AIs therapy are menopausal symptoms, musculoskeletal events, sexual disorders, impaired cognitive function and bone mineral density (BMD) decline [6]. Moreover, some studies have shown that therapy with aromatase inhibitors is also associated with an increased risk of cardiovascular events and the development of insulin resistance and diabetes [7–9]. Patients treated with aromatase inhibitors are more likely to develop hyperlipidemia, hypercholesterolemia and hypertension, which are known risk factors for cardiovascular disease, compared to patients receiving tamoxifen [10]. Some studies showed that treatment with AIs (compared with tamoxifen) was associated with an increased risk of heart failure and cardiovascular mortality [11]. The increase in the incidence of cardiovascular events in patients taking AIs is probably related to the cardioprotective effect of tamoxifen [7]. Nevertheless, the risk of cardiovascular events, dyslipidemia, insulin resistance or diabetes mellitus is increased in postmenopausal women [12]. Epidemiological data show that in the world, 69.1% of all breast cancer cases concern postmenopausal women (data for Western Europe indicate an even higher percentage of 81.4%). At the same time, it is postmenopausal patients who most often die from breast cancer—in the world, as much as 78.8% of all deaths from breast cancer concern postmenopausal patients, while in Western Europe this percentage is as high as 92.9% [13]. Thus, the assessment of whether the risk of developing these disorders additionally increases as a result of therapy with AIs therapy is a significant health concern.

We therefore performed a systematic review and meta-analysis of randomized control trials (RCTs) to determine whether AIs are associated with an increased risk of both cardiovascular and metabolic adverse effects, such as hyperglycemia, dyslipidemia and body weight gain.

2. Materials and Methods

This review was conducted in accordance with PRISMA guidelines. Details of the protocol for this systematic review have been registered on PROSPERO and are available at: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021270743 (accessed on 31 March 2022).

2.1. Data Sources and Literature Search

We searched the Cochrane Central Register of Controlled Trials, PubMed (Medline) and EMBASE up to 27 October 2020. The following search terms were used: #1 "breast tumor", #2 "aromatase inhibitor" OR "anastrozole" "arimidex" OR "letrozole" OR "femara" OR "exemestane" OR "aromasin", #3 "cardiovascular disease" OR "ischemic heart disease" OR "heart infarction" OR "cerebrovascular accident" OR "body weight" OR "obesity" OR "diabetes mellitus" OR "dyslipidemia" OR "glucose intolerance" OR

"insulin resistance" OR "hyperglycemia" OR "hypercholesterolemia" OR "hypertriglyceridemia" OR "metabolic syndrome X", #4 #1 AND #2 AND #3. No language limitations were applied. Search results in each query were included in supplementary materials (Supplementary Table S1). Reference lists of all included articles were searched to identify potentially relevant articles.

2.2. Study Selection and Data Collection Process

Two authors independently (KB and PP) conducted a review of abstracts and titles to remove duplicates and eliminate studies that did not meet the criteria for inclusion in the meta-analysis. Relevant articles were selected by reading the full texts. Disagreements were resolved by discussion among all authors. Inclusion criteria for this meta-analysis were: human, controlled randomized (phase II and III) clinical trials; studies reported on the prevalence of cardiovascular and/or metabolic adverse effects during treatment with third-generation aromatase inhibitor in postmenopausal women with hormone-dependent breast cancer. The comparators could be tamoxifen, placebo or no treatment. The exclusion criteria were: studies reporting on premenopausal women, estrogen or progesterone receptor-negative breast cancer, first/second generation aromatase inhibitors using in the study, reviews, expert opinions, guidelines and case studies. When multiple follow-up periods were reported for a given RCT, we selected the trial with the most comprehensive reporting of cardiovascular or metabolic events and/or the longest follow-up reported. The following data were extracted from included studies with the use of a prespecified data collection form: trial design, trial arm (n included in safety analysis), duration of treatment, characteristics of patients (age, disease stage, primary treatment) and reported adverse effects. In this systemic review, we focused in particular on the frequency of cardiovascular (arterial hypertension, cardiovascular events such as cardiac arrhythmia, ischemic heart disease, myocardial infraction, heart failure, atrial fibrillation) and metabolic adverse effects (hyperglycemia, body weight gain, dyslipidemia). Data were collected by two authors independently (KB and PP) and then compared. Disagreements was resolved by discussion among all authors.

2.3. Quality Assessment

The quality assessment was performed with the use of Cochrane Collaboration's tool [14]. Each RCT was evaluated for selection bias (random sequence generation, allocation concealment), performance bias (blinding of participants), detection bias (blinding of outcome), reporting bias (selective outcome) and other possible bias. Each domain was assigned a "high", "low" or "unclear" risk of bias independently by two reviewers (KB and PP), with disagreements resolved by discussion among all authors.

2.4. Statistical Analysis

A meta-analysis was performed using a random effects model. Odds ratios and 95% confidence intervals (95% CI) were calculated and illustrated using forest plot charts. All analyses were stratified by RCT design. In such a manner, we performed separate analyses depending on the treatment used. In the first analysis, we compared patients treated with aromatase inhibitors only vs. those treated with tamoxifen only (1). In the second analysis, we considered a group of patients who had been treated with aromatase inhibitors as monotherapy or in sequence with tamoxifen and compared them with a group of patients who had only used tamoxifen for treatment (2). The third analysis compared patients treated with aromatase inhibitors monotherapy with those treated with tamoxifen alone or in sequence with aromatase inhibitors (3). The fourth analysis compared patients treated with aromatase inhibitors with those treated with a placebo (4). The principle of selecting studies for each of the analyses is presented in Figure 1.

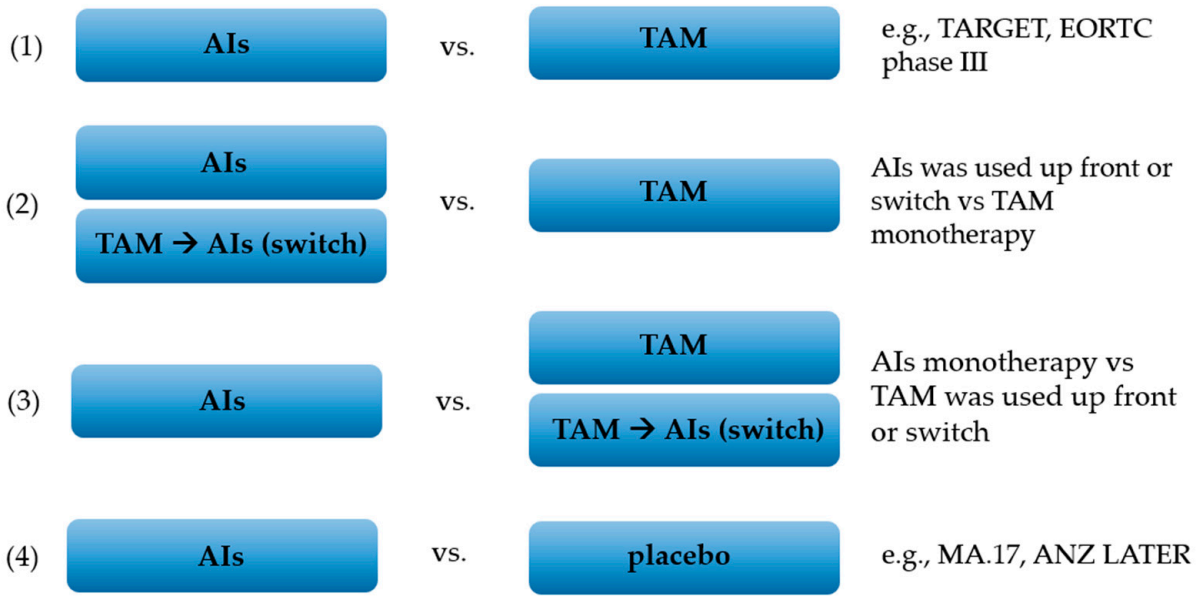


Figure 1. Visualization of groups in analyses. (1) Patients who were only using aromatase inhibitors or tamoxifen for treatment. (2) Group of patients who had been treated with aromatase inhibitors as monotherapy or in sequence with tamoxifen vs. group of patients who had only used tamoxifen for treatment. (3) Patients treated with aromatase inhibitors monotherapy vs. patents treated with tamoxifen alone or in sequence with aromatase inhibitors. (4) Patients treated with aromatase inhibitors vs. those treated with placebo.

The following outcomes, cardiovascular events, arterial hypertension, body weight gain and dyslipidemia, were assessed, provided they were reported in at least three studies. Statistical heterogeneity across the RCTs was estimated using the I^2 statistic. All analyses were made using the R-"meta" package version 4.19.0, and all tested p values < 0.05 were considered statistically significant.

3. Results

Details of the study selection are presented in Figure 2. In total, 614 articles were found. A primary screen of the titles and abstracts resulted in the exclusion of 556 records. A further 39 articles were excluded based on the full-text review. The most common reason for exclusion was inadequate outcomes. This selection produced 21 studies that met the inclusion criteria [15–35].

The study design and patient characteristics of included RCTs can be found in Table 1.

All of the patients were postmenopausal women undergoing treatment for hormone-dependent breast cancer. Most of the research concerned the treatment of early stages of breast cancer (71%). In 13 studies (62%), AIs were used as adjuvant therapy, in 3 studies extended adjuvant therapy, and in the remaining 5 studies first-line treatment for advanced breast cancer. Included studies had a different design; therefore, we decided to analyze the outcomes separately depending on the treatment used: (1) AIs (monotherapy) vs. TAM (monotherapy); (2) AIs (monotherapy) or AIs + TAM (sequence) vs. TAM (monotherapy); (3) AIs (monotherapy) vs. TAM (monotherapy) or AIs + TAM (sequence); and (4) AIs (monotherapy) vs. placebo. Among the studies included, there were three studies that compared the efficacy and safety of two aromatase inhibitors: FACE (letrozole vs. anastrozole), Iwata et al. (exemestane vs. anastrozole) and MA.27 (exemestane vs. anastrozole). However, these studies were not included in the quantitative analysis due to insufficient data to compare the side effect profile for individual aromatase inhibitors.

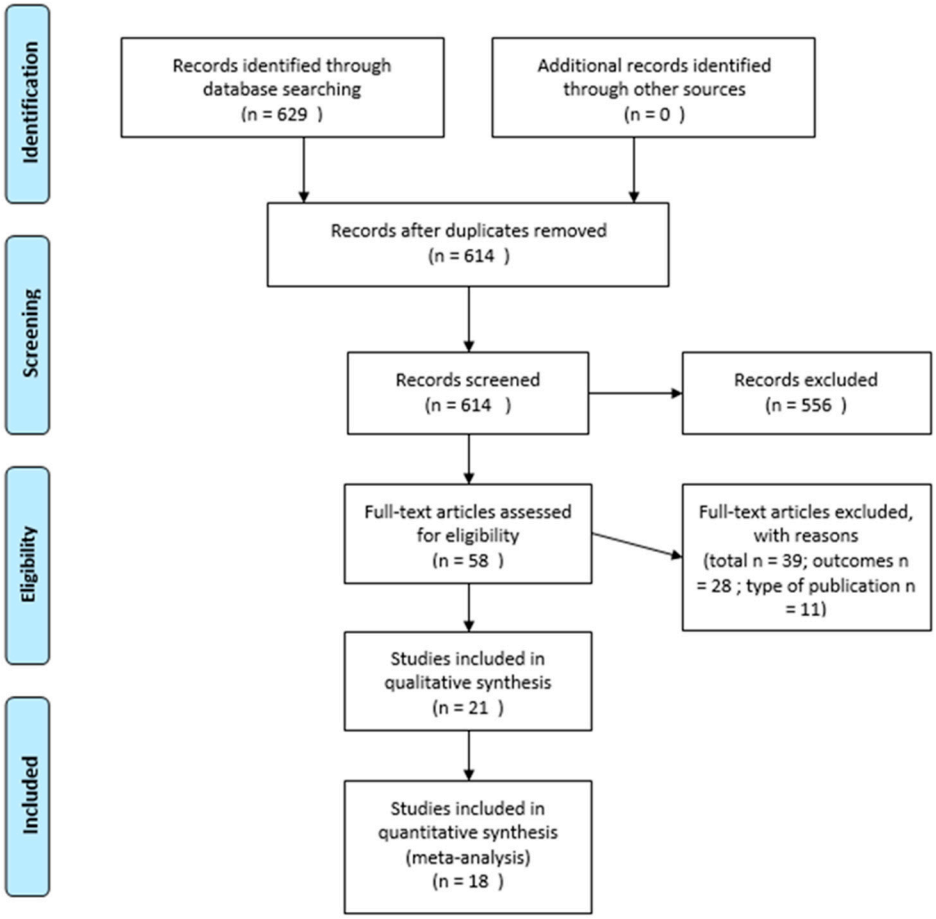


Figure 2. Details of the study selection—PRISMA flow chart of literature search.

3.1. Quality Assessment

A quality assessment of the included studies using of Cochrane Collaboration's tool is presented in Supplementary Table S2. The majority of RCTs were of a low risk of bias in different domains of Cochrane Collaboration's tool. The main limitations regarding the methodological quality were a lack of the blinding of participants and outcome.

3.2. Cardiovascular Events

Cardiovascular events included all reported events of ischemic heart disease, myocardial infarction, heart failure, atrial fibrillation or cardiac arrhythmia. Pooled analysis-of-odds ratios for cardiovascular events are presented in Figure 3 (Figure 3a–d). Thirteen studies reported adverse effects classified as cardiovascular events. A pooled analysis of five trials comparing AIs to tamoxifen showed the odds for cardiovascular events being 1.21 times higher for patients taking aromatase inhibitor, however not statistically significantly (OR = 1.21; 95% CI 0.99–1.48). The heterogeneity across studies was low, with I^2 = 7% (p = 0.37) (Figure 3a). In the ATAC trial, there were three arms–patients receiving aromatase inhibitor (anastrozole), patients receiving tamoxifen, or patients taking both anastrozole and tamoxifen. ATAC1 relates to the situation when aromatase inhibitor alone was compared with tamoxifen alone, ATAC2 relates to the situation when aromatase inhibitor used in combination with tamoxifen was compared with tamoxifen alone, and ATAC3 relates to the situation when aromatase inhibitor alone was compared with the combination of aromatase inhibitor and tamoxifen.

Table 1. Description of included studies. Legend: TAM—tamoxifen, EXE—exemestane, LET—letrozole, ANA—anastrozole, PBO—placebo, comb—combination, observ—observation, ND—no data.

TRIAL	TRIAL ARM (*n* Included in Safety Analysis)	TREATMENT	TRIAL DESIGN	AGE (Mean)	CANCER STAGE	PRIMARY TREATMENT		
						Surgery (%)	Radiotherapy (%)	Systemic Therapy (%)
FATA-GIM3 [15]	switch group = 1761 upfront group = 1766	adjuvant	upfront strategy vs. switch strategy; six treatment groups: ANA 1 mg, EXE 25 mg, LET 2,5 mg for 5 years; TAM 20 mg for 2 years followed by administration ANA or EXE or LET for 3 years	64	early	100%	1247 (67%)	712 (39%)
	upfront group = 1766					100%	1253 (68%)	703 (38%)
	ANA = 1175					100%	801 (65%)	469 (39%)
	EXE = 1177					100%	854 (69%)	474 (38%)
	LET = 1175					100%	845 (69%)	472 (39%)
SUCCESS C [16]	EXE = 54	adjuvant	5 years EXE vs. 2 years TAM + 3 years EXE	EXE-63	early	ND	ND	100%
	TAM-EXE = 54			TAM-EXE -60.5		ND	ND	100%
FACE [17]	LET = 2049	adjuvant	LET (2.5 mg) vs. ANA (1 mg) for 5 years	62	early	ND	652 (31.6%)	1294 (62.7%)
	ANA = 2062					ND	621 (29.9%)	1267 (61.1%)
Iwata et al, 2013 [18]	EXE = 149	first-line	EXE 25 mg vs. ANA 1 mg continued until disease progression, intolerable adverse event or death	EXE-63.4	advanced	ND	35 (23.5%)	103 (69.1%)
	ANA = 149			ANA-64		ND	28 (18.8%)	100 (67.1%)
MA.27 [19]	EXE = 3761	adjuvant	EXE 25 mg vs. ANA 1 mg for 5 years	EXE-63.9	early	3789 (100%)	ND	1163 (31%)
	ANA = 3759			ANA-64.3		3787 (100%)	ND	1164 (31%)
PROACT [20]	ANA = 48	neoadjuvant and adjuvant	pre-operative (3 months) and post-operative (5 years or until recurrence, withdrawal) treatment TAM (20 mg) vs. ANA (1 mg)	ANA-61.5	locally advanced	48 (100%)	18 (41.9%)	10 (23.3%)
	TAM = 48			TAM-61.6		49 (100%)	17 (39.5%)	20 (46.5%)
N-SAS BC04 [21]	EXE = 55	adjuvant	EXE for 5 years vs. 2.5–3 years TAM followed by EXE to a total of 5 years vs. ANA for 5 years	EXE-63.2	early	15 (27.3%)	35 (63.6%)	21 (38.2%)
	TAM = 56			TAM-63.0		18 (32.1%)	36 (64.3%)	23 (41.1%)
	ANA = 55			ANA-62.9		18 (32.7%)	34 (61.8%)	21 (38.2%)
TEAM [22]	TAM = 4814	adjuvant	25 mg EXE vs. TAM (20 mg) –> EXE for 5 years (EXE after 2.5–3 years TAM)	TAM $\geq$ 50–97%	early	4868 (100%)	3320 (68%)	1740 (36%)
	EXE = 4852			EXE $\geq$ 50–97%		4898 (100%)	3377 (69%)	1773 (36%)
N-SAS BC03 [23]	TAM = 349	adjuvant	TAM for 5 years vs. TAM (20 mg) for 1–4 years –> ANA (1 mg) to complete 5 years of hormone therapy	TAM-60.2	early	349 (100%)	ND	186 (53.3%)
	ANA = 347			ANA-59.5		347 (100%)	ND	187 (53.9%)
EORTC phase III, 2008 [24]	EXE = 182	first-line	TAM 20 mg vs. EXE 25 mg until disease progression or unacceptable toxicity occurred	EXE-63	metastatic	ND	75 (41.2%)	76 (41.7%)
	TAM = 189			TAM-62		ND	79 (41.8%)	79 (41.8%)
ARNO-95 [25]	ANA = 445	adjuvant	TAM for 5 years vs. TAM for 2 years –> ANA for 3 years	ANA-60.9	early	489 (100%)	326 (66.7%)	ND
	TAM = 452			TAM-60.5		490 (100%)	332 (67.8%)	ND
IES [26]	EXE = 2320	adjuvant	TAM 20 mg for 5 years vs. TAM 20 mg for 2 or 3 years, then switch to EXE 25 mg to complete a total of five years of adjuvant endocrine treatment	EXE-64.3	early	2349 (99.9%)	ND	766 (32.4%) chemoth.; 567 (24.0%) hormone-th.
	TAM = 2338			TAM-64.2		2365 (99.7%)	ND	765 (32.1%) chemoth.; 557 (23.4%) hormone-th.
ITA [27]	TAM = 225	adjuvant	TAM 20 mg (2–3 years) –> ANA 1 mg to complete 5-years treatment vs. TAM 20 mg for 5 years	63	early	225 (100%)	110 (49%)	150 (67%)
	ANA = 223					223 (100%)	120 (54%)	149 (67%)
BIG-98 [28]	LET (LET for 5 years; LET –> TAM) = 3975	adjuvant	LET (2.5 mg) vs. TAM (20 mg) vs. LET (2 years) –> TAM (3 years) vs. TAM (2 years) –> LET (3 years) for 5 years (this analysis compares the two groups assigned to receive LET initially with the two groups assigned to receive TAM initially)	61	early	4003 (100%)	2867 (71.6%)	1012 (25.3%)
	TAM (TAM for 5 years; TAM –> LET) = 3988					4007 (100%)	2870 (71.6%)	1012 (25.3%)

Table 1. *Cont.*

TRIAL	TRIAL ARM (*n* Included in Safety Analysis)	TREATMENT	TRIAL DESIGN	AGE (Mean)	CANCER STAGE	PRIMARY TREATMENT		
						Surgery (%)	Radiotherapy (%)	Systemic Therapy (%)
MA.17 [29]	LET = 2572	extended adjuvant	LET (2.5 mg) vs. placebo for 5 years	LET-62.0	early	1286 (50%)	1550 (60%)	1175 (46%)
	PBO = 2577			PBO-62.0		1301 (50%)	1528 (59%)	1177 (46%)
EORTC phase II trial [30]	EXE = 61	first-line	TAM 20 mg vs. EXE 25 mg; treatment was continued until progression of disease, unacceptable toxity, patient refusal or start of any new anti-cancer therapy	EXE-62	metastatic	ND	59%	42%
	TAM = 59			TAM-63		ND	59%	43%
ATAC [31]	ANA = 3092	adjuvant	ANA 1 mg + TAM placebo vs. ANA placebo + TAM 20 mg vs. ANA 1 mg + TAM 20 mg for 5 years	ANA-64.1	early	1494 (47.8%)	1978 (63.3%)	698 (22.3%)
	TAM = 3094			TAM-64.1		1474 (47.3%)	1946 (62.5%)	647 (20.8%)
	comb = 3097			comb-64.3		1502 (48.1%)	1936 (62.0%)	651 (20.8%)
TARGET [32]	ANA = 336	first-line	ANA 1 mg vs. TAM 20 mg; trial treatment was continued until disease progression	ANA-67	advanced	ND	ND	105 (30.8%)
	TAM = 329			TAM-66		ND	ND	97 (29.6%)
Nabholtz et al., 2000 [33]	ANA = 170	first-line	ANA 1 mg vs. TAM 20 mg; trial treatment was continued until disease progression	ANA-68	advanced	ND	ND	68 (39.8%)
	TAM = 182			TAM-67		ND	ND	70 (38.4%)
NRG Oncology/NSABP B-42 [34]	PBO = 1933	extended adjuvant	LET 2.5 mg vs. placebo for 5 years	ND	early	775 (39.1%)	ND	ND
	LET = 1941			ND		782 (39.4%)	ND	ND
ANZ 0501 LATER [35]	observ = 181	extended adjuvant	LET 2.5 mg for 5 years vs. observation	observ-64	early	67 (37.4%)	126 (70.4%)	86 (48.0%)
	LET = 176			LET- 65		64 (35.4%)	130 (71.8%)	75 (41.4%)

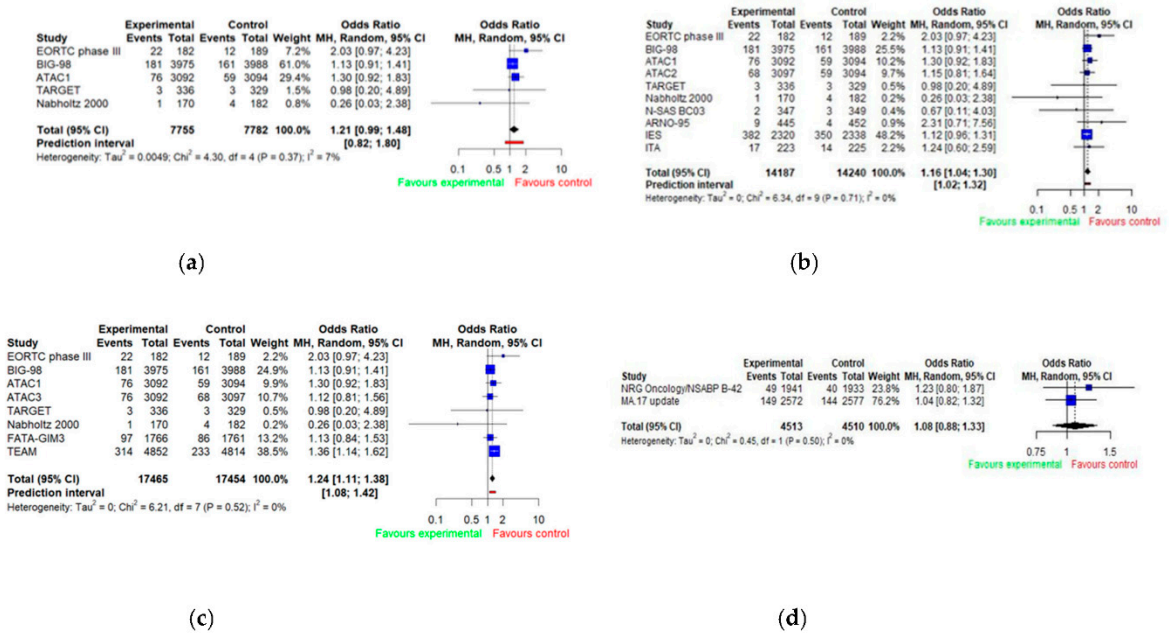


Figure 3. Forest plot of odds ratios for cardiovascular events with AIs by trial design (**a**–**d**). E (experimental group); C (control group). (**a**) E: AIs vs. C: tamoxifen (monotherapy); (**b**) E: AIs (monotherapy) or AIs + tamoxifen (sequence) vs. C: tamoxifen (monotherapy); (**c**) E: AIs (monotherapy) vs. C: AIs + tamoxifen (sequence) or tamoxifen (monotherapy); (**d**) E: AIs (monotherapy) vs. C: placebo/no treatment.

