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Ocena zmian epigenetycznych związanych z rozwojem oporności na insulinę w komórce tłuszczowej

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SPIS TREŚCI

1. WYKAZ PUBLIKACJI

1.1. "DNA methylation in adipocytes from visceral and subcutaneous adipose tissue influences insulin-signaling gene expression in obese individuals."

Aneta Cierzniak, Dorota Pawełka, Krzysztof Kaliszewski, Jerzy Rudnicki, Tadeusz Dobosz, Małgorzata Małodobra-Mazur
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1.2. "Histone modifications influence the insulin-signaling genes and are related to insulin resistance in human adipocytes."

Małgorzata Małodobra-Mazur, **Aneta Cierzniak**, Aneta Myszczyszyn, Krzysztof Kaliszewski, Tadeusz Dobosz
International Journal of Biochemistry and Cell Biology; 2021; 137; art.106031
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1.3. "The preliminary evaluation of epigenetic modifications regulating the expression of IL10 in insulin-resistant adipocytes."

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2. STRESZCZENIE

Insulinooporność i towarzysząca jej otyłość są poważnym i stale narastającym problemem współczesnej cywilizacji dotykającym zarówno osoby dorosłe, jak i dzieci. Poznanie molekularnych mechanizmów odpowiedzialnych za rozwój insulinooporności ze współistniejącą otyłością, która w sprzyjających warunkach może przyczynić się do rozwoju pełnoobjawowej cukrzycy typu 2, może stać się punktem wyjścia dla opracowania skutecznego leczenia.

Rozwojowi insulinooporności towarzyszą zmiany w ekspresji genów kluczowych dla szlaku insulinowego i metabolizmu glukozy. Modyfikacje epigenetyczne zarówno na poziomie DNA, jak i histonów mogą wykazywać znaczący wpływ na regulację transkrypcji genów. Regulacja epigenetyczna jest silnie powiązana z czynnikami zewnętrznymi i nie wynika ze zmian w sekwencji samego DNA, a modyfikacje epigenetyczne mogą pojawić się w trakcie życia organizmu. Jest to najważniejszy powód, dla którego analiza epigenomu stała się obiektem zainteresowania w analizie mechanizmów molekularnych rozwoju wielu chorób, w tym chorób metabolicznych. Coraz więcej doniesień naukowych wskazuje na znaczącą rolę modyfikacji epigenetycznych, przede wszystkim metylacji DNA, w patogenezie insulinooporności ze współistniejącą otyłością. Przeprowadzone w ostatnich latach badania dokumentują funkcjonalną zależność pomiędzy otyłością a metylacją DNA w obrębie promotorów genów regulujących wrażliwość na insulinę. Co więcej, opublikowane do tej pory wyniki badań wskazują na wpływ otyłości na metylację promotorów ponad 30 genów istotnych dla szlaku insulinowego.

Głównym celem pracy doktorskiej była ocena zmian na poziomie regulacji epigenetycznej związanych z indukcją insulinooporności przy współistniejącej otyłości zachodzących w ludzkiej tkance tłuszczowej z uwzględnieniem różnic metabolicznych pomiędzy jej frakcją trzewną i podskórną, oszacowanie szybkości pojawiania się zmian epigenetycznych w komórkach tłuszczowych w warunkach *in vitro*, a także ocena uniwersalności zaobserwowanych mechanizmów poprzez uwzględnienie modelu komórkowego w postaci linii mysich preadipocytów 3T3-L1 i przeprowadzenie na nich odpowiednich badań.

Pracę doktorską stanowią trzy publikacje o charakterze badawczym tworzące spójny cykl tematyczny realizujący wszystkie przedstawione założenia projektu badawczego.

Ludzki materiał biologiczny do przeprowadzenia badań stanowiły bioptaty brzusznej oraz podskórnej tkanki tłuszczowej pobrane od 47 pacjentów obojga płci w przedziale wiekowym 40-60 lat, w szerokim zakresie BMI w trakcie planowanych zabiegów chirurgicznych. Założone kryteria wykluczenia: zespoły metaboliczne przebiegające z insulinoopornością o innym podłożu (PCOS, zespół Cushinga itp.), miażdżyca, choroby tarczycy i wątroby, choroby zakaźne, neurologiczne, nowotworowe oraz przewlekłe stany zapalne. Wykluczone zostały osoby nadużywające alkoholu oraz abstynenci wtórni. Od każdego pacjenta uzyskano pisemną zgodę na włączenie jego materiału biologicznego do projektu badawczego. Pacjenci zostali podzielni na grupy badane na podstawie określonych parametrów oceniających stopień zaawansowania insulinooporności (HOMA-IR, QUICKI) oraz otyłości (BMI).

Do badań wykorzystano także hodowlę mysich preadipocytów komercyjnej linii 3T3-L1 oraz pierwotną hodowlę komórkową z wyizolowanych ludzkich preadipocytów pochodzących z frakcji podskórnej i trzewnej tkanki tłuszczowej, które zostały pobrane od trzech zdrowych mężczyzn w wieku 30-60 lat, mieszczących się w przedziale BMI 20-25 kg/m^2, niewykazujących zaburzeń metabolicznych w postaci otyłości czy insulinooporności. Od wszystkich pacjentów uzyskano pisemną zgodę na dołączenie ich materiału biologicznego do projektu badawczego.

Szczegółowe cele badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej:

1. Ocena zmian ekspresji genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji i adipogenezy w materiale badanym.
2. Ocena poziomu globalnej metylacji DNA w materiale badanym.
3. Ocena poziomu metylacji DNA w obrębie regionów promotorowych genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji i adipogenezy w materiale badanym.
4. Ocena modyfikacji epigenetycznych (metylacji, acetylacji) w obrębie histonów w materiale badanym.
5. Określenie mechanizmów rozwoju insulinooporności indukowanej otyłością poprzez zmiany na poziomie epigenetycznym w ludzkiej tkance tłuszczowej z uwzględnieniem różnic metabolicznych pomiędzy trzewną i podskórną tkanką tłuszczową.
6. Oszacowanie czasu powstawania modyfikacji epigenetycznych w adipocytach poprzez obserwacje przeprowadzone na hodowli ludzkich preadipocytów izolowanych z tkanki tłuszczowej trzewnej i podskórnej, które zostały poddane różnicowaniu do dojrzałych

adipocytów i indukcji insulinooporności z wykorzystaniem kwasu palmitynowego w odpowiednim stężeniu.

7. Ocena uniwersalności zaobserwowanych mechanizmów poprzez uwzględnienie modelu komórkowego w postaci linii mysich preadipocytów 3T3-L1 i przeprowadzenie na nich odpowiednich badań.

Kluczowe wnioski i obserwacje przedstawione w pracy doktorskiej:

1. Insulinooporności ze współistniejącą otyłością towarzyszą zaburzenia ekspresji genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji oraz kodujących adipokiny i enzymy biorące udział w generowaniu metylacji DNA w tkance tłuszczowej, zarówno w komponencie podskórnej jak i trzewnej.
2. Metylacja DNA może stanowić czynnik regulujący ekspresję genów u osób otyłych z insulinoopornością o czym świadczy zaobserwowana zwiększona metylacja DNA w adipocytach osób otyłych z insulinoopornością, zwiększony poziom metylacji regionów promotorowych genów kluczowych dla szlaku insulinowego (*SLC2A4*), a także regulacji adipogenezy i metabolizmu glukozy (*PPARG*, *ADIPOQ*), który korelował ujemnie z poziomem ekspresji tych genów. Analiza korelacji uzyskanych wyników ze wskaźnikiem BMI również sugeruje te zależność.
3. Modyfikacje epigenetyczne w obrębie histonów mają bezpośredni wpływ na regulację ekspresji genów kluczowych dla insulinooporności.
4. Obniżony poziom modyfikacji epigenetycznych u pacjentów z insulinoopornością i zaburzenia w ekspresji badanych genów został potwierdzony na modelu komórkowym bazującym na ludzkich komórkach frakcji trzewnej i podskórnej.
5. Większość zmian epigenetycznych zarówno na poziomie DNA, jak i histonów pojawia się po 72 h od wyidukowania insulinooporności w ludzkim i mysim modelu komórkowym.
6. Tkanka tłuszczowa trzewna wykazuje szybszą podatność na zmiany w regulacji o podłożu epigenetycznym niż jej komponenta podskórna co może świadczyć o jej zwiększonej podatności na zaburzenia o podłożu metabolicznym.
7. Mysi model 3T3-L1 wykazuje większą spójność z wynikami uzyskanymi z komórek komponenty podskórnej.
8. PPARγ jako czynnik transkrypcyjny odgrywa kluczową rolę w rozwoju insulinooporności u osób otyłych poprzez regulację ekspresji innych genów istotnych dla utrzymania wrażliwości na insulinę w komórkach tłuszczowych. Co więcej,

regulacja epigenetyczna ekspresji *PPARG* może stanowić pomost łączący współzależność molekularną pomiędzy otyłością i insulinoopornością.

9. IL-10 jako czynnik przeciwzapalny może pełnić kluczową rolę w poprawie wrażliwości komórek na insulinę. Ekspresja genu kodującego IL-10 może podlegać regulacji epigenetycznej, o czym świadczy obecność modyfikacji epigenetycznych na poziomie DNA i na poziomie histonów w komórkach tłuszczowych z wyindukowaną insulinoopornością.
10. Metylotransferaza DNA kodowana przez gen *DNMT1* i deacetylazy należące do grupy sirtuin (SIRT1 i SIRT7) mogą odgrywać kluczową rolę w regulacji epigenetycznej na poziomie DNA i histonów w rozwoju insulinooporności w komórkach tłuszczowych.

Podsumowując, w pracy doktorskiej wykazano znaczący wpływ regulacji epigenetycznej, zarówno na poziomie DNA jak i histonów, na zmiany w ekspresji genów kluczowych dla szlaku insulinowego, regulacji adipogenezy i metabolizmu glukozy. Zaburzenia w transkrypcji prowadzące do zmian w syntezie ważnych metabolicznie białek mogą bezpośrednio zakłócać działanie określonych szlaków, jednocześnie prowadząc do rozwoju chorób metabolicznych. Wykazano również, że w rozwoju insulinooporności w komórkach tłuszczowych istotną rolę odgrywa strategiczny czynnik transkrypcyjny w postaci PPARγ, którego ekspresja genu również podlega regulacji epigenetycznej.

3. SUMMARY

Insulin resistance and obesity are serious and growing problems of modern civilization affecting both adults and children. The understanding of molecular mechanisms responsible for the development of insulin resistance with coexisting obesity, which in favorable conditions may contribute to the development of type 2 diabetes, may become a starting point for the development of effective treatment.

The development of insulin resistance is accompanied by changes in the expression of genes crucial for the insulin pathway and glucose metabolism. Epigenetic modifications at both the DNA and histone levels can exhibit significant effects on the regulation of gene transcription. Epigenetic regulation is strongly linked to external factors and is not due to changes in the sequence of DNA itself. Because epigenetic modifications can occur during the life of an organism they become an object of interest in the analysis of molecular mechanisms of the development of many diseases, including metabolic diseases. An increasing number of scientific reports indicate a significant role of epigenetic modifications, primarily DNA methylation, in the pathogenesis of insulin resistance with coexisting obesity. Recent studies document a functional relationship between obesity and DNA methylation within the promoters of genes regulating insulin sensitivity. Moreover, results published so far indicate that obesity affects the methylation of promoters of more than 30 genes relevant to the insulin pathway.

The main objective of this doctoral thesis was to evaluate changes in human adipose tissue at the level of epigenetic regulation related to the induction of insulin resistance in the presence of coexisting obesity, taking into account metabolic differences between its visceral and subcutaneous fractions, to estimate the rate of occurrence of epigenetic changes in adipose cells in vitro, as well as to assess the universality of the observed mechanisms by taking into account a cell model in the form of mouse preadipocyte line 3T3-L1 and carrying out appropriate tests on them.

A doctoral thesis consists of three research publications forming a coherent thematic series realizing all presented assumptions of the research project.

The study material consisted of biopsy specimens of visceral and subcutaneous adipose tissue taken from 47 patients of both sexes, aged 40-60 years, in a wide range of BMI at the time of planned surgery. Exclusion criteria were established: metabolic syndromes running with insulin resistance of other causes (PCOS, Cushing's syndrome, etc.), atherosclerosis, thyroid

disease, hepatitis of various causes, infectious diseases, neurological diseases, cancer, and chronic inflammatory conditions. Alcohol abusers and secondary abstainers were also excluded. Written informed consent was obtained from each patient to include their biological material in the research project. Patients were divided into study groups based on specific parameters assessing the severity of insulin resistance (HOMA-IR, QUICKI) and obesity (BMI).

Mouse preadipocyte culture of the commercial 3T3-L1 line and primary cell culture from isolated human preadipocytes derived from the subcutaneous and visceral adipose tissue fractions, which were collected from three healthy men aged 30-60 years, falling within the BMI range of 20-25 kg/m^2, and not exhibiting metabolic disorders such as obesity or insulin resistance, were also used for this study. Written informed consent was obtained from all patients to include their biological material in the research project.

The specific aims of the research:

1. Evaluation of changes in the expression of genes that are essential for the insulin pathway, lipid metabolism, transcriptional regulation, and adipogenesis in the biological material.
2. Assessment of the level of global DNA methylation in the biological material.
3. Assessment of the level of DNA methylation within the promoter regions of genes involved in the insulin pathway, lipid metabolism, transcriptional regulation, and adipogenesis in the biological material.
4. Evaluation of epigenetic modifications (methylation, acetylation) of histones in the biological material.
5. Determine the mechanisms of obesity-induced insulin resistance development through epigenetic alterations in human adipose tissue, taking into account metabolic differences between visceral and subcutaneous adipose tissue.
6. Estimate of the timing frame of epigenetic modifications in adipocytes through observations made on cultures of human preadipocytes isolated from visceral and subcutaneous adipose tissue that have been differentiated into mature adipocytes in which insulin resistance was induced using palmitic acid at appropriate concentrations.
7. Evaluation of the universality of the observed mechanisms by considering a cellular model in the form of the 3T3-L1 mouse preadipocyte line and performing appropriate experiments on them.

Key findings and observations:

1. Insulin resistance with coexisting obesity is accompanied by impaired expression of genes that are essential for the insulin pathway, lipid metabolism, transcriptional regulation, and that encode adipokines and enzymes involved in the generation of DNA methylation in adipose tissue, both in the subcutaneous and visceral components.
2. DNA methylation may be a factor regulating gene expression in obese subjects with insulin resistance by the observed increased DNA methylation in adipocytes of obese subjects with insulin resistance, increased level of methylation of promoter regions of genes key for the insulin pathway (*SLC2A4*), and regulation of adipogenesis and glucose metabolism (*PPARG, ADIPOQ*), which correlated negatively with the expression level of these genes. The analysis of the correlation of the obtained results with the BMI index also suggests this relationship.
3. Epigenetic modifications within histones have a direct impact on the regulation of the expression of genes crucial for insulin resistance.
4. Reduced levels of epigenetic modifications in patients with insulin resistance and impaired expression of the genes studied have been confirmed in a cell model based on human visceral and subcutaneous fraction cells.
5. The majority of epigenetic changes at both DNA and histone levels appear 72h after insulin resistance in human and mouse cell models.
6. Visceral adipose tissue is more rapidly affected by epigenetic regulation than its subcutaneous component, which may reflect its increased susceptibility to metabolic disorders.
7. The mouse model of 3T3-L1 shows greater consistency with the results obtained from cells of the subcutaneous component.
8. PPARγ as a transcription factor plays a key role in the development of insulin resistance in obese individuals by regulating the expression of other genes important for maintaining insulin sensitivity in adipose cells. Moreover, epigenetic regulation of *PPARG* expression may bridge the molecular interdependence between obesity and insulin resistance.
9. IL-10 as an anti-inflammatory factor may play a key role in improving cellular insulin sensitivity. Expression of the gene encoding IL-10 may be epigenetically regulated, as evidenced by the presence of epigenetic modifications at the DNA level and the histone level in fat cells with induced insulin resistance.

10. DNA methyltransferase encoded by the *DNMT1* gene and deacetylases belonging to the sirtuin group (SIRT1 and SIRT7) may play a key role in epigenetic regulation at the DNA and the histone level in the development of insulin resistance in fat cells.

In summary, this doctoral thesis demonstrated the significant impact of epigenetic regulation, both at the DNA and histone levels, on changes in gene expression of genes crucial for the insulin pathway, regulation of adipogenesis, and glucose metabolism. Disturbances in transcription leading to changes in the synthesis of metabolically important proteins can directly interfere with specific pathways while leading to the development of metabolic diseases. It has also been shown that a strategic transcription factor PPARγ, whose gene expression is also epigenetically regulated, plays an important role in the development of insulin resistance in fat cells.

