Akademia Wychowania Fizycznego i Sportu im. Jędrzeja Śniadeckiego w Gdańsku

ROZPRAWA DOKTORSKA

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Molekularne mechanizmy adaptacji do wysiłku fizycznego - zmiany ekspresji wybranych genów w leukocytach

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Gdańsk 2023

Dziękuję prof. dr hab. Jędrzejowi Antosiewiczowi oraz dr hab. Małgorzacie Żychowskiej za dzielenie się wiedzą i doświadczeniem, wspólną pracę w laboratorium oraz opiekę merytoryczną.

Szczególnie dziękuję Rodzinie: Mężowi, Mamie oraz Teściom za nieocenioną pomoc, motywację oraz Wasze wsparcie w okresie pracy nad doktoratem.

Dziękuję również Kasi Anczykowskiej, dr Katarzynie Micielskiej, dr Mai Tomczyk oraz dr hab. Maciejowi Grzybkowi za współpracę i wsparcie, którym mnie obdarzaliście.

Pracę dedykuję moim Córkom.

Spis treści

1. Wykaz prac wchodzących w skład rozprawy

Niniejszą rozprawę doktorską oparto o cykl trzech publikacji pod wspólnym tytułem "Molekularne mechanizmy adaptacji do wysiłku fizycznego - zmiany ekspresji wybranych genów w leukocytach".

Łączna punktacja cyklu publikacji:
Impact Factor - 15,891
punktacja MEiN - 380

1. **Grzybkowska, A**., Anczykowska, K., Ratkowski, W., Aschenbrenner, P., Antosiewicz, J., Bonisławska, I., Żychowska, M. (2019). Changes in serum iron and leukocyte mRNA levels of genes involved in iron metabolism in amateur marathon runners-effect of the running pace. Genes, 10(6), 460. https://doi.org/10.3390/genes10060460
Genes 2019: 3,759 IF; 100 MEiN

2. Żychowska, M., **Grzybkowska, A**., Wiech, M., Urbański, R., Pilch, W., Piotrowska, A., Czerwińska-Ledwig, O., Antosiewicz, J. (2020). Exercise training and Vitamin C supplementation affects ferritin mRNA in leukocytes without affecting prooxidative/antioxidative balance in elderly women. International Journal of Molecular Sciences, 21(18), 6469. https://doi.org/10.3390/ijms21186469
IJSM 2020: 5,924IF; 140 MEiN

3. **Grzybkowska, A**., Anczykowska, K., Antosiewicz, J., Olszewski, S., Dzitkowska-Zabielska, M., & Tomczyk, M. (2023). Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans. International journal of molecular sciences, 24(7), 6734. https://doi.org/10.3390/ijms24076734
IJSM 2023: 6,208 IF; 140 MEiN

Badania finansowane z:
Grantu Ministerstwa Nauki i Szkolnictwa Wyższego N RSA4 06754: „*Rozwój sportu akademickiego*" (publikacje 1 i 2)
Grantu Preludium 16 Narodowego Centrum Nauki o numerze 2018/31/N/NZ7/02962 (publikacja 3).

2. Wprowadzenie

Wpływ wysiłku fizycznego na organizm ludzki jest przedmiotem badań od lat, a poziom sprawności fizycznej jest uznawany za wskaźnik jakości życia oraz prognozowanej długości życia [1]. Jednocześnie, siedzący tryb życia jest powiązany z częstszym występowaniem otyłości, cukrzycy typu drugiego, chorób kardiologicznych, neurologicznych oraz problemów ze zdrowiem psychicznym [2]. Jednak mimo wielu lat badań, molekularne podstawy zmian zachodzących pod wpływem wysiłku fizycznego pozostają niedostatecznie poznane. Jednym z wyzwań tego typu badań jest złożoność procesu adaptacji i odpowiedzi organizmu na wysiłek fizyczny w zależności od wielu czynników, m.in długości, intensywności, obciążenia i typu wysiłku. Dokładniejsze poznanie molekularnych mechanizmów adaptacji do wysiłku fizycznego umożliwia szczegółowe wskazanie szlaków metabolicznych, które są zmieniane pod wpływem danej aktywności fizycznej oraz może pozwolić dobrać rodzaj treningu tak, aby zmaksymalizować korzyści wynikające z jego stosowania. Ma to szczególne znaczenie dla osób wyczynowo uprawiających sport, ale także dla osób z ograniczeniami ruchowymi w tym osób starszych [3].

Wychodząc na przeciw potrzebie lepszego zrozumienia adaptacji do wysiłku fizycznego oraz suplementacji, w pracach naukowych wchodzących w skład niniejszej dysertacji wykorzystano zaawansowane techniki biologii molekularnej. Podstawą wszystkich przedstawionych badań była ilościowa reakcja łańcuchowa polimerazy w czasie rzeczywistym (ang. *quantitative real-time polymerase chain reaction* w skrócie: qRT-PCR). qRT-PCR to precyzyjna metoda laboratoryjna powielania oraz wykrywania materiału genetycznego. Dzięki metodzie ilościowej można dokładnie ocenić zmianę zawartości mRNA w próbce po zastosowanej interwencji. Zmiany widoczne w poziomach białek po wysiłku fizycznym pojawiają się na skutek wpływu wysiłku fizycznego na ekspresję genów [4]. Regulacja ekspresji genów może zachodzić na różnych etapach, w tym podczas transkrypcji (synteza pre-mRNA na matrycy DNA przez enzymy polimerazy DNA), obróbki posttranskrypcyjnej (zmiana pre-mRNA na dojrzały mRNA i translacji (powstawanie łańcucha polipeptydowego w oparciu o kod zapisany w mRNA) [5].

Dotychczas w badaniach molekularnych związanych z wysiłkiem fizycznym, opierających się na technice qRT-PCR, naukowcy głównie poddawali analizie tkankę mięśniową [6]. Biopsja mięśnia jest jednak inwazyjnym badaniem diagnostycznym i pozwala zbadać jedynie odpowiedź miejscową. W naszych badaniach poddaliśmy analizie odpowiedź komórek układu immunologicznego.

W porównaniu do badań na innych komórkach, np mięśniowych, enterocytach czy hepatocytach, badanie zmian ekspresji genów w leukocytach jest mniej inwazyjne i łatwiejsze do wykonania, ponieważ opiera się na pobraniu krwi żylnej [7]. Dzięki temu, sprawdzenie ekspresji genów było możliwe w niewielkich odstępach czasowych. Przykładowo, w badaniu opublikowanym wraz ze współautorami w 2019 w Genes [8], próbki krwi były pobierane od uczestników czterokrotnie w krótkim przedziale czasowym (na linii startu biegu, tuż po przekroczeniu mety, 3 godziny po oraz 24 godziny po przekroczeniu mety). Dodatkowo, leukocyty które krążą po całym organizmie pozwalają określić odpowiedź immunologiczną badanej osoby biorąc pod uwagę jednocześnie odpowiedź z wielu tkanek, co pozwala ocenić odpowiedź ogólnoustrojową. Zmiany w ekspresji genów w leukocytach stanowią również natychmiastową odpowiedź organizmu na wysiłek fizyczny, która następnie stymuluje adaptacyjną odpowiedź organizm [9].

Obszarami moich zainteresowań były zmiany w metabolizmie żelaza oraz związany z nim stan zapalny, a także suplementacja witaminą C oraz kwasami omega-3 w połączeniu z aktywnością fizyczną.
Żelazo jest niezwykle ważnym pierwiastkiem związanym z wysiłkiem fizycznym ze względu na jego bezpośrednią rolę w produkcji ATP oraz transporcie tlenu przez hemoglobinę i mioglobinę [10]. Jednocześnie dotychczas dostępne badania związane z metabolizmem żelaza i wysiłkiem fizycznym, dostarczają niejednoznacznych wyników, a dane z zakresu podstaw molekularnych są znikome [11,12]. W dostępnej literaturze, głównym obszarem zainteresowań w kontekście zmian molekularnych po wysiłku fizycznym jest odpowiedź zapalna, glikoliza i oksydacja kwasów tłuszczowych [13,14] oraz wpływ aktywności fizycznej na procesy starzenia [15]. Równocześnie udowodniono, że wiele genów związanych z metabolizmem żelaza ulega ekspresji w leukocytach, w tym geny kodujące białko transferynę (*TF*), transporter-1 metali dwuwartościowych (*DMT1*), podjednostka ciężka ferrytyny (*FTH*), podjednostka lekka ferrytyny (*FTL*), ferroportyna (*FPN*) [16].
Istotnym faktem jest to, że nadmiar niezwiązanej formy żelaza w tkankach jest toksyczny, zwiększa bowiem stres oksydacyjny i jest powiązany z apoptozą komórek. Jednocześnie istnieje ogrniczona liczba sposobów jego wydalania z organizmu ludzkiego [17]. Organizm chroni się więc przed toksycznością żelaza magazynując go w ferrytynie lub hemosyderynie - białkach wiążących żelazo w bezpieczny dla tkanki sposób. Synteza ferrytyny to proces regulowany w dużej mierze przez mechanizmy post-transkrypcyjne. Przy niedoborze żelaza w komórce, białko IRP (ang. iron regulatory protein) wiąże się z sekwencją IRE (iron response element) blokując syntezę ferrytyny. W przypadku gdy poziomy żelaza są ponad normą, białko IRP odłącza się od sekwencji IRE umożliwiając powstawanie białka ferrytyny. W ten sam sposób regulowana jest również

ekspresja ferroportyny, śródbłonowego białka transportującego żelazo z komórki [18].
Poziom żelaza w organizmie może być również kontrolowany poprzez hormony, m.in hepcydynę oraz erytroferron. Hepcydyna hamuje transport żelaza z enterocytów do krwi. Jej synteza wzrasta przy wysokich poziomach żelaza oraz stanie zapalnym. Erytroferron z kolei blokuje syntezę hepcydyny [19].
Borghini et al, 2015 oraz Carbonare et al., 2018 udowodnili, że wysiłek fizyczny wpływa na szlak IRP/IRE oraz poziomy hepcydyny poprzez indukcję stresu oksydacyjnego i odpowiedź zapalną [20, 21]. Regulacja poziomów żelaza w organizmie jest niezwykle ważnym złożonym procesem [22].

Przeprowadzone eksperymenty stanowią novum w literaturze, dlatego w przedstawionych pracach uwzględniono również metodologiczne aspekty prowadzenia badań, a dokładniej: określenie punktów czasowych od aktywności fizycznej do uzyskania zmian w ekspresji genów w leukocytach oraz eksperymentalne dobranie optymalnych genów referencyjnych dla wysiłku fizycznego w leukocytach.

Wszystkie badania realizowane w ramach doktoratu były przeprowadzane w oparciu o uzyskane uprzednio zgody Komisji Bioetycznej w Gdańsku (NKBBN/448/2016, KB-10/16 and NKBBN/628/2019).

3. Cele naukowe

I. Określenie wpływu wysiłku maratońskiego na ekspresję genów związanych z metabolizmem żelaza w leukocytach (*FTH, FTL, PCBP1, PCBP2, TFRC*)
II. Analiza związku pomiędzy uzyskaną liczbą transkryptów *FTH* i *FTL,* a poziomami białka ferrytyny we krwi
III. Określenie optymalnych punktów czasowych zmian w ekspresji badanych genów dla biegu maratońskiego
IV. Określenie wpływu treningu zdrowotnego w połączeniu z suplementacją witaminy C na ekspresję genów związanych z metabolizmme żelaza w leukocytach (*FTH, FTL, PCBP1, PCBP2, TFRC, FOXO3a*)
V. Znalezienie optymalnych genów referencyjnych do analizy zmian ekspresji genów dla treningu wytrzymałościowego

4. Omówienie prac oryginalnych wchodzących w skład rozprawy

4.1. Omówienie pracy "*Changes in serum iron and leukocyte mRNA levels of genes involved in iron metabolism in amateur marathon runners-effect of the running pace"*

W pracy "*Changes in serum iron and leukocyte mRNA levels of genes involved in iron metabolism in amateur marathon runners-effect of the running pace*" opublikowanej w 2019 roku w czasopiśmie Genes (IF=3,759, Q1) wraz ze współpracownikami zbadaliśmy wpływ wysiłku maratońskiego na zmiany w ekspresji genów związanych z metabolizmem żelaza w różnych odstępach czasowych od ukończenia biegu. Wybrane przez nas geny są związane z gospodarką żelazową i ulegały ekspresji w leukocytach: *PCBP1, PCBP2, FTL, FTH, CAT, TFRC* oraz *TUBB* jako gen referencyjny. Wymienione geny kodują białka opiekuńcze PCBP1 (ang. Poly(RC) Binding Protein 1) oraz PCPB2 (ang. ang. Poly(RC) Binding Protein 1), które są odpowiedzialne za transport żelaza, w obrębie komórki. Dostarczają cząsteczki żelaza do ferrytyny. Geny *FTL* (ang. ferritin light chain) i *FTH* (ang. ferritin heavy chain) kodują dwie podjednostki (odpowiednio lekką oraz ciężką), budujące białko magazynujące żelazo: ferrytynę. Gen *TFRC* (ang. transferrin receptor) z kolei jest podstawą do budowy receptora transferyny, białka odpowiedzialnego za transport żelaza do komórki, obecnego na błonach komórkowych. Poziomy mRNA *CAT* (ang. catalase), genu kodującego białko katalazę, dostarczały informacji dotyczącej stresu oksydacyjnego, również ściśle powiązanego z homeostazą żelazową. Krew była pobierana od mężczyzn, którzy ukończyli bieg maratoński w różnych odstępach czasowych w celu znalezienia najbardziej optymalnego momentu do uchwycenia zmian. Materiał pobierano przed biegiem, tuż po jego ukończeniu, 3 godziny po oraz 24 godzin po przebiegnięciu linii mety. Oprócz tego, krew była badana pod kątem zmian w morfologii, CRP, poziomach kwasu moczowego, kinazy kreatynowej, a także żelaza oraz ferrytyny. W analizie uwzględniliśmy również tempo biegu.

Badanie pozwoliło potwierdzić wpływ biegu maratońskiego na zmiany zarówno w biochemii krwi jak i w ekspresji genów związanych z metabolizmem żelaza i potwierdziło, że zmiany wsytępują w różnych odstępach czasowych na poziomie transkryptów oraz białek. Zgodnie z założeniami, zmiany w poziomach mRNA po ekstremalnym wysiłku jakim jest maraton, zachodziły wcześniej niż zmiany na poziomie białek. Istotną obserwacją w przedstawionym badaniu było znalezienie korelacji pomiędzy tempem biegu, a zmianami w ekspresji genów. U biegaczy wolniejszych (średnie tempo biegu 10.04 ± 0.52 km/h) tendencja do odchylenia od

poziomu wyjściowego była większa, a powrót do poziomu wyjściowego był dłuższy niż u biegaczy szybszych (średnie tempo biegu 12.18 ± 0.71 km/h). Ekspresja genów *PCBP1, PCBP2, FTH* oraz *CAT* była obniżona w momencie kiedy zawodnicy przekraczali linię mety. Natomiast już 3 godziny po ukończeniu maratonu poziomy mRNA *PCBP1, PCBP2, FTH* oraz *FTL* były wyższe w stosunku do wartości wyjściowych. Ferrytyna jest białkiem ostrej fazy, zwiększona ekspresja jest reakcją ochronną organizmu przy stanie zapalnym spowodowanym ekstremalnym wysiłkiem fizycznym jakim jest maraton. Ekspresja genu *TFRC* uległa obniżeniu po wysiłku maratońskim i obniżone wyniki utrzymywały się również 24h po ukończeniu biegu, jednak zmiany nie były istotnie statystyczne. Zwiększona ekspresja genów związanych z metabolizmem żelaza 3h po ukończeniu biegu sugeruje ochronną odpowiedź organizmu w celu przeciwdziałania toksyczności żelaza. Niezmienione poziomy *TFRC* (z tendencją do obniżenia ekspresji) sugerują stabilność w wewnętrzkomórkowej puli labilnego żelaza LIP (ang. labile iron pool) mimo ukończenia obciążającego wysiłku w postaci maratonu.

4.2. *Exercise training and Vitamin C supplementation affects ferritin mRNA in leukocytes without affecting prooxidative/antioxidative balance in elderly women*

W pracy "*Exercise training and Vitamin C supplementation affects ferritin mRNA in leukocytes without affecting prooxidative/antioxidative balance in elderly women*" opublikowanej w 2020 roku w czasopiśmie International Journal of Molecular Sciences (IF=5,924, Q1) wraz ze współautorami analizowaliśmy związek pomiędzy suplementacją witaminy C, wysiłkiem fizycznym i zmianami w ekspresji genów związanych z metabolizmem żelaza. W ramach badania, 15 zdrowych kobiet w wieku starszym (średnia wieku 69.2 ± 6.5 lat), które zostało podzielonych na dwie grupy. Grupa 1 w pierwszej części eksperymentu suplementowała 1000mg witaminy C dziennie oraz została poddana treningowi zdrowotnemu. Grupa 2 uczestniczyła w tym samym cyklu treningów, ale otrzymywała placebo. Po 6 tygodniach nastąpiła zamiana suplementacji w grupach. Podobnie jak w pierwszej publikacji [8], zbadano ekspresję genów: *FTH1, FTL, PCBP1, PCBP2* oraz *CAT*. Genem referencyjnym ponownie był *TUBB*. Ponadto, analizowaliśmy zmiany w ekspresji genu *FOXO3a* (ang. forkhead box O3a), który koduje białko o właściwościach regulujących apoptozę oraz kontrolującego ekspresję mRNA ferrytyny oraz katalazy.
Dodatkowo sprawdzone parametry u uczestniczek badania to: VO_2max w bezpośredniej próbie laboratoryjnej na cykloergometrze, stężenie witaminy C we krwi oraz profil oksydacyjny i antyoksydacyjny poprzez stosunek TOS/TOC (total

oxidative status/total oxidative capacity) i TAS/TAC(total antioxidative status/total antioxidative capacity).
Zastosowany trening nie spowodował zmian w VO_2max u uczestniczek. Ekspresja genu *FTH* uległa obniżeniu w obu grupach po ukończeniu 6 tygodni treningu połączonego z suplementacją witaminą C. Ekspresja genu *FTL* uległa obniżeniu w jednej grupie po ukończeniu 6 tygodni treningu zdrowotnego w połączeniu z suplementacją witaminy C. Nie stwierdzono zmian istotnie statystycznych dla ekspresji pozostałych genów.

