Marta Wołosowicz

Wpływ N-acetylocysteiny lub kwasu alfa-liponowego na ekspresję białkowych transporterów kwasów tłuszczowych oraz metaloproteinaz macierzy pozakomórkowej w trzewnej i podskórnej tkance tłuszczowej szczurów karmionych dietą bogatotłuszczową

Promotor pracy

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Wydział Lekarski z Oddziałem Stomatologii i Oddziałem Nauczania w Języku Angielskim

Uniwersytet Medyczny w Białymstoku

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Składam serdeczne podziękowania
mojemu Promotorowi za poświęcony czas,
cierpliwość, cenne wskazówki, wyrozumiałość,
a także nieograniczoną możliwość rozwoju naukowego.
Pragnę również podziękować wszystkim Pracownikom
Zakładu Fizjologii za owocną współpracę.
Dziękuję Rodzicom i Najbliższym za wiarę i wsparcie
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Spis treści

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Data i miejsce urodzenia: 20.10.1989, Białystok

Wykształcenie

05.2021 – 06.2022	Master of Business Administration w Ochronie Zdrowia, Uniwersytet Ekonomiczny we Wrocławiu, Polska
10.2018 – 05.2019	Studia podyplomowe, Promocja Zdrowia i Edukacja Zdrowotna, Uniwersytet Medyczny w Białymstoku, Polska
10.2018 – 05.2019	Studia podyplomowe, Epidemiologia, Uniwersytet Medyczny w Białymstoku, Polska
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10.2011 – 06.2014	Studia licencjackie, Zdrowie Publiczne, Uniwersytet Medyczny w Białymstoku, Polska
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Publikacje naukowe

1. **Wołosowicz M.,** Kamiński T.W.: *Współistniejąca cukrzyca i przewlekła choroba nerek jako problem zdrowotny XXI wieku.* Gazeta Farmaceutyczna, 2019:319:19-21. MEiN=5.
2. **Wołosowicz M.**, Łukaszuk B., Chabowski A.: *The causes of insulin resistance in type 1 diabetes mellitus: is there a place for quaternary prevention?* International Journal of Environmental Research and Public Health, 2020; 17(22):8651. IF=3.390, MEiN =140.

3. **Wołosowicz M.**, Łukaszuk B., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue.* Cellular Physiology and Biochemistry, 2022; 56, 166–179. IF=5.141, MEiN =140.
4. **Wołosowicz M.**, Dajnowicz-Brzezik P., Łukaszuk B., Żebrowska E., Maciejczyk M., Zalewska A., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue.* Advances in Medical Sciences; 2022; 67(2), 216–228. IF=3.287, MEiN =100.

Konferencje naukowe

1. **Wołosowicz M.**, Łukaszuk B., Kasacka I., Chabowski A.: *Impact of alpha-lipoic acid supplementation during high-fat diet regime on fatty acid transporters in adipose tissue.* 16th Bialystok International Medical Congress. Białystok, Polska, 2022.
2. **Wołosowicz M.**, Łukaszuk B., Kasacka I., Chabowski A.: I*mpact of N-acetylcysteine supplementation during high-fat diet regime on matrix metalloproteinase-2 and matrix metalloproteinase-9 in visceral and subcutaneous adipose tissue.* 16th Bialystok International Medical Congress. Białystok, Polska, 2022.
3. **Wołosowicz M.**, Maciejczyk M., Żebrowska E., Łukaszuk B., Chabowski A.: *Effect of N-acetylcysteine supplementation on fatty acid transporters in adipose tissue.* 28th Congress of the Polish Physiological Society. Gdańsk, Polska, 2021.
4. **Wołosowicz M.**: *Assessment of the occurrence of postural defects and back pain related to students carrying backpacks.* 6th International Conference "Medical Science Pulse", Integration of Science and Care: Innovation and Commercialization. Opole, Polska, 2019.
5. **Wołosowicz M.**: *Assessing HIV and AIDS knowledge in youth.* 6th International Conference "Medical Science Pulse", Integration of Science and Care: Innovation and Commercialization. Opole, Polska, 2019.
6. **Wołosowicz M.**, Kamiński T.W., Maciejczyk M., Żebrowska E., Łukaszuk B., Chabowski A.: *Wpływ suplementacji N-acetylocysteiny na białka transportujące kwasy tłuszczowe w tkance tłuszczowej w szczurzym modelu insulinooporności.* Kongres Zdrowia Publicznego 2019. Warszawa, Polska, 2019.

7. **Wołosowicz M.**, Kamiński T.W.: *Przewlekła choroba nerek i cukrzyca jako choroby współistniejące i wzajemnie się przenikające*. VI Ogólnopolska Konferencja Studentów Medycyny Laboratoryjnej i Młodych Diagnostów "Wschodząca Diagnostyka". Białystok, Polska, 2019.

Udział w projektach naukowych

01. – 12.2022 „Wpływ suplementacji kwasem alfa-liponowym na profil lipidowy tkanki tłuszczowej w szczurzym modelu insulinooporności". Kierownik projektu: mgr **Marta Wołosowicz**, Opiekun naukowy: prof. dr hab. Adrian Chabowski. Finansowanie projektu przez Uniwersytet Medyczny w Białymstoku, kwota: 10 000 PLN.

01. – 12.2021 „Wpływ suplementacji N-acetylocysteiną na transport białek w tkance tłuszczowej w szczurzym modelu insulinooporności". Kierownik projektu: mgr **Marta Wołosowicz**, Opiekun naukowy: prof. dr hab. Adrian Chabowski. Finansowanie projektu przez Uniwersytet Medyczny w Białymstoku, kwota: 10 000 PLN.

01.2020 – 06.2021 „Wpływ suplementacji N-acetylocysteiną na transport białek w tkance tłuszczowej w szczurzym modelu insulinooporności". Kierownik projektu: mgr **Marta Wołosowicz**, Opiekun naukowy: prof. dr hab. Adrian Chabowski. Finansowanie projektu przez Uniwersytet Medyczny w Białymstoku, kwota: 10 000 PLN

06.2019 – 06.2020 „Wpływ suplementacji N-acetylocysteiną na transport białek w tkance tłuszczowej w szczurzym modelu insulinooporności". Kierownik projektu: mgr **Marta Wołosowicz**, Opiekun naukowy: prof. dr hab. Adrian Chabowski. Finansowanie projektu przez Uniwersytet Medyczny w Białymstoku, kwota: 10 000 PLN.

Staże naukowe

01. – 04.2022 Staż naukowy w ramach IDUB11, University of Pittsburgh, Department of Medicine, Division of Endocrinology and Metabolism, Kershaw Lab, Pittsburgh, Pennsylvania, Stany Zjednoczone

07. – 09.2021 Staż naukowy w ramach IDUB11, University of Pittsburgh, Department of Medicine, Division of Endocrinology and Metabolism, Kershaw Lab, Pittsburgh, Pennsylvania, Stany Zjednoczone

03.2020 Staż naukowy w ramach PROM, UPMC Children's Hospital of Pittsburgh, University of Pittsburgh, Muzumdar Lab, Pittsburgh, Pennsylvania, Stany Zjednoczone

Udział w szkoleniach

01. – 04.2022 QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

11.2021 Podstawy techniki immunoenzymatycznej ELISA - kurs praktyczny, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska

07.2021 Basic Life Support, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 Working with Small Animals, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 Working with Mice in Research, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 GCP – Social and Behavioral Research Best Practices for Clinical Research, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 Use of Controlled Substances in Basic and Animal Research, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 Responsible Conduct of Research, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021 COI PHS Regulated Course, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone

07.2021	Responsible Conduct of Research, The Collaborative Institutional Training Initiative (CITI Program), University of Pittsburgh, Pittsburgh, Pennsylvania, Stany Zjednoczone
06.2021	Otyłość i zaburzenia metaboliczne - problemy zdrowotne XXI wieku, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
04.2021	Wpływ hormonów tarczycy na otyłość i zaburzenia metaboliczne - aspekty kliniczne i molekularne, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
03.2021	Rola mikroRNA w procesie nowotworzenia, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
12.2020	Przepływ informacji genetycznej od genu do białka, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
11.2020	Badania laboratoryjne a profilaktyka chorób cywilizacyjnych, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
02.2019	PCR klasyczny i real-time. Kurs dla początkujących i średniozaawansowanych, Centrum Medyczne Kształcenia Podyplomowego w Warszawie, Polska
02.2019	Kurs dla osób planujących doświadczenia, wykonujących procedury i uśmiercających zwierzęta, Polskie Towarzystwo Nauk o Zwierzętach Laboratoryjnych (PolLASA), Warszawa, Polska

Zestawienie publikacji

Rodzaj publikacji	Liczba	Impact Factor	Punktacja MEiN
Prace włączone do rozprawy doktorskiej	3	11.818	380
Prace, które nie zostały włączone do rozprawy doktorskiej	1	-	5
Streszczenia zjazdowe	7	-	-
Razem	11	11.818	385

Rozprawa doktorska

Wpływ N-acetylocysteiny lub kwasu alfa-liponowego na ekspresję białkowych transporterów kwasów tłuszczowych oraz metaloproteinaz macierzy pozakomórkowej w trzewnej i podskórnej tkance tłuszczowej szczurów karmionych dietą bogatotłuszczową

mgr Marta Wołosowicz

Promotor pracy: prof. dr hab. Adrian Chabowski

1. Artykuły stanowiące cykl prac włączonych do rozprawy doktorskiej

Nazwa czasopisma	Tytuł artykułu	Impact Factor	MEiN	Data publikacji	Rodzaj publikacji
International Journal of Environmental Research and Public Health	*The causes of insulin resistance in type 1 diabetes mellitus: is there a place for quaternary prevention?*	3.390	140	21.11.2020	Publikacja nr 1 – praca przeglądowa
Advances in Medical Sciences	*Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue*	3.287	100	08.05.2022	Publikacja nr 2 – praca oryginalna

Cellular Physiology and Biochemistry	*Diverse impact of N-acetylcysteine or alpha-lipoic acid supplementation during high-fat diet regime on matrix metalloproteinase-2 and matrix metalloproteinase-9 in visceral and subcutaneous adipose tissue.*	5.141	140	30.03.2022	Publikacja nr 3 – praca oryginalna

2. Wykaz stosowanych skrótów i oznaczeń

ALA (ang. *α-lipoic acid*) – kwas alfa-liponowy

ANOVA (ang. *analysis of variance*) – test analizy wariancji

BCA (ang. *bicinchoninic acid*) – kwas bicynchoninowy

BSA (ang. *bovine serum albumin*) – albumina surowicy bydlęcej

CTRL (ang. *control group*) – grupa kontrolna

CTRL SAT (ang. *control group, subcutaneous adipose tissue*) – grupa kontrolna, podskórna tkanka tłuszczowa

CTRL VAT (ang. *control group, visceral adipose tissue*) – grupa kontrolna, trzewna tkanka tłuszczowa

DAG (ang. *diacylglycerols*) – diacyloglicerole

DNA (ang. *deoxyribonucleic acid*) – kwas dezoksyrybonukleinowy

ECM (ang. *extracellular matrix*) – macierz zewnątrzkomórkowa

FA (ang. *fatty acids*) – kwasy tłuszczowe

FABPpm (ang. *plasma membrane fatty acid binding protein*) – błonowe białko wiążące kwasy tłuszczowe

FAME (ang. *fatty acid methyl esters*) – estry metylowych kwasów tłuszczowych

FAT/CD36 (ang. *fatty acid translocase*) – translokaza kwasów tłuszczowych

FATP1 (ang. *fatty acid transport protein 1*) – białko transportujące kwasy tłuszczowe 1

FATP4 (ang. *fatty acid transport protein 4*) – białko transportujące kwasy tłuszczowe 4

FFA (ang. *free fatty acids*) – wolne kwasy tłuszczowe

Fig. – figura

GLC (ang. *gas-liquid chromatography*) – chromatografia gazowo-cieczowa

GLUT4 (ang. *glucose transporter 4*) – transporter glukozy 4

H&E (ang. *haematoxylin and eosin staining*) – barwienie hematoksyliną i eozyną

HFD (ang. *high-fat diet*) – grupa z dietą bogatotłuszczową

HFD SAT (ang. *high-fat diet, subcutaneous adipose tissue*) – grupa z dietą bogatotłuszczową, podskórna tkanka tłuszczowa

HFD VAT (ang. *high-fat diet, visceral adipose tissue*) – grupa z dietą bogatotłuszczową, trzewna tkanka tłuszczowa

HFD+ALA (ang. *high-fat diet with α-lipoic acid*) – grupa z dietą bogatotłuszczową i kwasem alfa-liponowym

HFD+ALA SAT (ang. *high-fat diet with α-lipoic acid, subcutaneous adipose tissue*) – grupa z dietą bogatotłuszczową i kwasem alfa-liponowym, podskórna tkanka tłuszczowa

HFD+ALA VAT (ang. *high-fat diet with α-lipoic acid, visceral adipose tissue*) – grupa z dietą bogatotłuszczową i kwasem alfa-liponowym, trzewna tkanka tłuszczowa

HFD+NAC (ang. *high-fat diet with N-acetylcysteine*) – grupa z dietą bogatotłuszczową i N-acetylocysteiną

HFD+NAC SAT (ang. *high-fat diet with N-acetylcysteine, subcutaneous adipose tissue*) – grupa z dietą bogatotłuszczową i N-acetylocysteiną, podskórna tkanka tłuszczowa

HFD+NAC VAT (ang. *high-fat diet with N-acetylcysteine, visceral adipose tissue*) – grupa z dietą bogatotłuszczową i N-acetylocysteiną, trzewna tkanka tłuszczowa

HOMA-IR (ang. *Homeostatic Model Assessment – Insulin Resistance*) – wskaźnik insulinooporności

IKKβ (ang. *inhibitor of nuclear factor kappa-B kinase subunit beta*) – kinaza białkowych IκB

IL-1β (ang. i*nterleukin-1β*) – interlaukina 1

IL-6 (ang. *interleukin 6*) – interleukina 6

JNK (ang. *c-Jun N-terminal kinase*) – kinaza białka c-Jun

LCFA (ang. *long chain fatty acids*) – długołańcuchowe kwasy tłuszczowe

MMP (ang. *matrix metalloproteinase*) – metaloproteinaza macierzy pozakomórkowej

MMP2 (ang. *matrix metalloproteinase 2*) – metaloproteinaza macierzy pozakomórkowej 2

MMP9 (ang. *matrix metalloproteinase 9*) – metaloproteinaza macierzy pozakomórkowej 9

mRNA (ang. *messenger ribonucleic acid*) – matrycowy kwas rybonukleinowy

NAC (ang. *N-acetylcysteine*) – N-acetylocysteina

NFκB (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*) – czynnik jądrowego κB

OS (ang. *oxidative stress*) – stres oksydacyjny

RIPA (ang. *radioimmunoprecipitation assay buffer*) – bufor radioimmunoprecypitacyjny

RNA (ang. *ribonucleic acid*) – kwas rybonukleinowy

ROS (ang. *reactive oxygen species*) – reaktywne formy tlenu

SAT (ang. *subcutaneous adipose tissue*) – podskórna tkanka tłuszczowa

SDS-PAGE (ang. *sodium dodecyl sulfate-polyacrylamide gel*) – żel poliakrylamidowy z dodecylosiarczanem sodu

TAG (ang. *triacylglycerols*) – triacyloglicerole

TNFα (ang. *tumor necrosis factor* alpha) – czynnik martwicy nowotworów α

TLC (ang. *thin-layer chromatography*) – cienkowarstwowa chromatografia cieczowa

VAT (ang. *visceral adipose tissue*) – trzewna tkanka tłuszczowa

3. Wstęp

Nadwaga i otyłość, są uważane za globalne epidemie i należą do głównych przyczyn zgonów (ok. 2,8 miliona ludzi co roku umiera z powodu tych schorzeń) [1]. Wcześniejsze badania wykazały, że otyłość jest ściśle powiązana z zaburzeniami metabolicznymi, do których możemy zaliczyć insulinooporność czy cukrzycę typu 2 [2]. Otyłość jest głównym czynnikiem predysponującym do rozwoju powikłań sercowo-naczyniowych, oddechowych, neurologicznych, żołądkowo-jelitowych, wątrobowych, endokrynnych, kostnych i nerkowych, a także do znacznego obciążenia psychospołecznego i zwiększonej zachorowalności na nowotwory [3,4]. Ryzyko powikłań wynikających z otyłości jest związane z ilością tkanki tłuszczowej, jej lokalizacją (otyłość brzuszna lub otyłość pośladkowo-udowa) oraz czasem trwania choroby podstawowej [4].

Tkanka tłuszczowa zawiera w swoim składzie adipocyty, preadipocyty, tkankę nerwową, fibroblasty, komórki śródbłonka oraz komórki układu immunologicznego otoczone przez naczynia włosowate oraz sieci unerwienia, które funkcjonują jako zintegrowana jednostka[5]. Aktualnie tkanka tłuszczowa nie jest już postrzegana jedynie jako bierny magazyn lipidów oraz izolator termiczny, ale jako wielofunkcyjny, złożony i wysoce aktywny metabolicznie narząd, pełniący również funkcje odpornościowe i hormonalne. Narząd ten bezpośrednio moduluje bilans energetyczny i metabolizm całego organizmu [5]. Białą tkankę tłuszczową stanowi tkanka trzewna (VAT) i podskórna (SAT), które różnią się pod względem anatomicznym, ale i fizjologicznym. Wraz z rozwojem otyłości dochodzi do rozrostu tkanki tłuszczowej wskutek hipertrofii (wzrost wielkości adipocytów) i/lub hiperplazji (wzrost liczby adipocytów w wyniku rekrutacji nowych adipocytów). Dysfunkcji tkanki tłuszczowej, która jest wynikiem otyłości, początkowo towarzyszy przerost adipocytów i wydzielanie adipokin, które stymulują rektrutację komórek macierzystych tkanki tłuszczowej (preadipocytów), różnicujących się w dojrzałe adipocyty, działające jako „kompensacyjna ochrona” przez metabolicznymi konsekwencjami nadmiaru osoczowych kwasów tłuszczowych (FA). Podskórna tkanka tłuszczowa stanowi największy depozyt tkanki tłuszczowej, jednak przekroczenie jej możliwości magazynowania substratów energetycznych może wynikać z braku zdolności do wytwarzania wystarczającej ilości nowych adipocytów, poprzez ograniczoną hiperplazję, albo z braku zdolności do dalszej ekspansji istniejących już adipocytów, przez ograniczoną hipertofię [6,7]. Przedłużająca się nadmierna podaż energetyczna, głównie kwasów tłuszczowych, prowadzi do akumulacji triacylogliceroli w tkankach ektopowych, do których zaliczamy trzewną tkankę tłuszczową, ale również wątrobę,

mięśnie szkieletowe, czy serce. W konsekwencji nadmiernej wewnątrzkomórkowej akumulacji lipidów, będącej wynikiem nadmiernego napływu FA, dochodzi do rozwoju miejscowego stanu zapalnego oraz insulinooporności [6]. Tkanka tłuszczowa, magazynując nadmiar wolnych kwasów tłuszczowych (FFA) i glicerolu głównie w postaci triacylogliceroli (TAG), ale powstają w nadmiarze także inne frakcje lipidowe, takie jak diacyloglicerole (DAG). Drenaż wrotny kwasów tłuszczowych, odbywa się z trzewnej tkanki tłuszczowej, poprzez żyłę wrtoną, do wątroby, przez co zapewnia bezpośredni dostęp wątrobie do wolnych kwasów tłuszczowych i adipokin, wydzielanych przez adipocyty trzewnej tkanki tłuszczowej[5]. To z koleji aktywuje mechanizmy odpornościowe wątroby i wpływa na wytwarzanie mediatorów zapalnych, do których możemy zaliczyć białko C-reaktywne.

Otyłości towarzyszy szereg zmian strukturalnych i molekularnych w tkance tłuszczowej, co obejmuje również zmianę ekspresji białek transportujących kwasy tłuszczowe, a tym samym dokomórkowy transport FA i równowagę lipidową organizmu. Transport transbłonowy FA jest wspomagany głównie przez translokazę kwasów tłuszczowych (FAT/CD36), błonowe białko wiążące kwasy tłuszczowe (FABPpm) oraz białka transportujące kwasy tłuszczowe (FATP1 i FATP4). Powyższe białkowe transportery ułatwiają wychwyt długołańcuchowych kwasów tłuszczowych (LCFA) przez adipocyty i przyczyniają się – przy nadmiernej osoczowej dostępności FA – do akumulacji lipidów i dysfunkcji komórek [8].

Tkanka tłuszczowa odpowiada również za produkcję hormonów, do których możemy zaliczyć adipokiny, czynnik wzrostu, cytokiny i chemokiny. Wydzielanie adipokin jest uzależnione od stanu energetycznego tkanki tłuszczowej. Adipokiny pełnią bardzo ważną rolę w naszym organizmie, jako mediatory procesów metabolicznych, do których możemy zaliczyć utlenianie kwasów tłuszczowych, lipogenezę de novo, glukoneogenezę, wychwyt glukozy, sygnalizację insulinową [9]. Nadmierna akumulacja lipidów wewnątrz adipocytów zwiększa produkcję cytokin prozapalnych (rezystyna, TNFα, IL-6 i IL-1β), które są uwalniane w dużej ilości do osocza. To z kolei indukuje aktywację szlaków kompleksu kinaz białkowych IκB (IKKβ), czynnika jądrowego κB (NFκB) i kinazy białka c-Jun (JNK), prowadząc do rozwoju insulinooporności w samych adipocytach[5,9]. W wyniku tych procesów, reakcje zapalne zachodzące w tkance tłuszczowej nasilają się i mogą odgrywać kluczową rolę w rozwoju insulinooporności w całym organizmie. Nadmierna masa tkanki tłuszczowej prowadzi również do rozwoju insulinooporności poprzez nadmierną produkcję hormonów antagonistycznych wobec insuliny (hormonu wzrostu, glukagonu, kortyzolu i katecholaminy) oraz poprzez bezpośrednie wydzielanie zwiększonej ilości FFA do krwi. W przypadku podwyższonego poziomu FFA organizm zaczyna wykorzystywać je jako główne źródło energii zamiast

glukozy. W konsekwencji wzrasta poziom glukozy we krwi, a następnie zwiększa się wydzielanie insuliny w celu utrzymania prawidłowego poziomu glukozy we krwi. Ponadto przewlekle podwyższony poziom FFA we krwi może prowadzić do ich akumulacji w tkankach obwodowych, gdzie mogą zakłócać transdukcję sygnału insuliny [10]. Nadmierne nagromadzenie wewnątrzkomórkowych lipidów, zwłaszcza w bioaktywnych frakcjach, takich jak DAG, zaburza ścieżkę przewodnictwa sygnału insulinowego oraz homeostazę glukozy u osób z otyłością i insulinoopornością, poprzez inaktywację substratu receptora insulinowego 1 oraz obniżenie aktywacji 3-kinaza fosfatydyloinozytolu. Należy podkreślić, że trzewna tkanka tłuszczowa zawiera mniejszą liczbę receptorów insuliny, charakteryzuje się wyższą aktywnością lipolityczną i immunologiczną w stosunku do podskórnej tkanki tłuszczowej [9].

Ważnym składnikiem strukturalnym i funkcjonalnym tkanki tłuszczowej są cząsteczki macierzy zewnątrzkomórkowej (ECM), w tym proteoglikany i kolagen, które są wytwarzane w dużych ilościach podczas aktywnej przebudowy tkanki tłuszczowej, jaka ma miejsce w wyniku rozwoju otyłości [9]. W konsekwencji może to prowadzić do zwłóknienia tkanki tłuszczowej. Dotychczasowe badania funkcji ECM VAT były skoncentrowane na kolagenie, który tworzy swoiste rusztowanie, ograniczające rozrost adipocytów wywołany nadmierną akumulacją lipidów w komórkach tłuszczowych. Zwłóknienie tkanki tłuszczowej, prowadzi do potencjalnego złagodzenia ektopowej akumulacji lipidów, przyczyniającej się do rozwoju stresu oksydacyjnego, zaburzeń tolerancji glukozy i insulinooporności [11]. Ważną rolę odgrywają również makrofagi, które także regulują funkcję tkanki tłuszczowej oraz jej przebudowę, poprzez produkcję metaloproteinaz macierzy pozakomórkowej (MMP), takich jak MMP2 i MMP9, które biorą udział w adipogenezie i angiogenezie [12]. Komórki odpornościowe, takie jak makrofagi, które są źródłem prozapalnych cytokin, migrują do tkanki tłuszczowej. Prowadzić to może do dalszego rozwoju stanu zapalnego, powiększenia rozmiarów adipocytów, a także powstawania kolejnych związków prozapalnych, takich jak interleukina-6, białko C-reaktywne, oraz inhibitor 1 aktywatora plazminogenu [13,14]. Źródłem metaloproteinaz macierzy pozakomórkowej są bowiem także adipocyty, preadipocyty, fibroblasty oraz komórki śródbłonka [15]. Główną funkcją metaloproteinaz macierzy pozakomórkowej jest degradacja kolagenu typu IV, który jest głównym składnikiem błony podstawnej naczyń. Uszkodzenie tej bariery ułatwia migrację leukocytów do tkanki tłuszczowej, nasilając tym samym jej stan zapalny. Aktywacja leukocytów powoduje uwolnienie prozapalnych cytokin i metaloproteinaz macierzy pozakomórkowej, co prowadzi do nasilenia procesów zapalnych [16]. Przebudowa macierzy zewnątrzkomórkowej, w tym degradacja tkanki łącznej i białek błony podstawnej, jest konieczna na wczesnym etapie

angiogenezy. Makrofagi więc przyczyniają się do angiogenetycznej przebudowy tkanki tłuszczowej poprzez modulację ECM. Przebudowa ECM jest również związana z regulacją adipogenezy, podczas rozrostu tkanki tłuszczowej, kiedy to różnicowanie adipocytów jest regulowane przez odkładanie się kolagenu [12]. Podczas nadmiernej akumulacji lipidów wewnątrz adipocytów, a co za tym idzie wzrostu liczby lub wielkości adipocytów, dochodzi do zwiększonego unaczynienia tkanki tłuszczowej [5]. Nieprawidłowa przebudowa ECM, wynikająca z nadmiernej i szybkiej akumulacji lipidów wewnątrz adipocytów, prowadzi do obniżenia liczby naczyń włosowatych obecnych w tkance tłuszczowej osób z otyłością i insulinoopornością. Poprzez proteolizę elementów strukturalnych ECM, MMP9 wpływa na degradację jednego z łańcuchów kolagenu typu IV, co powoduje powstanie peptydu działającego hamująco na unaczynienie tkanki [12].

Dieta bogatotłuszczowa, przy zachowanym dodatnim bilansie energetycznym, prowadzi do rozwoju otyłości oraz powstawania stresu oksydacyjnego (OS) w komórkach, wzrost produkcji reaktywnych form tlenu (ROS) oraz postępu stanu zapalnego [17,18]. Wzrost ilości ROS w tkance tłuszczowej prowadzi do upośledzenia adipogenezy, rekrutacji i aktywacji makrofagów, wydzielania adipokin zapalnych czy uszkodzenia biologicznych struktur tkanki tłuszczowej. Stres oksydacyjny bezpośrednio zaburza również sygnalizację insuliny. Reaktywne formy tlenu mogą bowiem indukować substrat receptora insuliny fosforylacji seryny/treoniny, upośledzać redystrybucję komórkową komponentów sygnałowych insuliny oraz zmniejszać transkrypcję genu transportera glukozy typu 4 (GLUT4) [19]. Stres oksydacyjny, w powiązaniu z hiperglikemią, lipotoksycznością, i glukotoksycznością, jest więc również odpowiedzialny za rozwój insulinooporności. Dodatkowo, stres oksydacyjny powoduje wzrost produkcji cytokin prozapalnych, a tym samym indukcję stanu zapalnego, co prowadzi do dalszego rozwoju otyłości [20]. Wykazano, że stosowanie antyoksydantów, do których należą N-acetylocysteina (NAC) i kwas alfa-liponowy (ALA) mają korzystny wpływ na metaboliczne powikłania otyłości [21–25]. Oba te antyoksydanty mogą zmniejszać stres oksydacyjny, ilość wydzielanych cytokin prozapalnych, nacieki makrofagów i inne powikłania otyłości [26,27]. N-acetylocysteina jest pochodną cysteiny, która służy jako prekursor do syntezy zredukowanego glutationu, natomiast kwas alfa-liponowy jest niezbędnym endogennym kofaktorem kompleksu enzymatycznego biorącego udział w wytwarzaniu energii oraz inaktywacji wolnych rodników, a poprzez swoją zredukowaną formę także oddziałuje z reaktywnymi formami tlenu [22]. Wcześniejsze badania wykazały, że podawanie N-acetylocysteiny lub kwasu alfa-liponowego szczurom karmionym dietą bogatotłuszczową obniżyło poziom triacylogliceroli w osoczu i całkowitego cholesterolu, przywracając tym

samym, zaburzony przez otyłość, metabolizm lipidów osoczowych [28,29]. Niemniej jednak mechanizm działania NAC i ALA na metabolizm lipidów w tkance tłuszczowej (VAT i SAT) jest nadal nieznany. Ponieważ akumulacja lipidów często wynika ze zwiększonego transportu przezbłonowego LCFA, prawdopodobne jest, że NAC i ALA mogą wpływać na ten proces.