4. WPROWADZENIE

Cukrzyca, insulinooporność i towarzysząca jej otyłość są poważnym i stale narastającym problemem współczesnej cywilizacji dotykającym zarówno osoby dorosłe, jak i dzieci. Według najnowszych światowych raportów na cukrzycę typu 2 choruje 463 miliony dorosłych, a liczba ta wciąż się powiększa. Otyłość i nadwaga dotykają natomiast 2.2 miliarda kobiet i mężczyzn. Zaburzenia tolerancji komórek na insulinę przy współistniejącej otyłości mogą prowadzić do rozwoju cukrzycy typu 2[1,2]. Mając na uwadze ten mechanizm, niezwykle ważne jest, aby zapobiegać rozwojowi pełnoobjawowej cukrzycy już na pierwszym etapie zaburzeń metabolicznych, które mogą objawiać się wykształceniem oporności komórek na insulinę.

Kluczowym problemem związanym ze wspomnianymi zaburzeniami metabolicznymi jest brak skutecznych i długotrwałych metod terapeutycznych, które nie tylko zmniejszałyby objawy, ale także redukowały lub eliminowały przyczyny samej choroby. Powszechnie stosowane leczenie objawowe opiera się na wykorzystaniu metforminy i jej pochodnych, które zwiększają wrażliwość komórek na insulinę[3]. Poznanie molekularnych mechanizmów odpowiedzialnych za rozwój insulinooporności ze współistniejącą otyłością, która w sprzyjających warunkach może przyczynić się do rozwoju pełnoobjawowej cukrzycy typu 2, może stać się punktem wyjścia dla opracowania skutecznego leczenia przyczynowego.

Insulina jest hormonem produkowanym przez komórki β wysp trzustkowych Langerhansa. Jej główna rola polega na kontroli metabolizmu węglowodanów, białek i tłuszczów. Działając na komórki poprzez obecny w ich błonie receptor insulinowy hormon aktywuje molekularny szlak insulinowy regulujący dokomórkowe wchłanianie cząsteczek glukozy. Komórki organizmu wykazują zróżnicowaną ilość receptorów na swojej powierzchni, najwięcej występuje w obrębie błon hepatocytów, komórek mięśniowych i adipocytów. Receptor insuliny jest receptorem błonowym posiadającym aktywność kinazy tyrozynowej. Zbudowany jest z dwóch podjednostek: α i β. Związanie insuliny ze swoistym receptorem indukuje autofosforylację reszt tyrozynowych w obrębie jednostki β nadając jej właściwości aktywnej kinazy tyrozynowej umożliwiając tym samym aktywację dwóch głównych szlaków sygnalizacyjnych: szlaku kinazy białkowej aktywowanej mitogenami (*ang. mitogen-activated protein kinase*, MAPK) i szlaku 3-kinazy fosfatydyloinozytolu (*ang. phosphoinositide 3-kinase*, PI3K) poprzez fosforylację określonych białek substratowych IRS-1 (*ang. insulin receptor substrate 1*) i IRS-2 (*ang. insulin receptor substrate 2*). Szlak MAPK bierze udział w procesach proliferacji i różnicowania, w szczególności poprzez regulację aktywności

transkrypcyjnej jądra komórkowego. Natomiast szlak kinazy PIK3 bierze udział w wielu metabolicznych efektach działania insuliny, takich jak transport glukozy, glikoliza i synteza glikogenu. Prawidłowe funkcjonowanie całego szlaku warunkuje możliwość dokomórkowego pobierania glukozy z krwi za pomocą receptorów GLUT4 (*ang. glucose transporter type 4)* stanowiących ostatnie ogniwo szlaku insulinowego[4–6]. Mechanizm kontroli przekazywania sygnału jest wieloetapowy i podlega określonej regulacji. W funkcjonowanie szlaku zaangażowanych jest wiele białek, a zaburzenia w ich syntezie i transmisji sygnału komórkowego są molekularną podstawą rozwoju oporności na insulinę w komórkach. Insulinooporność jest klasyfikowana jako zaburzenie metaboliczne charakteryzujące się występowaniem stanu obniżonej wrażliwości tkanek na działanie insuliny przy jej prawidłowym, a nawet podwyższonym stężeniu w osoczu krwi. Nieprawidłowa odpowiedź tkanek na insulinę prowadzi do zaburzeń metabolizmu węglowodanów, lipidów oraz białek[7]. Na wykształcenie się zmniejszonej wrażliwości komórek na insulinę ma wpływ szereg czynników, zarówno o charakterze genetycznym, jak i środowiskowym. Jednym z najważniejszych jest otyłość, która jest wynikiem zaburzenia równowagi pomiędzy ilością zgromadzonej tkanki tłuszczowej, a faktycznym zapotrzebowaniem energetycznym organizmu. Patologiczne i nadmierne nagromadzenie się tkanki tłuszczowej, zarówno trzewnej jak i podskórnej, jest związane z czynnikami metabolicznymi, psychologicznymi, endokrynologicznymi i genetycznymi. Przede wszystkim otyłość jest jednak efektem nieprawidłowej diety, bogatej w tłuszcze i węglowodany, a także ograniczonej aktywności fizycznej[8,9]. Otyłość jest konsekwencją występowania dwóch procesów. Pierwszy, hipertrofia, związany jest z gromadzeniem się nadmiarów pokarmowych w postaci trójglicerydów (TG) w już istniejących komórkach tłuszczowych, co prowadzi do ich wzrostu. Drugi proces to hiperplazja, związana z pojawieniem się nowych adipocytów w tkance tłuszczowej[10]. Współistnienie otyłości i insulinooporności generuje ogromne ryzyko rozwoju chorób sercowo-naczyniowych oraz cukrzycy, a wystąpienie ich z innymi zaburzeniami definiowane jest jako złożony syndrom zwany zespołem metabolicznym[11,12].

Istnieje kilka potencjalnych mechanizmów wyjaśniających silną korelację otyłości z rozwojem insulinooporności w komórkach. Istotną rolę w tej zależności przypisuje się wolnym kwasom tłuszczowym (*ang. free fatty acids*, FFA). U osób otyłych obserwuje się podwyższone stężenie FFA zarówno we krwi, jak i w tkankach obwodowych. Głównym ich źródłem jest dieta bogata w tłuszcze. Podwyższone stężenie kwasów tłuszczowych w osoczu krwi stymuluje ich nadmierny wychwyt przez komórki, szczególnie hepatocyty, komórki mięśniowe i adipocyty.

W momencie nadmiernego gromadzenia się FFA w komórkach dochodzi do zaburzenia równowagi pomiędzy zdolnościami β oksydacyjnymi komórki, a ilością napływających kwasów z krwi, co generuje postępującą akumulację tych związków prowadząc bezpośrednio do zjawiska lipotoksyczności. Wpływ nadmiaru kwasów tłuszczowych na metabolizm komórki zależy od rodzaju tkanki. W przypadku mięśni szkieletowych przeładowanie wolnymi kwasami tłuszczowymi prowadzi do zaburzeń w prawidłowym dokomórkowym wychwytywaniu glukozy z krwi co jest związane z zaburzeniem funkcjonowania szlaków sygnałowych regulowanych przez diacyloglicerol (DAG). Ponadto obserwuje się podwyższoną aktywność kinaz serynowo-treoninowych (np. kinazy JNK), których aktywność indukowana jest stresem narastającym w środowisku komórki wynikającym z zaburzeń funkcjonowania szlaków metabolicznych i nagromadzenia się w mitochondriach metabolitów niepełnego utleniania lipidów. Fosforylacja reszt tyrozynowych receptora insulinowego oraz substratów IRS-1 i IRS-2 odgrywa główną rolę w przekazywaniu sygnału komórkowego pod wpływem insuliny i efektywny dokomórkowy transport glukozy. Natomiast fosforylacja reszt serynowych i treoninowych tych cząsteczek hamuje działanie insuliny[13]. Zwiększona aktywność kinaz indukowanych stresem prowadzi do fosforylacji wspomnianych reszt serynowych w obrębie białka IRS-1, co w konsekwencji blokuje całą ścieżkę przekazującą sygnał. Konsekwencją tego zjawiska jest przerwanie szlaku sygnałowego, brak aktywacji receptorów GLUT4 i ich translokacji do błony komórkowej. Narastające zaburzenie szlaków sygnałowych prowadzi do stopniowego nabywania przez komórkę oporności na insulinę przy jednocześnie narastającej hiperglikemii[14–17].

Otyłość może być również przyczyną przewlekłego ogólnoustrojowego i miejscowego stanu zapalnego o niskim stopniu nasilenia. W przypadku tkanki tłuszczowej, przy nadmiernej podaży pokarmów wysokotłuszczowych dochodzi do zwiększonego wychwytywania FFA z krwi i magazynowania w postaci TG w dojrzałych komórkach. Zwiększona aktywność lipogenezy prowadzi do postępującej hipertrofii tkanki tłuszczowej, której towarzyszy niewystarczająco szybkie tworzenie się nowych naczyń krwionośnych, w efekcie czego dochodzi do pojawiania się miejscowego niedotlenienia adipocytów. Zjawisko to jest główną przyczyną generowania stresu komórkowego, który podobnie jak w mięśniach szkieletowych, indukuje aktywność kinaz serynowo-treoninowych, których działanie prowadzi do dysregulacji szlaku insulinowego. Zaburzenia metaboliczne adipocytów wpływają również na funkcje endokrynne tkanki tłuszczowej. Dochodzi do zwiększonego wydzielania adipokin takich jak leptyna przy jednoczesnym spadku stężenia adiponektyny. Jest to istotne w patogenezie

rozwoju insulinooporności w tkankach obwodowych, ponieważ adiponektyna wpływa na zwiększenie insulinowrażliwości komórek wątroby, a także powoduje wzrost zużycia glukozy i oksydacji kwasów tłuszczowych w mięśniach szkieletowych[18–21]. Ponadto długotrwała ekspozycja wysp trzustkowych na podwyższone stężenie FFA w osoczu prowadzi do zmniejszenia wrażliwości komórek β, czego konsekwencją jest zahamowanie wydzielania insuliny. Jednocześnie towarzyszące otyłości podwyższone stężenie FFA w osoczu krwi wynika w dużej mierze z nasilonych procesów lipolizy w przeładowanych adipocytach. Hydroliza trójglicerydów pod wpływem aktywności lipazy hormonowrażliwej i uwolnienie wolnych kwasów tłuszczowych do osocza stymuluje ich wychwytywanie z krwi przez tkanki obwodowe, zamykając tym samym napędzające się wzajemnie koło obejmujące zaburzenia w obrębie tkanki tłuszczowej, mięśni szkieletowych, wątroby i trzustki. Insulinooporność tkanki tłuszczowej jest związana między innymi z obniżoną zdolnością do hamowania aktywności lipazy hormonowrażliwej przez insulinę, co przejawia się zwiększonym uwalnianiem kwasów tłuszczowych przez adipocyty, potwierdzając istotną rolę tkanki tłuszczowej w indukowaniu zmniejszonej wrażliwości na insulinę zarówno w adipocytach, jak i w innych komórkach tkanek obwodowych[22].

Molekularne mechanizmy leżące u podstaw rozwoju zaburzeń metabolicznych takich jak otyłość i insulinooporność oraz ich wzajemnej zależności są nadal niejasne. Rozwojowi insulinooporności towarzyszą zmiany w ekspresji genów kluczowych dla szlaku insulinowego i metabolizmu glukozy. Podejrzewa się, że duże znaczenie może w nich odgrywać regulacja epigenetyczna. Epigenetyka obejmuje kwestie dziedziczenia pozagenowego związanego z regulacją ekspresji genów, która nie wynika ze zmian w sekwencji DNA, a z obecności modyfikacji biochemicznych w obrębie jego łańcucha. Do najpowszechniejszych modyfikacji epigenetycznych zalicza się metylację, acetylację oraz fosforylację. Innym, równie istotnym mechanizmem jest interferencja ze strony microRNA. Zmianom epigenetycznym ulegają zarówno nici DNA, jak i białka histonowe. Generowane przez nie zmiany w ekspresji genów mogą być wynikiem zarówno indukcji transkrypcji, jak i jej wyciszania. Jedną z najlepiej poznanych zmian w epigenomie jest metylacja DNA, będąca wynikiem działania enzymów zaliczanych do grupy metylotransferaz (DNMT1, *ang. DNA Methyltransferase 1*; DNMT3a, *ang. DNA Methyltransferase 3a*; DNMT3b, *ang. DNA Methyltransferase 3b*). Głównym obiektem metylacji są cytozyny w obrębie tzw. wysp CpG, czyli regionów w obrębie DNA bogatych w dinukleotydy cytozyna-guanina. Znacząca ilość wysp CpG zlokalizowana jest w obrębie miejsc regulatorowych, w tym regionów promotorowych, mających znaczenie dla

prawidłowej ekspresji genów[23,24]. Regulacja epigenetyczna jest silnie powiązana z licznymi czynnikami środowiskowymi, takimi jak dieta, tryb życia, stres czy substancje toksyczne. Jest to najważniejszy powód, dla którego analiza epigenomu stała się obiektem zainteresowania w analizie mechanizmów molekularnych rozwoju wielu chorób, w tym chorób metabolicznych. Coraz więcej doniesień naukowych wskazuje na znaczącą rolę modyfikacji epigenetycznych, przede wszystkim metylacji DNA, w patogenezie insulinooporności ze współistniejącą otyłością[25,26]. Przeprowadzone w ostatnich latach badania dokumentują funkcjonalną zależność pomiędzy otyłością a metylacją DNA w obrębie promotorów genów regulujących wrażliwość na insulinę. Ponadto opublikowane do tej pory wyniki badań wskazują na wpływ otyłości na metylację promotorów ponad 30 genów istotnych dla szlaku insulinowego, co znacząco sugeruje potencjalny mechanizm indukowania zaburzeń na poziomie molekularnym poprzez regulację epigenetyczną[27,28]. W rozwoju oporności na insulinę w obrębie adipocytów kluczową rolę odgrywa przewlekły stan zapalnym. Szczególną rolę w tych procesach przypisuje się cytokinie prozapalnej TNFα (*ang. tumor necrosis factor α*). W zaburzeniach metabolicznych za czynnik prognostyczny uważa się zwiększoną ekspresję genu kodującego TNFα, szczególnie u osób otyłych z rozwijającą się insulinoopornością[29,30]. Ponadto wykazano dodatnią korelację pomiędzy zwiększoną podażą kwasów nasyconych z dietą a ekspresją genu i stężeniem TNFα, jednocześnie przy zwiększonej ekspresji genu zaobserwowano zmniejszoną metylację regionu promotorowego[31,32]. Czynnik prozapalne w postaci TNFα i interleukiny 6 (IL-6) mogą odgrywać znaczącą rolę w indukcji insulinooporności związanej z otyłością poprzez promowanie metylacji DNA indukując nadekspresję DNMT1[33].

Regulacja epigenetyczna odgrywająca rolę w indukcji insulinooporności przy współistniejącej otyłości może również przejawiać się wpływem modyfikacji epigenetycznych na poziomie histonów na ekspresję kluczowych genów. Dotychczasowe badania dokumentują zależność pomiędzy omawianymi zaburzeniami metabolicznymi a zmianami ekspresji genów kodujących białka o aktywności enzymatycznej, które odgrywają kluczową rolę w tworzenie profilu epigenetycznego w histonach, w tym deacetylazy histonowej SIRT1 (*ang. sirtuin 1*) czy HDAC3 (*ang. histone deacetylase 3*). Większość tych badań dotyczy jednak modeli zwierzęcych[34,35].

5. ZAŁOŻENIA I CELE PROJEKTU BADAWCZEGO

Szczegółowe cele badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej:

1. Ocena zmian ekspresji genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji i adipogenezy w materiale badanym.
2. Ocena poziomu globalnej metylacji DNA w materiale badanym.
3. Ocena poziomu metylacji DNA w obrębie regionów promotorowych genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji i adipogenezy w materiale badanym.
4. Ocena modyfikacji epigenetycznych (metylacji, acetylacji) w obrębie histonów w materiale badanym.
5. Określenie mechanizmów rozwoju insulinooporności indukowanej otyłością poprzez zmiany na poziomie epigenetycznym w ludzkiej tkance tłuszczowej z uwzględnieniem różnic metabolicznych pomiędzy trzewną i podskórną tkanką tłuszczową.
6. Oszacowanie czasu powstawania modyfikacji epigenetycznych w adipocytach poprzez obserwacje przeprowadzone na hodowli ludzkich preadipocytów izolowanych z tkanki tłuszczowej trzewnej i podskórnej, które zostały poddane różnicowaniu do dojrzałych adipocytów i indukcji insulinooporności z wykorzystaniem kwasu palmitynowego w odpowiednim stężeniu.
7. Ocena uniwersalności zaobserwowanych mechanizmów poprzez uwzględnienie modelu komórkowego w postaci linii mysich preadipocytów 3T3-L1 i przeprowadzenie na nich odpowiednich badań.