4.3. *Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans*

W pracy "*Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans*" opublikowanej w 2023 roku w czasopiśmie International Journal of Molecular Sciences (IF= 6,208, Q1) zbadałam zmiany w ekspresji 6 potencjalnych genów referencyjnych *GAPDH, ACTB, TUBB, RPS18, UBE2D2* oraz *HPRT1*. Geny zostały wybrane na podstawie przeglądu literatury. Celem badania było znalezienie genów, których ekspresja ulegnie jak najmniejszej zmianie po przeprowadzeniu 12-tygodniowego eksperymentu. Uczestnicy eksperymentu zostali poddani treningowi wytrzymałościowemu wraz suplementacją. Część uczestników otrzymywała wysoką dawkę kwasów omega-3 (2234 mg kwasu eikozapentaenowego (EPA) + 916 mg kwasu dokozaheksaenowego (DHA)), druga część natomiast, otrzymywała placebo w postaci 4000mg średniołańcuchowych kwasów tłuszczowych (MCT). Do porównania stabilności ekspresji genów użyłam narzędzia RefFinder, który wykorzystuje do obliczeń 4 algorytmy: BestKeeper, NormFinder, geNorm oraz metodę Delta-Ct. Udowodniłam, że genem o najwyższej stabilności dla zastosowanej interwencji jest *GAPDH*, natomiast w oparciu o międzynarodowe wytyczne MIQE (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments), dotyczące eksperymentów qRT-PCR, zaproponowałam, aby w podobnych eksperymentach, dla uzyskania jak najbardziej rzetelnych wyników, używać trzech genów referencyjnych o udowodnionej najwyższej stabilności, tj: *GAPDH, TUBB* oraz *HPRT1*.

5. Wnioski

I. Wysiłek fizyczny wpływa na ekspresję genów związanych z metabolizmem żelaza (*PCBP1, PCBP2, FTL, FTH, TFRC)*.
II. Ukończenie biegu maratońskiego wiąże się z obniżeniem ekspresji genów *PCBP1, PCBP2, FTH* i *CAT* bezpośrednio po wysiłku
III. Po biegu maratońskim, zmiany w ekspresji genów *FTH* oraz *FTL* nie korelują ze stężeniem ferrytyny w surowicy
IV. Wolniejsze tempo biegu wiąże się z większymi zmianami w poziomach mRNA oraz dłuższym powrotem do poziomów wyjściowych
V. Największe zmiany w ekspresji genów związanych z metabolizmem żelaza zachodzą po 3 godzinach od ukończenia biegu maratońskiego
VI. Trening zdrowotny w połączeniu z suplementacją 1000mg witaminy C powoduje obniżenie ekspresji genu *FTH*
VII. Geny referencyjne odpowiednie dla badań związanych z długotrwałym treningiem wytrzymałościowym to: *GAPDH, TUBB* oraz *HPRT1*

Gdansk University of Physical Education and Sport

DOCTORAL DISSERTATION

by

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Molecular mechanisms of adaptation to physical exercise - changes in gene expression in leukocytes

Dissertation Supervisor:
prof. dr hab. Jędrzej Antosiewicz

Gdańsk 2023

6. Abstract

The effect of physical exercise on human body has been the subject of the study for years, and the physical fitness is considered a great indicator of the quality of life and predictor of all-cause mortality [1]. Correspondingly, a sedentary lifestyle is associated with a higher incidence of obesity, type 2 diabetes, cardiovascular and neurological diseases as well as mental health problems [2]. However, despite many years of research, the molecular basis of changes occurring under the influence of physical exercise remains insufficiently understood. One of the challenges in this type of research is the complexity of the adaptation process and the body's response to exercise, which depends on many factors, including duration, intensity, load and type of exercise. A more detailed understanding of the molecular mechanisms of adaptation to physical exercise allows for a detailed indication of metabolic pathways affected by a given physical activity and may allow practitioners to choose optimal training protocols to maximise the beneficial effect. This is of particular importance for people practising sports professionally, but also for people with physical impairments including the elderly [3].

To better understand the process of adaptation to exercise and supplementation, advanced techniques of molecular biology were used in the research papers included in this dissertation. Quantitative real-time polymerase chain reaction (qRT-PCR) was the basis of all studies presented. qRT-PCR is a precise laboratory method for amplifying and detecting genetic material. Thanks to the quantitative method, it is possible to accurately assess the change in mRNA levels in the sample after the applied intervention. Changes that can be detected in protein levels after physical exercise appear as a result of the effect that physical exercise has on gene expression [4], and the regulation of this process may occur at various stages, including transcription (synthesis of pre-mRNA on the DNA template by DNA polymerase enzymes), post-transcription processing (change of pre-mRNA to mature mRNA), translation (formation of a polypeptide chain based on the code written in mRNA), protein folding, post-translational modifications (chemical changes in the protein, e.g. glycosylation, phosphorylation) [5].

Up to this date, molecular research related to physical exercise based on the qRT-PCR technique is mainly done on muscle tissue [6]. However, a muscle biopsy is an invasive diagnostic test that only allows for analysing the local response. In our research, we analysed the response of the cells of the immune system. Compared to tests on other cells, e.g. muscle, enterocytes or hepatocytes, studying changes in gene expression in leukocytes is less invasive and much easier to perform, since it is based on collecting venous blood [7]. Thanks to this, it was possible to check gene expression at short intervals. For example, in a study published with co-authors in 2019 in Genes [8], blood samples were collected

from participants 4 times in a short period of time (at the start line, right after crossing the finish line, 3 hours after and 24 hours after crossing the finish line). In addition, leukocytes that circulate throughout the body allowed us to determine the immune response of the tested person, taking into account the response from many tissues simultaneously, which reflects the systemic response. Changes in gene expression in leukocytes are also an immediate response of the body to exercise, which then stimulates the adaptive response of the body [9].

The main subject of my interest were changes in iron metabolism and the inflammation process associated with it, as well as supplementation with vitamin C and omega-3 acids in combination with physical activity.
Iron is an extremely important element associated with physical exercise due to its direct role in the production of ATP and the transport of oxygen by haemoglobin and myoglobin [10]. At the same time, the studies on iron metabolism and physical exercise available so far provide ambiguous results, and data on the molecular basis are scarce [11, 12]. In the current literature, the main area of interest in the context of molecular changes after physical exercise is the inflammatory response, glycolysis and oxidation of fatty acids [13, 14] and recently, the impact of physical activity on the ageing process [15]. At the same time, it has been proven that many genes related to iron metabolism are expressed in leukocytes, including genes encoding the protein transferrin (*TF*), divalent metal transporter-1 (*DMT1*), ferritin heavy subunit (*FTH*), ferritin light subunit (*FTL*), and ferroportin (*FPN*) [16].

It is worth mentioning, that the excess of unbound iron in tissues is toxic, as it increases oxidative stress and is associated with cell apoptosis. At the same time, there is a limited number of ways to excrete iron from the human body [17]. The body protects itself against iron toxicity by storing it in ferritin or hemosiderin - proteins that bind iron in a safe way. Ferritin synthesis is a process regulated largely by post-transcriptional mechanisms. During iron deficiency in the cell, the iron regulatory protein (IRP) binds to the iron response element (IRE) sequence, blocking the synthesis of ferritin. During iron overload, the IRP protein dissociates from the *IRE* sequence, allowing the formation of the ferritin protein. The expression of ferroportin, a transmembrane iron transport protein, is also regulated in the same way [18].
The level of iron in the body can also be controlled by hormones, including hepcidin and erythroferrone. Hepcidin inhibits the transport of iron from enterocytes into the blood. Its synthesis increases with high iron levels and inflammation. Erythroferrone, in turn, blocks the synthesis of hepcidin [19].
Borghini et al, 2015 and Carbonare et al., 2018 proved that physical effort affects the IRP/IRE pathway and hepcidin levels by stimulating oxidative stress and inflammatory response [20, 21]. The regulation of iron levels in the body is a complex and important process [22].

Due to the novel character of the presented research, we also took into consideration methodological aspects of research, namely: the determination of the best time points for research including physical activity, to obtain changes in gene expression in leukocytes as well as experimental selection of optimal reference genes for physical activity in leukocytes.

All research conducted as part of this dissertation was approved by the Bioethical Committee of Regional Medical Society in Gdansk (NKBBN/448/2016, KB-10/16 and NKBBN/628/2019).

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8. Załączniki

I. Oświadczenia współautorów

II. Publikacje wchodzące w skład rozprawy doktorskiej:

- Publikacja 1: "*Changes in serum iron and leukocyte mRNA levels of genes involved in iron metabolism in amateur marathon runners-effect of the running pace"*
- Publikacja 2: *"Exercise training and Vitamin C supplementation affects ferritin mRNA in leukocytes without affecting prooxidative/antioxidative balance in elderly women"*
- Publikacja 3: *"Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans"*

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

Grzybkowska, A., Anczykowska, K., Ratkowski, W., Aschenbrenner, P., Antosiewicz, J., Bonisławska, I., Żychowska, M. (2019). Changes in serum iron and leukocyte mRNA levels of genes involved in iron metabolism in amateur marathon runners-effect of the running pace. *Genes*, *10*(6), 460. https://doi.org/10.3390/genes10060460

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Grzybkowska, A., Anczykowska, K., Antosiewicz, J., Olszewski, S., Dzitkowska-Zabielska, M., & Tomczyk, M. (2023). Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans. International journal of molecular sciences, 24(7), 6734. https://doi.org/10.3390/ijms24076734

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

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genes

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Article

Changes in Serum Iron and Leukocyte mRNA Levels of Genes Involved in Iron Metabolism in Amateur Marathon Runners—Effect of the Running Pace

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Received: 28 April 2019; Accepted: 12 June 2019; Published: 15 June 2019

Abstract: Iron is essential for physical activity due to its role in energy production pathways and oxygen transportation via hemoglobin and myoglobin. Changes in iron-related biochemical parameters after physical exercise in athletes are of substantial research interest, but molecular mechanisms such as gene expression are still rarely tested in sports. In this paper, we evaluated the mRNA levels of genes related to iron metabolism (*PCBP1*, *PCBP2*, *FTL*, *FTH*, and *TFRC*) in leukocytes of 24 amateur runners at four time points: before, immediately after, 3 h after, and 24 h after a marathon. We measured blood morphology as well as serum concentrations of iron, ferritin, and C-reactive protein (CRP). Our results showed significant changes in gene expression (except for *TFRC*), serum iron, CRP, and morphology after the marathon. However, the alterations in mRNA and protein levels occurred at different time points (immediately and 3 h post-run, respectively). The levels of circulating ferritin remained stable, whereas the number of transcripts in leukocytes differed significantly. We also showed that running pace might influence mRNA expression. Our results indicated that changes in the mRNA of genes involved in iron metabolism occurred independently of serum iron and ferritin concentrations.

Keywords: iron metabolism; ferritin; gene expression; marathon runners; *PCBP1*; *PCBP2*; *TFRC*

1. Introduction

Iron is essential for physical activity due to its role in energy production pathways and oxygen transportation via hemoglobin and myoglobin [1]. Athletes are considered to be at greater risk of iron deficiency than the general population, although supportive data are inconclusive [2,3]. In one particular study, iron deficiency was found in 1.6% of recreational runners, but iron overload was found in 15% of the male participants [4].

Recently, research interest in iron metabolism during and after exercise has grown because physical activity can affect iron and iron-regulatory protein status in many ways, such as by inducing oxidative stress and inflammation [5–10]. In the case of intense running efforts, foot strike causes hemolysis as

an additional factor that contributes to disordered iron metabolism [11,12]. Terink et al. [13] reported that most of the studies related to iron metabolism were conducted on well-trained athletes, mainly during short and intensive efforts. Additionally, the changes in iron metabolism were determined only on protein concentrations in plasma or serum and usually showed an increase in blood ferritin values, although the findings were conflicting. To the best of our knowledge, there are no data focused on the effect of endurance exercise on the mRNA of genes related to iron metabolism.

The popularity of marathon running has increased in recent years especially in amateurs of different ages, sex and physical capabilities. It seems that the runner's age, running speed and level of adaptation to training are main influences of the physiological response to physical exercise. However, published studies also have some contradictory findings. For example, Jastrzębski et al. [14] reported that during a 100 km run, muscle and liver damage was age but not pace-dependent while negative metabolic changes were independent of age.

The aim of our study was to examine the changes in serum iron and ferritin concentrations together with the changes in leukocyte mRNA levels of genes encoding proteins involved in iron metabolism i.e., *PCBP1* (poly(rC) binding protein 1), *PCBP2* (poly(rC) binding protein 2), *FTH1* (ferritin heavy chain 1), *FTL* (ferritin light chain) and *TFRC* (transferrin receptor). The expression of these genes is expected to be affected by marathon running as the proteins they encode are involved in exercise-induced oxidative stress and inflammation [15–17]. We also aimed to evaluate the relationship between changes in gene expression and baseline serum iron and ferritin concentrations, and runner pace, during a run. We hypothesized that marathon running will induce an increase in the mRNA levels of genes associated with iron metabolism similarly to serum changes and that these changes will be pace-dependent.

2. Materials and Methods

2.1. Characteristics of the Subjects and Baseline Laboratory Parameters

A total of 28 healthy young men who reported regular physical activity involving a running program participated in our study. All participants were asked to refrain from changing their diet and to avoid nicotine and alcohol use, for one month prior to undertaking the study marathon run (42.2 km) at an athletic stadium (Gdansk University of Physical Education and Sport, Gdansk, Poland). The run was completed by 26 of the 28 participants, and a further two subjects were excluded as they had mRNA levels that were far from the average. Anthropometric data for the 24 included subjects are shown in Table 1. All the subjects were informed of the purpose of the study and the possible risks involved before giving written consent. The study was approved by the Bioethics Committee for Clinical Research at the Regional Medical Chamber in Gdansk (NKBBN/448/2016). The principles of the Helsinki Declaration were respected.

Table 1. Characteristic of the participants. Data are presented as a range or means ± standard deviation (SD).

Participant's Characteristic	
	Baseline (n = 24)
Age (years)	48.8 ± 6.5
Body mass (kg)	80.1 ± 8.5
Height (cm)	178.7 ± 5.3
BMI (kg/m^2)	25.1 ± 2.3
PBF (%)	15.5 ± 5.0
Pace during the run (km/h)	10.9 ± 1.4
Training units per week	Between 1 and 7
Training regimen (km/week)	Between 20 and 115
Training experience (years)	Between 4 and 24
Number of finished marathons	Between 1 and 62

BMI—body mass index, PBF—percentage body fat.

2.2. Experimental Procedure

Venous blood was collected and serum was obtained from Vacutest® Clot Activator tubes (Vacutest KIMA, Arzegrande, Italy) at four time points: before the run (pre-race), immediately after finishing the run (post-race), 3 h after the run (3 h post-race) and 24 h after the run (24 h post-race). The blood samples were analyzed for blood morphology, and serum concentrations of iron, ferritin, uric acid, creatinine kinase and C-reactive protein (CRP) at an accredited laboratory (Uniwersyteckie Centrum Kliniczne, Gdansk, Poland). Right before the run, the subjects' body weight, height, body mass index (BMI) and percentage of body fat (PBF) were determined using InBody 720 (Biospace Co., Ltd., Seoul, Korea) [18].

To assess gene expression, a further 2 mL of venous blood was collected using vacutainers spray-coated with K_3EDTA as an anticoagulant at the same four time points. The collected blood was mixed within 15 min with Red Blood Cell Lysis Buffer (RBCL) (A&A Biotechnology, Gdynia, Poland) and incubated on ice for at least 15 min. The samples were then spun at 3000× g at 4 °C for 10 min. The resulting pellet was washed again with the hemolysis buffer and the remaining white blood cells lysed using Fenozol (A&A Biotechnology, Gdynia, Poland), and immediately after stored at −20 °C for up to four months, with no freeze–thaw cycles.

2.3. RNA Extraction and Reverse Transcription

Isolation of total RNA was carried out by the modified Chomczynski and Sacchi method [19]. White blood cells diluted in fenozol were thawed at 50 °C for 5 min. Then 200 µL of chloroform (POCH, Gliwice, Poland) was added and the suspension was shaken. Samples were then centrifuged at 10,000 *g* for 30 min at 4 °C. The aqueous phase was collected and mixed with 500 µL of isopropanol (POCH, Gliwice, Poland) and left for at least 30 min to precipitate RNA. Samples were again spun at 10,000 g for 15 min at 4 °C. The aqueous phase was disposed, and the remained pellet was washed 2 times in 1 mL of 75% ethanol at 7500 g at 4 °C. After drying, the pellet was resuspended in 20 µL of PCR grade water. During the optimization period for tested genes, gel electrophoresis has been performed to check for the quality and integrity of RNA. RNA concentration and purity were determined by spectrophotometer (Multiskan Sky Microplate Spectrophotometer, ThermoFisher Scientific, Warszawa, Poland) by absorbance at UV 260/280, and a ratio >1.7 was accepted as pure RNA suitable for further analysis. RNA was then reverse transcribed to cDNA in Eppendorf Mastercycler Gradient 5331, using 0.2 µM oligo(dT) and a Transcriptor First Strand cDNA Synthesis Kit as per the manufacturer's instructions (Roche, Warszawa, Poland). For the analysis 1000 ng of RNA has been used. Thermal conditions used for this step were as follows: Incubation—60 min at 50 °C—followed by inactivation—5 min at 85 °C. Prepared samples were frozen immediately after the reverse transcription and then stored at −20 °C for up to one month, with no freeze–thaw cycles. For gene expression analysis, the obtained cDNA was diluted 10 times, just before the qRT-PCR step.

