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Analiza wybranych czynników regulujących zmiany adaptacyjne w odpowiedzi na podejmowaną aktywność fizyczną w grupie osób starzejących się

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Gdańsk, 2024

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WPROWADZENIE

Zaobserwowane w ostatnich latach zmiany demograficzne wskazują, iż nasze społeczeństwo jest społeczeństwem starzejącym się. Zgodnie z danymi Światowej Organizacji Zdrowia (WHO), grupa osób powyżej 60 roku życia zwiększa się w krajach zachodnich w najszybszym tempie (Rudnicka *et al.* 2020). Proces starzenia się związany jest z szeregiem niekorzystnych zmian, w tym ze zmniejszeniem masy mięśniowej (sarkopenia), kostnej (osteoporoza) oraz wieloma stanami patologicznymi takimi jak pogorszenie funkcji kognitywnych i fizycznych, przewlekły stan zapalny, a także nasilenie objawów chorób przewlekłych takich jak niewydolność układu sercowo-naczyniowego, insulino-oporność czy cukrzyca typu 2 (Wick *et al.* 2000; Thillainadesan *et al.* 2020). Zaburzenia funkcji kognitywnych u osób starszych nie tylko przyczyniają się do ograniczenia aktywności dnia codziennego ale są również związane ze zwiększonym ryzykiem upadku, które jest kolejnym istotnym problemem pogarszającym jakość życia seniorów (Ambrose *et al.* 2013). W konsekwencji w/w zmiany mogą prowadzić do zmniejszenia sprawności, ograniczenia niezależności i większej śmiertelności (Campisi *et al.* 2019).

Dotychczas zdefiniowano kilka mechanizmów mogących przyspieszać procesy starzenia. Jeden z nich jest związany ze zwiększeniem wolnorodnikowych uszkodzeń makromolekuł, do którego prowadzi nadprodukcja reaktywnych form tlenu (RFT) i zmniejszanie się efektywności ochrony antyoksydacyjnej (Bourgonje *et al.* 2021). Nasilony stres oksydacyjny wyrażony jest między innymi obniżeniem stężenia grup sulfhydrylowych (SH) w surowicy, które biorą udział w obronie antyoksydacyjnej organizmu i stanowią biomarker miejscowego i ogólnoustrojowego stresu oksydacyjnego (Bourgonje *et al.* 2021). Redukcja grup SH jest widoczna u osób starszych i powiązana z licznymi stanami patologicznymi takimi jak cukrzyca typu 2, stan zapalny, nadciśnienie, czy otyłość (Piwowar *et al.* 2009; Bourgonje *et al.* 2021).

Wraz z wiekiem pojawiają się także zaburzenia w uwalnianiu miokin – białek pochodzenia mięśniowego, które stymulowane są przez podejmowaną aktywność fizyczną. Sugeruje się, że biorą one udział w modyfikacji mechanizmów starzenia się poprzez działanie bezpośrednio w obrębie tkanki mięśniowej (efekt autokrynny), a także w narządach oddalonych (efekt parakrynny) oraz kontrolę licznych procesów takich jak metabolizm energetyczny, regulacja homeostazy glukozy, stresu oksydacyjnego czy stanu zapalnego (Pedersen, Febbraio 2012). Zmiany w stężeniach miokin, które pojawiają się wraz z wiekiem

mogą osłabiać efekt działania wysiłku fizycznego, a w konsekwencji mieć wpływ na pogorszenie się zarówno funkcji poznawczych jak i fizycznych. Nie wiadomo, czy jest to związane z procesem sarkopenii (Arancio, Chao 2007; Tsunemi, La Spada 2012; Chen *et al.* 2016) czy z innymi czynnikami. Biorąc pod uwagę ten fakt, poszukiwanie metod oraz mechanizmów, które odwracałyby niekorzystny wpływ starzenia się na uwalnianie miokin przez mięśnie szkieletowe, jest istotne, gdyż potencjalnie pozytywny efekt będzie zapobiegał wyżej wymienionym dysfunkcjom.

Obecnie, jednym z głównych wyzwań w zapobieganiu negatywnym skutkom starzenia się jest poprawa funkcji kognitywnych oraz fizycznych, co w konsekwencji prowadzi do polepszenia się jakości życia oraz zwiększenia niezależności, a tym samym redukcji obciążeń medycznych i ekonomicznych zarówno rodzin, jak i państwa (Chen *et al.* 2021).

Powszechnie wiadomo, że siedzący tryb życia jest powiązany z rozwojem licznych chorób cywilizacyjnych, takich jak otyłość, cukrzyca typu 2, insulino-oporność czy przewlekły stan zapalny (Arocha Rodulfo 2019). Powyższe stany patologiczne są czynnikiem ryzyka rozwoju chorób neurodegeneracyjnych, pogorszenia możliwości wysiłkowych, a także nasilają proces sarkopenii (Nishikawa *et al.* 2021; Tyagi, Pugazhenthi 2021). Niekorzystne zmiany związane z brakiem aktywności fizycznej są w szczególności nasilone u osób starszych, gdyż sam proces starzenia się dodatkowo przyspiesza pojawianie i nasilanie tych stanów patologicznych (Rebenson-Piano 1989). Dane z ostatnich lat wskazują, że około 75% osób powyżej 65 roku życia nie wypełnia minimalnego tygodniowego limitu aktywności fizycznej, niezbędnego do utrzymania zdrowia.

Jedną ze znanych strategii „zdrowego starzenia się" jest aktywność fizyczna. Badania ostatnich lat wskazują, że różne rodzaje regularnego treningu fizycznego takie tak trening nordic-walking, tai-chi, czy trening równowagi są skuteczną metodą przeciwdziałającą negatywnym skutkom starzenia się, poprawiającą sprawność fizyczną, funkcje kognitywne, możliwości wysiłkowe oraz zmniejszającą ryzyko upadku (Tsang *et al.* 2004; Gunendi *et al.* 2008; Gusi *et al.* 2012). Chociaż prozdrowotny efekt regularnej aktywności fizycznej jest dobrze znany, mechanizmy, które leżą u podstaw tych zmian są wciąż przedmiotem badań naukowców. Jeden z proponowanych mechanizmów pozytywnego wpływu wysiłku fizycznego uwzględnia modyfikację stężeń miokin – białek, cząstek czy peptydów uwalnianych przez mięśnie szkieletowe w odpowiedzi na różne rodzaje wysiłku fizycznego (Safdar, Tarnopolsky 2018). Sugeruje się, że mogą one odpowiadać za pozytywny wpływ aktywności fizycznej poprzez regulację odpowiedzi przeciwzapalnej, metabolizmu glukozy oraz stresu oksydacyjnego. W ostatnich latach stwierdzono również, iż w odpowiedzi na

intensywny i/lub przewlekły wysiłek fizyczny, uwalniane są molekuły nazwane exerkinami (*z języka ang. exerkines od słowa exercise*) – nie tylko przez mięśnie szkieletowe (miokiny), ale również przez serce (kardiokiny), wątrobę (hepatokiny), białą tkankę tłuszczową (adipokiny), brązową tkankę tłuszczową (baptokiny) i neurony (neurokiny) (Chow *et al.* 2022). Exerkiny mają potencjalny wpływ na poprawę funkcji układu sercowo-naczyniowego, odpornościowego i neurologicznego oraz sprawność metaboliczną, zapewniając tym samym tzw. zdrowe starzenie się (Chow *et al.* 2022).

Jedną z exerkin o udowodnionym działaniu prozdrowotnym jest neutroficzny czynnik pochodzenia mózgowego (BDNF) (Chow *et al.* 2022). BDNF to neutrofina zaangażowana głównie we wszystkie najważniejsze aspekty neuroplastyczności, od neurogenezy, wzrostu i przeżywalności neuronów po synaptogenezę, czy funkcje kognitywne (Mattson *et al.* 2004). Poza funkcją w ośrodkowym układzie nerwowym, BDNF reguluje metabolizm obwodowy. Wcześniejsze badania wykazały, że BDNF poprawia metabolizm glukozy poprzez stymulację jej wychwytu przez wątrobę i komórki mięśni szkieletowych oraz zwiększa wrażliwość na insulinę (Marosi, Mattson 2014). W ostatnich badaniach opartych na modelu zwierzęcym, pokazano nowy szlak sygnałowy biorący udział w regulacji stężenia insuliny. Autorzy wykazali rolę BDNF-u jako ligandu aktywującego receptor TrkB.T1 komórek β trzustki (Fulgenzi *et al.* 2020).

Kolejną dobrze znaną exerkiną jest iryzyna, odkryta przez Bostroma i wsp. (Bostrom *et al.* 2012). Jest to fragment białka FNDC5, a miejscem jej uwalniania są w głównie mięśnie szkieletowe. Czynnikami stymulującymi uwalnianie iryzyny jest zarówno wysiłek fizyczny, jak i ekspozycja na niskie temperatury (Bostrom *et al.* 2012; Lee *et al.* 2014). Jej główną funkcją jest konwersja białej tkanki tłuszczowej w brunatną poprzez zwiększanie ekspresji mitochondrialnego białka UCP1, zwiększając wydatek energetyczny i termogenezę (Novelle *et al.* 2013). Co więcej, iryzynie przypisywane są takie funkcje jak regulacja metabolizmu glukozy poprzez poprawę insulino-wrażliwości, a także zapobieganie utracie masy kostnej poprzez stymulację różnicowania się i tworzenia osteoblastów, a także hamowanie tworzenia się osteoklastów (Perakakis *et al.* 2017; Anastasilakis *et al.* 2019). Jednym z jej działań jest także działanie przeciwzapalne poprzez stłumienie produkcji cytokin prozapalnych, co wydaje się mieć szczególne znaczenie w procesie starzenia się, któremu towarzyszy przewlekły stan zapalny (*ang. inflamm-aging)*(Askari *et al.* 2018).

Ostatnie badania wykazały również, że zarówno iryzyna, jak i BDNF mogą pełnić funkcję regulatorów metabolizmu energetycznego w różnych tkankach, takich jak mózg czy tkanka mięśniowa. Huh i współautorzy wykazali, że iryzyna krążąca we krwi jest powiązana z

ilością adenozyno-5′-trifosforanu (ATP) i adenozyno-5′-difosforanu (ADP) w tkance mięśniowej, przez co posiada zdolność regulacji metabolizmu energetycznego zarówno w trakcie jak i po wysiłku (Huh *et al.* 2012). Z kolei BDNF ma zdolność zwiększania neuronalnej produkcji ATP (Cheng *et al.* 2012). Na modelu zwierzęcym wykazano, iż iryzyna może przechodzić przez barierę krew-mózg i stymulować syntezę BDNF (Wrann *et al.* 2013).

Regularny wysiłek fizyczny w starszym wieku ma nie tylko prowadzić do prewencji rozwoju sarkopenii, ale także utrzymywania lub zwiększenia masy i siły mięśniowej (Rodrigues *et al.* 2022). Odpowiedzialne za to mogą być szczególnie zmiany stężeń miokin takich jak iryzyna, miostatyna, czy C- końcowy fragment agryny (C-terminal agrin fragment – CAF)(Allen *et al.* 2011; Landi *et al.* 2016). Miostatyna przyczynia się do hamowania syntezy białek i procesów regeneracyjnych, co występuje w procesie sarkopenii wywołanej starzeniem się. Z kolei CAF kontroluje funkcje połączeń nerwowo-mięśniowych, a jego zwiększone stężenie w surowicy odzwierciedla stopień odnerwienia i zwyrodnienia włókien mięśniowych, co w konsekwencji prowadzi do dysfunkcji i atrofii mięśni (Pratt *et al.* 2021). Wyniki tych badań wymagają kontynuacji ze względu na fakt, iż aktywność fizyczna zapobiega zwyrodnieniu neuronów ruchowych, połączeń nerwowo-mięśniowych i odnerwieniu włókien mięśniowych, poprzez modyfikacje stężeń wyżej wymienionych białek; natomiast badania w tym obszarze nie są spójne (Fragala *et al.* 2014; Marcolin *et al.* 2021; Micielska *et al.* 2021).

Prozdrowotne oddziaływanie aktywności wiąże się z antyoksydacyjnym mechanizmem działania wysiłku fizycznego (Bachi *et al.* 2019), między innymi poprzez zwiększenie stężenia grup SH. Te zmiany zaobserwowano u osób młodych i w średnim wieku (Metin *et al.* 2003; de Oliveira *et al.* 2012), ale nie w grupie starzejących się osób. Wcześniejsze badania wykazały także redukcję stężenia dialdehydu malonowego (MDA) – markera peroksydacji lipidów – w wyniku treningu aerobowego i siłowo-aerobowego u osób starszych (Kortas *et al.* 2017; Mota *et al.* 2019). Można zatem przypuszczać, że odpowiedź antyoksydacyjna u osób starszych w następstwie regularnej aktywności fizycznej może hamować potencjalny negatywny wpływ RFT na zmiany związane z wiekiem.

Oprócz aktywności fizycznej, za istotny czynnik przyczyniający się do zdrowego starzenia się uznaje się optymalne stężenie witaminy D (25(OH)D). Powszechnie wiadomo, że niedobór tej witaminy jest częstym zjawiskiem, nie tylko wśród osób starszych. Dane światowe pokazują, że deficyt witaminy D może dotykać więcej niż 50% populacji seniorów (van Schoor, Lips 2011). Wykazano, że stężenie 25(OH)D poniżej 30 ng/ml związane jest ze zwiększonym ryzykiem upadków i złamań, a także nasilonym stresem oksydacyjnym, stanem

zapalnym (Krasowska *et al.* 2019), czy gorszymi możliwościami wysiłkowymi (Berridge 2017; Sizar *et al.* 2022). Witamina D pełni istotną funkcję regulującą wolnorodnikowe uszkodzenie komórek poprzez jej zdolność do kontrolowania komórkowej ekspresji antyoksydantów, a także poprzez utrzymywanie odpowiedniej funkcji mitochondriów (Dzik, Kaczor 2019). Sugeruje się, że wpływ optymalnego stężenia 25(OH)D na zapobieganie obniżaniu się możliwości wysiłkowych u osób starszych może być zależny od kilku czynników takich jak stężenie parathormonu (PTH), i/lub markerów obrotu kostnego. Niedobór 25(OH)D wiąże się ze wzrostem stężenia PTH oraz osteokalcyny, co w konsekwencji prowadzi do nasilonego obrotu kostnego, utraty masy kostnej i stanowi czynnik ryzyka rozwoju osteomalacji czy osteopenii (Kuchuk *et al.* 2009; Pludowski *et al.* 2013; Wang *et al.* 2019). W świetle tych danych, utrzymywanie optymalnego stężenia witaminy D może być niezbędne, aby zmniejszyć ryzyko upadków i złamań u osób w starszym wieku.

METODOLOGIA BADAŃ WŁASNYCH

1. Problem pracy, pytania i hipotezy badawcze

Prozdrowotne działanie regularnej aktywności fizycznej zostało w literaturze naukowej dobrze udokumentowane, niemniej jednak rola białek stymulowanych aktywnością fizyczną wymaga doprecyzowania. Obecnie jedną z najczęściej stosowanych form aktywności fizycznej w grupie starzejących się osób jest trening nordic - walking, czy trening oporowy. Skuteczność tych form wysiłku została dobrze udokumentowana i zweryfikowana w badaniach naukowych, jednakże efekty nie zawsze były jednoznaczne, stąd wciąż istnieje potrzeba analizowania innych, nowych rodzajów aktywności, takich jak trening funkcjonalny, czy zyskujący ostatnio na popularności trening tańca.

Celem niniejszej dysertacji jest ocena, jak wybrane formy aktywności fizycznej aplikowane w grupie osób starszych mogą poprawić wskaźniki odpowiedzialne za regulację masy mięśniowej, redukcję stresu oksydacyjnego czy stanu zapalnego. Analizie poddano skuteczność działania pojedynczej jednostki treningowej jak i długotrwałego treningu, na różnym poziomie zaawansowania, w regulacji stężenia wybranych miokin/exerkin w odniesieniu do indukowanych zmian adaptacyjnych.

W związku z powyższym postawiłam następujące pytania badawcze:

Pytanie 1: Czy uwalnianie białek/miokin/exerkin w odpowiedzi na podejmowane formy treningowe w grupie starzejących się osób reguluje zmiany adaptacyjne?

Pytanie 2: Która z form aktywności fizycznej wiąże się z korzystnym statusem uwalnianych białek/miokin/exerkin mających udział w procesie sarkopenii?

Pytanie 3: Czy działanie prozdrowotne i antyoksydacyjne wysiłku fizycznego regulowane jest przez wyjściowy poziom witaminy D?

Pytanie 4: Czy długotrwały trening fizyczny indukuje zmiany w metabolizmie krwinek czerwonych oraz czy miokiny/exerkiny mają wpływ na regulację tych zmian?

Pytania badawcze sformułowałam w oparciu o dotychczasowe wyniki badań, wskazujące na prozdrowotne oddziaływanie aktywności fizycznej związane z czynnością endokrynną mięśni szkieletowych.

Postawiłam następujące hipotezy:

Hipoteza 1: Zakładam, iż wybrane formy aktywności fizycznej (taniec, trening równowagi) będą efektywnie regulowały poziom białek biorących udział w regulacji procesu sarkopenii oraz potreningowych zmian adaptacyjnych w grupie starzejących się osób.

Hipoteza 2: Ze względu na dobrze udokumentowane przeciwzapalne i antyoksydacyjne działanie witaminy D, stawiam hipotezę, iż wyższe stężenie 25(OH)D będzie powiązane z lepszym statusem antyoksydacyjnym i możliwościami wysiłkowymi w grupie osób starzejących się, natomiast efekty treningowe wywołane treningiem wytrzymałościowo-siłowym będą niezależne od jej wyjściowego stężenia.

Hipoteza 3: Długotrwały trening fizyczny indukuje zmiany nie tylko dotyczące poziomu wydolności fizycznej, ale również metabolizmu krwinek czerwonych. Nie można wykluczyć udziału miokin/exerkin w regulacji tych zmian adaptacyjnych.

2. Procedura badań

2. 1. Charakterystyka osób badanych

Wyniki badań ujęte w publikacjach stanowiących rozprawę doktorską zostały przeprowadzone na osobach starszych (kobiety i mężczyźni, w wieku 55-82 lat). Do badań zrekrutowano 100 osób. Badane osoby zostały zakwalifikowane po kontroli medycznej. Były to osoby zdrowe, bez przeciwwskazań do podjęcia aktywności fizycznej. Z badań zostały wykluczone osoby z chorobami układu krążenia, niewydolnością serca, wszczepionym rozrusznikiem, nowotworami, depresją, urazami ortopedycznymi, chorobami neurologicznymi, autoimmunologicznymi i niedokrwistością. Z eksperymentów, w których zastosowano trening fizyczny zostały wykluczone osoby uczestniczące w przeciągu ostatniego pół roku w programach treningowych wytrzymałościowych lub siłowych. Eksperymenty te zostały zaakceptowane przez: Niezależną Komisję Bioetyczną ds. Badań

Naukowych przy Gdańskim Uniwersytecie Medycznym (NKBBN/55/2018: publikacja I), Komisję Bioetyczną przy Okręgowej Izbie Lekarskiej w Gdańsku (KB/34/18: publikacja II), Komisję Bioetyczną przy Uniwersytecie Medycznym w Poznaniu (KB/1252/18: publikacja III) i wykonane zgodnie z deklaracją Helsińską.

W poszczególnych eksperymentach wzięło udział:

1. **Publikacja I:** 41 osób (21 kobiet i 20 mężczyzn) w wieku 71.3 ± 5.5 lat. Badane osoby zostały podzielone na 3 grupy: poddaną treningowi równowagi (BG; n=15), poddaną treningowi tańca (TG; n=14) oraz grupę kontrolną (CG; n=12). Osoby z grupy BG i TG wykonywały treningi 3 razy w tygodniu (poniedziałek, środa, piątek) przez 12 tygodni. Osoby z grupy kontrolnej zostały poproszone o niezmienianie swojej dziennej aktywności.
2. **Publikacja II:** 37 kobiet w wieku 72.9 ± 5.2 lat. Zostały one podzielone na dwie grupy w zależności od przyjmowanej suplementacji: suplementowaną 2000 jednostkami lub więcej witaminy D dziennie przez przynajmniej 2 miesiące (SG; n=19) i niesuplementowaną (NSG; n=18). Po zmierzeniu stężenia witaminy D w osoczu, uczestniczki z grup SG oraz NSG zostały podzielone na grupę o stężeniu witaminy D wyższym niż 30 ng/ml (SD) oraz poniżej tego stężenia (ID) oraz poddane treningowi fizycznemu (ESD; n=10 oraz EID; n=9) oraz grupy kontrolne, nietrenujące (CSD; n=9 oraz CID; n=9). Osoby z grupy ESD oraz EID wykonywały trening siłowo – aerobowy 3 razy w tygodniu (poniedziałek, środa, piątek) przez okres 12 tygodni. Osoby z grup kontrolnych zostały poproszone o niezmienianie swojego trybu życia i dziennej aktywności fizycznej.
3. **Publikacja III:** 22 mężczyzn w wieku 58 ± 3.6 lat. Zostali oni podzieleni na dwie grupy: grupę trenującą, o wysokim poziomie wydolności fizycznej (n =12, VO_2max = 51.9 ± 2.7 mlO_2/kg/min, o stażu treningowym 38.0 ± 6.0 lat. Były to osoby regularnie uczestniczące w zawodach o randze europejskiej i światowej, w dyscyplinach o charakterze wytrzymałościowym. Do drugiej grupy zakwalifikowano mężczyzn nietrenujących (n=10, VO_2max=37.0±1.8 mlO_2/kg/min). Wszyscy uczestnicy wykonali jednorazowy wysiłek fizyczny w postaci testu do odmowy o wzrastającej intensywności na bieżni.

2. 2. Metody badań

W badaniach zastosowałam poniższe metody i narzędzia badawcze:

2.2.1. Metody pomiarów antropometrycznych

Analiza komponentów składu ciała osób biorących udział w badaniach dokonana została za pomocą dwóch metod. Oba badania były wykonywane w godzinach porannych (pomiędzy 7:00-12:00), na czczo, 12 godzin po ostatnim posiłku, przed wykonaniem pobrania krwi. W celu wykonania badania uczestnikom polecono rozebranie się do bielizny oraz zdjęcie metalowych elementów (biżuteria, zegarki itp.).

- Metoda absorpcjometrii podwójnej energii promieniowania rentgenowskiego (DEXA) wykonywana aparatem LunarProdigy (GE HealthCare, Madison, WI, USA). Podczas badania uczestnicy leżeli na plecach w bezruchu przez około 6-10 minut (Yu *et al.* 2016);
- Metoda bioimpedancji elektrycznej (BIA) wykonana przy pomocy aparatu InBody 720 (Biospace, Korea), z zastosowaniem sześciu różnych częstotliwości prądu (1 kHz, 5 kHz, 50 kHz, 250 kHz, 500 kHz i 1000 kHz). Pomiar był wykonywany przy pomocy 8-punktowej elektrody dotykowej. Podczas badania uczestnicy stali obunóż na wadze z dłońmi i stopami umieszczonymi na elektrodach przez około 60 sekund (McLester *et al.* 2020).

2.2.2. Metody pomiarów możliwości wysiłkowych i ryzyka upadku

- **Test wstań i idź (TUG)** – test TUG służy do oceny równowagi dynamicznej oraz mobilności, pośrednio ocenia także ryzyko upadku. Czas wykonania testu wynoszący 13,5 s lub więcej wynosi wiąże się z dwu- do trzykrotnie większym ryzykiem upadków (Shumway-Cook *et al.* 2000). Przed rozpoczęciem testu wszystkim badanym przekazano instrukcję jego wykonania. Polecono uczestnikom usiąść na krześle z oparciem i skrzyżować ręce na klatce piersiowej. Następnie badane osoby zostały poinstruowane, aby wstać z krzesła na komendę, przejść 3 m, zawrócić w oznaczonym miejscu, wrócić i usiąść ponownie na krześle. Czas wykonania zadania mierzono stoperem przez wykwalifikowanego fizjoterapeutę. Każdy uczestnik wykonał test dwukrotnie, najpierw powoli z badaczem w celu zapoznaniu się z procedurą. Następnie po 5 minutach odpoczynku, samodzielnie, tak szybko, jak to możliwe, bez biegania.

- **6 minutowy test chodu (6MWT)** – test oceniający dystans pokonany w ciągu 6 minut marszu. Zgodnie z danymi z piśmiennictwa wynik może obrazować sprawność funkcjonalną, a także wydolność układu oddechowego i krążenia osób starszych (Baddini-Martinez 2018).
- **Test ryzyka upadku (FRT)** – w celu oceny ryzyka upadku oraz zidentyfikowania osób mających predyspozycje do upadku w przyszłości użyto platformy balansowej BiodexBalance System. Test składał się z trzech prób, po 20 sekund każda. Podczas wykonywania każdej z prób badani mieli za zadanie utrzymać równowagę stojąc na niestabilnej platformie z nogami rozstawionymi na szerokość bioder. Uczestników poproszono o próbę stania bez podparcia, patrzenie przed siebie oraz skupienie się na informacji zwrotnej dotyczącej ustawienia ciała wyświetlanej na ekranie. Podczas przerw platforma wracała do zablokowanej, stabilnej pozycji. Wyniki badanych odnotowywane były jako wskaźnik stabilności (*stabillity index* -SI). Wyniki wyższe od wartości referencyjnych dla danego wieku wskazują na większe ryzyko upadku oraz zaburzenia równowagi.

