Akademia Wychowania Fizycznego i Sportu
im. Jędrzeja Śniadeckiego w Gdańsku

mgr Marta Flis

Zmiany stężenia czynnika wzrostu fibroblastów (FGF21) w odpowiedzi na wysiłek fizyczny i ogólnoustrojową krioterapię

promotorzy:
prof. dr hab. Ewa Ziemann
dr hab. Giovanni Lombardi

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Pragnę złożyć serdeczne podziękowania

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Spis treści

1. Wykaz stosowanych skrótów

WBC- krioterapia ogólnoustrojowa *(ang. whole body cryotherapy)*

HIIT- trening interwałowy wysokiej intensywności *(ang. high-intensity interval training)*

NW- Nordic Walking trening (*ang. nordic walking training)*

FGF21- czynnik wzrostu fibroblastów 21 *(ang. fibroblast growth factor 21)*

BAIBA- kwas β-aminoizomasłowy *(ang. β-aminoisobutyric acid)*

BDNF- neurotroficzny czynnik pochodzenia mózgowego *(ang. brain-derived neurotrophic fac*tor*)*

BMI- wskaźnik wagowo-wzrostowy prawidłowej masy ciała *(ang. body mass index)*

PBF- procentowa zawartość tkanki tłuszczowej *(ang. percentage of body fat)*

VFA- powierzchnia trzewnej tkanki tłuszczowej *(ang. visceral fat area)*

GIP- żołądkowy peptyd hamujący *(ang. gastric inhibitory peptide)*

GLP-1- glukagonopodobny peptyd 1 *(ang. glucagon-like peptide 1)*

TC- całkowity cholesterol *(ang. total cholesterol)*

HDL- lipoproteina o wysokiej gęstości *(ang. high-density lipoprotein)*

LDL- lipoproteina o niskiej gęstości *(ang. low-density lipoprotein)*

TG- trójglicerydy *(ang. triglicerydes)*

HOMA-S- wskaźnik wrażliwości na insulinę *(ang. The Homeostasis Model Assessment estimates insulin sensitivity)*

HOMA-IR- wskaźnik insulinooporności *(ang. The Homeostasis Model Assessment estimates insulin resistance)*

HOMA-B- wskaźnik funkcji komórek β-trzustki *(ang. The Homeostasis Model Assessment estimates β-cell function)*

HR- częstość skurczów serca *(ang. heart rate)*

BAT- brązowa tkanka tłuszczowa *(ang. brown adipose tissue)*

GLUT4- transporter glukozy, typ 4 (*ang. glucose transporter type 4*)

Glu-OC- niekarboksylowana postać osteokalcyny *(ang. undercarboxylated osteocalcin)*

Gla-OC- karboksylowana postać osteokalcyny *(ang. carboxylated osteocalcin)*

2. Wykaz prac wchodzących w skład rozprawy

Niniejszą rozprawę doktorską stanowi cykl publikacji naukowych pod wspólnym tytułem: ***„Zmiany stężenia czynnika wzrostu fibroblastów (FGF21) w odpowiedzi na wysiłek fizyczny i ogólnoustrojową krioterapię”***, na który składają się trzy prace opublikowane w recenzowanych czasopismach naukowych o sumarycznej punktacji Impact Factor 14.107 i MEiN 350 pkt.:

I. ***„Beneficial effects of whole-body cryotherapy on glucose homeostasis and amino acid profile are associated with a reduced myostatin serum concentration” (***2021); **Marta Kozłowska**, Jakub Kortas, Małgorzata Żychowska, Jędrzej Antosiewicz, Klaudia Żuczek, Silvia Perego, Giovanni Lombardi, Ewa Ziemann; Scientific Reports, DOI: 10.1038/s41598-021-86430-9; Impact Factor 4.379, MEiN 140 pkt.

II. ***“Short and long-term effects of high-intensity interval training applied alone or with whole-body cryostimulation on glucose homeostasis and myokine levels in overweight to obese subjects”*** (2021); **Marta Kozłowska-Flis**, Ewa Rodziewicz-Flis, Katarzyna Micielska, Jakub Kortas, Joanna Jaworska, Andżelika Borkowska, Veronica Sansoni, Silvia Perego, Giovanni Lombardi, Ewa Ziemann; Frontiers in Bioscience- Landmark, DOI:10.52586/5015; Impact Factor 4.009, MEiN 70 pkt.

III. ***“Nordic walking rather than high intensity interval training reduced myostatin concentration more effectively in elderly subjects and the range of this drop was modified by metabolites of vitamin D”*** (2021); Katarzyna Micielska†, **Marta Flis**†, Jakub Kortas, Ewa Rodziewicz-Flis, Jędrzej Antosiewicz, Krystian Wochna, Giovanni Lombardi, Ewa Ziemann; Nutrients, DOI: 10.3390/nu13124393; Impact Factor 5.719, MEiN 140 pkt.

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3. Wstęp

Brak aktywności fizycznej jest jednym z wiodących czynników zwiększonego ryzyka śmiertelności[1]. Prowadzi do akumulacji tkanki tłuszczowej, sprzyja rozwojowi otyłości, cukrzycy typu II, chorób sercowo- naczyniowych i wielu innych[1]. Z drugiej strony, aktywność fizyczna może łagodzić skutki bezczynności ruchowej i chronić organizm przed zaburzeniami metabolicznymi, pobudzając termogenezę, metabolizm, biogenezę mitochondrialną, β-oksydację czy wychwyt glukozy przez tkanki[2]. Nordic Walking (NW) to jedna z najpopularniejszych, dostępnych i powszechnych form aktywności fizycznej, niezależnie od kategorii wiekowej. Korzystny wpływ tego treningu wytrzymałościowego jest dobrze udokumentowany pod względem poprawy funkcji układu krążenia, gospodarki lipidowej[3] czy normalizacji stężenia glukozy we krwi[4]. Alternatywę dla tego rodzaju aktywności fizycznej może stanowić trening interwałowy wysokiej intensywności (HIIT), który indukuje podobne zmiany adaptacyjne w organizmie[5] przy jednoczesnej oszczędności czasu. Zainteresowanie naukowców treningami interwałowymi wynika z faktu istotnej redukcji codziennej, dowolnej aktywności fizycznej, co często jest tłumaczone brakiem czasu. Przeprowadzono szereg badań wykazujących prozdrowotne efekty tej formy aktywności ruchowej[6]. Udowodniono na przykład, iż jest on skuteczny w poprawie insulinowrażliwości wśród starszych pacjentów z cukrzycą typu II[7]. Drugą procedurą o korzystnym, prozdrowotnym działaniu jest ekspozycja na niskie temperatury. Oprócz dobrze udowodnionego działania przeciwzapalnego[8], może również stanowić metodę redukującą zaburzenia homeostazy glukozy poprzez zwiększenie masy i aktywności brązowej tkanki tłuszczowej (BAT)[9]. Aktywacja BAT w organizmie prowadzi do rozpraszania energii w postaci ciepła, co nie tylko zwiększa wydatek energetyczny, ale także przyspiesza utlenianie trójglicerydów (TG) i glukozy jako substratów energetycznych[10]. Po 2-godzinnej ekspozycji na temperaturę 19°C i okresowym schładzaniu kończyn dolnych okładami lodowymi zaobserwowano zwiększony wychwyt glukozy przez komórki BAT u młodych, zdrowych osób[11]. Ponadto u pacjentów z cukrzycą typu II, 10 dni aklimatyzacji w temperaturze 14-15°C zwiększyło obwodową wrażliwość na insulinę o ~43% poprzez wzrost translokacji transporterów glukozy (GLUT4) w mięśniach szkieletowych[10]. Terapia zimnem może mieć różne formy (okłady lodem, zanurzanie w zimnej wodzie, morsowanie, częściowa i ogólnoustrojowa krioterapia), które działają lokalnie lub ogólnoustrojowo[12]. Praca przeglądowa Lombardi i in. wskazuje, że krioterapia ogólnoustrojowa (3 minuty, −110ºC;−130ºC; WBC) wykazuje korzystne wielokierunkowe działanie: hormonalne, immunologiczne, antyoksydacyjne i metaboliczne[13]. Zabiegi WBC

mogą poprawiać profil lipidowy[14], redukować ogólnoustrojowy stan zapalny towarzyszący otyłości[15], zwiększać w erytrocytach aktywność enzymów antyoksydacyjnych takich jak dysmutaza ponadtlenkowa czy katalaza[16]. Przegląd badań wskazuje, że skuteczność WBC może być modyfikowana przez wiele czynników takich jak liczba przeprowadzonych zabiegów[14], wydolność krążeniowo-oddechową badanych[8] czy skład ich ciała[15]. Ekspozycja na niską temperaturę może wzmacniać działanie wysiłku fizycznego poprzez redukcję stanu zapalnego indukowanego samym wysiłkiem, czy też nadmiarem tkanki tłuszczowej[17,18]. Ponadto zaobserwowano poprawę profilu lipidowego u otyłych mężczyzn poddanych serii 20 zabiegów WBC połączonych z treningiem fizycznym[19]. Z drugiej strony wyniki badań pokazują, że terapia zimnem może tłumić zmiany adaptacyjne wywołane wysiłkiem fizycznym u mężczyzn[20,21]. Dlatego też poszukuje się najbardziej efektywnych protokołów łączących wysiłek z terapią zimnem i określenia ich skuteczności w poprawie profilu metabolicznego osób nieaktywnych fizycznie.

Dorosłe, nieaktywne fizycznie osoby, nawet jeśli są zdrowe, stanowią kluczowy cel interwencji, ponieważ bez zmiany stylu życia mogą być narażone na ryzyko pogorszenia stanu zdrowia w przyszłości. Warto zwrócić uwagę, iż proces starzenia i zmiany z tym związane korelują z niższym poziomem aktywności fizycznej, co przyczynia się do obniżenia całkowitego wydatku energetycznego[22] i może prowadzić do akumulacji tkanki tłuszczowej[23], zwłaszcza wisceralnej (VFA). W konsekwencji może dochodzić do zaburzeń metabolizmu węglowodanów i lipidów poprzez dysfunkcję endokrynną adipocytów (podwyższony poziom leptyny, obniżony poziom adiponektyny) oraz zaburzenia funkcji komórek β-trzustki i/lub zmniejszoną wrażliwość tkanek na działanie insuliny i wymienione hormony[24,25]. Uważa się, że stan ten poprzedza rozwój cukrzycy typu II o 10 do 15 lat[26], w związku z czym istotne jest wdrożenie strategii zapobiegających progresji dysfunkcji metabolicznych w odpowiednim czasie.

Aktualne badania sugerują, że zarówno wysiłek[27] jak i ekspozycja na zimno[13] wywołują podobne reakcje endokrynologiczne w organizmie poprzez wpływ na sekrecję miokin z mięśni i adipokin z tkanki tłuszczowej, zapewniając wzajemne oddziaływanie między różnymi tkankami i narządami[2,28]. Korzyści zdrowotne wynikające z podejmowanej aktywności fizycznej i/lub ekspozycji na zimno są związane między innymi ze zmianami stężenia we krwi białka irisin oraz czynnika wzrostu fibroblastów 21 (FGF21), którego źródłem, w zależności od warunków zewnętrznych i/lub wewnętrznych organizmu, może być nie tylko tkanka mięśniowa czy tłuszczowa, ale także wątroba[29]. Dzięki możliwości przekraczania bariery mózg-krew, białko irisin, może zmieniać stężenie neurotroficznego

czynnika pochodzenia mózgowego (BDNF), pełniąc wspólnie nie tylko rolę regulatorów funkcji metabolicznych[30,31], ale modyfikując w ten sposób czynność układu nerwowego[32]. FGF21 wywiera bezpośredni wpływ na stymulowany insuliną wychwyt glukozy przez mięśnie szkieletowe[33]. Ponadto FGF21 ma wiele podobieństw funkcjonalnych do adipokin, w tym adiponektyny, która w sposób endokrynny kontroluje homeostazę glukozy i lipidów w mięśniach szkieletowych i wątrobie. Udowodniono, że adiponektyna reguluje wpływ FGF21 na metabolizm energetyczny i wrażliwość na insulinę w tych tkankach[34,35]. FGF21 jest również istotnym regulatorem gospodarki hormonalnej, przyczyniając się do brązowienia białej tkanki tłuszczowej[36,37].

Ważnym biomarkerem wczesnych zaburzeń homeostazy glukozy są aminokwasy w surowicy krwi[38]. Zaobserwowano podwyższone poziomy tych związków organicznych we wszystkich stadiach cukrzycy, w tym także w poprzedzającej ją insulinooporności[39]. Ze względu na zmiany stężenia aminokwasów i zahamowanie działania insuliny, osoby z insulinoopornością mogą również wykazywać dysfunkcje mięśni szkieletowych i sarkopenię związaną z otyłością[40]. Jednym z czynników przyczyniających się do rozwoju sarkopenii jest miostatyna[41], która jest negatywnym regulatorem metabolizmu w kontekście indukowania atrofii mięśni[42], ale także hamowania utleniania glukozy w mięśniach szkieletowych[43]. Zahamowanie funkcji miostatyny u ludzi prowadzi do podwojenia masy mięśniowej[44] i pośrednio napędza brązowienie białej tkanki tłuszczowej u myszy[45]. Myszy ze znokautowanym genem kodującym miostatynę wykazują wzrost wrażliwości na insulinę i zwiększony wychwyt glukozy a także zmniejszenie masy tkanki tłuszczowej[46].

Korzystny wpływ regularnie podejmowanego wysiłku fizycznego w kontekście chorób metabolicznych znany jest od lat. Niemniej jednak zaobserwowano, że wielkość efektów prozdrowotnych zależy od rodzaju wysiłku, jego intensywności, czasu trwania czy wielkości obciążenia[47]. Biorąc pod uwagę powyższe doniesienia, istotne jest precyzyjne ustalenie efektów stosowania zabiegów ogólnoustrojowej krioterapii w połączeniu lub nie z wysiłkiem fizycznym na profil metaboliczny osób nieaktywnych fizycznie. Istotne jest także określenie czy i w jaki sposób zastosowane procedury zmienią stężenie FGF21 we krwi i innych miokin i adipokin, oraz czy zmiany te wpłyną na homeostazę glukozy i metabolizm badanych osób. Znalezienie efektywnego protokołu w kontekście wywołania zmian prozdrowotnych w grupie badanych osób może przyczynić się do powstania nowych możliwości terapeutycznych w chorobach metabolicznych. Wyniki prezentowanych badań uzupełniono o ocenę zmian stężenia innych czynników, takich jak inkretyn: żołądkowego peptydu hamującego (GIP) i glukagonopodobnego peptydu 1 (GLP-1); karboksylowanej (Gla-OC) i niekarboksylowanej

(Glu-OC) formy osteokalcyny a także decorin, które mogą mieć znaczenie dla regulacji metabolizmu i gospodarki węglowodanowej, mimo iż nie jest to ich pierwszorzędowa funkcja.

4. Cele badań i hipotezy badawcze

W świetle powyższych doniesień, określiłam następujące cele swoich badań:

I. Ustalenie wpływu WBC na spoczynkowy poziom glukozy, profil metaboliczny oraz czynniki je regulujące wśród osób nieaktywnych fizycznie.
II. Określenie wpływu połączenia obu procedur: wysiłku fizycznego i WBC na stężenie FGF21 we krwi i zmianę profilu metabolicznego osób nieaktywnych fizycznie.
III. Sprawdzenie, który z protokołów treningowych: HIIT czy NW w większym stopniu wpłynie na zmiany profilu metabolicznego osób nieaktywnych fizycznie.
IV. Określenie czy zmiany indukowane połączeniem zastosowanych procedur: WBC i HIIT utrzymają się do czterech tygodni po zakończeniu procesu interwencji.

Hipotezy badawcze

I. Krioterapia ogólnoustrojowa i wysiłek fizyczny, cyklicznie powtarzane, poprawią homeostazę glukozy w grupie osób nieaktywnych fizycznie.
II. Zaobserwowanym efektom będą towarzyszyły zmiany stężenia wybranych białek pochodzenia mięśniowego, tłuszczowego i wątrobowego we krwi, w tym FGF21.
III. Efekt połączenia procedur (WBC i wysiłku fizycznego) będzie bardziej korzystny w grupie nieaktywnych fizycznie osób w stosunku do zastosowania tych procedur oddzielnie.
IV. Zmiany indukowane połączniem procedur WBC i wysiłku fizycznego utrzymają się do czterech tygodni od zakończenia interwencji.
V. Trening HIIT wysokiej intensywności i małej objętości będzie mógł stanowić alternatywę dla treningu NW o średniej intensywności i dużej objętości.

5. Materiały i metody badań

5.1. Badani

W eksperymencie wzięły udział trzy grupy nieaktywnych fizycznie mężczyzn i kobiet (mniej niż 60 minut aktywności fizycznej tygodniowo oceniane za pomocą kwestionariusza). Warunkiem włączenia uczestników do badań był brak uczestnictwa w zabiegach WBC w ciągu 12 miesięcy poprzedzających eksperyment oraz brak przeciwwskazań do podjęcia wysiłku fizycznego.

I. Pierwszą grupę badanych stanowiło 35 zdrowych, nieaktywnych fizycznie mężczyzn. Zakwalifikowani mężczyźni zostali poddani dwuetapowemu podziałowi na grupy. W pierwszym etapie analiz, badani zostali losowo przypisani do jednej z grup: eksperymentalnej- uczestniczącej w serii 10 zabiegów WBC (WBC-EXP, n = 22; wiek = 40 ± 14 lat; BMI = 26.1 ± 3.9 $kg \cdot m^{-2}$) lub kontrolnej (WBC-CON, n = 13; wiek = 30 ± 7 lat; BMI = 23.5 ± 2.5 $kg \cdot m^{-2}$). W kolejnym etapie, grupa eksperymentalna WBC-EXP, została dalej podzielona na dwie kategorie wiekowe: osoby młode (YG; n = 9; wiek = 28 ± 7 lat) lub w średnim wieku (MG; n = 13; wiek = 51 ± 3 lata).

II. Do kolejnych badań włączono 65 nieaktywnych fizycznie kobiet i mężczyzn z nadwagą lub otyłością. Uczestnicy zostali losowo przypisani do grupy trenującej (TR, n = 27; wiek = 42 ± 13 lat; BMI = 31.4 ± 3.5 $kg \cdot m^{-2}$) lub łączącej treningi z ekspozycją na zimno (TR-WBC, n = 38; wiek= 45 ± 9 lat; BMI = 31.9 ± 5 $kg \cdot m^{-2}$).

III. Ostatnia grupa badanych obejmowała 32 zdrowe, nieaktywne fizycznie osoby w średnim i starszym wieku (wiek = 61 ± 12 lat; BMI = 27 ± 4.2 $kg \cdot m^{-2}$; PBF = 33 ± 7.7%). Badani charakteryzowali się dwoma wskaźnikami otyłości sarkopenicznej i obniżonej sprawności fizycznej[48]: stosunkiem ilości tkanki tłuszczowej do beztłuszczowej masy ciała BF/FFM na poziomie 0.47 ± 0.17 $kg \cdot kg^{-1}$ oraz stosunkiem całkowitej masy ciała do beztłuszczowej masy ciała BM/FFM na poziomie 1.47 ± 0.17 $kg \cdot kg^{-1}$. Ponadto osoby badane charakteryzowały się insulinoopornością wyrażoną wskaźnikiem wrażliwości na insulinę QUICKI = 0.358 ± 0.04 oraz wskaźnikiem insulinooporności HOMA-IR = 2.02 ± 1.42. Uczestnicy zostali losowo przydzieleni

do jednej z dwóch grup treningowych: grupy trenującej NW przez 12 tygodni (NW, n = 18, BMI = 26 ± 3.5 $kg \cdot m^{-2}$) oraz grupy, która wykonała 6 jednostek treningu HIIT (HIIT, n = 14, BMI = 29 ± 4.0 $kg \cdot m^{-2}$).

5.2. Metody

5.2.1. Pomiary antropometryczne i oznaczenia z krwi

I. Pomiary antropometryczne zostały przeprowadzone z wykorzystaniem dwóch metod:
   a) analizatora składu ciała wykorzystującego zjawisko bioimpedancji elektrycznej wielu częstotliwości (In Body 720, Biospace, Korea)[49].
   b) skanera całego ciała Lunar Prodigy (GE HealthCare, Madison, WI, USA) wykorzystującego zjawisko absorpcjometrii rentgenowskiej o podwójnej energii (DXA) oraz oprogramowania enCORE v16 SP1[50,51].

II. Stężenie białek indukowanych wysiłkiem i/lub krioterapią ogólnoustrojową w surowicy krwi oceniano przy użyciu zestawów immunologicznych ELISA.

III. Oznaczenie stężenia aminokwasów wykonano za pomocą wysoko wydajnej chromatografii cieczowej z odwróconą fazą jonową połączonej z tandemową spektrometrią mas (IP-RP HPLC-MS/MS TSQ Vantage Thermo Scientific, USA)[52].

IV. Poziom pozostałych mediatorów takich jak: peptydu C, inkertyn: greliny, GIP, GLP-1, a także glukagonu i prozapalnych cytokin: leptyny, rezystyny i wisfatyny oznaczono przy użyciu technologii multipleksowej immunofluorescencji Plex Pro Diabetes Assay Panels (Bio-Rad, USA).

V. Profil lipidowy w surowicy (cholesterol całkowity [TC], wysokiej [HDL] i niskiej gęstości [LDL], TG) oznaczono za pomocą testów immunoenzymatycznych przy użyciu komercyjnych zestawów (Alpha Diagnostics, Warszawa, Polska).

VI. Metabolity witaminy D oznaczono metodą analizy ilościowej przy użyciu chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (QTRAP®4500

Sciex, Framingham, MA, USA) i systemem Exion LC HPLC. Pomiar skorygowano o zmianę objętości osocza[53].

VII. W celu określenia homeostazy glukozy:

a) oznaczono stężenie glukozy przy użyciu analizatora Cobas 6000 (Roche Diagnostics, Warszawa, Polska) zgodnie z instrukcją producenta.

b) oznaczono stężenie insuliny za pomocą zestawu do testów immunologicznych (Diametra, Perugia, Włochy).

c) wykorzystano następujące wskaźniki w celu określenia wrażliwości i oporności na działanie insuliny w organizmie:

- QUICKI =1/(log insuliny w surowicy $\mu U \cdot mL^{-1}$ + log na czczo) stężenie glukozy w osoczu $mg \cdot dL^{-1}$)[54]
- HOMA-S, HOMA-B i HOMA-IR, które obliczono na podstawie sparowanych odczytów stężenia glukozy i insuliny/ peptydu C na czczo za pomocą kalkulatora HOMA2 v2.2.3 (University of Oxford; www .dtu.ox.ac.uk/homacalculator)[55] lub zastosowano wzór: HOMA-IR= insulina w surowicy na czczo $\mu U \cdot mL^{-1}$ × glikemia na czczo $mmol \cdot L^{-1}/22.5$[56].

5.2.2. Testy wysiłkowe i protokoły eksperymentalne

I. W pierwszym eksperymencie, pułap tlenowy badanych, nieotyłych mężczyzn oceniono za pomocą testu wysiłkowego do odmowy na ergometrze rowerowym (884E Sprint Bike Monark, Sweden). Podczas testu mierzono wymianę gazową w płucach przy wykorzystaniu analizatora gazów oddechowych MetaMax 3B (Cortex, Niemcy)[8].

II. Przed drugim i trzecim eksperymentem przeprowadzono pilotażowy test wysiłkowy obejmujący jedną jednostkę treningu HIIT na ergometrze rowerowym w celu ustalenia indywidualnej, wysiłkowej częstości skurczów serca (HR) i wielkości obciążenia treningowego. Zadaniem każdego uczestnika było utrzymanie kadencji na poziomie 80-100 obrotów/minutę z obciążeniem 1.5 $W \cdot kg^{-1}$ (kobiety) lub 2.0 $W \cdot kg^{-1}$ (mężczyźni), tak aby osiągnąć intensywność 90% HR_{max}[7] oszacowanego na podstawie wzoru HR_{max}= 220 – wiek[57]. W grupie trenującej NW, przed rozpoczęciem badań, przeprowadzono test marszu na dystansie 2000m w celu oszacowania indywidualnej intensywności wysiłkowej na poziomie 60-70% HR_{max}[58].

III. W drugim i trzecim eksperymencie nadzorowane sesje HIIT przeprowadzono zgodnie z protokołem Little i inni[7] trzy razy w tygodniu przez 2 tygodnie (łącznie 6 sesji). Każdy trening składał się z: (A) 3-minutowej rozgrzewki się przy obciążeniu 50W; (B) dziesięciu 60-sekundowych interwałów rowerowych (80-100 obrotów/minutę, indywidulane obciążenie odpowiadające 90% HR_{max}) przeplatanych 60-sekundowymi przerwami odpoczynkowymi (dowolna kadencja, obciążenie 50W); oraz (C) 2-minutowej fazy wyciszenia przy obciążeniu 50W. Cała sesja treningowa trwała 25 minut. W drugiej pracy, w grupie TR-WBC, treningi HIIT wykonywano w Pomorskim Centrum Reumatologicznym (Sopot, Polska) bezpośrednio przed 1, 3, 5, 6, 8 i 10 sesją WBC. Grupa TR (pub. II) oraz grupa HIIT (pub. III) wykonały sześć jednostek HIIT bez udziału w sesjach WBC.

IV. Zastosowany w trzecim eksperymencie program treningów Nordic Walking obejmował 36 sesji treningowych przeprowadzanych 3 razy w tygodniu przez 12 tygodni zgodnie ze wcześniej opublikowanym protokołem[58,59]. Każda jednostka treningu NW składała się z: (A) 10 minut rozgrzewki; (B) 45-55 minut głównego treningu NW z intensywnością 60-70% HR_{max}; oraz (C) 10-minutowej fazy wyciszenia.

V. W pierwszych dwóch eksperymentach sesje krioterapii ogólnoustrojowej przeprowadzane były pod medycznym nadzorem w specjalnej komorze kriogenicznej (Zimmer Medizin Systeme, Elecpol) znajdującej się w Pomorskim Centrum Reumatologicznym w Sopocie. Zabiegi były powtarzane codziennie w dni robocze z uwzględnieniem dwudniowej przerwy w weekend. Łącznie ukończono dziesięć sesji WBC w ciągu dwóch tygodni. Każdą sesję poprzedzała 30-sekundowa adaptacja organizmu w temperaturze −60°C w przedsionku kriokomory. Ekspozycja na temperaturę –110°C w komorze głównej trwała 3 minuty.

6. Wyniki publikacji wchodzących w skład rozprawy

6.1. Wyniki eksperymentu opublikowane w pracy *"Beneficial effects of whole-body cryotherapy on glucose homeostasis and amino acid profile are associated with a reduced myostatin serum concentration"*

Przed rozpoczęciem protokołu badawczego odnotowano istotne różnice stężenia markerów homeostazy glukozy w zależności od kategorii wiekowej uczestników badań. Starsi uczestnicy (MG) grupy eksperymentalnej (WBC-EXP) wykazywali cechy zaburzeń metabolicznych. Charakteryzowali się nieprawidłowościami w składzie ciała a także wyższym spoczynkowym stężeniem glukozy (106.5 ± 7.0 vs. 95.7 ± 8.6 mg·mL^{-1}; $p = 0.002$) oraz insuliny (9.0 ± 2.1 vs. 6.3 ± 2.5 μU·mL^{-1}; $p = 0.01$) we krwi, co w konsekwencji wpłynęło na podwyższenie wyjściowej wartości wskaźnika HOMA-IR (1.2 vs. 0.8) w porównaniu do młodszych uczestników grupy eksperymentalnej (YG). Różnicom tym towarzyszyły odmienne poziomy stężenia miokin we krwi. Wśród uczestników MG odnotowano podwyższony poziom miostatyny, która na początku badań korelowała z większością wskaźników homeostazy glukozy.

W kolejnym etapie, opis wyników pierwszej publikacji został podzielony na trzy części przedstawiające kolejno efekt krótkotrwały pojedynczych jednostek WBC: 1h po pierwszej i ostatniej oraz efekt całej serii zabiegów WBC.

Pierwsza sesja krioterapii ogólnoustrojowej spowodowała istotny statystycznie wzrost stężenia miokin: irisin ($p = 0.02$) oraz miostatyny ($p = 0.03$) w grupie eksperymentalnej WBC-EXP. Ta niekorzystna zmiana wystąpiła przede wszystkim u młodszych uczestników tej grupy (YG; $p = 0.01$). Co ciekawe zmianom tym towarzyszył wyraźny spadek stężenia białka BDNF. Pojedyncza ekspozycja WBC obniżyła także stężenie FGF21 (z 280.4 ± 160.5 do 239.7 ± 166.6 pg·mL^{-1}), jednak zmiana ta nie osiągnęła istotności statystycznej ($p = 0.07$). Poziom pozostałych miokin i adipokin nie uległ zmianie w wyniku pierwszej, pojedynczej ekspozycji na ekstremalnie niską temperaturę.

Ostatnia jednostka WBC spowodowała istotne obniżenie stężenia adiponektyny ($p = 0.04$) oraz FGF21 we krwi ($p < 0.01$) w stosunku do wartości wyjściowych. Wielkość efektu d-Cohena była duża dla obu białek (1.08 dla adiponektyny i 1.12 dla FGF21). W odniesieniu do aminokwasów, stężenie we krwi niemalże połowy z nich krótkotrwale wzrosło zarówno po pierwszej jak i ostatniej jednostce WBC. Stężenie pozostałych wskaźników nie zmieniło się.