2.4. Quantitative Polymerase Chain Reaction Assay

Quantitative real-time polymerase chain reaction (qRT-PCR) analyses were carried out on six genes of particular physiological significance in the context of iron metabolism. The AriaMx Real-Time PCR System (Agilent Technologies, Warszawa Poland) and FastStart Universal SYBR® Green Master (Rox) (Roche, Warszawa, Poland) were used, according to the manufacturer's protocol, on 96-well PCR plates in triplicate for each sample. 2 µL of diluted cDNA was used for qRT-PCR. The thermal cycling conditions comprised an activation step: 95 °C for 10 min followed by 40 cycles of annealing; and an extension step: 95 °C for 15 s and 60 °C for 1 min. Additionally, the melt curve analysis was performed for each reaction. *TUBB* (tubulin beta class I, NM_001293213) was chosen experimentally and used as the reference gene. The relative mRNA expression of *PCBP1* (NM_006196), *PCBP2* (NM_001128913), *FTH1* (NM_002032), *FTL* (NM_000146), *CAT* (catalase, NM_001752) and *TFRC* (NM_001128148) was calculated using qRT-PCR. The primer sequences were designed by the authors using the Primer3 Web

tool. In silico specificity screen has been performed using USCS genome browser. The primers were then ordered from Genomed, Warszawa, Poland. Primer sequences (5′-3′), were:
TUBB
Forward primer: TCCACGGCCTTGCTCTTGTTT
Reverse primer: GACATCAAGGCGCATGTGAAC
PCBP1
Forward primer: AGAGTCATGACCATTCCGTAC
Reverse primer: TCCTTGAATCGAGTAGGCATC
PCBP2
Forward primer: TCCAGCTCTCCGGTCATCTTT
Reverse primer: ACTGAATCCGGTGTTGCCATG
FTH1
Forward primer: TCCTACGTTTACCTGTCCATG
Reverse primer: CTGCAGCTTCATCAGTTTCTC
FTL
Forward primer: GTCAATTTGTACCTGCAGGCC
Reverse primer: CTCGGCCAATTCGCGGAA
CAT
Forward primer: GATGGACATCGCCACATGAAT
Reverse primer: AAGATCCCGGATGCCATAGTC
TFRC
Forward primer: TGCAGCAGTGAGTCTCTTCA
Reverse primer: AGGCCCATCTCCTTAACGAG

2.5. Statistical Analysis

2.5.1. Serum Parameters

Whole blood measurements were corrected for plasma volume shift using the Dill and Costill equation [20]. The normality of the distributions was checked for all parameters using the Shapiro–Wilk test. Values were compared statistically using the one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons test for parametric data, and Kruskal–Wallis test followed by Dunn's multiple comparisons test for nonparametric data.

2.5.2. mRNA Levels

Relative mRNA expression was determined using the Schmittgen and Livak delta delta C_t method [21] in Microsoft Excel (2017). The mRNA levels of the tested genes were described as the differences in the cycle threshold value normalized to the *TUBB* mRNA level, i.e., $\Delta C_T = C_T$ of gene—C_T of *TUBB*. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Relative mRNA expression data were linearly transformed and then the normality of the distribution was checked with the Shapiro–Wilk test. Results were analyzed using the Wilcoxon matched pairs [22] test for nonparametric variables. A p-value of <0.05 was considered significant.

3. Results

3.1. Blood Morphology

Statistically significant changes were observed in all white blood cells, uric acid and creatine kinase immediately after the run. Changes in other laboratory parameters occurred 3 h post-race, except for CRP which was significantly elevated after 24 h post-race. All values were corrected for changes in plasma volume (%Delta PV). All results are shown in Table 2.

Table 2. Changes in laboratory parameters after a marathon run (n = 24). $p < 0.05$ is statistically significant. Data are presented as the mean ± SD for the pre-race and the three following measurements. Values are corrected for plasma volume changes (%Delta PV). Statistical analyses were undertaken using Tukey's multiple comparisons test for the parametric values and Dunn's multiple comparisons test for nonparametric values, compared to the pre-race values. * $p < 0.05$.

	Pre-Race	**Post-Race**	**3 h Post-Race**	**24 h Post-Race**
%Delta PV	-	1.05%	11.58% *	12.43% *
	-	$p > 0.9999$	$p < 0.0001$	$p < 0.0001$
Hemoglobin (g/dL)	14.94 ± 0.85	14.78 ± 1.36	12.63 ± 1.06 *	12.41 ± 0.96 *
	-	$p = 0.9545$	$p < 0.0001$	$p < 0.0001$
RBC ($\times 10^{12}$/L)	4.97 ± 0.33	4.91 ± 0.44	4.19 ± 0.35 *	4.13 ± 0.32 *
	-	$p = 0.9445$	$p < 0.0001$	$p < 0.0001$
Hematocrite (%)	43.33 ± 2.17	42.61 ± 3.96	35.91 ± 3.09 *	35.91 ± 2.58 *
	-	$p > 0.9999$	$p < 0.0001$	$p < 0.0001$
Reticulocytes ($\times 10^9$/L)	59.44 ± 11.6	61.78 ± 13.77	49.38 ± 11.67 *	46.63 ± 12.52 *
	-	$p = 0.9088$	$p = 0.0261$	$p = 0.0027$
White blood cells ($\times 10^9$/L)	5.27 ± 1.22	14.74 ± 3.45 *	12.02 ± 2.07 *	6.35 ± 1.54
	-	$p < 0.0001$	$p < 0.0001$	$p = 0.9970$
Neutrophils ($\times 10^9$/L)	2.84 ± 0.9	12.2 ± 3.11 *	9.82 ± 1.97 *	3.58 ± 1.63
	-	$p < 0.0001$	$p < 0.0001$	$p > 0.9999$
Lymphocytes ($\times 10^9$/L)	1.7 ± 0.31	1.48 ± 0.47 *	1.26 ± 0.28	1.94 ± 0.45
	-	$p = 0.1485$	$p = 0.0007$	$p = 0.9166$
Monocytes ($\times 10^9$/L)	0.47 ± 0.14	0.95 ± 0.26 *	0.89 ± 0.21 *	0.56 ± 0.13
	-	$p < 0.0001$	$p < 0.0001$	$p > 0.9999$
Eosinophils ($\times 10^9$/L)	0.22 ± 0.14	0.04 ± 0.04 *	0.01 ± 0.01 *	0.19 ± 0.12
	-	$p < 0.0001$	$p < 0.0001$	$p > 0.9999$
Basophils ($\times 10^9$/L)	0.04 ± 0.02	0.06 ± 0.02 *	0.03 ± 0.01	0.04 ± 0.01
	-	$p = 0.0032$	$p > 0.9999$	$p > 0.9999$
CRP (mg/L)	1.4 ± 3.7	1.31 ± 3.26	1.87 ± 3.12	9.79 ± 7.28 *
	-	$p > 0.9999$	$p = 0.0807$	$p < 0.0001$
Uric acid (mg/dL)	5.26 ± 1.08	5.9 ± 1.07 *	5.71 ± 1.03 *	4.86 ± 0.96 *
	-	$p < 0.0001$	$p = 0.0007$	$p = 0.0113$
Creatine kinase (U/L)	171.56 ± 68.52	569.55 ± 490.71 *	871.04 ± 900.02 *	1410.66 ± 1444.06 *
	-	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

3.2. Serum Ferritin and Iron Concentrations

No significant changes in ferritin concentrations were observed immediately after the run or during the recovery period, compared with baseline. There was a slight tendency to an increase in ferritin immediately after the run compared with the pre-race rest value (113.1 versus 93 ng/mL, respectively). However, ferritin at 24 h after the run was essentially unchanged from the pre-race value (97 versus 93 ng/mL, respectively). The same direction of changes was observed in serum iron, but at 3 h after the run there was a significant decrease compared with baseline values (66.1 versus 102.3 µg/dL, respectively; $p = 0.002$). Between 3 h and 24 h post-race, serum iron increased and had returned to baseline by 24 h (Figure 1).

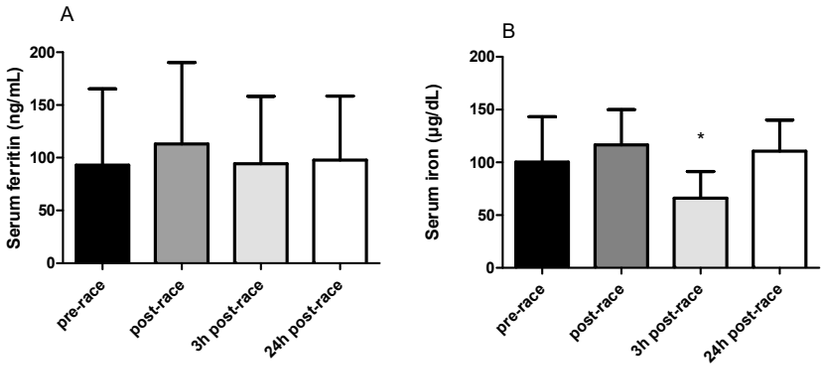

Figure 1. Serum ferritin (**A**) and iron (**B**) concentrations at different time points in the 24 participants. Values are presented as mean ± SD. * $p < 0.05$ compared to the pre-race value.

3.3. Effect of Exercise on mRNA Levels of Selected Genes

Out of six genes tested, five were down-regulated at the end of the race compared with baseline, with the differences for *PCBP1, PCBP2, FTH* and *CAT* achieving statistical significance ($p = 0.0359$, $p = 0.0443$, $p = 0.0158$ and $p = 0.0182$, respectively) (Figure 3). There was a trend for up-regulation in *PCBP1* and *PCBP2* ($p = 0.0826$ and $p = 0.2435$, respectively), and a significant up-regulation in *FTH* and *FTL* genes ($p = 0.0056$ and $p = 0.0064$, respectively) at 3 h after the marathon run. The mRNA levels of all genes except for *TFRC,* which remained insignificantly decreased, returned to baseline levels at 24 h after the run.

3.4. Relationship Between Baseline Levels of Serum Iron and Ferritin, and Changes in mRNA Levels with Exercise

There were no statistically significant differences in mRNA levels at any time point in participants with baseline serum iron concentrations below (serum iron ≤ 105 µg/dL) and above (serum iron >105 µg/dL) the median baseline value (data not shown). There were also no statistically significant differences in mRNA levels in participants with baseline serum ferritin concentrations below (serum ferritin ≤ 78.08 ng/mL) and above (serum ferritin > 78.08 ng/mL) the median baseline serum ferritin value (data not shown).

3.5. Effect of Running Pace on mRNA Levels of Selected Genes

To determine if the running pace had any effect on gene expression, the participants were divided into two groups (slow and fast) by the median split. The characteristic of two groups is shown in Table 3. A significant difference between groups was observed for pace ($p = 0.0001$), BMI ($p = 0.006$) and age of the participants ($p = 0.0001$). The mean ± SD pace value in the slow group was 10.0 ± 0.5 km/h and in the fast group was 12.2 ± 0.7 km/h ($p < 0.0001$). The mRNA levels of the genes tested in these two groups are shown in Figure 2.

Table 3. Characteristics of slow and fast groups. Data are presented as means ± standard deviation (SD). * $p < 0.05$ for comparison between two groups.

Slow and Fast Groups Characteristics		
	Slow ($n = 12$)	Fast ($n = 12$)
Pace during the run (km/h)	10.04 ± 0.52	12.18 ± 0.71 *
Age (years)	53.58 ± 5.45	44.25 ± 3.49 *
BMI (kg/m^2)	26.28 ± 1.88	23.83 ± 1.95 *
Training units per week	3.00 ± 1.04	4.58 ± 1.26
Training regimen (km/week)	43.58 ± 16.53	71.27 ± 25.22
Training experience (years)	10.83 ± 6.90	8.08 ± 5.02
Number of finished marathons	16.83 ± 20.85	13.58 ± 9.18
Baseline iron level (µg/dL)	112.75 ± 40.54	92.92 ± 42.44
Baseline ferritin level (ng/mL)	93.41 ± 36.58	88.18 ± 98.29

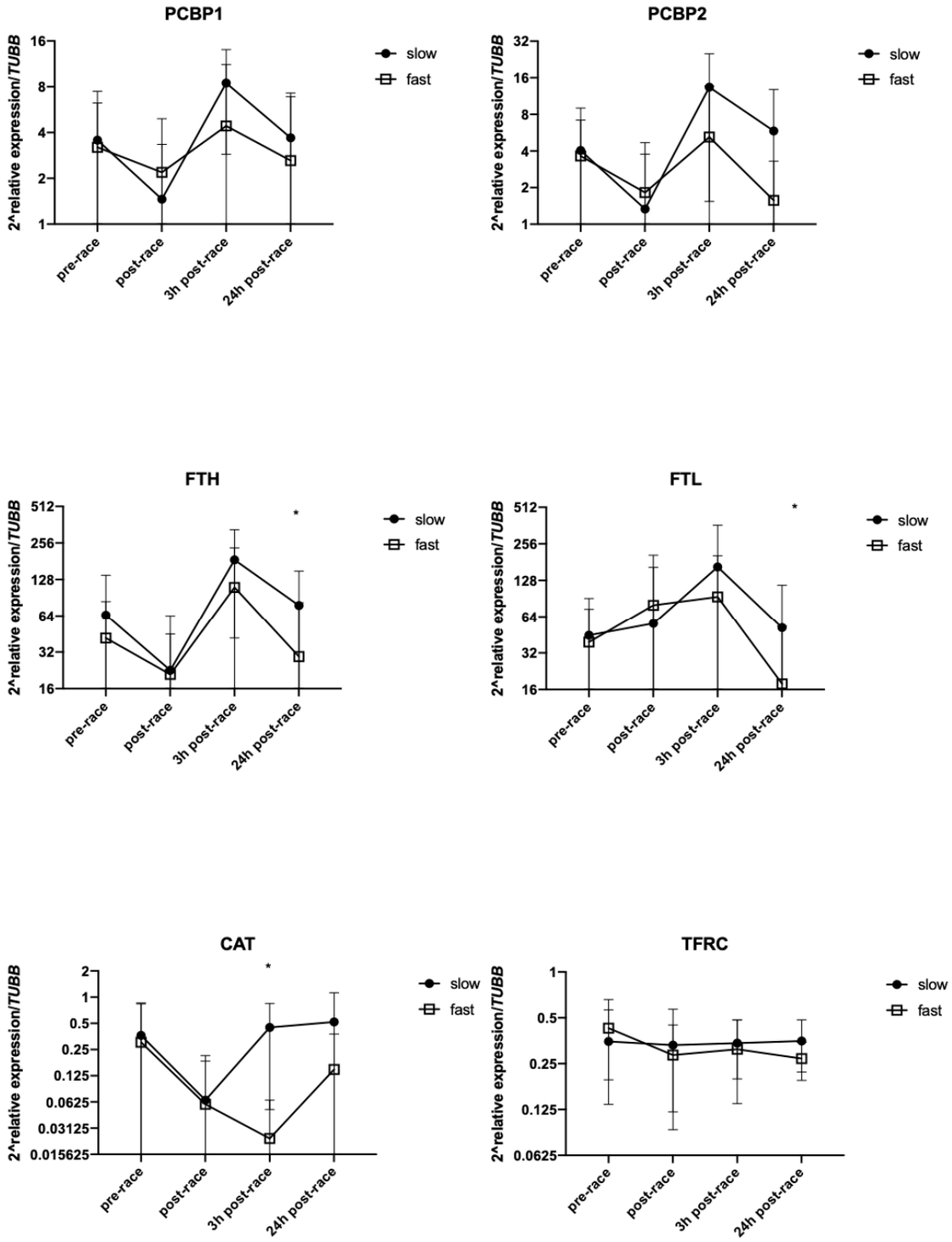

Figure 2. Changes in mRNA levels between slow and fast runners (n = 12 per group). Values are mean ± SD. * $p < 0.05$ for comparison between two groups.

The direction of change in *PCBP1*, *PCBP2* and *FTH* gene expression were the same i.e., a decrease immediately after the race and a statistically significant increase 3 h post-race. At 24 h post-race, the values returned almost to baseline (Figure 2). *FTL* mRNA levels were more stable than *FTH* mRNA levels between the end of the run and 3 h post-race. However, similarly to *FTH*, a significant difference in *FTL* mRNA levels between groups was observed 24 h after the race (p = 0.0245 and p = 0.0128, respectively). The slow group presented with higher levels than the fast group. The opposite changes

were observed in *CAT* mRNA levels at 3 h post-race. In the slow group *CAT* mRNA levels dropped, while they increased in the fast pace group ($p = 0.0017$).

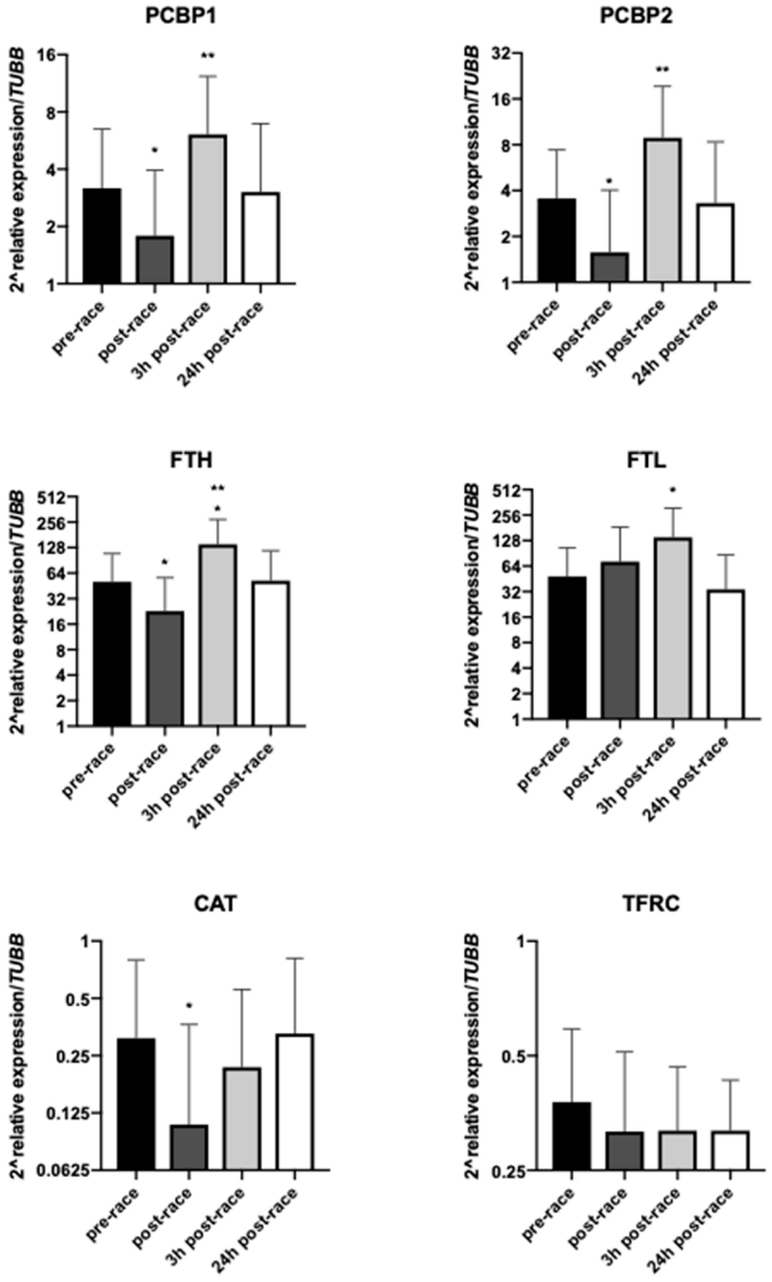

Figure 3. mRNA levels of selected genes at different time points ($n = 24$). Values are presented as mean ± SD. * $p < 0.05$ compared to the pre-race value and ** $p < 0.05$ compared to the post-race value.