2.2.4. Metoda izolacji erytrocytów

W celu wyizolowania erytrocytów, próbki krwi natychmiast odwirowano (1000 x g, 5 min, 4◦C; Universal 320R, Hettich Lab Technology, Tuttlingen, Niemcy). Kożuszek leukocytarny i osocze usunięto, a osocze zostało zamrożone w temperaturze -80°C do czasu analizy. Następnie erytrocyty zostały trzykrotnie przepłukane 0,9% roztworem NaCl i odwirowane (1000 x g, 5 min, 4°C). Po ostatnim płukaniu erytrocyty zawieszono w małej objętości PBS. Wyizolowane erytrocyty zostały odbiałczane (1,3 mol/l HClO4) i odwirowane (16 000 x g, 5 min, 4°C). Neutralizacja supernatantu (600 µl) została przeprowadzona za pomocą 130-160 µl 3 mol/l K3PO4 (do pH 5-7). Próbki ponownie odwirowano w taki sam sposób jak powyżej. Supernatant przechowywano w temperaturze -80°C do późniejszej analizy.

2.2.4. Metody oznaczeń biochemicznych

- **Wybrane miokiny i hormony** takie jak iryzyna, BDNF, interleukina 6 (IL-6), osteokalcyna, parathormon, miostatyna i CAF zostały oznaczone metodą immunoenzymatyczną ELISA przy użyciu gotowych zestawów firm: R&D, Phoenix Pharmaceutical Inc., Fine Test oraz Demeditec zgodnie z instrukcją producenta.

- **Stężenie nukleotydów purynowych** (ATP, ADP, AMP, IMP) w erytrocytach zostało oznaczone przy użyciu wysokosprawnej chromatografii cieczowej z detekcją UV-VIS (Merck-Hitachi/Agilent, Tokyo, Japan/Santa Clara, CA, United States) zgodnie z metodą opisaną wcześniej przez Smoleńskiego i współautorów (Smolenski *et al.* 1990). Stężenia oznaczanych nukleotydów wyrażono w stosunku do objętości erytrocytów i zaprezentowano jako μmol/L RBC. Wartość ładunku energetycznego (*ang. Adenylate energy charge - AEC)* została obliczona przy użyciu poniższego wzoru: AEC = [ATP] + 0,5 [ADP]/ [ATP] + [ADP] + [AMP]
- **Stężenie metabolitów purynowych** – hipoksantyny (Hx) i kwasu moczowego (UA) w osoczu zostało oznaczone przy użyciu wysokosprawnej chromatografii cieczowej z detekcją UV-VIS (Merck-Hitachi/Agilent, Tokyo, Japan/Santa Clara, CA, United States) zgodnie z metodą opisaną wcześniej przez Smoleńskiego i współautorów (Smolenski *et al.* 1990). Analizę ilościową przeprowadzono przy użyciu systemu ChemStation (Agilent, Stany Zjednoczone). Identyfikację substancji oparto na porównaniu czasów retencji z wzorcami dla Hx i UA. Pomiarów dokonano przy długości fali 254 nm dla Hx i 280 nm dla UA.
- **Markery stresu oksydacyjnego** zostały oznaczone spektrofotometrycznie. Grupy SH zmierzono zgodnie z metodą opisaną przez Kaszubowską i współautorów względem krzywej standardowej (standard – glutation zredukowany) (Kaszubowska *et al.* 2011). Najniższe stężenie glutationu zredukowanego w krzywej wzorcowej wynosiło 0,25 mmol/L, a jej zakres wynosił od 0 do 2 mmol/l. Stężenie MDA (marker peroksydacji lipidów) zostało zmierzone przy użyciu zestawu LPO-586 (OxisReserch, Portland, United States), zgodnie z instrukcją producenta, względem krzywej standardowej (standard– 1,1,3,3 Tetra-metoksypropan). Najniższe stężenie 1,1,3,3 tetra-metoksypropanu w krzywej wzorcowej wynosiło 2,5 μmol/L. Zakres krzywej wynosił od 0 do 150 μmol/L. Wartości grup SH i MDA wyrażono odpowiednio w mmol/l i μmol/l.
- **Witamina D –** stężenie całkowitej witaminy D (25(OH)D) zostało oznaczone metodą immunoenzymatyczną ELISA przy użyciu zestawu Demeditec, zgodnie z instrukcją producenta.

2.2.5. W celu określenia wskaźników homeostazy glukozy i stanu zapalnego:

- **Oznaczono stężenie glukozy** przy użyciu analizatora Cobas 6000 (Roche Diagnostics, Warszawa, Polska) zgodnie z instrukcją producenta, a także metodą kolorymetryczną (Randox manual gl2623),
- **Oznaczono stężenie insuliny** metodą immunoenzymatyczną ELISA przy użyciu zestawu producenta DiaMetra (Perugia, Włochy) oraz przy użyciu zestawu producenta Demeditec (Niemcy),
- **Wyliczono wskaźniki insulino-oporności i insulino-wrażliwości**: komputerowy model HOMA2 (HOMA 2 Calculator, wersja 2.2.3, The University of Oxford) wykorzystano do określenia markerów funkcji komórek β trzustki (HOMA-%B), wrażliwości na insulinę (HOMA-%S, QUICKI), oraz insulino-oporności (HOMA-IR). Wskaźniki te zostały wyliczone na podstawie stężeń glukozy i insuliny na czczo. Prawidłowe wartości to 100% dla HOMA-%B i HOMA-%S, 1,0 dla HOMA-IR oraz ≥ 0,34 dla OUICKI (Wallace *et al.* 2004),
- **Wyliczono wskaźnik stanu zapalnego - SII** (systemie immune-inflammation index) na podstawie wzoru zaproponowanego przez Chena i współpracowników: SII = (P × N)/L, gdzie P, N, and L odnosi się do liczny płytek krwi, neutrofili i limfocytów (Chen *et al.* 2017).

2.2.5. Procedura jednorazowego wysiłku fizycznego i protokołów treningowych

- **Jednorazowy wysiłek fizyczny do odmowy** – który stanowił wysiłek przeprowadzony na bieżni, aż do odmowy wykonywanej pracy, w celu określenia maksymalnego poboru tlenu (VO_2max). Test został przeprowadzony w godzinach porannych, 3 godziny po ostatnim posiłku. Wszyscy uczestnicy przed wysiłkiem zjedli jednakowe śniadanie o wartości kalorycznej około 300-400 kcal. Otrzymali także wodę do picia oraz zalecenie powstrzymania się do kawy i herbaty. Początkowa prędkość na bieżni wynosiła 4 km/h i po 3 minutach została zwiększona do 8 km/h. Następnie, prędkość wzrastała o 2 km/h co każde 3 minuty, aż do odmowy wykonywanej pracy. Pobór tlenu mierzono za pomocą analizatora gazów wydychanych (CortexMetaLyzer 3B, Lipsk, Niemcy) i analizowano przy użyciu oprogramowania MetasoftStudio v. 5.1.0 (CortexMetaLyzer3B; CortexBiophysik,

Lipsk, Niemcy). Polar Bluetooth Smart H6 (Polar Electro Oy, Kempele, Finlandia) był używany do rejestrowania tętna (bpm).

- **Trening siłowo – wytrzymałościowy**: 12-tygodniowy trening wytrzymałościowo-siłowy o umiarkowanej intensywności. Uczestnicy trenowali 3 razy w tygodniu (łącznie 36 jednostek treningowych), w poniedziałki, środy i piątki. Każdy z treningów trwał 50 minut i składał się z 5 minutowej rozgrzewki, 20 minut treningu aerobowego, 20 minut ćwiczeń siłowych i na zakończenie 5 minut rozluźnienia. Intensywność wysiłku podczas pierwszych 4 tygodni wynosiła 60% HRmax, natomiast podczas kolejnych 4 tygodni stopniowo wzrastała do 80% HRmax. Intensywność treningu była monitorowana przy użyciu pulsoksymetru (Polar Heart Rate Monitor, Polar Electro, Finland). Część wytrzymałościowa składała się z ćwiczeń fitness i równoważnych wykonywanych do muzyki, natomiast część siłowa składała się z ćwiczeń z obciążeniem własnego ciała oraz obciążeniem zewnętrznym (kettle, taśmy oporowe) mających na celu wzmocnienie mięśni brzucha oraz mięśni kończyn górnych i dolnych. Uczestnicy wykonywali po 12-25 powtórzeń każdego ćwiczenia, z 30-60 sekundową przerwą między ćwiczeniami, z obciążeniem 2-5kg. Intensywność ćwiczeń oporowych wzrastała co 4 tygodnie (12–15 powtórzeń w tygodniach 1–4, 15–20 powtórzeń w tygodniach 5–8, and 20–25 powtórzeń w tygodniach 9–12). Badani wykonywali takie ćwiczenia jak: zginanie ramion w podporze przodem na kolanach, przysiady, wykroki w przód i w tył, brzuszki, wznosy ramion, wyciskanie hantli i wspięcia na palce. Minimalna frekwencja na zajęciach wynosiła 80%, natomiast średnia frekwencja uczestników badania 89%.
- **Trening równowagi –** 12-tygodniowy trening równowagi. Wykonywany był 3 razy w tygodniu, przez 50-minut (36 jednostek treningowych). Każda sesja treningowa obejmowała 10 minut rozgrzewki, 30 minut treningu równowagi oraz 10 minut rozciągania i ćwiczeń oddechowych. Trening równowagi odbywał się przy użyciu niestabilnych urządzeń treningowych – dysków sensomotorycznych i piłek gimnastycznych. Intensywność treningu zwiększała się stopniowo w ciągu 12 tygodni poprzez zmniejszenie powierzchni podparcia, zwiększenie szybkości ruchu i stosowanie bardziej wymagających ćwiczeń równoważnych. Trudność treningu została zwiększana w celu uniknięcia przyzwyczajenia badanych do wykonywanych ćwiczeń równoważnych i zapewnienia optymalnej poprawy równowagi i kontroli postawy. Każda sesja treningowa odbywała się pod nadzorem fizjoterapeuty (magister fizjoterapii ze specjalnością prowadzenia programów treningowych z osobami

starszymi). Do analizy statystycznej zakwalifikowano jedynie tych uczestników, którzy uczestniczyli w co najmniej 80% treningów.

- **Trening tańca:** 50-minutowy trening taneczny w rytmie polskiej muzyki ludowej. Trening odbywał się w grupie na sali treningowej i wykonywany był 3 razy w tygodniu przez 12 tygodni. Każda sesja treningowa składała się z 10 minut rozgrzewki, 30 minut treningu tańca ludowego obejmującego naukę choreografii, wykonywanie figur tanecznych i ćwiczenia fitness. Trening kończył się 10-minutowymi ćwiczeniami rozciągającymi i relaksacyjnymi. Program taneczny obejmował poprawę umiejętności tanecznych, poprzez naukę nowych figur tanecznych i choreografii, a także wykonywanie ćwiczeń koordynacyjnych. Ze względu na zmiany zwyrodnieniowe stawów obecne u osób w starszym wieku, w programie treningowym unikano skoków i przeciążania stawów. Aby uzyskać lepszą adaptację treningową, trener prowadzący zajęcia zwiększał intensywność i trudność ćwiczeń po każdych 4 tygodniach interwencji treningowej, stosując szybszy rytm muzyki lub bardziej skomplikowane figury taneczne. Każda sesja treningowa odbywała się pod nadzorem wykwalifikowanego trenera tańca ludowego. Intensywność treningu była w zakresie 60-80% HRmax (odpowiednio początek i koniec okresu treningowego). Do analizy statystycznej zakwalifikowano jedynie tych uczestników, którzy uczestniczyli w co najmniej 80% treningów.

2.2.6. Analiza statystyczna

Analiza statystyczna została przeprowadzona przy użyciu programu Statistica (Statistica v. 13.0, StatSoft Inc.). Wyniki badań w opublikowanych pracach przedstawiono jako średnia ± odchylenie standardowe. W zależności od analizowanej grupy badanej dobrano i zastosowano odpowiednie testy statystyczne, które szczegółowo zostały opisane w publikacjach 1-3. Dodatkowo obliczono współczynniki korelacji oraz wielkość efektu uzyskanych zmian. Zastosowane metody statystyczne są szczegółowo opisane w publikacjach. Zastosowane metody były również weryfikowane i rozszerzane w zależności od przebiegu procesu recenzji.

GŁÓWNE WYNIKI PRZEPROWADZONYCH BADAŃ ORAZ WNIOSKI

3.1. Główne wyniki badań opisane w pracy pt. *Changes in selected exerkines concentration post folk-dance training are accompanied by glucose homeostasis and physical performance improvement in older adults*

Głównym celem pracy było zbadanie, czy 12 tygodniowy trening równowagi oraz trening tańca poprawią możliwości wysiłkowe, zmniejszą insulino-oporność, a także spoczynkowe ciśnienie krwi w grupie osób starszych. Dodatkowo porównałam skuteczność obu interwencji treningowych w poprawie insulino-wrażliwości i stężeń wybranych exerkin.

Wyniki badań uzyskane w tej pracy wskazują, że zarówno trening równowagi, jak i trening tańca spowodował poprawę sprawności funkcjonalnej oraz równowagi dynamicznej, co znajdowało odzwierciedlenie w lepszych wynikach testów – wstań i idź oraz 6-minutowego testu chodu.

Potreningowa poprawa insulino-wrażliwości została zaobserwowana w grupie poddanej treningowi tańca. W tej grupie zaobserwowaliśmy zmniejszenie wartości wskaźnika HOMA-IR, a z kolei zwiększenie HOMA-%S i QUICKI. W grupie, która wykonywała trening równowagi, jedynie odnotowano tendencję do poprawy tych wskaźników. Ponadto, zaobserwowaliśmy zmniejszenie nasilenia stanu zapalnego wyrażonego wskaźnikiem SII. Zmiana ta była istotna w grupie BG, natomiast tendencja do zmiany została obserwowana w grupie DG. Obie interwencje treningowe spowodowały istotne zmniejszenie ciśnienia skurczowego krwi, natomiast po treningu równowagi także ciśnienia rozkurczowego.

Tym pozytywnym zmianom w zakresie sprawności funkcjonalnej, insulino-wrażliwości, redukcji stanu zapalnego czy ciśnienia krwi towarzyszyły także modyfikacje stężeń badanych exerkin – iryzyny i BDNF. Zastosowane treningi spowodowały zmniejszenie stężenia białka BDNF oraz zwiększenie stężenia iryzyny we krwi. Zaobserwowane zależności zmian stężeń badanych exerkin z towarzyszącą poprawą wrażliwości na działanie insuliny mogą sugerować ich udział w zmianach indukowanych treningiem.

Ciekawy jest fakt, iż po zastosowanym 12-tygodniowym treningu tańca zaobserwowaliśmy istotną redukcję białka CAF, czemu towarzyszyła poprawa możliwości wysiłkowych i sprawności funkcjonalnej. Wyniki te mogą sugerować, że ta interwencja treningowa ma lepszy potencjał w prewencji sarkopenii oraz degeneracji połączeń nerwowo – mięśniowych u osób w starszym wieku.

Podsumowując, zastosowane 12 tygodniowe treningi zarówno tańca jak i równowagi są skutecznymi metodami poprawiającymi sprawność funkcjonalną oraz równowagę dynamiczną, a także obniżającymi ciśnienie krwi oraz stan zapalny. Z kolei trening tańca wydaje się być metodą skuteczniejszą w zwalczaniu insulino-oporności, a także zapobieganiu sarkopenii i zwyrodnieniu połączeń nerwowo-mięśniowych. Co więcej, te pozytywne efekty wywołane treningiem fizycznym były związany ze zmianami w stężeniach wybranych exerkin – iryzyny, BDNF, a także białka CAF.

3.2. Główne wyniki badań opisane w pracy pt. *12 weeks of physical exercise attenuates oxidative stress, improves functional tests performance, and reduces fall risk in elderly women independently on serum 25(OH)D concentration*

Moje badania wykazały zależność między spoczynkowym stężeniem witaminy D (25(OH)D), a możliwościami wysiłkowymi, ryzykiem upadku, markerami stresu oksydacyjnego i stanu zapalnego, a także metabolizmem kostnym. W publikacji szukałam odpowiedzi na pytanie czy początkowe stężenie 25(OH)D będzie istotnym czynnikiem regulującym zmiany adaptacyjne indukowane 12 tygodniowym treningiem fizycznym. Eksperyment ten opierał się na założeniu, że regularny trening siłowo-wytrzymałościowy będzie skuteczną metodą poprawy możliwości wysiłkowych, redukcji ryzyka upadku, a także redukcji stresu oksydacyjnego i stanu zapalnego u osób starszych. Uzyskane wyniki sugerują, że grupa osób starszych z wyższym, wyjściowym stężeniem 25(OH)D, charakteryzowała się niższą masą ciała, niższym poziomem tkanki tłuszczowej podskórnej oraz wisceralnej. Towarzyszyło temu niższe stężenie markerów obrotu kostnego – parathormonu i osteokalcyny, co może stanowić istotną rolę w prewencji rozwoju osteomalacji, zwiększonego ryzyka złamań, a także w redukcji incydentalnych upadków, często obserwowanych w tej grupie wiekowej. Co ciekawe, istotne różnice pomiędzy grupami zaobserwowałam wraz z zespołem w poziomie sprawności fizycznej przed rozpoczęciem programu treningowego. Osoby ze stężeniem witaminy D powyżej 30 ng/ml osiągały istotnie lepsze wyniki w teście wstań i idź, 6 minutowym teście chodu oraz w teście oceniającym ryzyko upadku. Można zatem sugerować, że niewystarczające stężenie witaminy D jest istotnym czynnikiem związanym z pogarszaniem się sprawności fizycznej oraz zwiększonym ryzykiem upadku u osób starszych. Niedobór 25(OH)D istotnie determinował także obecność stanu zapalnego w grupie badanych osób: charakteryzowały się one wyższym stężeniem IL-6. Biorąc pod uwagę uzyskane wyniki, potwierdziłam dane literaturowe wskazujące, iż utrzymanie optymalnego stężenia witaminy D u osób w starszym wieku jest istotne z punktu

widzenia poprawy jakości życia, czy też zapobiegania niesprawności, ale również może redukować stan zapalny związany z procesem starzenia się.

Wyniki uzyskane po przeprowadzeniu 12 – tygodniowego treningu wytrzymałościowo – siłowego wykazały poprawę możliwości wysiłkowych – skrócenie czasu wykonywania testu wstań i idź, wydłużenie dystansu pokonanego podczas 6 – minutowego testu chodu oraz zmniejszenie ryzyka upadku w obu trenujących grupach, zarówno z deficytem (EID) jak i z optymalnym stężeniem (ESD) witaminy D. W grupach kontrolnych takich zmian nie odnotowaliśmy. Po zakończeniu cyklu treningowego zaobserwowaliśmy ponadto zwiększenie stężenia grup SH zarówno w grupie EID jak i ESD, co świadczy o poprawie zdolności antyoksydacyjnych.

Z kolei zmiany w markerach metabolizmu kostnego: redukcja stężenia zarówno PTH jak i OC zostały wykazane jedynie w grupie z niedoborem 25(OH)D, która charakteryzowała się wyjściowo wyższym stężeniem tych białek. Wynik ten może być istotny z punktu widzenia osób z wtórną nadczynnością przytarczyc i zwiększonym stężeniem parathormonu spowodowanym niedoborem witaminy D.

Wyniki uzyskane w tej pracy sugerują, że 12 tygodni treningu o umiarkowanej intensywności poprawia możliwości wysiłkowe i sprawność funkcjonalną osób starszych oraz zwiększa ochronę antyoksydacyjną, niezależnie od wyjściowego stężenia witaminy D. To wskazuje, że pomimo niewątpliwego, korzystnego działania witaminy D w procesie starzenia się, najważniejszym czynnikiem poprawiającym jakość życia jest aktywność fizyczna o odpowiednio dobranej formie i intensywności.

3.3. Główne wyniki badań opublikowane w pracy pt. *Plasma concentration of irisin and brain-derived-neurotrophic factor and their association with the level of erythrocyte adenine nucleotides in response to long-term endurance training at rest and after a single bout of exercise*

Analiza danych dotyczących mechanizmów starzenia wykazała, iż niewiele jest badań dotyczących poziomu nukleotydów purynowych w erytrocytach oraz stężenia metabolitów purynowych w osoczu w grupie osób starzejących się. Żadna z publikacji nie podejmowała jak dotąd określenia zależności między poziomem białka BDNF i iryzyną, a stężeniem metabolitów purynowych w osoczu.

W badaniach wykorzystaliśmy wyjątkową grupę badaną – sportowców „Masters" biorących udział w międzynarodowych zawodach, których staż treningowy wynosił średnio 38 lat i porównaliśmy ją z wynikami grupy nietrenujących mężczyzn w podobnym wieku.

Wykazaliśmy po raz pierwszy, że długotrwały staż treningowy i wywołane nim zmiany adaptacyjne prowadzą do modyfikacji spoczynkowych stężeń exerkin i poziomu nukleotydów purynowych. Efekt wieloletniego treningu był wyrażony w wyższym statusie energetycznym erytrocytów (wyższy poziom ATP, ATP/ADP i AEC w czerwonych krwinkach), a wartości te były pozytywnie skorelowane z wydolnością fizyczną (VO_2max). Ponadto, osoby trenujące charakteryzowały się niższym stężeniem hipoksantyny (Hx) – produktu degradacji nukleotydów purynowych, niższym stężeniem insuliny oraz lepszymi parametrami insulino-wrażliwości (niższa wartość wskaźnika HOMA-IR i HOMA-%B oraz wyższa HOMA-%S). Wykazaliśmy także różnice w porównaniu z grupą kontrolną w stężeniu exerkin (wyższe stężenie białka BDNF i niższe iryzyny). Stężenie exerkin było również związane z poziomem wydolności fizycznej. Zaobserwowaliśmy negatywną korelację pomiędzy iryzyną oraz dodatnią pomiędzy stężeniem BDNF, a poziomem wydolności fizycznej mierzonej za pomocą VO_2max. Różnice te sugerowały nie tylko lepszy status metabolizmu krwinek czerwonych, ale również lepszą adaptację mięśni szkieletowych do wysiłku fizycznego u osób wysoko wytrenowanych.

Natomiast jednorazowy wysiłek fizyczny spowodował zmniejszenie stężenia białka BNDF, jednakże tylko w grupie trenującej. W obu grupach trenującej i kontrolnej zaobserwowano zmiany w poziomie nukleotydów purynowych w erytrocytach – zwiększenie poziomu ATP, wartości ATP/ADP oraz AEC, także znaczący wzrost Hx. Powyższe wskaźniki były również dodatnio skorelowane z poziomem VO_2max, u wszystkich badanych. Korelacja ta sugeruje, że wyższy status energetyczny erytrocytów prowadził do poprawy zaopatrzenia w tlen pracujących mięśni w warunkach zwiększonego zapotrzebowania energetycznego podczas maksymalnego wysiłku fizycznego. Zatem poziom ATP, stosunek ATP/ADP i AEC mogą być istotnymi wskaźnikami adaptacji do intensywnego wysiłku fizycznego.

Publikacja ta wykazała po raz pierwszy potencjalny wpływ zarówno iryzyny, jak i białka BNDF na metabolizm energetyczny erytrocytów. Zaobserwowaliśmy dodatnią korelację pomiędzy stężeniem BDNF, a statusem energetycznym erytrocytów – stosunku ATP/ADP oraz wskaźnikiem AEC. Z kolei ujemna korelacja została zaobserwowana pomiędzy stężeniem BDNF, a poziomem ADP w erytrocytach i stężeniem Hx w osoczu. Podobnie iryzyna była powiązana ze statusem energetycznym erytrocytów, co wyrażało się negatywną korelacją ze wskaźnikiem AEC oraz pozytywną z AMP oraz Hx. Wyniki te wskazują, że exerkiny również mogą być potencjalnymi regulatorami statusu energetycznego erytrocytów oraz mogą brać udział w regulacji mięśniowej puli nukleotydów adeninowych.

PODSUMOWANIE I WNIOSKI

Badania ostatnich lat skupiają się na poznaniu metod i mechanizmów wspomagających zdrowe starzenie się, ze względu na szybko rosnącą populację osób starszych, a w konsekwencji rosnące koszty ekonomiczne (Chen *et al.* 2021). Wcześniejsze badania wykazały, że regularna aktywność fizyczna wykonywana w starszym wieku może przyczynić się do zapobiegania niesprawności, obniżenia jakości życia, a także redukcji ryzyka rozwoju chorób związanych z wiekiem, takich jak cukrzyca typu 2, nadciśnienie czy demencja (Vagetti *et al.* 2014; McPhee *et al.* 2016). Dlatego też celem powyższej rozprawy doktorskiej była kontynuacja badań w celu poszukiwania najbardziej efektywnych metod aktywności fizycznej w grupie starzejących się osób, z uwzględnieniem mechanizmów z związanych ze zdrowym starzeniem się.

. Wszystkie zastosowane programy treningowe, zarówno 12 tygodniowy trening wytrzymałościowo – siłowy, trening równowagi, jak i trening tańca spowodowały istotną poprawę możliwości wysiłkowych, sprawności funkcjonalnej i równowagi dynamicznej u osób starszych, co jest szczególnie istotne z punktu widzenia sprawności i niezależności w starszym wieku. Z kolei poprawa równowagi dynamicznej może zapobiegać potencjalnym upadkom w przyszłości, a co za tym idzie zapobiegać niesprawności, ograniczeniu niezależności, jak i prowadzić do poprawy jakości życia.

