Uniwersytet Przyrodniczy we Wrocławiu
Wydział Medycyny Weterynaryjnej

PRACA DOKTORSKA

Doctoral Thesis

Analiza potencjału biologicznego ekstraktu polifenolowego ze skórek *Punica granatum* L. na szczurzym modelu zespołu metabolicznego.

Analysis of the biological potential of extract from *Punica granatum* L. peels on a rat model of metabolic syndrome.

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SPIS TREŚCI

1 STRESZCZEENIE I WYKAZ SKRÓTÓW

1.1 Wykaz skrótów

AF *atrial fibrilation*, migotanie przedsionków

AHSG *α2-Heremans–Schmid glycoprotein,* fetuina A

BMI *body mass index*, wskaźnik masy ciała

CAT *catalase,* katalaza

COX-2 *cyklooxygenase 2*, cyklooksygenaza 2

cTnI *troponin I*, troponina I

CVD *cardiovascular diseases,* choroby sercowo-naczyniowe

CYP *cytochrome P-450 enzymes*, monooksygenazy cytochromu P450

DNA *deoxyribonucleic acid,* kwas deoksyrybonukleinowy

EASD *European Association for the Study of Diabetes*, Kongres Europejskiego Towarzystwa Badań nad Cukrzycą

EFSA *European Food Safety Authority*, Europejską Agencję do Spraw Bezpieczeństwa Żywności i Żywienia

EPP *pomegranate peel extract* - ekstrakt ze skórek granatowca

FTO *fat mass and obesity associated gene*, gen związany z masą tkanki tłuszczowej i otyłością

GAL-3 *galectin 3*, galektyna 3

GPCRs *G protein-coupled receptors* receptory sprzężone z białkiem G

GPx *glutathione peroxidase,* peroksydaza glutationowa

GR *glutathione reductase,* reduktaza glutatnionowa

GST *glutathione transferase,* transferaza glutationowa

HDL *high-density lipoprotein,* lipoproteina o dużej gęstości

HFpEF *heart failure with preserved ejection fraction,* niewydolność serca z zachowaną frakcją wyrzutową

HR *heart rate,* częstotliwość pracy serca

ICAM-1 *intercellular adhesion molecule-1,* międzykomórkowa molekuła adhezyjna-1

IDF *International Diabetes Federation*, Międzynarodowa Federacja Diabetologiczna

IL *interleukin,* interleukina

iNOS *inducible nitric oxide synthase,* indukowalna syntaza tlenku azotu

LVEDV	*left ventricular end-diastiolic volume,* objętość końcoworozkurczowa lewej komory
MAFLD	*metabolic associated fatty liver disease,* choroba stłuszczeniowa wątroby
MAPK	*mitogen-activated protein kinases,* kinazy aktywowane mitogenami
MCHC	*mean corpuscular hemoglobin concentration*
MCV	*mean corpuscular volume*
MDA	*malondialdehyde*
MetS	*Metaolic Syndrome,* Zespół Metaboliczny
mFS	*midwall fractional shortening,* frakcja skracania włókien środkowych
MnSOD	*Mn-dependent superoxide dismutase,* izoforma mitochondrialna dysmutazy ponadtlenkowej
NADP	*nicotinamide adenine dinucleotide phosphate,* fosforan dinukleotydu nikotynoamino-adenionowego
NCEP-ATP III	*National Cholesterol Education Program Adult Treatment Panel III,* Trzeci Raport Narodowego Programu Edukacji Cholesteolowej
NFκβ	*nuclear factor kappa-light-chain-enhancer of activated B cells,* czynnik transkrypcyjny kappa β
Nrf2	*nuclear factor erythroid 2-related factor 2,* jądrowy czynnik transkrypcyjny pochodzenia erytroidalnego typu 2
OBS	*obstructive sleep apnea,* obturacyjny bezdech senny
PCOS	*polycystic ovary syndrome,* zespół policystycznych jajników
PGC-1α	*peroxisome proliferator-activated receptor gamma coactivator −1 α,* koaktywator receptorów aktywowanych proliferatorami peroksysomów 1α
PPAR	*peroxysome proliferator activator receptor,* jądrowe receptory aktywowane proliferatami peroksysomu
PVAT	*perivascular adipose tissue,* okołonaczyniowej tkanki tłuszczowej
RAA	*renin-angiotensin-aldosterone axis,* oś renina-angiotensyna-aldosteron
ROS	*reactive oxygen species,* reaktywne formy tlenu
SCD	*sudden cardiac death,* nagła śmierć sercowa
SOD	*superoxide dismutase,* dysmutaza ponadtlenkowa
STE	*standardized tomato extract,* wystandaryzowanego ekstraktu z pestek pomidorów
SV	*stroke volume,* objętość wyrzutowa
TG	*triglycerides,* trójglicerydy

TGF-	*trans forming growth factor* , transformujący czynnik wzrostu
TNF-α	*tumor necrosis factor α,* czynnik martwicy nowotworów
TOS	*total oxidative status*, całkowity status oksydacyjny
VCAM-1	*vascular cell adhesion molecule-1*, molekuła adhezyjna-1 komórki naczyniowej
WHO	*World Health Organization*, Światowa Organizacja Zdrowia
WOBASZ II	Wieloośrodkowe Ogólnopolskie Badanie Stanu Zdrowia Ludności w latach 2013-2014

1.2 Streszczenie w języku polskim

WSTĘP

Zespół metaboliczny (MetS) nie stanowi odrębnej jednostki chorobowej, a definiowany jest jako grupa wzajemnie ze sobą powiązanych nieprawidłowości, w skład których wchodzą otyłość typu brzusznego, hiperglikemia, insulinooporność, dyslipidemia oraz nadciśnienie tętnicze. Ich koincydencja znacznie zwiększa ryzyko rozwoju chorób układu sercowo-naczyniowego, cukrzycy typu 2, schorzeń nerek oraz wątroby. Novum w postępowaniu leczniczym jest próba implementacji związków polifenolowych do interwencji dietetycznej. Obiecującym ich źródłem zdają się być skórki granatowca stanowiące produkt uboczny przemysłu. Celem badania była ocena na modelu zwierzęcym potencjału biologicznego polifenoli pochodzących ze skórek granatowca w łagodzeniu głównych komponent zespołu metabolicznego, zwłaszcza w odniesieniu do układu sercowo-naczyniowego na modelu zwierzęcym.

MATERIAŁY I METODY

Szczurom Zucker Diabetic Faty (ZDF, fa/fa) oraz ich zdrowej kontroli (HC, fa/+) podawano ekstrakt ze skórek granatowca w dwóch dawkach: 100 mg/kg mc. oraz 200 mg/kg mc przez 8 tygodni. Wszystkie osobniki miały nieograniczony dostęp do wody oraz wysoko kalorycznej karmy Purina 5008. W trakcie trwania doświadczenia dokonano pomiarów masy ciała oraz parametrów echokardiograficznych. W celu oznaczenia morfologii, parametrów biochemicznych oraz wykonania rozmazów trzykrotnie pobrano próbki krwi. Po zakończeniu podaży ekstraktu szczury zostały poddane eutanazji, a następnie eksplantowano wycinki z serca oraz aorty. Uzyskany materiał został poddany ocenie histologicznej oraz posłużył do oznaczenia markerów stresu oksydacyjnego (CAT, SOD, MnSOD, GR, GST, GPx, TOS, SH, MDA) oraz biomarkerów niewydolności mięśnia sercowego (cTnI, GAL-3).

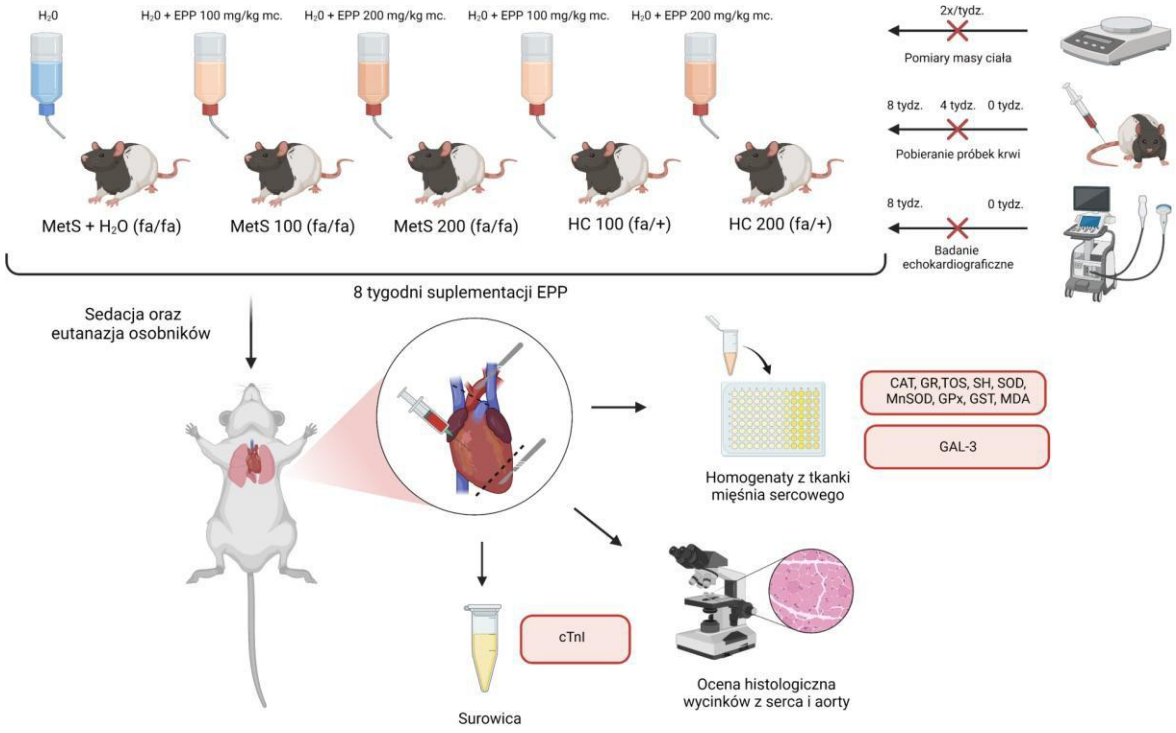
WYNIKI

Otrzymane wyniki wskazują na redukcję dynamiki przyrostu masy ciała mediowanej przez ekstrakt ze skórek granatowca (EPP). Nie wykazano wpływu EPP na profil glikemiczny oraz lipidowy. Dawka 200 mg/kg mc. w grupie osobników predysponowanych do MetS (MetS 200, fa/fa 200) wiązała się z poprawą kurczliwości mięśnia sercowego wyrażoną wzrostem frakcji skracania włókien środkowych (mFS, 95% CI: 0.69%-14.61%, p=0.032), zwiększeniem objętości końcoworozkurczowej lewej komory (LVEDV, 95% CI: 0.03-0.45, p=0.026) oraz istotnym zmniejszeniem częstotliwości pracy serca (HR, 95% CI: -79.29- - 8.38, p=0,017). W

homogenatach z mięśnia sercowego odnotowano istotny wzrost stężenia SH pod wpływem suplementacji EPP ($p<0,001$). Podaż 100 mg/kg mc. wiązała się także z istotnym zmniejszeniem TOS ($p<0.001$). Zależną od dawki redukcję stężenia MDA zaobserowoanao w grupach osobników bez genetycznej predylekcji do zespołu metabolicznego (HC 100: fa/+100, HC 200: fa/+ 200). Szczury MetS nie cechowały się podobną tendencją. W zakresie stężenia GR ($p=0,068$), SOD ($p=0,068$) oraz MnSOD ($p=0.363$) nie wykazano istotnych różnic. Uzupełnienie diety o ekstrakt nie wpłynęło także na zmiany w zakresie stężenia cTnI oraz GAL-3. Ocena histologiczna preparatów z mięśnia sercowego oraz aorty nie ujawniła toksycznego wpływu aplikowanych dawek.

WNIOSKI

Polifenole pochodzące ze skórek granatowca cechują korzystne właściwości biologiczne, zwłaszcza w zakresie protekcji układu sercowo-naczyniowego, które mogłyby zostać wykorzystane w prewencji lub leczeniu zespołu metabolicznego. Nie mniej jednak biodostępność tych związków u ludzi wymaga weryfikacji w toku badań klinicznych.



Ryc. 1 Schemat doświadczenia. Projekt został stworzony przy wykorzystaniu oprogramowania Biorender.com (dostęp 24.05.2023 r.) Skróty: EPP- ekstrakt ze skórek granatowca, mc.- masa ciała, MetS- zespół metaboliczny, fa/fa- szczury z mutacją w genie receptora leptyny predysponowane do MetS, fa/+- szczury Zucker (zdrowa kontrola), CAT- katalaza, GR- reduktaza glutationowa, TOS- całkowity status oksydacyjny, SH- grupy sulfhydrylowe, SOD- dysmutaza ponadtlenkowa, MnSOD- izoforma mitochondrialna dysmutazy ponadtlenkowej, GPx- peroksydaza glutationowa, GST-transferaza glutationowa, MDA- malonylodialdehyd, GAL-3- galektyna 3, cTnI- troponina I

1.3 Streszczenie w języku angielskim

INTRODUCTION:

Metabolic Syndrome (MetS) is not a separate disease but is defined as an array of correlative pathologies comprising central obesity, insulin resistance, hyperglycemia, dyslipidemia, and elevated blood pressure. Their coexistence rise pointedly the risk of cardiovascular diseases (CVD), diabetes mellitus, kidney failure, and liver insufficiency. Novel approach in the therapeutic management constitutes an implementation of polyphenols into the dietary intervention. Pomegranate peels, a by-product of food industry, seem to be a very promising source of these compounds. The present study aimed to evaluate the biological potency of polyphenol-rich pomegranate peel extract in mitigating the main components of metabolic syndrome, especially in the context of cardiovascular system in animal model.

METHODS:

Zucker Diabetic Fatty rats (ZDF, fa/fa) and their healthy control (fa/+) were supplemented with polyphenol-rich pomegranate peel extract (EPP) in two dosages: 100 mg/kg BW and 200 mg/kg BW for 8 weeks. All individuals had free access to water and a high-calorie diet, Purina 5008. During EPP administration performed body weight and echocardiography measurements. To asses morphology, biochemical parameters and smear image, the blood samples were collected three times. At the end of the study all individuals were euthanized and the heart and aorta sections were harvested. Obtained specimens was used in histology examination and to assess the oxidative status markers (CAT, SOD, MnSOD, GR, GST, GPx, TOS, SH, MDA) and biomarkers of heart failure (cTnI, GAL- 3).

RESULTS:

The results showed restrains the dynamic of body mass gain mediated via EPP administration. No influence on glucose and lipid profile was determined. The dose of 200 mg/kg BW in the group of rats predisposed to MetS (MetS 200, fa/fa 200) contributed to improvement of cardiac function reflected by increase in mid-wall fractional shortening (mFS, 95% CI: 0.69%-14.61%, p=0.032), enhancement of end-diastolic volume of left ventricule (LVEDV, 95% CI: 0.03-0.45, p=0.026) and relative decrease in heart rate (HR, 95% CI: -79.29- - 8.38, p=0,017). In homogenates of heart tissue the SH concentration increased significantly under the influence of EPP supplementation (p<0,001). Moreover, the extract administration in a dose of 100 mg/kg BW conduce to reduction of TOS level more efficiently compared to higher dose (p<0.001). The trend in MDA depletion in a dose-dependent manner was observed in rats without genetic

predilection to metabolic syndrome (HC 100: fa/+100, HC 200: fa/+ 200). MetS rats did not follow the same trend. No differences in in GR (P=0,068), SOD (p=0,068), and MnSOD (p=0.363) concentration were observed in groups treated with pomegranate peel extract. The EPP administration did not also affect the cTnI and GAL-3 concentration. In histology examination no toxic alternation was revealed in exposure to applied dosages.

CONCLUSION:

Phenolic compound from pomegranate peels possess health-promoting properties, chiefly in range of protection cardiovascular system and could be employ in prevention and treatment of Metabolic Syndrome. However, their bioavailability still requires further evaluation in clinical trials in humans.

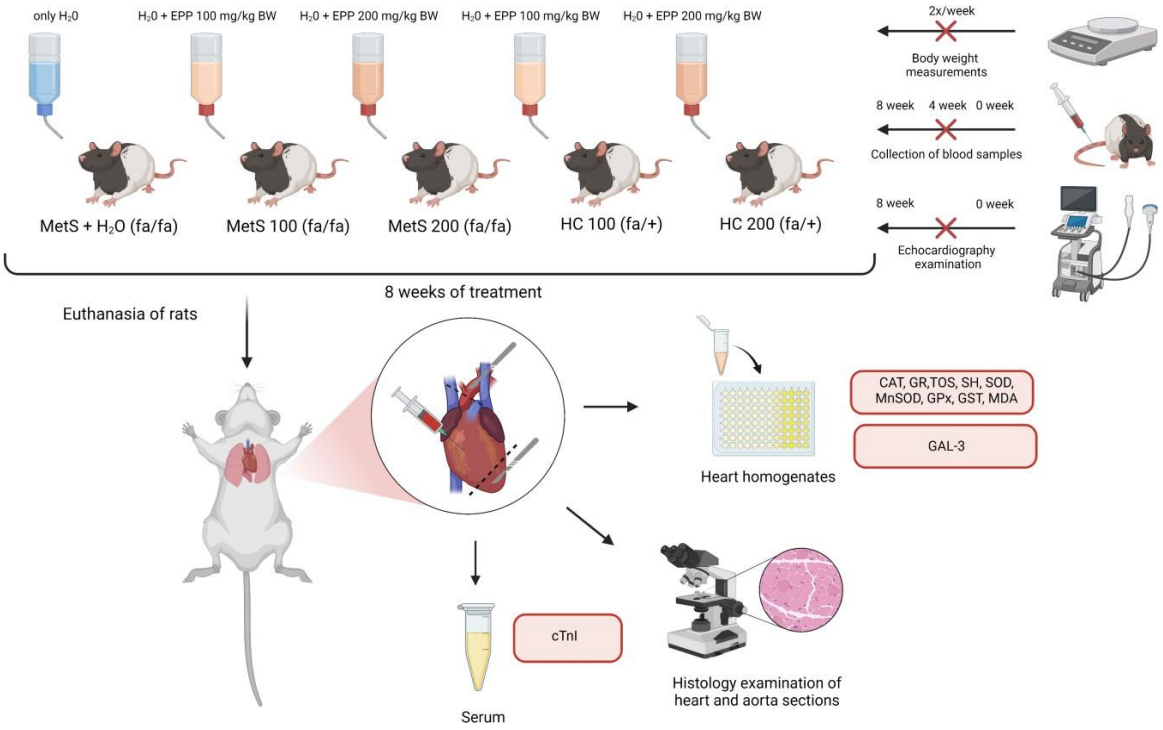


Fig. 1 Experimental design. The scheme was created with Biorender.com (accessed on 24.05.2023 r.). Abbreviations: EPP- extract from pomegranate peels, BW- body weight, MetS- metabolic syndrome, fa/fa- rats with mutation in leptin receptor gene predisposed to MetS, fa/+- Zucker rats, healthy control, CAT- catalase, GR- glutathione reductase, TOS- total oxidative status, SH- protein thiol groups, SOD- superoxide dismutase, MnSOD- Mn-dependent superoxide dismutase, GPx- glutathione peroxidase, GST- glutathione transferase, MDA- malondialdehyde, GAL-3- galectin 3, cTnI- troponin I

2 WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Niewiadomska J., Gajek-Marecka A., Gajek J. Noszczyk-Nowak A.: Biological Potential of Polyphenols in the Context of Metabolic Syndrome: An Analysis of Studies on Animal Models. Biology, 2022, *11*(4), 559. https://doi.org/10.3390/biology11040559

MNiSW: 100pkt; IF_{2022}: 5.168

Wkład w autorstwo: opracowanie koncepcji oraz metodologii, przeprowadzenie przeglądu piśmiennictwa w bibliograficznych bazach danych, analiza krytyczna źródeł, opracowanie pierwowzoru manuskryptu oraz ostatecznej jego wersji

Niewiadomska J., Kumiega E., Płóciennik M., Gajek J., Noszczyk-Nowak A.: Effects of Punica granatum L. peel extract suplementation on body weight, cardiac function, and haematological and biochemical parameters in an animal model of metabolic syndrome. Journal of Veterinary Research, 2023, 67. https://doi.org/10.2478/jvetres-2023-0031

MNiSW: 140pkt; IF_{2023}: 2.058

Wkład w autorstwo: opracowanie koncepcji, metodologii oraz przeprowadzenie eksperymentalnej części badania wraz z dbaniem o dobrostan zwierząt w trakcie doświadczenia, analiza statystyczna otrzymanych wyników, ich walidacja oraz opracowanie graficzne rezultatów, przygotowanie pierowozoru manuskryptu oraz ostatecznej jego wersji

Niewiadomska J., Kasztura M., Janus I., Chełmecka E., Stygar D.M., Frydrychowski P., Wojdyło A., Noszczyk-Nowak A.: Punica granatum L. extract show cardioprotective effects measured by oxidative stress markers and biomarkers of heart failure in an animal model of metabolic syndrome. Antioxidants, 2023, 12, 1152. https://doi.org/10.3390/antiox12061152

MNiSW: 100pkt; IF_{2023}: 7.675

Wkład w autorstwo: opracowanie koncepcji oraz metodologii, przeprowadzenie eksperymentalnej części badania wraz z dbaniem o dobrostan zwierząt w trakcie doświaczeenia, wykonanie części oznaczeń laboratoryjnych, analiza oraz walidacja otrzymanych wyników w tym opracowanie graficzne rezultatów oraz projektu badania, przygotowanie wstepnej oraz finalnej wersji manuskryptu

Sumaryczny IF cyklu prac: 14.901

Sumaryczna liczba punktów MNiSW cyklu prac: 340 pkt

3 WSTĘP

3.1 Zespół metaboliczny

Zespół metaboliczy (MetS) to stan kliniczny charakteryzujący się koincydencją otyłości typu brzusznego, zaburzeń gospodarki węglowodanowej oraz lipidowej, a także podwyższonymi wartościami ciśnienia tętniczego [1,2]. Oprócz głównych komponent obejmuje on także całe spektrum dodatkowych patologii takich jak: upośledzenie filtracji kłębuszkowej, metaboliczna choroba stłuszczeniowa wątroby (MAFLD, *metabolic associated fatty liver disease*), niewydolność serca z zachowaną frakcją wyrzutową (HFpEF, *heart failure with preserved ejection fraction*), obturacyjny bezdech senny (OBS, *obstructive sleep apnea*), oraz zespół policystycznych jajaników (PCOS, *polycystic ovary syndrome*) [1,3]. Nie stanowi oddzielnej jednostki chorobowej natomiast można go rozpatrywać w kategorii konstelacji współwystępowania wielu modyfikowalnych czynników ryzyka sercowo-naczyniowego. Pojęcie to ewoluowało na przestrzeni lat. Po raz pierwszy skojarzenie dysplipidemii, cukrzycy oraz łagodnego stopnia otyłości zostało określone mianem „zespołu plurimetabolicznego" na Kongresie Europejskiego Towarzystwa Badań nad Cukrzycą (EASD, *European Association for the Study of Diabetes*) w 1967 r. [4]. W nomenklaturze medycznej obecność zestawu metabolicznych czynników ryzyka kardiometabolicznego opisywano także pod pojęciami „zespół X", „zespół insulinooporności", „metaboliczny zespół X" czy barwny „śmiertelny kwartet" [5,6]. Nazwa „zespół metaboliczny" została wprowadzona w 1998 r. wraz z publikacją Światowej Organizacji Zdrowia (WHO, *World Health Organization*) prezentującą formalną definicję oraz ujednolicone kryteria rozpoznania [7].

Dane statystyczne wskazują na gwałtowny wzrost częstości występowania zespołu metabolicznego w ciągu ostatnich lat. Z analizy raportów badań kohortowych oraz ich metaanaliz wynika, iż może on dotyczyć nawet 10-40% populacji [8]. W Polsce przeprowadzone badanie WOBASZ II (Wieloośrodkowe ogólnopolskie Badanie Stanu Zdrowia Ludności) w latach 2013-2014 stwierdziło obecność MetS u 32.8% kobiet oraz 39% mężczyzn. Szacunkowo główną komponentą wśród kobiet była otyłość brzuszna (64.7%), natomiast u mężczyzn podwyższone wartości ciśnienia tętniczego (62%) [9]. Sukcesywny wzrost zachorowań rozpatrywany jest obecnie jako istotny problem kliniczny oraz społeczno-ekonomiczny, a udoskonalenie metod postępowania w zakresie prewencji jak i terapii może zapobiec zagrażającym życiu powikłaniom.

3.1.1 Kryteria rozpoznania

O ile panuje zgodność w zakresie podstawowych komponent MetS oraz ich związku ze wzrostem ryzyka sercowo-naczyniowego, o tyle diagnoza może być problematyczna. Wynika to z faktu współwystępowania wielu kryteriów rozpoznania oraz arbitralnych punków odcięcia. Ponadto rozbieżności w zakresie interpretacji MetS oraz mnogość stosowanych definicji utrudnia ocenę prewalencji zjawiska oraz analizę porównawczą pomiędzy różnymi krajami [8]. Pierwsza definicja zaproponowana przez WHO (1998 r.) podkreślała rolę insulinooporności jako czynnika patogenetycznego leżącego u podłoża rozwoju i eskalacji pozostałych patologii. Według niej konieczne było spełnienie dwóch założeń głównych dotyczących zaburzenia metabolizmu węglowodanów oraz następnie dwóch z pięciu kryteriów dodatkowych [7]. Popularność uzyskały także kryteria sformułowane w 2001 r. przez panel amerykańskich ekspertów Trzeciego Raportu Narodowego Programu Edukacji Cholesteolowej (NCEP-ATP III, *National Cholesterol Education Program Adult Treatment Panel III*). Raport NCEP-ATP III wyróżnił pięć składowych, a spełnienie dowolnych trzech z pięciu stanowiło o diagnozie zespołu metabolicznego. Nieprawidłowości, które wzięto pod uwagę obejmowały otyłość brzuszną, hiperglikemię, dyslipidemię aterogenną oraz podwyższone wartości ciśnienia tętniczego [10]. W 2005 r. Międzynarodowa Federacja Diabetologiczna (IDF, *International Diabetes Federation*) zaproponowała ujednoliconą delicję w wielu aspektach bazującą na kryteriach NCEP-ATP III, jednak w przeciwieństwie do nich wskazującą otyłość brzuszną jako składową *sine qua non* MetS upatrując w niej podłoża patofizjologicznego współwystępujących zaburzeń metabolicznych. Innowacyjność tych wytycznych wyrażała się również w założeniu uwzględniającym różne punkty odcięcia pomiarów obwodu pasa w zależności od pochodzenia etnicznego [11]. W Polsce w 2022 r. przedstawiono nową koncepcję zespołu metabolicznego przyjmując jako kryterium nadrzędne otyłość rozpoznaną na podstawie wartości BMI (*body mass index*) lub obwodu tali oraz dwa z trzech kryteriów dodatkowych wśród których wyróżniono nieprawidłowy metabolizm glukozy, dyslipidemię aterogenną oraz podwyższone wartości ciśnienia tętniczego [2]. Diagnostyczne narzędzia kliniczne opracowane przez różne organizacje ilustruje Tabela 1.

Tab. 1 Podsumowanie kryteriów klinicznych rozpoznania zespołu metabolicznego na podstawie wytycznych zaproponowanych przez różne organizacje.

	WHO (1998 r.)	NCEP-ATP III (2001 r.)	IDF (2005 r.)	Polska (2022 r.)
Insulinooporność	**Oporność na insulinę, IGT lub cukrzyca typu II**	-	-	-
Otyłość	BMI ≥30kg/m² lub WHR >0.90 ♂ , >0.85 ♀	otyłość brzuszna lub obwód pasa: >102 cm ♂, >88 cm ♀	**obwód pasa:** – **Europa, Bliski Wschód, Afryka: ≥94 cm ♂, ≥80 cm ♀** – **Ameryka Śr i Płd. Azja Płd., Chiny: ≥90 cm ♂, ≥80 cm ♀** – **Japonia: ≥85 cm ♂, ≥90 ♀**	**BMI ≥ 30kg/m² lub obwód pasa: ≥ 102 cm ♂, ≥88 cm ♀**
Glikemia	**IGT, IFG lub cukrzyca typu II**	≥100 mg/dl (5.6 mmol/l) lub cukrzyca typu II	≥100 mg/dl (5.6 mmol/l) lub cukrzyca typu II	≥100 mg/dl (5.6 mmol/l) lub IGT: ≥140 mg/dl po 2h OGTT lub HbA1c: ≥5.7 % lub leczenie hipoglikemizujące
TG	≥150 mg/dl (1.7 mmol/l)	≥150 mg/dl (1.7 mmol/l)	≥150 mg/dl (1.7 mmol/l)	-
cholesterol nie-HDL	-	-	-	≥130 mg/dl lub leczenie hipolipemizujące
HDL-C	<35 mg/dl (<0.90 mmol/l) ♂ <39 mg/dl (<1.00 mmol/l) ♀	<40 mg/dl (1.04 mmol/l) ♂ <50 mg/dl (1.33 mmol/l) ♀	<40 mg/dl (1.04 mmol/l) ♂ <50 mg/dl (1.33 mmol/l) ♀	<40 mg/dl (1.04 mmol/l) ♂ <50 mg/dl (1.33 mmol/l) ♀
CTK	≥140/90 mmHg	≥130/85 mmHg	≥130/85 mmHg	≥130/85 mmHg (pomiar gabinetowy) ≥130/80 mmHg (HBPM) lub leczenie hipotensyjne
Inne	mikroalbuminuria: stosunek albuminy do kreatyniny: ≥30 mg/g lub wydalanie albumin ≥20 µg/min			
Diagnoza	kryterium główne (upośledzenie metabolizmu glukozy lub oporność na insulinę) + 2 z 4 kryteriów dodatkowych	dowolne 3 z 5 kryteriów	kryterium główne (otyłość) + 2 z 4 kryteriów dodatkowych	kryterium główne (otyłość) + 2 z 3 kryteriów dodatkowych

♂- mężczyźni, ♀- kobiety, BMI- wskaźnik masy ciała, CTK- ciśnienie tętnicze krwi, HbA1c- hemoglobina glikowana, HBPM- pomiary domowe ciśnienia tętniczego, HDL-C- lipoproteina o dużej gęstości, IFG- nieprawidłowa glikemia na czczo, IGT- upośledzona tolerancja glukozy, TG- trój glicerydy, WHR- stosunek obwodu pasa do obwodu bioder

3.1.2 Patofizjologia

Szlaki metaboliczne scalające patomechanizm zespołu metabolicznego w dalszym ciągu nie zostały w sposób jednoznaczny scharakteryzowane [12]. Molekularne podłoże zespołu metabolicznego stanowi złożoną sieć interakcji pomiędzy genetycznymi, epigenetycznymi oraz środowiskowymi czynnikami. Głównymi elementami odgrywającymi rolę w patofizjologii zdaje się być rozrost tkanki tłuszczowej, insulinooporność, chroniczny stan zapalny, stres oksydacyjny, aktywacja osi renina-angiotensyna-aldosteron (RAA, *renin-angiotensin-aldosterone axis*) oraz dysfunkcja śródbłonka [13].