Wyniki eksperymentu pokazują, że seria ekspozycji na WBC spowodowała znaczny spadek stężenia glukozy ($p = 0.04$) i polepszyła większość mierzonych wskaźników jej homeostazy. Poprawa profilu metabolicznego była widoczna szczególnie wśród starszych uczestników badania, którzy charakteryzowali się większymi odchyleniami od normy na ich początku. Wśród MG odnotowano istotną redukcję wartości insuliny (z 9.0 ± 2.1 do 6.9 ± 2.1 $\mu mol \cdot L^{-1}$; $p = 0.01$) i HOMA-IR (z 1.2 ± 0.3 do 0.9 ± 0.3; $p = 0.01$). Stężenie FGF21 we krwi sukcesywnie obniżało się przez cały czas trwania interwencji. Wielokrotnej ekspozycji na WBC towarzyszył jednocześnie istotny wzrost stężenia adiponektyny (46.8%; $p = 0.05$) i spadek miostatyny u osób z grupy MG (–30%; $p < 0.01$). Profil lipidowy nie uległ zmianom pod wpływem zastosowanej procedury badawczej. Seria zabiegów WBC ostatecznie nie miała także wpływu na zmiany stężenia BDNF we krwi. Oznacza to, że zmiany odnotowane 1h po pierwszej krioterapii, były zmianami krótkotrwałymi, prawdopodobnie nie mającymi wpływu na metabolizm uczestników. Z kolei, podwyższony poziom irisin wywołany pierwszą sesją w kriokomorze utrzymał się w grupie eksperymentalnej, ale tylko wśród młodszych uczestników YG ($p = 0.04$). Stężenie aminokwasów: waliny i asparaginy obniżyło się istotnie w grupie WBC-EXP ($p < 0.01$) 24h po serii WBC w porównaniu do grupy kontrolnej WBC-CON. Co ciekawe w naszych badaniach odnotowano także dodatnią korelację między waliną a miostatyną w grupie WBC-EXP ($r = 0.60$). Stężenie pozostałych aminokwasów nie uległo zmianom w wyniku serii ekspozycji na ekstremalnie niską temperaturę.

6.2. Wyniki eksperymentu opublikowane w pracy *"Short and long-term effects of high-intensity interval training applied alone or with whole-body cryostimulation on glucose homeostasis and myokine levels in overweight to obese subjects"*

Na początku badań nie stwierdzono żadnych istotnych różnic pomiędzy parametrami charakteryzującymi uczestników. Zastosowane procedury badawcze nie zmieniły komponentów składu ciała uczestników.

W drugiej publikacji zaobserwowano, iż sześć jednostek treningu HIIT jest wystarczające, aby spowodować znaczny wzrost stężenia FGF21 (z 191.0 ± 91.8 do 275.0 ± 178.8 $pg \cdot mL^{-1}$; $p = 0.03$) we krwi nieaktywnych osób z nadwagą i otyłością. Zmianie tej towarzyszył istotny, w porównaniu do efektu odnotowanego w grupie łączącej HIIT z ekspozycją na WBC (TR-WBC), wzrost stężenia przeciwzapalnej adiponektyny ($p = 0.05$). Jednocześnie, w grupie trenującej (TR) nastąpiła poprawa profilu metabolicznego, poprzez obniżenie poziomu TG

(z 162.6 ± 131.2 do 129.0 ± 72.9 pg·mL^{-1}) i poprawę wartości wskaźnika HOMA-B (z 93.8 ± 36.9 do 83.7 ± 26.8%). Co ciekawe, HIIT w połączeniu z WBC, nie wywołał takich zmian w grupie TR-WBC (odpowiednio TR: –26.0% vs. TR-WBC: 0.9%; p = 0.07 dla TG oraz TR: –12.1% vs. TR-WBC: 6.0%; p = 0.02 dla HOMA-B). Protokół HIIT indukował także zmiany stężenia cytokin prozapalnych we krwi. Odnotowano spadek stężenia wisfatyny (z 5734.5 ± 2921.7 do 5107.4 ± 2713.6 pg·mL^{-1}; p < 0.05), leptyny oraz rezystyny. Zebrane dane sugerują, że sam HIIT jest bardziej skuteczny w obniżaniu poziomu wisfatyny niż HIIT stosowany razem z WBC, ponieważ efekty między grupami różniły się istotnie statystycznie (p = 0.04). Zastosowany protokół treningowy nie miał wpływu na pozostałe badane wskaźniki. Niemniej jednak stężenie peptydu C, zwanego też pro-insuliną, uwalnianego do krwi jako produkt uboczny wydzielania insuliny, wykazywał nieistotną statystycznie tendencję spadkową (–15.7%) i zmiana ta była dodatnio skorelowana ze zmianą wskaźnika HOMA-B (r = 0.74, p < 0.05).

Połączenie HIIT i WBC nie zmieniło znacząco profilu lipidowego w grupie TR-WBC, ale istotnie obniżyło spoczynkowe stężenie glukozy we krwi (z 99.2 ± 10.9 do 95.9 ± 9.9 mg·dL^{-1}; p < 0.05). Zmiana ta była istotnie różna od odnotowanej w grupie TR (p = 0.03). Niemniej jednak zimno stłumiło zmianę w poziomie FGF21 we krwi (z 204.0 ± 4.1 do 212.6 ± 113.7 pg·mL^{-1}) odnotowaną w grupie trenującej. Podobna sytuacja miała miejsce dla stężenia peptydu C (TR: –15.7% vs. TR-WBC: –2.3%), leptyny (TR: –16.2% vs. TR-WBC: –6.8%), rezystyny (TR: –7% vs. TR-WBC: –0.4%) czy wisfatyny (TR: –12.3% vs. TR-WBC: –1.6%). W żadnej z grup nie odnotowano zmian stężenia miostatyny, stąd, wynik ten nie został zaprezentowany w publikacji.

W przedstawionym badaniu, obserwowano wpływ zastosowanych procedur na zmiany stężenia inkretyn we krwi, w tym GIP i GLP-1. Nie wykryto istotnych zmian w poziomach tych czynników czy też glukagonu. Jednakże, we krwi grupy TR-WBC, zaobserwowano trend wzrostowy stężenia GIP. Zmiana ta była przeciwna do tendencji zaobserwowanej w grupie TR. Chociaż różnice między grupami nie były istotne statystycznie, należy podkreślić, że w grupie TR-WBC, wzrost poziomu GIP był dodatnio skorelowany ze spadkiem stężenia glukozy (r = 0.34, p < 0.05). Połączenie HIIT i WBC nie miało wpływu na pozostałe badane czynniki.

Cztery tygodnie po zakończeniu interwencji łączącej treningi z krioterapią ogólnoustrojową zarejestrowano podwyższoną masę mięśni szkieletowych oraz beztłuszczową masę ciała w grupie TR-WBC (p = 0.01 dla obu składowych). W tym momencie stężenie TC i frakcji HDL były znacznie wyższe niż w punkcie rozpoczęcia badań

(odpowiednio p = 0.03 i p = 0.02), ale nie różniły się statystycznie od wartości odnotowanych 24h po zakończeniu procedury badawczej. Spadek stężenia glukozy wywołany kombinacją HIIT i WBC nie utrzymał się 4 tygodnie po zakończeniu eksperymentu. W rzeczywistości stężenie glukozy wzrosło miesiąc po interwencji z 95.9 ± 9.9 do 98.7 ± 11.0 $mg \cdot dL^{-1}$ (p = 0.01), powracając w ten sposób do wartości wyjściowych.

6.3. Wyniki eksperymentu opublikowane w pracy *„Nordic walking rather than high intensity interval training reduced myostatin concentration more effectively in elderly subjects and the range of this drop was modified by metabolites of vitamin D”*

W celu weryfikacji protokołu HIIT w grupie osób w średnim i starszym wieku dokonano porównania jego skuteczności wobec bardzo popularnej formy ruchu w tej grupie wiekowej jakim jest trening NW. Zaobserwowano, że efekty indukowane tymi protokołami wysiłkowymi były przeciwne i różniły się istotnie statystycznie w odniesieniu do większości mierzonych cytokin. Protokół HIIT nie spowodował znaczących zmian w mierzonych wskaźnikach metabolizmu glukozy, profilu lipidowego ani stężeniu cytokin takich jak miostatyna, decorin czy osteokalcyna. Podobnie jak w grupie NW, nie zmienił się także wskaźnik otyłości sarkopenicznej i stężenie metabolitów witaminy D. Żaden z protokołów wysiłkowych nie wywołał także zmian stężenia FGF21 we krwi, stąd, wynik ten nie został zaprezentowany w publikacji.

Przeciwnie do treningów HIIT, seria 36 treningów NW znacznie obniżyła stężenie miostatyny w surowicy krwi (z 4.4 ± 1.9 do 3.9 ± 1.5 $ng \cdot mL^{-1}$; p = 0.05). Co ciekawe, większy zakres zmian stężenia tego białka w grupie NW odnotowano wśród osób z wyższym wyjściowym poziomem wszystkich mierzonych metabolitów witaminy D: $25(OH)D_3$ powyżej 25 $ng \cdot mL^{-1}$; $24,25(OH)_2D_3$ powyżej 2.0 $ng \cdot mL^{-1}$ i 3-epi-$25(OH)D_3$ ponad 1.3 $ng \cdot mL^{-1}$ (p < 0.01 dla wszystkich metabolitów). Wraz ze spadkiem miostatyny zaobserwowano wzrost stężenia decorin i zmiana ta była bliska istotności statystycznej (p = 0.06). Różnice delt w stężeniach tych miokin były istotnie i ujemnie skorelowane (r = –0.51; p = 0.04). Nieoczekiwanie, trening NW, spowodował także znaczny spadek stężenia obu form osteokalcyny: Glu-OC (z 10.3 ± 8.9 do 9.1 ± 7.5 $ng \cdot mL^{-1}$; p = 0.02) i Gla-OC (z 19.1 ± 5.9 do 16.8 ± 5.0 $ng \cdot mL^{-1}$; p < 0.01). Zmiany te nie zostały zaobserwowane w grupie HIIT.

Mimo, iż zmiany stężenia adiponektyny nie były istotne statystycznie w żadnej z grup, warto zwrócić uwagę na odwrotną tendencję zmian między HIIT i NW. Stężenie adiponektyny nieznacznie obniżyło się w odpowiedzi na trening interwałowy (z 8659.8 ± 6600.9 do 7842.8 ± 6069.3 $ng \cdot mL^{-1}$), podczas gdy trening NW wywołał nieznaczny wzrost stężenia tej adipokiny (z 11528.9 ± 9370.6 do 14173.6 ± 10504.2 $ng \cdot mL^{-1}$). Różnica między grupami była istotna statystycznie ($p < 0.01$). Zaobserwowane zmiany nie miały wpływu na gospodarkę węglowodanową, gdyż w żadnej z grup, mierzone cytokiny nie korelowały ze wskaźnikami metabolizmu glukozy.

7. Dyskusja

Regulacja metabolizmu związana jest z aktywnością białek uwalnianych do krwiobiegu z różnych narządów i tkanek: mięśniowej, tłuszczowej, czy kostnej, pod wpływem wysiłku fizycznego[60], ale także ekspozycji na niskie temperatury[61]. Białka te, zwane w zależności od źródła swojego pochodzenia miokinami, adipokinami lub osteokinami, tworzą endokrynne ścieżki sygnalizacyjne między odległymi tkankami[62]. Celem niniejszej rozprawy doktorskiej, będącej zbiorem trzech publikacji naukowych, było ustalenie wpływu krioterapii ogólnoustrojowej, wysiłku fizycznego i połączenia tych procedur na homeostazę glukozy oraz zmiany stężenia czynników ją regulujących wśród osób nieaktywnych fizycznie.

Wyniki przedstawionych prac wskazują, iż zarówno sesje krioterapii ogólnoustrojowej jak i cyklicznie powtarzany wysiłek fizyczny, mają pozytywny wpływ na homeostazę glukozy u nieaktywnych fizycznie osób. Zaobserwowane zmiany mogą zachodzić jednak na drodze różnych mechanizmów w zależności od zastosowanej procedury, a także od wieku i składu ciała uczestników. Zarówno samo WBC (pub. I) jak i połączenie z sześcioma jednostkami treningu HIIT (pub. II) spowodowało istotne obniżenie spoczynkowego stężenia glukozy we krwi badanych. Efekt ten okazał się jednak krótkotrwały i nie utrzymał się cztery tygodnie po zakończeniu interwencji. Poprawa metabolizmu pod wpływem WBC była zauważalna szczególnie wśród starszych badanych, charakteryzujących się wyższą wyjściową zawartością tkanki tłuszczowej podskórnej i wisceralnej w organizmie. W całej grupie WBC-EXP, spadkowi stężenia glukozy towarzyszyło obniżenie poziomu insuliny i wskaźnika HOMA-IR. Ponadto trening HIIT zastosowany oddzielnie (pub. II), mimo iż nie obniżył spoczynkowego stężenia glukozy we krwi to poprawił profil metaboliczny nieaktywnych osób w średnim wieku poprzez spadek stężenia TG oraz wskaźnika HOMA-B. Model HOMA-B

jest miarą aktywności komórek β-trzustki i odzwierciedla stopień wydzielania insuliny przez ten narząd[55]. Obniżenie wartości tego parametru w grupie HIIT (pub. II) istotnie korelowało z tendencją spadkową peptydu C, który jest powszechnie stosowany jako marker insulinooporności i zespołu metabolicznego[63]. Może to wskazywać na zmniejszone obciążenie metaboliczne komórek β-trzustki w celu utrzymania normoglikemii. Warto także zauważyć, że jedynie w grupie trenującej HIIT (pub. II) odnotowaliśmy spadek stężenia cytokin prozapalnych, w tym wisfatyny.

Zaobserwowanym efektom towarzyszyły zmiany stężenia FGF21 we krwi. Co istotne, kierunek zmian różnił się w zależności od zastosowanej procedury. Krioterapia ogólnoustrojowa wywołała obniżenie stężenia FGF21 we krwi, podczas gdy trening HIIT spowodował jego wzrost. Połączenie tych dwóch procedur stłumiło zaobserwowany efekt, co wydaje się sprzeczne z wcześniejszymi badaniami, w których WBC wykazuje swój prawdziwy potencjał tylko w połączeniu z programem ćwiczeń fizycznych[13,64]. FGF21 jest ważnym regulatorem metabolizmu. Udowodniono, że wpływa na ekspresję genów zaangażowanych w glukoneogenezę, lipogenezę, lipolizę i utlenianie kwasów tłuszczowych[65]. Jak niedawno donosił Sun i in.[66], FGF21 może działać jako hepatokina, adipokina i miokina, ale główne źródło krążącego w krwiobiegu FGF21 nie jest jasne i wydaje się, że zależy zarówno od warunków wewnętrznych jak i czynników zewnętrznych[67]. Według badań przeprowadzonych na modelu mysim, ekspresja i uwalnianie FGF21 do krwioobiegu z mięśni, wzrastają głównie w niekorzystnych warunkach, np. głodu, starzenia się czy otyłości[68]. W związku z tym obciążenie pracą o wysokiej intensywności w grupie trenującej HIIT u nieaktywnych osób mógł być czynnikiem generującym stres i przyczyniającym się do uwolnienia wspomnianego czynnika jako miokiny. Z drugiej strony, wzrost stężenia FGF21 może być również spowodowany zwiększoną ekspresją w wątrobie i białej tkance tłuszczowej[29]. Udowodniono, iż FGF21 pochodzenia wątrobowego poprawia tolerancję glukozy[69] i wzmaga utlenianie wolnych kwasów tłuszczowych[65]. W grupie TR (pub. II) zaobserwowano tendencję spadkową stężenia TG, podczas gdy poziom glukozy pozostał niezmieniony. Nie można jednak wykluczyć, że w tych warunkach wątroba mogła być głównym źródłem krążącego FGF21. W przeglądzie literatury, zmiany stężenia FGF21 na podejmowany wysiłek fizyczny zarówno pojedynczy jak i powtarzany są niespójne. We wcześniej opublikowanych badaniach odnotowano zarówno spadek[70], brak zmian[71] jak i wzrost[72] jego stężenia pod wpływem zastosowanych protokołów treningowych. Jednocześnie, zaobserwowano podwyższone spoczynkowe stężenie FGF21 we krwi osób starszych[73] i pacjentów z cukrzycą typu II[74], co często określane jest jako stan oporności

tkanek na działanie tego białka. W związku z tym, spadek stężenia FGF21 wywołany serią zabiegów WBC odnotowany w pierwszej publikacji z cyklu prac, może świadczyć o poprawie wrażliwości tkanek na FGF21, który posiada właściwości przeciwcukrzycowe, poprzez stymulacje wychwytu glukozy w adipocytach[69]. Poza tym udowodniono, że ekspozycja na zimno indukuje działalność adipocytowego FGF21 na drodze auto/parakrynnej[29], w związku z tym wzrost stężenia tego białka we krwi grupy WBC-EXP (pub. I) nie został zaobserwowowany. Wyniki ostatniej z zaprezentowanych publikacji nie potwierdziły zmian parametrów homeostazy glukozy pod wpływem różnych protokołów treningowych: HIIT i NW przeprowadzonych w grupie osób w średnim i starszym wieku. Co ciekawe, stężenie FGF21 we krwi w tym badaniu także nie zmieniło się. Z tego względu wynik ten nie został zaprezentowany.

W pierwszej publikacji niniejszego cyklu przeprowadzono analizę zmian stężenia aminokwasów na podstawie ich roli w procesach metabolicznych, w tym regulacji homeostazy glukozy. We wcześniejszych badaniach udowodniono, że podwyższone spoczynkowe stężenia aminokwasów rozgałęzionych we krwi (w tym waliny) mogą być predyktorem rozwoju cukrzycy typu II[75], zaś podwyższony poziom asparaginy może wiązać się z progresją tej choroby[76]. Po raz pierwszy przeprowadzono badanie, które oceniało zmiany stężenia aminokwasów we krwi w odpowiedzi na ekstremalnie niską temperaturę. Godzinę po pierwszej i ostatniej WBC zaobserwowano krótkotrwały wzrost stężenia niemalże połowy mierzonych aminokwasów, co mogło być spowodowane nasiloną proteolizą białek w wyniku ekspozycji na niską temperaturę[77]. Z kolei wielokrotne zastosowanie WBC spowodowało istotny statystycznie spadek waliny i asparaginy. WBC mogło zaindukować konwersję waliny do kwasu β-aminoizomasłowego (BAIBA), który jest miokiną zaangażowaną w brązowienie białej tkanki tłuszczowej[78]. Obserwowany spadek waliny mógł być zatem związany ze statystycznie istotnym spadkiem stężenia glukozy, któremu towarzyszył trend spadkowy insuliny i HOMA-IR odnotowany w wśród starszych uczestników grupy WBC-EXP (MG).

W zaprezentowanych badaniach odnotowano także spadek stężenia miostatyny we krwi nieaktywnych fizycznie osób, zarówno po całej serii zabiegów WBC (pub. I) jak i treningów NW (pub. III). Co ważne, zmiany te były zależne od wieku, a także od wyjściowego poziomu metabolitów witaminy D we krwi. Wzrost stężenia miostatyny jest charakterystyczny dla okresu starzenia się, podczas którego zmniejszona zostaje absorpcja glukozy[79], co potencjalnie przyczynia się do rozwoju insulinooporności. W związku z tym, odnotowany w badaniach spadek poziomu tego czynnika wśród starszych badanych (grupa MG, pub. I; grupa NW, pub. III), potwierdza korzystny wpływ zastosowanych procedur na profil

metaboliczny. Niemniej jednak tylko w wyniku zastosowania WBC oddzielnie, spadek miostatyny wiązał się z obniżeniem stężenia glukozy u badanych. Może mieć to związek z faktem, że ekspozycja na zimno prowadzi do zahamowania ekspresji miostatyny w BAT[80], która obok mięśni szkieletowych jest kolejnym, istotnym źródłem tego białka[80,81]. W grupie WBC-EXP (pub. I) odnotowano istotną korelację między miostatyną, a większością parametrów homeostazy glukozy, czego nie zaobserwowano w ostatniej z załączonych publikacji (pub. III). Niemniej jednak spadek miostatyny w grupie NW (pub. III) istotnie korelował ze wzrostem stężenia decorin we krwi osób w średnim i starszym wieku. Jest to zgodne z wcześniej opublikowanymi danymi, w których udowodniono, że decorin hamuje aktywność miostatyny[82]. Wynik ten jest istotny ze względu na fakt, iż starzenie się wiąże się ze obniżeniem stężenia decorin we krwi[83], a badanie przeprowadzone na myszach sugeruje, że białko to odgrywa ważną rolę w regulacji metabolizmu glukozy[84]. Z drugiej strony, istnieją doniesienia sugerujące związek podwyższonej ekspresji genu kodującego decorin z insulinoopornością[85,86]. W związku z tym, interpretacja wzrostu stężenia decorin w naszym badaniu nie jest jednoznaczna i może częściowo wyjaśniać brak poprawy parametrów homeostazy glukozy w odpowiedzi na zastosowany protokół wysiłkowy w III eksperymencie. Dodatkowo, znaczenie mógł mieć fakt, że stężenie mierzonych osteokin pochodzenia kostnego: Glu-OC i Gla-OC istotnie zmniejszyło się 24h po zakończeniu serii treningów NW (pub. III). Warto zauważyć, że osteokalcyna oprócz wpływu na funkcję mięśni[87], wykazuje potencjalne właściwości modulujące wrażliwość na działanie insuliny[88,89]. Zwłaszcza jej aktywna, niekarboksylowana forma Glu-OC, może regulować insulinowrażliwość adipocytów poprzez stymulowanie wydzielania adiponektyny[90].

Stężenie adiponektyny wzrosło zarówno pod wpływem serii zabiegów WBC (pub. I) jak i treningów HIIT (pub. II). Połączenie HIIT z WBC oraz protokoły treningowe zastosowane w ostatniej publikacji nie zmieniły istotnie stężenia tego białka. Adiponektyna jest uważana za marker ogólnoustrojowej wrażliwości na insulinę[91]. Wzrostowi stężenia tego białka towarzyszyły zmiany stężenia FGF21 i poprawa parametrów profilu metabolicznego badanych. Chociaż adiponektyna jest adipokiną[92], działa również jako miokina, ponieważ może być uwalniana przez mięśnie szkieletowe podczas ich skurczu[93], podobnie jak wcześniej opisany mediator FGF21. Co więcej, eksperymenty na modelach zwierzęcych dostarczyły informacji, iż FGF21, działając jako adipokina, w sposób auto/parakrynny może regulować ekspresję adiponektyny z adipocytów, co może stanowić mechanizm obronny przed ogólnoustrojową insulinoopornością[94].

W drugim z eksperymentów dokonano oceny stężenia inkretyn we krwi, ponieważ są to hormony regulujące wydzielanie insuliny i glukagonu przez komórki trzustki w sposób zależny od glukozy[95]. W grupie TR-WBC zaobserwowano nieistotną statystycznie tendencję wzrostową stężenia GIP, ale była ona skorelowana z istotnym spadkiem stężenia glukozy. Co ważne, w grupie TR, w której stężenie GIP wykazywało odwrotną tendencję, poziom glukozy we krwi nie zmienił się. Opierając się na wcześniej opublikowanych badaniach[96] wywnioskowano, że ekspozycja na zimno mogła pobudzić ekspresję GIP, co z kolei mogło przyczynić się do obniżenia stężenia glukozy we krwi grupy TR-WBC (pub. II).

Podsumowując, wyniki przedstawionych badań wskazują, że zarówno wysiłek fizyczny, jak i krioterapia ogólnoustrojowa wpływają na zmiany stężenia FGF21. Efekt ten wydaje się być stłumiony w przypadku połączenia zaproponowanych procedur. Niemniej jednak zastosowane protokoły eksperymentalne wpłynęły na poprawę parametrów homeostazy glukozy i zmodyfikowały stężenie wybranych czynników ją regulujących. Poprawa metabolizmu w grupie nieaktywnych fizycznie osób mogła wiązać zarówno z pobudzeniem układu mięśniowego jak i ekspresji tkanki tłuszczowej, w tym BAT.

8. Wnioski

Wyniki przeprowadzonych badań na grupie osób nieaktywnych fizycznie w różnym wieku pozwalają na wyciągnięcie następujących wniosków:

I. Krioterapia ogólnoustrojowa jak i wysiłek fizyczny modyfikowały stężenie FGF21 we krwi, przy czym kierunek tych zmian był odwrotny.

II. Obie interwencje poprawiły homeostazę glukozy.

III. Zmiany zależały od składu ciała uczestników oraz ich wieku.

IV. Uzyskane wyniki potwierdzają zastosowanie WBC i wysiłku fizycznego jako strategii profilaktycznych ograniczających poważne skutki chorób cywilizacyjnych takich jak insulinooporności i cukrzycy typu II poprzez wywołanie krótkotrwałej poprawy profilu metabolicznego.

V. Zmiany indukowane połączeniem procedur WBC i HIIT nie utrzymały się do czterech tygodni po zakończeniu interwencji.

VI. Trening NW okazał się bardziej skuteczny w wywoływaniu zmian stężenia miokin we krwi u osób starszych niż HIIT, przy czym zmiany te zależały od stężenia metabolitów witaminy D. Nie potwierdziło się nasze początkowe założenie, że trening HIIT o małej objętości i wysokiej intensywności może być skuteczną alternatywą dla treningu NW o dużej objętości i małej intensywności w wywoływaniu zmian prozdrowotnych u osób w średnim i starszym wieku.

9. Streszczenie

Brak aktywności fizycznej i siedzący tryb życia stanowią globalne obciążenie zdrowotne, które dotyka wszystkie populacje, niezależnie od dochodów. Brak aktywności fizycznej prowadzi do rozwoju nieprawidłowości w składzie ciała, które mogą predysponować do wystąpienia (a także pogorszyć) chorób przewlekłych związanych z dysfunkcjami metabolicznymi, w tym zaburzeniami homeostazy glukozy. Mimo, iż prozdrowotne skutki aktywności fizycznej jak i ekspozycji na niskie termeratury (jako metod o działaniu przeciwzapalnym) są dobrze udokumentowane, wciąż trwa poszukiwanie najskuteczniejszych protokołów, dostosowanych do wieku i/lub składu ciała badanych oraz identyfikacja czynników modyfikujących efekty tych procedur.

Ogólnym celem przedstawionych badań było ustalenie wpływu krioterapii ogólnoustrojowej (WBC) lub wysiłku fizycznego oraz ich połączenia na stężenie białek indukowanych tymi dwoma metodami (wysiłkiem lub WBC) oraz ich związku z metabolizmem i homeostazą glukozy. Ponadto badania miały na celu porównanie skuteczności dwóch rodzajów protokołów treningowych: treningu interwałowego wysokiej intensywności (HIIT) i Nordic Walking (NW) pod kątem reakcji organizmu na zastosowane procedury wśród osób nieaktywnych fizycznie oraz określenie czynników modyfikujących zakres tych zmian.

W badaniach wzięło udział łącznie 132 nieaktywnych fizycznie mężczyzn i kobiet w różnym wieku, w przedziale od 28 ± 7 do 61 ± 12 lat. Uczestnicy zostali losowo przydzieleni do różnych grup eksperymentalnych w zależności od zastosowanego protokołu badawczego, lub jak określono w pierwszym z przedstawionych badań, do grupy kontrolnej (WBC-CON). Grupa ta była proszona o niezmienianie swoich dotychczasowych, codziennych nawyków w czasie trwania interwencji. Osoby z grup eksperymentalnych poddane były: I. 10 sesjom WBC (grupa WBC-EXP), przeprowadzonych w kriokomorze (3 minuty, w temperaturze −110°C); II. 6 jednostkom treningu HIIT (grupy TR i HIIT); III. połączeniu obu procedur (grupa TR-WBC) oraz IV. 36 jednostkom treningu NW (grupa NW). Sesje WBC obywały 5 razy w tygodniu przez dwa tygodnie z dwudniową przerwą odpoczynkową w weekend. Protokoły treningowe odbywały się 3 razy w tygodniu. W grupie TR-WBC, treningi wykonywano bezpośrednio przed 1, 3, 5, 6, 8 i 10 sesją w kriokomorze. Dwadzieścia cztery godziny przed i 24h po każdej pierwszej i ostatniej interwencji, pobierano krew w celu oceny stężenia markerów homeostazy glukozy (glukozy, insuliny, peptydu C), profilu lipidowego (cholesterolu całkowitego [TC], wysokiej [HDL] i niskiej gęstości [LDL],

trójglicerydów [TG]), wybranych białek pochodzenia mięśniowego i wątrobowego (czynnika wzrostu fibroblastów [FGF]21, miostatyny, decorin), adipokin (adiponektyny, leptyny, rezystyny, wisfatyny), inkretyn (żołądkowego peptydu hamującego [GIP], glukagonopodobnego peptydu 1 [GLP-1]), karboksylowanej i niekarboksylowanej formy osteokalcyny (Glu-OC, Gla-OC), metabolitów witaminy D (25(OH)D3, 24,25(OH)2D3, 3-epi-25(OH)D3) oraz profilu aminokwasowego. Ponadto osoby te scharakteryzowano pod względem składu ciała uczestników oraz obliczonych wskaźników insulinooporności (HOMA-IR oraz QUICKI), insulinowrażliwości (HOMA-S) oraz funkcji komórek β-trzustki (HOMA-B). Dodatkowo, w pierwszym eksperymencie próbki krwi zebrano 1 godzinę po pierwszej i ostatniej sesji WBC, natomiast w drugim eksperymencie pomiary w grupie TR-WBC powtórzono 4 tygodnie od zakończenia procedury. Każdy protokół eksperymentalny został przeprowadzony pod nadzorem wykwalifikowanego trenera lub personelu medycznego.