Wykazano, że jednym z czynników, który odpowiada za pozytywny efekt treningu są białka uwalniane w odpowiedzi na wysiłek fizyczny, między innymi z tkanki mięśniowej, nazywane exerkinami (Chow *et al.* 2022). Zarówno 12 tygodniowy trening równowagi, jak i trening tańca stosowany w grupie osób starszych spowodował istotne zmniejszenie stężenia BDNF, a także zwiększenie stężenia iryzyny. Zmianom tym towarzyszyła istotna poprawa możliwości wysiłkowych, zmniejszenie wartości wskaźników insulino-oporności, redukcja ciśnienia krwi oraz stanu zapalnego. Poprawa w zakresie insulino-wrażliwości była szczególnie zaznaczona w grupie osób wykonujących trening tańca. Co również ciekawe, zaobserwowaliśmy istotne zmiany potreningowe w białku CAF, które stanowi marker funkcjonowania połączeń nerwowo – mięśniowych, a w związku z tym może być wskaźnikiem atrofii mięśni oraz obniżenia możliwości wysiłkowych. W naszych badaniach istotny spadek stężenia CAF został odnotowany w grupie trenującej taniec, równocześnie z poprawą wskaźników równowagi dynamicznej oraz możliwości wysiłkowych. Uzyskane

wyniki sugerują, że trening tańca jest skuteczniejszy w zapobieganiu degeneracji włókien nerwowo – mięśniowych, a potencjalny mechanizm tych zmian może leżeć u podstaw angażowania funkcji motorycznych, poznawczych, i zdolności koordynacyjnych w tańcu, co finalnie może przyczyniać się do poprawy neuroplastyczności mózgu (Teixeira-Machado *et al.* 2019). Ponadto, wyniki naszych badań wykazały, że osoby w starszym wieku posiadające długotrwały/wieloletni staż treningowy wykazują istotne różnice w stężeniach exerkin, zarówno w spoczynku, jak i po maksymalnym jednorazowym wysiłku fizycznym w porównaniu do osób nietrenujących.

Uzyskane modyfikacje stężeń exerkin wywołane regularnym treningiem fizycznym mogą być potencjalnymi mediatorami jego pozytywnych efektów u osób w starszym wieku, przyczyniając się do zdrowego starzenia się między innymi poprzez zmiany statusu antyoksydacyjnego, w tym zwiększenie stężenia grup SH we krwi.

Rola w zdrowym starzeniu się przypisywana jest także optymalnemu stężeniu witaminy D. Wcześniejsze badania sugerowały, że niedobór witaminy D może być powiązany ze zwiększonym ryzykiem wystąpienia osteoporozy i złamań, a także nasilonym stresem oksydacyjnym, stanem zapalnym, czy gorszymi możliwościami wysiłkowymi oraz większym ryzykiem upadku (Berridge 2017; Sizar *et al.* 2022). Wyniki powyższej rozprawy również wskazują na istnienie korelacji pomiędzy stężeniem witaminy D, a markerami obrotu kostnego, sprawnością fizyczną, ryzykiem upadku, a także stanem zapalnym. Niedobór 25(OH)D (<30 ng/ml) istotnie determinował gorsze wyniki w wyżej wymienionych parametrach. Pomimo obserwowanej korelacji, 12 tygodniowy trening wytrzymałościowo – siłowy poprawił sprawność fizyczną oraz zmniejszył ryzyko upadku wśród starszych kobiet, a także zwiększył ochronę antyoksydacyjną, niezależnie od tego, czy uczestniczki charakteryzowały się niedoborem czy optymalnym stężeniem witaminy D. Jedyne różnice dotyczyły stężenia osteokalcyny i parathormonu. Zmniejszenie wartości markerów obrotu kostnego po interwencjach treningowych może korzystnie wpłynąć na masę kostną, zwłaszcza u osób charakteryzujących się niedoborem witaminy D, co było już sugerowane we wcześniejszych badaniach (Wieczorek-Baranowska *et al.* 2012).

Podsumowując, skutecznej strategii zdrowego starzenia należy upatrywać w regularnej aktywności fizycznej o odpowiedniej formie i intensywności, a mechanizm jej działania może być powiązany ze zmianami stężeń exerkin, a także zmniejszeniem nasilenia stresu oksydacyjnego.

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STRESZCZENIE

Temat prozdrowotnego wpływu treningu fizycznego w zdrowym starzeniu się jest coraz lepiej poznawany. Jednak mechanizmy związane ze zmianami adaptacyjnymi indukowanymi przez różne formy wysiłku fizycznego nie są do końca poznane i wyjaśnione. Sugeruje się, że odpowiedzialne za pozytywny wpływ aktywności fizycznej są między innymi exerkiny, regulujące odpowiedź przeciwzapalną, metabolizm glukozy czy nasilenie stresu oksydacyjnego.

Celem przeprowadzonych przeze mnie badań była ocena wpływu zarówno jednorazowego wysiłku fizycznego jak i różnych form długotrwałych treningów wytrzymałościowych u osób starszych na stężenia wybranych exerkin. Kolejnym celem było także zweryfikowanie czy zaobserwowane zmiany będą miały wpływ na sprawność funkcjonalną, markery stanu zapalnego, metabolizmu energetycznego oraz insulino-oporności w grupie badanych osób.

W badaniach wzięło udział 100 osób w wieku 67.4 ± 4.8 lat. Uczestnicy losowo zostali przydzieleni do grup trenujących i kontrolnych. Osoby poddane jednorazowemu wysiłkowi fizycznemu były analizowane pod kątem adaptacji do wysiłku fizycznego. Przy użyciu wysokosprawnej chromatografii cieczowej analizie poddany został status energetyczny erytrocytów wyrażony poziomem ATP, ATP/ADP i wartością ładunku energetycznego – AEC oraz stężenie metabolitów purynowych – hipoksantyny i kwasu moczowego. W badaniach zastosowano 12 tygodniowy trening siłowo-wytrzymałościowy, równowagi lub trening tańca. Przed przystąpieniem do badań uczestnicy zostali poddani badaniu lekarskiemu w celu wykluczenia przeciwwskazań do treningu. Przed rozpoczęciem i po zakończeniu każdego z cykli treningowych, pobrano krew w celu oznaczenia stężenia wybranych miokin/exerkin takich jak neurotroficzny czynnik pochodzenia mózgowego (BDNF), iryzyna, C-końcowy fragment agryny (CAF), osteokalcyna, parathormon, oraz miostatyna metodą immunoenzymatyczną, markerów stresu oksydacyjnego – stężenie grup sulfhydrylowych (SH) i dialdehydu malonowego (MDA) spektrofotometrycznie. Wyliczono także wskaźniki insulino-oporności (QUICKI, HOMA-IR, HOMA-%B, HOMA-%S) oraz stanu zapalnego (systemie immune inflammation index – SII). Przeprowadzono również badanie sprawności funkcjonalnej przy pomocy wybranych testów sprawności takich jak test wstań i idź (TUG), czy 6-minutowy test chodu (6MWT) oraz testu ryzyka upadku.

Dodatkowo w przypadku treningu siłowo-wytrzymałościowego oceniono wpływ stężenia witaminy D na odpowiedź treningową.

Przeprowadzone badania wykazały, że długotrwały trening, zarówno taneczny jak i równoważny spowodował zmniejszenie stężenia białka BDNF oraz zwiększenie stężenia iryzyny. Trening tańca był skuteczny w redukcji białka odpowiedzialnego za rozwój atrofii - CAF. Zastosowane procedury treningowe spowodowały również zmniejszenie wskaźników insulino-oporności, a efekt ten był szczególnie widoczny w grupie tanecznej. Dodatkowo przeprowadzony 12-tygodniowy trening wytrzymałościowo-siłowy zwiększył stężenie grup SH, co świadczy o poprawie zdolności antyoksydacyjnych. Wszystkie powyższe interwencje treningowe spowodowały także poprawę możliwości wysiłkowych, sprawności funkcjonalnej oraz równowagi dynamicznej osób starszych. Istotnym wydaje się być również fakt, że poprawę adaptacji do wysiłku fizycznego obserwowaliśmy niezależnie od wyjściowego stężenia witaminy D.

Zmiany stężenia wybranych miokin w odpowiedzi na jednorazowy wysiłek fizyczny mogą być zależne od stopnia adaptacji do wysiłku fizycznego. Długoletni staż treningowy (38 lat) podnosi stężenia BNDF i iryzyny. Co również ciekawe, wyjściowe stężenie tych exerkin było związane z poziomem wydolności fizycznej. Zaobserwowaliśmy istotną korelację pomiędzy zarówno iryzyną, jak i BDNF, a poziomem VO_2max. Efekt treningowy był również wyrażony lepszym statusem energetycznym erytrocytów (wyższy poziom ATP, ATP/ADP i AEC w czerwonych krwinkach), a także zwiększoną insulino-wrażliwością.

Podsumowując, powyższe wyniki dowodzą skuteczności zastosowanych interwencji treningowych na poprawę sprawności funkcjonalnej osób starszych, a także na zapobieganie rozwojowi chorób przewlekłych związanych ze starzeniem się. Potencjalnie istotną rolę prowadzącą do korzystnych, prozdrowotnych tych efektów treningu mogą odgrywać exerkiny.

SUMMARY

The health – promoting effect of physical training is becoming more extensive and popular. However, the mechanisms of these positive changes induced by various training programs are not fully understood and explained. Last years it has been suggested that the beneficial effect of physical activity is mediated by exerkines regulating inflammation, glucose metabolism, or oxidative stress intensity.

The aim of this research was to assess the impact of both acute physical exercise and various forms of long-term endurance training on the concentrations of selected exerkines among older adults. We also want to verify if observed changes may affect functional mobility, as well as markers of oxidative stress, energy metabolism and insulin resistance.

In the study took part 100 adults aged 67.4 ± 4.8 . Participants were randomly assigned into trained and control groups. The effect of 12 weeks combined aerobic-resistance, balance or dance trainings were investigated. Additionally, subjects performed the acute physical exercise. They were analyzed in terms of long-lasting adaptation to physical exercise. High-performance liquid chromatography was used to assess the erythrocytes' energy status expressed by the level of ATP, ATP/ADP and the adenylate energy charge – AEC, as well as the concentration of purine metabolites – hypoxanthine and uric acid. The medical examination was carried out to exclude a contraindications to exercise. Before and after each training intervention the blood was collected to measure the concentrations of selected myokines/exerkines such as brain-derived neurotrophic factor (BDNF), irisin, C-terminal agrin fragment (CAF), osteocalcin, parathormon, and myostatin by immunoenzymatic method, indicators of oxidative stress – concentration of sulfhydryl groups (SH) and malondialdehyde (MDA) using spectrophotometric method. The markers of insulin-resistance (QUICKI, HOMA-IR, HOMA-%B, HOMA-%S) and inflammation (systemic immune inflammation index – SII) was also calculated. The functional mobility and fall risk tests were also performed by several physical tests such as up and go test (TUG) or 6-minute walk test (6MWT). Additionally, in case of aerobic-resistance training we analyzed the impact of baseline vitamin D concentration on physical training responses.

The current research showed that long-lasting training both dance and balance decreased BDNF and increased irisin concentration. Also CAF, a protein involved in muscle atrophy development, was reduced after dance program. What is more applied interventions attenuated insulin-resistance, and this effect was exacerbated in dance training group. In

addition performed 12-weeks combined aerobic-resistance training increased the concentration of SH groups, what may reflect improved antioxidant protection. All above mentioned training interventions ameliorate exercise capacity, functional mobility, and dynamic balance among older adults. What is also interesting, an enhancement in physical exercise adaptation was observed independently from baseline vitamin D concentration.

The response of myokines to a single exercise may depend on the adaptation to physical exercise. Long-lasting training experience (38 years) increase BDNF and irisin concentration. What's also interesting, the baseline exerkines concentration was related to level of physical performance. We observed a significant correlation between both irisin and BDNF, and VO2max status. The regular training effect was also reflected in higher erythrocytes energy status (increased ATP, ATP/ADP and AEC level in red blood cells), as well as higher status of insulin-sensitivity.

To sum up, the above results prove the effectiveness of applied training interventions on functional mobility improvement, and alsoon prevention the development of chronic diseases associated with aging. However, a potential, important role leading to the beneficial, health-promoting effects of training may play an exerkines.

SZKOŁA DOKTORSKA

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

Rodziewicz-Flis, E.A., Kawa, M., Kaczor J.J., Szaro-Truchan, M., Flis, D.J., Lombardi, G., Ziemann, E. (2023). Changes in selected exerkines concentration post folk-dancetraining are accompanied by glucose homeostasis and physical performance improvement in older adults. *ScientificReports, 13*, 1-11. https://doi.org/10.1038/s41598-023-35583-w

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Rodziewicz-Flis, E.A., Kawa, M., Flis, D.J., Szaro-Truchan, M., Skrobot, W., Kaczor J.J., (2022). 12 weeks of physical exercise attenuates oxidative stress, improves functional tests performance, and reduces fall risk in elderly women independently on serum 25(OH)D concentration. *Frontiers in physiology, 13*, 1-13. https://doi.org/10.3389/fphys.2022.809363

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OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

Rodziewicz, E.A., Król-Zielińska, M., Zieliński, J., Kusy, K., Ziemann, E. (2020). Plasma concentration of irisin and brain-derived-neurotrophic factor and their association with the level of erythrocyte adenine nucleotides in response to long-term endurance training at rest and after single bout of exercise. *Frontiers in Physiology, 11*, 1-10. https://doi.org/ 10.3389/fphys.2020.00923

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WYKAZ PRAC STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

Przedstawiona rozprawa doktorska pod tytułem: „Analiza wybranych czynników regulujących zmiany adaptacyjne w odpowiedzi na podejmowaną aktywność fizycznąw grupie osób starzejących się" składa się z cyklu trzech prac opublikowanych w czasopismach zagranicznych o łącznej punktacji Impact Factor (IF) łącznej 12.366 pkt oraz Ministerstwa Edukacji i Nauki (MEiN) równej 340 pkt:

Publikacja I: Changes in selected exerkines concentration post folk-dance training are accompanied by glucose homeostasis and physical performance improvement in older adults.(2023) Rodziewicz-Flis Ewa Aleksandra, Kawa Małgorzata Jolanta, Kaczor Jan Jacek, Szaro-Truchan Marzena, Flis Damian Józef, Lombardi Giovanni, Ziemann Ewa;*Scientific Reports*; DOI: 101038/s41598-023 35583-w; IF: 3.800; MEiN: 140.000

Publikacja II: 12 weeks of physical exercise attenuates oxidative stress, improves functional tests performance, and reduces fall risk in elderly women independently on serum 25(OH)D3 concentration (2022) Ewa Aleksandra Rodziewicz-Flis, Małgorzata Kawa, Damian Józef Flis, Wojciech Skrobot, Jan Jacek Kaczor; *Frontiers in Physiology*; DOI: 10.3389/fphys.2022.809363; IF: 4.000; MEiN:100

Publikacja III: Plasma concentration of irisin and brain-derived-neurotrophic factor and their association with the level of erythrocyte adenine nucleotides in response to long-term endurance training at rest and after a single bout of exercise (2020) Rodziewicz-Flis Ewa Aleksandra, Król-Zielińska Magdalena, Zieliński Jacek, Kusy Krzysztof, Ziemann Ewa; *Frontiers in Physiology*; DOI: 10.3389/fphys.2020.00923; IF: 4.566; MEiN: 100.000

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Changes in selected exerkines concentration post folk-dance training are accompanied by glucose homeostasis and physical performance improvement in older adults

Ewa Aleksandra Rodziewicz-Flis[1,6], Małgorzata Kawa[1,6], Jan Jacek Kaczor[2], Marzena Szaro-Truchan[1], Damian Józef Flis[3], Giovanni Lombardi[4,5] & Ewa Ziemann[5]✉

The study aimed to evaluate the impact of selected exerkines concentration induced by folk-dance and balance training on physical performance, insulin resistance, and blood pressure in older adults. Participants (n = 41, age 71.3 ± 5.5 years) were randomly assigned to folk-dance (DG), balance training (BG), or control group (CG). The training was performed 3 times a week for 12 weeks. Physical performance tests—time up and go (TUG) and 6-min walk test (6MWT), blood pressure, insulin resistance, and selected proteins induced by exercise (exerkines) were assessed at baseline and post-exercise intervention. Significant improvement in TUG (p = 0.006 for BG and 0.039 for DG) and 6MWT tests (in BG and DG p = 0.001), reduction of systolic blood pressure (p = 0.001 for BG and 0.003 for DG), and diastolic blood pressure (for BG; p = 0.001) were registered post-intervention. These positive changes were accompanied by the drop in brain-derived neurotrophic factor (p = 0.002 for BG and 0.002 for DG), the increase of irisin concentration (p = 0.029 for BG and 0.022 for DG) in both groups, and DG the amelioration of insulin resistance indicators (HOMA-IR p = 0.023 and QUICKI p = 0.035). Folk-dance training significantly reduced the c-terminal agrin fragment (CAF; p = 0.024). Obtained data indicated that both training programs effectively improved physical performance and blood pressure, accompanied by changes in selected exerkines. Still, folk-dance had enhanced insulin sensitivity.

Reduced daily physical activity, which has been further pushed by the SARS-CoV-2 pandemic period is observed both in young and older adults [1,2]. The sedentary lifestyle increased risk of developing many chronic diseases and pathological states such as obesity, insulin resistance, chronic inflammation, type 2 diabetes, and cardiovascular disease (CVD)[3]. these consequences are closely related to sarcopenia and physical performance deterioration[4] In addition, this series of problems mainly affect the elderly because, on the one hand, aging triggers and accelerates these pathological states and, on the other hand, by contextualizing this phenomenon into the current time, aged people are the group that mainly suffers from the "side effects" of the pandemics[5,6]. Thus, searching for potential effective methods that may ameliorate a condition of insulin resistance and counteract the development of type 2 diabetes seems to be essential in preventing dementia or sarcopenia, leading at the same time to healthy aging.

Physical exercise can convey a protective effect against aging-related inflammation, releasing proteins named myokines into the bloodstream during muscle contractions[7,8]. Although, the more than 600 myokines is known, biological function has been described for only 5% of them[9]. Available data have documented the role of selected myokines in lipid and glucose metabolism, or muscle hypertrophy. Therefore, they may be useful biomarkers

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in monitoring the effectiveness of regular physical activity. Myokines can interact between distant structures and stimulate other organs to release proteins in response to exercise, which are named exerkines[10]. Among several exerkines: brain-derived neurotrophic factor (BDNF)[10] and irisin[11] are known as promising exercise-dependent mediators with health-promoting effects. BDNF's primary function is to regulate neurogenesis and cognitive functions, but it also affects lipid and glucose metabolism[12,13] in both the central nervous system and the periphery. A recently published study identified a new β-cell signalling pathway regulating insulin secretion in mammals and demonstrated the role of BDNF as a soluble ligand activating TrkB.T1 receptor expressed by β-cells of the pancreas[14]. Most published studies indicated that circulating BDNF levels increase in response to single exercise bouts and regular physical activity[15,16]. Moreover, animal models revealed that irisin may cross the blood–brain barrier and enter the central nervous system, where it can stimulate the expression of BDNF[17]. It has also been suggested that irisin plays a crucial role in regulating glucose and lipid metabolism in adipose tissue and skeletal muscles[18].

Myostatin together with C-terminal agrin fragment (CAF) control skeletal muscle mass loss and have a particular meaning in counteracting the development of muscle atrophy and sarcopenia[19–22]. Myostatin is responsible for skeletal muscle wasting by inhibiting protein synthesis and regenerative processes, as occurs in aging. In turn, CAF through inducing muscle wasting is considered as a clinical biomarker for neuromuscular junction (NMJ) degeneration [23]. Its increased circulating concentration, secondary to enhanced agrin cleavage, reflects progressive myofibers denervation that finally contributes to muscle dysfunction and atrophy[24].. Although it is known that physical training prevents motor neuron and NMJ degeneration, muscle fiber denervation, and loss of motor units, along with the modification of circulating myostatin and CAF, the conclusions are not consistent[25–27].

One of the most popular forms of physical activity among the elderly is Nordic Walking (NW), still there is need to search for the most effective training methods and potential mechanisms leading to healthy aging. Nowadays, more attention is paid to dance-based interventions, that comprised several different stimuli, what may lead to improvement in physical fitness, balance, muscle strength and extended neuroplasticity in healthy older adults[28,29]. Thus, the aim of this study was to assess the effectiveness of two training programs—folk-dance and balance on older adults' physical performance, insulin resistance, blood pressure, inflammation, and lipid profile in conjunction with selected exerkines changes. We have assumed that folk-dance training will support healthy aging more effectively than other types of training—by improving physical performance, blood pressure and glucose metabolism. Moreover, we hypothesized that the positive effect of physical training will be accompanied by changes in blood concentrations of exerkines.

Results

Baseline participant's characteristics. The group's characteristics are presented in Table 1. All participants reported low daily physical activity levels measured by The Physical Activity Questionnaire—IPAQ (short version). There were significant baseline differences between BG and CG groups in the following parameters: height, fat-free mass (FFM), skeletal muscle mass (SMM), total cholesterol (TC),and low-density lipoprotein (LDL-C). The applied training intervention did not cause any change in the body composition.

Changes in physical performance after 12 weeks of training. The 12-week training programs positively impacted participants' physical performance, functional balance, and mobility. Women in both BG and DG groups showed significant improvement in the distance covered during the 6-min walking test (Table 1), whereas no significant differences in the evaluated parameter were noted in the CG. The distance increased from 359.5 ± 35.8 to 424.5 ± 43.8 m in the BG ($p = 0.0001$) and from 369.9 ± 16.7 to 434.1 ± 50.6 m in the DG ($p = 0.0001$). The final differences between BG and CG ($p = 0.020$) and between DG and CG ($p = 0.005$) were statistically significant. The time in the TUG test significantly decreased in both BG (from 7.1 ± 0.9 to 6.1 ± 1.0 s; $p = 0.0006$) and the DG (from 6.9 ± 0.7 to 5.9 ± 0.6 s; $p = 0.0039$) groups post-intervention (Table 1). There were significant differences between BG and CG ($p = 0.0005$) and between DG and CG ($p = 0.0002$) at the end of the experiment. No significant changes were observed in the CG after 12 weeks of intervention.

Alteration in glucose homeostasis markers after 12 weeks of interventions. The 12-week training programs improved glucose metabolism and insulin sensitivity in the cohort of elderly (Fig. 1). The most favorable changes were observed in response to dance training. In the DG group HOMA-IR was reduced from 3.1 ± 2.3 to 2.0 ± 1.2 ($p = 0.023$), HOMA-%S and QUICKI significantly increased from 86.8 ± 38.7 to 119.4 ± 47.1 ($p = 0.020$), and 0.33 ± 0.04 to 0.35 ± 0.03 ($p = 0.035$), respectively (Fig. 1A, C, D). In the BG, there were similar tendencies in HOMA-IR reduction (from 1.9 ± 0.8 to 1.7 ± 0.7) and HOMA-%S and QUCKI improvement (107.9 ± 31.3 to 119.0 ± 35.4, and 0.35 ± 0.02 to 0.36 ± 0.02, respectively) but these changes were not statistically significant. Further, the QUICKI index significantly differed between BG and CG after the intervention ($p = 0.034$). A slight decline in insulin level was noted in both training groups (from 7.6 ± 2.4 to 7.0 ± 2.0 μIU/ml in the BG and from 9.5 ± 3.2 to 7.4 ± 2.7 μIU/ml in the DG) (Fig. 1A). However, the post-intervention values significantly differed between BD and CG ($p = 0.019$) and between DG and CD ($p = 0.039$). After 12 weeks of training, no changes in glucose and insulin concentrations were noted (Figure S1).

Changes in lipid metabolism and inflammatory status after 12 weeks of training. The intervention induced a slight but insignificant reduction of TC concentration in both training groups (from 210.1 ± 31.7 to 199.9 ± 37.4 mg/dl in the BG and from 183.9 ± 28.5 to 168.3 ± 22.4 mg/dl in the DG) (Table 1). No changes were observed in the CG. After 12 weeks of dance training, but not balance training, a tendency to decrease in LDL-C was recorded (from 103.5 ± 25.6 to 91.4 ± 18.4 mg/dl). Any changes were also observed in HDL-C and triglycerides in each group participating in the experiment. Still, we recorded the significant amelioration of

Variables	BG (n = 15)			DG (n = 14)			CG (n = 12)		
	I	II	p*	I	II	p*	I	II	p*
Anthropometric parameters									
Weight (kg)	73.6 ± 15.0	73.3 ± 15.3	0.997	84.5 ± 22.6	83.0 ± 21.6	0.522	88.1 ± 13.5	87.7 ± 13.8	0.499
BMI (kg/m^2)	27.5 ± 4.0	27.8 ± 4.4	0.958	30.4 ± 6.4	29.9 ± 6.0	0.186	29.6 ± 3.7	29.5 ± 4.0	0.999
FFM (kg)	49.3 ± 10.4$^{\#}$	49.6 ± 10.4	0.976	52.7 ± 12.3	51.9 ± 11.8	0.999	60.3 ± 10.3	59.3 ± 9.7	0.584
SMM (kg)	27.0 ± 6.1$^{\#}$	27.3 ± 6.3	0.960	29.2 ± 7.4	29.3 ± 7.0	0.999	33.3 ± 6.0	33.0 ± 5.6	0.708
BFM (kg)	24.3 ± 9.2	24.0 ± 9.7	0.998	31.7 ± 13.6	30.8 ± 13.5	0.162	27.8 ± 8.5	28.7 ± 8.9	0.741
VFA (cm^3)	103.0 ± 34.8	97.2 ± 33.2	0.998	129.4 ± 46.1	118.8 ± 42.9	0.053	124.6 ± 32.0	126.5 ± 34.3	0.999
Lipid profile									
TC (mg/dL)	210.1 ± 31.7$^{\#}$	199.9 ± 37.4	0.555	183.9 ± 28.5	168.3 ± 22.4	0.164	171.0 ± 31.4	174.0 ± 41.7	0.997
TG (mg/dL)	109.9 ± 48.9	105.8 ± 58.7	0.997	116.8 ± 45.5	116.3 ± 31.2	0.999	110.8 ± 43.6	118.4 ± 51.6	0.969
LDL-C (mg/dL)	125.5 ± 28.1$^{\#}$	124.9 ± 60.6	0.998	103.5 ± 25.6	91.4 ± 18.4	0.881	94.4 ± 25.2	99.6 ± 27.9	0.997
HDL-C (mg/dL)	60.0 ± 12.7	64.8 ± 23.2	0.682	55.9 ± 7.0	54.7 ± 8.7	0.999	53.7 ± 12.0	56.3 ± 14.0	0.977
Blood pressure									
SBP (mmHg)	147.2 ± 9.9	133.9 ± 17.6	**0.001**	146.8 ± 7.6	133.8 ± 5.5	**0.003**	143.3 ± 8.8	144.2 ± 10.8	0.999
DBP (mmHg)	80.0 ± 9.4	70.2 ± 10.2	**0.001**	78.0 ± 2.7	72.0 ± 6.4	0.079	75.1 ± 6.9	75.3 ± 6.4	0.999
Functional fitness									
TUG (s)	7.1 ± 0.9	6.1 ± 1.0$^{\Delta}$	**0.001**	6.9 ± 0.7	5.9 ± 0.6$^{\Delta}$	**0.003**	7.4 ± 1.3	8.1 ± 1.8	0.087
6MWT (m)	359.5 ± 35.8	424.5 ± 43.8$^{\Delta}$	**0.001**	369.9 ± 16.7	434.1 ± 50.6$^{\Delta}$	**0.001**	357.4 ± 62.5	362.0 ± 69.4	0.997

Table 1. Baseline and post-training values of anthropometric parameters, lipid profile, blood pressure, and functional fitness. Values are given as mean ± SD. *BG* balance training group, *DG* dance training group, *CG* control group, *I* values at baseline, *II* values post training, *BMI* body mass index, *FFM* free fat mass, *SMM* skeletal muscle mass, *BFM* body fat mass, *VFA* visceral fat area, *TC* total serum cholesterol, *TG* triglycerides, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *TUG* time up and go test, *6MWT* 6-min walk test. Significant values are in bold. *p—p-value applying two-way repeated-measures ANOVA before and after intervention. $^{\#}p < 0.05$, a significant difference at baseline vs. CG. $^{\Delta}p < 0.05$, a significant difference after training vs. CG.