6. OPIS PUBLIKACJI

Pracę doktorską stanowią trzy publikacje o charakterze badawczym tworzące spójny cykl tematyczny realizujący wszystkie przedstawione założenia projektu badawczego.

Ludzki materiał biologiczny do przeprowadzenia badań stanowiły bioptaty brzusznej oraz podskórnej tkanki tłuszczowej pobrane od 47 pacjentów obojga płci w przedziale wiekowym 40-60 lat, w szerokim zakresie BMI w trakcie planowanych zabiegów chirurgicznych. Założone kryteria wykluczenia: zespoły metaboliczne przebiegające z insulinoopornością o innym podłożu (PCOS, zespół Cushinga itp.), miażdżyca, choroby tarczycy i wątroby, choroby zakaźne, neurologiczne, nowotworowe oraz przewlekłe stany zapalne. Wykluczone zostały osoby nadużywające alkoholu oraz abstynenci wtórni. Od każdego pacjenta uzyskano pisemną zgodę na włączenie jego materiału biologicznego do projektu badawczego. Pacjenci zostali podzielni na grupy badane na podstawie określonych parametrów oceniających stopień zaawansowania insulinooporności (HOMA-IR, QUICKI) oraz otyłości (BMI).

Do badań wykorzystano także hodowlę mysich preadipocytów komercyjnej linii 3T3-L1 oraz pierwotną hodowlę komórkową z wyizolowanych ludzkich preadipocytów pochodzących z frakcji podskórnej i trzewnej tkanki tłuszczowej, które zostały pobrane od trzech zdrowych mężczyzn w wieku 30-60 lat, mieszczących się w przedziale BMI 20-25 kg/m^2, niewykazujących zaburzeń metabolicznych w postaci otyłości czy insulinooporności. Od wszystkich pacjentów uzyskano pisemną zgodę na dołączenie ich materiału biologicznego do projektu badawczego.

Niedojrzałe preadipocyty mysie i ludzkie zostały poddane procesowi różnicowania do dojrzałych adipocytów zgodnie z opracowanym wcześniej protokołem. Następnie przeprowadzono w nich indukcję insulinooporności z wykorzystaniem kwasu palmitynowego w odpowiednim stężeniu.

Na przeprowadzenie badań wchodzących w zakres pracy doktorskiej otrzymano zgodę właściwej komisji bioetycznej. Numer zgody: KB-124/2017.

1. "*DNA methylation in adipocytes from visceral and subcutaneous adipose tissue influences insulin-signaling gene expression in obese individuals*."

   Aneta Cierzniak, Dorota Pawełka, Krzysztof Kaliszewski, Jerzy Rudnicki, Tadeusz Dobosz, Małgorzata Małodobra-Mazur

Głównym założeniem pracy była analiza wpływu otyłości na regulację epigenetyczną w postaci metylacji DNA na poziomie globalnym i miejscowo specyficznym, a także na dysregulację ekspresji genów kluczowych dla insulinooporności. Analizie poddano panel genów w skład, którego wchodziły geny istotne dla szlaku insulinowego (*INSR*, *IRS1*, *IRS2*, *PIK3R1*, *AKT*, *SLC2A4*), metabolizmu lipidów (*LPL*, *ACACA*, *FASN*, *ACSS2*, *SCD1*), stanu zapalnego (*IL10*, *IL1B*), kodujące czynniki transkrypcyjne (*CEBPA*, *CEBPB*, *PPARG*, *IGF2*, *PPARGC1A*, *TNFA*), adipokiny (*LEP*, *ADIPOQ*, *RBP4*) i kluczowe enzymy uczestniczące w tworzeniu zmian epigenetycznych (*DNMT1*, *DNMT3a*, *DNMT3b*). Przeanalizowano stopień metylacji DNA zarówno na poziomie globalnym, jak i miejscowo specyficznym, przede wszystkim w regionie promotorowym genów, w przypadku których zaobserwowano znaczące zmiany ekspresji (*PPARG*, *SLC2A4*, *ADIPOQ*, *INSR*, *IL6*). Ponadto dokonano szerokiej analizy korelacji otrzymanych wyników. Badania zostały wykonane z wykorzystaniem materiału biologicznego w formie tkanek tłuszczowych obu frakcji: trzewnej i podskórnej. Zrekrutowanych do badania pacjentów podzielono na trzy grupy badane na podstawie parametrów oceniających stopień zaawansowania insulinooporności (HOMA-IR, QUICKI) oraz otyłości (BMI):

1. LH – grupa nie wykazująca oznak otyłości i insulinooporności
2. OH – grupa wykazująca oznaki otyłości z prawidłową wrażliwością na insulinę
3. OR – grupa wykazująca oznaki otyłości i insulinooporności.

Insulinooporność była diagnozowana przy wartościach: HOMA-IR > 2.5 i QUICKI < 0.321, otyłość natomiast przy wartości wskaźnika BMI ≥ 25.

Kluczowe wyniki:

1. U pacjentów należących do grupy OR w tkance trzewnej i podskórnej zaobserwowano znaczne obniżenie ekspresji genów kluczowych dla szlaku insulinowego (*INSR*, *IRS1*, *IRS2*, *SLC2A4*), metabolizmu lipidów (*LPL*, *FASN*) i kodujących czynnik transkrypcyjny PPARγ (*ang. peroxisome proliferator activated receptor gamma*) (*PPARG*). W przypadku tkanki trzewnej zaobserwowano również spadek w ekspresji

genów kodujących adipokiny (*ADIPOQ, RBP4*) i transporter glukozy 4 (*SLC2A4*). W obu frakcjach zaobserwowano również znaczący wzrost ekspresji genu kodującej przeciwzapalną interleukinę 10 (*IL10*) i enzym o aktywności metylotransferazy (*DNMT1*).

2. U pacjentów należących do grupy OH w tkance trzewnej i podskórnej zaobserwowano znaczące spadki ekspresji genów kluczowych dla szlaku insulinowego (*INSR, IRS1*). W przypadku tkanki trzewnej zaobserwowano również obniżoną ekspresję genów kodujących: czynnik transkrypcyjny PPARγ (*PPARG*), adipokiny (*ADIPOQ*, *RBP4*) i podjednostkę regulacyjną alfa 3-kinazy fosfatydyloinozytolu (*PIK3R1*).
3. W obu frakcjach tkanki tłuszczowej pacjentów należących do grupy OR poziom globalnej metylacji DNA był znacznie podwyższony i dodatnio korelował z poziomem ekspresji genu kodującego enzym o aktywności metylotransferazy *DNMT1*.
4. Analiza miejscowo specyficznej metylacji w obrębie regionów promotorowych genów *PPARG, INSR, SLC2A4* wykazała wzrost poziomu metylacji skorelowany ujemnie z ekspresją tych genów u pacjentów w grupie OR. Dla *PPARG* jedynie w tkance trzewnej, a w przypadku dwóch pozostałych genów w obu frakcjach.
5. W tkance tłuszczowej trzewnej i podskórnej wskaźnik BMI korelował ujemnie z większością analizowanych genów. Dodatnią korelację zaobserwowano pomiędzy BMI a poziomem globalnej metylacji oraz BMI a poziomem ekspresji *DNMT1*. W tkance podskórnej zaobserwowano dodatnią korelację pomiędzy BMI a poziomem miejscowo specyficznej metylacji w obrębie regionu promotorowego *INSR, IL6* i *ADIPOQ*.
6. W tkance tłuszczowej trzewnej i podskórnej wskaźnik QUICKI dodatnio korelował z ekspresją *PPARG*, a ujemnie z poziomem globalnej metylacji DNA oraz ekspresją *DNMT1* i *IL10*.
7. W tkance tłuszczowej trzewnej i podskórnej wskaźnik HOMA-IR dodatnio korelował z poziomem globalnej metylacji DNA oraz ekspresją *DNMT1*. Jedynie w tkance trzewnej wskaźnik HOMA-IR dodatnio korelował z poziomem metylacji w obrębie regionu promotorowego genu *SLC2A4* i *IL10*.
8. Analiza korelacji wykazała powiązanie ekspresji genu kodującego czynnik transkrypcyjny PPARγ z ekspresją większości analizowanych genów zarówno w tkance trzewnej, jak i podskórnej.

Kluczowe wnioski:

1. Insulinooporności ze współistniejącą otyłością towarzyszą zaburzenia ekspresji genów kluczowych dla szlaku insulinowego, metabolizmu lipidów, regulacji transkrypcji oraz kodujących adipokiny i enzymy biorące udział w generowaniu metylacji DNA w tkance tłuszczowej, zarówno w komponencie podskórnej, jak i trzewnej. Wykazano znaczne obniżenie ekspresji wspomnianych genów u osób otyłych wykazujących oporność na insulinę.
2. Metylacja DNA stanowi czynnik regulujący ekspresję genów u osób otyłych z insulinoopornością o czym świadczy zaobserwowana zwiększona metylacja DNA w adipocytach osób otyłych z insulinoopornością, zwiększony poziom metylacji regionów promotorowych genów kluczowych dla szlaku insulinowego (*SLC2A4*), a także regulacji adipogenezy i metabolizmu glukozy (*PPARG*, *ADIPOQ*), który korelował ujemnie z poziomem ekspresji tych genów. Analiza korelacji uzyskanych wyników ze wskaźnikiem BMI również sugeruje te zależność.
3. PPARγ jako czynnik transkrypcyjny odgrywa kluczową rolę w rozwoju insulinooporności u osób otyłych poprzez regulację ekspresji innych genów istotnych dla utrzymania wrażliwości na insulinę w komórkach tłuszczowych. Co więcej regulacja epigenetyczna ekspresji *PPARG* może stanowić pomost łączący współzależność molekularną pomiędzy otyłością i insulinoopornością.
4. Metylotransferaza DNA kodowana przez gen *DNMT1* może odgrywać kluczową rolę w regulacji epigenetycznej powiązanej z otyłością i insulinoopornością.

2. "*Histone modifications influence the insulin-signaling genes and are related to insulin resistance in human adipocytes*."

   Małgorzata Małodobra-Mazur, Aneta Cierzniak, Aneta Myszczyszyn, Krzysztof Kaliszewski, Tadeusz Dobosz

Głównym założeniem pracy była ocena modyfikacji epigenetycznych na poziomie histonów w badanym materiale biologicznym. Analizie poddano potrójną metylację lizyny 4 (H3K4me3) i acetylację lizyny 9 i 14 (H3K9/14ac) w obrębie histonu 3 na poziomie globalnym oraz miejscowo specyficznym z uwzględnieniem regionów promotorowych genów *PPARG*, *SLC2A4* i *ADIPOQ*. Przeanalizowano również ekspresję panelu genów kluczowych dla regulacji wrażliwości na insulinę, adipogenezy, metabolizmu lipidów, a także kodujących czynniki transkrypcyjne i białka o aktywności enzymatycznej uczestniczące w tworzeniu modyfikacji epigenetycznych. Materiał badany stanowiły: ludzkie tkanki tłuszczowe frakcji trzewnej i podskórnej, a także materiał komórkowy z hodowli ludzkich preadipocytów i mysich preadipocytów komercyjnej linii 3T3-L1. Niedojrzałe komórki z obu hodowli zostały poddane procesowi różnicowania do dojrzałych adipocytów zgodnie z wcześniej opracowanym protokołem, a następnie sztucznie wyindukowano w nich insulinooporność za pomocą kwasu palmitynowego (16:0) we wcześniej określonym stężeniu wynoszącym 0,5 mM. Eksperyment indukcji insulinooporności prowadzono w dwóch punktach czasowych (48 h i 72 h), co pozwoliło na oszacowanie ram czasowych powstawania modyfikacji epigenetycznych w adipocytach. Po zebraniu komórek ich materiał genetyczny został poddany takiej samej analizie jaką przeprowadzono na ludzkich tkankach tłuszczowych, aby w przypadku ludzkiej hodowli potwierdzić otrzymane wyniki na modelu komórkowym, a w przypadku mysiej hodowli ocenić uniwersalność zaobserwowanych mechanizmów.

Pacjentów, podobnie jak materiał komórkowy podzielono na dwie grupy badane na podstawie parametrów oceniających stopień zaawansowania insulinooporności (HOMA-IR, QUICKI):

1. IS – grupa wykazująca prawidłową wrażliwość na insulinę
2. IR – grupa wykazująca oporność na insulinę

Kluczowe wyniki:

1. W obu frakcjach tkanki tłuszczowej globalny poziom metylacji i acetylacji histonów był obniżony u pacjentów należących do grupy IR (w przypadku tkanki podskórnej nie uzyskano istotności statystycznej przy obu modyfikacjach, a w przypadku tkanki trzewnej przy analizie acetylacji).
2. W trzewnej i podskórnej tkance tłuszczowej wykazano ujemną korelację pomiędzy wskaźnikiem HOMA-IR a obiema modyfikacjami epigenetycznymi na poziomie globalnym w grupie IR (brak istotności statystycznej w tkance trzewnej).
3. Poziom miejscowo specyficznej metylacji i acetylacji w obrębie promotora *PPARG*, *ADIPOQ* i *SLC2A4* był zmniejszony w grupie pacjentów z insulinoopornością w obu frakcjach tkanki tłuszczowej (brak istotności statystycznej dla *ADIPOQ*).
4. W trzewnej tkance tłuszczowej poziom acetylacji w odniesieniu do *SLC2A4* korelował ujemnie ze wskaźnikami BMI i HOMA-IR, a także dodatnio z QUICKI.
5. W trzewnej i podskórnej tkance tłuszczowej wykazano zmniejszoną ekspresję *PPARG*, *ADIPOQ* i *SLC2A4* w grupie pacjentów IR.
6. W trzewnej i podskórnej tkance tłuszczowej analiza ekspresji wykazała zmniejszoną ekspresję genu kodującego enzym o aktywności deacetylazy *SIRT7*. W tkance trzewnej wykazano również zmniejszoną aktywność innego genu kodującego białko należące do tej samej grupy: *SIRT1*. Ponadto zaobserwowano silną, dodatnią korelację pomiędzy ekspresją *SIRT7* i *SIRT1* a ekspresją licznych genów, w tym: *PPARG*, *INSR*, *SLC2A4*, *HDAC1*, *HDAC2*, *DNMT1*. Taki sam charakter korelacji wykazano także pomiędzy *SIRT1* i *SIRT7* a wskaźnikiem QUICKI (w przypadku *SIRT7* jedynie w tkance trzewnej).
7. Przeprowadzona analiza globalnego poziomu metylacji i acetylacji histonów w ludzkiej hodowli komórek wyizolowanych z frakcji trzewnej wykazała zmniejszony poziom obu modyfikacji w komórkach IR, ale jedynie w drugim punkcie czasowym eksperymentu, tzn. po 72 h (brak istotności statystycznej).
8. W komórkach z wyindukowaną opornością na insulinę w ludzkim modelu komórkowym obu frakcji zaobserwowano zmniejszony poziom metylacji i acetylacji histonów w obrębie miejsca promotorowego *PPARG*, a także metylacji w obrębie *ADIPOQ* i *SLC2A4* w obu punktach czasowych eksperymentu.
9. W komórkach z wyindukowaną opornością na insulinę w ludzkim modelu komórkowym frakcji podskórnej wykazano po 72 h od rozpoczęcia indukcji

insulinooporności spadek ekspresji genów kluczowych dla regulacji wrażliwości komórek na insulinę: *ADIPOQ, PPARG* i *SLC2A4*. Dla SLC2A4 zmiany były widoczne już po 48 h.