Comparing study arms in which aromatase inhibitors were used (alone or in sequence with tamoxifen) with the arms in which tamoxifen was used alone ($n = 9$) allowed for finding a statistically higher risk of cardiovascular events for AIs (OR = 1.16; 95% CI 1.04–1.30). The heterogeneity across studies was low: $I^2 = 0\%$ ($p = 0.71$) (Figure 3b). Similar results were observed when comparing patients taking aromatase inhibitors alone to patients taking tamoxifen alone or in sequence with aromatase inhibitor (OR = 1.24; 95% CI 1.11–1.38) (Figure 3c). Two studies that compared patients taking aromatase inhibitor as an extended adjuvant therapy with those on placebo showed no difference in relation to the occurrence of cardiovascular events (OR = 1.08; 95% CI 0.88–1.33) (Figure 3d).

3.3. Arterial Hypertension

Ten studies reported arterial hypertension as an adverse effect. Pooled analyses of odds ratios for arterial hypertension are presented in Figure 4 (Figure 4a–d). An analysis of five trials comparing adjuvant AIs to tamoxifen showed the odds for arterial hypertension being 1.31 times higher for patients taking aromatase inhibitor; however, this did not reach statistical significance (OR = 1.31; 95% CI 0.47–3.65). The studies showed high heterogeneity, with $I^2 = 70\%$ ($p = 0.04$) (Figure 4a).

There was no difference when comparing patients taking aromatase inhibitors alone or sequentially with tamoxifen to patients taking tamoxifen alone (OR = 1.15; 95% CI 0.72–1.83) (Figure 4b); patients taking aromatase inhibitors alone to patients taking tamoxifen alone or sequential with aromatase inhibitor (OR = 1.06; 95% CI 0.74–1.53) (Figure 4c); and, similarly, patients on aromatase inhibitor as extended adjuvant therapy to those on placebo (OR = 0.93; 95% CI 0.75–1.16) (Figure 4d). The highest OR for arterial hypertension of 1.40 (95% CI 1.17–1.67) was reported in the TEAM study.

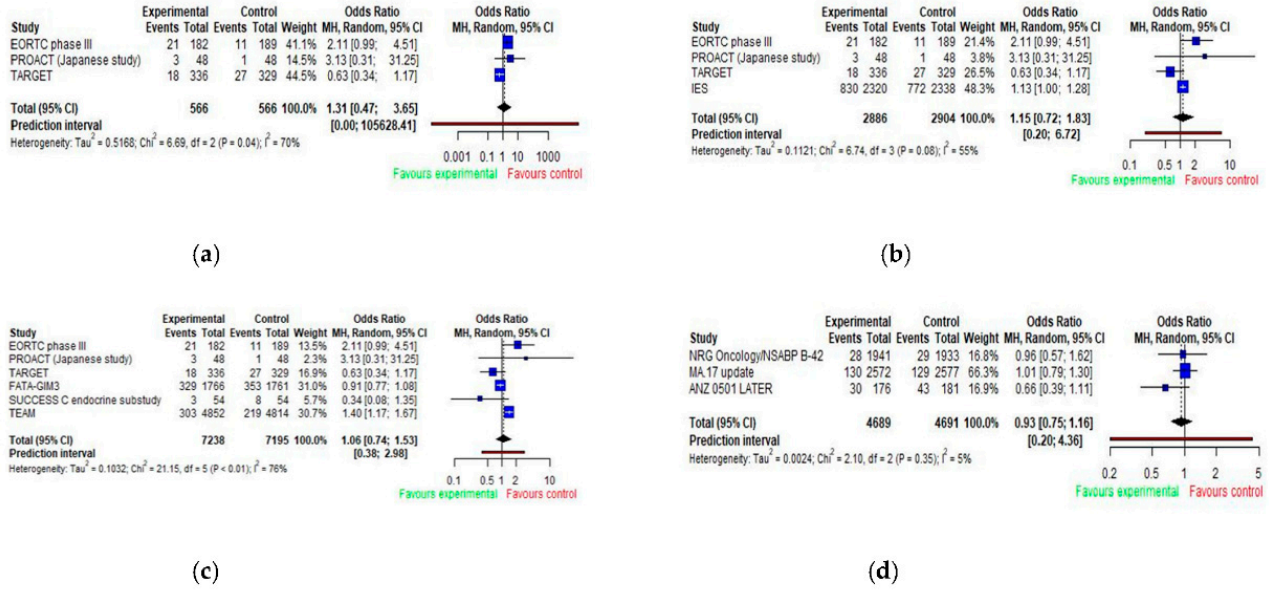


Figure 4. Forest plot of odds ratios for arterial hypertension with AIs by trial design (**a**–**d**). E (experimental group); C (control group). (**a**) E: AIs vs. C: tamoxifen (monotherapy); (**b**) E: AIs (monotherapy) or AIs + tamoxifen (sequence) vs. C: tamoxifen (monotherapy); (**c**) E: AIs (monotherapy) vs. C: AIs + tamoxifen (sequence) or tamoxifen (monotherapy); (**d**) E: AIs (monotherapy) vs. C: placebo/no treatment.

3.4. Body Weight Gain

Body weight gain as an adverse effect was reported in six studies. Pooled analyses of odds ratios for body weight gain are presented in Figure 5 (Figure 5a,b). The results were not statistically significant: OR = 1.30; 95% CI 0.85–3.33 when compared patients taking aromatase inhibitor to patients taking tamoxifen, and OR = 0.92; 95% CI 0.73–1.14 when compared patients taking aromatase inhibitor to patients taking tamoxifen alone or sequentially with aromatase inhibitor (Figure 5a,b).

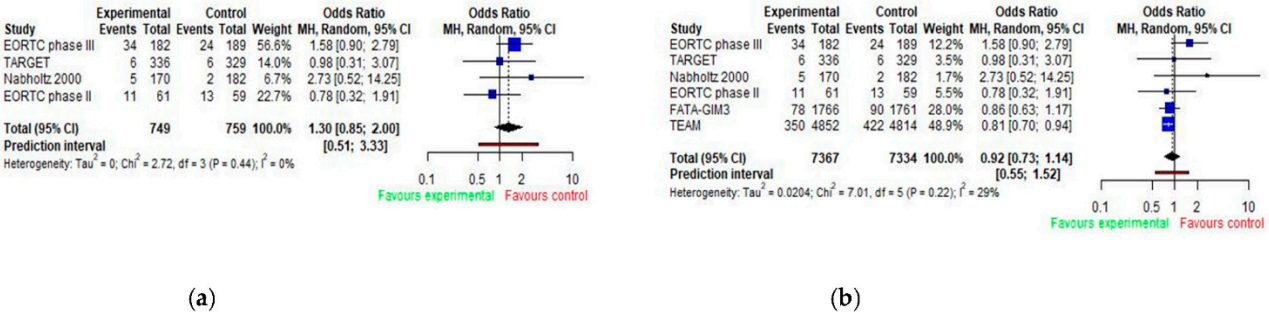
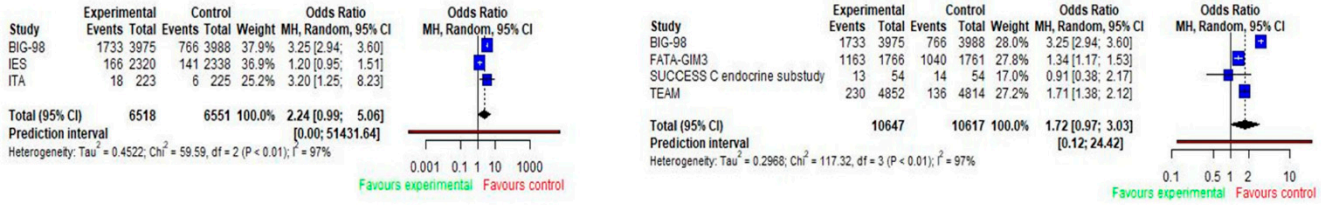


Figure 5. Forest plot of odds ratios for body weight gain with AIs by trial design (**a**,**b**). E (experimental group); C (control group). (**a**) E: AIs vs. C: tamoxifen (in monotherapy); (**b**) E: AIs (monotherapy) vs. C: AIs + tamoxifen (sequence) or tamoxifen (monotherapy).

3.5. Dyslipidemia

In our study we defined dyslipidemia as all lipid disorders, such as hyperlipidemia, hypercholesterolemia or hypertriglyceridemia. Dyslipidemia as an adverse effect was reported in six studies. Pooled analyses of odds ratios for dyslipidemia are presented in Figure 6 (Figure 6a,b). When data were pooled across trials, no evidence of difference was observed. However, these analyses were inconclusive due to wide 95% CIs (aromatase inhibitor alone or sequentially with tamoxifen vs. tamoxifen: OR = 2.24; 95% CI 0.99–5.06; aromatase inhibitor alone vs. tamoxifen alone or sequentially with aromatase

inhibitor: OR = 1.72; 95% CI 0.97–3.03) and high heterogeneity across studies with I2 = 97% (Figure 6a,b). The highest OR for dyslipidemia of 3.25 (95% CI 2.94–3.60) was reported in the BIG-98 study.



(**a**) (**b**)

Figure 6. Forest plot of odds ratios for dyslipidemia with AIs by trial design (**a**,**b**). E (experimental group); C (control group). (**a**) E: AIs (monotherapy) or AIs + tamoxifen (sequence) vs. C: tamoxifen (monotherapy); (**b**) E: AIs (monotherapy) vs. C: AIs + tamoxifen (sequence) or tamoxifen (monotherapy).

4. Discussion

The aim of this study was to determine whether treatment using AIs is associated with an increased risk of both cardiovascular and metabolic adverse effects, such as body weight gain, dyslipidemia, hyperglycemia or insulin resistance. Due to the fact that treatment with AIs is mainly used in postmenopausal women and given that these patients, due to their post-menopausal status, are more likely to suffer from cardiovascular events, lipid metabolism disorders or diabetes mellitus [12], this is a significant health concern.

To our knowledge, this is the most recent study to assess the cardiovascular and metabolic risk of AIs treatment in breast cancer patients. We found that treatment with AIs (alone or in sequence with tamoxifen) increases the risk for cardiovascular events (OR = 1.16; 95% CI 1.04–1.30) comparing with tamoxifen alone. Similar results were observed when patients taking aromatase inhibitors alone were compared to those taking tamoxifen alone or in sequence with aromatase inhibitor (OR = 1.24; 95% CI 1.11–1.38). Our findings are consistent with the results of the study by Khosrow-Khavar et al., who showed that the use of AIs compared to tamoxifen was associated with a 19% increase in the risk of cardiovascular adverse events (RR = 1.19, 95% CI 1.07–1.34) [7]. In contrast, we separately analyzed the studies depending on the treatment regimens of tamoxifen alone vs. AI alone (no relationship; OR = 1.21; 95% CI 0.99–1.48), AI alone vs. tamoxifen alone or sequentially with AI (e.g., the TEAM study), and AI alone or sequentially with tamoxifen vs. tamoxifen alone (e.g., the IES study). The lack of statistical significance in the comparison of tamoxifen alone vs. aromatase inhibitor alone could be explained by the small number of studies with this kind of design. Khosrow-Khavar et al. hypothesized that the increase in the incidence of cardiovascular events in patients treated with AIs is probably related to the cardioprotective effect of tamoxifen, which seems also be the case in our findings as there was no difference when the aromatase inhibitor treatment group was compared to the placebo treatment group.

The occurrence of cardiovascular events may also depend on the duration of therapy and the type of AI. Results from a population-based cohort study conducted by Sund et al. indicated an increased risk for arrhythmia and acute ischemic heart disease in patients treated for more than four years with AIs [36]. In turn, a network meta-analysis performed by Zhao et al. showed the total and severe cardiovascular events' risk ranking for letrozole, exemestane and anastrozole in descending order [37]. Different results were presented by He et al. [38], who suggested that patients treated with AIs do not have a significant risk of developing cardiovascular events in comparison with tamoxifen treatment (OR = 0.9940, 95% CI 0.8545–1.1562). However, in the same study it was found that almost all of the high-grade cardiovascular events occurred in patients treated with AIs [38].

Regardless of the treatment regimen, we did not show a significantly increased risk for arterial hypertension to be associated with AIs. Nevertheless, such a risk was reported in the TEAM study with an OR of 1.40 (95% CI 1.17–1.67) [22]. Blaes et al., examining vascular function in breast cancer patients, showed that compared to healthy post-menopausal women, women on AI had a higher mean systolic blood pressure (128.6 mmHg vs. 116.2 mmHg; $p = 0.004$) [39].

As with arterial hypertension, we did not show an increased risk for body weight gain in the AI-treated groups. Sestak et al. analyzed, for weight change, three large clinical trials (ATAC, IBIS-I, IBIS-II) and they reported that weight gain did not differ between AIs, tamoxifen and placebo [40], which is consistent with the results of the current meta-analysis. The body weight was compared at 2 years with that at diagnosis in 625 patients with breast cancer, and 31% had lost > 2 kg, 34% had a stable weight and 35% had gained >2 kg. Main factors associated with > 2 kg weight gain were pre-menopausal status and receiving any chemotherapy [41].

Patients treated with aromatase inhibitor tended to have a higher risk for dyslipidemia than those treated with tamoxifen, though the difference did not reach statistical significance. It could have been due to wide 95% CIs and high heterogeneity across studies ($I^2 = 97\%$). The highest risk was reported in the BIG-98 study (OR = 3.25; 95% CI 2.94–3.60). A total of 43.6% of patients in the letrozole group and 19.2% of patients in the tamoxifen group had hypercholesterolemia recorded at least once during treatment [28]. Wang et al. conducted a prospective single-center cohort study and found that steroidal aromatase inhibitor (exemestane) had a more favorable effect on lipid profiles than nonsteroidal aromatase inhibitors (anastrozole, letrozole). The cumulative incidence of lipid events in the steroidal and nonsteroidal groups at 24 months was 25.3% and 37.0%, respectively [42]. These findings are consistent with the results obtained in the BIG-98 study [28].

Data to assess the odds for AI-associated hyperglycemia was insufficient to perform a meta-analysis. Hyperglycemia was reported as an adverse event only in five studies having a different drug regimen. In the ITA study, hyperglycemia was observed in 1.3% of patients receiving tamoxifen therapy for 5 years and 4.5% of patients receiving tamoxifen and anastrozole sequentially [27]. In contrast, in the SUCCESS C study, hyperglycemia was significantly more frequent in patients receiving sequential therapy with tamoxifen and AI than in patients receiving therapy with exemestane alone (28% vs. 22%) [16]. In the study by Iwata et al., comparing exemestane and anastrozole in the first-line treatment of advanced breast cancer, the proportion of patients with hyperglycemia was significantly higher in the exemestane group, at 51.4% and 47.7%, respectively [18]. Despite the lack of data from large clinical trials on carbohydrate disturbances in patients using aromatase inhibitors, this seems to be an important issue. Gibb et al., in a case-control study, compared women with breast cancer diagnoses and receiving aromatase inhibitor therapy with age-matched healthy control subjects. They found that aromatase inhibitor therapy was associated with significantly lower insulin sensitivity, higher peak insulin concentration after oral glucose tolerance test, greater percentage of body fat and higher plasma leptin concentration [8]. In turn, Hamood et al. investigated the association between hormone therapy and diabetes risk in breast cancer survivors. Of 2246 breast cancer survivors, 324 developed diabetes over a mean follow-up of 5.9 years. They found the hazard for aromatase inhibitor use (HR = 4.27; 95% CI 1.42–12.84; $p = 0.010$) being higher than for the use of tamoxifen (HR = 2.25; 95% CI 1.19–4.26; $p = 0.013$) [9]. Different results come from the meta-analysis by Feng et al. exploring the association of hormone therapy (HT) and secondary diabetes in breast cancer patients. They showed that HT significantly increased the risk of developing diabetes mellitus. However, when analyzing specific HT medications, TAM use significantly enhanced the incidence of secondary diabetes mellitus, while AIs use did not have an influence [43].

Our study is not without limitations. First, the meta-analysis was based on the results of primary studies, not on individual patient data. We included studies with postmenopausal women with breast cancer of any stage–early, metastatic or advanced.

Patients with metastatic or advanced BC may have been exposed to a greater number of prior treatments, e.g., chemotherapy, which could also cause cardiotoxicity. To minimize the impact of this factor, we performed an additional analysis of early vs. advanced/metastatic breast cancer. The results of this analysis can be found in Supplementary Material S1. Then, there was a heterogeneity in reporting adverse effects between studies. The results of the sensitivity analysis can be found in Supplementary Material S2. There was also a heterogeneity across RCTs concerning the duration of follow-up and the trial design. However, to minimize the impact of the treatment regimen, we decided to conduct separate analyses in this respect. In addition, we did not have enough information characterizing patients at baseline in terms of the presence of cardiovascular or metabolic disorders. All of this may jeopardize our results to a certain extent.

5. Conclusions

In conclusion, our results suggest that postmenopausal women with breast cancer treated with AIs have an increased risk of cardiovascular events in comparison to those treated with tamoxifen, which is largely due to the cardioprotective effect of the latter compared to the cardiotoxicity of AIs. We were unable to find a similar association for hypertension, dyslipidemia, hyperglycemia, insulin resistance or weight gain. Further large, high-quality RCTs and post-marketing safety observational studies are still needed to definitively evaluate the impact of AIs on cardiovascular events and metabolic disorders in breast cancer patients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11113133/s1, Supplementary Table S1: Search results; Supplementary Table S2: Quality assessment of included studies. Supplementary Material S1: sensitivity analysis. Supplementary Material S2: additional analysis early vs. advanced/metastatic breast cancer.

Author Contributions: Conceptualization, K.B. and A.P.; methodology, P.P.; software, P.P.; formal analysis, K.B. and P.P.; investigation, K.B. and P.P.; resources, K.B. and P.P.; data curation, K.B. and P.P.; writing—original draft preparation, K.B.; writing—review and editing, P.P. and A.P.; visualization, P.P. and K.B.; supervision, A.P.; project administration, K.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data are available in the article and supplementary material. We will willingly share our knowledge, protocol and expertise when asked.

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

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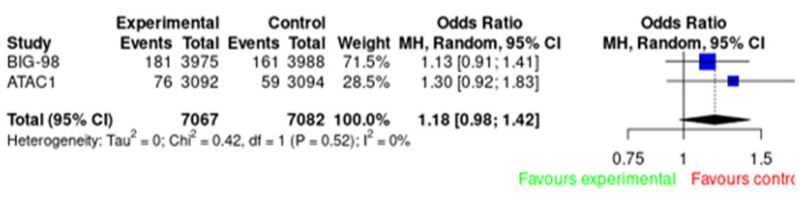
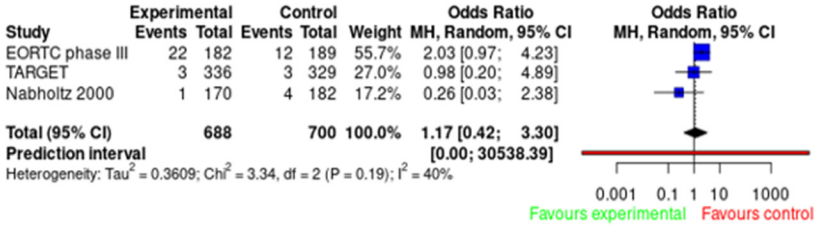
Supplementary Material S1: additional analysis early vs. advanced/metastatic breast cancer.

An additional analysis was performed only when the qualified studies included studies involving patients with early and advanced breast cancer.