Jednym z głównych czynników indukujących zmiany jest otyłość typu trzewnego. Nadmierny rozrost tkanki tłuszczowej przyczynia się do zachwiania stanu równowagi organizmu oraz zapoczątkowania szeregu przemian adaptacyjnych [14]. Tkanka tłuszczowa jako całość stanowi organ endokrynny. Adipocyty wydzielają liczne substancje bioaktywne określane mianem adipocytokin, biorących udział w utrzymaniu homeostazy ustroju. Do adipocytokin, których działanie może stymulować rozwój zespołu metabolicznego, najczęściej zalicza się rezystynę, leptynę, adiponektynę, czynnik martwicy nowotworów (TNF-α, *tumor necrosis factor* α) oraz interleukinę 6 (IL-6, *interleukin 6*). Szczególną rolę przypisuje się rezystynie, której nasilone wydzielanie przez adipocyty u osób otyłych koreluje ze zwiększoną opornością komórkową na insulinę [15]. W warunkach dodatniego bilansu energetycznego oraz chronicznego stanu zapalnego dochodzi do wzrostu cytoplazmatycznego stężenia wolnych kwasów tłuszczowych uwalnianych przez trzewną tkankę tłuszczową, co z kolei skutkuje

ekotopowym gromadzeniem się lipidów w różnych kompartymentach ustroju oraz przyczynia się do rozwoju lipotoksyczności [16]. W następstwie oksydacji lipidów w mięśniach szkieletowych dochodzi do zahamowania glikolizy, upośledzenia wychwytu glukozy oraz jej utylizacji, i tym samym rozwoju insulinooporności obwodowej. Z kolei nadmiar lipidów w wątrobie związany jest z nasileniem procesów glukoneogenezy i glikogenolizy, tym samym stymulując kompensacyjne uwalnianie insuliny i prowadząc do rozwoju tak zwanej insulinooporności wątrobowej [17]. Uważa się, że akumulacja trzewnej tkanki tłuszczowej poprzedza insulinooporność, a jej rola w zespole metabolicznym związana jest z sekrecją licznych mediatrorów promujących stan zapalny. On z kolei nierozerwalnie związany jest z patogenezą miażdżycy, co stanowi pomost łączący otyłość ze wzrostem ryzyka chorób sercowo-naczyniowych .

Istotę zespołu metabolicznego jest również wspomniana insulinooporność (IR, *insulin resistance*). Insulina jest hormonem odpowiedzialnym za szereg przemian anabolicznych w ustroju m.in. nasila wchłanianie glukozy oraz wolnych kwasów tłuszczowych do komórek, hamuje lipolizę oraz stymuluje glikogenogenezę i hamuje glukoneogenezę w wątrobie. Insulinooporność jest stanem osłabionej odpowiedzi na insulinę komórek tkanek insulinozależnych (hepatocyty, miocyty, adipocyty) w konsekwencji prowadząc do kompensacyjnego wzrostu syntezy insuliny oraz ostatecznie do stanu hiperinsulinemii [16]. Wśród mechanizmów odpowiedzialnych za insulinooporność w zespole metabolicznym oprócz wspomnianej lipotoksyczności wymienia się także: upośledzoną funkcję transporterów glukozy (głównie GLUT4, *glucose transporter type 4*), rozwój leptynooporności, zmniejszenie stężenia adiponektyny, chroniczny stan zapalny związany ze wzrostem stężenia TNF-α oraz IL-6, a także zaburzenie ekspresji genów regulowanych jądrowymi receptorami aktywowanymi proliferatami peroksysomu (PPAR, *peroxysome proliferator activator receptor*) [1,13]. Za rozwój insulinooporność w przebiegu otyłości typu trzewnego odpowiada również w dużej mierze stres oksydacyjny na jaki narażone są komórki [18]. Punktem wyjścia zachwiania reakcji oksydacyjno-redukcyjnych jest nadmierny napływ substancji energetycznych prowadzący do nasilenia syntezy fosforanu dinukleotydu nikotynoamino-adenionowego (NADP, *nicotinamide adenine dinucleotide phosphate*), co z kolei skutkuje wzmożoną biosyntezą reaktywnych form tlenu (ROS, *reactive oxygen species*). Komórki broniąc się przed tym zjawiskiem zamykają drogę wstępu dla związków energetycznych, w tym m.in. dla glukozy poprzez internalizacje receptorów GLUT. Zatem narastającą insulinooporność w MetS można rozpatrywać także w kategorii mechanizmu adaptacyjnego, chroniącego komórki przed uszkodzeniem oksydacyjnym [15,19,20].

U podłoża patofizjologicznego zespołu metabolicznego leży również proces zapalny obejmujący cały organizm [1,21]. Jego wykładnikami jest wzmożona biosynteza prozapalnych cytokin do których można zaliczyć interleukinę 1 (IL-1), IL-6, TNF-α, transformujący czynnik wzrostu β (TGF-β, *transforming growth factor β*), sekrecja adipocytokin takich jak leptyna czy rezystyna oraz białek ostrej fazy wśród których można wymienić białko C-reaktywne oraz fibrynogen [22]. W nadmiarze cytokin obecnych w następstwie nasilonej czynności endokrynnej adipocytów upatruje się przyczyny stanu zapalnego, ale także stanu prozakrzepowego, upośledzonej funkcji endothelium naczyniowego oraz insulinooporności [23]. Ponadto cytokiny uwalniane z tkanki tłuszczowej przyczyniają się do zwiększenia ekspresji na powierzchni komórek śródbłonka molekuł adhezyjnych takich jak międzykomórkowa molekuła adhezyjna-1(ICAM-1, *intercellular adhesion molecule-1*) oraz molekuła adhezyjna-1 komórki naczyniowej (VCAM-1, *vascular cell adhesion molecule-1*), co nie tylko podtrzymuje toczący się proces zapalny poprzez interakcję z krążącymi makrofagami ale uważane jest również za istotny czynnik inicjujący formowanie się blaszki miażdżycowej nasilając tym samym aterogenezę [21,24].

Także zachwianie homeostazy reakcji redox w organizmie jest jedną z głównych składowych tworzących łańcuch zaburzeń patofizjologicznych prowadzących do zespołu metabolicznego. Wolne rodniki są to małe, dyfuzyjne oraz wysoce reaktywne cząsteczki o dużym potencjale cytotoksycznym oraz genotoksycznym [25]. Powstają w trakcie naturalnych procesów metabolicznych odgrywając rolę w prawidłowym funkcjonowaniu komórek m.in. pełniąc rolę bakteriostatyczną i bakteriobójczą. Jednak nadmierna ich akumulacja prowadzi do inicjacji apoptozy komórkowej, utlenienia białek, peroksydacji lipidów i tym samym zaburzenia integralności ścian komórkowych oraz uszkodzenia jądrowego i mitochondrialnego kwasu deoksyrybonukleinowego (DNA, *deoxyribonucleic acid*). Stres oksydacyjny jest uważany za przyczynę uszkodzeń narządowych, mutagenezy, kancerogenezy, dysregulacji autonomicznej oraz wielu schorzeń w tym zapalnych, neurodegeneracyjnych i autoimmunologicznych [26,27]. W zespole metabolicznym nadprodukcja adipocytokin o charakterze prozapalnym i aktywacja makrofagów infiltrujących tkankę tłuszczową w głównej mierze odpowiadają za inicjację oraz nasilenie produkcji rodników nadtlenkowych [13].

Nieprawidłowa dystrybucja oraz ilość tkanki tłuszczowej niesie ze sobą również ryzyko zaburzeń hemodynamicznych. Przyczyn upatruje się w aktywacji osi renina-angiotensyna-aldosteron (RAA), sekrecji adipocytokin o odmiennym profilu ilościowo-jakościowym (głównie leptyny, TNF-α, IL-6) oraz nadreaktywności współczulnego układu nerwowego [28–

31]. Upośledzenie ośrodkowej regulacji pomiędzy współczulnym i przywspółczulnym układem autonomicznym, z promocją pierwszego, pociąga za sobą wzrost podstawowej częstości rytmu serca, objętości krwi krążącej, komorowej objętości końcowo rozkurczowej, a także rzutu serca, które to bezpośrednio bądź pośrednio poprzez pętlę sprzężenia zwrotnego z osią RAA mogą sprzyjać rozwojowi systemowego nadciśnienia tętniczego [32]. Ponadto aktywacja układu RAA w warunkach insulinooporności jest związana z retencją sodu skutkując wzrostem objętości wewnątrznaczyniowej co również jest przyczyną spoczynkowego ciśnienia tętniczego. Wzrost stężenia aldosteronu może zostać spotęgowany w wyniku nasilenia ekspresji genu angiotensynogenu w tkance wisceralnej. Przypuszcza się, że aktywacja układu RAA wynika ze stymulacji sekrecji angiotensynogenu w adipocytach w warunkach dużego stężenia adipokin o charakterze prozapalnym [33]. To właśnie lokalna stymulacja RAA w tkance tłuszczowej *per se* może być krytycznym czynnikiem odpowiedzialnym za rozwój nadciśnienia w przebiegu otyłości oraz zespołu metabolicznego.

Ponadto poszukując molekularnych przyczyn podłoża patogenetycznego zaburzenia wielosystemowego jakim jest zespół metaboliczny podkreśla się również rolę wzrostu stężenia fetuiny A (AHSG, *α2-Heremans–Schmid glycoprotein*), dysfunkcji mitochondrialnej, zmniejszonej aktywności koaktywatorów receptorów aktywowanych proliferatorami peroksysomów 1 α (PGC-1α, *peroxisome proliferator-activated receptor gamma coactivator −1 α*) oraz krążącego microRNA (miRNA) [34–37]. Ze względu na rosnącą prewalencję występowania zespołu metabolicznego wśród dzieci i młodzieży pod uwagę bierze się także predyspozycję genetyczną. Dotychczas raportowano wiele loci genów, które mogą wiązać się ze zwiększeniem ryzyka zaburzeń metabolicznych. Na szczególną uwagę zasługuje m.in. gen związany z masą tkanki tłuszczowej i otyłością (FTO, *fat mass and obesity associated gene*) zlokalizowany w rejonie chromosomalnym 16q12 oraz odmienności w ekspresji dwóch podtypów receptorów sprzężonych z białkiem G (GPCRs, *G protein-coupled receptors*), które mogą wynikać z imprintingu genetycznego [38,39]. Warto podkreślić także rolę metabolomiki. Faktem jest wpływ różnych amin na profil kardiometaboliczny. Wykazano związek pomiędzy patogenezą zespołu metabolicznego, a obecnością takich aminokwasów jak alanina, arginina, asparagina, cysteina, histydyna, glutamina, kwas asparaginowy, kwas glutaminowy, lizyna, metionina [40]. Zaangażowane są one m.in. w patogenezę insulinooporności (np. alanina). Ich profil możnaby wykorzystać jako czynnik predykcyjny wystąpienia cukrzycy typu 2 przed jej pojawieniem się u pacjentów z zespołem metabolicznym [41].

3.1.3 Zmiany w układzie sercowo-naczyniowym

Zmiany w układzie sercowo-naczyniowym są rozległe i skutkują rozwojem kardiomiopatii, zaburzeniem funkcji śródbłonka oraz uszkodzeniem mikrokrążenia [32,42]. Każda komponenta zespołu metabolicznego jest niezależnym i modyfikowalnym czynnikiem ryzyka kardiometabolicznego, a ich współwystępowanie prowadzi do znacznego wzrostu prawdopodobieństwa wystąpienia poważnego incydentu sercowo-naczyniowego. Badania wskazują na niezaprzeczalny związek zespołu metabolicznego z progresją miażdżycy, a także wzrostem odsetka ostrych zespołów wieńcowych czy niewydolności serca [43].

Kardiomiopatia występująca w przebiegu zespołu metabolicznego określana jest mianem związanej z otyłością, wyzwalanej insulinoporonością, lipotoksycznej, cukrzycowej lub metabolicznej [44]. Charakteryzuje się koncentryczną hipertrofią lewej komory mięśnia sercowego oraz upośledzoną kurczliwością miokardium. Remodeling mięśnia sercowego w wyniku toczącego się procesu zapalnego oraz upośledzenia mikrokrążenia wieńcowego w przebiegu otyłości prowadzi do zmniejszenia podatności mięśnia sercowego, przeciążenia objętościowego oraz dysfunkcji rozkurczowej [45,46]. Skutkuje to wystąpieniem niewydolności serca z zachowaną frakcją wyrzutową (HFpEF, *heart failure with preserved ejection fraction*), która koreluje ze zwiększonym ryzykiem zgonu i hospitalizacji [47]. W następstwie uszkodzenia kardiomiocytów upośledzeniu ulega również transmisja impulsów elektrycznych w układzie bodźcoprzewodzącym, której źródła upatruje się w zmianach w funkcjonowaniu kanałów jonowych, co może leżeć u podłoża rozwoju arytmii, zwłaszcza migotania przedsionków (AF, *atrial fibrilation*) czy zespołu długiego QT [48].

Zmiany czynnościowe w mikrokrążeniu dotyczą łożysk naczyniowych przede wszystkim występujących w sercu, mózgu, nerkach oraz w mięśniach szkieletowych [32]. Wynikają głównie z upośledzonej kontroli oporu naczyniowego i napięcia miogennego oraz zaburzenia homeostazy pomiędzy czynnikami promującymi wazokonstrykcję i wazodylatację. W zespole metabolicznym, występujące nadciśnienie tętnicze prowadzi także do dośrodkowej, eutroficznej przebudowy ściany naczyniowej polegającej na odmiennej organizacji przestrzennej komórek budujących naczynia krwionośne oraz zmniejszeniem ich światła [49]. Obecne jest również zjawisko przerzedzenia naczyń, zmniejszające rezerwę naczyniową wykorzystywaną w warunkach zwiększonego zapotrzebowania metabolicznego oraz tym samym upośledzające perfuzję obwodową [50]. Hipoksja tkanek w przebiegu zespołu metabolicznego wykazuje związek z uszkodzeniem kłębuszków nerkowych, zwłóknieniem

miąższowym macierzy zewnątrzkomórkowej różnych narządów, chorobą obwodową naczyń, zawałem serca oraz udarem niedokrwiennym [32].

Związek pomiędzy zespołem metabolicznym a miażdżycą nie budzi wątpliwości [51]. Przyśpieszona progresja zmian miażdżycowych w zespole metabolicznym powoduje znaczne zwiększenie globalnego ryzyka sercowo-naczyniowego [32]. W przypadku blaszki miażdżycowej zwężającej światło nasierdziowej tętnicy wieńcowej dochodzi do stopniowego spadku rezerwy wieńcowej, wynoszącej w prawidłowych warunkach nawet 300-600%, i tym samym pogłębiającego się niedokrwienia. Początkowo wzrost oporu generowany przez zwężenie kompensowany jest spadkiem oporu mikrokrążenia za zwężonym odcinkiem naczynia. Gdy zwężenie osiągnie wartość krytyczną (średnica światła tętnicy ulegnie zmniejszeniu o 80%, a pole przekroju o 90%) przepływ wieńcowy nie wystarcza do pokrycia wydatku energetycznego mięśnia sercowego, a konsekwencją nagłego zaburzenia równowagi między podażą, a zapotrzebowaniem na tlen może być zawał serca, który jest stanem zagrożenia życia [52]. Szacuje się, że przyczyną około 50% nagłej śmierci sercowej (SCD, *sudden cardiac death*) są nadżerki blaszki miażdżycowej prowadzące do powstawania materiału zatorowego stanowiącego przyczynę dysfunkcji mikrokrążenia [53,54]. Przyśpieszenie zmian miażdżycowych w zespole metabolicznym związane jest głównie z ekotopowym gromadzeniem się tkanki tłuszczowej naciekającej rejony okołonaczyniowe oraz uszkodzeniem śródbłonka naczyniowego, a czynnikami indukującymi zmiany są adipokiny [51,55]. Cytokiny uwalniane z okołonaczyniowej tkanki tłuszczowej (PVAT, *perivascular adipose tissue*) stanowią most molekularny łączący otyłość z chorobą naczyń, a do najważniejszych uczestniczących w etiopatogenezie miażdżycy należą leptyna, rezystyna, adiponektyna, wisfatyna oraz omentyna [56].

3.2 Związki polifenolowe

Polifenole są wtórnymi metabolitami roślinnymi o bardzo zróżnicowanej budowie, masie cząsteczkowej oraz właściwościach biologicznych [57]. Stanowią one jedne z najbardziej rozpowszechnionych substancji czynnych pochodzenia roślinnego obejmując grupę ponad 8 000 różnych związków [58]. Wspólną cechą polifenoli jest występowanie pierścienia benzoesowego połączonego z różną liczbą grup hydroksylowych. Bazując na strukturze chemicznej można je podzielić na cztery główne klasy: kwasy fenolowe, flawonoidy, stilbeny oraz lignany [59]. W świecie roślin ich wzmożona synteza następuje w warunkach stresu

abiotycznego pełniąc tym samym rolę naturalnej odpowiedzi obronnej przed zróżnicowanymi czynnikami takimi jak wirusy, bakterie, grzyby czy promieniowanie UV.

Spektrum bioaktywności tych związków jest szerokie. Przede wszystkim cechują się aktywnością antyoksydacyjną. Mechanizm działania przeciwutleniającego jest plejotropowy i uwarunkowany głównie obecnością dużej liczby grup hydroksylowych w cząsteczce [60]. Aktywność ta wynika z ich właściwości redukcyjnych jako dawców elektronów lub atomów wodoru, zdolności do wiązania wolnych rodników, inhibicji oksydaz (np. oksydazy ksantynowej, oksydazy NADPH), chelatacji jonów metali katalizujących reakcje utlenienia (np. Cu^{2+}, Fe^{2+}, Co^{2+}, Ti^{3+}, Cr^{5+}) oraz wsparcia enzymatycznej i nieenzymatycznej bariery antyoksydacyjnej [61]. Przez wzgląd na właściwości przeciwutleniające, polifenole były przedmiotem licznych badań pod kątem ich potencjalnego wykorzystania w prewencji chorób, w których etiopatogenezie stres oksydacyjny odgrywa główną rolę. Do takich schorzeń zalicza się m.in. choroby układu sercowo-naczyniowego, cukrzycę typu II, choroby neurodegeneracyjne oraz nowotwory.

Drugim filarem ich prozdrowotnego działania są właściwości przeciwzapalne. Wynikają one z modulacji ekspresji genów oraz komórkowych szlaków sygnałowych poprzez wpływ na aktywność czynników transkrypcyjnych. Na poziomie molekularnym hamują one czynnik transkrypcyjny kappa β (NFκβ, *nuclear factor kappa-light-chain-enhancer of activated B cells*) i kinazy aktywowane mitogenami (MAPK, *mitogen-activated protein kinases*), które odpowiadają za syntezę cytokin prozapalnych, a aktywują jądrowy czynnik transkrypcyjny pochodzenia erytroidalnego typu 2 (Nrf2, *nuclear factor erythroid 2-related factor 2*), który z kolei odpowiada za syntezę enzymów antyoksydacyjnych [61,62]. W ten sposób polifenole promują korzystny profil cytokinowy bazujący na zmniejszeniu uwalniania interleukin prozapalnych (Il-1 β, TNFα, IL-6, IL-8) oraz aktywności enzymów promujących reakcję zapalną (COX-2, *cyklooxygenase 2* oraz iNOS, *inducible nitric oxide synthase*), a wzroście czynników wyciszających stan zapalny (m.in. IL-10, IL-13) [63].

Polifenole cechują się także właściwościami przeciwalergicznymi, wazoprotekcyjnymi, kardioprotekcyjnymi, hepatoprotekcyjnymi, nefroprotekcyjnymi, neuroprotekcyjnymi, immunomodulującymi, przeciwalergicznymi, przeciwmutagennymi, antyangiogennymi oraz przeciwdrobnoustrojowymi [58]. Lista ich prozdrowotnych korzyści wciąż jest uzupełniana.

3.2.1 Rola związków polifenolowych w MetS

Możliwość wykorzystania biologicznie aktywnych naturalnych związków pochodzenia roślinnego w terapii chorób cywilizacyjnych cieszy się coraz większym zainteresowaniem, a praktyczny aspekt ich wdrożenia obejmuje m.in. fortyfikację (wzbogacenie) żywności, suplementy diety w postaci standaryzowanych ekstraktów oraz model odżywiania się oparty o produkty cechujące się dużą zawartością tych substancji. Przede wszystkim wynika to z faktu ich wielokierunkowego prozdrowotnego działania oraz bezpieczeństwa stosowania w porównaniu do syntetycznych farmaceutyków [64].

MetS jako schorzenie wieloukładowe wymaga interdyscyplinarnego oraz holistycznego podejścia. Postępowanie wstępne bazuje przede wszystkim na zmianie stylu życia, w tym m.in. interwencji dietetycznej [2]. Modyfikacja nawyków żywieniowych stanowi podstawę, która wpływa korzystnie na wszystkie składowe MetS. Badania naukowe dostarczają dowodów na słuszność implementacji związków polifenolowych do algorytmu terapeutycznego ze względu na ich bioaktywność pozwalającą na kontrolę współistniejących zaburzeń metabolicznych [65]. W tym aspekcie podkreśla się znaczenie aktywności antyoksydacyjnej, przeciwzapalnej, hipoglikemicznej, hipolipemizującej oraz ochronny wpływ na śródbłonek naczyniowy. Profil aktywności tych substancji obejmuje także w swym zakresie działanie antyaterogenne ograniczając nasilenie zmian miażdżycowych w naczyniach wieńcowych oraz obwodowych i tym samym prowadząc do zmniejszenia ryzyka kardiometabolicznego [66].

Kompozycja związków polifenolowych wykazuje rozbieżności w zależności od materiału roślinnego z którego zostały pozyskane. W odniesieniu do MetS dane literaturowe podają skuteczność zarówno złożonego składu polifenolowego występującego w winogronach, czerwonym winie, zielonej herbacie, oliwie z oliwek, jagodach czy żurawinie jak i wyekstrahowanych pojedynczych związków tj. naringenina, kurkumina, punicalagina, kwas kawowy oraz resweratrol [67,68].

O istotności klinicznej związków polifenolowych może świadczyć zaaprobowanie standaryzowanej kompozycji polifenolii bergamoty w terapii dyslipidemii przez Sekcję Farmakoterapii Sercowo-Naczyniowej Polskiego Towarzystwa Kardiologicznego w 2018 r. oraz wystandaryzowanego ekstraktu z pestek pomidorów (STE, *standardized tomato extract*) jako środka utrzymującego prawidłową agregację trombocytów przez Europejską Agencję do Spraw Bezpieczeństwa Żywności i Żywienia (EFSA, *European Food Safety Authority*) w 2009 r. [69,70].

3.2.2 Potencjał biologiczny ekstraktu ze skorek *Punica granathum* L.

Granatowiec właściwy (*Punica granathum* L.) to długowieczna roślina należąca do rodziny krwawnicowatych (*Lythraceae*). Na stanowiskach naturalnych występuje przede wszystkim na terenie Azji Środkowej (państwa Bliskiego Wschodu oraz Zakaukazia) i Półwyspu Indyjskiego, choć obecnie jej uprawa jest kultywowana na całym świecie. Nadal jednak głównymi eksporterami owoców pozostają Indie oraz Iran, a więc obszary jej rodzimego pochodzenia [71]. Poszczególne części rośliny stanowią bogate źródło rozmaitych związków. W owocach występują flawonoidy, antocyjaniny, fenolokwasy, a także garbniki i taniny. Olej z pestek zawiera kwas punicynowy oraz fitoestrogeny. Sok oraz skórka bogate są w liczne taniny, flawonoidy oraz katechiny. Kora i korzenie zawierają garbniki, terpeny, fenolokwasy i alkaloidy m.in. peletierynę oraz izopeletryninę działające przeciwpasożytniczo [72]. Właściwości terapeutyczne granatowca były znane już w starożytności gdzie głównie upatrywano w nim remedium na zaburzenia gastroenterologiczne. Współcześnie podkreśla się, iż ze względu na aktywność farmakologiczną zawartych w nim związków polifenolowych, garantowiec może być skuteczny w leczeniu wielu schorzeń, do których należą m.in. choroby sercowo-naczyniowe, neurodegeneracyjne, endokrynologiczne, autoimmunologiczne czy nowotworowe [73].

Skórka z granatowca jest produktem ubocznym powstającym w trakcie przemysłowej produkcji soku. Stanowi ona niemal 50% masy całego owocu i do niedawna była traktowana jedynie w kategorii bioodpadu. Jednak przeprowadzone w ostatnich latach badania udowodniły, że w samej skórce występują liczne prozdrowotne fitozwiązki do których należą m.in: polisacharydy, kwasy fenolowe (kwas elagowy, kwas galusowy, kwas kofeinowy), hydrolizujące taniny (ellagitaniny: punikalagina, punikalina; gallotaniny), falwonoidy (epikatechiny, gallokatechiny), antocyjanidyny oraz aktywne biologicznie metabolity np. powstałe po biotransformacji przez mikrobiotę jelitową z ellagitanin, urolityny [74]. Bogaty skład polifenolowy odpowiada za silne właściwości antyoksydacyjne, przeciwcukrzycowe, przeciwzapalne, przeciwpłytkowe, immunomodulujące, przeciwmiażdżycowe, przeciwnadciśnieniowe, przeciwdrobnoustrojowe, hipolipemizujące, przeciwosteoporotyczne, hepatoprotekcyjne oraz neuroprotekcyjne. Ponadto, polifenole zawarte w skórce granatowca cechują się aktywnością przeciwmutagenną oraz przeciwkancerogenną m.in. w odniesieniu do nowotworów jelita grubego, wątroby, piersi, prostaty, białaczki czy osteosarcomy [75]. Warto podkreślić także szeroki indeks terapeutyczny badanych ekstraktów, a zatem niską toksyczność i niewielkie ryzyko działań niepożądanych związanych z ich podażą. Jednak wiedza na temat ich famakokientyki oraz farmakodynamiki wciąż wymaga zgłębienia, a ingerencja

w liczne szalki metaboliczne niesie ryzyko groźnych powikłań w przypadku przyjmowania równocześnie innych substancji leczniczych np. poprzez inhibicję cytochromów CYP450 głównie CYP2C9 oraz CYP3A4 mogą zmieniać biodostępność i toksyczność innych leków, co wymagałoby weryfikacji przed wprowadzeniem standaryzowanych ekstraktów na rynek [76]. Ze względu na rosnącą stale ilość odpadów pochodzenia biologicznego istotnym zarówno dla środowiska jak i dla zdrowia ludzkości zdaje się być właściwa polityka zarządzenia ich gospodarką [77]. Odzysk bioodpadów w procesie recyklingu organicznego np. w charakterze nutraceutyków jest dobrym kierunkiem, który z jednej strony mógłby dostarczyć wartościowych surowców w interwencjach terapeutycznych różnych schorzeń, z drugiej natomiast ograniczyć obciążenie ekonomiczne związane z koniecznością ich utylizacji.

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5 CELE BADANIA

W związku z potwierdzeniem skuteczności ekstraktu ze skórek granatowca w ograniczaniu nasilenia poszczególnych komponent zespołu metabolicznego wysunięto hipotezę o jego efektywności również w przypadku ich współwystępowania.

Do głównych celów badania należało:

1. Określenie ilościowego oraz jakościowego profilu fitochemicznego związków polifenolowych otrzymanego ekstraktu ze skórek *Punica granatum* L. (odmiana Mollar de Elche) za pomocą układu LC/ESI-Qq-ToF składającego się z chromatografu cieczowego Aquity UPLC sprzężonego z spektrometrem mas typu kwadrupol z analizatorem czasu przelotu (Qq-ToF). Poszczególne związki fenolowe ekstraktu zostały scharakteryzowane na podstawie czasu retencji oraz mas molekularnych
2. Ocena wpływu ekstraktu ze skórek granatowca na pomiary masy ciała, wyniki morfologii i obraz rozmazu krwi obwodowej oraz profil glikemiczny i lipidowy
3. Analiza bioaktywności ekstraktu ze skórek granatowca (tożsama z działaniem kardioprotekcyjnym) w układzie sercowo-naczyniowym w oparciu o :
    a. badanie echokardiograficzne
    b. ocenę wybranych markerów stresu oksydacyjnego w homogenetach z mięśnia sercowego
    c. stężenie biomarkerów uszkodzenia/niewydolności serca (cTnI- w surowicy, GAL-3- w homogenatach z tkanki serca)
    d. ocenę histopatologiczną wycinków z lewej komory mięśnia sercowego oraz aorty wstępującej

6 MANUSKRYPT I

Niewiadomska J., Gajek-Marecka A., Gajek J. Noszczyk-Nowak A.: Biological Potential of Polyphenols in the Context of Metabolic Syndrome: An Analysis of Studies on Animal Models. Biology, 2022, *11*(4), 559. https://doi.org/10.3390/biology11040559

6.1 Streszczenie manuskryptu I

Zespół metaboliczny (MetS) jest nieprawidłowością patofizjologiczną o złożonej etiologii. Kryteria diagnostyczne definiują go jako koincydencję kilku zaburzeń metabolicznych do których należą: otyłość centralna, zaburzenia gospodarki węglowodanowej, dyslipidemia aterogenna oraz nieprawidłowe wartości ciśnienia tętniczego. Wraz z rozpowszechniającą się epidemią otyłości zwłaszcza w coraz młodszych grupach społecznych, rośnie także częstość występowania MetS, który aktualnie rozpatrywany jest jako problem cywilizacyjny.

Postępowanie terapeutyczne bazuje przede wszystkim na zmianie stylu życia, szczególną rolę przypisując zdrowemu modelowi odżywiania oraz poziomowi aktywności fizycznej. Przeprowadzono wiele badań, których celem było poszukiwanie nowych substancji, które stosowane jako dodatek do żywności mogłyby zmniejszać nasilenie, a nawet przyczynić się do regresji aktualnie występujących powikłań poprzez jednoczasowe oddziaływanie na wszystkie komponenty MetS. Obiecującymi naturalnymi związkami w tym kontekście zdają się być związki polifenolowe ze względu na ich właściwości antyoksydacyjne i przeciwzapalne oraz rolę jaką pełnią w normalizacji parametrów gospodarki węglowodanowej oraz lipidowej. W związku z tym upatruje się w nich potencjalnego *remedium* w profilaktyce i leczeniu MetS oraz zapobieganiu jego progresji w kierunku poważniejszych schorzeń.

Badanie zostało przeprowadzone w oparciu o bazy bibliograficzne PubMed oraz ScienceDirect. W PubMed publikacje filtrowano według następujących słów kluczowych: wybrane „animal models" AND „metabolic syndrome"AND („polyphenols" OR „phenols" OR „flavonoids" OR „falvonols"). W ScienceDirect badania wyselekcjonowano stosując pojęcia: wybrane „animal models" AND „polyphenols" AND „metabolic syndrome". Pod uwagę wzięło prace opublikowane w języku angielskim w latach 2000-2021. Dla każdego modelu zwierzęcego wyszukiwanie przeprowadzono oddzielnie w obu wyszukiwarkach baz danych.

Analiza wykazała, iż nie istnieje uniwersalny związek lub ekstrakt polifenolowy, który w sposób kompleksowy oddziaływałby na wszystkie składowe MetS. Większość z tych nich wpływa korzystnie na profil lipidowy oraz glikemię (czerwone wino, zielona herbata, qwecetyna, granatowiec, masa kakaowa, cynamon kurkumina). Niektóre wywierają efekt hipotensyjny (resweratrol, czerwone wino) lub ich stosowanie związane jest z hepatoprotekcją (zielona herbata, granatowiec, masa kakaowa, cynamon, kurkumina), nefroprotekcją (masa kakaowa), kardioprotekcją (czerwone wino, granatowiec, resweratrol) oraz modulacją mikrobioty (masa kakaowa). Niemniej jednak, ich zastosowanie w postaci żywności funkcjonalnej lub wystandaryzowanych pod względem składu suplementów mogłoby wpłynąć korzystnie na poprawę stanu zdrowia ludzi.