10. W komórkach z wyindukowaną opornością na insulinę w ludzkim modelu komórkowym frakcji trzewnej wykazano zarówno po 48 h jak i 72 h od rozpoczęcia indukcji insulinooporności spadek ekspresji genów kluczowych dla regulacji wrażliwości komórek na insulinę: *ADIPOQ, PPARG*, *INSR* i *SLC2A4*.
11. Przeprowadzona analiza globalnego poziomu metylacji i acetylacji histonów w mysiej hodowli wykazała zmniejszony poziom modyfikacji w komórkach z wyindukowaną insulinoopornością (IR), ale jedynie w drugim punkcie czasowym eksperymentu, tzn. po 72 h (istotność statystyczna jedynie przy acetylacji).
12. W komórkach z wyindukowaną opornością na insulinę w mysim modelu komórkowym wykazano w drugim punkcie czasowym, tj. po 72 h od rozpoczęcia indukcji insulinooporności spadek ekspresji genów kluczowych dla regulacji wrażliwości komórek na insulinę: *SLC2A4, ADIPOQ, PPARG*, a także spadek poziomu obu modyfikacji na poziomie histonów w obrębie regionu promotorowego *SLC2A4, ADIPOQ* i *PPARG*.
13. We wszystkich hodowanych komórkach (zarówno ludzkich obu frakcji, jak i mysich) wykazano zmniejszoną ekspresję genu *SIRT7* w obu punktach czasowych.

Kluczowe wnioski:

1. Modyfikacje epigenetyczne w obrębie histonów mają bezpośredni wpływ na regulację ekspresji genów kluczowych dla insulinooporności.
2. Insulinooporności towarzyszą zaburzenia w ekspresji genów kluczowych dla szlaku insulinowego (*SLC2A4*), kodujących czynnik transkrypcyjny PPARγ (*PPARG*) i adiponektynę (*ADIPOQ*).
3. Obniżony poziom modyfikacji epigenetycznych u pacjentów z insulinoopornością i zaburzenia w ekspresji badanych genów został potwierdzony na modelu komórkowym bazującym na ludzkich komórkach frakcji trzewnej i podskórnej.
4. Większość zmian epigenetycznych zarówno na poziomie DNA, jak i histonów pojawia się po 72 h od wyidukowania insulinooporności w modelu komórkowym ludzkich/mysich adipocytów.

5. Tkanka tłuszczowa trzewna wykazuje szybszą podatność na zmiany w regulacji o podłożu epigenetycznym niż jej komponenta podskórna co może świadczyć o jej zwiększonej podatności na zaburzenia o podłożu metabolicznym.
6. Mysi model 3T3-L1 wykazuje większą spójność z wynikami uzyskanymi z komórek komponenty podskórnej.
7. PPARγ jako czynnik transkrypcyjny odgrywa kluczową rolę w rozwoju insulinooporności w adipocytach. Co więcej, ekspresja *PPARG* podlega regulacji epigenetycznej zarówno na poziomie DNA, jak i histonów.
8. Deacetylazy należące do grupy sirtuin (SIRT1 i SIRT7) mogą odgrywać kluczową rolę w regulacji epigenetycznej na poziomie histonów w rozwoju insulinooporności w komórkach tłuszczowych.

3. "*The preliminary evaluation of epigenetic modifications regulating the expression of IL10 in insulin-resistant adipocytes*."

   Aneta Cierzniak, Krzysztof Kaliszewski, Małgorzata Małodobra-Mazur

Dotychczasowe doniesienia naukowe wskazują na ochronne działanie interleukiny 10 (IL-10) jako cytokiny przeciwzapalnej w przywracaniu wrażliwości na insulinę w komórkach. Głównym założeniem pracy była ocena modyfikacji epigenetycznych na poziomie histonów (H3K4me3; H3K9/14ac) i DNA (metylacja wysp CpG) związanych z regionem promotorowym genu kodującego interleukinę 10 i jej wpływ na ekspresję *IL10* w wykorzystanym materiale biologicznym. Materiał badany stanowiły: ludzkie tkanki tłuszczowe frakcji trzewnej i podskórnej, a także materiał komórkowy z hodowli ludzkich preadipocytów i mysich preadipocytów komercyjnej linii 3T3-L1. Niedojrzałe komórki z obu hodowli zostały poddane procesowi różnicowania do dojrzałych adipocytów zgodnie z wcześniej opracowanym protokołem, a następnie sztucznie wyindukowano w nich insulinooporność za pomocą kwasu palmitynowego (16:0) we wcześniej określonym stężeniu wynoszącym 0,5 mM. Eksperyment indukcji insulinooporności prowadzono w dwóch punktach czasowych (48 h i 72 h), co pozwoliło na oszacowanie ram czasowych powstawania modyfikacji epigenetycznych w adipocytach.

Kluczowe wyniki:

1. W komórkach z wyindukowaną opornością na insulinę w ludzkim modelu komórkowym frakcji podskórnej zaobserwowano wzrost ekspresji *IL10* w obu punktach czasowych. Po 48 h od wyindukowania w komórkach insulinooporności wzrost ten był bardzo wyraźny, w drugim punkcie czasowym jego siła wyraźnie zmalała. Zmianom ekspresji towarzyszyły zmiany metylacji na poziomie DNA w regionie promotorowym *IL10* (znaczący wzrost metylacji zaobserwowano po 72 h), a także na poziomie histonu 3 (H3K4me3), gdzie wykazano wyraźny wzrost w obu punktach czasowych, jednak jego siła w drugim punkcie czasowym podobnie jak przy ekspresji zmalała. Wykazano silną ujemną korelację pomiędzy poziomem metylacji regionu promotorowego a H3K4me3 w pierwszym punkcie czasowym i pomiędzy metylacją DNA a H3K9/14ac w drugim punkcie czasowym.
2. W komórkach z wyindukowaną opornością na insulinę w ludzkim modelu komórkowym frakcji trzewnej zaobserwowano spadek ekspresji *IL10* na przestrzeni obu punktów czasowych, przy czym w drugim punkcie był on większy. Zmianom

ekspresji towarzyszył wyraźny wzrost metylacji DNA regionu promotorowego dla tego genu, tendencja jednak była odwrotna w porównaniu do zmian ekspresji. Zaobserwowano dwunastokrotny wzrost metylacji w pierwszym punkcie czasowym i tylko półtorakrotny wzrost w drugim. Jednocześnie po 48 h wykazano przy jednoczesnym wzroście metylacji regionu promotorowego spadek metylacji histonu 3, a po 72 h spadkowi metylacji DNA towarzyszył wzrost metylacji histonu. Wykazano silną ujemną korelację pomiędzy poziomem metylacji regionu promotorowego a H3K4me3 i H3K9/14ac w drugim punkcie czasowym.

3. W komórkach z wyindukowaną opornością na insulinę w mysim modelu komórkowym wykazano wzrost ekspresji *IL10* w obu punktach czasowych przy jednoczesnym spadku poziomu wszystkich modyfikacji epigenetycznych, zarówno na poziomie DNA, jak i histonów. Wykazano również silną ujemną korelacje pomiędzy ekspresją *IL10* a metylacją histonu 3 w pierwszym punkcie czasowym oraz silną dodatnią korelację pomiędzy ekspresją genu a acetylacją na poziomie histonu po 72 h.

Kluczowe wnioski:

1. Miejscowo specyficzne modyfikacje epigenetyczne na poziomie DNA i histonów mogą mieć istotny wpływ na regulację ekspresji *IL10* w rozwoju insulinooporności w komórkach tłuszczowych.
2. Modyfikacje epigenetyczne mogą wpływać na siebie, co zaobserwowane zostało w ludzkim modelu komórkowym frakcji podskórnej i trzewnej, gdzie redukcji ilości jednej modyfikacji epigenetycznej towarzyszył wzrost drugiej. Oba zaobserwowane zjawiska zostały potwierdzone wykazaną ujemną korelacja między modyfikacjami epigenetycznymi na poziomie DNA i histonów.
3. Odmienny profil ekspresji *IL10* w modelach komórkowych pochodzenia ludzkiego obu frakcji tkanki tłuszczowej potwierdza różnice metaboliczne między jej komponentą podskórną i trzewną, szczególnie w kontekście podatności na stan zapalny.
4. Zaobserwowane zależności pomiędzy ekspresją *IL10* a zmianami na poziomie regulacji epigenetycznej w mysim modelu komórkowym mogą sugerować odmienne mechanizmy regulacji transkrypcji przez modyfikacje epigenetyczne u myszy.

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8. ZAŁĄCZNIKI

8.1. Załącznik nr 1. Publikacje stanowiące podstawę rozprawy doktorskiej.

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ARTICLE

Genetics and Epigenetics

DNA methylation in adipocytes from visceral and subcutaneous adipose tissue influences insulin-signaling gene expression in obese individuals

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Abstract

Objective Both obesity and insulin resistance are characterized by severe long-term changes in the expression of many genes of importance in the regulation of metabolism. Because these changes occur throughout life, as a result of external factors, the disorders of gene expression could be epigenetically regulated.

Materials/methods We analyzed the relationship between obesity and insulin resistance in enrolled patients by means of evaluation of the expression rate of numerous genes involved in the regulation of adipocyte metabolism and energy homeostasis in subcutaneous and visceral adipose tissue depots. We also investigated global and site-specific DNA methylation as one of the main regulators of gene expression. Visceral and subcutaneous adipose tissue biopsies were collected from 45 patients during abdominal surgery in an age range of 40–60 years.

Results We demonstrated hypermethylation of PPARG, INSR, SLC2A4, and ADIPOQ promoters in obese patients with insulin resistance. Moreover, the methylation rate showed a negative correlation with the expression of the investigated genes. More, we showed a correlation between the expression of PPARG and the expression of numerous genes important for proper insulin action. Given the impact of PPARγ on the regulation of the cell insulin sensitivity through modulation of insulin pathway genes expression, hypermethylation in the PPARG promoter region may constitute one of the epigenetic pathways in the development of insulin resistance in obesity.

Conclusions Our research shows that epigenetic regulation through excessive methylation may constitute a link between obesity and subsequent insulin resistance.

Introduction

Obesity is considered a strong risk factor in insulin resistance (IR) [1]. Excessive accumulation of adipose tissue, both visceral (VAT) and subcutaneous (SAT), is associated with metabolic, psychological, endocrine, and genetic factors [2]. Obesity causes changes in cell metabolism, which can lead to IR.

Metabolic disorders induced by obesity and IR are characterized by severe long-term changes in the expression of many genes important in metabolism regulation. Because these changes occur throughout life as a result of external factors (high-fat diet, sedentary lifestyle, stress), gene expression disorders have been regarded as being influenced by epigenetic modifications [3]. Epigenetics is defined as the heritable and reversible modification of gene expression without changes in the DNA sequence, maintained over a generation. One of the basic manifestations of epigenetics is

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tissue-specific gene regulation, which is mainly connected with the presence of methylation within DNA [4, 5].

The relationship between obesity and DNA methylation within both nuclear and mitochondrial DNA has been shown in numerous scientific reports [6–9]. An increased global methylation level was observed in the DNA of B cells from obese and type 2 diabetic patients, as compared to lean subjects [10]. Increased site-specific methylation in obese individuals was observed in the promoters of the genes regulating insulin sensitivity and the insulin-signaling pathway [7, 9, 11, 12].

In the present study, we analyzed the influence of obesity and obesity with concomitant IR on global and site-specific DNA methylation and the expression of genes involved in the insulin-signaling pathway, adipogenesis, lipid metabolism, inflammation, and the DNA methylation process in human adipose tissue. Both types of adipose tissue, VAT and SAT, were subjected to examination.

Materials and methods

The research protocols and all procedures were approved by the Ethical Review Board of Wroclaw Medical University, approval no. KB-124/2017.

Biological material

VAT and SAT biopsies were collected from 45 patients in an age range of 40–60 years during abdominal surgery, following written agreement. For each enrolled subject, the following parameters were assessed: fasting glucose, lipids panel, and body weight and height for calculation of BMI. In addition, a questionnaire regarding other metabolic diseases (type 2 diabetes, hypertension, sclerosis) and medications was completed.

Criteria for excluding patients from the study included other IR-related diseases (PCOS, Cushing's syndrome), thyroid dysfunction, hepatitis, chronic inflammatory or infectious diseases, tumors, heavy drinking or a positive history thereof, and use of insulin or metformin.

Insulin level

Insulin levels were measured in plasma, using a Human Insulin ELISA Kit (Sigma-Aldrich). Absorbance was read using a Victor3 1420 Multilabel Counter.

DNA and RNA isolation

DNA was isolated using a commercial column spin method kit, the QIAamp DNA Mini Kit (QIAGEN) according to protocol. Total RNA was isolated using a combination of the trizol method and commercial spin column kits (Promega). The tissues samples (10–20 mg) were homogenized in 1 ml of Trizol, after extraction, RNA was precipitated with isopropanol and applied on the silica membrane column. Further extraction was carried out according to protocol.

Reverse transcription reaction and gene expression level

Reverse transcription was performed with the use of a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), using 200 ng of total RNA. Gene expression was done using Real-Time PCR based on a SYBR Green assay (Applied Biosystems). Primers were manually designed to flank two exons of mRNA (Supplementary Table 1). The specificity of primers was checked using Primer-BLAST; secondary structures were analyzed using OligoAnalyzer. Prior to real-time PCR, the efficiency of the primers was analyzed using the standard curve method; specificity was checked based on the denaturation curve. Only primers characterized by efficiency values higher than $R^2 > 0.95$ were used for gene expression studies. A relative gene expression level, normalized to the housekeeping gene *β-actin*, was calculated using the delta-delta Ct (ΔΔCt) model.

Global DNA methylation analysis

The global methylation of DNA was measured using a commercial ELISA-based MethylFlash Methylated DNA Quantification Kit (Epigentek) in accordance with the manufacturer's protocol. Absorbance was read using a Victor3 1420 Multilabel Counter.

Site-specific DNA methylation analysis and prediction of CpG islands

Prediction of CpG islands in the promoter region was accomplished using a USCS (University of California Santa Cruz) Genome Browser and MethPrimer software (UCSF). The prediction criteria: CG content >55% (region 500 bp in length), ObsCpG/ExpCpG >0.65.

Site-specific DNA methylation within the promoter region of the analyzed genes was carried out via methylated DNA precipitation (meDIP) using a MagMeDIP qPCR Kit (Diagenode) followed by a percentage of input measurements in real-time PCR according to the algorithm provided by the manufacturer. Primers were designed to amplify representative CG clusters located in the promoter regions of the analyzed genes (Supplementary Table 2). The results of the meDIP analysis were confirmed by the bisulfite sequencing technique. Bisulfite treatment of genomic DNA (500 ng) was performed using an EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific). Amplification of the

CpG islands for the appropriate gene was done using a QIAGEN Multiplex PCR Kit (QIAGEN). The primers for PCR were selected based on data obtained during the prediction of the CpG islands using MethPrimer software (UCSF). Prior to the actual experiments, the temperature condition of PCR for each CpG island was determined. The amplification results were checked by means of gel electrophoresis. The amplified CpG islands were sequenced using the Sanger method. The results of sequencing were analyzed using QUMA (a quantification tool for methylation analysis) (Riken).

Assessment of IR and obesity

BMI was calculated as weight in kilograms divided by squared of height in meters [kg/m^2]. IR rate was assessed using IR ratios calculated according to the following formulas:

(1) HOMA-IR [(glucose [mmol/l] × insulin [μU/ml])/22.5],
(2) QUICKI [1/(log glucose [mg/dl] + log insulin [μU/ml])].

Statistical analysis

Statistical analysis was performed using STATISTICA 13.1 and Microsoft Office Excel 2007. ANOVA and a post-hoc test (the NIR-Fisher test) were used to assess the difference between studied groups. The correlation between numerical values was made using a correlation coefficient. Statistical significance was set at $p < 0.05$.

Results

The enrolled patients were divided into three study groups (LH—lean healthy, OH—obese healthy, with normal values of IR ratios, OR—obese with IR) depending on the BMI, HOMA-IR and QUICKI parameters. Patients with BMI > 25 were classified in the obese group. IR was diagnosed based on HOMA-IR > 2.5 and QUICKI < 0.321. We performed all analysis for both VAT and SAT tissues. We treated LH group as a control group and compared the other groups to it.

Study cohort characterization

The groups were characterized according to obesity level, IR ratios, lipid metabolism, age, and sex. The results are presented in Table 1.

Gene expression in IR and obesity

We measured the expression of genes important in adipocyte metabolism, i.e.: (1) insulin pathways: *INSR*, *IRS1*, *IRS2*, *PIK3R1*, *AKT*, *SLC2A4*; (2) lipid metabolism: *LPL*, *ACACA*, *FASN*, ACSS2, *SCD1*; (3) inflammation: *IL10*, *IL1B*; (4) adipokines: *LEP*, *ADIPOQ*, retinol-binding protein 4 (*RBP4*); (5) transcription factors: *CEBPA*, *CEBPB*, *PPARG*, *IGF2*, *PPARGC1A*, *TNFA*. We assessed the expression level of genes in all three study groups.