Dwa tygodnie ekspozycji na WBC spowodowało obniżenie stężenia FGF21 ($p = 0.06$), glukozy ($p = 0.04$) oraz poprawę większości mierzonych wskaźników homeostazy glukozy (pub. I), w tym redukcję poziomu insuliny ($p = 0.01$) i HOMA-IR ($p = 0.01$). Poprawa profilu metabolicznego była widoczna szczególnie wśród starszych uczestników poddanych WBC (MG). Wielokrotnej ekspozycji na WBC towarzyszył istotny wzrost stężenia adiponektyny ($p = 0.05$) i równoczesny spadek miostatyny ($p < 0.01$) w grupie MG. Profil lipidowy nie uległ zmianom pod wpływem zastosowanej procedury badawczej. Stężenie aminokwasów: waliny i asparaginy obniżyło się istotnie w grupie eksperymentalnej ($p < 0.01$).

Sześć jednostek treningu HIIT spowodowało istotny wzrost stężenia FGF21 we krwi ($p < 0.05$) nieaktywnych osób z nadwagą i otyłością (42 ± 13 lat; pub. II). Zmianie tej towarzyszył wzrost stężenia przeciwzapalnej adiponektyny i spadek mediatora prozapalnego wisfatyny ($p < 0.05$). Jednocześnie, w grupie trenującej (TR) nastąpiła poprawa profilu metabolicznego, czego odzwierciedleniem była redukcja poziomu TG oraz poprawa wskaźnika HOMA-B. Co ciekawe, HIIT w połączeniu z WBC nie wywołał podobnych zmian, ale istotnie obniżył spoczynkowe stężenie glukozy we krwi ($p < 0.05$). Niemniej jednak efekt ten nie utrzymał się do czwartego tygodnia od zakończenia interwencji.

Protokół HIIT, przeprowadzony wśród osób w średnim i starszym wieku (61 ± 12 lat), nie spowodował znaczących zmian w mierzonych wskaźnikach metabolizmu glukozy, profilu lipidowego, ani stężeniu cytokin (pub. III). Poziom metabolitów witaminy D we krwi nie zmienił się w żadnej z grup treningowych (HIIT lub NW). Ponadto żaden z protokołów wysiłkowych nie wywołał także zmian stężenia FGF21 we krwi. Niemniej jednak w grupie

NW zaobserwowano znaczny spadek stężenia miostatyny w surowicy krwi ($p = 0.05$), przy czym większy zakres zmian był związany z wyższym wyjściowym poziomem wszystkich mierzonych metabolitów witaminy D we krwi. Spadkowi miostatyny towarzyszył trend wzrostowy stężenia decorin, który był zbliżony, choć poniżej istotności statystycznej ($p = 0.06$).

Wyniki przestawionych publikacji wskazują, iż zarówno krioterapia ogólnoustrojowa jak i wysiłek fizyczny pozytywnie wpłynęły na homeostazę glukozy u osób nieaktywnych fizycznie, a efekt ten jest związany (i być może pośredniczny) ze zmianami stężenia FGF21 i innych czynników regulacyjnych. Jednak połączenie treningu i WBC wydaje się tłumić te efekty, ale potwierdzenie tej obserwacji oraz wyjaśnienie mechanizmu tego zjawiska wymagają dalszych badań. Wreszcie w grupie osób dorosłych w średnim i starszym wieku trening wytrzymałościowy NW okazał się bardziej skuteczny w poprawie profilu metabolicznego.

Gdansk University of Physical Education and Sport

M.Sc. Marta Flis

Changes in fibroblast growth factor (FGF21) circulating levels in response to physical exercise and whole body cryotherapy

Ph.D. thesis under the supervision of:

prof. dr hab. Ewa Ziemann

dr hab. Giovanni Lombardi

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10. Abstract

Physical inactivity and sedentary behaviors represent global health burden which affects all the populations, regardless the income. Physical inactivity leads to the development of abnormalities in body composition that may predispose to the onset of (and can worsen, too) chronic diseases associated with metabolic dysfunctions, including disorders of glucose homeostasis. Although the pro-health effects of a physically active behavior, as well as of exposure to extremely low temperatures (as the methods with anti-inflammatory action) are well documented, the searching for the most effective protocols, adjusted to age and/or body composition of the subjects, and the identification of the factors that modify the homeostatic effects of these procedures are still ongoing.

The overall aim of the presented studies was to determine the effect of either whole body cryotherapy (WBC) or training, and their combination on concentration of proteins induced by these two methods (exercise or WBC) and their relationship with metabolism and glucose homeostasis. Moreover, these studies aimed at comparing the effectiveness of two types of training protocol: high-intensity interval training (HIIT) and Nordic Walking (NW) on the response to applied procedures among physically inactive subjects and determine factors modifying the range of changes.

A total of 132 physically inactive male and female subjects of different ages, ranging from 28 ± 7 to 61 ± 12 years, took part in these studies. Participants were randomly assigned to different experimental groups depending on the applied experimental protocol or, as defined in the first study presented to the control group (WBC-CON). Specifically, this control group was asked not to change the daily habits during the intervention. People from the experimental groups completed: I. 10 sessions of WBC (WBC-EXP group), conducted in a special cryochamber (3 minutes, at a temperature of −110°C); II. 6 units of HIIT training (TR and HIIT groups); III. combination of both procedures (TR-WBC group) and IV. 36 units of NW training (NW group). WBC sessions were performed five times a week over two weeks, with a two-day rest period during the weekend. Exercise protocols were conducted 3 times a week. In the TR-WBC group, the exercise sessions were performed immediately before the 1st, 3rd, 5th, 6th, 8th and 10th exposures to extreme cold in the special cryochamber. Twenty-four hours before and 24h after every first and last intervention, blood samples were collected in order to assess the concentration of markers of glucose homeostasis (glucose, insulin, C-peptide), lipid profile (total cholesterol [TC], high [HDL] and low [LDL] density lipoproteins, triglycerides [TG]), selected proteins of muscle and liver origin (fibroblast

growth factor [FGF]21, myostatin, decorin), adipokines (adiponectin, leptin, resistin, visfatin), incretins (gastric inhibitory peptide [GIP], glucagon-like peptide 1 [GLP-1]), and carboxylated and undercarboxylated form of osteocalcin (Glu-OC, Gla-OC), vitamin D metabolites (25(OH)D3, 24,25(OH)2D3, 3-epi-25(OH)D3) and the amino acid profile. Moreover, these subjects were characterized in terms of body composition, and calculated indicators of insulin resistance (HOMA-IR and QUICKI), insulin sensitivity (HOMA-S) and pancreatic β-cell function (HOMA-B). Additionally, in the first experiment, blood samples were collected 1 hour after the first and last session of WBC, while in the second experiment, the measurements in the TR-WBC group were repeated 4 weeks after the end of the intervention. Each experimental protocol was carried out under the supervision of a qualified trainer or medical staff.

Two-week WBC exposure resulted in a decrease in the concentration of FGF21 ($p = 0.06$), glucose ($p = 0.04$) and improved most of the measured homeostasis indicators (pub. I), including decrease of insulin levels ($p = 0.01$) and HOMA-IR levels ($p = 0.01$). The improvement in the metabolic profile was particularly evident among older participants underwent WBC (MG). Multiple exposure to WBC was accompanied by a significant increase in adiponectin concentration ($p = 0.05$) and a simultaneous decrease in myostatin ($p < 0.01$) in the MG group. The lipid profile was not affected in response to the applied procedure. The concentration of the amino acids valine and asparagine decreased significantly in the experimental group ($p < 0.01$).

Six units of HIIT training resulted in a significant increase in blood concentrations of FGF21 ($p < 0.05$) of overweight-to-obese, inactive participants (42 ± 13 years old; pub. II). This change was accompanied by the increase in concentrations of the anti-inflammatory mediator adiponectin and the decrease in the pro-inflammatory mediator visfatin ($p < 0.05$). At the same time, in the training group (TR), the metabolic profile was improved as mirrored by reduction in TG levels and the improvement of the HOMA-B index. Interestingly, the combination of HIIT and WBC did not elicit similar changes but significantly decreased resting blood glucose concentration ($p < 0.05$). Nonetheless, this effect did not persist until the 4^{th} week post-intervention.

Elderly subjects (61 ± 12 years old) underwent the HIIT protocol, did not experience any improvement in measured indicators of glucose metabolism, lipid profile and cytokine concentrations (pub. III). The circulating level of vitamin D metabolites did not change in any of the training groups (HIIT or NW). Further, any of the exercise protocols induced changes in the blood concentration of FGF21. Nevertheless, in the NW group, a significant decrease

in serum myostatin concentration ($p = 0.05$) was observed, with a greater range of changes associated with higher baseline levels of all measured vitamin D metabolites in blood. The decrease of myostatin concentration was accompanied by an increasing trend in the concentration of decorin that was close, although below, the statistical significance ($p = 0.06$).

The results of the presented studies demonstrate that both whole body cryotherapy and exercise trainings positively affected the glucose homeostasis in physically inactive people, and this effect is associated to (and possibly mediated by) changes in the concentrations of FGF21 and other regulatory factors. However, the combination of training and WBC seem to suppress these effects, but a confirmation of this observation as well as the mechanistic explanation of this phenomenon merit further researches. Finally, in the group of middle-aged-to-elderly adults, endurance NW training proved to be more effective in improving the metabolic status.

11. Piśmiennictwo uwzględnione w autoreferacie

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12. Załączniki

I. Oświadczenia współautorów publikacji

II. Publikacje wchodzące w skład rozprawy doktorskiej

- Publikacja *„Beneficial effects of whole-body cryotherapy on glucose homeostasis and amino acid profile are associated with a reduced myostatin serum concentration”*

- Publikacja *“Short and long-term effects of high-intensity interval training applied alone or with whole-body cryostimulation on glucose homeostasis and myokine levels in overweight to obese subjects”*

- Publikacja *„Nordic walking rather than high intensity interval training reduced myostatin concentration more effectively in elderly subjects and the range of this drop was modified by metabolites of vitamin D”*

SZKOŁA DOKTORSKA

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Niniejszym oświadczamy, że indywidualny wkład w powstanie ww publikacji jest następujący:

autor	wkład %	opis*	podpis
Marta Kozłowska	65	ABCDEF	
Jakub Kortas	5	CD	
Małgorzata Żychowska	5	BD	
Jędrzej Antosiewicz	5	BD	
Klaudia Żuczek	5	BD	
Silvia Perego	5	BD	
Giovanni Lombardi	5	BD	
Ewa Ziemann	5	ABCDEFG	

* A – przygotowanie projektu badania, B – przeprowadzanie badań, C – analiza statystyczna, D – interpretacja wyników, E – przygotowanie publikacji, F – opracowanie piśmiennictwa, G – pozyskanie funduszy

podpis doktoranta

podpis promotora

Biuro Spraw Doktoranckich
szkola.doktorska@awf.gda.pl
+48 58 55 47 262, +48 603 766 393
ul. K. Górskiego 1, 80-336 Gdańsk

SZKOŁA DOKTORSKA

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

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Niniejszym oświadczamy, że indywidualny wkład w powstanie ww publikacji jest następujący:

autor	wkład %	opis*	podpis
Marta Kozłowska-Flis	60	ABCDEF	Marta Kozłowska-Flis
Ewa Rodziewicz-Flis	5	BD	Ewa Rodziewicz-Flis
Katarzyna Mielelska	5	BD	K. Mielelska
Jakub Kortas	5	CD	Jakub Kortas
Joanna Jaworska	5	BD	Jaworska Joanna
Andżelika Borkowska	2	BD	A. Borkowska
Veronica Sansoni	5	BD	Veronica Sansoni
Silvia Perego	3	BD	Silvia Perego
Giovanni Lombardi	5	BD	
Ewa Ziemann	5	ABCDEFG	Ziemann Ewa

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Marta Kozłowska-Flis

podpis doktoranta

podpis promotora

Ziemann Ewa

Biuro Spraw Doktoranckich
szkola.doktorska@awf.gda.pl
+48 58 55 47 262, +48 603 766 393
ul. K. Górskiego 1, 80-336 Gdańsk

SZKOŁA DOKTORSKA

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

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Niniejszym oświadczamy, że indywidualny wkład w powstanie ww publikacji jest następujący:

autor	wkład %	opis*	podpis
Katarzyna Micielska[†]	40	ABCDEF	K. Micielska
Marta Flis[†]	40	ABCDEF	Marta Flis
Jakub Antoni Kortas	3	CD	Jakub Kortas
Ewa Rodziewicz-Flis	5	BD	Ewa Rodziewicz-Flis
Jędrzej Antosiewicz	2	BD	J. Antosiewicz
Krystian Wochna	5	BD	K. Wochna
Giovanni Lombardi	2	ABDG	
Ewa Ziemann	3	ABCDEFG	Ziemann Ewa

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[†] Równorzędne pierwsze współautorstwo w publikacji

Marta Kozłowska - Flis
podpis doktoranta

podpis promotora
Ziemann Ewa

Biuro Spraw Doktoranckich
szkola.doktorska@awf.gda.pl
+48 58 55 47 262, +48 603 766 393
ul. K. Górskiego 1, 80-336 Gdańsk

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Beneficial effects of whole-body cryotherapy on glucose homeostasis and amino acid profile are associated with a reduced myostatin serum concentration

Marta Kozłowska[1], Jakub Kortas[2], Małgorzata Żychowska[3], Jędrzej Antosiewicz[4], Klaudia Żuczek[5], Silvia Perego[6], Giovanni Lombardi[6,7] & Ewa Ziemann[7]✉

The study investigated the effect of single and chronic (10 sessions) whole-body cryotherapy (WBC; 3-min, −110 °C) on amino acid (AA) profile, myostatin, fibroblast growth factor 21 (FGF21), and concentrations of brain-derived neurotrophic factor (BDNF), irisin and adiponectin in relation to glucose homeostasis. Thirty-five, healthy men were randomly split into experimental (young: 28 ± 7 years and middle-aged: 51 ± 3 years) and control groups. Blood samples were taken before and 1 h after the first and last (10th) WBC session. Baseline myostatin correlated significantly with visceral fat area, glucose, insulin, HOMA-IR and irisin (all $p < 0.05$). The single session of WBC induced temporary changes in AA profile, whereas chronic exposure lowered valine and asparagine concentrations ($p < 0.01$ and $p = 0.01$, respectively) compared to the baseline. The chronic WBC reduced fasting glucose ($p = 0.04$), FGF21 (− 35.8%, $p = 0.06$) and myostatin (-18.2%, $p = 0.06$). Still, the effects were age-dependent. The decrease of myostatin was more pronounced in middle-aged participants ($p < 0.01$). Concentrations of irisin and adiponectin increased in response to chronic WBC, while BDNF level remained unchanged. By improving the adipo-myokine profile, chronic WBC may reduce effectively the risk of the metabolic syndrome associated with hyperinsulinemia, increased levels of valine and asparagine, and muscle atrophy.

Insulin resistance (IR) occurs when higher circulating insulin levels are necessary to achieve the integrated glucose-lowering response[1]. IR results in a compensatory increased release of insulin by pancreatic β-cells and hyperinsulinemia, which is thought to precede the development of type 2 diabetes (T2DM) by 10 to 15 years[2]. Obesity, age and physical inactivity are the most prominent factors exacerbating the risk of developing IR[3]. These factors are codependent. Ageing is associated with a reduced activity, which contributes to lower total energy expenditure[4] and may lead to fat tissue accumulation[5], especially visceral fat area (VFA). This condition significantly affects development of the age-related IR[6]. Routine screening tests including fasting glucose concentration and glycated hemoglobin (HbA1C) are most commonly used to detect this condition[7]. The oral glucose tolerance test may also be applied for this purpose, but is performed less frequently due to being poorly tolerated by the patients as well as being time consuming[8].

Serum amino acids (AA's) are considered to be useful laboratory biomarkers in detecting early disruptions of glucose homeostasis[9]. Serving as an energy source, AA's can be used for gluconeogenesis during catabolic states[10], and influence insulin and glucagon secretion[11]. Increased levels of AA's have been observed in all stages of diabetes, including early pre-diabetic IR[12]. Insulin reduces concetrations of amino acids in circulation by stimulating their transport to cells[13]. In particular, the elevated circulating branched-chain amino acids (BCAA's)

[1]Department of Physiology and Biochemistry, Gdańsk University of Physical Education and Sport, Gdańsk, Poland. [2]Department of Sport, Gdansk University of Physical Education and Sport, Gdańsk, Poland. [3]Department of Sport, Bydgoszcz Kazimierz Wielki University, Bydgoszcz, Poland. [4]Department of Bioenergetics and Physiology of Exercise, Medical University of Gdansk, Gdańsk, Poland. [5]Department of Medical Biology and Genetics, Faculty of Biology, University of Gdańsk, Gdańsk, Poland. [6]Laboratory of Experimental Biochemistry and Molecular Biology, IRCCS Istituto Ortopedico Galeazzi, Milano, Italy. [7]Department of Athletics, Strength and Conditioning, Poznan University of Physical Education, Poznan, Poland. ✉email: ziemann.ewa@gmail.com

are considered to be reliable predictors of the T2DM development in normoglycemic subjects[14]. A cross-sectional study including both normoglycemic and T2DM individuals demonstrated higher concentrations of serum BCAA's and also the aromatic AA's (tyrosine and phenylalanine) in individuals with impaired fasting glycaemia and IR[15]. The authors reported a decrease in glycine in all T2DM individuals, contrary to the observed increase of AA's after the meal[15]. This result was attributed to an increased hepatic clearance of postprandial glycine to replenish a conjugated bile acid pool in the gall bladder[16]. Likewise, increased plasma AA's levels of alanine, proline and glutamate/glutamine were observed in a group of 263 men with different stages of diabetes, including early prediabetic IR[12].

Due to changes in AA's concentrations and inhibited insulin action, individuals with IR may also exhibit skeletal mass disfunction and obesity related sarcopenia[17]. Myostatin is one of the factors which contributes to the development of sarcopenia[18]. It is a skeletal muscle-derived member of the transforming growth factor β superfamily, which inhibits protein synthesis via an impaired mammalian target of rapamicyn (mTOR) signaling[19]. Circulating myostatin was previously demonstrated to be correlated with indices of IR[20]. A study in animal models showed that blocking the myostatin receptor induced an elevation of brown adipose tissue (BAT), an improvement of its mitochondrial function, and better cold tolerance, which altogether contributed to an enhanced energy expenditure[21]. Similarly, myostatin propeptide which inhibits its activity prevents the development of diet-induced obesity and insulin resistance in transgenic animals[17].

Together with physical activity[22], cold exposure might improve insulin sensitivity and counteract the inflammatory status associated with obesity. By increasing peripheral insulin sensitivity as well as BAT mass and activity, cold-induced adaptive thermogenesis may be a potential therapy for T2DM[23]. Similar to cold water immersion[24], WBC reduces superficial body temperature leading to changes in tissue blood flow. It does so by means of vasoconstriction at the skin and an increased metabolic rate caused by shivering to maintain a constant core temperature (around 37 °C)[25,26], ultimately affecting the expression of myokines[27] and adipokines[28]. These physiological responses provide a theoretical base for applying cold exposure as a possible therapeutic strategy in individuals with metabolic diseases[29].

Health benefits of cold exposure are releated to shifts in fibroblast growth factor 21 (FGF21) and irisin[30,31]. It has been proven that the secretion of FGF21 is stimulated by nonshivering thermogenesis and irisin, in turn, by shivering thermogenesis[30]. FGF21 regulates expression of genes involved in gluconeogenesis, lipogenesis, lipolysis and fatty acid oxidation[32]. It is also a metabolic regulator with anti-diabetic properties capable of stimulating enhanced glucose uptake in adipocytes[33]. FGF21 enhances energy expenditure by increasing the core body temperature and decreasing the respiratory quotient[34]. Dulian et al. (2015) noted an increase of irisin level in response to 10 sessions of WBC in obese, inactive men, which was also positively correlated withsubcutaneous fat tissue[31].

Data on the influence of cold exposure on AA profile's and myostatin are limited[35]. We previously reported that the effect of WBC on adipokines depended on participants' cardiorespiratory fitness[36] , expressed in relative maximal oxygen uptake (VO_2max). Therefore, for this study, we recruited only men with comparable levels of aerobic capacity. As such, the main purpose was to examine whether both acute and chronic WBC affected changes in blood AA's and myostatin concentration and the secondary purpose was to establish whether the induced changes were be associated with glucose homeostasis. We hypothesized that both a single and chronic WBC exposures would induce an imporvement in glucose metabolism, related to changes in blood myokines and adipokines concentrations, which would allow considering WBC as a preventative strategy against IR and development of T2DM.

Results

Significant differences in measured insulin sensivity indicators were noted among participants at baseline. Lower glucose (95.7 ± 8.6 vs 106.5 ± 7.0 mg·mL^{-1}; $p = 0.002$), insulin concentrations (6.3 ± 2.5 vs 9.0 ± 2.1 μU·mL^{-1}; $p = 0.01$) and HOMA-IR (0.8 vs 1.2) were evident in younger participants (YG) compared to middle-aged individuals respectively (MG; supplementary Table S1). These differences were also visible in myokine concentrations. At baseline, BDNF was significantly higher in YG than in MG subjects ($p = 0.01$), while the trend was opposite for myostatin ($p < 0.01$; Fig. 1a,b). Conversely, irisin and adiponectin concentrations did not differ at baseline between the two groups (Fig. 1c,d). In WBC-EXP group, baseline concentrations of BDNF and irisin was negatively correlated (r = -0.75, $p < 0.01$; Fig. 2a), which was not observed at the end of chronic WBC (r = -0.12; $p = 0.58$; Fig. 2b). In turn, irisin concentration correlated positively at baseline with the amount of fat tissue (percentage of body fat, PBF% as well in absolute kilograms) only in MG subjects (r = 0.58, $p = 0.01$; supplementary Table S2).

In the whole group of participants myostatin concentration, regardless of the age, correlated significantly with VFA (r = 0.70, $p < 0.01$), glucose homeostasis indicators such as glucose (r = 0.69, $p = 0.00$), insulin (r = 0.46, $p = 0.01$) and HOMA-IR (r = 0.53, $p < 0.01$) and irisin (r = 0.65, $p < 0.01$; Table 1).

Effects of a single session of WBC. In our assessment, we considered the analysis of blood samples collected before and 1hour after the first (acute) and the last (chronic) WBC exposure.

Changes in resposne to the first session of WBC. The effect of the first WBC session on myokines concentrations is presented in Fig. 1. Irisin ($p = 0.02$) and myostatin ($p = 0.03$) concentrations increased significantly in the WBC-EXP group. This was not the case for BDNF concentration. However, when considering the age groups, the first WBC session resulted in a pronounced drop of BDNF and a significant increase of irisin ($p = 0.01$) concentration in YG participants, but not in MG individuals (Fig. 1a,c). A single WBC exposure also decreased FGF21 level in the WBC-EXP group (from 280.4 ± 160.5 to 239.7 ± 166.6 pg·mL^{-1}, $p = 0.07$; Fig. 3a). Changes in AA profile's in response to a single session of WBC are presented in Table 2. Elevated levels of ala-

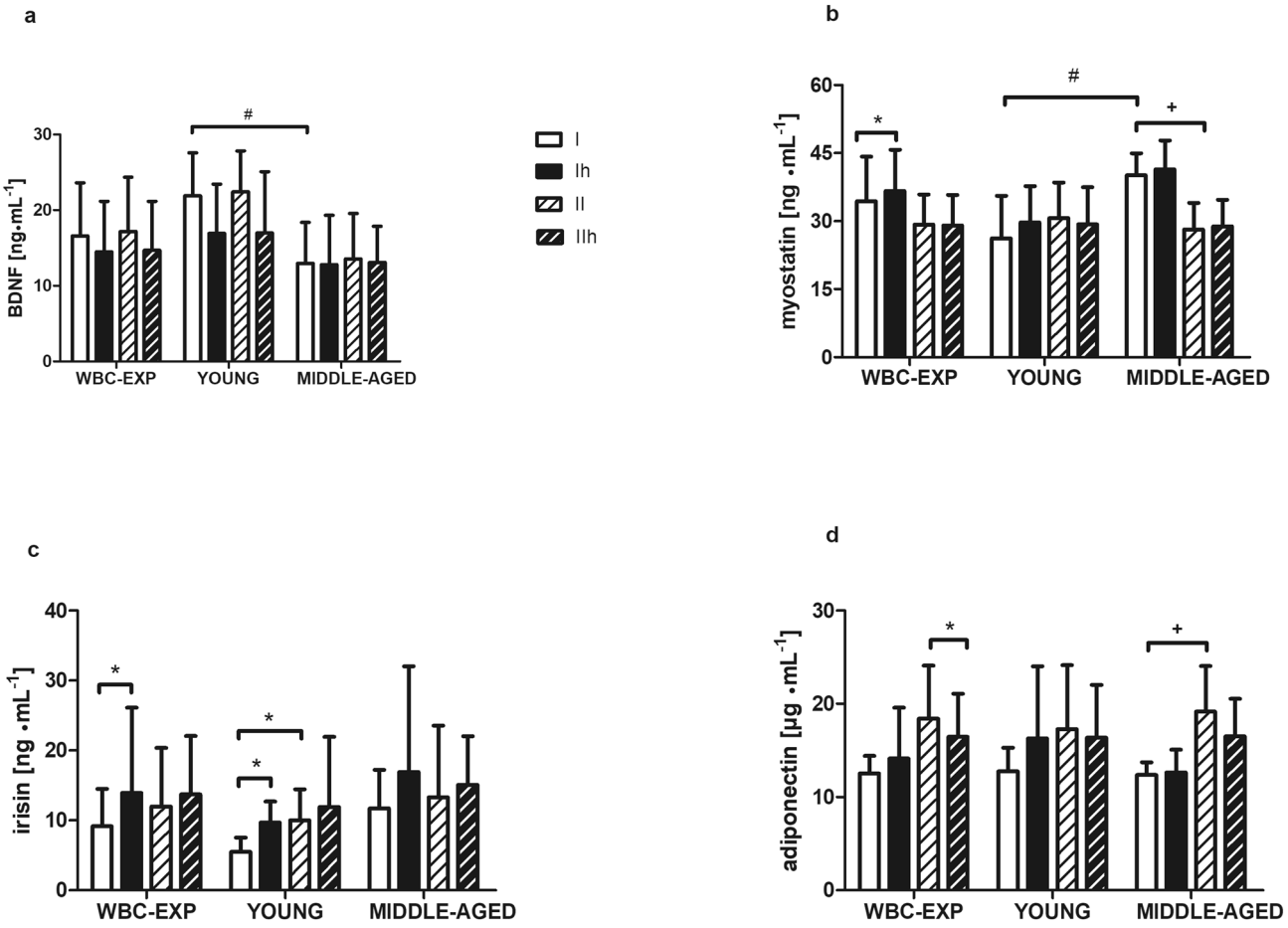

Figure 1. Group- and age-related changes post a single session of the WBC in concentrations of (**a**) BDNF; (**b**) myostatin; (**c**) irisin and (**d**) adiponectin; recorded before (I) and 1 h after the first (Ih) as well as before (II) and 1 h after the last (IIh) WBC session. WBC-EXP (n = 22) included young (YG, n = 9) and middle aged (MG, n = 13) participants. Data are presented as mean ± SD; *statistical significance in the group; #statistical difference between groups at a time point, +statistical significance in the group MG vs WBC-CON.

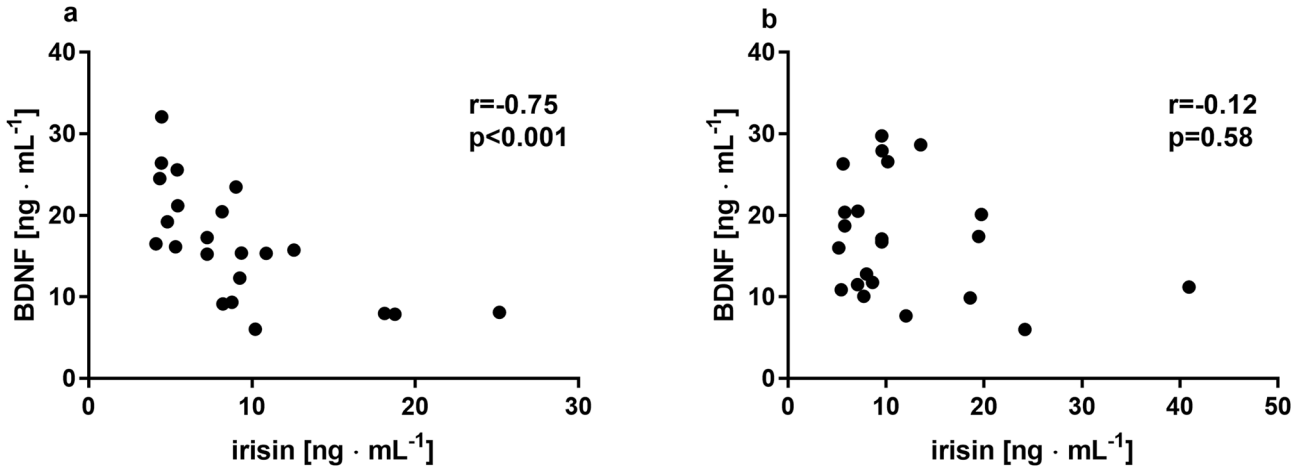

Figure 2. Correlation coefficients between BDNF and irisin in the WBC-EXP (n = 22) group (**a**) prior to and (**b**) after chronic WBC. Values are Spearman correlations, significant at $p < 0.05$.