1. **Cardiovascular events:**

A. AIs vs. tamoxifen monotherapy.

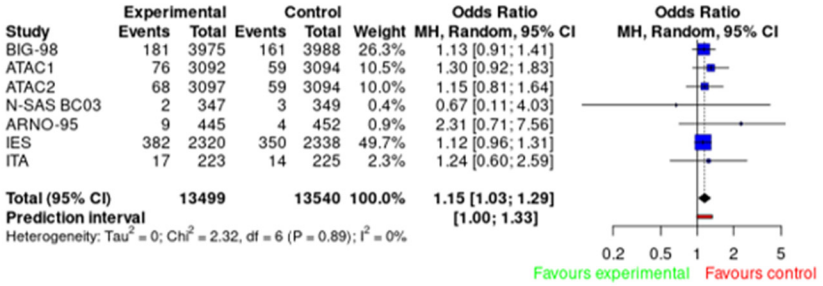


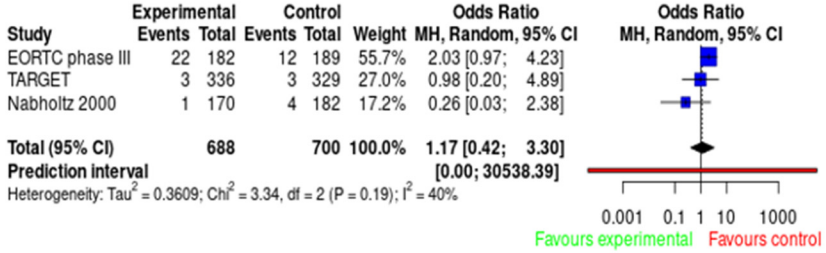


EARLY BREAST CANCER

ADVANCED/METASTATIC BREAST CANCER

B. AIs (monotherapy) or AIs+tamoxifen (sequence) vs. tamoxifen (monotherapy).

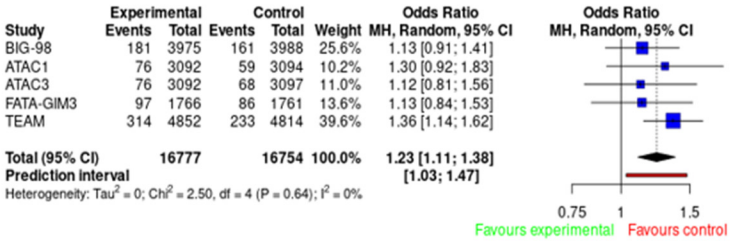




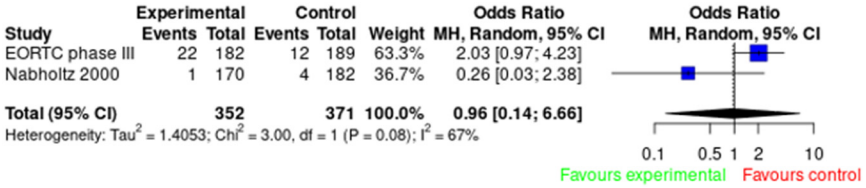
EARLY BREAST CANCER

ADVANCED/METASTATIC BREAST CANCER

C. AIs (monotherapy) vs. AIs+tamoxifen (sequence) or tamoxifen (monotherapy).



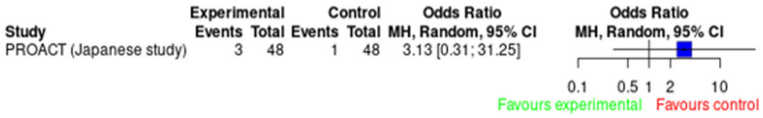
EARLY BREAST CANCER



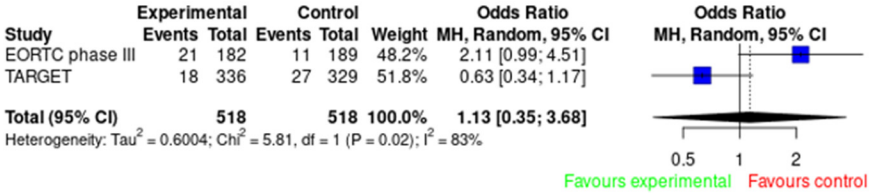
ADVANCED/METASTATIC BREAST CANCER

2. Arterial hypertension:

A. AIs vs. tamoxifen monotherapy.

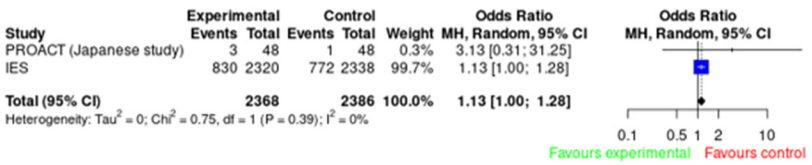


EARLY BREAST CANCER

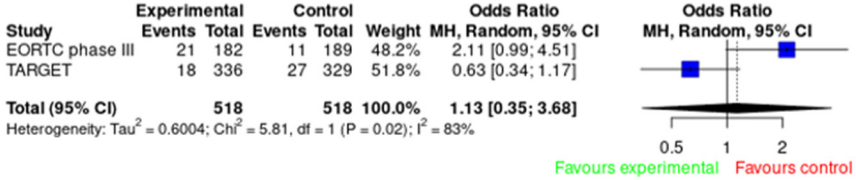


ADVANCED/METASTATIC BREAST CANCER

B. AIs (monotherapy) or AIs+tamoxifen (sequence) vs. tamoxifen (monotherapy).

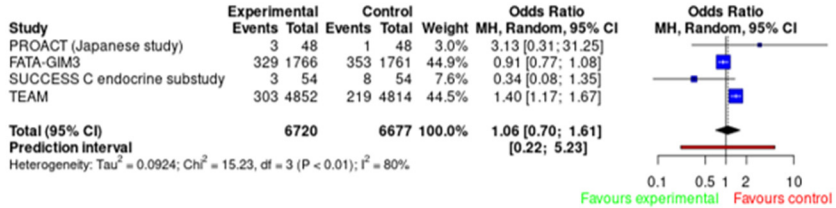


EARLY BREAST CANCER

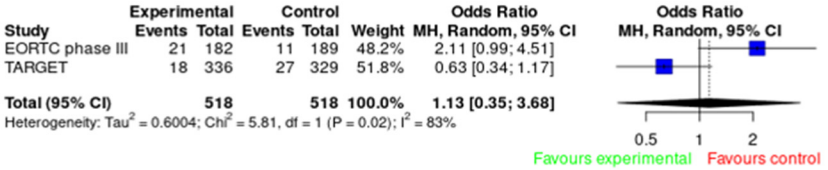


ADVANCED/METASTATIC BREAST CANCER

C. AIs (monotherapy) vs. AIs+tamoxifen (sequence) or tamoxifen (monotherapy).



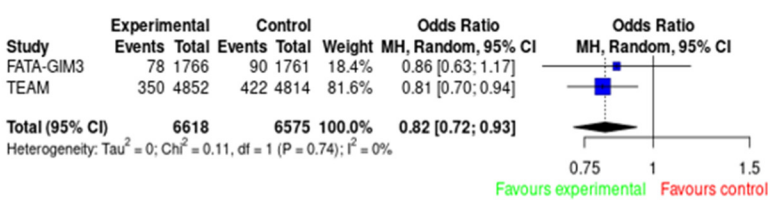
EARLY BREAST CANCER



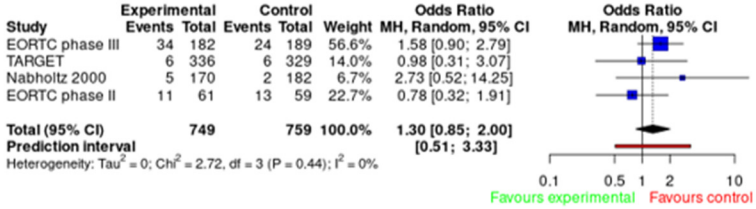
ADVANCED/METASTATIC BREAST CANCER

3. Body weight gain:

A. AIs (monotherapy) vs. AIs+tamoxifen (sequence) or tamoxifen (monotherapy)



EARLY BREAST CANCER



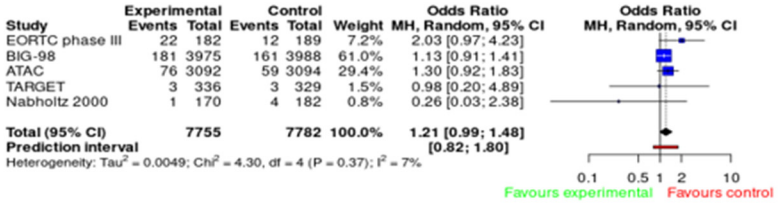
ADVANCED/METASTATIC BREAST CANCER

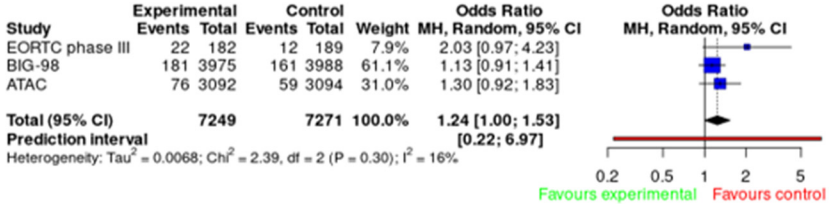
Supplementary Material S2: sensitivity analysis.

In the sensitivity analysis, were made the assumptions that studies with incidence rate of events < 0,01 (1%) will be exluded, therefore it only applies to cardiovascular events.

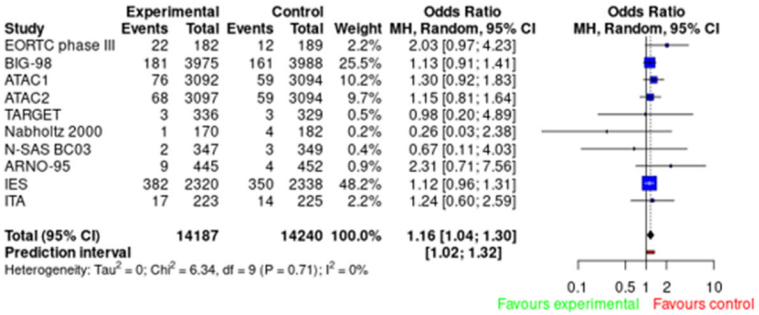
Cardiovascular events:

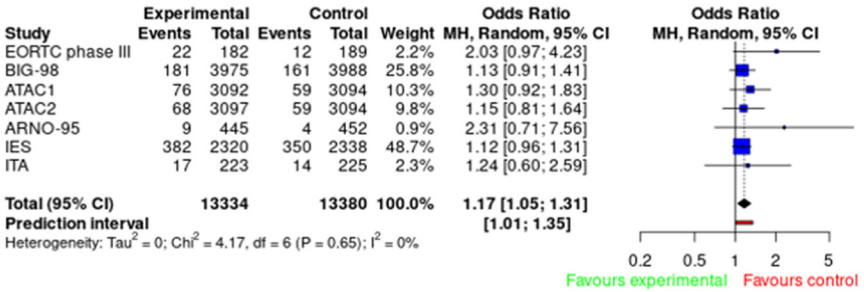
1. AIs vs. tamoxifen (monotherapy) – all (left) vs. without low incidence (right)



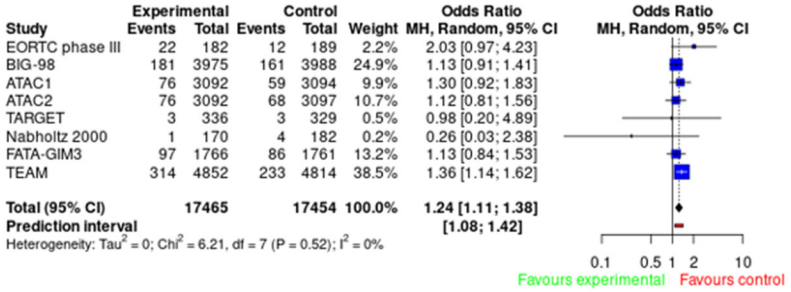


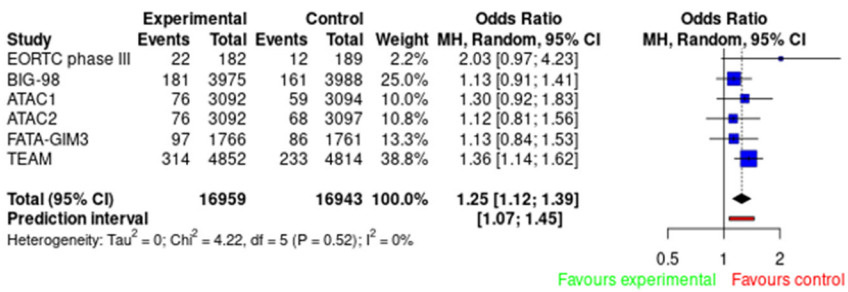
2. AIs (monotherapy) or AIs+tamoxifen (sequence) vs. tamoxifen (monotherapy) - all (left) vs. without low incidence (right)





3. AIs (monotherapy) vs. AIs+tamoxifen (sequence) or tamoxifen (monotherapy) - all (left) vs. without low incidence (right)





Supplementary Table S1. Search results in each query - cardiovascular and metabolic adverse events in postmenopausal women with breast cancer receiving aromatase inhibitors (on 27 Oct 2020)

		Embase + Medline
1	'breast tumor':ab,ti OR 'breast tumor'/exp OR 'breast tumor'	24 363
2	'aromatase inhibitor':ab,ti OR 'aromatase inhibitor'/exp OR 'anastrozole'/exp OR 'arimidex'/exp OR 'letrozole'/exp OR 'femara'/exp OR 'exemestane'/exp OR 'aromasin'/exp	3 008
3	'cardiovascular disease'/exp OR 'ischemic heart disease'/exp OR 'heart infarction'/exp OR 'cerebrovascular accident'/exp OR 'body weight'/exp OR 'obesity'/exp OR 'diabetes mellitus'/exp OR 'dyslipidemia'/exp OR 'glucose intolerance'/exp OR 'insulin resistance'/exp OR 'hyperglycemia'/exp OR 'hypercholesterolemia'/exp OR 'hypertriglyceridemia'/exp OR 'metabolic syndrome X'/exp	264 359
5	#1 AND #2 AND #3	629

Trial	Selection bias		Performance bias	Detection bias	Attrition bias	Reporting bias	Other bias
	Random Sequence Generation	Allocation Concealment	Blinding of participants	Blinding of outcome	Incomplete Outcome	Selective outcome	
FATA-GIM3 [13]	-	+	+	+	-	-	?
SUCCESS C [14]	?	?	+	+	?	?	-
FACE [15]	-	+	+	+	-	-	-
Iwata et al, 2013 [16]	-	?	-	-	-	-	?
MA.27 [17]	-	+	+	+	-	-	-
PROACT [18]	-	?	-	?	-	-	-
N-SAS BC04 [19]	?	?	+	+	-	-	-
TEAM [20]	-	-	+	+	-	-	-
N-SAS BC03 [21]	?	?	+	+	-	-	-
EORTC, phase III [22]	-	+	+	+	-	-	-
ARNO-95 [23]	-	-	+	+	-	-	-
IES [24]	-	-	-	-	-	-	-
ITA [25]	?	?	?	?	-	-	-
BIG-98 [26]	-	-	-	-	-	-	-
MA.17 [27]	?	?	-	-	-	-	-
EORTC, phase II [28]	-	+	+	+	-	-	-
ATAC [29]	-	-	-	-	-	-	-
TARGET [30]	-	?	-	-	-	-	-
Nabholtz et al, 2000 [31]	-	-	-	-	?	?	-
NRG Oncology/NSABP B-42 [32]	-	-	-	?	?	-	-
ANZ 0501 LATER [33]	-	+	+	+	-	-	-

Supplementary Table S2. Quality assessment of included studies. Legend: low risk of bias (-), high risk of bias (+), unclear risk of bias (?)

ZAŁĄCZNIK 3

cancers

MDPI

Article

The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model

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Simple Summary: Progressive industrialization, urbanization, and consumerism lead to increased contamination of the environment with endocrine-disrupting compounds (EDCs) which play an important role in the increased incidence of hormone-dependent cancers, e.g., breast cancer. EDCs include, among others, xenoestrogens—exogenous compounds that can bind to estrogen receptors and thus compete with, or mimic the action of endogenous estrogens (e.g., promote the proliferation of cancer cells). The aim of the study was to answer the question whether exposure to selected xenoestrogens, widespread in everyday life (aluminum in antiperspirants and deodorants; chromium (III) in dietary supplements and drugs) affects the effectiveness of drugs used in hormone therapy in breast cancer. We performed in vitro tests on a breast cancer cell model—MCF-7 and MCF-7/DOX cell lines exposed to selected xenoestrogens, drugs, and their combinations. Our results confirm that such exposure may negatively affect the effectiveness of breast cancer hormone therapy.

Abstract: Endocrine-disrupting compounds (EDC) play an important role in the increased incidence of breast cancer (BC). There are some 160 xenoestrogens that may be involved in the development of BC. Much less is known about the influence of xenoestrogens on the effectiveness of the treatment of BC. The aim of this study was to analyze the interaction of metalloestrogens (aluminum and chromium (III)) and drugs used in the treatment of hormone-dependent BC—aromatase inhibitors (AI)—letrozole and exemestane. A cell viability assay, a flow cytometer analysis of apoptosis and cell cycle phases, and protein activity of BAX and Bcl-2 were performed on two human breast cancer cell lines—MCF-7 and MCF-7/DOX. In MCF-7 cells, the lower concentration of exemestane and higher of letrozole, in combination with metalloestrogens, results in a decrease in the effectiveness of drugs. Additionally, in the MCF-7/DOX cell line, we observed that the combination of metalloestrogens and AI leads to a decrease in the drug's effectiveness due to an increase in the viability of breast cancer cells (both concentrations of letrozole and higher concentration of exemestane). In both cell lines, the reduction in the effectiveness of AI, in combination with metalloestrogens, is not related to the influence on the cell cycle. Our results confirm that exposure to metalloestrogens may negatively affect the effectiveness of hormone therapy with AI. Further studies are needed to fully explain the mechanism of these interactions.

Keywords: breast cancer; xenoestrogens; aromatase inhibitors; metalloestrogens; chromium (III); aluminum; interaction

Citation: Boszkiewicz, K.; Moreira, H.; Sawicka, E.; Szyjka, A.; Piwowar, A. The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model. *Cancers* **2023**, *15*, 457. https://doi.org/10.3390/cancers15020457

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1. Introduction

In 2020, breast cancer (BC) was diagnosed in 2.3 million women worldwide, and according to the latest prognosis of the American Cancer Society, in 2022 breast cancer

will continue to be the most common cancer among women [1,2]. Breast cancer is not a homogeneous disease depending on the expression of key receptors. Several subtypes are distinguished, which differ in the treatment method and prognosis [3]. One of the more common subtypes is steroid receptor-expressing hormone-dependent breast cancer, which is diagnosed in up to 70% of breast cancer patients [4]. In the treatment of hormone-dependent breast cancer, apart from surgery, radiotherapy, and chemotherapy, long-term use of hormone therapy is crucial to eliminate the proliferation-inducing effects of estrogens [3]. In early breast cancer, tamoxifen for 5–10 years is predominantly used in premenopausal women, and tamoxifen, aromatase inhibitors, or their sequence in postmenopausal women. Aromatase inhibitors or fulvestrant in combination with a cyclin-dependent kinase inhibitor CDK4/6, e.g., palbocyclib or alone—tamoxifen, aromatase inhibitors, or high doses (500 mg i.m.) of fulvestrant are used for hormonal treatment in patients with advanced breast cancer [5–7]. In recent years, treatment with aromatase inhibitors has become more and more popular due to their higher efficacy and a good tolerance profile compared to the current gold standard—tamoxifen. The mechanism of action of aromatase inhibitors is based on the inhibition of the activity of the aromatase enzyme, which is involved in the conversion of androgens to estrogens. The effect of their action is the elimination of estrogens stimulating the proliferation of cancer cells [5,8].

Xenoestrogens, also called endocrine-disrupting compounds (EDCs), are exogenous substances that disrupt the functioning of the endocrine system and exhibit estrogen-like effects, interacting with estrogen receptors (they act as their antagonists or agonists), interfering with the synthesis and metabolism of endogenous estrogens, and influencing the synthesis of estrogen receptors [9–11]. There are several major classes of xenoestrogens—phytoestrogens (e.g., genistein), mycoestrogens (e.g., zearalenone), pesticides, pharmaceuticals, and industrial chemicals (e.g., dichlorodiphenyltrichloroethane—DDT, diethylstilbestrol—DES), synthetic compounds and detergents (e.g., bisphenol A—BPA), and metalloestrogens (e.g., cadmium, chromium, and aluminum) [12]. The Endocrine Society highlights the link between different pathologies, including hormone-dependent cancers in women, and different EDCs, including BPA and dioxins [13–17]. Much less is known about the influence of xenoestrogens on the effectiveness of breast cancer treatment. Several studies have shown that genistein can reverse the therapeutic effect of tamoxifen and its active metabolite—4-hydroxytamoxifen [18–24]. Genistein and zearalenone reversed the inhibitory effect of palbociclib + letrozole combination on cancer cell proliferation, and BPA antagonizes the cytotoxicity of chemotherapeutic drugs (doxorubicin, cisplatin, and vinblastine) in both ER-positive and ER-negative breast cancer cells [25,26].

Despite the common use of metalloestrogens in everyday life, e.g., aluminum and chromium (III), there is no research on how these metalloestrogens affect the effectiveness of breast cancer hormone therapy [11]. Although the human population may be exposed to aluminum from a range of sources, including diet, antacids, and vaccine adjuvants, frequent application of antiperspirants with aluminum salts to the under-arm region adds a relatively high additional exposure directly to the local area of the human breast. Coincidentally, this is also the region of the human breast where there is a disproportionately high incidence of both breast cysts and breast cancer. Aluminum-based antiperspirants (mainly with aluminum chloride or aluminum chlorohydrate) are applied regularly, often to skin irritated by shaving, which further increases exposure to this metalloestrogen [27]. In addition, Darbre and her colleagues have shown that long-term exposure to aluminum (10^{-4} M aluminum chloride or aluminum chlorohydrate) can increase migratory and invasive properties of MCF-7 human breast cancer cells [28]. Of the two environmentally available forms of chromium, hexavalent and trivalent, the hexavalent form has been classified by IARC (the International Agency for Research on Cancer) as a human carcinogen and mutagen. There are conflicting results in the literature concerning the cytotoxicity and genotoxicity of chromium (III). Chromium (III) salts and chromium (III) compounds have been shown to induce DNA damage, sister chromatid exchange, centromere positive and negative micronuclei, oxidative damage, and Cr–DNA adducts [29]. Moreover, some

in vitro studies show that chromium chloride has estrogenic activity. Estrogenicity is defined as the property of producing biological responses qualitatively similar to those produced by the endogenous hormone, 17β-estradiol [16]. The effect of chromium as an endocrine disruptor is becoming increasingly important due to the popularity of trivalent chromium taken in the form of dietary supplements or over-the-counter medications. They are used to regulate glucose levels or to reduce weight; although the available data are inconclusive [30].

The purpose of this study was to analyze the interaction of selected metalloestrogens, aluminum and chromium (III), and drugs used in the treatment of hormone-dependent breast cancer—aromatase inhibitors (AI): non-steroidal AI—letrozole and steroidal AI—exemestane. Our research was conducted on the two estrogen-dependent breast adenocarcinoma cell lines: MCF-7 and MCF-7/DOX (doxorubicin-resistant cell lines). Due to widespread exposure to metalloestrogens, as well as a steady increase in the incidence of BC, examining their impact on the effectiveness of therapies used in the treatment of hormone-dependent BC is becoming a clinically important issue.

2. Materials and Methods

2.1. Materials

DMEM, low-glucose (Dulbecco's modified eagle's medium), FBS (fetal bovine serum), penicillin-streptomycin (10×) solution, and PBS (phosphate-buffered saline) were purchased from Biological Industries, Haemek, Israel. Aluminum chloride hydrate, chromium (III) chloride hexahydrate, letrozole, exemestane, testosterone, DMSO (dimethyl sulfoxide), deionized water, and ethanol were obtained from Sigma Aldrich, Burlington, MA, USA. TrypLE™ Express and GlutaMAX™ were from Gibco, Waltham, MA, USA. Annexin V-FITC Apoptosis Kit and FxCycle™ PI/RNase Staining Solution were purchased from Invitrogen, Waltham, MA, USA. Cell Proliferation Kit II (XTT) was from Roche Diagnostics, Mannheim, Germany. The Halt™ Protease Inhibitor Coctail (100×) and Pierce™ BCA Protein Assay Kit were obtained from Thermo Fisher Scientific, Waltham, MA, USA. The lysis buffer for ELISA, Nori Human Apoptosis Regulator BAX ELISA Kit, and Nori Human Bcl-2 ELISA Kit were purchased from Genorise Scientific, Glen Mills, PA, USA. Accutase™ Cell Detachment was obtained from BD Biosciences, San Jose, CA, USA.

2.2. Methods

2.2.1. Cell Culture

Estrogen-dependent breast adenoma cell line, MCF-7, was purchased in CLS Cell Lines Service GmbH, Eppelheim, Germany. The MCF-7/DOX (an MCF-7 cell line with P-gp overexpressing; a doxorubicin-resistant cell line) was derived from an MCF-7 cell line by 3-month cultivation in the presence of a low doxorubicin concentration. Cells were cultured in complete DMEM growth medium (DMEM, low-glucose with glucose concentration 5.5 (5) mM), supplemented with fetal bovine serum (FBS)—10% v/v, 2 mM L-glutamine, anti-biotics streptomycin (10,000 U/mL), penicillin (10 mg/mL), and 10^{-9} M testosterone, at 37 °C in a humidified atmosphere with 5% CO_2. The cells were subcultured twice a week using TrypLE™ Express.

2.2.2. Drugs and Metalloestrogens Solutions

Letrozole (LET) and exemestane (EXE) were dissolved in DMSO as 100 mM stock solution (LET: 285 mg in 10 mL DMSO and EXE: 296 mg in 10 mL DMSO) and stored at −20 °C. Aluminum chloride hydrate (AL) and chromium (III) chloride hexahydrate (CR) were dissolved in deionized water as 1 mM stock solution and stored at −20 °C. The working solutions were freshly prepared before each experiment by dilution of stock solution in a culture medium. To prepare the highest concentration of EXE (200 μM) and LET (100 μM), we used 10 μL of stock solution of EXE and 5 μL of stock solution of LET, respectively, so the final DMSO concentration was less than 0.2% in EXE and less than 0.1% in LET at the highest aromatase inhibitor concentration tested.

2.2.3. Cell Viability Assay

The cytotoxic effect of letrozole, exemestane, chromium (III) salt, aluminum (III) salt, and their combinations on MCF-7 and MCF-7/DOX cells was determined using the XTT assay. The XTT assay is based on the cleavage of the yellow tetrazolinum salt XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolinum]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) to form an orange formazan dye by metabolic active cells. Briefly, 1×10^4 cells were seeded in a 96-well plate and treated in triplicates with various concentrations of letrozole (10 nM–100 µM), exemestane (12.5–200 µM), and chromium (III)/aluminum (III) salt (5–250 µM) for 72 h. To determine the cytotoxicity of the drug–metalloestrogen combination, we treated cells with the drugs (in two different doses) and metalloestrogen (EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM) + 100 µM CR/AL for 72 h. Untreated control cells were also included. Cells supplemented with 0.2% DMSO were considered as a control. After incubation, the XTT assay was performed according to the manufacturer's instructions. Absorbance was determined at λ = 450 nm, with a reference wavelength at 650 nm using Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VA, USA. The untreated control served as the 100% reference.