In the VAT samples of OR patients, we observed a statistically significant reduction in the expression rate of the following genes: *INSR* ($p = 0.001$), *IRS1* ($p = 0.012$), *IRS2* ($p = 0.050$), *PIK3R1* ($p = 0.009$), *SLC2A4* ($p = 0.018$), *ACSS2* ($p = 0.014$), *ACACA* ($p = 0.019$), *LPL* ($p = 0.030$), *FASN* ($p = 0.002$), *PPARG* ($p = 0.001$), *ADIPOQ* ($p = 0.031$), *RBP4* ($p = 0.001$). Contrastingly, we observed a significant increase of *IL10* expression level in OR group ($p = 0.017$).

A similar profile of gene expression was noticed in VAT depots in OH group. Among others, reduction in expression

Table 1 Study cohort characterization.

	LH	OH	OR	*p* value
N [female/male]	3/12	2/10	4/14	–
BMI [kg/m^2]	21.80 ± 0.71	27.38 ± 0.77	29.50 ± 0.63	0.000
Glucose [mg/dl]	87.63 ± 7.72	88.71 ± 8.63	106.06 ± 7.25	n.s.
Insulin [mg/dl]	8.60 ± 4.42	9.24 ± 5.32	32.73 ± 5.04	0.002
HOMA-IR	1.83 ± 1.41	2.06 ± 1.69	8.32 ± 1.61	0.010
QUICKI	0.350 ± 0.005	0.343 ± 0.006	0.297 ± 0.006	0.000
Triglycerides [mg/dl]	86.90 ± 26.37	174.50 ± 26.37	203.08 ± 23.13	0.008
Cholesterol [mg/dl]	211.67 ± 11.97	199.57 ± 13.57	219.91 ± 10.82	n.s.
LDL [mg/dl]	132.70 ± 9.81	131.40 ± 9.81	148.18 ± 9.36	n.s.
HDL [mg/dl]	60.70 ± 4.83	43.60 ± 4.83	43.00 ± 4.23	0.019
Age	48 ± 3	49 ± 3	51 ± 3	n.s.

The *p* value is related to the analysis of variance between all study groups.

LH lean healthy, *OH* obese healthy, with normal values of insulin resistance ratios, *OR* obese with insulin resistance.

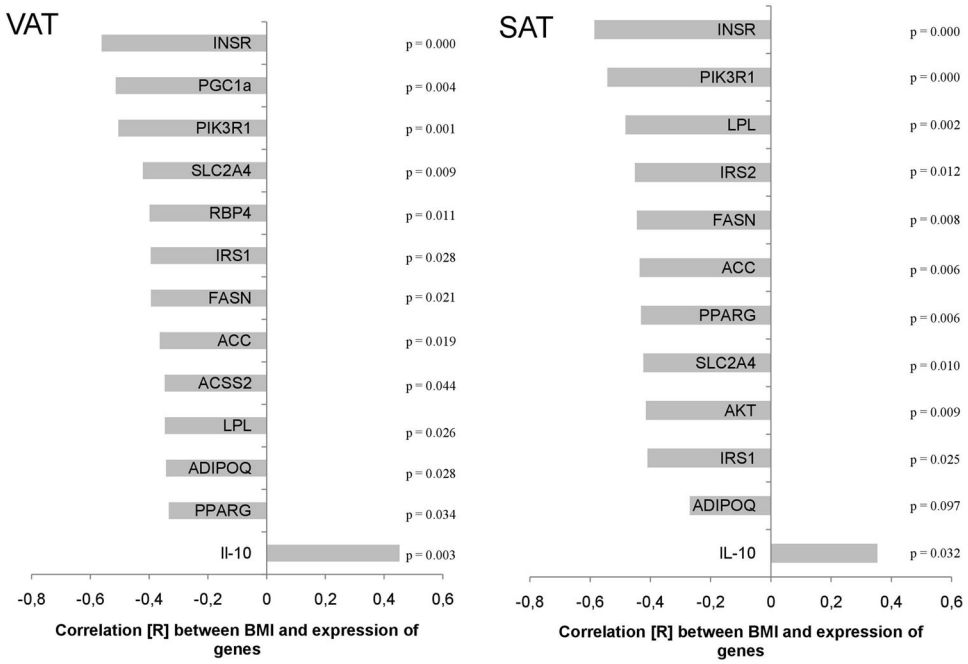

Fig. 1 A correlation between BMI and the expression level of genes with a *p* value. The results of correlation between BMI and the expression level of genes in visceral adipose tissue (VAT) and subcutaneous (SAT) adipose tissue, respectively.

rate was diagnosed mainly for *INSR* ($p = 0.008$), *IRS1* ($p = 0.025$), *PIK3R1* ($p = 0.014$), *FASN* ($p = 0.069$), *PPARG* ($p = 0.057$), *ADIPOQ* ($p = 0.091$) and *RBP4* ($p = 0.022$).

In SAT samples in OR group, we observed statistically significant reductions in expression rate for the following genes: *INSR* ($p = 0.001$), *SLC2A4* ($p = 0.013$), *IRS1* ($p = 0.062$), *IRS2* ($p = 0.015$), *LPL* ($p = 0.039$), *FASN* ($p = 0.036$), and *PPARG* ($p = 0.017$). Moreover, *IL10* gene was overexpressed in the OR group ($p = 0.034$). The only genes that were downregulated in the SAT depots of the OH group were *INSR* ($p = 0.008$), *SLC2A4* ($p = 0.053$), and *IRS1* ($p = 0.021$).

Next, we analyzed the correlation between the expression of all genes and BMI, HOMA-IR, and QUICKI values. The observed correlation between patients' BMI and expression of numerous genes are presented in Fig. 1.

A negative correlation was observed between QUICKI value and expression of *IL10* (VAT: $R = 0.410$; $p = 0.022$, SAT: $R = 0.435$; $p = 0.016$). On the other hand, a positive correlation was seen between QUICKI and expression of *PPARG* (VAT: $R = 0.312$; $p = 0.082$, SAT: $R = 0.353$; $p = 0.048$), *INSR* (SAT: $R = 0.306$; $p = 0.088$), *LPL* (SAT: $R = 0.308$; $p = 0.087$), and *ADIPOQ* (SAT: $R = 0.376$; $p = 0.034$). In the case of HOMA-IR, we did not observe statistically significant correlations.

Global DNA methylation

We found differences in global DNA methylation between studied groups. We observed the highest level of DNA methylation in OR group in both fat depots (VAT: $p = 0.000$, SAT: $p = 0.007$). DNA methylation was also higher in the OH compared to the LH group, without, however, being significant (Fig. 2).

Furthermore, in both VAT and SAT samples, we observed a positive correlation between the global DNA methylation level and BMI (VAT: $R = 0.591$; $p = 0.000$; SAT: $R = 0.574$; $p = 0.000$) as well as between the global DNA methylation level and HOMA-IR value (VAT: $R = 0.380$; $p = 0.061$; SAT: $R = 0.431$; $p = 0.032$). A negative correlation was observed between the global DNA methylation level and QUICKI value (VAT: $R = -0.360$; $p = 0.077$; SAT: $R = -0.509$; $p = 0.009$).

We also assessed the expression level of gene encoding DNA methyltransferases (*DNMT1*, *DNMT3a*, and *DNMT3b*). In the case of *DNMT3a* and *DNMT3b*, we did not observe significant differences in gene expression between the study groups (data not shown). We showed that *DNMT1* was overexpressed in OR patients in both fat depots (VAT: $p = 0.039$, SAT: $p = 0.045$; Fig. 2). Furthermore, we observed a positive correlation between the DNA methylation rate and the expression level of *DNMT1* in both fat depots (VAT: $R = 0.380$; $p = 0.029$; SAT: $R = 0.298$; $p = 0.087$). We observed a similar relationship in the case of the expression level of *DNMT1* and BMI (VAT: $R = 0.346$; $p = 0.031$; SAT: $R = 0.372$; $p = 0.015$). Moreover, the expression rate of *DNMT1* correlated positively with IR assessed based on HOMA-IR value (VAT: $R = 0.647$; $p = 0.000$; SAT: $R = 0.598$; $p = 0.000$) and QUICKI (VAT: $R = -0.441$; $p = 0.013$; SAT: $R = -0.487$; $p = 0.005$).

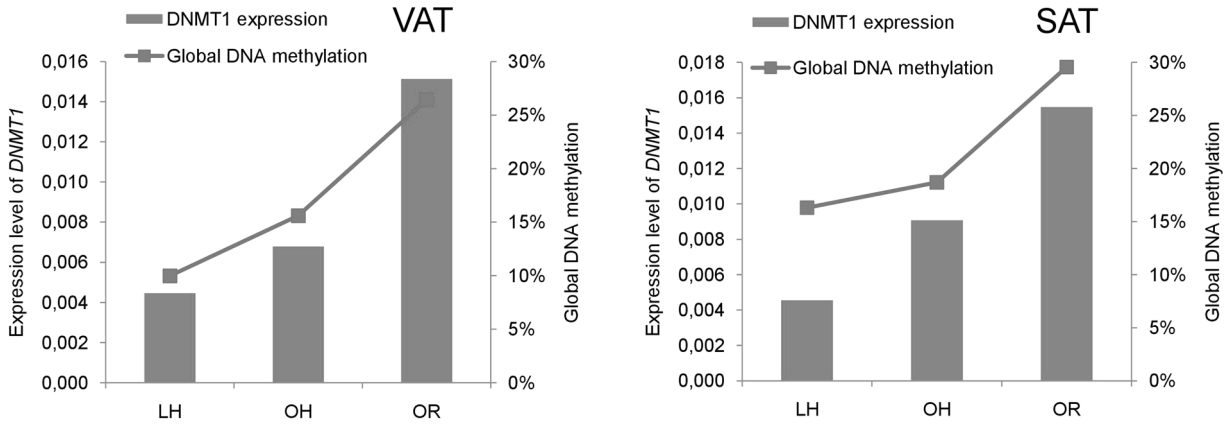

Fig. 2 The results of the comparative analysis regarding the expression of DNMT1 gene and global DNA methylation level between three groups (LH, OH, and OR). The results of *DNMT1* gene expression are compared with the results of global DNA methylation in visceral (VAT) and subcutaneous (SAT) adipose tissue, respectively. Statistical significance in the comparative analysis was demonstrated for the OR group both in the analysis of *DNMT1* gene expression (VAT: $p = 0.039$; SAT: $p = 0.046$) and global methylation (VAT: $p = 0.001$; SAT: $p = 0.007$).

Methylation of gene promoter region

Next, the site-specific methylation pattern within the promoter region of the selected genes was analyzed.

In VAT samples, we noted a significant increase in the methylation level of *PPARG* promoter in OR compared to the LH group ($p = 0.009$). What is more, the methylation rate of the *PPARG* promoter negatively correlated with the expression rate in this fat depot ($R = -0.4702$; $p = 0.049$). In both VAT and SAT samples, we observed an increased methylation rate of *SLC2A4* (VAT: $p = 0.041$; SAT: $p = 0.078$) and *ADIPOQ* (VAT: $p = 0.080$; SAT: $p = 0.013$) promoter in the OR group. On the other hand, methylation of *INSR* increased in both obese groups in both VAT (OH: $p = 0.002$, OR—no statistical significance) and SAT (OH $p = 0.016$, OR—no statistical significance), which corresponded to expression rate of this gene. In SAT, we also observed a negative correlation between the *INSR* promoter methylation level and the expression of *INSR* ($R = –0.3967$; $p = 0.068$). The results are presented in Fig. 3A.

We also wished to correlate the site-specific methylation pattern with clinical parameters of enrolled patients, such as BMI, HOMA-IR, QUICKI, and lipids panel.

In the case of VAT samples, we observed a negative correlation between the QUICKI value and level of promoter methylation of *SLC2A4* ($R = -0.4239$; $p = 0.055$) and *IL10* ($R = -0.5010$; $p = 0.021$). A positive correlation was seen between the HOMA-IR value and level of promoter methylation of *SLC2A4* ($R = 0.4841$; $p = 0.026$) and *IL10* ($R = 0.5265$; $p = 0.014$).

In SAT samples, we observed a positive correlation between BMI and the level of methylation of the promoters of the following genes: *INSR* ($R = 0.5781$; $p = 0.006$), *ADIPOQ* ($R = 0.4784$; $p = 0.028$), and *IL6* ($R = 0.8387$; $p = 0.000$). The methylation rate of the promoter of *INSR* ($R = 0.8341$; $p = 0.000$) and *IL6* ($R = 0.8436$; $p = 0.001$) correlated positively with triglyceride level in serum, similar to methylation of *ADIPOQ* promoter that correlated positively with the LDL cholesterol level in serum ($R = 0.5697$; $p = 0.027$).

To confirm the results of differentially methylated promoters, we performed a bisulfite sequencing study of representative samples from three investigated groups. In samples from both fat depots, VAT and SAT, we observed an increased site-specific methylation level within the promoter region of *PPARG* in OR compared to the LH group (Fig. 3B).

Gene expression and transcription factor

The nuclear peroxisome proliferator-activated receptor gamma (PPARγ) is a crucial transcription factor regulating adipocyte development and normal metabolism. Thus we were interested as to whether PPARγ could regulate the expression of genes necessary for normal adipocyte function. In both types of tissue, we observed a correlation between *PPARG* gene expression and several genes. The results are presented in Fig. 4.

Discussion

In the present study, we analyzed the influence of obesity on global and site-specific DNA methylation as well as the potential influence of these epigenetic modifications on the expression of genes involved in the insulin-signaling pathway, adipogenesis, lipid metabolism, inflammation, and DNA methylation process in human adipose tissue. The link

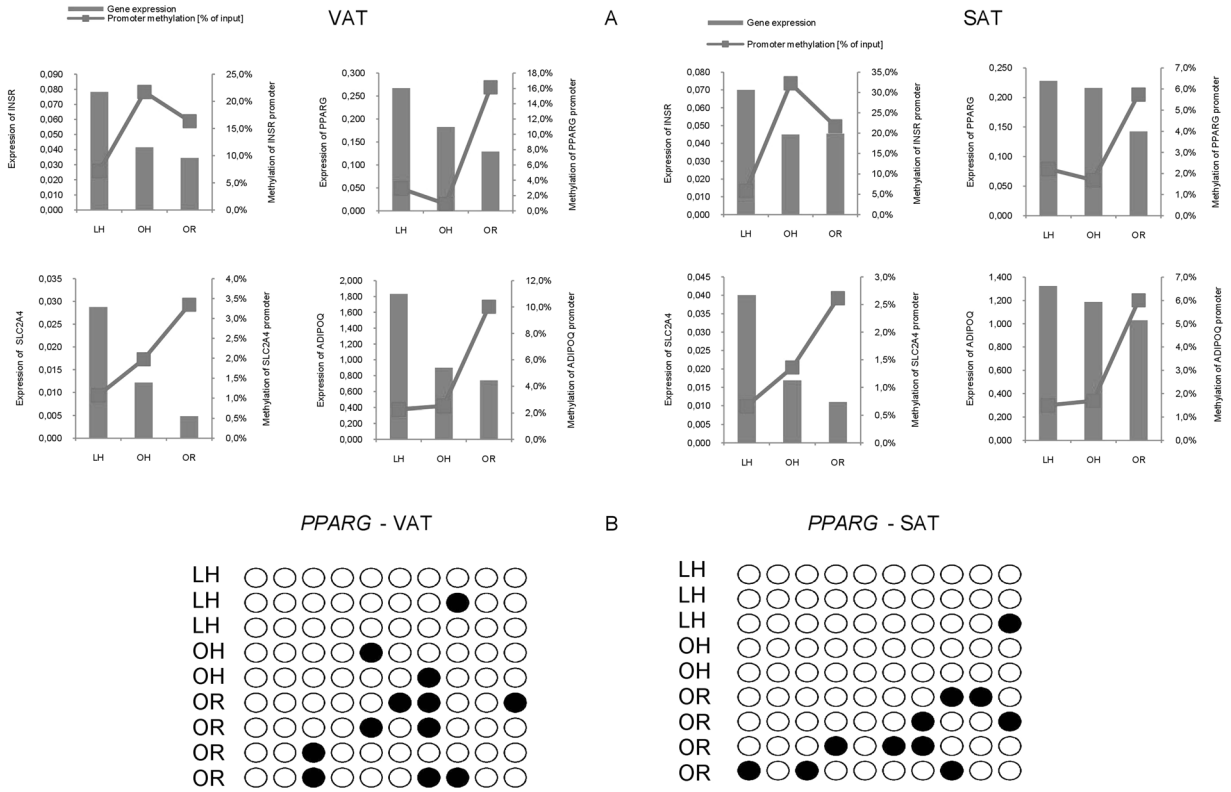

Fig. 3 The results of the analysis regarding to the site-specific methylation of the promoter region of the selected genes. The results of the comparative analysis regarding the expression of *INSR*, *SLC2A4*, *PPARG*, and *ADIPOQ* and level of methylation of this gene promoter between three groups (LH, OH, and OR) in visceral (VAT) and subcutaneous (SAT) adipose tissue, respectively (**A**). The site-specific DNA methylation within the promoter region of *PPARG* gene in VAT and SAT—the results of the bisulfite sequencing technique (**B**).