	Visceral Fat Area (cm^{-2})			Glucose ($mg \cdot dL^{-1}$)			Insulin ($\mu U \cdot mL^{-1}$)			HOMA-IR			BDNF ($ng \cdot mL^{-1}$)			Irisin ($ng \cdot mL^{-1}$)			Valine ($\mu mol \cdot L^{-1}$)		
	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP	ALL	WBC-CON	WBC-EXP
Myostatin ($ng \cdot mL^{-1}$)																					
Before	0.70*	0.40	0.57*	0.69*	0.41	0.59*	0.46*	0.16	0.68*	0.53*	0.31	0.70*	− 0.52*	− 0.19	− 0.65*	0.65*	0.66*	0.66*	0.06	0.07	− 0.14
After	0.11	0.10	0.06	0.08	0.38	− 0.05	0.34*	− 0.34	− 0.33	− 0.31	− 0.23	− 0.33	− 0.21	− 0.01	− 0.23	− 0.09	− 0.12	− 0.09	0.21	− 0.21	0.60*
p	**0.01**	0.48	**0.04**	**0.00**	0.94	**0.02**	0.57	0.26	**0.00**	**0.00**	0.23	**0.00**	0.15	0.69	**0.05**	**0.00**	**0.05**	**0.01**	0.54	0.53	**0.01**

Table 1. Correlation coefficients of myostatin and visceral fat area; glucose; insulin; HOMA-IR; BDNF; irisin and valine among ALL participants: WBC-CON and WBC-EXP group recorded before and after whole procedure. Values are Spearman correlation; *statistically significant correlations; *p*-difference between the correlations; $p < 0.05$; Statistically significant differences between correlations are bold.

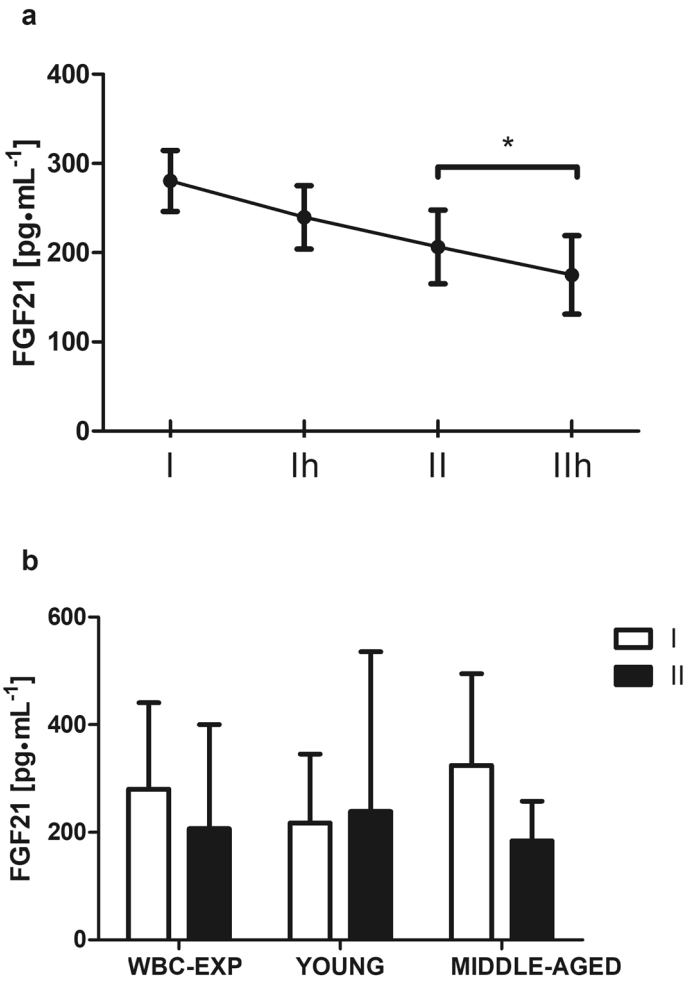

Figure 3. Changes in the concentration of FGF21 (data are presented as mean ± SEM) recorded: (**a**) at each point of blood collection: (I) before WBC, (Ih) 1 h after the first WBC, (II) before the last WBC and (IIh) 1 h after the last WBC; (**b**) in the WBC-EXP group with age-dependent changes before the first (I) and the last (II) session of WBC. *$p < 0.05$ significant differences between time point measurements.

nine, isoleucine, tryptophan, lysine, tyrosine, phenylalanine, methionine, arginine and threonine were recorded. The effect size expressed by Cohen's d value ranged from medium to large.

Changes in resposne to the last session of WBC. Before the last WBC session, the circulating level of irisin remained elevated in YG ($p = 0.01$) but not MG subjects (Fig. 1c). Additionally, in MG individuals, the concentration of irisin correlated significantly with PBF% ($r = 0.58$, $p < 0.01$; supplementary Table S2). In YG subjects, a positive relationship between skeletal muscle mass (SMM) and irisin concentration was observed 1 h after the last exposure ($r = 0.78$, $p < 0.01$; supplementary Table S2). Blood analysis of the last WBC session

	First session				Last session			
	Before	1 h post	*p*- value	Cohen's-d	Before	1 h post	*p*- value	Cohen's-d
AA's- the most important gluconeogenic precursors								
Alanine (μmol·L^{-1})	115.6 ± 26.2	147.2 ± 37.2*	**0.00**	0.79	116.4 ± 23.9	144.2 ± 43.1*	**0.00**	0.70
Glutamine (μmol·L^{-1})	251.1 ± 57.5	265.4 ± 51.5	0.32	0.24	265.5 ± 62.4	267.5 ± 46.8	0.27	0.27
AA's- after deamination form keto acid like acetyl-CoA								
Isoleucine (μmol·L^{-1})	84.4 ± 30.6	117.4 ± 47.7*	**0.00**	0.86	96.5 ± 32.3	88.2 ± 26.5	0.33	0.11
Leucine (μmol·L^{-1})	112.9 ± 70.3	115.5 ± 43.9	0.35	0.04	92.3 ± 30.7	157.8 ± 67.8*	**0.00**	0.53
Tryptophan (μmol·L^{-1})	38.1 ± 11.5	45.5 ± 15.1*	**0.00**	0.94	35.1 ± 11.1	36.4 ± 8.7	0.53	0.17
Lysine (μmol·L^{-1})	68.4 ± 13.7	85.6 ± 23.7*	**0.01**	0.67	71.9 ± 15.1	84.4 ± 16.8*	**0.02**	0.77
AA's- after deamination form keto acid like fumarate								
Valine (μmol·L^{-1})	97.8 ± 26.1	111.7 ± 31.5	0.09	0.39	88.7 ± 21.9	119.2 ± 27.7*	**0.00**	0.75
Asparagine (μmol·L^{-1})	41.3 ± 12.7	45.9 ± 12.4	0.25	0.26	37.1 ± 6.2	45.2 ± 8.2*	**0.00**	0.27
Aspartic acid (μmol·L^{-1})	3.6 ± 1.5	3.7 ± 2.2	0.91	0.05	4.7 ± 2.7	4.3 ± 1.6	0.55	0.39
Tyrosine (μmol·L^{-1})	56.9 ± 12.4	78.1 ± 24.3*	**0.00**	0.88	67.1 ± 21. 5	69.1 ± 18.6	0.56	0.87
Phenylalanine (μmol·L^{-1})	45.7 ± 10.0	63.0 ± 26.2*	**0.00**	0.72	49.8 ± 22.6	51.9 ± 20.4	0.32	0.33
AA's- after deamination form keto acid like alpha-ketoglutarate								
Glycine (μmol·L^{-1})	121.1 ± 34.0	120.9 ± 44.4	0.99	0.00	143.0 ± 47.6	110.1 ± 30.9*	**0.01**	0.26
Glutamic acid (μmol·L^{-1})	19.3 ± 9.8	21.1 ± 9.7	0.51	0.17	18.1 ± 7.7	21.6 ± 8.3	0.11	0.19
Proline (μmol·L^{-1})	111.5 ± 33.3	126.1 ± 30.0	0.05	0.42	110.3 ± 27.5	138.9 ± 29.5*	**0.00**	0.64
Methionine (μmol·L^{-1})	14.8 ± 5.8	19.1 ± 9.8*	**0.04**	0.55	14.9 ± 4.6	15.1 ± 6.8	0.62	0.05
Histidine (μmol·L^{-1})	55.9 ± 15.8	57.7 ± 15.0	0.68	0.09	61.5 ± 14.5	60.4 ± 7.7	0.64	0.32
Arginine (μmol·L^{-1})	52.2 ± 8.1	66.7 ± 16.8*	**0.00**	0.82	55.8 ± 12.1	61.8 ± 12.8	0.13	0.66
AA's- after deamination form keto acid like pyruvate								
Serine (μmol·L^{-1})	89.3 ± 22.4	97.4 ± 36.5	0.61	0.23	87.1 ± 20.9	87.6 ± 30.4	0.91	0.05
Threonine (μmol·L^{-1})	40.0 ± 9.7	46.9 ± 13.4*	**0.01**	0.55	41.1 ± 12.5	39.5 ± 9.5	0.47	0.04

Table 2. The effect of single session of the whole-body cryotherapy on amino acid profile. Data are presented as mean ± SD; AAs- amino acids; *statistically significant difference (before vs 1 h); Statistically significant differences are bold; Cohen's d- effect size: > 0.2 small, > 0.5-medium, > 0.8-large.

revealed a continued decrease of FGF21 ($p < 0.01$; Fig. 3a). Adiponectin also tended towards a decrease ($p = 0.04$) contrary to the effect observed after the chronic WBC exposure (Fig. 1d). The Cohen's d effect size was large for both proteins (1.08 for adiponectin and 1.12 for FGF21). The last WBC session affected circulating levels of alanine, leucine, lysine, valine, asparagine, glycine and proline, which increased significantly with the exception of glycine concentration, which declined ($p = 0.01$; Table 2). Moreover, at this time point, the level of alanine was significantly higher in MG subjects compared to YG individuals (163.5 ± 44.9 vs 116.3 ± 19.3 μmol·L^{-1}, respectively, $p = 0.05$). The Cohen's d effect size for the AA's was medium (> 0.5 but < 0.8), except for asparagine and glycine, where the observed change was small (0.27 and 0.26, respectively).

Effects of chronic WBC. Our assessment of the effect of chronic WBC is based on the analysis of blood samples collected at rest before the first and the last exposure (completing nine sessions, before 10th session). A comparison of PBF% before and after chronic WBC exposure showed a reduction in the WBC-EXP group (19.3 ± 6.1 to 18.8 ± 6.0%, $p = 0.03$,$\eta_p^2 = 0.14$). Chronic WBC exposure also resulted in a reduction of VFA (88.74 ± 40.39 to 84.41 ± 39.56 cm^2, $p = 0.03$, $\eta_p^2 = 0.13$). Changes in glucose homeostasis indicators (glucose, insulin, HOMA indicators) and the lipid profile from the initial to the final WBC session are presented in Table 3. Fasting glucose level significantly decreased ($p = 0.04$, $\eta_p^2 = 0.13$), whereas the lipid profile was not affected. Additionally, a significant reduction of insulin (from 9.0 ± 2.1 to 6.9 ± 2.1 μmol·L^{-1}, $p = 0.01$, $\eta_p^2 = 0.28$) and HOMA-IR (from 1.21 ± 0.3 to 0.92 ± 0.3, $p = 0.01$, $\eta_p^2 = 0.28$) was recorded only in MG subjects (Supplementary Table S1). In the WBC-EXP group, HOMA-S increased by 19.6% compared to the baseline for all participants ($p = 0.08$). HOMA-B increased significantly only in YG individuals (from 71.6 ± 13.1 to 90.4 ± 21.4%, $p = 0.01$, $\eta_p^2 = 0.3$; Supplementary Table S1).

Table 4 presents changes in biochemical markers and AA profile's recorded at baseline and in blood collected before the last WBC session. Ulike following acute WBC exposure, chronic WBC did not affect BDNF. The elevated level of irisin induced by the first cryosession was maintained among YG participants ($p = 0.04$). FGF21 concentration continued to drop throughout the intervention (baseline WBC $p = 0.57$ vs final WBC session $p < 0.01$; Fig. 3a,b). Chronic WBC exposure was also accompanied by a significant increase of adiponectin (46.8%, $p = 0.05$, $\eta_p^2 = 0.09$) in comparison to the WBC-CON group. Further, chronic WBC caused a decline in the circulating myostatin concentration but only in MG subjects (-30%, $p < 0.01$; effect size was equal 0.58; Fig. 1b). The opposite- upward trend was noted in the whole WBC-CON group. Interestingly, chronic WBC exposure blunted the difference in myostatin concentration recorded at baseline between YG and MG subjects.

	WBC-EXP		WBC-CON		ANOVA	
	Before	After	Before	After	p	η_p^2
Total cholesterol (mg·dL^{-1})	191.8 ± 34.6	173.4 ± 31.3	183.2 ± 35.2	157.2 ± 15.5	0.08	0.02
HDL (mg·dL^{-1})	55.5 ± 13.4	55.7 ± 16.5	56.7 ± 11.2	54.2 ± 7.7	0.38	0.02
LDL (mg·dL^{-1})	110.3 ± 29.0	96.8 ± 32.8	106.3 ± 27.7	83.3 ± 16.3	0.36	0.03
Triglycerides (mg·dL^{-1})	130.0 ± 68.3	104.4 ± 18.4	100.9 ± 57.1	98.9 ± 29.2	0.94	0.05
Glucose (mg·dL^{-1})	102.1 ± 9.3	93.3 ± 10.6*	94.2 ± 6.2	89.4 ± 5.8*	**0.04**	**0.13**
Insulin (μmol·L^{-1})	7.9 ± 2.6	6.7 ± 2.4	7.7 ± 2.9	7.0 ± 2.0	0.53	0.01
HOMA-S (%)	109.1 ± 49.7	130.1 ± 53.4	114.2 ± 50.2	119.5 ± 37.4	0.08	0.06
HOMA-B (%)	74.4 ± 13.9	81.7 ± 21.7	87.1 ± 25.4	91.1 ± 21.2	0.08	0.01
HOMA-IR	1.1 ± 0.4	0.9 ± 0.3	1.0 ± 0.4	0.9 ± 0.3	0.40	0.06

Table 3. The effect of chronic whole-body cryotherapy on lipid profile and glucose homeostasis indicators among WBC-EXP (n = 22) and WBC-CON (n = 13). Data are presented as mean ± SD; *statistically significant difference between before and after measurements in the group, $p < 0.05$; η_p^2- effect sizes (partial eta squared): ≥ 0.01 small, ≥ 0.06 medium and ≥ 0.14 large effect; HDL: high density lipoprotein; LDL: low density lipoprotein; HOMA: The Homeostasis Model Assessment estimates: HOMA-B- β-cell function; HOMA-S: insulin sensitivity as percentages of a normal reference population and HOMA-IR: insulin resistance. Statistically significant group x time interaction are bold.

	WBC-EXP		WBC-CON		ANOVA	
	Before	After	Before	After	p	η_p^2
BDNF (ng·mL^{-1})	16.6 ± 7.0	17.2 ± 7.2	19.2 ± 5.5	17.3 ± 5.1	0.24	0.04
Myostatin (ng·mL^{-1})	34.5 ± 9.8#	29.2 ± 6.7^{+}	25.3 ± 6.5	28.4 ± 7.7	**0.02**	**0.16**
Irisin (ng·mL^{-1})	9.2 ± 5.4	12.0 ± 8.4	6.9 ± 2.7	9.0 ± 2.7*	**0.03**	**0.11**
Adiponectin (μg·mL^{-1})	12.5 ± 1.9	18.4 ± 5.7#	12.2 ± 3.5	12.6 ± 4.0	**0.05**	**0.09**
FGF21 (pg·mL^{-1})	280.4 ± 160.5	206.5 ± 193.6	246.4 ± 149.0	184.2 ± 161.6	0.06	0.01
°Alanine (μmol·L^{-1})	115.6 ± 26.2	116.4 ± 23.9	124.2 ± 31.9	121.0 ± 31.0	0.92	0.01
°Glutamine (μmol·L^{-1})	251.1 ± 57.5	265.5 ± 62.4	282.1 ± 62.4	301.5 ± 33.3	0.08	0.01
■Isoleucine (μmol·L^{-1})	84.4 ± 30.6	96.5 ± 32.3	100.9 ± 60.6	102.5 ± 37.2	0.17	0.01
■Leucine (μmol·L^{-1})	112.9 ± 70.3	92.3 ± 30.7	85.3 ± 21.2	106.9 ± 36.4	0.81	0.09
■Tryptophan (μmol·L^{-1})	38.1 ± 11.5	35.1 ± 11.1	36.1 ± 10.9	37.2 ± 14.5	0.22	0.04
■Lysine (μmol·L^{-1})	68.4 ± 13.7	71.9 ± 15.1	83.9 ± 27.2	78.1 ± 18.8	0.11	0.03
▲Valine (μmol·L^{-1})	97.8 ± 26.1	88.7 ± 21.9#	91.6 ± 22.5	122.9 ± 25.1*	**0.00**	**0.28**
▲Asparagine (μmol·L^{-1})	41.3 ± 12.7	37.1 ± 6.2#	41.4 ± 12.2	49.6 ± 8.2	**0.01**	**0.17**
▲Aspartic acid (μmol·L^{-1})	3.6 ± 1.5	4.7 ± 2.7	4.9 ± 1.3	6.6 ± 2.5	0.59	0.00
▲Tyrosine (μmol·L^{-1})	56.9 ± 12.4	67.1 ± 21.5	65.7 ± 16.9	66.9 ± 24.5	0.19	0.05
▲Phenylalanine (μmol·L^{-1})	45.7 ± 10.0	49.8 ± 22.6	48.6 ± 13.3	51.3 ± 11.0	0.42	0.01
♦Glycine (μmol·L^{-1})	121.1 ± 34.0	143.0 ± 47.6	118.1 ± 30.6	144.0 ± 46.0	0.80	0.01
♦Glutamic acid (μmol·L^{-1})	19.3 ± 9.8	18.1 ± 7.7	14.2 ± 8.4	22.1 ± 8.3	0.18	0.06
♦Proline (μmol·L^{-1})	111.5 ± 33.3	110.3 ± 27.5	108.8 ± 47.2	130.4 ± 47.7	0.27	0.09
♦Methionine (μmol·L^{-1})	14.8 ± 5.8	14.4 ± 4.6	13.9 ± 3.9	13.5 ± 6.3	0.74	0.01
♦Histidine (μmol·L^{-1})	55.9 ± 15.8	61.6 ± 14.5	49.9 ± 10.6	62.3 ± 13.9	0.16	0.06
♦Arginine (μmol·L^{-1})	52.2 ± 8.1	55.8 ± 12.1	59.4 ± 15.2	57.2 ± 10.9	0.68	0.03
●Serine (μmol·L^{-1})	89.3 ± 22.4	87.1 ± 20.9	84.7 ± 25.0	89.1 ± 22.8	0.45	0.02
●Threonine (μmol·L^{-1})	40.0 ± 9.7	41.1 ± 12.5	39.5 ± 12.9	39.9 ± 10.3	0.94	0.05

Table 4. The effect of chronic whole-body cryotherapy on biochemical indicators and amino acid profile among WBC-EXP (n = 22) and WBC-CON (n = 13). Data are presented as mean ± SD; *statistically significant difference between before and after measurements in the group; # statistically significant difference between groups at a time point, $^{+}$ statistically significant difference between before and after measurements MG from WBC-EXP vs WBC-CON $p < 0.05$;η_p^2- effect sizes (partial eta squared): ≥ 0.01 small, ≥ 0.06 medium and ≥ 0.14 large effect; Amino acids after deamination form keto acid like: °the most important gluconeogenic precursors in liver ; ■acetyl-CoA; ▲fumarate; ♦alpha-ketoglutarate; ●pyruvate, which are further metabolized in gluconeogenesis process in the Krebs-cycle. Statistically significant group x time interaction are bold.

Regarding changes in AA profile's, the concentrations of valine ($p < 0.01$) and asparagine ($p < 0.01$) were significantly lower in the WBC-EXP than in the WBC-CON upon the last session of WBC. At this point in time, a positive correlation between valine and myostatin was recorded in the WBC-EXP group (r = 0.60; Table 1). The remaining AA's were not affected by the intervention (Table 4).

Discussion

Our results demonstrate that chronic WBC exposure had a positive effect on glucose homeostasis in normoglycemic participants. This exposure caused a significant decrease of blood glucose concentration and ameliorated most of the measured indicators of glucose homeostasis. There was also a significant reduction of glucose, evident in the WBC-CON, but still the decrease noted among experimental WBC-EXP group was two-fold higher compared to the WBC-CON group. Significant reductions of insulin and HOMA-IR values were particularly visible among MG participants subject to WBC. The level of these factors was elevated at baseline compared to YG subjects, thus the effect of the intervention in MG participants was more pronounced. Beneficial changes in glucose homeostasis may be connected with the activation of the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system[27]. A recently published paper by Yoneshiro et al. (2019) revealed that cold exposure significantly reduced plasma concentrations of valine, leucine and isoleucine. The authors relied on plasma metabolomics in obese mice and measured the acitivity of BAT, which displayed the highest valine oxidation in cold exposure, relative to other metabolic organs. In a follow up study, these observations were also verified on humans[37]. Therefore, a WBC induced reduction in glucose concentration evident in our study may have modified the activity of white as well as BAT resulting in reduced PBF% and VFA.

To the best of our knowledge, our study is the first to assess strictly the effect of WBC on blood concentrations of AA's in men. Previously, only one study demonstrated a significant drop of tryptophan and valine after 10 sessions of WBC combined with volleyball training[38]. In the present study, we assessed AA concentrations based on their role in glucose homeostasis. Similar to previous research in animal models (Yoneshiro et al. 2019), we noted a drop of valine in the WBC-EXP group following chronic WBC exposure compared to the WBC-CON group. In the present study, the observed decrease in valine following WBC likely occurred either because WBC could have induced the conversion of valine to β-aminoisobutyric acid, which is a myokine involved in the browning of fat[39]. Or because cold treatment stimulated the activity of mitochondrial BCAA enzymes such as the branched-chain α-keto acid dehydrogenase complex in the white adipose tissue[37]. Hence, the observed decrease of valine could have been associated with a statistically significant decrease of glucose concentration accompanied by the downward trend of insulin and HOMA-IR recorded in the MG part of the WBC-EXP group. At the same time, we noted a significant drop of VFA in the whole WBC-EXP group. This reduction in the amount of VFA might have had also a diminishing effect on its endocronical action.

Circulating concentrations of almost half of the AA's increased significantly 1 h after the first WBC session. It is possible that, at this point in time, the protein breakdown peaked and AA's were released into the bloodstream. This hypothesis is supported by the fact that this trend of change was also observed 1 h after the last session of WBC. Among all of the AA's only changes in alanine followed the same trend in response to first as well last session of WBC. Increased metabolism of BCAA in skeletal muscle during WBC, which is manifested by a decrease in serum valine, may lead to increased alanine formation[40]. Thus, alanine can be transported to the liver to act as a substrate in the gluconeogenesis process. Nevertheless, chronic WBC exposure did not affect alanine expression in the present study. Meanwhile, only concentrations of valine and asparagine were reduced following the chronic WBC. This response might be beneficial in IR individuals because a previous study revealed that high concentrations of BCAA, phenylalanine, tyrosine, alanine, ornithine and lysine were associated with an increased risk of T2DM[41]. Further, valine and asparagine belong to an AAs signature associated with T2DM risk and progression. Particularly, while increased valine levels together with isoleucine and leucine predict T2DM risk, increased asparagine is associated with a progression of diabetes (along with aspartic acid, glutamine and glutamate)[42]. It is possible to hypothesize that if chronic WBC exposure is capable of reducing AA expression in normoglycemic participants, a similar response in hyperglycemic individuals would be beneficial. Therefore, the beneficial effects of WBC on metabolism can be marked by the improved AA profile.

Together with the improvement of AA profile's, we noted a drop of myostatin among MG participans of WBC-EXP group. In addition to regulating muscle cell growth, myostatin has been shown to inhibit glucose uptake[43], which suggests that it may contribute to systemic IR. Elevated myostatin levels were registered in pathological conditions characteristic of the metabolic deregulations such as obesity, T2DM and aging[44]. Our results are consistent with those findings. We observed a significant correlation between myostatin and most of the glucose homeostasis indicators at baseline. Also, at baseline, MG exhibited higher concentrations of myostatin than YG ones. These data are consistent with the findings of Yarasheski et al. (2002), who reported elevated serum myostatin in advance-aged men compared with younger subjects[45]. Interestingly, following chronic WBC exposure, serum myostatin dropped in MG subjects to the level recorded in their YG counterparts. This response could be beneficial, particularly in MG individuals at risk of hyperglycaemia.

In addition to skeletal muscles, BAT is a significant source of myostatin[46,47]. Cold exposure has been shown to up-regulate the transcription of interferon regulatory factor 4 (IRF4) in BAT, leading to inhibition of the myostatin expression[46]. In comparison, heat exposure (30 °C) or loss of IRF4 function have been reported to result in an elevation of serum myostatin[46]. Report of Kong et al. (2018) revealed that BAT can secrete significant amounts of myostatin into the blood; therefore, cold treatment can be expected to inhibit the secretion of myostatin from BAT[46]. In contrast, Zak et al. (2018) previously observed that the synthesis of myostatin in skeletal muscles was not sensitive to temperature[48]. Based on these reports, our data suggest that the effects of WBC were related to its impact on BAT rather than skeletal muscles. Importantly, the changes observed in serum myostatin were age-dependent. Sliwicka et al. (2020) observed that shifts in myostatin induced by cold treatment and/or

physical exercise were only temporary in young men and returned to the baseline level within 24 h following cryotherapy/exercise[27,49]. In the present study, changes in myostatin concentration were more pronounced in MG participants, subject to chronic WBC, after which they exhibited serum myostatin at the level observed in YG subjects at baseline.

Myostatin acts through the inhibition of Akt kinase, which can lead to the activation of FOXO3a, a transcription factor that induces the expression of atrogin-1 gene encoding for a protein strongly linked to muscle atrophy. Thus, reducing the expression of myostatin through chronic WBC exposure can possibly improve the uptake of AA's in MG individuals, and indirectly, ameliorate insulin sensitivity[50].

The improvement of glucose homeostasis was accompanied by changes in FGF21 concentration in the present study. These results are partly comparable to those reported by Shabkhiz et al. (2020)[51]. They observed a decrease of FGF21 and myostatin, which suppressed IR in elderly men after 12-weeks of resistance training[51]. On the other hand, elevated circulating levels of FGF21 have been reported in the elderly[52] and in T2DM patients[53]. In the present study MG participants demonstrated an elevated concentration of FGF21 compared to YG counterparts at baseline. WBC induced a drop of FGF21 among all participants, however, these changes were age- dependent. Previously, Hollstein aet al. (2020) also observed a decrease in plasma FGF21 after a long-term cooling session (24 h inside a calorimeter at 19.0 ± 0.3 °C) in overweight and obese participants[54]. Others have reported conflicting effects on FGF21; with one study reporting an increased secretion of FGF21 (12 h exposure to 24 °C or 19 °C in a whole-room indirect calorimeter)[55], while a second study reported a decrease in FGF21 (cooling vest ~ 14.5 °C for 1-2 h)[28]. The disparity in the aforementioned results could be a result of the cooling protocols which differed significantly from the extremely low temperature applied in our WBC intervention. Furthermore, the variations in FGF21 concentrations can also be attributed to the different time points at which FGF21 was measured particulary because the circadian rhythm modulates a nightly increase and daily decrease in FGF21[56]. In order to standardize our data collection, and in an attempt to mitigate the impact of circadian rhythm on FGF21 concentration as well as other tested markers[57,58], we collected blood samples at the same time of day on each day of our data collection.

Together with FGF21, irisin represents a link between myostatin and glucose metabolism[59]. Lee et al. (2014) demonstrated that both irisin and FGF21 are cold-modulated factors that participate in the regulation of glucose metabolism[30]. In the present study, we observed a significant increase in serum irisin 1 h after the first WBC session, particularly among YG participants. This effect was sustained throughout the study protocol. We also observed two-fold higher values of irisin at baseline in MG participants compared to YG. This observation is consistent with that of Huth et al. (2015), who found a positive correlation between irisin, age and obesity markers, which all correlated inversely with insulin sensitivity[60]. Changes in irisin concentration in response to WBC can be linked with the two sources of this protein: skeletal muscles[30] and fat tissue[31,61]. The correlations recorded in WBC-EXP group in our study would suggest that the origin of irisin during cold exposure depended on body composition. In our previous study, we concluded that the effect of WBC on irisin concentration depended on participants' physical fitness level[31], thus men with a similar level of relative VO_2max were recruited for this experiment. Chronic WBC caused significant increase in irisin concentration, but we did not observe any correlations of this change with body composition or fitness level.

It is worth noting that the elevated concentration of irisin at baseline was accompanied by a lower level of BDNF in MG participants, who were also characterized by higher adiposity compared to the YG individuals. This relationship was also confirmed by a statistically significant, inverse correlation between irisin and BDNF in the whole experimental group. BDNF is hypothesized to be a growth factor with a strong influence on peripheral metabolism, including fat oxidation and the subsequent effect on adipose tissue[62]. Krabble et al. (2007) noted low levels of circulating BDNF in individuals with both obesity and T2DM[63]. Moreover, Pedersen et al. (2009) observed an inverse correlation between plasma BDNF and glucose, which raises a possibility that high plasma glucose levels would negatively influence BDNF concentration[62]. Significantly higher glucose concentration among MG subjects recorded at baseline in our study, partly confirm these findings. BDNF did not change in response to the single and chronic exposure to WBC.