Słowa kluczowe: zespół metaboliczny, polifenole, modele zwierzęce

biology

MDPI

Review

Biological Potential of Polyphenols in the Context of Metabolic Syndrome: An Analysis of Studies on Animal Models

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Simple Summary: Polyphenols are a family of widespread organic compounds occurring in heterogeneous plants. The antioxidative properties of polyphenols are widely known. However, they also possess unquestionably anti-inflammatory, antiatherosclerotic, anti-microbial, antiallergic, immunomodulatory, anti-carcinogenic, and antimutagenic activity. As evidenced by recent studies, diets abundant in polyphenol-rich plant materials induce positive effects in preventing and treating non-communicable chronic diseases, such as metabolic syndrome, which is currently recognized as a crucial public health problem. This study aims to review the literature on the role of polyphenols in the aspect of metabolic syndrome in studies on animal models and highlight the most promising micronutrients. Positive results in this field can lead to the development of innovative functional food products, which can contribute to the health improvement of the population.

Abstract: Metabolic syndrome (MetS) is a disease that has a complex etiology. It is defined as the co-occurrence of several pathophysiological disorders, including obesity, hyperglycemia, hypertension, and dyslipidemia. MetS is currently a severe problem in the public health care system. As its prevalence increases every year, it is now considered a global problem among adults and young populations. The treatment of choice comprises lifestyle changes based mainly on diet and physical activity. Therefore, researchers have been attempting to discover new substances that could help reduce or even reverse the symptoms when added to food. These attempts have resulted in numerous studies. Many of them have investigated the bioactive potential of polyphenols as a "possible remedy", stemming from their antioxidative and anti-inflammatory effects and properties normalizing carbohydrate and lipid metabolism. Polyphenols may be supportive in preventing or delaying the onset of MetS or its complications. Additionally, the consumption of food rich in polyphenols should be considered as a supplement for antidiabetic drugs. To ensure the relevance of the studies on polyphenols' properties, mechanisms of action, and potential human health benefits, researchers have used laboratory animals displaying pathophysiological changes specific to MetS. Polyphenols or their plant extracts were chosen according to the most advantageous mitigation of pathological changes in animal models best reflecting the components of MetS. The present paper comprises an overview of animal models of MetS, and promising polyphenolic compounds whose bioactive potential, effect on metabolic pathways, and supplementation-related benefits were analyzed based on in vivo animal models.

Keywords: polyphenols; laboratory animal models; metabolic syndrome

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1. Introduction

Metabolic syndrome (MetS), a risk factor for cardiovascular diseases (CVD) and type 2 diabetes, affects a significant part of the population worldwide, with a prevalence of 10–30%. It is a clustering of interrelated metabolic disorders, which include insulin [1–6] resistance, central obesity, hypertriglyceridemia, lowered HDL cholesterol concentration, and hypertension [1,2]. MetS has had different criteria over the years, mainly associated with distinguishable definitions of abdominal obesity, with the World Health Organization (WHO), the National Cholesterol Education Program Adult Treatment Panel (NCEP/ATP111), the American Association of Clinical Endocrinologists (AACE), and the European Group for Study of Insulin Resistance (EGSIR) all proposing their own diagnostic criteria [3,4]. Finally, in 2005, the International Diabetes Federation (IDF) provided a standardized consensus. The proposed definition includes waist circumference as a precondition for the identification of MetS and embraces the standard features of the previous definition, such as the assessment of triglyceride (TG) level, high-density lipoprotein cholesterol (HDL), blood pressure, and fasting glucose [5,6].

Metabolic pathways comprising the pathomechanism of MetS have not yet been clearly characterized. However, this is a tedious process due to the wide range of different pathophysiological mechanisms needing to be considered. Evidence suggests that various factors may predispose one to the development of MetS, such as genetics, diet, lifestyle, and gut microbiome [7,8]. A syndrome, which is more of a clinical term than a disease entity, suggests an association with other disorders. The current research shows that MetS predisposes one to cardiovascular dysfunctions via, e.g., atherosclerotic changes [9–11] and type 2 diabetes [12]. The correlation with other disorders is based on oxidative stress's role in the pathomechanism. Studies have indicated that there may be an association between MetS and Parkinson's disease [13], obstructive sleep apnea [14], and the progression and development of different cancers, such as colon cancer or gastric cancer [15–17]. Treatment is mainly based on lifestyle changes involving increased physical activity and a balanced diet. Researchers are currently looking for new substances that could significantly mitigate the severity and progression of MetS symptoms by affecting the metabolic pathways involved in the MetS pathophysiology. Numerous studies based on animal models have demonstrated the existence of a relationship between the intake of polyphenol-rich products and the mitigation of individual components of MetS. A beneficial effect was obtained via a reduction in body weight, blood pressure, blood glucose levels, and improved lipid metabolism.

The aim of this study is to review the contemporary literature on the role of polyphenols in the aspect of metabolic syndrome in studies on animal models, which provide information that cannot be obtained in humans.

1.1. Pathophysiology of the Metabolic Syndrome

The main molecular changes in MetS result from a complex interaction between genetic and environmental factors. Visceral adipose tissue endocrine mediation, insulin resistance, and hypertension have been included as pathophysiological elements. The literature also highlights the contribution of endothelial dysfunction, systemic inflammation, and oxidative stress to MetS pathogenesis. However, it is difficult to identify individual pathophysiological mechanisms due to overlapping changes, where one pathology generates the next one, which determines yet another, and so forth.

1.1.1. Obesity

One of the main change-inducing factors is visceral obesity. An excess of adipose tissue contributes to the disruption of the body's homeostasis and the initiation of adaptive changes [18]. The state of positive energy balance and low-grade chronic inflammation leads to increased plasma FFA levels, which result in ectopic lipid storage and lipotoxicity. It is believed that the accumulation of visceral adipose tissue precedes the development of insulin resistance, and its role in MetS is associated with the secretion of numerous

inflammatory mediators. Inflammation, in turn, is inextricably linked to the pathogenesis of atherosclerosis, forming a link between obesity and increased risk of CVD [18]. Adipose tissue as a whole is an endocrine organ. Adipocytes secrete numerous bioactive substances called adipocytokines, which maintain systemic homeostasis. An altered profile of adipocytokines may stimulate the development of MetS and play a crucial role in cellular dysfunction. The cytokines that predominantly contribute to abnormalities are resistin, leptin, adiponectin, TNF-α, and IL-6. A unique role is played by resistin, whose increased secretion by adipocytes in obese individuals correlates with increased cellular insulin resistance [19].

1.1.2. Insulin Resistance

The core element of MetS is insulin resistance (IR). Insulin resistance decreases the ability of various organs, for example, the liver, skeletal muscle, or adipose tissue—to respond to insulin [20]. Insulin regulates a wide range of biological processes by the activation of two crucial post-receptor transduction signaling cascades, PI3K (phosphatidylinositide 3 kinase) and RAS-MAPK (mitogen-activated protein kinase) [21]. PI3K cascade activation is responsible for the insulin effect on the metabolism by adjusting the activity of transcription factors responsible for cell proliferation and apoptosis. This pathway in vascular endothelium enhances nitric oxide production, inducing vasodilatation [22,23]. The RAS-MAPK signaling cascade plays a role in cell growth and proliferation and results in vasoconstriction [24,25]. The molecular alterations caused by insulin resistance are based on the downregulation of the PI3K pathway and the upregulation of RAS-MAPK [26,27]. In MetS, the insulin resistance that seems to emerge from positive energy balance is mainly caused by the oxidative stress to which cells are exposed. An excessive intake of energy-providing substances leads to an increased synthesis of nicotinamide adenine dinucleotide phosphate (NADP), which promotes the biosynthesis of reactive oxygen species (ROS). As a means of defense, cells block the entry of energy-providing compounds, including, for example, glucose. Therefore, it may be concluded that insulin resistance in MetS serves as an adaptive mechanism protecting cells from potential damage related to the excessive generation of free radicals, thus exacerbating pathological changes in carbohydrate metabolism [2,19,28].

1.1.3. Free Radicals

Free radicals are small, diffusible, and highly reactive molecules marked by cytotoxic and genotoxic effects. The production of reactive oxygen species (ROS) is many associated with dysfunctional homeostasis, though some of these, called bioradicals, originate from the physiological process [17,29]. The excess accumulation of free radicals leads to chronic inflammation and an imbalance in cellular apoptosis and proliferation via the altered hyper- or hypo-activation of some cellular signaling pathways [17]. In MetS, visceral obesity contributes to the overproduction of adipokines [30,31]. Abnormal adipokine levels yield a persistent increase in systemic inflammation, and the infiltration of macrophages in visceral adipose tissue has collectively been indicated as a possible factor enhancing reactive oxygen species production. ROS contribute directly to autonomic balance dysregulation and, in turn, to inadequate blood pressure control [32,33].

1.1.4. Renin–Angiotensin–Aldosterone System (RAA)

The cardiovascular system is also subject to alterations related to MetS. Essential roles in hemodynamic pathophysiology are played by the activation of the renin–angiotensin–aldosterone system [34]; differing levels of adipocytokine secretion—i.e., leptin, tumor necrosis factor (TNF-α), and interleukin 6 (IL-6) [35,36]; and the hyperactivity of the sympathetic nervous system [37,38]. The hyperactivity of the sympathetic nervous system alone contributes to an increase in heart rate, circulating blood volume, ventricular end-diastolic volume, and cardiac output, which can directly—or indirectly via a feedback loop with the RAA system—lead to the development of hypertension [10]. The activation of the

RAA system in the insulin resistance state is closely related to sodium retention, which leads to increased intravascular volume. However, an increase in the aldosterone serum level may also occur due to the upregulation of the angiotensinogen gene in adipose tissue. Studies suggest that increased proinflammatory adipokine secretion contributes to RAA activation by stimulating angiotensinogen production in adipocytes. The local stimulation of RAA in visceral adipose tissue may be critical in the pathogenesis of hypertension in obesity and metabolic syndrome [34,39].

1.2. Cardiovascular Consequences

The changes that occur in the cardiovascular system are extensive and lead to cardiomyopathy, microcirculation damage, and endothelial function impairment [9,10]. Each component of MetS is an independent risk factor for cardiovascular diseases. Studies indicate an association between MetS and the elevated risk of atherosclerosis, myocardial infarction, and heart failure. Obesity-associated cardiomyopathy is characterized by concentric left ventricular hypertrophy and systolic or diastolic dysfunction. In addition, myocardial contractility, systolic velocity, and left ventricular shortening have been proven to be impaired [40,41]. Changes in microvascular tone and density are attributable to the non-equilibrium between oxygen delivery and tissue metabolism in miscellaneous vascular beds. These alternations in MetS are mainly caused by the significant variations existing in the control of arteriolar resistance [42]. Endothelial dysfunction is associated with decreased nitric oxide bioavailability in the setting of MetS. The underlying mechanism contributing to endothelial pathology is the augmented production of vasoconstrictors, including endothelin-1 (ET-1), thromboxane A_2 (TXA_2), and prostaglandin H_2 (PGH_2) [42]. The progression of atherosclerosis occurs over the years and is strongly correlated with age. The relation between the metabolic abnormalities occurring in MetS and atherosclerotic disease is undoubted. MetS leads to accelerated and more advanced atherosclerotic disease, which is correlated with a greater incidence of myocardial infractions. The adipose-derived hormones and adipokines released from fat depots, including perivascular adipose tissue, are considered to be the core of the pathological process and thought to mediate vascular calcification [43,44].

1.3. Polyphenols

Polyphenols are the most widespread bioactive compounds derived from plants. The basic monomer forming these secondary metabolites is a phenolic ring. According to their diverse chemical structures, polyphenols are classified into two major groups, flavonoids and nonflavonoids, as well as many subsequent groups [45,46]. The most widely known polyphenol substances include phenolic acids, flavonoids, stilbenes, lignans, and phenolic alcohols. Fruits and beverages constitute the core sources of polyphenolic compounds. Plants contain mixtures of polyphenols. They are considered to play a crucial role in adapting plants to their environment. In addition, they represent a significant source of bioactive pharmaceuticals [47]. Their health-promoting properties are mainly attributed to their antioxidant activity. However, polyphenols also possess pronounced anti-inflammatory, antiatherosclerotic, antiallergic, anti-microbial, anti-carcinogenic, and antimutagenic activities [48]. Considering that chronic progressive inflammation is a feature of MetS, polyphenols appear to be promising dietary supplements for preventing the progression of the disease and minimizing the effects of MetS.

1.4. Usability of Animal Models in MetS Research

As the MetS pathophysiology is involved plenty of genetic variations and environmental factors, it is troublesome to discover one by one particular underlying components in such a complex system as a human being. Scientists have established many animal models that mimic the metabolic complications in humans to look for a solution. They are fundamental in discovering the genetic fundamentals of diverse parameters, characteristic of MetS, and their dependence on each other. Given the same environment, we can explore

how certain conditions impact metabolic changes and analyze the effect of environment on phenotype. In vivo models render genetic and environmental factors controlled, adjusted, and monitored in organisms of known origin. Understanding pathophysiology in animal models is crucial for implementing appropriate therapies and prevention strategies. Animal subjects, providing stable conditions, are thought to be small-scale follow-up research embracing relevant human studies [49,50].

2. Methods

Numerous studies on animal models have been conducted to identify the mechanisms of polyphenols' impact on biochemical pathways in MetS. Multiple compounds have been investigated, including quercetin, epigallocatechin gallate, naringenin, resveratrol, and extracts obtained from polyphenol-rich foods. The study aims to point out the natural polyphenols or polyphenols themselves that are of the utmost importance on the course of the disease. Studies on selected animal models mirroring the components of MetS were taken into consideration.

2.1. Search Strategy

This research was carried out in the PubMed and ScienceDirect search libraries. The search keywords used in PubMed were: "selected animal model" and "metabolic syndrome" and ("polyphenols" or "phenols" or "flavonoids" or "flavonols"). The search terms used in ScienceDirect were as follows: "selected animal model" and "polyphenols" and "metabolic syndrome"; filtered: years: 2000–2021; article type: research articles. For each animal model, the search in both databases was conducted separately. Only papers in English were analyzed. The time interval considered comprised the years 2000 to 2021.

2.2. Inclusion Criteria

1. The research performed on preferable animal models in the context of metabolic syndrome (Zucker Fatty Rat (fa/fa), Zucker Diabetic Fatty Rat, Spontaneously Hypertensive Rat (SHR), animal models with induced diabetes/obesity/hypertension (pathophysiological changes playing an essential role in metabolic syndrome)).
2. The presence of a control group and the comparison between the polyphenol's intervention group and the placebo group were incorposed. The administration route and the dosage of polyphenols were not restricted.

2.3. Exclusion Criteria

1. Studies without indicating a statistically significant difference between the control and experimental group.
2. Papers with incomplete data and duplicated publications.
3. Studies on animal models differed from those mentioned in the inclusion criteria.

The studies were selected from 695 publications found. Two independent researchers reviewed all eligible papers (JN and AGM). Based on the results obtained for each animal model, the most promising substances were chosen.

3. Animal Model of Obesity Zucker Fatty Rats (fa/fa)

Laboratory ZF rats are used in human disease studies as a model of obesity with accompanying hyperlipidemia and hypertension. While this model is most widely used in studies on genetic obesity, ZF rats are also used in studies on MetS and non-insulin-dependent obesity-related diabetes. ZF rats are characterized by a recessive mutation in the leptin receptor gene (called "fa"), which leads to polyphagia, with the consequent development of obesity at around four weeks of age. The causes of obesity in ZF rats also include hypertrophy and adipocyte hyperplasia, which are linked to their genetic predisposition. Other conditions observed in ZF rats include hyperinsulinemia and impaired glucose tolerance, which do not lead to overt diabetes [51,52].

Studies conducted using this animal model have shown that many polyphenolic substances have potentially beneficial metabolic effects in extracts or individual compounds.

3.1. Red Wine

Grapes and red wine are rich sources of phenolic acids, flavonols, quercetin, (+)-catechin, dihydroflavonols, anthocyanins, catechins, and stilbenes [53,54]. Red wine polyphenols were first noticed as very useful with the identification of the theory called the "French Paradox". This theory pointed out that the high amount of red wine polyphenols consumed by the French every year is responsible for the comparatively low level of coronary heart disease (CHD) among the French population [55,56]. The French concept later became the reason for investigating the role of red wine constituents as cardioprotective factors. Following these findings, scientific studies proved their protective action in the vascular system. Moreover, compounds from red wine protect against cerebrovascular incidents [57]. Additionally, in vitro studies evidenced that the supplementation of red wine polyphenols reduced inflammation and NADPH oxidase activity and increased endothelial nitric oxide production [58,59]. In vivo, animal studies seem to confirm these findings. An animal model study investigated dietary supplementation with red wine polyphenol extract on metabolic, circulatory, and vascular changes. The analysis found that the polyphenol extracts improved glucose metabolism by reducing serum glucose levels and improved lipid profiles by lowering triglyceride and LDL cholesterol levels. In turn, echocardiographic measurements showed an increase in fractional shortening and cardiac output. The analysis also showed an increase in nitric oxide (NO) bioavailability associated with increased endothelial NO-synthase (eNOS) activity and, consequently, a reduction in peripheral arterial resistance. In turn, the decreased expression of NADPH oxidase inhibited the release of superoxide anions [60]. The decline in vascular tone is probably linked to the modulation of the expression of cyclooxygenase (COX) and COX-derived vasoconstrictive agents via a mechanism that involves the NF-κB pathway. The vasoprotective effect of the dietary supplementation of red vine polyphenols is also associated with a reduction in the release of vasoconstrictive factors such as thromboxane-A2 and 8-isporostone [61,62].

3.2. Green Tea

Green tea is a rich source of catechins, including epigallocatechin gallate (EGCG), an organic chemical compound belonging to the polyphenol family. Green tea extract, as well as EGCG alone, were analyzed in studies on ZF rats to verify their impact on weight, lipid profile, and glucose metabolism. One study investigated the protective effects and molecular mechanisms of action of green tea polyphenols in non-alcoholic fatty liver disease (NAFLD). In that study, pathological metabolic changes in hepatocytes identical to those seen in humans with NAFLD were induced in ZF rats by a high-fat diet. A decrease in body weight and a statistically significant reduction in visceral fat (31.0%, $p < 0.01$) were observed in rats treated with green tea polyphenols compared with controls. Moreover, significant decreases in fasting insulin, glucose, and lipid levels were observed. The observed reduction in hepatic lipogenesis was linked to the upregulation of the AMPK pathway [63]. In another study, an intraperitoneal injection of green tea catechin extract, mainly containing EGCG, was found to reduce food intake and body weight and lead to a number of changes in the endocrine system, including a reduction in the blood levels of testosterone, estradiol, leptin, insulin, IGF-1, LH, glucose, cholesterol, and triglycerides. This experiment was performed on Sprague Dawley and Zucker Fatty rats. Similar effects were observed in both groups, suggesting that the effect of EGCG on appetite control is independent of leptin. The effective dose of EGCG was approximately 30–50 mg/kg BW. The loss in body weight was reversible. When the administration of EGCG was stopped, the rats regained their weight [64]. The beneficial effect of green tea polyphenols on weight gain attenuation, the reduction in visceral fat accumulation, and the decline in insulin level and fasting serum glucose may be associated with molecular changes in the expression of insulin signaling protein in skeletal muscle. The polyphenols from green tea administered

to Zucker Fatty (ZF) rats fed a high-fat diet at a dose of 200 mg/kg of body weight for 8 weeks resulted in lower insulin resistance. Immunoblotting revealed that the expression and translocation of glucose transporter-4 were enhanced in skeletal muscle. The insulin-stimulated glucose uptake by isolated muscle in ZF rats treated with green tea polyphenols increased as well. Moreover, a decrease in the activation of the inhibitory protein kinase isoform, PKC-θ, which is muscle-specific, was also observed. This outcome shows that the effects of polyphenols from green tea may be associated with the impact on skeletal muscle insulin sensitivity [65]. The ingestion of green tea polyphenols can ameliorate the metabolic abnormalities linked with MetS and promote favorable molecular effects.

3.3. Quercetin

Quercetin is a bioflavonol found in numerous plant foods, such as beans, red onion, lettuce, broccoli, citrus, tea, wine, and herbs. It is believed that quercetin has significant antioxidant potential and thus shows protective effects concerning osteoporosis, cardiovascular disease, neuropathy, and even some types of cancer, for example, breast cancer, lung cancer, and colon cancer [66–70]. Moreover, it is characterized by anti-inflammatory, antiobesity, antihyperlipidemic, antihypercholesterolemic, neuroprotective, antihypertensive (via vasodilator effects), and antiatherosclerotic properties [67,71,72]. The role of quercetin in organism metabolism is assumed to be mediated via the activation of transcription factors such as PPAR-γ, AMPk, NF-κB, or SIRT1 [69,73]. A daily quercetin dose of 2 mg/kg BW or 10 mg/kg BW administered to obese ZF rats for ten weeks resulted in reduced dyslipidemia, hypertension, and insulin resistance. However, only the use of the higher dose reduced body weight and produced anti-inflammatory effects by lowering TNF-alpha production in visceral adipose tissue. The decline in body weight was associated with an increase in the plasma concentration of adiponectin, reduced levels of which are observed in obesity, type 2 diabetes, and hypertension. The vasoprotective effects of quercetin were shown to be mediated by an increase in eNOS expression [74].

4. Animal Model of Diabetes: Zucker Diabetic Fatty (ZDF) Rats

Laboratory ZDF rats are derived from the Zucker Fatty strain. A spontaneous mutation that occurred in ZF rats resulted in a diabetic phenotype. The inbreeding of ZF rats carrying the desired mutation led to the development of a new strain called the Zucker Diabetic Fatty strain. The cause of the genetic defect leading to impaired beta-cell function is not clear. However, a number of changes in the expression of pancreatic islet genes have been described, including a reduced expression of the GLUT2 transporter and increased activity of glucokinase and hexokinase. ZDF rats have higher insulin resistance and are less obese compared with the parental strain [51]. Male ZDF rats develop diabetes at eight weeks of age, which is associated with changes in the morphology of pancreatic islets. Female rats do not develop overt diabetes, despite their significant level of insulin resistance, except when they are fed a high-fat diet [75]. In male ZDF rats, diabetes-related cataract is observed at 15 weeks of age, first as angiogenesis changes at the periphery of the lens, which progress to the development of a mature cataract at 21 weeks of age [76,77]. Laboratory ZDF rats provide, in particular, a suitable animal model of type 1 and type 2 diabetes and its complications, including retinopathy, cardiomyopathy, and diabetic nephropathy. In addition, due to its specificity (hyperglycemia, hyperinsulinemia, impaired glucose metabolism), the strain can be used in studies on MetS [49].

The polyphenolic compounds analyzed in studies conducted using this animal model include pomegranate extracts and cocoa flavonols.

4.1. Pomegranate

The pomegranate (Punica granathum L.) is a long-lived plant that belongs to the Lythraceae family. Its various parts are rich sources of a variety of compounds. Pomegranate seed oil contains punicinic acid (a polyunsaturated fatty acid) and phytoestrogens. The juice and peel are rich in numerous polyphenolic compounds, especially tannins and flavonoids.

Pomegranate tannins include ellagitannins, such as punicalagin and punicalin, whereas pomegranate flavonoids include, in particular, anthocyanins and flavonols. Moreover, pomegranate juice and peel contain numerous catechins. In turn, pomegranate bark and roots are sources of alkaloids, including pelletierine and isopelletierine, which are used in folk medicine as anthelmintics. Pomegranate polyphenols have antioxidant properties, as they indirectly inhibit inflammatory markers. They also present anti-carcinogenic effects [78,79]. Pomegranate extracts seem to have a beneficial effect on changes characteristic of MetS, as confirmed by the findings from the available studies. ZDF rats treated with pomegranate flower extract (500 mg/kg, p.o. × six weeks) had milder symptoms of diabetes- and obesity-related hepatic steatosis (lower liver weight, lower triglyceride levels, and lower lipid droplet content) compared with controls. This was due, at least in part, to the enhanced expression of genes associated with the oxidation of fatty acids, including peroxisome proliferator-activated receptors (PPAR-α), acyl-CoA oxidase, and carnitine palmitoyltransferase-1 [80]. A 6-week treatment with pomegranate extract (500 mg/kg p.o.) was also found to reduce the collagen deposit area in the left ventricle as well as the perivascular collagen deposit areas. The reduction in cardiac fibrosis was mediated by the modulation of the endothelin-1 (ET-1) and nuclear factor kappa B (NF-κB) pathways. The diminished cardiac fibrosis was accompanied by reduced hyperglycemia and hyperlipidemia [81]. In another study on ZDF rats with insulin resistance and hyperlipidemia, *Punica granathum* extract reduced cardiac triglyceride accumulation and decreased circulating triglyceride and cholesterol levels. In that study, the improvement in cardiac lipid metabolism was mediated by the activation of, e.g., PPAR-alpha [82].

4.2. Cocoa

The cocoa tree (*Theobroma cacao* L.) is a tree in the family *Malvaceae* native to the forests of South and Central America. Its seeds, commonly known as cocoa, are used in many food products. The chemical composition of cocoa includes numerous polyphenolic compounds, including proanthocyanins, catechins, flavan-3-ols, and anthocyanins. It is currently being investigated whether it may play a significant role as a dietary intervention to reduce cardiovascular risk in type 2 diabetes, given that it reduces plasma lipid levels and promotes the production of nitric oxide (NO) [83]. Recent studies have shown that cocoa polyphenols also have beneficial effects on carbohydrate metabolism. In one study, male ZDF rats fed a cocoa powder-rich diet (10%) for ten weeks showed improved glucose tolerance and insulin resistance. Moreover, the consumption of a diet rich in cocoa products had protective effects against diabetes-induced structural alterations in the kidneys. The antihyperglycemic effects of cocoa, protecting against diabetic nephropathy, were mediated through the inhibition of the synthesis of gluconeogenic enzymes, i.e., phosphoenolpyruvate-carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase), and glucose transporters (i.e., sodium-glucose-co-transporter-2 (SGLT-2) and glucose-transporter-2 (GLUT-2)) in the renal cortex [84]. In both in vivo (ZDF rats) and in vitro (HepG2 cells) models, cocoa flavonols, especially (−)-epicatechin, improved lipid metabolism by reducing body weight gain and lipid accumulation in liver cells [85]. Cocoa intake also improves the gut microbiota via interactions that may contribute to its antidiabetic effect. Male ZDF rats fed with 10% cocoa presented more acetate-producing bacteria and had a reduced amount of lactate-producing bacteria compared to the lean group. The modified gut microbiota was associated with an improvement in glucose homeostasis and intestinal integrity and with a reduced expression of pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-a) or interleukin-6 (IL-6), in the colon of rats [86].

5. Animal Model of Hypertension: Spontaneously Hypertensive Rat (SHR)

SHR rats were produced by outbreeding Wistar–Kyoto rats, followed by the selective inbreeding of their offspring with the highest blood pressure values. These rats are mainly used in studies on cardiovascular diseases, especially hypertension, metabolic diseases that lead to insulin resistance, hypertriglyceridemia, hyperinsulinemia, hypercholesterolemia,

and renal dysfunction. SHR rats develop hypertension at 6–7 weeks of age, reaching a stable level with accompanying insulin resistance and hyperinsulinemia at 12 weeks of age. The development of hypertension in these rats is probably mediated by the renin–angiotensin axis and the activity of the sympathetic nervous system [49,51]. SHR rats do not develop hypercholesterolemia and hyperlipidemia, except where a specific dietary regimen induces these changes. For many years, these rats have been used in studies on heart failure due to the changes that occur in their cardiac muscle, including the progressive hypertrophy of the left ventricle, which is evident over the first 9 months of life and progresses to systolic dysfunction by 12 months of age. A variation of SHR rats is SHR stroke-prone (SHRSP) rats. SHRSP rats develop malignant hypertension and die from stroke within a few weeks [51].

As this animal model is well suited for assessing the impact of a given substance on the cardiovascular system, one of the most exciting polyphenols studied using SHR rats is resveratrol, a compound with cardioprotective properties. The characterization of metabolic changes in genetic animal models of metabolic syndrome is presented in Table 1.

Table 1. The characterization of metabolic changes in genetic animal models of metabolic syndrome.

Strain	Mutation/Genetic Background	Metabolic Changes	Model	References
Zucker Fatty rats (ZF)	missense mutation on the leptin receptor gene (fa/fa)	1. obesity 2. hypertension 3. hyperinsulinemia 4. insulin resistance 5. hypercholesterolemia 6. hypertriglyceridemia	obesity, type II diabetes, MetS	[51,52]
Zucker Diabetic Fatty rats (ZDF)	non-functional leptin receptor (selective in-bread rat strain)	1. obesity 2. hypertension 3. hyperinsulinemia 4. insulin resistance 5. hyperglycemia 6. hypercholesterolemia 7. hypertriglyceridemia	type I and II diabetes, MetS	[49,51,75–77]
Spontaneously Hypertensive rats (SHR)	-	1. hypertension 2. hyperinsulinemia 3. insulin resistance	hypertension, heart failure, renal dysfunction	[49,51]

Resveratrol

Resveratrol is an organic chemical compound that is a member of a polyphenol family called viniferins. It is present in numerous plants, such as eucalyptus, mulberry, peanut, bilberry, strawberry, grape, rhubarb, and cranberry [87]. Grapes are considered one of the most abundant sources of resveratrol, as they have the highest levels of this compound, with their concentration in peel and seeds reaching 50–100 μg/g [88]. This stilbene derivative exhibits significant biological potential. In natural conditions, resveratrol is produced by some plants in response to injury. The available studies on this topic have shown that it has estrogenic, anti-inflammatory, cerebroprotective, cardioprotective, anti-angiogenic, antioxidant, and anti-cancer properties [88,89]. The effects of resveratrol are concentration-dependent, as it acts as a chemoprotective agent at a specific dose and promotes apoptotic cell death when used at a higher concentration [90]. In one study, the administration of resveratrol in drinking water to SHR rats for 10 weeks reduced the development of hypertension, as indicated by the lower blood pressure of the treated rats compared with the controls. Resveratrol-treated rats showed reduced hydrogen peroxide (H_2O_2) content and increased superoxide dismutase activity, thus reducing their oxidative stress. Moreover, the rats displayed the normalization of endothelium-dependent vasorelaxation [91]. Resveratrol may also prevent hypertension-induced cardiac dysfunction. In one experiment, a ten-week treatment with resveratrol significantly reduced concentric hypertrophy and systolic

dysfunction in SHR rats. The cardioprotective effects of resveratrol are probably mediated by a lowering of oxidative stress levels in the cardiac muscle tissue [92]. Resveratrol may also play a role in electrophysiological alternations via its influence on chromaffin cells and Ca^{2+} signaling, exerting antihypertensive effects through these mechanisms. Systolic blood pressure decreased in a group of SHR rats exposed to trans-resveratrol treatment in drinking water (50 mg/L/v.o.) for 28 days. However, no reversal of cardiac hypertrophy was observed. The study exhibited an increase in outward voltage-dependent potassium currents (I_K), a reduction in inward voltage-dependent sodium (I_{Na}), calcium (I_{Ca}), and nicotinic (I_{Ach}) currents, and an attenuation of cytosolic Ca^{2+} concentration overload in chromaffin cells from SHR rats. Based on these findings, the modulation of the sympathoadrenal axis functionality may be a new target that could account for resveratrol's antihypertensive effect [93].