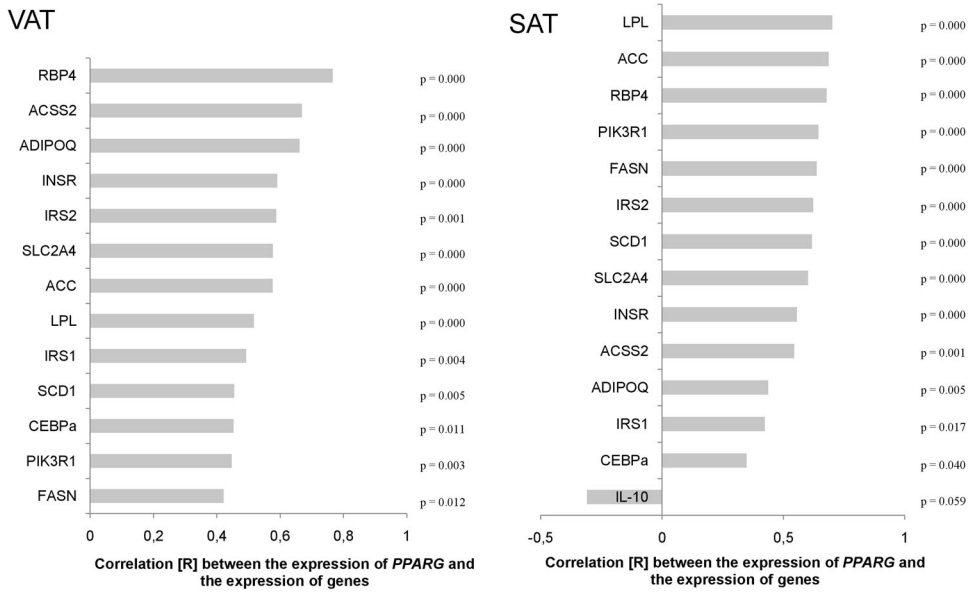

Fig. 4 A correlation between the expression of *PPARG* and the expression of other genes with a *p* value. The results of correaltion between *PPARG* expresiion and the expression of other genes in visceral (VAT) and subcutaneous (SAT) adipose tissue, respectively.

between obesity and IR, which is reflected in disorders in the expression of genes relevant to the insulin-signaling pathway, is well described. Indeed, in the present study, we demonstrated a strong negative correlation between BMI and expression of genes important for the insulin pathway (*INSR*, *IRS1*, *IRS2*, *PIK3R1*, *AKT*, *SLC2A4*). The normal

expression of all of these genes is extremely important for correct insulin signaling [13].

Numerous factors may be responsible for aberrant gene expression, ranging from intracellular to extracellular factors such as nutrition, age, or physical activity. Considering that obesity and IR have genetic and environmental backgrounds, we hypothesized that epigenetic modification might also be involved. In the present study, we showed the association between global DNA methylation level and obesity, which had also been confirmed previously [7, 9, 14, 15]. The observed increased level of methylation with increased BMI suggests a link between epigenetic modifications and obesity. What is more, we also demonstrated positive correlations between global DNA methylation and *DNMT1* expression and between BMI and *DNMT1* expression, which would explain the potential role of this enzyme in creating epigenetic modification in adipocyte DNA.

We observed a similar relationship between DNA methylation and IR (a positive correlation between DNA methylation and HOMA-IR value and a negative correlation between DNA methylation and QUICKI value). Moreover, comparative analysis between the groups showed that in both tissues the level of DNA methylation was significantly higher in the group of obese people with IR compared to lean, healthy people, which may suggest the role of epigenetic regulation in insulin sensitivity disorders in obese patients.

The detected relationships between increased global DNA methylation level in adipocytes and obesity and IR led us to take a closer look at site-specific methylation, especially within the promoter region of genes important for the insulin pathway or genes connected with the regulation of this pathway. We showed increased methylation levels within the promoter region of the *INSR* and *SLC2A4* genes in the group of obese patients with IR compared to the lean group, in both VAT and SAT. The insulin receptor encoded by the *INSR* gene constitutes the first stage of the insulin pathway, but *SLC2A4* encodes the glucose transporter 4, the major insulin-regulated glucose intercellular transporter, which is the last stage of this pathway. The expression level of these genes was also significantly reduced in the OR group, which may explain the silencing role of DNA methylation on gene expression. This is also confirmed by the demonstrated a significant negative correlation between methylation of the *INSR* promoter region and expression of this gene in SAT. Interestingly, in SAT we also observed a positive correlation between the methylation promoter level of *INSR* and BMI, suggesting that epigenetic modifications are connected with obesity, and in the next step lead to IR development via methylation of the promoter region of the genes, which are important for the insulin pathway, such as INSR.

PPARγ is considered a transcriptional regulator of adipogenesis and lipid and glucose metabolism. The *PPARG* gene is expressed especially in both white and brown adipose tissue [16, 17]. What is more, its synthetic ligands, such as glitazones, are used in the treatment of diabetes, as they improve insulin and glucose parameters and increase insulin sensitivity [18, 19]. Dysregulation of PPARγ can lead to the development of obesity, IR, and type 2 diabetes [17]. Studies have shown that changes in *PPARG* expression, e.g., gene knockout, cause IR and dysregulation of adipogenesis in mice [20, 21]. In our research, we also observed a relationship, in both VAT and SAT, between PPARγ and obesity and IR, which is shown by a negative correlation between *PPARG* expression and BMI as well as by a positive correlation between *PPARG* expression and QUICKI value. Moreover, our comparative analysis also confirms the association of both diseases with PPARγ. The expression of *PPARG* was significantly reduced in the OR group. Interestingly, we also demonstrated that changes in *PPARG* expression are based on epigenetic regulation, which confirms the very high level of methylation within the promoter region of this gene. The level of methylation was at least several times (in VAT about 5.5 times, in SAT 2.5 times) higher in the OR group compared to the LH group. What is more, with an increase of methylation within the promoter region of *PPARG*, we observed decreased expression of this gene (a negative correlation between the level of methylation and expression of *PPARG*), which confirms that *PPARG* is subject to epigenetic regulation. PPARγ as a transcription factor can influence regulation of the expression of other genes important for the insulin pathway or adipogenesis. In order to better elucidate the regulation of its role, we performed correlation analysis. We observed a strong positive correlation between the expression of PPARγ and numerous genes. It has been shown that PPARγ takes part in the regulation of *INSR*, *IRS1*, *IRS2*, *PIK3R1*, and *SLC2A4*, which make up a significant part of the genes involved in the transmission of the insulin pathway signal, and also in the regulation of genes involved in lipid metabolism, namely *LPL*, *ACACA*, *ACSS2*, *SCD1*, and *FASN*, and a gene coding, a transcriptional factor important for adipogenesis, *CEBPA*. These results show the important role of PPARγ in regulating metabolic pathways and in potential disorders. Changes in *PPARG* expression caused by epigenetic modifications can significantly interfere with the expression of other genes relevant for the insulin pathway, lipogenesis, or adipogenesis.

In addition, we examined the effect of epigenetic regulation on adiponectin, because PPARγ directly regulates expression of the *ADIPOQ* gene, as we have shown in this study (a strong positive correlation between the expression of *PPARG* and of *ADIPOQ*) as was proved earlier [22]. Adiponectin, one of the adipokines produced by adipocytes

of white adipose tissue, is involved in insulin sensitivity, glucose uptake, and lipid metabolism. Some studies have shown that adiponectin improves insulin sensitivity by reducing the amount of intercellular fat and enhancing the insulin receptor substrate [23–25]. Other studies have shown that adiponectin gene expression is downregulated in IR and obesity [26–29]. We also demonstrated an association between BMI and *ADIPOQ* expression (negative correlation) and between QUICKI value and *ADIPOQ* expression (positive correlation). What is more, we showed that the expression level of the adiponectin gene is strongly downregulated in the OR and OH group compared to LH group, however only in VAT depots. Interestingly, we also demonstrated that changes in *ADIPOQ* expression are based on epigenetic regulation, which confirms the very high level of methylation within the promoter region of *ADIPOQ*. The level of methylation was at least several times (in VAT about 4.5 times, in SAT 4 times) higher in the OR group compared to the LH group. A published study also demonstrated a decrease in *ADIPOQ* expression in mice with hypermethylation of the promoter region of the *ADIPOQ* gene [30].

We also took a closer look at another potential regulatory factor of insulin sensitivity in obesity. RBP4 has been identified as an adipokine with potential involvement in the development of impaired glucose metabolism. It has been shown that the serum level of RBP4 positively correlates with obesity and IR, and induces IR through preventing insulin-initiated phosphorylation of insulin receptor substrate 1 [31–33]. Interestingly, in the present study, we demonstrated completely different relationships, namely, a negative correlation in VAT samples between BMI and *RBP4* expression. More significantly, the downregulation of the expression of this gene in OR compared to LH group indicates that RBP4 may play an important role in regulating insulin sensitivity in obese patients with IR. Moreover, we showed an association between the expression of *PPARG* and *RBP4*. The strong positive correlation between these genes could suggest that RBP4, similar to adiponectin, may be under the control of PPARγ, which is considered a positive regulator of insulin sensitivity. It is, therefore, possible that the nature of the effect of RBP4 on the development of IR should be investigated further.

Noteworthy are also the slight differences we observed in the expression of genes relevant to the insulin pathway between the group of obese patients with normal glucose tolerance and the obese group with IR. These small differences may indicate that obesity itself generates disorders in the insulin pathway at the molecular level that are visible in gene expression but are not yet visible in metabolic parameters such as QUICKI and HOMA-IR. We suggest that a very important role in this process may be played by epigenetic regulation, as we observed a higher level of site-specific methylation in the *INSR* gene in OH compared to OR. Thus obese patients may experience disorders in the insulin pathway long before their clinical manifestation and complete development of IR.

The value and strength of presented expression results would be increased by the western blot analysis of the protein; however, the limited amount of biological material made it impossible to perform the research. In the future, we plan to take care of the increased amount of collected biological material to extend the research to protein analysis while enhancing the power of the results.

Summarizing, our research shows that epigenetic regulation through excessive methylation may constitute a link between obesity and subsequent IR. Moreover, our research confirms that, in obese patients with co-existing IR, the expression of genes relevant to the insulin pathway is significantly reduced compared to lean healthy patients, and the expression was shown to be epigenetically regulated. Interestingly, the differences in gene expression between the group of obese patients (with normal insulin sensitivity and with IR) were not very large except in the case of the *SLC2A4* gene. This observation shows how obesity adversely affects the insulin pathway, creating the necessary conditions for the development of cells' resistance to insulin through disorder in gene expression, even where IR has not yet been diagnosed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary Table 1. The primer pairs used in a study for gene expression analysis (Real-Time PCR).

Symbol of gene	Official full name of gene	Forward sequence	Reverse sequence	R^2 value
DNMT1	*DNA methyltransferase 1*	AGGCGGCTCAAAGATTTG	CTCCTTCACACATTCCTT	0.98
INSR	*insulin receptor*	TTCGAGGAGGCAACAATCTG	CGTAGGATCGGCGGATTTTT	0.95
IRS1	*insulin receptor substrate 1*	CCCCAACGGTCACTACATTT	ACTGGCTGCTTCATCCCCA	0.99
IRS2	*insulin receptor substrate 2*	AACACCTACGCCAGCATTGA	TCACTCTTTCACGATGGTGG	0.99
PIK3R1	*phosphoinositide-3-kinase regulatory subunit 1*	TGCCTGCTCTGTAGTGGTG	GCCATAGCCAGTTGCTGTTT	0.98
AKT	*AKT serine/threonine kinase 1*	TTCCTCACAGCCCTGAAGTA	GTTGGCGTACTCCATGACAA	0.99
SLC2A4	*solute carrier family 2 member 4*	AGCAGCTCTCTGGCATCAAT	ACCAACAACACCGAGACCAA	0.99
LEP	*leptin*	TTCACACACGCAGTCAGTCT	GCATACTGGTGAGGATCTGT	0.98
ADIPOQ	*adiponectin, C1Q and collagen domain containing*	GGAGATCCAGGTCTTATTGG	TGGGCATGTTGGGGATAGTA	0.99
RBP4	*retinol binding protein 4*	TACTCCTTCGTGTTTTCCCG	CGCAGTAACCGTTGTGGAC	0.99
LPL	*lipoprotein lipase*	TCACTCTGCCTGAAGTTTCC	TGCTCCACCAGTCTGACCA	0.99
ACACA	*acetyl-CoA carboxylase alpha*	CGTCCTCACCCAACCCAAA	TCTACCAACCACCACAGTCT	0,98
FASN	*fatty acid synthase*	GGCATCAATGTCCTGCTGAA	TACCCATTCCCCGCTGTGT	0.99
ACSS2	*acyl-CoA synthetase short chain family member 2*	CTGTCACCAAGCATAGCCG	CCTCAGGGTTGATGGGTTC	0.96
SCD1	*stearoyl-CoA desaturase*	CCAGAGGAGGTACTACAAAC	AAATACCAGGGCACAAGCGT	0.99
CEBPA	*CCAAT enhancer binding protein alpha*	GCCAAGAAGTCGGTGGACA	GCGGTCATTGTCACTGGTC	0.98
CEBPB	*CCAAT enhancer binding protein beta*	AGCACCACGACTTCCTCTC	AGTTCTTGCCCCCGTAGTC	0.99
PPARG	*peroxisome proliferator activated receptor gamma*	TAATGCCATCAGGTTTGGGC	GGTCAGCGGACTCTGGATT	0.96
IGF2	*insulin like growth factor 2*	GGCTTCTACTTCAGCAGGC	AGCACAGTACGTCTCCAGG	0.97
PPARGC1A	*PPARG coactivator 1 alpha*	GATCCTCTTCAAGATCCTGC	TCGTAGCTGTCATACCTGGG	0.97
IL10	*interleukin 10*	GGACTTTAAGGGTTACCTGG	CTGGGTCTTGGTTCTCAGC	0.99
IL1B	*interleukin 1 beta*	AACAGATGAAGTGCTCCTTC	TGGTGGTCGGAGATTCGTAG	0.99
TNFA	*tumor necrosis factor alpha*	GTTGTAGCAAACCCTCAAGC	TGGTTATCTCTCAGCTCCAC	0.99
ACTB	*β-actin*	GAGAAGATGACCCAGATCA	TAGCACAGCCTGGATAGCAA	0.99

Supplementary Table 2. The primer pairs used in a study for gene expression analysis in me-DIP.

Symbol of gene	Forward sequence	Reverse sequence	Source
INSR	GGTAGAGAAAGGATCTGTG	GAGTCTCCTCCAGTTTCAG	self-designed
SLC2A4	TTGTGGCTGTGGGTCCCAT	CTCGTCTTAGAATAGCTGGA	self-designed
ADIPOQ	GCTGTTCTACTGCTATTAGC	GATCTCCTTTCTCACCCTTC	self-designed
PPARG	AAACTTCGGATCCCTCCT	GCTACCTGGTGTCGTTTG	[34]
IL6	CCTGCATTAGGAGGTCTTTG	CTGACACCAGCAAAGGATAA	[34]
IL10	ACTGCTCTGTTGCCTGGTC	GTCTTCACTCTGCTGAAGG	self-designed

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Histone modifications influence the insulin-signaling genes and are related to insulin resistance in human adipocytes

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ABSTRACT

Insulin resistance (IR) is a state when the physiological amount of insulin is not sufficient to evoke proper action, that is, glucose uptake. Numerous conditions lead to IR, including epigenetic components. Epigenetic modifications, associated with obesity and IR are one of the main mechanisms leading to IR pathogenesis.

The adipose tissue samples (subcutaneous (SAT) and visceral (VAT)) were collected during abdominal surgery from 40 patients of a wide range of BMI, age, and insulin resistance ratios (F = 9, M = 31). IR was induced in 3T3-L1 adipocytes and human adipocytes collected from SAT and VAT of healthy subjects. Global and site-specific histone modifications (H3K4me3 and H3K9/14ac) were determined.