Chronic cold exposure caused an increase of the level of adiponectin in the WBC-EXP group, yet no significant changes were observed in the WBC-CON group. Similarly, Imbeault et al. (2009) observed an increase in adiponectin levels in young healthy men during a 2 h period of cold exposure (both 4 °C and 10 °C) which was inhibited by glucose ingestion[64]. Adiponectin is considered a marker of systemic insulin sensitivity[65]. In the present study, the elevation in adiponectin concentration was accompanied by a decrease of insulin and glucose in the WBC-EXP group at the conclusion of the tenth WBC exposure. Despite the differences in PBF% among YG and MG participants, the mean change of adipokine during WBC exposure did not differ significantly between these two cohorts. Nevertheless, a trend towards an increase in adiponectin among MG individuals compared with YG individuals (mean increase of 54.6% vs 35.5%, respectively) was noticed. Adiponectin is one of the most abundant adipokines secreted by adipocytes[65]. Our findings suggest that the amount of body adipose tissue may have affected the relative increase of circulating adiponectin during WBC.

To conclude, the both the acute and chronic WBC protocol led to an improvement in glucose homeostasis indicators together with a reduction of valine and asparagine (Fig. 4). These changes were accompanied by a decline of serum myostatin concentration. This effect was more pronounced amongst the MG participants. Our intervention is not without limitations. We did not perform fat or muscle tissue biopsy, which means that we cannot clearly determine the source of the indicators observed in the blood. Further research could address these limitations, in particular determine the longevity of the WBC-induced effects on reduce myostatin level, changes in blood AA profile, improvement of glucose homeostasis and explore other factors modulating these effects. Overall, our results support the use of WBC to induce at least a short-term improvement in the metabolic profile that may feed into more complex preventive strategies, including physical activity and eventually, pharmacologic interventions, against the risk of development of IR and T2DM.

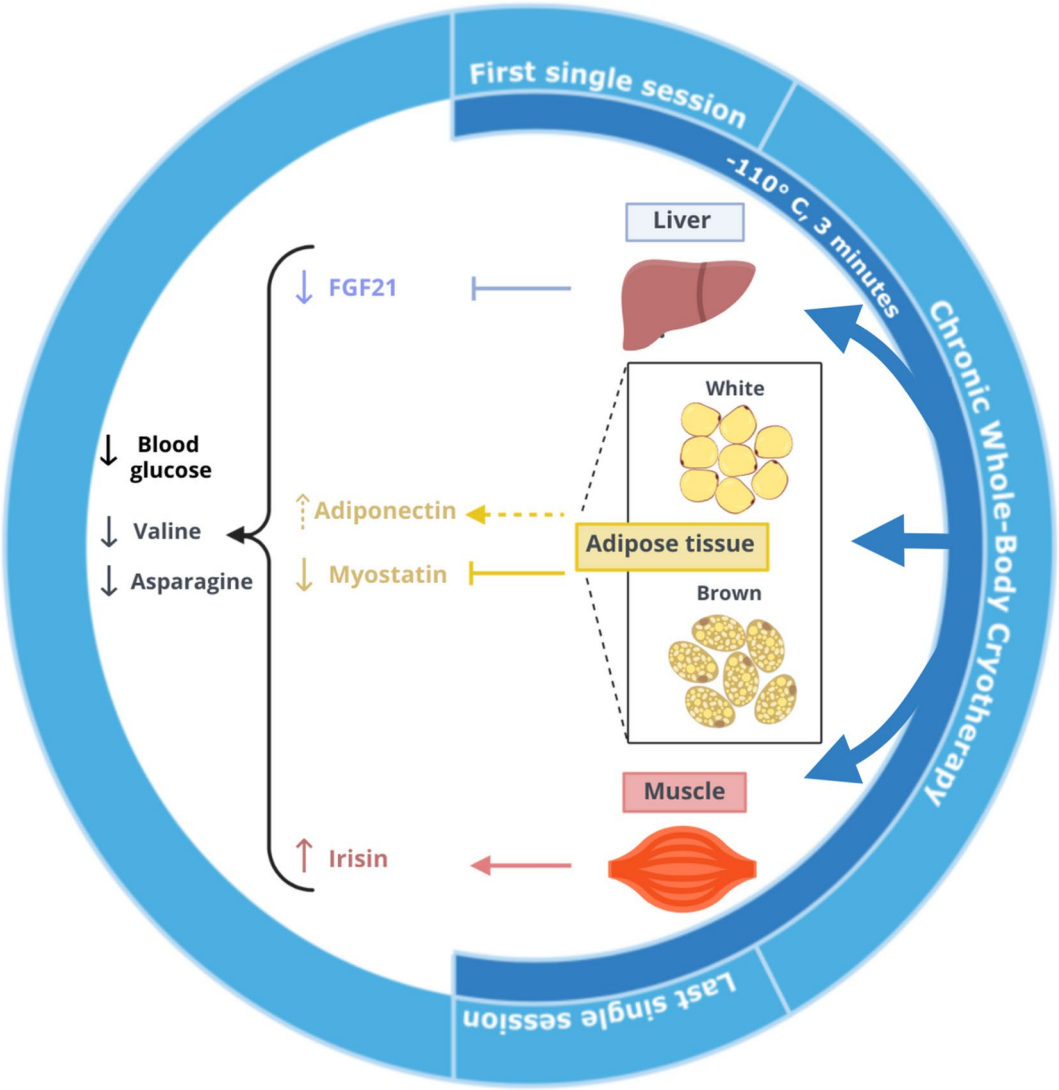

Figure 4. Graphical conclusion–proposed interpretation of the findings. WBC inhibited FGF21 in the liver, triggered skeletal muscle mass shivering, which lead to an increased release of irisin, and stimulated brown fat tissue to reduce myostatin and white fat tissue to release adiponectin.

Methods

Subjects. Thirty-five healthy, untrained, male participants, who had not experienced WBC in the previous 12 months, took part in the experiment. Prior to participation in the study, volunteers completed a medical screening in order to eliminate those with contraindications to cold exposure (e.g. cardiovascular disease, blood pressure > 160/100 mmHg, stroke or cold intolerance)[66]. Using an online software https://www.graphpad.com/quickcalcs/randMenu/), the participants were randomly assigned to either the experimental WBC group (WBC-EXP, n = 22; age = 40 ± 13.5 years; BMI = 26.1 ± 3.9 kg·m^2; PBF% = 19.3 ± 6.1%) or the control group (WBC-CON, n = 13; age = 30.1 ± 7.4 years; BMI = 23.5 ± 2.5 kg·m^2; PBF% = 17.2 ± 5.9%). The WBC-EXP group was further divided intoeither young (YG; n = 9; age = 28 ± 7 years) or middle-aged (MG; n = 13; age = 51 ± 3 years). Considering previous reports, which revealed that the effect of WBC on circulating myokines and adipokines was depended on participants' cardiorespiratory fitness[36], that the participants in the present study all had a similar relative VO_2max (WBC-CON 47.4 ± 4.6 mL kg^{-1} min^{-1}; WBC-EXP 46.5 ± 5.1 mL kg^{-1} min^{-1}). The study protocol was approved by the Bioethical Committee of the Regional Medical Society in Gdansk KB-28/17 and was conducted in accordance with the Declaration of Helsinki. This experiment was conducted as an arm of the clinical trial registered in the ClinicalTrials.gov: NCT04375969 on 6 May 2020. A written, informed consent was obtained from all subjects. A schematic representation of the experimental protocol is presented in Fig. 5.

Body composition assessment. Body mass and body composition, skeletal muscle mass, PBF% and VFA[67,68] were estimated using a multi-frequency impedance analyser (In Body 720, Biospace, Korea). Measurements were taken on the first day of data collection and after the final session of WBC with the participants in a

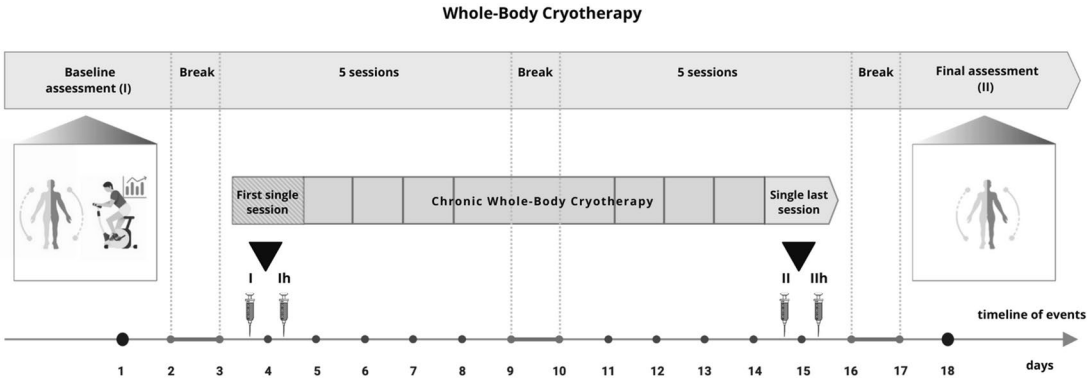

Figure 5. The experiment schedule. Blood collection: (I) before the first WBC session, (Ih) 1 h after the first WBC, (II) before the last WBC session and (IIh) 1 h after the last WBC session. Baseline assessment: body composition assessment and cardiorespiratory fitness measurement. Final assesment: body composition asesssement.

fasted state. During the measurement, subjects wore only shorts and remained barefoot. The impedance of segments of different body parts (trunk, arms and legs) was measured at six different frequencies (1, 5, 50, 250,500, and 1000 kHz) using an eight-polar tactile-electrode. This method can be used as a surrogate of dual-energy X-ray absorptiometry[69] because of greater availability and smaller individual error produced by InBody analyzer, which makes it equally precise.

Cardiorespiratory fitness measurement. In order to standarize the study group in terms of cardiorespiratory fitness, participants performed a graded cycle test on a cycle ergometer (884E Sprint Bike Monark, Sweden) to determine their VO_2max. The test was conducted three days prior to the start of the first WBC exposure. The VO_2max test began with a 5-min warm up at a workload of 1.5 $W \cdot kg^{-1}$ and a pedalling cadence of 60 rpm. The load increased progressively by 25 $W \cdot min^{-1}$ until an individual reached the point of volitional exhaustion. Pulmonary gas exchange was measured during the test (MetaMax 3B, Cortex, Germany)[36]. The highest value of relative oxygen uptake was taken into consideration when assigning experiment and control groups.

Blood analysis and collection. Blood samples were taken on the first day of the WBC treatment (both prior to and 1 h after the first session) and on the last day of the final WBC session (also both prior to and 1 h after the last, 10th session). Samples (approx. 20 ml per person during each collection) were collected from the antecubital vein uding a needle into vacutainer tubes with K3EDTA (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) for plasma analysis, and into vacutainer's with sodium fluoride to estimate glucose concentration and SSTTM II Advance for serum analysis. Samples were centrifuged at 2000 g at 4 °C for 10 min and then stored at −80 °C.

Serum FGF21, myostatin and BDNF were determined by enzyme immunoassay methods using commercial kits (R&D Systems, USA; catalog no. DF2100, DGDF80 and DBD00, respectively) in accordance with manufacturer's instructions. The detection limits were 8.69 $pg \cdot mL^{-1}$ for FGF21, 2.25 $pg \cdot mL^{-1}$ for myostatin and < 20 $pg \cdot mL^{-1}$ for BDNF. The average intra-assay coefficient of variation (CV) was 3.5–3.9% for FGF21 and 5% for myostatin and BDNF. For myostatin measurements, samples were diluted in a 1:4 ratio (in 1 N HCL, 1.2 N NaOH/0.5 M HEPES and Calibrator Diluent RD5-26) prior to the analysis according to the manufacturer's instruction.

Quantification of serum irisin and plasma adiponectin was determined via the enzyme immunoassay method using commercially available kits from Phoenix Pharmaceuticals Inc, USA (catalog no. EK 067–29 and EK- ADI-01, respectively) according to the manufacturer´s protocol. For irisin, intra-assay CV was 4–6% and inter-assay CV was 8–10%. For adiponectin intra-assay and inter-assay CV's were < 10% and < 15% respectively, and detection sensitivity was 5.32 pg mL^{-1}. AA profile was conducted based on the ion-pair reversed phase high performance liquid chromatography combined with the tandem mass spectrometry IP-RP HPLC–MS/MS (TSQ Vantage Thermo Scientific, USA). The procedure was executed following the protocol already described by Gmiat et al.[70].

Glucose level was assessed using the Cobas 6000 analyser. To determine insulin concentration the immunoassay kit from DiaMetra (catalogue no DKO076, Perugia, Italy) was used. The intra-assay CV was ≤ 5% and the inter-assay CV was ≤ 10%. Homeostasis model assessments for insulin sensitivity (HOMA-S), β-cell function (HOMA-B) and insulin resistance (HOMA-IR) were obtained from paired fasting glucose and insulin levels using the updated software HOMA calculator, version 2.2.3, copyright by The University of Oxford (www.dtu.ox.ac.uk/homacalculator). Normal values are 100% for HOMA-S and HOMA-B and 1.0 for HOMA-IR[71].

Whole-body cryotherapy procedure. WBC sessions took place in a cryogenic chamber (Zimmer MedizinSysteme, Elecpol) at the Pomeranian Rheumatologic Centre in Sopot, Poland. The treatments were performed five days in a row, with a two-day rest period, followed by five more consecutive days, for a total of 10 sessions completed over two weeks (Fig. 5). Sessions took place at the same time of day (in the morning between 7:30 am and 8:00 am after a light breakfast). Each session was preceded by a 30-s adaptation in the chamber at

– 60 °C. The cryotherapy exposure in the main chamber lasted 3 min at – 110 °C. Participants wore shorts, socks, gloves and a hat to protect their hands, feet and ears againts frostbite. According to the instructions, they moved slowly on a circle, changing direction of the motion every 1 minute[72]. Participants did not engage in any other recovery treatment, throughout the duration of the study.

Statistical analysis. Statistical analyses were performed using a dedicated software package (Statistica 13.1 software, TIBCO Software, Palo Alto, California, USA). The sample size of the study group was predetermined using a power calculation in the software G*power version 3.1.9.4[73] (a priori repeated-measures within-between interaction, $\alpha = 0.05$, $1\text{-}\beta = 0.95$, $r = 0.6$, $f = 0.25$, $\varepsilon = 1$, with a further 20% surcharge due to the possibility of the participant not completing the experiment). Shapiro–Wilk tests were used to assess the homogeneity of dispersion from normal distribution. Brown–Forsythe test was used to evaluate the homogeneity of variance. To analyse the effect of a single cryotherapy session, we used paired tests for a homogenous sample. For a heterogeneous sample, Wilcoxon signed-rank test was used. In the second phase of analysis, for lipid profile, amino acid profile and glucose homeostasis indicators two (group: WBC-EXP, WBC-CON) x two (time: before and after 2 weeks) analyses of repeated measurements of variances (ANOVA) were calculated. In case of a significant group x time interaction, for homogenous results Tukey's post hoc tests for unequal sample sizes were performed to identify significantly different results. For heterogeneous results, ANOVA Friedman's test and Dunn-Bonferroni post-hoc test were used. The effect size (partial eta squared, η_p^2) was also calculated, with $\eta_p^2 \geq 0.01$ indicating a small effect; ≥ 0.059 indicating a medium effect; and ≥ 0.138 indicating a large effect[74]. A similar analysis was done for age groups (YG and MG) in the WBC-EXP treatment group. Relationships between variables were evaluated using the Spearman correlation coefficient. Additionally, the effect size (Cohen's d) was calculated, with $d \geq 0.2$ indicating small effect; ≥ 0.5 indicating medium effect; and ≥ 0.8 indicating large effect. The level of significance was set at $p < 0.05$. In the descriptive analysis, data are reported as a mean ± standard deviation (SD).

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Author contributions

MK, EZ designed the study and performed the research. MK, EZ, JA, SP, GL performed the research and wrote the paper. MK, GL, MŻ, JA, EZ designed, drafted, and critically revised the manuscript. MK, EZ, SP, KŻ, JK analysed the data. MŻ, JK, KŻ, SP 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

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Correspondence and requests for materials should be addressed to E.Z.

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Original Research

Short and long-term effects of high-intensity interval training applied alone or with whole-body cryostimulation on glucose homeostasis and myokine levels in overweight to obese subjects

Marta Kozłowska-Flis[1], Ewa Rodziewicz-Flis[2], Katarzyna Micielska[3], Jakub Kortas[4], Joanna Jaworska[3], Andżelika Borkowska[5], Veronica Sansoni[6], Silvia Perego[6], Giovanni Lombardi[6,7], Ewa Ziemann[7,*]

[1]*Department of Physiology, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland,* [2]*Department of Physiotherapy, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland,* [3]*Department of Physical Education and Lifelong Sports, Poznan University of Physical Education, 61-871 Poznan, Poland,* [4]*Department of Health and Natural Science, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland,* [5]*Department of Bioenergetics and Physiology of Exercise, Medical University of Gdansk, 80-210 Gdansk, Poland,* [6]*Laboratory of Experimental Biochemistry & Molecular Biology, Istituto Ortopedico Galeazzi (IRCCS), 20161 Milano, Italy,* [7]*Department of Athletics, Strength and Conditioning, Poznan University of Physical Education, 61-871 Poznan, Poland*

TABLE OF CONTENTS

1. Abstract

Background: COVID-19 pandemic has exacerbated the problem of physical inactivity and weight gain. Consequently, new strategies to counteract weight gain are being sought. Because of their accessibility, interval training and cold therapy are the most popular such strategies. We here aimed to examine the effect of 6 units of high-intensity interval training (HIIT), applied alone or in combination with 10 sessions of whole-body cryotherapy (WBC; 3 min at –110 °C per session) on incretins, myokines, and adipokines levels. **Materials and methods**: The study involved 65 subjects (body mass index of approximately 30 $kg \cdot m^{-2}$). The subjects were randomly divided into training group (TR; n = 27) and training supported by WBC group (TR-WBC; n = 38). Blood samples were collected before, immediately following, and 4 weeks after the intervention. **Results**: Fibroblast growth factor 21 (FGF21) levels sig-

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nificantly increased (p = 0.03) and adiponectin levels increased in the TR group (p = 0.05) compared with those recorded in TR-WBC group 24 h after the end of experimental protocol. Beneficial changes in the lipid profile (p = 0.07), a significant drop in visfatin levels ($p < 0.05$), and the improvement in β-cell function (HOMA-B; p = 0.02) were also observed in the TR group in the same time point of study. While TR-WBC did not induce similar changes, it ameliorated blood glucose levels (p = 0.03). Changes induced by both interventions were only sustained for 4 weeks after treatment. **Conclusion**: Collectively, HIIT, alone and in combination with WBC, positively affects metabolic indicators, albeit, most likely, different mechanisms drive the beneficial effects of different treatments.

2. Introduction

According to global estimates prior to the outbreak of COVID-19 pandemic, 27.5% of adults [1] and 81% of adolescents were physically inactive [2], and did not meet the recommended 150 min weekly dose of physical activity (PA). According to some authors, pandemic-related lockdown induced additional, major changes in lifestyle behavior among adults, with a 43% decrease in PA and 19% increase in unhealthy food consumption, ultimately resulting in weight gain [3]. The average weight gain among adults associated with the COVID-19 pandemic is 4.7 kg (unpublished, statistic data). Obese individuals with low cardiorespiratory fitness are typically a challenging population to be treated; in the presence of accompanying diseases, these individuals struggle to survive [4]. Further, obesity increases the risk of severe infection with SARS-CoV-2, the virus that causes COVID-19 [5]. Excessive fat accumulation, especially as visceral adipose tissue, impairs glucose homeostasis [6] and results in a low-grade inflammation that may, over time, lead to insulin resistance and type 2 diabetes (T2DM). Nonetheless, during the ongoing COVID-19 pandemic, home-based PA programs supported by digital solutions are commonly used to maintain an adequate level of PA and weight balance [7]. Further, limited access to fitness centers and infrastructure has focused the attention on intermittent forms of PA that could be performed at home, e.g., high-intensity interval training (HIIT). Among the different forms of physiotherapy, cold exposure is thought to enhance the beneficial effects of exercise. Consequently, cold water immersion in the sea or lake became popular in the winter of 2020/2021, when access to professional physiotherapy was limited. For these reasons, in this project we aimed to evaluate short- and long-term effects of interval training in combination with exposure to extreme cold, considering pro-health changes in the lipid profile, myokine profile and glucose homeostasis among overweight to obese, inactive participants.

Studies suggest that exercise [8] and cold exposure [9] elicit comparable muscle contractions, the latter in association with shivering, and induce similar endocrine responses, by stimulating the release of muscle-derived peptides. These act as endocrine-like factors, such as myokines [10] and exerkines [11], and are involved in the prevention or reversion of the negative effects of high food intake, being overweight, and obesity, as well as several other pathological conditions [12]. Fibroblast growth factor 21 (FGF21) [13] and irisin [14] are myokines modulated by cold exposure. They are important metabolic regulators that stimulate glucose uptake by adipocytes and myofibers, and improve glucose homeostasis. However, the physiology of the effects of physical exercise and cold exposure on myokine expression, in particular, that of FGF21, is still only marginally understood.

Costello *et al*. [15] studied the effect of whole-body cryotherapy (WBC; –110 °C, 3 min exposure) and cold-water immersion (8 °C, 4 min) on the temperature of different body parts. They observed comparable changes in the muscle and core temperature, but not in the skin temperature. Further, we have previously demonstrated that regular WBC causes a drop in FGF21 blood levels, but only among middle-aged participants [16]. The decrease in FGF21 levels was accompanied by an improvement of glucose homeostasis-related parameters, and a reduction of valine and asparagine levels [16]. The latter effect is particularly important since valine and asparagine are considered to be early markers of glucose homeostasis disturbance [17]. Other studies involving human subjects have focused on mild cold exposure: 12 h exposure to 19 °C [18] and 12 min exposure to 18 °C, lowered by 2 °C every 3 min to 12 °C [19]. The latter treatment enhanced circulating FGF21 levels and brown adipose tissue mass [19]. Further, treadmill exercise test performed over 2 weeks, following the Bruce's protocol, induces a significant increase in serum FGF21 levels in young, inactive women, and this is accompanied by an increase in free fatty acid levels in the blood, heart rate (HR), and energy expenditure during exercise, as well as changes in epinephrine levels [20].

Considering the above, one may hypothesize that a combination of HIIT and cryostimulation would positively impact metabolic homeostasis, consequently improving the inflammatory (i.e., myokine) status and glucose metabolism. As maintaining a good health status may reduce the risk of developing a severe disease associated with diverse infections, the search for accessible and effective pro-health, non-pharmacological strategies during the ongoing COVID-19 pandemic offers a valuable insight. To the best of our knowledge, this specific aspect and in particular, the HIIT- and cryostimulation-dependent changes in FGF21 levels (as an emerging pivotal mediator of metabolic homeostasis), as well as myokine, incretins, and appetite-controlling hormone levels, have not yet been investigated. Accordingly, the aim of the current study was to understand if, and how, the combination of HIIT and extreme cold exposure, vs. the HIIT alone, affects FGF21 serum levels,

and the adipo-myokine profile and metabolic status of overweight to obese subjects.

3. Materials and methods

3.1 Subjects

Sixty-five inactive, overweight to obese participants [body mass index (BMI) of approximately 30 kg·m^{-2}], who had not undergone WBC in the preceding 12 months, took part in the study. Eligible subjects underwent physical examination to evaluate their global health status, to exclude individuals with contraindications to cold exposure (e.g., acute cardiovascular and respiratory disease, unstable hypertension, blood pressure >160/100 mmHg, stroke or cold intolerance, circulatory and deep veins disorders, claustrophobia, cryoglobulinemia, hypothyroidism, neuropathies, Reynaud disease, and pregnancy) [21, 22]. Other than being overweight or obese, the inclusion criteria were: age >18 years, functional autonomy and physical inactivity (less than 60 minutes PA a week) assessed by the questionnaire. The exclusion criteria were: taking insulin or other chronic medications, immune-mediated pathologies, T2DM, and traumatic fractures in the preceding 2 years. Participants were randomly assigned to either the training group (TR, n = 27; BMI = 31.4 ± 3.5 kg·m^{-2}; age = 42 ± 13 years) or training combined with WBC group (TR-WBC, n = 38; BMI = 31.9 ± 5 kg·m^{-2}; age = 45 ± 9 years). Anthropometric data for the participants is shown in Fig. 2.

Body composition analyses were performed and the blood was collected 1 week prior to the study, and 24 h directly after and 4 weeks after completion of the intervention. The training workload for each subject was determined before the first HIIT session. The participants were asked to maintain and not to change their usual daily habits during their participation in the study.

The study protocol was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (approval number KB-28/17) and the study was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent for the publication of any associated data after being informed about the procedures.

3.2 Baseline assessment

Body mass and composition (lean body mass, BMI, body fat, and visceral adipose tissue) were determined using dual energy X-ray absorptiometry (DXA) with a Lunar Prodigy whole-body scanner (GE HealthCare, Madison, WI, USA) and enCORE v16 SP1 software (version 3.1.9.4, Heinrich Heine University, Düsseldorf, Germany). Subjects were assessed using DXA in the morning, after an overnight fast, prior to blood collection, usually within 1 h of arrival for clinical assessment and after medical check-up. The day before each assessment, DXA was calibrated using phantoms, according to the manufacturer's guidelines. Scanning mode was automatically chosen by the DXA apparatus. The subjects were exposed to a radiation dose of approximately 2 μSv per scan; the scan took approximately 6–11 min. During DXA assessment, the subjects were lying on the scanning table in supine position, wearing light indoor clothing, and with no metal objects on their body [23]. The DXA measurements were performed three times: at baseline, and immediately after and 4 weeks after completion of the intervention.

Prior to the experiment, a pilot HIIT test was performed to establish individual HR and the training workload. Each individual pedaled at 80–100 rpm with a load of 1.5 W·kg^{-1} (women) or 2.0 W·kg^{-1} (men), so as to achieve intensity of 90% of HR$_{max}$.

Supervised HIIT sessions were performed according to a protocol of Little *et al.* [24], three times a week for 2 weeks (6 sessions in total). In the TR-WBC group, exercise training was performed at the Pomeranian Rheumatologic Centre (Sopot, Poland) directly before WBC sessions 1, 3, 5, 6, 8, 9, and 11. Each training comprised: (A) 3 min warm up at 50 W; (B) 10 × 60 s cycling intervals interspersed with 60 s of recovery; and (C) 2 min cooling down at 50 W. The entire session lasted 25 min. During recovery, the subjects were allowed to rest by slowly pedaling against a resistance of 50 W. The TR group performed the 6 HIIT units without WBC treatment.

WBC exposure took place at the Pomeranian Rheumatologic Centre. The center is equipped with an electric cryochamber (Zimmer Medicine System, Cryochamber ELECPOL, Poznan, Poland), located in a temperature- and humidity-controlled room. The study schedule involved 10 treatments over 2 weeks, with a 2-day rest during the weekend. Sessions took place at the same time of day (in the morning, between 8:30 AM and 9:00 AM, after a light breakfast). In the TR-WBC group, the WBC session was conducted directly after the HIIT session, after careful sweat removal from the body by wiping. During WBC, the participants were minimally dressed (e.g., bathing suit, socks, clogs, headband, and a surgical mask), spent 30 s in a vestibule at –60 °C to allow the body to adapt to low temperature, and then moved to the cryochamber maintained at –110 °C, where they stayed for 3 min. Blood pressure was measured before each WBC session to exclude participants with an elevated blood pressure caused by the activation of sympathetic nervous system (blood pressure ≥130/90 mmHg). Access to the cryochamber was allowed only under the supervision of skilled personnel in control of the procedures.

3.3 Blood collection and analysis

Blood samples were collected by standard venipuncture by a trained nurse, before the study protocol was initiated, at the completion of the intervention, and 4 weeks after the completion of the intervention. However,

most participants from the TR group did not attend the sampling at the third time point; therefore, only participants from the TR-WBC group (n = 35) were considered in the ensuing analysis (Fig. 1). At each sampling time, 14 mL of blood was drawn into two plain serum and two K_2EDTA tubes (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). After mixing by inverting 10 times, the serum in the plain tubes was allowed to clot, in vertical position, for 45 min at approximately 20 °C, while the contents of the K_2EDTA tubes were homogenized for 15 min. The tubes were then centrifuged at 2000 × *g* at 4 °C for 10 min to separate the serum and the plasma, and stored at –80 °C until analysis.

The serum lipid profile [total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG)] was determined by enzyme immunoassays using commercial kits (Alpha Diagnostics, Warsaw, Poland). Glucose levels were determined using Cobas 6000 analyzer (Roche Diagnostics, Warsaw, Poland) according to the manufacturer's instructions. Insulin levels were assessed using an immunoassay kit (Diametra, catalog no. DKO076, Perugia, Italy). The intra-assay coefficient of variation (CV) for insulin was ≤5.0% and the detection limit was 0.25 μIU·mL^{-1}. Insulin sensitivity (HOMA-S), β-cell function (HOMA-B), and insulin resistance (HOMA-IR) were calculated from paired fasting glucose and C-peptide level readings using HOMA2 calculator v2.2.3 (University of Oxford; www.dtu.ox.ac.uk/homacalculator). The reference values are 100% for HOMA-S and HOMA-B, and 1.0 for HOMA-IR [25].

Serum FGF21 levels were determined by enzyme immunoassay using a commercial kit (R&D Systems, Minneapolis, USA, catalog no. DF2100), following the manufacturer's recommendations. The detection limit was 8.69 pg·mL^{-1} and the average intra-assay CV was 3.9%. Serum irisin levels were assessed using an immunoassay kit from Phoenix Pharmaceuticals Inc., Burlingame, USA (catalog no. EK 067-29). The intra-assay CV and the detection limits were <10.0% and 1.29 ng·mL^{-1}, respectively.