6. Animal Models with Induced Diabetes/Obesity/Hypertension

Animal models with a relevant genetic setup are not the only way to analyze the biological effects of polyphenolic compounds. Pathophysiological changes typical of MetS may also be induced by dietary manipulation or the administration of drugs. The nutritional approaches studied involved administering a single type of diet or a combination of diets to modify metabolic pathways, especially those related to carbohydrate and lipid metabolism, to induce changes that best reflect those observed in people with MetS. To cause hypertension, obesity, hyperglycemia, or dyslipidemia in laboratory animals, they can be fed a diet including large doses of carbohydrates, including fructose and sucrose, or a high-fat diet. The percentage content of carbohydrates or fat in a diet necessary to induce relevant effects varies. For example, in one study, the dose of fructose used to cause hypertension, insulin resistance, and glucose intolerance exceeded 60% of caloric intake [94], whereas the administration of 30% sucrose solution to male Wistar rats was sufficient to induce hypertension as well as an increase in body weight, insulin content, and total lipids [95]. The fat content used in the experiments ranged from 20% to 60% of the total energy demand [96,97]. The most commonly used strains in diet-induced models of MetS are Sprague Dawley rats, Wistar rats, C57BL/6 J mice, and Syrian hamsters [49]. MetS can also be induced in laboratory animals using drugs such as glucocorticoids. In medicine, glucocorticoids comprise the primary treatment for various conditions, including autoimmune disorders, dermatological conditions, and cancer. However, they have certain side effects, which determine their usefulness in triggering a cascade of changes leading to the development of MetS in laboratory animals. Glucocorticoids act on different tissues and organs by, e.g., stimulating the differentiation of preadipocytes into mature adipocytes; increasing lipolysis, glucose intolerance, and body weight gain; and disturbing calcium metabolism. Experiments involving animal models use the effects of both exogenous and endogenous glucocorticoids [98,99].

Studies investigating the properties of various polyphenols have used animal models with induced changes characteristic of MetS. These polyphenols include cinnamon compounds and curcumin. Table 2 summarizes the beneficial effects on metabolic changes by propitious polyphenolic compounds gathered in this paper.

6.1. Cinnamon

Cinnamon is primarily known as a spice obtained from the bark of the Cinnamomum tree. It has numerous medicinal properties. Different parts of the plant are enriched in different chemicals, including eugenol, cinnamaldehyde, camphor, and numerous polyphenols [100]. Cinnamon has been found to present anti-inflammatory, anti-microbial, antiviral, antifungal, antioxidant, cardioprotective, hepato-protective, analgesic, wound-healing, and epithelialization-promoting effects, as well as many other properties [100,101]. Cinnamon compounds also have an important impact on carbohydrate metabolism. The supplementation of a diet with cinnamon reduces insulin resistance due to the tannin content by increasing the expression of PPAR-α and PPAR-γ and stimulating the β-subunits of the

insulin receptors of adipocytes [102,103]. In vivo studies on animals seem to confirm that cinnamon compounds also have beneficial effects on MetS. A study on Wistar rats fed a high-fat/high-fructose diet for 12 weeks found that a diet containing 20 g of cinnamon improved insulin sensitivity and reduced peritoneal fat accumulation without achieving a statistically significant reduction in body weight compared with controls. The improved insulin sensitivity is probably mediated mainly by the trimeric and tetrameric type A polyphenols present in cinnamon [104]. The wide range of biological activities of cinnamon was confirmed in a study on male Sprague Dawley rats, in which obesity was induced by a high-fat diet, whereas diabetes was induced by the subcutaneous injection of alloxan. A reduction in body weight and fat mass and a decrease in serum leptin levels were observed in rats whose diet included cinnamon extract. Moreover, the administration of cinnamon extract resulted in normalized levels of liver enzymes and reduced blood glucose levels. Furthermore, it produced a dose-dependent antioxidant effect [105]. The findings of many in vivo studies on animals allow the hypothesis that supplementation with cinnamon also has a beneficial impact on MetS [106].

6.2. Curcumin

Curcumin is a polyphenolic compound that is naturally present in turmeric rhizomes. It has anti-inflammatory, anti-carcinogenic, and antioxidant properties. Moreover, it shows antibacterial, antiviral, and antifungal effects. Its mechanism of action inhibits the expression of the NF-kB transcription factor, which in turn regulates the expression of numerous proteins involved in the initiation and maintenance of inflammation, which underlies multiple conditions [107]. In a study on male albino Wistar rats, in which a high-fat diet induced diabetes with a dose of streptozotocin, the administration of curcumin for 8 weeks (80 mg/kg BW/day) lowered glucose levels and reduced insulin resistance, dyslipidemia, and lipid peroxidation. Moreover, the administration of curcumin significantly increased the expression of the GLUT-4 gene, which regulates insulin-dependent glucose transport in muscles and adipose tissue, compared with the control group. The regulation of this transporter is altered under pathological conditions, including, among others, type 2 diabetes. The administration of curcumin was found to stimulate the expression of GLUT4, thus normalizing glucose metabolism in the treated group [108]. Curcumin can also be used as a dietary intervention against lipid accumulation and liver fibrosis. It acts via the stimulation of lipogenic gene expression and, in this way, induces lipolysis and inhibits lipogenesis. In groups of Wistar rats administered curcumin at a dose of 100 mg/kg for 4 weeks, lipid imbalance was induced by bile duct ligation. A reduction in hepatic fat accumulation via AMPK upregulation was observed. AMPK is a serine/threonine-protein kinase that is responsible for lipid metabolism. Its dysregulation may lead to the development of hepatic injury. Curcumin seems to improve the expression of AMPK and hepatic redox potential and attenuate lipid peroxidation. A curcumin-treated group also showed protective effects against hepatic fibrosis. The hepatic protection was also associated with a reduction in the lipid level in serum by curcumin [109].

Table 2. The effects of selected polyphenolic compounds with promising bioactive potential on MetS.

Substance	Animal Model	Dose (Time)	Metabolic Effect	Mechanism	References
Red wine	Zucker Fatty rats	20 mg/kg BW (8 weeks)	↑ FS ↑ CO ↓ serum glucose ↓ LDL cholesterollevel ↓ TG level ↓ peripheral arterial resistance ↓ superoxide anions ↓ thromboxane A2 ↓ 8-isoprostane	↑ NO bioavailability ↑ eNOS activity ↓ NADPH oxidase expression	[60]

Table 2. *Cont.*

Substance	Animal Model	Dose (Time)	Metabolic Effect	Mechanism	References
Green tea	Zucker Fatty rats	200 mg/kg BW (8 weeks)	↓ body weight ↓ visceral fat ↓ hepatic lipogenesis ↓ insulin level ↓ glucose level ↓ lipids level	↑ expression AMPK-Thr172 ↑ expression phosphorylated acetyl-CoA carboxylase (ACC) ↑ sterol regulatory element-binding protein 1c (SREBP1c)	[63]
	Zucker Fatty rats, Sprague Dawley rats	15 mg, 20 mg, 40 mg (7 days, 4 days)	↓ food intake ↓ testosterone level ↓ estradiol level ↓ LH level ↓ leptin level ↓ insulin level ↓ glucose level ↓ IGF-1 level ↓ cholesterol level ↓ TG level	↓ food intake (hypothalamic neuropeptide gene expression alternation?, changes in bilirubin, alkaline phosphatase activity?)	[64]
	Zucker Fatty rats	200 mg/kg BW (8 weeks)	↓ body weight ↓ visceral fat ↓ insulin level ↓ glucose level ↓ insulin resistance	modulation of insulin signaling protein in skeletal muscle ↑ expression and translocation of GLUT-4 in skeletal muscle ↓ activation of the inhibitory protein kinase isoform- PKC-θ	[65]
Quercetin	Zucker Fatty rats	2 mg/kg BW 10 mg/kg BW (10 weeks)	↓ dyslipidemia ↓ hypertension ↓ insulin resistance ↓ weight (only dose 10mg/kg BW) + anti-inflammatory effect	↑ eNOS expression ↑ adiponectin level in plasma ↓ TNF-alpha production in visceral tissue	[74]
Pomegranate	Zucker Diabetic Fatty rats	500 mg/kg BW (6 weeks)	↓ TG level ↓ lipid droplet content in liver	↑ expression PPAR-α ↑ expression acyl-CoA oxidase ↑ expression CPT1	[80]
	Zucker Diabetic Fatty	500 mg/kg BW (6 weeks)	↓ hyperglycemia ↓ hyperlipidemia ↓ cardiac fibrosis	↓ NF- κB activation in macrophages ↓ expression ET-1	[81]
	Zucker Diabetic Fatty	500 mg/kg BW (6 weeks)	↓ cardiac TG accumulation ↓ TG level ↓ cholesterol level	↑ cardiac expression PPAR-α ↑ cardiac expression CPT-1 ↑ cardiac expression ACO ↑ cardiac expression AMPKαK ↓ cardiac expression acetyl-CoA carboxylase (ACC)	[82]

Table 2. *Cont.*

Substance	Animal Model	Dose (Time)	Metabolic Effect	Mechanism	References
Cocoa	Zucker Diabetic Fatty	10% cocoa-rich diet (10 weeks)	↑glucose tolerance ↓ body weight ↓ insulin resistance ↓ glucose level ↓ insulin level + nephroprotective effect	↓ renal synthesis PEPCK ↓ renal synthesis G-6-P ↓ expression of glucose transporters (SGLT-2, GLUT-2) in the renal cortex	[84]
	Zucker diabetic Fatty	10% cocoa-rich diet (9 weeks)	↓ body weight ↓ lipid accumulation in liver cells	↑ phosphorylated AMPK level in liver ↑ phosphorylated protein kinase B (AKT) level in liver ↓ phosphorylated protein kinase C (PKCζ) level in liver	[85]
	Zucker diabetic Fatty	10% cocoa-rich diet (10 weeks)	↑ glucose homeostasis ↑ intestinal integrity + modification of gut microbiota	↓ amount of lactate-producing bacteria ↓ expression TNF-α ↓ expression IL-6	[86]
Resveratrol	Spontaneously Hypertensive rats	dissolved in drinking water (concentration 50 mg/L), ad libitum (10 weeks)	↓ hypertension ↓ oxidative stress	↓ H_2O_2 content ↓ SOD activity ↓ eNOS uncoupling ↓ NO scavenging	[91]
	Spontaneously Hypertensive rats	2.5 mg/kg BW (10 weeks)	↓ concentric heart hypertrophy ↓ systolic heart dysfunction	↓ oxidative stress in cardiac muscle tissue	[92]
	Spontaneously Hypertensive rats	50 mg/kg BW (28 days)	↓ SBP	↑ outward voltage-dependent potassium currents (I_K) ↓ inward voltage-dependent sodium currents (I_{Na}), ↓ inward voltage-dependent calcium currents (I_{Ca}) ↓ inward voltage-dependent nicotinic currents (I_{Ach})	[93]
Cinnamon	Wistar rats (high-fat/high-fructose diet)	20 g cinnamon-rich/kg of diet (12 weeks)	↓ insulin resistance ↓ peritoneal fat accumulation	↑ peroxisome proliferators-activated receptors activity?	[104]
	Sprague Dawley rats (high-fat diet + subcutaneous injection of alloxan)	200 mg/kg BW 400 mg/kg BW (6 weeks)	↑ HDL cholesterol level ↓ body weight ↓ LDL cholesterol level ↓ leptin level ↓ glucose level ↓ liver enzymes levels + antioxidant effect	↓ the intestinal absorption of cholesterol? ↓ appetite? ↓ oxidative stress?	[105]

Table 2. *Cont.*

Substance	Animal Model	Dose (Time)	Metabolic Effect	Mechanism	References
Curcumin	Wistar rats (high-fat diet + streptozotocin)	80 mg/kg BW (8 weeks)	↓ glucose level ↓ insulin resistance ↓ lipid level ↓ lipid peroxidation	↑ expression GLUT-4	[108]
	Wistar rats (bile duct ligation)	100 mg/kg BW (4 weeks)	↓ hepatic fat accumulation ↓ lipid peroxidation ↓ hepatic fibrosis	↑expression AMPK ↑expression CPT-1a	[109]

7. Conclusions

Currently, over one-third of the world's population is obese. The prevalence rates of hypertension, dyslipidemia, and insulin resistance are similarly high. With the concurrence of these conditions becoming increasingly common, MetS affects a growing number of people. Thus, it is necessary to find new compounds that may mitigate its symptoms and prevent the progression of this disease, as well as develop animal models that closely mirror all the changes characteristic of MetS.

The establishment of animal models with the desired metabolic changes is crucial in order to understand the molecular mechanisms of MetS. However, the advantage of MetS animal models is that they allow the impact of new compounds on morphological, histological, biochemical, and functional changes to be investigated. In contrast, such a broad scope of monitoring is practically impossible in human studies. The clarification of the underlying mechanisms of MetS may result in the development of more effective therapies or dietary interventions, possibly based on natural compounds, in the future.

Researchers currently place their hopes in polyphenolic compounds naturally present in various plants. Numerous substances contained in tea, berries, grapes, blueberries, and many other foods have potential ameliorating effects on particular components of MetS. In vivo studies on animals confirm that the dietary intake of specific polyphenols or their complex mixtures reduces MetS symptoms. However, it is still necessary to carry out relevant analyses on humans. Despite the effects observed in in vivo human studies, dietary interventions have shown that some compounds do not have good bioavailability, leaving their usefulness in question. The development of appropriate preparations could make it possible to overcome this obstacle. Further studies may provide a better understanding of the impact of plant polyphenols on changes characteristic of MetS, and animal models, which undoubtedly most accurately reflect these changes, are necessary to enhance our knowledge of these compounds.

In conclusion, the findings presented in this paper show that there is no universal compound that can improve all pathological effects. Most of them ameliorate effects on the lipid and glucose profiles (red wine, green tea, quercetin, pomegranate, cocoa, cinnamon, curcumin). The consumption of resveratrol and red wine can decrease hypertension. Moreover, additional effects are associated with hepatoprotection (pomegranate, green tea, cocoa, cinnamon, curcumin), nephroprotection (cocoa), cardioprotection (red wine, pomegranate, resveratrol), the modulation of the gut microbiota (cocoa), anti-inflammatory effects (quercetin), and antioxidant effects (resveratrol, cinnamon). The administration of individual polyphenols leads to particular changes, while the use of a combination of related polyphenols may provide synergistic effects and lead to more significant benefits. Further studies on the bioavailability of polyphenols, the most advantageous forms of administration, the best formulations, and the synergy of polyphenols are necessary in order to clarify the true potential of plant polyphenols in the treatment of metabolic syndrome.

These studies are of significant clinical importance because they may lead to the development of the so-called functional food, which food, unlike typical pharmaceuticals,

can contribute to the improvement in the health of the population by influencing common metabolic disorders [110].

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7 MANUSKRYPT II

Niewiadomska J., Kumiega E., Płóciennik M., Gajek J., Noszczyk-Nowak A.: Effects of Punica granatum L. peel extract suplementation on body weight, cardiac function, and haematological and biochemical parameters in an animal model of metabolic syndrome. Journal of Vetrinary Research, 2023, 67. https://doi.org/10.2478/jvetres-2023-0031

7.1 Streszczenie manuskryptu II

Zespół metaboliczny (MetS) stanowi grupę wzajemnie ze sobą powiązanych modyfikowalnych czynników ryzyka sercowo-naczyniowego. Koncepcja zespołu wielosystemowego wnikającego ze współistniejących zaburzeń metabolicznych została dobrze opisana u ludzi. Jednak u zwierząt towarzyszących wciąż stanowi novum i wymaga badań, celem ustalenia precyzyjnego algorytmu diagnostycznego oraz terapeutycznego. Aktualnie w leczeniu postuluje się wykorzystanie bioaktywnych naturalnych związków. Obiecującym materiałem roślinnym bogatym w nutraceutyki, które można by wykorzystać w interwencji dietetycznej w MetS, jest skórka z *Punica granatum* L. Przeprowadzone badanie miało na celu oszacowanie biopotencjału ekstraktu ze skórek granatowca w łagodzeniu poszczególnych komponent MetS na przykładzie modelu zwierzęcego.

Ekstrakt ze skórek granatowca (EPP) podawano szczurom Zucker Diabetic Fatty (ZDF-Leprfa/Crl, fa/fa), cechujących się mutacją typu missense w genie receptora leptyny *Lepr* oraz ich zdrowej kontroli, szczurom Zucker (ZUC-*Lepr*^{fa}, fa/+) w dwóch dawkach: 100 mg/kg mc. oraz 200 mg/kg mc. W celu indukcji MetS wszystkie osobniki otrzymywały *ad libitum* karmę wysokokaloryczną. Ekstrakt podawano przez 8 tygodni. Masę ciała monitorowano dwukrotnie w ciągu każdego tygodnia trwania doświadczenia. Krew pobrano trzykrotnie: przed rozpoczęciem podawania EPP, po 4 tygodniach oraz po 8 tygodniach. Pomiary echokardiograficzne przeprowadzono przed rozpoczęciem suplementacji ekstraktem oraz po 8 tygodniach badania.

Ekstrakt nie wpłynął istotnie na redukcję masy ciała w ostatnim punkcie pomiarowym, jednak jego podaż wiązała się z tendencją do ograniczenia dynamiki jej przyrostu. Nie zaobserwowano poprawy w zakresie profilu glikemicznego oraz lipidowego. Dawka 200 mg/kg mc. w grupie osobników predysponowanych do MetS (fa/fa 200) doprowadziła do istotnego zmniejszenia częstotliwości rytmu serca (95% CI: -79.29- - 8.38, p=0,017), zwiększenia objętości końcoworozkurczowej lewej komory (95% CI: 0.03-0.45, p=0.026) oraz poprawy w zakresie frakcji skracania włókien środkowych (95% CI: 0.69%-14.61%, p=0.032) względem grupy kontrolnej otrzymującej tylko wodę (fa/fa H_2O). W zakresie większości parametrów morfologicznych nie odnotowano zmian pomiędzy grupami. Jedyna istotna różnica dotyczyła odsetka eozynofilii, który był istotnie wyższy w grupach szczurów otrzymujących ekstrakt w odniesieniu do grupy kontrolnej.

Ekstrakt ze skórek granatowca posiada korzystne właściwości prozdrowotne m.in. wyrażone poprzez aktywność kardioprotekcyjną, dlatego jego zaimplementowanie do algorytmu terapeutycznego zespołu metabolicznego mogłoby przyczynić się do ograniczenia ryzyka kardiometabolicznego oraz powikłań z nim związanych. Nie mniejsze znaczenie ma także jego znikoma toksyczność. Niemniej jednak biodostępność związków polifenolowych wymaga dalszych badań zarówno wśród ludzi jak i zwierząt.

Słowa kluczowe: polifenole, skórka z granatowca, szczury Zucker Diabetic Fatty, zespół metaboliczny

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Effects of *Punica granatum* L. peel extract supplementation on body weight, cardiac function, and haematological and biochemical parameters in an animal model of metabolic syndrome

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Abstract

Introduction: Metabolic syndrome (MetS) is a cluster of pathological conditions well described in humans but still investigated insufficiently in animals. A novel approach in its management is the utilisation of nutrients from natural sources. Recent studies suggested that phenolic compounds from pomegranate peel could be a promising dietary intervention for MetS. This study evaluated the potency of polyphenol-rich pomegranate peel extract (EPP) in mitigating some MetS components in an animal model. **Material and Methods:** Zucker diabetic fatty rats (with an fa/fa missense mutation in the *Lepr* leptin receptor gene) and their healthy counterparts (fa/+) as controls were fed a high-calorie diet to induce MetS and supplemented with EPP at two doses: 100 mg/kg body weight (b.w.) and 200 mg/kg b.w. The extract was administered for eight weeks. The rats' body weights were monitored twice per week, and blood samples were taken before EPP administration after four weeks and eight weeks of study. Echocardiography measurement was performed at the beginning and at the end of the study. **Results:** The extract restrained the dynamic of weight gain. A cardioprotective effect of the highest dose of EPP supplementation was manifested in a relative decrease in heart rate and improved mid-fractional shortening, representing myocardial contractility. No improvement in fasting blood glucose or lipid profile was observed. **Conclusion:** Pomegranate peel extract possesses beneficial health properties that could be useful in dietary intervention in MetS. However, its bioavailability still requires further investigation in clinical trials in humans and animals suffering from endocrine and metabolic disorders.

Keywords: polyphenols, pomegranate peel, Zucker diabetic fatty rats, metabolic syndrome.

Introduction

Metabolic syndrome (MetS) is a pathological condition defined as a set of several metabolic disorders comprising visceral obesity, insulin resistance, dyslipidaemia and hypertension (5). It is defined by diagnostic criteria with parametric cut-off points varying as appropriate for particular target populations. Any three out of the five following risk factors must be present to make a diagnosis: central obesity (abnormal waist circumference or body mass index), insulin resistance (impaired glucose tolerance, impaired fasting glucose or type 2 diabetes requiring treatment), dyslipidaemia (hypertriglyceridaemia, low concentration of high-density lipoprotein cholesterol (HDL) or hyperlipidaemia requiring treatment) and hypertension (high resting blood pressure or need for antihypertensive drugs) (39). Additionally, apart from the main components, MetS is also associated with impaired kidney function, polycystic ovary syndrome, hyperuricaemia, fatty liver disease, obstructive sleep apnoea and heart failure with preserved ejection fraction (EF) (47). The pathogenesis is complex and still not

completely elucidated. However, it seems that the primary roles are played by insulin resistance, chronic inflammation and neurohormonal activity (45). Early diagnosis and treatment are vital to prevent the development of more severe conditions such as atherosclerotic cardiovascular disease and to modify the risk factors. The first line treatment of MetS is based on lifestyle changes promoting physical activity, sleep hygiene, reduced alcohol consumption, and dietary intervention (12, 47). Drugs such as antihypertensives, statins and metformin are used when non-medical strategies prove ineffective. Nonetheless, their application is limited by drug-related adverse effects emerging over the course of long-term therapy (12).

In veterinary clinics, MetS is well described in horses but still requires further investigation in obese dogs and cats and in individuals with hypercortisolaemia. Some reports have already shown the connection between obesity, hyperlipidaemia and insulin resistance. The occurrence of these disorders in humans escalates the risk of cardiovascular diseases such as atherosclerosis, a fatal condition well known in humans but unusual in small animals or horses. Establishing diagnostic criteria for MetS in veterinary medicine will facilitate its diagnosis and help in the early implementation of appropriate treatment.

A novel approach to the management of MetS involves plant food supplements. Nutraceuticals are natural dietary components with proven health benefits. Studies show that compounds derived from plants display potentially propitious features in MetS (48). Polyphenols in particular, which are biomolecules present in flowers, seeds, juice, arils, roots and leaves, are believed to be integral to the future of the food industry as natural food additives (48). Studies have shown that polyphenols exhibit antioxidative, anti-inflammatory, antihypertensive, antimicrobial, antiatherogenic, antiaging and antimutagenic effects. Favourable medical features also include the promotion of weight loss, antidiabetic properties, improvement of lipid profile, and cardioprotective, hepatoprotective and nephroprotective activity (13, 48). Therefore, they are considered potential therapeutic agents in MetS. Consumption of functional food enriched with phenolic compounds could improve public well-being and help prevent diseases of affluence such as cardiovascular diseases, type 2 diabetes and obesity (22). Additionally, incorporating phenolic compounds in animal food may promote health and serve as a component of pre-emptive veterinary medicine.

Pomegranate (*Punica granatum* L.) is a rich source of phenolic compounds. Numerous studies highlight its high concentration of health-promoting phytochemicals and promising pharmaceutical properties in dietary supplementation (1, 21). It is predominantly consumed fresh or in the form of juice. Health benefits are bestowed not only by juice and the flesh of the fresh fruit but also by the uneatable parts, which contain a wide range of bioactive compounds (19, 37). Pomegranate peel, a by-product of industrial juice processing, contains a high concentration of polyphenol fractions such as phenolic acids (ellagic acid, caffeic acids, hydroxybenzoic acid, hydroxycinnamic acid and gallic acid), hydrolysable tannins (catechin, ellagitannins, gallotannins and gallagyl esters), flavonols (epicatechin and gallocatechin) and anthocyanins (9). The bioactivity of pomegranate peel is anti-inflammatory, antioxidant, antimutagenic, anticancerogenic, antidiabetic and antimicrobial (4, 7). Pomegranate peel's content of phytochemicals with nutraceutical and medical significance make it an auspicious natural source in the development of functional food products.

The main objective of the present study was to assess the potential health benefits from pomegranate peel extract (EPP) supplementation in an animal MetS model.

Material and Methods

Animals. The study was carried out on Zucker diabetic fatty rats with missense mutations in the *Lepr* leptin receptor gene (ZDF-Leprfa/Crl, fa/fa) and their healthy counterparts as controls (fa/+). The rats were purchased from Charles River Laboratories, Research Models and Services (Sulzfeld, Germany). The room where the animals were kept was maintained on a 12-h light-dark cycle at 20°C ± 2°C. After two weeks of acclimatisation, individuals were randomly assigned into five groups as follows: 1) a control group designated fa/fa H_2O (ZDF, fa/fa, n = 6) receiving only water, 2) a study group designated fa/fa 100 (ZDF, fa/fa, n = 6) receiving EPP in a dose of 100 mg/kg body weight (b.w.), 3) a study group designated fa/fa 200 (ZDF, fa/fa, n = 6) receiving EPP in a dose of 200 mg/kg b.w., 4) a study group designated fa/+ 100 (healthy controls (HC), fa/+, n = 6) receiving EPP in a dose of 100 mg/kg b.w., and 5) a study group designated fa/+ 200 (HC, fa/+, n = 6) receiving EPP in a dose of 200 mg/kg b.w. Extract of pomegranate peel was administered daily by oral gavage using water as a vehicle. All individuals were maintained on Purina 5008 (LabDiet, Richmond, IN, USA). The extract from pomegranate peel was administered for eight weeks. The study project was approved by the Ethics Committee for Experiments on Animals at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in the Polish Academy of Sciences, Wrocław, Poland (Resolution 53/2017).

Procedure for extraction of polyphenols from pomegranate peel. The polyphenolic extract was obtained from *Punica granatum* L. peel (Mollar de Eche cultivar) delivered from Spain. The dried peel of pomegranates was shredded in a Thermomix domestic kitchen appliance. The resulting material (1 kg) was extracted and re-extracted twice with 50% ethanol. The extraction process was conducted in an ultrasonic bath for 25 min. The extract was subsequently concentrated

with a Rotavapor rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) in a water bath at 40°C. The concentrated ethanol extract from the pomegranate peel was passed through a column with Amberlite XAD-16 resin (Brenntag, Essen, Germany), and the column was washed with distilled water to rinse out the organic acids, sugars and other undesirable compounds. Polyphenols were eluted with 80% ethanol. The collected fractions were dried in an SPT-200 vacuum oven (Zeamil, Kraków, Poland).

Identification and quantification of polyphenolic compounds by liquid chromatography–mass spectrometry coupled with quadrupole time-of-flight photodiode array detection. Identification and quantification of polyphenolic compounds were performed on an Acquity ultra-performance liquid chromatography (UPLC) system, coupled with a Synapt quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) instrument (Waters Corp., Milford, MA, USA), with an electrospray ionisation source and photodiode array detector. Separation was obtained on the Acquity bridged ethylene hybrid C18 column (100 mm × 2.1 mm i.d., 1.7 m, Waters Corp.). The water phase was a mixture of 0.1 % (v/v) aqueous formic acid (A) and acetonitrile (B). The gradient programme was as follows: the initial conditions were 1% B in A, 12 min of 25% B in A, 12.5 min of 100% B in A and 13.5 min of 1% B in A. The flow rate was 0.45 mL/min, and the injection volume was 10 µL. The column was operated at 30°C. Ultraviolet-visible absorption spectra were recorded online during UPLC analysis, and the spectral measurements were made in the wavelength range of 200–600 nm in steps of 2 nm. The primary operating parameters for the Q-TOF MS were set as follows: capillary voltage of 2.5 kV, cone voltage of 30 V, cone gas flow of 11 L/h, collision energy 28–30 eV, source temperature of 100°C, desolvation temperature of 250°C, argon collision gas, nitrogen desolvation gas flowing at 300 L/h, data acquisition range m/z 100–2000 Da, and negative ionisation mode. The data were collected with MassLynx v. 4.1 software (Waters Corp). The results of the analysis are presented in Table 1.

Body weight measurement. Body weight was recorded twice per week. Measurements for each individual in a single survey point were taken three times, and the mean was calculated and subsequently incorporated into the statistical analysis. The weight of individuals was measured using an SW-II certified calibrated scale (CAS Poland Sp. z.o.o., Warsaw, Poland).

Blood sampling and analyses. The blood samples were obtained at three time points: before starting EPP administration, after four weeks, and after eight weeks of study. Blood was drawn from the lateral tail vein after warming the tail to increase the obtainable blood volume, and was collected into microtubes containing 1.6 mg/mL liquid ethylenediaminetetraacetic acid (EDTA) and serum tubes. Serum samples were separated by centrifugation (4,000 × *g* for 5 min) and stored at −80°C for biochemical assays. A SCIL VET ABC animal blood counter analytical haematology system (Horiba ABX, Montpellier, France) was employed to determine the following morphological parameters: white blood cell count (WBC), red blood cell count (RBC), haematocrit, haemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelets (PLT). Peripheral blood smear images were examined manually by the same laboratory technician.

Table 1. Mass spectrum characteristic and content of phenolic compounds in pomegranate peel extract

Rt (min)	MS $(M\text{-}H)^-$ (*m/z*)	MS/MS $(M\text{-}H)^-$ (*m/z*)	Name of compounds	Polyphenol content
1.67	331	271/169	Galloyl-glucose	2.00 ± 0.03
1.73	781	721/601	Punicalin α/A	3.11 ± 0.06
2.02	1083	611/331/146	HHDP-galloyl-hexoside (punicalagin)	4.20 ± 0.09
2.12	1083	781/622/301	Punicalagin isomer	14.82 ± 1.04
2.33	933	631/450/301	Ellagitannin	4.71 ± 0.40
2.87	1083	781/301	HHDP-gallagyl-hexoside (punicalagin)	93.91 ± 2.05
3.12	1085	907/783/301	Ellagic acid derivative	2.49 ± 0.53
3.69	1083	781/301	HHDP-gallagyl-hexoside (punicalagin)	157.00 ± 2.65
3.89	799	301	Granatin A	4.74 ± 0.32
5.08	783	481/301	Ellagitannin	25.86 ± 1.53
6.20	1085	933/301	Digalloyl-gallagyl-hexoside	10.37 ± 0.65
6.25	783	481/301	Ellagitannin	13.51 ± 0.99
6.38	463	301	Ellagic acid-hexoside	33.63 ± 1.23
6.89	951	907/635/301	Galloyl-HHDP-DHHDP-hex (granatin B)	2.68 ± 0.11
			Total (mg/g dry weight)	373.05

Rt – retention time; MS – mass spectrometry; $(M\text{-}H)^-$ – deprotonated molecule; *m/z* – mass-to-charge ratio; MS/MS – tandem mass spectrometry; HHDP – hexahydroxydiphenic acid; DHHDP – dehydrohexahydroxydiphenic acid

The leukocyte quantities were determined by a Schilling differential cell count. Erythrocyte abnormalities of size and shape were recorded and reticulocytes were counted in a microscopic examination. After staining EDTA blood with 1% new methylene blue with 1.6% potassium oxalate anticoagulant and 1% brilliant cresol blue in saline (ANALAB Sp. z o.o., Warsaw, Poland), the percentage of reticulocytes per 1,000 non-nucleated red blood cells was calculated. Blood biochemical indices were investigated with an Epoll 300 analyser (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA). Serum levels of glucose and the lipid panel (total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides) were established.