2.2.4. Apoptosis and Necrosis Assay

Apoptosis and necrosis were detected with flow cytometry. Cells were stained with an Annexin V-FITC Apoptosis Kit, which contains annexin V conjugated to fluorescein (FITC, annexin V) and propidium iodide (PI). The staining allows for the distinguishing of living cells, early and late apoptotic cells, and necrotic cells. Briefly, 1×10^6 cells were seeded in a 6-well plate and treated with letrozole (LET 1 = 10 µM; LET 2 = 100 µM), exemestane (EXE1 = 100 µM; EXE2 = 200 µM), Cr(III)/Al(III) salt (100 µM) and their combination for 72 h. The untreated control was also prepared and cells supplemented with 0.2% DMSO were considered as a control. Following incubation, the cells were detached with Accutase™ Cell Detachment and washed in cold PBS. The cells were resuspended in 100 µL 1 × annexin-binding buffer and stained with 5 µL of FITC annexin V and 1 µL of PI. After the incubation period (15 min in the dark at room temperature) 400 µL 1 × annexin-binding buffer was added and samples were immediately analyzed in the flow cytometer (CyFlow® SPACE flow cytometer, Sysmex, Kobe, Japan).

2.2.5. Cell Cycle Analysis

To study the anti-proliferative effects induced by the AIs, metalloestrogens, and their combinations, cell cycle analysis was performed with flow cytometry. Cells were stained with FxCycle™ PI/RNase Staining Solution, which allows for the measurement of DNA content and cell distribution among three major phases of the cell cycle: G0/G1, S, and G2/M. The number of cells, the concentrations used, and the incubation time was the same as for the apoptosis and necrosis assay (Section 2.2.4). After incubation, the cells were detached with TrypLE™ Express solution and washed with cold PBS. The cells were then fixed with ice-cold 70% ethanol and kept on ice for 1 h. After two washing steps with cold PBS, the cells were resuspended in 500 µL of PI solution and incubated for 30 min, protected from light. Following incubation, samples were analyzed in the flow cytometer (CyFlow® SPACE flow cytometer, Sysmex, Kobe, Japan).

2.2.6. Flow Cytometric Analysis

All cytometric analyzes were performed on CyFlow® SPACE flow cytometer (Sysmex, Japan). The laser excitation 488 nm (50 mW) and the filter 536/40 (BP) were used for fluorescence measurement of FITC. Propidium iodide fluorescence was measured using laser excitation 488 nm (50 mW) and the filter 675/20 (BP). The MultiCycle™ DNA analysis model was used for cell cycle analysis. All results were analyzed using FCS Express 7 Cytometry software (De Novo Software, Pasadena, CA, USA).

2.2.7. Preparation of Cell Lysates

Briefly, 1×10^6 cells per T-25 flask were seeded and treated with letrozole (LET 1 = 10 µM; LET 2 = 100 µM), exemestane (EXE1 = 100 µM; EXE2 = 200 µM), CR/AL (100 µM), and their combination for 48 h. The untreated cell control was also prepared and cells supplemented with 0.2% DMSO were considered as a control. Following incubation, the cells were detached with TrypLE™ Express solution, washed with cold PBS, and transferred to a microfuge tube. The cells were centrifuged to pellet the cells and then any remaining buffer was removed. A 0.5 mL lysis buffer, supplemented with a protease inhibitor cocktail, was added to the cell pellet, vortexed, and incubated for 30 min on ice. Then, samples were centrifuged at 10,000× *g* for 10 min. The supernatants were transferred to a clean tube and stored at −80 °C for further analysis.

2.2.8. Determination of Total Protein Concentration in Cell Lysates

Total protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The assay was performed according to the manufacturer's instructions. Absorbance was determined at λ = 562 nm using a Synergy HTX Multi-Mode Microplate Reader, BioTek, USA.

2.2.9. ELISA Assays for Bcl-2 and BAX Proteins Detection

ELISA was used to detect and quantify from cell lysates (details in Section 2.2.7) Bcl-2 and BAX apoptotic proteins involved in the cell death pathway. A Nori Human Apoptosis Regulator BAX ELISA Kit and a Nori Human Bcl-2 ELISA Kit (Genorise Scientific, Glen Mills, PA, USA) were performed, according to the manufacturer's instructions. Each sample was assayed in duplicate and expressed relative to the total protein concentration in the same sample. Absorbance was determined at λ = 570 nm using a Synergy HTX Multi-Mode Microplate Reader, BioTek, USA. The four-parameter logistic fitted standard curves for calculating the concentration of Bcl-2 and BAX protein were generated from the Arigo Biolaboratories website (https://www.arigobio.com/elisa-analysis, accessed on 15 September 2022). Bcl-2 and BAX concentrations were calculated per 100 µL of total protein in the sample, and the Bcl-2/BAX ratio was then calculated.

2.2.10. Statistical Analysis

Results were analyzed using GraphPad Prism 9 (GraphPad Software, Boston, MA, USA) using one-way ANOVA, followed post hoc by Tukey's (or Dunnett's tests in case of cell viability assay for alone compounds) multiple comparisons tests. The results were expressed as the mean and standard deviation of the mean (SD). Each experiment was repeated three times. Significant differences among means were estimated at $p < 0.05$.

3. Results

3.1. Effect of Metalloestrogens, Aromatase Inhibitors, and Their Combination on Cell Viability

3.1.1. Metalloestrogens and Aromatase Inhibitors Alone

Results obtained for individual compounds—exemestane (12.5–200 µM), letrozole (10 nM–100 µM), chromium (III), and aluminum (5–250 µM), are in the Supplementary Materials (Figures S1 and S2). Exemestane and letrozole were more cytotoxic to MCF-7 cells than to MCF-7/DOX cells, with exemestane being more potent in both cell lines. Aluminum and chromium (III) had no cytotoxic effect; they stimulated cell proliferation and there were more MCF-7 cells than MCF-7/DOX cells.

3.1.2. Metalloestrogens and Aromatase Inhibitors in Combination

The results are shown in Figure 1.

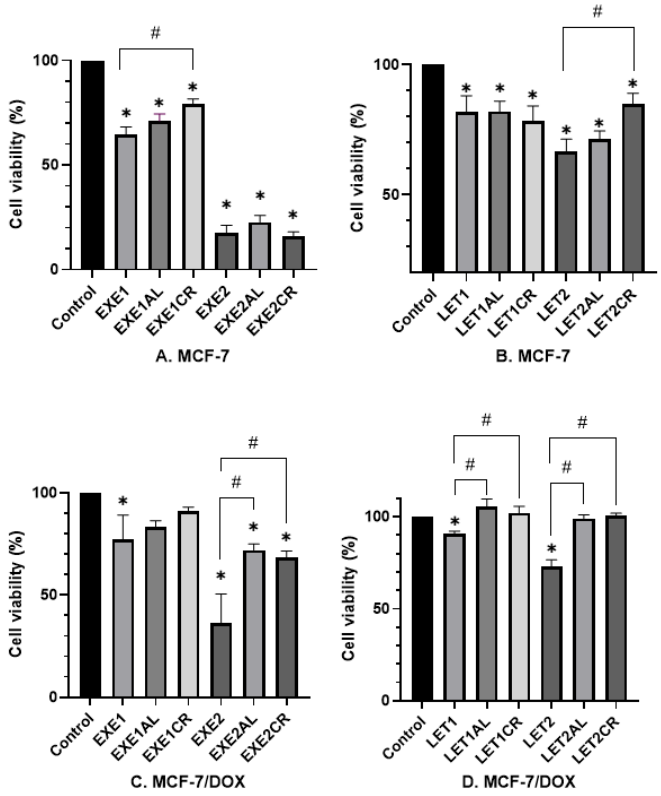

Figure 1. Effect of metalloestrogens and aromatase inhibitors in combination on cell viability. The viability of MCF-7 cells treated with exemestane (**A**) and letrozole (**B**), alone or in combination with aluminum or chromium (III). The viability of MCF-7/DOX cells treated with exemestane (**C**) and letrozole (**D**), alone or in combination with aluminum or chromium (III). EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr(III) = 100 μM. The results are presented as mean ± SD, $n = 3$; $p < 0.05$; * statistically significant difference from the control; # statistically significant difference from the aromatase inhibitor.

- Effects on MCF-7 cells.

The combination of exemestane (100 μM) with both aluminum and chromium (III) increased the viability of MCF-7 cells (EXE1—65%; EXE1AL—71%; and EXE1CR—79%, respectively). The result for chromium (III) is statistically significant ($p = 0.0004$). The combination of exemestane at 200 μM with metalloestrogens had no significant effect (EXE2 18% vs. EXE2AL 23%, $p = 0.3888$; vs. EXE2CR 16%, $p = 0.997$) on MCF-7 cells viability. Letrozole (10 μM) in combination with aluminum or chromium (III) had no statistically significant effect on MCF-7 cell viability (LET1 82% vs. LET1AL 82%, $p > 0.9999$; vs. LET1CR 78%, $p = 0.9645$). However, a combination letrozole at a concentration of 100 μM with chromium (III) and aluminum increased cell viability (LET2—67%; LET2CR—85%; and LET2AL—70%, respectively). The difference between LET2 and LET2CR was statistically significant ($p = 0.0028$).

- Effects on MCF-7/DOX cells.

Aromatase inhibitors were less cytotoxic to MCF-7/DOX cells than MCF-7 cells. The combination of EXE1 with aluminum or chromium (III) increased cell viability, but the results were not statistically significant (EXE1 77% vs. EXE1AL 83%, $p = 0.9331$; vs. EXE1CR 91%, $p = 0.3006$). The combination of EXE2 with metalloestrogens was statistically significant and increased the viability of MCF-7/DOX cells—EXE2 37% vs. EXE2AL 72%, $p = 0.0006$; vs. EXE2CR 68%, $p = 0.0017$. The combination of letrozole (in both concentrations) with metalloestrogens significantly increased the viability of MCF-7/DOX cells: LET1

91% vs. LET1AL 106%, $p = 0.0001$; vs. LET1CR 102%, $p = 0.0022$; LET2 73% vs. LET2AL 99%, $p < 0.0001$; vs. LET2CR 100%, $p < 0.0001$.

3.2. The Effect of Metalloestrogens on Proapoptotic and Necrotic Effects of Aromatase Inhibitors

Proapoptotic and necrotic effects of aromatase inhibitors and their combination with metalloestrogens were studied after 72 h of incubation in MCF-7 and MCF-7/DOX cells using staining with Annexin V-FITC and PI. Results are presented as a percentage of early apoptotic (Annexin V-FITC+, PI−), late apoptotic (Annexin V-FITC+, PI+), and necrotic (Annexin V-FITC−, PI+) cells in Figures 2–5. The gating strategy for flow cytometry analysis of apoptosis and necrosis assays is in the Supplementary Materials (Figure S3). A representative cytogram for the controls is in the Supplementary Materials (Figures S4 and S5).

3.2.1. Effects on MCF-7 Cells

The combination of 100 μM exemestane with metalloestrogens significantly reduced the percentage of cells undergoing apoptosis and necrosis (EXE1 vs. EXE1AL, $p = 0.0152$; vs. EXE1CR, $p = 0.0353$). For exemestane at the higher concentration (200 μM), no statistically significant effect of metalloestrogens on the aromatase inhibitor efficacy was observed (EXE2 vs. EXE2AL, $p = 0.9973$; vs. EXE2CR, $p = 0.9305$). The results are shown in Figure 2.

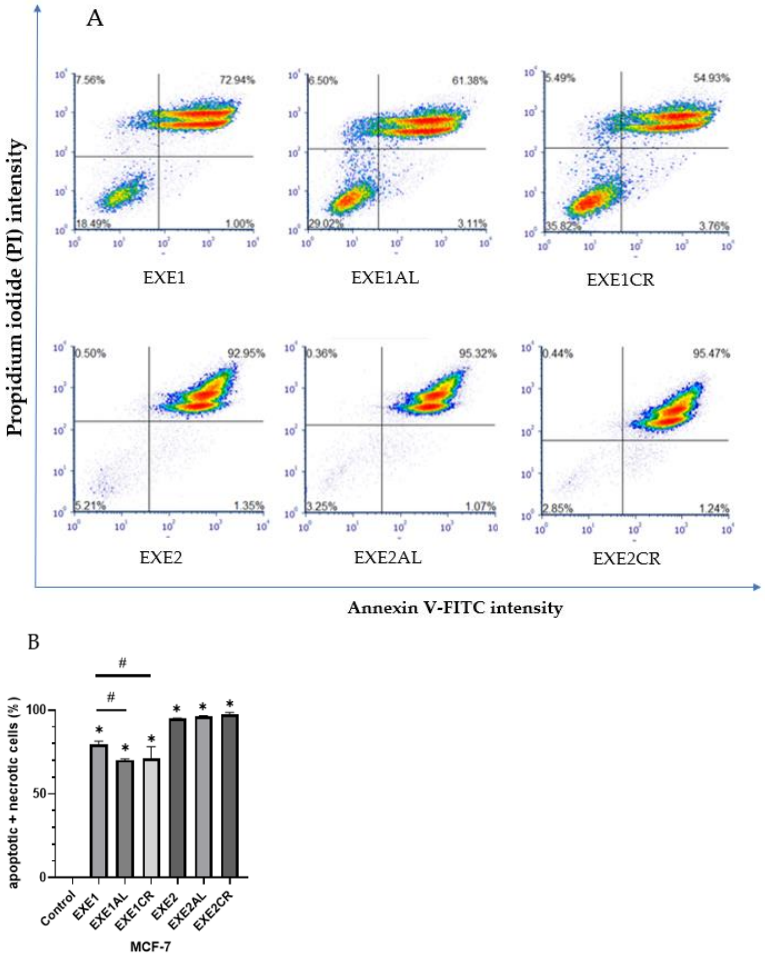

Figure 2. Effect of exemestane alone and in combination with metalloestrogens on MCF-7 cell death. (**A**) Representative cytograms of flow cytometric are shown. EXE1 = 100 μM; EXE2 = 200 μM; Al./Cr(III) = 100 μM. (**B**) Percentage of both apoptotic (early and late apoptotic) and necrotic cells. The results are presented as mean ± SD, $n = 3$; $p < 0.05$; * statistically significant difference from control; # statistically significant difference from aromatase inhibitor alone.

The combination of 10 μM of letrozole with metalloestrogens had no effect on the proapoptotic and necrotic activity of aromatase inhibitor (LET1 vs. LET1AL, $p = 0.9540$; vs. LET1CR, $p = 0.9982$). However, the combination of a higher concentration of letrozole (100 μM) with metalloestrogens significantly reduced the percentage of necrotic and apoptotic cells, thus reducing the effectiveness of the drug (LET2 vs. LET2AL, $p = 0.0224$; vs. LET2CR, $p = 0.0125$). The results are shown in Figure 3.

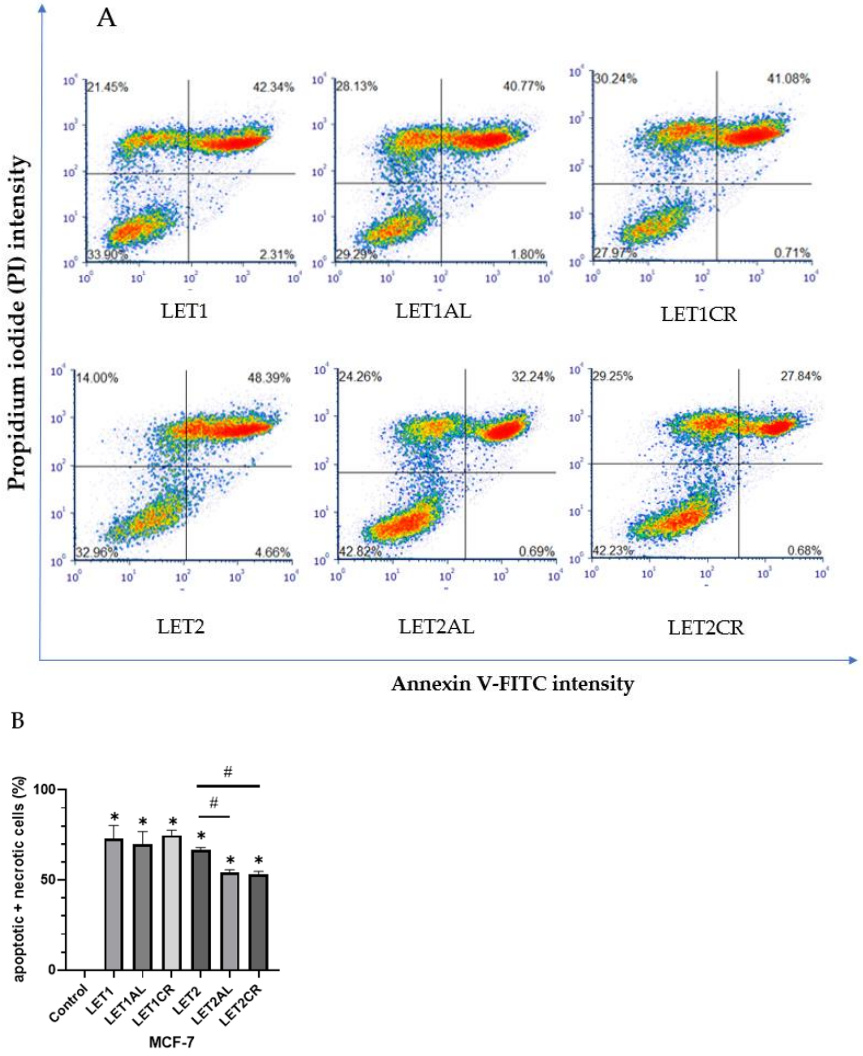

Figure 3. Effect of letrozole alone and in combination with metalloestrogens on MCF-7 cell death. (**A**) Representative cytograms of flow cytometric are shown. LET1 = 10 μM; LET2 = 100 μM; Al./Cr(III) = 100 μM. (**B**) Percentage of both apoptotic (early and late apoptotic) and necrotic cells. The results are presented as mean ± SD, $n = 3$; $p < 0.05$; * statistically significant difference from control; # statistically significant difference from aromatase inhibitor alone.

3.2.2. Effects on MCF-7/DOX Cells

The combination of exemestane at 100 μM with metalloestrogens significantly reduced the number of apoptotic/necrotic MCF-7/DOX cells (EXE1 vs. EXE1AL, $p = 0.0152$; vs. EXE1CR, $p = 0.0353$). No similar relationship is observed when combining a higher concentration of exemestane (200 μM) and metalloestrogens (EXE2 vs. EXE2AL, $p = 0.1097$; vs. EXE2CR, $p = 0.2246$). The results are shown in Figure 4.

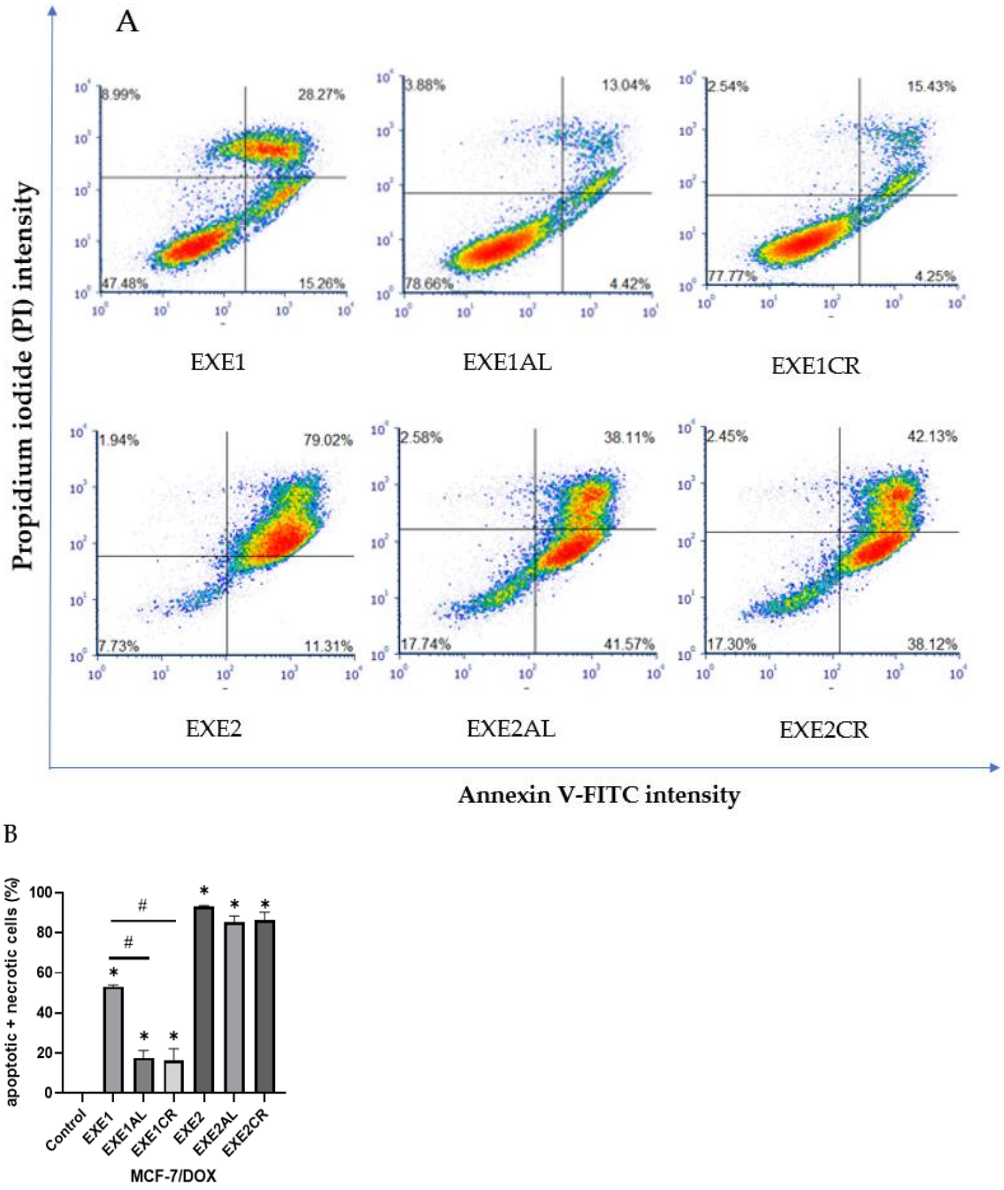

Figure 4. Effect of exemestane alone and in combination with metalloestrogens on MCF-7/DOX cell death. (**A**) Representative cytograms of flow cytometric are shown. EXE1 = 100 μM; EXE2 = 200 μM; Al./Cr(III) = 100 μM. (**B**) Percentage of both apoptotic (early and late apoptotic) and necrotic cells. The results are presented as mean ± SD, $n = 3$; $p < 0.05$; * statistically significant difference from control; # statistically significant difference from aromatase inhibitor alone.

Letrozole, at both concentrations, in combination with metalloestrogens, did not have a statistically significant effect on the proapoptotic/necrotic effects of the drug (LET1 vs. LET1AL, $p = 0.9960$; vs. LET1CR, $p = 0.9992$; LET2 vs. LET2AL, $p > 0.9999$; vs. LET2CR, $p = 0.9989$). The results are shown in Figure 5.