The levels of other mediators [adiponectin, C-peptide, ghrelin, gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, leptin, resistin, and visfatin] were assayed using multiplex immunofluorescence technology and Bio-Plex Pro Diabetes Assay Panels (Bio-Rad, USA, catalog no. 171A7002M for adiponectin and 171A7001M for others). The detection limits were 31.0 pg·mL^{-1} for adiponectin; 4.0 pg·mL^{-1} for C-peptide; 3.0 pg·mL^{-1} for ghrelin, GIP, and leptin; 12.0 pg·mL^{-1} for GLP-1; 47.0 pg·mL^{-1} for glucagon; 1.0 pg·mL^{-1} for resistin; and 8.0 pg·mL^{-1} for visfatin. The average intra-assay CV was 3.0% for adiponectin, C-peptide, GIP, GLP-1, glucagon, resistin, and visfatin; and 4.0% for ghrelin and leptin.

3.4 Statistical analysis

The sample size of the study group was predetermined using power calculations with the software G*power (version 3.1.9.4, Heinrich Heine University, Düsseldorf) [26] (*a priori* repeated-measures, within–between interaction; α = 0.05, 1-β = 0.95, r = 0.8, η_p^2 = 0.06, ε = 1; with a further 20% surplus for the possibility that a participant would not complete the intervention course).

Statistical analyses were performed using the statistics software package Statistica v13.1 (TIBCO Software, Palo Alto, CA, USA). Shapiro-Wilk test was used to assess the homogeneity of dispersion from normal distribution. Brown-Forsythe test was used to evaluate the homogeneity of variance. Repeated measures analysis of variance (rANOVA) was calculated. In case of a significant time × group interaction, *post hoc* tests for unequal sample sizes were performed to identify significantly different results. The effect size (partial eta squared) was also calculated, with $\eta_p^2 \geq 0.01$ indicating a small effect; ≥0.059 indicating a medium effect; and ≥0.138 indicating a large effect [27]. Paired tests were used to analyze the prolonged effect of cryotherapy in the TR-WBC group. For a homogenous sample, paired *t*-test analysis was performed to identify significant changes; for a heterogeneous sample, Wilcoxon signed-rank test was used. In addition, 95% confidence interval was calculated for changes within each study group. The level of significance was set at $p < 0.05$.

4. Results

Anthropometric data are presented in Fig. 2. No significant differences were noted among the participants at baseline. The interventions did not affect the participants' body composition.

4.1 Short-term changes induced by HIIT

The HIIT protocol, performed alone, lowered TG levels from 162.6 ± 131.2 to 129.0 ± 72.9 pg·mL^{-1} (TR: –26.0%, Δ = –33.5, Cl = –67.9; 0.9 vs. TR-WBC: 0.9%, Δ = 1.2, Cl = –22.0; 22.5, p = 0.07, ES = 0.05; Fig. 3C) and HOMA-B values from 93.8 ± 36.9 to 83.7 ± 26.8% (TR: –12.1%, Δ = –10.2, Cl = –23.4; 3.0 vs. TR-WBC: 6.0%, Δ = 5.8, Cl = –1.2; 12.9, p = 0.02, ES = 0.07; Fig. 3B) in comparison to HIIT protocol with 10 WBC sessions (TG: from 125.5 ± 80.7 to 126.7 ± 66.5 pg·mL^{-1}; HOMA-B: from 91.1 ± 19.0 to 96.9 ± 22.9%). Other indicators of the lipid profile and glucose homeostasis (i.e., glucose, insulin, HOMA-S, HOMA-R, and glucagon) remained unaltered after the completion of the HIIT protocol (Fig. 4C, Tables 1,2). The levels of C-peptide, released into the blood as a by-product of insulin secretion, showed a downward trend in the TR group (–15.7%; Table 2). Pre-to-post changes (Δ) in HOMA-B and C-peptide levels were strongly and positively correlated (r = 0.74; Table 3).

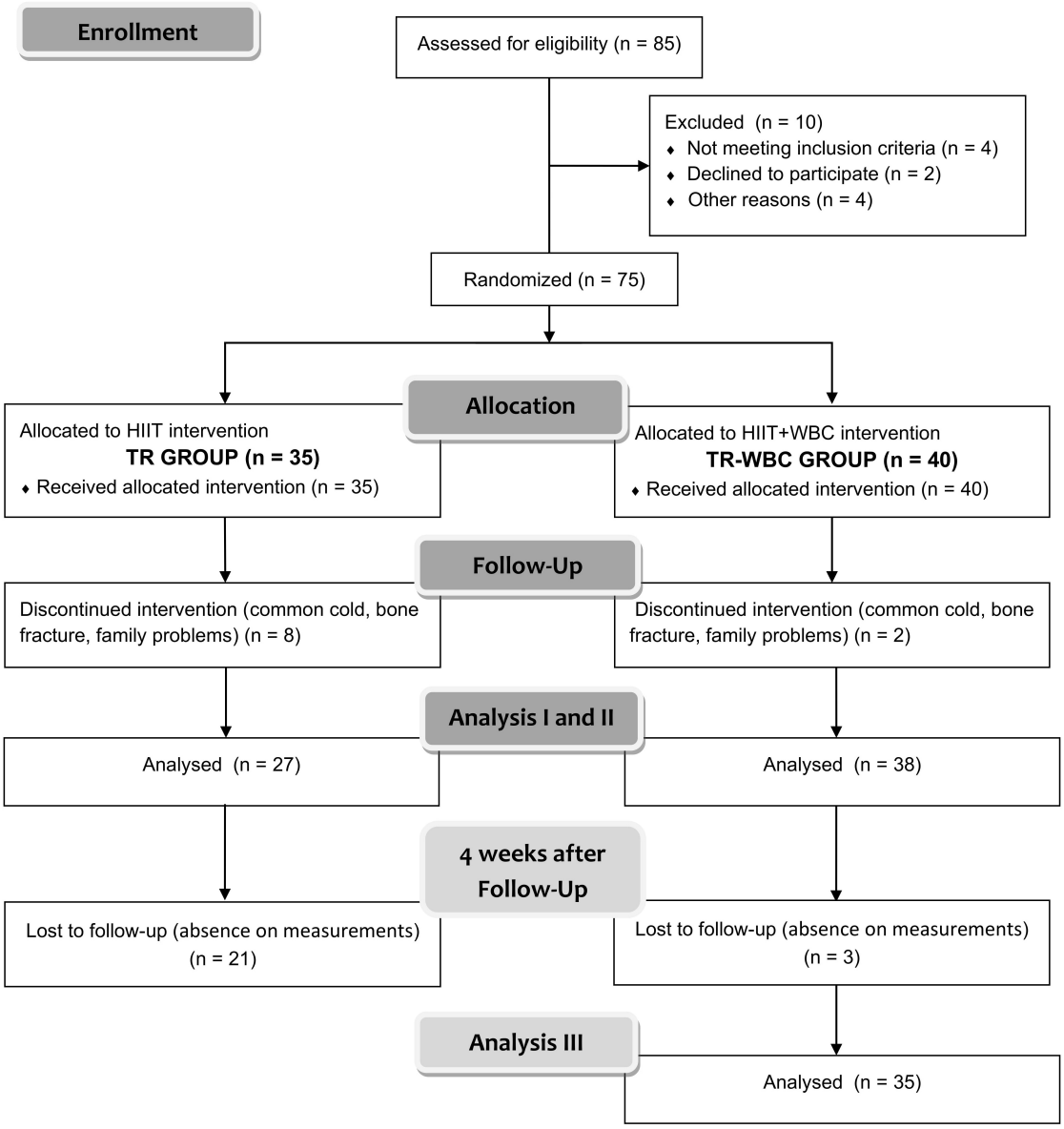

Fig. 1. The schedule of examinations. Analysis: (I) before protocol, (II) directly after protocol and (III) 4 weeks post finishing the whole experimental protocol only in training with whole-body cryotherapy group (TR-WBC).

The HIIT protocol resulted in a significant increase in FGF21 blood levels, from 191.0 ± 91.8 to 275.0 ± 178.8 pg·mL^{-1} (Δ = 83.9, Cl = 13.4; 154.4, $p < 0.05$). Shifts in FGF21 and adiponectin levels in the TR group differed significantly from the values recorded for the TR-WBC group ($p = 0.03$, ES = 0.08 for FGF21; Fig. 4A; $p = 0.05$, ES = 0.06 for adiponectin; Fig. 4B). In the TR-WBC group, FGF21 levels remained unchanged, while adiponectin levels decreased.

The HIIT protocol induced changes in the levels of proinflammatory cytokines, namely, a drop in the visfatin from 5734.5 ± 2921.7 to 5107.4 ± 2713.6 pg·mL^{-1} (Δ = –627.2, Cl = –1103.1; –151.2, $p < 0.05$; Fig. 3D), leptin, and resistin levels (Table 2), although statistical significance in comparison to the TR-WBC group was only reached for visfatin ($p = 0.04$, ES = 0.07; Fig. 3D). The remaining factors were not affected by the training intervention (Table 2).

4.2 Short-term changes induced by the HIIT–WBC combination

The combination of HIIT and WBC did not significantly alter the lipid profile (Table 1) but it improved glucose homeostasis indicators.

Nonetheless, the observed upward trend in HDL levels in the TR-WBC group (from 55.6 ± 15.9 to 57.8 ± 17.3 mg·dL^{-1}, Δ = 2.2, Cl = –0.2; 4.2) was significantly different from the response in the TR group (decrease from 52.7 ± 13.7 to 51.3 ± 14.6 mg·dL^{-1}, Δ = –1.4, Cl = –

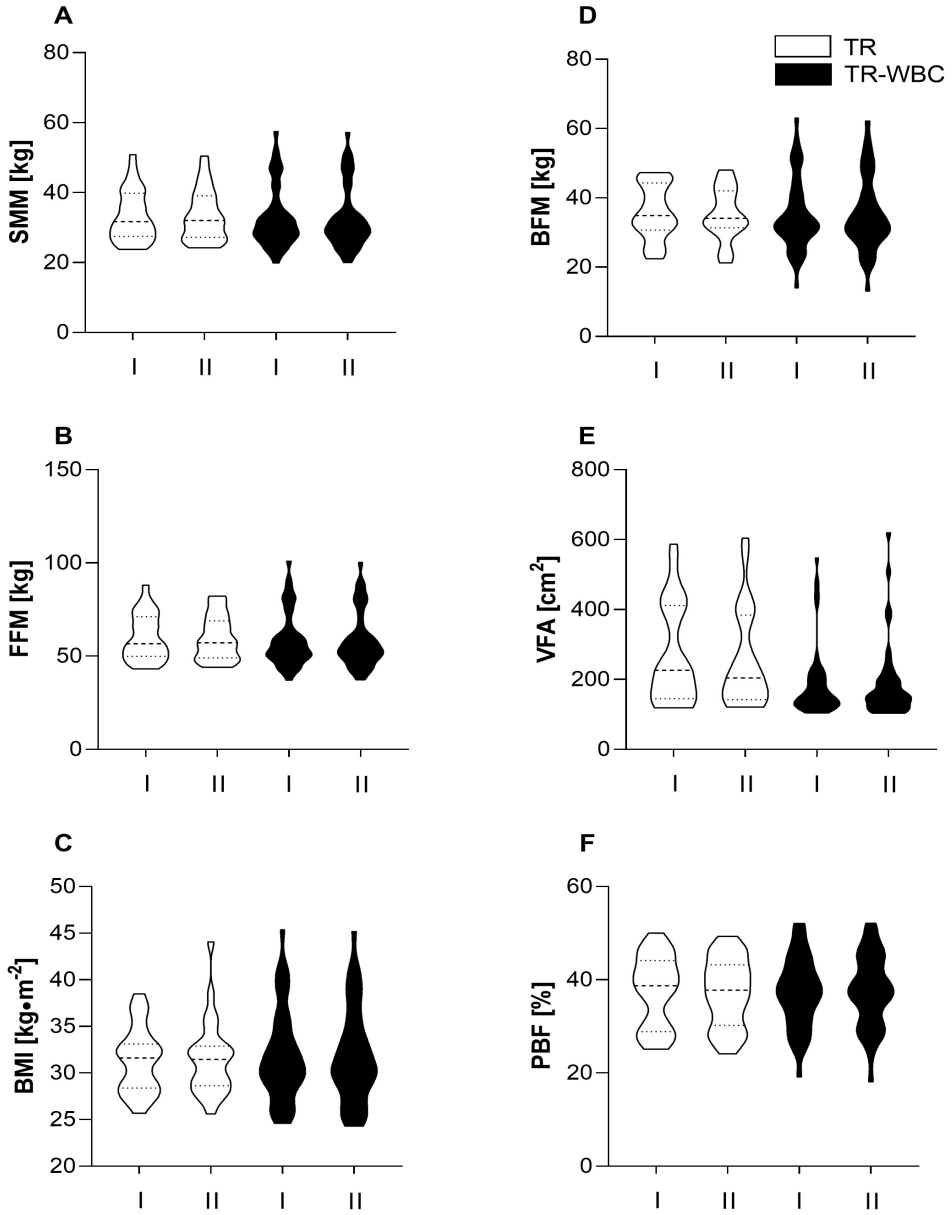

Fig. 2. Anthropometric characteristics of participants. (A) Skeletal muscle mass (SMM). (B) Free fat mass (FFM). (C) Body mass index (BMI). (D) Body fat mass (BFM). (E) Visceral fat area (VFA) and (F) percent of body fat (PBF) recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38). Data are presented as median and range.

3.8; 1.0, p = 0.02, ES = 0.08; Fig. 3A). Resting glucose levels were significantly reduced from 99.2 ± 10.9 to 95.9 ± 9.9 mg·dL^{-1} (Δ = –3.3, CI = –5.5; –1.1) in the TR-WBC group ($p < 0.05$) and this shift was significantly different from values recorded for the TR group (p = 0.03, ES = 0.07; Fig. 4C).

Of note, changes in TG (Fig. 3C) and FGF21 levels (from 204.0 ± 4.1 to 212.6 ± 113.7 pg·mL^{-1}, Δ = 8.6; Fig. 4A) recorded in the TR-WBC group were relatively blunted. Similar trends were noted for C-peptide (TR: –15.7% vs. TR-WBC: –2.3%), leptin (TR: –16.2% vs. TR-WBC: –6.8%), resistin (TR: –7% vs. TR-WBC: –0.4%, Table 2) and visfatin levels (TR: –12.3% vs. TR-WBC: –1.6%, from 6220.9 ± 2516.9 to 6125.2 ± 2683.6 pg·mL^{-1}, Δ = –95.8, CI = –389.0; 197.5; Fig. 3D).

Considering the diabetic panel markers, an upward trend in the levels of ghrelin (TR-WBC: 8.5% vs. TR: –0.6%) and GIP (TR-WBC: 7.9% vs. TR: –9.6%) was noted in the TR-WBC group, and the opposite was observed in the TR group. The differences were not statistically significant (Table 2); however, in the TR-WBC group, ΔGIP positively correlated with Δglucose (r = 0.34; Table 3) and negatively correlated with ΔFGF21 (r = –0.33; Table 3). The remaining factors were not affected by the combination of HIIT and WBC (Tables 1,2).

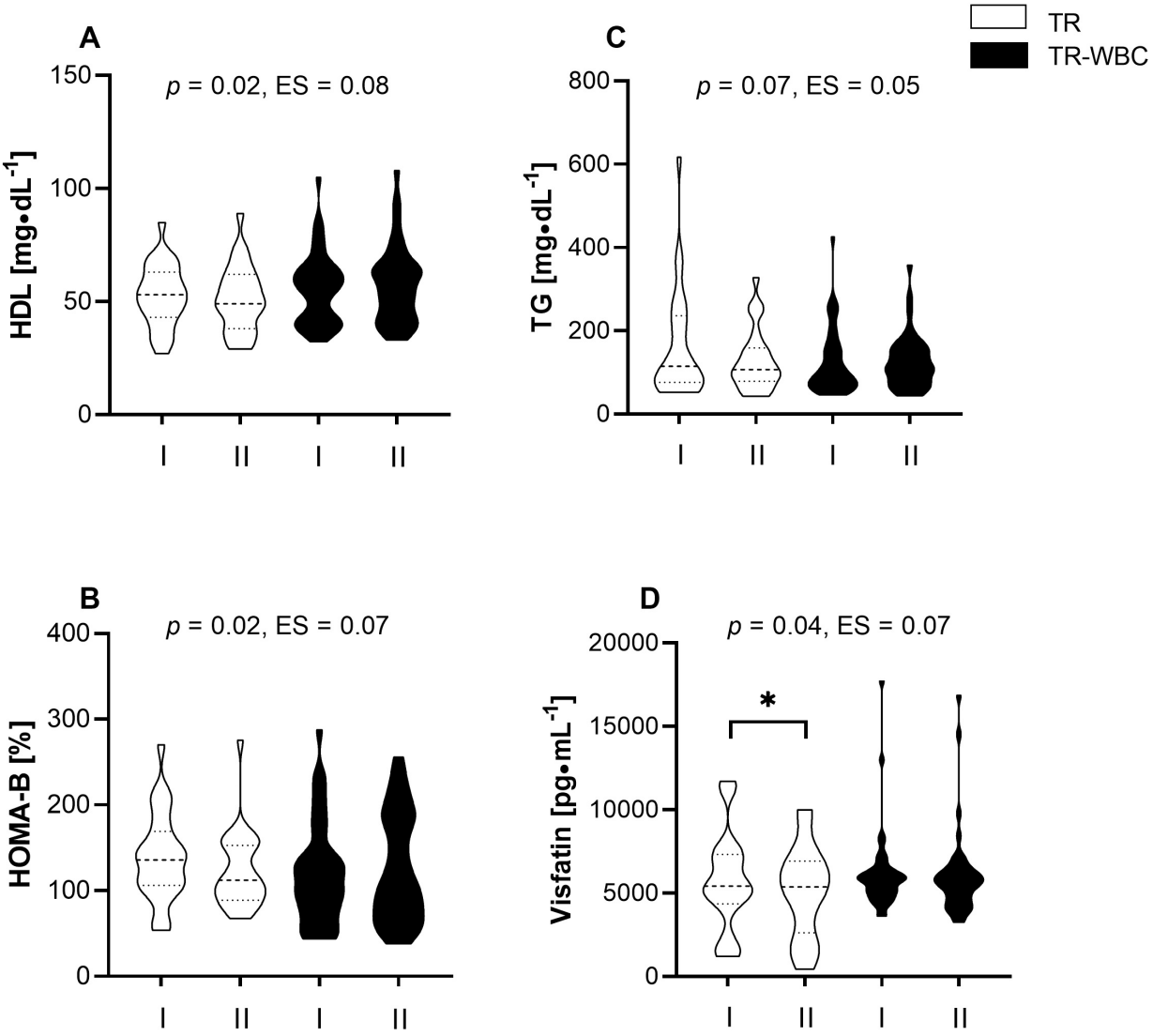

Fig. 3. Changes in the concentration of selected metabolic indicators. (A) High density lipoprotein cholesterol (HDL). (B) The level of Homeostasis Model Assessment estimates β-cell function as percentages of a normal reference population (HOMA-B). (C) Triglycerides (TG) and (D) Visfatin recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38). Data are presented as median and range; *statistical significance in the group, $p < 0.05$; ES, effect size (partial eta squared).

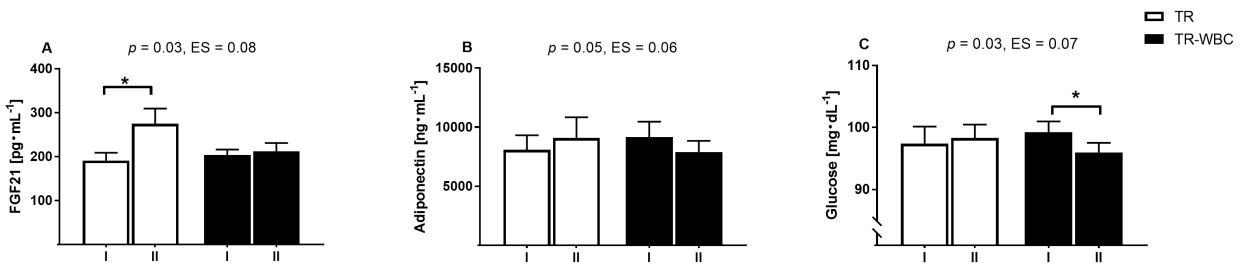

Fig. 4. Changes in adipokines concentration and glucose level before and after intervention. (A) Fibroblast growth factor 21 (FGF21). (B) Adiponectin and (C) glucose recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole- body cryotherapy (TR-WBC; n = 38). Data are presented as mean $\pm$ SD; *statistical significance in the group, $p < 0.05$; ES, effect size (partial eta squared).

Table 1. The effect of interventions on lipid profile and glucose homeostasis indicators among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	TR				TR-WBC				ANOVA	
	Before	After	Δ	95% CI	Before	After	Δ	95% CI	Group × time	ES
TC [mg·dL^{-1}]	196.2 ± 44.6	192.7 ± 39.0	–3.56	–11.96; 4.85	194.5 ± 42.6	197.9 ± 38.3	3.39	–4.12; 10.91	0.22	0.02
LDL [mg·dL^{-1}]	112.6 ± 43.1	112.5 ± 34.7	1.23	–8.38; 10.84	113.8 ± 35.4	114.7 ± 31.7	0.93	–5.39; 7.24	0.95	0.00
Insulin [µIU·mL^{-1}]	17.5 ± 11.0	16.1 ± 9.8	–2.2	–5.67; 1.27	18.3 ± 12.2	17.6 ± 10.0	–0.7	–3.63; 2.24	0.50	0.01
HOMA-S [%]	106.5 ± 56.4	114.2 ± 58.0	7.66	–11.56; 26.89	93.1 ± 48.6	91.2 ± 36.9	–1.87	–13.1; 9.4	0.36	0.01
HOMA-IR	1.3 ± 0.9	1.1 ± 0.5	–0.17	–0.46; 0.13	1.3 ± 0.5	1.3 ± 0.5	–0.05	–0.2; 0.08	0.42	0.01

Data are presented as mean ± SD; Δ, difference between after and before measurements; 95% CI, 95% confidence interval; ANOVA, analysis of variance with repeated measure; ES, effect size (partial eta squared); TC, total cholesterol; LDL, low density lipoprotein cholesterol; HOMA, The Homeostasis Model Assessment estimates; HOMA-S, insulin sensitivity as percentages of a normal reference population; HOMA-IR, insulin resistance.

Table 2. The effect of training and whole-body cryotherapy on metabolic indicators among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	TR				TR-WBC				ANOVA	
	Before	After	Δ	95% CI	Before	After	Δ	95% CI	Group × time	ES
C-Peptide [pg·mL^{-1}]	1687.1 ± 1104.2	1457.8 ± 680.3	–229.3	–619.9; 161.4	1716.5 ± 659.2	1678.4 ± 602.4	–38.1	–206.4; 1302	0.31	0.02
Ghrelin [pg·mL^{-1}]	918.4 ± 527.6	923.8 ± 573.0	5.3	–74.8; 85.5	601.9 ± 512.1	657.7 ± 656.5	55.8	–15.3; 126.9	0.34	0.01
GIP [pg·mL^{-1}]	430.1 ± 524.0	392.3 ± 297.9	–50.6	–362.3; 261.1	224.0 ± 185.9	243.1 ± 292.7	19.1	–54.8; 93.0	0.52	0.01
GLP-1 [pg·mL^{-1}]	289.8 ± 143.2	255.1 ± 92.9	0.9	–30.9; 32.7	297.4 ± 110.1	294.9 ± 144.7	–2.5	–22.0; 16.9	0.85	0.00
Glucagon [pg·mL^{-1}]	1087.0 ± 430.9	1047.7 ± 427.2	–39.3	–108.8; 30.1	1413.8 ± 336.2	1428.4 ± 403.1	14.6	–25.1; 54.4	0.15	0.03
Leptin [pg·mL^{-1}]	13637.6 ± 12742.4	11736.5 ± 10775.9	–1901.1	–4278.0; 475.8	11494.9 ± 8614.3	10766.7 ± 8407.2	–728.2	–2051.7; 595.2	0.35	0.01
Resistin [pg·mL^{-1}]	8311.8 ± 2648.4	7769.5 ± 2175.8	–542.3	–1703.0; 618.4	8809.4 ± 4635.1	8777.9 ± 4953.8	–31.5	–939.3; 876.3	0.48	0.01
Irisin [ng·mL^{-1}]	26.1 ± 14.5	24.1 ± 13.7	–2.1	–4.4; 0.2	23.4 ± 13.9	24.6 ± 13.3	1.1	–2.4; 4.6	0.16	0.03

Data are presented as mean ± SD; Δ, difference between after and before measurements; 95% CI, 95% confidence interval; ANOVA, analysis of variance with repeated measure; ES, effect size (partial eta squared); GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide 1.

Table 3. Correlation coefficients of ΔHOMA-B, ΔGlucose, ΔFGF21 and ΔC-peptide, ΔGIP among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	ΔHOMA-B [%]		ΔGlucose [mg·dL^{-1}]		ΔFGF21 [pg·mL^{-1}]	
	TR	TR-WBC	TR	TR-WBC	TR	TR-WBC
ΔC-peptide [pg·mL^{-1}]	0.74*	0.69*	0.10	0.26	0.10	–0.13
ΔGIP [pg·mL^{-1}]	0.46	0.33*	0.14	0.34*	–0.13	–0.33*

Values are Spearman correlation; *statistically significant correlations, $p < 0.05$; GIP, gastric inhibitory peptide; HOMA-B, β-cell function; FGF-21, fibroblast growth factor 21.

4.3 Prolonged effects of the HIIT–WBC combination

Four weeks after the end of the training intervention, elevated skeletal muscle mass (SMM) and free fat mass (FFM) were registered in the TR-WBC group (p = 0.01 for both components, accordingly; Fig. 5A,B). At that time point, the TC and HDL levels were significantly higher than the baseline levels (194.5 $\pm$ 42.6 vs. 197.9 $\pm$ 38.0 mg·dL^{-1} at baseline, p = 0.03, and 55.6 $\pm$ 15.9 vs. 58.2 $\pm$ 17.4 mg·dL^{-1} at baseline, p = 0.02, accordingly), but were not statistically different from the values immediately after the end of the training intervention (Fig. 5C,D). The decrease in glucose levels induced by the HIIT-WBC combination was not maintained. In fact, glucose levels 1 month after the intervention increased from 95.9 $\pm$ 9.9 to 98.7 $\pm$ 11.0 mg·dL^{-1} (p = 0.01), i.e., returned to baseline values (Fig. 5E). Four weeks after the training intervention, irisin levels tended to decrease from the level recorded 24 h after last HIIT-WBC procedure (from 24.6 $\pm$ 13.3 to 21.7 $\pm$ 10.4 ng·mL^{-1}, p = 0.08; Fig. 5F).

5. Discussion

Physical exercise is an important and effective strategy for counteracting metabolic imbalance in overweight and obese individuals; this is of particular relevance in periods, such as the ongoing COVID-19 pandemic that sees overweight, obese and metabolically dysfunctional patients as one of the more vulnerable groups. In recent years, WBC has been described as a valuable form of physiotherapy because of its exercise-mimicking effects. However, as reported by different studies, WBC manifests its real potential only when combined with a physical exercise program [28, 29]. Accordingly, in the current study, we set out to determine the effect of HIIT in combination with WBC vs. HIIT alone, on FGF21 serum levels, adipo-myokine profile, and metabolic status of overweight to obese individuals as a preventative strategy against the most severe outcomes of SARS-CoV-2 infection.

Data presented in the current study only partially support the claim that the beneficial effects of WBC are fully realized only in combination with physical exercise. The main finding of the study is that 6 units of HIIT training (the TR intervention) suffice to cause a significant increase in FGF21 levels in obese inactive individuals. These changes were associated with an increase in the circulating levels of the anti-inflammatory adipokine adiponectin. At the same time, the metabolic profile improved, i.e., TG levels dropped and HOMA-B values improved. Of note, HIIT in conjunction with WBC did not induce such changes. As recently reported by Sun *et al.* [30], FGF21 acts as a hepatokine, adipokine, and myokine; however, the main tissue source of circulating FGF21 that mediates the effect of exercise is not known. Further, FGF21 responses to exercise are inconsistent, and different studies have reported a decrease [31], no change [32], and increase [33] in its levels upon exercise. Micielska *et al.* [34] demonstrated that 15 units of high-intensity circuit resistance training induce a drop and an increase in FGF21 levels. Only the drop was associated with an amelioration and impairment of cognitive function. The mechanisms underlying the diverse effects of exercise remain unclear and warrant further research.

Physical inactivity exerts a catabolic effect on muscle tissue [35]. Data on the effect of physical inactivity alone on the blood levels of FGF21 are limited. However, Asle *et al.* [36] reported that FGF21 levels increase after 12 weeks of HIIT (3 sets of 10 $\times$ 60 s, 3 times/week) in obese non-active participants particularly in conjunction with low carbohydrate diet but also in normal diet and low fat diet groups except for the high fat diet group where decrease of FGF21 level was noted. This partially agrees with the changes in FGF21 levels observed herein. According to a recent study in a mouse model, the expression and systemic release of muscle-derived FGF21 are very low in normal healthy muscle, and mainly increase under stress conditions (e.g., starvation, aging, and obesity) [37]. Exercise sensitizes adipose tissue to FGF21, which is the basis for a multi-organ crosstalk coordination responsible for maintaining metabolic homeostasis [38]. Therefore, the source of FGF21 release into the bloodstream depends on both, internal and external stimuli [39].

