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ROZPRAWA DOKTORSKA

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Wpływ 12-tygodniowej suplementacji kwasami tłuszczowymi omega-3 na metabolizm L-argininy i wydolność tlenową u biegaczy długodystansowych

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DOCTORAL DISSERTATION

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The effect of 12-week omega-3 fatty acid supplementation on L-arginine metabolism and aerobic capacity in long-distance runners

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1. Wykaz skrótów (Abbreviations)

ADMA – (ang. Asymmetric dimethylarginine) – asymetryczna dimetyloarginina

ALA – (ang. Alpha linolenic acid) – kwas α-linolenowy

DHA – (ang. Docosahexaenoic acid) – kwas dokozaheksaenowy

EE – (ang. Exercise economy) – ekonomia wysiłkowa

eNOS – (ang. Endothelial nitric oxide synthase) – śródbłonkowa syntaza tlenku azotu

EPA – (ang. Eicosapentaenoic acid) – kwas eikozapentaenowy

HR – (ang. Heart rate) – częstość skurczów serca

L-arg/ADMA – (ang. L-arg/ADMA ratio) – współczynnik L-arginina/ADMA

LT – (ang. Lactate threshold) – próg mleczanowy

n-3 PUFAs – (ang. Omega-3 polyunsaturated fatty acids) - wielonienasycone kwasy tłuszczowe omega-3

NO – (ang. Nitric oxide) – tlenek azotu II

O3I – (ang. Omega-3 Index) – indeks omega-3

OUE – (ang. Oxygen uptake efficiency) – wydajność poboru tlenu

OUE@VAT – (ang. Oxygen uptake efficiency at the ventilatory anaerobic threshold) – wydajność poboru tlenu na wentylacyjnym progu anaerobowym

OUEP – (ang. Oxygen uptake efficiency plateau) – plateau wydajności poboru tlenu

PPARs – (ang. Peroxisome proliferator-activated receptors) – receptory aktywowane przez proliferatory peroksysomów

RE – (ang. Running economy) – ekonomia biegowa

RER – (ang. Respiratory exchange ratio) – współczynnik wymiany oddechowej

VAT – (ang. Ventilatory anaerobic threshold) – wentylacyjny próg anaerobowy

Ve – (ang. Minute ventilation) – wentylacja minutowa

VO_{2max} – (ang. Maximal oxygen uptake) – maksymalny pobór tlenu

VO_{2peak} – (ang. Peak oxygen uptake) – szczytowy pobór tlenu

VT – (ang. Ventilatory threshold) – próg wentylacyjny

2. Autoreferat w języku polskim

2.1. Wykaz prac wchodzących w skład rozprawy

1. Maja Tomczyk, Zbigniew Jost, Maciej Chroboczek, Robert Urbański, Philip C**.** Calder, Helena L. Fisk, Mateusz Sprengel, Jędrzej Antosiewicz. **Effects of 12 Weeks of Omega-3 Fatty Acid Supplementation in Long-distance Runners**; Medicine & Science in Sports & Exercise, 2023; 55(2):216-224.
   doi:10.1249/MSS.0000000000003038
   Impact Factor: 4.1; punktacja MEiN: 140
   Wkład doktoranta (30%): przygotowanie projektu badania, przeprowadzenie badań, interpretacja wyników, przygotowanie publikacji, opracowanie piśmiennictwa

2. Zbigniew Jost, Maja Tomczyk, Maciej Chroboczek, Philip C. Calder, Radosław Laskowski. **Improved Oxygen Uptake Efficiency Parameters Are Not Correlated with VO_{2peak} or Running Economy and Are Not Affected by Omega-3 Fatty Acid Supplementation in Endurance Runners**; International Journal of Environmental Research and Public Health 2022, 19(21):14043.
   doi.org/10.3390/ijerph192114043
   Punktacja MEiN: 140
   Wkład doktoranta *(55%)*: *przygotowanie projektu badania, przeprowadzenie badań, analiza statystyczna, interpretacja wyników, przygotowanie publikacji, opracowanie piśmiennictwa*

3. Zbigniew Jost, Maja Tomczyk, Maciej Chroboczek, Philip C. Calder, Helena L. Fisk, Katarzyna Przewłócka, Jędrzej Antosiewicz. **Increased Plasma L-Arginine Levels and L-Arginine/ADMA Ratios after Twelve Weeks of Omega-3 Fatty Acid Supplementation in Amateur Male Endurance Runners;** Nutrients 2022, 14(22):4749.
   doi.org/10.3390/nu14224749
   Impact Factor: 5.9; punktacja MEiN: 140
   Wkład doktoranta (35%): przygotowanie projektu badania, przeprowadzenie badań, interpretacja wyników, przygotowanie publikacji, opracowanie piśmiennictwa

Łączna punktacja cyklu publikacji: Impact Factor: 10; punktacja MEiN: 420

2.2. Wprowadzenie

Wielonienasycone kwasy tłuszczowe omega-3 (*ang. omega-3 polyunsaturated fatty acids*; n-3 PUFAs) takie jak kwas α-linolenowy (*ang. alpha linolenic acid*; ALA), kwas eikozapentaenowy (*ang. eicosapentaenoic acid*; EPA) oraz kwas dokozaheksaenowy (*ang. docosahexaenoic acid*; DHA) stanowią ważną część diety człowieka z racji na ich niewystarczającą endogenną syntezę (Calder 2018). Pomimo znacznej wiedzy dotyczącej korzyści będących efektem suplementacji n-3 PUFAs, wciąż wiele źródeł wskazuje na ich niedobory w diecie wśród dzieci i młodzieży (Rahmawaty i wsp. 2013), osób dorosłych (Howe i wsp. 2006) oraz sportowców (Ritz i wsp. 2020). Według literatury światowej n-3 PUFAs korzystnie wpływają na funkcjonowanie organizmu ulegając inkorporacji do błony śluzowej jelit (Sorensen i wsp. 2014), błon komórkowych miocytów (McGlory i wsp. 2014), czy też kardiomiocytów (Harris i von Schacky 2004). Jednak co ciekawe, to wzbogacenie krwinek czerwonych (erytrocytów) w n-3 PUFAs (w szczególności kwasów EPA i DHA) odzwierciedla ich zawartość w organizmie. Stąd, Harris i von Schacky jako pierwsi opracowali indeks omega-3 (*ang. omega-3 index;* O3I) - wskaźnik, który definiowany jest jako procent wagowy kwasów EPA i DHA wszystkich kwasów tłuszczowych w erytrocytach (Harris i von Schacky 2004). Ich niskie wartości tj. <4% wskazują na niedostateczne zdolności kardioprotekcyjne i prozdrowotne, zaś wartości >8% wskazuje się jako wartości docelowe - wzmacniające homeostazę organizmu. Niemniej jednak, badania z udziałem sportowców uwzględniające O3I są niewystarczające, aby precyzyjniej określić mechanizmy ich oddziaływania na poziomie subkomórkowym i ogólnoustrojowym.

Liczne badania wskazują na znaczącą poprawę zdolności wysiłkowych i regeneracyjnych wśród osób aktywnych fizycznie w następstwie suplementacji n-3 PUFAs (Buckley i wsp. 2009; Macartney i wsp. 2019; Żebrowska i wsp. 2015). Odbywa się to poprzez obniżenie ilości tlenu wymaganej do uzyskania submaksymalnej mocy (Kawabata i wsp. 2014) lub prędkości biegu, które określa się poprawą odpowiednio - ekonomii wysiłkowej (*ang. exercise economy;* EE) lub biegowej (*ang. running economy*; RE) (Saunders i wsp. 2004). W eksperymencie Peoplesa i wsp. suplementacja n-3 PUFAs trwająca 8 tygodni w dawce 800 mg EPA i 2400 mg DHA dziennie spowodowała obniżenie zużycia tlenu podczas 60-minutowej jazdy na cykloergometrze rowerowym (Peoples i wsp. 2008). W badaniu Hingley'a i wsp. w następstwie suplementacji w dawce 140 mg EPA i 560 mg DHA dziennie przez 8 tygodni odnotowano poprawę EE podczas 10-minutowej jazdy ze stałym obciążeniem (Hingley i wsp. 2017). Jednak większość dostępnych w literaturze badań eksploruje maksymalnie 8-tygodniowe protokoły suplementacyjne n-3 PUFAs i nie uwzględnia biegaczy długodystansowych. Z molekularnego punktu widzenia, maksymalne wysycenie błon erytrocytów kwasem EPA obserwowane jest dopiero po 180 dniach suplementacji n-3 PUFAs (Katan

i wsp. 1997). Stąd, dłuższe protokoły wydają się być odpowiednimi do maksymalizacji efektów, jakie można uzyskać w następstwie ich podaży.

Najpopularniejszym i najbardziej obiektywnym testem do oceny wydolności tlenowej wśród sportowców jest test z narastającym obciążeniem tzw. „do odmowy wykonywania pracy" (Poole i Jones 2017; Raghuveer i wsp. 2020). Aby precyzyjnie określić zdolności wysiłkowe, podczas tego badania wymaga się od sportowców wykonania wysiłku z intensywnością maksymalną celem wyznaczenia szczytowego/maksymalnego poboru tlenu ($VO_{2peak/max}$) (Levine 2008; Taylor i wsp. 1955). Z drugiej strony w literaturze światowej istnieją dobrze poznane submaksymalne parametry określające wydolność tlenową: próg mleczanowy (*ang. lactate threshold;* LT) lub próg wentylacyjny *(ang. ventilatory threshold;* VT), kinetyka poboru tlenu i RE (Jones i Carter 2000; Whipp i wsp. 1982). Jednak co ważne, prawidłowy i dokładny pomiar może zostać wykonany metodami inwazyjnymi - metodami, podczas których od badanego pobiera się krew włośniczkową (Feliu i wsp. 1999; Forsyth i Farrally 2000). Stąd poszukiwanie wiarygodnych, nieinwazyjnych i przede wszystkim submaksymalnych parametrów odzwierciedlających wydolność krążeniowo-oddechową jest wciąż fascynującym, choć nie do końca zbadanym tematem. W 2012 roku Sun i wsp. zaproponowali powiązanie między poborem tlenu, a wentylacją minutową (*ang. minute ventilation;* Ve), które nazwano wydajnością poboru tlenu (*ang. oxygen uptake efficiency;* OUE) (Sun i wsp. 2012). Autorzy opisali również dwa inne submaksymalne pomiary - plateau wydajności poboru tlenu (*ang. oxygen uptake efficiency plateau;* OUEP) oraz wydajność poboru tlenu na poziomie wentylacyjnego progu anaerobowego (*ang. oxygen uptake efficiency at the anaerobic ventilatory threshold;* OUE@VAT) (Sun i wsp. 2012). Wykorzystanie tych parametrów dotyczy głownie osób z przewlekłą chorobą nerek (Wilkinson i wsp. 2020), chorobami układu oddechowego (Guo i wsp. 2016) oraz wśród dzieci (Bongers i wsp. 2016). Do dziś nie poznano użyteczności tych parametrów w ocenie wydolności tlenowej wśród sportowców, jak również ich czułości w odpowiedzi na suplementację substancjami bioaktywnymi, jak np. n-3 PUFAs.

Jednym z proponowanych mechanizmów odpowiedzialnych za poprawę zdolności wysiłkowych wśród sportowców jest zwiększenie biodostępności/syntezy tlenku azotu II (*ang. nitric oxide*; NO) w śródbłonku naczyniowym (Higashi i wsp. 1999). NO będący produktem tlenowej przemiany L-argininy w L-cytrulinę pełni funkcję wazodylatacyjną względem naczyń krwionośnych dzięki rozkurczowi mięśni gładkich budujących te naczynia (Álvares i wsp. 2011). Co ciekawe, aktywność śródbłonkowej syntazy tlenku azotu (*ang. endothelial nitric oxide synthase;* eNOS) - enzymu katalizującego tę reakcję może być hamowana w obecności asymetrycznej dimetyloargininy (*ang. asymmetric dimethylarginine*; ADMA) (Surdacki i wsp. 1999). ADMA jest inhibitorem kompetycyjnym dla eNOS, a wzrost jej stężenia w osoczu jest ściśle powiązany z osłabionym

efektem wazodylatacyjnym naczyń krwionośnych, co w konsekwencji prowadzi do zmniejszonej pojemności minutowej serca (Kielstein i wsp. 2004).

Jednakże z diagnostycznego punktu widzenia, to współczynnik L-arginina/ADMA (*ang. L-arg/ADMA ratio*) stanowi solidny i powtarzalny wskaźnik wydolności układu sercowo-naczyniowego. Jego niskie wartości potęgują ryzyko upośledzenia funkcji śródbłonka naczyniowego, tym samym prowadząc do zwiększenia wskaźnika hospitalizacji i śmiertelności (Anderssohn i wsp. 2012). W badaniu, w którym wzięło udział 785 osób wykazano, że współczynnik L-arg/ADMA może być czułym markerem miażdżycy (Notsu i wsp. 2015). Co więcej, obniżone wartości współczynnika L-arg/ADMA obserwowane po długotrwałym wysiłku fizycznym zmniejszają zdolności regeneracyjne poprzez obniżenie możliwości organizmu do syntezowania NO (Nyborg i wsp. 2021). Stąd odnalezienie egzogennego modulatora metabolitów L-argininy, ADMA oraz współczynnika L-arg/ADMA wydają się być kluczowe nie tylko dla osób prowadzących siedzący tryb życia, ale również osób zdrowych i sportowców.

Wpływ suplementacji n-3 PUFAs w stanach patologicznych, takich jak choroby układu sercowo-naczyniowego (Shen i wsp. 2022), układu nerwowego (AlAmmar i wsp. 2021), czy też w chorobach metabolicznych, takich jak cukrzyca typu II (Delpino i wsp. 2022) jest dobrze udokumentowany. Z drugiej strony jej stosowanie wśród sportowców dotyczy głównie właściwości regeneracji powysiłkowej oraz dyscyplin, w których nie uwzględnia się biegaczy długodystansowych (Hingley i wsp. 2017; Kawabata i wsp. 2014; Philpott i wsp. 2019). Pomimo znacznej wiedzy dotyczącej korzyści płynących ze stosowania suplementacji n-3 PUFAs, mechanizmy odpowiedzialne za te zmiany są niedostatecznie poznane. Stąd też niniejsza praca stanowi próbę oceny wpływu suplementacji n-3 PUFAs na zdolności wysiłkowe powiązane z O3I, jak również próbę poznania mechanizmu odpowiedzialnego za modulację tych zmian na poziomie subkomórkowym i ogólnoustrojowym.

2.3. Cele pracy i hipotezy

2.3.1. Cele pracy

1) Sprawdzenie wpływu 12-tygodniowej suplementacji n-3 PUFAs u biegaczy długodystansowych na:
   a) indeks omega-3 (O3I), szczytowy pobór tlenu (VO_{2peak}) i ekonomię biegową (RE),
   b) parametry wydolności tlenowej mierzone na poziomie submaksymalnej intensywności: OUEP i OUE@VAT.
2) Ocena zmian w metabolizmie L-argininy i ADMA i ich zestawienie ze zmianami RE.

2.3.2. Hipotezy badawcze

1) 12-tygodniowa suplementacja n-3 PUFAs u biegaczy długodystansowych zwiększy O3I i VO_{2peak} oraz poprawi RE.
2) Podjęta interwencja suplementacyjna przyczyni się do poprawy OUEP oraz OUE@VAT.
3) 12- tygodniowa suplementacja n-3 PUFAs zwiększy spoczynkowy poziom L-argininy, jednocześnie obniżając poziom ADMA w osoczu, które przyczynią się do:
   a) wzrostu współczynnika L-arg/ADMA,
   b) negatywnej korelacji między współczynnikiem L-arg/ADMA a RE.

2.4. Materiał i metody badań

Niniejsza rozprawa doktorska została przygotowana na podstawie trzech powiązanych ze sobą, oryginalnych prac opublikowanych w recenzowanych czasopismach o zasięgu międzynarodowym. Badania zostały przeprowadzone za zgodą Lokalnej Komisji Bioetycznej w Gdańsku (NKBBN/628/2019). Doświadczenie zostało sfinansowane ze środków Narodowego Centrum Nauki w ramach grantu Preludium 16 nr. 2018/31/N/NZ7/02962.

2.4.1. Charakterystyka uczestników i pobór krwi

W badaniach uczestniczyło 40 biegaczy amatorów. Troje uczestników (*n*=3) z powodu niewystarczającej ilości ukończonych treningów (<80%), dziewięcioro ze względów zdrowotnych (*n*=9), jeden z powodów osobistych (*n*=1) i jeden z grupy kontrolnej (*n*=1), który zwiększył spożycie n-3 PUFAs zostało wykluczonych z dalszych analiz. Stąd 26 biegaczy (37 ± 3 lat; 77 ± 9 kg masy ciała; VO_{2peak}: 54.2 ± 6 $ml \cdot kg^{-1} \cdot min^{-1}$) ukończyło 12-tygodniowy program suplementacyjno-treningowy. Uczestnicy zostali losowo podzieleni na dwie grupy: eksperymentalną „OMEGA" (*n*=14), która spożywała n-3 PUFAs w dawce 2234 mg kwasu EPA i 916 mg kwasu DHA dziennie. Grupa kontrolna „MCT" (*n*=12) spożywała placebo w postaci średniołańcuchowych kwasów tłuszczowych w dawce 4000 mg dziennie. Od uczestników z żyły łokciowej pobrano dwukrotnie krew na czczo: przed rozpoczęciem i po ukończeniu programu. Następnie pobrana krew została zwirowana przy 4000 × *g* przez 10 min w 4 °C. Po zwirowaniu osocze oraz erytrocyty zostały umieszczone w oddzielnych probówkach i ulokowane w -80 °C w oczekiwaniu na oznaczenia. Oznaczenia stężeń kwasów EPA i DHA w erytrocytach wykonano metodą chromatografii gazowej, a poziomy L-argininy i jej matabolitów w osoczu metodą chromatografii cieczowej z tandemową spektrometrią mas. Szczegółowa metodologia oznaczeń n-3 PUFAs w erytrocytach oraz metabolitów L-argininy w osoczu została szczegółowo opisana w cyklu publikacji.