Cardiac function. Heart size and function assessment was performed before EPP administration and eight weeks after it. Echocardiography measurements were performed in the Department of Internal Medicine and Clinic of Diseases of Horses, Dogs and Cats in the Faculty of Veterinary Medicine at Wroclaw University of Environmental and Life Sciences. The echocardiographic measurements were taken by the same researcher over at least three consecutive cardiac cycles using an Arietta echocardiograph (Aloka Company, Tokyo, Japan) and a 7.5–10 MHz transducer according to the guidelines of the American Society for Echocardiography. The following cardiac dimensions were determined: the relative left atrial size (from the left atrial diameter to aortic root diameter ratio (LA/Ao), end-diastolic and end-systolic thickness of the interventricular septum, left ventricle posterior wall, and the internal left ventricular dimensions at end diastole and end systole (LVIDd and LVIDs). Estimates of left ventricular systolic function were obtained from the index of circumferential myocardial contraction and fractional shortening (FS) using the Teicholz formula ((LVIDd−LVIDs/LVIDd) × 100%). Estimates of left ventricular end-diastolic volume, end-systolic volume, stroke volume, and EF were calculated by the echocardiographic software.

Statistical analysis. The results are displayed as a mean ± standard deviation. All statistical analyses were performed using the R statistical computing environment (version 4.1.1.; 32). If the data followed a normal distribution, parametrical tests were used. The non-normal data were analysed with the use of nonparametric tests. For three or more groups of variables with normal distribution, one-way analysis of variance was applied, and to compare two groups of normal data, Welch's *t*-statistic was calculated. Welch's formula for one-way ANOVA was applied in order to preserve type I error robustness for unequal variances of groups. The effect size was estimated applying Field's convention for the comparison of more than two groups (estimator ω^2_p) and Cohen's criteria for the comparison of two groups (estimator $\hat{g}_{Hedges}$). Violin plots were drawn to visualise the distribution of the numerical data. To examine the effect size of repeated measurements associated with differences between interventional and control groups, the multilevel growth model proposed by Feingold was applied. Statistical analyses were conducted at a significance level of $P < 0.5$.

Results

Effect of pomegranate peel extract on body weight. Body weight assessments were analysed at 16 time points. No significant differences were noted at the last time point between the control group fa/fa H_2O (mean = 407.14 g, standard deviation (SD) = 32.68 g) and the experimental group fa/+ 200 (mean = 337.69 g, SD = 29.34g), F_{Welch} = 4.56 using the value 4 as adjustment for degrees of freedom, 11.68 as adjustment for error degrees of freedom, and 0.02 as the rectified p-value according to the Welch formula. Similarly, no significant effect of pomegranate peel extract on body weight in other groups was observed (Table 2 and Fig. 1).

The dynamic of body weight increase was registered at 16 time points. Graphic representations of data are presented in Fig. 2. The data show clear tendencies, despite slight differences between individuals at the beginning of the study. Body weights in all groups trended upwards. However, in groups without MetS (fa/+), the increase was more pronounced than in rats predisposed to the syndrome (fa/fa), and the gain in these groups was considerably quicker (Fig. 3). Experimental groups with MetS were characterised by smaller weight gains than the group of control rats with MetS administered only water. The body weight gain at each time point in group fa/fa 100 was 1.37 g less and in group fa/fa 200 was 1.22 g less than that in group fa/fa H_2O. In groups of rats without MetS, the body mass increase was higher at each time point in comparison to the increase in group fa/fa H_2O, in group fa/+ 100 being so by about 3.09 g, and in group fa/+200 by about 2.25 g.

Table 2. Mean body weights for individual groups at the final 16th time point

group	fa/fa H_2O	fa/fa 100	fa/fa 200	fa/+ 100	fa/+ 200
n	6	6	6	6	6
Mean	407.14	382.08	392.75	358.97	337.69
Standard deviation	32.68	35.48	36.28	12.90	29.34

fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP

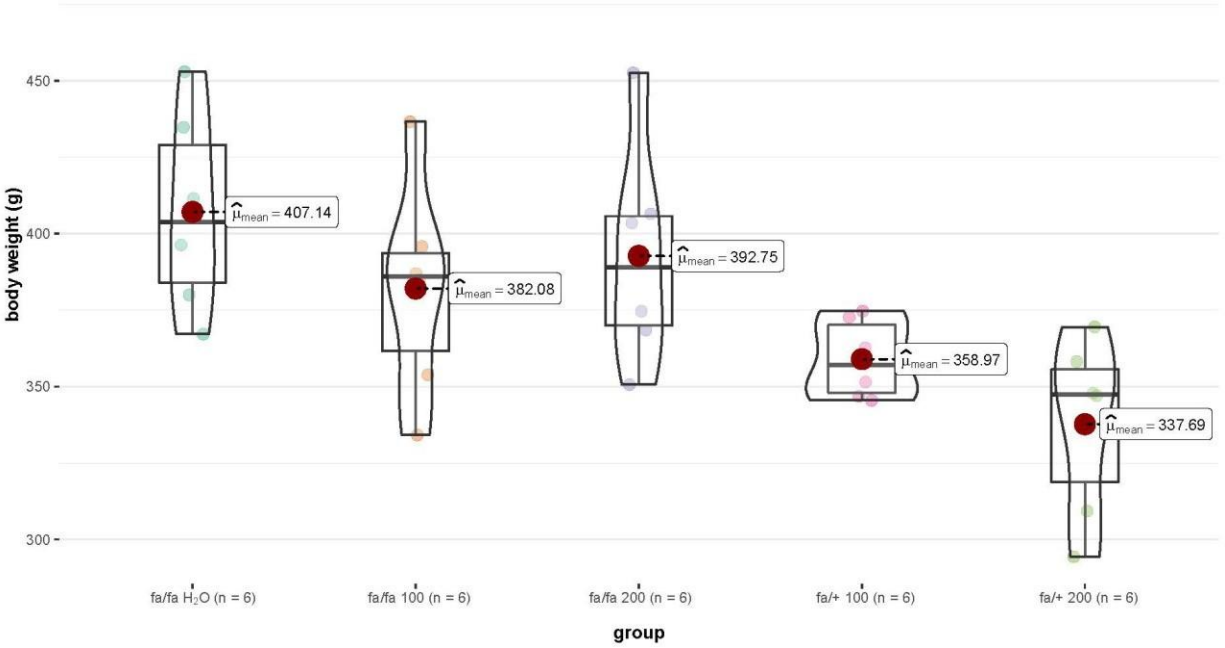


Fig. 1. Violin plots of body weight with regard to individual groups at the final 16th-time point. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; $\hat{\mu}$ – predicted value of group means

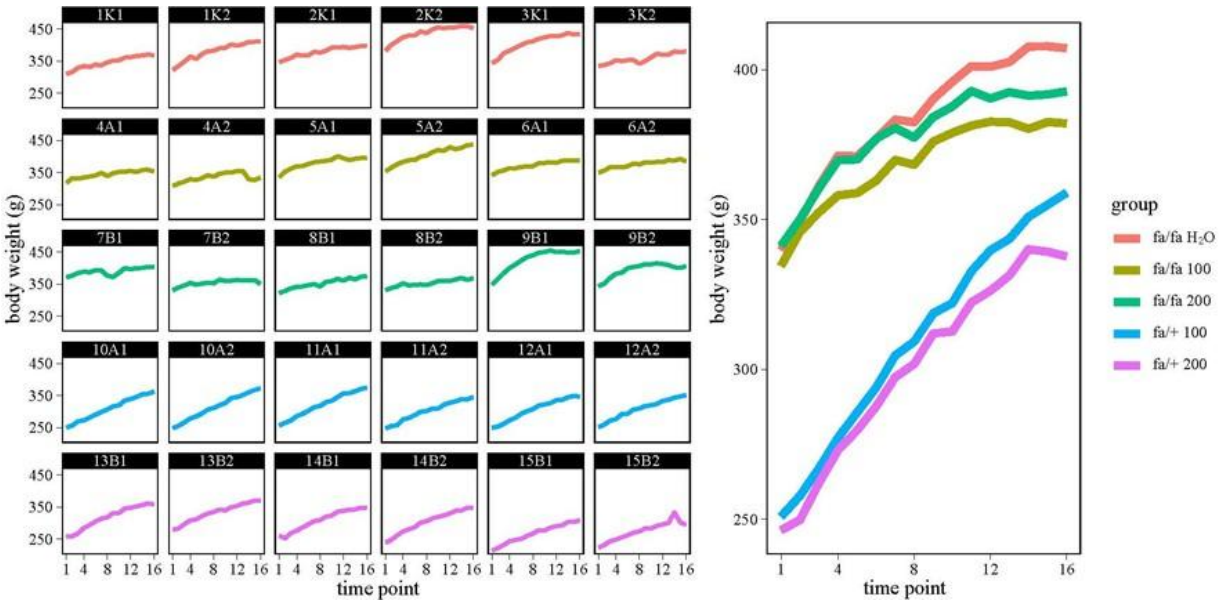


Fig. 2. The dynamics of body weight increases of individual rats (left side) and of group mean weight increases (right side) during the study period. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP

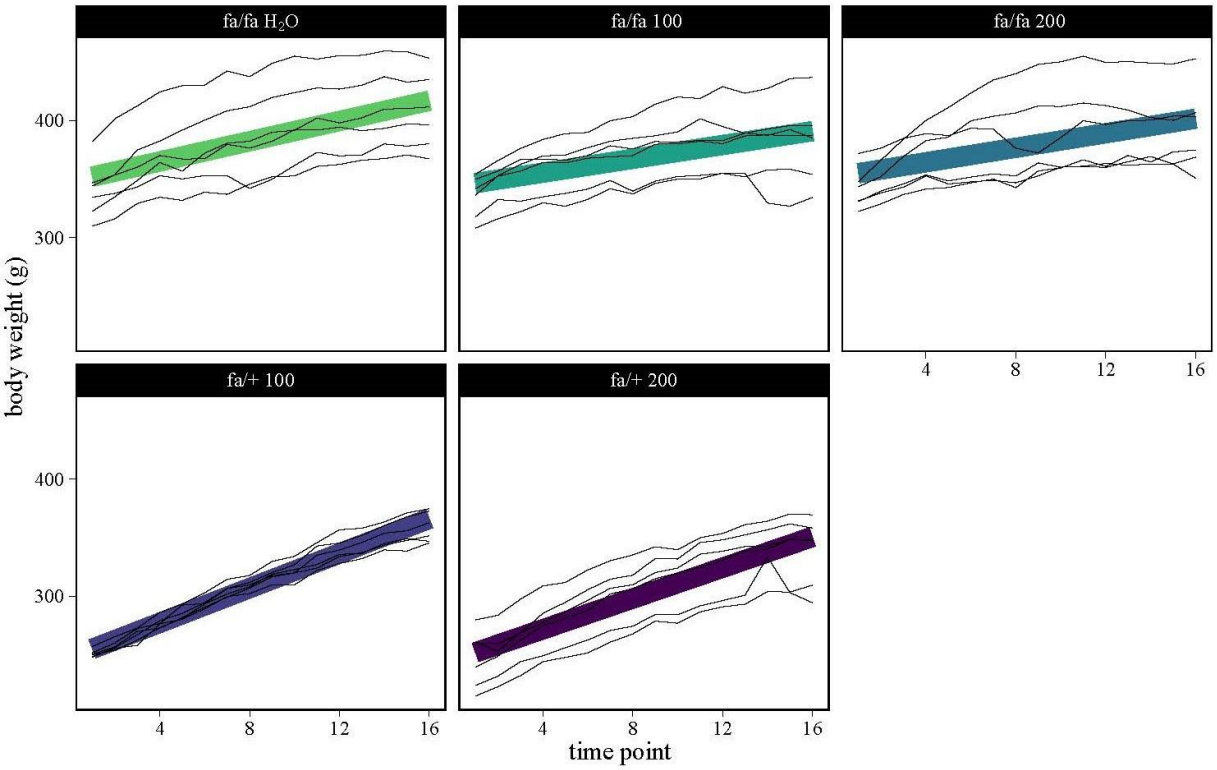


Fig. 3. Trajectories of individual rats' body weight variation and group average variations. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP

Effects of pomegranate peel extract on echocardiographic parameters. Echocardiographic parameters were determined to evaluate the influence of EPP administration on cardiac function. The measurements were made twice: before the beginning of the study and after its termination (Table 3). The analysis was based on the design control group pretest-posttest approach. The results indicate that there were no significant differences between groups with EPP supplementation and the group receiving only water in most of the analysed parameters. However, some of them showed changes versus the fa/fa H_2O group. At the end of the study, the heart rate in experimental groups was significantly lower – by 54.67 bpm (95% CI: −88.19–−21.15, P = 0.002) – than at the beginning. Moreover, the heart rate in the fa/fa 200 group was markedly decreased – by 43.83 bpm – compared to the control group (CI: −79.29–−8.38, P = 0.017). The value of end-diastolic volume in the fa/fa 200 group was significantly altered when compared with the fa/fa H_2O group (95% CI: 0.03–0.45, P = 0.026). Cardiac output was diminished in all groups after eight weeks by 0.03 L/min (95% CI −0.06–−0.01, P = 0.015). Also LA/Ao was lower in all groups by 0.11 without regard to EPP supplementation (95% CI: −0.19–−0.04, P = 0.005). Mid-wall fractional shortening (mFS) representing myocardial contractility was better by 7.65% in the fa/fa 200 group in comparison to the control group (95% CI: 0.69%–14.61%, P = 0.032).

Effects of pomegranate peel extract on blood morphology and smear results. Regarding the results of blood morphology, no explicit differences between tested groups were found (Table 4). However, in all groups, some tendencies were revealed. During the course of the study, the WBC, HGB, PLT, MCV, MCH, MCHC and lymphocytes decreased, in contrast to the RBC and neutrophil count, which increased. The only marked difference noted in blood morphology between the control and experimental groups was the eosinophilic granulocyte count, which was relevantly elevated in individuals obtaining EPP. The results of blood smears and determination of abnormal cells showed a higher number of acanthocytes and schistocytes in rats without MetS (fa/+ 100 and fa/+ 200) than in individuals with genetically programmed MetS (fa/fa H_2O, fa/fa 100 and fa/fa 200) regardless of EPP supplementation. The findings are presented in Figs 4 and 5. Images of peripheral blood smears containing acanthocytes and schistocytes are shown in Fig. 6. No blast cells or other abnormalities were reported.

Table 3. Echocardiographic parameters at two time points: at the beginning of the study (t1) and after eight weeks of pomegranate peel supplementation (t2)

Index	fa/fa H_2O		fa/fa 100		fa/fa 200		fa/+ 100		fa/+ 200	
	t1	t2	t1	t2	t1	t2	t1	t2	t1	t2
HR (bpm)	289.00 ± 28.08	234.33 ± 37.03	265.00 ± 21.02	232.25 ± 45.85	245.17 ± 21.34	226.83 ± 32.62	264.60 ± 20.40	230.60 ± 46.55	275.20 ± 11.82	245.80 ± 31.40
LVIDs (mm)	2.52 ± 0.54	2.65 ± 1.03	3.35 ± 1.61	2.65 ± 1.03	3.35 ± 1.23	2.90 ± 1.01	3.36 ± 0.69	2.36 ± 1.19	2.80 ± 0.70	2.28 ± 0.42
LVIDd (mm)	6.48 ± 0.51	6.08 ± 1.15	6.65 ± 0.83	5.82 ± 0.74	6.87 ± 1.25	6.52 ± 0.95	6.08 ± 1.13	5.56 ± 1.39	5.84 ± 0.77	5.68 ± 0.69
LVPWs (mm)	2.93 ± 0.32	3.00 ± 0.39	2.72 ± 0.43	3.00 ± 0.55	2.62 ± 0.39	2.77 ± 0.67	2.68 ± 0.49	3.32 ± 0.72	2.38 ± 0.49	3.18 ± 0.72
LVPWd (mm)	1.80 ± 0.39	1.95 ± 0.42	1.78 ± 0.39	2.22 ± 0.38	1.88 ± 0.28	2.03 ± 0.43	1.98 ± 0.56	1.86 ± 0.19	1.62 ± 0.30	1.94 ± 0.42
IVSs (mm)	3.32 ± 0.38	3.15 ± 0.62	3.10 ± 0.55	3.67 ± 0.71	3.68 ± 0.55	3.22 ± 0.41	3.04 ± 1.10	3.24 ± 0.58	3.30 ± 0.71	3.38 ± 0.36
IVSd (mm)	1.77 ± 0.16	1.77 ± 0.29	1.73 ± 0.13	2.00 ± 0.27	1.92 ± 0.23	1.80 ± 0.43	1.96 ± 0.55	1.62 ± 0.46	1.78 ± 0.19	1.86 ± 0.30
LVEDV (mL)	0.63 ± 0.15	0.55 ± 0.26	0.68 ± 0.22	0.48 ± 0.22	0.80 ± 0.38	0.65 ± 0.26	0.54 ± 0.26	0.44 ± 0.37	0.50 ± 0.16	0.44 ± 0.15
LVESV (mL)	0.05 ± 0.05	0.07 ± 0.05	0.15 ± 0.17	0.05 ± 0.10	0.13 ± 0.12	0.08 ± 0.08	0.10 ± 0.07	0.04 ± 0.09	0.06 ± 0.09	0.02 ± 0.04
SV (mL)	0.60 ± 0.14	0.50 ± 0.24	0.58 ± 0.10	0.40 ± 0.22	0.65 ± 0.31	0.57 ± 0.20	0.44 ± 0.21	0.40 ± 0.28	0.40 ± 0.16	0.38 ± 0.13
CO (L/min)	0.17 ± 0.04	0.12 ± 0.07	0.15 ± 0.02	0.09 ± 0.04	0.16 ± 0.07	0.14 ± 0.06	0.12 ± 0.06	0.09 ± 0.04	0.11 ± 0.04	0.10 ± 0.03
EF (%)	92.85 ± 3.36	89.83 ± 6.38	83.50 ± 14.01	84.12 ± 18.64	85.80 ± 8.92	89.55 ± 5.71	80.68 ± 7.18	90.22 ± 6.40	87.00 ± 7.26	92.64 ± 1.78
FS (%)	61.25 ± 6.78	57.72 ± 12.38	51.52 ± 18.55	53.75 ± 21.09	52.07 ± 12.09	56.62 ± 10.47	44.80 ± 8.12	58.58 ± 13.68	52.18 ± 8.29	59.94 ± 3.22
mFS (%)	25.40 ± 3.67	22.05 ± 3.24	21.50 ± 7.14	18.10 ± 12.31	20.95 ± 4.67	25.25 ± 3.25	18.84 ± 3.43	15.22 ± 3.37	19.92 ± 1.76	19.80 ± 7.22
LAD (mm)	4.22 ± 0.71	3.57 ± 0.47	4.34 ± 1.08	3.82 ± 0.36	4.87 ± 0.53	3.83 ± 0.69	4.30 ± 0.58	4.15 ± 0.42	4.58 ± 0.42	3.76 ± 0.29
AOD (mm)	3.37 ± 0.38	3.23 ± 0.35	3.34 ± 0.73	3.44 ± 0.25	3.85 ± 0.23	3.12 ± 0.51	3.28 ± 0.39	3.65 ± 0.83	3.44 ± 0.22	3.12 ± 0.33
LA/Ao	1.25 ± 0.19	1.10 ± 0.08	1.30 ± 0.11	1.12 ± 0.13	1.26 ± 0.11	1.24 ± 0.17	1.31 ± 0.08	1.17 ± 0.18	1.34 ± 0.18	1.21 ± 0.13

fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; HR – heart rate; LVIDs – left ventricular internal dimension at end systole; LVIDd – left ventricular internal dimension at end diastole; LVPWs – left ventricular posterior wall thickness at end systole; LVPWd – left ventricular posterior wall thickness at end diastole; IVSs – interventricular septum thickness at end systole; IVSd – interventricular septum thickness at end diastole; LVEDV – left ventricular end-diastolic volume; LVESV – left ventricular end-systolic volume; SV – stroke volume; CO – cardiac output; EF – ejection fraction; FS – fractional shortening; mFS – mid-wall fractional shortening; LAD – left atrial diameter; AOD – aortic diameter; LA/Ao – left atrial to aortic root ratio. Values are presented as mean ± standard deviation

Table 4. Blood cell counts at two time points: at the beginning of the study (t1) and after eight weeks of EPP supplementation (t2)

Index	fa/fa H_2O		fa/fa 100		fa/fa 200		fa/+ 100		fa/+ 200	
	t1	t2	t1	t2	t1	t2	t1	t2	t1	t2
WBC (K/µL)	10.40 ± 1.63	7.05 ± 1.96	10.50 ± 1.26	6.02 ± 2.33	11.26 ± 2.56	6.40 ± 1.29	8.52 ± 0.86	5.00 ± 1.41	9.38 ± 0.99	3.73 ± 1.25
RBC (G/µL)	7.49 ± 0.21	8.36 ± 0.69	7.63 ± 0.23	8.59 ± 0.44	7.60 ± 0.13	8.19 ± 0.53	7.53 ± 0.64	8.07 ± 0.54	7.69 ± 0.24	7.82 ± 0.63
HGB (g/L)	16.13 ± 0.37	14.53 ± 0.99	16.10 ± 0.37	14.80 ± 0.65	15.90 ± 0.34	14.32 ± 0.73	15.70 ± 0.73	13.88 ± 0.88	16.05 ± 0.42	13.90 ± 0.35
HCT (%)	43.47 ± 1.25	43.23 ± 3.31	39.18 ± 12.49	44.52 ± 2.32	44.12 ± 0.92	42.22 ± 2.66	41.88 ± 3.76	41.86 ± 2.61	42.75 ± 1.48	39.75 ± 3.18
PLT (K/µL)	1,069.33 ± 207.16	748.00 ± 76.58	1033.83 ± 74.09	658.67 ± 67.82	1078.80 ± 76.59	718.20 ± 52.11	905.80 ± 53.27	656.60 ± 244.39	911.67 ± 60.07	738.00 ± 39.33
MCV (fL)	58.00 ± 1.10	51.83 ± 0.75	57.67 ± 0.52	51.83 ± 0.41	58.00 ± 1.00	51.60 ± 0.55	55.60 ± 1.14	51.80 ± 0.84	55.50 ± 0.55	51.00 ± 0.00
MCH (pg)	21.55 ± 0.58	17.43 ± 0.63	21.15 ± 0.22	17.25 ± 0.29	20.88 ± 0.40	17.50 ± 0.42	20.90 ± 1.07	17.18 ± 0.26	20.90 ± 0.19	17.87 ± 1.11
MCHC (g/dL)	37.15 ± 0.69	33.65 ± 1.13	36.50 ± 0.49	33.30 ± 0.62	36.02 ± 0.29	33.92 ± 0.73	37.62 ± 2.00	33.20 ± 0.17	37.58 ± 0.42	35.08 ± 2.23
RET (%)	2.17 ± 0.87	1.85 ± 1.56	2.72 ± 0.31	1.15 ± 0.82	3.60 ± 0.67	0.92 ± 0.33	2.34 ± 0.61	1.12 ± 1.11	1.48 ± 0.69	0.53 ± 0.30
NEU (%)	13.33 ± 6.92	25.50 ± 4.76	19.33 ± 8.04	21.50 ± 4.59	20.40 ± 5.08	33.20 ± 5.72	16.25 ± 7.37	20.75 ± 3.95	12.83 ± 5.53	18.67 ± 4.50
LYM (%)	81.17 ± 5.00	71.50 ± 5.68	76.83 ± 10.26	74.83 ± 6.11	78.40 ± 5.13	61.80 ± 4.76	81.00 ± 8.68	75.75 ± 5.68	84.67 ± 4.63	79.50 ± 4.46
MONO (%)	2.50 ± 3.51	0.83 ± 0.98	1.00 ± 0.89	0.33 ± 0.52	0.20 ± 0.45	2.00 ± 1.58	0.50 ± 1.00	0.25 ± 0.50	1.83 ± 1.33	0.17 ± 0.41
EOS (%)	1.83 ± 0.75	0.67 ± 0.52	1.50 ± 1.38	2.33 ± 2.42	0.60 ± 0.55	1.60 ± 1.34	2.00 ± 1.15	2.50 ± 1.29	0.33 ± 0.52	1.33 ± 1.03

fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; WBC – white blood cell count; RBC – red blood cell count; HGB – haemoglobin concentration; HCT – hematocrit; PLT – platelet count; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular hemoglobin concentration; RET – percent of reticulocytes; NEU –- percent of neutrophils; LYM – percent of lymphocytes; MONO – percent of monocytes; EOS – percent of eosinophils. Values are presented as mean ± standard deviation

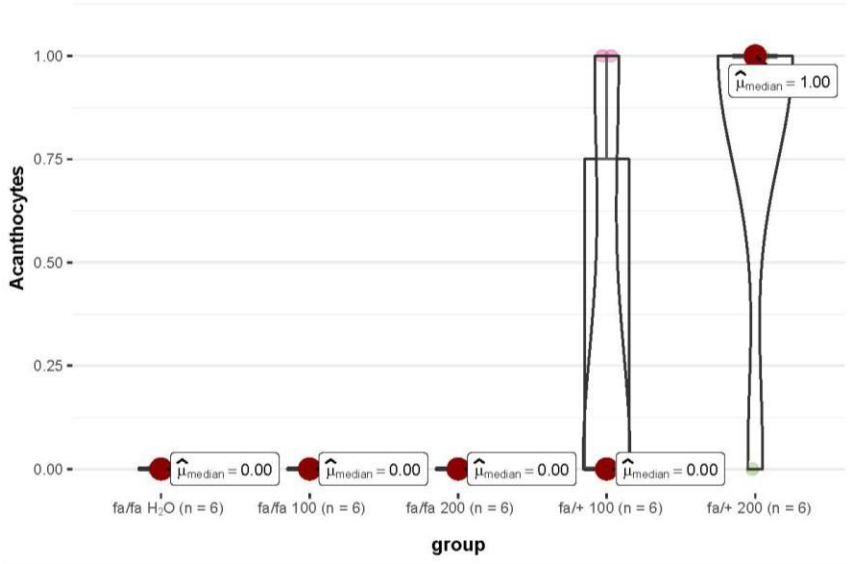


Fig. 4. Violin plot of the level of acanthocytes by group after eight weeks of pomegranate peel supplementation. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; $\hat{\mu}$ – predicted value of group means

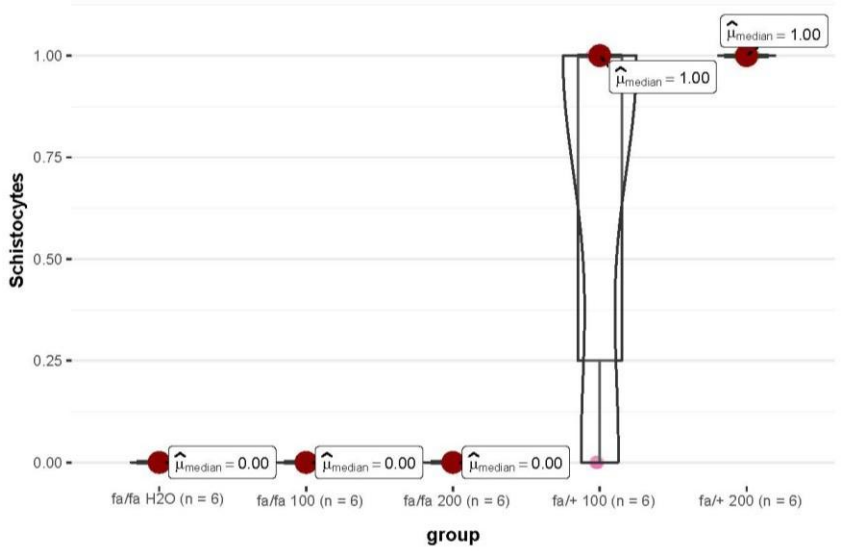


Fig. 5. Violin plots of the level of schistocytes by group after eight weeks of pomegranate peel supplementation. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; $\hat{\mu}$ – predicted value of group means

Effects of pomegranate peel extract on glucose and lipid profile. The levels of fasting blood glucose and serum lipidic indices are presented in Table 5. Regarding the glucose level, the EPP supplementation did not improve the glycaemic status of rats. The glucose concentration in individuals with MetS (fa/fa H_2O, fa/fa 100 and fa/fa 200) was significantly elevated compared to rats without MetS (fa/+ 100 and fa/+ 200), as was to be expected (Fig. 7). The same pattern was observed in the lipid profiles. No significant differences ($P > 0.05$) were found in the total cholesterol (CHOL), LDL, HDL or triglyceride concentrations among the tested groups (Fig. 8).