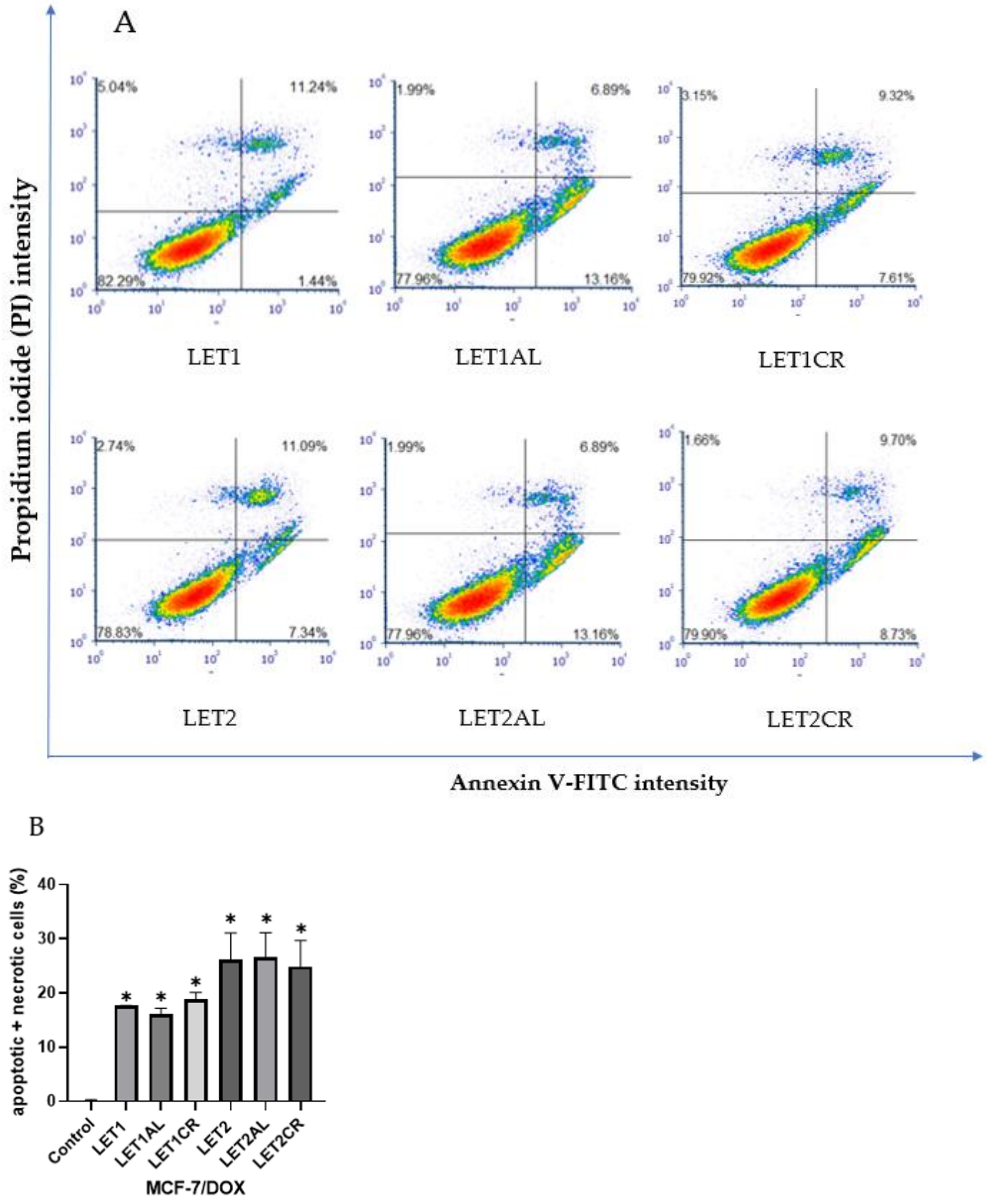

Figure 5. Effect of letrozole alone and in combination with metalloestrogens on MCF-7/DOX cell death. (**A**) Representative cytograms of flow cytometric are shown. LET1 = 10 μM; LET2 = 100 μM; Al./Cr(III) = 100 μM. (**B**) Percentage of both apoptotic (early and late apoptotic) and necrotic cells. The results are presented as mean ± SD, $n = 3$; $p < 0.05$; * statistically significant difference from control.

3.3. The Effect of Aromatase Inhibitors and Their Combination with Metalloestrogens on the Cell Cycle

To determine whether the combination of aromatase inhibitors and metalloestrogens may disturb the cell cycle progression, we further evaluated their effects on the cell cycle. For this purpose, after 72 h of treatment, the cells were stained with PI and the percentage of cells in the G0/G1, S, and G2/M phases were determined using flow cytometry.

3.3.1. Exemestane

Exemestane induces cell cycle arrest in the G0/G1 phase and G2/M phase, and reduces the number of cells in the S phase. In the MCF-7 cell line, a combination of exemestane (100 μM) with aluminum results in an increase in the number of cells in the S phase (and a decrease in the G2/M phase). A combination of metalloestrogens (both aluminum and chromium (III) with exemestane in higher concentration (200 μM) results in an increase

in the percentage of cells in the G2/M phase (compared to EXE2). In the MCF-7/DOX cell line, a combination of exemestane (200 μM) with aluminum or chromium (III) results in an increase in the number of cells in the S phase. However, in both lines, MCF-7 and MCF-7/DOX, changes in the distribution of cells in individual phases of the cell cycle are non-statistically significant. The results are presented in Table 1.

Table 1. Effect of exemestane and its combination with metalloestrogens on cell cycle in MCF-7 and MCF-7/DOX cell lines. EXE1 = 100 μM; EXE2 = 200 μM; and Al/Cr(III) = 100 μM. The results are presented as mean, *n* = 3.

Sample	%G1 (G0/G1)	%S	%G2 (G2/M)
MCF-7			
Control	73.19	24.03	2.78
EXE1	79.51	0.23	20.26
EXE1AL	81.72	7.95	10.33
EXE1CR	84.78	1.65	13.57
EXE2	82.96	6.09	10.95
EXE2AL	76.18	5.70	18.12
EXE2CR	80.43	5.01	14.56
MCF-7/DOX			
Control	63.73	29.22	7.05
EXE1	75.86	0.07	24.07
EXE1AL	76.81	0.00	23.20
EXE1CR	76.14	0.00	23.86
EXE2	71.61	0.62	27.77
EXE2AL	71.70	5.94	22.36
EXE2CR	72.07	3.64	24.29

3.3.2. Letrozole

Letrozole also induces cell cycle arrest in the G0/G1 phase. In both cell lines, MCF-7 and MCF-7/DOX, a combination of letrozole with metalloestrogens results in non-statistically significant changes in the distribution of cells in phases of the cell cycle. In the MCF-7/DOX cell line, the combination of letrozole (10 μM) with aluminum increases the percentage of cells in the G2/M phase, but the result is not statistically significant. The results are presented in Table 2.

Table 2. The effect of letrozole and its combination with metalloestrogens on the cell cycle in MCF-7 and MCF-7/DOX cell lines. LET 1 = 10 μM; LET 2 = 100 μM; and Al/Cr(III) = 100 μM. The results are presented as mean, *n* = 3.

Sample	%G1 (G0/G1)	%S	%G2 (G2/M)
MCF-7			
Control	73.19	24.03	2.78
LET1	83.00	12.70	4.30
LET1AL	85.48	13.14	1.38
LET1CR	82.03	13.54	4.43
LET2	81.68	14.02	4.30
LET2AL	82.09	17.46	0.45
LET2CR	86.90	13.10	0.00
MCF-7/DOX			
Control	63.73	29.22	7.05
LET1	77.05	21.54	1.41
LET1AL	74.72	18.39	6.88
LET1CR	78.62	20.30	1.08
LET2	73.74	19.79	6.48
LET2AL	77.73	13.93	8.35
LET2CR	80.23	10.47	9.30

3.4. The Effect of Aromatase Inhibitors and Their Combination with Metalloestrogens on Bcl-2/BAX Ratio

Bcl-2 and BAX concentrations were calculated per 100 µL of total protein in the sample; then the Bcl-2/BAX ratio was calculated. In the case of the MCF-7/DOX cell line, lower concentrations of BAX protein (pro-apoptotic protein) and similar (to MCF-7 cell line) concentrations of Bcl-2 protein (anti-apoptotic protein) were observed, resulting in higher Bcl-2/BAX ratios. An increase in the Bcl-2/BAX ratio indicates a reduced susceptibility of cells to apoptosis, and thus a reduction in the effectiveness of the aromatase inhibitor.

3.4.1. The Effect of the Combination of Exemestane and Metalloestrogens on the Bcl-2/BAX Ratio

In both lines, the combination of a lower concentration of exemestane (100 µM) with metalloestrogens resulted in a statistically significant increase in the Bcl-2/BAX ratio. In the case of a higher concentration of exemestane (200 µM), a statistically significant increase in the Bcl-2/BAX ratio was observed only in the MCF-7/DOX line. The results are shown in Figure 6 and Table 3.

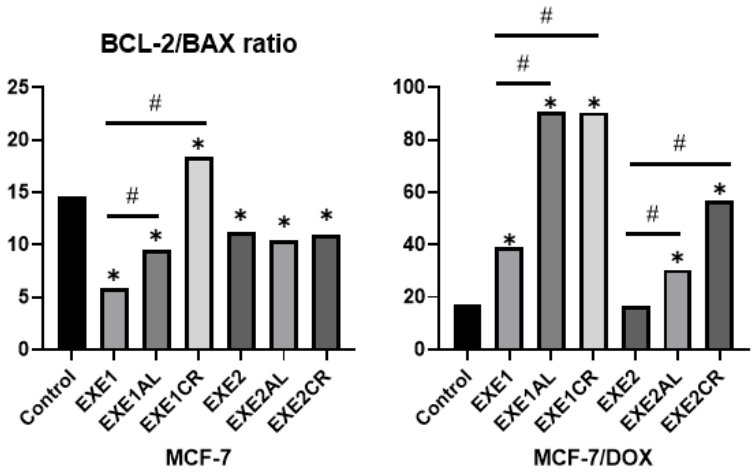

Figure 6. The effect of exemestane and its combination with metalloestrogens in the Bcl-2/BAX ratio. EXE1 = 100 µM; EXE2 = 200 µM; and Al/Cr(III) = 100 µM. The results are presented as mean, $n = 3$; $p < 0.05$; * statistically significant difference from control; # statistically significant difference from aromatase inhibitor alone.

Table 3. Bcl-2/BAX ratio values in MCF-7 and MCF-7/DOX cell lines after exposure to exemestane alone or in combination with metalloestrogens. The results are presented as mean, $n = 3$; $p < 0.05$.

Sample	Bcl-2/BAX Ratio	*p*
MCF-7		
Control	14.59	
EXE1	5.88	
EXE1AL	9.47	0.0084 * [1]
EXE1CR	18.44	<0.0001 * [1]
EXE2	11.22	
EXE2AL	10.46	0.9609 [2]
EXE2CR	10.99	>0.9999 [2]
MCF-7/DOX		
Control	17.28	
EXE1	39.15	
EXE1AL	90.72	<0.0001 * [1]
EXE1CR	90.42	<0.0001 * [1]
EXE2	16.70	
EXE2AL	30.48	<0.0001 * [2]
EXE2CR	56.90	<0.0001 * [2]

[1] vs. EXE1; [2] vs. EXE2; * statistically significant result.

3.4.2. The Effect of the Combination of Letrozole and Metalloestrogens on the Bcl-2/BAX Ratio

Incubation of MCF-7 cells with the combination of letrozole (at both concentrations) and metalloestrogens did not result in a statistically significant increase in the Bcl-2/BAX ratio, while in MCF-7/DOX cells, the combination of higher concentrations of letrozole with metalloestrogens increased it. The results are shown in Figure 7 and Table 4.

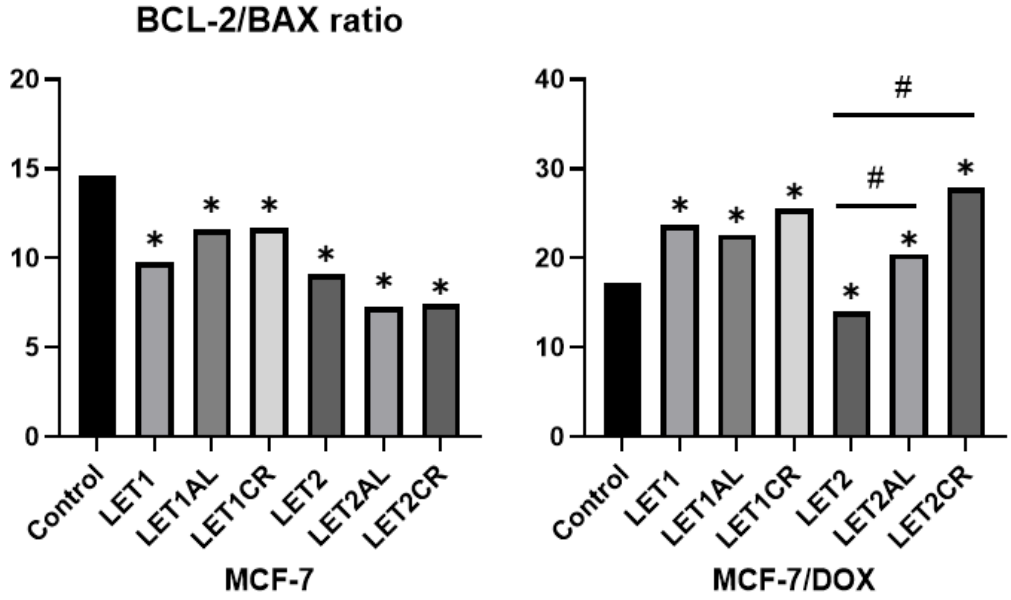

Figure 7. Effect of letrozole and its combination with metalloestrogens in the Bcl-2/BAX ratio. LET1 = 10 μM; LET2 = 100 μM; and Al/Cr(III) = 100 μM. The results are presented as mean, $n = 3$; $p < 0.05$; * statistically significant difference from control; # statistically significant difference from aromatase inhibitor alone.

Table 4. Bcl-2/BAX ratio values in MCF-7 and MCF-7/DOX cell lines after exposure to letrozole alone or in combination with metalloestrogens. The results are presented as mean, n = 3; $p < 0.05$.

Sample	Bcl-2/BAX Ratio	*p*
MCF-7		
Control	14.59	
LET1	9.76	
LET1AL	11.58	0.3400 [1]
LET1CR	11.65	0.3020 [1]
LET2	9.09	
LET2AL	7.27	0.3400 [2]
LET2CR	7.44	0.4440 [2]
MCF-7/DOX		
Control	17.28	
LET1	23.71	
LET1AL	22.66	0.8474 [1]
LET1CR	25.59	0.3073 [1]
LET2	13.97	
LET2AL	20.44	<0.0001 * [2]
LET2CR	27.87	<0.0001 * [2]

[1] vs. LET1; [2] vs. LET2; * statistically significant result.

4. Discussion

Hormone-dependent breast cancer is the most commonly diagnosed subtype, especially in postmenopausal women [4]. Most patients with hormone-dependent breast cancer,

except for hormone therapy, also receive preoperative or postoperative chemotherapy based on the sequential use of multi-drug regimens based on cytostatics—anthracyclines (e.g., doxorubicin and epirubicin) and taxoids (e.g., docetaxel and paclitaxel). Doxorubicin is currently the most effective and popular chemotherapeutic drug used to treat breast cancer. Unfortunately, resistance to this agent is common, representing a major obstacle to successful treatment [31]. In our study, we performed tests on two cell lines: MCF-7 and MCF-7/DOX, lines resistant to doxorubicin, because patients using hormone therapy often had prior chemotherapy which could lead to the selection of cancer cells resistant to doxorubicin. Therefore, we want to compare the response of both cell lines to the aromatase inhibitors, metalloestrogens, and their combinations. Studies by Devajaran et al. Indicate that MCF-7/DOX cells were exquisitely sensitive to apoptotic stimuli. Thus, treatment with staurosporine (50 nM for 48 h) consistently exerted higher cytotoxicity against MCF-7/DOX cells when compared to the MCF-7 cells under similar conditions, indicating a differentiated response between lines to the same stimuli [32]. We confirmed that the MCF-7 and MCF-7/DOX cell lines reacted differently to the same stimuli; however, in our studies it was the MCF-7/DOX cells that were less susceptible to the cytotoxic effects of drugs (as well as their combination with metalloestrogens) and to apoptosis. It was manifested, among others, by lower concentrations of the BAX protein and an increased Bcl-2/BAX ratio, which indicates a lower susceptibility to apoptosis [33]. At the same time, MCF-7/DOX cells were less susceptible to the stimulating effect of metalloestrogens.

In this study, we attempted to assess whether there is an interaction between metalloestrogens: aluminum, chromium (III), and aromatase inhibitors (exemestane and letrozole) that reduces the effectiveness of the drugs. To the best of our knowledge, similar issues have not been studied so far, so we can only refer to the effects of single substances or their combinations with other xenoestrogens.

The results of this study showed that a combination of metalloestrogens with exemestane leads to a reduction in its cytotoxicity. In the MCF-7 cell line, a statistically significant decrease in the activity of the drug was visible in the combination of chromium (III) with a lower concentration of exemestane (100 μM), while in the MCF-7/DOX cell line with a the combination of both chromium (III) and aluminum with a higher concentration of exemestane (200 μM). Letrozole had a lower cytotoxic effect than exemestane, but in the case of letrozole, we observed a decrease in drug activity after the addition of metalloestrogens. In the MCF-7 cell line, a decrease in letrozole activity was observed when combining a higher concentration with chromium (III); while in the MCF-7/DOX cell line, a decrease in drug activity was observed in all analyzed combinations. We have identified several studies that investigated the effects of xenoestrogens (from the phytoestrogen group) on the effectiveness of aromatase inhibitors. Ju et al. Studied the effect of genistein on therapy with an aromatase inhibitor, letrozole, as an animal model. While letrozole was effective in inhibiting tumor growth, adding genistein to the mice's diet reversed this effect [19]. Van Duursen et al. Studied the effect of genistein and 8-prenylnarigenin alone, as well as four multicomponent dietary supplements on the effectiveness of letrozole. Genistein and 8-prenylnarigenin, as well as all tested supplements containing various combinations of phytoestrogens, have been shown to activate an estrogen-dependent increase in MCF-7 cell proliferation that was not inhibited by letrozole [34]. Warth et al. Studied the influence of dietary xenoestrogens (zearalenone and genistein) on the effectiveness of treatment with letrozole and palbociclib. An in vitro study on breast cancer cell lines showed that the combination of letrozole and palbociclib effectively inhibited tumor cell proliferation, but the addition of both genistein and zearalenone counteracted this effect [25]. Different results, i.e., lack of interaction between formestane, a second-generation steroid aromatase inhibitor (currently no longer used in the treatment of breast cancer), and phytoestrogens derived from the root bug extract, were shown in the animal model by Nißlein et al. [35].

Apoptosis is the programmed cell death that occurs in response to various environmental stimuli and can be induced through intrinsic and extrinsic pathways. The intrinsic pathway is regulated by the Bcl-2 family of proteins—a balance between pro-apoptotic

proteins, such as BAX, and anti-apoptotic proteins, e.g., Bcl-2 is a key apoptosis regulator in numerous types of cells [36,37]. Previous studies have shown that some xenoestrogens, such as estrogens, can reduce the rate of apoptosis, e.g., bisphenol A (BPA) via increasing the Bcl-2/BAX ratio and di(2-ethylhexyl) phthalate (DEHP) via activating the Akt and NF-κB pathways [14,38,39]. Most drugs used in anticancer therapy, including aromatase inhibitors, lead to the elimination of cancer cells through apoptosis and cell cycle dysregulation [40,41]. Exemestane (a steroidal aromatase inhibitor) decreased MCF-7 cell proliferation, induced cell cycle arrest at G0/G1 and G2/M, as well as apoptosis through the mitochondrial pathway and cytoprotective autophagy [42]. Letrozole (a non-steroidal aromatase inhibitor) also inhibited the growth of breast cancer cells by induced cell cycle arrest at the G0/G1 phase (but without G2/M arrest) and apoptosis by the intrinsic pathway, which was associated, among others, with the decreased Bcl-2 protein expression and the increased BAX protein expression. Non-steroidal aromatase inhibitors (except letrozole and anastrozole), unlike exemestane, did not induce autophagy [41,43].

Our research showed that exemestane induces apoptosis in a dose-dependent manner and this effect is more potent in MCF-7 cells than in MCF-7/DOX cells. In MCF-7 cells, this is associated with a significant decrease in the Bcl-2/BAX ratio. Any agent that decreased the Bcl-2/BAX ratio may promote apoptosis [44]. In MCF-7 cells, the addition of both metalloestrogens, aluminum or chromium (III), to exemestane (100 μM) reduces the percentage of cells undergoing apoptosis. According to our research, responsible for this effect is an increase in the Bcl-2/BAX ratio, mainly related to a decrease in the concentration of the pro-apoptotic protein BAX. Similar relationships are not observed at high concentrations of exemestane (200 μM), whose activity is high regardless of the combination with metalloestrogens, which also had no effect on lowering the Bcl-2/BAX ratio. We obtained similar results in the MCF-7/DOX cell line, where a combination of a lower concentration of exemestane with metalloestrogens resulted in a decrease in the number of cells undergoing apoptosis, followed by an increased Bcl-2/BAX ratio. In the case of high concentrations of exemestane, we did not observe such a relationship. The effect of letrozole, a non-steroidal aromatase inhibitor, also leads to apoptosis of hormone-dependent breast cancer cells and, as in the case of exemestane, this effect is stronger in MCF-7 cells than in MCF-7/DOX, which may be explained by lower concentrations of the pro-apoptotic protein BAX in the MCF-7/DOX cell line (a higher Bcl-2/BAX ratio). Interestingly, we observed that the combination of letrozole and metalloestrogens resulted in a lower negative effect on apoptosis than in exemestane, and a decrease in the number of apoptotic cells was significant only in the MCF-7 line when letrozole (100 μM) was combined with aluminum or chromium (III).

To study the anti-proliferative effects induced by Ais and their combination with metalloestrogens, cell cycle progression was evaluated by flow cytometry. Estrogens induce cell proliferation by stimulating progression through the G0/G1 phase of the cell cycle. We confirmed that both Ais (steroidal and non-steroidal), by blocking the effects of estrogens action, arrest the cell cycle in phase G0/G1 (exemestane and in G2/M), which is in line with what has been previously observed by other authors [41–43]. However, we did not observe that the simultaneous exposure to metalloestrogens and aromatase inhibitors changed the distribution of cells in the phases of the cell cycle. This means that the reduction in the activity of aromatase inhibitors in combination with metalloestrogens is not related to the influence on the cell cycle.

5. Conclusions

The widespread exposure to xenoestrogens and the constantly increasing number of cases of BC lead to more and more detailed studies of the influence of EDC, not only on the carcinogenesis process, but also on the effectiveness of drugs used in the treatment of breast cancer. In the present study, we aimed to evaluate whether exposure to metalloestrogens commonly present in everyday human life, aluminum and chromium (III), may reduce the effectiveness of aromatase inhibitors used in hormone therapy of breast cancer.

We have shown that the MCF-7 and MCF-7/DOX cell lines reacted differently to the same stimuli, and the MCF-7/DOX cells were less susceptible to the cytotoxic effects of the drugs (as well as their combination with metalloestrogens), and to apoptosis. In MCF-7 cells, the lower concentration of exemestane and higher of letrozole, in combination with metalloestrogens, results in a decrease in the effectiveness of drugs (increases cell viability and reduces apoptosis). Additionally, in the MCF-7/DOX cell line, we observed that the combination of metalloestrogens and aromatase inhibitors led to a decrease in the drug's effectiveness due to an increase in the viability of breast cancer cells (both concentrations of letrozole and higher concentrations of exemestane). However, in the case of the MCF-7/DOX cell line, the regulation of apoptosis was less likely to be responsible for this effect than in the case of MCF-7 cells. In both cell lines, the reduction in the effectiveness of aromatase inhibitors, in combination with metalloestrogens, is not related to the influence on the cell cycle.

Our results indicate that exposure to metalloestrogens may negatively affect the effectiveness of hormone therapy with aromatase inhibitors. They also show that this is a complex issue. Therefore, further research is needed to fully explain these interactions and be able to effectively counteract them in the treatment of patients with hormone-dependent breast cancer.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cancers15020457/s1, Figure S1: The effect of individual metalloestrogens and aromatase inhibitors on MCF-7 cell viability. Figure S2: The effect of individual metalloestrogens and aromatase inhibitors on MCF-7/DOX cell viability. Figure S3: Gating strategy for flow cytometry analysis of apoptosis and necrosis assay. Figure S4: Representative cytogram for MCF-7 cell control. Figure S5: Representative cytogram for MCF-7/DOX cell control.

Author Contributions: Conceptualization, A.P., K.B. and E.S.; methodology, H.M., A.S. and K.B.; software, K.B. and H.M.; validation, A.S., H.M. and K.B.; formal analysis, K.B.; investigation, K.B., A.S.; resources, H.M., A.S. and K.B.; data curation, K.B. and H.M.; writing—original draft preparation, K.B.; writing—review and editing, A.P., H.M. and E.S.; visualization, K.B.; supervision, A.P.; project administration, K.B.; funding acquisition, A.P., E.S. and K.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data are available in the article and Supplementary Materials. We will willingly share our knowledge, protocol, and expertise when asked.

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

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SUPPLEMENTARY MATERIALS for:

Article

Effect of metalloestrogens on the effectiveness of aromatase inhibitors in a hormone-dependent breast cancer cell model.