4. Cele pracy

Zmiany strukturalne tkanki tłuszczowej, towarzyszące otyłości, wpływają na ekspresję metaloproteinaz (MMP), jak i białek transportujących lipidy, a tym samym na wielkość transportu długołańcuchowych kwasów tłuszczowych (LCFA). Transport transbłonowy LCFA w tkance tłuszczowej jest wspomagany głównie przez translokazę kwasów tłuszczowych (FAT/CD36), błonowe białko wiążące kwasy tłuszczowe (FABPpm) oraz białka transportujące kwasy tłuszczowe (FATP1 i FATP4). Ostatnie badania wykazały także, że szczególnie metaloproteinazy 2 (MMP2) i 9 (MMP9) biorą udział w degradacji i przebudowie macierzy pozakomórkowej, zmieniając strukturę tkanki tłuszczowej, co może wpływać na transport LCFA.

Dieta bogatotłuszczowa skutkuje rozwojem licznych nieprawidłowości metabolicznych, także w obrębie tkanki tłuszczowej, co może przyczyniać się do zwiększonej produkcji reaktywnych form tlenu oraz zwiększonego stresu oksydacyjnego/nitrozacyjnego, natomiast związki przywracające homeostazę redoks takie jak N-acetylocysteina (NAC) i kwas alfa-liponowy (ALA) mogą poprawiać metabolizm lipidów. Wewnątrzkomórkowa akumulacja lipidów wynika głównie ze zwiększonego transportu transbłonowego LCFA, prawdopodobne jest więc, że NAC i ALA mogą wpływać na ten proces.

Mając na uwadze powyższe, celem zrealizowanych badań była:

1. Ocena wpływu N-acetylocysteiny lub kwasu alfa-liponowego podczas stosowania diety bogatotłuszczowej na wybrane parametry osocza (insulina, glukoza, FFA, DAG, TAG).
2. Ocena wpływu N-acetylocysteiny lub kwasu alfa-liponowego podczas stosowania diety bogatotłuszczowej na histologię trzewnej i podskórnej tkanki tłuszczowej.
3. Ocena wpływu N-acetylocysteiny lub kwasu alfa-liponowego podczas stosowania diety bogatotłuszczowej na ekspresję (zarówno na poziomie mRNA, jak i białka) transporterów kwasów tłuszczowych (FAT/CD36, FABPpm, FATP1, FATP4) oraz na następczą akumulację kwasów tłuszczowych w wybranych frakcjach lipidowych (FFA, DAG, TAG) w trzewnej i podskórnej tkance tłuszczowej.
4. Ocena wpływu N-acetylocysteiny lub kwasu alfa-liponowego podczas stosowania diety bogatotłuszczowej na ekspresję (zarówno na poziomie mRNA, jak i białka) metaloproteinaz macierzy pozakomórkowej (MMP2 i MMP9) w trzewnej i podskórnej tkance tłuszczowej.

5. Materiały i metody

Szczegółowe informacje dotyczące zastosowanego modelu doświadczalnego i metodyki badań znajdują się w niżej wymienionych pracach włączonych do rozprawy:

a) Publikacja nr 2 – Wołosowicz M., Dajnowicz-Brzezik P., Łukaszuk B., Żebrowska E., Maciejczyk M., Zalewska A., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue.* Advances in Medical Sciences; 2022; 67(2), 216–228.

b) Publikacja nr 3 – Wołosowicz M., Łukaszuk B., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue.* Cellular Physiology and Biochemistry, 2022; 56, 166–179.

5.1. Model doświadczalny

Badania z wykorzystaniem modelu zwierzęcego zostały przeprowadzone na samcach szczurów rasy Wistar (masa początkowa=50-72g). Zgoda na przeprowadzenie badań na zwierzętach została udzielona przez Komisję Bioetyczną w Olsztynie (nr 21/2017). Po sześciu dniach aklimatyzacji zwierzęta zostały losowo podzielone na cztery grupy:

1) grupa kontrolna (CTRL) (n=10) – szczury były karmione dietą standardową przez 10 tygodni. Po 6 tygodniach doświadczenia zwierzęta otrzymywały dodatkowo przez kolejne 4 tygodnie roztwór soli fizjologicznej;
2) grupa z dietą bogatotłuszczową (HFD) (n=10) – szczury były karmione dietą bogatotłuszczową przez 10 tygodni. Po 6 tygodniach doświadczenia zwierzęta otrzymywały dodatkowo przez kolejne 4 tygodnie roztwór soli fizjologicznej;
3) grupa z dietą bogatotłuszczową i N-acetylocysteiną (HFD+NAC) (n=10) – szczury były karmione dietą bogatotłuszczową przez 10 tygodni. Po 6 tygodniach doświadczenia zwierzęta otrzymywały dodatkowo roztwór NAC przez 4 tygodnie;
4) grupa z dietą bogatotłuszczową i kwasem alfa-liponowym (HFD+ALA) (n=10) – szczury były karmione dietą bogatotłuszczową przez 10 tygodni. Po 6 tygodniach doświadczenia zwierzęta otrzymywały dodatkowo roztwór ALA przez kolejne 4 tygodnie.

Roztwory NAC i ALA były podawane przez zgłębnik żołądkowy szczurom z odpowiednich grup. Zostały podane dawki 500 mg/kg masy ciała NAC i 30 mg/kg masy ciała ALA, ponieważ są to najczęściej stosowane, nietoksyczne dawki o udowodnionym działaniu

antyoksydacyjnym. W podobny sposób podaliśmy sól fizjologiczną (2 ml/kg masy ciała) szczurom kontrolnym i na diecie bogatotłuszczowej. Masę ciała szczurów oraz spożycie pokarmu codziennie monitorowaliśmy.

Po 10 tygodniach eksperymentu szczury zostały znieczulone dootrzewnowym wstrzyknięciem fenobarbitalu (80 mg/kg masy ciała). Następnie z okolic brzucha pobraliśmy próbki VAT i SAT, natomiast osocze z żyły głównej brzusznej. Tkanki oraz osocze natychmiast zamroziliśmy w ciekłym azocie, a następnie umieściliśmy w zamrażarkach, w temperaturze -80^{o}C do czasu dalszych analiz biochemicznych.

Szczegółowe badania w eksperymencie na modelu zwierzęcym objęły ocenę:

a) zawartości insuliny i glukozy w osoczu przy użyciu metod kolorymetrycznych oraz ELISA,
b) zawartości wolnych kwasów tłuszczowych (FFA), diacylogliceroli (DAG) i triacylogliceroli (TAG) w osoczu za pomocą metody chromatografii gazowo-cieczowej (GLC),
c) stopnia akumulacji makrofagów i zmian morfologii adipocytów w trzewnej i podskórnej tkance tłuszczowej za pomocą barwienia hematoksyliną + eozyną (H&E), przeciwciałem CD68 oraz zestawem Toluidine Blue Stain w preparatach histologicznych,
d) całkowitej ekspresji mRNA transporterów kwasów tłuszczowych, takich jak: translokaza kwasów tłuszczowych (FAT/CD36), białko wiążące kwasy tłuszczowe (FABPpm) oraz białka transportujące kwasy tłuszczowe w błonie komórkowej (FATP1 i FATP4) w trzewnej i podskórnej tkance tłuszczowej metodą real-time PCR,
e) całkowitej ekspresji białek transporterów kwasów tłuszczowych, takich jak: translokaza kwasów tłuszczowych (FAT/CD36), białko wiążące kwasy tłuszczowe (FABPpm) oraz białka transportujące kwasy tłuszczowe w błonie komórkowej (FATP1 i FATP4) w trzewnej i podskórnej tkance tłuszczowej za pomocą metody Western blot,
f) akumulacji kwasów tłuszczowych w wybranych frakcjach lipidowych, takich jak: wolne kwasy tłuszczowe (FFA), diacylogliceroli (DAG) oraz triacylogliceroli (TAG) w trzewnej i podskórnej tkance tłuszczowej z użyciem metody chromatografii gazowo-cieczowej (GLC),
g) całkowitej ekspresji mRNA metaloproteinaz macierzy pozakomórkowej, takich jak: metaloproteinazy 2 (MMP2) i 9 (MMP9) w trzewnej i podskórnej tkance tłuszczowej metodą real-time PCR,
h) całkowitej ekspresji białek metaloproteinaz macierzy pozakomórkowej, takich jak: metaloproteinazy 2 (MMP2) i 9 (MMP9) w trzewnej i podskórnej tkance tłuszczowej za pomocą metody Western blot.

5.2. Analiza wybranych parametrów osocza

Zawartość glukozy i insuliny w osoczu oznaczono przy użyciu zestawu kolorymetrycznego (Glucose Colorimetric Assay Kit II; BioVision Inc., Milpitas, CA, USA) oraz ELISA (Rat Insulin ELISA Kit; Mercodia, Uppsala, Szwecja), odpowiednio, zgodnie z protokołami producenta. Absorbancję zmierzono spektrofotometrycznie przy 450 nm za pomocą czytnika mikropłytek (Synergy H1TM, BioTek Instruments, Winooski, USA). Następnie, z uzyskanych krzywych wzorcowych, obliczono wartości parametrów. Homeostatyczny model oceny insulinooporności (HOMA-IR) obliczono na podstawie wartości glukozy (FPG) i insuliny (FPI) – HOMA-IR = (FPG × FPI/22,5).

5.3. Analiza histologiczna

W celu określenia zmian morfologicznych adipocytów pobrano próbki VAT i SAT od każdego szczura z każdej badanej grupy (CTRL, HFD, HFD+NAC, HFD+ALA). Fragmenty tkanek natychmiast utrwalono w 10% buforowanej formalinie i poddano rutynowej obróbce w celu zatopienia w parafinie. Bloki parafiny pocięto na skrawki 4 μm, przymocowano do dodatnio naładowanych szkiełek (Superfrost Plus; Menzel Gläser, Braunschweig, Niemcy) i wybarwiono hematoksyliną i eozyną (H&E).

Zatopione w parafinie próbki tkanki tłuszczowej odparowano i uwodniono w czystych alkoholach. Do barwienia makrofagów zastosowano zestaw Toluidine Blue Stain (nr kat.: SS057 BioGenex; 49026 Milmont Drive, Fremont, CA, USA). W skład zestawu wchodziły: nadmanganian potasu, pirosiarczyn potasu, roztwór Scotta, roztwór błękitu toluidynowego. Barwienie przeprowadzono zgodnie z instrukcjami producenta.

Barwienie immunohistologiczne przeprowadzono przy użyciu następującego protokołu: w celu odzyskania antygenu próbki poddano wstępnej obróbce w komorze ciśnieniowej i ogrzewano stosując roztwór Target Retrieval Solution (S 1699 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA, USA). Po schłodzeniu do temperatury pokojowej próbki inkubowano z roztworem blokującym peroksydazę przez 10 minut w celu zablokowania aktywności endogennej peroksydazy. Próbki tkanek tłuszczowych inkubowano przez 1 godzinę w temperaturze pokojowej, z monoklonalnym przeciwciałem pierwszorzędowym CD68 (M0876, Agilent Technologies). Surowicę odpornościową uprzednio rozcieńczono w Antibody Diluent Background Reducing (S 3022 Agilent Technologies) w stosunku 1:50 dla CD68. Następnie, przez 30 minut, próbki były inkubowane z przeciwciałem drugorzędowym (EnVision FLEX, High pH (Link), HRP, Królik/Mysz (K800021-2 Agilent Technologies).

Kolejno inkubowano próbki przez 1 minutę z chromogenem DAB Flex, w celu uwidocznienia związanych przeciwciał. Próbki ostatecznie wybarwiono kontrastowo hematoksyliną QS (H-3404, Vector Laboratories; Burlingame, USA), osadzono i oceniono pod mikroskopem świetlnym. Swoistość przeciwciał potwierdzono przy użyciu kontroli negatywnej, co obejmowało zastąpienie przeciwciał rozcieńczalnikiem przeciwciał (bez barwienia) i kontrolę pozytywną, która obejmowała barwienie CD68 ludzkiego migdałka.

Ocena wielkości komórek i liczby makrofagów została przeprowadzona przez dwóch doświadczonych histologów (niezależnych od siebie) i przeanalizowana za pomocą oprogramowania ImageJ (The National Institutes of Health, USA). Obrazy zostały przekonwertowane do 8-bitowej skali szarości, a tło zostało odjęte. Następnie ustawiono binarną funkcję progową, aby oddzielić komórki od barwienia tła. Całkowitą powierzchnię komórek obliczono jako całkowitą liczbę pikseli na obrazach z ustalonym progiem. Wyniki wykreślono w GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Wyniki przedstawiono jako średnią ± odchylenia standardowe. Poziom istotności statystycznej ustalono na $p<0,05$.

5.4. Analiza metodą real-time PCR

Całkowity kwas rybonukleinowy (RNA) wyizolowano z VAT i SAT przy użyciu TriReagent RNA Isolation Reagent zgodnie z protokołem producenta (Sigma-Aldrich, Darmstadt, Niemcy). Całkowita ilość i jakość RNA została zbadana spektrofotometrycznie przez pomiar współczynników absorbancji próbki (260/280 nm i 260/230 nm). Syntezę komplementarnego kwasu dezoksyrybonukleinowego (DNA) przeprowadzono z użyciem uniwersalnego zestawu Master cDNA EvoScript (Roche Molecular Systems, Boston, USA). Specyficzne startery użyte w badaniu przedstawiono w Tabeli 1. Analizę real-time PCR przeprowadzono przy użyciu termocyklera LightCycler 96 System Real-Time Thermal z cyklerem z FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Niemcy). Warunki cykli były następujące: 15s denaturacji w 95°C; wygrzewanie 15s w 58°C dla β-aktyny, FATP1, FATP4 i MMP2, 59°C dla MMP9, 61°C dla FABPpm, 62°C dla FAT/CD36, następnie 15 s wydłużanie w 72°C przez 45 cykli. Analizę krzywych topnienia przeprowadzono przed każdą reakcją, aby zweryfikować specyficzność produktu PCR. Poziomy matrycowego kwasu rybonukleinowego (mRNA) genów docelowych znormalizowano do poziomu β-aktyny szczurów i obliczono zgodnie z metodą Pfaffla.

Tabela 1. Sekwencje starterów do analizy real-time PCR

Gene	Primer Sequence		Annealing temperature
	Forward	Reverse	
β-actin	*5'*-ACGGTCAGGTCATCACTATCG-*3'*	*5'*-GGCATAGAGGTCTTTACGGATG-*3'*	58°C
FAT/CD36	*5'*-GCCTCCTTTCCACCTTTTGT-*3'*	*5'*-GATTCAAACACAGCATAGATGGAC-*3'*	62°C
FABPpm	*5'-T*CATCCTTTGTCTCCAGCTTTT-*3'*	*5'*-CCTATGCCATGCTGACAGGT-*3'*	61°C
FATP1	*5'*-GGGTTTGCAAGCCAGAGA-*3'*	*5'*-CAAAGCAGCCCCAATGAG-*3'*	58°C
FATP4	*5'-TTGCCTGAGCTGCACAAAAC-3'*	*5'*-AGTGCAACATAGCAGCCTGT-*3'*	58°C
MMP2	*5'*-AAAGGAGGGCTGCATTGTGAA-*3'*	*5'*-CTGGGGAAGGACGTGAAGAGG-*3'*	58°C
MMP9	*5'*-AGGTGCCTCGGATGGTTATCG-*3'*	*5'*-TGCTTGCCCAGGAAGACGAA-*3'*	59°C

5.5. Analiza metodą Western Blot

W celu zbadania ekspresji białek zastosowano rutynową procedurę Western blot (tj. FAT/CD36, FABPpm, FATP1, FATP4, MMP2, MMP9 i β-aktyna). VAT i SAT zhomogenizowano (przy uzyciu homogenizatora eletrycznego) w lodowatym buforze radioimmunoprecypitacyjnym (RIPA) zawierającym koktajl inhibitorów proteazy i fosfatazy (Roche Diagnostics GmbH, Mannheim, Niemcy). Całkowite stężenie białka oznaczono za pomocą kwasu bicynchoninowego (BCA) z albuminą surowicy bydlęcej (BSA), jako standardem. W oparciu o odczytaną ilość białka, do próbek dodano odpowiednią ilość merkaptoetanolu w buforze, a następnie powstały roztwór inkubowano w temperaturze 90°C.

Homogenat tkankowy zawierający 20 µg białka poddano elektroforezie w żelu poliakrylamidowym (SDS-PAGE) (200 V przez 60 minut), a następnie transferowi na membrany nitrocelulozowe (100 V przez 30 minut). W kolejnym etapie membrany nitrocelulozowe były inkubowane w temperaturze pokojowej przez 60 minut w 5% roztworze odtłuszczonego mleka w proszku w soli fizjologicznej buforowanej tris z tween 20. Następnie membrany były inkubowane przez noc z pierwszorzędowymi przeciwciałami przeciwko FAT/CD36 (1:1000, nr kat. ab252922, Abcam, Cambridge, Wielka Brytania), FABPpm (1:5000, nr kat. ab171739, Abcam, Cambridge, Wielka Brytania), FATP1 (1:1000, nr kat. LS-C373484-100, LSBio, Seattle, USA), FATP4 (1:1000, nr kat. ab200353, Abcam, Cambridge, Wielka Brytania), MMP2 (1:2500, nr kat. ab92536, Abcam, Cambridge, UK) i MMP9 (1:10000, nr kat. ab76003, Abcam, Cambridge, UK). Następnie membrany były inkubowane z przeciwciałem drugorzędowym anti-rabbit IgG sprzężonym z peroksydazą chrzanową (Santa Cruz Biotechnology, Heidelberg, Niemcy). Następnie prążki białka uwidoczniono za pomocą

substratu chemiluminescencji (Thermo Scientific, Waltham, MA, USA) i oznaczono ilościowo za pomocą densytometrii (Bio-Rad Systems, Hercules, USA). Ekspresje białek znormalizowano do ekspresji β-aktyny (1:200, nr kat. ab115777, Abcam, Cambridge, UK).

5.6. Analiza metodą chromatografii gazowo-cieczowej (GLC)

Poziom frakcji wolnych kwasów tłuszczowych (FFA), diacylogliceroli (DAG) i triacylogliceroli (TAG) zmierzono za pomocą chromatografii gazowo-cieczowej. W pierwszym etapie, wybrane frakcje lipidowe wyekstrahowano metodą Bligha i Dyera. Następnie, lipidy rozdzielono metodą chromatografii cienkowarstwowej (TLC).

Poszczególne estry metylowe kwasów tłuszczowych (FAME), obecne w każdej frakcji, zidentyfikowano i oznaczono ilościowo, zgodnie z czasami retencji wzorców metodą GLC (chromatograf cieczowy Hewlett-Packard5890 Series II, kolumna kapilarna HP-INNOWax; Agilent Technologies, Santa Clara, Kalifornia, USA). Całkowite ilości FFA, DAG i TAG oszacowano jako sumę poszczególnych rodzajów FA w ocenianej frakcji i wyrażono w nanomolach na mg białka.

5.7. Analiza statystyczna

Analizy przeprowadzono przy użyciu oprogramowania GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Normalność rozkładu wyników sprawdzono za pomocą testu Shapiro–Wilka. Przeprowadzono test analizy wariancji (ANOVA) w celu porównania średnich między wszystkimi 4 grupami oraz test t-Studenta w celu porównania średnich między tylko 2 testowanymi grupami (CTRL i HFD; CTRL i HFD+NAC; CTRL i HFD+ALA; HFD i HFD+NAC; HFD i HFD+ALA; HFD+NAC i HFD+ALA). Jeżeli założenia powyższych testów nie zostały spełnione, zastosowano test Kruskala–Wallisa z testem post hoc U Manna–Whitneya w parach. Dodatkowo, ze względu na wielokrotne porównania, dla uzyskanych wartości *p* zastosowano poprawkę Benjaminiego-Hochberga. Wyniki przedstawiono jako średnie ± odchylenie standardowe (SD). Poziom istotności statystycznej ustalono na $p<0,05$.

6. Wyniki

Szczegółowe informacje dotyczące uzyskanych wyników badań znajdują się w niżej wymienionych pracach włączonych do rozprawy:

a) Publikacja nr 2 – Wołosowicz M., Dajnowicz-Brzezik P., Łukaszuk B., Żebrowska E., Maciejczyk M., Zalewska A., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue.* Advances in Medical Sciences; 2022; 67(2), 216–228.

b) Publikacja nr 3 – Wołosowicz M., Łukaszuk B., Kasacka I., Chabowski A.: *Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue.* Cellular Physiology and Biochemistry, 2022; 56, 166–179.

W opisie wyników wykorzystano odniesienia do figur zamieszczonych w wyżej wymienionych publikacjach włączonych do rozprawy.

6.1. Ogólna charakterystyka modelu badawczego i zmian wybranych parametrów osoczowych

Zastosowanie diety bogatotłuszczowej spowodowało istotny wzrost masy ciała szczurów z grupy HFD (+22%, $p<0,0001$, HFD vs. CTRL, Fig. 1a, b), jak również w grupach HFD+NAC (+24%, $p<0,0001$, HFD+NAC vs. CTRL, Fig. 1a, b) i HFD+ALA (+18%, $p<0,0001$, HFD+ALA vs. CTRL, Fig. 1a, b) w stosunku do grupy kontrolnej (Publikacja nr 3). Zwierzęta w grupach HFD+NAC (-30%, $p<0,0001$) i HFD+ALA (-14%, $p=0,0220$) ważyły mniej niż szczury z grypy na diecie bogatotłuszczowej (Tabela 2, Publikacja nr 2). Przeprowadzone badania wykazały, że stężenie glukozy i insuliny oraz wartości HOMA-IR były istotnie podwyższone we wszystkich grupach z zastosowaną dietą bogatotłuszczową w stosunku do grupy CTRL (HOMA-IR: +26%, $p=0,014$, HFD vs. CTRL; +123%, $p=0,0010$, HFD+NAC vs. CTRL; +8-krotnie, $p=0,0021$, HFD+ALA vs. CTRL; Publikacja nr 3). Stężenia glukozy i insuliny oraz HOMA-IR były istotnie niższe w grupie HFD+NAC (-28%, $p=0,0016$; -51%, $p=0,0042$; -87%, $p=0,0023$; odpowiednio; HFD+NAC vs. HFD) oraz w grupie HFD+ALA (-19%, $p=0,0210$; -48%, $p=0,0024$; -88%, $p=0,0022$; odpowiednio; HFD+ALA vs. HFD), kiedy porównano je z grupą z dietą bogatotłuszczową (Publikacja nr 3). Co ciekawe, zaobserwowano również istotne różnice w poziomie glukozy pomiędzy grupami, w których

zastosowano antyoksydanty (+12%, p=0,0172, HFD+ALA vs. HFD+NAC; Publikacja nr 3). Stosowanie NAC podczas utrzymanej diety bogatotłuszczowej spowodowało istotne obniżenie zawartości DAG (-50%, p<0,0001, Fig. 1e) i TAG (-45%, p=0,009, Fig. 1f) w osoczu w porównaniu z grupą CTRL (HFD+NAC vs. CTRL; Publikacja nr 3). Ponadto badanie wykazało istotne obniżenie zawartości DAG (-37%, p=0,0045, Fig. 1e) i TAG (-53%, p=0,0080, Fig. 1f) w osoczu, kiedy porównaliśmy grupy HFD+NAC vs. HFD (Publikacja nr 3). Zaobserwowano również istotne zmiany w zawartości DAG w osoczu szczurów z grupy HFD+ALA w stosunku do szczurów na diecie bogatotłuszczowej (+35%, p=0,0491, HFD+ALA vs. HFD, Fig. 1e) oraz pomiędzy dwiema grupami z podawanymi antyoksydantami (+115%, p<0,0001, HFD+ALA vs. HFD+NAC, Fig. 1e) (Publikacja nr 3).

6.2. Zmiany histologiczne trzewnej i podskórnej tkanki tłuszczowej

Badanie histologiczne wycinków tkanki tłuszczowej w grupie HFD ujawniło istotnie większe rozmiary adipocytów, w odniesieniu do grup kontrolnych (p<0,0001, HFD VAT vs. CTRL VAT, Fig. 2a, 4a; p=0,0165, HFD SAT vs. CTRL SAT, Fig. 2b, 4b; Publikacja nr 3). Analizy immunohistochemiczne w tej grupie wykazały również wzrost liczby komórek CD68 immunopozytywnych (p=0,0061, HFD SAT vs. CTRL SAT, Fig. 3b, 4d; Publikacja nr 3). Najwięcej jednak makrofagów infiltrujących stwierdzono w VAT, gdzie tworzyły duże skupiska w grupach CTRL, HFD, HFD+NAC oraz HFD+ALA (Fig. 3a; Publikacja nr 3). Adipocyty VAT i SAT w grupach HFD+NAC były nieco mniejsze niż komórki tłuszczowe w grupach z zastosowaną dietą bogatotłuszczową (Fig. 2a i b, 4a i b; Publikacja 3). W obu grupach HFD+NAC zaobserwowano mniejszą liczbę makrofagów (Fig. 3a i b), z większą liczbą komórek CD68 immunopozytywnych w VAT (p=0,0358, HFD+NAC VAT vs. HFD VAT, Fig. 3a, 4c) w porównaniu z grupami na diecie bogatotłuszczowej (Publikacja 3). Wielkość adipocytów w grupach HFD+ALA była mniejsza w stosunku do tych w grupach z dietą bogatotłuszczową (p=0,0003, HFD+ALA VAT vs. HFD VAT, Fig. 2a, 4a; p=0,0209, HFD+ALA SAT vs. HFD SAT, Fig. 2b, 4b; Publikacja nr 3). Liczba komórek CD68 immunopozytywnych była wyższa zarówno w VAT, jak i SAT, w odniesieniu do grup z zastosowaną dietą bogatotłuszczową (p=0,0088, HFD+ALA VAT vs. HFD VAT, Fig. 3a, 4c; Publikacja nr 3). Podaż ALA (HFD+ALA) skutkowało większą liczbą makrofagów, w VAT i SAT, w porównaniu z grupami na diecie bogatotłuszczowej (Fig. 2a i b; Publikacja 3). Co ciekawe, wielkość adipocytów w obu tkankach tłuszczowych, była nieco mniejsza w grupach HFD+ALA, niż w grupach HFD+NAC (p=0,0010, HFD+ALA VAT vs. HFD+NAC VAT, Fig. 2a, 4a; Publikacja nr 3). Z drugiej jednak strony, liczba komórek CD68 immunopozytywnych

w grupie HFD+ALA była wyższa w obu depozytach tkanki tłuszczowej w porównaniu z HFD+NAC (p=0,0358, HFD+ALA VAT vs. HFD+NAC VAT, Fig. 3a, 4c; Publikacja nr 3).