4. Discussion

The results of this study did not confirm our hypothesis associated with serum iron, ferritin and expression of genes involved in iron metabolism. Serum ferritin concentrations remained almost unchanged at all time points. Iron status immediately and 24 h after completion of a marathon also did not differ from baseline but there was a significant decrease 3 h after the run. Moreover, changes in iron and ferritin did not correlate with each other (data not shown). Interestingly, a significant decrease in *FTH*, *PCBP1*, *PCBP2* and *CAT* mRNA was observed immediately after the run, and a significant increase in *PCBP1*, *PCBP2*, *FTH* and *FTL* mRNA was seen at 3 h after the run. *TFRC* mRNA remained

unchanged. Furthermore, changes in serum indicators and gene expression in leukocytes occurred at different time points.

4.1. Changes in Serum Iron and Ferritin Concentrations

Interindividual variability was observed in baseline serum iron (39–196 μg/L) and ferritin (8.2–367.9 ng/mL) concentrations. The literature on the changes in iron status induced by endurance exercise is equivocal. A decrease in serum iron concentrations 24 h after a marathon was reported by Roecker et al. [23], by Terink et al. [13] after repeated walking and by Chiu et al. [24] after an ultramarathon. On the other hand, an increase in iron concentrations was reported by Peeling et al. [25] after a triathlon and by Buchman et al. [26] after a marathon. According to Terink et al. [13], these differences could be associated with changes in plasma volume and whether this parameter was taken into consideration before the analysis of the results. We corrected for changes in plasma volume and our findings were similar to those reported by Duca et al. [27]. These authors found no change in serum iron or ferritin at 24 h after a half-marathon. Similar findings in serum iron and ferritin concentrations at 24 and 48 h after a marathon were also reported by Weight et al. [28]. Unfortunately, there appear to be no studies in which ferritin and iron concentrations were determined at 3 h after exercise. It is important to note that at this time point increased serum hepcidin was observed [25], and it can be assumed that this would be accompanied by a drop in serum iron, which is consistent with our data. At 24 h after the marathon, basal values had been attained in the participants of our study. In contrast to our results, at the same time point (1 day after prolonged walking) Terink et al. [13] reported decreased iron concentrations. These authors also corrected their results for the change in plasma volume. Lack of a significant correlation between serum ferritin and iron was observed earlier by Galanello et al. [29]. These authors reported that after a stressful event such as a marathon run, the serum ferritin concentrations could not accurately reflect body iron status. Moreover, the observed nonsignificant changes in ferritin concentrations at 24 h after a marathon are in agreement with data reported by Terink et al. [13]. Indirectly, the nonsignificant changes in ferritin in our study might indicate low or no inflammation in the study participants (since Peeling et al. [16] reported an increase in ferritin during exercise-induced inflammation), low or no oxidative stress [15] and minimal damage including damaged blood cells [8].

4.2. Changes in the mRNA of Genes Involved in Iron Metabolism

The genes related to iron metabolism that were selected for analysis are easily induced by stressful conditions, and sensitive to intracellular iron concentrations, oxidative stress and hypoxia [30,31]. To the best of our knowledge, this is the first study in which changes in the mRNA of these genes were examined after a marathon run. The significant decrease in mRNA of *PCBP1* and *PCBP2* (expression partners) and *FTH* was observed post-race while at 3 h after the race an up-regulation occurred in *PCBP1* and *PCBP2* as well as in *FTH* and *FTL*. Furthermore, 24 h after the run the gene mRNA levels returned to baseline values. Unfortunately, discussion about these changes is hard since, as mentioned before, there are no data on this topic in the current literature. We assumed that the increase in the mRNA of genes involved in apoptosis and inflammatory response reported earlier [32] would cause long-term up-regulation in our tested genes i.e., that remained up-regulated 24 h after a marathon run. Unfortunately, this was not confirmed by our results. According to the literature, PCBP1 and PCBP2 proteins are iron chaperones that deliver iron to ferritin, the iron storage protein [17,33]. Thus, it is expected that an increase in the expression of these genes might play a protective role against iron toxicity. The mRNA levels of *TFRC* remained unchanged during the marathon run and in the recovery period (with a slight tendency to decrease compared to basal values) suggesting that the intracellular labile iron pool was kept under control. In turn, the *CAT* mRNA level decreased after the marathon run but also returned to baseline after 24 h. The results at 3 h after the marathon showed a significant increase in mRNA levels. It is established that during exercise, changes in many plasma or serum parameters influence intracellular homeostasis. Oxidative stress is another indicator of tested

gene expression; thus, we evaluated the mRNA levels of C*AT* for additional information on changes in intracellular oxidative stress. One of the functions of catalase is an increase in antioxidative capacity (Sureda et al. [34]), thus its expression indirectly shows the level of oxidative stress in the cell.

4.3. Relationship Between the mRNA of Genes Involved in Iron Metabolism and Running Speed

Generally, the same direction of changes in *PCBP1*, *PCBP2*, *FTH*, *FTL* and *TFRC* mRNA was observed in both groups, indicating a tendency to decrease immediately after the run and increase 3 h post-race. However, significant differences in *FTH* and *FTL* mRNA were observed between the slow and fast groups at 24 h after finishing the marathon. In faster participants, the mRNA levels of these genes were significantly lower compared to slower participants. According to Jastrzębski et al. [14], based on organ damage indicators, our findings could be caused by a better adaptation to a long-lasting effort in the faster group. The cited authors concluded that participants choose their running speed to individual possibilities determined by changes in tested parameters. In our opinion, the results obtained in our experiment, regarding changes in gene expression, indicating that this hypothesis could be true. Additionally, significant differences between groups (slow and fast) indicated that faster runners were significantly younger than slower runners. This finding indicated possibilities of influence of age to obtained results during marathon run. However, after dividing the participants of the run into two groups by the median split of age (younger and older), no significant differences between groups have been observed.

5. Conclusions

We concluded that marathon running induced changes in biochemical parameters and the expression of genes involved in iron metabolism, but these changes occurred at different time points. Interestingly, in faster runners, the return to basal values occurred faster than in slower runners. Generally, the amateurs could adjust the pace of the run to their capabilities.

Author Contributions: Conceptualization, J.A, W.R., A.G. and M.Ż.; methodology, M.Ż., A.G. and K.A.; validation, M.Ż., K.A., I.B. and A.G.; formal analysis, A.G.; investigation, I.B., W.R. and P.A.; resources, W.R., M.Ż. and P.A.; writing—original draft preparation, M.Ż. and A.G.; writing—review and editing, J.A.; visualization, K.A. and A.G.; supervision, J.A. and M.Ż.; project administration, J.A. and M.Ż.; funding acquisition, W.R and J.A.

Funding: This research was funded by the knowledge grant of the Polish Ministry of Science and Higher Education N RSA4 06754.

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

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Exercise Training and Vitamin C Supplementation Affects Ferritin mRNA in Leukocytes without Affecting Prooxidative/Antioxidative Balance in Elderly Women

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Received: 3 July 2020; Accepted: 1 September 2020; Published: 5 September 2020

Abstract: Physical training and antioxidant supplementation may influence iron metabolism through reduced oxidative stress and subsequent lowering of mRNA levels of genes that are easily induced by this stress, including those responsible for iron homeostasis. Fifteen elderly women participated in our 12-week experiment, involving six weeks of training without supplementation and six weeks of training supported by oral supplementation of 1000 mg of vitamin C daily. The participants were divided into two groups (n = 7 in group 1 and n = 8 in group 2). In group 1, we applied vitamin C supplementation in the first six weeks of training, while in group 2 during the remaining six weeks of training. In both phases, the health-related training occurred three times per week. Training accompanied by vitamin C supplementation did not affect prooxidative/antioxidative balance but significantly decreased ferritin heavy chain *(FTH)* and ferritin light chain (*FTL*) mRNA in leukocytes (for *FTH* mRNA from 2^64.24 to 2^11.06, p = 0.03 in group 1 and from 2^60.54 to 2^16.03, p = 0.01 in group 2, for *FTL* mRNA from 2^20.22 to 2^4.53, p = 0.01 in group 2). We concluded that vitamin C supplementation might have caused a decrease in gene expression of two important antioxidative genes (*FTH*, *FTL)* and had no effect on plasma prooxidative/antioxidative balance.

Keywords: supplementation; ageing; ferritin gene expression; FOXO3a; catalase; oxidative stress

1. Introduction

Oxidative stress can influence iron metabolism and iron-regulatory protein status [1]. Genes associated with cellular stress response and iron metabolism, such as *FTH1* (ferritin heavy chain 1), *FTL* (ferritin light chain), *PCBP1* (poly(rC)-binding protein 1), *PCBP2* (poly(rC)-binding protein 2), *FOXO3a* (forkhead box O3A), or *CAT* (catalase) are easily induced by oxidative stress. Oxidants can

directly induce ferritin gene expression by targeting conserved regions of these genes [2] as well as by regulating the activity of mRNA at transcriptional levels, and proteins at post-transcriptional levels [3]. For example, inactivation of iron-regulating protein 1 (IPR1) by oxidative stress can be associated with not blocking ferritin mRNA [4]. Moreover, expression of these genes is directly regulated by cellular iron status.

All genes investigated in this study are involved in iron homeostasis and are easily induced by stressors. *FTH* and *FTL* encode "acute phase" proteins. It is well known that these genes represent the level of the labile iron pool (LIP), and their upregulation or downregulation is associated with free iron levels [5,6].

In the literature PCBP1 and PCBP2 proteins are described as iron chaperones that deliver iron to ferritin [7,8]. Thus, an increase in expression of these genes may appear concurrently with a rise in the labile iron pool. However, little is known about how the factors that increase oxidative stress or antioxidant capacity affect mRNA levels of genes associated with iron metabolism.

An augmentation in oxidation accompanies exercise training but also ageing and some diseases. An easy way to increase antioxidant capacity is to supplement antioxidant vitamins such as vitamin C. This vitamin plays a crucial role in preventative therapy against ageing [9] and diseases [10,11]. However the data on its beneficial effects are conflicting and the findings vary with dose, age, and type of exercise [12–14].

It is well documented that supplementation with vitamins C and E during acute exercise did not attenuate oxidative stress in muscle cells [15]. Additionally, vitamin C also influences iron uptake by increasing its absorption from the gastrointestinal tract. These data support the close association between vitamin C, iron or ferritin concentration, and exercise.

According to Mankowski et al. [16], antioxidant supplementation can improve training effects by the reduction in exercise production of reactive oxygen species (ROS) but may also impair training adaptation. Moreover, antioxidative supplements could block an increase in exercise level through reduced expression of some genes (such as PGC-1α) associated with ROS-stimulated mitochondrial biogenesis. It is also unclear whether antioxidant supplementation influences a decrease of enzymatic protection against oxidative stress.

Unfortunately, to this day, there are no published data showing the effects of vitamin C supplementation combined with exercise in terms of adaptation of ferritin mRNA in leukocytes. Furthermore, there are no data which could answer the question of whether introducing supplementation of ascorbic acid during the training period is important in regard to iron metabolism. Moreover, in current data, there are many studies associated with antioxidant supplementation, but usually in healthy and active men or as an adjunct to treatment of cancer and other diseases. Thus, we decided to investigate the influence of 1000 mg vitamin supplementation used in elderly (postmenopausal) healthy women.

The aim of this study was to evaluate the effects of sessions of six-week endurance training with and without six-week oral vitamin C supplementation (1000 mg daily) on plasma oxidative stress and antioxidant capacity, as well as on genes associated with cellular stress response and iron metabolism. We assumed that an increase in antioxidant capacity, induced by training and vitamin C supplementation, would have resulted in a decrease in mRNA concentration of the tested genes. Moreover, we assumed that there were no differences in mRNA levels of genes associated with iron metabolism regardless of the timing of supplementation during training.

2. Methods

2.1. Subjects

The study protocol was completed by 15 women (from the total of 24 who started the trial) with a mean age of 69.2 ± 6.5 years and body weight of 72.9 ± 12.7 kg. We excluded from the analysis the participants who had less than 90% attendance in training. All participants were volunteer students at the University of the Third Age at the Gdansk University of Physical Education and had never trained

professionally before. The volunteers had to meet the following inclusion criteria: age between 60 and 80 years, a medical certificate confirming that there were no contraindications to participation in the research, no participation in other projects, a low level of physical activity, and a properly-balanced diet in terms of energy content and basic nutrients. We excluded from the study women who were under 60 or over 80 years of age; had movement limitations that disrupted training or who had ongoing injuries; had endoprostheses or other conditions that limited the possibility of performing tests (including InBody); had poor health in general, including neoplastic diseases, advanced cardio-respiratory diseases, arrhythmias, history of arterial congestion, hypertension (>160/100 mm Hg), transient ischemic attacks, thyroid malfunction, diabetes, were smokers, had high total cholesterol levels (>300 mg/dL), were on a weight-loss diet, or who were taking anti-inflammatory drugs. None of the women had a disease requiring constant treatment and all avoided supplementation for 3 months before the experiment. Additionally, only those women whose dietary intake of energy, protein, fat, and carbohydrates was within the norms established for a given age group for the Polish population qualified to participate in the project.

The women were divided randomly into two groups (simple randomization). Group 1 (n = 7, mean ± SD age of 68.75 ± 7.5 years and body weight of 74.26 ± 16.87 kg) received oral supplementation with vitamin C (1000 mg daily) for the first six weeks of training while group 2 (n = 8, mean ± SD age of 67.7 ± 5.6 years and bodyweight of 71.46 ± 5.39 kg) received cellulose. During the next 6 weeks, the groups received the alternative supplement. The study was double-blinded (products in caps, looking similar). Body mass analysis was performed using the InBody 720 composition analysis (Inbody, Biospace Co. LTD., Seoul, Korea). The anthropometric characteristics of the two tested groups are summarized in Table 1.

Table 1. Anthropometric characteristics of participants at baseline (n = 15).

Parameter	I (Baseline)		
	Group 1	Group 2	*p*
VO_2 max. (mL/kg/min)	20.69 ± 4.05	19.73 ± 2.45	0.61
Body mass (kg)	74.26 ± 16.87	71.46 ± 5.39	0.68
Fat (kg)	29.86 ± 13.86	29.74 ± 3.12	0.98
Muscle mass (kg)	24.07 ± 3.76	22.46 ± 2.17	0.34

2.2. Ethics Approval and Consent to Participate

This study was approved by the Bioethics Committee for Clinical Research at the Regional Medical Chamber in Gdansk (permission number KB-10/16, Gdansk, 12.04.2017) and conducted according to the Declaration of Helsinki. All participants gave their written, informed consent prior to participation and were informed about the possibility of withdrawal at any time for any reason.

2.3. Diet Analysis

All participants recorded their dietary intake for 5 days—3 working days and 2 holiday days at the beginning of the experiment—before starting the training and vitamin C supplementation. The obtained data were analyzed using the Diet 5.0 software, developed by the Food and Nutrition Institute (Warszawa, Poland). Meal sizes were estimated using the photo album of products and dishes [17]. On this basis, the average amount of energy consumed per day, as well as the percentage of proteins, fats, and carbohydrates, were calculated. Participants were asked not to change their nutritional habits during the study. The study took place between November and February i.e., in the winter season, and there was no seasonal change in nutrition.

2.4. Training

The training program was implemented using the basic principles of health-related training. The 15 women participated in a 12-week multidisciplinary training program (2 × 6-week periods, three times per week for 60 min) consisting of water gymnastics, gyrokinesis, stabilization training, and Nordic walking at moderate intensity. Each 6-week training period involved 1080 min of exercise (total duration of 2160 min). During each session, participants maintained their heart rate (Polar H1) at less than 130 beats/min.

2.5. Supplementation

In the first 6 weeks of training, group 1 received 1000 mg of Vitamin C (Max VitaC 1000, Colfarm, Poland) and group 2 received cellulose in tablets (Colfarm, Poland). In the following 6 weeks of the training period, participants received the alternative supplement (vitamin C or placebo). Neither they nor the people carrying out the experiment knew what supplements had been given. The choice of vitamin C dose was due to its easy availability and high frequency of consumption in the Polish population.

2.6. Report of Determination of VO_2 Max

The VO_2 max. was determined directly with the cycloergometer, Ergoline Ergoselect 150p (Jaeger OxyconPro) and a gas analyzer (Jaeger OxyconPro).

Each participant was tested according to the following procedure: 2 min for registration of resting values followed by 5 min of a warm-up with 30 W load and 60 rpm cadence; then the commencement of the appropriate exercise test with a gradual increase of the load by 10 W in intervals of 1 min. Each participant's test was interrupted when they were exhausted and unable to continue with a given power and the required term of 60 rpm, or after the occurrence of symptoms indicative of the need to end the trial. The results of the VO_2 max. are presented in Table 1.

2.7. Blood Collection, Analysis of Vitamin C Concentration, Total Oxidative Status (TOS)/Total Oxidative Capacity (TOC), Total Antioxidative Status (TAS)/Total Antioxidative Capacity (TAC) Analysis and Genetic Research

Three blood samples (5 mL) were collected from each participant—at baseline and 24 h after the first and second training periods.