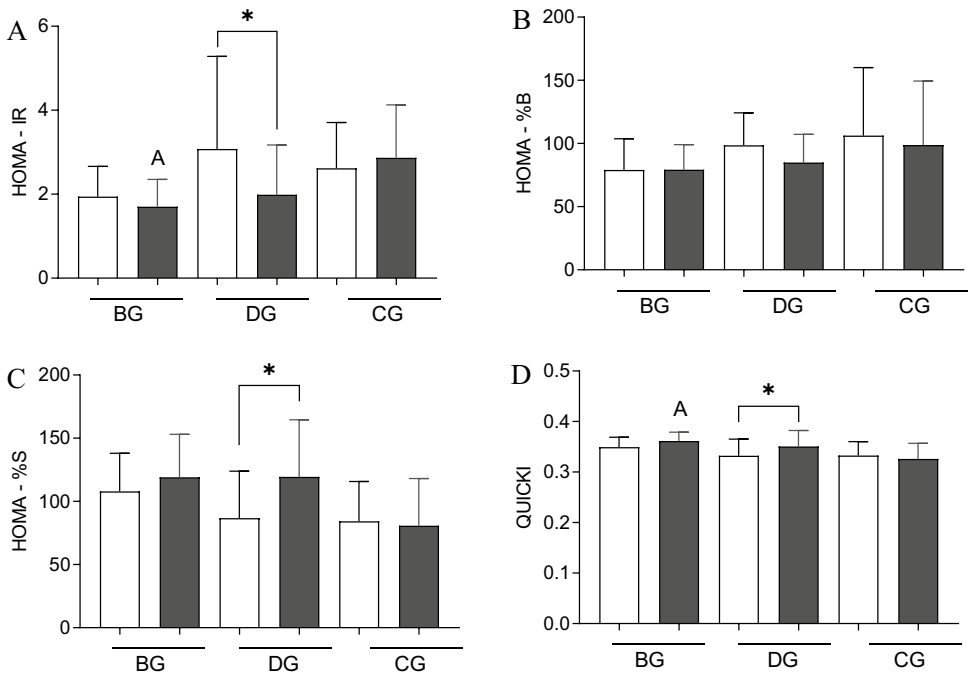

Figure 1. Post-training changes in insulin sensitivity and resistance indicators. (**A**) HOMA-IR; (**B**) HOMA-%B; (**C**) HOMA-%S; (**D**) QUICKI. There were significant differences between the indicated time points: *p < 0.05; between the groups: A—< 0.05 after training vs. CG. The data are presented as the means ± SEM; white before and black color after the intervention.

SII after 12 weeks of training in the BG (from 429.1 ± 116.7 to 353.3 ± 90.4; p = 0.017), and the same tendency in the DG group (from 440.5 ± 156.2 to 379.0 ± 154.3) (Fig. 2A). No effect of either group or time was noted on IL-18 concentration (Fig. 2B). 25-(OH)D concentration were homogenous among the groups (23.8 ± 10.8 – 29.0 ± 15.3 ng/ml).

The effect of the 12 weeks of a training procedure on blood pressure. The training programs significantly improved systolic (SBP) and diastolic blood pressure (DBP) (Table 1). SBP was significantly reduced in BG (from 147.2 ± 9.9 to 133.9 ± 17.6 mmHg; p = 0.0003), as well as in the DG (from 146.8 ± 7.6 to 133.8 ± 5.5 mmHg; p = 0.0035). In the BG, also a significant reduction in DBP was noted (from 80.0 ± 9.4 to 70.2 ± 10.2 mmHg; p = 0.0002), and the same tendency was noticed in the DG (from 78.0 ± 2.7 to 72.0 ± 6.4 mmHg). No changes in both SBP or DBP were recorded in the CG group.

Exerkines profile at baseline and post the 12 weeks of training. BDNF concentration significantly decreased in both BG (from 18.1 ± 10.7 to 2.1 ± 3.1 ng/ml; p = 0.0002) and DG (from 19.5 ± 10.9 to 6.7 ± 10.6 ng/ml; p = 0.0025), while it did not change in the CG (Fig. 3A). Irisin concentration significantly increased in both training groups (from 14.5 ± 3.5 to 16.4 ± 4.4 ng/ml; p = 0.029 for BG, and from 15.6 ± 4.3 to 17.6 ± 4.5 ng/ml; p = 0.022 for DG), whereas its concentration remained unchanged in the CG group (Fig. 3B). The training

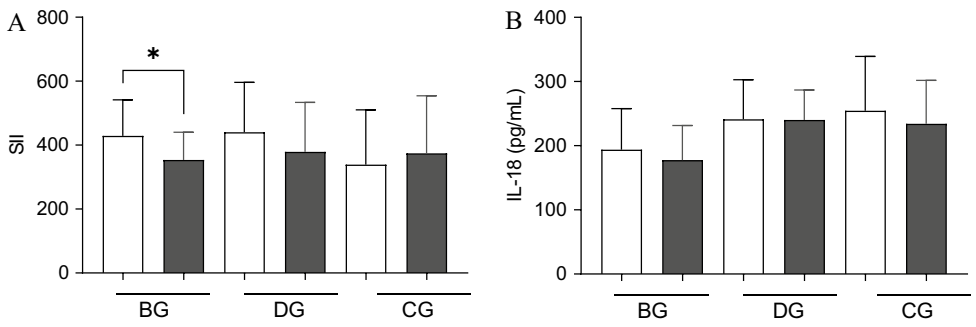

Figure 2. Post-training changes in inflammatory status. (**A**) SII index; (**B**) interleukin-18. There were significant differences between the indicated time points: *p < 0.05. The data are presented as the means ± SEM; white before and black color after the intervention.

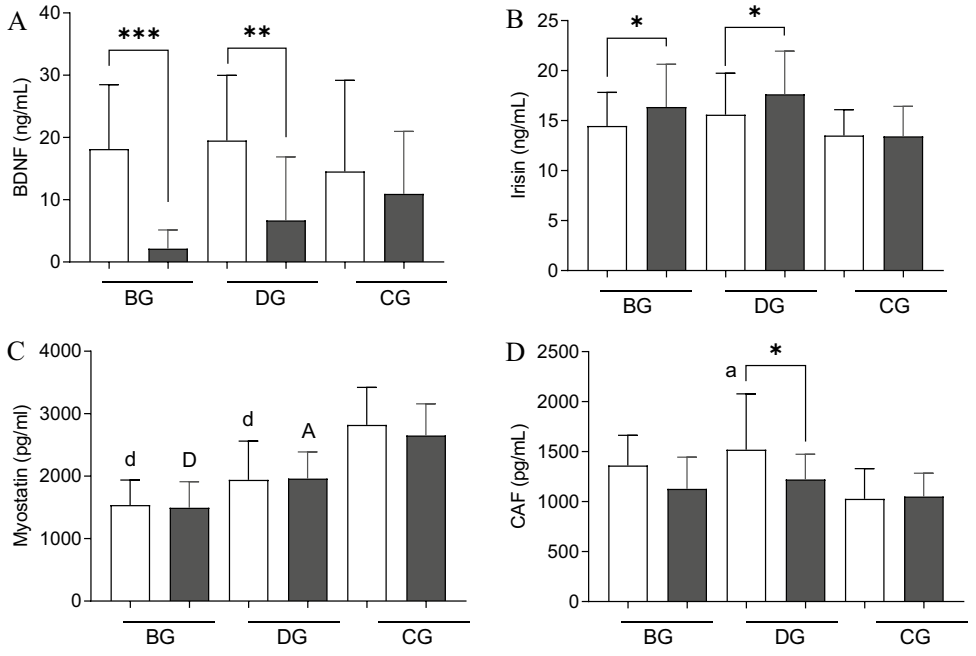

Figure 3. Post-training changes in exerkines concentrations. (**A**) BDNF; (**B**) irisin; (**C**) myostatin; (**D**) CAF. There were significant differences between the indicated time points: *p < 0.05, **p < 0.01, ***p < 0.001; between the groups: a—p < 0.05 before training, A—< 0.05 after training; d—p < 0.001 before training; D—p < 0.001 after training vs. CG. The data are presented as the means ± SEM; white before and black color after the intervention.

interventions did not induce changes in myostatin concentration (Fig. 3C). Significant changes were, instead, observed in CAF. In the DG, the significant reduction in CAF concentration was noted after training (from 1519.3 ± 581.6 to 1221.3 ± 262.2 pg/ml; p = 0.024), whereas, in the BG, only the same tendency was recorded (from 1362.5 ± 313.1 to 1127.5 ± 329.9 pg/ml; p = 0.073). Moreover, at the end of the intervention, CAF levels differed between DG and CG (p = 0.026) (Fig. 3D).

Discussion

The main finding of the current study is that a 12-week folk-dance training performed by older adults significantly improved insulin sensitivity indicators with a significant drop in BDNF and the rise of irisin concentrations. Additionally, CAF, a potential marker for muscle atrophy, resulted in a reduced association with improved physical performance. It is worth to note that such beneficial effects in insulin sensitivity were pronounced, especially among those subjects who presented the worse condition (i.e., frank insulin resistance) at baseline.

The improvement in insulin sensitivity and resistance indicators was visible mainly in HOMA-IR, HOMA-%S, and QUICKI. The positive effects were more pronounced in the folk-dance training group compared to the balance training group, among whom only a tendency to improve insulin sensitivity indicators was observed. The discrepancies between groups may result from slight, although not significant, differences in baseline values of these parameters. These results reflect the findings of previously published research, where the positive effect of physical training was more pronounced among people with type 2 diabetes, hypertension, hyperlipidemia, or metabolic syndrome. Indeed, the recorded reductions in total cholesterol, LDL, fasting insulin, and HOMA-IR were not as pronounced in healthy subjects[20]. Further, the latest studies indicated that the cut-off point of HOMA-IR for predicting the prevalence of metabolic syndrome is 1.8, and a value of 1.62 was obtained for identifying individuals at risk of IR[30,31]. Both training groups in the current study are above this cut-off point. Thus, we could summarize that moderate-intensity aerobic exercise effectively counteracts risk factors for CVD and glucose metabolism indexes.

Together with beneficial changes in insulin sensitivity registered in our trained groups, we noted the reduction of blood pressure. Although the awareness of the need for physical activity is growing, the elderly may be afraid of undertaking regular physical activity. Both resistance and long-term aerobic training have been commonly suggested to reduce blood pressure in middle-aged individuals with pre-hypertension and frank hypertension[32]. The magnitude of blood pressure reduction observed after physical training might be comparable to those induced by first-line anti-hypertensive drugs[33]. Our results supported these observations and proved that both moderate-intensity training programs effectively reduce blood pressure. The potential mechanism underlying this effect may be the aerobic exercise-induced increases in nitric oxide (NO) bioavailability, consequently improving endothelial function and endothelium-dependent vasodilation[32].

Additionally, we hypothesize that the amelioration in insulin resistance was associated with a reduction in BDNF concentrations.The primary function of BDNF is to regulate neurogenesis in the brain; however, it has also been shown to have a pivotal role in the regulation of peripheral metabolism, especially energy balance and insulin sensitivity[34]. Although the mechanism of action of BDNF in the central nervous systems quite well known, the peripheral pathways are not well understood. A novel metabolic pathway has been presented by Fulgenzi et al. It has been proposed in a mouse model that the BDNF receptor TrkB.T1 is expressed by pancreatic β-cells where it regulates insulin release[14]. Previous human research has shown that low circulating concentrations of BDNF are associated with insulin resistance, type 2 diabetes, and cognitive impairments[12]. However, Boyuk and co-workers have also observed contradictory results, who found higher serum BDNF concentration in T2M patients and a positive correlation with HOMA-IR and triglycerides[35]. It has been suggested that BDNF, through crossing the blood–brain barrier, may shift to the central nervous system and ameliorate the detrimental effects of insulin resistance in the brain as an antioxidant and neurotrophic factor[36,37].

The results from studies conducted on humans are ambiguous regarding the relationship between BDNF and long-term exercise protocols. No effects[38], enhancement in circulating BNDF concentration[15,39], and decrease have been observed[40].Previously published studies indicated that the post-exercise increase in BDNF concentration was associated with improved mood, cognitive functions, and quality of life[41,42]. However, the elevation of BDNF and improvement of cognitive functions depended on the subjects' age, the intensity of exercise, and metabolic factors, including, for instance, peripheral lactate concentration, insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor levels (VEGF)[43]. When considering the high-intensity exercise modality, most of these observations were about young, healthy subjects. In agreement with previous results[40], our study indicates that serum BDNF concentration decreased after aerobic training, regardless of the type, associated with the reduction in HOMA-IR and the increase in QUICKI indexes.

Another exerkine that may regulate insulin resistance in response to physical training is irisin. Irisin is expressed in skeletal muscle and other tissues and seems to induce a brown-like phenotype in some white adipocytes, which improves multiple metabolic parameters by increasing energy expenditure[44]. Therefore, irisin could potentially protect against different conditions such as cardiovascular diseases, type 2 diabetes mellitus, or fatty liver disease. In the current study, we observed an amelioration in insulin resistance, especially in the dance training group, with a positive association with circulating irisin, supporting the hypothesis of an insulin-like action of irisin. Irisin, indeed, enhances the expression of genes involved in glucose transport and lipid metabolism in myocytes (GLUT4, HK2, and PPARA), inhibits the expression of genes that are involved in glycogenolysis or gluconeogenesis and downregulates proteins associated with insulin resistance pathway[45,46].

Along with insulin sensitivity improvement, the reduction of the systemic immune-inflammation index was evidenced in the balance group and a trend to reduction in the folk-dance group. In the last published review, authors pointed out that irisin-mediated alterations in cytokine production results in reduced macrophage migration and infiltration, vascular leukocyte adhesion and migration, and acute phase inflammatory response[47].

Thus, it cannot be ruled out that the drop in SII was the effect of irisin's action. The reduction of the SII index and changes in irisin have particular meaning due to the potential role of irisin in obesity-related cancer prevention as well as in osteoporosis and neurodegenerative diseases- commonly noted among aged people[48]. Noteworthy, sarcopenia's pathophysiological mechanism(s) contemplates the deterioration of the homeostatic systems, including the immune system. In opposition to the previous observation, any alternations were recorded in other pro-inflammatory cytokine IL-18[49] in the current study. Also, Gomarasca et al. did not indicate changes in circulating IL-18 concentrations in obese men and women following a 12-week moderate-intensity aerobic NW training program[50]. However, among our subjects at baseline, a significant positive correlation was noted between IL-18 and visceral fat area, insulin, HOMA-IR, and triglycerides, and a negative correlation with HOMA-%S and QUICKI index. These results suggest that IL-18 could take part in the pathogenesis of obesity and insulin resistance, but it may not be sensitive to exercise intervention.

Although we did not determine muscle mass, our study focused on motor and functional balance test results (TUG and 6MWT). In both training groups, together with shifts of BDNF and irisin, the amelioration of these muscle functions was recorded. As irisin has been suggested as muscle wasting and muscular performance biomarker[23], these changes are significant since sarcopenia (the decline of fibers numbers and its size reduction) and dynapenia (reduction of strength) are highly prevalent in the elderly[51]. The scientific data point out that dynapenia precedes the process of sarcopenia, which is expressed by weakening motor abilities. Thus, the attenuation of this drop and the improvement of glucose homeostasis can be considered a preventive strategy against sarcopenia. Therefore, changes in these two proteins may indicate a positive effect of this training on preventing age-related loss of muscle mass.

Among the proteins considered as biomarkers of muscle atrophy and sarcopenia analyzed in this study—myostatin and CAF, only the latter substantially changed. Although previous studies showed a reduction in myostatin concentration, we did not observe such an effect. However, different results have been reported depending on sex, age, and exercise type (aerobic or resistance)[52]. The myostatin decrease was mainly observed after resistance forms of training[53]. Significant post-training changes have been noted in CAF—one of the best indicators of neuromuscular junction integrity and thus may be a potential biomarker for the progression of age-related functional decline and muscle atrophy[20].CAF is associated with one of the mechanisms of functional deterioration in aging—a decline in neuromuscular junction integrity. This pathology leads to sarcopenia development and worsening in physical performance[24]. The results of our study suggest that dance training may be effective in coping with functional decline related to aging via the reduction of CAF concentration. Only a few studies assess changes in circulating CAF levels following physical exercise[20,25]. It should be indicated that previous researches show both increases, no effects, and decrease in circulating CAF concentration after exercise interventions. One of the previous researches indicated that one year of physical activity intervention combining moderate-intensity walking, strength, and balance exercises did not reduce CAF concentration[20]. On the other hand, Fragala and co-workers observed that a 6-week resistance training increased CAF concentration in a group of elderly[25]. However, this study assessed the resistance form of training. One study confirmed our observations and indicated that recreationally active aged individuals, who regularly practiced dance for more than 3 years, had significantly lower CAF concentration compared to their counterparts involved in other aerobic physical activities. Additionally, elderly dancers demonstrated better dynamic balance and functional performance[26]. In the current study, a significant decrease in CAF concentration was observed in the dance training group, with an improvement in dynamic balance and physical performance, measured by TUG and 6MWT tests. Somehow, in the balance training group, a tendency to reduction in circulating CAF was also observed; however, this result was not statistically significant. The obtained results suggested that dance training is more effective in protecting from NMJ degeneration. We are far from speculating, but dance training may induce greater neuroplasticity and NMJ health than resistance or aerobic training since it involves both motor and sensory, cognitive and coordinative[54]. Moreover, dance training belongs to creative activities.

This study presents some limitations that should be highlighted. Future studies should include assessing cognitive functions to evaluate if the changes in exerkines are related to cognition improvement. However, for this purpose, previous studies, including ours, indicated that dance training is an effective method to improve cognitive abilities[29] due to the engagement in sensory, cognitive, and coordinative functions[54].

Overall obtained data indicated that both training programs effectively improve physical performance, dynamic balance, and inflammatory status and reduce high blood pressure. However, folk dance appears to be more beneficial in ameliorating insulin resistance. The changes in response to training interventions are connected with shifts in BDNF, irisin, and CAF concentrations. Since there is limited data regarding the direct connection between BDNF, irisin, and CAF in humans, obtained results have particular meaning and fill the gaps in this research area.

Materials and methods

Participants. Fifty-six community-dwelling older female (n = 30) and male (n = 26) participants (age 71.3 ± 5.5 years, range 65–85) were enrolled in the study (Fig. 4). Participants were recruited from advertisements in community centres in Gdansk, Poland. All participants lived and functioned independently without any severe cognitive or physical impairments. The inclusion criteria for the study were age ≥ 65 years and low-to-moderate physical activity assessed by the International Physical Activity Questionnaire-IPAQ (short version). The exclusion criteria were any diseases accompanied by contraindication to exercise and require specialized treatment, coronary disease, type 1 or 2 diabetes, grade II or III obesity (measured by BMI), arrhythmia, implanted pacemaker, heart failure, depression, cancer, significant orthopaedic injuries. Participants who take medications for depression, osteoporosis, heart diseases, lipid-lowering, or estrogenic replacement drugs were also excluded and those who regularly participated in exercise programs for 12 months before the study. Dur-

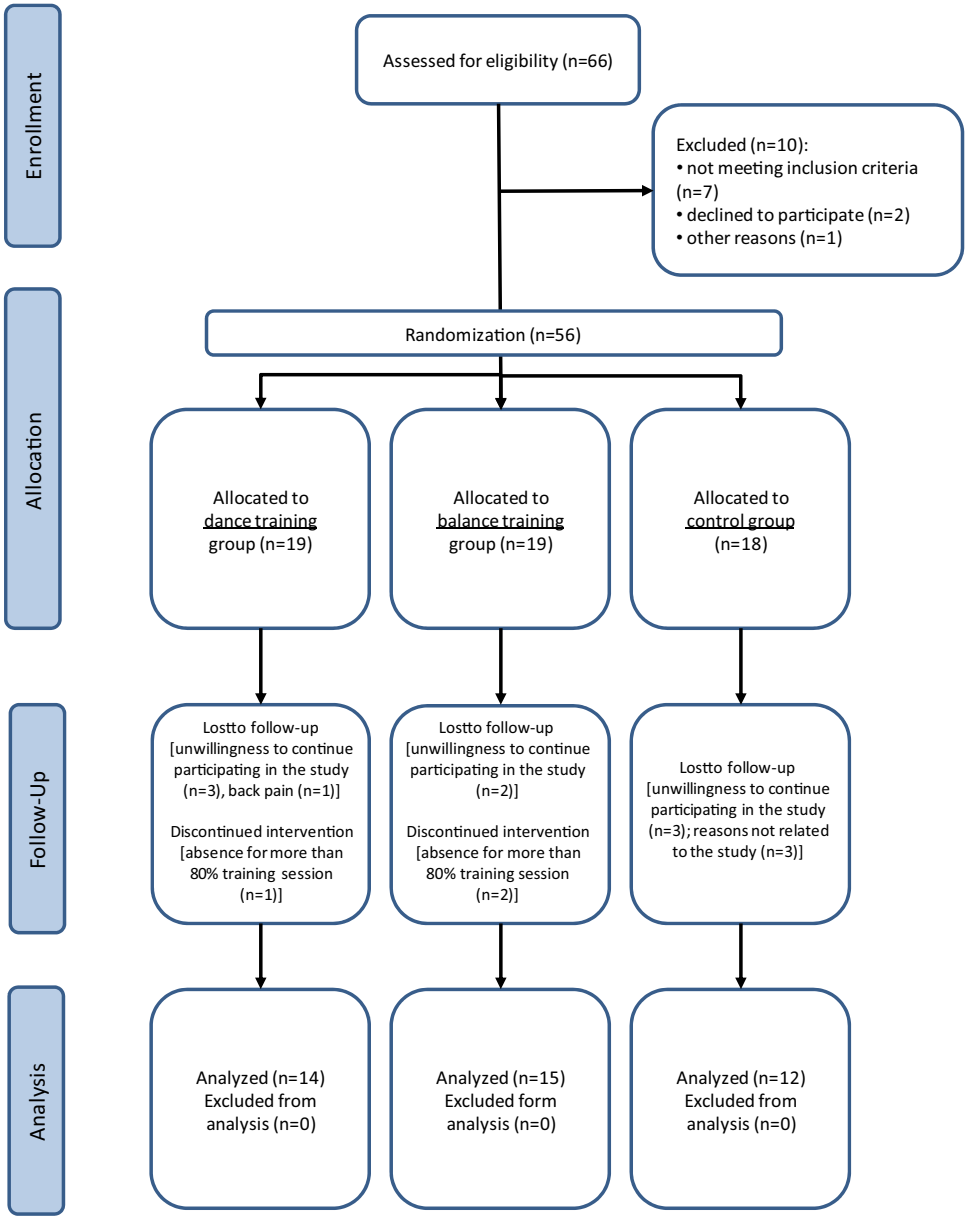

Figure 4. CONSORT flow diagram of the study.

ing the follow-up, 15 of the 56 participants withdrew from the study. Forty-one older adults (21 females and 20 males) completed the study. All participants were informed about the risks and study purpose and were familiarized with the research methods. Written informed consent was obtained from the participants of the study. The study was conducted in accordance with the Declaration of Helsinki. The experiment was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (KB-34/18).