6.3. Zmiany ekspresji białkowych transporterów kwasów tłuszczowych w trzewnej i podskórnej tkanki tłuszczowej (mRNA i białek)

Szczury karmione dietą bogatotłuszczową charakteryzowały się istotnie niższymi poziomami ekspresji FAT/CD36 (-43%, p=0,0130, Fig. 3a) i FABPpm (-45%, p=0,0030, Fig. 3c) na poziomie mRNA w VAT w stosunku do szczurów kontrolnych (HFD VAT vs. CTRL VAT; Publikacja nr 2). Czterotygodniowa podaż NAC, z utrzymanym reżimem diety bogatotłuszczowej, spowodowała dalsze istotne obniżenie poziomu ekspresji FAT/CD36 mRNA w VAT (-56%, p=0,0007, HFD+NAC VAT vs. HFD VAT, Fig. 3a) i SAT (-50%, p=0,009, HFD+NAC SAT vs. HFD SAT, Fig. 3b), w porównaniu z grupami na diecie bogatotłuszczowej (Publikacja nr 2). Podobną redukcję zaobserwowano w poziomie ekspresji FATP1 mRNA w SAT (-80%, p=0,0001, HFD+NAC SAT vs. HFD SAT, Fig. 3f; Publikacja nr 2). Zastosowanie ALA poskutkowało wzrostem poziomu ekspresji FABPpm mRNA w VAT (+61%, p=0,0389, HFD+ALA VAT vs. HFD VAT, Fig. 3c) oraz FATP1 mRNA w SAT (+146%, p=0,0223, HFD+ALA SAT vs. HFD SAT, Fig. 3f) w porównaniu z odpowiednimi grupami na diecie bogatotłuszczowej (Publikacja nr 2). Przeprowadzone badania wykazały istotne zmniejszenie poziomu ekspresji FATP1 mRNA w VAT, w stosunku do tej uzyskanej w grupie z dietą bogatotłuszczową (-62%, p=0,0061, HFD+ALA VAT vs. HFD VAT, Fig. 3e; Publikacja nr 2). Porównanie grup z zastosowanymi antyoksydantami, wykazało istotnie wyższy poziom eskpresji FAT/CD36 (+133%, p=0,0066, Fig. 3a) i FABPpm (+130%, p=0,0031, Fig. 3c) mRNA w VAT szczurów z podażą ALA (HFD+ALA VAT vs. HFD+NAC VAT; Publikacja nr 2). Podobne zmiany wystąpiły również dla obu grup w SAT w przypadku poziomu ekspresji FAT/CD36 (+101%, p=0,0072, Fig. 3b) i FATP1 (+11-krotnie, p=0,0062, Fig. 3f) mRNA (HFD+ALA SAT vs. HFD+NAC SAT; Publikacja nr 2). Wykazano również zmiany pomiędzy działaniem antyoksydantów, w postaci różnicy poziomów ekspresji FATP1 mRNA w VAT (-46%, p=0,0451, HFD + ALA VAT vs. HFD + NAC VAT, Fig. 3e; Publikacja nr 2). Dziesięciotygodniowe stosowanie diety bogatotłuszczowej spowodowało wzrost ekspresji białek FAT/CD36 (+113%, p=0,0268, Fig. 4a), FABPpm (+113%, p=0,027, Fig. 4c), FATP1 (+11-krotny, p=0,0027, Fig. 4e) oraz FATP4 (+152%, p=0,0005, Fig. 4g) w VAT w odniesieniu do grupy kontrolnej (HFD VAT vs. CTRL VAT; Publikacja 2). Natomiast w SAT nastąpił jedynie wzrost ekspresji białka FAT/CD36, porównując do grupy kontrolnej (+110%, p=0,0036, HFD SAT vs. CTRL SAT, Fig. 4b; Publikacja nr 2). Stosowanie NAC doprowadziło

do istotnych zmian ekspresji białek FAT/CD36 (-74%, p=0,0008, Fig. 4b) i FATP4 (+10-krotnie, p=0,05, Fig. 4h) w SAT (HFD+NAC SAT vs. HFD SAT, Fig. 4b) oraz wzrostu ekspresji białka FATP4 (+15-krotnie, p=0,0157, HFD+NAC VAT vs. HFD VAT; Fig. 4g) w VAT w stosunku do odpowiednich grup z zastosowaną dietą bogatotłuszczową (Publikacja nr 2). Podobnie w grupie HFD+ALA zaobserwowano obniżenie ekspresji białka FAT/CD36 w SAT (-44%, p=0,0112, HFD+ALA SAT vs. HFD SAT, Fig. 4b; Publikacja nr 2). Porównanie grup z zastosowanymi różnymi antyoksydantami wykazało niższą ekspresję białka FATP4 w HFD+ALA, w VAT (-95%, p=0,030, HFD+NAC VAT vs. HFD+ALA VAT, Fig. 4g) i SAT (-91%, p=0,0279, HFD+NAC SAT vs. HFD+ALA SAT, Fig. 4h), zaś wzrost ekspresji białka FAT/CD36 (+118%, p=0,0252, HFD+NAC SAT vs. HFD+ALA SAT, Fig. 4b) w SAT (Publikacja nr 2).

6.4. Zmiany akumulacji kwasów tłuszczowych w wybranych frakcjach lipidowych w trzewnej i podskórnej tkanki tłuszczowej

Zbadano również całkowitą zawartość wybranych frakcji lipidowych (FFA, DAG i TAG) w pobranych próbkach tkanki tłuszczowej trzewnej i podskórnej. Zastosowanie diety bogatotłuszczowej wpłynęło na wzrost poziomu FFA (+77%, p<0,05, HFD VAT vs. CTRL VAT, Fig. 5a), DAG (+52%, p<0,05, HFD VAT vs. CTRL VAT, Fig. 5c) i TAG (+52%, p<0,05, HFD VAT vs. CTRL VAT, Fig. 5e) w VAT oraz FFA (+39%, p<0,05, HFD SAT vs. CTRL SAT, Fig. 5e) w SAT w porównaniu z odpowiednimi grupami kontrolnymi (CTRL VAT, CTRL SAT, Publikacja nr 3). Całkowity poziom FFA był istotnie mniejszy w grupie z zastosowaną NAC w VAT (-28%, p<0,05, HFD+NAC VAT vs. HFD VAT, Tabela 3, Fig. 5a) i SAT (-20%, p<0,05, HFD+NAC SAT vs. HFD SAT, Tabela 4, Fig. 5b) w porównaniu do grup na diecie bogatotłuszczowej (Publikacja nr 2). Szczury, którym podawano ALA miały niższy całkowity poziom FFA, jedynie w VAT w stosunku do grupy z reżimem diety bogatotłuszczowej (-44%, p<0,05, HFD+NAC VAT vs. HFD VAT, Tabela 3, Fig. 5a; Publikacja nr 2). Co ciekawe, kiedy porównano między sobą grupy z zastosowanymi antyoksydantami, zauważono istotne różnice między całkowitą zawartość FFA w VAT (-22%, p<0,05, HFD+ALA VAT vs. HFD+NAC VAT, Tabela 3, Fig. 5a) i SAT (+47%, p<0,05, HFD+ALA SAT vs. HFD+NAC SAT, Tabela 4, Fig. 5b) (Publikacja nr 2). Przeprowadzone badania dowiodły, że NAC wpływa na całkowity poziom DAG w VAT (-26%, p<0,05, HFD+NAC VAT vs. HFD+NAC VAT, Tabela 5, Fig. 5d) i SAT (-35%, p<0,05, HFD+NAC SAT vs. HFD+NAC SAT, Tabela 6, Fig. 5e) (Publikacja nr 2). Porównanie wyników z grup HFD+ALA i HFD+NAC wykazało pewne statystycznie istotne różnice w ilości DAG w SAT

(+45%, p<0,05, HFD+ALA SAT vs. HFD+NAC SAT, Tabela 6, Fig. 5e; Publikacja nr 2). Zastosowanie NAC (-16%, p<0,05, HFD+NAC VAT vs. HFD VAT) i ALA (-37%, p<0,05, HFD+ALA VAT vs. HFD VAT) wywołało również istotne zmiany w całkowite ilości TAG w VAT, kiedy porównano je ze zmianami uzyskanymi u szczurów na diecie bogatotłuszczowej (Tabela 7, Fig. 5g; Publikacja nr 2).

6.5. Zmiany ekspresji metaloproteinaz macierzy pozakomórkowej w trzewnej i podskórnej tkanki tłuszczowej (mRNA i białek)

W przeprowadzonych badaniach oceniono również ekspresję MMP2 i MMP9 mRNA w obu depozytach tkanki tłuszczowej badanych szczurów. Podaż diety bogatotłuszczowej wpłynęła na wzrot ekspresji MMP2 w VAT (+2-krotny, p<0.0001, HFD VAT vs. CTRL VAT, Fig. 6a) i MMP9 w VAT (+3-krotny, p=0.0018, HFD VAT vs. CTRL VAT, Fig. 6c) i SAT (+2-krotny, p=0.0052, HFD SAT vs. CTRL SAT, Fig. 6d) w stosunku do odpowiednich grup kontrolnych (Publikacja nr 3). Co ciekawe, czterotygodniowe podaż NAC z utrzymaną dietą bogatotłuszczową spowodowało znaczne obniżenie poziomu ekspresji MMP9 mRNA w VAT (-70%, p=0,009, HFD+NAC VAT vs. HFD VAT, Fig. 5d) i SAT (-92%, p=0,0150, HFD+NAC SAT vs. HFD SAT, Fig. 5e) w odniesieniu do odpowiednich grup na diecie bogatotłuszczowej (Publikacja nr 3). Porównanie grup z zastosowanymi antyoksydantami wykazało wielokrotnie wyższy poziom ekspresji MMP9 mRNA w SAT, w grupie z zastosowaną ALA niż NAC (+6-krotny, p=0,0162, HFD+ALA SAT vs. HFD+NAC SAT, Fig. 5e; Publikacja nr 3). Stwierdzono istotne różnice w poziomach ekspresji MMP2 (+97%, p<0,0001, HFD SAT vs. HFD VAT, Fig. 5c) i MMP9 (+2-krotnie, p=0,0089, HFD SAT vs. HFD VAT, Fig. 5f) mRNA pomiędzy tkankami, na korzyść SAT, porównując do wyników uzyskanych w grupach z dietą bogatotłuszczową (Publikacja nr 3). Wystąpiła również istotna różnica poziomów ekspresji MMP9 mRNA w grupach HFD+ALA pomiędzy badanymi tkankami (+1,5-krotny, p=0,0349, HFD+ALA SAT vs. HFD+ALA VAT, Fig. 5f; Publikacja nr 3). W toku prowadzonych badań została również dokonana ocena ekspresja białek MMP2 i MMP9 w tkance tłuszczowej, we wszystkich badanych grupach. W porównaniu z grupami kontrolnymi, stosowanie diety bogatotłuszczowej skutkowało wzrostem ekspresji białka MMP2 (+2-krotny, p<0,0001, HFD VAT vs. CTRL VAT, Fig. 5a) w VAT oraz MMP9 w VAT (+3-krotny, p=0,0018, HFD VAT vs. CTRL VAT, Fig. 5c) i SAT (+2-krotnie, p=0,0052, HFD SAT vs. CTRL SAT, Fig. 5d) (Publikacja nr 3). Czterotygodniowe stosowanie NAC podczas kontynuowania diety bogatotłuszczowej wpłynęło na istotny wzrost ekspresji białek MMP9 w VAT w porównaniu do grupy z samą dietą bogatotłuszczową (+3-krotny, p=0,0004, HFD+NAC VAT vs. HFD

VAT, Fig. 5c; Publikacja nr 3). Zastsowanie HFD+ALA istotnie wpłynęło na ekspresję białkek MMP2 i MMP9 w VAT (MMP2: -50%, *p*=0,0059, Fig. 5a; MMP9: -76%, *p*=0,0061, Fig. 5c; HFD+ALA VAT vs. HFD VAT) i SAT (MMP2: +4-krotnie, *p*=0,0071, Fig. 5b; MMP9: -66%, *p*=0,0126, Fig. 5d; HFD+ALA SAT vs. HFD SAT) porównując do grup z dietą bogatotłuszczową (Publikacja nr 3). Porównanie pomiędzy grupami z zastosowanymi antyoksydantami wykazało wyższą ekspresję białka MMP2 w SAT (+7-krotnie, *p*=0,011, HFD+ALA SAT vs. HFD+NAC SAT, Fig. 5b) i niższą ekspresję białka MMP9 w VAT (-94%, *p*=0,0003, HFD+ALA VAT vs. HFD+NAC VAT, Fig. 5c), w grupie HFD+ALA (Publikacja nr 3).

7. Wnioski

1. N-acetylocysteina i kwas alfa-liponowy obniżają poziom osoczowej insuliny oraz glukozy i wpływają na redukcję masy ciała.
2. Podaż N-acetylocysteiny podczas stosowania diety bogatotłuszczowej wpływa na obniżenie zawartości diacylogliceroli i triacylogliceroli w osoczu, natomiast kwas alfa-liponowy w połączeniu z reżimem diety bogatotłuszczowej nie wywołuje takiego efektu.
3. Stosowanie diety bogatotłuszczowej prowadzi do zwiększenia wielkości adipocytów oraz liczby komórek CD68 immunopozytywnych w obu depozytach tkanki tłuszczowej.
4. Zastosowanie N-acetylocysteiny lub kwasu alfa-liponowego, podczas utrzymanej diety bogatotłuszczowej, zmniejszyło wielkość adipocytów, jednocześnie zwiększając liczbę komórek CD68 immunopozytywnych w trzewnej i podskórnej tkance tłuszczowej.
5. Dieta bogatotłuszczowa prowadzi do wzrostu ekspresji białek FAT/CD36, FABPpm, FATP1, FATP4 w trzewnej tkance tłuszczowej, ale tylko FAT/CD36 również w podskórnej tkance tłuszczowej.
6. Stosowanie N-acetylocysteiny w trakcie reżimu diety bogatotłuszczowej prowadzi do obniżenia ekspresji FAT/CD36, FABPpm, FATP1 i FATP4 na poziomie mRNA, w obu depozytach tkanki tłuszczowej, oraz obniżenia ekspresji białka FAT/CD36 w podskórnej tkance tłuszczowej i białka FATP4 w trzewnej i podskórnej tkance tłuszczowej.
7. Stosowanie diety bogatotłuszczowej wpływa na wzrost zawartości wolnych kwasów tłuszczowych, diacylogliceroli i triacylogliceroli w trzewnej tkance tłuszczowej oraz wzrost poziomu wolnych kwasów tłuszczowych w podskórnej tkance tłuszczowej.
8. Podaż zarówno N-acetylocysteiny, jak i kwasu alfa-liponowego, w czasie stosowania diety bogatotłuszczowej, prowadzi do obniżenia akumulacji kwasów tłuszczowych (wolnych kwasów tłuszczowych, diacylogliceroli i triacylogliceroli) w obu depozytach tkanki tłuszczowej.
9. Ekspresja białka MMP2 jest obniżona pod wpływem stosowania N-acetylocysteiny, podczas gdy kwas alfa-liponowy nie wpływa na zmianę ekspresji białka MMP2. Natomiast ekspresja białka MMP9 jest zredukowana tylko przez podaż kwasu alfa-liponowego. Zmiany te zachodzą podczas podaży diety bogatotłuszczowej.

8. Publikacja nr 1

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Review

The Causes of Insulin Resistance in Type 1 Diabetes Mellitus: Is There a Place for Quaternary Prevention?

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Abstract: Diabetes mellitus was the first non-communicable disease that was recognized by the United Nations as a 21st-century pandemic problem. Recent scientific reports suggest that people with type 1 diabetes mellitus also develop insulin resistance, which is generally considered to be a distinctive feature of type 2 diabetes mellitus. The causes of insulin resistance in type 1 diabetes mellitus were explored, but there was a lack of publications that connected the risk factors of insulin resistance in type 1 diabetes mellitus with the proposition of repair mechanisms that are offered by quaternary prevention. Toward this end, the present review is an attempt to combine the previous reports on the causes of insulin resistance in type 1 diabetes mellitus and a brief review of quaternary prevention. The destructive effect of insulin resistance on many physiological processes that predisposes the individual to chronic diabetes complications creates an urgent need to introduce effective therapeutic methods for preventing the development and progression of this pathology.

Keywords: type 1 diabetes; insulin resistance; quaternary prevention; metformin; screening; diagnosis; risk factors; multidimensional approach

1. Introduction

Since the beginning of civilization, humanity has struggled with many epidemics of infectious diseases. After they had been defeated, mankind faced another problem of epidemic or rather pandemic proportions, i.e., non-communicable diseases. Currently, non-communicable diseases are the main cause of morbidity and mortality around the world. Diabetes mellitus (DM) was the first non-communicable disease that was recognized by the United Nations as a 21st-century pandemic problem [1]. DM is a metabolic disease, characterized by an inappropriately elevated blood glucose level. DM is divided into several types that are based on the pathogenesis of the disease. The two most common forms of the condition are type 1 and type 2 DM. Table 1 provides a concise comparison of these two types of DM.

Type 1 diabetes mellitus (T1DM), which was previously known as insulin-dependent, or childhood diabetes, is characterized by a deficiency in insulin production and, thus, requires constant administration of exogenous insulin. Recent scientific reports suggest that people with T1DM also develop insulin resistance (IR), a phenomenon that is generally considered to be a distinctive feature of T2DM. Therefore, this review will focus on the pathogenesis of IR in T1DM, as well as its quaternary prevention and treatment [2].

Interestingly, the majority of people with diagnosed DM live in the Western Pacific (131 million) and South-East Asian Regions (96 million) [3]. However, the population of people with DM in the European (64 million) and American (62 million) Regions is also quite large [3]. This spatial distribution may be due to the increase in the number of overweight and obese people in those regions [4]. According to the WHO report, nowadays, one in three adults is overweight, and more than one in 10 is

obese [3]. In 2014, it was estimated that 422 million adults (8.5% of the world adult population) live with DM, when compared to 108 million in 1980 (4.7% of the world adult population) [3]. DM is the seventh leading cause of death and a major cause of costly and debilitating complications, such as heart attack, stroke, kidney failure, blindness, and lower-limb amputation.

Table 1. Comparison of type 1 and type 2 diabetes mellitus.

Feature	T1DM	T2DM
body weight at the time of diagnosis	within the normal range or underweight	overweight or obese
cause of the disease	insulin deficiency as a result of β-cells damage	insulin resistance
presence of antibodies	anti-GAD, ICA, IA2, IAA, ZnT8	not found
disease onset	acute with accompanying diabetic ketoacidosis	mild onset
basic pharmacotherapy	insulin	initially metformin and other orally administered medications, in some cases insulin
Does the occurrence of the disease depend on a patient's lifestyle?	no	yes

anti-GAD: anti-glutamic acid decarboxylase; ICA: islet cell antibodies; IA2: islet antigen 2; IAA: insulin autoantibodies; ZnT8: zinc transporter 8.

2. Insulin

Insulin is a highly-anabolic endocrine hormone that is secreted by β-cells of the Islets of Langerhans located in the pancreas. It plays a wide spectrum of metabotropic roles, e.g., it stimulates transmembrane transport of glucose, drives the glycolysis process, triggers glycogen synthesis, and abolishes its breakdown [5]. Moreover, insulin silences lipolytic processes within adipose tissue and increases the amount of non-esterified fatty acids (NEFA) in the bloodstream and some other tissues, while simultaneously decreasing their oxidation in skeletal muscle and the liver. This hormone mainly plays a role in protein metabolism by amplification of their biosynthesis and lowering their degradation in the liver, skeletal muscle, and adipose tissue [5].

3. What Does It Mean to Be Insulin Resistant?

Insulin resistance is clinically defined as a reduced response of target tissues to stimulation by insulin. The phenomenon of IR is accompanied by a pathological insulin secretion after a meal, which is called hyperinsulinemia. Interestingly, long-lasting hyperinsulinemia leads to aggravated IR. In line with that notion, a chronically elevated insulin level (e.g., due to improper insulin injections) produces an adaptive reduction in the number of plasma membrane receptors for the hormone (due to their adaptive internalization and degradation) [6]. Consequently, greater insulin dosage is required to elicit the same physiological effect, hence IR begins. Moreover, secondary alterations in target tissues are also possible. Marban et al. demonstrated that transgenic mice over-expressing insulin showed diminished insulin responsiveness despite fasting normoglycaemia and proper body weight [7]. This could be explained by an impaired binding of insulin to its receptors and/or stem from hypertriglyceridemia, which may impair insulin signal transduction [8]. Hyperinsulinemia may also promote weight gain, since insulin overdose results in severe hypoglycaemia and polyphagia (excessive eating) [9]. This leads to the formation of a specific vicious circle, i.e., hyperinsulinemia propels IR and weight gain, which, in turn, require higher insulin dosage for compensation. Moreover, also defects in the insulin receptor structure, a reduction in the density of the receptors at the cell surface, or improper action of the immune system, may lead to an abnormal response of target tissues to insulin.

The possibility of the existence of reduced tissue insulin sensitivity in children, adolescents, and adults that are diagnosed with T1DM is no longer put into question. The development of IR in T1DM may be triggered by many risk factors (presented in Figure 1). A key role in the initiation of IR in

T1DM is attributed to the complex interactions between the genetic predisposition of an individual and their lifestyle. The human leukocyte antigen (HLA) accounts for most of the aforementioned genetic susceptibility to IR in T1DM. A study conducted by Todd et al. suggests that only the amino acid in position 57 of the DQB3-chain is strongly correlated with IR in T1DM [10]. IR is often associated with being overweight and/or obese. Therefore, priority has been given to non-pharmacological approaches that can inhibit the development of obesity in T1DM. Excessive adipose tissue mass leads to the development of IR by the overproduction of hormones antagonistic to insulin (growth hormone, glucagon, cortisol, and catecholamines), and through a direct secretion of increased amount of free fatty acids (FFA) into the blood. In the case of exorbitant FFA level, the body begins to use them as an energy source instead of glucose. Consequently, glucose is not underoxidized in the tissues and, thus, its level in the blood increases. Subsequently, insulin secretion is increased in order to maintain a normal blood glucose level. Moreover, chronically elevated FFA level in the blood may lead to their accumulation in peripheral tissues, where they may interfere with insulin signal transduction [11]. This is especially true for skeletal muscle, i.e., a tissue accountable for even 80% of insulin stimulated glucose uptake that takes place after a meal [12]. Under physiological conditions, insulin binds to its receptor and initiates the embedding of glucose transporters type 4 (GLUT-4) into the myocyte plasma membrane. The process is guided by a cascade of secondary signal transducers, the most important of which are: IRS-1, PI3K, PKB/Akt, and AS160 [13,14]. An excessive intracellular lipid build-up, especially into bioactive diacylglycerol (DAG) and ceramide (CER) species, disrupts this pathway. DAG, for instance, is known to activate protein kinase C (e.g., PKC θ) that inactivates IRS-1 (insulin receptor substrate 1). Ceramide, on the other hand, disables both IRS-1 (via activation of JNK) and protein kinase B (PKB/Akt) (via its proxy PP2A) [13,14]. Interestingly, Conway et al., in the Pittsburgh Epidemiology of Diabetes Complications Study, showed that, amongst adults with T1DM, the prevalence of overweight and obese individuals increased from 29% to 42%, and from 3% to 23%, respectively [15]. The participants were first seen in 1986–1988 (mean age and DM duration, were 29 and 20 years, respectively), and then after 18 years. Conway et al. suggested that the predictors of weight gain were: higher baseline hemoglobin A1c (HbA1c) concentration (directly related), symptomatic autonomic neuropathy (inversely related), overt nephropathy (inversely related), and intensive insulin therapy during follow-up (directly related) [15]. Fellinger et al. showed that patients with T1DM have higher BMI values when compared to the general population of Austria. However, the higher BMI values were observed for a group of the T1DM patients aged 30–49. This may be due to the fact that, in this age group, insulin therapy was conducted from the beginning of the diagnosis of T1DM. The higher BMI values were not related to 10.2% poorer glycemic level, which was examined by HbA1c [16]. Table 2 outlines a summary of the mechanisms that are involved in the etiopathogenesis of IR related to obesity. Unsurprisingly, it seems that, with worse metabolic stability of diabetes, IR severity also increases. The toxic effects of hyperglycemia and some lipid fractions also favor an increase in the resistance of insulin-dependent tissues to the action of insulin, thus enhancing IR and its metabolic consequences.

Table 2. The mechanisms involved in the etiopathogenesis of insulin resistance related to obesity [17,18].

Pre-Receptor	Receptor	Post-Receptor
↓ access of insulin to muscle secondary to free fatty acids excess	insulin receptor downregulation secondary to hyperinsulinemia	inhibition of the intracellular cascades by several adiposity-related factors (e.g., ↑free fatty acids, impaired adipokines, and/or cytokines secretion)
abnormal hormone structure	↓ affinity of the receptor for the hormone	glucose transporter abnormalities
the presence of insulin binding antibodies		
insulin degradation		
the presence of insulin antagonists such as glucagon, cortisol, thyroid hormones		

Oxidative stress interferes with insulin signaling, since reactive oxygen species (ROS) may induce insulin receptor substrate (IRS) serine/threonine phosphorylation, impair cellular redistribution of insulin signaling components, reduce *GLUT4* gene transcription, or alter mitochondrial activity [19]. Mechanisms, such as oxidative stress that is associated with hyperglycemia, lipotoxicity, and glucotoxicity are also responsible for the development of IR in T1DM. Oxidative stress causes an increase in the production of pro-inflammatory cytokines and, thus, the induction of inflammatory processes. In T1DM immune cells, such as macrophages, which are the source of pro-inflammatory cytokines, migrate to pancreatic islet cells. Chronic inflammation can also be included among the factors that contribute to the development of IR in T1DM. Adipose cell enlargement leads to a pro-inflammatory state and the formation of pro-inflammatory compounds, such as interleukin-6, C-reactive protein, and plasminogen activator inhibitor-1, which, in turn, contribute to the death of β-cells, modulation of β-cell regeneration processes, and IR [20,21]. Studies conducted by Gunawardana and Piston [22,23] seem to be in line with that notion. The authors investigated therapeutical effects of embryonic BAT (brown adipose tissue) transplants on the reversal of STZ- (streptozotocin) and autoimmune-mediated T1DM. The team was able to improve whole body glucose metabolism in mice. They attributed the success to the post-transplant increased the level of plasma insulin-like growth factor-I (IGF-I), a hormone with well-known anti-inflammatory properties [22,24].

The development of IR is also related to the gender of T1DM patients. Millstein et al. found that T1DM affected adipose and skeletal muscle insulin sensitivity to a greater extent in women than in men [25]. Participants in the control groups presented higher Morbus values (M-values) than those with DM, regardless of gender. Interestingly, women had a higher M-value than men from the respective control groups, whereas there were no differences by sex in M-values among the participants with T1DM. This result may suggest a greater deficit in the whole-body insulin sensitivity in women than in men with T1DM. Additionally, during the third stage of the glucose uptake study, some differences were observed for the values between women with and without T1DM [25]. During the first and second stages of the hyperinsulinemic-euglycemic clamp, the difference in FFA concentration by T1DM status was greater in women than in men during the least-squares means stages. These changes in IR in skeletal muscle and adipose tissue may be associated with disturbances in the hypothalamic-pituitary-gonadal axis in T1DM [25,26].

The severity of IR is variable and it depends on the influence of many factors. It is also related to the duration of T1DM [27–30]. A family history of T2DM has also been shown to be associated with greater IR in T1DM [27,31,32]. IR, as measured by the estimated glucose disposal rate (eGDR), is greater in racial/ethnic minorities as non-Hispanic blacks, Hispanics than in non-Hispanic whites [33,34].