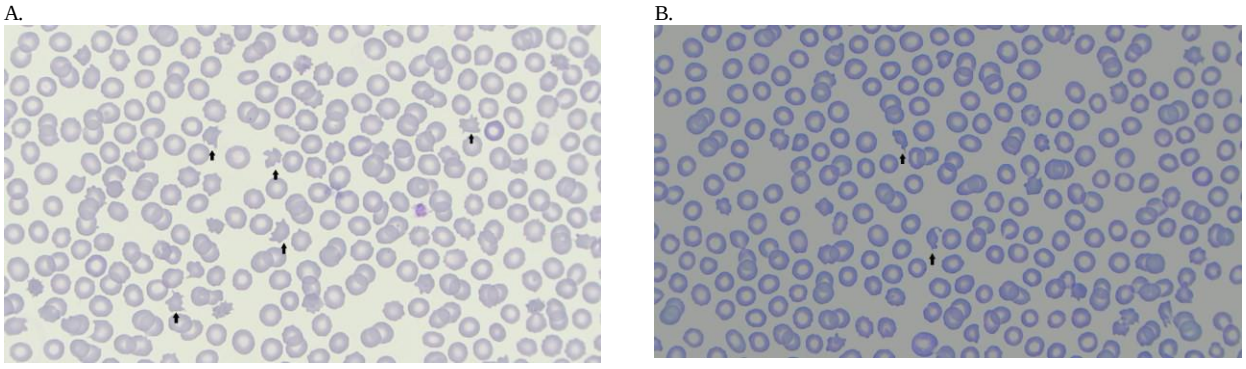


Fig. 6. A – Acanthocytes and B – schistocytes found in smears of rat peripheral blood. Abnormal cells are indicated by arrows

Table 5. Glucose and lipid profile at the beginning of the study (t1) and after eight weeks of pomegranate supplementation (t2)

Index	fa/fa H_2O		fa/fa 100		fa/fa 200		fa/+ 100		fa/+ 200	
	t1	t2	t1	t2	t1	t2	t1	t2	t1	t2
GLU (mmol/L)	17.50 ± 5.90	59.40 ± 11.30	21.40 ± 6.40	63.00 ± 12.80	21.10 ± 8.50	57.80 ± 8.50	7.60 ± 0.60	23.00 ± 3.70	7.70 ± 1.20	22.90 ± 2.80
CHOL (mmol/L)	4.30 ± 0.30	5.10 ± 0.70	4.30 ± 0.20	4.80 ± 0.50	4.10 ± 0.10	5.10 ± 1.00	3.40 ± 0.20	2.20 ± 0.30	3.60 ± 0.10	2.40 ± 0.10
LDL (mmol/L)	2.50 ± 0.10	2.90 ± 0.40	2.40 ± 0.20	3.00 ± 0.40	2.40 ± 0.20	3.20 ± 0.60	1.70 ± 0.10	1.30 ± 0.20	1.80 ± 0.10	1.40 ± 0.10
HDL (mmol/L)	2.20 ± 0.10	2.80 ± 0.20	1.90 ± 0.10	2.80 ± 0.20	1.90 ± 0.20	2.80 ± 0.40	1.20 ± 0.20	1.20 ± 0.20	1.20 ± 0.10	1.10 ± 0.10
TG (mmol/L)	11.50 ± 2.70	5.60 ± 5.60	12.70 ± 3.40	5.70 ± 2.90	12.50 ± 2.80	5.90 ± 1.30	1.30 ± 0.30	1.20 ± 0.60	1.30 ± 0.30	0.90 ± 0.60

fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; GLU – glucose concentration; CHOL – total cholesterol concentration; LDL – low-density lipoprotein concentration; HDL – high-density lipoprotein concentration; TG – triglyceride concentration. Values are presented as mean ± standard deviation

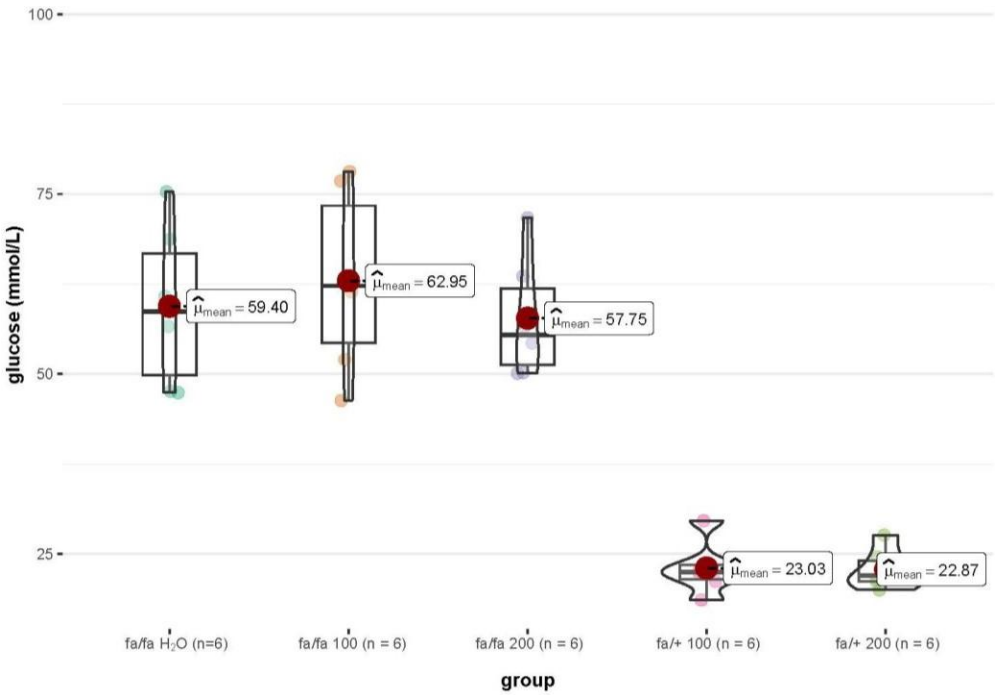
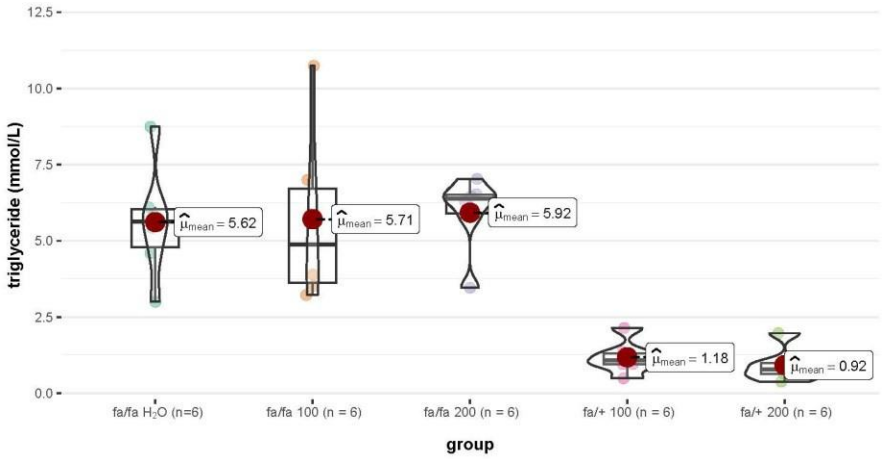
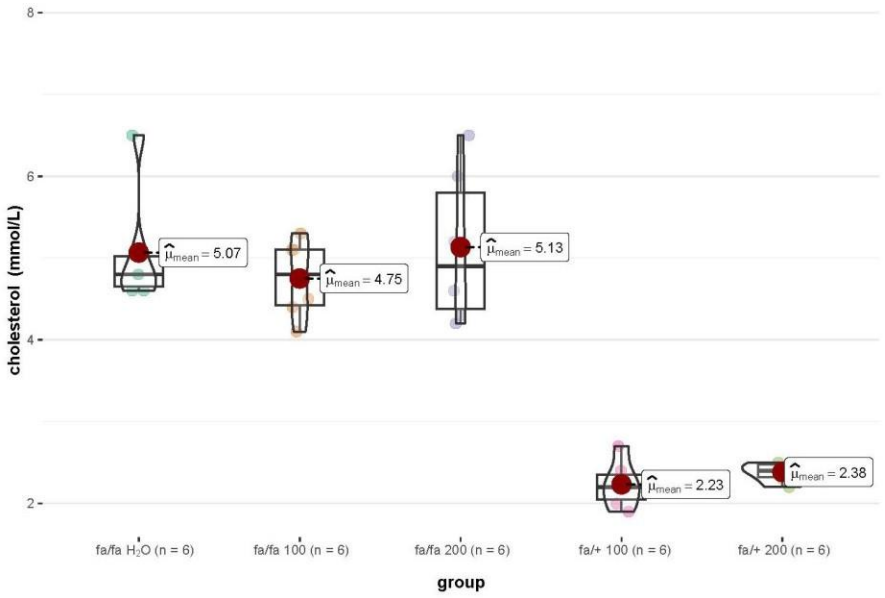


Fig. 7. Violin plots of fasting blood glucose concentrations by group after eight weeks of pomegranate peel supplementation. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; $\hat{\mu}$ – predicted value of group means

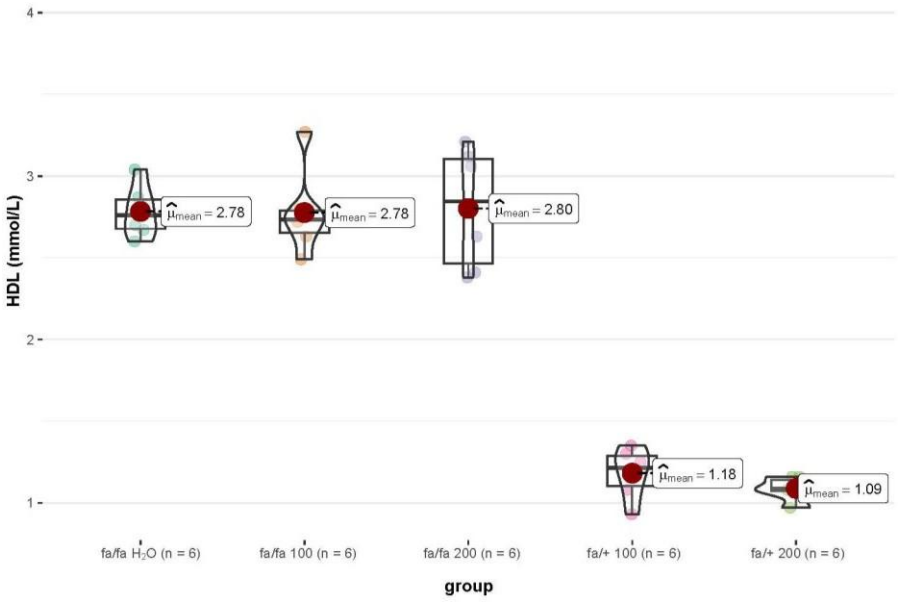
A.


triglyceride (mmol/L)
12.5
10.0
7.5
5.0
2.5
0.0
$\hat{\mu}_{mean}$ = 5.62
$\hat{\mu}_{mean}$ = 5.71
$\hat{\mu}_{mean}$ = 5.92
$\hat{\mu}_{mean}$ = 1.18
$\hat{\mu}_{mean}$ = 0.92
fa/fa H2O (n=6)
fa/fa 100 (n = 6)
fa/fa 200 (n = 6)
fa/+ 100 (n = 6)
fa/+ 200 (n = 6)
group

B.


cholesterol (mmol/L)
8
6
4
2
$\hat{\mu}_{mean}$ = 5.07
$\hat{\mu}_{mean}$ = 4.75
$\hat{\mu}_{mean}$ = 5.13
$\hat{\mu}_{mean}$ = 2.23
$\hat{\mu}_{mean}$ = 2.38
fa/fa H2O (n = 6)
fa/fa 100 (n = 6)
fa/fa 200 (n = 6)
fa/+ 100 (n = 6)
fa/+ 200 (n = 6)
group

C.


HDL (mmol/L)
4
3
2
1
$\hat{\mu}_{mean}$ = 2.78
$\hat{\mu}_{mean}$ = 2.78
$\hat{\mu}_{mean}$ = 2.80
$\hat{\mu}_{mean}$ = 1.18
$\hat{\mu}_{mean}$ = 1.09
fa/fa H2O (n = 6)
fa/fa 100 (n = 6)
fa/fa 200 (n = 6)
fa/+ 100 (n = 6)
fa/+ 200 (n = 6)
group

D.

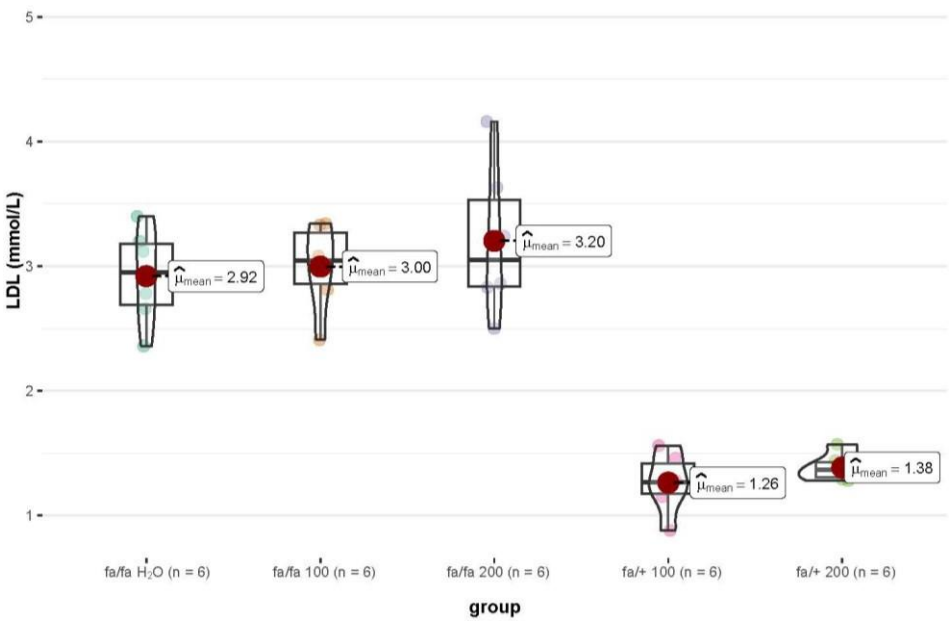


Fig. 8. Violin plots of lipid profiles by group after eight weeks of pomegranate peel supplementation. A – serum triglyceride concentration; B – serum total cholesterol concentration; C – serum high-density lipoprotein concentration; D – serum low-density lipoprotein concentration. fa/fa H_2O – control group of Zucker diabetic fatty (ZDF) rats with missense mutation in the *Lepr* leptin receptor gene administered only water; fa/fa 100 – experimental group of ZDF rats with this mutation administered 100 mg/kg body weight (b.w.) pomegranate peel extract (EPP); fa/fa 200 – experimental group of ZDF rats with this mutation administered 200 mg/kg b.w. EPP; fa/+ 100 – experimental group of ZDF rats without this mutation administered 100 mg/kg b.w. EPP; fa/+ 200 – experimental group of ZDF rats without this mutation administered 200 mg/kg b.w. EPP; $\hat{\mu}$ – predicted value of group means

Discussion

Pomegranate (*Punica granatum* L.) is a promising source of polyphenols that could help prevent or mitigate the course of MetS. The flesh excluded, pomegranates contain a substantial amount of active phenolic compounds. These compounds scavenge various reactive oxygen species (ROS) (14). For many years juice was believed to contain the most bioactive particles that could be utilised as supplements and nutraceuticals in miscellaneous diseases (2). However, studies investigating the health benefits of pomegranate peel showed that polyphenols in this material possess even higher antioxidant activity than the pulp itself (14, 24). Its nutritional value has been explored in many *in vivo* studies on animal models, and these proved that pomegranate peel extract reduces obesity (38), dyslipidaemia (34), hypertension (11), and hyperinsulinaemia and insulin resistance (49), and also ameliorates chronic inflammatory status (30). In the presented study, we aimed to evaluate the health benefits of pomegranate peel extract supplementation in a model of MetS and healthy control rats maintained on a high-calorie diet.

The role of pomegranate peel extract in preventing obesity has been previously reported (30). Our result consonantly indicates that phenolic compounds derived from pomegranate peel possess bioactivity that could be helpful in the prevention or mitigation of body weight gain. The ability of pomegranate peel to prevent obesity depends on the impact of polyphenol metabolites in the expression of four proteins: an adipose-formulation–related one such as adiponectin, peroxisome proliferator–activated receptor γ, glucose transporter type 4, and fatty acid–binding protein 4 (23). It has also been proposed that oral supplementation with polyphenols modulates gut microbiota, which elicits an obesity control effect (30, 38). *In vivo* studies have shown that phenolic compounds could promote beneficial bacterial species, such as *Bifidobacterium* spp. and *Lactobacillus* spp., as well as suppress potentially pathogenic bacteria (46). Targeting microbiota with the pomegranate by-product phenolic compounds which have probiotic properties encourages the proliferation of *Bacteroides* spp. and significantly reduces the abundance of *Firmicutes* spp. and *Proteobacteria* spp. (38). Inverse proportionality of these species of bacteria was reported to be a likely diagnostic biomarker of gut dysbiosis correlated with the risk of metabolic diseases (36). One benefit of phenolic compounds is that they promote the abundance of beneficial bacterial genera, and a separate positive effect is that the fermentation of polyphenols by bacteria contributes to the generation of metabolite products, especially urolithin A, that inhibit triglyceride accumulation and suppress the gene expression involved in adipogenesis (23, 41). The relationship between the consumption of polyphenols and gut microbiota is complex and certainly requires further investigation.

Obesity is associated with many pathological conditions, such as insulin resistance, atherosclerosis

and lipid metabolism disorders (18). Pomegranate peel extract also improves the glycaemic and lipid profiles. It has been proved that EPP lowers the serum concentration of triglycerides, total cholesterol and LDL-cholesterol in obese hamsters and an obese mice model (25, 30, 38). The mechanisms lying behind this propitious impact on lipid metabolism were partially revealed. The outcomes elucidated how phenolic compounds derived from pomegranate peel upregulated liver X receptor α, peroxisome proliferator-activated receptor α, peroxisome proliferator-activated receptor γ and gene ATP-binding cassette transporter A1, downregulated fatty acid synthase through inhibition of the keto-acetyl synthase and acetyl/malonyl transferase domains, and supported cholesterol removal by enhancing faecal bile acid (25, 26). Interestingly, in the study on hamsters, the effect of pomegranate ellagic acid extracted from peel on lipid metabolism was stronger than that of simvastatin alone in raising HDL and lowering LDL (25). Contrary to these findings, our research indicated no change in lipid profile in individuals treated with pomegranate peel extract. However, some other *in vivo* studies with results consistent with ours also reported no difference in serum levels of triglycerides and cholesterol fractions between the control group and pomegranate-treated groups on a high-fat, high-sucrose diet (16).

Metabolic syndrome also leads to improper insulin utilisation and production (47). As evidenced by recent studies, many natural plant derivatives classified as flavonoids, alkaloids, terpenoids and phenolics display antidiabetic properties (35). Phenolic components from pomegranate peels, of which punicalagin, gallic acid and ellagic acid are examples, have been proved to exhibit antidiabetic, antihyperglycemic and antiglycation effects (28, 29). Pomegranate peel diminishes fasting blood glucose concentration and improves insulin sensitivity through various mechanisms. Research findings highlighted the crucial involvement of pomegranate phenolics in carbohydrate regulation (44). The principal mechanisms are the inhibition of α-glucosidases and α-amylases (20), inhibition of advanced glycation end-product formation (8), and mitigation of hyperglycaemic-induced oxidative stress (10). Pomegranate seed oil extract improved insulin tolerance and reduced serum fasting blood glucose in diet-induced obese mice (16). However, in the same study, extracts from pomegranate flowers and peel did not invigorate carbohydrate metabolism and in this were inferior to rosiglitazone; the extracts did nevertheless decrease the plasma level of proinflammatory cytokines IL-6 and TNF-α, which also protects against dysregulation of glucose metabolism (10, 16). Furthermore, anthocyanins extracted from pomegranate peel favourably altered the insulin signalling pathway. These findings implied that the supplementation with polyphenols from pomegranate peel might alleviate insulin resistance (38). In the present study, EPP did not lower the glucose level in the serum. The data from other research on animal models are also at some variance on this point.

The cardiovascular consequences of MetS also comprise an alteration in cardiac function associated with obesity. This cardiac failure in MetS was described as "cardiomyopathy of obesity" (43). It is characterised in humans by the development of concentric left ventricular hypertrophy and mild diastolic or systolic dysfunction with normal or elevated EF (3). Impairment in myocardial contractility also has been reported in animal models of obesity (27). Furthermore, changes occurring in MetS disrupt the equilibrium between coronary blood flow and myocardial metabolism, significantly increasing the risk of myocardial infarction and mortality (42). The pleiotropic effect of phenolic compounds on cardiovascular diseases comprises vasodilatative activity and anti-inflammatory, antithrombotic and antiatherogenic effects (32). Cardiovascular protection is also provided with polyphenols from pomegranate peel. A study in a spontaneously hypertensive rat model found the consumption of EPP to reduce systolic blood pressure, coronary angiotensin-converting enzyme activity and oxidative stress level, and prevent vascular remodelling (11). Evidence suggests that phenolics from peel may find application in mitigating coronary heart disease by attenuation of electrocardiographic changes, myocardial tissue damage and heart weight increase. Notably, the phenolics' role as a cardioprotective agent arises from their upregulating of endothelial nitric oxide synthase expression, leading to intensification of antioxidant mechanisms and inhibition of apoptosis (15). In addition, punicalagin suppresses cardiac fat accumulation by stimulating the cardiac adenosine monophosphate–activated protein kinase signalling pathway. Concomitantly, it prevents mitochondrial loss by enhancing mitochondrial biogenesis and ameliorating oxidative stress (6). In our study, we also noted a cardioprotective effect of the largest EPP supplementation in the relative decrease in heart rate. Likewise, EPP improves mFS, representing myocardial contractility.

Blood morphological and haematological indices represent the general state of health of individuals. The assessment of these parameters could indicate metabolic abnormalities or the noxious effect of xenobiotics (31). Changes in blood morphology and haematology may also suggest pathological conditions such as anaemia, infection, thrombotic state and bone marrow impairment. In this context, red blood cell morphology is a very sensitive marker of exposure to ROS (17). Polyphenols are characterised by low toxicity and, in recent years, have gained attention as natural radioprotective and cytoprotective agents (40). Our findings demonstrated that no toxicity levels manifested by haematological changes were observed in individuals treated with EPP. In the blood smears, the only alterations were the increased number of acanthocytes and schistocytes in rats without genetically programmed MetS, which may have resulted from diet-induced

obesity and its consequences. No blast cells nor anaemic state were observed.

The present study suggests that phenolic compounds from pomegranate peel have a potentially beneficial effect in dietary intervention in metabolic syndrome. Together these results confirm the promise of pomegranate peel as a nutrient, especially in restricting body weight gain. However, studies on humans and animals suffering from MetS are needed in order to determine the bioaccessibility of bioactive constituents and metabolites and to indicate their actual effectiveness in ameliorating individual abnormalities involved in the pathogenesis of MetS. Polyphenols incorporated into the diet of humans and animals could help maintain health and counteract metabolic syndrome.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The experiment was conducted in accordance with the local Ethical Committee rules and regulations.

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8 MANUSKRYPT III

Niewiadomska J., Kasztura M., Janus I., Chełmecka E., Stygar D.M., Frydrychowski P., Wojdyło A., Noszczyk-Nowak A.: Punica granatum L. extract show cardioprotective effects measured by oxidative stress markers and biomarkers of heart failure in an animal model of metabolic syndrome. Antioxidants, 2023, 12, 1152 https://doi.org/10.3390/antiox12061152

8.1 Streszczenie manuskryptu III

Zespół metaboliczny (MetS) jest to zbiór wzajemnie ze sobą powiązanych zaburzeń metabolicznych obejmujących otyłość wisceralną, hiperglikemię, inuslinooporność, podwyższone wartości ciśnienia tętniczego oraz dyslipidemię. Ich współwystępowanie koreluje ze zwiększeniem ryzyka wystąpienia chorób sercowo-naczyniowych, które stanowią wiodącą przyczynę śmiertelności ogólnej. Wprowadzenie na rynek naturalnych standaryzowanych nutraceutyków o właściwościach kardioprotekcyjnych mogłoby przyczynić się do ograniczenia występowania incydentów sercowo-naczyniowych, a tym samym zmniejszenia związanej z nimi umieralności i niepełnosprawności. Zaprezentowane badanie miało na celu ocenę kardioprotekcyjnych właściwości związków polifenolowych pochodzących ze skórek granatowca w zespole metabolicznym na modelu zwierzęcym.

Doświadczenie przeprowadzono na szczurach Zucker Diabetic Fatty rats (ZDF, fa/fa), które otrzymywały etanolowy ekstrakt ze skórek granatowca (EPP) w dwóch dawkach: 100 mg/kg mc. (MetS 100) oraz 200 mg/kg mc. (MetS 200). Kontrolę stanowiły szczury otrzymujące tylko wodę (MetS control). Ekstrakt podawano przez 8 tygodni. W trakcie badania wszystkie osobniki miały stały dostęp do wody oraz wysokokalorycznej karmy Purina 5000. Po 8 tygodniach, szczury zostały poddane eutanazji, a następnie pobrano krew oraz wycinki lewej komory mięśnia sercowego i aorty wstępującej. Oceniono wpływ związków polifenolowych ze skórek *Punica granatum* L. na stężenie wybranych markerów stresu oksydacyjnego (CAT, SOD, MnSOD, GR, GST, GPx, TOS, SH, MDA) oraz biomarkerów niewydolności serca (cTnI, GAL-3). Analizie poddano także preparaty histologiczne.

Suplementacja ekstraktem wiązała się z zależnym od dawki wzrostem stężenia SH w homogenatach z mięśnia sercowego ($p<0,001$). Aplikacja 100 mg/kg mc. EPP w porównaniu do 200 mg/kg mc. EPP wiązała się z istotną redukcją TOS ($p<0.001$). Stężenia CAT i GST były znamiennie wyższe w odpowiedzi na dawkę 100 mg/kg mc. EPP w porównaniu do grupy kontrolnej, MetS control ($p<0.001$). Wśród osobników otrzymujących EPP w ilości 200 mg/kg mc. nie zaobserwowano podobnej tendencji. Nie stwierdzono różnic w stężeniach GR ($p=0.063$), SOD ($p=0.455$), MnSOD ($p=0.155$) oraz MDA ($p=0.790$) po ekspozycji na związki polifenolowe ze skórek granatowca. Podaż ekstraktu nie miała wpływu na stężenie cTnI oraz GAL-3. Ocena histologiczna preparatów z serca oraz aorty nie ujawniła toksyczności związanej z aplikacją ekstraktu.

Uzyskane rezultaty potwierdzają korzystny wpływ związków polifenolowych ze skórek *Punica granatum* L. na potencjał oksydacyjno-redukcyjny miokardium. Nie zaobserwowano złagodzenia remodelingu tkanki mięśnia sercowego oraz ograniczenia stopnia uszkodzenia kardiomiocytów.

Słowa kluczowe: skórki *Punica granatum* L., kardioprotekcja, stres oksydacyjny, zespół metaboliczny

antioxidants

MDPI

Article

Punica granatum L. Extract Shows Cardioprotective Effects Measured by Oxidative Stress Markers and Biomarkers of Heart Failure in an Animal Model of Metabolic Syndrome

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Abstract: Metabolic syndrome (MetS) significantly increases the risk of cardiovascular diseases (CVD), a leading cause of death globally. The presented study investigated the cardioprotective role of dietary polyphenols found in pomegranate peels in an animal model of metabolic syndrome. Zucker diabetic fatty rats (ZDF, MetS rats, fa/fa) were supplemented with polyphenol-rich pomegranate peel extract (EPP) at two dosages: 100 mg/kg BW and 200 mg/kg BW. The extract was administered for 8 weeks. The effect of ethanolic peel extract on the concentration of oxidative stress markers (CAT, SOD, MnSOD, GR, GST, GPx, TOS, SH, and MDA), biomarkers of heart failure (cTnI, GAL-3), and alternations in tissue architecture was assessed. The results showed a significant increase in SH concentration mediated via EPP supplementation ($p < 0.001$). Treatment with a 100 mg/kg BW dosage reduced the TOS level more efficiently than the higher dose. Interestingly, the CAT and GST activities were relevantly higher in the MetS 100 group ($p < 0.001$) compared to the MetS control. The rats administered EPP at a dose of 200 mg/kg BW did not follow a similar trend. No differences in the GR ($p = 0.063$), SOD ($p = 0.455$), MnSOD ($p = 0.155$), and MDA ($p = 0.790$) concentration were observed after exposure to the pomegranate peel extract. The administration of EPP did not influence the cTnI and GAL-3 levels. Histology analysis of the heart and aorta sections revealed no toxic changes in phenolic-treated rats. The findings of this study prove that the extract from pomegranate peels possesses free radical scavenging properties in the myocardium. The effect on alleviating ventricular remodeling and cardiomyocyte necrosis was not confirmed and requires further investigation.

Keywords: *Punica granatum* L. peels; cardioprotection; oxidative stress; metabolic syndrome

1. Introduction

Cardiovascular diseases (CVDs) are still the leading cause of mortality worldwide [1,2]. This group of heart and blood vessel disorders comprises principally coronary heart disease, peripheral arterial disease, cerebrovascular disease, and thrombo-embolic disease [3]. A crucial part of preventing CVDs is lifestyle modification, especially based on developing

healthy eating patterns and physical activity. To control the burden of CVD-related mortality and disability, clinical strategies aimed at declining modifiable risk factors, including dyslipidemia, hypertension, diabetes, alcohol consumption, smoking, and obesity, must be implemented [4]. However, the global trend of increasing cardiovascular risk factors in most world regions has been observed, mainly linked to an epidemic of obesity [5].

One cluster of pathologies that significantly contributes to increased heart disease, stroke, and diabetes is metabolic syndrome (MetS). These conditions include central obesity, elevated blood pressure, hyperglycemia, insulin resistance, and dyslipidemia [6,7]. Each constitutes a single risk factor conducive to cardiovascular disorders. However, their co-occurrence increases the risk to an extremely high level. Obesity is believed to play a prominent role in MetS pathogenesis, and constitutes a trigger agent for other components. MetS is also associated with pro-thrombotic and proinflammatory states [8]. Cardiovascular changes occurring in MetS comprise a multifaceted image of overlapping pathologies. Obesity-associated alternations in heart muscle anatomy and function have been defined as "cardiomyopathy of obesity" [9]. The cardiac phenotype in obesity comprises concentric left ventricular hypertrophy, diastolic and systolic dysfunction, myocardial fibrosis, and microvascular dysfunction. Finally, the deterioration in myocardial function leads to heart failure [10,11]. Using biomarkers in the detection of heart impairment seems to be pivotal in verifying cardiac acute and chronic alternations. One such biomarker that is associated with the disruption of myocardial integrity is troponin I (cTnI), reflecting acute cardiac injury [12,13]. Galectin-3 (GAL-3), in turn, can be used to obtain insight into the progression and severity of myocardial architecture remodeling, including fibrosis [14,15].

Polyphenols are secondary metabolites produced by plants through metabolic pathways acting as protective agents against biotic and abiotic stresses and contributing to plants' growth and development [16,17]. The nutrient value of phytochemicals is guaranteed by antioxidant, anti-inflammatory, antibacterial, antifungal, antiallergic, antiangiogenic, anticoagulant, immunomodulatory, anticancerogenic, and antimutagenic properties [18,19]. Previously conducted studies have proven that the supplementation of phenolic compounds can mitigate or even reverse the pathological changes in cardiovascular diseases (coronary heart disease, stroke, heart failure, and thrombo-embolic disease) and endocrine disorders (diabetes mellitus and osteoporosis) [20,21].

The parley on the cardiovascular protective role of polyphenols remains open. The relevancy of polyphenol intake and CVD outcomes has been investigated in many studies. Some natural sources of phenolic compounds and individual particles, such as resveratrol, purple grapes, green tea, and coca, exert a favorable effect on cardiovascular health [21]. Phenolic compounds' mechanism of action in reference to cardiovascular protection comprises the inhibition of platelet aggregation and activation; boosting of the bioavailability of nitric oxide for the endothelium, resulting in vasodilatation; free radical scavenging properties; and the inhibition of LDL oxidation [18,22].

Pomegranate (*Punica granatum* L.) has been used as a traditional remedy for centuries in the Middle East. Different parts of the plant contain various phytochemicals. In particular, fruit consumed as juice, pulp or beverage was believed to possess more bioactive compounds than the non-editable parts. However, health benefits are also attributed to peels, leaves, or seeds [23,24]. It has been proven that pomegranate peels, a by-product of juice extraction, are abundant in phenolic compounds such as flavonoids, ellagitannins, and anthocyanidins, and metabolites such as urolithins [25]. Compared to the juice and seeds, pomegranate peel extract exhibits comparable antioxidant and anti-inflammatory activity. Moreover, it also displays antidiabetic, antiatherosclerotic, antihypertensive, antihyperlipidemic, antimicrobial, and anticancer properties [26]. In this context, peels appear to be valuable biowaste products with promising biological and pharmaceutical activities.

The present study focused on the role of dietary polyphenols found in pomegranate peels as cardiovascular protective agents regarding the oxidative status in heart tissue, the level of biomarkers of heart failure, and histopathologic changes occurring in the heart and aorta in an animal model of metabolic syndrome.

2. Materials and Methods

2.1. Animals and Diet

The study project was approved by the Ethics Committee for Experiments on Animals at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wroclaw, Poland (Resolution 53/2017). Zucker diabetic fatty rats (metabolic syndrome rats (MetS), ZDF-Leprfa/Crl, fa/fa) were purchased from Sulzfeld (Charles River Laboratories, Research Models and Services, Germany GmbH). The rats were housed in groups of two per cage and maintained on a 12 h light–dark cycle at a temperature of 20 °C ± 2 °C and a relative humidity of 40–60%. All animals had free access to water and Purina 5008 feed (LabDiet, Charles River Laboratories, Wilmington, MA, USA).