2.8. Genetic Evaluation

RNA was isolated using the procedure of Chomczynski and Sacchi [18] as described previously by Grzybkowska et al. [6]. Venous blood (2 mL) was collected into special Vancouver tubes with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. To eliminate erythrocytes, blood samples were treated with red blood cell lysis buffer (RBCL) (A&A Biotechnology, Gdynia, Poland) on ice (20 min) and centrifuged (3000× *g* at 4 °C for 10 min). The obtained leukocytes were lysed using Fenozol (A&A Biotechnology, Gdynia, Poland). After 5 min incubation 200 µL of chloroform (POCH, Gliwice, Poland) was added and the suspension was shaken. The water phase was moved to another Eppendorf tube, and to precipitate RNA, 500 µL of isopropanol (POCH, Gliwice, Poland) was added. Then samples were centrifuged again (10,000× *g*, 30 min, 4 °C). The obtained pellet of RNA was washed two times in 1 mL of 75% ethanol and centrifuged one more time (7500× *g* at 4 °C). Then, ethanol was removed and the pellet was set aside to dry. Dry RNA was diluted in 20 µL molecular grade water. Purity and quality were evaluated spectrophotometrically (BioPhotometer Plus, Eppendorf, Germany). For reverse transcription, 1000 ng of pure RNA (A260/280 ≥ 1.7) was used (AffinityScript QPCR cDNA Synthesis Kit: Agilent, Poland) on Eppendorf Mastercycler Gradient 5331. The profile of the reaction was in accordance with the manufacturer's instructions. Obtained cDNA was diluted 10-fold indirectly before the PCR reaction. Each gene's expression was detected using quantitative real-time PCR (qRT-PCR, Aria, Agilent, Department Poland). To amplify gene expression the following primers were used:

- For *TUBB* (tubulin beta class I, NM_001293213): forward primer: TCCACGGCCTTGCTCTTGTTT and reverse primer: GACATCAAGGCGCATGTGAAC;
- For *FTH1* (NM_002032): forward primer: TCCTACGTTTACCTGTCCATG and reverse primer: CTGCAGCTTCATCAGTTTCTC;
- For *FTL* (NM_000146): forward primer: GTCAATTTGTACCTGCAGGCC and reverse primer: CTCGGCCAATTCGCGGAA;
- For PCBP1 (NM_006196): forward primer: AGAGTCATGACCATTCCGTAC and reverse primer: TCCTTGAATCGAGTAGGCATC;
- For PCBP2 (NM_001128913): forward primer: TCCAGCTCTCCGGTCATCTTT and reverse primer: ACTGAATCCGGTGTTGCCATG;
- For *CAT* (NM_001752): forward primer: GATGGACATCGCCACATGAAT and reverse primer: AAGATCCCGGATGCCATAGTC;
- For *FOXO3A*: forward primer: TTCAAGGATAAGGGCGACAGC and reverse primer: CCCATCAGGGTTGATGATCCA.

2.9. Vitamin C Plasma Concentration Measurement

Plasma vitamin C concentrations were determined using the method of Robitaille and Hoffer [19]. In brief, cold trichloroacetic acid (TCA) 20% (0.4 mL) and cold dithiothreitol (DTT) 0.2% (0.4 mL) were added to 0.2 mL of plasma. The samples were then vortexed for 2 min, centrifuged (10,000× *g* at 4 °C for 10 min), and the resultant supernatant was frozen at −80 °C for further analysis. Analysis of vitamin C concentrations was performed in an accredited laboratory in Krakow. Concentrations were measured using high-performance liquid chromatography (HPLC) with UV detection (wavelength: 245 nm). Chromatography was performed on a reverse-phase chromatographic column (RP-18) (Merck, Darmstadt) with a length of 25 cm, diameter 4.6 mm, and a grain diameter of 5 µm, and the mobile phase pH was 2.7. A 2140 Rap1d Spectral Detector Optical Unit UV detector (LKB Bromma, Sweden) and Rheodyne® (Model 7010) injector (Rheodyne, Germany) were used. The retention time of ascorbic acid was 3 min. [19].

2.10. TOS/TOC and TAS/TAC Analysis

For TOS/TOC and TAS/TAC analysis, venous blood was collected into BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged (3000× *g* at 4 °C for 10 min), and the obtained plasma was stored immediately at −80 °C for further analysis. To evaluate TOS/TOC a photometric PerOx assay kit was applied (Immundiagnostik AG, Germany), and for TAS/TAC an ImAnOx assay kit (Immundiagnostik AG, Germany) was applied. Prooxidative/antioxidative balance was also calculated. The photometric PerOx test was applied (Immunodiagnostic, Department Poland); for TAS, ImAnOx (Immunodiagnostic, Department Poland) was applied.

2.11. Statistical Analysis

For all results, the presence of a normal distribution was checked with the Shapiro–Wilk test. Results before and after each six-week training period were compared with parametric or non-parametric (Wilcoxon) tests, as appropriate. To evaluate within-person changes, the paired *t*-test and ANOVA one-way test were applied. The prooxidative/antioxidative balance was calculated as the prooxidative/antioxidative ratio. The genetic data were collected and relative gene expressions were analyzed in Microsoft Excel 2015. The level of mRNA was calculated using the comparative method of Schmittgen and Livak [20]. The mRNA levels of the tested genes were described as the differences in the cycle threshold values normalized to the TUBB mRNA levels, i.e., $\Delta C_T = C_T$ of gene $- C_T$ of TUBB. The data were transformed into linear values, and statistical significance was evaluated using the Shapiro–Wilk test to assess for normal distribution and the Wilcoxon test for comparison of results before and after each training period. To determine the significance of differences between

the groups, *t*-test and ANOVA two-way were applied. All calculations and graphics were performed using GraphPad Prism 6.0. Differences were considered statistically significant at a level of $p \leq 0.05$.

3. Results

No changes in VO_2 max. were observed during the experiment, both during the training period supported by supplementation and without supplementation (see Table 2 for reference). Body mass significantly decreased in group 2 after the training period with 1000 mg Vitamin C supplementation, however, in both groups, the tendency to decrease was noted in the second six weeks of training. Fat mass significantly decreased in both groups after 12 weeks of training (compared to baseline). Interestingly, muscle mass significantly decreased in group 2 after 12 weeks of training. No significant differences between groups were observed in tested parameters, independently on supplementation time (first or second six weeks of training, Table 2).

Table 2. Anthropometric characteristics of participants after 6 (II) and 12 (III) weeks of training (n = 15).

Parameter		II	*p* (I vs. II)	III	*p* (I vs. III)	*p* (II vs. III)
VO_2 max. (mL/kg/min)	Group 1	20.96 ± 4.47	0.67	21.63 ± 4.66	0.13	0.49
	Group 2	19.62 ± 2.04	0.79	20.62 ± 1.48	0.30	0.27
	***p* (Group 1 vs. Group 2)** = 0.51			***p* (Group 1 vs. Group 2)** = 0.62		
Body mass (kg)	Group 1	73.33 ± 16.15	0.55	71.79 ± 16.24	0.13	0.07
	Group 2	70.07 ± 7.12	0.38	65.50 ± 6.95 *,#	0.03	0.04
	***p* (Group 1 vs. Group 2)** = 0.63			***p* (Group 1 vs. Group 2)** = 0.36		
Fat (kg)	Group 1	26.87± 10.67	0.14	24.98 ± 14.37 *	0.02	0.45
	Group 2	26.60 ± 6.18	0.12	23.07 ± 7.68 *	0.02	0.13
	***p* (Group 1 vs. Group 2)** = 0.95			***p* (Group 1 vs. Group2)** = 0.75		
Muscle mass (kg)	Group 1	25.58 ± 4.79	0.55	25.89 ± 4.75 *	0.04	0.85
	Group 2	23.57 ± 2.24	0.07	23.07 ± 1.91	0.45	0.45
	***p* (Group 1 vs. Group 2)** = 0.60			***p* (Group 1 vs. Group 2)** = 0.15		

Baseline values are summarized in Table 2 (I). II—24 h after 6 weeks of training and III—24 h after 12 weeks of training. Values are means (±SD); * significant differences between I and III; # significant differences between II and III.

3.1. Gene Expression

No significant differences in mRNA levels were observed between groups at baseline. However, mRNA levels of the two ferritin genes significantly decreased after six weeks of training supported by Vitamin C supplementation (in group 1 from 2^64.24 to 11.06, $p = 0.03$ and in group 2 from 2^53.77 to 2^16.03, $p = 0.03$, Figure 1A). At the same time, the decrease in *FTL* mRNA was observed in both tested groups however, significant changes were observed only in group 2 (for group 1 from 2^14.94 to 2^7.64 and for group 2 from 2^18.53 to 2^4.53, $p = 0.01$, Figure 1B). No significant changes were observed for other tested genes, although a slight increase in *PCBP1* and *PCBP2* mRNA and decrease in *FOXO3A* and *CAT* were observed in both groups (Figure 1C–F). There were low levels of *CAT* and *FOXO3a* mRNA throughout the study (Figure 1E,F).

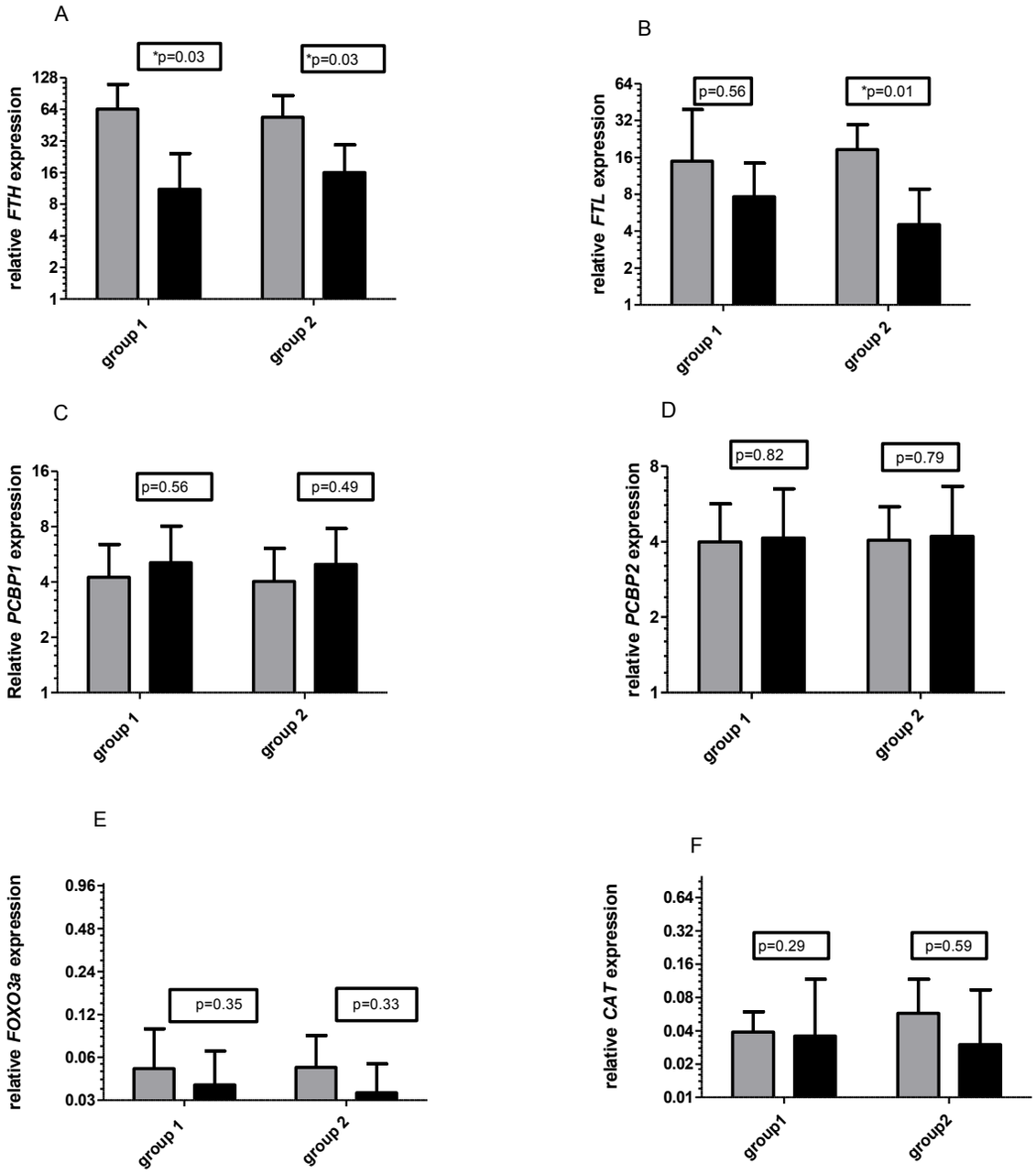

Figure 1. Changes in relative expression (2^) of ferritin heavy chain (*FTH*) (**A**), ferritin light chain (*FTL*) (**B**), poly(rC)-binding protein 1 (*PCBP1*) (**C**), poly(rC)-binding protein 2 (*PCBP2*) (**D**), forkhead box O3A *(FOXO3a*) (**E**), and catalase (*CAT*) (**F**) before (grey bars) and after 6 weeks (dark bars) training period supported by Vitamin C supplementation. All mRNA expressed as 2^ relative expression/TUBB. * significant differences within the group, compared to the baseline value.

No significant differences in the mRNA of tested genes were observed during six weeks of the unsupplemented training period (Figure 2). At the end of the experiment independently of the time of supplementation (at first or second six weeks of training), there were no differences between groups in RNA levels. As for the effect of supplementation in different periods, the differences in *FTH* mRNA between groups before the unsupplemented period were significant ($p = 0.01$).

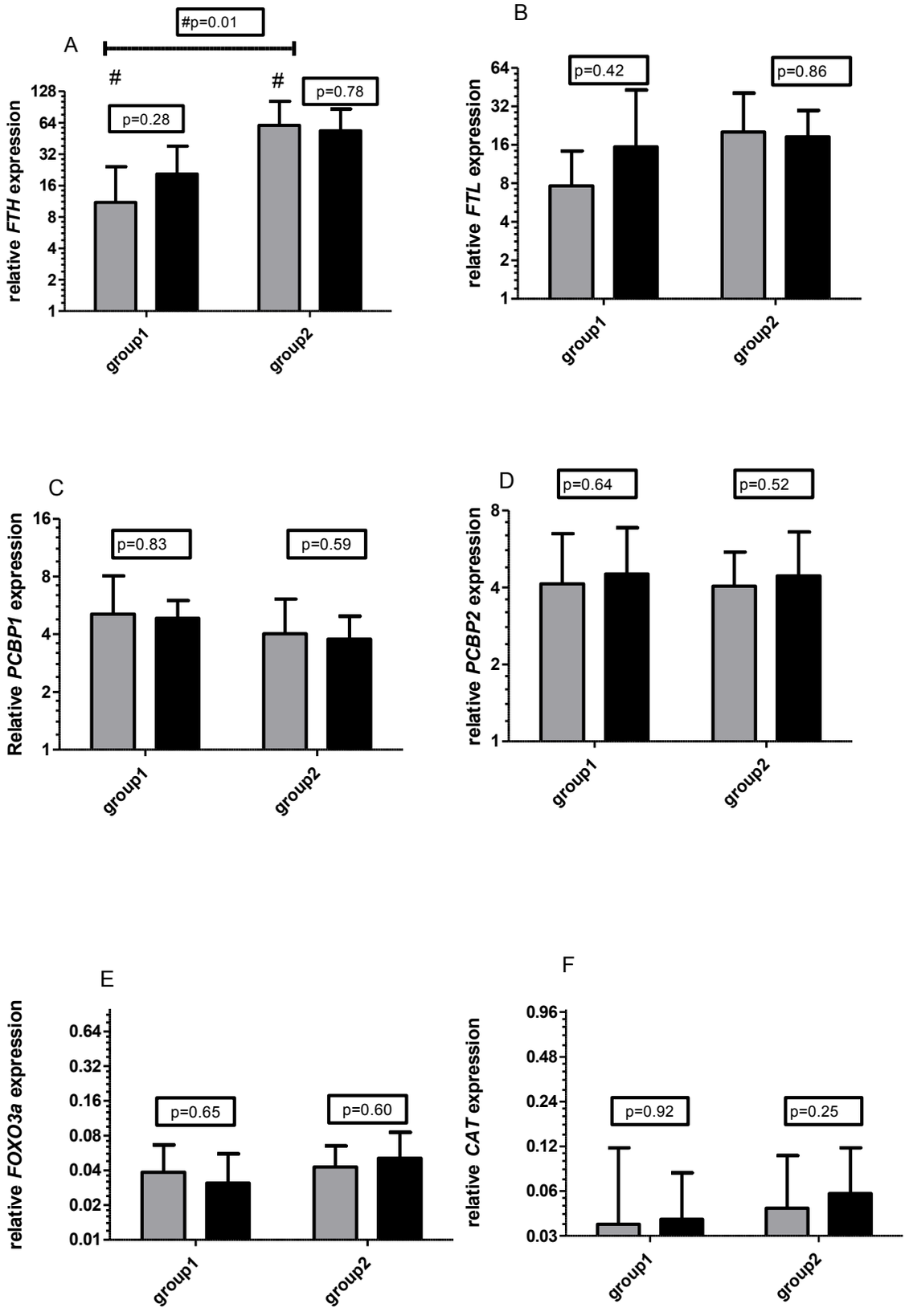

Figure 2. Changes in relative expression (2^) of *FTH* (**A**), *FTL* (**B**), *PCBP1* (**C**), *PCBP2* (**D**), *FOXO3a* (**E**), and *CAT* (**F**) before (grey bars) and after 6 weeks (dark bars) unsupplemented training period. # significant differences between groups. All mRNA expressed as 2^ relative expression/TUBB.

3.2. Control of Diet and Vitamin C Concentration

Dietary analysis indicated energy shortages in group 2 deviating from standards for the Polish population amounting to 1900 kcal/day. Protein intake accounted for 17% of the caloric value of the diet in both groups and was within the norm of 15–20% of energy. Fat intake differed in both groups from the norm for women with the same body weight and age of 79 g/day. Consumption of carbohydrates in group 1 accounted for 50% of the calorie diet value in group 2 and constituted 47% of the calorific value and was in the norm of 45–65% of energy [21] (Table 3).

Table 3. Average daily energy values and nutrients of the female diet.

Diet Analysis	Group 1	Group 2
Energy (kcal)	2069 ± 702	1634 ± 245
Proteins (g/day)	90 ± 32	71 ± 20
Fat (g/day)	75 ± 29	62 ± 12
Carbohydrates (g/day)	260 ± 130	196 ± 22
Vit C (mg)	154 ± 105	141 ± 56
Vi C (mg/kg b. m.)	2.13 ± 1.5	2.00 ± 0.79

Further, the participants achieved the recommended daily intake of vitamin C of around 100 mg per day via consumption of foods like peppers, apples, and oranges. Participants in group 2 had lower consumption of kilocalories per day resulting from a significantly-lower amount of fat in their diet. Both groups had a similar intake of vitamin C per kg of body weight.

The volunteers in group 1 had a mean basal vitamin C plasma concentration of 12.2 mg/L, while those in group 2 had a mean concentration of 14.8 mg/L. All participants had basal vitamin C concentrations within the physiologically normal range (6–20 mg/L). After six weeks of training with supplementation, the vitamin C plasma concentration in both groups increased significantly (in group 1 mean value changed from 12.2 to 14.9 mg/L and in group 2 from 14.8 to 19.5 mg/L, $p = 0.03$). Six weeks of training without vitamin C supplementation did not affect the plasma concentration of vitamin C. There were no significant differences between groups in the plasma vitamin C concentrations during the whole experiment (Figure 3A,B).