A decreased insulin response is reported in adolescence when hormonal changes are observed, mainly in the growth hormone and sex hormones levels. It is often said that growth hormone has an anti-insulin effect because it impairs the ability of insulin to stimulate glucose uptake in peripheral tissues and enhances hepatic glucose synthesis. Growth hormone secretion is pulsatile throughout the day, but almost 50% of the daily secretion of this hormone occurs during the night. The dawn phenomenon happens as a result of increased blood glucose levels in the early morning hours (between 3 and 5 am) and it results in a significant increase in glucose levels on awakening. Physiologically speaking, between these hours the insulin level drops, which results in unblocking growth hormone secretion. A healthy person has a compensating mechanism in the form of additional insulin release, in people with DM this mechanism is disturbed, which leads to the appearance of pathology in the form of the dawn phenomenon. This event is most often observed in patients with T1DM, especially in children in their puberty period. It is related to the increased secretion of growth hormone by the pituitary gland during the night [35].

However, in adults, growth hormone deficiency may occur. It manifests itself by increased visceral obesity, IR, dyslipidemia, and hyperglycemia, and it contributes to increased cardiovascular morbidity and mortality. Insulin-like growth factor (IGF-1), which, like insulin, reduces blood glucose level,

has anti-inflammatory properties, and it is important for the regulation of glucose uptake by peripheral tissues [36].

Growth hormone therapy has an antagonistic effect on insulin with respect to peripheral tissues, such as the liver, skeletal muscle, and adipose tissue. It increases glucose uptake by skeletal muscle and the liver and reduces it in adipose tissue. To compensate for the increase in circulating glucose level, following the administration of growth hormone, insulin production increases. As a result of growth hormone supplementation, an increase in lipolysis in visceral adipose tissue is observed, which leads to an increased number of FFA in the blood, which, in turn, causes the disruption of insulin signaling pathway and directly exerts toxic effect on β-cells [36].

Increased IR may be due to the presence of autoantibodies, i.e., antibodies against endogenous insulin and/or antibodies that are directed against the insulin receptor [37]. In case of some patients undergoing insulin therapy, the antibodies are produced against exogenous insulin [38,39]. This situation increases the risk of allergic reactions, large fluctuations in blood glucose levels, and increased insulin requirement.

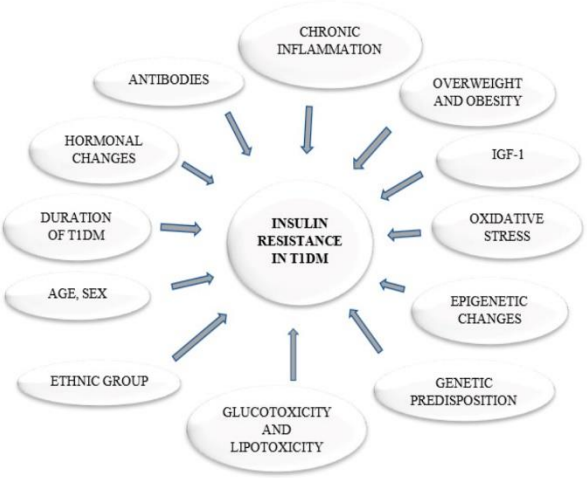

Figure 1. The causes of insulin resistance in type 1 diabetes.

IR severity/presence can be estimated by the: oral glucose tolerance test, the HOMA method for calculating the IR index (HOMA-IR), and the hyperinsulinemic-euglycemic clamp methods.

4. Quaternary Prevention

A Belgian general practitioner, Marc Jamoulle, coined the concept of quaternary prevention in 1995 [40]. According to the Wonca International Dictionary for General/Family Practice Quaternary Prevention was defined as: 'action taken to identify a patient at risk of overmedicalization, to protect him from new medical invasion, and to suggest to him interventions, which are ethically acceptable' [41]. The concept of quaternary prevention reflects more traditional levels of preventive medicine: primordial, primary, secondary, and tertiary preventions [42], as described below.

4.1. Primordial Prevention

Primordial prevention strategy encompasses all of the activities that aim to limit the occurrence of unhealthy behaviors and promote the correct ones. Primordial prevention includes actions targeted to whole society. Currently, there is no way to delay T1DM, but a reduction in the risk of IR development in T1DM is plausible [43]. Therefore, early risk reduction plays a key role in prophylaxis of the IR occurrence in T1DM. In 1989, at the forty-second World Health Assembly, WHO presented a health

promotion proclamation and the introduction of multi-level coordinated DM prevention, which is supported by the International Diabetes Federation (IDF) [44]. This proclamation calls on the Member States to keep reliable statistics on the epidemiological status of DM. The declaration provides a support for the Member States and their activities targeted at the prevention of DM and its complications [44]. This is important for planning and implementing preventive measures, tailored to the needs of local communities, which aimed to counteract and monitor the development of IR.

A very important element of primordial prevention is the implementation of a coordinated international policy in health education, trade, agriculture, transport, and urban planning; this would enable the members of a society to make healthy choices and learn healthy habits. Healthy choices can be promoted in schools, workplaces, and homes, which will have an impact on the health of society [45]. This can also be achieved by the decision regarding the amount of tax that is imposed on food products, including sweets, sweetened drinks, as well as alcohol and cigarettes. It is also worth emphasizing that the prices of vegetables, fruit, and fish should be set so that the products are more accessible to residents [46]. Modern lifestyle is characterized by a lack of physical activity and a sedentary lifestyle. The IDF recommends that a person stays physically active three to five times a week for at least 30–45 min [47]. Therefore, during urban planning, a sufficient number of playgrounds, open-air gyms, and bicycle paths should be ensured.

IR is not a direct cause of T1DM, but rather an accompanying phenomenon. Nevertheless, it is a burden, since people with this type of DM and concomitant IR will need higher insulin doses in order to keep their blood glucose level stable (as compared to the people with DM, but without IR). The availability and price of insulin is currently a major concern for patients, their families, healthcare professionals, insurers, and employers. Between 2007 and 2016, as prices rose and more expensive insulin products were introduced, the average total Medicare Part D spending per insulin user increased by 358%, from $862 to $3949. In the same years, the out-of-pocket expenses on insulin increased from $236 million to $968 million. This was due to both the increase in insulin prices and number of users. An average per capita insulin spending increased from $324 to $588, which is an increase of 81% [48]. In 2018, the American Diabetes Association conducted a survey [49]. The results indicated that 25% of people with DM rationed their insulin stock, because they could not afford to take the whole prescribed amount. Among the respondents, 23% reported the need to change the insulin to a cheaper one or a different brand. Interestingly, 23% and 20% of the patient submitted to take 1–2 required doses of insulin per week or month, respectively. Worryingly, 36% of the respondents were forced to choose between purchasing a medicament or paying for other medical services. On account of the type of insurance, 22% of the respondents had to change insulin due to its price [48,49].

The problem of rising insulin prices is quite complex and it is influenced by many factors. Only three companies control 90% of the global insulin market (Eli Lilly, Novo Nordisk, and Sanofi). Often, only one of these companies supplies insulin in a given country, which permits them to dictate the prices of their products. The key factor behind the price of insulin is the existence of patents that give companies a monopoly on specific inventions, usually for up to 20 years. Previous-generation insulin, as well as older animal-derived insulins, are currently excluded from the patent. However, pharmaceutical companies use loopholes in the patent system to patent other elements that are related to a basic drug. This decreases the competition on the market and keeps insulin prices high [48]. The above is of particular importance for the uninsured and the high-split insured individuals. If current trends are maintained, the annual cost of insulin treatment could reach $121.2 billion by 2024. This suggests a necessity to change the current pricing practices [48].

It turns out that a device that almost every inhabitant of the globe has can play an important role within the primordial prevention framework. In 2013, the WHO, in collaboration with the International Telecommunications Union, introduced the mDiabetes service on a large scale. The utility was originally intended to help countries, such as Senegal. The initiative Be He@lthy, Be Mobile was aimed at designing, implementing, and expanding services for the prevention and treatment of DM and a range of non-communicable diseases [50]. Through SMSs, their recipients had easier

access to information regarding the disease and health education. This allows for a reduction in morbidity and treatment costs and lets the patients live longer and in better health. Senegal was the first country, which, in 2014, launched a targeted mDiabetes campaign to help people manage fasting during Ramadan. In 2016, the application was launched in India, where it currently supports over 96,000 users. The WHO also conducts annual campaigns in Egypt. In 2017, the campaign reached over 175,000 people worldwide [50]. As Dr. Douglas Bettcher, director of the WHO's NCD Prevention Department, emphasizes, it is important to provide information to the public in a form that is both simple and action-oriented. That way, it is easier to incorporate the recommendations into a person's daily activities and make positive changes to their diet, exercise, and habits. It is also very important for patients to be able to take care of their health during time periods between visits to the doctor or health care professional. This is essential for improving the quality of life and treatment outcomes [50].

4.2. Primary Prevention

Primary prevention aims to avert the occurrence of a specific disease by reducing the risk of the disease by changing people's behaviors or their exposure to risk factors that in turn may lead to the disease development. This type of prophylaxis is aimed at a specific endangered group of people. At this level of prevention, activities at the local stage play a very important role, where we can see cooperation and intermingling of activities of local governments with non-governmental organizations (NGOs) [51]. There are programs in the field of counteracting alcoholism and tobacco smoking. Workshops are often held in order to learn to cook healthier versions of familiar foods. In the line with the IDF recommendations for a healthy diet for the general society, individuals are endorsed to replace fruit juices, soda, and other sugar-sweetened beverages with water, tea, or coffee. Additionally, they are urged to eat at least three servings of raw vegetables a day and fruit up to three servings a day. Suggestions are also put forward to choose lean meat, fish and seafood, whole wheat bread, and unsaturated fats. The above is often supported by classes that teach how to read the labels on food products.

The Trial to Reduce Insulin Dependent Diabetes Mellitus (IDDM) in the Genetically at Risk Study (TRIGR), which is an international, randomized, and double-blinded trial, is an interesting example of primary prevention. The research was conducted in 78 study centers from 15 countries, between May 2002 and January 2007, and follow-up continued until the youngest participant reached 10 years of age in February 2017. A total of 2159 newborn infants who had a first-degree relative with T1DM and defined human leukocyte antigen (HLA) genotypes were recruited to this research [52]. The study aimed to test "whether hydrolyzed infant formula compared to cow's milk-based formula decreases the risk of developing T1DM in children with increased genetic susceptibility". The TRIGR Study Group found that weaning to the hydrolyzed formula did not reduce the risk of T1DM in children with increased disease risk. The finding indicates that there is no need for revision of the dietary recommendations for a newborn at risk for T1DM [53].

Pacaud et al., in a secondary analysis of the TRIGR study, compared the clinical characteristics and development of β-cell autoantibodies in patients with a family history of T1DM. The research involved 2074 children from families with a single family member (mother, father, or sibling) affected by T1DM [54]. The study showed that the risk to develop β-cell autoimmunity was significantly lower ($p < 0.001$) in children with maternal T1DM than with other family members with T1DM. This indicates that more attention should be paid to the other genetic and epigenetic risk factors or the immunological mechanisms. That approach should allow for better identifying those at risk of developing T1DM and to better plan future prevention strategies.

Other findings from the TRIGR primary prevention study include that early postnatal vitamin D may confer protection against the development of T1DM [55]. Krischer et al. also showed evidence of the relationships between atopic eczema, allergic rhinitis, or persistent asthma and diabetes-related autoimmunity. Research proved that, for eczema, the interaction depends upon the appearance of the autoantibody [56].

4.3. Secondary Prevention

The aim of secondary prevention is the early detection and treatment of pathological changes while maintaining control over the development of the disease. Screening tests are the first step in implementing early interventions, which, when considering the condition of economy, are much more profitable than later treatment. Once disease screening tests detect a disease that is in the acute clinical phase, secondary prevention aims to improve the patient's quality of life.

These activities can be carried out at the level of a family doctor, which, in Poland, is often additionally scored by the National Health Fund. The family doctor can order the examination of blood pressure, urine, sugar level, lipid profile, and BMI. These tests belong to the foundation of basic tests ordered by a general practitioner and, although cheap, are often sufficient for predicting the risk of developing IR in a patient with T1DM.

A good example of secondary prevention could be research that was conducted by TrialNet, which is the largest clinical trial network for T1DM [57]. The Teplizumab Prevention Study was the first study conducted in humans in order to show that clinical T1DM could be delayed for two years in children and adults with a high risk of developing the disease [58]. The study was conducted from 2011 to 2018 in the United States, Canada, Australia, and Germany. The research was conducted among nondiabetic relatives of patients with T1DM; they were at least eight years of age and at a high risk of developing clinical DM. Each participant received a 14-day treatment with teplizumab or saline, administered intravenously. Although the research results are very promising, they have some limitations. The study was based on a very small cohort group, 76 people, relatives of people with T1DM, and non-Hispanic white participants. Future research should involve not only the relatives of people with T1DM, but also those who are at risk of developing DM. Different ethnic groups should be also included. The participants of this study only received one cycle of teplizumab administration. Perhaps multiple administration would provide additional benefits and exert its effect on more people. Nevertheless, the research that was conducted by TrialNet is a step to achieving their goal of "a future without T1DM" [57].

4.4. Tertiary Prevention

Tertiary prevention with its activities aims to stop the progression of the disease and reduce its complications. These activities should take place at the outpatient level. This requires the cooperation of the patient, doctors, and specialists in many fields, like a diabetologist, a medical rehabilitation physician, a neurologist or an ophthalmologist, and a dietitian and physiotherapist [59]. Patients are often referred to specialized sanatorium centers and, after their return, they are still under the care of specialists. Tertiary prevention is focused on preventing further adverse effects of IR, including disability. The rehabilitation aims to restore the lost functions. The health education of a patient and his relatives is a very important element that may help the patient to cope with their limitations and prevent secondary social isolation that can lead to secondary disability and the formation of a vicious circle [60]. Health education shall focus on teaching how to: (a) self-monitor blood glucose level, (b) determine the appropriate time for blood glucose measurement, (c) eat healthy, and (d) properly inject insulin. The health education training should be repeated.

Another important element of health education while using of the appropriate language concerning people with T1DM. Proper language habits help to avoid stigmatization, not only of people with T1DM, but also of their relatives and closest social environment. Dickinson et al. paid special attention to the language and gave recommendations in order to facilitate cooperation with people with DM. The language, which we use, should be respectful, person-oriented, and devoid of stigmatization. The publication provides recommendations consistent with the guidelines of the American Psychological Association and should be used by diabetologists, diabetes educators, researchers, or those who deal with people with diabetes in their environment [61]. Language has power and it can significantly affect our perception or behavior. Dunning et al. also emphasized that the way we talk to the people with diabetes and about them is of great importance and can

help improve their involvement in the process of diabetes treatment. Moreover the language may better treatment outcomes, and has a positive impact on an individual psycho-social well-being [62]. Future diabetes prevention programs should include educational classes that will teach the language that will not stigmatize a person with T1DM. LaManna et al. also emphasized the importance of health education as an important resource that helps to reduce the risk and incidence of hypoglycemia and improve the quality of life of people with T1DM [63].

5. Treatment

Metformin is a drug of choice in T2DM due to its safety and efficacy, as well as its multi-metabolic effects. Not only does it lower glycemic stability by sensitizing tissues to the impact of insulin, but it also increases glucose uptake by adipose tissue and supports the process of FFA re-esterification. Through these processes, it prevents lipolysis and the release of FFA to the blood. This drug increases the activity of lipoprotein lipase, lowers the concentration of triacylglycerol, as well as total and LDL cholesterol. It also has anti-inflammatory and antioxidant properties.

Many studies have been conducted on the appropriateness of introducing metformin to the pharmacotherapy of patients with T1DM. The results indicated that these patients obtained an improvement in total insulin dose, basal insulin dose, a modest reduction in weight, or lipids level (total and LDL cholesterol), but only during short term observation [64–67]. Some studies also confirmed that metformin may lower HbA1c level [65,66]. The above-mentioned positive effects are no longer observed with long-term drug supplementation. During many years of research, no significant changes in body weight, total daily insulin dose, basal insulin dose, improvement in lipids level, or fasting plasma glucose or HbA1c level were observed in the patients [67–70]. Some studies also indicated the possible side effects of metformin application, such as an increased risk of adverse gastrointestinal effects [64,69,71,72]. Some studies also report vitamin B12 deficiency in patients with T1DM [66,72]. For patients with T1DM, this vitamin is important due to the reduction of the high homocysteine level, which is considered to be a major risk factor for the formation of atherosclerotic plaque. Vitamin B12 also participates in the formation of myelin sheaths of neurons, which are necessary for the process of nerve impulses conduction [73]. Its deficiency can lead to nerve damage.

Metformin is successfully used for the treatment of people with T2DM; however, not much is known regarding its impact on T1DM. A longitudinal study conducted by Staels et al. that encompassed 10 years timespan proved the lack of long-term beneficial effects of metformin therapy on weight loss, HbA1c level reduction, or diminishing insulin dosage requirements. Therefore, this drug is not expected to bring satisfactory effects, even after a long-term pharmacotherapy of patients with T1DM [68].

An ideal drug should meet several criteria, as Drzeworski emphasized. First of all, it should reduce IR in T1DM and protect β-cells, and, thus, reduce the need for insulin. Secondly, it should also reduce hyperglycemia, body weight, oxidative stress, and the risk of cardiovascular complications that stem from many years of its administration [74]. Important features of the ideal drug also include: its affordable price, high safety, improvement of the patient's life quality, and extension of its duration. Although metformin meets many of these criteria, future studies in search for an ideal medication for IR in T1DM are still warranted [75].

However, we should remember the words of the doctor of medicine, the court physician of Polish kings, Wojciech Oczko, who often repeated that "Movement can replace almost any medicine, but all medicines taken together can't replace movement".

6. Conclusions

The existence of reduced tissue insulin sensitivity in children, adolescents, and adults diagnosed in T1DM is no longer being questioned. Our present analysis, which focused on IR in T1DM, presented that many factors influence the development of this disorder. A better understanding of the risk factors for the development of IR in T1DM is still needed in order to improve quaternary prevention. Research has

showed that the inclusion of prophylaxis may prevent IR in T1DM or reduce its negative effects. Probably, with respect to primary and secondary prevention of IR in T1DM, it would not matter if the actions that were carried out under primordial prevention were more effective and covered a larger number of the world's populations. To be effective, prevention requires commitment and organized action at all levels, from the activities of international organizations to the involvement of people with T1DM.

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9. Publikacja nr 2

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Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue.

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Diverse impact of N-acetylcysteine or alpha-lipoic acid supplementation during high-fat diet regime on fatty acid transporters in visceral and subcutaneous adipose tissue

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ABSTRACT

Purpose: Adipose tissue's (AT) structural changes accompanying obesity may alter lipid transport protein expression and, thus, the fatty acids (FAs) transport and lipid balance of the body. Metabolic abnormalities within AT contribute to the elevated production of reactive oxygen species and increased oxidative/nitrosative stress. Although compounds such as N-acetylcysteine (NAC) and α-lipoic acid (ALA), which restore redox homeostasis, may improve lipid metabolism in AT, the mechanism of action of these antioxidants on lipid metabolism in AT is still unknown. This study aimed to examine the impact of NAC and ALA on the level and FA composition of the lipid fractions, and the expression of FA transporters in the visceral and subcutaneous AT of high-fat diet-fed rats.
Materials and methods: Male Wistar rats were randomly divided into four groups. The mRNA levels and protein expression of FA transporters were assessed using real-time PCR and Western Blot analyses. The collected samples were subjected to histological evaluation. The level of lipids (FFA, DAG, and TAG) was measured using gas-liquid chromatography.
Results: We found that antioxidants affect FA transporter expressions at both the transcript and protein levels, and, therefore, they promote changes in AT's lipid pools. One of the most remarkable findings of our research is that different antioxidant molecules may have a varying impact on AT phenotype.
Conclusion: NAC and ALA exert different influences on AT, which is reflected in histopathological images, FA transport proteins expression patterns, or even the lipid storage capacity of adipocytes.

1. Introduction

Obesity, a condition caused by excessive caloric intake together with a concomitant lack of physical activity, is a growing public health problem in industrialized countries. Based on previous research, we know that it is closely related to several metabolic disorders, such as insulin resistance, type 2 diabetes, and cardiovascular diseases [1]. Furthermore, the above-mentioned comorbidities depend not only on the mass of adipose tissue (AT) but also its localization in the body. Although both visceral (VAT) and subcutaneous (SAT) adipose tissue store lipids, mainly in the form of triacylglycerols (TAG), they still differ morphologically and functionally. Many studies indicate that fatty acid (FA) turnover, lipolysis [2], and insulin sensitivity are correlated with VAT's mass, which could explain the increased morbidity observed in patients with visceral obesity.

Obesity is often accompanied by structural changes in AT; the changes may alter the expression of lipid transport proteins and, thus, FA transport and lipid balance of the body. FA transmembrane transport in AT is facilitated mainly by fatty acid translocase (FAT/CD36), plasma membrane fatty acid-binding protein (FABPpm), and fatty acid transport proteins (FATP1 and FATP4). FAT/CD36 facilitates the uptake of long-chain fatty acids by adipocytes and contributes - under excessive fat supply - to lipid accumulation and metabolic dysfunction [3]. The FABPpm is a peripheral membrane protein that is localized to the

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mitochondrion and the plasma membrane. Research conducted by Schwieterman et al. [4] proved that antibodies directed against rat liver FABPpm were found to inhibit FA uptake by adipocytes by 50–75%. FATP1 is an integral plasma membrane protein expressed in tissues with high levels of FA uptake for metabolism or storage, such as skeletal muscle or AT [5]. The privileged targeting of FAs, captured by FATP1, to triglyceride synthesis may indicate a functional relationship between FATP1-mediated FA absorption and lipid storage [6]. FATP4 is an integral membrane protein expressed in the plasma and internal membranes, highly expressed in AT and skeletal muscle, and closely related to FATP1 [6]. A very important element of this puzzle is a beta-hydroxyacid dehydrogenase (β-HAD) that encodes the β-subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial β-oxidation of FAs [7]. It was previously shown that a high-fat diet (HFD) may cause a greater influx of FA to muscle and AT probably via up-regulated FATP1 expression [7,8]. Furthermore, patients with obesity are often characterized by an increased expression of the messenger ribonucleic acid (mRNA) of FAT/CD36 in AT. Thus, one may postulate that an increase in the activity of the above-described proteins may alleviate the metabolic consequences of obesity.

Metabolic abnormalities within AT can contribute to the complications of obesity, e.g., an elevated production of reactive oxygen species (ROS) and an increased oxidative/nitrosative stress [9]. Therefore, the compounds that restore redox homeostasis may improve lipid metabolism in "obese AT". N-acetylcysteine (NAC) and α-lipoic acid (ALA) are antioxidants with well-documented beneficial effects on the metabolic complications of obesity [10–14]. NAC is a cysteine derivative that serves as a precursor to reduced glutathione (GSH) synthesis, whereas ALA is an essential endogenous co-factor of an enzymatic complex involved in energy generation as well as the inactivation of free radicals, and by its reduced form also interacts with ROS [11]. Some studies have shown that the administration of NAC/ALA in rats fed with an HFD reduced their plasma triglycerides and total cholesterol levels, thus restoring lipid metabolism disturbed by obesity [15,16]. Nevertheless, the mechanism of NAC and ALA actions on lipid metabolism in AT is still unknown. Since lipid accumulation often stems from increased long-chain FA transmembrane transport, it is plausible that NAC and ALA can influence the process. Therefore, we are the first to examine the impact of NAC and ALA on the level and FA composition of the selected lipid fractions: free fatty acids (FFAs), diacylglycerols (DAGs), and TAGs, as well as the expression (both at the mRNA and protein levels) of FA transporters (FAT/CD36, FABPpm, FATP1, and FATP4) in the VAT and SAT of HFD-fed rats. By assessing β-HAD expression, we estimated the oxidation level of FAs.

2. Materials and methods

2.1. Animals and study design

The experiment was carried out on 4-week-old male Wistar rats (n = 40) with initial body mass of 50–72 g. The animals were kept in an animal facility with stable housing conditions (21 °C±2 °C; 12 h light/dark cycle), with free access to water and fodder. After six days of acclimatization to the trial conditions, the rats were divided into 4 groups (n = 10 per group):

1) Control group (CTRL)—rats fed a control diet (10.3% fat, 24.2% proteins, and 65.5% carbohydrates; Agropol, Motycz, Poland);
2) High-fat diet group (HFD)—rats fed a HFD (59.8% fat, 20.1% proteins, 20.1% carbohydrates; Research Diet, USA, catalog number: D12492);
3) High-fat diet and N-acetylcysteine group (HFD+NAC)—rats fed the HFD plus NAC;
4) High-fat diet and α-lipoic acid group (HFD+ALA)—rats fed the HFD plus ALA.

Once daily, each morning between 8 and 9 a.m., NAC (at a dose of 500 mg/kg body weight, Sigma-Aldrich, catalog number: A9165) and ALA (at a dose of 30 mg/kg body weight, Sigma-Aldrich, catalog number: PHR2561-1G) were prepared by dissolving the substances in saline solution and immediately administered intragastrically by gastric gavage to rats from the HFD+NAC and HFD+ALA groups, respectively. The doses were based on a literature analysis [17–19]. Similarly, saline (2 ml/kg body weight) was administered to the CTRL and HFD groups. The intragastric administration of antioxidants ensured that rats received a full dose calculated for their body weight. According to each rat's body weight, which was controlled every two days, the dose of antioxidants administration was adjusted.

The rats were anesthetized with an intraperitoneal injection of phenobarbital (80 mg/kg body weight). The animals were placed on a heating pad (37°C). Then, the samples of AT (VAT and SAT) were obtained from the abdominal area, whereas blood was collected from the abdominal vena cava. The tissues were immediately frozen using aluminum forceps pre-cooled in liquid nitrogen. All the procedures were performed by the same experienced technician. All the samples (AT and blood plasma) were stored at −80°C until further analysis.

2.2. Ethical issues

All procedures were approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok, Poland (approval no.: 21/2017).

2.3. Adipose tissue histopathology

The samples of VAT and SAT were obtained and immediately fixed in 10% buffered formalin and processed routinely for embedding in paraffin. The paraffin blocks were cut into 4 μm sections, attached to positively charged glass slides (Superfrost Plus; Menzel Gläser, Braunschweig, Germany), and stained with hematoxylin and eosin (H&E).

Paraffin-embedded sections were deparaffinized and hydrated in pure alcohols. A Toluidine Blue Stain kit (cat.-no.: SS057 BioGenex; 49026 Milmont Drive, Fremont, CA, USA) was used to stain macrophages. The kit included: potassium permanganate, potassium metabisulphite, Scott's solution, and Toluidine blue solution. The staining was performed following the manufacturer's instructions.

Immunostaining was performed using the following protocol: for antigen retrieval, the sections were subjected to pre-treatment in a pressure chamber and heated using Target Retrieval Solution (S 1699 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA, USA). After cooling down to room temperature, the sections were incubated with Peroxidase-Blocking Solution for 10 min to block endogenous peroxidase activity. The sections were finally counterstained in hematoxylin QS (H-3404, Vector Laboratories; Burlingame, CA, USA), mounted, and evaluated under a light microscope.

The assessment of the cell size and number of macrophages was performed by two experienced histologists (independent from each other) and analyzed with ImageJ software (The National Institutes of Health, MD, USA). Images were converted to 8-bit grey scale, and the background was subtracted. Then, binary threshold function was adjusted to separate the cells from background staining. The total cells area was calculated as the total number of pixels in images with a set threshold [20,21]. Results were plotted in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Results are depicted as mean ± standard deviations. The statistical significance level was set as $p < 0.05$.

2.4. Quantitative real-time polymerase chain reaction (real-time PCR)

Total ribonucleic acid (RNA) was isolated using the TriReagent RNA Isolation Reagent according to the manufacturer's protocol (Sigma-

Aldrich, Darmstadt, Germany). The total RNA amount and quality were spectrophotometrically verified by measuring the sample's absorbency ratios (260/280 nm and 260/230 nm) [22]. The synthesis of complementary deoxyribonucleic acid (DNA) was performed using the EvoScript universal cDNA master kit (Roche Molecular Systems, Boston, MA, USA). Specific primers used in this study are presented in Table 1. Real-time PCR was carried out using the LightCycler 96 System Real-Time thermal cycler with FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany). Cycling conditions were: 15s denaturation at 95°C; 15s annealing at 58°C for β-actin, FATP1, and FATP4 and at 60°C for Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Beta (β-HAD); 61°C for FABPpm; 62°C for FAT/CD36; and 15s extension at 72°C for 45 cycles. Melting curve analysis was performed before each reaction to verify PCR product specificity. The mRNA levels of target genes were normalized to the rats' β-actin levels and calculated according to the Pfaffl method [23].