2.2. Extraction Procedure of Pomegranate Peel Phenolic Compounds

The fresh pomegranate fruit *Punica granatum* L. (cultivar Mollar de Eche) was delivered from Spain. The peels were collected, dried, and shredded in Thermomix. Subsequently, the obtained peel powder in the total amount of 1 kg was extracted and reextracted twice with 50% ethanol in an ultrasonic bath for 25 min. After the extraction procedure, the mixtures were centrifugated, and the supernatants were filtered. The resulting filtrate was concentrated by a Rotavapor rotary evaporator in a water bath at 40 °C. The concentrated extract from pomegranate peel was passed through the column filled with Amberlite XAD-16 resin (Brenntag, Essen, Germany) and eluted with distilled water to remove the organic acids, sugars, and other compounds. The polyphenol elution procedure was carried out with an 80% aqueous ethanol solution. Afterward, the gathered fractions were dried in an SPT-200 vacuum oven (Zeamil, Krakow, Poland).

2.3. Identification of Polyphenolic Compounds by LC-PDA – QTOF/MS and Quantification via the UPLC-PDA Method

The identification and quantification of polyphenols from pomegranate peels were performed with the use of an Acquity ultra-performance liquid chromatography (UPLC) system, consisting of a photodiode array detector (PDA; Waters Corporation, Milford, MA) coupled with a G2 mass detector quadrupole time-of-flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters Corp., Milford, CT, USA), equipped with an electrospray ionization (ESI) source. Separation was carried out on the Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm, Waters Corp., Milford, CT, USA). The samples were dissolved in MeOH/H_2O/ascorbic acid (30:68:1, *v*/*v*/*m*) with a 1% hydrochloric acid (37%) mixture. The volume injected was 10 µL. The mobile phase was composed of a mixture of 0.1% (*v*/*v*) aq. formic acid (A) and acetonitrile (B). The gradient program was set as follows: the initial phase consisted of 99% of A for 1 min; subsequently, an 11 min linear gradient of 1% to 40% of B was applied, and after 12 min, B was increased to 100%, continued to 2 min, and then returned to inductive conditions (99% of A) at a 2 min step. For elution, a mobile phase flow rate of 0.45 mL/min was used. The operating parameters for the Q-TOF MS were as follows: capillary voltage of 2.5 kV, cone voltage of 30 V, cone gas flow of 11 L/h, collision energy of 28–30 eV, source temperature of 100 °C, desolvation temperature of 250 °C, collision gas, argon, desolvation gas (nitrogen) flow rate of 300 L/h, data acquisition range of m/z 100–2000 Da, and a negative ionization mode. The data from LC-MS were analyzed with the MassLynx 4.0 ChromaLynx Application Manager software. Individual phenolic compounds were characterized via the retention time and exact molecular masses. Identification was based on MS/MS analysis and comparison of the obtained results with literature data. The quantification of the derivatives of ellagic acid was performed using UPLC-PDA. The spectra were measured in the 200–600 nm wavelength range in steps of 2 nm. The findings are presented as milligrams per 100 g dry matter (dm).

2.4. Experimental Design

After 2 weeks of acclimatization, the rats were divided into three groups as follows: (1) MetS control—these control-group (ZDF) rats received only water; (2) MetS 100—this study group (ZDF) received an extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW; and (3) MetS 200—this study group (ZDF) received EPP at a dose of 200 mg/kg BW. Each group consisted of six animals. The extract from the pomegranate peels was administered daily by oral gavage using water as a vehicle. During the study, body weight in all groups increased pointedly, reaching 407.14 ± 32.68 g in MetS control, 382.08 ± 35.48 g in MetS 100, and 392.75 ± 36.28 g in Mets 200 by the end of the study. The performed biochemistry panel assessing the glycemic and lipid profile proved the hyperglycemic and hyperlipidemic status of the animals used in the study. After 8 weeks, the rats were fasted overnight and euthanized via an intraperitoneal injection of pentobarbital. Before the termination procedure, every individual was sedated using a mixture of intramuscular anesthetics as follows: ketamine at a dose of 60 mg/kg and medetomidine at a dose of 0.3 mg/kg. The heart and aorta samples were subsequently harvested. The dissected tissues were immersed in liquid N_2 and subsequently stored at −80 °C. Serum samples were collected at three consecutive time points: before EPP administration, after 4 weeks, and after 8 weeks of study (Figure 1).

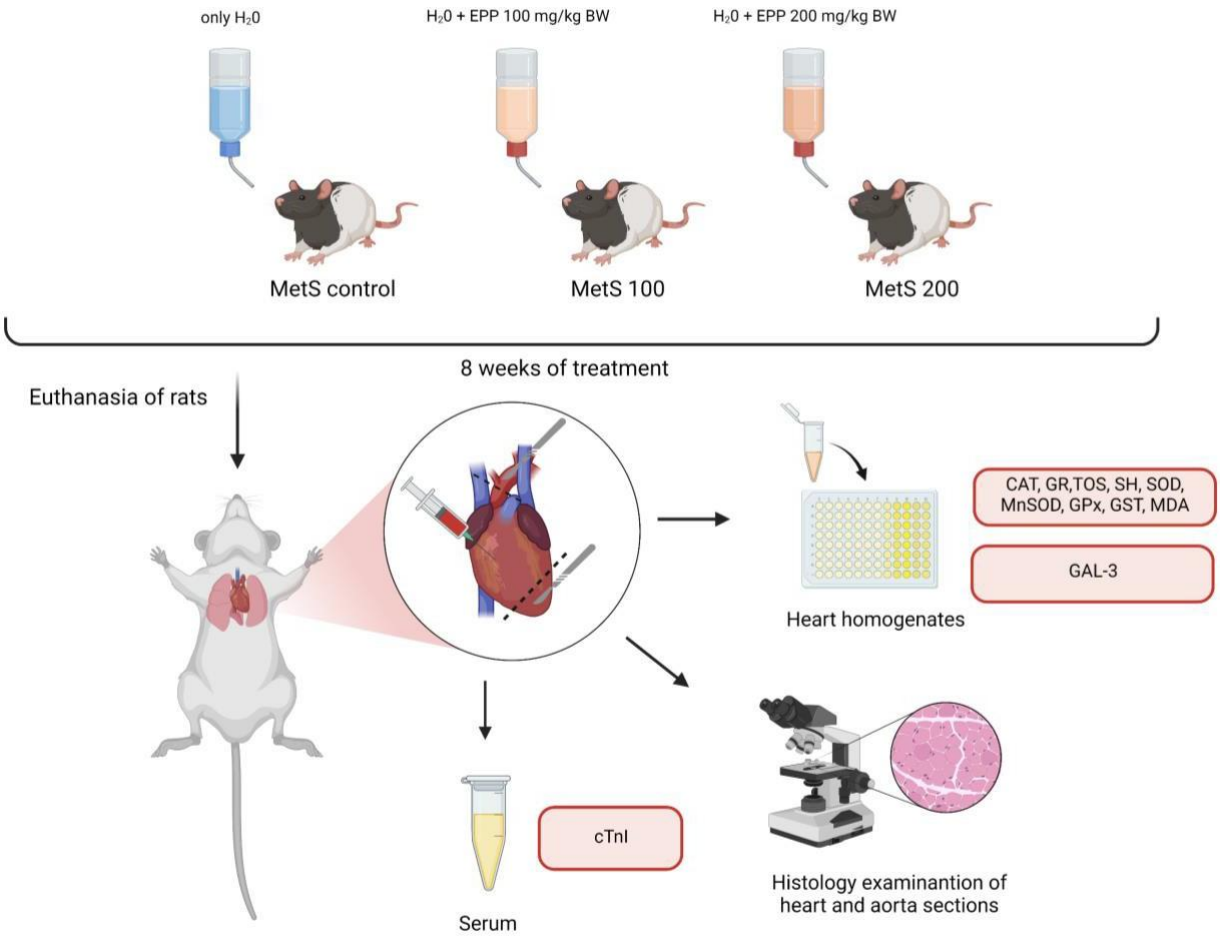

Figure 1. Experimental design. This scheme was created using Biorender.com (accessed on 11 May 2023). Abbreviations: EPP—extract from pomegranate peels, BW—body weight, MetS—rats with a mutation in the leptin receptor gene predisposed to metabolic syndrome, CAT—catalase, GR—glutathione reductase, TOS—total oxidative status, SH—protein thiol groups, SOD—superoxide dismutase, MnSOD—Mn-dependent superoxide dismutase, GPx—glutathione peroxidase, GST—glutathione transferase, MDA—malondialdehyde, GAL-3—galectin 3, and cTnI—troponin I.

2.5. Preparation of Homogenates from Heart Tissue

The fragments of cardiac tissue from the left ventricle were weighted (about 100 mg) and homogenized (1:10 *w*/*v*) in PBS with protease inhibitors using tissueruptor (Qiagen). The lysates were then incubated for 10 min on ice cold water and centrifuged for 15 min at 14,000× *g* at 4 °C. The protein concentration in the supernatants was assessed according to the Lowry method [27]. The protein samples were frozen and stored in liquid nitrogen for further analysis.

2.6. Assessment of Oxidative Stress Markers

The evaluation of antioxidant capacity was analyzed in the heart tissue. The following biomarkers were determined: the activity of total catalase (CAT, IU/g protein), superoxide dismutase (SOD, NU/mg protein), glutathione reductase (GR, IU/g protein), glutathione transferase (GST, IU/g protein), glutathione peroxidase (GPx, IU/g protein), and Mn-dependent superoxide dismutase (MnSOD, NU/mg protein). Moreover, the protein thiol groups (SH, µmol/g protein), to indicate the redox potential and total oxidative status (TOS, µmol/g protein), were estimated to assess the entire antioxidants in the samples. The malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation secondary to increased oxidative stress. For the measurement of the change in absorbance in spectral assays, a Perkin Elmer Victor X3 reader was applied (PerkinElmer, Inc., Waltham, MA, USA).

2.6.1. Catalase Activity (CAT, EC 1.11.1.6)

Catalase activity was assessed following the Aebi protocol [28]. Following the method principles, the heart tissue homogenate was mixed with phosphate buffer (50 mM TRIS/HCl), and subsequently, the reaction was initiated by adding hydrogen peroxide (H_2O_2). The enzyme activity was measured using the UV spectrophotometric method, monitoring the alternation of 240 nm absorbance, as the decomposition of H_2O_2 is followed by a decrease in absorbance. CATactivity was displayed per 1 g of protein (IU/g protein).

2.6.2. Superoxide Dismutase Activity (SOD, EC 1.15.1.1)

Superoxide dismutase activity was determined using the Oyanagui method [29]. In this protocol, xanthine oxidase catalyzes the reaction, generating the superoxide anion free radical (O_2^-), which oxidases hydroxylamine (which turns into the nitrile form). The nitro anion, together with a chromogenic agent, gives a colored product. In the presence of SOD in samples, the reaction of the formation of nitro anion is reduced because of the inhibitory effect of the enzyme on the superoxide anion-free radical. The SOD activity was measured spectrophotometrically at 550 nm and expressed as nitrile units (NU) per 1 mg of protein (NU/mg protein).

MnSOD activity was assessed by adding potassium cyanide to the reaction mixture, which is responsible for the inactivity of CuZnSOD. The total MnSOD activity was presented in nitrile units per 1 mg of protein (NU/mg protein).

2.6.3. Glutathione Reductase Activity (GR, EC 1.8.1.7)

GR activity was determined using the kinetic method described by Calberg and Manervick [30]. The core of the method is based on the NDPH-dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH). The produced beta-nicotinamide dinucleotide phosphate (NDPH) as a hydrogen donor in the regeneration of GSH was measured spectrophotometrically at 340 nm. GR activity was presented per 1 g of protein (IU/g protein).

2.6.4. Glutathione Peroxidase Activity (GPx, EC 1.11.1.9)

GPx activity was assessed using the kinetic method, according to Mannervik [31]. The enzyme catalyzes the reduction of hydroperoxide (ROOH) coupling with the oxidation of glutathione (GSH) to glutathione disulfide (GSSG), and beta-nicotinamide dinucleotide

phosphate (NDPH) to nucleoside-diphosphate (NDP). In this method, the decrease in NDPH absorbance measured at 340 nm correlated with GPx activity, which was expressed per 1 g of protein (IU/g protein). As a substrate, tert-Butyl hydroperoxide was used.

2.6.5. Glutathione S-Transferase Activity (GST, EC 2.5.1.18)

GST activity was measured using the kinetic method established by Habig and Jakoby [32]. In this spectral assay of the reduced glutathione and analyzed sample, the substrate 1-chloro-2,4-dinitrobenzene was added. The principle of the method arises from the change in a specific quantity of substrate after its conjugation with glutathione (GSH). After incubating the reaction mixture at room temperature, the change in absorbance at 340 nm was measured. GST activity was displayed per 1 g of protein (IU/g protein).

2.6.6. Protein Thiol Groups (SHs)

Protein thiol group (SH) concentration was determined according to Sedlak and Lindsay [33]. This spectrophotometric protocol is based on Elman's reagent, 5,5^{t}-dithiobis-(2,-nitrobenzoic acid), i.e., DTNB. Aliquot tissue homogenates were mixed with tris buffer and DTNB. Subsequently, the proteins were precipitated by incubation with methanol. The absorbance of relatively clear supernatants was read at 412 nm, and the SH concentration was presented in µmol/g protein.

2.6.7. Total Oxidative Status (TOS)

Total oxidative status (TOS) determination followed the colorimetric method according to Erel [34]. The technique is grounded on the oxidation of the ferrous ion–o-dianisidine complex to ferric ion by the oxidants in the analyzed sample. In an acid medium, the produced ferric ion reacts with xylenol orange, making a colored complex that can be measured spectrophotometrically at a 560 nm wavelength. Hydrogen peroxide was used for calibration. The TOS result was expressed in µmol/g protein.

2.6.8. Malondialdehyde Concentration (MDA)

Malondialdehyde concentration was determined using the previously described protocol by Ohkawa et al. [35]. The procedure of assessing lipid peroxides in animal tissue is based on their reaction with thiobarbituric acid. As the pH affects the reagents mixture, the pH value was set at 3.5. The measurements were conducted spectrophotometrically and compared with a standard curve. Tetramethoxypropane (TMP) was used as the external standard. The MDA concentration was displayed in µmol/g protein.

2.7. Assessment of Heart Failure Biomarkers

Galectin-3 (GAL3, pg/mL) and troponin I (cTnI, pg/mL) were used as the biomarkers of heart failure. These proteins correlate with heart muscle remodeling and impairment, including fibrogenesis, inflammation, and myocardial necrosis. Commercial ELISA assays were applied.

2.7.1. Galectin-3 (GAL-3)

Galectin-3 concentration in heart tissue homogenates was measured using a quantitative Rat Galectin-3 (LGALS3) ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA). The myocardial tissue samples were homogenized in PBS and centrifugated. An ELISA assay was carried out according to the manufacturer's instructions. GAL-3 in the analyzed samples, after incubating with immobilized monoclonal antibodies, was detected using biotin-conjugated anti-GAL3 polyclonal antibodies. Then, streptavidin horseradish peroxidase and 3,3^{t},5,5^{t}-tertamethylbenzidine (TMB) were added to initiate the reaction. The absorbance was read within 30 min after adding the stop solution at 450 nm, and the GAL-3 concentration was calculated based on the standard curve and expressed in pg/mL.

2.7.2. Troponin T (cTnI)

Troponin I in the serum was assessed using quantitative the Rat Cardiac Troponin I (ab246529) ELISA Kit (Abcam, Eugene, OR, USA), applying the manufacturer's protocol. After adding to appropriate wells, the samples and standards were incubated with an antibody cocktail for an hour. The reaction was developed using a 3,3′,5,5′-tertamethylbenzidine (TMB) substrate solution. The results were read on a microplate reader set at a wavelength of 450 nm. The cTnI concentration was determined by interpolating the absorbance values against the standard curve and presented in pg/mL.

2.8. Histological Analysis

Histology examination was performed on the heart and aorta tissue samples. The specimens were fixed in 7% buffered formalin, dehydrated, embedded in paraffin blocks, and cut into 6 µm sections. The sections were stained with hematoxylin and eosin (HE), Masson-Goldner trichrome (MGt), and Picro Sirius Red stain (PSR). The heart and aorta specimens were evaluated on a semi-quantitative scale from 0 (no changes) to 3 (severe changes). The examined changes in the heart samples included the presence and intensity of parenchymal degeneration, the presence and intensity of fibrosis, and the presence and intensity of inflammatory infiltration. In the specimens obtained from the aorta, the fiber disarrangement was evaluated.

2.9. Statistical Analysis

The data are displayed as the mean ± standard deviation (SD) for biochemical analysis or median (min–max) for histological analysis. Statistical analyses were conducted using Statistica 12.5 PL. The one-way parametric ANOVA, followed by post hoc Dunnett testing was applied for variables with a normal distribution. The figures show the mean values and 95% confidence intervals. The distribution and variance homogeneity were evaluated using the Shapiro–Wilk and Levene's tests, respectively. The outliers in the data were detected and not considered in statistical analysis; $p < 0.05$ was considered significant.

3. Results

3.1. Identification and Quantification of Phenolic Compounds in Pomegranate Peel Extract

Ellagic acid and its derivatives were found in the analyzed sample of extract from pomegranate peels. Among the 14 ellagic acid derivatives, the most abundant were: punicalagin isomer (R_t = 2.12 min), HHDP-gallagyl-hexoside (R_t = 2.87 min), HHDP-gallagyl-hexoside (R_t = 3.69 min), ellagitannin (R_t = 5.08 min), and ellagic acid-hexoside (R_t = 6.08 min). Hydrolyzable tannins possessed a higher concentration and seemed to be the main antioxidant compound in pomegranate peels. The results of the analysis are presented in Table 1.

Table 1. Mass spectrum characteristic and content of phenolic compounds in pomegranate peel extract.

T pRt	MS $[M-H]^-$ (*m/z*)	MS/MS $[M-H]^-$ (*m/z*)	Name of Compound	Polyphenol Content
1.67	331	271/169	Galloyl-glucose	2.00 ± 0.03
1.73	781	721/601	Punicalin α/A	3.11 ± 0.06
2.02	1083	611/331/146	HHDP-galloyl-hexoside (punicalagin)	4.20 ± 0.09
2.12	1083	781/622/301	Punicalagin isomer	14.82 ± 1.04
2.33	933	631/450/301	Ellagitannin	4.71 ± 0.40
2.87	1083	781/301	HHDP-gallagyl-hexoside (punicalagin)	93.91 ± 2.05
3.12	1085	907/783/301	Ellagic acid derivative	2.49 ± 0.53
3.69	1083	781/301	HHDP-gallagyl-hexoside (punicalagin)	157.0 ± 2.65
3.89	799	301	Granatin A	4.74 ± 0.32

Table 1. *Cont.*

T pRt	MS [M-H]$^-$ (*m*/*z*)	MS/MS [M-H]$^-$ (*m*/*z*)	Name of Compound	Polyphenol Content
5.08	783	481/301	Ellagitannin	25.86 ± 1.53
6.20	1085	933/301	Digalloyl-gallagyl-hexoside	10.37 ± 0.65
6.25	783	481/301	Ellagitannin	13.51 ± 0.99
6.38	463	301	Ellagic acid-hexoside	33.63 ± 1.23
6.89	951	907/635/301	Galloyl-HHDP-DHHDP-hex (granatin B)	2.68 ± 0.11
			Total (mg/g dw)	373.05

3.2. Impact on Oxidative Stress Markers

There were significant differences in myocardial CATactivity between the groups (Figure 2). Statistical analysis exhibited that in the groups with MetS, the CAT concentration was higher in the experimental group MetS 100 treated with 100 mg/kg EPP than in the control MetS group ($p < 0.001$). No statistical significance was observed in the experimental group MetS 200 treated with 200 mg/kg EPP in comparison to rats receiving only water ($p < 0.364$).

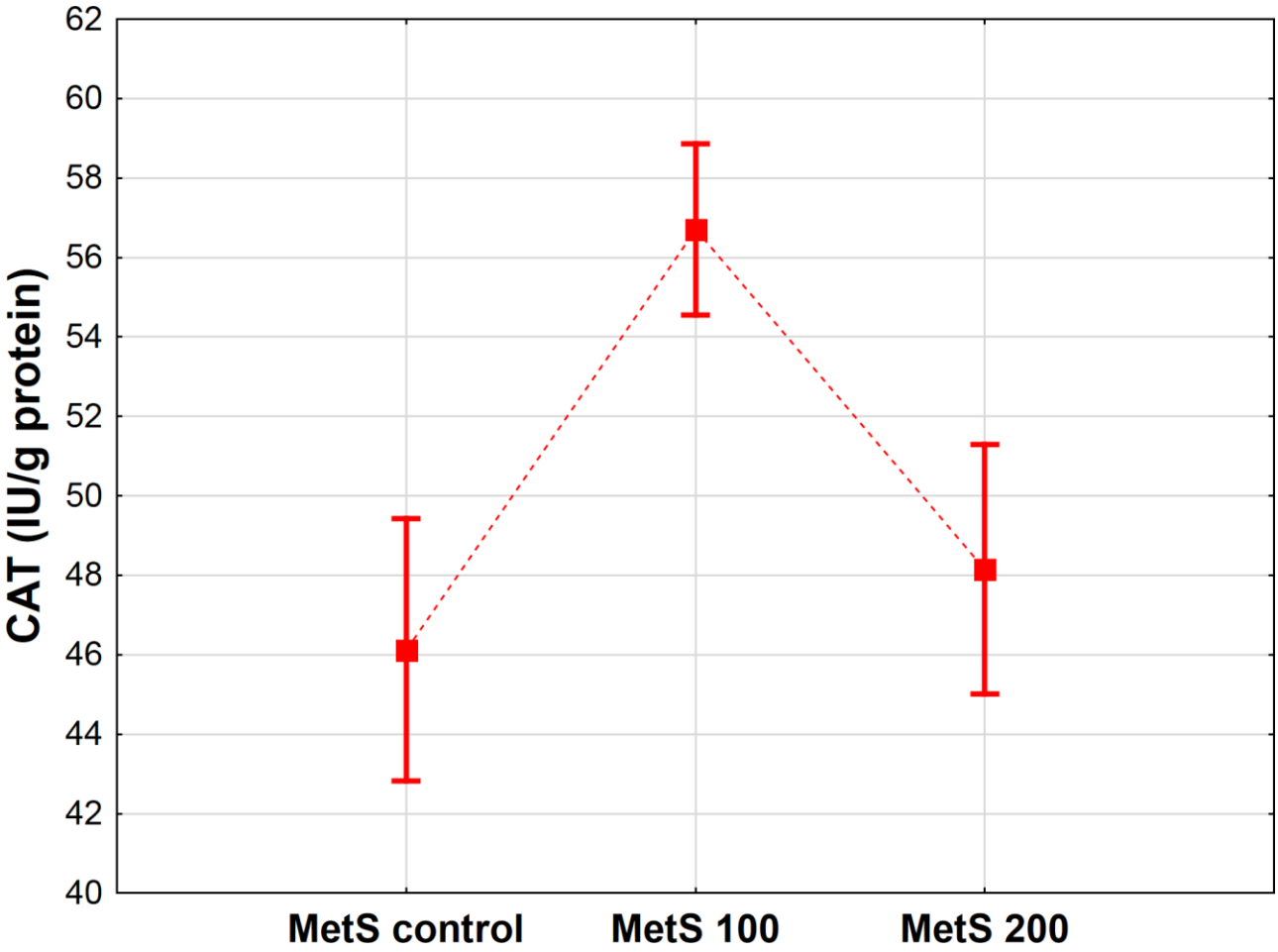

Figure 2. Mean catalase activity (CAT, IU/g protein) in heart homogenates of rats from the groups administered only water, as well as the extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW, or 200 mg/kg BW. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). Vertical lines represent 95% confidence intervals, and the points reflect the mean of the group. For the reader's convenience, the markers were connected by dashed lines.

The TOS concentration was decreased in rats with MetS treated with 100 mg/kg EPP (MetS 100) in comparison to the control group (MetS control, $p < 0.001$). No explicit differences between the control group and the rats supplemented with 200 mg/kg were found ($p = 0.227$). The results are presented in Figure 3.

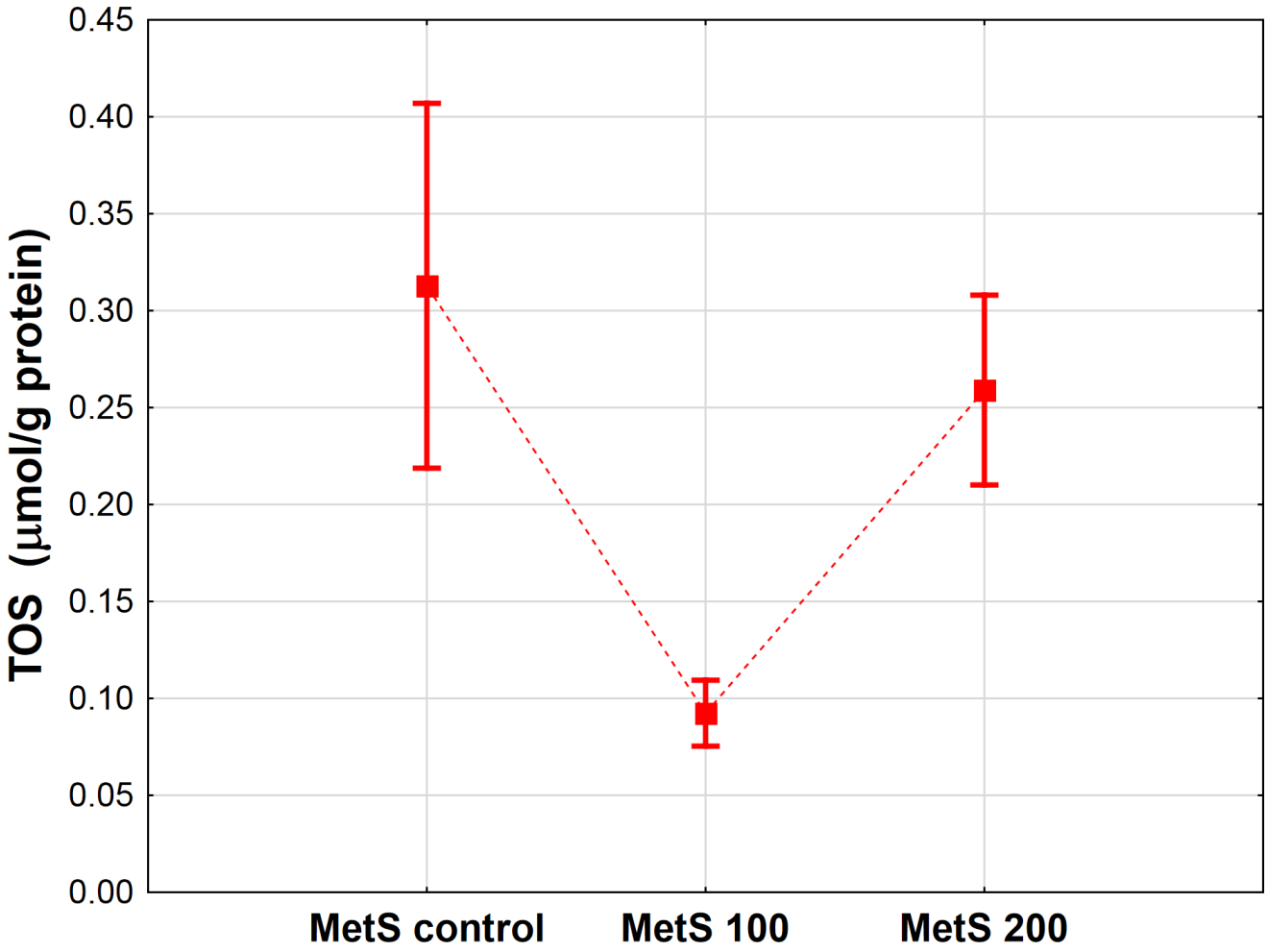

Figure 3. Mean total oxidative status (TOS, µmol/g protein) in heart homogenates of rats from the groups administered only water, as well as the extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW, or 200 mg/kg BW. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). Vertical lines represent 95% confidence intervals, and the points reflect the mean of the group. For the reader's convenience, the markers were connected by dashed lines.

As shown in Figure 4, a higher extract dosage led to a significant elevation in the SH concentration. A phenolic extract dosage of 200 mg/kg compared to the control group resulted in an upswing in free sulfhydryl compounds, which correlated with antioxidant capacity in MetS rats ($p < 0.001$). Correspondingly, a significant variation was noted between the MetS rats from the group treated with 100 mg/kg EPP and the control group ($p < 0.001$).

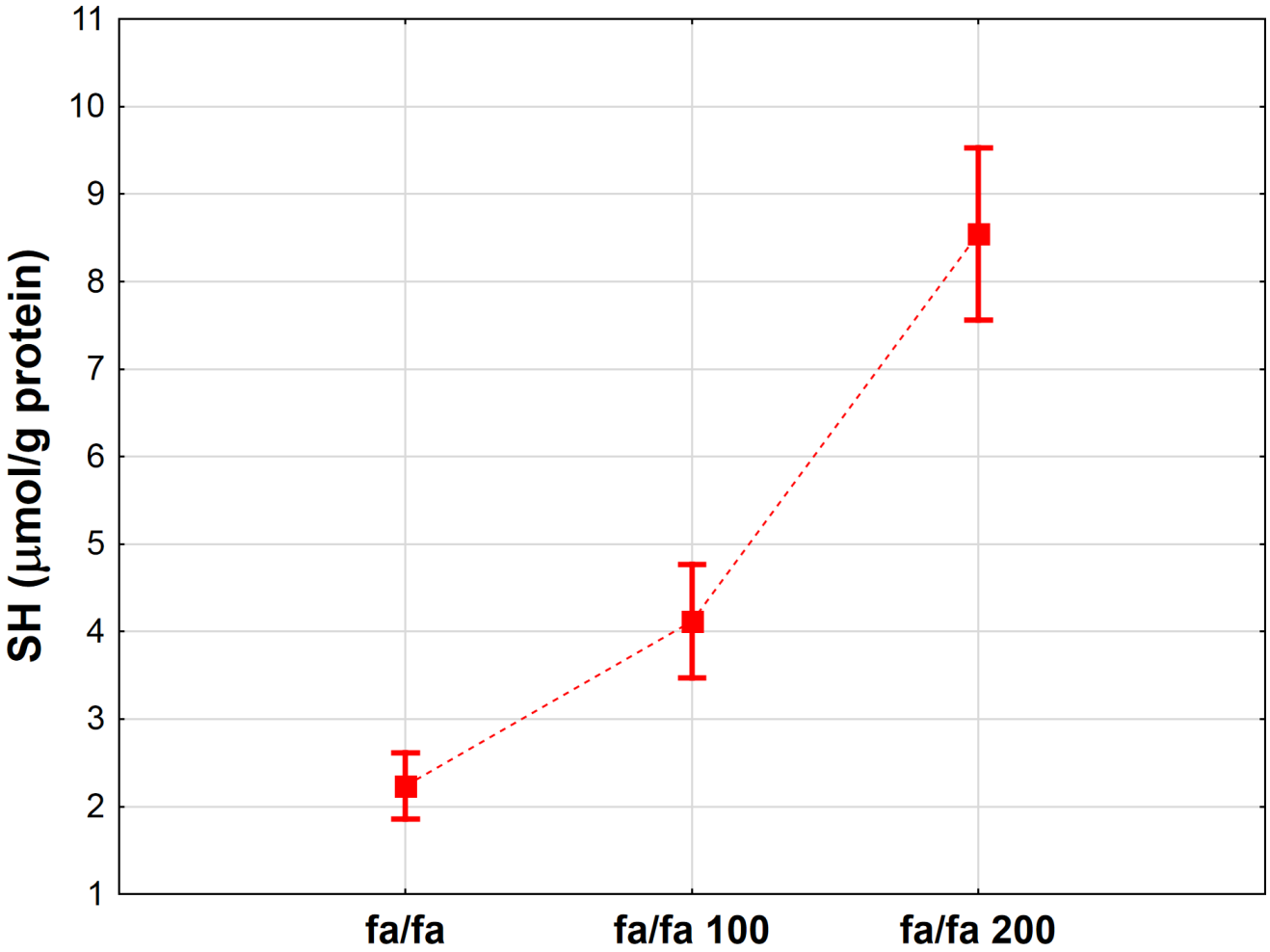

Figure 4. Mean protein thiol group concentration (SH, µmol/g protein) in heart homogenates of rats from the groups administered only water, as well as the extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW or 200 mg/kg BW. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). Vertical lines represent 95% confidence intervals, and the points reflect the mean of the group. For the reader's convenience, the markers were connected by dashed lines.