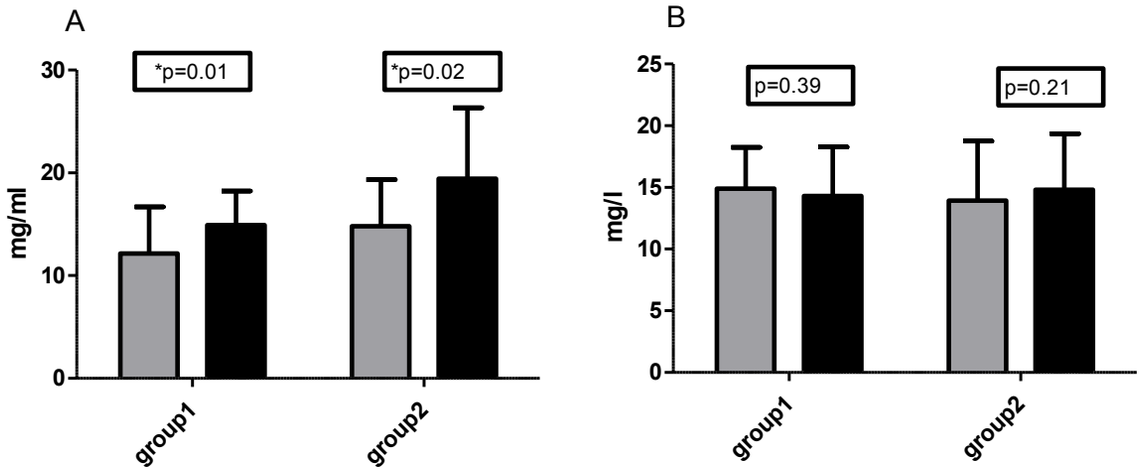

Figure 3. Changes in Vitamin C concentration before (grey bars) and after 6 weeks of training (dark bars). (**A**) Training period supported by supplementation and (**B**) unsupplemented training period. * significant differences compared to the baseline value ($p < 0.05$).

3.3. TOS/TOC and TAS/TAC Analysis

High levels of total oxidative status were maintained throughout the study in both groups (≥350 umol/L for plasma). Little change in TOS was noted during the experiment, although the mean values after supplementation were slightly higher in both groups (for Group 2 the increase was from 465 to 509 umol/L, Figure 4A). In the supplementation period, TAS/TAC status, as well as prooxidative/antioxidative ratio, remained unchanged (Figure 4B,C). The TAS level in participants was around the average or less of the physiological norm.

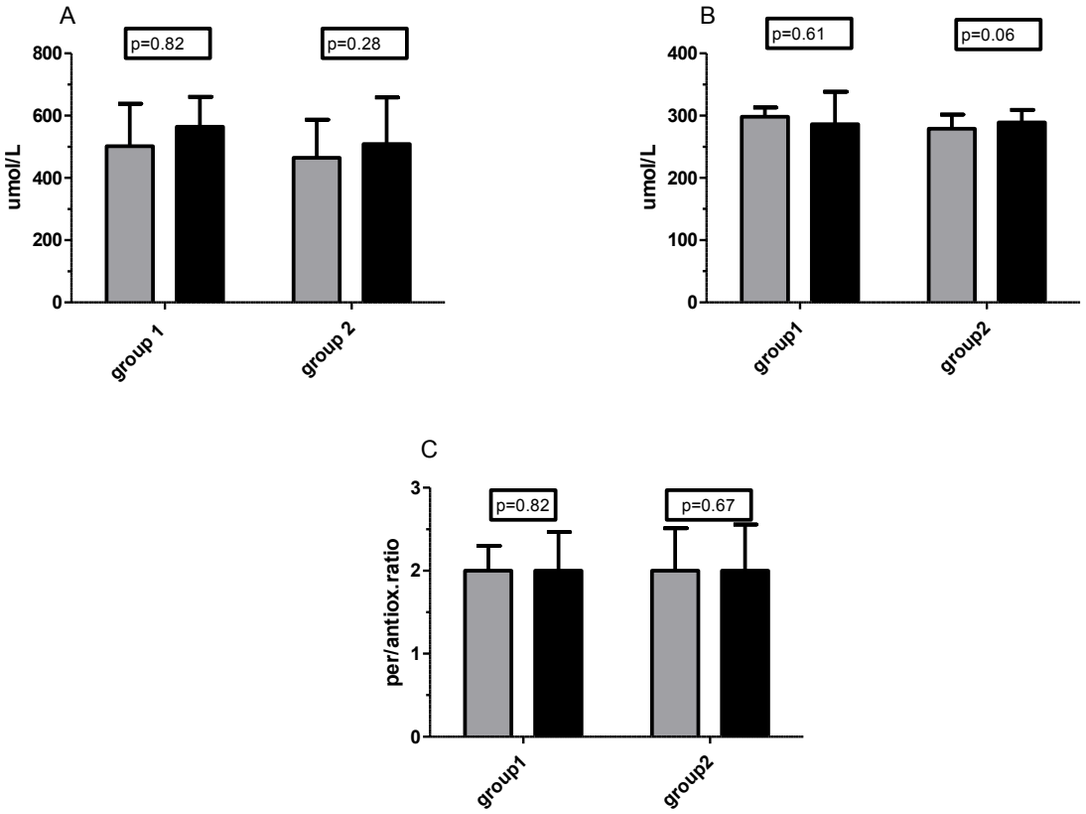

Figure 4. Changes in total oxidative status (TOS)/total oxidative capacity (TOC) (**A**), total antioxidative status (TAS)/total antioxidative capacity (TAC) (**B**), and prooxidative/antioxidative ratio (**C**) before (grey bars) and after 6 weeks of training (dark bars) supported by Vitamin C supplementation.

During six weeks of training without supplementation, there were also no significant changes in total oxidative/antioxidative status as well in prooxidative/antioxidative ratio (Figure 5A–C).

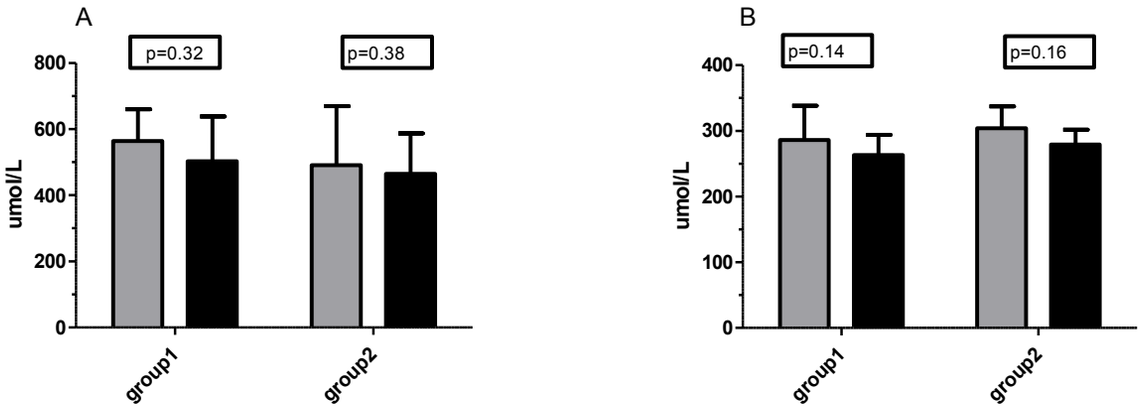

Figure 5. *Cont.*

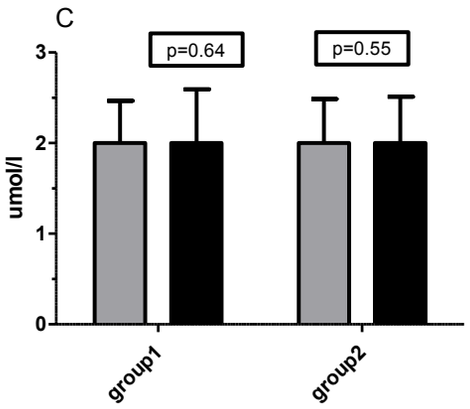

Figure 5. Changes in TOS/TOC (**A**), TAS/TAC (**B**), and prooxidative/antioxidative ratio (**C**) before (grey bars) and after 6 weeks of the unsupplemented training period (dark bars).

4. Discussion

In our study, we hypothesized that vitamin C would increase antioxidant capacity and simultaneously decrease oxidative stress and that the expression of genes associated with oxidative stress, such as *FTH*, *FTL,* and *FOXO3a* in leukocytes would decrease. Our findings only partially confirmed these hypotheses. First, after vitamin C supplementation, neither group experienced an increase in TAS/TAC, although both showed a tendency to an increase in TOS/TOC. This means that vitamin C could have a prooxidative effect. During the study period, the prooxidative/antioxidative status remained unchanged at a value of 2.0, indicating high oxidative stress according to reference values. At the same time, plasma vitamin C concentration increased as an effect of supplementation. Second, the decrease in mRNA of *FTH* and *FTL* (both significant) as well as of CAT and FOXO3a, and a slight tendency to increase in *PCBP1* and *PCBP2* mRNA caused by vitamin C did not occur when the participants trained without supplementation. Moreover, the results suggest that the influence of vitamin C on genes associated with iron metabolism is much more important than the applied training.

According to the literature, antioxidants may influence both oxidative stress and iron metabolism [22]. The labile iron pool (LIP) is strictly regulated as it is crucial for cell survival, and to prevent iron toxicity [22,23]. One of the most important proteins involved in the prevention of iron toxicity is ferritin, which is encoded by *FTH1* and *FTL* genes. The main function of ferritin is to store iron intracellularly [24], thus it is a key molecule that limits the oxidative stress [3] and is involved in iron homeostasis [25]. Moreover, ferritin gene expression and post-transcriptional regulation of mRNA activity are sensitive to oxidants [23]. Post-transcriptional activity by oxidants is associated with inactivation of IRP1 through reversible oxidation of critical cysteine residues [2].

Some published studies showed a decrease in plasma iron and ferritin after 12 and 32 weeks of Nordic-walking training [26,27]. The findings of these studies indicate that observed changes in iron metabolism are an important part of adaptation to the training. Unfortunately, in our study, we did not observe significant changes in mRNA levels of tested genes after the applied training. Significant decrease in *FTH* and *FTL* mRNA levels occurs only after the training period supported by vitamin C supplementation.

To our knowledge, there are no published studies investigating the effects of training supported by vitamin C supplementation on *FTH*, *FTL*, *FOXO3a, PCBP1, PCBP2,* or *CAT* mRNA levels in human leukocytes. Our results showed that independent of the length of the training period, a significant decrease in *FTH* mRNA was observed after vitamin C supplementation. A decrease in *FTL* mRNA may indicate or lower oxidative stress within cells in subjects after vitamin C supplementation. Genes encoding iron chaperones (*PCBP1* and *PCBP2)* did not change their expression significantly. This may suggest that intracellular free iron level has not changed. Furthermore, *FOXO3a* and *CAT* have apoptosis-regulation properties [28,29]. Recently it has been shown that FOXO3a controls the expression of ferritin and catalase mRNA [30,31], thus the next goal of this study was to determine the effect of

training and supplementation on the expression of these genes. Unfortunately, no significant changes were observed for *FOXO3a* and *CAT* mRNA. Although there were no statistically-significant differences, we observed a similar tendency of changes in *CAT* mRNA levels after vitamin C supplementation compared to *FTH* and *FTL*. In summary, the effect of vitamin C supplementation on leukocytes supports the decrease in intracellular oxidative stress (tendency to decrease *FOXO3a* and *CAT* mRNA, and a significant decrease of *FTH* and *FTL* mRNA) and should be considered positive in regard to human health.

Interestingly, a significant difference between groups was observed in *FTH* mRNA levels before the unsupplemented period. This difference could be associated with the fact, that group 1 had foregone vitamin C supplementation for six weeks before going through the unsupplemented period. Moreover, this effect could still be observed at the end of the experiment (after another six weeks). It is possible that the use of antioxidant supplementation has a long-term effect. However, the duration of the supplementation effect requires further research.

Vitamin C Concentration and Plasma Pro-/Antioxidant Capacity

Plasma vitamin C concentrations of the participants of our study were in the middle and (after the supplementation) upper range of normal. According to Pearson et al. [32], women have higher vitamin C status compared to men. Moreover, these authors postulated that people with higher vitamin C status exhibit lower weight, body mass index and waist circumference and that their indicators of metabolic health were better. In our study, we have observed a significant decrease in body mass after training in both groups and a substantial decrease in mean body mass after supplementation in group 2. However, after the supplementation, there was a tendency towards increased TOS/TOC in both groups. During our study, participants had high oxidative stress and supplementation did not change the prooxidative/antioxidative balance. The same results were obtained by Bunpo and Anthony [33]. These authors evaluated the changes in oxidative status in healthy young people after 12 weeks of moderate exercise (three times a week) supported by vitamin C in doses of 250 and 500 mg per day. They observed no change in TOS/TOC or TAS/TAC and a slight reduction in antioxidative enzymes (including CAT) in erythrocytes. As a result of this, in the presence of Fe^{3+} or Cu^{2+}, vitamin C could promote a generation of the reactive oxygen species i.e., OH, O^{2-}, H_2O_2, and ferryl ion [34]. In the current literature, some published reports have described a prooxidative role of vitamin C [34]. Hydrogen peroxide formation increased under the influence of high dose vitamin C and in the presence of an appropriate concentration of iron ions [35,36]. Further, Tsuma-Kaneko et al. [37] suggested that this could exert anti-cancer effects. On the other hand, in patients with chronic lymphocytic leukaemia, vitamin C in a dose of 1000 mg/day had a positive influence on the incidence of infectious complications [38].

Vitamin C is not the only antioxidant that can manifest antioxidant and prooxidant properties. There are some studies in which both effects were observed, not only in humans but also in other species [39,40]. According to Tofolean et al. [41] another antioxidant, epigallocatechin-3-gallate (EGCG), could influence the prooxidative or antioxidative effects, and these effects were strongly associated with used dose [41]. This study was conducted on Jurkat T-cells in human leukaemia.

Gheorghe et al. [42] postulated that the benefits of antioxidant supplementation are dependent on initial antioxidant concentration. The authors suggested that in low dose, antioxidants may improve liver function and in high dose, substance-specific adverse effect was detected [15]. Similar findings associated with supplementation of another vitamin which is associated with antioxidative action (vitamin D) were reported by Zhang et al. [43]. Authors postulated that the relationship between serum vitamin D and the risk of liver cancer could be inverse.

Our study was conducted on healthy elderly women. Ageing is accompanied by an increase in oxidative stress. However, the interpretation of changes in oxidative stress and its influence on ageing is difficult because increased ROS production is associated with healthy ageing as well as ageing caused by inflammation. In the literature, the results related to oxidative stress and ageing obtained by

various authors are inconclusive. Some authors postulated an oxidative damage theory of mammalian ageing [44] and indicated that permanent metabolic slowing accompanied by a reduction in oxidative stress could prevent ageing. Further, a healthy diet and regular exercise can be useful to prevent this kind of ageing. According to Bhatti et al. [45], anti-ageing strategies are mainly focusing on reducing mitochondrial dysfunction and oxidative stress. Thus, lifestyle, diet, supplementation, and physical exercise can influence ageing, e.g., through overexpression of sirtuins, which may be inhibited by oral antioxidant supplementation [46]. El Assar et al. [47] postulated that there are two main mechanisms of ageing-related endothelial dysfunction (a determinant factor for cardiovascular disease and health status in the elderly)—oxidative stress and inflammation. They also indicated that chronic activation of NF-κB and downregulation of sirtuins and SOD2 intensify the cellular response to acute ROS generation. Interventions focused on the recovery of endogenous antioxidant capacity and cellular stress response, rather than exogenous antioxidants, could reverse oxidative stress inflammation. This opinion is consistent with our results. In our study, volunteers using supplementation did not manifest a reduction in oxidative stress. In another study investigating the influence of supplementation on adaptation to training, Yfanti et al. [14] concluded that a dose of 1000 mg of vitamin C daily did not alter endurance training adaptation, which is compatible with our observation. The authors observed an increase in VO_2 max but no difference in metabolic indicators in muscle cells between the supplemented and unsupplemented group. In our study, there was no significant change in VO_2 max, but changes in body composition were associated with the length of training.

Thus, in our opinion Vitamin C in a dose of 1000 mg/day did not bring the expected effect in a reduction in oxidative stress and its influence on this parameter was neutral. Despite the influence that vitamin C has on oxidative status, changes in ferritins mRNA levels were observed during the supplementation period, however in group 1 these lasted until the end of training. This could indicate a positive effect of vitamin C supplementation in terms of adaptation to training, but further research is needed to confirm this theory.

5. Conclusions

Our findings indicated that six weeks of training supported by daily supplementation of 1000 mg of vitamin C did not influence prooxidative/antioxidative balance but did cause a significant decrease in *FTH* and *FTL* mRNA levels in elderly women. We concluded that vitamin C supplementation had a greater effect on gene expression (and could indirectly indicate lower oxidative stress within the cells), compared with its influence on plasma prooxidative/antioxidative balance. The influence on ferritin mRNA levels could indicate a positive effect of supplementation on adaptation to training in elderly women. Additionally, this effect can be observed during the following six weeks of training. Further, the use of supplementation later in the training period caused major changes in body mass, muscle mass, and *FTL* mRNA, but there were no significant differences between the groups. In our opinion, in the absence of plasma vitamin C levels indicative of deficiency, the need for its supplementation is questionable.

Study Limitations

Our study has some limitations. We investigated the changes caused by training supported by vitamin C supplementation in healthy, elderly women, and thus our findings cannot be extrapolated with confidence to the women of different ages or males. Moreover, only one dose (1000 mg per day) of vitamin C was used. The effects of supplementation may be dose-dependent.

Author Contributions: Conceptualization, M.Ż., W.P., and J.A.; methodology, M.Ż., J.A., W.P., O.C.-L., and A.P.; software, R.U. and M.Ż.; validation, M.Ż., A.G., and W.P.; formal analysis, M.Ż.; investigation, A.G., M.W., M.Ż., R.U., O.C.-L., and J.A.; data curation, M.W., R.U., O.C.-L., A.G., M.Ż., and A.P.; writing—original draft preparation, M.Ż. and A.G.; writing—review and editing, M.Ż., A.G., and J.A.; visualization, M.Ż.; A.P.; and O.C.-L.; supervision, J.A.; project administration, J.A.; funding acquisition, J.A., W.P., and M.Ż. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Polish Ministry of Science and Higher Education grant number No. NRSA 406,754 and the APC was funded by 169/BS/KK/2018.

Acknowledgments: The authors thank Ewa Sztupecka for providing the equipment and technical assistance in obtaining the results.

Conflicts of Interest: The authors declare that they have no competing interests.