Kamila Boszkiewicz, Helena Moreira, Ewa Sawicka, Anna Szyjka and Agnieszka Piwowar

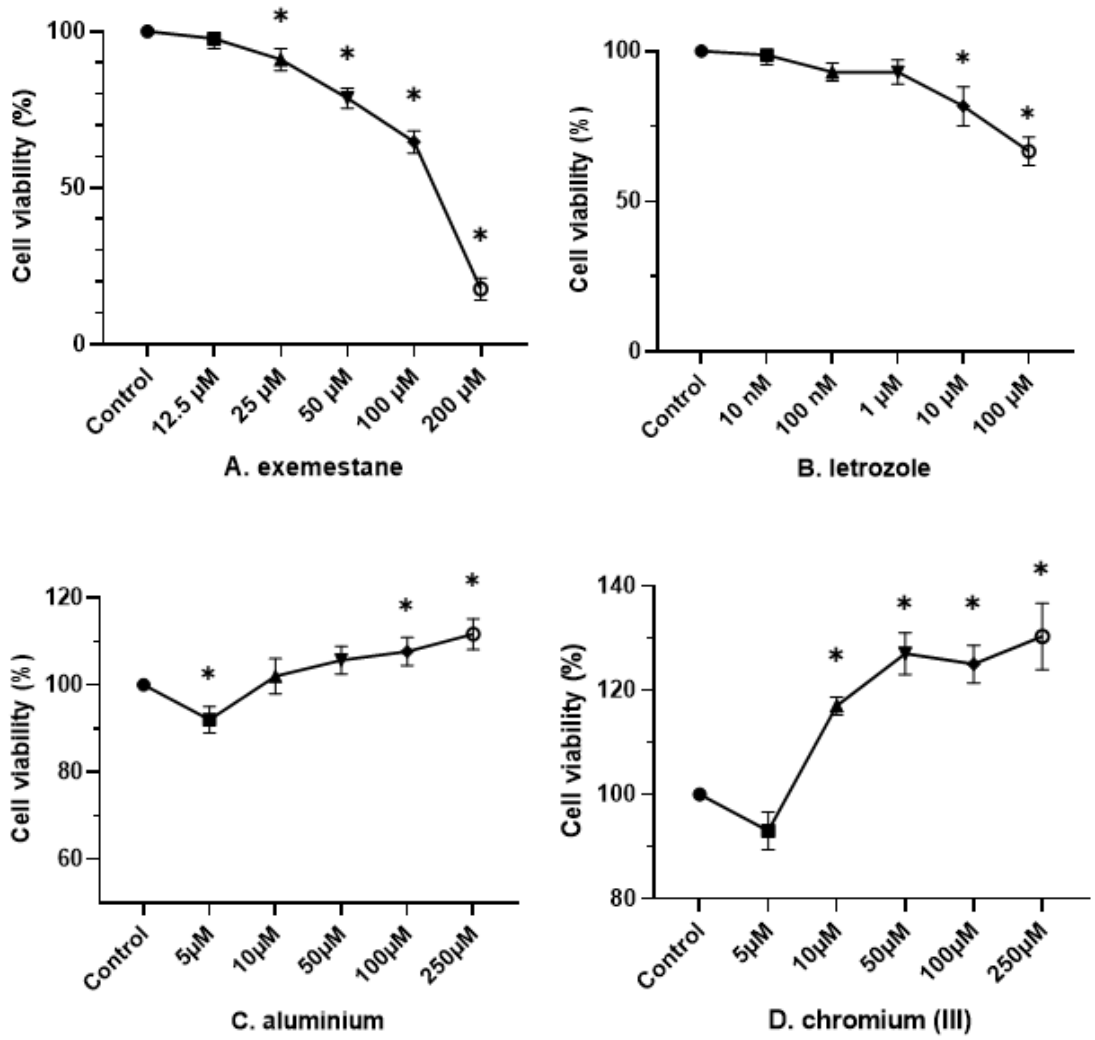

Figure S1. Effect of individual metalloestrogens and aromatase inhibitors on MCF-7 cells viability. The viability of MCF-7 exposed for 72 h for: A. exemestane (12.5-200 μM); B. letrozole (10 nM-100 μM); C. aluminium (5-250 μM) and D. chromium (III) (5-250 μM). The results are presented as mean ± SD, n = 3; $p<0.05$; * statistically significant difference from control.

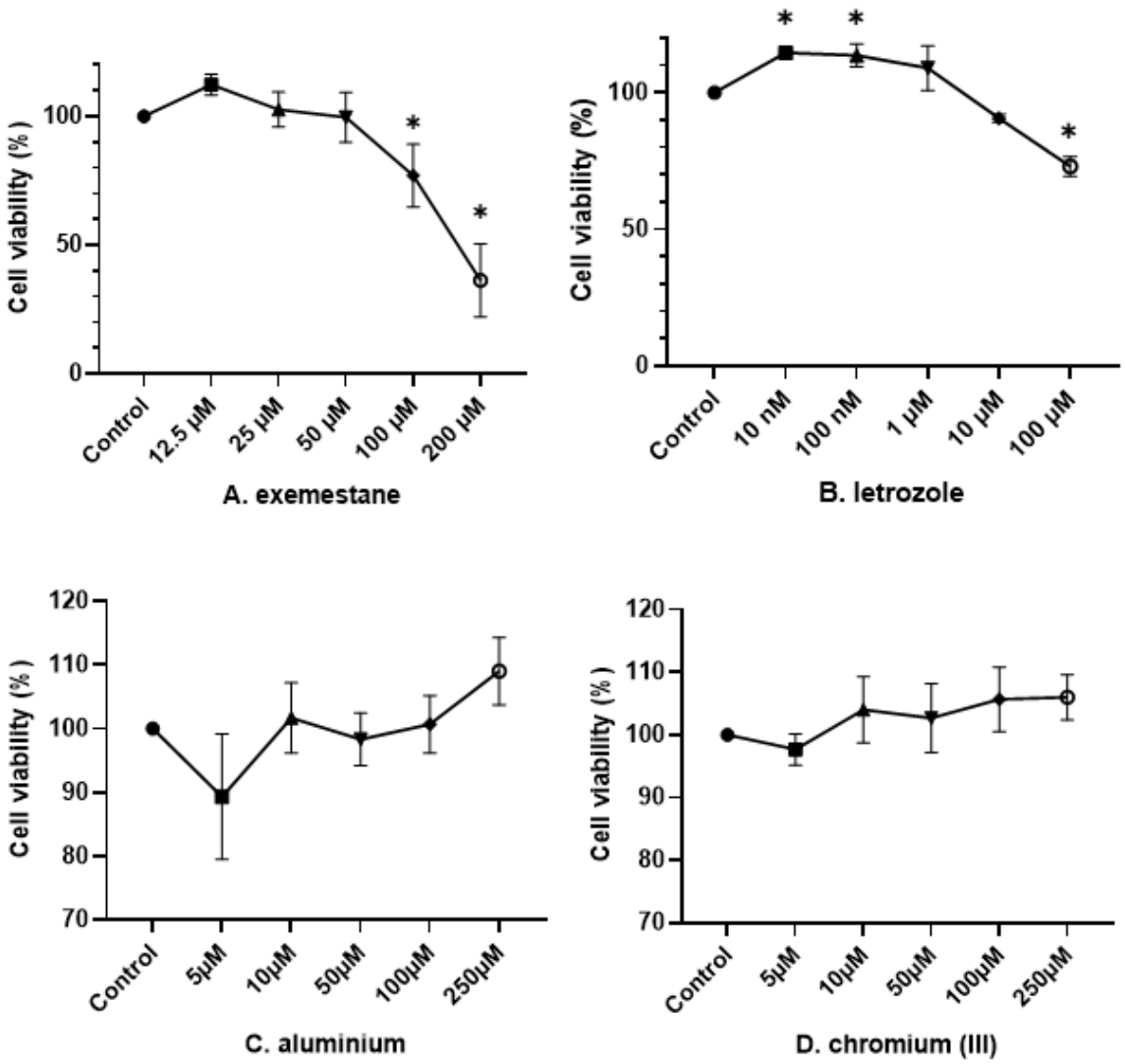

Figure S2. Effect of individual metalloestrogens and aromatase inhibitors on MCF-7/DOX cells viability. The viability of MCF-7/DOX exposed for 72 h for: A. exemestane (12.5-200 μM); B. letrozole (10 nM-100 μM); C. aluminium (5-250 μM) and D. chromium (III) (5-250 μM). The results are presented as mean ± SD, n = 3; $p<0.05$; * statistically significant difference from control.

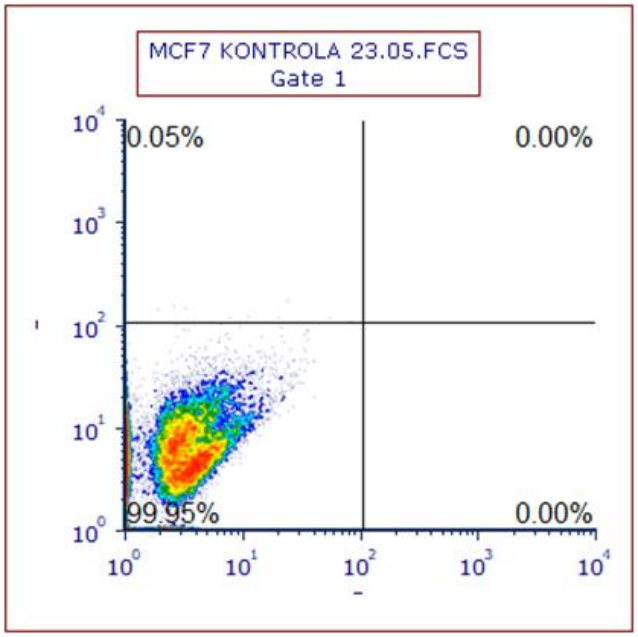

Figure S3. Representative cytogram for MCF-7 cells control.

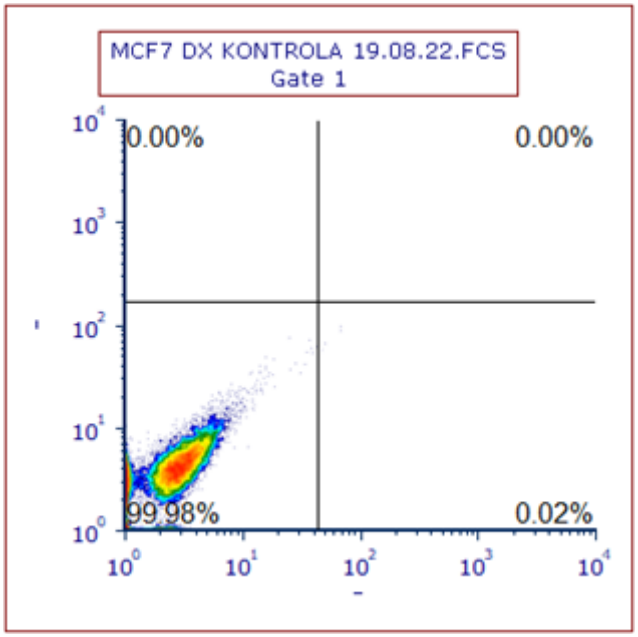

Figure S4. Representative cytogram for MCF-7/DOX cells control.

ZAŁĄCZNIK 4

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Drug Research

Decision: **accept without changes**

June 14, 2023
APPDR-01453-2023-02
High glucose reduces anti-tumor activity of aromatase inhibitors in a hormone-dependent breast cancer cell model.

Dear Kamila Boszkiewicz,

I am pleased to inform you that your manuscript, entitled: High glucose reduces anti-tumor activity of aromatase inhibitors in a hormone-dependent breast cancer cell model., has been accepted for publication in our journal.

Thank you for submitting your work to us.

Kind regards,
Michał Tomczyk
Editor-in-Chief
Acta Poloniae Pharmaceutica - Drug Research

ACTA POLONIAE PHARMACEUTICA
Drug Research

Original article

High glucose reduces anti-tumor activity of aromatase inhibitors in a hormone-dependent breast cancer cell model.

Keywords
breast cancer, diabetes, hyperglycemia, estrogens, aromatase inhibitors, metalloestrogens

Abstract
One third of breast cancer patients also suffer from diabetes, which is associated with a 40% higher risk of mortality. Patients undergoing anti-cancer treatment are also exposed to xenoestrogens present in everyday life, which may affect the effectiveness of the therapies used. The purpose of our study was to analyzed the effect of metalloestrogens (Al, Cr[III]) on the effectiveness of aromatase inhibitors (AIs) under high glucose conditions prevailing in the cellular model. On two human breast cancer cell lines—MCF-7 and MCF-7/DOX—a cell viability assay, a flow cytometer analysis of apoptosis, and protein activity of BAX, Bcl-2, and VEGF-A by ELISA were carried out. Results were analyzed using one-way ANOVA, followed post hoc by Tukey's multiple comparisons tests. High glucose conditions reduced the effectiveness of AIs in both cell lines (decreased cytotoxicity, inhibition of apoptosis, increase in Bcl-2/BAX ratio and angiogenesis), regardless of the combination with metalloestrogens. Hyperglycemia may affect the effectiveness of AIs to a greater extent than metalloestrogens alone - the activity of drugs in the presence of high glucose concentrations was significantly lower, regardless of the combination with metalloestrogens, which indicates the key role of hyperglycemia in attenuating their activity. Therefore controlling hyperglycemia and individualized treatment regimens in cancer patients with diabetes may have important therapeutic implications.

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Explanation letter
We send an explanation letter as an attachment.

Response to Reviewer 1 Comments_31.05.23.odt

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HIGH GLUCOSE REDUCES ANTI-TUMOR ACTIVITY OF AROMATASE INHIBITORS IN A HORMONE-DEPENDENT BREAST CANCER CELL MODEL.

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Abstract: One third of breast cancer patients also suffer from diabetes, which is associated with a 40% higher risk of mortality. Patients undergoing anti-cancer treatment are also exposed to xenoestrogens present in everyday life, which may affect the effectiveness of the therapies used. The purpose of our study was to analyzed the effect of metalloestrogens (Al, Cr[III]) on the effectiveness of aromatase inhibitors (AIs) under high glucose conditions prevailing in the cellular model. On two human breast cancer cell lines—MCF-7 and MCF-7/DOX—a cell viability assay, a flow cytometer analysis of apoptosis, and protein activity of BAX, Bcl-2, and VEGF-A by ELISA were carried out. Results were analyzed using one-way ANOVA, followed post hoc by Tukey's multiple comparisons tests. High glucose conditions reduced the effectiveness of AIs in both cell lines (decreased cytotoxicity, inhibition of apoptosis, increase in Bcl-2/BAX ratio and angiogenesis), regardless of the combination with metalloestrogens. Hyperglycemia may affect the effectiveness of AIs to a greater extent than metalloestrogens alone - the activity of drugs in the presence of high glucose concentrations was significantly lower, regardless of the combination with metalloestrogens, which indicates the key role of hyperglycemia in attenuating their activity. Therefore controlling hyperglycemia and individualized treatment regimens in cancer patients with diabetes may have important therapeutic implications.

Keywords: breast cancer; diabetes; hyperglycemia; aromatase inhibitors; estrogens; metalloestrogens

1. Introduction

Breast cancer is still one of the most frequently diagnosed malignancies in women. About 1 in 8 of US women is going to develop invasive breast cancer in the course of their life and about 1 in 39 will die from breast cancer [1]. Most patients with breast cancer require, in addition to surgical treatment, adjuvant treatment including radiotherapy, chemotherapy or hormonal therapy. Endocrine therapy is used in the treatment of hormone-dependent breast cancer, which accounts for approximately 75% of all cases. Of all adjuvant treatment methods, hormone therapy has been used by patients for the longest time – according to the guidelines from 5 to 10 years, which makes the safety profile of drugs used in endocrine therapy extremely important for maintaining adherence and well-being of patients during treatment [2-4]. The purpose of hormone therapy is to eliminate the stimulating effect of estrogens on cancer cells, and the most commonly used drugs are aromatase inhibitors (anastrozole, letrozole, exemestane) and tamoxifen. Aromatase inhibitors work by inhibiting the activity of the aromatase enzyme, which is responsible for the conversion of androgens to estrogens [5].

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Tamoxifen, a selective estrogen receptor modulator (SERM), selectively binds to estrogen receptors, producing both estrogenic and anti-estrogen effects depending on the binding site. In the breast tissue, it antagonistically competes with estrogen for binding sites, which results in antiestrogenic and antitumor effects [6].

Despite effectiveness of tamoxifen and aromatase inhibitors, it has also been shown that women using endocrine therapy were significantly more likely to develop among others cardiovascular diseases, insulin resistance and diabetes [7-11]. Estrogens modulate insulin resistance and may exert a direct protective effect against various injuries on the pancreatic β-cell islets that produce insulin. Both insulin resistance and pancreatic β-cell dysfunction are central disorders in the pathogenesis of diabetes. Endocrine treatment with tamoxifen or aromatase inhibitors, which block estrogen action or production, can disrupt the estrogen-insulin interplay and elevate the risk of diabetes [12]. Hamood et al. showed that the use of hormone therapy is associated with an increased risk of diabetes – of 2246 breast cancer survivors, 324 developed diabetes over a mean follow-up of 5.9 years - they found the hazard for aromatase inhibitor use (HR = 4.27; 95% CI 1.42–12.84; p = 0.010) being higher than for the use of tamoxifen (HR = 2.25; 95% CI 1.19–4.26; p = 0.013) [10]. Ye et al. also showed that endocrine therapy significantly increased the risk of developing diabetes mellitus [13]. In clinical trials, hyperglycemia was a frequently reported side effect during endocrine therapy - in the SUCCESS C study, hyperglycemia occurred in 28% of patients using tamoxifen and aromatase inhibitor in sequential hormone therapy, and in 22% of patients treated with exemestane, while in a study conducted by Iwata et al., proportion of patients with hyperglycemia was 51.4% in the exemestane group and 47.7% in the anastrozole group [14,15].

It is estimated that up to one third of breast cancer patients also suffer from diabetes [16]. Diabetes is called the epidemic of the 21st century - according to the data of the International Diabetes Federation, in 2021 approximately 537 million adults lived with diabetes in the world. This number is projected to increase to 643 million by 2030 and 783 million by 2045 [17]. Diabetes is a chronic metabolic disease characterized by hyperglycemia, which can lead to serious damage to many organs, especially the kidneys, heart, as well as blood vessels and nerves [18]. Diabetes has been linked to an increased risk of developing many types of cancers including breast cancer [19-23]. Hyperglycemia can affect cancer progression in many ways – metabolic reprogramming and molecular alterations; increasing proliferation and apoptosis inhibition as well as metastasis. Cancer cells has ability to reprogram metabolism, including glucose metabolism. They use much more glucose than normal cells - the enhanced glucose metabolism in cancer cells is referred to as the Warburg effect, which is defines as an increase in the rate of aerobic glycolysis and preferential production of lactate, even in the presence of oxygen [24,25]. Data from many studies strongly implicate that hyperglycemia, as an additional fuel source, leading to enhanced cell proliferation [20]. In normal cells, high glucose concentrations induced apoptosis; however, in cancer, hyperglycemia protects cancer cells from apoptosis. In addition, studies shown that hyperglycemic conditions could enhance the migration of cells, what result that cancer patients with hyperglycemia have a higher proportion of metastasis [23].

Diabetes and hyperglycemia not only increases the risk of developing breast cancer, but are also associated with a 40% higher risk of mortality after breast cancer than women without diabetes [16]. One of the possible explanation for the increased mortality in patients with diabetes, is that treatment is less effective in a hyperglycemic tumor environment. Gerards et

al. studied the impact of hyperglycemia on chemotherapy efficacy in systematic review of preclinical studies. In most studies, hyperglycemia was associated with the attenuation of chemotherapy efficacy (decreasing the antiproliferative effect, interference with apoptotic signaling). In breast cancer, hyperglycemia attenuated efficacy of treatment in estrogen receptor (ER) positive, but not in ER negative breast cancer cells [26].

We recently discovered and published that exposure to metalloestrogens, which are present in our everyday life (aluminum, chromium [III]), under normoglycemic conditions (NG, glucose concentration 5.5 mM), can reduce the efficacy of aromatase inhibitor-based hormone therapy by increasing cell viability and resistance to apoptosis [27]. Data from the literature indicate that glucose concentration plays an important role in many processes related to the pathogenesis, course and treatment of breast cancer, so in the same time - we also conduct our research under conditions reflecting hyperglycemia - HG (glucose concentration 25 mM). Considering that the majority of patients using hormone therapy with aromatase inhibitors are postmenopausal patients, and the menopause itself predisposes to a higher incidence of glycemic disorders, but also that endocrine therapy alone can cause hyperglycemia, it seems that taking into account the factor related to the glucose conditions in cell lines studies may provide additional, valuable information. In our experiment we use, except MCF-7 cell lines, also cell line resistant to doxorubicin – MCF-7/DOX, because patients, before hormonotherapy, frequently underwent prior chemotherapy, which may have contributed to the selection of cancer cells resistant to doxorubicin. Our previous research has shown that these cancer cell lines have distinct responses to apoptotic stimuli [27].

The purpose of this study, which was carried out on two estrogen-dependent breast cancer cell lines - MCF-7 and MCF-7/DOX (doxorubicin-resistant cell lines), was to analyzed the effect of metalloestrogens (aluminum, chromium [III]), commonly found in the human environment, on the effectiveness of aromatase inhibitors (exemestane, letrozole) under high glucose conditions prevailing in the cellular model. Mutual interactions of metalloestrogens, aromatase inhibitors and hyperglycemia have not been analyzed so far, which means that the presented research has an innovative value.

2. Experimental.

2.1. Materials.

DMEM (Dulbecco's Modified Eagle's Medium) high-glucose/low-glucose, without phenol red, FBS (fetal bovine serum), Penicillin-Streptomycin (10x) Solution, PBS (Phosphate-Buffered Saline) were obtained from Biological Industries, Israel. Exemestane, letrozole, chromium (III) chloride hexahydrate, aluminum chloride hydrate, testosterone, DMSO (dimethyl sulfoxide) and deionized water were provided by Sigma Aldrich, USA. Annexin V-FITC Apoptosis Kit were obtained from Invitrogen, USA. Cell Proliferation Kit II (XTT) was from Roche Diagnostics, Germany. Halt™ Protease Inhibitor Coctail (100x) and Pierce™ BCA Protein Assay Kit were obtained from Thermo Fisher Scientific, USA. TrypLE™ Express and GlutaMAX™ were from Gibco, USA. Accutase™ Cell Detachment was purchased from BD Biosciences, USA. Genorise Scientific, USA, provided the ELISA lysis buffer, Nori Human Apoptosis Regulator BAX ELISA Kit, Nori Human Bcl-2 ELISA Kit and Nori Human VEGF-A ELISA Kit.

2.2. Methods.

2.2.1. Cell culture.

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MCF-7, an estrogen-dependent breast cancer cell line, was obtained from CLS Cell Lines Service GmbH, Germany (certificate of analysis available upon request, CLS Lot No. 300273-2120). MCF-7/DOX (MCF-7 cell line with P-gp overexpression; doxorubicin resistant cell line) was derived by cultivating MCF-7 cells for three months in the presence of a low doxorubicin concentration. Cells were cultured in complete DMEM growth medium with high glucose concentration (25 mM) and with low glucose concentration (5.5 mM) – only for VEGF-A ELISA test, without phenol red, supplemented with fetal bovine serum (FBS) - 10% v/v, 2 mM L-glutamine, antibiotics streptomycin (10,000 U/mL and penicillin (10 mg/mL) and 10^{-9} M testosterone, at 37ºC in a humidified atmosphere with 5% CO2. TrypLE™ Express was used to subculture the cells twice a week.

2.2.2. Stock solutions for drugs and metalloestrogens.

Letrozole (LET) and exemestane (EXE) were dissolved in DMSO and stored at -20°C as a 100 mM stock solution. Aluminum chloride hydrate and chromium [III] chloride hexahydrate were dissolved in deionized water and stored at -20°C as a 1 mM stock solution. Before each experiment, the working solutions were freshly prepared by dilution of a stock solution in a culture medium.

2.2.3. Cell viability assay.

The XTT assay was used to determine the cytotoxicity of LET, EXE, metalloestrogens, and their combinations on MCF-7 and MCF-7/DOX cells. In a 96-well plate, 1 x 10^4 cells were seeded and treated in triplicates for 72 hours with letrozole (LET1 = 10 µM; LET2 = 100 µM), exemestane (EXE1 = 100 µM; EXE2 = 200 µM), metalloestrogens (100 µM Cr[III]/Al [III] salt) or their combinations. Control cells that had not been treated were also included. Following incubation, the XTT assay was carried out according to the manufacturer's instructions. Absorbance was measured at λ=450 nm with a reference wavelength at 650 nm using Synergy HTX Multi-Mode Microplate Reader, BioTek, USA. Untreated control served as the 100% reference.