Study design. Participants were randomly assigned into three groups: balance training group (BG; n = 15), dance training group (DG; n = 14), and control group (CG; n = 12). The BG and the DG groups performed training three times a week (Monday, Wednesday, and Friday) for 12 weeks. All measurements (body composition analysis, blood pressure measurement, blood collection, and functional tests—6-min walk test (6MWT) and timed up and go test (TUG) were performed 2 days before and 2 days after the intervention. Blood samples were taken to determine blood irisin, myostatin, IL-18, BDNF, CAF, 25-(OH)D3, insulin, and glucose concentration. Before the beginning of the training protocols, a medical examination was performed to exclude those participants with significant contraindications to exercise. The physician also assayed the blood pressure (BP) before and after the intervention. BP was measured in the seated position, on the left arm, thrice with 2-min intervals by the auscultator technique using a conventional mercury sphygmomanometer with an appropriately sized cuff. Measurements were obtained in the morning between 8:00 AM and 10.00 AM, after a 5-min of rest.

The fitness tests were performed by specialists who were blinded to the group assignments, exercise program, and control group status of the participants.

Body composition analysis. Body composition analysis was estimated by a precise multi-frequency impedance body composition analyzer (InBody 720, Biospace, Korea), using six different frequencies (1 kHz, 5 kHz, 50 kHz, 250 kHz, 500 kHz, and 1000 kHz) at each of five segments of the body (Right Arm, Left Arm, Trunk, Right Leg, Left Leg). The measurement was performed with the 8-point tactile electrode method. InBody assesses parameters of body composition as follows: body weight, free fat mass (FFM), body fat mass (FM), skeletal muscle mass (SMM), and visceral fat (VFA). The body composition analysis was performed by one researcher for all participants in a fasted state, 12 h after the last meal and drink, between 9:00 and 10:00 in the morning after blood collection)[55].

Blood collection. Blood samples for irisin, myostatin, IL-18, BDNF, CAF, 25-(OH)D3, insulin, and glucose assessment were collected between 7:00 and 9:00 AM after overnight fasting at two time-points: at baseline and after completion of the 12-week intervention. A qualified nurse took venous blood from the antecubital vein into vacutainer tubes (Vacutainer SSTTM II Advance) for serum separations and tubes with EDTA as an anticoagulant for plasma separation. Samples were centrifuged at 2000*g* for 10 min at 4 °C. The separated plasma and serum samples were frozen and stored at − 80 °C until later analysis.

Biochemical assays and insulin sensitivity indicators. Serum glucose was measured by the colorimetric enzymatic method (Randox manual gl2623). Serum insulin concentration was assessed by the immunoezymatic (ELISA) method (Demeditec diagnostics, Germany, no. DE2935). The maximal intra-assay coefficient of variability (CV) was 2.6–1.8%, and the inter-assay CV was 2.9–6.0%. The assay sensitivity was 1.76 µIU/ml. The following formulas calculated insulin sensitivity according to QUICKI and HOMA-IR: QUICKI = 1/(log(fasting insulin [µU/ml] + log(fasting glucose [mg/dl])), HOMA-IR = (fasting serum insulin µU/ml × fasting plasma glucose mmol/l/22.5). The updated computer HOMA2 model was used to determine the β-cell function (HOMA-%B) and insulin sensitivity (HOMA-%S) from paired fasting glucose and insulin level. The HOMA-indexes were obtained by the software HOMA 2 Calculator, version 2.2.3, copyrighted by The University of Oxford. In order to assess if the inflammation status has modified exercise-induced response, the systemic immune-inflammation index (SII) was calculated based on the formula proposed by Chen and co-workers: SII = (P × N)/L, where P, N, and L refer to peripheral platelet, neutrophil, and lymphocyte counts[56].

Exerkines blood concentration. Irisin and myostatin concentrations were determined by immunoenzymatic (ELISA) method using commercially available kits(Phoenix Pharmaceuticals Inc, no. EK067-29 for irisin, and R&D, United States&Canada, no. DGDF80 for myostatin) according to the manufacturer's protocol. The maximal intra-assay CV was 4–6%, and inter-assay CV was 8–10% for irisin and 1.8–5.4%, and 3.1–6.0% for myostatin. The assay sensitivity was 1.29 ng/ml for irisin and 5.32 pg/ml for myostatin. BDNF was assessed in serum using ELISA Kit (R&D, United States&Canada, no. DBNT00). The maximal intra-assay CV was 3.2–3%, and inter-assay CV was 7.2–4.7% for BDNF, and the assay sensitivity was 1.35 pg/ml.IL-18 was assessed in serum using ELISA Kit (R&D, United States&Canada, No. QK318). The maximal intra-assay and inter-assay CV for IL-18 were 2.5–4% and 5.4–7.9%, respectively, and the assay sensitivity was 4.57 pg/ml. Serum CAF was determined using Fine Test ELISA Kit—no. EH4820. The assay sensitivity was 28.125 pg/ml for CAF. The maximal intra-assay CV was < 8%, and inter-assay was < 10% for CAF. According to the manufacturer's protocol, serum 25(OH)D3 was assessed by ELISA Kit (Demeditec diagnostics, Germany, No. DE1971). The maximal intra-assay CV was 2.5–7.8%, and the inter-assay was 7.4–9.2%. The assay sensitivity was 2.81 ng/ml. The concentration of 25-(OH)D3 below 30 ng/ml was classified as insufficient.

Physical performance assessment. The time up and go test (TUG) test assessed functional balance and mobility and identified potential fallers. It was shown that a test time of 13.5 s or above is associated with a two- to three-fold higher risk of falls[57]. 6-min walk test (6MWT)assessed the walking distance of patients within 6 min. The test result could indicate the functional status and elders' cardiovascular and locomotor systems[58]. The functional tests were performed as described previously[59].

Balance training protocol. Two days after the completion of baseline measurements, women from the BG participated in the training program. The training was conducted three times a week for 12 weeks, with 36 training units as described previously[29]. Each training session lasted 50 min, including 10 min warm-up, 30 min balance training, and 10 min stretching and respiratory activity. Each training session was performed under the supervision of a physiotherapist (Master's degree in physiotherapy, specializing in conducting exercise programs with older adults). All training sessions were performed for the entire group in the training hall. Only those participants who attended at least 80% of training sessions were qualified for statistical analysis.

Dance training protocol. Two days after baseline measurements, women from the DG group participated in the dance training program as described in our previous study[29]. Participants assigned to the DG performed 50 min of dance training to Polish folk music. The training was performed in the group in a training hall and was conducted three times a week for 12 weeks. Each training session lasted for 50 min and included 10 min warm-up, 30 min of folk-dance training. Each exercise session was performed under the supervision of a qualified dance coach. The same rules of participants' attendance were concerned.

Control group. The control group did not participate in any training intervention. Participants were instructed not to change their daily habits during the intervention phase.

Statistical analysis. Statistical analyses were performed using the Statistica v.13 software package. The results are expressed as the mean ± standard deviation (SD). The Shapiro–Wilk and Brown-Forsyth tests were performed to test the normality of parameter distribution and group variances equality. For data with normal distribution and equal variations, the baseline differences between groups were tested using the one-way ANOVA model. For data without normal distribution and equal variations, the non-parametric Kruskal–Wallis test was used to test between-group differences at baseline. The changes in mean scores were tested using the two-way repeated-measures ANOVA models; if a difference was detected in the ANOVA models, the significant differences were determined using Tukey's post-hoc test. The results were considered statistically significant when $p \leq 0.05$. A Pearson product-moment correlation coefficient was computed to assess the relationship between the obtained results.

Data availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Received: 25 January 2023; Accepted: 20 May 2023
Published online: 26 May 2023

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Acknowledgements

This work has been funded by a grant from National Science Centre OPUS15, Project No 2018/29/B/NZ7/02094. We would like to thank all participants for their engagement in the training interventions and physical performance assessment.

Author contributions

E.A.R.F. and E.Z. designed the study. E.A.R.F., E.Z., D.J.F., and J.J.K. wrote the paper. E.A.R.F., M.K., E.Z., G.L., M.S.T., D.J.F., and J.J.K. designed, drafted, and critically revised the manuscript. E.A.R.F., E.Z., M.K., and J.J.K. analyzed the data. E.A.R.F., M.K., J.J.K., M.S.T., D.J.F. and E.Z. performed the research. All authors have read and approved the final version of the manuscript and agree with the order of presentation of the authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-35583-w.

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ORIGINAL RESEARCH
published: 20 April 2022
doi: 10.3389/fphys.2022.809363

12 Weeks of Physical Exercise Attenuates Oxidative Stress, Improves Functional Tests Performance, and Reduces Fall Risk in Elderly Women Independently on Serum 25(OH)D Concentration

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: 04 November 2021
Accepted: 05 April 2022
Published: 20 April 2022

Citation:
Rodziewicz-Flis EA, Kawa M, Flis DJ, Szaro-Truchan M, Skrobot WR and Kaczor JJ (2022) 12 Weeks of Physical Exercise Attenuates Oxidative Stress, Improves Functional Tests Performance, and Reduces Fall Risk in Elderly Women Independently on Serum 25(OH)D Concentration. Front. Physiol. 13:809363. doi: 10.3389/fphys.2022.809363

The study aimed to evaluate if the 25(OH)D concentration is related to physical training responses. Moreover, to determine the association between serum 25(OH)D concentration and older women's physical performance, oxidative stress markers, inflammation, and bone metabolism. 37 older women (age 72.9 ± 5.2 years) were assigned into two groups: supplemented (SG) and non-supplemented (NSG). Then, the participants from SG and NSG were randomly assigned into exercised and non-exercised groups: exercise sufficient vitamin D group (ESD; n = 10), exercise insufficient vitamin D group (EID; n = 9), control sufficient vitamin D group (CSD; n = 9), and control insufficient vitamin D group (CID; n = 9). To assess the study aims time up and go test (TUG), 6 min walk test (6MWT), fall risk test (FRT), blood osteocalcin (OC), parathormone (PTH), calcium (Ca^{2+}), sulfhydryl groups (SH), malondialdehyde (MDA), and interleukin-6 (IL-6) were performed. The results showed that a higher 25(OH)D concentration was in line with better physical performance and bone metabolism as well as lower inflammation. After 12 weeks of training we noted an improvement in 6MWT (from 374.0 ± 17.3 to 415.0 ± 18.8; p = 0.001 and from 364.8 ± 32.8 to 419.4 ± 32.3; p = 0.001 for EID and ESD, respectively), TUG (from 7.9 ± 0.5 to 6.8 ± 0.8; p = 0.001 and from 7.3 ± 1.5 to 6.4 ± 0.9; p = 0.002, for EID and ESD, respectively), reduction of fall risk (from 2.8 ± 0.8 to 1.9 ± 0.4; p = 0.003 and from 2.1 ± 1.1 to 1.6 ± 0.5; p = 0.047, for EID and ESD, respectively) and increase in SH groups (from 0.53 ± 0.06 to 0.58 ± 0.08; p = 0.012 and from 0.54 ± 0.03 to 0.59 ± 0.04; p = 0.005, for EID and ESD, respectively), regardless of the baseline 25(OH)D concentration. A decrease in PTH and OC concentration was observed only in EID group

Abbreviations: PTH, parathormone; BTM, bone turnover markers; OC, osteocalcin; ROS, reactive oxygen species; IL-6, interleukin-6; SH, sulfhydryl groups; MDA, malondialdehyde; 6MWT, 6 min walk test; TUG, time up and go test; FRT, fall risk test; FFM, free fat mass; FM, body fat mass; SMM, skeletal muscle mass; VFA, visceral fat area; SI, stability index.

(from 57.7 ± 15.7 to 49.4 ± 12.6; $p = 0.013$ for PTH and from 27.9 ± 17.2 to 18.0 ± 6.2; $p = 0.004$ for OC). To conclude, vitamin D concentration among older women is associated with physical performance, fall risk, inflammation, and bone metabolism markers. Moreover, 12 weeks of training improved physical performance and antioxidant protection, regardless of baseline vitamin D concentration.

Keywords: healthy ageing, fall risk, aerobic training, vitamin D, bone metabolism

1 INTRODUCTION

Aging is associated with many chronic diseases and pathological states, like sarcopenia, osteoporosis, chronic inflammation, and oxidative stress. In consequence, it may lead to disability, higher mortality, geriatric syndrome, and accelerated aging process (Campisi et al., 2019). One factor that may contribute to healthy aging is vitamin D status (Berridge, 2017). It is well known that vitamin D deficiency is a common health problem not only in the elderly. The prevalence of vitamin D deficiency is 50% or more among the senior population worldwide (van Schoor and Lips, 2011). The International Society for Clinical Densitometry and International Osteoporosis Foundation recommend a serum concentration of 25(OH)D at least 30 ng/ml to minimize the risk of fall and fractures in older individuals (Sizar et al., 2021). Serum 25(OH)D deficiency in the elderly may lead to increased inflammation, oxidative stress, muscle weakness, as well as more significant declines in physical performance as getting older (Bischoff-Ferrari et al., 2004; Wicherts et al., 2007; Berridge, 2017); however, the associations between vitamin D and physical performance remain controversial (Verreault et al., 2002; Annweiler et al., 2009).

The effect of vitamin D status on the risk of falls and physical performance may be related to several factors such as parathormone (PTH), bone turnover markers (BTM), cytokine concentration, and others. It is known that low vitamin D level leads to increased PTH concentration by impaired calcium metabolism. This phenomenon contributes to an increase in bone turnover and bone loss and is a risk factor for rickets and osteomalacia (Pludowski et al., 2013). Moreover, it has been shown that a higher level of PTH is related to increased risk of fracture and higher fall risk (de Franca et al., 2019). Consequently, maintaining an optimal vitamin D level may be essential to reduce fall risk and fractures. Osteocalcin (OC) is one of the BTM also related to vitamin D status, and it is produced by bone osteoblasts and partly released into the bloodstream (Schwetz et al., 2012). A previous study indicates that serum OC concentration decreased while serum mean 25(OH)D increased (Kuchuk et al., 2009). Moreover, a human with osteoporosis and osteopenia has been shown to have elevated serum OC concentration (Wang et al., 2019).

Furthermore, it has been proposed that vitamin D may affect aging processes also by controlling the activity of several cellular processes, among others oxidative stress or inflammation. These are one of the main drivers of aging and can be enhanced in individuals with vitamin D deficiency (Berridge, 2017). Insufficient serum vitamin D concentration may be related to increased cell damage induced by reactive oxygen species (ROS) through its ability to control the expression of cellular antioxidants (Dzik and Kaczor, 2019). Sufficient vitamin D concentration reduces inflammation by decreasing pro-inflammatory cytokines (Krasowska et al., 2019).

One of the well-known markers of chronic systemic inflammation in older adults is an interleukin-6 (IL-6) (Maggio et al., 2006). The circulating concentration of IL-6 higher than 2.5 pg/ml is a cut-off point indicating low-grade inflammation and a higher risk of functional decline (Ferrucci et al., 1999). Moreover, higher IL-6 concentration negatively affects bone metabolism by stimulating osteoblasts precursors differentiation and osteoclasts formation *via* an increase in RANKL expression by osteoblasts. Thus, it may be associated with the pathogenesis of postmenopausal osteoporosis; however, this data remains controversial (Kaji, 2016). On the other hand, it has also been suggested that IL-6 might be related to physical performance in older adults, but these data are both limited and inconsistent (Custodero et al., 2020; Grosicki et al., 2020).

Besides vitamin D status, physical activity also contributes to healthy aging by reducing the risk of chronic diseases, slowing aging-related processes, or preventing loss of independence (Campisi et al., 2019). The positive impact of exercise on age-related chronic diseases and physical performance may be associated with modifying both muscle and bone-derived proteins or hormones concentrations (Safdar and Tarnopolsky, 2018). Systemic humoral factors produced from muscle or bone tissue in response to exercise affect each other and may mediate the positive impact of physical exercise (Kaji, 2016). Furthermore, physical activity is known for its anti-inflammatory and antioxidant effects (Bachi et al., 2019). Nevertheless, little is known if vitamin D insufficiency may regulate training responses and training adaptations. It has been previously indicated that vitamin D status is related to physical performance only at baseline; however, its supplementation in addition to physical training did not enhance training responses. Previous studies indicated that vitamin D supplementation before and during the resistance training program did not show additive effects compared to physical training applied alone in male and female elders (Antoniak and Greig, 2017; Molmen et al., 2021). On the other hand, it has been suggested that responses to resistance training, especially muscle mass gains, are related not to blood vitamin D concentration but enhanced vitamin D receptor expression in skeletal muscle (Bass et al., 2020).

The first purpose of this study was to determine if the baseline concentration of 25(OH)D is related to physical training

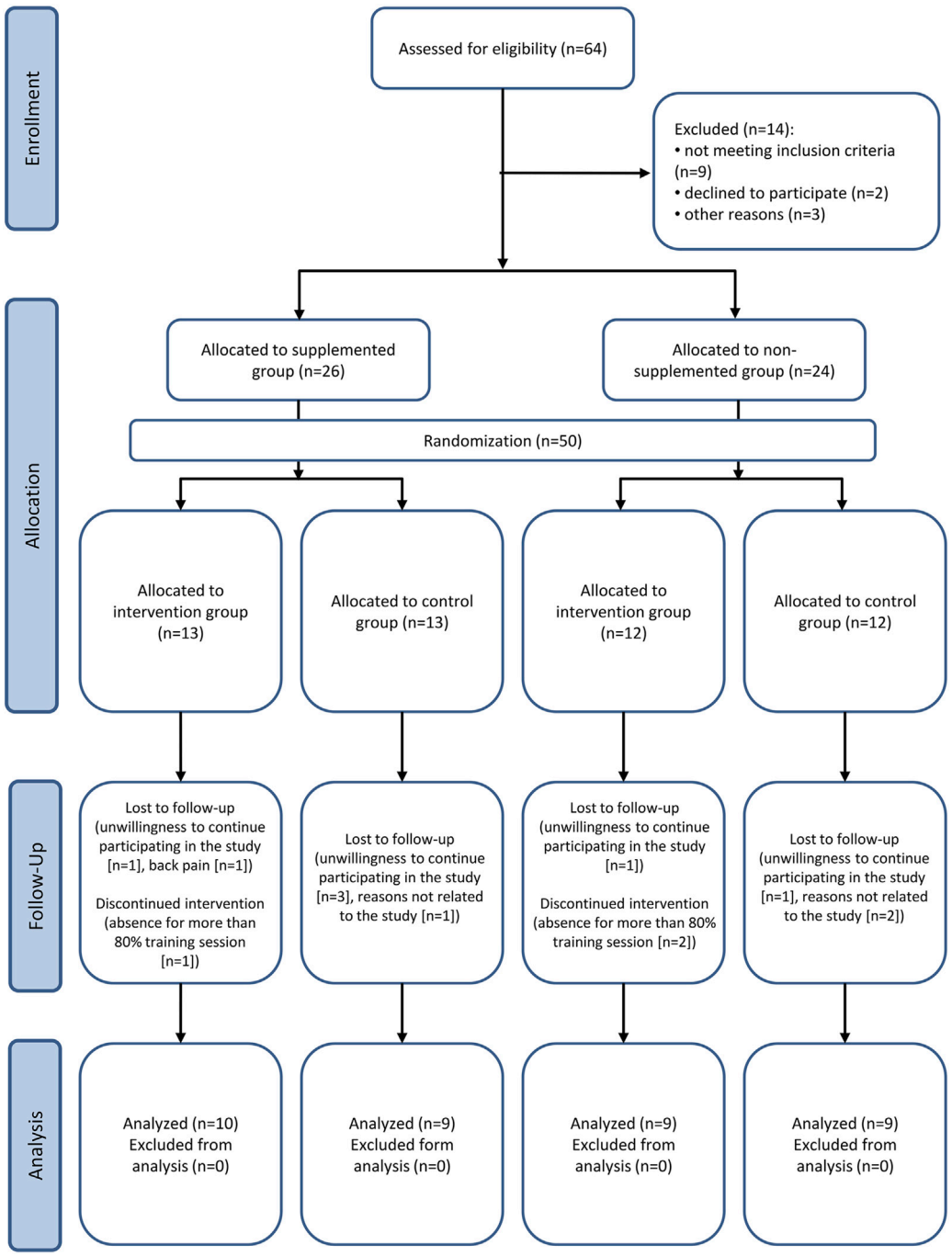

FIGURE 1 | CONSORT flow diagram of the study.

responses. The second was to estimate the association between serum 25(OH)D concentration and older women's physical performance, fall risk, oxidative stress markers, inflammation, and bone metabolism. We hypothesized that regular physical activity decreases oxidative stress and inflammation markers, improves functional tests performance, and reduces fall risk in elderly women independently on serum 25(OH)D concentration.

2 MATERIALS AND METHODS

2.1 Participants

Fifty community-dwelling female participants (age 72.9 ± 5.2 years, range 65–82) were enrolled in the study (**Figure 1**). Participants were recruited from advertisements in community centers in Gdansk, Poland. All participants were lived and

functioned independently, in good health, without severe cognitive or physical impairments. The inclusion criteria for the study were: female, age of 65 or older, and low to moderate physical activity assessed by International Physical Activity Questionnaire—IPAQ (short version). This questionnaire measures the recreational activity, housework, and physical activity performed over the past years. Before the study, participants also completed the questionnaire regarding medications and supplements taken, including vitamin D. The exclusion criteria were: any diseases accompanied by contraindication to exercise and required specialized treatment, coronary disease, arrhythmia, implanted pacemaker, heart failure, depression, cancer, significant orthopedic injuries, disabling dyspnea, taking medications for depression and heart diseases. We also excluded participants who regularly participated in exercise programs such as strength training, balance training, or aerobic training for 12 months before the study. Of the 50 participants included, 13 withdrew from the study during the follow-up. Finally, thirty-seven older women completed the study (**Figure 1**). All women were informed about the research's risks and purpose and familiarized with the study methods. The study was approved by the local institutional Bioethical Committee in Gdansk (NKBBN/455/2018) and conformed to the Declaration of Helsinki. Written informed consent was obtained from the participants of the study. The trial was registered at ClinicalTrials.gov (Identifier: NCT03417700).

2.2 Study Design

The study was a single-blind, randomized trial. All assessments (body composition analysis, functional tests, fall risk test, blood analysis) at baseline and after training intervention were performed by researchers blinded to the group allocation. At first, based on participants answer to the question: whether they use vitamin D supplements of 2,000 units or more, two groups were performed: supplemented with vitamin D (SG); $n = 19$ or not (NSG); $n = 18$ in order to evaluate if there are differences in physical performance, fall risk, inflammation, oxidative stress, and bone metabolism among groups at baseline of the experiment. The criterion that qualified a person to the supplemented group was taking 2,000 units or more of vitamin D per day for at least 2 months. Additionally, after the baseline assessments, participants from SG and NSG were randomly assigned into exercised and non-exercised groups: exercise sufficient vitamin D group (ESD; $n = 10$), exercise insufficient vitamin D group (EID; $n = 9$), control sufficient vitamin D group (CSD; $n = 9$), and control insufficient vitamin D group (CID; $n = 9$). The ESD and EID performed training three times a week (Monday, Wednesday, Friday) for 12 weeks. The training procedure started 2 days after the baseline measurements (blood collection, body composition analysis, 6-min walk (6MWT), time up and go (TUG), and fall risk (FRT) tests). Also, 2 days after the last training session, all of the measurements mentioned above were repeated. The blood was collected from the participants to assess OC, PTH, 25(OH)D, IL-6, sulfhydryl (SH) groups, malondialdehyde (MDA), and calcium (Ca^{2+}) concentration. Participants were asked not to change their daily habits during the experiment, including supplementation.

2.3 Intervention

2.3.1 Experimental Group

Exercise groups completed 12 weeks of combined aerobic-resistance training with moderate intensity, supervised by a physiotherapist. Participants trained 3 days a week for 50 min per session (150 min of activity per week), totaling 36 training sessions. The training was conducted on Mondays, Thursdays, and Fridays. Participants performed aerobic exercises combined with resistance exercises. The exercise intensity was prescribed at 60% of maximum heart rate during the first 4 weeks, then gradually increased to 80% of HRmax during the last 4 weeks. The heart rate was measured by pulsometer (Polar Heart Rate Monitor, Polar Electro, Finland) and was between 100–140 beats per minute during the training sessions. Each training session consisted of 5 min of warm-up, 20 min of aerobic exercise, 20 min of resistance exercise, and 5 min of cool-down. The aerobic training periods contained standard fitness and balance exercises performed to the music. During the resistance part of the training, participants performed exercises with their body weight and with external load (dumbbells, resistance band) for upper and lower limbs and abdominal muscles. Exercises were performed with a 2–5 kg load and were designed to improve strength endurance. Participants performed about 12–25 repetitions of each exercise with a 30–60 s break. Resistance training intensity increased every 4 weeks (12–15 repetitions in weeks 1–4, 15–20 repetitions in weeks 5–8, and 20–25 repetitions in weeks 9–12). The exercises used in resistance training were, for example, as follows: knee push-ups, squats, forward lunges, back lunges, crunches, standing dumbbell press, dumbbell lateral raise, dumbbell bench press, toe lifts. The muscles trained in the resistance training periods were: biceps brachii, triceps brachii, deltoid, pectoralis, abdominal muscles, back muscles, gluteus muscles, and lower limbs muscles. The minimal training attendance was 80%, and the mean training attendance was 89%.

2.3.2 Control Group

The control group did not participate in any training intervention. Participants were instructed not to change their daily habits and maintain current activity during the 12-weeks study period.

2.4 Measurements

2.4.1 Body Composition Analysis

Body composition analysis was estimated by a precise multi-frequency impedance plethysmography body composition analyzer (InBody 720, Biospace, Korea), using six different frequencies (1 kHz, 5 kHz, 50 kHz, 250 kHz, 500 kHz, and 1000 kHz) at each of five segments of the body (Right Arm, Left Arm, Trunk, Right Leg, Left Leg). The measurement was performed with the 8-point tactile electrode method. InBody measures body composition by segment, and test duration is approximately 60 s. Parameters as body weight, free fat mass (FFM), body fat mass (FM), skeletal muscle mass (SMM), and visceral fat (VFA) have been assessed. The body composition

analysis was performed by one researcher for all participants in a fasted state, 12 h after the last meal and drink, between 9:00–10:00 in the morning after blood collection, at baseline (2 days before first training session) and 2 days after the last training session. Before contacting with electrodes, participants disinfected their hands and feet with alcohol. During the assessment, women were barefoot, dressed only in underwear and without jewelry, watches, etc. The impedance measurements were made standing in an upright position, with feet and hands centered on the electrodes, and the upper limbs positioned around 30° away from the trunk. This position was held for the test duration (McLester et al., 2020).