The high-intensity workload featured in the HIIT protocol applied in the current study to overweight to obese inactive individuals, could be a stress-generating factor contributing to the increase in blood FGF21 levels, with the growth factor most likely released from the muscle. On the other hand, FGF21 expression in the muscle is reportedly elevated during mitochondrial dysfunction, and protects against obesity and insulin resistance [40, 41]. We here observed a strong, positive correlation between the reduction of HOMA-B values and the downward trend of C-peptide levels in the TR group participants. The changes in HOMA-B values were significantly different from those in the TR-WBC group, where the FGF21 levels remained unchanged. C-peptide is commonly used as a marker of insulin resistance and metabolic syndrome [42]. The rate of C-peptide secretion is more constant than that of insulin and, hence, it is a reliable marker of pancreatic β-cell function [43]. The observed drop in HOMA-B values to those close to the reference (100%) [25] in the TR group may indicate a reduced metabolic load on β-cells, to maintain normoglycemia.

The increase in FGF21 levels may be also caused by its enhanced expression in the liver [44] and white adipose tissue [45]. Indeed, liver-derived FGF21 improves glucose tolerance [46] and enhances oxidation of free fatty acids [47]. In the TR group, we observed a downward trend in the TG levels, while glucose levels remained unchanged. Therefore, we cannot rule out the possibility that the liver was the main source of FGF21 under these conditions. This

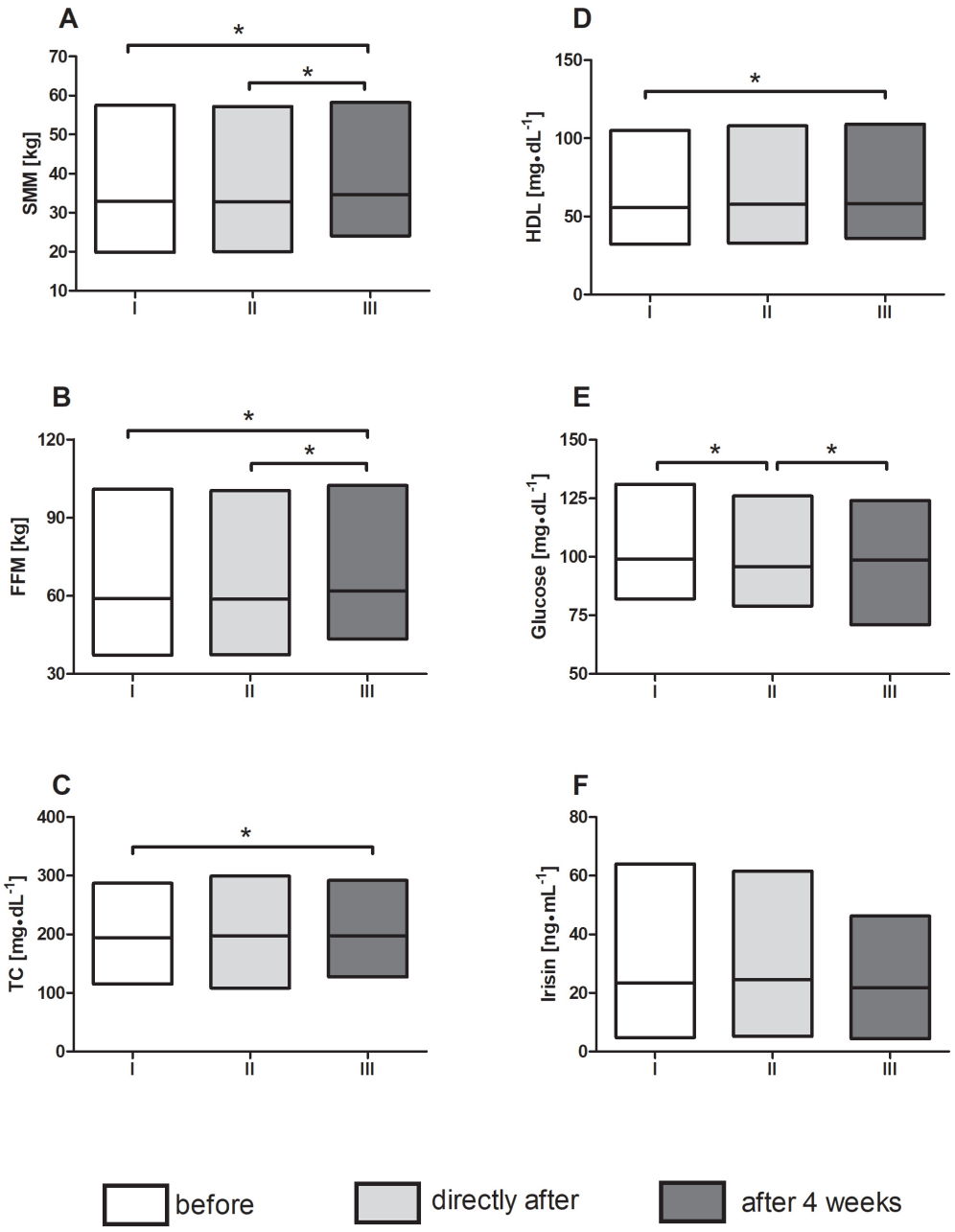

Fig. 5. Changes of body composition and selected metabolic indicators. Changes in the (A) skeletal muscle mass (SMM). (B) Free fat mass (FFM). Concentration of (C) total cholesterol (TC). (D) High- density lipoprotein cholesterol (HDL). (E) Glucose and (F) irisin at each point of blood collection: (I) before protocol, (II) directly after protocol and (III) 4 weeks post finishing the whole experimental protocol in training with whole-body cryotherapy group (TR-WBC). Data are presented as mean, min and max value; *significant differences between time point measurements, $p < 0.05$.

response differed from the effect in the TR-WBC group. Savikj *et al.* [48] reported that the effect of HIIT training on blood glucose levels depends on the time of day the training is performed. Specifically, in their study, afternoon HIIT was more effective in improving blood glucose levels in men with T2DM than morning HIIT; by contrast, morning HIIT had an opposite effect, increasing blood glucose levels [48]. In the current study, the training sessions took place in the morning but we did not observe any changes in glucose levels. However, it is worth noting that blood glucose levels significantly decreased in the TR-WBC group. Hence, the beneficial changes in glucose homeostasis noted in this group may be associated with the activation of the sympathetic nervous system either by cold exposure [49, 50] or by physical activity. We have previously reported that changes in glucose levels may be related to fluctuating FGF21 levels [16]. Fisher *et al.* [51] reported that cold exposure induces expression of endogenous FGF21 in different adipose de-

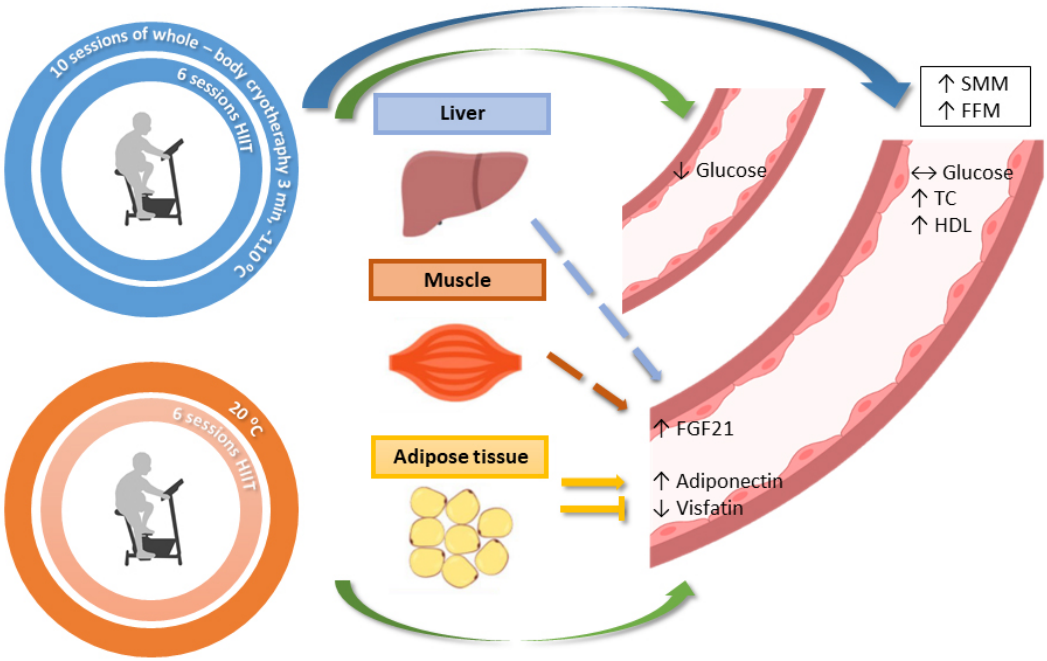

Fig. 6. Graphical conclusion of induced changes recorded in blood and body mass composition. Green arrows indicate short-term effects, purple long-term effects observed 4 weeks after the end of the intervention. The dashed arrows indicate the likely source of FGF21 (liver or muscle as a possible) released to bloodstream. The drop-in glucose level in the training with whole-body cryotherapy group turned out to be short-term effect and did not last until 4 weeks after the end of the protocol. HIIT, high intensity interval training; FGF21, fibroblast growth factor 21; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; SMM, skeletal muscle mass; FFM, free fat mass.

pots or browning of white adipose tissue (WAT). FGF21 was originally described as a factor that enhances insulin-independent glucose uptake in cultured adipocytes, acting via glucose transporter 1 (Glut1) [46]. Therefore, FGF21 can increase the uptake of glucose by adipose tissue in an autocrine manner, independently of insulin, causing its concentration to drop.

In the current study, cold exposure in conjunction with HIIT led to an increase in HDL cholesterol levels but did not affect TG levels. This partially agrees with previous observations of Lubkowska *et al.* [52], who noted a significant decrease of the LDL/HDL ratio after at least 10 WBC sessions. In the current study, changes in HDL levels were significantly different in the two experimental groups. Since HDL cholesterol levels did not change in the TR group, it is likely that WBC contributed to the changes in the TR-WBC group lipid profile. Similar, Rymaszewska *et al.* [53] showed that WBC positively affects the lipid profile, especially in individuals with high BMI. TG, TC, and LDL levels were reduced after WBC [53]. Consequently, it has been suggested that cryotherapy could be an effective treatment for lipid disorders.

Together with increased FGF21 levels, we here observed an increase in adiponectin levels in the TR group. While adiponectin is an adipokine [54], it also acts as a myokine as it is expressed by skeletal muscle during contraction [55], similarly to the previously described mediator, FGF21. Based on the data from a mouse model, FGF21 regulates adiponectin expression in adipocytes in endocrinal manner, de facto coupling FGF21 activity in WAT with metabolic effects in the liver and muscle [56]. Further, circulating FGF21 upregulates adiponectin expression in different fat depots (subcutaneous and visceral adipose tissue) and serum level in obese mouse, as a protective mechanism against systemic insulin resistance [57]. In the TR group in the present study, the increase in adiponectin and FGF21 levels was accompanied by a decrease in HOMA-B values, and a downward trend of C-peptide levels. Furthermore, we noted a decrease in proinflammatory adipokine levels (visfatin, leptin, and resistin) in the TR group. However, only changes in visfatin levels were statistically significant in comparison with those in the TR-WBC group.

Visfatin levels increase with obesity and elevated BMI [58]. In a recently published review, Kumari and Yadav [58] concluded that visfatin modulates obesity-related pathophysiological activities, contributing to the development of disease, such as diabetes (by regulating pancreatic β-cell function), cardiovascular disorders, or even some forms of cancer. Studies involving obese subjects confirmed that while PA reduces blood visfatin levels, this effect is mainly induced by aerobic [59] and resistance training [60]. No data regarding the effect of HIIT training on visfatin levels in obese adults are available. Studies with

young participants revealed some changes in visfatin levels in response to interval training [61, 62]. In the current study, a decrease in visfatin levels was recorded only in the TR group. Previously, Ziemann *et al.* [9] reported no changes in adiponectin levels and a reduction of visfatin levels (7.4%) in individuals with low cardiorespiratory fitness in response to 10 sessions of WBC. Data presented herein suggest that HIIT alone is more effective in reducing visfatin levels than HIIT applied together with WBC.

Irisin is another factor that regulates glucose homeostasis [63]. It inhibits the development of obesity-related inflammatory phenotype in adipocytes and macrophages *in vitro* [64]. Accordingly, we evaluated irisin and inflammatory marker levels in the current study. Levels of circulating irisin are modulated by diet, obesity, exercise, and pharmacological agents [65]. Of note, Dulian *et al.* [66] showed that resting irisin levels increase in response to WBC in obese subjects. Nevertheless, in the current study, 10 sessions of WBC combined with HIIT, and HIIT alone, did not impact circulating levels of this myokine.

Incretins, including GIP and GLP-1, were also assayed herein, since their secretion and activity are dysregulated in obesity and diabetes [67]. Incretins are hormones that regulate insulin and glucagon secretion by pancreatic cells in a glucose-dependent manner. Despite the significant decrease in blood glucose levels in the TR-WBC group, no significant changes in GIP, GLP-1, or glucagon levels were detected. These observations are in line with a report of Hindsø *et al.* [68], who showed that fasting and oral glucose-stimulated incretins levels are not affected in inactive and overweight to obese individuals after 6 weeks of low-volume 3-time per week HIIT. However, in the current study, an upward tendency in GIP levels was apparent in the TR-WBC group. This was opposite to the changes observed in the TR group. A similar tendency was observed for glucagon levels in the TR (a decrease) and TR-WBC (an increase) groups. Although the differences between the groups were not statistically significant, it is important to note that changes in GIP levels were positively correlated with changes in glucose levels in the TR-WBC group. Hence, an increase in GIP levels either causes a significant reduction in blood glucose levels or the relationship is opposite. In rat models, hyperglycemia reduces GIP receptor expression in β-cells [69]. Accordingly, in the current study, the increase in circulating GIP levels could have been stimulated by the decrease in blood glucose levels. On the other hand, in animal models, cold acclimation (4 ± 1 °C for 42 days) increases brown adipose tissue mass, improves glycemic response to oral glucose, and significantly reduces insulin responses [70]. These changes are associated with increased intestinal levels of GIP and GLP-1. These observations indicate that in rat, changes in GIP secretion and activity may be involved in the metabolic adaptation to cold acclimation [71]. Accordingly, we conclude that cold exposure may contribute to the upward trend of GIP levels in the TR-WBC group, consequently leading to a reduction in glucose levels.

Ghrelin is a peptide-hormone that, similarly to GIP and GLP-1, is mainly secreted by enteroendocrine cells [72], and plays an important role in the development of obesity and metabolic-related disorders. It also promotes feeding in cold environments [73, 74], a response associated with an increase in ghrelin levels and a reduction in leptin levels [74, 75]. In the current study, ghrelin levels were not affected in a statistically significant manner by either intervention; however, we observed a tendency of ghrelin blood levels to increase following WBC. This observation was partially in agreement with that of Kojima and co-workers [76], who demonstrated that post-exercise WBC (–140 °C for 3 min) does not affect plasma ghrelin and serum leptin levels, but significantly increases energy intake in human. This might suggest that the tendency of ghrelin levels to increase after WBC leads to an increased energy uptake [77]. Nevertheless, we were unable to evaluate the effect on the study participants' appetite because this aspect was not tested in the current study.

The current study has some limitations that should be addressed in the future. First, we did not assess the effects of WBC alone in the current study. This can be addressed by including a third, WBC-only, group in the study design, and comparing the effects of WBC on the various metabolic parameters with those of other interventions. Another potential limitation is the choice of the evaluated analytes. It is possible that the investigated panel of mediators, although broad, did not provide a complete overview of the body's response to the tested interventions. Although we detected some metabolic and endocrine changes, it is possible that the effect would have been more pronounced upon a longer training program and, above all, additional WBC sessions. Hence, we are unable to recommend the minimum therapeutic number of cold exposures at this time. Secondly, we did not conduct any monitoring of changes in participants' fitness level during and at the end of HIIT protocol which could have revealed more granular variation in the individual cardiorespiratory response to exercise protocol. On the other hand, in preliminary phase of our experiment, based on previously published studies [9, 78], we tried to select a group uniform in terms of body composition and the level of physical activity. Finally, we did not explore the mechanisms underlying the observed effects of cryostimulation and HIIT on the metabolic homeostasis.

Still, to the best of our knowledge, this study is the first to assess long-term effects of cold exposure applied in conjunction with physical training on adipo-myokine profile, and metabolic status of overweight to obese individuals. The findings presented herein indicate that the observed decrease in glucose levels induced by HIIT–WBC was reversed 1 month after the treatment, as a return to daily habits eroded the beneficial effects of the intervention.

6. Conclusions

To summarize, the HIIT protocol, both alone and in combination with WBC, affected the metabolic indicators and myokines' concentrations. These impacts manifested differently, likely due to the different underlying mechanisms. Training alone caused significant changes in FGF21 concentration, whereas in combination with WBC, it abolished this effect. Similarly, different responses in adiponectin were observed; it increased in response to the HIIT alone but it decreased in response to the combination of HIIT and WBC. Moreover, the combined approach of training and WBC induced beneficial, yet temporary, effects on glucose concentration and glucose homeostasis among obese participants (Fig. 6). In practice, although short-term, the presented effects support the use of pro-health procedures, such as physical activity and cold exposure, as preventative strategies to limit the severe effects of other incident diseases.

7. Author contributions

Conceptualization—MKF and EZ; methodology—MKF, ERF and VS; validation—MKF, KM, SP and GL; formal analysis—JK; investigation—MKF, ERF, JJ and KM; resources—JJ and AB; data curation—MKF; writing-original draft preparation—MKF, EZ; writing-review and editing—MKF, AB, SP, GL and EZ; visualization—MKF and JK; supervision—JK and VS; project administration—EZ; funding acquisition—EZ All authors have read and agreed to the published version of the manuscript and agree with the order of presentation of the authors.

8. Ethics approval and consent to participate

The study protocol was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (approval number KB-28/17) and was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent for the publication of any associated data after being informed about the procedures.

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11. Conflict of interest

The authors declare no conflict of interest.

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Abbreviations: HIIT, high intensive interval training; WBC, whole-body cryotherapy; TR, training group; TR-WBC, training supported by WBC group; FGF21, fibroblast growth factor 21; HOMA-IR, The Homeostasis Model Assessment estimates insulin resistance; HOMA-β, The Homeostasis Model Assessment estimates β-cell function; HOMA-S, The Homeostasis Model Assessment estimates insulin sensitivity as percentages of a normal reference population; T2DM, type 2 diabetes; PA, physical activity; HR, heart rate; BMI, body mass index; CV, coefficient of variation; DXA, dual energy X-ray absorptiometry; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GIP, gastric inhibitory peptide; GLP-1, glucagon- like peptide 1; rANOVA, analysis of variance; ES, effect size (partial eta squared); SMM, skeletal muscle mass; FFM, free fat mass; WAT, white adipose tissue; Glut1, glucose transporter 1.

Keywords: Fibroblast growth factor 21; Adiponectin; Cold exposure; Physical activity; COVID-19

Send correspondence to: Ewa Ziemann, Department of Athletics, Strength and Conditioning, Poznan University of Physical Education, 61-871 Poznan, Poland, E-mail: ziemann@awf.poznan.pl

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Nordic Walking Rather Than High Intensity Interval Training Reduced Myostatin Concentration More Effectively in Elderly Subjects and the Range of This Drop Was Modified by Metabolites of Vitamin D

Katarzyna Micielska [1,2,†], Marta Flis [3,†], Jakub Antoni Kortas [4], Ewa Rodziewicz-Flis [5], Jędrzej Antosiewicz [6], Krystian Wochna [7], Giovanni Lombardi [8,9] and Ewa Ziemann [9,*]

1 Department of Physical Education and Lifelong Sports, Poznan University of Physical Education, 61-871 Poznan, Poland; micielska.katarzyna@gmail.com
2 Doctoral School, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland
3 Department of Physiology, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland; kozl.marta@gmail.com
4 Department of Health and Life Sciences, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland; jakubantonikortas@gmail.com
5 Department of Physiotherapy, Gdansk University of Physical Education and Sport, 80-336 Gdansk, Poland; ewu.rodziewicz@wp.pl
6 Department of Bioenergetics and Physiology of Exercise, Medical University of Gdansk, 80-210 Gdansk, Poland; jant@gumed.edu.pl
7 Laboratory of Swimming and Water Lifesaving, Poznan University of Physical Education, 61-871 Poznan, Poland; kwochna@awf.poznan.pl
8 Laboratory of Experimental Biochemistry and Molecular Biology, IRCCS Istituto Ortopedico Galeazzi, 20161 Milan, Italy; giovanni.lombardi@grupposandonato.it
9 Department of Athletics, Strength and Conditioning, Poznan University of Physical Education, 61-871 Poznan, Poland
* Correspondence: ziemann@awf.poznan.pl
† These authors contributed equally to this work.

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Abstract: The COVID-19 pandemic and subsequent self-isolation exacerbated the problem of insufficient amounts of physical activity and its consequences. At the same time, this revealed the advantage of vitamin D. Thus, there was a need to verify the effects of those forms of training that can be performed independently. In this study, we examined the effects of Nordic walking (NW) and high intensity interval training (HIIT) with regard to the impact of the metabolite vitamin D. We assigned 32 overweight adults (age = 61 ± 12 years) to one of two training groups: NW = 18 and HIIT = 14. Body composition assessment and blood sample collection were conducted before starting the training programs and a day after their completion. NW training induced a significant decrease in myostatin ($p = 0.05$) concentration; however, the range was dependent on the baseline concentrations of vitamin D metabolites. This drop was accompanied by a significant negative correlation with the decorin concentration. Unexpectedly, NW caused a decrement in both forms of osteocalcin: undercarboxylated (Glu-OC) and carboxylated-type (Gla-OC). The scope of Glu-OC changes was dependent on a baseline concentration of $25(OH)D_2$ ($r = -0.60$, $p = 0.01$). In contrast, the HIIT protocol did not induce any changes. Overall results revealed that NW diminished the myostatin concentration and that this effect is more pronounced among adults with a sufficient concentration of vitamin D metabolites.

Keywords: decorin; aging; myokines; osteokines; $25(OH)D_3$; $24,25(OH)_2D_3$; 3-epi-$25(OH)D_3$

1. Introduction

An insufficient level of physical activity, along with a sedentary lifestyle, is particularly common among middle-aged and elderly persons, which in consequence contributes to chronic

diseases' development and to premature mortality [1,2]. With the onset of the COVID-19 pandemic and the related extended periods of self-isolation, this problem was intensified, meaning that two global pandemics coexisted at the same time [3,4]. Paradoxically, Sallis et al. reported that inactivity is a modifiable variable for severe post-COVID-19 outcomes. Among adult patients who had suffered from COVID-19, consistent inactivity (documented in their electronic health record, before the pandemic) represented a significant risk factor for hospitalisation and mortality [5]. Additionally, it has been suggested that a low concentration of 25-hydroxyvitamin D (25(OH)D) contributes to low physical performance, muscle weakness [6] and higher rates of COVID-19 infection and mortality [7,8]. Consequently, the role of these two factors, exercise (especially forms that accessible in periods of lock-down/isolation) and level of vitamin D, is particularly significant.

Nordic walking (NW) is one of the most popular forms of physical activity that can be performed individually. Its beneficial impact is well documented in terms of an improved quality of life and motor skills development [9,10]. One report linked beneficial effects of NW training with the baseline vitamin D concentration of > 20 $ng \cdot mL^{-1}$. A reduction of pro-inflammatory markers was more pronounced than among subjects characterized by a lower vitamin D concentration [11]. Alternatively, high intensity interval training (HIIT) was also proposed as a time-saving and advantageous practice to induce adaptive changes [12,13], reduce fall and fracture risk [14] and improve glucose homeostasis and insulin sensitivity among elderly diabetic patients [15]. What is more, HIIT was shown to be an effective way to prevent sarcopenia onset and progression [16].

Regulation of metabolism is associated with proteins released into the bloodstream from different tissues e.g., muscles (myokines), bone cells (osteokines) or adipose tissue (adipokines), which are collectively referred to as exerkines when released in response to physical activity. Exerkines create endocrine-like signalling pathways between distance tissues, by means of which they are considered to mediate adaptation changes to exercise [17]. However, a detailed mechanism of their secretion and action following training is not fully understood. Decorin is one of the myokines that might be released in response to exercise. Although Kanzleiter et al. described a significantly higher decorin expression in response to regular resistance and endurance training among men, as well on an animal model [18], a direct impact of exercise on decorin concentration in women is debated. It was reported that 5 weeks of high intensity circuit training did not alter decorin concentration in middle-aged women [19]. However, decorin was demonstrated to bind with and inhibit myostatin activity, a strong negative regulator of muscle growth [20] and one of the potential serum biomarkers of sarcopenia [21]. Another exerkine associated with muscle function is osteocalcin. The signalling of osteokine in myofibers prevents muscle function deterioration during aging, as well determines muscle adaptation to exercise [22]. Still, the effect of exercise on myostatin and osteocalcin is indistinct. It is worth noting that osteocalcin is under investigation for its potential insulin sensitivity-modulating properties [23,24], especially by its active form, the undercarboxylated osteocalcin (Glu-OC), as it might regulate the insulin sensitivity of adipocytes, just through the secretion of adiponectin [25]. Nonetheless, it was revealed that the beneficial effects of exercise on exerkines can be modulated by vitamin D and possibly by other factors [11].

Since both HIIT and NW can be used interchangeably and can be performed in home/safe-distance conditions, they are considered particularly convenient exercise options in the COVID-19 pandemic. Thus, the aim of this study was to compare the effects of these two training protocols (HIIT vs NW) on myokines' and osteokines' secretion, including the impact of some metabolites of vitamin D: 25(OH)D_3, 25(OH)D_2, 24,25$(OH)_2D_3$ and 3-epi-25(OH)D_3. The study also aimed to assess whether short-term HIIT can be an effective alternative to long-term NW with respect to pro-health changes.

2. Materials and Methods

2.1. Design of the Study

The study was performed just after the summer holiday (at the beginning of September). One week prior to the start of the experiment, as well as 24 h after the completion of each training protocol, the subjects were tested for body composition analysis and underwent blood collection. The study was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (KB-34/18) in accordance with the Declaration of Helsinki.

2.2. Subjects

Thirty-two healthy middle-aged-to-elderly adults (nine men and twenty-three women; age = 61 ± 12 years; body mass = 78 ± 17.5 kg; body mass index, BMI = 27 ± 4.2 kg·m^{-2}; percent body fat, PBF = 33 ± 7.7%) took part in the study. The participants were characterized by two adiposities to muscle ratios (Table 1) that are good indicators of sarcopenic obesity and physical disability [26]. Moreover, the subjects were characterized by hepatic insulin resistance expressed by a quantitative insulin sensitivity check index, QUICKI = 0.358 ± 0.04, homoeostasis model assessment of insulin resistance, HOMA-IR = 2.02 ± 1.42 and bone mineral content, BMC = 3.0 ± 0.7 kg. The recruitment was encouraged through advertisements in the local and social media. Before the start of the intervention, all participants underwent a medical examination in order to exclude any contraindications to physical activity, such as bone disease, diabetes, uncontrolled hypertension, cardio-respiratory disorders or any orthopaedic issues. Participants included in the study reported that they did not use vitamin D or other supplements; however, some reported using prophylactic medications. Moreover, participants were asked not to change this or other daily habits during the intervention. Subjects were familiarized with the experimental procedures and a written, informed consent was obtained from all of them. Enrolled individuals were assigned to one of two training groups: NW group (n = 18, BMI = 26 ± 3.5 kg·m^{-2}, PBF = 32 ± 7.7%) and HIIT group (n = 14, BMI = 29 ± 4.0 kg·m^{-2}, PBF = 34 ± 8.0%). The NW group completed 12-week training programs, whereas the program for the HIIT group lasted 2 weeks. One week before the start of the experiment, the individual workload for the participants from the HIIT group was determined. Each participant had to perform a preliminary test on the cycle-ergometer by pedalling for 1 min with a cadence of 80–100 rpm, at 90% of the maximum heart rate (HR_{max}), with an individually established load (1.5–2.5 W·kg^{-1}) to determine the proper workload. Between each bout, a 1 min rest was applied. Participants from the NW group received and were instructed on how to use a sport-tester device (Polar Electro Oy, Professorintie 5, Kempele, Finland M200) for their own cardiovascular and training unit intensity control. Only subjects whose training attendance was at 100% in the HIIT group and at 90% in the NW group were included in the statistical analysis.

Table 1. Characteristic of participants.

	NW (n = 18)		**HIIT (n =14)**		**rANOVA Group × Time Interaction**
	I	**II**	**I**	**II**	
Sarcopenic Obesity Indicators					
BF/FFM [kg·kg^{-1}]	0.47 + 0.17	0.47 + 0.16	0.51 ± 0.18	0.50 ± 0.19	0.28
BM/FFM [kg·kg^{-1}]	1.47 + 0.17	1.47 + 0.16	1.51 ± 0.18	1.50 ± 0.19	0.28

Data are presented as mean ± SD; rANOVA- analysis of variance with repeated measure; NW—Nordic walking group, HIIT—high intensity interval training group, I—before the intervention, II—24 h after completing training procedures; BF—body fat, FFM—free fat mass, BM—body mass.

2.3. Body Composition Assessment

Body mass (BM), body mass index (BMI), bone mineral content (BMC), body fat (BF) and free fat mass (FMM) were evaluated in a fasted state in the morning by using a multi-frequency impedance (1, 5, 50, 250, 500, and 1000 kHz) with the analyser InBody 720

(Biospace, Seoul, Korea). Percent of body fat (PBF) mass repeated measurement precision was expressed as the coefficient of variation, on average, 0.6% [27]

2.4. Blood Collection

Blood samples were taken at baseline and 24 h after completing training protocols. Blood collected from the antecubital vein into vacutainer tubes by a professional nurse was centrifuged at 2000× *g* for 10 min at 4 °C and stored at −80 °C until assayed.