2.2.4. Flow cytometric analysis.

The CyFlow® SPACE flow cytometer was used for all cytometric analyses (Sysmex, Japan). For FITC fluorescence measurement, the laser excitation 488 nm (50mW) and the filter 536/40 (BP) were used. The fluorescence of propidium iodide was measured using a laser excitation of 488 nm (50mW) and a filter of 675/20 (BP). FCS Express 7 Cytometry software was used to analyze all of the results (De Novo Software, USA).

2.2.5. Apoptosis and necrosis assay.

Flow cytometry was used to detect apoptosis and necrosis. The Annexin V-FITC Apoptosis Kit was used to stain the cells, which allows for the differentiation of living cells, early and late apoptotic cells, and necrotic cells. In a 6-well plate, 1 x 10^6 cells were seeded and treated for 72 hours with letrozole (LET1 = 10 µM; LET2 = 100 µM), exemestane (EXE1 = 100 µM; EXE2 = 200 µM), Cr[III]/Al [III] salt (100 µM) and their combination. A control group that had not been treated was also prepared. Following incubation, the cells were detached with Accutase™ Cell Detachment and washed in cold PBS. The cells were resuspended in 100 µL 1 x annexin-binding buffer before being stained with 5 µL of FITC annexin V and 1 µL of PI. Following a 15-minute incubation in the dark at room temperature and the addition of 400 µL 1 x annexin-binding buffer, samples were immediately analyzed with a flow cytometer.

2.2.6. Preparation of cell lysates.

In brief, 1 x 10^6 cells were seeded per T-25 flask and treated for 48 hours with letrozole (LET1 = 10 µM; LET2 = 100 µM), exemestane (EXE1 = 100 µM; EXE2 = 100 µM), Cr[III]/Al [III] salt (100 µM) and their combination. A control group that had not been treated was also prepared. The cells were detached with TrypLE™ Express solution after incubation, washed with cold PBS, and transferred to a microfuge tube. The cells were centrifuged to pellet them, and any remaining buffer was removed. To the cell pellet, 0.5 ml lysis buffer supplemented with protease inhibitor cocktail was added, vortexed, and incubated for 30 minutes on ice. The samples were then centrifuged for 10 minutes at 10,000 x g. The supernatants were transferred to clean tubes and kept at -80°C for future use.

2.2.7. Determination of total protein concentration in cell lysates.

The Pierce™ BCA Protein Assay Kit was used to determine total protein concentration (Thermo Fisher Scientific, USA). The assay was carried out in accordance with the manufacturer's instructions. The absorbance was measured at λ=562 nm using Synergy HTX Multi-Mode Microplate Reader, BioTek, USA.

2.2.8. ELISA assays for Bcl-2, BAX and VEGF-A detection.

ELISA was used to detect and quantify from cell lysates: Bcl-2 and BAX - apoptotic proteins involved in the cell death pathway and VEGF-A – vascular endothelial growth factor A, which is a potent angiogenic factor. Nori Human Apoptosis Regula-tor BAX ELISA Kit, Nori Human Bcl-2 ELISA Kit and Nori Human VEGF-A ELISA Kit (Genorise Scientific, USA) were used as directed. Each sample was tested in duplicate and expressed relative to the total protein concentration in the same sample. The absorbance was measured at λ= 570 nm using Synergy HTX Multi-Mode Microplate Reader, BioTek, USA. The four-parameter logistic fitted standard curves for calculating Bcl-2, BAX, and VEGF-A concentrations were generated using the Arigo Biolaboratories website (https://www.arigobio.com/elisa-analysis). The concentrations of Bcl-2, BAX, and VEGF-A were calculated per 100 µl of total protein in the sample, and the Bcl-2/BAX ratio was then calculated.

2.2.9. Statistical analysis.

The data was analyzed with GraphPad Prism 9 (GraphPad Software, USA) using one-way ANOVA, followed by Tukey's multiple comparisons tests post hoc. We also use two-way ANOVA, followed by Tukey's multiple comparisons tests post hoc for analyzing data from VEGF-A ELISA test obtained in normoglycemia vs hyperglycemia. The mean and standard deviation of the mean were used to express the results (SD). Each experiment was carried out three times. Significant differences among means were estimated at $p<0.05$.

3. Results

In the results section, we studied the effect of monotherapy, as well as a combination of aromatase inhibitors (letrozole, exemestane) and metalloestrogens (aluminum, chromium [III]) on the studied parameters in two cell lines - MCF-7 and MCF-7/DOX in hyperglycemic conditions. To simplify the notation of results, we have used abbreviations: EXE1 = 100 µM; EXE2 = 200 µM; LET1 = 10 µM; LET2 = 100 µM; AL = 100 µM; CR = 100 µM; EXE1AL = combination of 100 µM of exemestane and 100 µM of aluminum etc. Since the experiment under high glucose conditions was conducted in the same time also in normoglycemic conditions (results have been published previously [27], except the VEGF-A results), we

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performed an additional comparison of results obtained in normoglycemia vs hyperglycemia conditions only in case of VEGF-A.

3.1. Under high glucose conditions metalloestrogens do not reduces the cytotoxicity of aromatase inhibitors.

3.1.1. MCF-7 cell line.

Results are shown in the Figure 1. Under high glucose conditions, the addition of metalloestrogens did not reduce efficacy of exemestane at both concentrations - EXE1 89% vs EXE1AL 81%, p=0.0712; vs EXE1CR 91%, p=0.9985. EXE2 39% vs EXE2AL 38%, p=0.9997; vs EXE2CR 37%, p=0.9723. Letrozole has a lower effect on MCF-7 cell viability than exemestane, but we also did not observe a decrease in its activity after the addition of metalloestrogens – LET1 85% vs LET1AL 88%, p=0.9073; vs LET1CR 93%, p=0.1513 and LET2 83% vs LET2AL 87%, p=0.8013; vs LET2CR 90%, p=0.2247.

3.1.2. MCF-7/DOX cell line.

Results are shown in the Figure 2. Both aromatase inhibitors were less cytotoxic to MCF-7/DOX cells than MCF-7 cells, regardless of the metalloestrogen combination, which showed no negative impact on their efficacy - EXE1 94% vs EXE1AL 90%, p=0.5562; vs EXE1CR 95%, p>0.9999. EXE2 81% vs EXE2AL 80%, p=0.9955; vs EXE2CR 81%, p>0.9999. Activity of letrozol under high glucose conditions was not different from control, regardless of the metalloestrogen combination.

3.2. High glucose concentration, independently of metalloestrogens, has a negative effect on the number of cells undergoing apoptosis and necrosis after exposure to an aromatase inhibi-tor.

The effect of aromatase inhibitors, metalloestrogens and their combination under high glucose conditions on apoptosis and necrosis was studied after 72 h of incubation in MCF-7 and MCF-7/DOX cells using Annexin V-FITC and PI staining. The percentages of early apoptotic (Annexin V-FITC+, PI+), late apoptotic (Annexin V-FITC+, PI+), and necrotic (Annexin VFITC,PI+) cells are shown.

3.2.1. MCF-7 cell line.

Results are shown in the Figure 3. Combination of metalloestrogens and aromatase inhibitors under high glucose conditions did not change significantly the activity of drugs –

Exemestane:

- EXE1 49.7% vs EXE1AL 54.3%, p=0.7924; vs EXE1CR 48.3%, p=0.9995
- EXE2 57% vs EXE2AL 53%, p=0.8790; vs EXE2CR 56.3%, p>0.9999.

Letrozole:

- LET1 15.63% vs LET1AL 11.67%, p=0.1384; vs LET1CR 11.33%, p=0.0928
- LET2 15.67% vs LET2AL 14%, p=0.8875; vs LET2CR 16.30%, p=0.9991.

Letrozole has a lower effect on number of cells undergoing apoptosis or necrosis.

3.2.2. MCF-7/DOX cell line.

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Results for MCF-7/DOX cell line are shown in the Figure 4. In MCF-7/DOX cell line, significantly fewer cells undergo apoptosis or necrosis under the influence of aromatase inhibitors. The differences resulting from the addition of a metalloestrogen to an aromatase inhibitor (both exemestane and letrozole) are not statistically significant, indicating that in hyperglycemic conditions, the addition of a metalloestrogen does not reduce the activity of the aromatase inhibitor (EXE1 19.24% vs EXE1AL 22.53%, p=0.9061; vs EXE1CR 24.16%, p=0.6278; EXE2 17.73% vs EXE2AL 13.81%, p=0.8179; vs EXE2CR 16.35%, p=0.9988 and LET1 16.38% vs LET1AL 13.20%, p=0.8349; vs LET1CR 13.76%, p=0.9225; LET2 20.95% vs LET2AL 14.29%, p=0.1551; vs LET2CR 14.14%, p=0.1408).

3.3. Combination of aromatase inhibitors and metalloestrogens under high glucose conditions did not change the Bcl-2/BAX ratios.

Bcl-2 and BAX concentrations were determined by ELISA after 48 h incubation with aromatase inhibitors, metalloestrogens or their combination. Concentrations were calculated per 100 µL of total protein in the sample; then the Bcl-2/BAX ratio was calculated. In the case of the MCF-7/DOX cell line, we found higher Bcl-2/BAX ratios, which corresponded to a lower susceptibility to apoptosis.

3.3.1. MCF-7 cell line.

Results for MCF-7 cell line are presented on Figure 5. In conditions of hyperglycemia - differences in Bcl-2/BAX ratio after adding metalloestrogens were not statistically significant: EXE1 20.89 vs EXE1AL 24.21, p=0.2193; vs EXE1CR 23.58, p=0.4284; EXE2 20 vs EXE2AL 18.33, p=0.8521; vs EXE2CR 19.64, p>0.9999 and LET1 16.30 vs LET1AL 15.69, p=0.9990; vs LET1CR 18.1, p=0.8070; LET2 14.9 vs LET2AL 17, p=0.6855; vs LET2CR 15.75, p=0.9936.

3.3.2. MCF-7/DOX cell line.

As shown in Figure 6, in MCF-7/DOX as in the MCF-7 cell line, we did not observed statistically significant changes in Bcl-2/BAX ratios after combine aromatase inhibitors with metalloestrogens (EXE1 36.39 vs EXE1AL 31.92, p=0.1550; vs EXE1CR 34.74, p=0.9423; EXE2 20.91 vs EXE2AL 23.62, p=0.6419; vs EXE2CR 23.92, p=0.5342 and LET1 16.89 vs LET1AL 16.87, p>0.9999; vs LET1CR 16.40, p=0.9941; LET2 14.49 vs LET2AL 15.75, p=0.6623; vs LET2CR 17.10, p=0.0518). In MCF-7/DOX we found higher Bcl-2/BAX ratios, which corresponded to a lower susceptibility this cell lines to apoptosis.

3.4. High glucose conditions increases VEGF-A concentrations.

We assessed the concentrations of VEGF-A (vascular endothelial growth factor A) - a potent angiogenic factor, in MCF-7 and MCF-7/DOX cells under normal and high glucose conditions. We have shown that in both cell lines, under hyperglycemic conditions, VEGF-A concentrations were higher, and additionally the MCF-7/DOX line (regardless of the conditions) was characterized by higher VEGF-A levels. Results are shown on Figure 7 (MCF-7 cell line) and 8 (MCF-7/DOX cell line).

3.4.1. MCF-7 cell line.

In the MCF-7 cell line, the concentration of VEGF-A decreased under the influence of both aromatase inhibitors, and the decrease in concentration was greater in normoglycemic conditions. Under normal glucose conditions, the combination of exemestane (100 µM) with

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metalloestrogens resulted in a statistically significant increase in VEGF-A concentration (EXE1 NG 148.5 pg/ml vs EXE1AL NG 331.5 pg/mL; p<0.0001/EXE1CR NG 245.5 pg /mL; p<0.0001), which was not observed in the conditions of hyperglycemia (EXE1 HG 383.5 pg/mL vs EXE1AL HG 353 pg/mL; p=0.4608/EXE1CR HG 384.5 pg/mL; p>0.9999). In the case of letrozole, it was similar - the combination of letrozole (100 µM) with metalloestrogens in normoglycemic conditions led to an increase in VEGF-A concentrations (LET2 NG 163 pg/mL vs LET2AL NG 237.5 pg/mL; p<0.0001/LET2CR NG 223 pg /mL; p=0.0006), and under high glucose conditions these difference was not statistically significant (LET2 HG 395.5 pg/mL vs LET2AL HG 421.5 pg/mL; p=0.2944/LET2CR HG 415 pg/mL; p=0.6658).

3.4.2. MCF-7/DOX cell line.

In the MCF-7/DOX line, we also observed that the concentration of VEGF-A decreased under the influence of both aromatase inhibitors. As in the MCF-7 line, under normoglycemic conditions, we observed interactions between aromatase inhibitors and metalloestrogens, which resulted in an increase in VEGF-A concentrations after adding a metalloestrogens to aromatase inhibitor (e.g. EXE1 NG 229.5 pg/mL vs EXE1AL NG 358 pg/mL; p<0.0001/EXE1CR NG 362.5 pg/mL; p<0.0001), which is not observed in conditions of hyperglycemia, where the concentration of VEGF-A was at a similar level regardless of the presence of metalloestrogens (e.g. EXE1 HG 409 pg/mL vs EXE1AL HG 393 pg/mL; p=0.9949/EXE1CR HG 418.5 pg/mL; p>0.9999).

4. Discussion

Incidence of diabetes continues to increase worldwide, and hyperglycemia (the most important feature of diabetes, defined as increased glucose level in blood) or diabetes is associated with an increased risk of developing many types of cancer, including breast cancer [19-23]. In addition breast cancer mortality is higher in diabetic patients, probably because hyperglycemia may contribute to cell proliferation, apoptosis, metastasis and chemotherapy resistance [28]. It is also worth remembering that some anti-cancer therapies can lead to hyperglycemia or diabetes [10]. Therefore, we believe that glycemic conditions should be taken into account at every stage of research on the effectiveness of anticancer therapy. In studies on cell lines, normo- and hyperglycemic conditions can be achieved by culture medium, e.g. DMEM, containing 5.5 mM (normoglycemic conditions) and 25 mM (hyperglycemic conditions) respectively, which was also used in our studies [28-31].

Hyperglycemia has been shown in vitro to stimulate cell proliferation in breast cancer cells. Sun et al. demonstrated that supplementation with 25 mM of glucose significantly promoted proliferation of both MCF-7 and MDA-MB-231 cells, in comparison with 5.5 mM glucose in the normal culture medium, which implicated a critical role of high blood glucose in the tumor biology of breast cancer. Moreover the presence of high glucose concentration in the medium significantly stimulated cell migration and invasive capacities of both cell lines [32]. Hou et al. obtained similar results - high glucose concentration (25 mM glucose) significantly increase the proliferation of breast cancer cells (MDAMB231, SKBR3 and MCF-7 cells) compared to low glucose condition (5 mM glucose) [33]. Kansestani et al. revealed that hyperglycemia increased cell proliferation, angiogenesis (increase VEGF) and decreased apoptosis (increase Bcl-2 - anti-apoptotic protein) in MCF-7 cell line. Probable mechanism, indicated by the authors, is based on increasing reactive oxygen species (ROS) which activate NF-κB directly. NF-κB, the transcription factor, has a crucial role in cancer metabolism.

Target genes for this factor are e.g. Bcl-2 (cell apoptosis mediator) and VEGF (vascular endothelial growth factor) – angiogenesis inducer [34]. Zielinska et al. found, using MCF-7 and T47D cell lines, that hyperglycemia promoted the Warburg effect, among others, by upregulating glucose uptake and lactate release [35]. On the other hand aerobic glycolysis has been shown to associate with the resistance to chemotherapeutic agents, e.g. doxorubicin or tamoxifen in ER-positive breast cancer cells [36]. Woo et al. showed that tamoxifen resistance may be driven by HIF-1α (hypoxia-inducible factor 1α), hyperactivation via modulation of Akt/mTOR and/or AMPK signaling pathways caused by increased aerobic glycolysis [37].

In our recent work we have shown that exposure to metalloestrogens may reduce the efficacy of aromatase inhibitors under normoglycemic conditions [27]. High glucose concentration reduces the cytotoxicity of aromatase inhibitors in both cell lines and this results were statistically significant (except LET1 in MCF-7 cell line). In normoglycemic conditions we have shown that the addition of a metalloestrogens to aromatase inhibitor reduces the effectiveness of the drug (e.g. combination EXE1 with chromium [III] in MCF-7 cells or both concentrations of letrozole with aluminum/chromium [III]) [27], but we did not observe this relationship in hyperglycemia. Aromatase inhibitors activity under hyperglycemic conditions was lower regardless of the combination with metalloestrogens. We also showed that, independently of metalloestrogens, high glucose conditions has a negative impact on the number of cells undergoing apoptosis and necrosis after exposure to aromatase inhibitor. In MCF-7 cell line this was accompanied by a statistically significant increase in Bcl-2/BAX ratio (due to increase of anti-apoptotic protein – Bcl-2). Studies by Vaughn et al. suggest that high glucose concentration protects cytochrome c-mediated apoptosis, however we did not test the level of cytochrome c in our studies [38].

Our findings are consistent with the observations of Wahdan-Alaswad et al. who studied the activity of metformin at various concentrations of glucose in breast cancer cell lines. There is a growing number of research showing that metformin has potent anticancer activity, particularly against breast cancer. However, Wahdan-Alaswad et al. showed that high-glucose conditions (10 mmol/L or above) significantly abrogated the effects of metformin – promotes cell growth and increases the EC50 of metformin, especially in luminal and HER2 breast cancer cell lines. They also showed that hyperglycemia inhibits metformin-induced apoptosis and cell cycle arrest [39]. Varghese et al. reported similar findings, demonstrating that high glucose (25 mM) concentrations, which mimicked diabetes, significantly reduced the effect of metformin on cell proliferation, cell death, and cell cycle arrest in triple-negative breast cancer [40]. Ambrossio et al. proved that sensitivity to tamoxifen was reduced by 2-fold by high glucose conditions compared with low glucose conditions [41].

VEGF is the most effective factor in promoting angiogenesis. Among seven isostructural forms of VEGF, VEGF-A is the strongest stimulating factor of angiogenesis. Studies shown that VEGF expression is increasing in tumors and is related to hypoxia, which promotes the activation of HIF-1α, thereby upregulating VEGF [42,43]. In our research, we confirmed that in both cell lines, under hyperglycemic conditions, VEGF-A concentrations were higher, and additionally the MCF-7/DOX line (regardless of the conditions) was characterized by higher VEGF-A levels. We also showed that the concentration of VEGF-A decreased under the influence of both aromatase inhibitors in both cell lines, and the decrease in concentration was greater in normoglycemic conditions. Under normoglycemic conditions, we also observed interactions between aromatase inhibitors and metalloestrogens, which resulted in an increase in VEGF-A concentrations after adding a metalloestrogens to aromatase inhibitor, which was

not observed in conditions of hyperglycemia, where the concentration of VEGF-A was at a similar level regardless of the presence of metalloestrogens.

Our study is not without limitations. We assessed the effect of metalloestrogens on the activity of aromatase inhibitors under conditions of hyperglycemia in short-term exposure (72 hours), while exposure to environmental metalloestrogens and/or high concentrations of glucose is usually long-term. In addition, in our research we used two-dimensional (2D) in vitro cell cultures - 2D cultures have many limitations, the most important of which is the disruption of interactions between the cellular and extracellular environment. All of this may jeopardize our results to some extent, but it also sets the course for further action. The obtained results may be the basis for further research - in vivo on an animal model or observational studies, undertaken in cooperation with a clinical center dealing with the treatment of patients with hormone-dependent breast cancer.

5. Conclusion

Our research has shown for the first time that the effectiveness of aromatase inhibitors – letrozole and exemestane, can be reduced under the influence of metalloestrogens [27] or high concentrations of glucose (decreased cytotoxicity, inhibition of apoptosis, increase in Bcl-2/BAX ratio and increase angiogenesis). We have also proved that the influence of hyperglycemia is crucial and may affect the effectiveness of drugs used in the treatment of hormone-dependent breast cancer to a greater extent than metalloestrogens alone. The constantly growing number of patients with diabetes, and thus also the increase in patients with diabetes and breast cancer, makes the treatment of breast cancer with abnormal metabolism a major clinical challenge. Controlling hyperglycemia and individualized treatment regimens in these patients may have important therapeutic implications.

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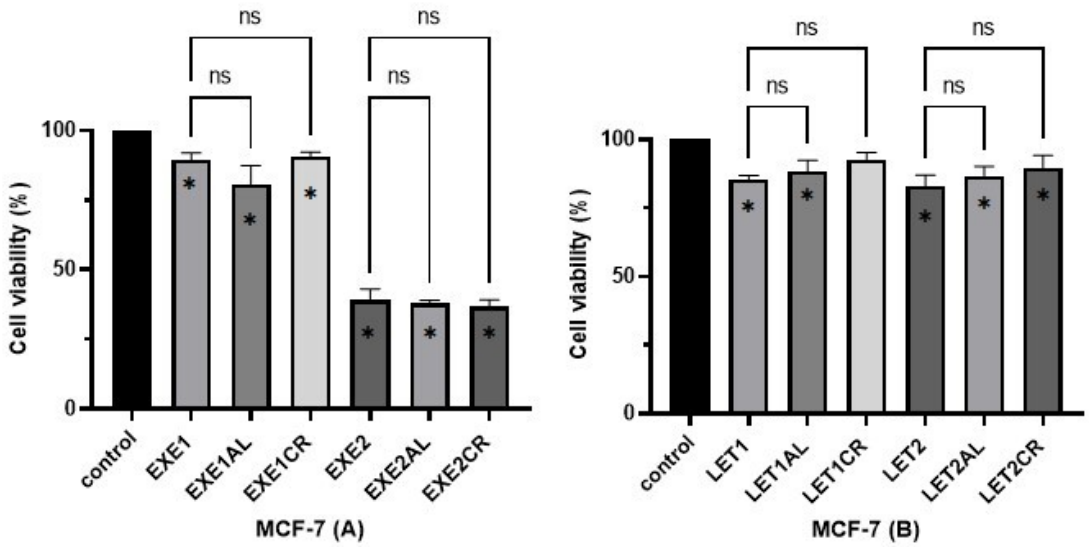

Figure 1. Effect of metalloestrogens, aromatase inhibitors and their combination on cell viability of MCF-7 cell line under high glucose (HG) conditions. The viability of MCF-7 cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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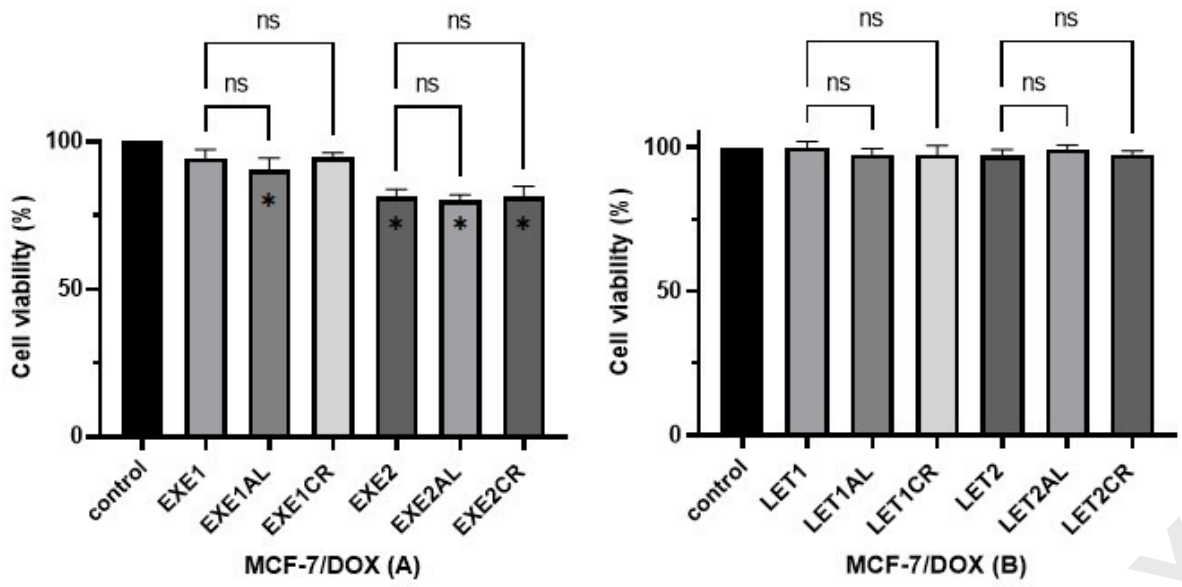