2.4.2 Blood Analysis and Collection

Blood samples for 25(OH)D, OC, PTH, Ca^{2+}, SH groups, MDA, and IL-6 assessment were collected between 8:00–9:00 after overnight fasting at two time-points: at baseline (2 days before the intervention) and 2 days after completion the 12-weeks training procedure. A qualified nurse took venous blood from the antecubital vein into vacutainer tubes (Vacutainer SSTTM II Advance) for serum separations and tubes with EDTA as an anticoagulant for plasma isolation. The samples were centrifuged at 2000 × g for 10 min at 4°C. The separated plasma and serum samples were frozen and stored at −80°C until later analysis. Plasma OC and PTH (hormone regulating calcium metabolism) concentrations were determined using the immunoenzymatic (ELISA) method using commercially available kits (R&D, Unites States & Canada, No. DSTCN0 and Demeditec diagnostics, Germany, No. DE3645, respectively) according to the manufacturer's protocol. The maximal intra-assay coefficient of variability (CV) was 1.6%–3.4%, and inter-assay CV was 6.1%–6.9% for OC and 3.68%–6.08% and 2.8%–3.6% for PTH. The assay sensitivity was 0.898 ng/ml for OC and 0.761 pg/ml for PTH. IL-6 was assessed in serum also by immunoenzymatic method using ELISA Kit - R&D, United States & Canada, No. HS600B). The maximal intra-assay and inter-assay CV for IL-6 was 6.9%–7.4% and 6.5%–9.6%, and the assay sensitivity was 0.11 pg/ml. Ca^{2+} concentration was assessed by plate-based colorimetric method (Cayman chemical, United States, No. 701220). The maximal intra-assay and inter-assay CV for Ca^{2+} was 3% and 5.96%, and the assay sensitivity was 0.25 mg/dl. Serum 25(OH)D was assessed by ELISA Kit (Demeditec diagnostics, Germany, No. DE1971) according to the manufacturer's protocol. The maximal intra-assay CV was 2.5%–7.8%, and inter-assay was 7.4%–9.2%. The assay sensitivity was 2.81 ng/ml. The concentration of 25(OH)D below 30 ng/ml was classified as insufficient.

2.4.3 Manifestation of Oxidative Stress

Plasma SH groups (a marker of protein peroxidation) were measured spectrophotometrically according to Kaszubowska and coworkers (Kaszubowska et al., 2011) against the standard curve (standard–reduced glutathione). The lowest concentration of reduced glutathione in the standard curve was 0.25 mmol/L. The range of the standard curve was between 0 and 2 mmol/L. The MDA concentration (a marker of lipid peroxidation) was measured spectrophotometrically using the LPO-586 assay (OxisReserch, Portland, United States) according to the manufacturer's instructions, against the standard curve (standard–1,1,3,3 Tetramethoxypropane). The lowest concentration of 1,1,3,3 Tetramethoxypropane in the standard curve was 2.5 μmol/L. The range of the standard curve was between 0 and 150 μmol/L. The SH groups and MDA values were expressed as mmol/L and as μmol/L, respectively.

2.4.4 Time Up and Go Test (TUG) and 6 min Walk Test (6MWT)

The TUG test is used to assess functional balance, mobility, and indirectly also a risk of falls. The test time of 13.5 s or above is associated with a two- to three-fold higher risk of falls (Shumway-Cook et al., 2000). All women were provided with standardized verbal instructions before the test started. Participants were asked to sit on a back-supported chair with arms crossed on the chest. Then, participants were instructed to get up from the chair on command, walk 3 m, turn in a signed place, walk back and sit down again. The time taken to perform the task was measured by a stopwatch by a qualified physiotherapist. Every participant performed the test twice, first slow with the researcher's assistance to familiarize herself with the procedure. Then, after 5 min of rest, independently, as fast as possible, without running.

6MWT assessed the walking distance of patients within 6 min. The test result could indicate the functional status and elders' respiratory, cardiovascular, and locomotor systems (Baddini-Martinez, 2018). The 6MWT was performed in a 30 m hallway. Women were asked to begin walking at a command "start" and walk back and forth as much as possible in 6 min with no running. The distance was measured by the measuring wheel. One researcher tested each participant. The TUG and 6MWT tests were separated by 10 min of rest.

2.4.5 Fall Risk Test (FRT)

To evaluate the fall risk, participants received baseline and post-treatment assessments using the Biodex Balance System. The FRT is designed to identify potential fallers. This test protocol gives the age-adjusted normative data to assess the patient's risk of falling. During the test, participants performed three trials of 20 s. Each trial began with an initial platform setting of 6 and ended with 2. Before the first test trial, the screen provided 3 s countdown. Between each trial, participants had 10 s rest. During the rest period, the platform returned to the locked, stable position. In order to perform the test, patients should stand in a comfortable position on the platform bilaterally with feet shoulder-width apart over the midline of the platform. Three test trials were used to avoid excessive balance deviations. Participants were asked to stand without support, look straight and focus on the visual feedback screen. After the test, the results are shown on the screen and compared to the normative data. The patients' performance is noted as a stability index (SI). Scores higher than normative age values suggest a higher fall risk, indicating balance impairments. Lowering SI after the training program indicates balance control improvement.

2.5 Statistical Analysis

Statistical analyses were performed using the Statistica v.13.software package (StatSoft Inc., Tulsa, OK, United States).

TABLE 1 | Characteristics of the participants.

Variables	NSG (*n* = 19)	SG (*n* = 18)	*P* value	Cohen's d	Mean differences
Age (years)	72.8 ± 5.3	72.9 ± 5.1	ns	0.02	0.12
Height (cm)	1.61 ± 0.04	1.59 ± 0.04	ns	0.40	0.02
Weight (kg)	73.4 ± 8.1	66.5 ± 8.2*	0.015	**0.88**	7.17
BMI (kg/m^2)	27.9 ± 3.4	26.0 ± 3.1	ns	**0.59**	2.21
FFM (kg)	45.9 ± 4.7	43.5 ± 4.4	ns	**0.51**	2.34
BFM (kg)	29.0 ± 7.7	23.9 ± 5.5*	0.026	**0.76**	5.13
SMM (kg)	24.9 ± 2.7	23.6 ± 2.6	ns	0.48	1.28
VFA (cm^2)	115.9 ± 28.4	98.4 ± 21.1*	0.033	**0.61**	15.31
Vitamin D (ng/ml)	16.9 ± 7.7	43.2 ± 10.2*	0.001	**2.92**	26.32
PTH (pg/ml)	59.1 ± 16.6	37.2 ± 9.7*	0.001	**1.61**	21.91
OC (ng/ml)	29.7 ± 20.7	22.1 ± 10.0	ns	0.47	7.57
IL-6 (pg/ml)	2.96 ± 1.8	1.96 ± 0.8*	ns	**0.72**	1.01
Ca (mg/dl)	12.1 ± 1.5	12.5 ± 1.8	ns	0.22	0.36
SH (mmol/L)	0.57 ± 0.09	0.56 ± 0.05	ns	0.12	0.01
MDA (μmol/L)	0.079 ± 0.03	0.069 ± 0.03	ns	0.37	0.01
TUG (s)	8.48 ± 1.3	7.50 ± 1.4*	0.032	**0.74**	0.98
6MWT (m)	333.8 ± 60.9	364.7 ± 38.2	ns	**0.61**	30.96
Fall risk	2.89 ± 0.8	2.14 ± 1.2*	0.030	**0.75**	0.75

Values are given as mean ± SD. Ns, non-significant differences between groups; Cohen's d, the Cohen's effect size [effect sizes as small (d = 0.2), medium (d = 0.5), large (d = 0.8), and very large (d = 1.3), moderate to very large effect size have been highlighted in bold]; mean differences, mean differences between SG and NSG group. NSG, non-supplemented group; SG, supplemented group; BMI, body mass index; FFM, free fat mass; BFM, body fat mass; SMM, skeletal muscle mass; VFA, visceral fat area; PTH, parathormone; OC, osteocalcin; IL-6, interleukin-6; Ca, calcium; SH, sulfhydryl groups; MDA, malondialdehyde; TUG, time up and go test; 6MWT, 6 min walk test.
p < 0.05, a significant difference between supplemented and non-supplemented group at baseline.

The participants' characteristic were analyzed using descriptive statistics and is presented as mean ± standard deviation (SD). The Shapiro-Wilk test was determined for parameters distribution normality. For the baseline analysis the Mann-Whitney U (M-WU) test was used if the distribution was abnormal (for age, IL-6, SH, MDA, OC, Ca, 6MWT). If the parameters presented normal distribution, equality of group variances *via* the Brown-Forsyth test was verified. All groups presented equal variances; therefore, the unpaired student t-test was used for data with normal distribution. The differences between values before and after the training program were tested using the two-way repeated measurements of ANOVA. The Fisher's Least Significant Difference (LSD) post-hoc test determined significant differences if a difference was detected in the ANOVA model. A Pearson product-moment correlation coefficient was computed to assess the relationship between the obtained results. Multivariate regression analysis was carried out to recognize the strength of each parameter predicting functional tests performance, fall risk, and IL-6 concentration. The results were considered statistically significant for p values less than 0.05.

3 RESULTS

3.1 Baseline Characteristic of Participants From Supplemented and Non-Supplemented Group

The baseline group's characteristic is presented in **Table 1**. None of the participants took osteoporosis drugs such as bisphosphonates before or during the study. All women had low to moderate daily physical activity levels measured by The Physical Activity Questionnaire–IPAQ (short version). The mean age of the participants from the supplemented group was 72.9 ± 5.1, and the non-supplemented group was 72.8 ± 5.3 years. We observed significant differences between groups in 25(OH)D concentration. The supplemented group's 25(OH)D serum concentration was 43.2 ± 10.2 ng/ml, and in the non-supplemented group, the mean concentration was 16.9 ± 7.7 ng/ml (p = 0.001). The SG had significantly lower body mass, BFM, and VFA when compared to NSG (p = 0.015; p = 0.026 and p = 0.033, respectively). Moreover, the SG had a substantially lower PTH plasma concentration than the NSG (p = 0.001) at baseline. Before the intervention, no significant differences had been found in OC concentration. At baseline, the group with higher serum 25(OH)D concentration tends to have lower inflammation marker–IL-6 (p = 0.080 (M-WU); cohen's d = 0.72). No differences were detected in oxidative stress markers between groups. Interestingly, significant differences were observed between SG and NSG groups in physical performance. Before the training procedure, the SG was characterized by better results in TUG (p = 0.032), lower fall risk (p = 0.030), and tend to have better results in 6MWT (p = 0.071; cohen's d = 0.61).

3.2 Pearson's Correlations of 25(OH)D, a Marker of Inflammation, Bone Metabolism Markers, and Physical Performance

The negative correlation between vitamin D and body mass was found (r = −0.33; p = 0.003). We also found a significant negative correlation between 25(OH)D and IL-6 among all study groups (r = −0.34; p = 0.002). Moreover, the higher 25(OH)D concentration was associated with lower PTH concentration (r = −0.59; p = 0.001)

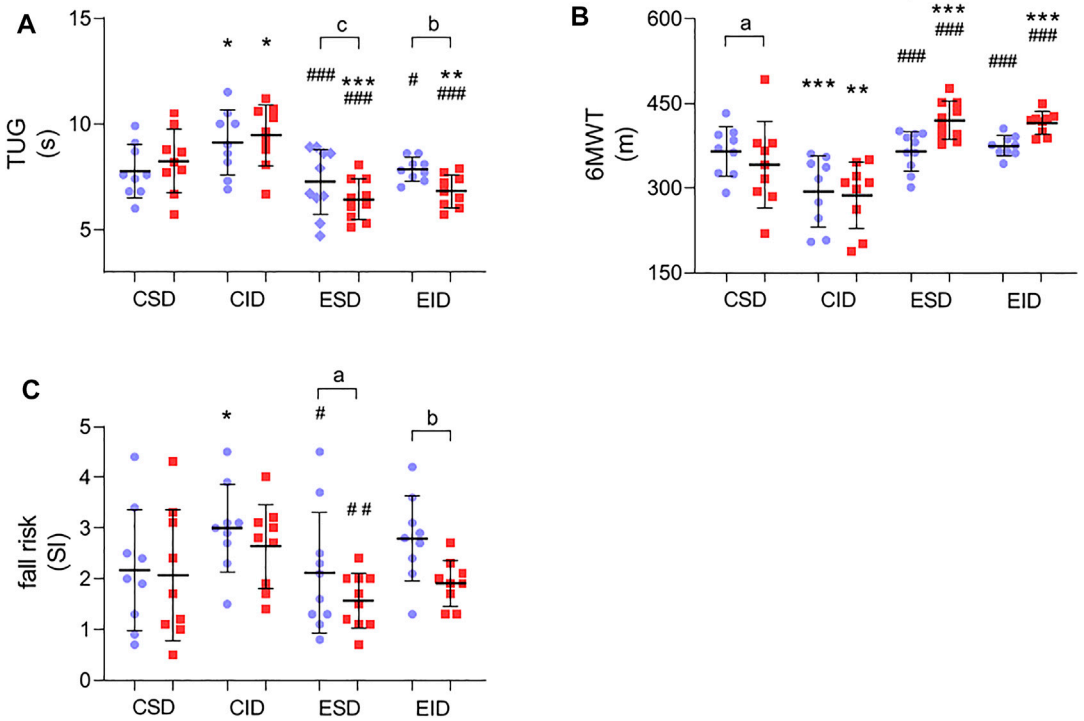

FIGURE 2 | Post-training changes in physical performance and fall risk. **(A)** Time up and go test; **(B)** 6-minute walk test; **(C)** fall risk test. There were significant differences between the indicated time points: **a** – $p < 0.05$, **b** – $p < 0.01$, **c** – $p < 0.001$; between the groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CSD; #$p < 0.05$, ##$p < 0.01$; ###$p < 0.001$ vs. CID. The data are presented as the means ± SEM; plain—before the intervention; strips—after the intervention.

and lower OC (r = −0.28; $p = 0.01$). A significant association between 25(OH)D concentration and physical performance was also observed. A negative correlation was noted between 25(OH)D and TUG test time (r = −0.46; $p = 0.001$) as well as fall risk (r = −0.26; $p = 0.017$). Furthermore, 25(OH)D concentration positively correlates with the distance performed during 6MWT (r = 0.33; $p = 0.003$). The association between inflammation and body composition as well as physical performance was also observed. We noted the positive correlation between IL-6 concentration and TUG and BFM (r = 0.45; $p = 0.001$; r = 0.29; $p = 0.008$, respectively) and a negative correlation between IL-6 and 6MWT (r = −0.34; $p = 0.002$).

3.3 Multivariate Regression Analysis

Multivariate regression analyses were performed to investigate factors measured in the blood associated with functional test performance, fall risk, and IL-6 concentration. Multivariate analysis for TUG as a dependent variable showed that concentration of 25(OH)D and IL-6 are strong predictors (β = −1.171, $p = 0.006$ for 25(OH)D and β = 1.069, $p = 0.001$ for IL-6). Multivariate analysis for 6MWT as a dependent variable showed that concentration of 25(OH)D and IL-6 are strong predictors (β = 1.473, $p = 0.001$ for 25(OH)D and β = −0.933, $p = 0.003$ for IL-6). No significant associations were observed for FR as a dependent variable; however, 25(OH)D and OC pretend to be predictors (β = −0.864, $p = 0.074$ for 25(OH)D and β = −0.772, $p = 0.063$ for OC). Multivariate analysis for IL-6 as a dependent variable showed that 25(OH)D concentration, as well as BMI are a strong predictors (β = −1.305, $p = 0.005$ for 25(OH)D and β = −3.769, $p = 0.047$ for BMI).

3.4 Post-Training Changes in Physical Performance and Fall Risk

After 12 weeks of a training program, we found a significant improvement in physical performance (**Figure 2**). For the TUG, there was a non-significant effect for time [$F_{(1,37)} = 3.866$, $p = 0.057$, $\eta 2 = 0.095$], but a significant group × time interaction [$F_{(3,37)} = 8.741$, $p = 0.0002$, $\eta 2 = 0.415$. A Fishers LSD post-hoc test showed a significant amelioration in TUG test time in both exercise groups ($p = 0.002$ for ESD and $p = 0.001$ for EID) (**Figure 2A**). For the 6MWT, there was a main effect for time [$F_{(1,37)} = 8.734$, $p = 0.005$, $\eta 2 = 0.191$] and a significant group × time interaction [$F_{(3,37)} = 11.748$, $p = 0.00001$, $\eta 2 = 0.488$] (**Figure 2A**). A used post-hoc test revealed an increase in the performed distance during the 6MWT in the ESD group ($p = 0.001$) and EID group ($p = 0.001$). Moreover, the decrease in the performed distance was found in the CSD group after 12 weeks of the experiment ($p = 0.039$) (**Figure 2B**). For the fall risk, there was a main effect for time [$F_{(1,37)} = 11.295$, $p = 0.0018$, $\eta 2 = 0.234$] but a non-significant group × time interaction [$F_{(3,37)} = 1.339$, $p = 0.277$, $\eta 2 = 0.098$]. The lower FR was found in the EID ($p = 0.003$) and ESD ($p = 0.047$) in Fishers LSD post-hoc test (**Figure 2C**). No changes were observed in the control groups.

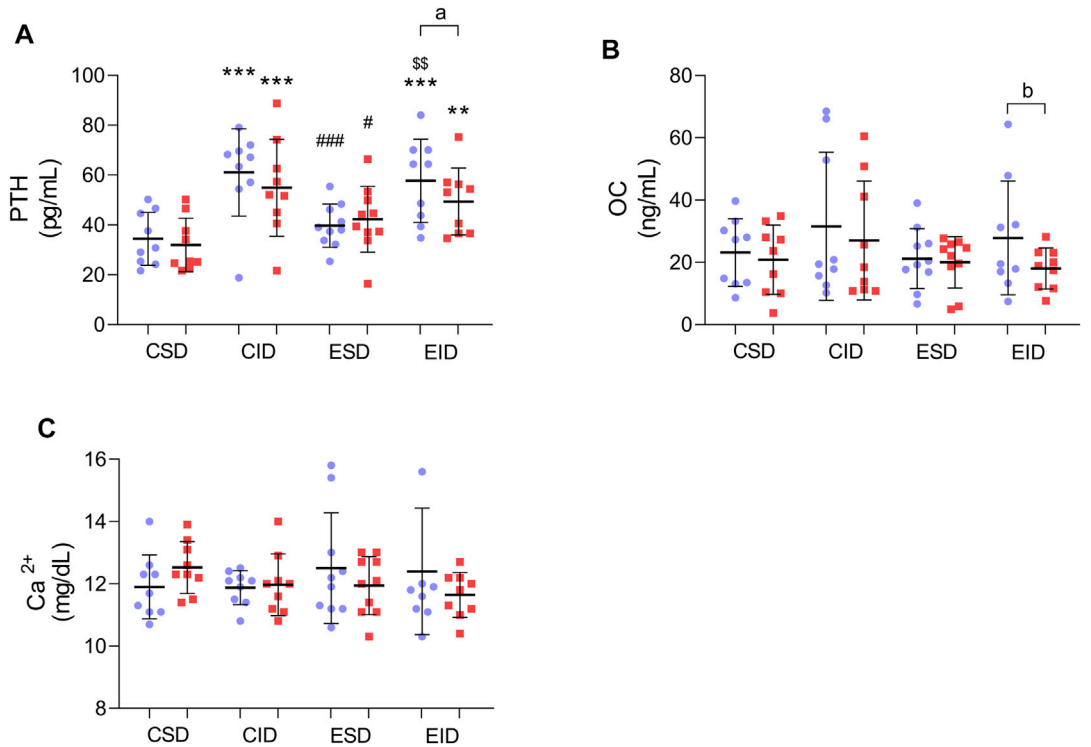

FIGURE 3 | Post-training changes in bone metabolism and PTH. **(A)** PTH concentration; **(B)** OC concentration; **(C)** Ca^{2+} concentration. There were significant differences between the indicated time points: **a** – $p < 0.05$, **b** – $p < 0.01$; between the groups: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. CSD; #$p < 0.05$, ###$p < 0.001$ vs. CID; $$$p < 0.01$ vs. ESD. The data are presented as the means ± SEM; plain—before the intervention; strips—after the intervention.

3.5 Post-Training Changes in Bone Metabolism Markers and PTH

For the PTH, there was no main effect for time [$F_{(1,37)} = 3.859$, $p = 0.057$, $\eta 2 = 0.094$] and for a group × time interaction [$F_{(3,37)} = 1.729$, $p = 0.178$, $\eta 2 = 0.123$]. Nevertheless, a Fishers LSD post-hoc test revealed a significant reduction in PTH concentration after 12 weeks of training, but only in the exercise group with insufficient 25(OH)D concentration ($p = 0.031$). In the ESD group, we did not find any significant changes (**Figure 3A**). For the OC, there was a main effect for time [$F_{(1,37)} = 7.776$, $p = 0.008$, $\eta 2 = 0.174$] but no effect for a group × time interaction [$F_{(3,37)} = 1.429$, $p = 0.250$, $\eta 2 = 0.104$]. An alteration in OC concentration was shown by a post-hoc test. Its reduction was noted only in the EID group ($p = 0.004$) (**Figure 3B**). For the calcium, there was neither effect for time [$F_{(1,37)} = 0.418$, $p = 0.522$, $\eta 2 = 0.011$], nor for a group × time interaction [$F_{(3,37)} = 2.017$, $p = 0.128$, $\eta 2 = 0.141$]. A slight but insignificant reduction in Ca^{2+} concentration was observed among both groups (**Figure 3C**).

3.6 Post-Training Changes in Inflammation Marker and Oxidative Stress

We did not distinguish any significant changes in IL-6 concentration after the training program (**Figure 4A**). For the IL-6, there was an insignificant effect for time [$F_{(1,37)} = 2.750$, $p = 0.106$, $\eta 2 = 0.069$] and for a group × time interaction [$F_{(3,37)} = 0.399$, $p = 0.754$, $\eta 2 = 0.031$]. The 12-weeks of training also did not change the MDA concentration in any of the training groups. For the MDA, there was neither effect for time [$F_{(1,37)} = 0.155$, $p = 0.696$, $\eta 2 = 0.004$] nor a group × time interaction [$F_{(3,37)} = 1.752$, $p = 0.173$, $\eta 2 = 0.124$] (**Figure 4B**). For the SH groups, there was a main effect for time [$F_{(1,37)} = 10.243$, $p = 0.003$, $\eta 2 = 0.217$] and an insignificant group × time interaction [$F_{(3,37)} = 2.154$, $p = 0.110$, $\eta 2 = 0.149$]. The Fishers LSD test revealed an increase in SH groups concentration in both exercised participants–EID ($p = 0.012$) and ESD ($p = 0.005$) (**Figure 4C**).

4 DISCUSSION

To our knowledge, for the first time, this research assessed if, among healthy, older women, the physical training responses are related to baseline vitamin D concentration. Our study confirmed an association between older women's serum 25(OH)D concentration, a marker of inflammation, and physical performance. Moreover, this study pointed out the positive impact of moderate-intensity regular training on older women's physical performance, fall risk, and oxidative stress regardless of baseline 25(OH)D concentration, indicating the physical activity as the most crucial factor leading to healthy aging.

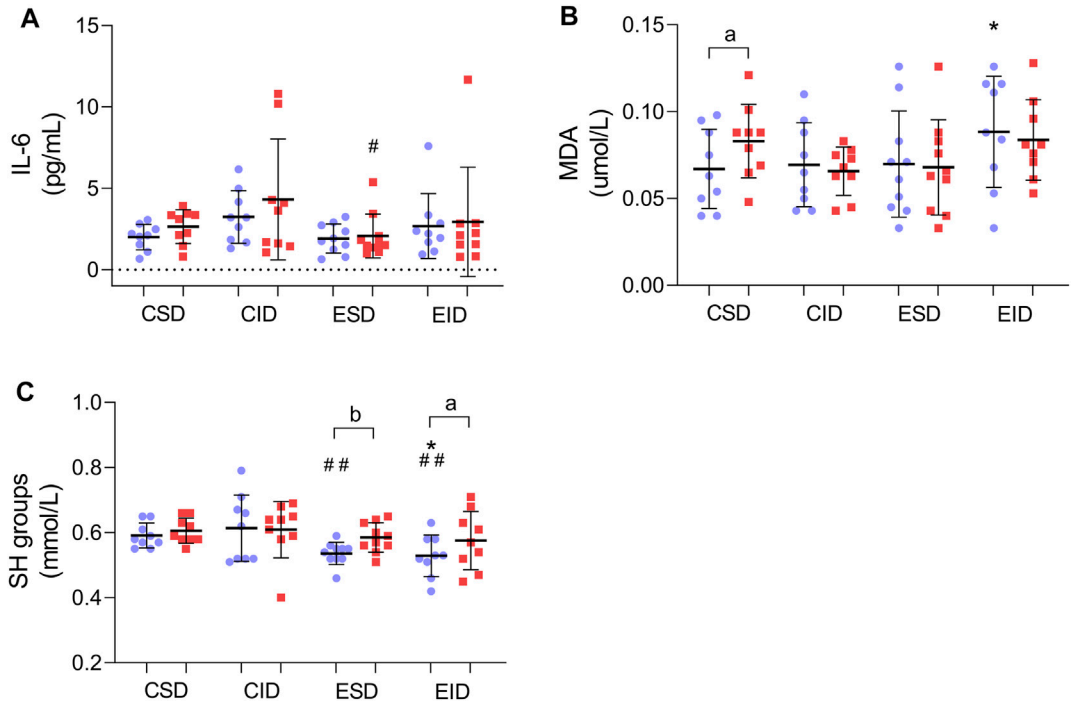

FIGURE 4 | Post-training changes in inflammation marker and oxidative stress. **(A)** IL-6 concentration; **(B)** MDA concentration; **(C)** SH concentration. There were significant differences between the indicated time points: **a** – $p < 0.05$, **b** – $p < 0.01$; between the groups: *$p < 0.05$ vs. CSD; #$p < 0.05$, ##$p < 0.01$ vs. CID. The data are presented as the means ± SEM; plain—before the intervention; strips—after the intervention.