We found lower histone modifications in adipose tissue of IR patients. Furthermore, numerous genes regulating insulin action (*PPARG, SLC2A4, ADIPOQ*) were differently marked by histone methylation and acetylation. Moreover, we noticed that epigenetic changes appear as soon as 72 h following IR induction. The epigenetic changes appeared to be mediated through the *SIRT* family.

Based on obtained results, the histone marks related to insulin resistance mostly concerned *PPARG* and *SLC2A4* genes. Furthermore, our results proved a vital role of the SIRT family in insulin action and IR pathogenesis.

1. Introduction

Insulin resistance (IR) is a state when the physiological amount of insulin is not sufficient to evoke proper action, that is, glucose uptake by peripheral tissues like the liver, muscles, and adipose tissue (Lebovitz, 2001). IR and the consequently developed hyperglycemia are serious conditions leading to numerous cardiovascular system complications, nephropathy, retinopathy, or neuronal disorders (Lebovitz, 2001). Furthermore, it is a considerable epidemiologic problem affecting millions of people each year, mainly in highly industrialized countries (Engin, 2017; Saklayen, 2018).

The pathomechanism of IR remains unclear. Although several compounds can induce IR in experimental animals or culture cells, the metabolic regulation leading to IR is not clear. Moreover, the way of overcoming IR is yet to be developed (Abdul-Ghani and DeFronzo, 2010; McCracken et al., 2018).

Numerous factors lead to IR, including a sedentary lifestyle, inappropriate diet rich in saturated fats and carbohydrates, and excess calorie intake (González-Becerra et al., 2019; Jiménez-Chillarón et al., 2012). Genetic components that predispose or increase IR induction risk also play an important role in IR pathogenesis (Brown and Walker, 2016; Lebovitz, 2001). In patients with impaired insulin sensitivity, severe abnormalities in the gene expression involved in insulin action and/or insulin sensitivity regulation can be detected. The gene expression regulation can be exerted at either transcriptional level, post-transcriptional level, or epigenetic mechanisms. Epigenetics is defined as the heritable and reversible gene expression modification without changing the DNA sequence maintained over a generation (Deans and Maggert, 2015).

Epigenetics refers to gene expression regulation arising from chromatin marks, including DNA methylation, histone methylation, acetylation, ubiquitination, or phosphorylation. DNA and histone modifications induce chromatin remodeling toward either euchromatin and induction of gene expression or heterochromatin and gene silencing.

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Histone methylation, depending on the site of modification, might either activate gene transcription (H3K4, H3K36, and H3K79) or silence gene expression (H3K9 and H3K27) (Stillman, 2018). Histone acetylation reduces the positive charge and concurrently decreases histones affinity to DNA, causing increased accessibility of various transcription factors and gene expression induction. On the other hand, histone deacetylation is considered with chromatin condensation and depression of transcription factor assembly to DNA, leading to repression of transcription (Stillman, 2018).

Numerous studies reported dysregulation in histone marks enrichment of insulin-related genes in insulin resistance (Castellano-Castillo et al., 2019; Emamgholipour et al., 2020; Wang et al., 2017). Moreover, the inhibitors of histone deacetylases are promising agents for the treatment of insulin resistance (Sun and Zhou, 2008). However, most of the available data are based on cell culture or animal or cell models. There is a limited number of studies that analyze the epigenetic changes in adipose tissue, the primary tissue responsible for glucose utilization.

In the present study, we compared histone modifications in two fat depots: subcutaneous (SAT) and visceral (VAT) adipose tissue of healthy and insulin-resistant patients. We also wanted to assess the mechanism of epigenetic changes underlying IR, and for this reason, we induced IR in human adipocytes and 3T3-L1 cell lines.

2. Material and methods

The study protocol was approved by the Ethics Committee Board of Wroclaw Medical University, Approval No. KB-124/2017.

2.1. Study cohort and clinical samples collection

The adipose tissue samples (subcutaneous (SAT) and visceral (VAT)) were collected during abdominal surgery from 40 patients between the 2018–2020 years. Patients meeting the following criteria were recruited: age 40–60 years without active cancer disease, chronic inflammation, thyroid dysfunction, and other condition related to IR (eg. PCOS, Cushing Diseases). Alcohol abusing patients were also excluded. The study cohort was divided into two groups based on IR ratios (HOMA-IR and QUICKI (Małodobra-Mazur et al., 2019)): insulin-sensitive (IS), and insulin-resistant (IR). The characterization of the study group is presented in Table 1.

Immediately after biopsy collection, the adipose tissues were placed in cold PBS (ITD) supplemented with protease inhibitor mix (PI, 200x, BioShop) and transported to a laboratory. The adipose tissue intended for DNA/RNA extraction was placed in *RNA*Later (Invitrogen) The *RNA*Later was discarded, and tissues were stored at −80 °C until analysis.

Table 1
Anthropometric and biochemical characterization of studied groups.

Parameter	IS group (mean ± SD)	IR group (mean ± SD)	P-value (TTest)
Sex (F/M)	5/19 (24)	4/12 (16)	
BMI [kg/m^2]	25,2 ± 4,0	28,2 ± 4,8	0.0455
Glucose [mg/dl]	91 ± 9,7	98,8 ± 16,7	n.s.
Insulin [μU/mL]	6,9 ± 2,3	25,5 ± 23,1	0.0003
HOMA-IR	1,5 ± 0,5	6,5 ± 7,33	0.0019
QUICKI	0,363 ± 0,03	0,308 ± 0,03	0.0000
CHOL [mg/dl]	232,1 ± 72,4	210,0 ± 36,5	n.s.
TG [mg/dl]	127,4c± 71,6	173,2 ± 93,3	n.s.
HDL [mg/dl]	57,0 ± 21,4	42,8 ± 5,4	0.0421
LDL [mg/dl]	151,9 ± 47,2	136,8 ± 32,9	n.s.
Age [years]	45,6 ± 7,8	48,3 ± 10,2	n.s.

SD – standard deviation.
n.s. – not significant.
IS – Insulin sensitive group; IR – Insulin resistant group.

The blood was collected before abdominal surgery in a fastened state and transported to the laboratory. The plasma was collected by centrifugation at 4000 rpm for 5 min and kept at −80 °C until analysis.

2.2. Human preadipocyte collection and differentiation

Human preadipocytes in the form of mesenchymal stem cells (MSCs) of the stromal fraction of white adipose tissue were extracted from SAT and VAT collected from three patients undergoing abdominal surgery, however without any signs of metabolic disorders. The additional patients that donated the tissue for MSCs extraction were additional patients not belonging to the *in vivo* study group. The classification criteria were normal BMI, normal insulin and glucose levels, and lipids metabolism, the age between 30–50 years (the mean age of patients was 44 years). Furthermore, patients declared no type 2 diabetes diagnosed in close relatives. All patients that denoted the adipose tissue samples for MSCs collection were men. The reason for the surgery for all three patients was an abdominal hernia.

The collected tissues were placed in PBS supplemented with PI and transported to the laboratory. Next, the tissues were dissected with scissors, and blood vessels were removed, then digested with collagenase (1 mg/mL medium) with the addition of BSA (10 mg/mL medium) until complete digestion (approx. an hour). Next, the cells were centrifuged for 5 min at 2000 rpm; the supernatant was discarded, and the cells were washed twice with ice-cold PBS, followed by washing with DMEM/F12 (50:50, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Corning) supplemented with antibiotics ((50 U/mL of penicillin and 50 μg/mL of streptomycin, Corning). After the final centrifugation, the cells were suspended in DMEM/F12 supplemented with 10 % FCS (Sigma-Aldrich) and antibiotics.

The medium was changed every second day until confluent. The differentiation was induced by a differentiation cocktail (DMEM/F12 (50:50) supplemented with 10 % FCS, penicillin (50 U/mL), streptomycin (50 μg/mL), IBMX (115 μg/mL), dexamethasone (390 ng/mL), insulin (10 μg/mL), pioglitazone (0.1 μg/mL), human transferrin (10 μg/mL), all purchased in Sigma-Aldrich. After three days, the medium was replaced with one that sustains differentiation containing DMEM/F12 (50:50) supplemented with 10 % FCS, penicillin (50 U/mL), streptomycin (50 μg/mL), insulin (10 μg/mL), pioglitazone (0.1 μg/mL), human transferrin (10 μg/mL) for the next three days. At the end of differentiation, the medium contained DMEM/F12 (50:50) and 10 % FCS and antibiotics. After the following three-four days, the cells were fully mature adipocytes. Experiments were run in triplicates.

2.3. 3T3-L1 cell line culture and differentiation

3T3-L1 were differentiated after achieving 100 % confluence. The differentiation medium contained DMEM (Dulbecco's Modified Eagle Medium, Corning), 10 % fetal bovine serum (FBS, Corning), antibiotics (penicillin, 50 U/mL; streptomycin, 50 μg/mL, Corning), 3-isobutyl-1-methylxanthine (115 μg/mL, Sigma-Aldrich), dexamethasone (390 ng/mL, Sigma-Aldrich) and insulin (10 μg/mL, Sigma-Aldrich), After three days, the medium was changed to DMEM with antibiotics, 10 % FBS and insulin (10 μg/mL). After three more days, the medium was changed to DMEM with antibiotics, 10 % FBS, and further cultured for additional two days to achieve a fully mature phenotype. Experiments were run in triplicates.

2.4. Insulin resistance induction

The IR was induced by 0.5 mM palmitic acid (16:0) added to the medium after differentiation. The cells were cultured for 48 h and 72 h. After each time point, the glucose uptake test was performed. Glucose uptake was analyzed using the Glo-Glucose Uptake (Promega) according to protocol. Before testing, the cells were starved in an FCS-free medium. Before the test, the medium containing glucose was removed and cells

were washed with PBS twice. Next, some of the cells were stimulated by 1μM insulin for 15 min; after insulin stimulation, the deoxyglucose (1 mM) was added and incubated for another 15 min, followed by adding the reaction mixtures and luminescence measurements.

2.5. Genetic material extraction

DNA was extracted using phenol: chloroform (BioShip) extraction followed by ethanol precipitation. DNA was suspended in nuclease-free water and stored at −20 °C. The adipose tissues were first homogenized using the MagNa Lyser (Roche) and then treated with proteinase K (10 mg/mL, Qiagen), next subjected to phenol:chloroform extraction.

RNA was extracted using the TriReagent (Sigma-Aldrich) reagent followed by isopropanol (Sigma-Aldrich) precipitation. After drying, the RNA was suspended in nuclease-free water and stored at −80 °C. The adipose tissues were first homogenized using the MagNa Lyser (Roche) directly in Trizol, next subjected to trizol extraction.

2.6. Gene expression

The RNA (200 ng) was transcribed into cDNA using the High Capacity Reverse Transcription Kit (ThermoFisher). Gene expression was analyzed in Real-Time PCR using the Fast SYBR Green Master Mix (ThermoFisher). Primers were designed manually, the efficiency of primers was checked by standard curve. The sequence of primers was published previously (Cierzniak et al., 2021). The normalization was done to the housekeeping gene (β-actin) and calculated according to the ΔΔCt algorithm.

2.7. Global and site-specific histone modifications

Adipose tissues were first dissected with scissors and cross-linked at 37 °C with 1% formaldehyde (FA, Sigma-Aldrich) supplemented with PI (200x, BioShop) and 1 mM PMSF (Sigma-Aldrich). After 10 min, the reaction was stopped by glycine (final concentration of 125 mM, Diagenode), next centrifuged for 5 min at 2500 rpm at 4 °C. The supernatant was discarded, and tissue was further processed by homogenization (MagNa Lyser, Roche) in adipocyte lysis buffer (500 mM PIPES, 50 mM KCl, and 1% Igepal) and incubation in ice for an additional 15 min (vortexed every 5 min). After centrifugation for 5 min at 2500 rpm at 4 °C, the nuclei pellet was suspended in 300 μL of SDS Lysis Buffer (as part of ChIP Assay Kit, Millipore) supplemented with PI and PMSF, incubated additionally for 15 min and subjected to sonication ((30 s ON, 45 s OFF) x 15) using the Bioruptor®Plus sonicator (Diagenode). The purpose of the sonication was the fragmentation of chromatin in the range of 200bp – 1000bp and this step requires prior optimization. Next, the procedure was performed according to the Chromatin Immunoprecipitation protocol (ChIP) Assay Kit (Millipore).

The preparation of cultured cells started with treatment with 1% FA with PI and 1 mM PMSF for 10 min at 37 °C. Next, the cells were washed three times with PBS with PI and 1 mM PMSF. Later, the cells were scraped and suspended in 300 μL of SDS Lysis Buffer supplemented with PI and PMSF and sonicated according to the above parameters to the final chromatin fragmentation between 200bp – 1000bp. Next, the procedure was carried out according to the protocol instruction of Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore).

Before the precipitation stage, 1% of the final volume dedicated to precipitation with specific antibodies was collected as the input and stored frozen until the stage of DNA extraction. Next, to the rest sonicated lysate H3K4me3, H3K9/14ac, and IgG rabbit antibodies (all purchased from Diagenode) were added. The chromatin immunoprecipitation was carried at 4 °C overnight, followed by several washing steps. After the decross-link by adding 20 μL of 5 M NaCl, DNA was extracted as described in section 2.5.

The enrichment of specific histone modification was analyzed by the ChIP-Real-Time PCR according to the protocol described in section 2.6. Primers for Real-Time PCR were designed manually (Table 2), to amplify the region related to the transcription start site (TSS). Results were calculated as the percentage of input (% of input) that is the whole lysate, not precipitated with an appropriate antibody in relation to the precipitated part of lysate, according to the following formula

$$\%\ \text{of input} = 100{*}2^{CtAI-CtIP}$$

CtAI – Ct value of adjusted input (calculated as Ct value – 6.644), CtIP – Ct value of IP samples.

2.8. Statistical analysis

Statistical analysis was done using Statistica13.1 (StatSoft). For analysis of differences between groups, one-way ANOVA was used. To assess the correlation between numerical characteristics, the correlation of coefficient was used. The gene expression was calculated using the ΔΔCt algorithm. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Global epigenetic changes in insulin-resistant adipocytes

The histone modifications at the global level showed no statistically significant divergences between IS and IR patients in SAT. Both analyzed markers (H3K4me3 and H3K9/14ac) were lower in IR patients compared to IS subjects but without statistical significance (Fig. 1A). However, both markers showed a negative correlation between the enrichment and IR (HOMA-IR) in SAT (H3K4me3, R=-0.41, $p = 0.0115$; H3K9/14ac, R=-0.57, $p = 0.023$, Fig. 1B). In VAT, the methylation of H3K4me3 residue was lower in IR patients than in IS healthy subjects ($p = 0.0101$; Fig. 1A). The H3K9/14ac residue was lower in IR patients, but without being statistically significant. Similarly, a negative correlation between histone markers (H3K4me3 and H3K9/14ac) in VAT and IR (assessed based on HOMA-IR and QUICKI) was noticed, however without being significant.

We also checked the expression of epigenetic modifying genes. We found downregulation of *SIRT7* in both fat depots (SAT, $p = 0.0346$; VAT, $p = 0.0984$) and *SIRT1* in SAT ($p = 0.0457$). Furthermore, a strong positive correlation between the expression of *SIRT1* and *SIRT7* and the expression of numerous genes regulating insulin sensitivity, adipogenesis, lipids metabolism as well as between expression of other epigenetic regulatory genes, including *PPARG*, *INSR*, *SLC2A4, HDAC1, HDAC2, DNMT1* was found in both analyzed fat depots (Fig. 1C, Table 3). A mutual positive correlation was also observed for both examined *SIRT* genes (*SIRT1* and *SIRT7*) in both adipose tissues (SAT: $R = 0.43$, $p = 0.012$; VAT: $R = 0.65$, $p < 0.000$; Table 3). Particular attention should also be paid to the correlations between the *SIRT* genes

Table 2
Primers sequences used for epigenetic modification analysis.

Gene	Species	Sequence [3′ → 5′]		Region* [bp]	PCR size [bp]
PPARG	Human	F	CTGTTATGGGTGAAACTCTGG	−6 ÷ 53	58
		R	GTGAAGGAATCGCTTTCTGG		
SLC2A4	Human	F	TTGTGGCTGTGGGTCCCAT	−154 ÷ -1	153
		R	CTCGTCTTAGAAGAGCTGGA		
ADIPOQ	Human	F	GCTGTTCTACTGCTATTAGC	20 ÷ 216	196
		R	GATCTCCTTTCTCACCCTTC		
Pparg	Mouse	F	ACACCAGTGTGAATTACAGC	−41 ÷ 38	79
		R	TCTGGGTCAACAGGAGAAATC		
Slc2a4	Mouse	F	CAAGCGGGTCTCACTAGATC	−195 ÷ -19	176
		R	AGACTCAGGCGCTGCAATAA		
Adipoq	Mouse	F	CCTGTTCCTCTTAATCCTGC	20 ÷ 116	96
		R	CAAGTTCCCTTGGGTGGAG		

* Location of the primers binding sites concerning TSS (transcription start site).