2.4.2. Pomiar wydolności tlenowej

Zarówno przed jak i po ukończeniu programu uczestników poddano testowi wydolności tlenowej „do odmowy wykonywania pracy" na bieżni mechanicznej (h/p Cosmos, Saturn, Niemcy). Przed wysiłkiem testowym badani stali na bieżni przez 2 minuty, aby upewnić się, że aparatura badawcza działa poprawnie. Następnie, uczestnicy maszerowali przez 5 min z prędkością 5 $km \cdot h^{-1}$ z nachyleniem bieżni 1.5% w ramach rozgrzewki. Każdy kolejny etap trwał 3 min, a prędkość bieżni była zwiększana od 8 $km \cdot h^{-1}$ do 12 $km \cdot h^{-1}$ co 1 $km \cdot h^{-1}$. Potem nachylenie bieżni zostało zwiększane do 5%, 10% i 15% przy prędkości 12 $km \cdot h^{-1}$ aż do wolicjonalnego wyczerpania. Podczas obydwu testów częstość skurczów serca (*ang. heart rate*; HR) była mierzona za pomocą pulsometru (Polar

RS400; Polar Electro Oy, Kempele, Finlandia). Pobór tlenu (VO_2), objętość wydychanego dwutlenku węgla (VCO_2), Ve, oraz współczynnik wymiany oddechowej (*ang. respiratory exchange ratio;* RER) były mierzone „oddech-za-oddech" za pomocą analizatora gazów oddechowych (Oxycon Pro, Jaeger, Niemcy), który kalibrowano przed każdym testem zgodnie z zaleceniami producenta. Pomiary w raporcie zostały uśrednione do 10-sekundowych interwałów. VO_{2peak} wyznaczono jako średnia trzech najwyższych pomiarów z rzędu, a RE jako średnia poboru tlenu z ostatnich 50 sekund podczas biegu z prędkością 12 $km \cdot h^{-1}$ (Jones i wsp. 2021). Dodatkowo, OUEP wyznaczono jako 90-sekundowa średnia najwyższych z rzędu pomiarów OUE (VO_2 ($mL \cdot min^{-1}$) / Ve ($L \cdot min^{-1}$)) oraz OUE@VAT jako 60-sekundowa średnia najwyższych z rzędu pomiarów OUE bezpośrednio przed lub po uzyskaniu wentylacyjnego progu anaerobowego (*ang. ventilatory anaerobic threshold*; VAT) (Sun i wsp. 2012). VAT został wyznaczony metodą „V-slope" (Beaver i wsp. 1986).

2.4.3. Program treningowy

Biegacze amatorzy zostali poddani 12-tygodniowemu, jednolitemu planowi treningowemu (Costa i wsp. 2019). Program treningowy polegał na wykonaniu tygodniowo 3 sesji biegowych o charakterze aerobowym oraz jednej oporowej z własną masą ciała, której celem było wzmocnienie mięśni szkieletowych (de Blaiser i wsp. 2018). Co ważne, uczestnicy trenowali w trzech strefach intensywności podyktowanej częstością skurczów serca i odpowiadającym im progom wentylacyjnym wraz z progresywnym wzrostem objętości treningowej. Wszystkie aktywności były rejestrowane za pomocą monitorów (Polar M430, Kempele, Finlandia) oraz czujnika pracy serca H9 zakładanego na klatkę piersiową. Szczegółowa metodologia programu treningowego została opisana w cyklu publikacji.

2.4.4. Analiza statystyczna

We wszystkich trzech pracach analizę statystyczną przeprowadzono za pomocą programu statystycznego GraphPad Prism 7 (San Diego, CA, Stany Zjednoczone). Obliczono średnie arytmetyczne, odchylenia standardowe (SD) oraz poziomy istotności pomiędzy średnimi. W pracach wykonano dwukierunkową analizę wariancji (ANOVA) z powtarzanymi pomiarami aby zbadać istotności różnic między grupami i czasem. Istotne efekty były następnie analizowane przy użyciu skorygowanego *post hoc* testu Bonferroniego (praca nr 1 i nr 2) oraz testu *post hoc* Sidaka w pracy nr 3. Korelacje pomiędzy zmiennymi oceniono za pomocą współczynników korelacji Pearsona i/lub Spearmana. We wszystkich analizach zastosowano poziom istotności $p<0.05$.

2.5. Omówienie prac wchodzących w skład rozprawy

2.5.1. Omówienie pracy nr 1

W pracy nr 1 pt. „**Effects of 12 Weeks of Omega-3 Fatty Acid Supplementation in Long-distance Runners”** (Autorzy: Tomczyk M., Jost Z., Chroboczek M., Urbański R., Calder P.C, Fisk H.L., Sprengel M., Antosiewicz J.) opublikowanego w Medicine & Science in Sports & Exercise głównym celem było sprawdzenie wpływu 12-tygodniowej suplementacji n-3 PUFAs na O3I i wydolność tlenową u biegaczy długodystansowych.

Wykazano istotny statystycznie wzrost O3I w grupie eksperymentalnej do wartości docelowych tj. >8%, czego nie odnotowano w grupie kontrolnej. Ponadto w grupie, która spożywała n-3 PUFAs zaobserwowano istotny statystycznie wzrost VO_{2peak} oraz poprawę RE, podczas, gdy podobnych zmian nie obserwowano w grupie spożywającej placebo. Istotność statystyczna została również odnotowana w delcie (różnicy) pomiędzy obydwiema grupami w poborze tlenu podczas biegu z prędkością 12 $km \cdot h^{-1}$ (w grupie eksperymentalnej - spadek; w kontrolnej - wzrost), co świadczyło o odmiennej odpowiedzi organizmu na zastosowane interwencje suplementacyjne. Jednak co ważne, po skumulowaniu wyników wszystkich uczestników zarówno przed, jak i po badaniu zaobserwowano ścisłą korelację pomiędzy deltą O3I a RE. Okazało się, że wzrost O3I jest negatywnie skorelowany z poprawą RE.

Po raz pierwszy w tym badaniu sprawdzono wpływ spożycia kwasów EPA i DHA wśród aktywnych mężczyzn (biegaczy amatorów). Ten eksperyment uwypukla zasadność stosowania długotrwałej suplementacji n-3 PUFAs, aby w optymalnej ilości uległy one inkorporacji w błony erytrocytów. Obserwowane w tym doświadczeniu wypadkowe wysycenia krwinek czerwonych kwasami EPA i DHA tj. wzrost VO_{2peak} oraz poprawa RE stanowi solidny fundament dla dalszych badań w sprecyzowaniu: a) podaży optymalnej dziennej dawki kwasów EPA i DHA, b) poszukiwania mechanizmu odpowiedzialnego za poprawę zdolności wysiłkowych wśród sportowców w następstwie suplementacji n-3 PUFAs.

2.5.2. Omówienie pracy nr 2

W pracy nr 2 pt. „**Improved Oxygen Uptake Efficiency Parameters Are Not Correlated with VO_{2peak} or Running Economy and Are Not Affected by Omega-3 Fatty Acid Supplementation in Endurance Runners**” (Autorzy: Jost Z., Tomczyk M., Chroboczek M., Calder P.C., Laskowski R.) opublikowanej w International Journal of Environmental Research and Public Health głównym celem było wykazanie: a) czy OUEP oraz OUE@VAT mogą być wiarygodnymi, submaksymalnymi i przede wszystkim nieinwazyjnymi parametrami oceny wydolności tlenowej;

b) czy powyższe parametry ulegają zmianom w odpowiedzi na 12-tygodniową suplementację n-3 PUFAs.

W tym badaniu zaobserwowano słabą korelację lub jej brak pomiędzy OUEP a VO_{2peak} po połączeniu wyników uzyskanych przez wszystkich uczestników. Podobne wyniki uzyskano w przypadku OUE@VAT, ponieważ korelacja była bardzo słaba lub tej korelacji nie odnotowano względem VO_{2peak}. Co warte podkreślenia ani zmiany OUEP, ani OUE@VAT nie odzwierciedlały zmian w RE odnotowanej już w pracy nr 1. Wzrost OUEP oraz OUE@VAT zaobserwowano wśród uczestników niezależnie od podjętej interwencji suplementacyjnej (w grupach OMEGA i MCT łącznie).

Po raz pierwszy w tej pracy podjęto próbę odnalezienia pomiarów, które mogłyby być submaksymalnymi parametrami czułymi na zmiany VO_{2peak} oraz RE. Niestety, jak wcześniej opisano, OUEP i OUE@VAT nie oddają pełnego obrazu zmian na poziomie szczytowego poboru tlenu i ekonomii biegowej, toteż nie powinny być uważane jako pomiary zastępcze dla VO_{2peak} i RE. Jednak co interesujące, wzrost OUEP i OUE@VAT był niezależny od przydziału do grup (eksperymentalnej lub kontrolnej). Wskazuje to raczej na adaptację biegaczy amatorów do treningu o charakterze wytrzymałościowym niż efekt spożywania n-3 PUFAs.

Stąd, OUEP oraz OUE@VAT można uznać za parametry, które umożliwiają obiektywną ocenę zmian adaptacyjnych, lecz nie stanowią niezawodnego narzędzia w ocenie zmian w następstwie suplementacji n-3 PUFAs. W tym miejscu należy podkreślić nowatorską, aczkolwiek częściowo nieudaną próbę poszukiwania innych parametrów wydolności tlenowej. O ile mogą one przysłużyć się w ocenie zmian adaptacyjnych do treningu, o tyle czułość wskazania efektów suplementacyjnych jest w tym badaniu mocno zakwestionowana. Niewątpliwie jest to ciekawy kierunek w obszarze fizjologii wysiłku fizycznego i konieczne są kolejne badania, by w pełnym stopniu odrzucić lub przyjąć zaproponowaną użyteczność opisywanych parametrów.

2.5.3. Omówienie pracy nr 3

W pracy nr 3 pt. **„Increased Plasma L-Arginine Levels and L-Arginine/ADMA Ratios after Twelve Weeks of Omega-3 Fatty Acid Supplementation in Amateur Male Endurance Runners”** (Autorzy: Jost Z., Tomczyk M., Chroboczek M., Calder P.C., Fisk H.L., Przewłócka K., Antosiewicz J.) opublikowanego w Nutrients głównym celem było: a) sprawdzenie zmian w metabolizmie L-argininy i ADMA w osoczu oraz współczynnika L-arginina/ADMA w odpowiedzi na 12-tygodniową suplementację n-3 PUFAs; b) sprawdzenie, czy zmiany w metabolizmie L-argininy i jej metabolitów korelują ze zmianami RE u biegaczy amatorów.

W tym badaniu zaobserwowano istotny statystycznie wzrost poziomu L-argininy w osoczu

w grupie eksperymentalnej, czego nie odnotowano w grupie kontrolnej. Poziom ADMA w osoczu w obydwu grupach w następstwie interwencji nie uległ zmianom. Istotny statystycznie wzrost wskaźnika L-arg/ADMA zaobserwowano w grupie eksperymentalnej, czego nie odnotowano w grupie kontrolnej. W tej pracy przeprowadzono również próbę korelacji pomiędzy poziomami L-argininy w osoczu i wskaźnikiem L-arg/ADMA a RE. Co interesujące, nie odnotowano istotnej statystycznie korelacji pomiędzy deltami wyżej wymienionych zmiennych.

Za odkrycia w tym badaniu niewątpliwie trzeba uznać zmiany w metabolizmie L-argininy, przy jednoczesnym braku zmian w metabolizmie ADMA w osoczu u biegaczy amatorów. Po raz pierwszy podjęto próbę charakterystyki/odszukania mechanizmu odpowiedzialnego za poprawę zdolności wysiłkowych (mierzonej jako RE) w następstwie suplementacji n-3 PUFAs. Zgodnie z aktualnym stanem wiedzy wzrost L-argininy w osoczu jest pośrednio powiązany z większą biodostępnością/syntezą NO. To z kolei prowadzi do uruchomienia kaskady reakcji biochemicznych umiejscowionych w śródbłonku naczyniowym. Stąd, wzrost stężenia NO skutkuje efektem wazodylatacyjnym mięśni gładkich budujących te naczynia. Z diagnostycznego punktu widzenia, wskaźnik L-arg/ADMA stanowi solidny indykator łączący dwie zmienne kluczowe dla określenia kondycji śródbłonka naczyniowego i/lub wskazania zmian adaptacyjnych wśród sportowców. Co fascynujące, nie odnaleziono korelacji pomiędzy metabolitami kształtującymi ten wskaźnik a RE. Dlatego zakłada się, że mechanizm leżący u podstaw poprawy zdolności wysiłkowych nie jest powiązany z metabolizmem tlenku azotu, który to wzrost pośrednio wskazaliśmy w tej pracy.

2.6. Wnioski

1) 12-tygodniowa suplementacja n-3 PUFAs u biegaczy długodystansowych powoduje wzrost VO_{2peak} a także O3I, który bezpośrednio wpływa na poprawę RE.
2) OUEP i OUE@VAT nie uległy poprawie w następstwie suplementacji n-3 PUFAs.
3) 12-tygodniowa suplementacja n-3 PUFAs zwiększa spoczynkowy poziom L-argininy w osoczu, jednocześnie nie wywołując zmian w poziomie ADMA. Ponadto, wzrost współczynnika L-arg/ADMA nie przyczynia się bezpośrednio do poprawy RE.

Wnioski uzyskane w ramach cyklu publikacji potwierdzają zasadność stosowania n-3 PUFAs wśród sportowców (Hingley i wsp. 2017; Kawabata i wsp. 2014; Peoples i wsp. 2008), jednak po raz pierwszy u biegaczy długodystansowych. Pomimo wciąż niedostatecznie wyjaśnionego mechanizmu odpowiedzialnego za te zmiany warto podkreślić, że wzrost L-argininy w osoczu i wskaźnika L-arg/ADMA w następstwie suplementacji n-3 PUFAs w tym przypadku nie jest przyczyną poprawy zdolności wysiłkowych, co nie potwierdza wcześniej założonej hipotezy.

Stąd sugeruje się, aby dalsze badania zmierzały w kierunku prac nad innym czynnikiem, który będzie katalizować syntezę NO - receptorami aktywowanymi przez proliferatory peroksysomów (*ang. peroxisome proliferator-activated receptors;* PPARs), które, jak wskazują badania mogą być zaangażowane w odpowiedź organizmu na suplementację n-3 PUFAs (Moradi i wsp. 2021; Wang i wsp. 2022).

2.7. Piśmiennictwo (References)

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3. Streszczenie

Wprowadzenie: Wielonienasycone kwasy tłuszczowe omega-3 (n-3 PUFAs) w szczególności kwas eikozapentaenowy (EPA) i kwas dokozaheksaenowy (DHA) posiadają właściwości prozdrowotne, jak również wspomagające regenerację powysiłkową wśród sportowców. Swoim działaniem wspierają płynność błon komórkowych, śródbłonka naczyniowego oraz wspomagają funkcjonowanie erytrocytów. Niemniej jednak, wiedza dotycząca ich pełnego oddziaływania względem organizmu jest niedostateczna. Stąd, celem niniejszej pracy było sprawdzenie wpływu 12-tygodniowej suplementacji n-3 PUFAs na metabolizm L-argininy i wydolność tlenową u biegaczy długodystansowych. **Materiały i metody:** 26 biegaczy amatorów ukończyło 12-tygodniowy program suplementacyjno-treningowy. Przydzielono ich losowo do jednej z dwóch grup: eksperymentalnej (OMEGA; n=14) dziennie spożywającej n-3 PUFAs w dawce 2234 mg kwasu EPA i 916 mg kwasu DHA lub do grupy kontrolnej (MCT; n=12) dziennie spożywającej 4000 mg średniołańcuchowych kwasów tłuszczowych (MCTs). Uczestnicy zostali poddani jednolitemu treningowi wytrzymałościowemu, który wykonywano trzy razy w tygodniu oraz jednemu o charakterze oporowym z własną masą ciała. Zarówno przed, jak i po interwencji uczestników poddano próbie wydolności tlenowej „do odmowy wykonywania pracy" na bieżni mechanicznej oraz pobrano od nich krew na czczo z żyły łokciowej. Oznaczenia stężeń kwasów EPA i DHA w erytrocytach wykonano metodą chromatografii gazowej, a poziomy L-argininy i jej metabolitów w osoczu metodą chromatografii cieczowej z tandemową spektrometrią mas. **Wyniki:** 12-tygodniowa interwencja spowodowała wzrost indeksu omega-3 (O3I) i szczytowego poboru tlenu (VO_{2peak}) w grupie eksperymentalnej, czego nie obserwowano w grupie kontrolnej. Co więcej, wzrost O3I był negatywnie skorelowany z ekonomią biegową (RE). Nie odnotowano jednak poprawy plateau wydajności poboru tlenu (OUEP) oraz wydajności poboru tlenu na poziomie wentylacyjnego progu anaerobowego (OUE@VAT) w następstwie suplementacji n-3 PUFAs. Niemniej jednak poprawę tych parametrów odnotowano u uczestników niezależnie od przydziału do grupy. Co ciekawe, zaobserwowano istotny statystycznie wzrost poziomu L-argininy przy jednoczesnym braku zmian w poziomie asymetrycznej dimetyloargininy (ADMA) i wzrost wskaźnika L-arginina/ADMA w osoczu w grupie eksperymentalnej, czego nie obserwowano w grupie kontrolnej. Mimo tego wzrost zarówno L-argininy, jak i L-arg/ADMA nie były skorelowane z poprawą RE. **Wnioski:** 12-tygodniowa suplementacja n-3 PUFAs powoduje wzrost VO_{2peak}; a także O3I, który bezpośrednio wpływa na poprawę RE, jednocześnie nie wpływając na OUEP i OUE@VAT. Podjęta interwencja przyczynia się do wzrostu L-argininy w osoczu i współczynnika L-arg/ADMA pośrednio wskazując na zwiększoną biodostępność tlenku azotu (NO) nie przyczyniając się do poprawy RE u biegaczy długodystansowych.