2.5. Proteins analysis

A routine Western blotting procedure was used to examine protein expression (i.e., FAT/CD36, FABPpm, FATP1, FATP4, β-HAD, and β-actin). Briefly, the VAT and SAT were homogenized (using an electric homogenizer) in an ice-cold radioimmunoprecipitation assay (RIPA) buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The total protein concentration was determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as a standard. Based on the protein quantity read, an appropriate amount of mercaptoethanol in a buffer was added, and the total solution was incubated at 90°C.

Western blot technique was used to detect FA transport proteins according to previously described procedures [24]. A tissue homogenate containing 20 μg of protein was subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (200V for 60 min), and transferred to nitrocellulose membranes (100V for 30 min). Then, the membranes were incubated at room temperature for 60 min in a 5% non-fat dry milk solution in tris buffered saline with tween 20. Subsequently, the membranes were incubated overnight with primary antibodies against FAT/CD36 (1:1000, cat. no. ab252922, Abcam, Cambridge, UK), FABPpm (1:5000, cat. no. ab171739, Abcam, Cambridge, UK), FATP1 (1:1000, cat. no. LS-C373484-100, LSBio, Seattle, USA), FATP4 (1:1000, cat. no. ab200353, Abcam, Cambridge, UK), and β-HAD (1:1000, cat. no. ab230667, Abcam, Cambridge, UK). The membranes were then incubated with the appropriate anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). Next, the protein bands were visualized with a chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA) and quantified by densitometry (Bio-Rad Systems, Hercules, USA). The protein expressions were normalized to β-actin (1:200, cat. no. ab115777, Abcam, Cambridge, UK) expression.

2.6. Lipids analysis

The level of lipids (FFA, DAG, and TAG) was measured using gas–liquid chromatography, as described previously [25,26]. The selected lipid fractions were extracted using Bligh and Dyer's method [27]. Then, lipids were separated by thin-layer chromatography (TLC).

Individual fatty acid methyl esters (FAMEs) present in each fraction were identified and quantified according to the retention times of the standards by gas–liquid chromatography (Hewlett-Packard5890 Series II gas chromatograph, HP-INNOWax capillary column; Agilent Technologies, Santa Clara, CA, USA). The total amounts of FFA, DAG, and TAG were estimated as the sum of individual FA species in the evaluated fraction and expressed in nanomoles per mg of protein.

2.7. Statistical analysis

The analyses were conducted using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The normality of the results' distribution was tested using the Shapiro–Wilk test. We carried out the analysis of variance (ANOVA) test to compare the means among all 4 groups, and Student's t-test to compare the means between only 2 tested groups (CTRL and HFD; CTRL and HFD+NAC; CTRL and HFD+ALA; HFD and HFD+NAC; HFD and HFD+ALA; HFD+NAC and HFD+ALA). If the assumptions of the above tests were not satisfied, a Kruskal–Wallis test with a post hoc pairwise Mann–Whitney *U* test was applied. Additionally, due to multiple comparisons, a Benjamini–Hochberg correction for the obtained *p*-values was applied. The results were presented as means ± standard deviation (SD). The statistical significance level was set as $p < 0.05$.

3. Results

3.1. General characteristics

The HFD regime applied in this study resulted in a statistically significant increase in body mass (HFD vs. CTRL, $p < 0.05$). As expected, the rats supplemented with NAC (HFD+NAC) and ALA (HFD+ALA) weighed less than the rats from the HFD group ($p < 0.05$). The HFD treatment also resulted in a statistically significant increase in plasma-free fatty acid

Table 2
The effects of N-acetylcysteine (NAC) and α-lipoic acid (ALA) supplementation on body weight, plasma metabolic parameters, and food intake.

	CTRL	HFD	HFD + NAC	HFD + ALA
Final body weight	281.4 ± 11.51	381.6 ± 37.58*	319.5 ± 35.65*#	309.5 ± 32.93#
Plasma free fatty acids (μmol/L)	429.2 ± 70.84	430.4 ± 115.2	448.5 ± 63.68	513.6 ± 79.61*
Plasma diacylglycerols (μmol/L)	74.96 ± 13.27	59.32 ± 21.08	37.15 ± 4.906*#	79.85 ± 22.40#^
Plasma triacylglycerols (μmol/L)	2139 ± 670.1	2512 ± 1361	1187 ± 348.7*#	1728 ± 793.7*#^
Food intake (mg/day)	19.98 ± 5.095	12.57 ± 7.802*	10.46 ± 5.889*#	13.91 ± 5.52*#

Abbreviations: CTRL—control rats; HFD—high-fat diet-fed rats; HFD+NAC—high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA—high-fat diet-fed rats that received α-lipoic acid.
*$p < 0.05$ vs. control.
#$p < 0.05$ vs. HFD; ^$p < 0.05$ vs. HFD+NAC.

Table 1
Primer sequences for real-time PCR analysis.

Gene	Primer Sequence		Annealing temperature
	Forward	Reverse	
β-actin	5′-ACGGTCAGGTCATCACTATCG-3′	5′-GGCATAGAGGTCTTTACGGATG-3′	58°C
FAT/CD36	5′-GCCTCCTTTCCACCTTTTGT-3′	5′-GATTCAAACACAGCATAGATGGAC-3′	62°C
FABPpm	5′-TCATCCTTTGTCTCCAGCTTTT-3′	5′-CCTATGCCATGCTGACAGGT-3′	61°C
FATP1	5′-GGGTTTGCAAGCCAGAGA-3′	5′-CAAAGCAGCCCCAATGAG-3′	58°C
FATP4	5′-TTGCCTGAGCTGCACAAAAC-3′	5′-AGTGCAACATAGCAGCCTGT-3′	58°C
β-HAD	5′-TATCTGGGGCGGATCACTCT-3′	5′-CATAGCATGACCCTGTCCTCC-3′	60°C

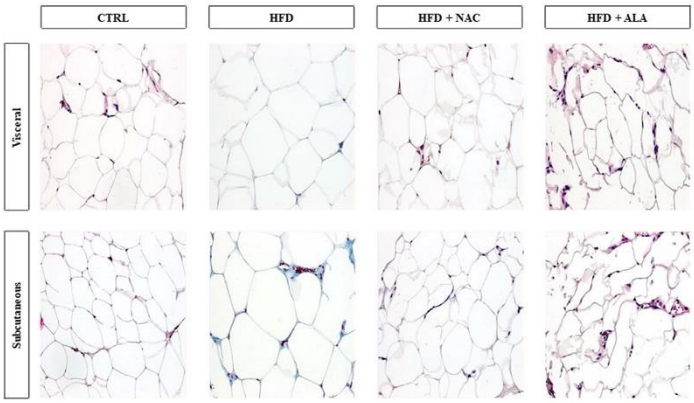

Fig. 1. Representative microphotographs of visceral and subcutaneous adipose tissue. Samples were stained by hematoxylin and eosin (H&E). Images were taken using 200× magnification.
Abbreviations: CTRL—control rats; HFD—high-fat diet-fed rats; HFD+NAC—high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA—high-fat diet-fed rats that received α-lipoic acid.

levels ($p < 0.0001$) compared to the CTRL group. Moreover, NAC (HFD+NAC) administration caused the normalization of plasma FFA concentrations to values comparable with those observed in the CTRL group. The rats from the HFD ($p < 0.05$), HFD+NAC ($p < 0.05$) and HFD+ALA ($p < 0.05$) groups consumed statistically significantly less chow compared to the rats from the CTRL groups (Table 2).

3.2. Histological changes in AT

The histological examination of the AT samples in the HFD group revealed greater volumes of fat cells in VAT ($p < 0.0001$; Figs. 1 and 2a) and SAT ($p = 0.0165$; Figs. 1 and 2b), when compared with the respective CTRL groups. In the animals supplemented with NAC (HFD+NAC), the adipocytes from both VAT and SAT were slightly smaller than their counterparts in the HFD group. Moreover, the former (HFD+NAC VAT) was characterized by a slightly folded cell membrane with a thin rim of the cytoplasm (Fig. 1). The cells from the animals treated with NAC also had a lower number of macrophages visible when compared to the HFD group ($p < 0.05$; Fig. 1). The shape of the fat cells in the HFD+ALA group was similar to that found in the CTRL group, while the size of the adipocytes was slightly smaller than their counterparts from the HFD group in VAT ($p = 0.0003$; Figs. 1 and 2a) and SAT ($p = 0.0209$; Figs. 1 and 2b). Furthermore, the cells from the HFD+ALA group had a slightly higher number of macrophages present in comparison to the respective CTRL group ($p < 0.05$; Fig. 1). Interestingly, the size of the adipocytes, in both VAT and SAT, was slightly smaller in the case of the animals treated with ALA (HFD+ALA) than in those that received NAC (HFD+NAC) (in VAT, $p = 0.0010$, Fig. 2a; Fig. 1).

3.3. The effect of NAC and ALA on the mRNA levels of FAT/CD36, FABPpm, FATP1, and FATP4 in the AT of the HFD-fed rats

The rats fed with the HFD were characterized by statistically significant lower mRNA levels of FAT/CD36 and FABPpm in VAT in comparison to the CTRL group (−43%, $p = 0.0130$, Fig. 3a; and −45%, $p = 0.0030$, Fig. 3c, respectively). The injections with NAC resulted in decreased mRNA levels of FAT/CD36 and FABPpm in VAT in comparison to the CTRL group (−75%, $p = 0.0001$, Fig. 3a; and −62%, $p < 0.0001$, Fig. 3c, respectively). Interestingly, the four-week administration of NAC alongside the HFD regime resulted in a statistically significant decrease in the mRNA expression of FAT/CD36 in VAT (−56%, $p = 0.0007$, Fig. 3a) and SAT (−50%, $p = 0.0009$, Fig. 3b) compared to the HFD alone. A similar reduction was observed in FATP1 mRNA expression in SAT (−80%, HFD+NAC vs. NAC, $p = 0.0001$, Fig. 3f). Moreover, the supplementation of the HFD-fed rats with ALA also resulted in a drop in FAT/CD36 mRNA expression in VAT in comparison with the CTRL group (−41%, $p = 0.0222$, Fig. 3a). Interestingly, the combination of ALA with the HFD regime caused a statistically significant increase in FABPpm mRNA expression in VAT (+61%, $p = 0.0389$, Fig. 3c), and in FATP1 mRNA expression in SAT when compared to the HFD group (+146%, $p = 0.0223$, Fig. 3f). However, we observed a statistically significant

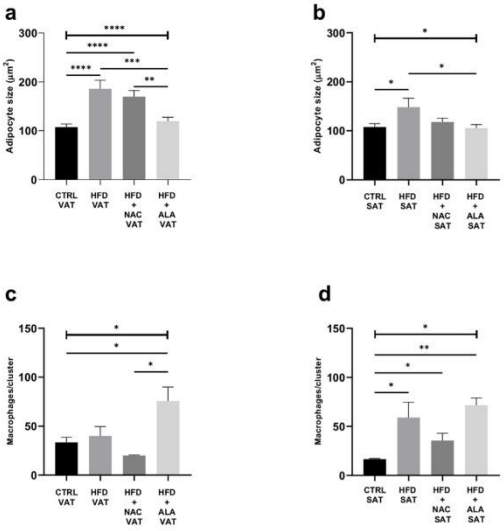

Fig. 2. The effects of NAC and ALA on: (**a**, **b**) adipocyte size and (**c**, **d**) macrophages number in adipose tissue.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.
Abbreviations: CTRL VAT—control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; CTRL SAT—control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

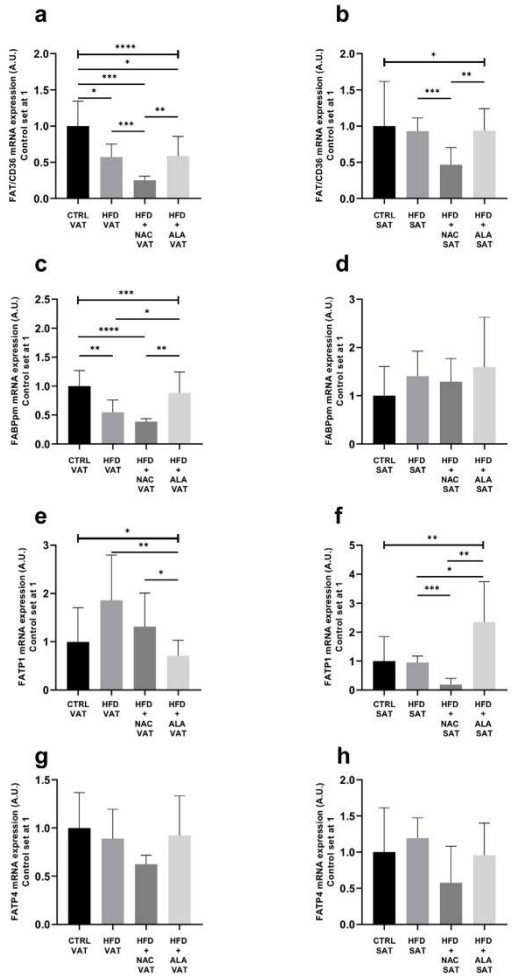

Fig. 3. The effects of NAC and ALA on: (**a**, **b**) FAT/CD36, (**c**, **d**) FABPpm, (**e**, **f**) FATP1, and (**g**, **h**) FATP4 mRNA levels in adipose tissue.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.
Abbreviations: CTRL VAT—control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; CTRL SAT—control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

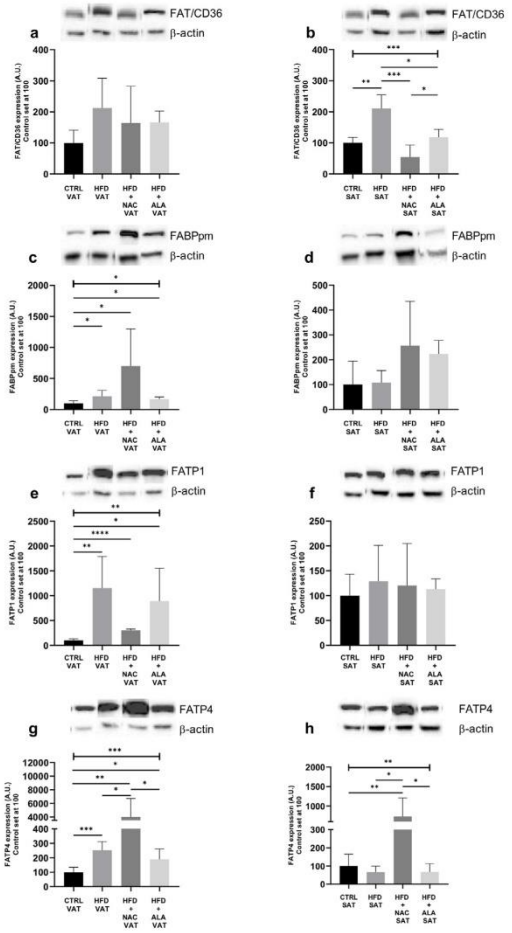

Fig. 4. The effects of NAC and ALA on (**a**, **b**) FAT/CD36, (**c**, **d**) FABPpm, (**e**, **f**) FATP1, and (**g**, **h**) FATP4 protein expressions in adipose tissue during the HFD regime.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.
Abbreviations: CTRL VAT - control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; CTRL SAT—control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

reduction in FATP1 mRNA expressions in VAT (−62%, HFD+ALA vs. HFD, $p = 0.0061$, Fig. 3e). A comparison between HFD+NAC and HFD+ALA showed statistically significant higher FAT/CD36 and FABPpm mRNA levels in the VAT of the latter group (+133%, $p = 0.0066$, Fig. 3a; and +130%, $p = 0.0031$, Fig. 3c, respectively). Similar changes were also observed for both of the groups in SAT in the case of FAT/CD36 and FATP1 mRNA expression (+101%, $p = 0.0072$, Fig. 3b; and +11-fold, $p = 0.0062$, Fig. 3f, respectively). However, we found a decrease in FATP1 mRNA expression in the VAT (−46%, HFD+ALA vs. HFD+NAC, $p = 0.0451$, Fig. 3e).

3.4. The effect of NAC and ALA on protein levels of FAT/CD36, FABPpm, FATP1, and FATP4 in the AT of the HFD-fed rats

The administration of the HFD resulted in increased expression levels of FAT/CD36, FABPpm, FATP1, and FATP4 proteins in VAT in comparison with the CTRL group (+113%, $p = 0.0268$; +113%, $p = 0.027$; +11-

fold, $p = 0.0027$; +152%, $p = 0.0005$; respectively; Fig. 4a, c, e and g). In the case of SAT, only FAT/CD36 protein expression was up-regulated (+110%, HFD vs. CTRL, $p = 0.0036$, Fig. 4b). Interestingly, the four-week administration of NAC alongside the HFD regime resulted in statistically significant increases in the expression of FABPpm, FATP1, and FATP4 proteins in VAT in comparison to the CTRL (+6-fold, $p = 0.0351$; +2-fold, $p < 0.0001$; +40-fold, $p = 0.0064$; respectively; Fig. 4c, e, and g). On the contrary, only FATP4 protein expression was greater in SAT (+6-fold, HFD+NAC vs. CTRL, $p = 0.0095$; Fig. 4h). Interestingly, in comparison to the HFD group alone, the addition of NAC led to statistically significant changes in FAT/CD36 and FATP4 protein expressions in SAT (−74%, $p = 0.0008$; +10-fold, $p = 0.05$; respectively; Fig. 4b and h), and an up-regulated FATP4 protein level in VAT (+15-fold, $p = 0.0157$; Fig. 4g). Comparing the HFD+ALA group with the CTRL group, HFD+ALA group demonstrated increases in the expression levels of FABPpm, FATP1, and FATP4 proteins in VAT (+66%, $p = 0.0320$; +8-fold, $p = 0.0168$; +89%, $p = 0.0306$; respectively; Fig. 4c, e and g). On the contrary, the comparison of the HFD+ALA group with the HFD only group alone revealed a statistically significant drop in the expression of the FAT/CD36 protein level in SAT (−44%, $p = 0.0112$; Fig. 4b). The juxtaposition of the NAC treatment with the ALA application (HFD+NAC vs. HFD+ALA) showed lower protein expressions of FATP4 in VAT and SAT of the latter group (−95%, $p = 0.030$, Fig. 4g; −91%, $p = 0.0279$, Fig. 4h; respectively). Contrarily, we found elevated FAT/CD36 protein expression in the SAT of the HFD+ALA rats (+118%, HFD+NAC vs. HFD+ALA, $p = 0.0252$; Fig. 4b).

3.5. The effect of NAC and ALA on the AT's FFA level and composition in the HFD-fed rats

The total FFA level was statistically significant reduced in the HFD+NAC group in VAT and SAT in comparison to the HFD alone (−28%, Table 3, Fig. 5a; −20%, Table 4, Fig. 5b; respectively; $p < 0.05$). Additionally, the rats supplemented with ALA had lower total FFA levels in VAT (−44%, HFD+ALA vs. HFD, Table 3, $p < 0.05$, Fig. 5a). As regards the amount of FFA unsaturated FAs, we noticed that the supplementation with NAC resulted in decreases in their amounts in both VAT and SAT when compared to the HFD group (−39%, Table 3; −22%, Table 4; respectively; $p < 0.05$). The same pattern was observed for the two groups in the case of monounsaturated fatty acids (MUFAs) (−36%, Table 3; −21%, Table 4; respectively; $p < 0.05$), and polyunsaturated fatty acids (PUFAs) (−41%, Table 3, $p < 0.05$, VAT only). When contrasted wi th the HFD group, the rats supplemented with ALA presented decreased levels of saturated FFAs (−33%, $p < 0.05$; Table 3) and unsaturated fatty acids in VAT (−55%, $p < 0.05$; Table 3). The above was accompanied by changes in PUFA (−74%, $p < 0.05$; Table 3) and MUFA (−30%, $p < 0.05$; Table 3) in VAT. Interestingly, in the HFD+ALA group vs. HFD group, SAT was characterized by elevated concentrations of saturated fatty acids (+46%, $p < 0.05$; Table 4) and MUFA (+37%, $p < 0.05$; Table 4), with a concomitant decrease in the level of PUFA (−49%, $p < 0.05$; Table 4). Interestingly, contrasting the HFD+ALA with the HFD+NAC group showed a statistically significant smaller amount of total FFA in VAT, and also their greater volume in SAT (−22%, Table 3, Fig. 5a; +47%, Table 4, Fig. 5b; respectively; $p < 0.05$). A further comparison of the groups demonstrated lower levels of FFA saturated fatty acids and PUFA in VAT (−19% and −56%, respectively; $p < 0.05$; Table 3). The amount of PUFA was also lower in the SAT of the animals supplemented with ALA; however, their saturated fatty acid amount was greater (−33% and +72%, respectively; $p < 0.05$; Table 4).

3.6. The effect of NAC and ALA on the AT's DAG level and composition in the HFD-fed rats

The total DAG level was lower in the HFD+NAC group in VAT and SAT in comparison to the HFD alone (−26%, Table 5, Fig. 5d; −35%, Table 6, Fig. 5e; respectively; $p < 0.05$). Moreover, the rats from the HFD+NAC group also had decreased amounts of DAG saturated fatty acids (−19%, Table 5; −33%, Table 6; respectively; $p < 0.05$) and PUFA (−33%, Table 5; −37%, Table 6; respectively; $p < 0.05$) in both VAT and SAT. In comparison with the HFD group, HFD+ALA had a lower DAG PUFA level in VAT and SAT (−44%, Table 5; −51%, Table 6; respectively; $p < 0.05$). A comparison of the results from the HFD+ALA and HFD+NAC groups revealed some statistically significant differences in the amount of DAG and its composition. Concerning SAT, the total DAG level was greater in the rats supplemented with ALA (+45%, HFD+ALA vs. HFD+NAC; Table 6; $p < 0.05$; Fig. 5e). Interestingly, HFD+ALA was also characterized by an elevated amount of DAG MUFA in both VAT and SAT (+73%, Table 5; +118%, HFD+ALA vs. HFD+NAC, Table 6;

Table 3
The effects of NAC and ALA on the free fatty acid (FFA) level and the FA composition in visceral adipose tissue (VAT).

Acid	CTRL VAT	HFD VAT	HFD + NAC VAT	HFD + ALA VAT
Myrystic (14:0)	292.4 ± 65.46	287.5 ± 109.6	270.3 ± 53.58	226 ± 50.92
Palmitic (16:0)	1550 ± 301.6	2683 ± 519.7*	2203 ± 307*	1476 ± 178.6#^
Palmitooleic (16:1)	100.9 ± 15.87	443.2 ± 168.3*	270.2 ± 41.91*	109.1 ± 16.06#^
Stearic (18:0)	661.6 ± 113.7	665.7 ± 114.9	526.4 ± 71.94*#	734.6 ± 87.84^
Oleic (18:1n9c)	1040 ± 150.7	1265 ± 212.4	807.8 ± 140.3*#	1082 ± 133.9^
Linoleic (18:2n6c)	457.5 ± 54.41	1805 ± 293.9*	1031 ± 272.5*#	483.8 ± 70.4#^
Arachidic (20:0)	22.27 ± 6.578	17.42 ± 3.21	20.72 ± 4.225	18.28 ± 2.971
Linolenic (C18n3)	46.75 ± 7.837	262.7 ± 54.68*	152.4 ± 37.83*#	46.95 ± 7.742#^
Behenic (22:0)	14.37 ± 3.704	15.72 ± 3.389	17.93 ± 4.935	11.67 ± 2.01#^
Arachidonic (20:4n6)	33.55 ± 4.972	118.1 ± 34.97*	74.43 ± 22.18*#	27.44 ± 5.761#^
Lignoceric (24:0)	49.7 + 19.25	35.38 + 12.65	49.87 + 13.93	19.62 ± 4.617*#^
Eicosapentaenoic (20:5n3)	20.96 ± 7.018	20.22 ± 5.017	31.83 ± 7.785*#	18.76 ± 3.903^
Nervonic (24:1)	17.26 ± 0.293	7.884 ± 1.847*	13.94 ± 2.616*#	8.549 ± 1.893*^
Docosahexaenoic (22:6n3)	17.9 ± 7.127	37.61 ± 12.78*	33.04 ± 6.366*	11.13 ± 2.2*#^
n-3/n-6	0.132 ± 0.022	0.155 ± 0.011	0.169 ± 0.011*	0.114 ± 0.009#^
16:0/16:1	15.55 ± 3.15	6.39 ± 1.164*	8.177 ± 0.331*#	13.6 ± 0.833*#^
18:0/18:1	0.635 ± 0.050	0.527 ± 0.049*	0.656 ± 0.043#	0.682 ± 0.065#
Saturated	2590 ± 481.1	3704 ± 740.8*	3088 ± 447.7	2486 ± 294.8#^
Unsaturated	1733 ± 222.1	3960 ± 717.4*	2415 ± 508.1*#	1788 ± 233#^
Polyunsaturated	576.7 ± 70.45	2244 ± 395.7*	1323 ± 333.7*#	582.9 ± 87.55#^
Monounsaturated	1157 ± 153	1716 ± 379.3*	1092 ± 179.9#	1200 ± 150#
Total	**4323 ± 697.7**	**7664 ± 1431***	**5503 ± 943.5#**	**4274 ± 511.9#^**

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
* $p < 0.05$, difference versus CTRL VAT.
# $p < 0.05$, difference versus HFD VAT.
^ $p < 0.05$, difference versus HFD + NAC VAT.

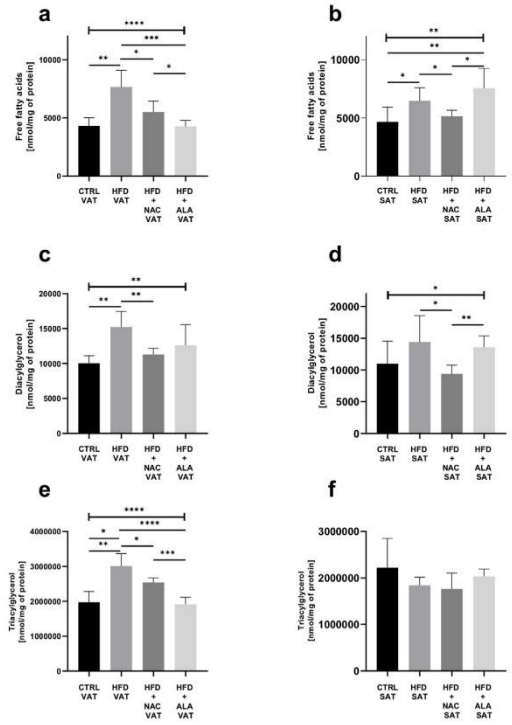

Fig. 5. The effects of NAC and ALA on (**a**, **b**) FFA, (**c**, **d**) DAG, and (**e**, **f**) TAG levels in adipose tissue.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.
Abbreviations: CTRL VAT—control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; CTRL SAT—control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

respectively; $p < 0.05$). Moreover, ALA supplementation increased the level of DAG saturated fatty acids, but only in SAT (+66%, HFD+ALA vs. HFD+NAC, $p < 0.05$; Table 6).

3.7. The effect of NAC and ALA on the AT's TAG level and composition in the HFD-fed rats

The total level of TAG in SAT was relatively stable among all the examined groups. However, some changes were noticed in the case of VAT. For instance, HFD+NAC and HFD+ALA had lower total TAG levels in VAT compared to the HFD group (−16% and −37%, respectively; Table 7; $p < 0.05$; Fig. 5g). Moreover, HFD+NAC had smaller concentrations of TAG unsaturated fatty acids (−24%, $p < 0.05$; Table 7), and PUFA (−27%, $p < 0.05$; Table 7) in VAT in comparison to the HFD alone. We also observed a statistically significant reduction in the total TAG in the HFD+ALA group in VAT in comparison to the HFD group (−37%, $p < 0.05$; Table 7; Fig. 5g). When contrasted with the HFD alone, HFD+ALA was characterized by a lower level of saturated (−39%, $p < 0.05$; Table 7), and unsaturated fatty acids (−35%, $p < 0.05$; Table 7) in VAT. In the case of PUFA, the declines were visible in both VAT and SAT (−60%, Table 7; −34%, Table 8; respectively; $p < 0.05$). Interestingly, the tissues (VAT and SAT) from the rats treated with ALA had lower TAG PUFA levels compared with the animals supplemented with NAC (−45%, Table 7; −29%, Table 8; respectively; $p < 0.05$).