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International Journal of
Molecular Sciences

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Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans

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Abstract: The quantitative polymerase chain reaction (qRT-PCR) technique gives promising opportunities to detect and quantify RNA targets and is commonly used in many research fields. This study aimed to identify suitable reference genes for physical exercise and omega-3 fatty acids supplementation intervention. Forty healthy, physically active men were exposed to a 12-week eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation and standardized endurance training protocol. Blood samples were collected before and after the intervention and mRNA levels of six potential reference genes were tested in the leukocytes of 18 eligible participants using the qRT-PCR method: GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), ACTB (*Beta actin*), TUBB (*Tubulin Beta Class I*), RPS18 (*Ribosomal Protein S18*), UBE2D2 (*Ubiquitin-conjugating enzyme E2 D2*), and HPRT1 (*Hypoxanthine Phosphoribosyltransferase 1*). The raw quantification cycle (Cq) values were then analyzed using RefFinder, an online tool that incorporates four different algorithms: NormFinder, geNorm, BestKeeper, and the comparative delta-Ct method. Delta-Ct, NormFinder, BestKeeper, and RefFinder comprehensive ranking have found GAPDH to be the most stably expressed gene. geNorm has identified TUBB and HPRT as the most stable genes. All algorithms have found ACTB to be the least stably expressed gene. A combination of the three most stably expressed genes, namely GAPDH, TUBB, and HPRT, is suggested for obtaining the most reliable results.

Keywords: gene expression; mRNA levels; polyunsaturated fatty acids; n-3 PUFAs; endurance training

Citation: Grzybkowska, A.; Anczykowska, K.; Antosiewicz, J.; Olszewski, S.; Dzitkowska-Zabielska, M.; Tomczyk, M. Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans. *Int. J. Mol. Sci.* **2023**, *24*, 6734. https://doi.org/10.3390/ijms24076734

Academic Editor: Alessandro Fatica

Received: 1 March 2023
Revised: 29 March 2023
Accepted: 30 March 2023
Published: 4 April 2023

1. Introduction

Quantitative polymerase chain reaction (qRT-PCR) is a versatile technique that is widely used in many research fields to determine the relative change in mRNA levels of tested genes. Gene expression analysis has become more affordable and accessible over the last 15 years [1]. Evaluating gene expression in human leukocytes has been previously used in, e.g., cancer [2], multiple sclerosis research in human cells [3], as well as in other species [4]. However, its application in studies involving physical training and supplementation is limited. A salient feature of qRT-PCR is the determination of relative gene expression results represented as a quantification cycle (Cq) value. The Cq value indicates the PCR cycle number at which the fluorescent signal generated by the amplification of the target gene surpasses the background fluorescence level. Obtaining relative results means that data need to be normalized with at least two or three stably expressed genes, called reference genes. However, the Cq value is not the only result obtained from qRT-PCR and should be taken into consideration together with other key measurements, such as amplification efficiency and melting curve analysis.

The identification of stably expressed genes in human cells, that could be used as reference genes in research, is fundamental for obtaining reliable and reproducible results, yet the use of a single reference gene without proof of validation is common in literature [5].

It has also been demonstrated that reference genes must vary for different types of cells and interventions, as no single gene could be used as a reference [6]. Hence, the use of multiple (usually two or three) reference genes should be adopted as a gold standard, as it significantly reduces the risk of producing artefactual data [7,8]. MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines state that reference genes should be chosen and validated according to the specific experimental design [9]. Using several mathematic algorithms is in line with this recommendation. Physical activity can influence leukocytes, as they are powerful mediators that produce chemokines, cytokines, and growth factors, which are crucial for recovery and adaptive processes [10]. Similarly, omega-3 polyunsaturated fatty acids (n-3 PUFAs) are proven to influence DNA (Deoxyribonucleic acid) methylation changes in leukocytes, which is one way of regulating gene expression [11]. These two factors are considered to be promising targets for improving human health and could influence a great number of mRNA levels in leukocytes. There is growing evidence that increasing the amount of omega-3 fatty acids in the diet, particularly EPA and DHA acids, can promote a number of health benefits, including but not limited to the regulation of vascular and immune functions or inflammation [12]. However, there are few long-term studies in physically active individuals evaluating the effects of omega-3 fatty acids supplementation along with endurance training, on physical performance indicators [13], which was the aim of our previous work [14]. The pleiotropic effect of omega-3 fatty acids in the human body has been confirmed by published papers [15]. It has also been proven on a molecular level. In this regard, Bouwens et al., found that high doses of EPA and DHA supplementation altered the expression of 1040 genes [16]. This indicates the complexity and importance of finding genes that are not affected by omega-3 ingestion and thus, present stable expression in qRT-PCR research.

The purpose of the present study was to identify stably expressed genes in leukocytes obtained from healthy, physically active men to be used as solid reference genes. Genes were analyzed before and after 12 weeks of endurance training, combined with omega-3 fatty acids supplementation, to evaluate their effects on physical performance [14].

In silico analysis was performed using RefFinder, a web multi-tool that incorporates five methods: NormFinder, BestKeeper, geNorm, comparative ΔCq value, and RefFinder comprehensive ranking. Each method uses a different algorithm. NormFinder results are presented with a stability value (S), whereby a lower stability value represents relatively more stable expression [17]. The BestKeeper algorithm is based on the standard deviation (SD) and coefficient of variance and results with lower (SD) values are thought to provide better reference genes [18]. The GeNorm method is based on the exclusion of the least stable reference gene and results are presented as expression stability (M value), where lower M values indicate better stability [7]. Comparative ΔCq ranking calculates the average of standard deviation (Average STDEV), and again, lower Average STDEV values translate into more stable expression of the tested gene [19]. The most recent tool is RefFinder, which incorporates all of the aforementioned algorithms and enables a comprehensive analysis based on the geomean of ranking values (GM). A lower GM indicates greater stability [20]. We chose six potential reference genes for further analysis, based on published literature: GAPDH, ACTB, TUBB, RPS18, UBE2D2, and HPRT1. We aimed to compare commonly used reference genes with alternative genes identified in recent literature. GAPDH, ACTB, and RPS18, which are widely used in research due to their historical use in Northern Blots, were also assessed, even though their stability has been questioned by some [21]. Our results suggest that GAPDH, TUBB, and HPRT1 are the most stably expressed genes after an endurance training intervention and n-3 PUFAs supplementation period. ACTB consistently proved to be the least stably expressed. These results could enable other researchers to choose their reference genes more accurately in further studies, with regards

to both physical activity and/or supplementation trials, which would make published data more accurate and easier to reproduce.

2. Results

2.1. mRNA Levels of the Candidate Reference Genes

The Cq value was obtained for six potential reference genes using the qRT-PCR method in both the omega-3 supplemented and placebo groups. The range of expression levels in the supplemented and control groups are presented in Figure 1 for all tested genes.

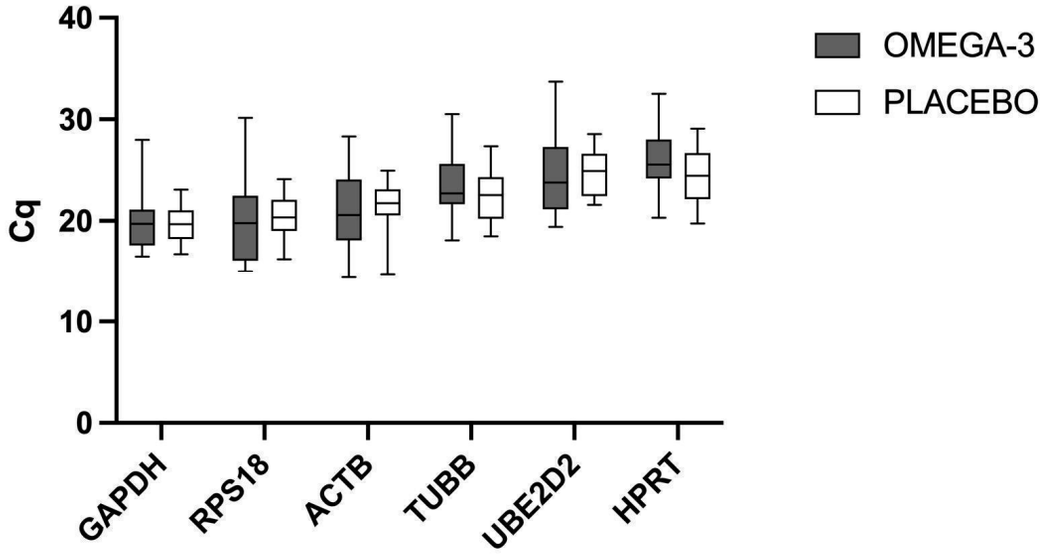

Figure 1. Comparison of Cq values for omega-3 supplemented vs. placebo group. The box plot for each reference gene represents the median, interquartile range, and the upper and lower range of raw Cq values for each experimental group. No statistically significant differences were found between groups.

The observed Cq values of all tested genes ranged from 14.4 (ACTB) to 33.7 (UBE2D2). Lower Cq values suggest a notable abundance of a tested gene within the analyzed samples. GAPDH showed the lowest Cq values with a mean value of 20.07, while the HPRT gene showed the highest mean value of 25.15. The biggest range within the gene was identified for RPS18 (range = 15.14), which can be a preliminary indicator of stability, as mentioned by Giri A. and Sundar I.K [22]. No statistically significant differences between tested groups were found.

2.2. Evaluation of Candidate Reference Genes' Expression after 12-Week Intervention

Since no statistically significant differences in Cq values were found between the placebo and omega-3-supplemented groups, all sample data were used for RefFinder in silico analysis. According to NormFinder, the most stable gene is GAPDH with a stability value (S) of 0.73. Other genes showed higher stability values: TUBB (S = 1.62), RPS18 (S = 1.86), UBE2D2 (S = 2.05), HPRT (S = 2.14), and the least stable ACTB (S = 5.48).

BestKeeper showed similar results when considering the SD [± crossing point values] with values presented in descending order, as follows: GAPDH (SD = 1.79), TUBB (SD = 1.84), HPRT (SD = 1.88), RPS18 (SD = 2.32), UBE2D2 (SD = 2.68), and ACTB (SD = 3.85).

The geNorm stability value (M) presented different results, identifying TUBB and HPRT as the most stably expressed genes with the same stability value of M = 0.74. GAPDH was ranked as being less stable with a result of M = 1.42, followed by RPS18 (M = 1.86), and UBE2D2 (M = 2.32). Still, geNorm also listed ACTB (M = 3.46) as the least stably expressed gene. As described above, ACTB consistently proved to be the least stably expressed gene by all integrated methods. GAPDH was the most stable according to all methods except for the geNorm algorithm.

Comparative delta-Ct ranking presented as the Average of standard deviation (Average STDEV) places GAPDH first, with the most stable result of Average STDEV = 2.64. The second most stably expressed gene was TUBB with a result of 2.83, followed by RPS18 (Average STDEV = 3.05), HPRT (Average STDEV = 3.09) and UBE2D2 (Average STDEV = 3.40). ACTB was listed at the bottom of the ranking with an Average STDEV number of 5.74. Nevertheless, RefFinder comprehensive ranking, which calculates the geomean of ranking values (GM), rated GAPDH as the best reference gene with a GM value of 1.32. The next suitable gene was TUBB (GM = 1.68), then HPRT (GM = 2.78), RPS18 (GM = 3.46), UBE2D2 (GM = 4.73), and ACTB (GM = 6.00). The results are presented in Figure 2.

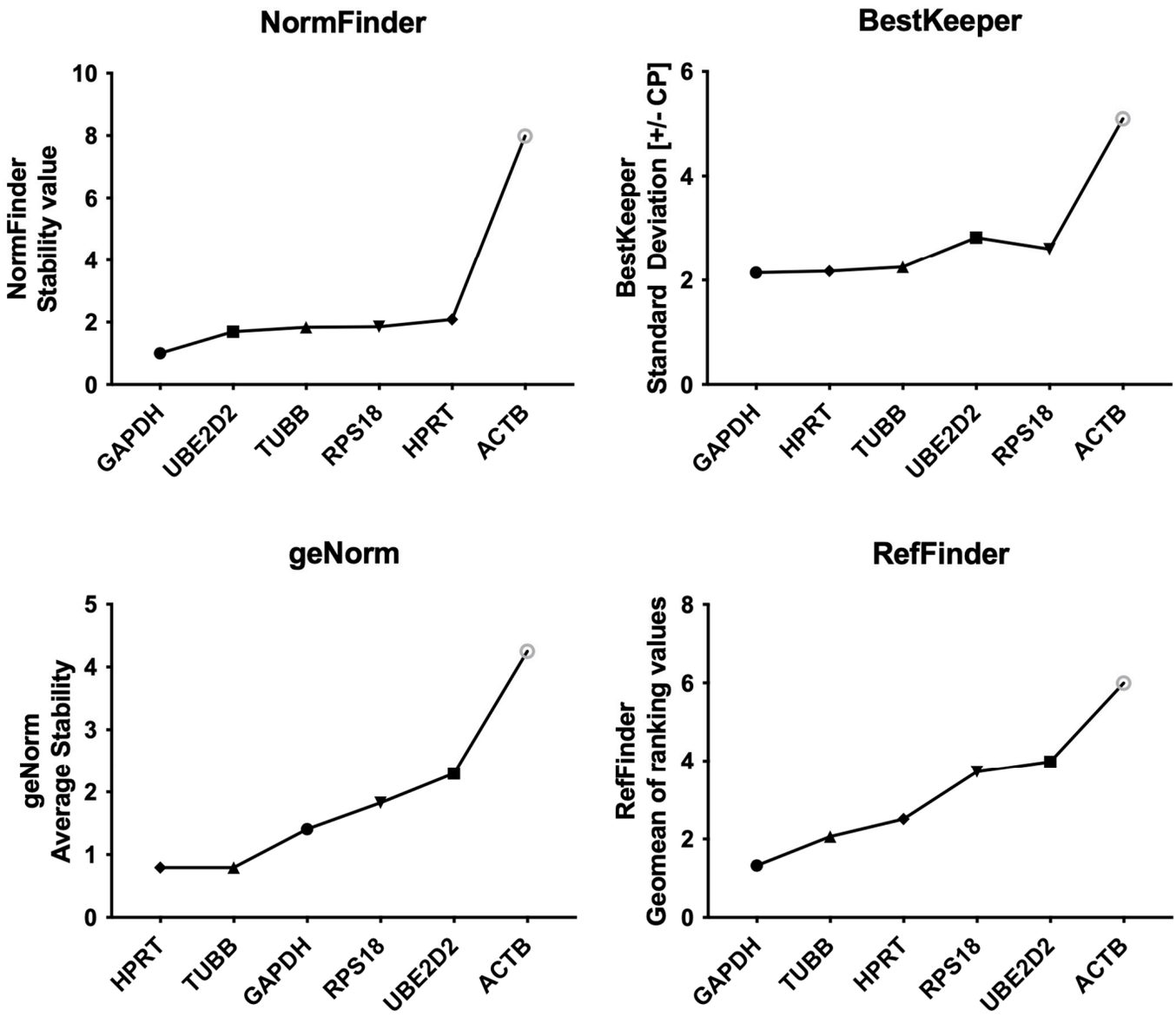

Figure 2. A comparison of results obtained via the RefFinder tool. The most stably expressed genes are represented by lower values obtained by three algorithms: NormFinder, BestKeeper, geNorm, and a comprehensive ranking of all algorithms together with the comparative Delta-Ct method using RefFinder.

3. Discussion

The effects of omega-3 supplementation and physical activity on human health and performance have been extensively researched over the last 10 years. Both of these factors have proven to be beneficial for human health, especially in regard to the prevention and

management of civilization diseases, such as obesity, cardiovascular diseases, and mental health disorders [23,24].

Yet, few of these studies are based on long-term (>7–12 weeks), high-dose supplementation, even though both of these factors seem to be crucial to promote notable changes.

It has been previously demonstrated by Browning et al., 2012 that the amount of time needed for EPA and DHA incorporation in platelets varies between 4 and8 weeks, in the erythrocyte membrane after a minimum of 8 weeks, and in blood mononuclear cells after 6–9 months [25]. This study underscores the importance of an extended supplementation period. We have obtained similar results regarding fatty acid composition in erythrocytes. Both EPA and DHA as % of fatty acids in erythrocytes increased after a 12-week omega-3 supplementation period to a level that is considered within a target range (specific data has been shown and discussed by Tomczyk et al., 2023 [14]). This proves a physiological change and confirms the efficacy of the used dosage amount and duration in the participants of our study.

The effect of omega-3 fatty acids consumption, specifically on gene expression, is also well described in the literature; however, long-term and high-dose studies are scarce. For instance, Myhrstad et al., 2014, conducted a study in which 36 subjects ingested 8 g of either fish oil, including 1.6 g of DHA + EPA (n = 17) or sunflower oil (n = 19) for 7 days [26]. Microarray analysis was used to investigate the effect of fish oil supplementation on the transcriptome profile in PBMCs, before and after the 1-week experimental period. According to the authors, subjects were also tested after 3 weeks of supplementation. Interestingly, the authors claim that their results varied more between groups after 1 week of supplementation than after 3 weeks, which stands in opposition to data demonstrated by Browning et al., 2012 [25].

A long-term study was performed by Schmidt et al., who used qRT-PCR and microarrays to test whole-genome gene expression profiles after a 12-week exposure to high doses of n-3 PUFAs (1.14 g DHA and 1.56 g EPA) in normo- and dyslipidemic men [27]. In this study, identification of the composition of fatty acids in red blood cell membranes showed no statistically significant differences. This finding differs from the results found in the subjects of our study [14]. For qRT-PCR, Schmidt et al., [27] chose GAPDH and ribosomal protein S2 (RPS2) as reference genes based on the geNorm algorithm. The authors found increased expression of genes encoding antioxidative enzymes and a decrease in the expression of genes encoding prooxidative enzymes.

More extensive and detailed research was conducted by Bouwens et al., 2009, which involved a 26-week intervention. They tested the influence of EPA and DHA as well as high-oleic acid sunflower oil on gene expression. PBMCs from a total of 111 subjects were tested at two different doses of omega-3 fatty acids: 1.8 g EPA + DHA/d (n = 36), 0.4 g EPA + DHA/d (n = 37) and a placebo group: 4.0 g high-oleic acid sunflower oil (HOSF)/d (n = 38) [16]. A high intake of EPA + DHA was effective in altering 1040 genes, while a lower intake of fish oil influenced the expression of 298 genes. The affected genes highlight the possible anti-inflammatory and antiatherogenic properties of fish oil consumption, which is commensurate with the studies mentioned above. Moreover, this research clearly demonstrated the importance of finding stably expressed genes, since ingesting n-3 PUFAs affects the expression of a great number of genes. Normalization of the mRNA levels by suitable reference genes is a crucial step and one that affects the final reported results, as shown in many published reports [22,28,29].