4. Abstract

Introduction: Omega-3 polyunsaturated fatty acids (n-3 PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have pro-health properties as well as support post-exercise regeneration among athletes. With their action, they support the fluidity of cell membranes, vascular endothelium and support the functioning of erythrocytes. Nevertheless, knowledge about their full impact on the body is insufficient. Therefore, the aim of this study was to test the effect of 12-week n-3 PUFAs supplementation on L-arginine metabolism and aerobic capacity in long-distance runners. **Materials and methods:** 26 amateur runners completed a 12-week supplementation and training program. They were randomly assigned to one of two groups: experimental (OMEGA; *n*=14) daily consuming n-3 PUFAs in a dose of 2234 mg of EPA and 916 mg of DHA, or the control group (MCT; *n*=12) daily consuming 4000 mg of medium-chain fatty acids (MCTs). Participants were subjected to a uniform endurance training, which was performed three times a week, and one resistance training with their own body weight. Both before and after the intervention, the participants underwent an aerobic capacity test 'until volitional exhaustion' on a mechanical treadmill and fasting blood was taken from the cubital vein. Determination of concentrations of EPA and DHA acids in erythrocytes were performed by gas chromatography method, and the levels of L-arginine and its metabolites in plasma by liquid chromatography with tandem mass spectrometry method. **Results:** The 12-week intervention resulted in an increase in omega-3 index (O3I) and peak oxygen uptake (VO_{2peak}) in the experimental group, which was not observed in the control group. Moreover, the increase in O3I was negatively correlated with running economy (RE). However, there was no improvement in the plateau of oxygen uptake efficiency (OUEP) and oxygen uptake efficiency at the ventilatory anaerobic threshold (OUE@VAT) as a result of n-3 PUFAs supplementation. Nevertheless, improvement in these parameters was noted in participants regardless of group assignment. Interestingly, a statistically significant increase in the level of L-arginine was observed with no changes in the level of asymmetric dimethylarginine (ADMA) and an increase in the L-arginine/ADMA ratio in plasma in the experimental group, which was not observed in the control group. However, increases in both L-arginine and L-arg/ADMA were not correlated with improvement in RE. **Conclusions:** 12-week n-3 PUFAs supplementation causes an increase in VO_{2peak}; and O3I, which directly affects the improvement of RE, while not affecting OUEP and OUE@VAT. The undertaken intervention contributes to the increase of L-arginine and L-arg/ADMA ratio indirectly indicating increased bioavailability of nitric oxide (NO) without contributing to the improvement of RE in long-distance runners.

5. Załączniki (Attachments)

- Oświadczenia współautorów,
- Publikacja nr 1. „Effects of 12 Weeks of Omega-3 Fatty Acid Supplementation in Long-distance Runners”,
- Publikacja nr 2. „Improved Oxygen Uptake Efficiency Parameters Are Not Correlated with VO_{2peak} or Running Economy and Are Not Affected by Omega-3 Fatty Acid Supplementation in Endurance Runners”,
- Publikacja nr 3. „Increased Plasma L-Arginine Levels and L-Arginine/ADMA Ratios after Twelve Weeks of Omega-3 Fatty Acid Supplementation in Amateur Male Endurance Runners”.

SZKOŁA DOKTORSKA

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

Tomczyk M., Jost Z., Chroboczek M., Urbański R., Calder P.C., Fisk H.L., Sprengel M., Antosiewicz J., 2023. **Effects of 12 Weeks of Omega-3 Fatty Acid Supplementation in Long-distance Runners.** *Medicine & Science in Sports & Exercise, 2023;* 55(2): 216-224; doi:10.1249/MSS.0000000000003038

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Maciej Chroboczek	6	B, C	
Robert Urbański	3	B	
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Helena L. Fisk	5	B	
Katarzyna Przewłócka	5	E	
Jędrzej Antosiewicz	10	A, G	

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Effects of 12 Wk of Omega-3 Fatty Acid Supplementation in Long-Distance Runners

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BASIC SCIENCES

ABSTRACT

TOMCZYK, M., Z. JOST, M. CHROBOCZEK, R. URBAŃSKI, P. C. CALDER, H. L. FISK, M. SPRENGEL, and J. ANTOSIEWICZ. Effects of 12 Wk of Omega-3 Fatty Acid Supplementation in Long-Distance Runners. *Med. Sci. Sports Exerc.*, Vol. 55, No. 2, pp. 216–224, 2023. **Purpose:** This study aimed to investigate the effects of 12 wk of omega-3 fatty acid supplementation during endurance training on omega-3 index (O3I) and indicators of running performance in amateur long-distance runners. **Methods:** Twenty-six amateur male long-distance runners ≥29 yr old supplemented omega-3 fatty acid capsules (OMEGA group, n = 14; 2234 mg of eicosapentaenoic acid and 916 mg of docosahexaenoic acid daily) or medium-chain triglycerides capsules as placebo (medium-chain triglyceride [MCT] group, n = 12; 4000 mg of MCT daily) during 12 wk of endurance training. Before and after intervention, blood samples were collected for O3I assessment, and an incremental test to exhaustion and a 1500-m run trial were performed. **Results:** O3I was significantly increased in the OMEGA group (from 5.8% to 11.6%, $P < 0.0001$). A significant increase in $\dot{V}O_{2peak}$ was observed in the OMEGA group (from 53.6 ± 4.4 to 56.0 ± 3.7 mL·kg^{-1}·min^{-1}, $P = 0.0219$) without such change in MCT group (from 54.7 ± 6.8 to 56.4 ± 5.9 mL·kg^{-1}·min^{-1}, $P = 0.1308$). A positive correlation between the change in O3I and the change in running economy was observed when data of participants from both groups were combined (−0.1808 ± 1.917, $P = 0.0020$), without such an effect in OMEGA group alone ($P = 0.1741$). No effect of omega-3 supplementation on 1500-m run results was observed. **Conclusions:** Twelve weeks of omega-3 fatty acid supplementation at a dose of 2234 mg of eicosapentaenoic acid and 916 mg of docosahexaenoic acid daily during endurance training resulted in the improvement of O3I and running economy and increased $\dot{V}O_{2peak}$ without improvement in the 1500-m run trial time in amateur runners. **Key Words:** OMEGA-3 INDEX (O3I), POLYUNSATURATED FATTY ACIDS, RUNNING PERFORMANCE, ENDURANCE TRAINING, RUNNING ECONOMY

Omega-3 fatty acids include α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), characterized by the first double bond on the third carbon atom from the methyl end of the fatty acyl chain. There is growing evidence that synthesis *de novo* of EPA and, in particular, DHA is limited in the human body, and sources of preformed EPA and DHA, e.g., seafood, especially fatty fish or supplements should be consumed (1,2). Despite this, athlete's intake of sources of omega-3 fatty acids is often inadequate (3,4). Harris and von Schacky (5) proposed the so-called omega-3 index (O3I) as a valid indicator of omega-3 PUFA status, reflecting both intake of these fatty acids and their biological and health effects. O3I is the sum of EPA and DHA expressed as a percent of total fatty acids in erythrocytes. It is proposed that values >8% are associated with the greatest cardioprotection, whereas values <4% are associated with the least (5). O3I has been recognized as the best marker of omega-3 PUFA status associated with many health indicators and outcomes in the general population (6); however, its relation with physical performance indicators in athletes is poorly understood. Observations on amateur and competitive athletes confirm low O3I values. For example, in 106 German elite winter endurance athletes, only one had an

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O3I in the target range of >8%, and the average O3I value of the others was 4.97% ± 1.19% (7). Analysis conducted on collegiate athletes, professional basketball players, and trained but not professional endurance athletes confirm low values of the O3I and its increase after supplementation with omega-3 PUFA (4,8,9). A recent systematic review summarizing randomized placebo-controlled trials in athletes revealed that omega-3 PUFA supplementation improved cognitive function (e.g., reduction of reaction time and improvement of mood state), promoted skeletal muscle recovery, and attenuated proinflammatory cell responses (10).

The effect of omega-3 fatty acid supplementation on exercise performance is unclear, although several studies show positive effects on oxygen kinetics: cycling efficiency or maximal oxygen uptake (10). To date, the longest study where physical performance parameters were analyzed lasted 10 wk with the applied dose of 1.60 g of EPA and 1.04 g of DHA daily (11). The length and the dose of omega-3 fatty acid supplementation seem to be crucial because of the incorporation of EPA + DHA into target tissues, which would be reflected in erythrocyte membranes and O3I. Maximal incorporation of EPA and DHA into erythrocytes is related to erythrocyte turnover: in a 12-month controlled intervention trial conducted on healthy individuals, Browning and coauthors (12) revealed that it takes 55 and 136 d for EPA and DHA, respectively, to achieve peak incorporation into erythrocytes in the case of a supplementation dose of 3.27 g of EPA + DHA for 4 $d \cdot wk^{-1}$.

Given the paucity of long-term studies using omega-3 fatty acid supplements in athletes showing relation between O3I values and physical performance indicators, there is a need for further work in this area. Accordingly, we determined the effects of 12 wk of EPA + DHA supplementation (2234 mg and 916 $mg \cdot d^{-1}$, respectively) compared with medium-chain triglycerides (MCT) as placebo in dose 4000 $mg \cdot d^{-1}$ during endurance training on O3I and physical performance indicators in amateur runners. We hypothesize that this duration and dosage of omega-3 PUFA will result in significant incorporation of EPA and DHA into erythrocytes membranes and increase O3I to values considered as a target range (i.e., >8%). Moreover, using the longest duration and the highest dose of supplementation of the studies conducted so far, we hypothesize that this will increase $\dot{V}O_{2peak}$ and improve running economy (RE) to a degree that will translate into better running performance.

METHODS

Ethical approval. The study was approved by the Bioethical Committee of Regional Medical Society in Gdańsk (NKBBN/628/2019) and conducted according to the Declaration of Helsinki. After comprehensive details of the study protocol were explained orally and in writing, all participants provided their written informed consent.

Participants. Forty amateur male long-distance runners were recruited through advertisements on the Internet. Inclusion criteria included age between 29 and 42 yr and completion of an official 10 km race over the 2016 and 2020 time period with a time result between 37 and 57 min. The exclusion criteria included chronic diseases, cigarette smoking, or use of prescribed medications or dietary supplements, including omega-3 fatty acids. On the day of familiarization with the laboratory conditions and the treadmill test, participants were allocated sequential numbers that were then used as the identifiable characteristic. Assignment to each group (OMEGA or MCT) using an online randomizer (http://www.randomizer.org) took place on the first day of the actual exercise tests. All participants agreed to carry out only the training courses included in the program and were instructed to continue with their habitual dietary patterns for the duration of the intervention.

Overview of study design. The trial was conducted in the Laboratory of Physical Exercise and Department of Biochemistry of the Academy of Physical Education and Sport in Gdansk. After inclusion, participants were randomly assigned to one of the two groups: OMEGA or MCT providing either omega-3 fatty acids or MCT. All participants completed a progressive endurance training supervised by a track and field coach. The parallel randomized trial consisted of three 4-wk phases, for a total of 12 wk together with simultaneous supplementation. A graded exercise test to exhaustion with assessment of $\dot{V}O_{2peak}$, RE, and a 1500-m run trial was carried out before and after completion of the exercise training program. Each test was preceded by a standardized breakfast for all participants consumed 1 h before the test began. Blood collection and weight assessment were performed when participants were in a fasting state. Figure 1 outlines the experimental protocol.

Omega-3 PUFA supplementation. Throughout the study, all participants took four identical-looking capsules each day (two in the morning and two in the evening) containing either omega-3 fatty acids or MCT. The omega-3 capsules provided 2234 mg of EPA and 916 mg of DHA daily (Omega-3 double plus, NAMED SPORT, Italy), whereas the MCT capsules contained 4000 mg of MCT (MCT Oil; Now Foods, Bloomingdale, IL). The dose of omega-3 fatty acids is consistent with the dosage applied in the study of Browning and coauthors (12). To maintain certainty of the amount of each fatty acid and the general quality of the supplements containing omega-3 fatty acids, a product certified by the International Fish Oil Standard was selected. The International Fish Oil Standard program verifies the amount of each fatty acid and the content of heavy metals, dioxins, and rate of oxidation. A publicly available batch report of the supplements used in the study indicated that the amounts of individual acids were in accordance with the manufacturer's claims, and content of heavy metals, dioxins, and rate of oxidation did not exceed accepted standards. Moreover, both supplements were certified by the informed-sport program, under which products are tested for substances banned by the World Anti-Doping Agency. To avoid a potential recognition of supplements, participants were informed that they were all taking omega-3 fatty acids in one of two chemical forms. On the day of arrival at the laboratory, 1 h before the graded exercise test and the 1500-m run trial, participants consumed the same standardized breakfast. Breakfast was a

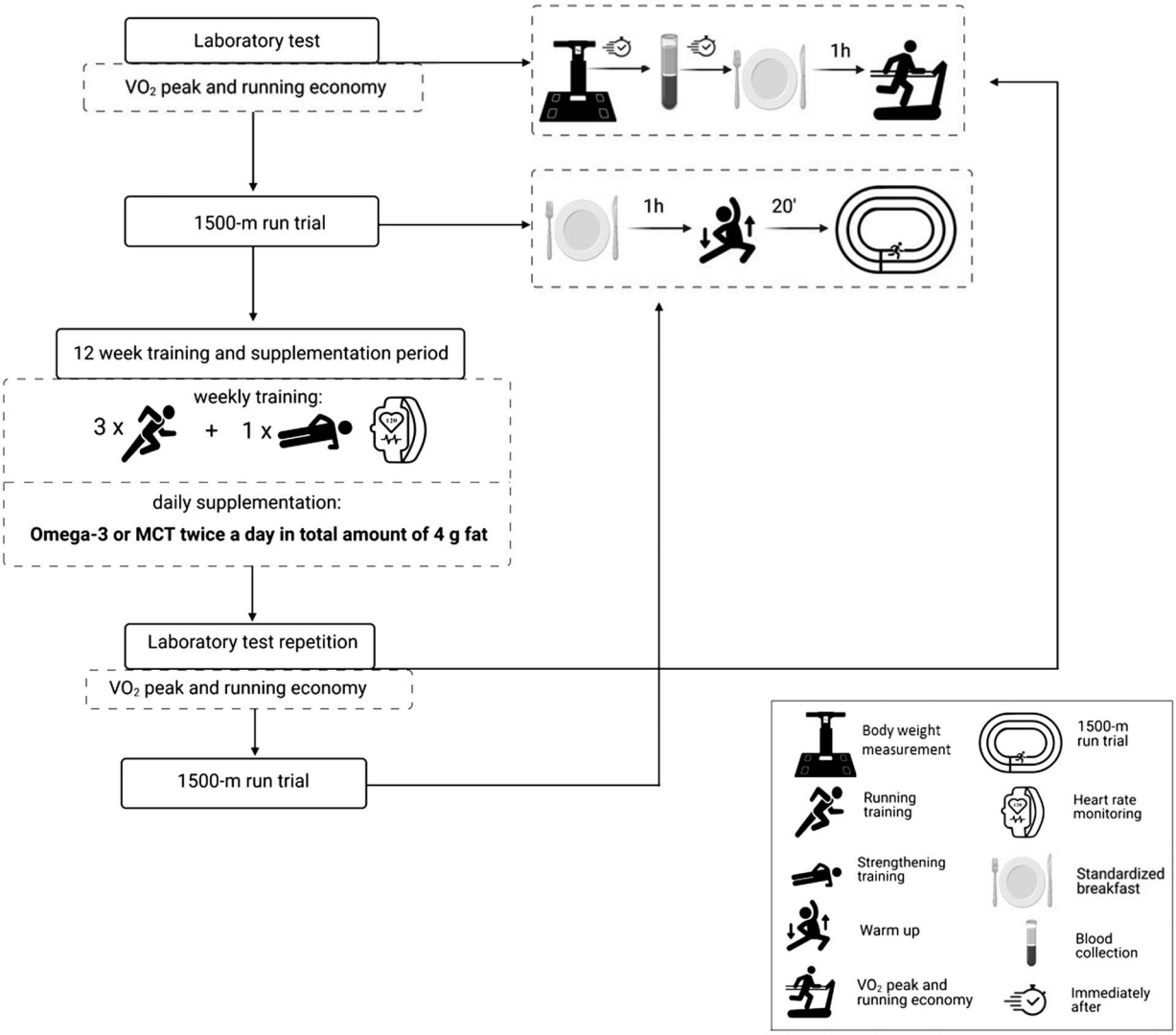

FIGURE 1—General experimental design.

replication of a typical prestart meal and consisted of wheat roll with butter and jam and half a banana.

Total energy value and amount of carbohydrate, protein, and fat was 290 kcal, 49 g, 5 g, and 8 g, respectively. Dietary intake over 3 d (2 d from week and 1 d from weekend) was recorded in the first and the last week of the program. Participants used the MyFitnessPal mobile application to record the meals they consumed. Before using the app for the first time, the basic functions were demonstrated to all participants. Moreover, the Web site ilewazy.pl was presented to participants, so they could more easily estimate the portions they consumed when kitchen scales were not available. If recorded meals were not precise, participants were asked to clarify the information. Collected dietary records where then analyzed using nutrition analysis software (Kcalmar.pro, Poland). Every food item in meals, with the consumed amount, was entered to the nutrition analysis software, and total dietary energy, carbohydrate, protein, and fat content were calculated.