3.8. The effect of NAC and ALA on AT β-HAD mRNA expressions in the HFD-fed rats

Compared to the CTRL, the HFD rats had a statistically significantly lower β-HAD mRNA expression in VAT (−43%, p = 0.0004; Fig. 6a) and a much greater level in SAT (+7-fold, p < 0.0001; Fig. 6b). In comparison to the CTRL groups, the rats treated with NAC showed a build-up of β-HAD mRNA expression in VAT (+3-fold, p < 0.0001; Fig. 6a) and SAT (+5-fold, p = 0.0288; Fig. 6b). Interestingly, the four-week administration of NAC alongside the HFD regime resulted in statistically significantly increased β-HAD mRNA expression in VAT compared to the HFD alone (+6-fold, p < 0.0001; Fig. 6a). The addition of ALA increased β-HAD mRNA expressions in SAT when contrasted with the CTRL (+8-fold, p = 0.0068; Fig. 6b). The juxtaposition of the results of the two antioxidants in VAT revealed a lower β-HAD mRNA expression in the HFD+ALA group (−81%, p < 0.0001, HFD+ALA vs. HFD+NAC; Fig. 6a).

3.9. The effect of NAC and ALA on AT β-HAD protein expression in the HFD-fed rats

The HFD group had a greater β-HAD protein expression in VAT when compared to the CTRL group (+4-fold, $p = 0.0004$; Fig. 7a). Interestingly, four-week ALA supplementation during the HFD regime resulted in a statistically significantly higher β-HAD protein level in VAT when compared to the CTRL (+3-fold, $p = 0.0012$; Fig. 7a). Moreover, the NAC treatment during HFD resulted in a statistically significantly lower β-HAD protein level in VAT when compared to the HFD alone (−21%, $p = 0.05$; Fig. 7a).

4. Discussion

We examined, for the first time, the effects of two antioxidant agents (NAC and ALA) on FA transporter expression in the VAT and SAT of rats with obesity induced by HFD. We found the antioxidants affected FA transporter expressions at both the transcript and protein levels and, therefore, they promoted changes in AT's lipid pools. One of the most remarkable findings of our research is that different antioxidant molecules may have varying effects on the AT's phenotype.

The chronic positive energy balance found in obesity contributes to the formation of oxidative stress (OS) in cells, the production of ROS, and the development of inflammation [28]. The unstable reactive molecules can oxidize DNA, proteins, and lipids [29,30]. As regards AT, ROS are involved in the maintenance of metabolic homeostasis, the proliferation of pre-adipocytes (via the insulin-dependent pathway) [30–32], and adipocyte differentiation [33,34].

In line with our results, Ma et al. [35] also demonstrated that NAC inhibited HFD-induced weight gain and meaningfully blocked the increase in fat pads. The histological examinations confirmed that NAC reduced the size of adipocytes in SAT, thus proving that NAC supplementation blocks lipid accumulation in AT [35]. The effects of ALA supplementation are also corroborated by the previous observations of Prieto-Hontoria et al. [36] and Kim et al. [37]. This ALA-induced reduction in body weight is mainly due to a considerable reduction in the body mass of total white AT, along with a decrease in the size of adipocytes in VAT and SAT. Interestingly, of the two molecules tested, ALA appears to have a greater therapeutic effect on VAT and SAT morphology.

Our present research showed that the HFD feeding resulted in increased FA transport into adipocytes, which was also expressed in the up-regulation of the transporter protein expression in VAT and SAT. We proved that the FA transporters - tested by us - work independently from

Table 4
The effects of NAC and ALA on the free fatty acid (FFA) level and the FA composition in subcutaneous adipose tissue (SAT).

Acid	CTRL SAT			HFD SAT			HFD + NAC SAT			HFD + ALA SAT		
Myrystic (14:0)	335.8	±	134.1	281.6	±	52.21	246.0	±	33.27	449.7	±	166.5$^{\wedge}$
Palmitic (16:0)	1631	±	443.7	2301	±	391.1*	1947	±	45.95	3002	±	743.7*$^{\wedge}$
Palmitooleic (16:1)	119	±	32.32	285.7	±	64.05*	248.2	±	45.48*	320.2	±	151.4*
Stearic (18:0)	743.7	±	248	607	±	84.96	480.8	±	8.581$^{\#}$	1209	±	339.4*$^{\#\wedge}$
Oleic (18:1n9c)	1041	±	288.8	1036	±	187.4	829.9	±	23.2	8.51480	±	235.2*$^{\#\wedge}$
Linoleic (18:2n6c)	553.1	±	154.9	1570	±	374.7*	1166	±	129.1*	748.1	±	244.8$^{\#\wedge}$
Arachidic (20:0)	23.91	±	5.275	16.44	±	3.414	17.20	±	5.125	30.85	±	16.05
Linolenic (C18n3)	56.63	±	12.85	201.9	±	47.78*	153.3	±	14.03*	73.16	±	25.24$^{\#\wedge}$
Behenic (22:0)	18.01	±	7.271	12.56	±	3.106	13.86	±	1.767	15	±	3.658
Arachidonic (20:4n6)	36.54	±	8.855	83.35	±	13.32*	78.36	±	11.14*	48.31	±	25.92$^{\#\wedge}$
Lignoceric (24:0)	50.65	±	23	35.3	±	11.15	45.02	±	12.93	44.65	±	12.79
Eicosapentaenoic (20:5n3)	25.21	±	7.201	12.06	±	2.356*	22.11	±	0.883$^{\#}$	26.96	±	7.768$^{\#}$
Nervonic (24:1)	17.65	±	7.875	8.132	±	1.882	13.44	±	2.903	19.42	±	10.8
Docosahexaenoic (22:6n3)	16.25	±	7.443	18.84	±	4.899	24.01	±	5.579	28.56	±	21.80
n-3/n-6	0.125	±	0.017	0.134	±	0.002	0.14	±	0.011	0.11	±	0.014$^{\#\wedge}$
16:0/16:1	13.68	±	0.938	8.35	±	1.95*	7.69	±	1.14*	10.45	±	3.419
18:0/18:1	0.73	±	0.16	0.59	±	0.05	0.58	±	0.02	0.83	±	0.24$^{\wedge}$
Saturated	2802	±	761	3254	±	501.2	2769	±	47.01	4756	±	1213*$^{\#\wedge}$
Unsaturated	1865	±	505.9	3216	±	643.9*	2499	±	236.9*$^{\#}$	2791	±	654.6*
Polyunsaturated	687.8	±	185.9	1887	±	438.7*	1446	±	150.9*	971.7	±	350.5$^{\#\wedge}$
Monounsaturated	1178	±	323.9	1330	±	217.7	1053	±	130.8$^{\#}$	1819	±	384.5*$^{\#\wedge}$
Total	**4667**	**±**	**1258**	**6471**	**±**	**1124***	**5148**	**±**	**504.2$^{\#}$**	**7547**	**±**	**1712*$^{\wedge}$**

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
*p < 0.05, difference versus CTRL SAT.
#p < 0.05, difference versus HFD SAT.
^ p < 0.05, difference versus HFD + NAC SAT.

Table 5
The effects of NAC and ALA on the diacylglycerol (DAG) level and the FA composition in visceral adipose tissue (VAT).

Acid	CTRL VAT			HFD VAT			HFD + NAC VAT			HFD + ALA VAT		
Myrystic (14:0)	410.2	±	58.99	614.3	±	144.7*	441.3	±	73.72$^{\#}$	258.7	±	43.11*$^{\#}$
Palmitic (16:0)	3160	±	377.3	4774	±	756.8*	3886	±	259.9*#	3561	±	783.5$^{\#}$
Palmitooleic (16:1)	386.4	±	59.61	1077	±	311.1*	854.6	±	90.85*	439.7	±	112.1$^{\#\wedge}$
Stearic (18:0)	663.4	±	64.88	575.2	±	99.48	470.6	±	47.1*	877.5	±	194.6*$^{\#\wedge}$
Oleic (18:1n9c)	3003	±	343.1	2593	±	460.2	1861	±	163.7*$^{\#}$	4273	±	1207*$^{\#\wedge}$
Linoleic (18:2n6c)	2112	±	216.2	4935	±	533.2*	3246	±	278.7*$^{\#}$	2789	±	569*$^{\#}$
Arachidic (20:0)	24.95	±	7.437	23.6	±	6.604	31.4	±	6.562	26.04	±	6.336
Linolenic (C18n3)	124.3	±	11.91	447.9	±	54.66*	279.8	±	27.4*$^{\#}$	151.9	±	41.17$^{\#\wedge}$
Behenic (22:0)	19.81	±	5.399	14.92	±	4.17	22.74	±	5.372$^{\#}$	16.37	±	1.901$^{\wedge}$
Arachidonic (20:4n6)	40.49	±	11.63	97.78	±	25.74*	80.41	±	13.73*	68.16	±	34.2
Lignoceric (24:0)	22.11	±	11.66	24.76	±	5.193	26.29	±	8.858	16.44	±	3.257
Eicosapentaenoic (20:5n3)	36.32	±	12.64	13.22	±	4.109	38.41	±	12.01	31.59	±	6.309
Nervonic (24:1)	14.22	±	6.208	9.888	±	1.525	17.45	±	7.431	14.5	±	3.211
Docosahexaenoic (22:6n3)	17.66	±	4.335	36.40	±	8.802*	41.05	±	10.34*	18.12	±	4.223$^{\#\wedge}$
n-3/n-6	0.066	±	0.005	0.096	±	0.002*	0.096	±	0.005*	0.059	±	0.007$^{\#\wedge}$
16:0/16:1	8.245	±	0.814	4.579	±	0.737*	4.565	±	0.26*	8.15	±	0.478$^{\#\wedge}$
18:0/18:1	0.222	±	0.017	0.223	±	0.02	0.253	±	0.019*$^{\#}$	0.209	±	0.024$^{\wedge}$
Saturated	4301	±	492.3	6026	±	1010*	4878	±	368.5$^{\#}$	4808	±	1105
Unsaturated	5734	±	617.2	9210	±	1250*	6419	±	554.1$^{\#}$	7799	±	1925*
Polyunsaturated	2331	±	227.4	5530	±	614*	3686	±	320.3*$^{\#}$	3063	±	642.2*$^{\#}$
Monounsaturated	3404	±	392.3	3680	±	765.7	2733	±	257.3*$^{\#}$	4728	±	1311$^{\wedge}$
Total	10035	±	1101	15237	±	2238*	11297	±	903.3$^{\#}$	12607	±	2973

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
*p < 0.05, difference versus CTRL VAT.
#p < 0.05, difference versus HFD VAT.
^ p < 0.05, difference versus HFD + NAC VAT.

the aforementioned FAT/CD36 and FATP1 and fulfill different physiological roles in adipocytes. Previous research revealed that FAT/CD36 is a key long-chain fatty acid transporter in AT, whereas FABPpm is more uniformly expressed in a wide variety of tissues [38]. We observed a decrease of the FAT/CD36 in groups with antioxidant supplementation (HFD+NAC and HFD+ALA), which can be explained by the lipid level, especially TAG, in AT. On the contrary, we found statistically significant differences between the adipocytes from VAT and SAT concerning FAT/CD36, FABPpm, FATP1, and FATP4 mRNA expression. These inter-tissue disparities may stem from the distinctive developmental origin and metabolic properties of the adipocytes [39–43].

In many biological systems, protein expression does not follow the mRNA level [44]. Interestingly, this study revealed differences between the mRNA and protein levels of fatty-acids transporters in AT. Both real-time PCR and Western blot were used to test steady-state mRNA and protein levels, respectively, so anything that occurred regarding the relationship between the transcription rate and mRNA stability or translation efficiency versus protein stability was not detected. Presumably, there are at least three reasons for the weak correlation between mRNA and protein levels [45]. First, there are many complex and varied post-transcriptional mechanisms involved in converting mRNA into protein that are not yet sufficiently well-defined for us to calculate

Table 6
The effects of NAC and ALA on the diacylglycerol (DAG) level and the FA composition in subcutaneous adipose tissue (SAT).

Acid	CTRL SAT			HFD SAT			HFD + NAC SAT			HFD + ALA SAT		
Myrystic (14:0)	378.7	±	148.7	748.3	±	186	419.5	±	27.31	830.9	±	522.1
Palmitic (16:0)	3390	±	1166	4428	±	1327	3014	±	491.8	4461	±	776
Palmitooleic (16:1)	374.9	±	118.4	702.6	±	294.1	509.3	±	158.6	528.4	±	231.6
Stearic (18:0)	711	±	250.4	599.3	±	177.4	439	±	47.31*	1137	±	240.2*#^
Oleic (18:1n9c)	3370	±	1061	2384	±	758.2	1496	±	302.7*#	4201	±	503.6#^
Linoleic (18:2n6c)	2440	±	772.5	4871	±	1301*	3050	±	325.8#	2424	±	1013#
Arachidic (20:0)	25.93	±	5.441	29.35	±	13.9	21.75	±	5.114	44.41	±	23.55
Linolenic (C18n3)	132.3	±	39.48	432.5	±	128.3*	252.3	±	36.84*#	120.6	±	39.29#^
Behenic (22:0)	21.93	±	6.821	19.04	±	8.205	15.81	±	4.010	25.42	±	15.20
Arachidonic (20:4n6)	51.92	±	15.98	93.84	±	29.98	67.52	±	4.894	64.95	±	28.76
Lignoceric (24:0)	19	±	4.039	35.84	±	17.49	22.29	±	6.801	12.98	±	3.387*#^
Eicosapentaenoic (20:5n3)	36.58	±	11.98	19.04	±	11.78*	30.60	±	5.144	69.21	±	47.30#
Nervonic (24:1)	14.7	±	2.508	15.97	±	10.96	12.81	±	3.441	15.67	±	3.622
Docosahexaenoic (22:6n3)	22.14	±	4.442	29.11	±	13.79	26.20	±	3.537	16.50	±	3.616*#^
n-3/n-6	0.063	±	0.003	0.093	±	0.004*	0.088	±	0.004*	0.054	±	0.003*#^
16:0/16:1	8.99	±	0.999	6.59	±	1.34	6.19	±	1.10	10.78	±	7.381
18:0/18:1	0.209	±	0.024	0.256	±	0.045	0.301	±	0.055*	0.257	±	0.045
Saturated	4546	±	1566	5859	±	1690	3932	±	549.5#	6511	±	1264*^
Unsaturated	6442	±	2015	8548	±	2473	5467	±	806.1	7088	±	2220
Polyunsaturated	2683	±	839.2	5445	±	1470*	3450	±	356.6#	2685	±	1007#
Monounsaturated	3759	±	1178	3103	±	1038	2018	±	461.3*	4392	±	1236^
Total	10988	±	3555	14408	±	4144	9399	±	1355#	13599	±	1756^

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
*$p < 0.05$, difference versus CTRL SAT.
#$p < 0.05$, difference versus HFD SAT.
^ $p < 0.05$, difference versus HFD + NAC SAT.

Table 7
The effects of NAC and ALA on the triacylglycerol (TAG) level and the FA composition in visceral adipose tissue (VAT).

Acid	CTRL VAT			HFD VAT			HFD + NAC VAT			HFD + ALA VAT		
Myrystic (14:0)	29959	±	5087	57542	±	11922*	49744	±	1569*	27531	±	2706#^
Palmitic (16:0)	511005	±	76243	898319	±	114158*	799761	±	36161*	492172	±	51331#^
Palmitooleic (16:1)	77548	±	19351	205695	±	48858*	191413	±	5747*	69840	±	8213#^
Stearic (18:0)	108213	±	14080	79414	±	10082*	67990	±	11252*	108057	±	13129#^
Oleic (18:1n9c)	781934	±	126002	626534	±	75704*	532192	±	26240*	756659	±	83529#^
Linoleic (18:2n6c)	425251	±	67153	993569	±	125160*	721719	±	128309*#	421962	±	46172#^
Arachidic (20:0)	994.8	±	130.5	1369	±	284.1*	1190	±	269.1	856.5	±	203#^
Linolenic (C18n3)	31269	±	4717	116927	±	15585*	83922	±	15006*#	27702	±	2672#^
Behenic (22:0)	530.3	±	105.7	706.5	±	171.9	776.5	±	183.8*	385.5	±	92.97*#^
Arachidonic (20:4n6)	3782	±	769.9	19383	±	5128*	14784	±	2658*	5858	±	1029*#^
Lignoceric (24:0)	396.8	±	76.69	1214	±	487*	905.1	±	104*	435	±	113.4#^
Eicosapentaenoic (20:5n3)	1217	±	225.7	1944	±	692.8	2548	±	345.4*	485.3	±	103.3*#^
Nervonic (24:1)	258.7	±	102.5	209.8	±	35.52	370.2	±	113.3#	177.3	±	54.51^
Docosahexaenoic (22:6n3)	886.1	±	204.3	9048	±	2791*	7125	±	1344*	1120	±	200.9#^
n-3/n-6	0.075	±	0.001	0.124	±	0.002*	0.124	±	0.001*	0.068	±	0.003*#^
16:0/16:1	6.755	±	0.921	4.473	±	0.627*	4.20	±	0.11*	7.064	±	0.386#^
18:0/18:1	0.14	±	0.014	0.127	±	0.011	0.137	±	0.001	0.143	±	0.010
Saturated	651098	±	93890	1038565	±	131985*	925602	±	41362*	629438	±	65595#^
Unsaturated	1322146	±	217414	1973308	±	226445*	1503514	±	262986#	1283805	±	139733#
Polyunsaturated	462406	±	72930	1140870	±	148338*	830097	±	147285*#	456980	±	48909#^
Monounsaturated	859740	±	144584	832438	±	118146	724020	±	31008	826676	±	90954
Total	**1973245**	**±**	**310035**	**3011874**	**±**	**351861***	**2541726**	**±**	**128774*#**	**1913243**	**±**	**204359#^**

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
*$p < 0.05$, difference versus CTRL VAT.
#$p < 0.05$, difference versus HFD VAT.
^ $p < 0.05$, difference versus HFD + NAC VAT.

protein concentrations from mRNA. We also know that a cell can control protein levels at a transcriptional and/or translational level [46,47]. Previous studies have concluded that genes show minimal variability in their mRNA expression throughout the cell cycle, and are more likely to have weak or no correlation with final protein levels [48]. A second reason for the lack of correlation between mRNA and protein levels may be that proteins have very different half-lives, due to the different synthesis and degradation of proteins. The presence of oxidative stress can also affect protein damage or degradation [49–51]. Third, there was a considerable amount of error and noise in the mRNA and protein experiments that limited the ability to obtain a clear picture [52]. These results suggest that protein abundance is under more complex genetic control than mRNA abundance [52]. We also already know that post-transcriptional mechanisms can shape protein levels independently of mRNA abundance [52–55]. There are also studies suggesting the existence of mechanisms that buffer protein levels against changes in the mRNA abundance [56,57].

A recent study shows that, although white AT does not contain numerous mitochondria, their functioning is still necessary for the proper synthesis of adenosine 5′-triphosphate (ATP), which is needed to cover the energy demand of adipocytes for processes such as lipid metabolism, adipocyte differentiation, and maturation [58]. Increased levels of FFAs in AT may contribute to greater OS due to the enhanced production of ROS, which leads to endothelial damage and the activation of the

Table 8
The effects of NAC and ALA on the triacylglycerol (TAG) level and the FA composition in subcutaneous adipose tissue (SAT).

Acid	CTRL SAT			HFD SAT			HFD + NAC SAT			HFD + ALA SAT		
Myrystic (14:0)	39071	±	10477	50221	±	4534	41874	±	6737	31013	±	2912$^{\#\wedge}$
Palmitic (16:0)	587826	±	163200	529288	±	37898	517579	±	121950	517071	±	44959
Palmitooleic (16:1)	73157	±	21566	91646	±	16245	97910	±	33113	70908	±	7954
Stearic (18:0)	126329	±	36703	55919	±	8266*	57229	±	10745*	116802	±	7125$^{\#\wedge}$
Oleic (18:1n9c)	860072	±	247903	383898	±	27967*	370519	±	79737*	811568	±	61829$^{\#\wedge}$
Linoleic (18:2n6c)	490376	±	139104	643657	±	97895	592811	±	79273	448596	±	35907$^{\#\wedge}$
Arachidic (20:0)	1421	±	328.5	994.3	±	169.9*	855.5	±	192.2*	1045	±	149.1*
Linolenic (C18n3)	33992	±	9146	70773	±	10547*	66414	±	10007*	26534	±	1630$^{\#\wedge}$
Behenic (22:0)	787.8	±	231.3	564.3	±	147.6	681.2	±	156.4	445.4	±	131.1$^{*\wedge}$
Arachidonic (20:4n6)	5226	±	1069	10669	±	2024*	11609	±	1397*	4489	±	1494$^{\#\wedge}$
Lignoceric (24:0)	568.4	±	170.2	455.8	±	140.4	572.9	±	118.3	421.6	±	74.1
Eicosapentaenoic (20:5n3)	1198	±	339.1	1192	±	323.2	1922	±	467.9$^{*\#}$	406.3	±	115$^{*\#\wedge}$
Nervonic (24:1)	281.1	±	74.78	172.7	±	37.75	275.3	±	88.58	221.3	±	98.44
Docosahexaenoic (22:6n3)	1122	±	266.8	3272	±	696.1*	4127	±	467*	797.7	±	116.5$^{*\#\wedge}$
n-3/n-6	0.071	±	0.001	0.113	±	0.002*	0.117	±	0.002$^{*\#}$	0.061	±	0.002$^{*\#\wedge}$
16:0/16:1	8.074	±	0.704	5.963	±	1.337*	5.472	±	0.694*	7.337	±	0.617$^{\#\wedge}$
18:0/18:1	0.147	±	0.01	0.145	±	0.013	0.155	±	0.005	0.146	±	0.007
Saturated	756003	±	210505	637442	±	48304	618792	±	139458	666924	±	54963
Unsaturated	1465424	±	418481	1205280	±	125906	1145587	±	202036	1365167	±	105682
Polyunsaturated	531914	±	149728	729564	±	111297*	676883	±	91087	482322	±	39582$^{\#\wedge}$
Monounsaturated	933510	±	269100	475716	±	17771*	468704	±	112801*	882676	±	67909$^{\#\wedge}$
Total	2221426	±	628554	1842722	±	173958	1764379	±	341151	2032091	±	159610

The values (nmol mg^{-1} of protein) are expressed as: mean SD.
*$p < 0.05$, difference versus CTRL SAT.
$^{\#}p < 0.05$, difference versus HFD SAT.
$^{\wedge}p < 0.05$, difference versus HFD + NAC SAT.

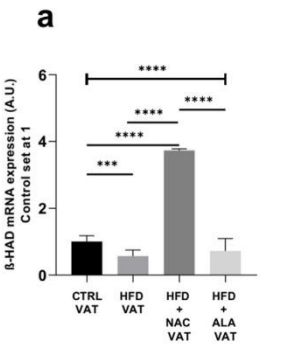

Fig. 6. The effects of NAC and ALA on (**a**, **b**) β-HAD mRNA expression in adipose tissue.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.
Abbreviations: CTRL VAT—control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; CTRL SAT - control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

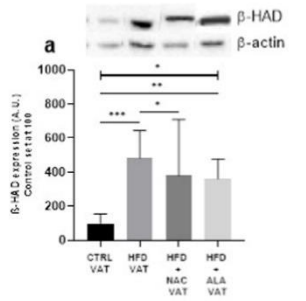

Fig. 7. The effects of NAC and ALA on (**a**, **b**) β-HAD protein expressions in adipose tissue.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Abbreviations: CTRL VAT—control rats, visceral adipose tissue; HFD VAT—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC VAT—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA VAT—high-fat diet-fed rats that received α-lipoic acid. visceral adipose tissue; CTRL SAT—control rats, subcutaneous adipose tissue; HFD SAT—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC SAT—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA SAT—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue.

pro-inflammatory cascade in AT [59]. An HFD-induced lipid overload contributes to adipocyte OS, obesity, and subsequent obesity-related metabolic disorders, such as hyperglycemia, insulin resistance, and cardiovascular diseases [60–62]. During obesity, the concentration of OS

indicators, such as highly sensitive C-reactive protein (CRP) and oxidized LDL increases [60]. As expected, the supplementation of the HFD-fed rats with NAC resulted in lower levels of FFA, DAG, and TAG in their VAT and SAT. The drops were caused by the declines in the levels of unsaturated fatty acid species. We believe that the current study is the first to investigate the effects of ALA on the FAs level in the VAT and SAT of rats with diet-induced obesity. Interestingly, animals in the HFD+ALA group also had reduced levels of FFA, DAG and TAG, but only in the VAT. An indirect effect of OS is an increased amount of circulating FFAs in the serum, and the presence of FFA in adipocytes [63]. Antioxidants reduce the disorders associated with HFD, which consequently leads to better use of available FAs and prevents their accumulation in adipocytes. Yang et al. [35] also confirmed the beneficial effect of NAC supplementation on the lipid profile of adipocytes. NAC, in addition to its antioxidant and anti-inflammatory properties, is intensively investigated as a candidate drug for the treatment of metabolic disorders [64]. In our research, we noticed that NAC supplementation statistically significantly lowered blood lipids levels, which may suggest its beneficial effects on HFD-induced obesity. Our previous study demonstrated that NAC (HFD+NAC) and ALA (HFD+ALA) also improved glucose and insulin levels in the study groups compared to the HFD group [65]. Kim et al. [37] observed in ALA-treated experimental animals increased insulin sensitivity, restoration of glucose tolerance, lower body weight and reduced food intake. The changes were attributed to the reduced appetite of the rodents [37]. The above was also reflected in our results. The aforementioned appetite reduction could be caused by the inactivation of hypothalamic 5′ adenosine monophosphate-activated protein kinase (AMPK) caused by the ALA supplementation. This seems to be likely since previous studies have demonstrated that ALA-treated rats are characterized by increased energy expenditure, lower hypothalamic AMPK activity, and reduced plasma glucose, insulin, FFAs, and leptin levels [37,66,67]. Golbidi et al. [66] suggest that hypothalamic AMPK is involved in appetite regulation and ALA exerts anorexic, anti-obesity effects by suppressing hypothalamic AMPK activity.

FAs regulate the β-oxidation process in white and brown AT and the liver. In our present study, β-HAD mRNA expression was statistically significantly increased in the SAT and decreased in the VAT of the HFD-fed rats. We also observed differences in response to the antioxidants applied, which can be explained by the fact that NAC can more readily pass through cell membranes than ALA. These differences may have also resulted from the dose of the administered antioxidants. Intensified β-oxidation is likely one of the cell's protective mechanisms against excessive lipid accumulation and its lipotoxic effects [68].

5. Conclusions

In summary, we present the first report on the effects of NAC and ALA supplementation on the lipid profile of the VAT and SAT of the rats with HFD-induced obesity. Moreover, our study is the first to compare the influence of the two antioxidants on FA transporter expression in both of the aforementioned tissues. Current studies clearly demonstrate that the antioxidants, i.e., NAC and ALA, exert different influences on AT. This varying impact is reflected in histopathological images, the FA transport protein expression pattern, or even in the lipid storage capacity of adipocytes. Our results provide direct evidence to support the thesis that antioxidant supplementation can be considered as effective means to block diet-induced obesity and its complications.

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Data statement/availability of data and material

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare no conflict of interests.

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10. Publikacja nr 3

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Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue.