The association of vitamin D concentration with older adults' physical performance remains controversial and conflicting. The research in recent years showed an association between vitamin D deficiency and sarcopenia, increased fall risk, impaired muscle strength, and physical performance (Dhaliwal and Aloia, 2017; Remelli et al., 2019). The highest prevalence of lower muscle strength and physical performance decline was noted among older adults with a serum 25(OH)D concentration below 10 ng/ml (Aspell et al., 2019). Our study confirmed a correlation between physical performance and vitamin D concentration. Women with a 25(OH)D concentration higher than 30 ng/ml performed significantly better in the TUG and fall risk test and tended to have better results in 6MWT, indicating vitamin D status as a significant factor related to poor physical performance and higher fall risk in the elderly. Moreover, multivariate regression analysis indicated vitamin D concentration as one of the most important predictors of functional test performance and predicts fall risk. Therefore, vitamin D may be a potentially modifiable factor associated with maintaining physical performance and preserving independence as getting older. It is also known that vitamin D insufficiency leads to increased inflammation and intensified production of pro-inflammatory cytokines (Berridge, 2017). Our study is in line with these observations. We found a significant negative correlation between 25(OH)D and IL-6–a marker of systemic inflammation. The participants with 25(OH)D concentrations below 30 ng/ml had IL-6 concentrations higher than 2.5 pg/ml, a cut-off point indicating low-grade inflammation (Ferrucci et al., 1999). The multivariate regression analysis performed in the current study revealed that an insufficient 25(OH)D concentration strongly predicts inflammation development. Although the effect of vitamin D on muscle tissue is well known, the mechanism by which vitamin D acts positively on physical exercise is not fully understood. One of the potential mechanism(s) might be associated with suppressed production of pro-inflammatory cytokines while the vitamin D concentration is sufficient.

The higher concentration of IL-6 in the elderly is also suggested to induce physical performance decline, especially when combined with insufficient vitamin D concentration (Kositsawat et al., 2020). A negative correlation between IL-6 and functional test results was also observed. Therefore, a higher level of inflammation marker is associated with worse physical performance. The multivariate regression analysis confirmed that IL-6 and 25(OH)D concentrations are the strongest predictors of functional test performance.

The association of vitamin D status with fall risk and physical performance presented in our study might also be related to its impact on PTH and OC—a bone turnover marker. We found a significant negative correlation between the above-mentioned bone metabolism markers and 25(OH)D concentration. It has been previously proven that vitamin D deficiency may impair calcium metabolism and increase PTH concentration (Pludowski et al., 2013; Niculescu et al., 2020). A similar relationship was previously presented between vitamin D and OC. A study conducted by Kuchuk and coworkers indicated that in

humans with lower vitamin D concentration, a higher concentration of OC is observed (Kuchuk et al., 2009). The concentration of circulating osteocalcin may also be increased in hyperparathyroidism. Previous research showed a significant correlation between OC and PTH (Delmas et al., 1986); however, this correlation was insignificant in our study. Besides that, increased PTH is associated with a higher risk of fractures, bone loss, and osteomalacia (Pludowski et al., 2013). It is also a risk factor for falls and impaired balance among elders (de Franca et al., 2019). Previous studies indicated that elevated PTH concentration is associated with worse performance in the TUG test, lower gait speed, higher risk of sarcopenia, increased instability, and fall risk (Visser et al., 2003; Montero-Odasso et al., 2016). Our results are in line with these observations. A positive correlation between PTH concentration with fall risk and a negative correlation with physical performance measured by TUG and 6MWT has been found. Interestingly, multivariate regression analysis revealed that also OC tends to be a predictor of fall risk. Although no previous studies assessed the association of total OC and fall risk, a study conducted by Vitale J.A. and coworkers indicated that carboxylated osteocalcin is positively correlated with fall risk among older, fractured women (Vitale et al., 2021).

It is well known that in addition to vitamin D insufficiency, another factor associated with impairments in physical performance and higher fall risk is lack of physical activity and a sedentary lifestyle. Several training strategies such as resistance, balance, or tai chi training are recommended for the elderly to reduce the risk of disability and falls (Papa et al., 2017; Hewitt et al., 2018; Penn et al., 2019). The multi-component training programs—including balance, functional, strengthening, and endurance exercises are suggested to be the most effective in improving gait, balance, muscle strength, coordination, and overall physical functioning (El-Khoury et al., 2013). The training procedure applied in the current study also appears successful in improving physical performance. Consequently, it may contribute to healthy aging cause after 12 weeks of moderate-intensity aerobic training combined with resistance exercises, significant improvement in performed functional tests (TUG and 6MWT) and fall risk reduction was observed. Moreover, to the best of our knowledge, no previous studies assess if physical performance improvement after training is related to baseline 25(OH)D concentration. Several studies have assessed whether vitamin D supplementation will enhance the training effect. One study performed on high-level, well-trained athletes indicated that 8 weeks of high-intensity interval training combined with vitamin D supplementation did not induce better training responses than training alone (Jastrzebska et al., 2016). Also, a study by Kirsti Uusi-Rasi and coworkers indicated that both multimodal training and training supported by vitamin D supplementation improved muscle strength, balance, and mobility of older adults. Vitamin D did not enhance exercise effects on physical functioning. Still, some positive effects of supplementation were observed—vitamin D reduced bone loss at the femoral neck and increased trabecular density at the distal tibia (Uusi-Rasi et al., 2015). In the present study, no differences between groups in physical performance improvement and fall risk reduction were pointed out. Both training groups performed better in TUG, 6MWT, and fall risk tests. Therefore, physical training may be the most effective strategy for preventing falls and improving physical performance in older adults. However, there were discrepancies in PTH and OC concentration changes; PTH and OC concentrations were reduced after 12 weeks of training but only in the insufficient vitamin D concentration group. The studies investigating post-exercise changes in PTH concentration are still inconclusive. The review of Lombardi and coworkers indicated that after a single bout of exercise, mainly an increase in PTH secretion was observed, especially after long-lasting and moderate to high-intensity exercises. On the other hand, long-term training may have the opposite effect and limit PTH secretion, particularly among older adults (Lombardi et al., 2020). After 12 weeks of nordic-walking training, a decrease in PTH concentration was observed among postmenopausal women with baseline vitamin D insufficiency (below 18.6 ng/ml) (Nowak et al., 2020), which corresponds to the results obtained in our study. Therefore, this effect may benefit older adults with secondary hyperparathyroidism related to vitamin D deficiency. The impact of aerobic exercise on OC concentration is not fully understood. Both an increase (Kim et al., 2015; Kortas et al., 2020) no changes (Nowak et al., 2020) and a decrease (Wieczorek-Baranowska et al., 2012) was observed. On the one hand, OC is an indicator of bone tissue synthesis, but on the other, its high level reflects intense bone metabolism, leading to increased tissue degradation. A study conducted by Iki and coworkers indicated that subjects at risk of osteoporosis progression, especially the lumbar spine, had significantly higher serum OC concentration (Iki et al., 2006). The current study revealed a decrease in OC concentration, but only in older women with insufficient vitamin D concentration. Among participants with sufficient vitamin D concentration, no changes were observed. It has been previously suggested that reducing the bone turnover rate after training interventions may be favorable for bone mass, especially in people with vitamin D insufficiency (Wieczorek-Baranowska et al., 2012). Nevertheless, future studies are needed to evaluate the effect of exercise on OC concentration and its' associations with bone metabolism.

Oxidative stress and ROS generation play an important role in aging and age-related diseases—diabetes, neurodegenerative diseases, cardiovascular diseases, sarcopenia, and frailty (Liguori et al., 2018). Physical activity is suggested to contribute to healthy aging by its antioxidant effect (Bachi et al., 2019). In the current study, a significant increase in SH groups concentration in both training groups, which refers to better antioxidant capacity, was observed. There is a lack of studies assessing post-training modifications of the SH groups among older adults; however, studies on young (Metin et al., 2003) and middle-aged participants (de Oliveira et al., 2012), as well as animal studies (Dos Santos et al., 2021), confirmed our observations. It is well known that intensified oxidative stress is associated with a reduced level of serum SH groups, which are components of extracellular antioxidant machinery (Bourgonje et al., 2021). Therefore, regular physical training, particularly aerobic, may elevate antioxidant enzymes content and activity,

regardless of age. Among elders, it may also attenuate the effect of oxidative stress on age-related changes (Bachi et al., 2019). Moreover, it has been shown that reduced serum SH groups may be a risk factor for cardiovascular events, type 2 diabetes, and rheumatoid arthritis (Giustarini et al., 2005; Piwowar et al., 2009; Bourgonje et al., 2021). Thus, we are very far from any speculations, but an increase in serum SH groups in our study after 12 weeks of training may have a protective effect on age-related diseases. Unfortunately, this study did not note any significant decrease in MDA concentration as observed in previous studies on older adults after 12 weeks of nordic-walking training (Kortas et al., 2017) and after 16 weeks of combined aerobic-resistance training (Mota et al., 2019).

The study's limitations should be discussed in the context of the reported results and findings. Firstly, the number of participants was limited. Secondly, the vitamin D supplements were taken by the participants themselves (2,000 units or more per day). Nowadays, the LC-MS/MS HPLC method is the gold standard to measure 25(OH)D; thus, this method should be performed instead of ELISA in future studies. Another limitation is that also other BTM's such as sclerostin, serum C-terminal telopeptide of type I collagen, or serum procollagen type I N propeptide, as well as bone mineral density, should be investigated further to assess the effect of training and vitamin D status on bone metabolism and bone mass. However, in this research, we focused only on healthy, older women, verification whether observed changes are sex-dependent should be considered in the future. Also, applying this type of training to elders with comorbidities seems essential to investigate whether this type of training acts in the same way as in healthy women.

In conclusion, vitamin D concentration among older women was related to physical performance, fall risk, inflammation, and bone metabolism markers. Consequently, maintaining the optimal vitamin D concentration may be a potential, modifiable factor protecting from declines in physical performance and loss of independence among elders. Moreover, obtained results indicate that 12 weeks of combined training effectively improved elders' physical performance and increased antioxidant protection, regardless of baseline vitamin D status, indicating the physical activity as the most crucial factor leading to healthy aging. Still, the favorable effect of regular training on bone metabolism tends to be more pronounced in participants with vitamin D insufficiency. Future research is needed to evaluate the role of vitamin D in enhancing physical functioning in humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local institutional Bioethical Committee in Gdansk. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ER-F and JK conceived and designed the research and wrote the manuscript. ER-F, MK, DF, MS-T, and WS conducted the experiments. ER-F, MK, DF, and JK analyzed the data. MK, DF, WS, and JK reviewed and revised the manuscript. All authors read and approved the manuscript.

FUNDING

This research was funded by the Polish Ministry of Science and Higher Education (DS_RiK_2018_KF).

ACKNOWLEDGMENTS

We would like to thank all participants for their engagement in the training intervention and physical performance assessment.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 31 July 2020
doi: 10.3389/fphys.2020.00923

Plasma Concentration of Irisin and Brain-Derived-Neurotrophic Factor and Their Association With the Level of Erythrocyte Adenine Nucleotides in Response to Long-Term Endurance Training at Rest and After a Single Bout of Exercise

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Edited by:
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: 27 April 2020
Accepted: 09 July 2020
Published: 31 July 2020

Citation:
Rodziewicz E, Król-Zielińska M, Zieliński J, Kusy K and Ziemann E (2020) Plasma Concentration of Irisin and Brain-Derived-Neurotrophic Factor and Their Association With the Level of Erythrocyte Adenine Nucleotides in Response to Long-Term Endurance Training at Rest and After a Single Bout of Exercise. Front. Physiol. 11:923. doi: 10.3389/fphys.2020.00923

The study aimed to assess the effect of a single bout of incremental exercise on irisin and BDNF plasma concentrations as related to erythrocyte purine nucleotides concentration at rest and after exercise. Master endurance master athletes (training experience 38 ± 6 years) and a group of untrained participants completed a single bout of progressive incremental exercise test until exhaustion. The dual-energy x-ray absorptiometry and blood collection were performed. Blood was taken twice at rest and 10 min after exercise. Concentrations of ATP, ADP, and AMP were assessed in the erythrocytes. Hypoxanthine and uric acid were determined in plasma using the high-performance liquid chromatography. Plasma concentrations of irisin and BDNF were assessed through the immunoenzymatic method. The ATP level, ATP/ADP ratio and AEC value were significantly higher in the athletic group. A significantly higher concentration of BDNF was it also noted in the trained group that correlated with the erythrocyte energy status at rest. The single session of exercise induced a significant increase in ATP erythrocyte levels in both groups. Both exerkines significantly correlated at rest with red blood cell adenine nucleotides and degradation products (BDNF positively and irisin negatively). The blood concentration of BDNF and irisin, in response to exercise, was not significantly different between groups. Obtained data revealed a higher erythrocyte energy status and lower purine degradation products concentration in master athletes. Also resting plasma exerkines differed substantially between groups. In conclusion, long-term training resulted in exercise adaptation reflected by a higher erythrocyte energy status, lower purine degradation products concentration and modified concentration of

exerkines (higher BDNF and lower irisin blood concentrations). Therefore, we consider the training-induced adaptations in master athletes to be beneficial and significant. The moderate level of physical activity in the untrained group, even if sufficient in terms of general health, did not cause any discernible changes.

Keywords: adenylate metabolites, purine degradations, long-term endurance training, lactate, exerkines

INTRODUCTION

Muscle cells are highly metabolically active, mainly to deliver energy at rest and during exercise. Skeletal muscles communicate with other organs by producing and releasing myokines (Pedersen, 2019). One of them is irisin, a myokine newly discovered by Bostrom et al. (2012). It is produced from the fibronectin type III domain containing protein 5 and expressed mainly by skeletal muscles (Bostrom et al., 2012). Physical exercise is one of the main factors stimulating the release of irisin (Bostrom et al., 2012) thus, this protein belongs to muscle-derived exerkines – a group of proteins stimulated by exercise (Safdar and Tarnopolsky, 2018).

Despite the initial controversy around irisin, many years of research have since well documented its function. The major action of irisin is the conversion of white adipose tissue into brown adipose tissue by increasing the expression of mitochondrial uncoupling protein 1, thereby increasing energy consumption, thermogenesis, and promoting weight loss (Novelle et al., 2013). It has been also proposed that irisin plays a key role in regulating glucose and lipid metabolism in adipose tissue and skeletal muscles (Lee et al., 2015; Xin et al., 2016). Irisin is further suggested to improve glucose homeostasis by reducing insulin resistance (Perakakis et al., 2017). In addition, it can be a regulator of the cross-talk between muscles and bones. It prevents bone loss by enhancing osteoblast differentiation through the Wingless (Wtn)/β-catenin pathway, increasing the osteoblasts numbers and suppressing osteoclast formation (Anastasilakis et al., 2019). The physiological concentration of circulating irisin is affected by several factors such as age (Huh et al., 2014), metabolic syndrome, insulin resistance (Park et al., 2013), and body composition (Huh et al., 2012). Recently published data revealed that irisin can be also considered as a factor modifying adaptive changes in response to training (Fatouros, 2018).

Irisin was found to cross the blood-brain barrier, enter the central nervous system and stimulate the expression of the brain-derived neurotrophic factor (BDNF) (Jin et al., 2018). Thus, BDNF can also belong to the group of proteins stimulated by exercise, called exerkines (Pedersen, 2019). The main functions of BDNF are associated with the regulation of neurogenesis and neuron growth and survival by increasing their resistance to damage (Mattson et al., 2004). BDNF is not only involved in the regulation of neurogenesis but it also affects other tissues and regulates peripheral metabolism. Previous animal studies indicated that BDNF stimulates glucose uptake by the liver and skeletal muscle cells, improves glucose metabolism and increases insulin sensitivity (Marosi and Mattson, 2014).

In recent years, it was suggested that irisin and BDNF are regulators of energy metabolism in different tissues such as brain or muscle tissue. A study conducted by Huh et al. (2012) indicated that circulating irisin is associated with muscle ATP and ADP content and thus regulates muscle metabolism before and after exercise. Moreover, an animal study showed that treatment with irisin improves energy metabolism in the prefrontal cortex by enhancing ATP level and up-regulating ATP level in astrocyte cells via the AMPK pathway (Wang and Pan, 2016). Previous data showed that also BDNF regulates cellular bioenergetics and increases neuronal ATP production (Cheng et al., 2012). Pospieszna et al. (2019) demonstrated that in competitive athletes, an increment in the RBC energy status occurred across an annual training cycle, as assessed by the increments in AEC and ATP/ADP and ADP/AMP ratios. This suggests that red blood cell metabolism adjusts to increased physical requirements of consecutive training phases. Still, there are no data about the association of irisin or BDNF with erythrocyte energy status in human.

Chronic physical training leads to many adaptive changes in blood and RBC, e.g., regulation of oxygen delivery and uptake (Mairbaurl, 2013). Therefore, chronic training as well as a single bout of physical exercise has an impact on erythrocyte energy status and purine nucleotides metabolism in RBC. An *in vitro* study conducted by Berman et al. (1988) indicated that conditions associated with an intense physical exercise (low pO_2, decreased pH in RBC and increased intracellular inorganic phosphate-Pi concentration) lead to changes in purine nucleotide metabolism: a decrease in ADP together with an increase in erythrocyte ATP and IMP level. However, human studies evaluating the effect of a single bout of exercise and chronic training on purine nucleotides concentration in red blood cells and RBC energy status are limited and inconsistent. On the one hand, changes in ATP concentration with a concomitant decrease in ADP and AMP levels were observed in response to a single session of exercise (Wagner et al., 1991; Dudzinska et al., 2010; Dudzinska et al., 2018). On the other hand, Markiewicz et al. (1980) noted an increase in erythrocyte ATP levels in young swimmers. The erythrocyte ATP levels together with purine metabolites-hypoxanthine (Hx) and uric acid (UA)-are also considered as potential indicators of a training status and physical capacity (O'Brien et al., 2015; Dudzinska et al., 2018). Previous studies indicated that athletes

Abbreviations: ADP, adenosine 5′-diphosphate; AEC, adenylate energy charge; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; ATP/ADP, adenosine 5′-triphosphate/adenosine 5′-diphosphate; HOMA-IR, homeostatic model assessment; HOMA-%B, β-cell function assessment; HOMA-%B, insulin sensitivity assessment; Hx, hypoxanthine; LA, lactate; RBC, red blood cells; UA, uric acid; VO_{2max}, maximal oxygen uptake.

undergoing lifelong sprint training have lower resting and post-exercise plasma Hx and UA concentrations (Zielinski et al., 2019). There is no available data regarding the relationship between exerkines, ATP status and purine metabolites. Diverse forms of exercise can change concentrations of the above biomarkers. In addition, an increase in some exerkines and a drop in its concentration was noted in response to both a single bout of exercise and long-term training (Bostrom et al., 2012; Huh et al., 2014; Norheim et al., 2014; Perakakis et al., 2017; Kim and Kim, 2018). For example, a drop in irisin and BDNF concentration suggests their faster uptake by different tissues. Moreover, changes in blood BDNF concentration in response to exercise do not necessarily reflect changes in BDNF in the brain. They may have also too be associated with an increased uptake by the central nervous system (Knaepen et al., 2010). The recently published paper by Kujach et al. (2019) revealed that changes in peripheral BDNF in response to sprint interval training were accompanied by an increase in lactate levels (LA). Another study pointed out that LA is the "missing exercise factor" inducing BDNF synthesis (El Hayek et al., 2019).

Therefore, this study aimed to assess the effect of a single bout of incremental exercise on irisin and BDNF plasma concentrations and erythrocyte purine nucleotide levels. We also wanted to investigate the link between the two exerkines and erythrocyte purine nucleotides concentration at rest and after exercise.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the local ethics committee at the Poznań University of Medical Sciences and was conformed to the Declaration of Helsinki. Before commencing the study, all participants were informed about the study protocol and related risks and signed written informed consent.

Participants

Twenty two men aged 58 ± 3.6 years participated in the study. All subjects were healthy, normotensive, with an optimal body mass index and they had no contraindications to exercise. The characteristic of participants is presented in **Table 1**.

Study Design

Participants were assigned to two groups. The highly trained group (n = 12, VO_{2max} = 51.9 ± 2.7 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$, training experience = 38.0 ± 6.0 years), consisted of still active endurance master athletes who regularly participated in European and world championships and were classified between 1st and 10th position in their age categories. The second group consisted of untrained participants (n = 10, VO_{2max} = 37.0 ± 1.8 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$, without any training experience). All participants performed a single bout of progressive incremental exercise test on a treadmill until exhaustion. Before the test, participants underwent the dual-energy x-ray absorptiometry measurement to assess body composition (Nana et al., 2015). At rest and 10 min after exercise, blood samples were collected. The erythrocyte concentrations of ATP, ADP, and AMP were assessed. Hypoxanthine (Hx) and uric acid (UA) were determined in plasma by the high-performance liquid chromatography method. Plasma concentrations of irisin and BDNF were assessed by the immunoenzymatic method.

Exercise Protocol

Participants performed a maximal physical capacity test, i.e. an incremental treadmill exercise test until volitional exhaustion to determine maximal oxygen uptake (VO_{2max}). Subjects were instructed not to participate in any high-intensity or long-duration training sessions at least 24–48 h before testing. The test was performed in the morning, 3 h after a light breakfast (no coffee or tea). All participants had the same breakfast of calorific value of about 300–400 kcal including water to drink. During all examinations, the ambient temperature was maintained at 20–21°C. The incremental exercise test was performed on a mechanical treadmill (H/PCosmosPulsar, Sports & Medical, Nussdorf-Traunstein, Germany). Initial speed during was set at 4 $km \cdot h^{-1}$ and was increased after 3 min to 8 $km \cdot h^{-1}$. Then, the treadmill speed increased by 2 $km \cdot h^{-1}$ every 3 min until volitional exhaustion. Respiratory parameters were measured (breath by breath) by an ergospirometer (Cortex MetaLyzer 3B, Leipzig, Germany) and analyzed using MetasoftStudio v. 5.1.0 Software (Cortex MetaLyzer 3B; Cortex Biophysik, Leipzig, Germany). The polar Bluetooth Smart H6 (Polar Electro Oy, Kempele, Finland) heart ratemonitor was used to record the heart rate (bpm).

Sample Collection

Blood samples were collected at two-time points: before and 10 min after incremental exercise. Venous blood was taken from the antecubital vein by a qualified nurse into vacutainer tubes (Vacutainer SST^{TM} II Advance) for serum analysis, the tubes with sodium fluoride to assess glucose concentration and with lithium heparinate (4.9 mL) as an anticoagulant (S-monovette, Sarstedt, Nümbrecht, Germany) to assess erythrocyte purine nucleotides (ATP, ADP, and AMP), plasma Hx and UA.

Erythrocytes Isolation

To isolate the erythrocyte samples from the lithium heparinate, the tubes were immediately centrifuged (1000 × g, 5 min, 4°C; Universal 320R, Hettich Lab Technology, Tuttlingen, Germany). Buffy coat and plasma were removed and then plasma was frozen at −80°C until the analysis. Next, erythrocytes were washed three times with buffered 0.9% NaCl solution and centrifuged (1000 × g, 5 min, 4°C). After the last wash, the erythrocyte pellet was resuspended with a small volume of PBS. The erythrocytes were then collected in Modulohm glass capillaries (volume 20 μL, length 75 mm) to obtain hematocrit (Htc) values. Then, isolated erythrocytes were deproteinized with a volume of 1.3 mol/L HClO4, mixed, and centrifuged at 16,000 × g for 5 min at 4°C. The neutralization of the supernatant (600 μL) was performed with 130–160 μL of 3 mol/L K_3PO_4 (to pH 5–7). The samples were centrifuged again in

TABLE 1 | Characteristics of the participants from trained and control group.