Exercise testing. Before (week 0) and after completion (week 13) of the exercise training program, participants were submitted to a graded exercise test to exhaustion on a motorized treadmill (h/p Cosmos, Saturn, Germany) to determine whether omega-3 fatty acids combined with endurance training might positively affect the endurance potential of runners. Before the intervention, the participant's body weight and height were measured (analyzer InBody 720 and stadiometer Seca 213, respectively), then they were familiarized with the laboratory conditions and the treadmill test.

First, participants stood on the treadmill for 2 min to make sure the measuring equipment was ready and to measure the resting values. Thereafter, runners walked for 5 min at 5 $km \cdot h^{-1}$ speed and with a 1.5% inclination as a warm-up before starting the test. Every next stage lasted 3 min aimed to reach steady-state $\dot{V}O_2$ (13), and the treadmill belt was accelerated starting from 8×1 $km \cdot h^{-1}$ per stage up to 12 $km \cdot h^{-1}$. Then the inclination of the treadmill was increased to 5%, 10%, and 15% at 12 $km \cdot h^{-1}$

speed until volitional exhaustion. During both tests, heart rate (HR) was monitored (Polar RS400; Polar Electro Oy, Kempele, Finland) to define the highest value (HR_{max}) during each test. Minute ventilation ($\dot{V}_E$), oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), and RER were continuously measured using a breath-by-breath analyzer (Oxycon Pro, Jaeger, Germany), which was calibrated before each test following the manufacturer's recommendations. Measurements were averaged in 10-s intervals. $\dot{V}O_{2peak}$ was obtained as the highest 30-s mean value recorded during the test. Running economy was measured as an oxygen cost from the last 50 s of each stage to 12 $km \cdot h^{-1}$ speed and was expressed as milliliters per kilogram per minute (14), and RE analysis was performed up to RER <1. All measurements were performed at similar time of day ±2 h and constant environmental conditions (18°C–20°C and humidity 40%–45%). Additionally, participants were informed to avoid strenuous exercise for 24 h before and caffeine and alcohol consumption for 12 h before laboratory tests. One week after the graded exercise test, participants took part in a 1500-m run time trial on an indoor 200-m track. The time was recorded with a handheld stopwatch to the nearest 0.1 s. During both tests, participants received strong verbal encouragement.

Training protocol. The training protocol lasted 12 wk and was built based on undulatory load manipulation 3:1, which was suggested to be effective to prevent overtraining and stress due to oscillations between volume/intensity according to Costa et al. (15) with slight modifications. Hence, participants performed endurance training 3 times per week. One additional training per week aimed to strengthen core muscles to reduce the risk of lower extremity injuries was also included in protocol (16). Training intensity was prescribed according to the first ventilatory threshold and ventilatory anaerobic threshold (VT1 and VAT), respectively, and their associated HR values were obtained during the laboratory testing. The threshold-based method was described as better than the HR reserve–based method to design more individualized exercise prescriptions that will enhance training efficacy and limit training unresponsiveness (17). Consequently, participants trained in three HR zones: [Z1: ≤HR@VT1 + 5 bpm; Z2: (>HR@VT1 + 5 bpm) to (≤HR@VAT-5 bpm); Z3: >HR@VAT-5 bpm], and their average training times spent in every mesocycle were (~80%–15%–5%) in zones (Z1–Z2–Z3), respectively, accordingly to previous authors (18) with slight modifications. On the last (12th) week, the tapering procedure was performed, whereby the training load was reduced to 70% from the volume obtained in the 11th week to reduce accumulated fatigue. Participant's training activity (training volume, intensity, and energy expenditure) was monitored by a Polar M430 wristwatch and an H9 HR chest sensor. All running tests and training procedures were supervised by a track and field coach.

Erythrocyte fatty acid analysis. Fasting blood samples were collected from participants by a nurse into 4-mL sodium citrate vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ) and centrifuged at 4°C (4000*g* for 10 min). After centrifugation, erythrocytes were collected with a disposable pasteur pipette and transferred into eppendorfs, which were stored in a − 80°C freezer until further analysis. Erythrocyte EPA and DHA were assessed using gas chromatography as described elsewhere (19). Briefly, erythrocyte lipids were extracted into chloroform–methanol, and fatty acid methyl esters (representing the erythrocyte fatty acids) were formed by heating the lipid extract with methanolic sulfuric acid. The fatty acid methyl esters were separated by gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column using the settings and run conditions described elsewhere (19). Fatty acid methyl esters were identified by comparison with run times of authentic standards. Data are expressed as weight % of total fatty acids. O3I was calculated by summing the percentages of EPA and DHA according to Harris and von Schacky (5).

Statistical analysis. The sample size calculation was based on changes in oxygen consumption during graded exercise test to exhaustion assessed as $\dot{V}O_{2peak}$, as this was the primary outcome of the study. A typical value for $\dot{V}O_{2peak}$ in population of recreational long-distance runners is about 54 $mL \cdot kg^{-1} \cdot min^{-1}$ with an SD of about 5 (20).

It is considered that an 8% increase in $\dot{V}O_{2peak}$ is meaningful in amateur runners (21). A sample size of 18 participants per group (i.e., 36 participants in total) would give 70% power to detect this difference as significant with alpha = 0.05. In order to account for a dropout rate of 10%, 40 participants were recruited. Statistical analysis was performed using the tools of GraphPad Prism 7. Arithmetic means, SD, and significance levels of differences between means were calculated. A two-way repeated-measures ANOVA was used to investigate the significance of differences between groups and time. Significant main effects were further analyzed using the Bonferroni corrected *post hoc* test. Changes (Δ) in both groups were compared using an independent samples *t*-test. Correlations between variables were evaluated using the Pearson correlation coefficient. All analyses used a significance level of $P < 0.05$.

RESULTS

Participant flow through the study. Participants excluded from the final analysis completed insufficient (<80%) training sessions ($n = 3$) or withdrew from the study for health ($n = 9$) or personal reasons ($n = 1$). Moreover, one participant from MCT group increase intake of omega-3 fatty acids during study; therefore, he was also excluded from statistics. Participant flow through the study is presented in Figure 2. From the 40 participants enrolled, 26 completed the entire study and their characteristic is shown in Table 1.

Erythrocyte EPA, DHA, and O3I. The percentage values of erythrocyte EPA, DHA, and O3I pre- and postintervention in the OMEGA and MCT groups are presented in Figures 3 and 4. There was no difference in baseline values of either omega-3 PUFA or O3I between the groups (OMEGA group: 1.1% EPA, 4.7% DHA, 5.8% O3I; MCT group: 1.2% EPA, 4.4% DHA, 5.6% O3I; all $P > 0.9999$). Twelve weeks of omega-3 fatty acid supplementation during endurance training

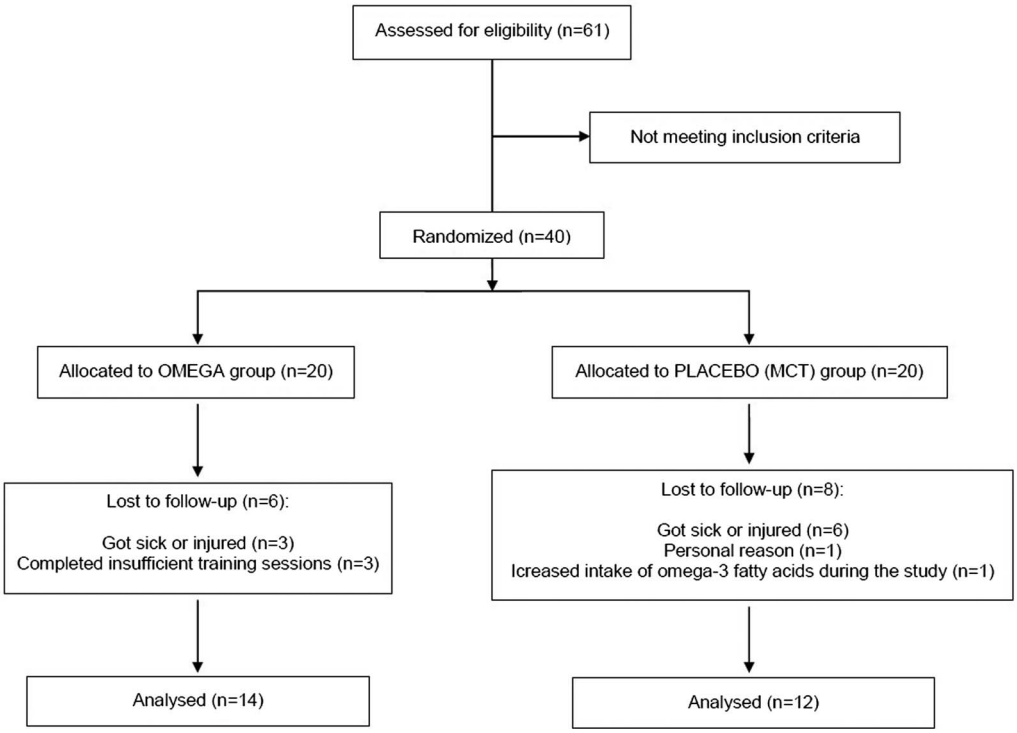

FIGURE 2—Flow of participants through the study.

increased both omega-3 PUFA and O3I in the OMEGA group (to 4.9% ± 1.1% EPA, 6.7% ± 0.8% DHA, 11.6% ± 1.7% O3I; all $P < 0.0001$) without significant changes in the MCT group (to 1.1% EPA, 4.5% DHA, 5.6% O3I; all $P > 0.9999$). At the end of the intervention period EPA, DHA and O3I were significantly higher in OMEGA group than in MCT group (all $P < 0.0001$).

$\dot{V}O_{2peak}$, RE, and 1500-m run trial. There was no significant difference between groups in change in $\dot{V}O_{2peak}$ over the 12-wk intervention period ($P = 0.6764$) (Fig. 5B). However, a significant increase in $\dot{V}O_{2peak}$ from pre- to postintervention in OMEGA group was observed (from 53.6 ± 4.4 to 56.0 ± 3.7 $mL \cdot kg^{-1} \cdot min^{-1}$, $P = 0.0219$) with no significant change in MCT group (from 54.7 ± 6.8 to 56.4 ± 5.9 $mL \cdot kg^{-1} \cdot min^{-1}$, $P = 0.1308$) (Fig. 5A). Increase in $\dot{V}O_{2peak}$ was seen in 13 (93%) out of 14 participants in the OMEGA group, whereas in the MCT group, improvements were visible in 9 (75%) out of 12 runners.

Moreover, oxygen uptake at 12 $km \cdot h^{-1}$ changed in both groups: the RE increased significantly in the OMEGA group (from 47.6 ± 1.8 to 46.5 ± 2.4 $mL \cdot kg^{-1} \cdot min^{-1}$, $P = 0.0295$), whereas it decreased in the MCT group (from 47.7 ± 3.3 to 48.7 ± 2.9 $mL \cdot kg^{-1} \cdot min^{-1}$, $P = 0.1127$) (Fig. 5C). The change in oxygen uptake over the 12-wk intervention period was significantly different between groups ($P = 0.0033$) (Fig. 5D). When results before and after the 12-wk intervention from all participants were combined, correlation highlighted the relationship between O3I and oxygen cost of submaximal running (Fig. 6A, $P = 0.0338$; Fig. 6B, $P = 0.0020$). There was significant improvement in completion of the 1500-m run trial in both groups from pre- to postintervention; however, results did not differ between groups over the study period (OMEGA group from 356.3 to 344.9 s, $P = 0.0002$; MCT group from 362.1 to 347.3 s, $P < 0.0001$; pre- to postintervention between groups, $P > 0.9999$).

Physiological and nutritional variables. Table 2 summarizes physiological and nutritional variables obtained from the participants at the beginning and after completing the intervention program. There was no difference in weekly training volume ($P = 0.7399$), energy expenditure ($P = 0.1828$), and HR_{max} ($P = 0.4624$) between the groups. However, in both groups, there was a significant increase in HR_{max} at VAT (%) postintervention compared with preintervention (OMEGA group from 91.7 ± 2.6 to 93.9 ± 2.8, $P = 0.0331$; MCT group from 90.8 ± 3.9 to 95.2 ± 3.7, $P = 0.0001$). Total energy ($kcal \cdot d^{-1}$), carbohydrate, and protein ($g \cdot kg^{-1} \cdot d^{-1}$) intake did not differ pre- to postintervention within either group (OMEGA group $P > 0.9999$, $P = 0.5442$, $P = 0.5777$; MCT group $P = 0.1973$, $P > 0.9999$, $P = 0.7721$, respectively).

There was a statistically significant difference in fat intake between the two groups with a significantly higher fat intake in the OMEGA group (from 83.4 ± 25.9 to 91.9 ± 25.9 g,

TABLE 1. Characteristics of participants who completed the study.

Variable	Omega ($n = 14$)	MCT ($n = 12$)
Age (yr)	37 ± 3	37 ± 4
Body mass (kg)	76.3 ± 11	78.0 ± 8
Height (cm)	181 ± 7	180 ± 4
$\dot{V}O_2$ peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	53.6 ± 4	54.7 ± 7
Personal best in 10-km run between 2016 and 2020 (min)	45 ± 4	46 ± 5

Data are presented as mean ± SD.

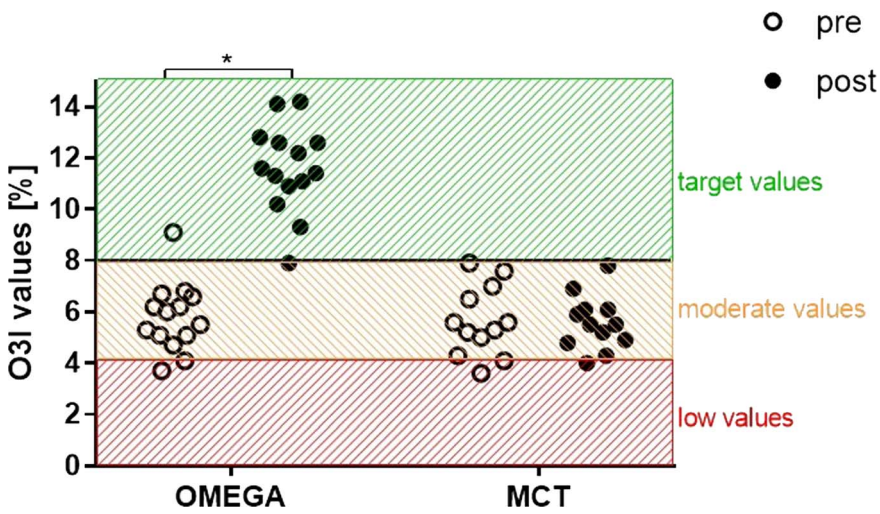

FIGURE 3—Effect of supplementation with omega-3 PUFA or MCT on individual values of O3I before and after the 12-wk intervention. *$P < 0.0001$.

$P = 0.0321$) and lower; however, not significant fat intake in the MCT group ($P = 0.0943$).

DISCUSSION

The main finding of the study is that 12 wk of supplementation with omega-3 fatty acids at a dose of 2234 mg of EPA and 916 mg of DHA daily shifts erythrocyte O3I to values considered as a target range for cardiovascular health. Moreover, this duration and dose of supplementation during endurance training increased $\dot{V}O_{2peak}$ and improved RE at velocity 12 km·h^{-1} with no effect on 1500-m run trial results. Insufficient values of O3I in active individuals are well described. In a study including vegan and omnivorous endurance athletes, Cradock et al. (8) showed suboptimal O3I in both groups: 4.13% in vegans and 5.40% in omnivores, respectively. Similarly, O3I below the desirable values was demonstrated in German national elite winter endurance athletes (4.97% ± 1.19%), professional basketball players from the NBAG League (5.02% ± 1.19%), and collegiate athletes, representing diverse disciplines throughout the United States (4.33% ± 0.81%) (4,7,22). Our observations are in agreement with these reports, indicating that amateur runners had mean baseline O3I of around 5.7% (5.8% and 5.6% in OMEGA and MCT groups, respectively).

Twelve weeks with omega-3 fatty acid supplementation at a dose of 2234 mg of EPA and 916 mg of DHA daily during

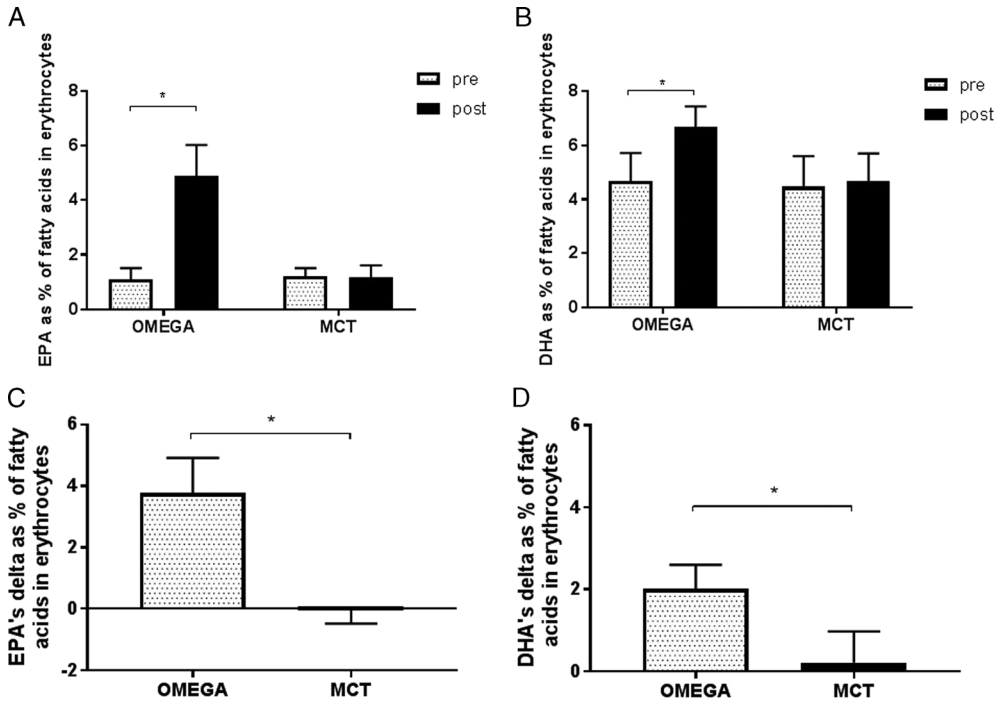

FIGURE 4—Effect of supplementation with omega-3 PUFA or MCT on erythrocyte EPA (A) and DHA (B) before and after the 12-wk intervention and change from baseline in EPA (C) and DHA (D) compared between the two groups. Data are expressed as mean. Error bars indicate ± SD, *$P < 0.0001$.