The concentrations of myostatin, adiponectin and decorin were determined via ELISA kits, according to the manufacturer's instructions. For serum myostatin and adiponectin (R&D Systems, Minneapolis, USA catalogue no. DGDF80 and DRP300, respectively) maximal intra-assay coefficient of variability (CV), inter-assay CV and detection sensitivity were 5%, 6%, and 5.32 pg·mL^{-1} and 5%, 7%, and 0.891 ng·mL^{-1}, respectively. Decorin was assessed by Human Decorin DuoSet ELISA (R&D Systems, Minneapolis, USA, catalogue no. DY143, and Ancillary Reagent Kit catalogue no. DY008). Serum undercarboxylated osteocalcin (Glu-OC) and carboxylated-type of osteocalcin (Gla-OC) were assessed by enzyme immunoassay commercial kits Takara Bio Inc., Kusatsu, Japan (catalog no.#MK118 and #MK111, respectively). The detection limits and CV for Glu-OC were 0.25 ng·mL^{-1} and 4.6%, and 0.2 ng·mL^{-1} and 3.3% for Gla-OC.

Insulin was assessed using an immunoassay kit from DiaMetra, Perugia, Italy (catalogue no. DKO076) within intra-assay CV ≤5% and the inter-assay CV ≤10%.

Glucose concentration was assayed on Cobas 6000 (Roche Diagnostics, Warsaw, Poland). In order to define each participant's insulin sensitivity and insulin resistance, the two well-described indexes were used as follow: the quantitative insulin sensitivity check index (QUICKI; used formula: QUICKI = 1/(log serum insulin μU·mL^{-1} + log fasting plasma glucose mg·dL^{-1} [28]) and homoeostasis model assessment of insulin resistance (HOMA-IR; used formula: HOMA-IR = fasting serum insulin μU·mL^{-1} × fasting plasma glucose mmol·L^{-1}/22.5 [29]).

Total cholesterol (TC), high-density (HDL), and low-density lipoproteins (LDL) cholesterol, and triglycerides (TG) were assessed with commercially available kits using enzymatic methods (Alpha Diagnostics, Warsaw, Poland).

Vitamin D metabolites: 25(OH)D_3, 25(OH)D_2, 24,25$(OH)_2D_3$ and 3-epi-25(OH)D_3 concentrations were determined and corrected to change in plasma volume, as was previously described by Mieszkowski and co-workers [30]. It was performed by quantitative analysis using liquid chromatography, coupled with tandem mass spectrometry (QTRAP®4500 (Sciex, Framingham, MA, USA) coupled with ExionLC HPLC system). The measurement of serum samples was performed in the positive ion mode, using electrospray ionization. The Analyst® software was used to collect raw data and MultiQuant® (Sciex, Framingham, MA, USA) was used to process and quantify it. The reagents used in the procedure were as follows: derivatization agent: 4-(40 -Dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) synthesized by Masdiag Laboratory (Warsaw, Poland); water; ethyl acetate (POCh S.A., Gliwice, Poland) and methanol (Honeywell, Sigma-Aldrich, Gillingham, Dorset, UK). For the mobile phases acetonitrile (ACN) (Honeywell, Sigma-Aldrich, Gillingham, Dorset, UK), water (POCh S.A., Gliwice, Poland) and formic acid (FA) (Merck KGaA, Darmstadt, Germany) were used. All solvents were of LC-MS grade.

2.5. Applied Training Protocol

2.5.1. NW Training Protocol

The applied NW training program included 36 training sessions, 3 times per week for 12 weeks, and was based on previous published protocol [31,32]. Each NW unit was performed as follows: 10-min warm-up, 45–55-min main NW training and 10-min cool-down. Training sessions were conducted by a qualified NW instructor, who demonstrated and taught proper walking with the pools technique and monitored the intensity of the training workload. Each training unit was performed at 60–70% HR_{max} intensity.

2.5.2. HIIT Protocol

HIIT training procedure was based on Little et al. [15] and consisted of 6 supervised training units performed 3 times per week for 2 weeks. The single HIIT duration time was 25 min, including: 3-min warm-up, 10 × 1-min cycling intervals separated by 10 × 1-min of rest and 2-min cool-down at the end of session. The load of warm-up and cool-down was established at 50 W; however, interval workload was the same as individually determined one week prior to the start of the experiment. Cycling intervals were performed at 80–100 rpm·min^{-1} with an intensity of 90% HR_{max}. While performing HIIT, participants had constant biofeedback from a screen showing their pedalling cadence and HR so that they could keep those indicators maintained. During the 1-min recovery period, adults could rest cycling freely.

2.6. Statistical Calculation

Statistical analyses were performed by using a statistics software package (Statistica 13.1 software, TIBCO Software, Palo Alto, USA). The Shapiro–Wilk tests were used to assess the homogeneity of dispersion from normal distribution. The Brown–Forsythe test was used to evaluate the homogeneity of variance. Repeated measures analyses of variances (rANOVA) were calculated. In case of a significant time × group interaction, post hoc tests for unequal sample sizes were performed to identify significantly different results. The variable's relationships were measured using the Spearman correlation coefficient. The level of significance was set at $p < 0.05$.

3. Results

3.1. NW Trainining Program

The applied NW sessions did not change sarcopenic obesity indicators (Table 1), nor were glucose homeostasis indicators, the lipid profile and vitamin D metabolites altered (Table 2). Significant changes were observed in cytokines' concentrations (Figure 1). NW training caused a significant reduction of the serum myostatin concentration (Figure 1A), whereas values in HIIT participants remained unchanged. The range of changes in the NW group was noted among those subjects with a higher baseline level of all vitamin D metabolites (Figure 2). Subjects with 25(OH)D_3 over 25 ng·mL^{-1} had a slightly higher impact of a drop of myostatin ($p < 0.01$; Figure 2A), than those with 24,25$(OH)_2D_3$ over 2.0 ng·mL^{-1} ($p < 0.01$; Figure 2B) and 3-epi-25(OH)D_3 over 1.3 ng·mL^{-1} ($p < 0.01$; Figure 2C). Together with the myostatin decrement, the increase of decorin concentration was observed (Figure 1B) and this result was accompanied by the negative correlation between delta changes in the concentrations of these myokines (Figure 3). Unexpectedly, NW training induced a significant drop of both forms of osteocalcin: Glu-OC and Gla-OC (Figure 1C,D, respectively). Among all participants, regardless of the group, the shifts of myostatin modified the range of the osteocalcin decrease, especially for Gla-Oc ($p = 0.04$). Still, it is worth noting that the baseline concentration of 25$(OH)D_2$ inversely correlated with the delta osteocalcin change Gla-Oc ($r = -0.59$, $p = 0.01$, Figure 4).

NW training induced a slight elevation of adiponectin concentration (from 11,528.89 ± 9370.57 to 14,173.56 ± 10,504.19 ng·mL^{-1}). None of the measured exerkines correlated with glucose metabolism indicators.

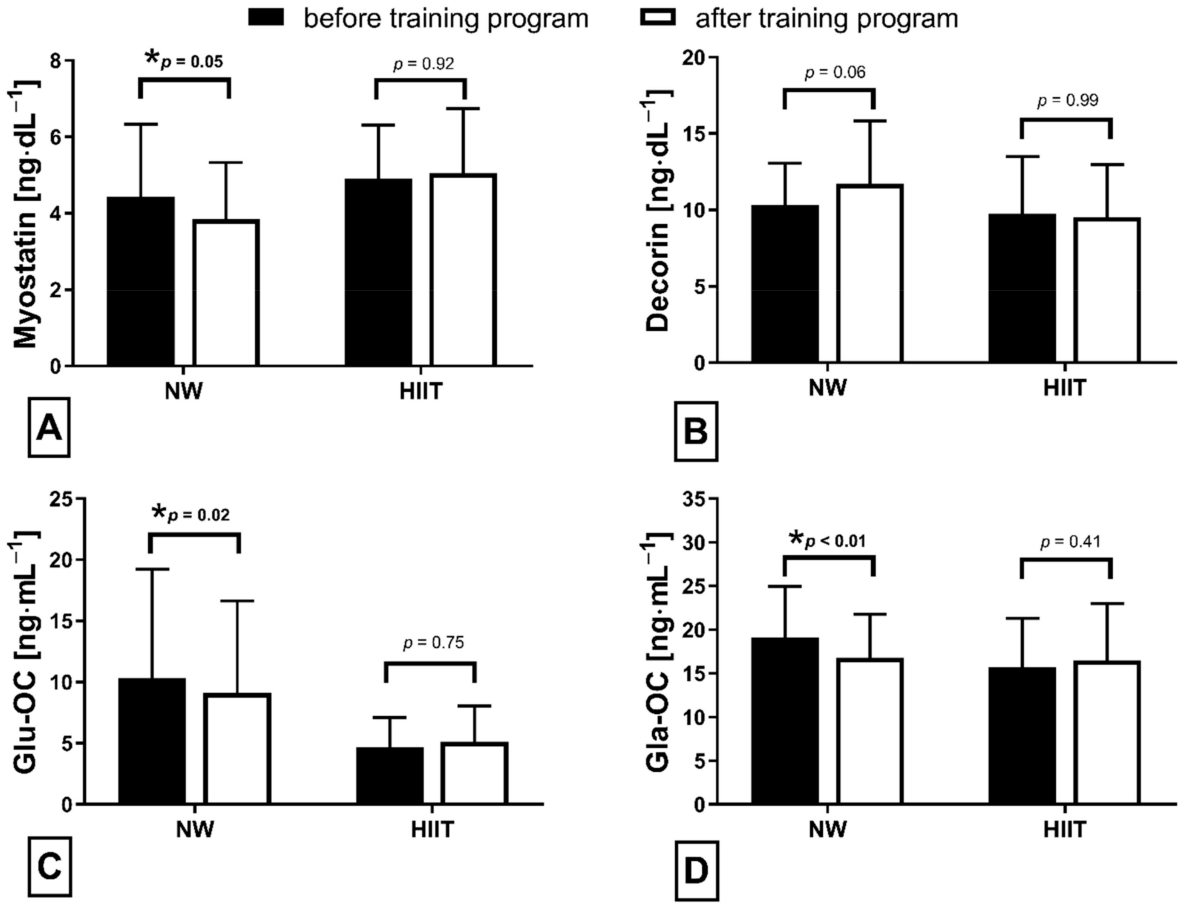

Figure 1. Myokines (**A**,**B**) and osteokines (**C**,**D**) concentration changes in response to applied training protocols: Nordic walking (NW; n = 18) and high intensity interval training (HIIT; n = 14). Data are presented as mean ± SD; * statistically significant result (post hoc tests); Glu-OC—undercarboxylated osteocalcin; Gla-OC—carboxylated-type of osteocalcin.

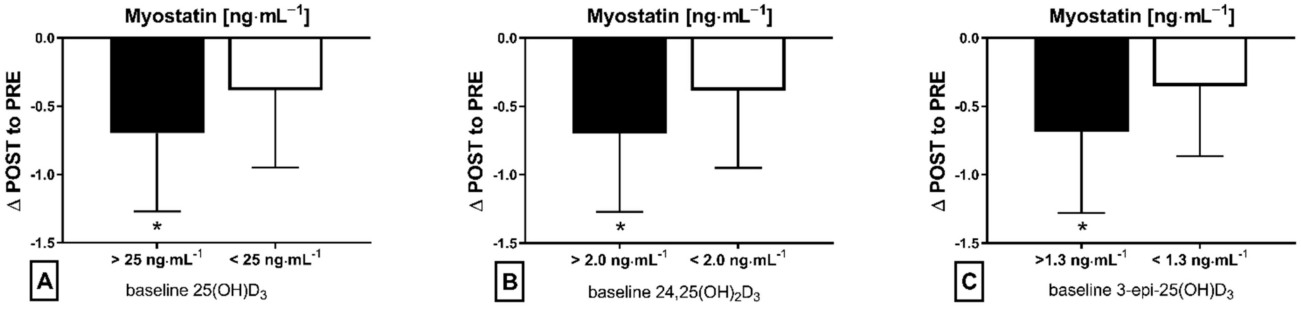

Figure 2. Myostatin concentration delta changes (Δ POST to PRE) dependant on baseline level of metabolite vitamin D in NW training group: (**A**) baseline 25(OH)D_3, (**B**) baseline 24,25$(OH)_2D_3$ and (**C**) baseline 3-epi-25(OH)D_3; * statistically significant result (post hoc tests)—$p < 0.05$.

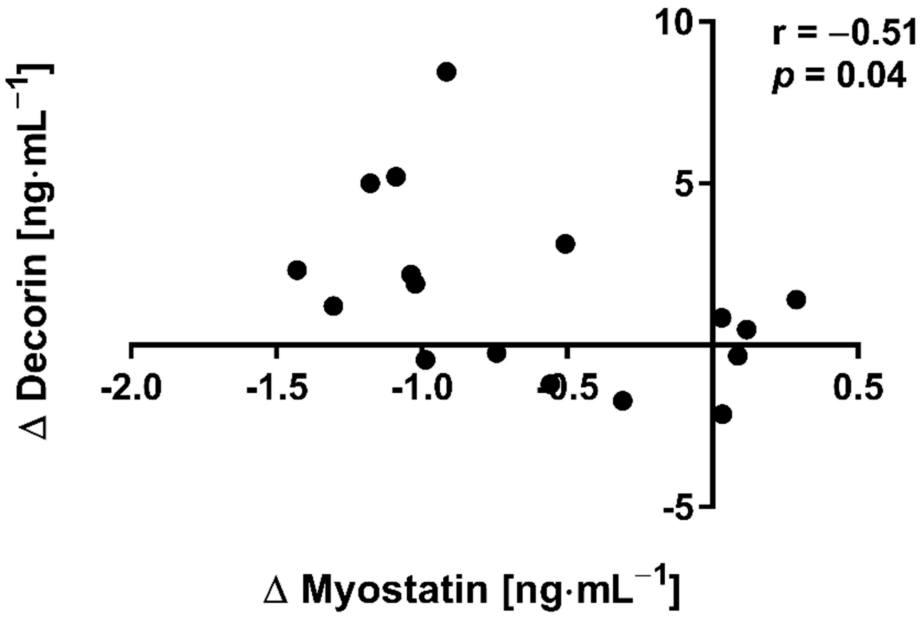

Figure 3. Correlation of myostatin and decorin concentration delta changes (Δ POST to PRE) in response to NW training.

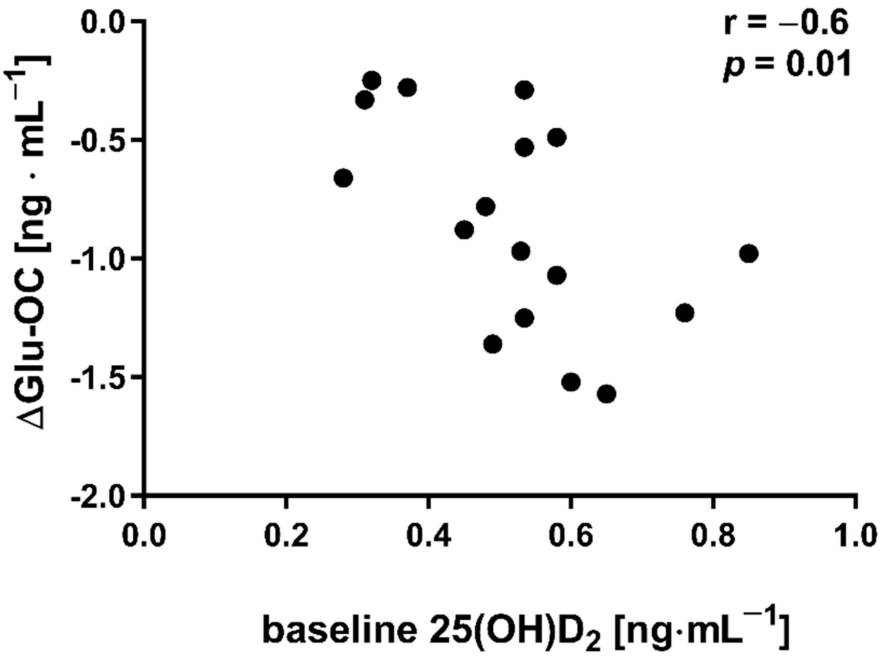

Figure 4. Correlation between baseline concentration of 25(OH)D_2 and delta changes (Δ POST to PRE) in undercarboxylated osteocalcin (Glu-OC) among participants from NW group.

Table 2. Glucose, lipids and metabolite of vitamin D parameters before and after NW and HIIT trainings programs.

	NW (n = 18)		HIIT (n = 14)		rANOVA Group × Time Interaction
	I	II	I	II	
Glucose homeostasis indicators					
Glucose [mg·dL^{-1}]	100.83 ± 21.92	93.69 ± 6.01	100.36 ± 8.70	96.29 ± 8.88	0.73
Insulin [μIU·mL^{-1}]	7.80 ± 4.87	8.19 ± 4.15	7.92 ± 4.90	8.19 ± 6.02	0.62
QUICKI	0.359 ± 0.04	0.357 ± 0.03	0.358 ± 0.04	0.362 ± 0.04	0.42
HOMA-IR	2.02 ± 1.51	1.88 ± 1.10	2.02 ± 1.38	2.01 ± 1.64	0.48
Lipid profile					
Total cholesterol [mg·dL^{-1}]	232.50 ± 35.05	231.06 ± 35.68	178.93 ± 43.71	176.86 ± 31.34	0.94
HDL cholesterol [mg·dL^{-1}]	77.75 ± 29.39	73.75 ± 20.43	54.45 ± 15.52	54.79 ± 15.27	0.27
LDL cholesterol [mg·dL^{-1}]	133.44 ± 37.36	133.5 ± 41.35	104.94 ± 35.48	101.71 ± 28.21	0.63
Triglycerides [mg·dL^{-1}]	105.63 ± 35.34	118.75 ± 39.74	97.07 ± 61.73	101.43 ± 44.04	0.54
Vitamin D metabolites					
25(OH)D_3 [ng·mL^{-1}]	27.61 ± 10.82	27.78 ± 7.86	23.8 ± 5.18	25.54 ± 7.06	0.21
25(OH)D_2 [ng·mL^{-1}]	0.52 ± 0.15	0.47 ± 0.11	0.44 ± 0.16	0.45 ± 0.15	0.08
24,25(OH)$_2D_3$ [ng·mL^{-1}]	2.62 ± 1.59	2.68 ± 1.43	2.12 ± 0.71	2.16 ± 0.83	0.94
3-epi-25(OH)D_3 [ng·mL^{-1}]	1.34 ± 0.59	1.61 ± 0.68	1.23 ± 0.41	1.37 ± 0.63	0.27

Data are presented as mean ± SD; rANOVA—analysis of variance with repeated measure; I—before the intervention, II—24 h after completing training procedures; QUICKI—quantitative insulin sensitivity check index; HOMA-IR—homoeostasis model assessment of insulin resistance; HDL cholesterol—high density lipoprotein cholesterol; LDL cholesterol—low density lipoprotein cholesterol, 25(OH)D_3-25-hydroxyvitamin D_3, 25(OH)D_2—25-hydroxyvitamin D_2, 24,25(OH)$_2D_3$—24,25-dihydroxyvitamin D_3, 3-epi-25(OH)D_3—3 epimer of 25-hydroxyvitamin D_3.

3.2. HIIT Program

The HIIT protocol did not induce significant changes in measured factors. Similarly to the NW group, adiposity to muscle ratios (Table 1), glucose metabolism, lipid profile indicators and vitamin D metabolites (Table 2) remained unchanged. Furthermore, concentrations of resting myostatin and decorin did not shift in response to the HIIT program (Figure 1A,B). Both Glu-OC and Gla-OC forms showed an upward trend (Figure 1C,D, respectively); however, these changes were not statistically significant. Serum adiponectin concentration 24 h after the HIIT program's completion revealed an opposite tendency in comparison to NW training, with the change between groups being statically significant (Figure 5). The concentration of adiponectin declined in response to HIIT (from 8659.79 ± 6600.87 to 7842.79 ± 6069.32 ng·mL^{-1}), this drop being insignificant. Although there was a significant, negative correlation between adiponectin and triglycerides ($r = -0.67$, $p = 0.01$) after the intervention, no significant correlations between glucose homeostasis indicators and exerkines were observed. Comparing the effects of NW and HIIT procedures, significant and opposite changes in all measured exerkines concentrations were recorded (Figure 5).

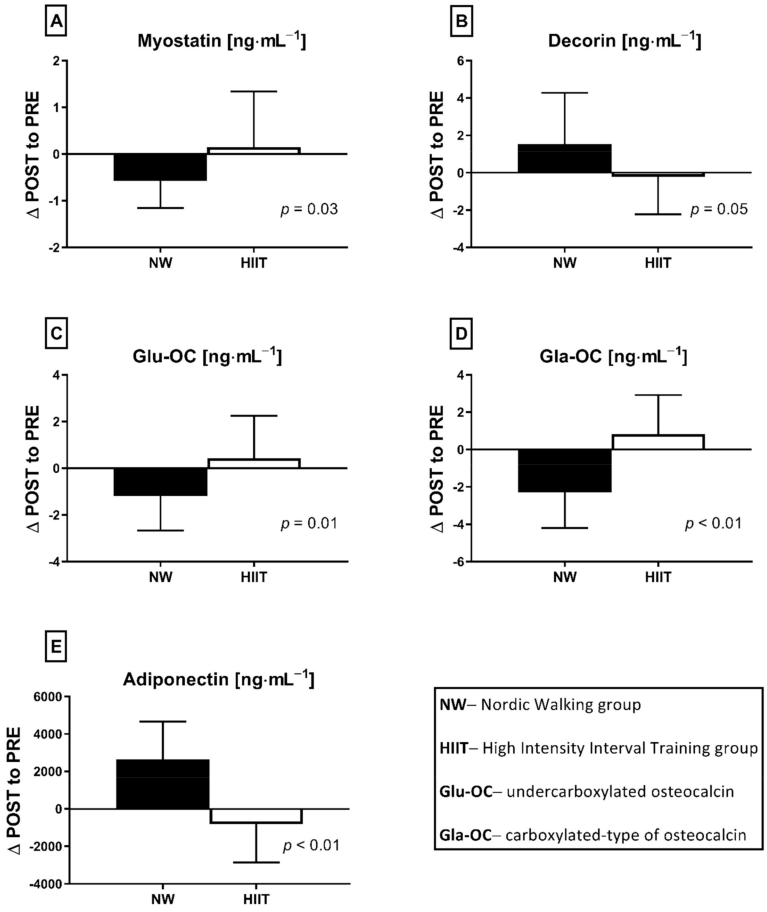

Figure 5. The differences between NW (n = 18) and HIIT (n = 14) training programs expressed as delta changes (Δ POST to PRE) in myokines (**A**,**B**,**E**) and osteokines (**C**,**D**) concentration, before and after applied interventions. Values are statistically significant. Analysis of variance (rANOVA) was used.

4. Discussion

The main finding of the current study is that NW proved to be more effective in inducing changes in blood exerkines' concentrations in elderly people than HIIT. Thus, our initial assumption that a low-volume and high-intensity HIIT training could be an effective alternative to a high-volume and low-intensity NW training in inducing pro-health changes has not been confirmed. NW training significantly reduced the myostatin concentration in comparison to HIIT training, with this drop correlating inversely with an increase in the decorin concentration. The range of changes of myostatin in the NW group was higher among those subjects who were characterised by a higher concentration of vitamin D metabolites at baseline: 25(OH)D_3 (over 25 ng·mL^{-1}), 24,25$(OH)_2D_3$ (over 2.0 ng·mL^{-1}) and 3-epi-2(OH)D_3 (over 1.3 ng·mL^{-1}). By demonstrating how the attenuation of skeletal muscle mass and function can be potentiated in middle-aged and elderly participants, the obtained results are insightful in connection with the COVID-19 pandemic, when access to physical activity may be limited [33]. They also highlight the importance of maintaining the adequate level of vitamin D in every season, where insufficient vitamin D_3 levels were found to correlate with the severity of COVID-19 and increased rates of hospitalization [34]. Vitamin D is known to have a multitude of non-calcemic actions. This is due in part to the presence of the vitamin D receptor (VDR) in most tissues and cells, including the skeletal muscle, adipose tissue, endocrine pancreas, immune cells, blood vessels, brain and others. While vitamin D deficiency impairs muscle function in both young athletes and elderly people, where it is thought to predispose falls [35]. In the current study, a higher concentration of vitamin D metabolites correlated with the drop of myostatin among those subjects who had trained NW. Although there were no associations between serum

markers of vitamin D and decorin, delta changes of myostatin were determined by delta changes of decorin.

Regarding the fact that aging is associated with an elevated level of myostatin and a decline in the concentration of decorin [36], the data obtained here have particular significance. It is worth noting that the impact of diverse forms of exercise on decorin concentration is not well established yet. Previous studies have shown that resistance training did not alter plasma decorin concentration, neither with [37] nor without [19] nutritional intervention in people of different gender and age (men and women, 21–50 years old). Moreover, Kanzleiter et al. indicated an increase in decorin muscle expression observed in healthy, middle-aged men (40–65 years old) in response to a regular, prolonged (12 weeks) combination of resistance and endurance training [18]. Despite the diversity of the decorin interacting network, two main roles emerge as prominent themes in itsfunction: maintenance of cellular structure and outside-in signalling, culminating in anti-tumorigenic effect [38]. Interestingly, it has been suggested that overexpression of decorin causes a significant growth inhibition of breast cancer tumor xenografts in MDA-MB-231 cells [39]. Thus, our data support a growing body of evidence that regular exercise can enhance the circulating decorin concentration; however, more evidence is required to explain its mechanism of beneficial metabolic regulation.

Additionally, Kortas et al. demonstrated that the myostatin concentration among elderly women decreased significantly in response to 12 weeks of NW training, accompanied by a significant increase in osteocalcin and adiponectin concentrations, with serum iron and ferritin determining the effects of training [32]. This study indicated that, in addition to iron, vitamin D can modulate a response to NW training. Circulating myostatin is basically increased in obese subjects; its concentration correlates positively with insulin resistance/pancreatic β-cell dysfunction indicators and negatively with insulin sensitivity indicators [40,41]. The findings of this study do not indicate the amelioration of glucose homeostasis (only slight shifts) and, contrary to data presented by Kortas, the bone-derived osteokines (Glu-OC and Gla-OC) decreased significantly a day after NW training had been completed. It is documented that, in osteoporosis, osteocalcin concentration in elderly women increased in response to 12 weeks of comprehensive physical activity [42]. On the contrary, Wieczorek-Baranowska et al. revealed that 8-week cycle-ergometer training, with a ventilatory threshold intensity at 70–80%, induced a significant decrement of circulating osteocalcin in postmenopausal women [43], which is in line with our study's findings. Still, the above-mentioned studies did not measure Glu-OC, but rather only the circulating osteocalcin concentration. An elevated Glu-OC concentration was recorded in response to acute exercise [44]. In the current study, NW training induced a decrease in Glu-OC, inversely dependant on the baseline concentration of $25(OH)D_2$. At present, we don't have a credible explanation for this observation and we therefore recommend further research into this area.

Although circulating concentrations of adiponectin, the anti-inflammatory mediator, did not change significantly in response to NW or HIIT programs, the trends of change between groups were opposite and statistically significant. It has been proven that adiponectin can act as a myokine, as it is also expressed by skeletal muscles during contraction [45]. The obtained results suggest that NW is more effective than HIIT in inducing beneficial shifts of adiponectin.

In our study, contrary to the many well-documented beneficial changes of HIIT protocols [46,47], the desired effect was not observed. A previously published study reported an improvement of glucose homeostasis via an increment of skeletal muscle mitochondrial content, mitofusin 2 protein content and an elevation of the number of glucose transporters 4 (GLUT4) among older diabetic patients (mean age of 63 $\pm$ 8 years) in response to a 2-week HIIT protocol [15]. In contract, Shaban et al. did not observe any changes in glucose homeostasis indicators, such as serum insulin and HOMA-IR level, in response to HIIT training among obese subjects with type 2 diabetes [48]. In our study, we did not collect blood immediately after exercise, but only at rest before starting and 24 h

after completing exercise protocols. For this reason, we may not have noticed short-term changes in the blood glucose concentration.

Several limitations of the study warrant mentioning. Firstly, both training procedures were different types of exercise and their application was based on previously published data. Still, it should be considered to compare the effect of applying those procedures in the same time period. Secondly, the participants' diet was only the same on the days of blood collection. For the remainder of the study, their diet was not controlled, and participants only were instructed not to change their daily habits.

To sum up, the obtained results show that the beneficial effects of a diminishing myostatin concentration were induced only by the NW training; however, the range of changes was modified by the concentration of vitamin D metabolites.

5. Conclusions

In conclusion, this study is the first to demonstrate that endurance exercise in the form of NW induced a decrease in myostatin and an increase in decorin, with these changes being modulated by the vitamin D status. It also suggests that elderly people can experience more benefits from a high-volume and low-intensity NW than high-intensity and low-volume HIIT.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethical Committee of the Regional Medical Society in Gdansk (KB-34/18).

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Adnotacja

Gdańsk, 2022.02.01

Zaznajomiliśmy się z niniejszą rozprawą doktorską przygotowaną pod naszą opieką przez doktorantkę mgr Martę Flis. Praca została przez nas zaakceptowana.

Promotorzy:

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prof. dr hab. Ewa Ziemann

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dr hab. Giovanni Lombardi