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Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue

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Key Words
N-acetylcysteine • Alpha-lipoic acid • Oxidative stress • Matrix metalloproteinase-2 • Matrix metalloproteinase-9

Abstract
Background/Aims: The high-fat diet (HFD) regime causes obesity and contributes to the development of oxidative stress in the cells by the production of reactive oxygen species and the occurrence and progress of inflammation. Despite years of studies, there is no data explaining the mechanism of action of N-acetylcysteine (NAC) or alpha-lipoic acid (ALA) on matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) in visceral and subcutaneous adipose tissue of HFD-fed rats. Our experiment aimed to evaluate for the first time the influence of chronic antioxidants administration on MMPs biology after an HFD regime as a potential therapeutic strategy for obesity-related complications prevention. ***Methods:*** Male Wistar rats were fed a standard rodent chow or an HFD with intragastric administration of NAC or ALA for ten weeks. The collected samples were subjected to pathohistological evaluation. Real-time PCR and western blot approaches were used to check whether NAC or ALA impacts MMP2/9 expression. ***Results:*** Antioxidant supplementation markedly reduced the number of circulating inflammatory cytokines, and tissue macrophage infiltration. Moreover, NAC and ALA have a divergent impact on MMP2 and MMP9 expression in different adipose tissue localization. ***Conclusion:*** Based on our results, we speculate that NAC and ALA have a prominent effect on the MMP2/9 functions under obesity conditions.

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Introduction

Nowadays, overweight and obesity are considered global epidemics and are the leading causes of death (roughly 2.8 million people die each year because of the conditions) [1]. Obesity is the main factor predisposing to the development of cardiovascular, respiratory, neurological, gastrointestinal, hepatic, endocrine, skeletal, and renal complications, as well as to a significant psychosocial burden or an increased incidence of cancer [2, 3]. The risk of obesity-related complications is associated with the amount of adipose tissue, its location (abdominal obesity or gluteal-femoral obesity), and the duration of the underlying disease [3].

Recent studies have demonstrated that matrix metalloproteinases (MMPs) are involved in processes taking place in adipose tissue, such as adipogenesis and angiogenesis. The sources of MMPs include adipocytes, preadipocytes, fibroblasts, endothelial cells, as well as immune cells [4]. MMPs are involved in physiological and pathological complications of obesity through the degradation and remodeling of the extracellular matrix (ECM) molecules [5]. In this research, we were focused on metalloproteinases 2 (MMP2) and 9 (MMP9), otherwise known as gelatinases A and B, which belong to type IV collagenases. The main function of the gelatinases is the degradation of type IV collagen, which is the main component of the vascular basement membrane. Damage to this barrier facilitates the migration of leukocytes to adipose tissue, thus enhancing its inflammation. The activation of the leukocytes causes the release of pro-inflammatory cytokines and MMPs, the above drives the vicious circle even further [6].

A high-fat diet (HFD) regime, and thus a positive energy balance, causes obesity and contributes to the development of oxidative stress in the cells, the production of reactive oxygen species (ROS), and the progress of inflammation [7, 8]. The increase in the amount of ROS in adipose tissue leads to impaired adipogenesis, recruitment, and activation of macrophages, secretion of inflammatory adipokines, or damage to the tissue's biological structures. The coexistence of oxidative stress and inflammation in adipose tissue contributes to the subsequent development of obesity and the formation of a vicious circle [9]. It was proved that antioxidants supplementation, e.g. with N-acetylcysteine (NAC) or alpha-lipoic acid (ALA), can reduce oxidative stress, the number of inflammatory cytokines, macrophage infiltration, and ultimately other complications of obesity [9, 10]. Hence, alteration in MMPs metabolism (e.g., MMP2 and MMP9) may be a therapeutic strategy for obesity and its complication. Thus, the goal of this study was to elucidate changes in adipose tissue matrix metalloproteinases, especially MMP2 and MMP9 after chronic antioxidants administration (NAC and ALA) after an HFD regime as a potential therapeutic strategy for obesity-related complications prevention.

Materials and Methods

Animals and Study Design

After six days of adaptation to the conditions in an animal facility, male Wistar rats were divided into the following four groups – control (CTRL), high-fat diet (HFD), a high-fat diet supplemented with N-acetylcysteine (HFD+NAC), and a high-fat diet supplemented with α-lipoic acid (HFD+ALA) (10 rats in each group). The CTRL group was formulated as the control for HFD, HFD+NAC, and HFD+ALA. Forty male Wistar rats were housed under standard conditions (21°C ± 2°C, 12h reverse light/dark cycle) with ad libitum access to a control standard chow (LSM, Agropol, Motycz, Poland; containing 10.3% fat, 24.2% protein, and 65.5% carbohydrate) or a high-fat diet (Research Diet, USA, catalog number D12492; containing 59.8% fat, 20.1% protein, 20.1% carbohydrate; 279.6 mg/kg of the cholesterol) from weaning until sacrifice. Wistar rats were fed a high-fat diet not isocaloric relative to control animals. After six weeks of the experiment, once daily, each morning between 8 and 9 am, the animals in the HFD+NAC group received N-acetylcysteine at a dose of 500 mg/kg body weight, whereas the rats from the HFD+ALA group received α-lipoic acid solution at a dose of 30 mg/kg body weight (once a day, every day for the consecutive 4 weeks). The solutions of NAC (Sigma-

Aldrich, catalog number: A9165) and ALA (Sigma-Aldrich, catalog number: PHR2561-1G) were prepared by dissolving the substances in the saline solution and immediately applied intragastrically by gastric gavage to rats from appropriate groups. The doses were based on the literature analysis [11, 12]. We decided to use 500 mg/kg body weight of NAC and 30 mg/kg body weight of ALA as those are the most frequently used, non-toxic doses with proven antioxidative effects [11–13]. Similarly, saline (2 ml/kg body weight) was administered to the CTRL and HFD-fed rats. Body weight was monitored every day and the amount of NAC, ALA, or saline solution was adjusted accordingly. The intragastric administration of antioxidants ensured that rats obtained a full dose calculated for their body weight. According to each rat's body weight, which was controlled every two days, the dose of antioxidants administration was adjusted. After ten weeks, the rats fasted twelve hours and were anesthetized by intraperitoneal phenobarbital injection (80 mg/kg body weight). The rats were placed lying down on a heating pad (37°C). Blood was drawn from the abdominal aorta and was immediately centrifuged to obtain plasma. Samples of adipose tissue (visceral and subcutaneous) were taken from the abdominal area. The harvested tissues were immediately frozen using aluminum forceps precooled with liquid nitrogen. All the obtained samples (adipose tissue and plasma) were stored at -80^{0}C until further analysis. All the experimental procedures were approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok, Poland.

Adipose tissue Histopathology

The samples of visceral and subcutaneous adipose tissue of the rats were taken and immediately fixed in 10% buffered formalin and processed routinely for embedding in paraffin. The paraffin blocks were cut into 4 µm sections, attached to positively charged glass slides (Superfrost Plus; Menzel Gläser, Braunschweig, Germany), and stained with hematoxylin and eosin.

Paraffin-embedded sections were deparaffined and hydrated in pure alcohols.

Toluidine Blue Stain Kit (no cat. SS057 BioGenex; 49026 Milmont Drive, Fremont, CA 94538 USA) was used to stain mast cells. The kit included: potassium permanganate, potassium metabisulphite, Scott's solution, Toluidine Blue Solution. The staining was done by following the manufacturer's instructions attached to the kit.

Immunostaining was performed by the following protocol: for antigen retrieval, the sections were subjected to pretreatment in a pressure chamber and heated using Target Retrieval Solution (S 1699 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA 95051, USA). After cooling down to room temperature, the sections were incubated with Peroxidase-Blocking Solution for 10 minutes to block endogenous peroxidase activity.

The sections with the monoclonal mouse primary antibody (Agilent Technologies, Inc.), CD68 (M0876), were incubated for 1 hour at RT in a humidified chamber. The antiserum was previously diluted in Antibody Diluent, Background Reducing, Ready-to-use diluent (S 3022 Agilent Technologies, Inc.) in a ratio of 1:50 for CD68.

The procedure was followed by incubation (30 minutes) with secondary antibody (EnVision FLEX, High pH (Link), HRP. Rabbit/Mouse. (K800021-2 Agilent Technologies, Inc. 5301 Stevens Creek Blvd Santa Clara, CA 95051, USA). The bound antibody was visualized by 1-min incubation with DAB Flex chromogen. Finally, the sections were counterstained in hematoxylin QS (H-3404, Vector Laboratories; Burlingame, CA), mounted, and evaluated under the light microscope. Appropriate washing with Wash Buffer (S 3006 Agilent Technologies, Inc.) was performed between each step (3 times for 2 minutes). Sections were dehydrated with absolute alcohol followed by xylene, and coverslipped with Entellan (Merck). The specificity of the antibody was confirmed using a negative control, which involved replacing the antibody with the Antibody Diluent (no staining), and positive control, which involved staining a human tonsil with CD68.

The assessment of the cell size and number of CD68 was performed by two experienced histologists (independent from each other) and analyzed with ImageJ software (The National Institutes of Health, MD, USA). Images were converted to 8-bit greyscale, and the background was subtracted. Then, the binary threshold function was adjusted to separate the cells from background staining. The total cells area was calculated as the total number of pixels in images with a set threshold [14, 15]. Results were plotted in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Results are depicted as mean ± standard deviations. The statistical significance level was set as $p<0.05$.

Table 1. Primers sequences for real-time PCR analysis

Gene	Primer Sequence		Annealing temperature
	Forward	Reverse	
β-actin	5′-ACGGTCAGGTCATCACTATCG-3′	5′-GGCATAGAGGTCTTTACGGATG-3′	58°C
MMP2	5′-AAAGGAGGGCTGCATTGTGAA-3′	5′-CTGGGGAAGGACGTGAAGAGG-3′	58°C
MMP9	5′-AGGTGCCTCGGATGGTTATCG-3′	5′-TGCTTGCCCAGGAAGACGAA-3′	59°C

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the rats' visceral and subcutaneous adipose tissue using the TriRreagent RNA Isolation Reagent according to the manufacturer's protocol (Sigma-Aldrich). The total RNA amount was determined by spectrophotometry and RNA quality was verified by measuring the sample's absorbencies at 260 and 280 nm [16]. The synthesis of the complementary DNA was done using the EvoScript universal cDNA master kit (Roche Molecular Systems, Boston, MA, USA). Specific primers used in this study are presented in Table 1. Real-time PCR was carried out using the LightCycler 96 System Real-Time thermal cycler with FastStart Essential DNA Green Master (Roche Molecular Systems). Cycling conditions were: 15s denaturation at 95°C, 15s annealing at 58°C for β-actin, MMP2, and 59°C for MMP9, and 15s extension at 72°C for 45 cycles. Melting curve analysis was performed before each reaction to verify PCR product specificity. The mRNA levels of the target genes were normalized to the rat's β-actin and calculated according to the Pfaffl method [17].

Proteins Analysis

To investigate the protein expression of various MMPs in visceral and subcutaneous adipose tissue extracts, we used Western blot analysis. The samples were homogenized in an ice-cold RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The total protein concentration was determined using the BCA method with bovine serum albumin (BSA) as a standard. Next, homogenates (20 μg of the total protein) were reconstituted in Laemmli buffer, separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated overnight with antibodies i.e., MMP2 (1:2500, cat. no. ab92536, Abcam, Cambridge, UK), MMP9 (1:10000, cat. no. ab76003, Abcam, Cambridge, UK), and β-actin (1:200, cat. no. ab115777, Abcam, Cambridge, UK). Thereafter, the PVDF membranes were incubated with secondary antibodies conjugated with anti-rabbit IgG conjugated to horseradish peroxidase (cat. no. 7074S, Cell Signaling). Protein bands were visualized using an enhanced chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA) and quantified densitometrically (ChemiDoc visualization system EQ, Bio-Rad Systems). Equal protein concentrations were loaded in each lane, which was confirmed by Ponceau S staining. Protein expression (Optical Density Arbitrary Units) was normalized to β-actin expression. Finally, the control was set to 100, and the experimental groups were expressed relative to the control.

Lipids Analysis

The content of plasma lipids (FFA, DAG, TAG, and PL) was measured using gas-liquid chromatography as described previously [18, 19]. The selected lipid fractions were extracted using Bligh and Dyer's method [20]. Then, the lipids were separated by thin-layer chromatography (TLC) into specific fractions. Next, they were fractionated on Silica Gel Plates (silica plate 60, 0.25 mm; Merck).

Individual fatty acid methyl esters (FAMEs) present in each fraction were identified and quantified according to the retention times of the standards by gas-liquid chromatography (Hewlett-Packard5890 Series II gas chromatograph, HP-INNOWax capillary column; Agilent Technologies, Santa Clara, CA, USA). The total amount of FFA, DAG, TAG, and PL was estimated as the sum of individual fatty acid species in the evaluated fraction and expressed in nanomoles per mg of protein.

Statistical Analysis

The obtained results were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Briefly, in the first step, the assumptions of the statistical methods were checked. The normality of the results' distribution was tested using the Shapiro-Wilk test. Analysis of variance (ANOVA) followed by post-hoc pairwise Student's *t*-tests was carried out to determine the existence of differences between the studied groups. For all analyses, p values < 0.05 were considered to be statistically significant. The results are expressed as mean ± SD.

Results

Supplementation with antioxidants during a high-fat diet regime affects body weight and plasma metabolic parameters

In the present study, high-fat diet feeding was associated with greater body mass and metabolic parameters. To determine the effects of the antioxidants supplementation during the HFD regime, we evaluated weekly weight gain, glucose and insulin levels, as well as HOMA-IR value, and also a plasma lipids content in male Wistar rats, under the nutritional conditions (CTRL and HFD) and in response to the antioxidants supplementation during the HFD regime (HFD+NAC and HFD+ALA). The animals' body weight was significantly increased after HFD alone (+22%, $p<0.0001$, HFD vs. control group; Fig. 1a, b) as well as in the rats at HFD regime with NAC or ALA (+24%, $p<0.0001$; +18%, $p<0.0001$; HFD+NAC and HFD+ALA vs. control group; respectively; Fig. 1a, b). Our previous study established that the glucose and insulin levels, as well as HOMA-IR values, were significantly increased in HFD groups (+26%, $p=0.014$, HFD; +123%, $p=0.0010$, HFD+NAC; +8-fold, $p=0.0021$, HFD+ALA; vs. control group; respectively) [21]. Glucose and insulin levels, as well as HOMA-IR values were lower in HFD+NAC (-28%, $p=0.0016$; -51%, $p=0.0042$; -87%, $p=0.0023$; HFD+NAC vs. HFD group; respectively) and in HFD+ ALA groups (-19%, $p=0.0210$; -48%, $p=0.0024$; -88%, $p=0.0022$; HFD+ALA vs. HFD groups; respectively) [21]. Interestingly, we also observed significant differences in glucose level between the groups treated with the antioxidants (+12%, $p=0.0172$, HFD+ALA vs. HFD+NAC) [21]. As expected, the HFD caused an increase in the plasma phospholipid content (+31%, $p=0.0009$, vs. control group; Fig. 1d). There was a relevant decrease in the plasma diacylglycerols and triglycerides content in the NAC-treated group (-50%, $p<0.0001$, Fig. 1e; -45%, $p=0.0009$, Fig. 1f; respectively; vs. control group). Moreover, comparison between HFD and HFD+NAC revealed a pronounced decrease in the plasma phospholipids, diacylglycerols, and triglycerides content in the latter group (-23%, $p=0.0040$, Fig. 1d; -37%, $p=0.0045$, Fig. 1e; -53%, $p=0.0080$, Fig. 1f; respectively; vs. HFD

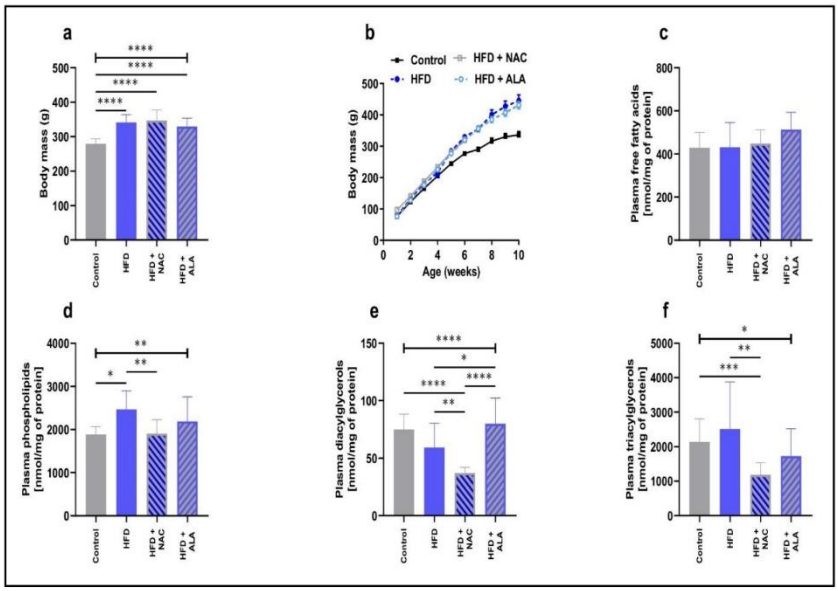

Fig. 1. The effects of N-acetylcysteine (NAC) and α-lipoic acid (ALA) supplementation on body mass, glucose and insulin levels, as well as HOMA-IR value, and plasma metabolic parameters. Control – control rats; HFD – high-fat diet-fed rats; HFD+NAC – high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA – high-fat diet-fed rats that received α-lipoic acid; HOMA-IR – homeostatic model assessment of insulin resistance; *p<0.05 vs. control; #p<0.05 vs. HFD; ^p<0.05 vs. HFD+NAC.

group). On the other hand, we observed significant changes in the plasma diacylglycerols content in the NAC+ALA group (+35%, p=0.0491, vs. HFD group, Fig. 1e) as well as between the two groups with antioxidants treatment (+115%, p<0.0001, HFD+ALA vs. HFD+NAC group, Fig. 1e). Thus, the antioxidants supplementation during the HFD regime affects body weight, plasma glucose, and insulin levels, as well as HOMA-IR value, and plasma lipids content.

Histological changes in adipose tissue

Histological examination of the adipose tissue sections in the HFD group revealed significantly larger dimensions of the fat cells, especially in SAT (Fig. 2b; p<0.0001, HFD V vs. Control V, Fig. 4a; p=0.0165, HFD S vs. Control S, Fig. 4b), when compared to the control. Immunohistochemical analyses in this group showed also an increase in the number of CD68 immunopositive cells (HFD S vs Ctrl S, Fig. 3; p=0.0061, 4d). However, the greatest number of infiltrating macrophages was found in VAT, where they formed large clusters (Fig. 3a). The adipocytes in the VAT and SAT of the HFD+NAC group were slightly smaller than the fat cells of the HFD group, and their cell membrane had a thin rim of folded cytoplasm (Fig. 2, 4a, and b). There was a lower number of macrophages observed in HFD+NAC when compared to HFD (Fig. 3), with more CD68 immunopositive cells compared to VAT (Fig. 3a; p=0.0358, 4c). The shape of the fat cells in the HFD+ALA group was similar to that found in the control group, while the size of the adipocytes was slightly smaller than in the HFD (Fig. 2; p=0.0003, HFD+ALA V vs. HFD V, Fig. 4a; p=0.0209, HFD+ALA S vs. HFD S, Fig. 4b). The number of CD68 immunopositive cells was higher in both VAT and SAT of HFD+ALA compared to HFD (Fig. 3; p=0.0088, HFD+ALA V vs. HFD V, Fig. 4c; Fig. 4d). In the HFD+ALA group, a slightly higher number of macrophages was found in the adipose tissue sections, from both the locations (Fig. 2) compared to the respective control groups (Fig. 2). The number of CD68 immunopositive cells in the HFD+ALA group was higher in both types of adipose tissue compared to HFD+NAC (Fig. 3; p=0.0358, HFD+ALA V vs. HFD+NAC V, Fig. 4c; Fig. 4d). On the other hand, in HFD+ALA the size of the adipocytes, in VAT and SAT, was slightly smaller compared to HFD+NAC (Fig. 2; p=0.0010, HFD+ALA V vs. HFD+NAC V, Fig. 4a; Fig. 4b).

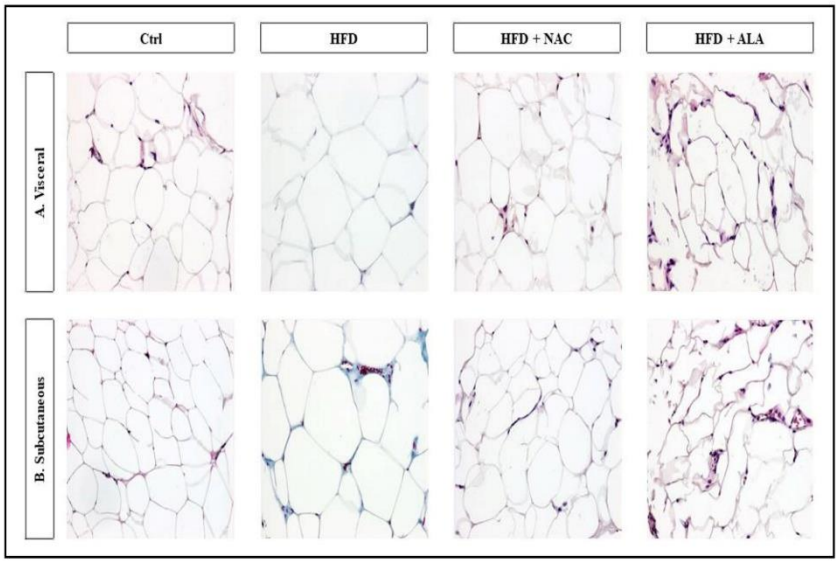

Fig. 2. Representative microphotographs of visceral and subcutaneous adipose tissue. The samples were stained with hematoxylin and eosin (H&E). Magnification: 200x. Ctrl – control rats; HFD – high-fat diet-fed rats; HFD+NAC – high-fat diet-fed rats that received N-acetylcysteine; HFD+ALA – high-fat diet-fed rats that received α-lipoic acid.

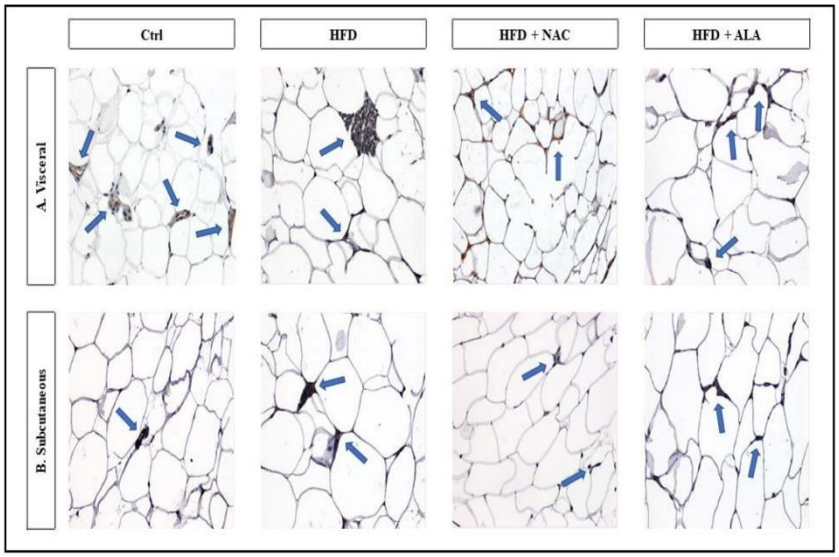

Fig. 3. Representative microphotographs of visceral and subcutaneous adipose tissue. The samples were stained with the pro-inflammatory marker (CD68). Magnification: 200x. Ctrl – control rats; HFD – rats fed a high-fat diet; HFD + NAC - rats fed high-fat diet + N-acetylcysteine; HFD + ALA – rats fed high-fat diet + α-lipoic acid.

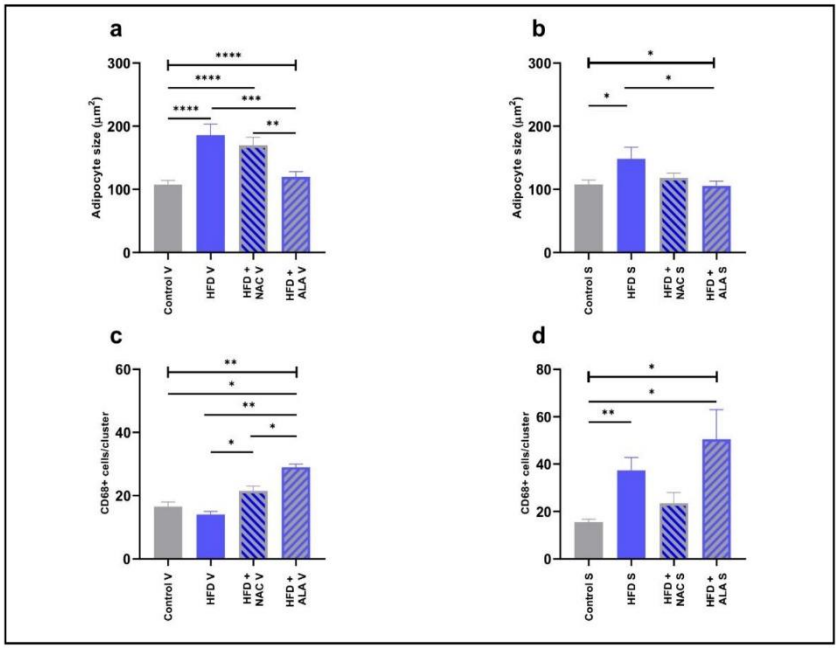

Fig. 4. The effects of NAC and ALA on: (a, b) adipocyte size and (c, d) CD68 cells number in adipose tissue. Control V—control rats, visceral adipose tissue; HFD V—high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V—high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V—high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; Control S—control rats, subcutaneous adipose tissue; HFD S—high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S—high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S—high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

The effects of NAC and ALA on the mRNA levels of MMP2 and MMP9 in the adipose tissues of HFD-fed rats

We determined MMP2 and MMP9 mRNA expression levels in VAT and SAT of the rats fed with HFD with/without antioxidants supplementation. The NAC treatment resulted in the decreased MMP9 mRNA expression in VAT and SAT (-66%, p=0.0254, Fig. 5d; -73%, p=0.0009, Fig. 5e; vs. control group; respectively). Interestingly, four-week-long administration of NAC alongside the HFD regime resulted in a significant decrease in the MMP9 mRNA expression in VAT and SAT when compared to HFD alone (-70%, p=0.0009, Fig. 5d; -92%, p=0.0150, Fig. 5e; vs. HFD group; respectively). A comparison between HFD+NAC and HFD+ALA showed a significantly higher MMP9 mRNA expression level in the SAT of the latter group (+6-fold, p=0.0162, Fig. 5e). Overall, we found greater MMP2 and MMP9 mRNA expressions in SAT compared to VAT (+97%, p<0.0001, Fig. 5c; +2-fold, p=0.0089, Fig. 5f; HFD S vs. HFD V; respectively). There was also a significant difference between the tissues in HFD+ALA with respect to MMP9 mRNA expression (+1.5-fold, p=0.0349, HFD+ALA S vs. HFD+ALA V, Fig. 5f).