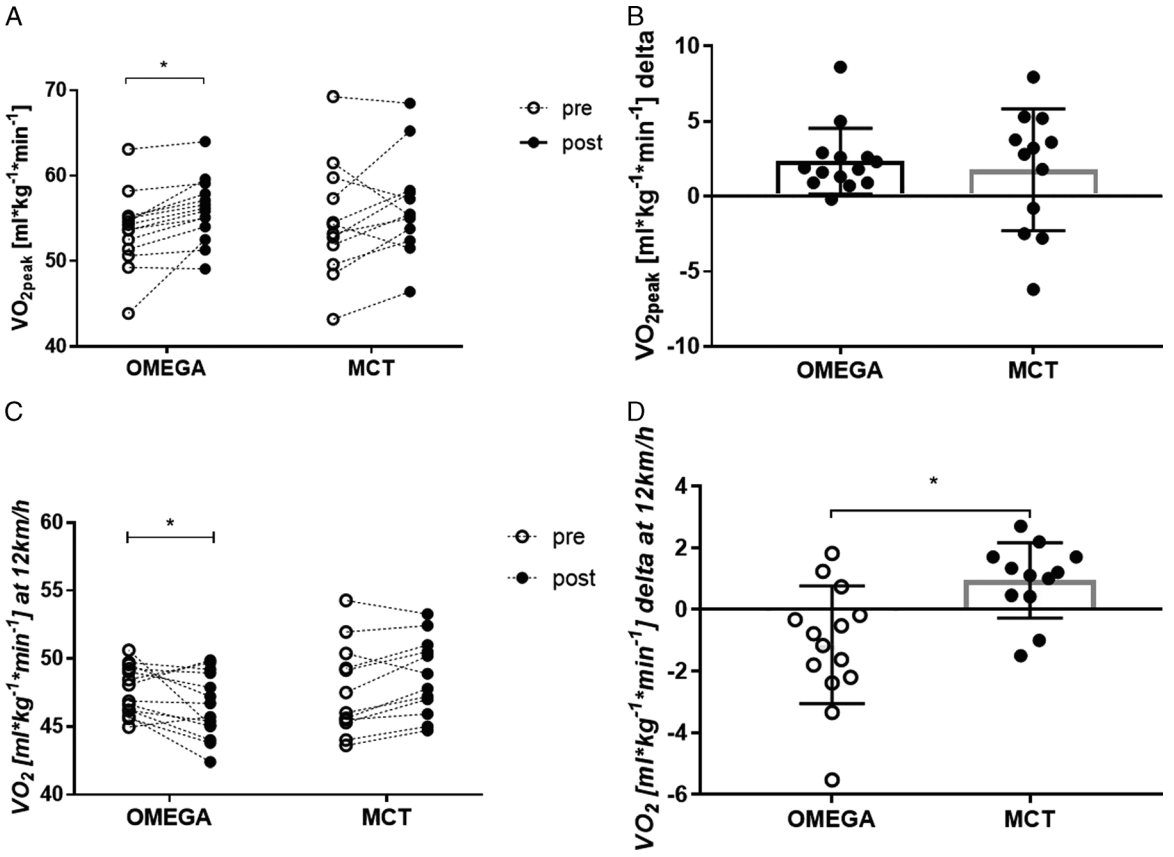

FIGURE 5—Effect of training and supplementation on peak oxygen consumption (A) (mL·kg^{-1}·min^{-1}) before and after the 12-wk intervention, change in peak oxygen consumption (B) (mL·kg^{-1}·min^{-1}) in the two groups over the 12-wk intervention, oxygen utilization (C) (mL·kg^{-1}·min^{-1}) during submaximal treadmill running at 12 km·h^{-1} before and after the 12-wk intervention, change in oxygen utilization (D) (mL·kg^{-1}·min^{-1}) in the two groups over the 12-wk intervention. Data are expressed as mean. Error bars indicate ± SD, *$P < 0.05$.

endurance training increased O3I in all but one participant in OMEGA group to mean of 11.4%, which is considered to be well within the O3I target range (5). Moreover, an increase in O3I correlated with an increase in RE at velocity 12 km·h^{-1} when results post- minus pre-12-wk intervention of participants from both groups were combined. Improvements in exercise economy as an effect of supplementation with omega-3 fatty acids have previously been shown in both amateur and competitive athletes (9,23,24). In an 8-wk double-blind, parallel design study in well-trained cyclists, Peoples et al. (23) showed that 3.2 g·d^{-1} of omega-3 fatty acids reduced whole-body O_2 consumption throughout 60 min of sustained submaximal cycling. Contrary to our observations, peak oxygen consumption in these cyclists was not changed, which may be related to their high level of training status or quite high compared with other data (above 9%) baseline O3I values (23). Improved economy of cycling during the physiologically demanding time trial in trained cyclists and runners was also revealed by Hingley et al. (9) after 8 wk of supplementation with a dose of 560 mg of DHA + 140 mg of EPA a day. Despite an elevation in O3I (from 4.7% ± 0.2% to 6.3% ± 0.3%), the values did not achieve the recommended O3I >8%, which may be related to the low dose of EPA + DHA used. A study conducted by Kawabata et al. (24) with recreational players of American football, rugby, baseball, and basketball is consistent with other observations in trained individuals: 8 wk of daily supplementation with 914 mg of EPA and

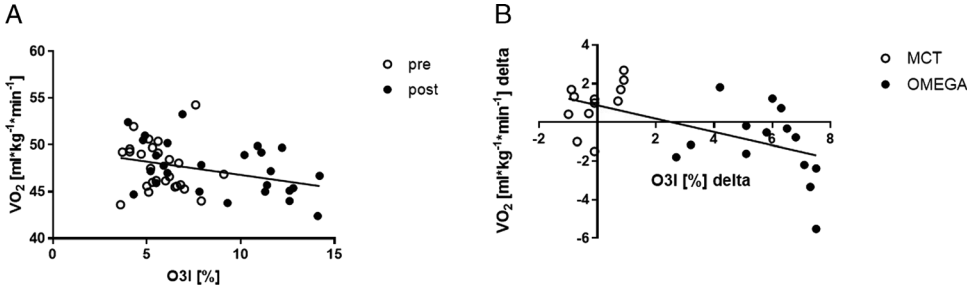

FIGURE 6—Correlation between O3I and oxygen cost of submaximal running when: OMEGA and MCT groups were combined before and after the 12-wk intervention (A). B. Results postintervention minus preintervention (Δ) in OMEGA and MCT groups were combined.

TABLE 2. Physiological and nutritional variables according to treatment group.

Variable		Omega		MCT
Weekly training volume (km)		30.95 ± 2.47		31.5 ± 5.51
Energy expenditure (kcal·d^{-1})		2515 ± 445		2748 ± 415
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	Pre	53.61 ± 4.36	Pre	54.66 ± 6.76
	Post	55.96 ± 3.72*	Post	56.44 ± 5.89
HR_{max} (bpm)	Pre	190 ± 9	Pre	186 ± 9
	Post	189 ± 9	Post	184 ± 7
HR_{max} at VAT (%)	Pre	91.71 ± 2.65	Pre	90.81 ± 3.95
	Post	93.89 ± 2.77*	Post	95.20 ± 3.69**
Body mass (kg)	Pre	76.30 ± 10.96	Pre	78.03 ± 7.70
	Post	76.55 ± 11.32	Post	77.0 ± 7.35*
Energy and nutrient intake (per day)				
Energy (kcal)	Pre	2393 ± 453	Pre	2456 ± 587
	Post	2429 ± 420	Post	2338 ± 627
Carbohydrate (g)	Pre	301 ± 63	Pre	310 ± 111
	Post	289 ± 46	Post	302 ± 127
Protein (g)	Pre	98 ± 20	Pre	99 ± 20
	Post	102 ± 17	Post	95 ± 17
Fat (g)[a]	Pre	83 ± 27	Pre	86 ± 18
	Post	92 ± 27*	Post	79 ± 15

Data are presented as mean ± SD.
*$P < 0.05$ for post- vs preintervention value.
**$P < 0.01$ for post- vs preintervention value.
[a]Statistically significant difference in groups (Δ) with a trend of higher intake in the O3I group and lower intake in the MCT group.

399 mg of DHA increased exercise economy during a steady-state submaximal cycloergometer test. In one crossover study with trained cyclists, researchers observed an increase in $\dot{V}O_{2max}$ after 3 wk of supplementation with a daily dose of 660 mg of EPA and 440 mg of DHA (25).

In contrast to this report, an earlier study conducted by Raastad et al. (11) showed no changes in $\dot{V}O_{2max}$ and running performance in well-trained soccer players receiving 1.60 g of EPA and 1.04 g of DHA a day through 10-wk period. Exercise economy together with $\dot{V}O_{2max}$, lactate threshold, and critical power are all strongly related to endurance exercise performance (26). Therefore, studies showing increased exercise economy, $\dot{V}O_{2max}$, or $\dot{V}O_{2peak}$ provide a rationale to further explore this topic together with the potential underlying mechanisms. Supplementation with omega-3 fatty acids reduces exercise-induced inflammation in athletes through decreasing in proinflammatory omega-6 fatty acids (27) and AA/EPA ratio (28). Given the large cross-sectional study indicating that inverse relationship between $\dot{V}O_{2max}$ and C-reactive protein is modified by omega-3 fatty acid levels (29), this may be the case. Moreover, an increase in insulin sensitivity due to unsaturation of skeletal muscle membranes (30), improved calcium handling by skeletal muscle sarcoplasmic reticulum (23), and improved endothelial function via increase in NO release (25) should be taken into account in searching for potential mechanisms of action. Of note, in the present study, 13 out of 14 participants in the OMEGA group showed an improved $\dot{V}O_{2peak}$ compared with a variable response in the MCT group, in which only 9 out of 12 runners improved their results. This may suggest better adaptation to endurance training in response to omega-3 fatty acid supplementation, as has been observed with several other dietary supplements (31). Still, neither our nor previous reports support the hypothesis that long-term supplementation with omega-3 fatty acids enhances exercise performance. Duration and dose of omega-3 supplementation are crucial factors determining the amount of fatty acids incorporated into erythrocyte membranes, and more than 4 months is needed to reach the highest concentration of DHA in case of a supplementation dose of 1.5 g of EPA and 1.77 g of DHA for 4 d·wk^{-1} (12).

Compared with previous studies in which performance indicators were assessed, our supplementation protocol (2234 mg of EPA and 916 mg of DHA daily for 12 wk) was a higher dose over a longer supplementation period (9,23–25). However, what values of O3I are sufficient for amateur and competitive athletes to optimize athletic performance remains a question to be answered in future studies.

Our study has some limitations that must be highlighted. Running economy is typically determined by measuring the consumption of oxygen when the steady state of $\dot{V}O_2$ is observed (13). We recognized steady-state conditions when runners had RER <1 during treadmill running (13,32); however, the concentration of lactic acid was not assessed. Considering that lactate threshold (LT) is one of the indicators of disturbance in $\dot{V}O_2$ steady state (26,33), it should be included in future research. Animal studies showed that DHA is incorporated into the membranes of fast-oxidative glycolytic fibers (type IIA) of skeletal muscle (34). These muscle fibers have both a high oxidative and glycolytic capacity, and because of their increased activation during moments of high energy demand (35), we decided to perform a 1500-m run trial. Our participants typically perform distances from 10 km to a marathon; therefore, lack of experience and unfamiliarization at such a short distance as 1500-m may influence the outcome of the run trial, and this must be taken into consideration when interpreting our findings. Future studies with omega-3 supplementation should also consider prescreening, during which individuals with similar baseline O3I should be selected (36).

CONCLUSIONS

In conclusion, 12 wk of omega-3 fatty acid supplementation at a dose of 2234 mg of EPA and 916 mg of DHA daily during an endurance running program increased O3I to values currently considered as a target range. This duration and dose of supplementation combined with endurance training increased peak oxygen consumption and improved RE in amateur runners without affecting their performance.

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Competing interests: None of the authors of this paper has a competing interest.

Conception and design of the experiments were undertaken by M.T., Z. J., P. C. C., and J. A. Collection, assembly, analysis, and interpretation of data were undertaken by M. T., Z. J., M. C., R. U., H. L. F., P. C. C., M. S., and J. A. Drafting the work or revising it critically for important intellectual content was undertaken by M. T., Z. J., M. C., P. C. C., and J. A. All authors have approved the final version of the manuscript and agree to be

accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Clinical registry: The study was registered at https://www.clinicaltrials.gov/ with identifier NCT04780451.

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Improved Oxygen Uptake Efficiency Parameters Are Not Correlated with VO_{2peak} or Running Economy and Are Not Affected by Omega-3 Fatty Acid Supplementation in Endurance Runners

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Abstract: Peak oxygen uptake (VO_{2peak}) is one of the most reliable parameters of exercise capacity; however, maximum effort is required to achieve this. Therefore, alternative, and repeatable submaximal parameters, such as running economy (RE), are needed. Thus, we evaluated the suitability of oxygen uptake efficiency (OUE), oxygen uptake efficiency plateau (OUEP) and oxygen uptake efficiency at the ventilatory anaerobic threshold (OUE@VAT) as alternatives for VO_{2peak} and RE. Moreover, we evaluated how these parameters are affected by endurance training and supplementation with omega-3 fatty acids. A total of 26 amateur male runners completed a 12-week endurance program combined with omega-3 fatty acid supplementation or medium-chain triglycerides as a placebo. Before and after the intervention, the participants were subjected to a treadmill test to determine VO_{2peak}, RE, OUE, OUEP and OUE@VAT. Blood was collected at the same timepoints to determine eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in erythrocytes. OUE correlated moderately or weakly with VO_{2peak} ($R^2 = 0.338$, $p = 0.002$) and ($R^2 = 0.226$, $p = 0.014$) before and after the intervention, respectively. There was a weak or no correlation between OUEP, OUE@VAT, VO_{2peak} and RE despite steeper OUE, increased OUEP and OUE@VAT values in all participants. OUE parameters cannot be treated as alternative parameters for VO_{2peak} or RE and did not show changes following supplementation with omega-3 fatty acids in male amateur endurance runners.

Keywords: peak oxygen uptake; oxygen uptake efficiency plateau; running economy; omega-3 fatty acids; endurance runners

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1. Introduction

There are many cardiopulmonary exercise tests (CPETs) that aim to assess parameters related to human performance, such as peak oxygen uptake (VO_{2peak}) or maximal oxygen uptake (VO_{2max}). VO_{2max} is considered the best indicator of potential in endurance events, being a 'gold standard' measurement of integrated cardiopulmonary-muscle oxidative function [1–3]. Although heart rate (HR), respiratory exchange ratio (RER), and minute ventilation (Ve) are considered cardiovascular, respiratory, and pulmonary parameters, respectively, their comprehensive function is often difficult to evaluate. Therefore, there is a need to identify alternative validated and reliable parameters for assessing cardiorespiratory fitness.

Sun and co-authors [4] determined the relationship between oxygen uptake (VO_2) and Ve, called oxygen uptake efficiency (OUE). They noted that OUE increases linearly with time during early exercise, but becomes non-linear as Ve increases faster than VO_2.

This curvilinear relationship during an exercise test is not as appropriate for assessing aerobic capacity as VO_{2peak}. Thus, the authors described other physiological parameters that can be determined from respiratory gases during CPET, i.e., oxygen uptake efficiency at the ventilatory anaerobic threshold (OUE@VAT) and oxygen uptake efficiency plateau (OUEP) in healthy subjects. It was observed that both OUE@VAT and OUEP are simple measurements that do not require maximum effort. Moreover, they are also easy to visualise, recognise and calculate [4], making them potentially robust parameters for assessing physical fitness. It is worth noting that there is still scarce evidence of improvements in OUE parameters after physical training and no evidence of improvements after supplementation with bioactive compounds such as the omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)).

Although some studies show no improvements in cardiopulmonary-muscle oxidative function following supplementation with fish oil containing omega-3 fatty acids [5,6], several studies do indicate a positive effect. For example, long-term EPA and DHA supplementation may contribute to the improvement in VO_{2max} [7] or to the reduction in the cost of aerobic exercise in trained cyclists [8,9]. Moreover, our recent study showed that 12-week supplementation with omega-3 fatty acids improved running economy (RE) in amateur runners [10]. These studies focus mainly on VO_{2max} and RE, and other submaximal oxygen kinetics parameters need to be further explored.

The aim of our study was to determine whether OUE, OUEP and OUE@VAT can be considered as a robust measurements of endurance capacity. Moreover, we verify if those parameters are sensitive to changes after omega-3 fatty acid supplementation. The main hypothesis of this research was that OUE will be sensitive to changes in VO_{2peak}. We also hypothesised that OUE@VAT and OUEP can be used as non-invasive, submaximal parameters of oxygen kinetics replacing VO_{2peak} and RE. We also evaluated whether twelve-week of endurance training combined with omega-3 fatty acid supplementation can alter these parameters in male amateur endurance runners.