Variables	Trained group (n = 12) Mean ± SE	Control group (n = 10) Mean ± SE	p-value
Age (years)	58.6 ± 4.3	57.4 ± 2.9	Ns
High (cm)	174.7 ± 1.5	177.0 ± 1.3	Ns
Weight (kg)	73.7 ± 1.9	75.4 ± 1.6	Ns
BMI ($kg \cdot m^{-2}$)	24.2 ± 0.5	24.0 ± 0.4	Ns
FM (%)	14.8 ± 0.9	22.5 ± 1.1	0.001
LBM (kg)	61.4 ± 1.9	61.6 ± 1.5	Ns
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	51.9 ± 2.7	37.0 ± 1.8	0.001
HR_{max} (bpm)	167.3 ± 2.4	174.8 ± 2.4	0.009
Distance (m)	1899.2 ± 197.1	1281.5 ± 121.8	0.02
Training experience (years)	38 ± 6.0	0.0 ± 0.0	0.001
Glucose ($nmol \cdot L^{-1}$)	5.2 ± 0.2	5.1 ± 0.2	Ns
Insulin ($pmol \cdot L^{-1}$)	40.4 ± 3.0	69.7 ± 3.0	0.001
HOMA-%B	80.3 ± 7.8	124.9 ± 7.5	0.001
HOMA-%S	112.9 ± 12.2	68.2 ± 3.5	0.004
HOMA-IR	0.89 ± 0.1	1.5 ± 0.1	0.001
Cholesterol ($mmol \cdot L^{-1}$)	46.8 ± 1.6	49.8 ± 1.1	Ns
LDL ($mmol \cdot L^{-1}$)	2.9 ± 0.2	2.9 ± 0.1	Ns
HDL ($mmol \cdot L^{-1}$)	1.5 ± 0.1	1.7 ± 0.1	Ns
TG ($mmol \cdot L^{-1}$)	0.75 ± 0.1	0.85 ± 0.1	Ns

Values are given as mean ± SE. Ns, non-significant differences between groups; BMI, body mass index; FM, fat mass; LBM, lean body mass; VO_{2max}, maximal oxygen uptake; HR_{max}, maximum heart rate; distance, total distance achieved by participants during the incremental treadmill exercise test; HOMA-%S, insulin sensitivity assessment; HOMA-%B, β-cell function assessment; HOMA-IR, Homeostasis Model Assessment; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.

the same way as above. The supernatant was stored at −80°C until the analysis.

Erythrocyte Purine Nucleotides Concentration

The purine nucleotide (ATP, ADP, AMP, and IMP) concentrations were assessed in erythrocytes using high-performance liquid chromatography with UV-VIS detection (Merck-Hitachi/Agilent, Tokyo, Japan/Santa Clara, CA, United States) according to the method used and described in details by Smolenski et al. (1990) and Pospieszna et al. (2019). To determine ATP, ADP, and AMP levels, the aliquots of 100 μL were injected into the sample loop. Purine nucleotides were separated using a gradient elution system at a 1 mL/min flow rate. Peaks were detected by absorbance at 254 nm. The concentrations of nucleotides being determined were expressed in relation to erythrocyte volume and expressed as μ mol/L RBC.

The values of adenylate energy charge (AEC) were calculated using the following formula (Dudzinska et al., 2006):

$$\mathrm{AEC} = \frac{[\mathrm{ATP}] + 0.5\ [\mathrm{ADP}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]}$$

Plasma Purine Metabolites (Hx, UA) Concentration

The concentration of purine metabolites was assessed by high-performance liquid chromatography method in neutralized plasma using UV-VIS detection (Merck-Hitachi/Agilent, Japan/United States) according to the method previously described in details by Smolenski et al. (1990) and used by our team (Zielinski et al., 2019). The system contains high-pressure gradient pump L-6200, 1050 diode array detector and autosampler AS 2000A with a thermostatic cooler set at 4°C. Separations were achieved by the analytical column BDS Hypersil C18, 150 mm × 4.6 mm × 3 μm (Thermo Finnigan, United States) protected with guard column 20 mm × 4 mm (Phenomenex, United States). Quantitative analysis was conducted using the ChemStation data system (Agilent, United States) operating on a PC. The substances identification was based on comparison retention times with standards of Hx and UA. The measurement was done at wavelength 254 nm for Hx and 280 nm for UA by comparison with external standards. The within-run/between-run %CVs were 3.1/4.1, 3.3/4.4 and 2.7/3.2% for Hx, X and UA, respectively.

Plasma BDNF and Irisin Concentration

All of the samples were immediately placed at 4°C and, within 10 min, the samples were centrifuged at 2000 × g for 10 min at 4°C. The separated plasma samples were frozen and stored at −80°C until later analysis. Plasma BDNF concentration was determined before and after exercise by immunoassay method using Elisa Kit (R&D, United States&Canada, catalog no. DBD00) according to the manufacturer's protocol. The maximal intra-assay coefficient of variability (CV) 5–6.2% and inter-assay CV 11.3–8.1%. Plasma irisin concentration was also determined before and

after exercise by immunoassay method using Elisa Kit (Phoenix Pharmaceuticals Inc., Germany, catalog no. EK06729) according to the manufacturer's protocol. The maximal intra-assay CV was 4–6% and inter-assay CV was 8–10%.

Biochemical Assays and HOMA Assessment

Serum glucose was measured by the colorimetric enzymatic method using the diagnostic kit of PZ Cormay S.A. (Warsaw, Poland). The sensitivity of the method was 0.03 $mmol \cdot L^{-1}$, the intra-essay CV was 2.20% and the inter-assay CV was 2.10%. The serum insulin concentration was analyzed with an immunoradiometric assay based on coated-tube separation (INS-IRMA kit, Biosource, Belgium). The sensitivity of the method was 1 $\mu IU \cdot mL^{-1}$, the intra-essay CV was 1.9% and the inter-assay CV was 6.3%.Both methods were previously described by Kusy et al. (2013).

The updated computer HOMA2 model was used to determine the β-cell function (HOMA-%B), insulin sensitivity (HOMA-%S), insulin resistance (HOMA-IR) from paired fasting glucose and insulin level. The normal values are 100% for HOMA-%B and HOMA-%S and 1.0 for HOMA-IR (Wallace et al., 2004). The HOMA-indexes were obtained by the software HOMA 2 Calculator, version 2.2.3, copyrighted by The University of Oxford[1].

Total cholesterol (TCh) was assessed by the reaction of cholesterol esters with cholesterol esterase and oxidase. Low-density lipoprotein cholesterol (LDL-c) was determined with phosphotungstic acid and magnesium ions. High-density lipoprotein cholesterol (HDL-c), remaining in the supernatant, was assessed enzymatically. Triglycerides (TG) assay was based on the reaction with glycerophosphate oxidase. The sensitivities of the methods were 0.026, 0.10, and 0.023 $mmol \cdot L^{-1}$ for TCh, HDL-c and TG, respectively. Intra-/inter assay CVs were 1.7/1.7%, 1.20/0.93% and 1.4/1.6%, respectively. The method was also previously described in detail by Kusy et al. (2013).

Lactate (LA) in whole blood (20 μL) was assayed using the spectrophotometric enzymatic method (Biosen C-line, EKF Diagnostics, Barleben, Germany).

Statistical Analysis

Statistical analyses were performed using the Statistica v.13. software package. The values were presented as mean values ± standard error (SEM). The baseline differences between groups were tested using the Student *t*-test. The differences between values before and after exercise were tested using the two-way repeated-measures ANOVA. If a difference was detected in the ANOVA model, significant differences were determined using Tukey's *post hoc* test. The results were considered statistically significant when $p \leq 0.05$. A Pearson product-moment correlation coefficient was computed to assess the relationship between the obtained results.

[1]https://www.dtu.ox.ac.uk/homacalculator

RESULTS

Participant Characteristics

There were no significant between-group differences in demographic and anthropometric characteristics. The exception was the amount of the fat mass. The higher level was observed among untrained participants. The trained group had higher values of physical capacity indicators in the untrained group a good level of aerobic capacity and fatigue tolerance was shown. As expected, significantly higher insulin concentration, HOMA-%B and HOMA-IR but lower HOMA-%S were observed in the untrained group. The concentration of resting glucose did not differ between groups (**Table 1**).

Resting Values

At rest, we observed statistically significant differences in the erythrocyte energy status between the trained and untrained groups (**Table 2**). The ATP level, ATP/ADP ratio and the AEC value were significantly higher in trained than in untrained participants. These results were reflected in the significant positive correlation between erythrocytes energy status and VO_{2max} ($r = 0.83$; $p = 0.001$ for ATP; $r = 0.61$; $p = 0.003$ for ATP/ADP ratio; $r = 0.65$; $p = 0.001$ for AEC) for the combined group of participants. We also noted a significantly higher Hx plasma concentration in the untrained group. Moreover, Hx concentration was negatively correlated with VO_{2max} ($r = -0.84$; $p = 0.001$) and ATP level ($r = -0.87$; $p = 0.001$). The UA concentration did not differ between the groups and was independent of the participants' VO_{2max}. A significantly higher concentration of plasma BDNF was noted in the trained compared to the trained group. The serum irisin concentration tended to be higher in the untrained compared to the trained group, which was supported by a negative correlation between irisin and VO_{2max} ($r = -0.44$; $p = 0.004$). At rest, irisin correlated positively with glucose ($r = 0.43$; $p = 0.047$), insulin ($r = 0.46$, $p = 0.032$) and HOMA-IR ($r = 0.52$; $p = 0.012$) in both groups. Also, erythrocyte ATP level correlated with the parameters of insulin resistance parameters: a negative correlation with insulin ($r = -0.83$; $p = 0.001$) and HOMA-IR ($r = -0.81$; $p = 0.001$) was observed for pre-exercise values.

Exerkines Modified by a Single Bout of Intense Exercise

The single bout of exercise did not cause any significant changes in irisin serum concentration in either group (**Table 2**). Some non-significant tendencies were noted: irisin slightly (18.4%) decreased in the untrained group and increased (12.5%) in the trained group in response to exercise. BDNF blood concentration significantly decreased in the trained group while in the untrained group no detectable changes were observed. Moreover, we observed a significant negative correlation between BDNF and LA in the combined group ($r = -0.35$; $p = 0.021$) but this relationship was more pronounced in the trained group ($r = -0.56$; $p = 0.004$).

TABLE 2 | Red blood cell adenine nucleotides (ATP, ADP, and AMP), cell energy status (AEC, ATP/ADP, and ADP/AMP), plasma purine metabolites (Hx, UA), myokines and lactate in control and trained group at baseline and 10 min after acute exercise.

Variables	Group	Baseline	After exercise	*p*-value (Time change)
ATP RBC (umol•L^{-1})	Control	1394.7 ± 28.4	1429.9 ± 26.9	0.001
	Trained	1610.3 ± 30.0*	1662.0 ± 26.0*	0.001
ADP RBC (umol•L^{-1})	Control	213.1 ± 1.9	195.2 ± 2.6	0.001
	Trained	179.4 ± 6.0*	166.2 ± 5.5*	0.001
AMP RBC (umol•L^{-1})	Control	17.6 ± 0.3	16.3 ± 0.2	0.001
	Trained	15.9 ± 0.4*	14.9 ± 0.4*	0.001
ATP/ADP	Control	6.6 ± 0.2	7.3 ± 0.2	0.001
	Trained	9.0 ± 0.2*	10.1 ± 0.2*	0.001
ADP/AMP	Control	12.1 ± 0.1	12.0 ± 0.1	Ns
	Trained	11.2 ± 0.1*	11.2 ± 0.1*	Ns
AEC	Control	0.92 ± 0.1	0.93 ± 0.1	0.001
	Trained	0.94 ± 0.1*	0.95 ± 0.1*	0.001
Hx (umol•L^{-1})	Control	5.1 ± 0.3	34.6 ± 2.1	0.001
	Trained	1.4 ± 0.2*	27.1 ± 1.4*	0.001
UA (umol•L^{-1})	Control	317.8 ± 8.8	421.3 ± 9.1	0.001
	Trained	313.5 ± 6.3	381.4 ± 8.3*	0.001
Irisin (ng•mL^{-1})	Control	9.0 ± 1.7	7.3 ± 1.0	Ns
	Trained	6.1 ± 0.2	6.8 ± 0.5	Ns
BDNF (ng•mL^{-1})	Control	17.2 ± 0.2	16.8 ± 0.1	Ns
	Trained	20.8 ± 0.2*	16.2 ± 0.5	0.02
LA (mmol•L^{-1})	Control	1.0 ± 0.3	7.5 ± 1.6	0.001
	Trained	1.1 ± 0.2	7.9 ± 1.4	0.001

*Values are given as mean ± SE. *p < 0.05, significant difference between trained and control groups at indicated time point. Ns, non-significant differences between indicated time points; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; AEC, adenylate energy charge; ATP/ADP, adenosine-5′-triphosphate/adenosine-5′-diphosphate ratio; ADP/AMP, adenosine-5′-diphosphate/adenosine-5′-monophosphate ratio; Hx, hypoxanthine; UA, uric acid; BDNF, brain derived neurotrophic factor; LA, lactate. AEC (adenylate energy charge) was evaluated according to the formula by Atkinson AEC = [(ATP) + 0.5(ADP)] / [(ATP) + (ADP) + (AMP)].*

Erythrocyte Purine Nucleotides After Exercise

The single session of an acute bout of exercise induced a significant increase in ATP erythrocyte levels in both trained and untrained groups (**Table 2**), still, the level was higher in the trained group. Erythrocyte ADP and AMP levels decreased after exercise in both groups. The drop in ADP and AMP levels was accompanied by a significant increase in AEC in both groups. The exercise performed induced an increase in the ATP/ADP ratio in both trained and untrained groups. The ATP level, ATP/ADP ratio and AEC value in erythrocytes after exercise were also positively associated with VO_{2max} among all participants (r = 0.83; p = 0.001; r = 0.54; p = 0.01; r = 0.59; p = 0.004, respectively).

Post-exercise Changes in Purine Degradation Products

The applied incremental exercise induced a significant increase in Hx concentration in both trained and untrained groups (**Table 2**). We observed a negative correlation between Hx concentration and VO_{2max} (r = −0.79; p = 0.001) and erythrocyte ATP levels (r = −0.79; p = 0.001) after exercise. Moreover, the single bout of exercise elicited an increase in UA concentration in both trained and untrained groups. The latter group had higher UA and Hx concentration after exercise compared to trained participants. The ATP level (r = −0.69; p = 0.001) and VO_{2max} (r = −0.72; p = 0.001) after exercise correlated negatively with UA.

Correlations of Exerkines With Purine Nucleotides

A significant positive correlation was observed between resting BDNF and two indicators of erythrocyte energy status, ATP/ADP ratio (r = 0.52; p = 0.012) and AEC (r = 0.5; p = 0.016) in the combined group of participants. Moreover, before exercise, we observed a negative correlation between BDNF and ADP level in erythrocytes (r = −0.45; p = 0.03). Also, purine degradation product Hx correlated with BDNF at rest (r = −0.46; p = 0.031). Likewise, irisin correlated with erythrocyte energy status at baseline as confirmed by the significant negative correlation between irisin and AEC (r = −0.46; p = 0.032). We also observed a positive correlation between irisin and AMP (r = 0.48; p = 0.025) and Hx (r = 0.46; p = 0.031). After exercise, we did not observe any correlations between exerkines and purine nucleotides. The correlations are shown in **Table 3**.

DISCUSSION

In this study, we have shown for the first time that the training and long-term training adaptation (38 ± 6.0 years

TABLE 3 | The correlations between exerkines, purine nucleotides and its derivatives and physical performance before exercise.

	BDNF ($ng \cdot mL^{-1}$)	Irisin ($ng \cdot mL^{-1}$)	ATP RBC ($umol \cdot L^{-1}$)
Red blood cell adenine nucleotides and degradation products			
ATP RBC ($umol \cdot L^{-1}$)	0.37	−0.37	1.00
ADP RBC ($umol \cdot L^{-1}$)	−0.45*	0.34	−0.28
AMP RBC ($umol \cdot L^{-1}$)	−0.34	0.48*	−0.18
ATP/ADP	0.52*	−0.42	0.70*
AEC	0.51*	−0.46*	0.75*
Hx ($umol \cdot L^{-1}$)	−0.46*	0.46*	−0.87*
UA ($umol \cdot L^{-1}$)	0.20	0.22	−0.29
Physical performance			
VO_{2max} ($ml \cdot kg^{1} \cdot min^{1}$)	0.44*	−0.44*	0.83*
Training hours/week	0.51*	−0.38	0.72*
LA ($mmol \cdot L^{-1}$)	−0.35*	−0.08	0.20

*Values represent a Pearson product-moment correlation coefficient. *$p < 0.05$, statistically significant correlation; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate, AMP – adenosine 5′-monophosphate; AEC, adenylate energy charge; ATP/ADP, adenosine-5′-triphosphate/adenosine-5′-diphosphate ratio; Hx, hypoxanthine; UA, uric acid; BDNF, brain-derived neurotrophic factor; VO_{2max}, relative maximal oxygen consumption; LA, lactate.*

of training experience) modifies exerkines blood concentration, purine nucleotides level in erythrocytes and purine degradation products in middle-aged individuals. Resting and post-exercise plasma exerkines (BDNF and irisin) and purine nucleotides levels substantially differed in the highly trained group in comparison to untrained participants. To evaluate if training status has an impact on the above indicators, we assessed their concentration in two conditions: at rest and after acute exercise. Since the effect of chronic exercise training on resting BDNF concentrations is not clear, we decided to involve a group of participants with exceptional training experience (Dinoff et al., 2016). Obtained data revealed higher resting erythrocyte energy status, expressed as ATP level, ATP/ADP ratio and adenylate energy charge (AEC), in the trained than untrained individuals. What is more, a positive correlation between erythrocyte energy status and VO_{2max}. was noted. This suggests that higher erythrocyte energy status leads to better oxygen delivery to working muscles in the conditions of increased oxygen demand during maximal physical exercise. Thus, ATP level, ATP/ADP ratio and AEC could be significant indicators of adaptation to a single acute session of exercise.

Purine nucleotide degradation products (Hx and UA) are considered as training status indicators. In this study, significant differences in Hx and UA concentrations between trained and untrained individuals were recorded at rest and after exercise. A negative correlation between these two parameters and VO_{2max} was also observed among all participants after exercise. This result is in line with previously demonstrated data that purine degradation products concentration at rest and after exercise are related to the training status and physical capacity (Saiki et al., 1999; Zielinski et al., 2019). Moreover, both a long-lasting sprint and endurance training lead to a decrease in plasma Hx and UA concentrations (Zielinski and Kusy, 2012, 2015). This suggests that chronic training causes a reduction in muscle adenine nucleotide pool loss and hence, improves muscle adaptation. Such an affect was also observed after a single acute bout of exercise (Zielinski et al., 2019).

Our study aimed to assess whether irisin affects erythrocyte energy metabolism. At baseline, we observed a significant negative correlation between irisin and AEC and a positive correlation between irisin and AMP. These results suggest that there is an association between irisin and erythrocyte energy status as well as metabolism. So far, there have been no studies on an association between irisin and purine degradation products. However, we observed a positive correlation between irisin and Hx, suggesting for the first time a potential association between irisin and regulation of muscle adenine nucleotide pool loss and thus, muscle training adaptation. These phenomena may be related to better training adaptation and higher muscle capacity in trained individuals, reflected by lower irisin concentration, higher AEC value and lower Hx concentration. Animal and human studies showed that irisin has an impact on energy metabolism and ATP level in muscle tissue, prefrontal brain cortex and astrocyte cells. Wang and Pan proved that irisin administration at a concentration of 100 ng/mL improved energy metabolism in the prefrontal cortex by enhancing ATP level. They observed an increase in the activity of mitochondrial complexes I, II, and IV and an up-regulation of ATP level in astrocyte cells via the AMPK pathway (Wang and Pan, 2016). Only in one human study, was an association between irisin and ATP level observed. Huh and coworkers noted that when ATP concentration in muscle decreases, irisin production is upregulated. This relation was observed after a sprint exercise. Authors suggested that irisin could play a role in regulating muscle metabolism and muscle metabolite content (Huh et al., 2012). In our study, we didn't observe any association between irisin and erythrocyte energy metabolism, thus future studies on larger populations are needed.

Jedrychowski et al. (2015) indicated that healthy humans have circulating irisin levels in the range between 3 and 5 $ng \cdot mL^{-1}$, and that these values increase with exercise. In our study, the values measured in the trained group were similar to the recommended range, whereas in the untrained group an elevation of irisin levels was noted. What is more, the study by Huh et al. (2014) revealed that a baseline concentration of irisin was lower in physically active compared to sedentary subjects and noted a negative correlation between baseline values of irisin and VO_{2max}. Literature data also indicate that a higher concentration of plasma irisin is observed in pre-diabetes middle-aged males, compared with controls (Norheim et al., 2014). The same tendency was observed in our study, where by a significant negative correlation between irisin concentration and VO_{2max} was obtained. It is noteworthy that after 12 weeks of training, irisin concentration decreased both in pre-diabetic and healthy participants (Norheim et al., 2014). A reduction in irisin concentration related to a response to long-term training, we have observed, is in line with this result. Our study

demonstrates that a lower concentration of irisin in trained individuals is associated with higher muscle adaptation, physical capacity and a higher erythrocyte energy status. However, Kerstholt et al. (2015) observed a positive correlation between physical capacity and irisin among female middle-aged subjects, while in males of the same age, the correlation was negative. Therefore, it cannot be excluded that irisin concentration is sex-dependent.

Previous data also showed that irisin is related to glucose metabolism and insulin resistance. Human studies revealed that there is a positive association between circulating irisin concentration and whole-body mass, BMI, fat mass and insulin resistance (Perakakis et al., 2017). The results of our study confirm these data by revealing a significant positive correlation between irisin concentration and fasting glucose, insulin and HOMA-IR. Participants from the trained group, as expected, were characterized by significantly lower insulin concentration and HOMA-IR than the untrained group. Lower insulin resistance was accompanied by lower irisin concentration. An increase in irisin concentration could be related to a compensatory mechanism called the "irisin resistance syndrome," activated to overcome glucose metabolism disturbances and is associated with insulin resistance (Huh et al., 2015).

Most of the publications so far have focused on changes in irisin concentration in response to physical effort (time, intensity or mode) (Pekkala et al., 2013; Briken et al., 2016; Kabak et al., 2018) but only a few investigated irisin uptake that can modify energetic status. In our participants, irisin was not significantly affected by exercise but different tendencies emerged depending on the training status. A slight decrease in the untrained and an increase in the trained group was noted. Although irisin did not change significantly, we observed a significant post-exercise increase in ATP levels in both groups. The ATP level was higher in the trained group in response to the performed exercise. It was previously found that the erythrocyte ATP level is a regulator of hemoglobin affinity for oxygen. Thus, an increase in ATP level possibly leads to an increase in Hb-A p50 values, resulting in reduced erythrocyte affinity to oxygen and better tissue oxygenation (O'Brien et al., 2015). In the current study, ADP and AMP concentrations post-exercise were significantly lower than before exercise, while ATP/ADP ratio increased. An *in vitro* study indicated that a decrease in ADP level, accompanied by an increase in ATP level and ATP/ADP ratio in erythrocytes, could be caused by a decrease in pH, low pO_2 with a concomitant rise in intracellular inorganic phosphate-Pi in red blood cells – the conditions characteristic of an intense physical exercise (Berman et al., 1988).

Both regular training and a single bout of exercise are suggested to be the main factors that modify BDNF and irisin concentrations. There have been attempts to elucidate the impact of a single session of exercise on BDNF concentration. A large variety of protocols have been used and subjects of diverse training and health status and age have been involved. Nevertheless, the results remained inconclusive as discordant findings have been revealed (Knaepen et al., 2010). On the other hand, a recently published paper revealed that BDNF concentration increased after regular long-lasting endurance training (Mrowczynski, 2019). We also demonstrated a significantly higher BDNF concentration in the trained group and, what is more, observed a positive correlation between BDNF and VO_{2max}. Since, according to the available data, BDNF is also considered as one of the regulators of energy metabolism, we decided to verify if its concentration is related to ATP concentration in erythrocytes. Until now, an association between BDNF and ATP was assessed only in neurons. The study by Cheng et al. (2012) found out that neurons treated with BDNF (20 $ng{\cdot}mL^{-1}$) exhibited an increased ATP production (by about 20%) compared to control neurons, showing that there could be some association between BDNF and energy metabolism. Our study also showed a correlation between BDNF and energy metabolism, this time in human red blood cells. We observed a positive correlation between BDNF and ATP/ADP ratio and adenylate energy charge (AEC). Moreover, a negative correlation between BDNF and Hx was also noted. Both of these associations were observed before exercise. Therefore, BDNF also regulates muscle adenine nucleotide pool loss and muscle adaptation to training. Our results suggest that the higher peripheral BDNF concentration, the higher the erythrocyte energy status and the lower the muscle nucleotide pool loss.

Recently published papers revealed a significant correlation between BDNF and lactate concentration during exercise (El Hayek et al., 2019; Kujach et al., 2019). The results presented by Kemppainen et al. (2005) suggest that with a decreasing glucose supply during a high-intensity exercise, lactate is used by the brain to compensate for the increased energy required to maintain neuronal activity (Kemppainen et al., 2005). In our study, correlation between BDNF and lactate was inverse at rest but absent after exercise. It is worth noting that the experiment by Kemppainen et al. involved young participants, thus we cannot rule out that the training experience of our subjects and the energetic status of their erythrocytes may have compensated for this relationship. Further investigations are still needed. Our work has been partly limited by the factors ranging from the number of subjects involved to the lack of assessment of cognitive function to show an impact of exerkines on cognitive processes. Notwithstanding, the goal of this study was mainly to assess the baseline and post-exercise exerkines concentrations in relation to erythrocyte energy status. Future studies to evaluate cognitive functions and their association with exerkines are essential and needed.

CONCLUSION

In conclusion, long-term training results in training adaptation reflected by higher erythrocyte energy status, lower purine degradation products concentration and modified concentration of exerkines (higher BDNF and lower irisin blood concentration).

Our study offers an assessment of the energy status in erythrocytes in association with exerkines concentrations in an exceptional cohort of master athletes. It is rare to involve people with such a long training experience in the study, hence, the obtained results are particularly valuable.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Poznań University of Medical Sciences, Decision No. 1252/18. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ER and EZ conceived and designed the research and wrote the manuscript. JZ, MK-Z, KK, ER, and EZ conducted the experiments. ER, KK, and MK-Z analyzed the data. EZ, MK-Z, JZ, and KK reviewed and revised the manuscript. All authors read and approved the manuscript.

FUNDING

This work was funded by the National Science Centre, Poland, within the OPUS 14 program (application and Grant No: 2017/27/B/NZ7/02828).

ACKNOWLEDGMENTS

We would like to thank all participants for their engagement in the physical performance assessment.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.