In this study, we tested the stability of six potential reference genes in leukocytes obtained from healthy men, who were exposed to 12 weeks of endurance training, coupled with a high dose of omega-3 fatty acids supplementation (2.234 g EPA and 0.916 g DHA). As mentioned before, despite the abundance of literature, it is impossible to identify a single reference gene adequate for different interventions. However, we believe that the identification of possible candidate genes can, across replicate studies, allow researchers to better target reference genes in their own experimental work and may also guide interventional strategies based on the genes identified.

To the best of our knowledge, this is the first in vivo study to identify optimal reference genes in human leukocytes after a standardized endurance training and omega-3 supplementation protocol. To do this, we employed an online tool (RefFinder) that incorporates four different algorithms in order to compare the Cq values of GAPDH, ACTB, TUBB, RPS18, UBE2D2, and HPRT1.

One of the most widely used reference genes is the GAPDH gene, which encodes glyceraldehyde 3-phosphate dehydrogenase. This enzyme is involved in the process of glycolysis and in several non-metabolic processes, such as activation of transcription, initiation of apoptosis [30], or rapid axonal or axoplasmic transport [31]. Likewise, the β-ACTIN gene (ACTB) is characterized by stable expression, as it encodes a highly conserved protein that is involved in cell mobility, structure, and integrity [32]. HGPRTase, encoded by the HPRT1 gene, plays a crucial role in recovering purines from degraded DNA to reintroduce them into purine synthesis pathways [33]. The RPS18 gene carries information about the ribosomal protein, a component of the 40S subunit, and is involved in the binding of fMet-tRNA and thus, initiation of the translation process [34]. Another candidate gene is TUBB. It encodes beta tubulin protein, which is implicated in maintaining the structure of microtubules [35]. The Ubiquitin-conjugating enzyme E2 D2 is a protein that in humans is encoded by the UBE2D2 gene. Protein ubiquitination regulates the degradation of misfolded, damaged, or short-lived proteins and is mediated by a cascade of enzymes that includes E2 (ubiquitin coupling) enzyme. UBE2D2 is claimed to be one of the most stable reference genes [36].

Apart from geNorm, all of the algorithms included in the RefFinder tool showed that the single most stably expressed gene in this study was GAPDH. The MIQE guidelines suggest using more than one reference gene for more reliable results [9]. The need for using several reference genes was also discussed by Leal et al., 2015 [28].

The comprehensive data from all four software algorithms showed that GAPDH, TUBB, and HPRT are the most stable genes and using all three could be beneficial to obtain the most valid results. Moreover, GAPDH, HPRT, and TUBB are genes from different functional classes, which minimizes the risk of co-regulation. According to Vandesompele et al. [7], this diversity adds to the study's robustness. The credibility of a single software package for choosing optimal reference genes is inconclusive and thus, RefFinder was chosen as it offers the benefits of applying and comparing multiple algorithms simultaneously. Some authors reported identical results for NormFinder, BestKeeper, and those algorithms used by RefFinder, while some show substantially different outputs for all three algorithms in and outside of RefFinder [37–39]. However, it should be noted that RefFinder does not take qRT-PCR efficiency data into account and De Spiegelaere et al., 2015 found that the results are similar to those that assume 100% efficiency of input data. This could possibly hinder the current findings and must be taken into consideration. Even though ACTB has been thoroughly tested as a reference gene, published data regarding its stability is inconclusive [40,41]. Our results consistently identified ACTB as the most unstable gene for the n3-PUFAs supplementation and endurance training intervention. For all that, our suggestion is that the contradictions found in the data regarding ACTB might be caused largely by the problem with the primer design, which was discussed in detail by Sun et al., 2012 [42].

As mentioned above, published data regarding the selection of appropriate reference genes is inconclusive. This might be due to the nature, specificity, and vulnerability of the PCR method [43]; hence, it is highly recommended to use more than one reference gene to obtain the most reliable results, preferably two or three. Other authors have emphasized the importance of choosing the right reference genes immediately prior to experimentation, with necessary adjustments to the cells, tissue, and methods being used [37,44]. It must also be stressed that there is no universally stable reference gene. In addition, results from our study cannot be directly applied to other studies, but they can help other researchers find the most suitable reference genes to normalize their data.

4. Materials and Methods

4.1. Ethics

The study was approved by the Bioethical Committee of Regional Medical Society in Gdansk (NKBBN/628/2019). The protocol was constructed according to the Declaration of Helsinki. All study participants were given an oral and written explanation of the study aims and written consent was obtained from each participant prior to the experiment.

4.2. Study Setting and Subjects

This study is part of a larger research project with details presented elsewhere [14]. Briefly, the effect of 12 weeks of endurance training with simultaneous omega-3 fatty acids supplementation was studied in healthy men. Participants received either omega-3 fatty acids or medium chain triglycerides (MCTs) in a daily dose of 2234 mg of eicosapentaenoic acid (EPA) + 916 mg of docosahexaenoic acid (DHA) (OMEGA-3 group) or 4000 mg of MCTs (PLACEBO group). Before and after intervention, blood samples were collected for omega-3 index (O3I) assessment, a sum of EPA and DHA expressed as a percent of total fatty acids in erythrocytes, which is a valid biomarker of omega-3 PUFA status. Moreover, a graded exercise test to exhaustion with assessment of VO_2peak, running economy (RE), and a 1500-m run trial, was conducted. Out of 40 eligible participants, 26 male runners (37 ± 4 years old; 77 ± 10 kg body weight; VO_2peak 54.2 ± 6 mL·kg^{-1}·min^{-1}) completed the protocol. Blood samples for gene expression analysis were collected in a fasted state, before and after the 12-week experimentation period. In the final gene analyses, 18 individuals were included (n = 10 in the OMEGA-3 group and n = 8 in the PLACEBO group). Detailed data on the inclusion and exclusion criteria are described by Tomczyk et al., 2023 [14]. The research protocol and the exclusion criteria for the gene analyses are presented in Figure 3.

4.3. Blood Collection, RNA Extraction and Reverse Transcription

A modification of previously described protocols for blood collection and RNA extraction were employed [45,46], with different reagents and lab equipment used in the current study. To obtain leukocytes, 2 mL of venous blood was collected from each participant into vacutainers spray-coated with K3EDTA. Within 15 min of collection, the blood was mixed with Red Blood Cell Lysis Buffer (RBCL) (A&A Biotechnology, Gdynia, Poland), incubated for 15 min, and centrifuged at $3000\times$ *g* at 4 °C for 10 min. The obtained platelet was washed and later lysed using Fenozol (A&A Biotechnology, Gdynia, Poland). Finally, samples were stored at −20 °C for up to 3 months. Further RNA isolation was carried out using the modified Chomczynski and Sacchi method [47]. A total of 200 µL of chloroform (POCH, Gliwice, Poland) was used, samples were centrifuged, and the aqueous phase was mixed with 500 µL of isopropanol (POCH, Gliwice, Poland) and spun again. The obtained platelet was washed with ethanol, dried, and resuspended with PCR-grade water. Gel electrophoresis was performed to check for the quality and integrity of selected RNA. Nucleic Acid purity and concentration were determined. At UV 260/280, a ratio of 1.75–2.2 was accepted and at UV 260/230, a ratio >1.8 was accepted as pure RNA suitable for further analysis, based on available data [40,48]. At this stage, 4 samples from the supplemented group and 4 samples from the placebo group were excluded from further analysis due to the unacceptable UV 260/280 ratio or insufficient amount of obtained material.

Reverse transcription was performed using the AffinityScript qPCR cDNA synthesis kit (Agilent Technologies, Warszawa, Poland) and applied according to the manufacturer's protocol with 1000 ng of RNA. The obtained cDNA was immediately frozen at −20 °C and stored for up to 1 month without repeated freeze-thaw cycles. cDNA was later diluted 1:10 with PCR-grade water immediately before the qRT-PCR step.

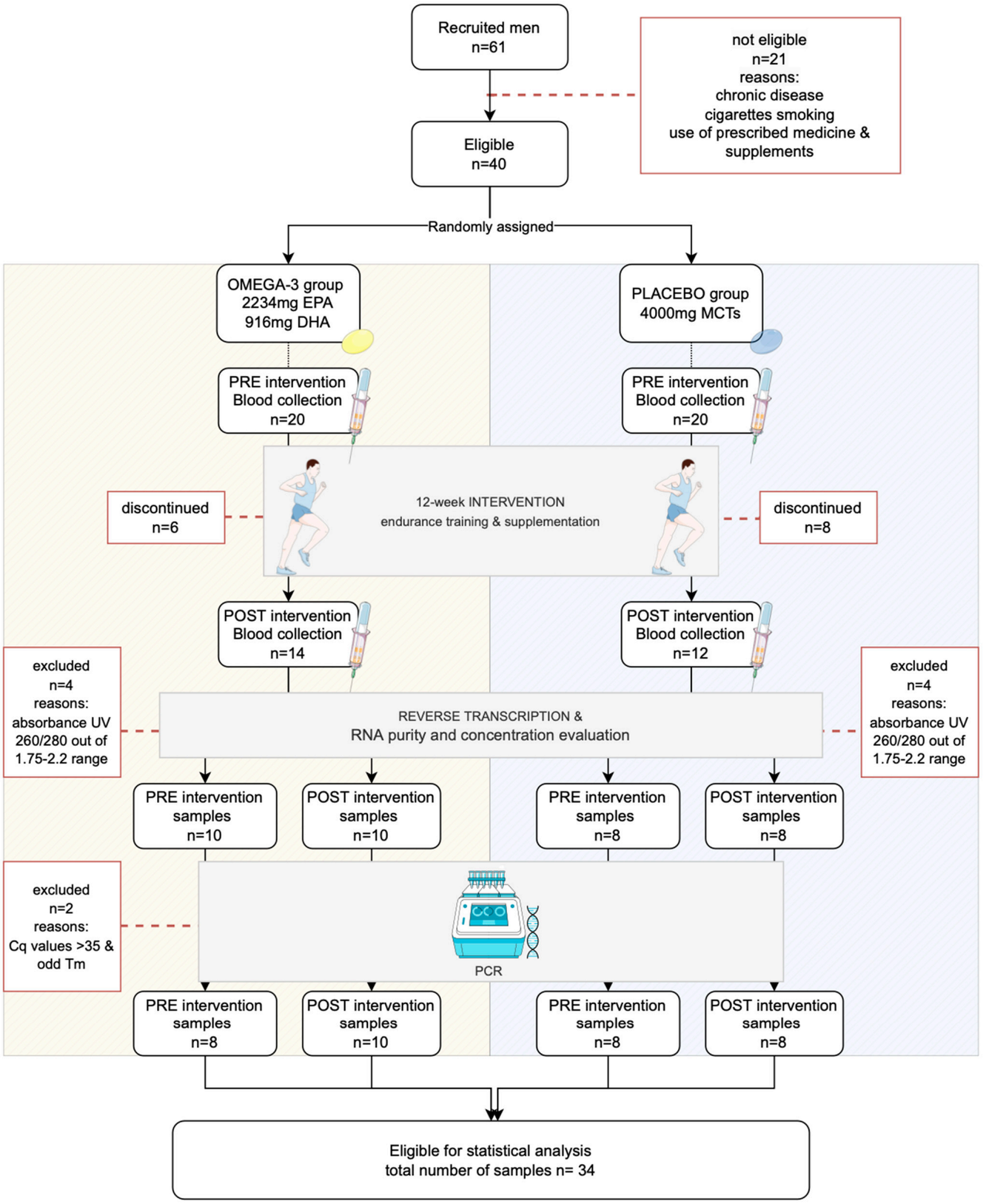

Figure 3. Flow diagram of the study process and group sizes.

4.4. Selection of Potential Reference Genes and Primer Design

Potential reference genes (RGs) were chosen according to the data presented in published papers [3,40,41,49–54]. The genes tested in this study are listed in Table 1.

Table 1. List of candidate reference genes evaluated in this study. Function information based on data published at the human genome database https://www.genecards.org/ (accessed on 15 November 2022).

Gene Symbol	Gene Accession Number	Name	Function
ACTB	NM_001101	Beta actin	Cytoskeletal protein
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
RPS18	NM_022551.3	Ribosomal Protein S18	Encodes a ribosomal protein that is a component of the 40S subunit
TUBB	NM_001293212.2	Tubulin Beta Class I	Forms a dimer with alpha-tubulin and acts as a structural component of microtubules
UBE2D2	NM_003339.3	Ubiquitin-conjugating enzyme E2 D2	Degradates misfolded, damaged, or short-lived proteins in eukaryotes
HPRT1	NM_000194.3	Hypoxanthine Phosphoribosyltransferase 1	Plays a central role in the generation of purine nucleotides through the purine salvage pathway

Candidate reference genes primers were either obtained from published literature or the real-time PCR primer database (PrimerBank https://pga.mgh.harvard.edu/primerbank/ (accessed on 15 November 2022)). Primer sequence, product length, and source are listed in Table 2. An efficiency of 100% was assumed for all used primers.

Table 2. Primer sequences, amplicon size and source for the sequences for each of the tested genes.

Symbol	Primer Sequence	Amplicon Size	Source
ACTB	F: GAGAAAATCTGGCACCACACC	177	Chen et al., 2018 [55]
	R: GGATAGCACAGCCTGGATAGCAA		
GAPDH	F: TCTCCTCTGACTTCAACAGCGAC	126	Andersen et al., 2004 [17]
	R: CCCTGTTGCTGTAGCCAAATTC		
RPS18	F: GCGGCGGAAAATAGCCTTTG	139	Spandidos et al., 2010 [56]
	R: GATCACACGTTCCACCTCATC		
TUBB	F: CTAGAACCTGGGACCATGGA	191	Żychowska et al., 2021 [57]
	R: TGCAGGCAGTCACAGCTCT		
UBE2D2	F: GTACTCTTGTCCATCTGTTCTCTG	120	Roy et al., 2020 [40]
	R: CCATTCCCGAGCTATTCTGTT		
HPRT1	F: CGAGATGTGATGAAGGAGATGG	97	Jeon et al., 2019 [41]
	R: TGATGTAATCCAGCAGGTCAGC		

The specificity of potential reference genes and the obtained material was randomly checked through 2.0% agarose gel electrophoresis and later (for all samples) using a melt curve analysis.

4.5. Quantitative Real-Time Polymerase Chain Reaction

The AriaMx Real-Time PCR System (Agilent Technologies, Warszawa, Poland) and Brilliant III Ultra-Fast QPCR Master Mix—Agilent (Agilent Technologies, Warszawa, Poland) were used to perform qRT-PCR analyses. A total of 10 samples from the omega-3

supplemented group and 8 samples from the placebo group at 2 time points were analyzed at this stage. For the analysis, 2 µL of diluted cDNA of each sample was loaded in triplicates into 96-well PCR plates previously filled with 8 µL of MasterMix each. The thermal cycling conditions comprised an activation step: 95 °C for 10 min followed by 40 cycles of annealing, and an extension step: 95 °C for 15 s and 60 °C for 1 min. Additionally, the melt curve analysis was performed for each reaction to confirm the specific amplification of the target genes. At this point, 2 PRE-intervention samples from the supplemented group were excluded due to probable contamination (seen as Cq values > 35 and odd Tm values). On each plate, negative controls were included to verify the absence of contamination.

4.6. Evaluation of Stable Reference Genes for leukocytes

To compare the Cq value between the placebo and experimental groups, the D'Agostino–Pearson Normality Test was applied using GraphPad Prism version 9 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com (accessed on 21 January 2023). Subsequently, an unpaired t test was performed to check for statistical differences. Since no statistically significant differences were found, all Cq values were included in further analysis. In accordance with the Real-time PCR Data Markup Language (RDML), we have used the abbreviation for quantification cycle value (Cq) instead of the cycle threshold value (Ct) [58].

To evaluate the most stable reference genes, the RefFinder tool was used [20]. The RefFinder is an online software tool that integrates algorithms from the NormFinder, BestKeeper, and geNorm programs, as well as the comparative delta-Ct method.

5. Conclusions

Our results show that GAPDH, TUBB, and HPRT could be suitable reference genes for studies involving physical exercise and omega-3 supplementation in humans. We do not recommend using ACTB as a reference gene, based on current findings, as well as data presented in the literature. We believe there is a strong need for long-term (>7–12 weeks) molecular studies on this topic to accommodate the expected time course of adaptation. The information gained would enable a better understanding of the interplay between n-3 PUFAs supplementation and endurance training, and how these factors co-regulate changes in mRNA levels that, ultimately, mediate functional aspects of human health and performance.

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

Funding: This research was funded by the National Science Center (Poland), grant number 2018/31/N/NZ7/02962.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethical Committee of Regional Medical Society in Gdansk (NKBBN/628/2019, approval date 3 December 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: Authors would like to thank Maciej Chroboczek for his helpful advice regarding data curation.

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

Abbreviations

ACTB	beta-actin
Average STDEV	Average of standard deviation
Cq	quantification cycle
Ct	cycle threshold
DHA	docosahexaenoic acid
DNA	Deoxyribonucleic acid
EPA	eicosapentaenoic acid
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
MCTs	Medium Chain Triglycerides
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
n-3 PUFAs	omega-3 polyunsaturated fatty acids
PBMC	Peripheral Blood Mononuclear Cell
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RBCL	Red Blood Cell Lysis Buffer
RDML	Real-time PCR Data Markup Language
RGs	reference genes
RPS18	Ribosomal Protein S18
SD	standard deviation
TUBB	Tubulin Beta Class I
UBE2D2	Ubiquitin-conjugating enzyme E2 D2
VO_2peak	peak oxygen uptake

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9. Publikacje nie włączone do rozprawy doktorskiej

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10. Wyróżnienia i osiągnięcia

Stypendium dla najlepszych doktorantów AWFiS Gdańsk w latach 2017-2021

Stypendium z dotacji podmiotowej dla najlepszych doktorantów AWFiS Gdańsk w latach 2017-2021

Naukowa nagroda zespołowa II stopnia JM Rektora Gdańskiego Uniwersytety Medycznego za rok 2020

Podwójny staż naukowo-dydaktyczny na Universitat de Barcelona pod kierunkiem prof. Prof. Ginés Viscor w laboratorium Zakładu Fizjologii oraz Dr. Daniel Grinberg w laboratorium Zakładu Genetyki, Mikriobiologii i Statystyki, listopad-grudzień 2018