2. Materials and Methods

2.1. Participants

A total of 26 male amateur runners (37 ± 3 years old; 77 ± 9 kg body weight; VO_{2peak}: 54.2 ± 6 $mL*kg^{-1}*min^{-1}$) completed the 12-week experimental study as previously described [10], which tested the effect of supplementation with omega-3 fatty acids on exercise capacity in male amateur endurance runners. Participants were not taking medication and all were in good health, as confirmed by a medical check. The study was approved by the Bioethical Committee of Regional Medical Society in Gdańsk (NKBBN/628/2019) and conducted according to the Declaration of Helsinki (2013). All participants provided their written informed consent prior participating in the study. Detailed participant characteristics and project design are shown in Table 1 and Figure 1, respectively.

Table 1. Characteristics of participants.

Variable		MCT (n = 12) Mean ± SD	OMEGA (n = 14) Mean ± SD
Age [y]		37 ± 4	37 ± 3
Body mass [kg]		78.0 ± 8	76.3 ± 11
Height [cm]		180 ± 4	181 ± 7
EPA [% of total erythrocyte fatty acids]	Pre	1.2 ± 0.3	1.1 ± 0.4
	Post	1.2 ± 0.3	4.9 ± 1.1 *,^
DHA [% of total erythrocyte fatty acids]	Pre	4.4 ± 1.1	4.7 ± 1.0
	Post	4.5 ± 0.8	6.7 ± 0.8 *,^

Table 1. *Cont.*

Variable		MCT (n = 12) Mean ± SD	OMEGA (n = 14) Mean ± SD
HRmax [beats*min^{-1}]	Pre	186 ± 9	190 ± 9
	Post	184 ± 7	189 ± 9
VO_{2peak} [mL*kg^{-1}*min^{-1}]	Pre	54.7 ± 6.8	53.6 ± 4.4
	Post	56.4 ± 5.9	56.0 ± 3.7 *
RE [mL*kg^{-1}*min^{-1}]	Pre	47.7 ± 3.3	47.6 ± 1.8
	Post	48.7 ± 2.9	46.5 ± 2.4 ^

EPA—eicosapentaenoic acid; DHA—docosahexaenoic acid; HRmax—maximal heart rate; RE—running economy; data are presented as mean ± SD; * $p < 0.05$ for post vs. pre value ^ $p < 0.05$ for MCT vs. OMEGA.

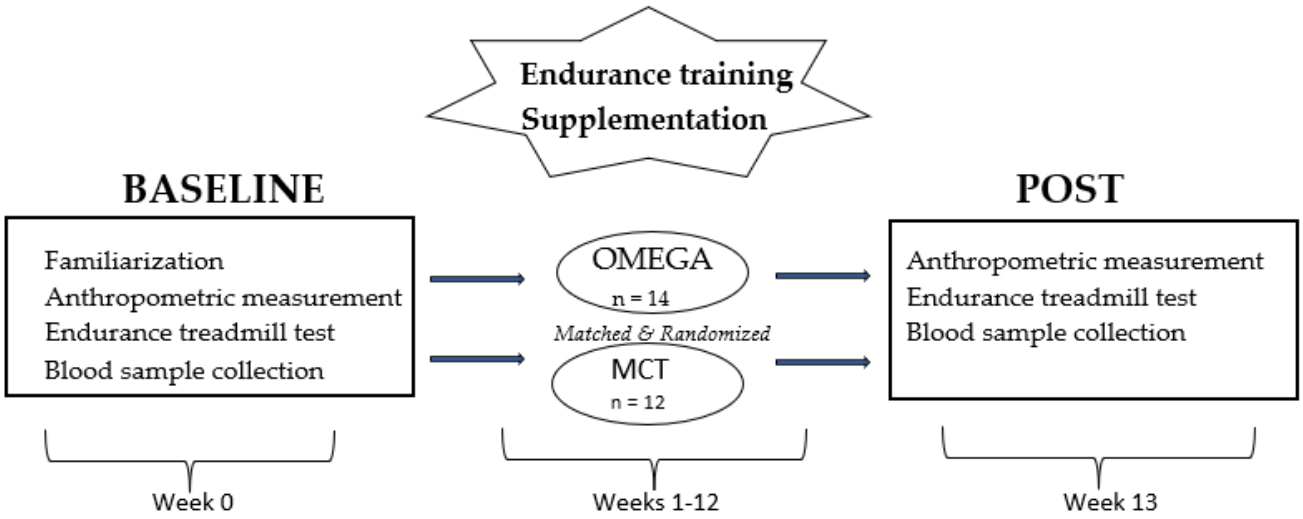

Figure 1. Procedure design.

2.2. Supplementation

Participants were randomly assigned to one of two groups with the final characteristics as follows: OMEGA (37 ± 3 years; 76.3 ± 11 kg body weight; VO_{2peak}: 53.6 ± 4 mL*kg^{-1}*min^{-1}) or medium-chain triglycerides (MCT) as placebo (37 ± 4 years; 78 ± 8 kg body weight; VO_{2peak}: 54.7 ± 7 mL*kg^{-1}*min^{-1}). The division of participants into two groups was performed to check the difference in the response of OUE parameters to supplementation. Hence, participants supplemented four capsules per day, providing a total of 2234 mg of EPA + 916 mg of DHA (OMEGA group) or 4000 mg of MCT (MCT group). The capsules were provided in coded, identical-looking packages to avoid a potential recognition. To maintain the quality of supplements consisting of omega-3 fatty acids and their respective dosages, materials adhering to the International Fish Oil Standard (IFOS) were used.

2.3. Treadmill Exercise Testing

Exercise tests were conducted under controlled environmental conditions (18–20 °C and humidity 40–45%) and were performed at similar time of day ± 2 h. Before carrying out the exercise tests, the participants performed a familiarization trial. The participants were informed to refrain from strenuous exercise for 24 h and from caffeine or alcohol consumption for 12 h prior to the tests. Before and after twelve weeks of the training program, participants undertook a ramp exercise test to volitional exhaustion on a treadmill (*h*/*p* Cosmos, Saturn, Nussdorf-Traunstein, Germany). First, participants stood on the treadmill for 2 min to make sure the measuring equipment was ready and to measure the resting parameters. Thereafter, runners walked for 5 min at 5 km/h speed and with a 1.5% inclination as a warm-up prior to starting the test. Every next stage lasted 3 min, and the treadmill belt was accelerated starting from 8 km/h by 1 km/h per stage up to 12 km/h.

Then, the inclination of the treadmill was increased to 5%, 10% and 15% at 12 km/h speed until volitional exhaustion, despite strong verbal encouragement. During both tests, heart rate (HR) was monitored (Polar RS400, Kempele, Finland). RE was measured as an oxygen cost from last 50 s of each stage to 12 km/h speed and was expressed as $mL*kg^{-1}*min^{-1}$ [11].

2.4. Respiratory Gas Measurements

During both laboratory tests, the exhaled air was continuously measured using a breath-by-breath analyser (Oxycon Pro, Jaeger, Hoechberg, Germany). Before the tests, the analyser was calibrated in accordance with the manufacturer's instructions. All measurements were averaged to 10 s intervals and included: oxygen uptake (VO_2), carbon dioxide output (VCO_2), minute ventilation (Ve), end-tidal pressure of oxygen ($P_{ET}O_2$) and end-tidal pressure of carbon dioxide ($P_{ET}CO_2$).

2.5. Determination of Oxygen Uptake Efficiency and Ventilatory Thresholds

The OUE was individually determined for each participant by calculating the regression slope from the linear relationship of absolute VO_2 ($mL*min^{-1}$) plotted as a linear function of Ve ($L*min^{-1}$) (VO_2 = Ve + b), as previously described by Sun et al. [4]. After calculating the OUE individually for each participant from the formula, the OUE was correlated with the true VO_{2peak} and normalized, and the original OUE values ("b") were compared for the slope of the linear regression of the oxygen uptake efficiency. OUEP was calculated as the 90 s average of the highest consecutive measurements of VO_2 ($mL*min^{-1}$)/Ve ($L*min^{-1}$) and OUE at the ventilatory anaerobic threshold (OUE@VAT), as the 60 s average of consecutive measurements at and immediately before the VAT accordingly to Sun et al. [4]. First, ventilatory threshold (VT_1) was determined as increase in both the ventilatory equivalent of oxygen (Ve/VO_2) and end-tidal pressure of oxygen ($P_{ET}O_2$) with no concomitant increase in the ventilatory equivalent of carbon dioxide (Ve/VCO_2) [12]. The ventilatory anaerobic threshold (VAT) was measured by the V-slope method [13]. Peak oxygen uptake (VO_{2peak}) was obtained as the last 30 s oxygen uptake mean value recorded during the test [14].

2.6. Training Program

All participants underwent 12 weeks of an endurance training program. The participants performed endurance training of varying intensity three times a week according to Costa et al. [15] with slight modifications. Additionally, participants performed training once a week, which aimed to strengthen the central stabilization muscles and to reduce the risk of injury [16]. The training intensity was distributed among 3 heart-rate zones (Z1-Z2-Z3). They were determined according to the first ventilatory threshold (VT_1), ventilatory anaerobic threshold (VAT) and the corresponding values of the heart rate [Z1: $\leq$HR@VT_1 + 5 bpm; Z2: (>HR@VT_1 + 5 bpm) to ($\leq$HR@VAT-5 bpm); Z3: >HR@VAT-5 bpm]. Average training times spent in every mesocycle were (~80%-15%-5%) in zones (Z1-Z2-Z3), respectively. In the last week, the training volume was reduced to reduce the accumulated fatigue. All trainings were monitored by Polar M430 (Kempele, Finland) wrist watches and H9 heart-rate chest sensor and the supervision over the participants was carried out by a certified track and field coach.

2.7. Erythrocyte Fatty Acid Analysis

Sample collection and fatty acid determination were outlined elsewhere [10]. In brief, blood samples were collected into 4 mL sodium citrate vacutainer tubes and centrifuged at 4 °C (4000× *g* for 10 min). After centrifugation, plasma was collected with a disposable Pasteur pipette, transferred into separate Eppendorf probes and stored in a −80 °C freezer until further analysis. Erythrocyte lipids were extracted into chloroform:methanol and fatty acid methyl esters (representing the erythrocyte fatty acids) were formed by heating the lipid extract with methanolic sulphuric acid. The fatty acid methyl esters were separated

by gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column using the settings and run conditions described by Fisk et al. [17]. Fatty acid methyl esters were identified by comparison with runtimes of authentic standards and data were expressed as weight % of total fatty acids.

2.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (San Diego, CA, USA). Arithmetic means, standard deviation (SD), and significance levels of differences between means were calculated. Two-way analysis of variance (ANOVA), with repeated measures, was used to investigate the significance of differences between groups and time. Significant main effects were further analyzed using the Bonferroni corrected post hoc test. Correlations between variables were evaluated using the Pearson and Spearman correlations coefficients. All analyses used a significance level of $p < 0.05$.

3. Results

3.1. Predicted VO_{2peak} from OUE Equation

Predicted VO_{2peak} calculated from the OUE formula both before and after the supplementation intervention was moderately correlated with peak oxygen uptake ($R^2 = 0.338$, $p = 0.002$; Figure 2A) for all participants before the study. Moreover, the results without grouping also showed a correlation after 12 weeks of intervention ($R^2 = 0.226$, $p = 0.014$; Figure 2B), but the correlation was weak.

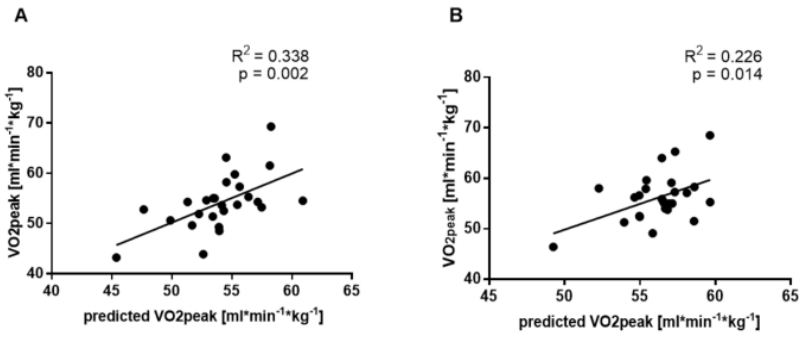

Figure 2. The linear relationship between VO_{2peak} and predicted VO_{2peak} before (**A**) and after (**B**) twelve weeks of combined endurance training and supplementation (OMEGA and MCT groups; n = 26).

3.2. Oxygen Uptake Efficiency Plateau

Pre-intervention OUEP values weakly correlated with VO_{2peak} ($R^2 = 0.247$, $p = 0.01$; Figure 3A). After twelve weeks of intervention, no correlation was found between these two indicators ($R^2 = 0.077$, $p = 0.17$, Figure 3B).

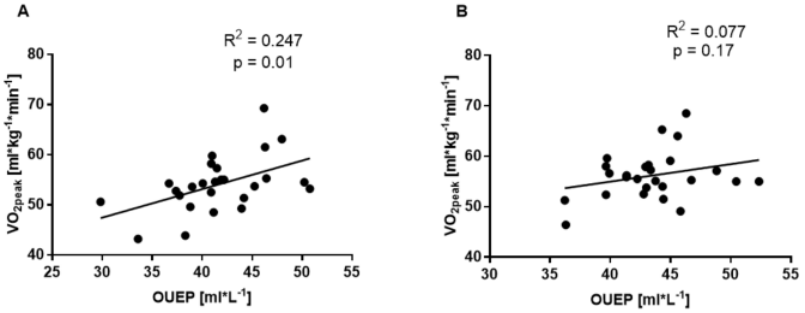

Figure 3. The linear relationship between VO_{2peak} and OUEP before (**A**) and after (**B**) twelve weeks of combined endurance training and supplementation (OMEGA and MCT groups; n = 26).

3.3. Oxygen Uptake Efficiency at the Ventilatory Anaerobic Threshold

OUE@VAT poorly correlated with the peak oxygen uptake (VO_{2peak}) before the study ($R^2 = 0.179$, $p = 0.031$, Figure 4A) and there was no correlation after the 12-week intervention ($R^2 = 0.082$, $p = 0.154$, Figure 4B) in all participants.

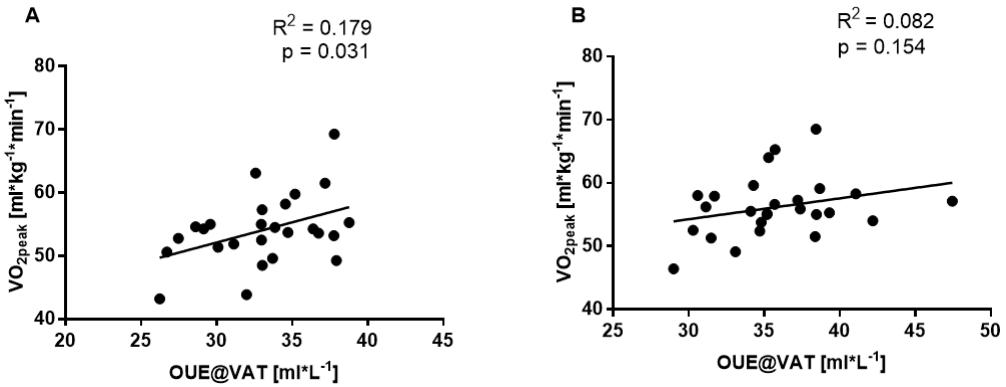

Figure 4. The linear relationship between VO_{2peak} and OUE@VAT before (**A**) and after (**B**) twelve weeks of combined endurance training and supplementation (OMEGA and MCT groups; n = 26).

3.4. Correlation between OUEP, OUE@VAT and RE

The changes observed in RE (presented as VO_2 delta [%] at 12 km/h) were not correlated with the change in OUEP ($R^2 = 0.018$, $p = 0.511$; Figure 5A). Similar results were observed in the correlation between RE and OUE@VAT (r = 0.079, $p = 0.699$; Figure 5B) in all participants.

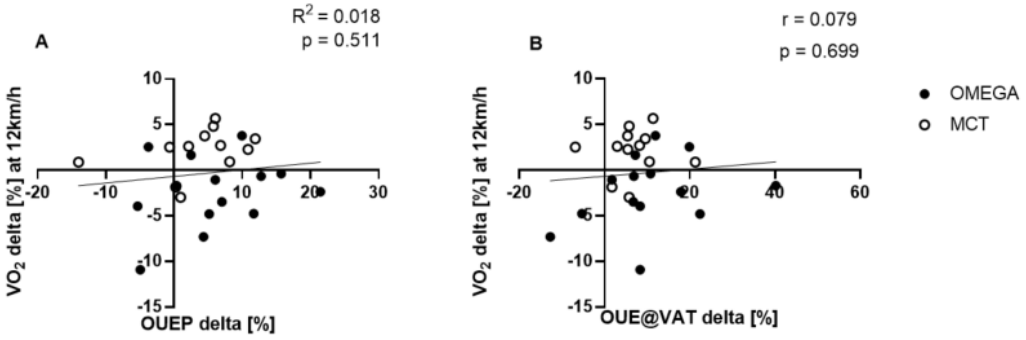

Figure 5. Correlation between changes in RE and OUEP (**A**) and OUE@VAT (**B**) after twelve weeks of combined endurance training and supplementation (OMEGA and MCT groups; n = 26).

3.5. Omega-3 Fatty Acids Supplementation

Baseline levels of EPA and DHA did not differ between the groups (OMEGA group: 1.1% EPA, 4.7% DHA; MCT group: 1.2% EPA, 4.4% DHA, both $p > 0.999$). Post-intervention values of EPA and DHA increased in OMEGA group (4.9% EPA, 6.7% DHA, both $p < 0.001$). Changes were not observed in MCT group (1.2% EPA, $p > 0.999$; 4.7% DHA, $p = 0.551$). All results are provided in Table 1.