The effects of NAC and ALA on protein levels of MMP2 and MMP9 in the adipose tissue of HFD-fed rats

We measured the total expression of MMP2 and MMP9 proteins in VAT and SAT in the obese rats after an HFD with/without antioxidants supplementation. In comparison to the control, the administration of the HFD resulted in an increased protein expression of MMP2 in VAT, and MMP9 in VAT and SAT (+2-fold, p<0.0001, Fig. 6a; +3-fold, p=0.0018, Fig. 6c; +2-fold, p=0.0052, Fig. 6d; vs. control group; respectively). Four-week-long administration of

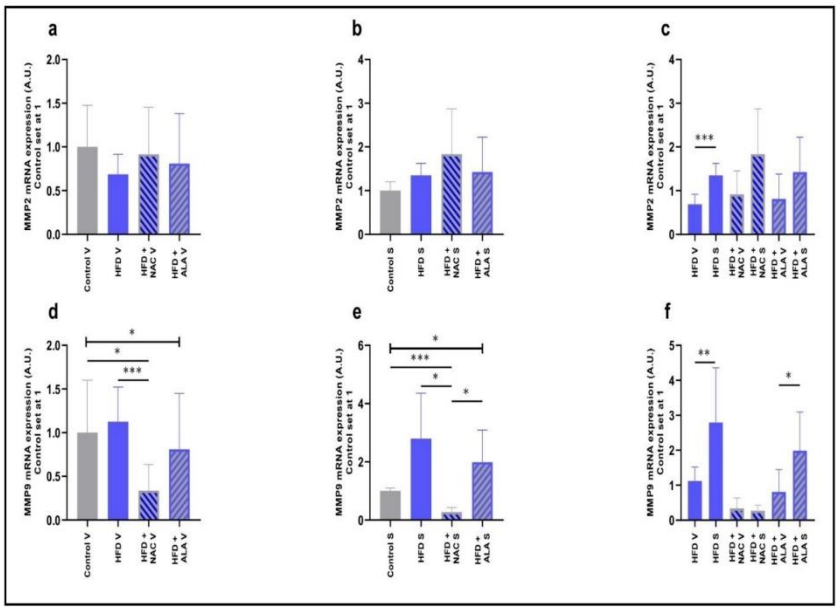

Fig. 5. The effects of NAC and ALA on: (a, b) MMP2 and (c, d) MMP9 mRNA levels in adipose tissue. The effect of NAC and ALA between tissue levels of: (c) MMP2 and (f) MMP9 mRNA. Control V – control rats, visceral adipose tissue; HFD V – high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V – high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V – high-fat diet +fed rats that received α-lipoic acid, visceral adipose tissue; Control S - control rats, subcutaneous adipose tissue; HFD S – high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S – high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S – high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; * p<0.05; ** p<0.01; *** p<0.001.

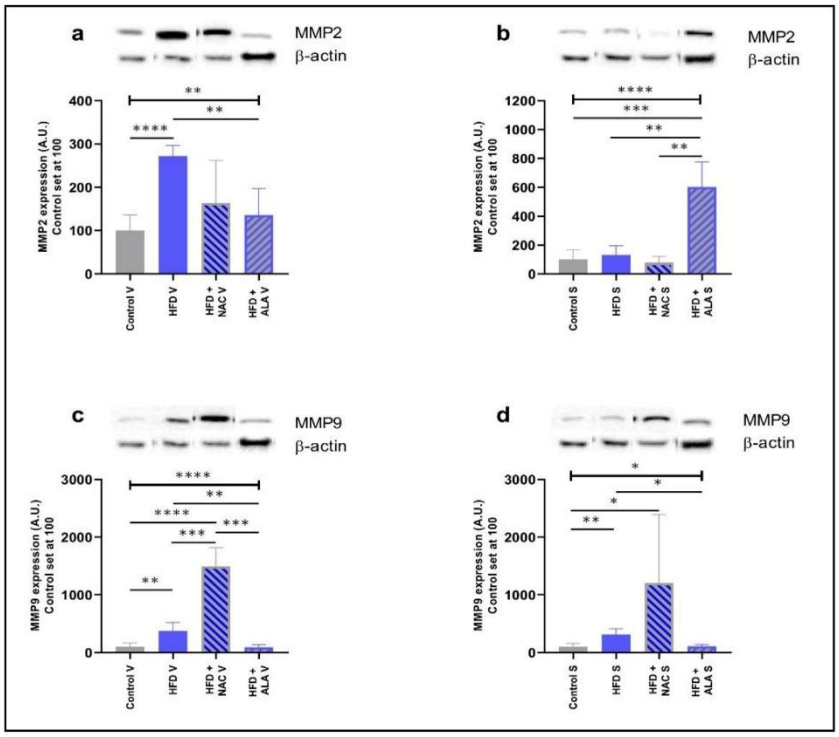

Fig. 6. The effects of NAC and ALA on (a, b) MMP2 and (c, d) MMP9 protein expressions in adipose tissue during the HFD regime. Control V - control rats, visceral adipose tissue; HFD V – high-fat diet-fed rats, visceral adipose tissue; HFD+NAC V – high-fat diet-fed rats that received N-acetylcysteine, visceral adipose tissue; HFD+ALA V – high-fat diet-fed rats that received α-lipoic acid, visceral adipose tissue; Control S - control rats, subcutaneous adipose tissue; HFD S – high-fat diet-fed rats, subcutaneous adipose tissue; HFD+NAC S – high-fat diet-fed rats that received N-acetylcysteine, subcutaneous adipose tissue; HFD+ALA S – high-fat diet-fed rats that received α-lipoic acid, subcutaneous adipose tissue; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

NAC alongside the HFD regime resulted in significant increases in the expression of MMP9 proteins in VAT and SAT when contrasted with the control (+14-fold, p<0.0001, Fig. 6c; +11-fold, p=0.0469, Fig. 6d; vs. control group; respectively). On the other hand, only MMP2 protein expression was greater in SAT when juxtaposing HFD+ALA with the control (+5-fold, p=0.0002, HFD+ALA vs. control, Fig. 6b). The administration of NAC alongside the HFD regime also resulted in a significant increase in the expression of MMP9 proteins in VAT when compared to HFD alone (+3-fold, p=0.0004, vs. HFD, Fig. 6c). Interestingly, the addition of ALA during the HFD regime led to significant changes in MMP2 (VAT: -50%, p=0.0059, Fig. 6a; SAT: +4-fold, p=0.0071, Fig. 6b; vs. HFD; respectively) and MMP9 (VAT: -76%, p=0.0061, Fig. 6c; SAT: -66%, p=0.0126, Fig. 6d; vs. HFD; respectively) protein expression. The juxtaposition of the NAC treatment with the ALA application showed a higher protein expression of MMP2 in SAT and a lower protein expression of MMP9 in VAT, of the latter group (+7-fold, p=0.011, Fig. 6b; -94%, p=0.0003, Fig. 6c; HFD+NAC vs. HFD+ALA; respectively).

Discussion

In the present study we examined, presumably for the first time in the literature, the effects of two anti-oxidative agents (N-acetylcysteine and α-lipoic acid) supplementation on matrix metalloproteinase-2 and matrix metalloproteinase-9 expression in the visceral and subcutaneous adipose tissue of the rats with obesity induced by a high-fat diet. Herein, we found that the anti-oxidants affect the matrix metalloproteinases expressions (MMP2 and MMP9), as confirmed by measurements of the genes' mRNA and protein expression levels. This indicates that NAC and ALA may promote changes in the structure of adipose tissue. One of the most remarkable findings of our research is that different anti-oxidative molecules may have a dissimilar impact on adipose tissue's phenotype, as reflected in a specter of parameters ranging from histological images to the proteins (MMP2 and MMP9) expression levels.

Chronic HFD feeding leads to the development of obesity, which is a health burden itself, but also contributes to the development of many life-threatening comorbidities, including inflammation, oxidative stress, type 2 diabetes mellitus, or insulin resistance [22]. Therefore, unsurprisingly our 10-week high-fat diet regime resulted in a significant increase in the body weight of the rats, changes in their plasma lipids content, and decreased whole-body insulin sensitivity. The existence of the last phenomenon was confirmed by significantly higher blood insulin and glucose levels as well as the HOMA-IR index found in the HFD-fed rats as compared to the control animals. NAC and ALA affect body mass, plasma glucose, insulin, and lipid profile, therefore they might become potential therapeutic agents against the onset of oxidative stress and its complications induced by HFD-feeding [23, 24]. Interestingly, the body masses of the rats from the HFD groups (with or without the antioxidants) did not differ. Still, we observed a reduction in the levels of hyperglycemia, hyperinsulinemia, and HOMA-IR in the groups with antioxidants when compared to the HFD alone. The addition of NAC and ALA restored the values of the above-mentioned parameters to the range observed in the control group.

Histological examination has shown that the HFD regime leads to an increase in the size of adipocytes (especially in SAT), an increase in the number of CD68 immunopositive cells, and an increase in the number of macrophages, especially in VAT. This is reflected in the research results obtained also by Gollisch et al. [25]. These changes are caused by adipose tissue remodeling and the penetration of immune cells into the tissue [26]. Consequently, it leads to the development of obesity, accompanied by adipocyte hypertrophy and hyperplasia [27–29]. Previous studies also reported that HFD feeding was able to induce obesity in rodents within 8 weeks [30]. A positive energy balance accompanying the development of obesity contributes to stress in the cell, production of reactive oxygen species (ROS), and inflammation [7]. Oxidative stress leads to the development of obesity by stimulating the deposition of white adipose tissue and changing the amount of food intake [31]. Studies conducted by two independent teams have shown that oxidative stress contributes to an increase in preadipocyte proliferation, adipocyte differentiation, and influences the size of mature adipocytes [32, 33]. It has also been shown that adipocytes produce huge amounts of reactive oxygen species that damage cells and their inner biological structures. Thus, they contribute to the aggravation of the already existing inflammation, creating a vicious circle of the inflammatory response and further growth of adipose tissue [34]. The use of antioxidant supplementation during the HFD regime contributed to a reduction in the size of adipocytes, an increase in the number of CD68 immunopositive cells, and an increase in the number of macrophages in VAT and SAT, compared to the HFD group, which is confirmed also by the results of other studies [23, 35–37]. Additionally, we observed histological differences between the groups with antioxidant supplementation. After ALA supplementation, we observed fewer CD68 immunopositive cells and the adipocytes were larger in comparison to the results obtained from the NAC group. Our data show that both NAC and ALA supplementation is capable of diminishing lipids accumulation in both VAT

and SAT. Interestingly, the results show that ALA supplementation has a greater therapeutic effect on the morphology of adipose tissue in both localizations.

The quantitative real-time polymerase chain reaction revealed that the NAC supplementation during application of the HFD regime led to a decreased MMP9 mRNA expression in VAT and SAT when compared to the HFD alone. We also observed significant differences in the effects of NAC and ALA supplementation during HFD on MMP9 mRNA expression in SAT. In contrast, we found no significant changes (between any of the examined groups) in MMP2 mRNA expression in VAT or SAT. Still, inter-tissue comparison of HFD revealed that VAT adipocytes had greater levels of MMP2 and MMP9 mRNA than their SAT counterparts. This is also reflected in the histological results, where we noted that in SAT, of the HFD group, adipocytes had greater size and the number of CD68 immunopositive cells (macrophages), in relation to VAT. Our findings are in agreement with previous studies [38]. Tomita et al. showed that there is a correlation between oxidative stress and VAT or MMP9 mRNA in HFD-feed rats. They also observed a lipid deposition, immunostaining of CD68, and MMP9 mRNA expression was observed in the aorta's intima in the HFD-feed group [38]. Supplementation with NAC and ALA enhanced adipocyte differentiation and expansion, showing that antioxidants may play a role in downregulating pathways associated with collagen accumulation and abnormal adipocyte growth to attenuate the pathogenic "obesity phenotype".

In many biological systems, protein expression does not follow its mRNA levels. The difference in the obtained results may also be due to the greater sensitivity of the real-time PCR method than the Western blot. Application of the HFD regime led to an increase in MMP2 protein expression in VAT, and MMP9 protein expression in VAT and SAT. Despite the application of the HFD, NAC and ALA were able to increase MMP2 and MMP9 protein levels in AT. Despite that, each of the antioxidants, NAC or ALA, supplemented during the HFD regime has various influences on MMP2 and MMP9 protein expression in VAT and SAT, compared to respective HFD groups.

Unfortunately, so far, no study results have been published, where scientists check the direct activity of MMP2 and MMP9, in the treatment's context of ALA or NAC, in an obesity model. However, Uemura et al. proved, by using the gelatin zymography and Western blot, that the activity and expression of MMP9, but not MMP2 were significantly increased in vascular tissue and plasma of two distinct rodent models of diabetes mellitus. Enhanced MMP-9 activity was significantly reduced by treatment with the NAC [39]. In other research, Liu et al. investigated the role of MMP2 in pancreatic β-cell injury induced by oxidative stress. Intracellular MMP2 expression and activity were determined by real-time PCR, Western blotting, and zymography. They proved that NAC treatment inhibited MMP2 expression and activity, and partially reversed cell apoptosis and insulin secretion [40]. Bogani et al. sought to determine whether the antioxidants as NAC or ALA affect gelatinase production and secretion. The results show that thiol compounds affect MMPs' expression and activity in different ways. MMP2 activity is directly inhibited by NAC, while ALA is ineffective. On the other hand, MMP9 expression is inhibited by ALA at a pretrascriptional level [41].

Conclusion

In the current study, we presented presumably the first report about the effects of NAC and ALA supplementation on the MMP2 and MMP9 levels in visceral and subcutaneous adipose tissue of the rats with high-fat diet-induced obesity. Moreover, we believe our study to be the first one that compares the influence of the two antioxidants on the matrix metalloproteinases expression in white adipose tissue. Our data illustrate a potential role for NAC and ALA supplementation in the modulation of matrix metalloproteinases expression in visceral and subcutaneous adipose tissue of the animals fed with an HFD. The antioxidants treatment can help to protect adipose tissue against oxidative stress by regulating MMP2 and MMP9 expression.

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Author Contributions

Conceptualization, M.W., A.C.; data curation, M.W., B.L., I.K., A.C.; formal analysis, M.W., A.C.; funding acquisition, M.W.; investigation, M.W., I.K.; methodology, M.W., A.C.; material collection, M.W.; supervision, A.C., B.L.; validation, M.W., B.L., A.C.; visualization, M.W.; writing—original draft, M.W.; writing—review and editing, A.C., B.L..

All authors have read and agreed to the published version of the manuscript.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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11. Streszczenie w języku polskim

Otyłość jest głównym czynnikiem predysponującym do rozwoju wielu powikłań, m.in. sercowo-naczyniowych, oddechowych, neurologicznych i endokrynnych. Ryzyko wystąpienia tych powikłań jest związane z ilością tkanki tłuszczowej, jej lokalizacją oraz czasem trwania choroby podstawowej. Towarzyszące otyłości zmiany strukturalne tkanki tłuszczowej, prowadzą do zmian ekspresji białek transportujących kwasy tłuszczowe i równowagi lipidowej organizmu. Transport transbłonowy kwasów tłuszczowych w tkance tłuszczowej jest wspomagany głównie przez translokazę kwasów tłuszczowych (FAT/CD36), błonowe białko wiążące kwasy tłuszczowe (FABPpm) oraz białka transportujące kwasy tłuszczowe (FATP1 i FATP4). Nadmierna masa tkanki tłuszczowej prowadzi również do rozwoju insulinooporności, poprzez nadprodukcję hormonów antagonistycznych wobec insuliny oraz bezpośrednie wydzielanie zwiększonej ilości wolnych kwasów tłuszczowych do krwi.

Metaloproteinazy macierzy pozakomórkowej, biorą między innymi udział w adipogenezie i angiogenezie. Enzymy te uczestniczą w rozwoju powikłań otyłości poprzez degradację i przebudowę cząsteczek macierzy zewnątrzkomórkowej tkanki tłuszczowej.

Długotrwały dodatni bilans energetyczny prowadzi do rozwoju otyłości oraz powstawania stresu oksydacyjnego w komórkach, nadmiernej produkcji reaktywnych form tlenu oraz nasilenia stanu zapalnego. Wykazano, że stosowanie antyoksydantów, takich jak N-acetylocysteina i kwas alfa-liponowy mają korzystny wpływ na metaboliczne powikłania otyłości. N-acetylocysteina jest pochodną cysteiny, która służy jako prekursor do syntezy zredukowanego glutationu, natomiast kwas alfa-liponowy jest niezbędnym endogennym kofaktorem kompleksu enzymatycznego biorącego udział w wytwarzaniu energii oraz inaktywacji wolnych rodników, a poprzez swoją zredukowaną formę także oddziałuje z reaktywnymi formami tlenu.

Celem pracy była ocena wpływu N-acetylocysteiny lub kwasu alfa-liponowego podczas stosowania diety bogatotłuszczowej na: wybrane parametry osocza (insulina, glukoza, FFA, DAG, TAG); histologię trzewnej i podskórnej tkanki tłuszczowej; ekspresję (na poziomie mRNA, jak i białka) transporterów kwasów tłuszczowych (FAT/CD36, FABPpm, FATP1, FATP4) oraz na następczą akumulację kwasów tłuszczowych w wybranych frakcjach lipidowych (FFA, DAG, TAG) w trzewnej i podskórnej tkance tłuszczowej; oraz ekspresję (na poziomie mRNA, jak i białka) metaloproteinaz macierzy pozakomórkowej (MMP2 i MMP9) w trzewnej i podskórnej tkance tłuszczowej.

Samce szczurów rasy Wistar podzielono losowo na cztery grupy (10 szczurów w każdej grupie): grupa kontrolna (CTRL), grupa z dietą bogatotłuszczową (HFD), grupa z dietą bogatotłuszczową i N-acetylocysteiną (HFD+NAC), grupa z dietą bogatotłuszczową i kwasem alfa-liponowym (HFD+ALA). Przy pomocy metod kolorymetrycznych i ELISA oznaczono stężenie insuliny i glukozy w osoczu. Pobrane próbki tkanki tłuszczowej poddano ocenom immunohistologicznym. Poziomu ekspresji mRNA i białek, FAT/CD36, FABPpm, FATP1, FATP4, MMP2 i MMP9, oceniono odpowiednio za pomocą metod real-time PCR i Western blot. Poziom zawartości lipidów (FFA, DAG i TAG) w osoczu i tkance tłuszczowej oszacowano przy użyciu metody chromatografii gazowo-cieczowej.

Z przeprowadzonych badań wynika, że podaż antyoksydantów podczas stosowania diety bogatotłuszczowej prowadzi do obniżenia masy ciała, poziomów glukozy, insuliny oraz lipidów w osoczu badanych szczurów. Zastosowanie antyoksydantów wpłynęło również na zmniejszenie wielkości adipocytów i ilości makrofagów oraz wzrost ilości komórek CD68 immunopozytywnych, w trzewnej i podskórnej tkance tłuszczowej. Ponadto, stosowanie antyoksydantów istotnie wpłynęło na wystąpienie zmian w ekspresji mRNA i białek białkowych transporterów kwasów tłuszczowych w obu depozytach tkanki tłuszczowej. Czterotygodniowa podaż antyoksydantów istotnie obniżyła zawartość lipidów w badanych tkankach tłuszczowych. Wykazano również, że stosowanie antyoksydantów podczas reżimu diety bogatotłuszczowej, istotnie wpłynęło na ekspresję metaloproteinaz macierzy pozakomórkowej na poziomie mRNA i białka.

Biorąc pod uwagę przedstawione wyniki można stwierdzić, że dieta bogatotłuszczowa prowadzi do istotnych zmian w obrazie histologicznym adipocytów, na co się również przekładają zmiany ekspresji białkowych transporterów kwasów tłuszczowych, lipidów oraz metaloproteinaz macierzy pozakomórkowej. N-acetylocysteina i kwas alfa-liponowy mają różny wpływ na trrzewną i podskórną tkankę tłuszczową. Stosowanie antyoksydantów może pomóc w ochronie tkanki tłuszczowej przed szkodliwym wpływem stresu oksydacyjnego, poprzez regulację ekspresji, zarówno transporterów kwasów tłuszczowych, jak i metaloproteinaz macierzy pozakomórkowej.

12. Streszczenie w języku angielskim

Obesity is the main factor predisposing to the development of many complications, including cardiovascular, respiratory, neurological, and endocrine. The risk of these complications is related to the amount of adipose tissue, its location, and the duration of the underlying disease. The structural changes in adipose tissue accompanying obesity lead to changes in the expression of fatty acid transport proteins, fatty acids transport, and lipids balance. The transmembrane transport of fatty acids in adipose tissue is promoted mainly by the fatty acid translocase (FAT/CD36), the plasma membrane fatty acid-binding protein (FABPpm), and the fatty acid transport proteins (FATP1 and FATP4). Excessive adipose tissue mass also leads to the development of insulin resistance through the overproduction of insulin antagonists and the direct secretion of increased levels of free fatty acids into the blood.

Matrix metalloproteinases are involved in adipogenesis and angiogenesis. These enzymes participate in the development of obesity complications through the degradation and remodeling of adipose tissue extracellular matrix molecules.

Long-term positive energy balance leads to the development of obesity and the formation of oxidative stress in cells, excessive production of reactive oxygen species, and intensification of inflammation. N-acetylcysteine and alpha-lipoic acid are antioxidants with well-documented beneficial effects on the metabolic complications of obesity. N-acetylcysteine is a cysteine derivative that serves as a precursor to reduced glutathione synthesis, whereas alpha-lipoic acid is an essential endogenous co-factor of an enzymatic complex involved in energy generation, as well as the inactivation of free radicals, and by its reduced form, also interacts with reactive oxygen species.

The aim of the study was to evaluate the effect of N-acetylcysteine or alpha-lipoic acid on a high-fat diet on selected plasma parameters (insulin, glucose, FFA, DAG, TAG); histology of visceral and subcutaneous adipose tissue; expression (at the mRNA and protein level) of fatty acid transporters (FAT/CD36, FABPpm, FATP1, FATP4) and the subsequent accumulation of fatty acids in selected lipid fractions (FFA, DAG, TAG) in visceral and subcutaneous adipose tissue; and expression (at the mRNA and protein level) of extracellular matrix metalloproteinases (MMP2 and MMP9) in visceral and subcutaneous adipose tissue.

Male Wistar rats were divided into four groups (10 rats per group): control group (CTRL), high-fat diet group (HFD), high-fat diet with N-acetylcysteine group (HFD + NAC), high-fat diet group with alpha-lipoic acid (HFD + ALA). Plasma insulin and glucose were determined by colorimetric and ELISA methods. The collected adipose tissue samples were

subjected to immunohistological evaluation. MRNA and protein expression levels, FAT/CD36, FABPpm, FATP1, FATP4, MMP2, and MMP9, were assessed by real-time PCR and Western blot methods, respectively. The level of lipids (FFA, DAG, and TAG) in plasma and adipose tissue was estimated using the gas-liquid chromatography method.

The conducted research shows that the supply of antioxidants during the use of a high-fat diet leads to a reduction in body weight, glucose, insulin, and lipid levels in the plasma of the tested rats. The use of antioxidants also reduced the size of adipocytes and the number of macrophages, as well as increased the number of immunopositive CD68 cells in visceral and subcutaneous adipose tissue. Moreover, the use of antioxidants significantly influenced the occurrence of changes in the expression of mRNA and protein proteins of fatty acid transporters in both adipose tissue deposits. The four-weeks long use of antioxidants significantly lowered the lipid level in the adipose tissues. It was also shown that the use of antioxidants during the high-fat diet regimen significantly influenced the expression of mRNA and proteins of matrix metalloproteinases.

In summary, we can conclude that a high-fat diet leads to significant changes in the histological images of adipocytes, which is also reflected in changes in the expression of fatty acid transporter proteins, lipids and matrix metalloproteinases. N-acetylcysteine and alpha-lipoic acid have different effects on visceral and subcutaneous adipose tissue. Using antioxidants can help protect adipose tissue from the harmful effects of oxidative stress by regulating the expression of fatty acid transporter proteins and matrix metalloproteinases.

13. Oświadczenie współautorów

The causes of insulin resistance in type 1 diabetes mellitus: is there a place for quaternary prevention?		
Autorzy	Udział w przygotowaniu publikacji	Procentowy udział
doktorantka – mgr Marta Wołosowicz	Stworzenie koncepcji pracy, wykonywanie oznaczeń, analiza i interpretacja wyników, przygotowanie rycin, zebranie piśmiennictwa, napisanie manuskryptu oraz pozyskanie finansowania.	70%
prof. dr hab. Adrian Chabowski	Stworzenie koncepcji pracy, ocena merytoryczna i korekta manuskryptu.	15%
dr n. med. Bartłomiej Łukaszuk	Ocena merytoryczna i korekta manuskryptu.	15%

Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue		
Autorzy	Udział w przygotowaniu publikacji	Procentowy udział
doktorantka – mgr Marta Wołosowicz	Stworzenie koncepcji badań, pozyskanie finansowania, wykonywanie oznaczeń, analiza i interpretacja wyników, zebranie danych, analiza statystyczna, interpretacja wyników, zebranie piśmiennictwa i napisanie manuskryptu.	65%
prof. dr hab. Adrian Chabowski	Stworzenie koncepcji badań, pozyskanie finansowania, zebranie danych, analiza statystyczna, interpretacja wyników, ocena merytoryczna i korekta manuskryptu.	11%
prof. dr hab. Irena Kasacka	Zebranie danych, interpretacja wyników i napisanie manuskryptu.	5%
prof. dr hab. Anna Zalewska	Stworzenie koncepcji badań i zebranie danych.	3%
dr n. med. Bartłomiej Łukaszuk	Zebranie danych, analiza statystyczna, interpretacja wyników, ocena merytoryczna i korekta manuskryptu.	5%
dr n. biol. Ewa Żebrowska	Zebranie danych, ocena merytoryczna i korekta manuskryptu.	4%
dr n. med. Mateusz Maciejczyk	Stworzenie koncepcji badań i zebranie danych.	3%

mgr Patrycja Dajnowicz-Brzezik	Zebranie danych oraz piśmiennictwa i napisanie manuskryptu.	4%

Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation during High-Fat Diet Regime on Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 in Visceral and Subcutaneous Adipose Tissue		
Autorzy	Udział w przygotowaniu publikacji	Procentowy udział
doktorantka – mgr Marta Wołosowicz	Stworzenie koncepcji badań, integracja danych, zebranie materiału, metodologia, wykonywanie oznaczeń, analiza i interpretacja wyników, przygotowanie rycin, pozyskanie finansowania, zebranie piśmiennictwa i napisanie manuskryptu.	70%
prof. dr hab. Adrian Chabowski	Stworzenie koncepcji badań, integracja danych, analiza wyników, metodologia, ocena merytoryczna i korekta manuskryptu.	15%
prof. dr hab. Irena Kasacka	Integracja danych, wykonywanie oznaczeń, analiza i interpretacja wyników.	5%
dr n. med. Bartłomiej Łukaszuk	Integracja danych, ocena merytoryczna i korekta manuskryptu.	10%

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Białystok, 12.05.2022

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Oświadczam, iż mój udział w przygotowaniu publikacji:

„*The causes of insulin resistance in type 1 diabetes mellitus: is there a place for quaternary prevention?*" autorów: Marta Wołosowicz, Bartłomiej Łukaszuk, Adrian Chabowski, opublikowanej w International Journal of Environmental Research and Public Health, wchodzącej w skład rozprawy doktorskiej: „Wpływ N-acetylocysteiny lub kwasu alfa-liponowego na ekspresję białkowych transporterów kwasów tłuszczowych oraz metaloproteinaz macierzy pozakomórkowej w trzewnej i podskórnej tkance tłuszczowej szczurów karmionych dietą bogatotłuszczową", wynoszący 15%, polegał na stworzeniu koncepcji pracy, ocenie merytorycznej i korekcie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie przez mgr Martę Wołosowicz publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

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Białystok, 12.05.2022

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

„*Diverse Impact of N-Acetylcysteine or Alpha-Lipoic Acid Supplementation During High-Fat Diet Regime on Fatty Acid Transporters in Visceral and Subcutaneous Adipose Tissue*" autorów: Marta Wołosowicz, Patrycja Dajnowicz-Brzezik, Bartłomiej Łukaszuk, Ewa Żebrowska, Mateusz Maciejczyk, Anna Zalewska, Irena Kasacka, Adrian Chabowski, opublikowanej w Advances in Medical Sciences, wchodzącej w skład rozprawy doktorskiej: „Wpływ N-acetylocysteiny lub kwasu alfa-liponowego na ekspresję białkowych transporterów kwasów tłuszczowych oraz metaloproteinaz macierzy pozakomórkowej w trzewnej i podskórnej tkance tłuszczowej szczurów karmionych dietą bogatotłuszczową", wynoszący 4%, polegał na zebraniu danych oraz piśmiennictwa i napisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie przez mgr Martę Wołosowicz publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Patrycja Dajnowicz-Brzezik

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Bartłomiej Łukaszuk

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