EPP supplementation also affected GPx activity in the analyzed samples ($p < 0.05$). According to the results, the GPx content showed an upward tendency in the group of MetS rats treated with a lower dose (MetS 100) compared to the MetS control ($p = 0.071$). No alternations were detected in phenolic-treated MetS groups exposed to higher doses versus the MetS control (MetS 200 vs. MetS control, $p = 0.876$). A graphical representation of the obtained data is displayed in Figure 5.

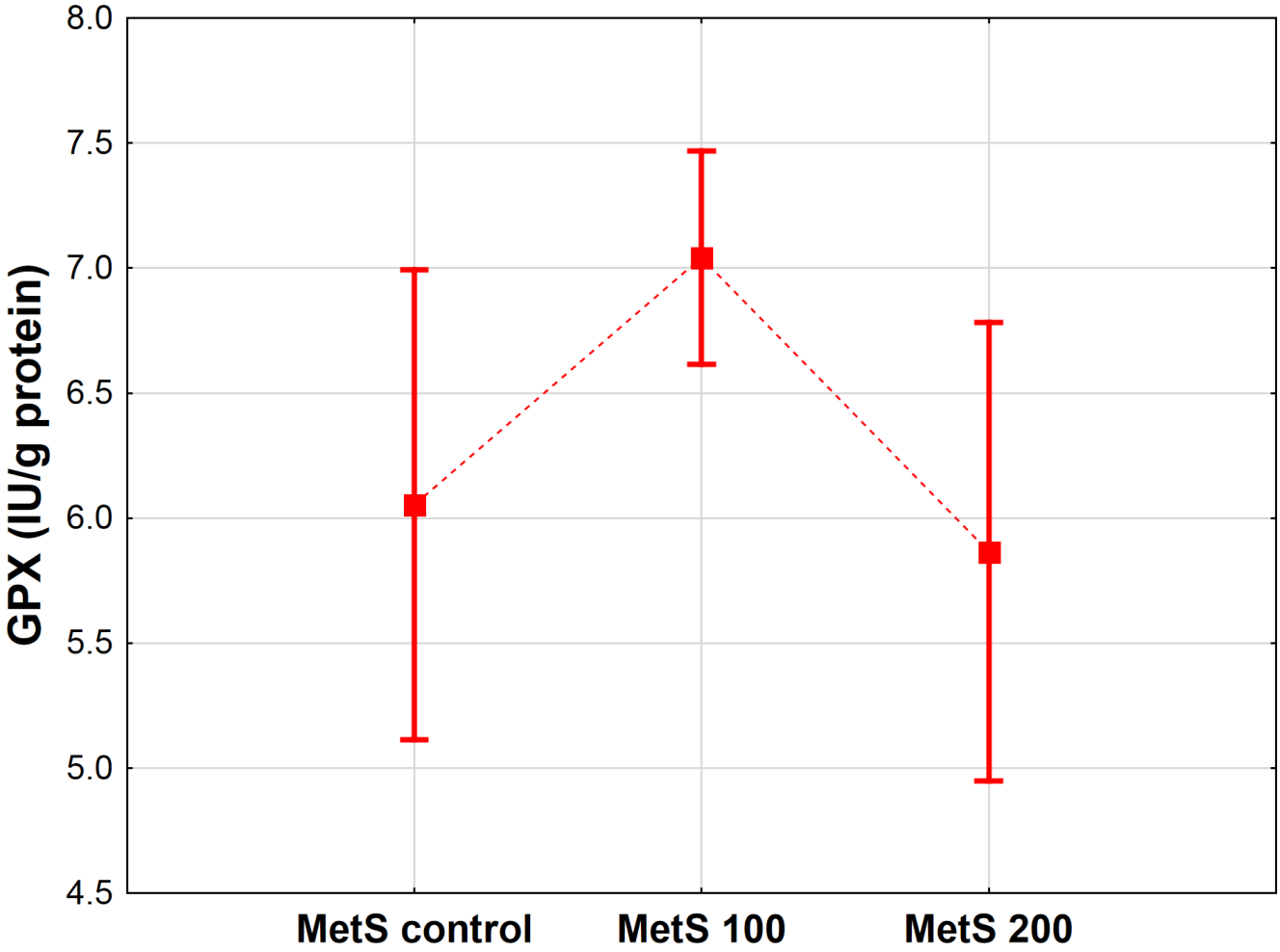

Figure 5. Mean glutathione peroxidase concentration (GPx, IU/g protein) in heart homogenates of rats from groups administered only water, as well as the extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW or 200 mg/kg BW. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). Vertical lines represent 95% confidence intervals, and the points reflect the mean of the group. For the reader's convenience, the markers were connected by dashed lines.

The results displayed in Figure 6 also revealed changes in GST activity in exposure to pomegranate peel extract ($p < 0.05$). Likewise, the GPx content and GST showed a significant difference only in the group of rats administered EPP at a dose of 100 mg/kg BW versus the MetS control, and the same uptrend in GST activity was observed ($p < 0.05$). In the rats treated with 200 mg/kg BW EPP, no statistically substantial variation was observed (MetS 200 vs. MetS control, $p = 0.991$).

No differences in the GR ($p = 0.063$), SOD ($p = 0.455$), MnSOD ($p = 0.155$), and MDA ($p = 0.790$) concentration were observed after exposure to pomegranate peel extract. The differences in oxidative stress markers by the group are presented in Table 2.

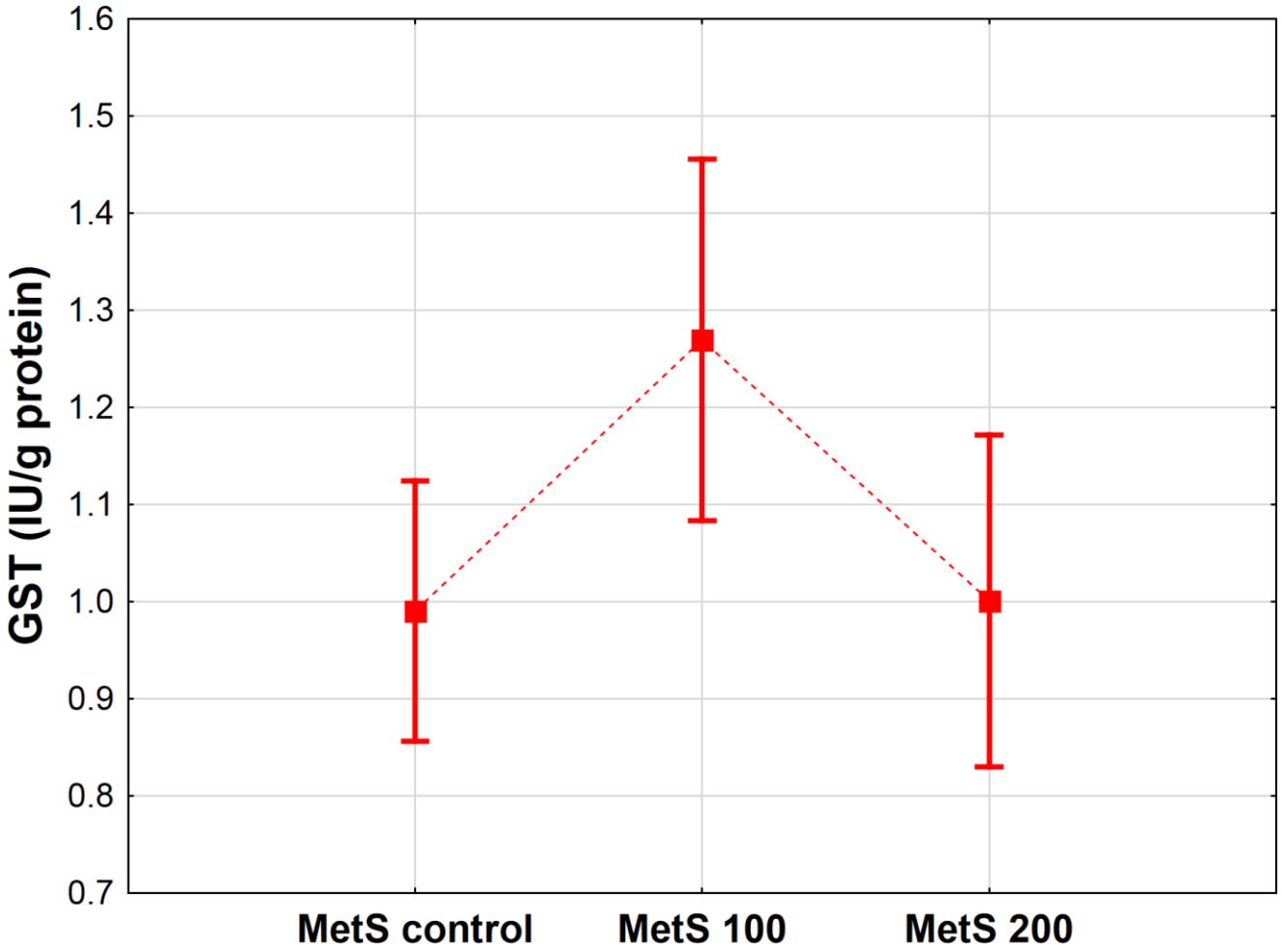

Figure 6. Mean glutathione transferase concentration (GST, IU/g protein) in heart homogenates of rats from groups administered only water, as well as the extract from pomegranate peels (EPP) at a dose of 100 mg/kg BW or 200 mg/kg BW. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). Vertical lines represent 95% confidence intervals, and the points reflect the mean of the group. For the reader's convenience, the markers were connected by dashed lines.

Table 2. Determination of oxidative stress marker concentration in the heart tissue by the group. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). The data are displayed as the mean ± standard deviation (SD).

	MetS Control	MetS 100	MetS 200	p_{ANOVA}	$p_{control\ vs.\ 100}$	$p_{control\ vs.\ 200}$
CAT (IU/g protein)	46.1 ± 3.14	56.7 ± 2.1	48.2 ± 3.0	<0.001	<0.001	0.364
GR (IU/g protein)	11.0 ± 1.4	12.0 ± 1.2	10.2 ± 1.2	0.063	–	–
TOS (µmol/g protein)	0.31 ± 0.09	0.09 ± 0.01	0.26 ± 0.05	<0.001	<0.001	0.227
SH (µmol/g protein)	2.2 ± 0.4	4.1 ± 0.6	8.5 ± 0.9	<0.001	<0.001	<0.001
SOD (NU/mg protein)	40.0 ± 1.4	41.6 ± 1.6	40.9 ± 3.1	0.455	–	–
MnSOD (NU/mg protein)	37.6 ± 1.1	39.2 ± 1.5	39,7 ± 2.5	0.155	–	–
GPx (IU/g protein)	6.1 ± 0.9	7.0 ± 0.4	5.9 ± 0.9	<0.05	0.071	0.876
GST (IU/g protein)	0.99 ± 0.13	1.27 ± 0.18	1.00 ± 0.16	<0.05	<0.05	0.991
MDA (µmol/g protein)	6.5 ± 0.7	6.1 ± 1.1	6.3 ± 1.4	0.790	–	–

Abbreviations: CAT—catalase, GR—glutathione reductase, TOS—total oxidative status, SH—protein thiol groups, SOD—superoxide dismutase, MnSOD—Mn-dependent superoxide dismutase, GPx—glutathione peroxidase, GST—glutathione transferase, and MDA—malondialdehyde.

3.3. Changes in Serum Troponin I Levels (cTnI) and Galectin-3 (GAL-3) under EPP Administration

The troponin I level did not change in phenolic-treated rats (Table 3). The cTnI level in all groups was markedly elevated, which corresponds to heart injury in the course of obesity. The concentration of galectin-3, a marker of fibrosis and an indicator of myocardial remodeling, also did not change relevantly in exposure to polyphenolic extract between the groups.

Table 3. Determination of heart failure markers concentration in heart tissue (GAL-3) and in serum (cTnI) by the group. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW). The data are displayed as mean ± standard deviation (SD).

	MetS Control	MetS 100	MetS 200	p_{ANOVA}
cTnI (pg/mL)	338.3 ± 92,1	359.1 ± 125.3	501.2 ± 97.9	0.244
GAL-3 (pg/mL)	30.7 ± 1.1	30.3 ± 0.9	30.7 ± 0.9	0.670

Abbreviations: cTnI—troponin I, GAL-3—galectin 3.

3.4. Effect of EPP on the Heart and Aorta Tissue Composition

Histology analysis did not reveal any relevant effect of pomegranate peel extract on the anatomical rearrangement in the analyzed tissues. As shown in Table 3, the heart sections were characterized by mild to severe parenchymal degeneration and slightly expressed fibrosis. Inflammatory infiltration was absent in the majority of the samples. The phenolic extract also did not significantly modify aortic fiber disarrangement (Tables 4 and 5). Representative myocardial and aortic sections are presented in Figure 7.

Table 4. Evaluation of changes in the heart specimens on a semi-quantitative scale (0—no changes, 3—severe changes. Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW).

	MetS Control	MetS 100	MetS 200
Parenchymal degeneration	1 (0–2)	2 (2–3)	2 (1–3)
Fibrosis	0 (0–0)	0 (0–1)	0 (0–2)
Inflammatory infiltration	0 (0–0)	0 (0–0)	0 (0–3)

Table 5. Evaluation of changes in aorta specimens in a semi-quantitative scale (0—no changes, 3—severe changes). Groups: MetS control (only water), MetS 100 (EPP at a dose of 100 mg/kg BW), and MetS 200 (EPP at a dose of 200 mg/kg BW).

	MetS Control	MetS 100	MetS 200
Fiber disarrangement	0 (0–1)	1 (1–3)	1 (0–2)

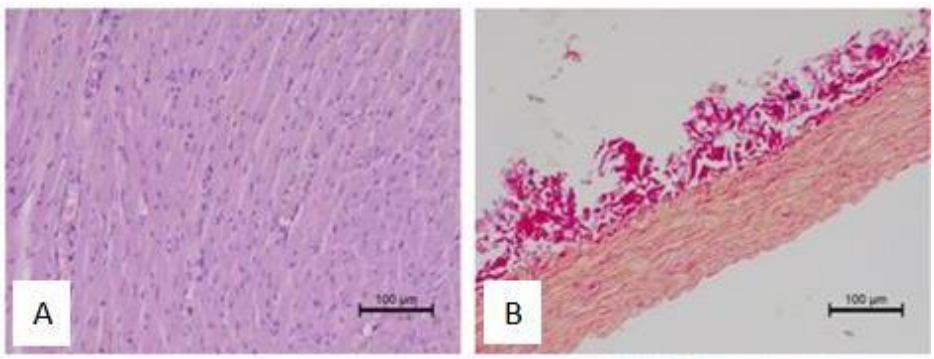

Figure 7. *Cont.*

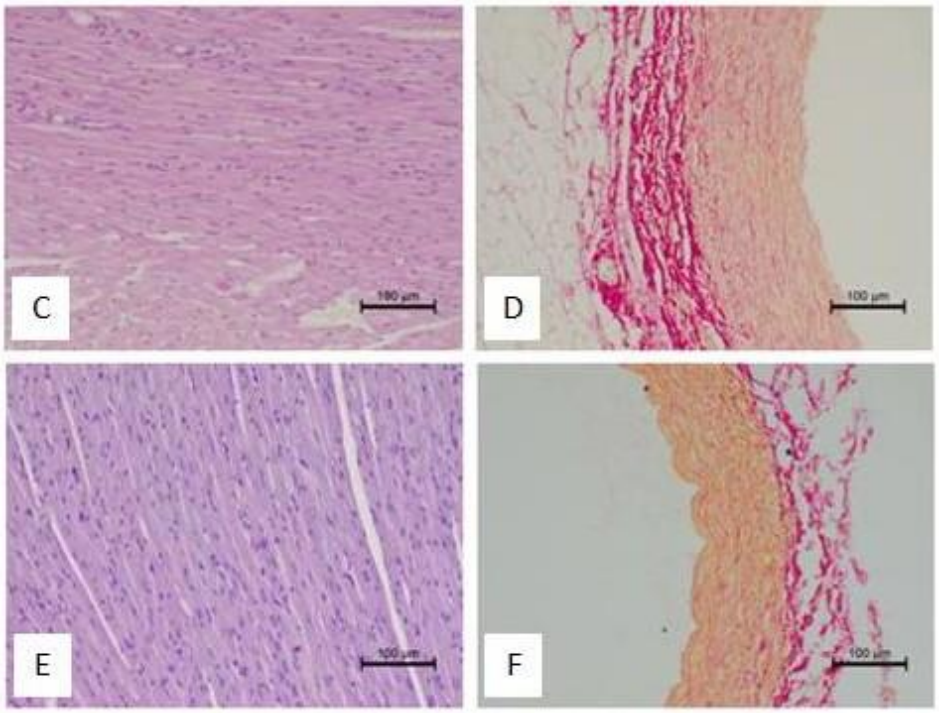

Figure 7. Hematoxylin–eosin-stained sections of the heart and picrosirius red staining of the aortic sections sampled from rats in various experimental groups: (**A**) heart, MetS control—MetS rat receiving only water; (**B**) aorta, MetS control—MetS rat receiving only water; (**C**) heart, MetS 100—MetS rats treated with 100 mg/kg EPP; (**D**) aorta, MetS 100—MetS rats treated with 100 mg/kg EPP; (**E**) heart, MetS 200—MetS rats treated with 200 mg/kg EPP; (**F**) aorta, MetS 200—MetS rats treated with 200 mg/kg EPP. The histological examination revealed no toxic changes in the phenolic-treated rats.

4. Discussion

The current study evaluated the substantial cardioprotective potential of phenolic compounds from pomegranate peels in animal models of MetS, both genetically programmed and induced by diet.

The beneficial health-promoting role of dietary polyphenols in chronic diseases such as MetS has been investigated in many studies. Pomegranate peels seem to be a good source of a wide range of beneficial phytochemicals [36,37]. However, the content of phenolic constituents differs in various pomegranate peel extracts [38]. The quantity of bioactive compounds, in particular, is grounded in the storage conditions, pomegranate cultivar, maturity stage of fruits, and extraction procedure [39]. Polyphenols are susceptible to high temperatures, light, pH, and processing, and all these factors can readily degrade the phenolic content, possibly losing their antioxidant properties [40]. It was proven that the ultimate scope of beneficial phytochemicals is present in pomegranate peels that originated in Turkey [41]. Similarly, *Punica granatum* L. varieties cultivated in Spain are also characterized by a high antioxidative polyphenol content [38]. When comparing the various solvents (i.e., methanol, ethanol, distilled water) used in the extraction procedure, methanol is the most effective in extracting potent antioxidant compounds. However, water and ethanol also seem to be good choices in heterodox studies [42,43].

Based on the studied literature, the ethanolic extract from pomegranate peels (cultivar Mollar de Elche, Spain) used in this investigation possessed valuable phytochemicals [38]. The main class of identified compounds was ellagic acid derivatives, which positively correlate with the high antioxidant properties of the pomegranate peel extract [44]. Precisely, cardiovascular health benefits were accredited to bioactivity exhibited by individual phenolic compounds present in pomegranate peels. Ellagic acid demonstrates an antihypertensive effect, protection against arrhythmia, and cardiac muscle hypertrophy during myocardial

infarction [45,46]. It also mitigates doxorubicin-induced cardiotoxicity and ameliorates myocardial diastolic dysfunction occurring in diabetes mellitus [47,48]. Moreover, ellagic acid is accredited to antiatherosclerotic activity by lowering lipid peroxidation [45,49]. The cardiovascular protection of punicalagin evinces itself in moderation of myocardial infarct reperfusion tissue injury, endothelial dysfunction, and pulmonary hypertension [50,51]. Ellagitannins presented at a high concentration in the obtained peel extract as well, contributing to suppressing inflammation, which also plays a crucial role in MetS pathogenesis and cardiovascular consequences [52,53].

Oxidative stress is a crucial component in the pathogenesis of metabolic syndrome and its cardiovascular consequences, including stroke, myocardial infarction, or atherosclerotic disease [54,55]. In obese individuals suffering from MetS, the persistent overproduction of reactive oxygen species (ROS) corresponds with chronic low-grade inflammation affecting miscellaneous organs and results in deleterious effects on their function. Importantly, oxidative stress leads to the formation of insulin resistance in adipocytes, which also plays a vital role in MetS pathophysiology [56]. The imbalance in redox equilibrium aggravates the endogenous enzymatic and non-enzymatic antioxidant defense. The ultimate components of the ROS scavenging system comprise catalase (CAT), superoxide dismutases (SOD), glutathione peroxidases (GPxs), and glutathione transferase (GST) [57]. These antioxidant enzymes provide an oxidation–reduction balance, and the elevation level of these enzymes indicates a boost in the endogenous active oxidative species [58].

In the context of MetS-induced free radical production, the protective influence of the extract from pomegranate peels was observed in the liver and kidneys [59,60]. The antioxidant-promoting effect was demonstrated by reduced oxidative enzyme activity, lipid peroxidation, and increased GSH level in the liver [61,62], as well as decreased reactive oxygen and nitrogen species (ROS/RNS) levels, oxidized LDL (oxLDL) concentration, and SOD activity in the kidneys [59].

The results provided by this study support the hypothesis that phenolic compounds originating from pomegranate peels have superoxide-scavenging activity in myocardial tissue. Especially in terms of the protein thiol group concentration (SH), the molecules marked by the capacity to neutralize the reactive oxygen species were increased in MetS rats treated with pomegranate peel extract in a dose-dependent manner. Data also suggest that ethanolic peel extract increased glutathione disulfide redox buffer in doxorubicin-induced cardiotoxicity [63]. The antioxidative potential of the pomegranate peel extract also enforced an upward tendency inCAT, GPX, and GST activity observed in the rats treated with lower doses, which arise from their mechanism of action. In the presence of reactive oxygen species, these enzymes act as free radical scavengers and convert into inactive forms leading to a decrease in their levels before renewal. Similar results were observed in Wistar rats administered pomegranate peel extract derived from Bosnia and Hercegovina (100 mg/kg BW) for 7 days [64]. As reported previously, the methanol pomegranate peel extract in chlorpyrifos-exposed rats reduces cardiac MDA and increases SOD levels [65]. Our study did not record a favorable influence of ethanol extract from pomegranate peel on their concentration. Total oxidative status (TOS) is useful in assessing the overall redox status [66]. Our findings indicated that a 100 mg/kg BW dose inversely correlated with the TOS concentration. In contrast, in rats treated with 200 mg/kg BW, the alike effect was not determined.

Studies have shown a relationship between pomegranate peel extract supplementation and protection against myocardial tissue failure [25]. As evidenced in isoproterenol-induced myocardial infarction (MI) in Wistar rats, the *Punica granatum* L. peel extract attenuated electrocardiographic changes, myocardial hypertrophy, and lipid peroxidation, and decreased the serum markers of MI. The maximum result was provided with the highest analyzed dosage, 200 mg/kg BW. The upregulation of myocardial expression of endothelial nitric oxide synthase (eNOS), activating nitric-oxide-mediated Nrf2, was concluded to be responsible for the cardioprotection of extract treatment [67]. Similarly, *Punica granatum* L. peel tended to protect the myocardium against toxic agents, demonstrated by the mitigation

of ECG disturbances and biochemical parameters [63,65]. Contrary to these outcomes, we did not observe a marked alternation of cTnI level in the rats treated with ethanolic extract. Interestingly, a prominent troponin elevation was detected in healthy rats receiving the phenolic extract. Therefore, it may be concluded that an 8-week EPP exposure might not be enough to moderate the chronic progression of heart failure.

The metabolic consequences also comprise leptin-induced ROS production in heart muscle, leading to lipotoxicity [68]. The results from various studies indicated that *Punica granatum* L. peel extract reduced vascular remodeling and heart tissue damage [67,69]. Our observations did not indicate a marked amendment of the GAL-3 concentration in ethanolic-peel-treated groups, both with MetS and diet-driven obesity. At this point, fibrosis in the histopathological heart section was observed. As mentioned above, the 8-week course of the study might be insufficient for driving collagen fiber deposition in heart tissue. Therefore, the estimation of *Punica granatum* L. peel extract in prevention against myocardial fibrosis linked with cardiac failure requires further investigations.

The implications of obesity and MetS in cardiovascular tissue reconstruction incorporate a wide range of pathologies, including cardiomyocytes necrosis, interstitial fibrosis, increase in connective tissue deposition, myocardial hypertrophy, adipose tissue infiltration, and coronary vessels sclerosis and narrowing [70,71]. Alternations in cardiac muscle equal its stiffness and diastolic dysfunction [72]. The proposed molecular mechanism of heart failure is pyroptosis, marked by inflammatory-regulated cell death triggered by metabolic alternations [73]. Evidence has shown that pomegranate peel extract can reduce the hypertrophied coronary arterial walls induced via the downregulation of angiotensin-converting enzyme activity (ACE) [69]. Of note, referring to cardiac muscle, phenolic extract from peels improved myofibril composition via the reduction in separated and disorientated muscle fibers with pyknotic nuclei, inhibition in cytoplasmatic lipid deposition, and vacuolar degeneration of cardiomyocytes [63,67,74]. In our study, histopathological data revealed considerable alternation, such as parenchymal degeneration in heart sections and fiber displacement in the aorta cross-sectional area in all the groups of rats, which are linked to the course of metabolic imbalance, especially co-occurring obesity and diabetes mellitus in MetS. The administration of EPP did not result in notable improvements and reduction in structural variations. However, in an animal model of streptozotocin-induced diabetic cardiomyopathy, the administration of pomegranate peel extract (150 mg/kg BW) exhibited a significant reduction in myocardial fibrosis, reflected by lowering the cardiac myofibrils reactivity against TGF-β and collagen fiber deposition [75]. No necrosis or toxicity effect in cardiomyocytes in all EPP-administered groups showed that ethanol extract from pomegranate peels did not lead to myocardium damage, and its usage is safe in the context of the cardiovascular system.

Albeit, some limitations of the study must be taken into account. Primarily, each group of the MetS rats consists only of six individuals. Nonetheless, before our research, the minimal sample size in terms of the analyzed values was calculated to warrant statistically relevant data. Reduction in the scrutinized population refers to the principle of the 3Rs in animal experimentation (replacement, reduction, and refinement), improving the welfare of animals used in science [76]. The EPP composition is not standardized, and the phenolic constitution, both quantitative and qualitative, depends on miscellaneous variables, such as the fruit cultivar used, maturity stage, and storage conditions, which also act as a disadvantage. Finally, even though Zucker diabetic rats proved to be a suitable animal model for metabolic syndrome, further double-blinded and randomized trials are essential for proving Punica granatum L. peel bioavailability and efficacy in humans.

5. Conclusions

The observations presented in this study support the hypothesis that pomegranate peels play a role as an antioxidant agent in heart tissue. The polyphenolic extract had a significant impact on the protein thiol group concentration (SH) and total oxidative status (TOS). The influence on inhibiting the myocardium architecture remodeling and the

myofibril necrosis reflected by troponin I (cTnI) and galectin 3 concentration (GAL-3) was not confirmed. Importantly, no toxic effect of the applied dosages on cellular integrity and tissue structure in the heart and aorta sections was observed. These findings support the beneficial role of pomegranate peel phenolic composition as a nonhazardous, antioxidative agent in heart tissue. However, to comprehensively divulge the usability of phenolic constituents extracted from pomegranate peels, further studies on humans must be conducted to determine the real nutritional and medical value.

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9 WNIOSKI

1. Główną komponentą polifenolową był kwas elagowy oraz jego pochodne. Spośród 14 zidentyfikowanych związków do najliczniejszych należały: HHDP-galoilo-heksozyd, heksozyd kwasu elagowego, elagitanina oraz izomer punikalaginy. Hydrolizujące taniny, zarówno należące do elagotanin, jak i galo tanin, były dominującymi związkami antyoksydacyjnymi.

2. Nie wykazano toksyczności związanej z przyjmowaniem ekstraktu na podstawie obrazu rozmazu i morfologii krwi obwodowej oraz analizy histologicznej pobranych wycinków tkanki mięśnia sercowego oraz aorty.

3. Potwierdzono zależne od dawki kardioprotekcyjne działanie ekstraktu odzwierciedlone poprzez poprawę statusu oksydacyjnego w homogenatach z mięśnia sercowego oraz korzystne zmiany w zakresie pomiarów echokardiograficznych głównie w grupach szczurów predysponowanych do MetS (zmniejszenie HR, zwiększenie LVEDV oraz kurczliwości miokardium). Aktywność antyoksydacyjna związana była przede wszystkim ze zwiększeniem stężenia grup sulfhydrylowych, zmniejszeniem całkowitego statusu oksydacyjnego oraz wzrostem aktywności katalazy, peroksydazy glutationowej oraz transferazy glutationowej.

4. Nie zaobserwowano istotnej statystycznie redukcji masy ciała w ostatnim punkcie pomiarowym, jednak suplementacja ekstraktem wiązała się z tendencją do ograniczenia dynamiki jej przyrostu.

5. Nie odnotowano poprawy profilu glikemicznego i lipidowego pod wpływem podaży ekstraktu zawierającego polifenole ze skórek *Punica granatum* L.

6. Suplementacja ekstraktem zawierającym polifenole ze skórek *Punica granatum* L. nie miała wpływu na przebudowę miocardium oraz stopnień uszkodzenia kardiomiocytów.

10 PODSUMOWANIE

W cyklu artykułów składających się na pracę doktorską poddano ocenie bioaktywność ekstraktu ze skórek granatowca w zakresie mitygacji poszczególnych komponent zespołu metabolicznego na modelu zwierzęcym, ze szczególnym uwzględnieniem wpływu na układ sercowo-naczyniowy. Kardioprotekcyjne działanie zostało potwierdzone zarówno na poziomie molekularnym mając odzwierciedlenie w korzystnych zmianach koncentracji wybranych markerów stresu oksydacyjnego w homogenatach pochodzących z mięśnia sercowego, jak i w układzie makro na podstawie pomiarów echokardiograficznych w zakresie funkcji pracy serca. Nie zaobserwowano znaczącego wpływu podaży ekstraktu na redukcję masy ciała oraz normalizację gospodarki węglowodanowej oraz lipidowej. Co istotne w toku doświadczenia nie wykazano także cech toksyczności związanych z suplementacją ekstraktu.

Uzyskane rezultaty mogą stanowić wstęp do dalszych badań nad prozdrowotnymi właściwościami ekstraktu ze skórek *Punica granatum* L. przede wszystkim w zakresie kontroli czynników ryzyka sercowo-naczyniowego oraz opracowania standaryzowanych ekstraktów lub żywności funkcjonalnej, która mogłaby zostać implementowania do algorytmów terapeutycznych chorób z różnych kręgów etiopatogenetycznych. Wykorzystanie w tym celu skórek z granatowca byłoby także istotne w kontekście odzysku bioodpadów, a tym samym zmniejszenia obciążenia ekonomicznego związanego z gospodarką odpadami oraz zanieczyszczenia środowiska wynikającego z problemów z ich utylizacją.