3.5.1. Oxygen Uptake Efficiency

At the end of the 12-week supplementation period, there was an increase in the slope of oxygen uptake efficiency in the OMEGA group from 35.4 ± 3.3 to 37.6 ± 3.0 and in the MCT group from 35.5 ± 3.7 to 37.2 ± 3.1; (both $p < 0.001$). OUE increased when groups were combined from 35.5 ± 3.4 to 37.4 ± 3.0; ($p < 0.001$, Table 2).

Table 2. Comparison of effects omega-3 fatty acid supplementation with placebo controlled on cardiorespiratory fitness (CRF) parameters.

Variable	MCT (n = 12) Mean ± SD		OMEGA (n = 14) Mean ± SD		ALL (n = 26) Mean ± SD	
	Pre	Post	Pre	Post	Pre	Post
OUE [mL*L^{-1}]	35.5 ± 3.7	37.2 ± 3.1 ***	35.4 ± 3.3	37.6 ± 3.1 ***	35.5 ± 3.4	37.4 ± 3.0 ***
OUEP [mL*L^{-1}]	41.8 ± 5.2	42.9 ± 3.8	41.3 ± 4.6	43.6 ± 4.0 *	41.6 ± 4.8	43.2 ± 3.9 **
OUE@VAT [mL*L^{-1}]	33.2 ± 3.8	35.4 ± 3.5 **	32.7 ± 3.6	35.9 ± 4.7 *	32.9 ± 3.7	35.7 ± 4.1 ***
Ve [L*min^{-1}]	93.8 ± 11.6	90.7 ± 9.3 **	92.9 ± 20.4	87.4 ± 20.2 *	93.3 ± 16.4	88.9 ± 15.7 *

OUE—oxygen uptake efficiency; OUEP—oxygen uptake efficiency plateau; OUE@VAT—oxygen uptake efficiency at the ventilatory anaerobic threshold; Ve—minute ventilation; * $p < 0.05$ for post to pre value; ** $p < 0.01$ for post to pre value; *** $p < 0.001$ for post to pre value; data are presented as mean ± standard deviation (SD).

3.5.2. Oxygen Uptake Efficiency Plateau

Oxygen uptake efficiency plateau values increased in the OMEGA group from 41.3 ± 4.6 to 43.6 ± 4.0; ($p = 0.017$). There were no changes in the MCT group ($p = 0.2$). Moreover, the analysis of the two groups together (regardless of the supplementation that was undertaken) showed that OUEP increased from 41.6 ± 4.8 to 43.2 ± 3.9; ($p = 0.007$, Table 2).

3.5.3. Oxygen Uptake at Ventilatory Anaerobic Threshold

There was an increase in OUE@VAT in the OMEGA group from 32.7 ± 3.6 to 35.9 ± 4.7; ($p = 0.012$) and in the MCT group from 33.2 ± 3.8 to 35.4 ± 3.5; ($p = 0.003$). The results, regardless of the supplementation undertaken, showed that OUE@VAT increased from 32.9 ± 3.7 to 35.7 ± 4.1; ($p < 0.001$, Table 2).

4. Discussion

This is the first study to report the correlations between OUE, OUEP, OUE@VAT and VO_{2peak} as well as OUEP and OUE@VAT and RE. They were analyzed in terms of reliability and repeatability, and whether they could be non-invasive substitute measurements for VO_{2peak} and RE. Additionally, we investigated whether these parameters were altered following supplementation with omega-3 fatty acids.

The true VO_{2max} value is mainly achievable during a laboratory progressive exercise test to exhaustion where large muscle groups are involved. Simultaneously, the observed kinetics of oxygen supply/utilization in the muscles must be without significant changes: the so-called plateau [18]. It is known that this phenomenon occurs when a high intensity is met, and the primary criteria for achieving this parameter (VO_{2max}) during CPET are: (1) reaching a VO_2 plateau or (2) levelling-off the oxygen uptake (VO_2) [19–21]. Thus, in Sun and co-authors' study, OUE, OUEP and OUE@VAT comprehensively reflected cardiovascular functions as an alternative for parameters assessing CRF without the need for maximum effort [4]. A steeper OUE (VO_2/Ve) and higher values of OUEP and OUE@VAT show more efficient oxygen uptake and utilization in the working skeletal muscles. OUE showed an improvement, but, for both groups, this occurred after 12 weeks of intervention. Hence, it is believed that the increase in slope/higher OUE values was the result of endurance training. Moreover, in our study, weak or no correlation was observed between OUEP and peak oxygen uptake. In a study by Bongers et al. [22], in which 214 children participated, OUEP was weak-to-moderately correlated with VO_{2peak} (r = 0.646), which is inconsistent with our results. However, children and adults respond differently to exercise, which might explain this difference. Another study also confirms that OUEP does not accurately predict VO_{2max} in male adolescents and should not replace VO_{2max}, when assessing CRF [19]. In our study, OUE@VAT also demonstrated no correlation with VO_{2peak} before and after 12 weeks of intervention. In contrast to our results, one study revealed that ventilatory anaerobic threshold (VAT) strongly correlated with VO_{2peak} (r = 0.831) [23]. However, there is a difference between the compared parameters, because OUE@VAT is the

60-s average of consecutive measurements at and immediately before the VAT. On the other hand, VAT is a single measurement and is not free from intra-observer and inter-observer variability [24]. Hence, both OUEP and OUE@VAT may be more stable measurements than VAT; however, the results of our study did not confirm this.

Endurance capacity also has a stable predictor in the form of RE [25]. However, as earlier authors suggest, an accurate measurement of RE can be carried out with the use of invasive lactate measurement, which is one of the disturbances in VO_2 steady-state indicators [26,27]. Therefore, in this study, an attempt was made to replace RE with OUEP and OUE@VAT and to check whether they can be a solid, non-invasive predictor of RE in recreational runners. Despite the increase in the efficiency of oxygen uptake in all participants, the linear regression did not show any correlation between OUEP, OUE@VAT and RE. Hence, the RE measurement should not be replaced with OUEP and OUE@VAT, as they are not related.

The assessment of adaptive changes following supplementation with omega-3 fatty acids is also not fully known. The health-promoting effects of n-3 PUFA supplementation are well-established [28–30]. These effects are related to the incorporation of EPA and DHA into the erythrocyte cell membrane [31], skeletal muscles [32] and heart [33]. Furthermore, the systemic response to supplementation with omega-3 fatty acids as exemplified by maximum oxygen uptake [7], exercise economy [9,10] or anaerobic endurance capacity [34] is well-known. Nevertheless, in our study, for the first time, an attempt was made to link the effect of supplemental EPA + DHA to changes in OUEP and OUE@VAT. However, the OUE parameters increased in both groups. Therefore, changes in OUEP and OUE@VAT following 12 weeks of intervention are dictated by adaptation to endurance training rather than changes caused by EPA and DHA supplementation.

Limitations and Future Perspectives

Despite some valuable information coming from this study, there are some limitations. First, the small number of participants could distort the estimate of correlations between the variables. Second, this study was conducted in male runners only; therefore, these findings cannot be generalized and extrapolated to females. Future studies should include a larger number of participants and include females.

5. Conclusions

In conclusion, the results obtained in this study do not support the use of OUEP and OUE@VAT as an alternative parameter to VO_{2peak} and RE. Additionally, the 12-week supplementation of omega-3 fatty acids at a dose of 2234 mg of EPA and 916 mg of DHA daily did not reveal changes in OUEP and OUE@VAT. Hence, the suitability of using OUEP and OUE@VAT as alternative, non-invasive CRF parameters for VO_{2peak} and RE can be questioned.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethical Committee of Regional Medical Society in Gdańsk (NKBBN/628/2019).

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 on request from the corresponding author.

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

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Increased Plasma L-Arginine Levels and L-Arginine/ADMA Ratios after Twelve Weeks of Omega-3 Fatty Acid Supplementation in Amateur Male Endurance Runners

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Abstract: It is not fully understood how supplementation with omega-3 fatty acids affects the metabolism of amino acids required for the bioavailability/synthesis of NO, i.e., L-arginine (L-arg), asymmetric dimethylarginine (ADMA), their metabolites, and the L-arg/ADMA ratio and their impact on running economy (RE) in runners. Thus, 26 male amateur endurance runners completed a twelve-week study in which they were divided into two supplemented groups: the OMEGA group (n = 14; 2234 mg and 916 mg of eicosapentaenoic and docosahexaenoic acid daily) or the MCT group (n = 12; 4000 mg of medium-chain triglycerides daily). At the same time, all participants followed an endurance training program. Before and after the 12-week intervention, blood was collected from participants at two time points (at rest and immediately post-exercise) to determine EPA and DHA in red blood cells (RBCs) and plasma levels of L-arg, ADMA, and their metabolites. RBC EPA and DHA significantly increased in the OMEGA group ($p < 0.001$), which was related to the resting increase in L-arg ($p = 0.001$) and in the L-arg/ADMA ratio ($p = 0.005$) with no changes in the MCT group. No differences were found in post-exercise amino acid levels. A total of 12 weeks of omega-3 fatty acid supplementation at a dose of 2234 mg of EPA and 916 mg of DHA daily increased levels of L-arg and the L-arg/ADMA ratio, which indirectly indicates increased bioavailability/NO synthesis. However, these changes were not associated with improved RE in male amateur endurance runners.

Keywords: omega-3 fatty acids; L-arginine; ADMA; nitric oxide; running economy; endurance runners

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1. Introduction

Supplementation with omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has effects that include, but are not limited to, a reduction in the risk of cardiovascular diseases [1,2], nervous system diseases [3] and metabolic diseases such as diabetes mellitus [4]. Moreover, in healthy, trained and/or untrained subjects, supplementation with omega-3 fatty acids has been shown to enhance muscle function and recovery [5,6]. Evidence for performance improvement in endurance athletes following omega-3 fatty acid supplementation is scarce; however, our recent study showed that 12-week supplementation with omega-3 fatty acids in amateur runners increased the so-called omega-3 index (O3I) (expressed as a sum of % EPA and % DHA levels in red blood cells (RBCs)) which was associated with improved running economy (RE) [7]. Nonetheless, the underlying mechanism appears to be complex and is not fully understood. Among the proposed mechanisms is an increase in the release of nitric oxide (NO) by the vascular

endothelium, which is characteristic of, among others, aerobic physical training [8]. This phenomenon is possibly due to the metabolism of L-arginine (L-arg) into L-citrulline via endothelial nitric oxide synthase (eNOS); among the products of this transformation is NO [9]. As a result, there is an increase in cyclic guanosine monophosphate (cGMP), which leads to the relaxation of smooth muscle and vasodilation [10].

On the other hand, the vasodilator effect is antagonized in the presence of asymmetric dimethylarginine (ADMA) in plasma, a competitive inhibitor for eNOS [11,12]. Both ADMA and the second amino acid from the methylarginase family, symmetric dimethylarginine (SDMA) negatively correlate with the bioavailability of NO, although the latter weakly and indirectly inhibits NO synthesis [13]. Increased plasma ADMA and/or SDMA levels are related to an impairment of vascular functions, thus becoming a factor increasing the risk of cardiovascular diseases [14,15]. Previous research suggests the L-arg/ADMA ratio as among the robust tools for assessing vascular endothelial function [16]. Low values of the ratio increase the risk of impaired vascular endothelial function, and therefore enhance the rate of hospitalization and mortality [17]. Decreased levels of L-arg and a lower L-arg/ADMA ratio observed after strenuous exercise may result in reduced ability to synthesize NO [18]. Hence, finding an exogenous modulator of these amino acids seems to be important not only for the sedentary, but also for healthy, physically active people and athletes. Despite the positive effect of supplementation with omega-3 fatty acids on the exercise capacity of endurance athletes [19,20], deficiencies of omega-3 fatty acids are still observed, among others, in the diet of NCAA athletes [21].

Mechanisms responsible for changes in amino acid metabolism following supplementation with omega-3 fatty acids are not comprehensively understood, and the effect on L-arg metabolites and the L-arg/ADMA ratio seems to be crucial in understanding the effect of omega-3 fatty acids among athletes. Thus, the aim of this study was twofold—firstly, to investigate the effect of 12-week supplementation with omega-3 fatty acids on the plasma levels of L-arg, ADMA, the L-arg/ADMA ratio and related metabolites and, secondly, to assess whether the aforementioned markers correlate with RE in male amateur endurance athletes.

2. Materials and Methods

2.1. Participants

Twenty-six male runners (37 ± 3 years old; 77 ± 9 kg body weight; VO_{2peak}: 54.2 ± 6 mL*kg^{-1}*min^{-1}) completed a randomized controlled trial, approved by the Bioethical Committee of Regional Medical Society in Gdańsk (NKBBN/628/2019) and conducted according to the Declaration of Helsinki.

2.2. Study Design

This study was part of a larger research project with details outlined elsewhere [7], and characteristics of the participants are shown in Table 1. Briefly, participants were randomly assigned to one of two groups with the final characteristics as follows: OMEGA (age: 37 ± 3 years; body weight: 76 ± 11 kg; VO_{2peak}: 53.8 ± 5 mL*kg^{-1}*min^{-1}) or medium-chain triglycerides (MCT) (age: 37 ± 4 years; body weight: 78 ± 8 kg; VO_{2peak}: 54.7 ± 7 mL*kg^{-1}*min^{-1}). All participants completed a 12-week programme that included 4 training sessions per week (3 running sessions + 1 core strengthening session). The training structure was based on the ventilatory threshold (VT) and ventilatory anaerobic threshold (VAT) method with corresponding three heart rate (HR) zones: [Z1: ≤HR@VT1 + 5 bpm; Z2: (>HR@VT1 + 5 bpm) to (≤HR@VAT-5 bpm); Z3: >HR@VAT-5 bpm]. Simultaneously, participants ingested 4 capsules per day providing a total of 2234 mg of EPA + 916 mg of DHA (OMEGA group) or 4000 mg of MCTs (MCT group). Before and after the 12-week period, VO_{2peak} during an incremental treadmill test was measured on a motorized treadmill (h/p Cosmos, Saturn, Germany) and blood samples were taken twice: before starting and immediately after finishing the test. The test consisted of a few stages: first, participants walked for 5 min at 5 km/h speed and with a 1.5% incline as a warm-up.

Second, the treadmill belt was accelerated starting from 8 km/h by 1 km/h per stage up to 12 km/h with every next stage duration of 3 min. Then, the incline of the treadmill was increased to 5%, 10% and 15% at 12 km/h speed until volitional exhaustion. During both tests, heart rate (HR) was monitored (Polar RS400, Kempele, Finland). Additionally, oxygen uptake (VO_2), carbon dioxide output (VCO_2), minute ventilation (Ve) and respiratory exchange ratio (RER) were continuously measured using a breath-by-breath analyzer (Oxycon Pro, Jaeger, Hoechberg, Germany). VO_{2peak} was obtained as the highest 30 s mean value recorded during the test. RE was measured as an oxygen cost from last 50 s as previously described [22] with slight modifications accordingly to Tomczyk et al., 2022 [7].

Table 1. Characteristics of participants.

Variable		MCT (n = 12) Mean ± SD		OMEGA (n = 14) Mean ± SD
Age (years)		37 ± 4		37 ± 3
Body mass (kg)		78 ± 8		76 ± 11
Height (cm)		180 ± 4		181 ± 7
VO_{2peak} ($mL*kg^{-1}*min^{-1}$)		54.7 ± 7		53.6 ± 4
RE ($mL*kg^{-1}*min^{-1}$)	Pre	47.7 ± 3.3	Pre	47.6 ± 1.8
	Post	48.7 ± 2.9	Post	46.5 ± 2.4 †
EPA (% of total RBC fatty acids)	Pre	1.2 ± 0.3	Pre	1.1 ± 0.4
	Post	1.2 ± 0.3	Post	4.9 ± 1.1 *†
DHA (% of total RBC fatty acids)	Pre	4.4 ± 1.1	Pre	4.7 ± 1.0
	Post	4.5 ± 0.8	Post	6.7 ± 0.8 *†
O3I	Pre	5.6 ± 1.4	Pre	5.8 ± 1.3
	Post	5.6 ± 1.1	Post	11.6 ± 1.7 *†
Test duration (min: s)	Pre	1091 ± 144	Pre	1111 ± 70
	Post	1137 ± 84 *	Post	1138 ± 85

* $p < 0.05$ post vs. pre; † $p < 0.05$ MCT vs. OMEGA; SD—standard deviation; EPA—eicosapentaenoic acid; DHA—docosahexaenoic acid; RBC—red blood cell; O3I—Omega-3 index.

2.3. Sample Collection

Blood samples were collected into 4 mL sodium citrate vacutainer tubes and centrifuged at 4 °C (4000× *g* for 10 min). After centrifugation, plasma and RBCs were collected with a disposable Pasteur pipette and transferred into separate Eppendorf probes and stored in a −80 °C freezer until further analysis.

2.4. Fatty Acid Analysis

Concentrations of EPA and DHA in red blood cells (RBCs) were measured using gas chromatography [23]. Briefly, RBC lipids were extracted into chloroform methanol and fatty acid methyl esters (representing the RBC fatty acids) were formed by heating the lipid extract with methanolic sulphuric acid. The fatty acid methyl esters were separated by gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column. Fatty acid methyl esters were identified by comparison with run times of authentic standards. Fatty acids are expressed as a % of total fatty acids present.

2.5. Amino Acid Assessment

Determinations of plasma L-arginine, ornithine, L-citrulline, DMA, ADMA and SDMA concentrations were performed using high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) with prior protein precipitation and derivatization. To 50 µL of plasma, 200 µL of protein precipitation reagent was added (mixture of internal standards in water and methanol, 20:80). The sample was stirred for 15 min (1100× *g* rpm) and centrifuged (3000× *g* rpm, 10 min). A volume of 10 µL of supernatant was transferred to a new insert vial and subjected to AccQ-Tag (Waters Co, Milford, MA, USA) derivatization in accordance with the manufacturer's recommendations. After derivatiza-

tion, samples were diluted 1:1 with ultrapure water and subjected to LC-MS/MS analysis accordingly to Carling et al. [24] with slight modifications.