Figure 2. Effect of metalloestrogens, aromatase inhibitors and their combination on cell viability of MCF-7/DOX cell line under high glucose (HG) conditions. The viability of MCF-7/DOX cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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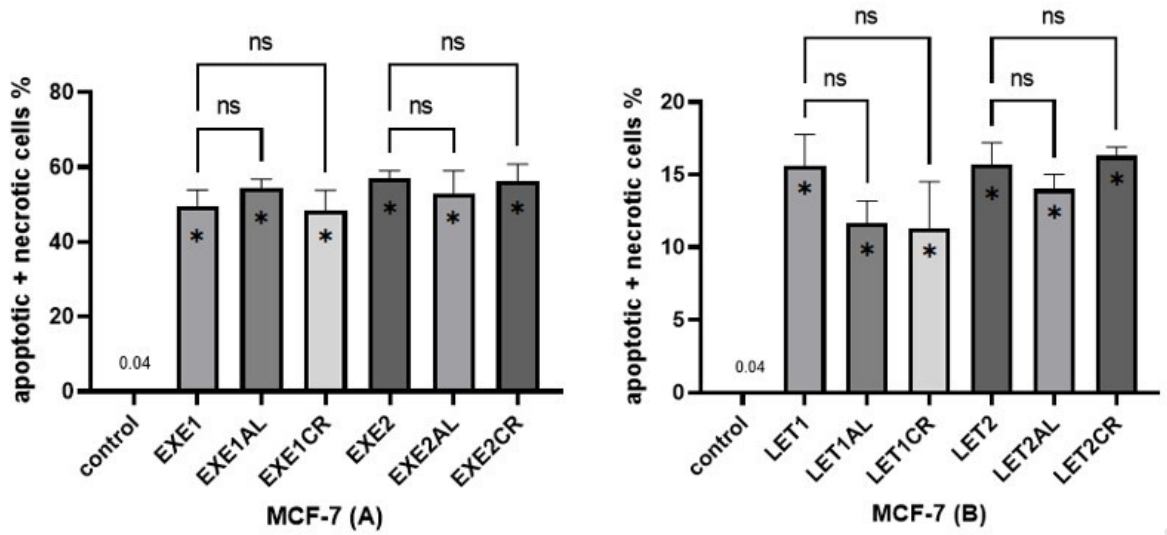

Figure 3. Effect of metalloestrogens, aromatase inhibitors and their combination on MCF-7 cell death under high glucose (HG) conditions. Percentage of both apoptotic (early and late apoptotic) and necrotic cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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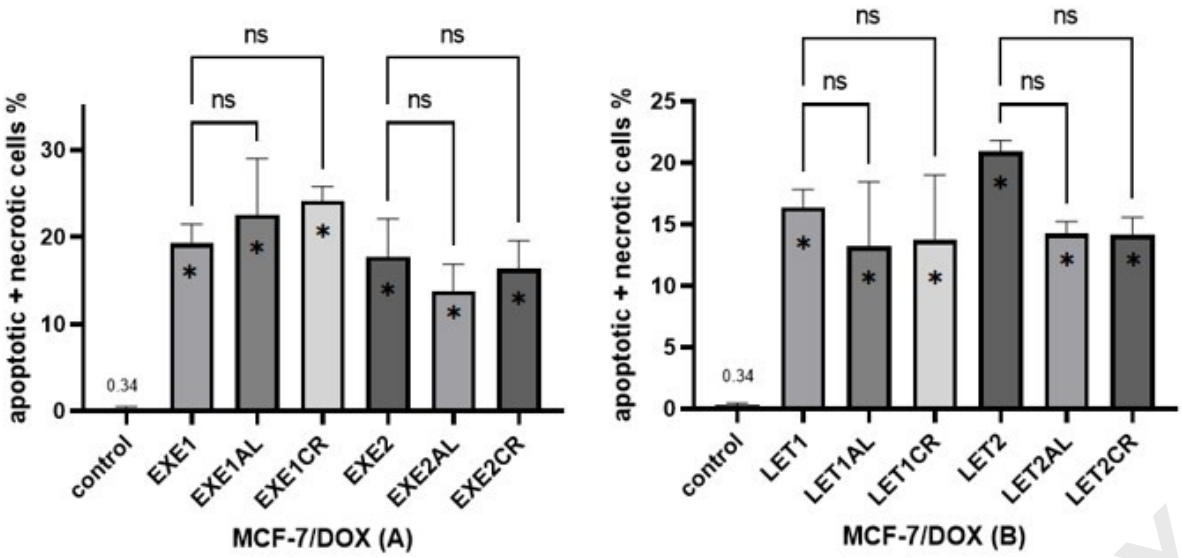

Figure 4. Effect of metalloestrogens, aromatase inhibitors and their combination on MCF-7/DOX cell death under high glucose (HG) conditions. Percentage of both apoptotic (early and late apoptotic) and necrotic cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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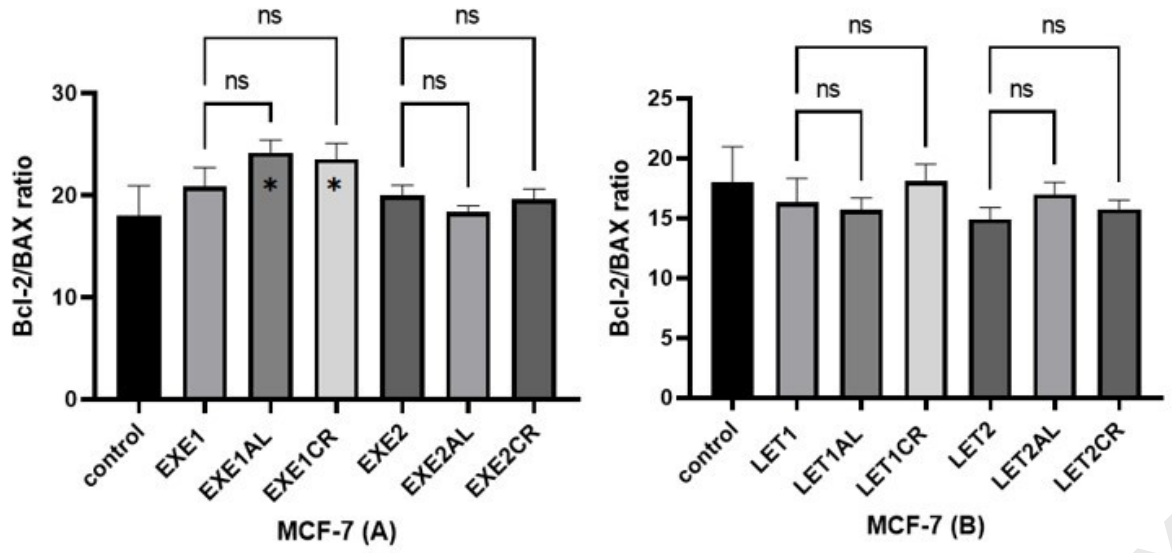

Figure 5. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the Bcl-2/BAX ratio in MCF-7 cell line under high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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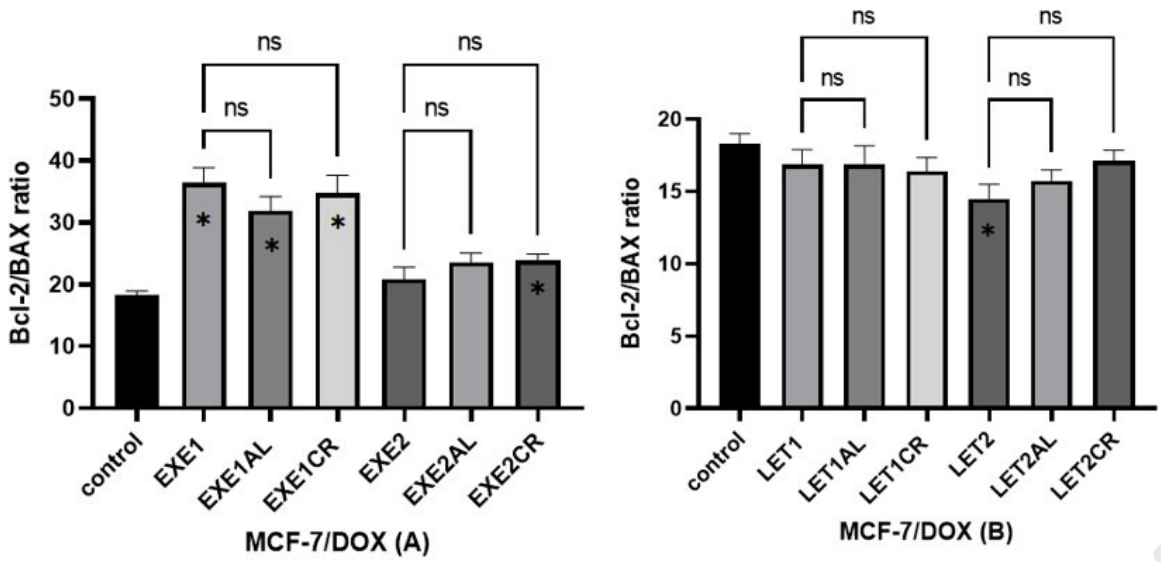

Figure 6. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the Bcl-2/BAX ratio in MCF-7/DOX cell line under high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$. * statistically significant difference from control.

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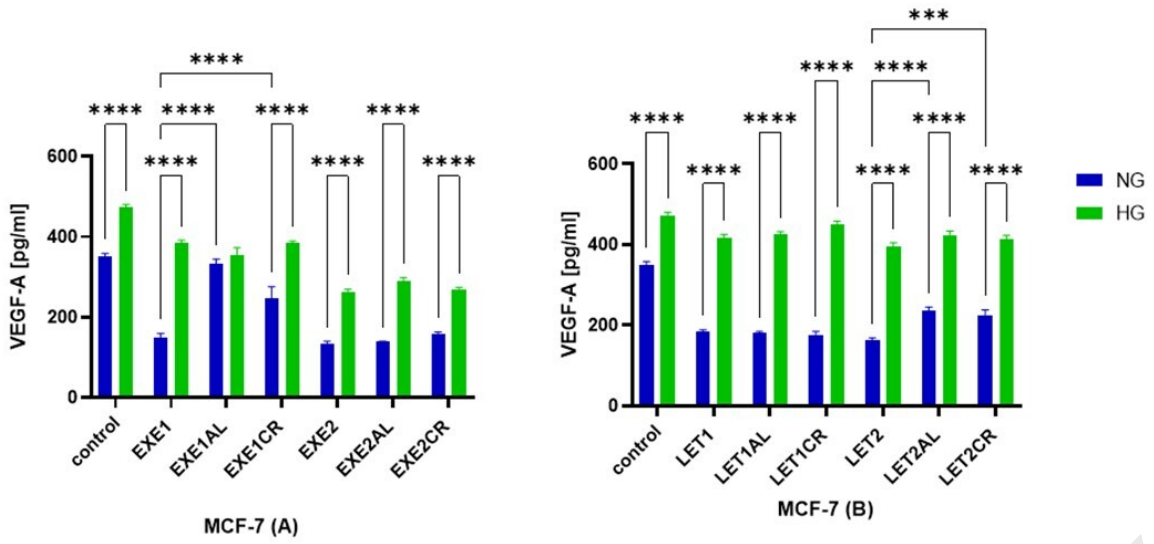

Figure 7. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the VEGF-A concentration in MCF-7 cell line under normal (NG) and high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$; *p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001. Only statistically significant differences are marked on the chart.

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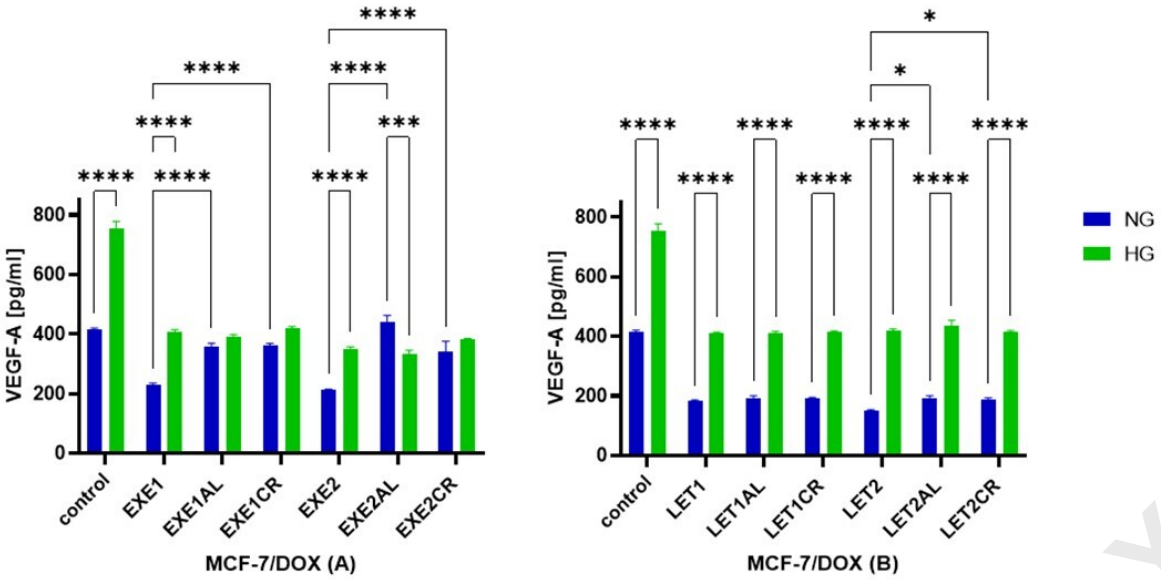

Figure 8. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the VEGF-A concentration in MCF-7/DOX cell line under normal (NG) and high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$; *p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001. Only statistically significant differences are marked on the chart.

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Figures

Figure 1 - Download source file (43.2 kB)
Figure 1. Effect of metalloestrogens, aromatase inhibitors and their combination on cell viability of MCF-7 cell line under high glucose (HG) conditions. The viability of MCF-7 cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 2 - Download source file (40.77 kB)
Figure 2. Effect of metalloestrogens, aromatase inhibitors and their combination on cell viability of MCF-7/DOX cell line under high glucose (HG) conditions. The viability of MCF-7/DOX cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 3 - Download source file (52.03 kB)
Figure 3. Effect of metalloestrogens, aromatase inhibitors and their combination on MCF-7 cell death under high glucose (HG) conditions. Percentage of both apoptotic (early and late apoptotic) and necrotic cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 4 - Download source file (44.24 kB)
Figure 4. Effect of metalloestrogens, aromatase inhibitors and their combination on MCF-7/DOX cell death under high glucose (HG) conditions. Percentage of both apoptotic (early and late apoptotic) and necrotic cells treated with exemestane (A) and letrozole (B), alone or in combination with aluminum or chromium [III]. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 5 - Download source file (45.05 kB)
Figure 5. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the Bcl-2/BAX ratio in MCF-7 cell line under high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 6 - Download source file (55.67 kB)
Figure 6. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the Bcl-2/BAX ratio in MCF-7/DOX cell line under high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05. * statistically significant difference from control.

Figure 7 - Download source file (77.42 kB)
Figure 7. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole (B) and their combination on the VEGF-A concentration in MCF-7 cell line under normal (NG) and high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; p<0.05; *p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001. Only statistically significant differences are marked on the chart.

Figure 8 - Download source file (82.75 kB)
Figure 8. Effect of metalloestrogens, aromatase inhibitors – exemestane (A) or letrozole

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(B) and their combination on the VEGF-A concentration in MCF-7/DOX cell line under normal (NG) and high glucose (HG) conditions. EXE1 = 100 μM; EXE2 = 200 μM; LET1 = 10 μM; LET2 =100 μM; Al/Cr[III] = 100 μM. The results are presented as mean ± SD, n=3; $p<0.05$; *p=0.0332; **p=0.0021; ***p=0.0002; ****p<0.0001. Only statistically significant differences are marked on the chart.

ZAŁĄCZNIK 5

Mgr Kamila Boszkiewicz Wrocław, 14.06.2023 r.

WYKAZ PUBLIKACJI

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1	Kowalczyk Adam, Bodalska Agnieszka, **Boszkiewicz Kamila**, Karłowicz-Bodalska Katarzyna: Use of the herbal OTC products and dietary supplements by patients receiving chemotherapy: survey-based study, Indian Journal of Pharmaceutical Education and Research, 2017, vol. 51, nr 4 suppl., S675-S678, DOI:10.5530/ijper.51.4s.98	0,351	15
2	Karłowicz-Bodalska Katarzyna, Głowacka Krystyna, **Boszkiewicz Kamila**, Han Stanisław, Wiela-Hojeńska Anna: Safety of oral nifuroxazide - analysis of data from a spontaneous reporting system, Acta Poloniae Pharmaceutica, 2019, vol. 76, nr 4, s. 745-751, DOI:10.32383/appdr/105805	0,456	100
3	**Boszkiewicz Kamila**, Sawicka Ewa, Piwowar Agnieszka: The impact of xenoestrogens on effectiveness of treatment for hormone-dependent breast cancer - current state of knowledge and perspectives for research, Annals of Agricultural and Environmental Medicine, 2020, vol. 27, nr 4, s. 526-534, DOI:10.26444/aaem/124165	1,447	100
4	Sawicka Ewa, **Boszkiewicz Kamila**, Wolniak Martyna, Piwowar Agnieszka: Znaczenie ekspozycji środowiskowej na wybrane ksenoestrogeny w patogenezie raka piersi, Postępy Higieny i Medycyny Doświadczalnej, 2020, vol. 74, s. 155-170, DOI:10.5604/01.3001.0014.1542	0,270	40
5	**Boszkiewicz Kamila**, Piwowar Agnieszka, Petryszyn Paweł: Aromatase inhibitors and risk of metabolic and cardiovascular adverse effects in breast cancer patients - a systematic review and meta-analysis, Journal of Clinical Medicine, 2022, vol. 11, nr 11, art.3133 [14 s.], DOI:10.3390/jcm11113133	4,964*	140
6	**Boszkiewicz Kamila**, Moreira Helena, Sawicka Ewa, Szyjka Anna, Piwowar Agnieszka: The effect of metalloestrogens on the effectiveness of aromatase inhibitors in a hormone-dependent breast cancer cell model, Cancers, 2023, vol. 15, nr 2, art.457 [18 s.], DOI:10.3390/cancers15020457	6,575*	140
	Podsumowanie	14,063	535

** IF2021*

1.2 Publikacje w czasopiśmie bez IF -

2. Monografie naukowe

2.1 Książka autorska -

2.2 Książka redagowana -

2.3 Rozdziały -

3. Varia -

4. Abstrakty

Lp	Opis bibliograficzny
1	**Wiszyńska Kamila**, Andrzejewska Barbara: Biegunki zapalne i niezapalne - diagnostyka przesiewowa, W: XVI Ogólnopolska Konferencja Studenckich Kół Naukowych Uczelni Medycznych; Ist International Student Conference of Young Medical Researchers. Wrocław, 1-2 kwietnia 2011 roku, Wrocław 2011, Akademia Medyczna im. Piastów Śląskich, 130-131 poz.11, ISBN 978-83-7055-587-0
2	Jermakow Katarzyna, Pajączkowska Magdalena, **Wiszyńska Kamila**: Clostridium difficile - czy można opanować wzrost zachorowań na terenie szpitala?, W: III Ogólnopolska Konferencja Naukowo-Szkoleniowa "Wektory i patogeny w przeszłości i przyszłości". [Wrocław], 20-21 listopada 2014 2014, 45 poz. P12
3	Kowalczyk Adam, Bodalska Agnieszka, **Boszkiewicz Kamila**, Karłowicz-Bodalska Katarzyna: OTC herbal products and dietary supplements using by patients receiving chemotherapy - survey study, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 101 poz.P47, ISBN 978-83-65932-64-8
4	**Boszkiewicz Kamila**, Wiśniewska Daria, Rackiewicz Joanna, Karłowicz-Bodalska Katarzyna: Zastosowanie ilościowych badań marketingowych w farmacji na przykładzie badania CATI I PAPI, W: IV Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 12 kwietnia 2019 r. Książka abstraktów 2019, [16], [[Dostęp 18.04.2019]. Dostępny w: http://www.farmacja.wroclaw.pl/images/ksia%CC%A8z%CC%87ka-abstrakto%CC%81w-kwiecien%CC%81-2019.pdf]
5	**Boszkiewicz Kamila**, Sawicka Ewa, Piwowar Agnieszka: The impact of xenoestrogens on the effectiveness of treatment of hormone-dependent breast cancer, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 88-90, ISBN 978-83-66489-37-0
6	**Boszkiewicz Kamila**, Sawicka Ewa, Piwowar Agnieszka: Zastosowanie suplementów diety i leków OTC z fitoestrogenami w łagodzeniu objawów menopauzy - analiza rynku aptecznego w Polsce, W: V Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". [Online], 27 listopada 2020 r. Książka abstraktów 2020, s. 18-19

Impact factor: 14,063

Punktacja ministerialna: 535,00

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Boszkiewicz K, Sawicka E, Piwowar A. The impact of xenoestrogens on effectiveness of treatment for hormone-dependent breast cancer - current state of knowledge and perspectives for research. Ann Agric Environ Med. 2020 Dec 22;27(4):526-534

Boszkiewicz K, Piwowar A, Petryszyn P. Aromatase Inhibitors and Risk of Metabolic and Cardiovascular Adverse Effects in Breast Cancer Patients-A Systematic Review and Meta-Analysis. J Clin Med. 2022 May 31;11(11):3133

Boszkiewicz K, Moreira H, Sawicka E, Szyjka A, Piwowar A. The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model. Cancers (Basel). 2023 Jan 11;15(2):457

Boszkiewicz K, Piwowar A. High glucose reduces anti-tumor activity of aromatase inhibitors in a hormone-dependent breast cancer cell model. Acta Poloniae Pharmaceutica – Drug Research, vol.80 No.3, 2023.

mój udział polegał na współuczestniczeniu w opracowaniu koncepcji publikacji, interpretacji wyników oraz ocenie merytorycznej i korekcie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w prac przez mgr Kamilę Karolinę Boszkiewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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Boszkiewicz K, Moreira H, Sawicka E, Szyjka A, Piwowar A. The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model. Cancers (Basel). 2023 Jan 11;15(2):457

mój udział polegał na pomocy w planowaniu, wykonywaniu i analizowaniu badań wykonanych przy pomocy cytometru przepływowego oraz na korekcie tekstu manuskryptu.

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Oświadczam, że w pracy:

Boszkiewicz K, Piwowar A, Petryszyn P. Aromatase Inhibitors and Risk of Metabolic and Cardiovascular Adverse Effects in Breast Cancer Patients-A Systematic Review and Meta-Analysis. J Clin Med. 2022 May 31;11(11):3133

mój udział polegał na pomocy w planowaniu, wykonywaniu i analizowaniu badań, wykonaniu analizy statystycznej, wizualizacji danych oraz na korekcie manuskryptu.

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Oświadczam, że w pracy:

Boszkiewicz K, Sawicka E, Piwowar A. The impact of xenoestrogens on effectiveness of treatment for hormone-dependent breast cancer - current state of knowledge and perspectives for research. Ann Agric Environ Med. 2020 Dec 22;27(4):526-534

mój udział polegał na pomocy w analizie i interpretacji danych oraz na korekcie manuskryptu.

W pracy:

Boszkiewicz K, Moreira H, Sawicka E, Szyjka A, Piwowar A. The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model. Cancers (Basel). 2023 Jan 11;15(2):457

mój udział polegał na pomocy w planowaniu badań oraz na korekcie manuskryptu.

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Boszkiewicz K, Moreira H, Sawicka E, Szyjka A, Piwowar A. The Effect of Metalloestrogens on the Effectiveness of Aromatase Inhibitors in a Hormone-Dependent Breast Cancer Cell Model. Cancers (Basel). 2023 Jan 11;15(2):457

mój udział polegał na pomocy w prowadzeniu hodowli komórkowej, a także pomocy w planowaniu i wykonywaniu badań.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Kamilę Karolinę Boszkiewicz jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

podpis współautora