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7. Each variable was subjected to normal distribution analysis using the Shapiro–Wilk test. Arithmetic means, standard deviation and significance levels were calculated. When the distribution of the variable was normal, the paired *t*-test was used, while when the distribution was not normal the non-parametric Wilcoxon test was used. Then, two-way analysis of variance (ANOVA) with repeated measures to investigate the significance of differences between groups and time was used. Significant main effects were further analyzed using the Sidak post hoc test. Correlations between variables were evaluated using the Spearman correlation coefficient. Significance for all analyses was assumed at $p < 0.05$.

3. Results

3.1. Omega-3 Polyunsaturated Fatty Acids in RBCs

Baseline levels of EPA and DHA and the O3I did not differ between the two groups (OMEGA group: 1.1% EPA, 4.7% DHA, 5.8% O3I; MCT group: 1.2% EPA, 4.4% DHA, 5.6% O3I, all $p > 0.999$). Post-intervention values of EPA, DHA and O3I increased in the OMEGA group to 4.9% EPA, 6.7% DHA, 11.6% O3I (all $p < 0.001$). Changes were not observed in the MCT group (1.2% EPA, $p > 0.999$; 4.7% DHA, $p = 0.551$; 5.8% O3I, $p > 0.999$).

3.2. Plasma L-arginine and Its Metabolites at Resting Conditions

The plasma levels of L-arg and its metabolites for both groups at rest are provided in Table 2 and Figure 1. For L-arg, a statistically significant increase was noted in the OMEGA group ($p = 0.001$), while there was no change ($p = 0.109$) in the MCT group after 12 weeks of supplementation. The level of ornithine was significantly decreased from pre to post in both groups ($p < 0.001$ and $p = 0.007$ for the OMEGA and MCT groups, respectively). Additionally, the L-arg/ADMA ratio was increased in the OMEGA group from pre to post ($p = 0.005$), while there was no change in the MCT group ($p = 0.077$).

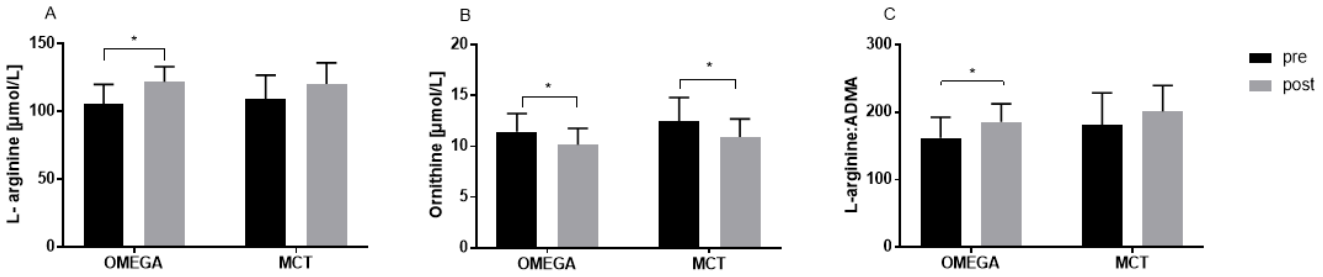

Figure 1. Resting plasma L-arginine (**A**) and ornithine (**B**) levels and L-arginine/ADMA ratios (**C**) pre- and post-12 weeks of supplementation (* $p < 0.05$- pre vs. post).

Table 2. The effect of 12-week omega-3 fatty acid supplementation on resting plasma levels of L-arginine and its metabolites.

	MCT ($n = 12$) Mean ± SD	OMEGA ($n = 14$) Mean ± SD	Diff	95% CI		*p*
				Lower	Upper	
		L-arginine (µmol/L)				
Before	109.4 ± 17.53	105.4 ± 14.67	−4.003	−17.4	9.394	0.744
After	120.4 ± 15.55	122.0 ± 11.12	1.621	−11.78	15.02	0.952
Change	11.00 ± 17.21	16.63 ± 14.87				
p	0.109	0.001				

Table 2. *Cont.*

	MCT (n = 12) Mean ± SD	OMEGA (n = 14) Mean ± SD	Diff	95% CI		p
				Lower	Upper	
			ADMA (µmol/L)			
Before	0.618 ± 0.082	0.669 ± 0.147	0.051	−0.059	0.161	0.496
After	0.611 ± 0.095	0.673 ± 0.139	0.062	−0.482	0.172	0.360
Change	−0.007 ± 0.086	0.004 ± 0.054				
p	0.883	0.819				
			SDMA (µmol/L)			
Before	0.255 ± 0.03	0.262 ± 0.036	0.007	−0.025	0.04	0.851
After	0.259 ± 0.038	0.264 ± 0.038	0.004	−0.028	0.037	0.940
Change	0.004 ± 0.031	0.001 ± 0.031				
p	0.963	0.868				
			DMA (µmol/L)			
Before	1.334 ± 0.148	1.301 ± 0.241	−0.033	−0.267	0.202	0.937
After	1.361 ± 0.275	1.394 ± 0.325	0.033	−0.200	0.268	0.934
Change	0.027 ± 0.336	0.092 ± 0.314				
p	0.865	0.509				
			L-citrulline (µmol/L)			
Before	33.73 ± 6.184	34.97 ± 9.323	1.237	−5.842	8.315	0.903
After	35.36 ± 7.092	33.8 ± 7.905	−1.553	−8.632	5.526	0.852
Change	1.626 ± 3.268	−1.164 ± 3.736				
p	0.113	0.265				
			Ornithine (µmol/L)			
Before	12.49 ± 2.314	11.45 ± 1.771	−1.048	−2.744	0.649	0.295
After	10.91 ± 1.773	10.17 ± 1.598	−0.740	−2.437	0.956	0.536
Change	−1.582 ± 1.857	−1.274 ± 0.991				
p	0.007	<0.001				
			L-Arginine:ADMA			
Before	180.9 ± 47.61	162.1 ± 30.45	−18.84	−51.52	13.85	0.343
After	201.5 ± 38.18	185.7 ± 26.54	−15.73	−48.42	16.95	0.470
Change	20.56 ± 41.54	23.66 ± 23.48				
p	0.077	0.005				

3.3. Plasma L-arginine and Its Metabolites Post-Exercise

The post-exercise plasma levels of L-arg and its metabolites for both groups are provided in Table 3 and Figure 2. For L-arg, a statistically significant change was observed in both groups after 12 weeks of supplementation ($p < 0.001$ and $p = 0.016$ for the OMEGA and MCT groups, respectively). Additionally, change in the L-arg/ADMA ratio was significant for both groups ($p < 0.001$ and $p = 0.021$ for the OMEGA and MCT groups, respectively). However, there were no differences between the OMEGA and MCT groups in post-exercise levels.

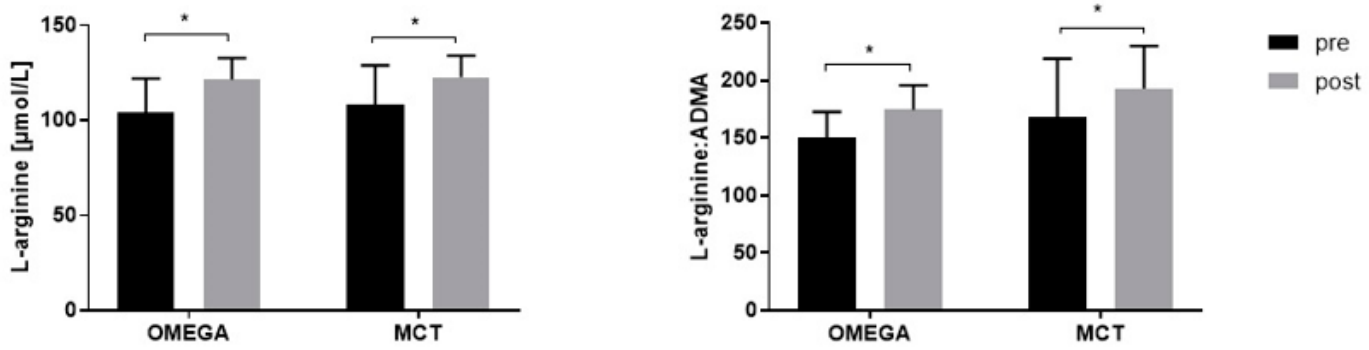

Figure 2. Post-exercise plasma L-arginine levels and L-arginine/ADMA ratios pre- and post-12 weeks of supplementation (* $p < 0.05$- pre vs. post).

Table 3. The effect of 12-week omega-3 fatty acid supplementation on post-exercise plasma levels of L-arginine and its metabolites.

	MCT (n = 12) Mean ± SD	**OMEGA (n = 14) Mean ± SD**	**Diff**	**95% CI**		p
				Lower	**Upper**	
			L-arginine (µmol/L)			
Before	108.1 ± 20.8	104.3 ± 17.67	−3.809	−18.1	10.49	0.790
After	122.7 ± 11.41	121.5 ± 11.24	−1.157	−15.45	13.14	0.978
Change	14.55 ± 17.71	17.20 ± 13.75				
p	0.016	<0.001				
			ADMA (µmol/L)			
Before	0.663 ± 0.095	0.701 ± 0.139	0.038	−0.0611	0.137	0.615
After	0.65 ± 0.089	0.706 ± 0.102	0.056	−0.043	0.155	0.361
Change	−0.013 ± 0.078	0.004 ± 0.064				
p	0.566	0.797				
			SDMA (µmol/L)			
Before	0.256 ± 0.03	0.272 ± 0.045	0.016	−0.019	0.051	0.489
After	0.265 ± 0.035	0.28 ± 0.039	0.015	−0.02	0.05	0.545
Change	0.009 ± 0.034	0.008 ± 0.032				
p	0.374	0.381				
			DMA (µmol/L)			
Before	1.505 ± 0.213	1.593 ± 0.374	0.088	−0.249	0.425	0.797
After	1.628 ± 0.373	1.742 ± 0.461	0.115	−0.222	0.452	0.682
Change	0.123 ± 0.341	0.149 ± 0.462				
p	0.338	0.248				
			L-citrulline (µmol/L)			
Before	34.69 ± 9.013	34.65 ± 11.18	−0.046	−8.486	8.394	>0.999
After	36.98 ± 7.893	34.17 ± 8.511	−2.813	−11.25	5.627	0.693
Change	2.288 ± 3.382	−0.479 ± 4.157				
p	0.052	0.952				
			Ornithine (µmol/L)			
Before	13.18 ± 2.459	12.25 ± 1.754	−0.932	−2.564	0.07	0.35
After	11.66 ± 1.38	11.78 ± 1.456	0.117	−1.516	1.75	0.983
Change	−1.52 ± 2.546	−0.471 ± 1.497				
p	0.063	0.26				
			L-Arginine:ADMA			
Before	167.5 ± 51.38	150.8 ± 22.14	−16.78	−47.92	14.35	0.391
After	192.9 ± 37.03	174.6 ± 21.33	−18.33	−49.47	12.8	0.328
Change	25.35 ± 42.21	23.8 ± 17.42				
p	0.021	<0.001				

3.4. Plasma L-arginine, the L-arg/ADMA Ratio and Running Economy

The correlations between plasma L-arg, the L-arg/ADMA ratio and RE are provided in Figure 3. There was no correlation between L-arg and RE (R^2 = 0.037, p = 0.348) and between the L-arg/ADMA ratio and RE (R^2 < 0.001, p = 0.92) after 12 weeks of supplementation.

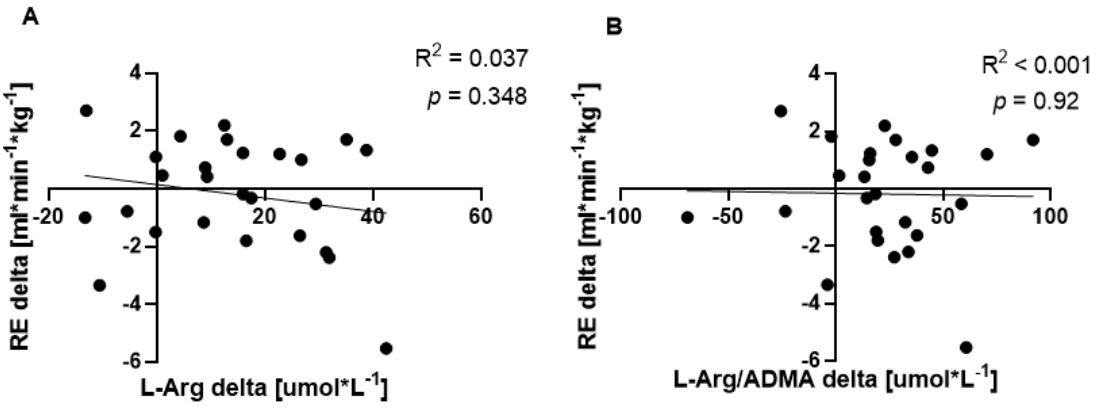

Figure 3. Correlation between resting plasma L-arginine levels (**A**) and L-arginine/ADMA ratios (**B**) and running economy.

4. Discussion

To date, most research has focused on the potential role of omega-3 fatty acids as a vasodilator of the vascular endothelium by increasing nitric oxide (NO) synthesis [25–27]. The mechanisms responsible for this phenomenon are not fully understood. However, potential changes in the metabolism of L-arg, ADMA and their metabolites seem to be crucial in understanding these mechanisms. Therefore, in this paper we present for the first time the effect of 12 weeks of supplementation with omega-3 fatty acids in runners on levels of L-arg, ADMA, and their metabolites.

In our study, in response to daily supplementation with 2234 mg of EPA and 916 mg of DHA, we observed an increase in resting plasma L-arg concentration with no change in ADMA concentration. These results are in line with a previous report in non-athletes [28]. As previously mentioned, the mechanism behind this is not fully understood, although it was originally thought that omega-3 fatty acids could decrease plasma ADMA concentrations; however, the evidence for this is scarce and inconsistent. A study with patients with obesity supplemented with EPA and DHA for 8 weeks showed decreased plasma ADMA levels [29]. On the other hand, a study involving trained cyclists showed no changes in plasma ADMA level after three weeks of omega-3 fatty acid supplementation [30]. Other studies have shown that the ADMA level in response to other supplementation interventions is difficult to assess [31,32] due to disturbances resulting from amino acid metabolism/gluconeogenesis and various levels of skeletal muscle damage [33]. Previous studies involving animals [34] and humans [35] identify that it is an increase in L-arg that increases the L-arg/ADMA ratio rather than changes in ADMA concentration; our results are consistent with this. In addition, a higher L-arg/ADMA ratio is positively related to endothelium-dependent vasodilation [36], but this ratio has not previously been used to assess athletes' exercise capacity. In our previous research we observed improvement in RE in the group supplementing omega-3 fatty acids [7]. In this study, for the first time, according to the authors' knowledge, the relationships between plasma L-arg, the L-arg/ADMA ratio and RE were investigated. However, increased plasma L-arg levels were not correlated with RE, which is consistent with a study where acute supplementation with 6 g L-arg did not alter oxygen cost of exercise or exercise tolerance in healthy subjects [37]. Nevertheless, these outcomes relate to the acute effect of an increase in plasma L-arginine where NO is rapidly oxidized to its final forms- NO_2^- and NO_3^- [38]. Therefore, it is considered that high levels of L-arg in plasma during resting may be an adaptation of the organism as a result of long-term supplementation with omega-3 fatty acids. While the resting L-arg level is a robust factor influencing the L-arg/ADMA ratio, post-exercise changes in the level of amino acids should be analyzed with caution due to omega-3 fatty acids ability to amplify the effect of exercise [39,40]. Indeed, previous research indicates that 15 min of exercise promotes an increase in L-arg levels in the plasma of athletes [41,42]. Simultaneously, these studies show no changes in ornithine levels after exercise, which is also consistent with

our results. Therefore, it seems that the assessment of the level of amino acids (in this case, L-arg and ADMA) after supplementation with omega-3 fatty acids should be performed under resting conditions, which is crucial in the context of studying ergogenic effects. Still, the mechanisms responsible for these changes are the subject of much research, although it is known that omega-3 fatty acids may also act as peroxisome proliferator-activated receptors (PPARs) agonists [43].

The pleiotropic nature of PPARs also includes regulation of the metabolism of amino acids, such as L-arg, thus increasing the bioavailability/synthesis of NO [44]. Interestingly, recent research points to the involvement of omega-3 fatty acids, especially EPA and DHA, in activation of PPARs in rats [45], while omega-3 fatty acids also upregulate PPARγ mRNA expression in blood mononuclear cells in athletes [46]. For this reason, it is believed that PPARγ expression is critical in regulating the metabolism of amino acids such as L-arg. Nevertheless, more research on this topic is needed to understand the changes that occur following omega-3 fatty acid supplementation.

Our study has some limitations. First, the small number of participants means that the observed effects should be treated cautiously. Second, analysis of PPARγ mRNA and protein expression were not performed but would add mechanistic insight into our observations.

5. Conclusions

In conclusion, twelve weeks of omega-3 fatty acid supplementation at a dose of 2234 mg of EPA and 916 mg of DHA daily increased plasma L-arg concentration with no change in plasma ADMA levels. The omega-3 intervention promotes an increase in plasma L-arg and the L-arg/ADMA ratio, which indirectly indicates increased bioavailability/NO synthesis. However, our results do not support the relevance of the L-arg/ADMA ratio as a factor improving running economy in male amateur endurance athletes.

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Zapoznałem się z niniejszą rozprawą doktorską przygotowaną pod moją opieką przez doktoranta mgra Zbigniewa Jost. Praca została przeze mnie zaakceptowana.

Radosław Laskowski

Elektronicznie podpisany przez Radosław Laskowski
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Prof. dr hab. Radosław Laskowski