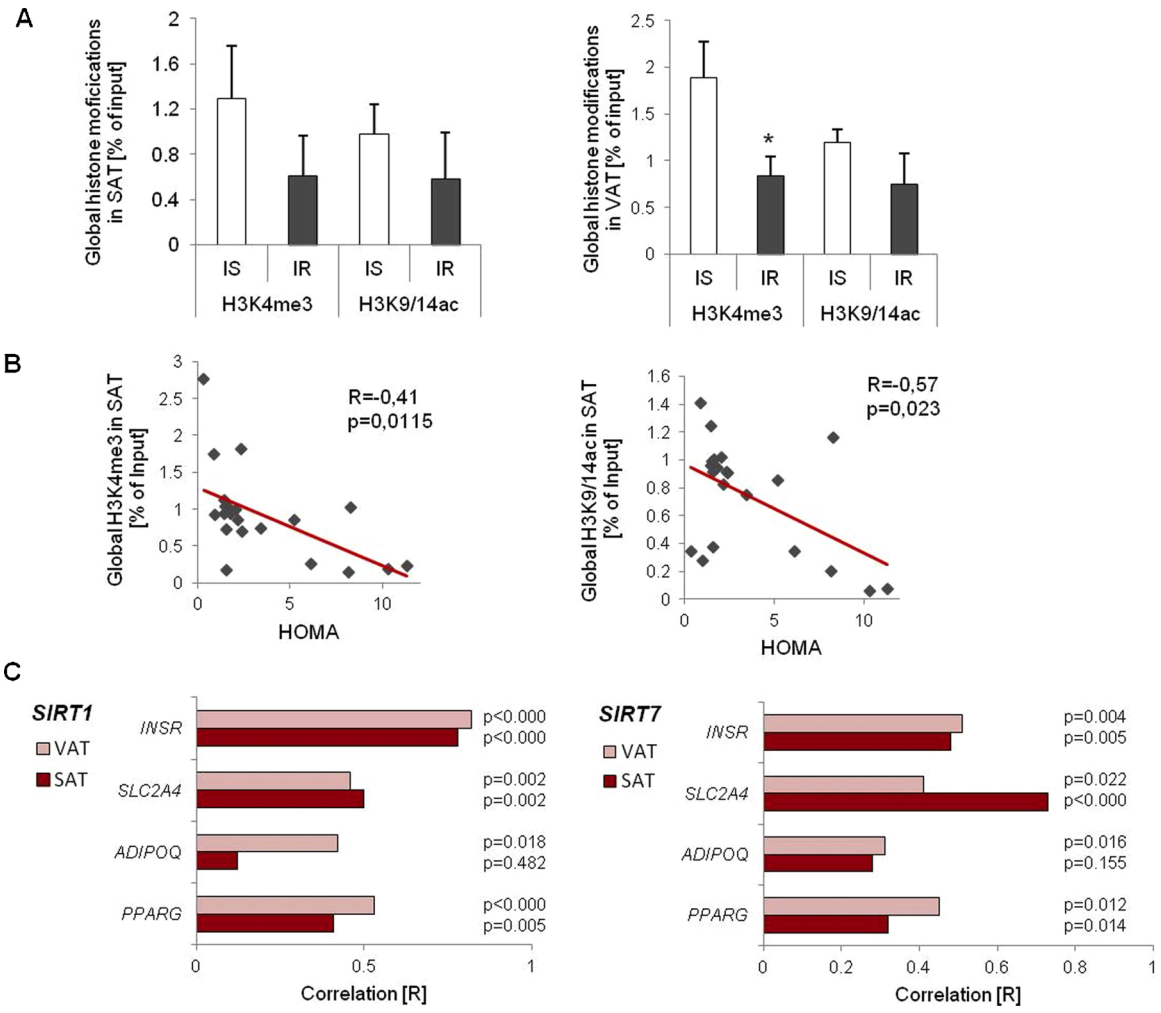

Fig. 1. Global epigenetic changes in insulin-sensitive (IS) and insulin-resistant (IR) patients. **A** –global histone modifications (H3K4me3, H3K9/14ac) in SAT and VAT, **B -** the correlation between H3K4me3 and H3K9/14ac and insulin resistance (HOMA) in SAT, **C** – correlation between *SIRT1* and *SIRT7* expression and the expression of insulin signaling genes in SAT and VAT.

and other epigenome-regulating genes. The expression of *SIRT1* positively correlated with the expression of genes belonging to the histone deacetylase family (*HDAC1* and *HDAC2*), while the expression of *SIRT7* with the expression of DNA methyltransferases, however only in the case of *DNMT1* (Table 3). The above dependence concerned both adipose tissues. What is more, the expression of both *SIRT1* and *SIRT7* positively correlated with the QUICKI ratio (R = 0,38, p = 0.031; R = 0,40, p = 0.028, respectively) in SAT samples. In VAT samples the correlation with IR (QUICKI) was noticed only for *SIRT1* (R = 0.45, p = 0.011).

To confirm the dependency between IR and histone modifications, we induced IR in the 3T3-L1 cell line and human adipocytes collected from VAT and SAT. The IR was induced by 0.5 mM of palmitic acid (16:0) for 48 h and 72 h. The glucose uptake test confirmed that the cells treated with palmitic acid were resistant to insulin. In cells treated with palmitic acid (16:0), the glucose uptake rate after insulin stimulation was at the same level compared to basal glucose uptake in treated cells, unlike in cells with proper insulin sensitivity (data not shown).

The histone modifications observed in insulin-resistant cells were similar to those seen in adipose tissue of IR patients, however only after 72 h of IR induction. In 3T3-L1 adipocytes, both analyzed histone modifications (H3K4me3 and H3K9/14ac) decreased after 72 h of IR induction, but only for H3K9/14ac, the decrease was statistically significant (p = 0.0480, Fig. 2A).

In SAT-derived human adipocytes, the global histone modifications (H3K4me3 and H3K9/14ac) did not differ after 48 h and 72 h in IR cells compared to controls. In VAT-derived adipocytes after 72 h of IR induction, the global changes of both markers were shown to be decreased compared to control cells. However, none of the registered changes were statistically significant (Fig. 2B and C).

Regarding the expression of epigenetic regulatory genes, we found a *SIRT7* downregulation in IR adipocytes in all experimental cells (Fig. 2A, B, and C). We detected downregulation of *Sirt7* in 3T3-L1 adipocytes with IR compared to controls (48 h: p = 0.0181; 72 h: p = 0.0161). Similarly, in insulin-resistant human adipocytes, we displayed decreased expression of *SIRT7* compared to controls (48 h: SAT p = 0.0096; VAT p = 0.0018; 72 h: SAT p = 0.0002; VAT p = 0.0651; Fig. 3). No other histone-modified genes were shown to be dysregulated.

3.2. Site-specific epigenetic changes in adipose tissue of IR and IS patients

To assess the site-specific epigenetic changes, first, we analyzed the expression level of the insulin pathway genes or genes encoding proteins relevant to insulin sensitivity. We found aberrant expression of numerous genes in adipose tissue of IR patients compared to the healthy subject, that is, *PPARG* (SAT, p = 0.016; VAT, p = 0.0060), *ADIPOQ* (SAT, p = 0.0481; VAT, p = 0.0354), and *SCL2A4* (SAT, p = 0.0454; VAT, p = 0.1361; Fig. 3A).

The H3K4me3 enrichment at the promoter of *PPARG* was statistically reduced in SAT depot of IR patients (p = 0.0440), the H3K9/14ac was decreased, however close to being significant. In VAT samples of IR

Table 3
The correlation between expression of various genes with the expression of *SIRT1* and *SIRT7* measured in SAT and VAT of enrolled patients.

Pathway	Gene	SAT				VAT			
		SIRT1		*SIRT7*		*SIRT1*		*SIRT7*	
		R	*p*	R	*p*	R	*p*	R	*p*
Epigenetic modifying enzymes	*HDAC1*	**0.58**	**0.000**	−0.04	n.s.	**0.37**	**0.020**	0.24	n.s.
	HDAC2	**0.55**	**0.000**	0.13	n.s.	**0.55**	**0.000**	0.56	0.001
	SIRT1	———	———	**0.43**	**0.012**	———	———	**0.64**	**0.000**
	SIRT7	**0.43**	**0.012**	———	———	**0.65**	**0.000**	———	———
	DNMT1	−0.01	n.s.	**0.41**	**0.018**	−0.05	n.s.	**0.55**	**0.001**
	DNMT3a	0.03	n.s.	0.05	n.s.	−0.02	n.s.	0.04	n.s.
	DNMT3b	0.41	n.s.	0.18	n.s.	0.42	n.s.	0.41	n.s.
Insulin signaling pathway	*INSR*	**0.68**	**0.000**	**0.48**	**0.005**	**0.78**	**0.000**	**0.51**	**0.004**
	PIK3R1	**0.64**	**0.000**	0.20	n.s.	**0.60**	**0.000**	**0.36**	**0.049**
	PTPN1	**0.80**	**0.000**	0.49	n.s.	**0.84**	**0.000**	0.53	n.s.
	AKT	**0.83**	**0.000**	0.31	n.s.	**0.79**	**0.000**	**0.57**	**0.001**
	SLC2A4	**0.49**	**0.002**	**0.72**	**0.000**	**0.49**	**0.002**	**0.41**	**0.022**
MAPK pathway	*ERK1*	**0.53**	**0.014**	**0.55**	**0.038**	**0.61**	**0.002**	**0.66**	**0.014**
	ERK2	0.12	n.s.	0.26	n.s.	**0.48**	**0.019**	0.45	n.s.
	GRB2	0.28	n.s.	0.41	n.s.	**0.70**	**0.000**	0.49	n.s.
Cytokines and adipokines	*IL6*	−0.17	n.s.	−0.08	n.s.	−0.18	n.s.	−0.19	n.s.
	IL10	−0.11	n.s.	−0.11	n.s.	−0.07	n.s.	0.04	n.s.
	ADIPOQ	015	n.s.	0.32	n.s.	**0.32**	**0.018**	**0.38**	**0.016**
	LEP	0.13	n.s.	0.13	n.s.	0.18	n.s.	0.31	n.s.
	TNFα	−0.09	n.s.	−0.17	n.s.	0.20	n.s.	−0.16	n.s.
Lipids metabolism	*ACC*	**0.40**	**0.012**	**0.35**	**0.050**	**0.41**	**0.008**	**0.52**	**0.002**
	SCD1	**0.41**	**0.015**	0.29	n.s.	0.31	n.s.	**0.47**	**0.008**
	FASN	**0.44**	**0.007**	**0.38**	**0.031**	**0.54**	**0.001**	**0.55**	**0.002**
	ACSS2	**0.54**	**0.001**	**0.37**	**0.046**	**0.69**	**0.000**	**0.51**	**0.004**
	LPL	**0.32**	**0.050**	0.27	n.s.	0.29	n.s.	**0.55**	**0.001**
Adipogenic transcription factors	*PPARG*	**0.44**	**0.019**	**0.45**	**0.014**	**0.56**	**0.002**	**0.38**	**0.012**
	CEBPA	−0.08	n.s.	0.15	n.s.	0.19	n.s.	0.31	n.s.
	CEBPB	0.06	n.s.	0.18	n.s.	0.15	n.s.	−0.05	n.s.
	CEBPD	−0,19	n.s.	0,13	n.s.	0,16	n.s.	0,01	n.s.
Others	*HIF3a*	0.26	n.s.	0.28	n.s.	**0.59**	**0.000**	**0.42**	**0.020**
	PGC1a	−0.08	n.s.	007	n.s.	−0.08	n.s.	−0,06	n.s.

R – the correlation of coefficient.
p - significance level.

subjects, enrichment of both histone modifications at the *PPARG* promoter site were significantly reduced in IR patients compared to controls (H3K4me3, p = 0.0334; and H3K9/14ac, p = 0.0134; Fig. 3B). The enrichment of histone modifications at the promoter sites of *ADIPOQ* were reduced in IR patients, however, none of the changes were statistically significant in either adipose tissue depots in IR patients. The enrichments measured in VAT at the promoter site of *SLC2A4* were significantly reduced in IR patients compared to IS subjects (H3K4me3, p = 0.0477; and H3K9/14ac, p = 0.0010; Fig. 3B). Similarly, in SAT samples of IR patients, the analyzed histone modifications of *SLC2A4* were reduced, but only the H3K9/14ac was found to be statistically significant (p = 0.0469). Additionally, the H3K9/14ac of *SLC2A4* gene promoter in VAT negatively correlated with BMI (R= -0,59, p = 0015), HOMA-IR (R= -0,53, p = 0.013), and positively with QUICKI (R = 0,63, p = 0.046, Fig. 3C).

3.3. Site-specific epigenetic changes in insulin-resistant 3T3-L1 and human adipocytes

In 3T3-L1 cells after 48 h of IR induction, we did not find any significant changes in the expression rate of insulin pathway genes or lipid metabolism genes, or genes regulating insulin sensitivity. However, after 72 h of IR induction, we observed downregulation of the following genes in IR cells compared to controls: *Slc2a4* (p = 0.0022), *Adipoq* (p = 0.0000), *Pparg* (p = 0.0002; Fig.4A).

When analyzing the enrichment of histone modifications (H3K4me3, H3K9/14ac) in IR 3T3-L1 cells, we saw lower methylation and acetylation of H3 at the *Adipoq* promoters' sites after 72 h of IR induction compared to controls (p = 0.0136 and p = 0.0351, respectively; Fig. 4B). Similarly, the *Pparg* promoter was also characterized by a lower enrichment of histone modifications in IR cells after 72 h of IR induction (H3K4me3 p = 0.0137 and H3K9/14ac p = 0.0190, Fig. 4B). The enrichment of the *Slc2a4* promoter's histone modifications was statistically reduced after 72 h of IR induction (H3K4me3, p = 0.0100; H3K9/14ac, p = 0.0155). Additionally, the reduced H3K9/14ac was detected after 48 h of IR induction (p = 0.0125; Fig. 4B).

In human IR adipocytes collected from SAT, the *ADIPOQ* and *PPARG* expressions were shown to be decreased after 72 h of IR induction (p = 0.0010 and p = 0.0020, respectively; Fig. 5A). Additionally, the *SLC2A4* gene was downregulated after 48 h and 72 h of IR induction (p = 0.0440 and p = 0.0068, respectively).

In VAT-derived adipocytes changes in expression of analyzed genes were detected at both time points: after 48 h and 72 h. The expression of *SLC2A4* gene (48 h: p = 0.0154, 72 h: p = 0.0015), *ADIPOQ* gene (48 h: p = 0.0130, 72 h: p = 0.0120) and *PPARG* gene (48 h: p = 0.0020, 72 h: p = 0.0041; Fig. 6A) was decreased in IR adipocytes compared to controls. Additionally, the *INSR* gene was also downregulated after 48 h (p = 0.0465) and 72 h (p = 0.0022) of IR induction (data not shown).

The enrichment of histone modifications at the *PPARG* promoter were shown to be reduced in SAT-derived insulin resistant adipocytes (48 h: H3K4me3, p = 0.0062; H3K9/14ac, p = 0.0452; 72 h: H3K4me3, p = 0.0004; H3K9/14ac, p = 0.0341; Fig. 6C) and in VAT-derived insulin resistant adipocytes (48 h: H3K4me3, p = 0.0223; H3K9/14ac, p = 0.0224; 72 h: H3K4me3, p = 0.0054; H3K9/14ac, p = 0.0443; Fig. 5B). In the expression regulation of *ADIPOQ*, only the H3K4me3 seemed to be implemented. At the promoter of *ADIPOQ*, the enrichment of H3K4me3 was considerably lower in IR cells both collected from SAT (48 h: p = 0.0009, 72 h: p = 0.0011) and VAT (48 h: p = 0.0373, 72 h: p = 0.0007).

The epigenetic modifications related to *SLC2A4* promoter regulation were shown to be similar to *ADIPOQ*, only the enrichment of H3K4me3 was differently marked in IR adipocytes. Considerably lower % of input of H3K4me3 was detected in SAT adipocytes at both time points of IR

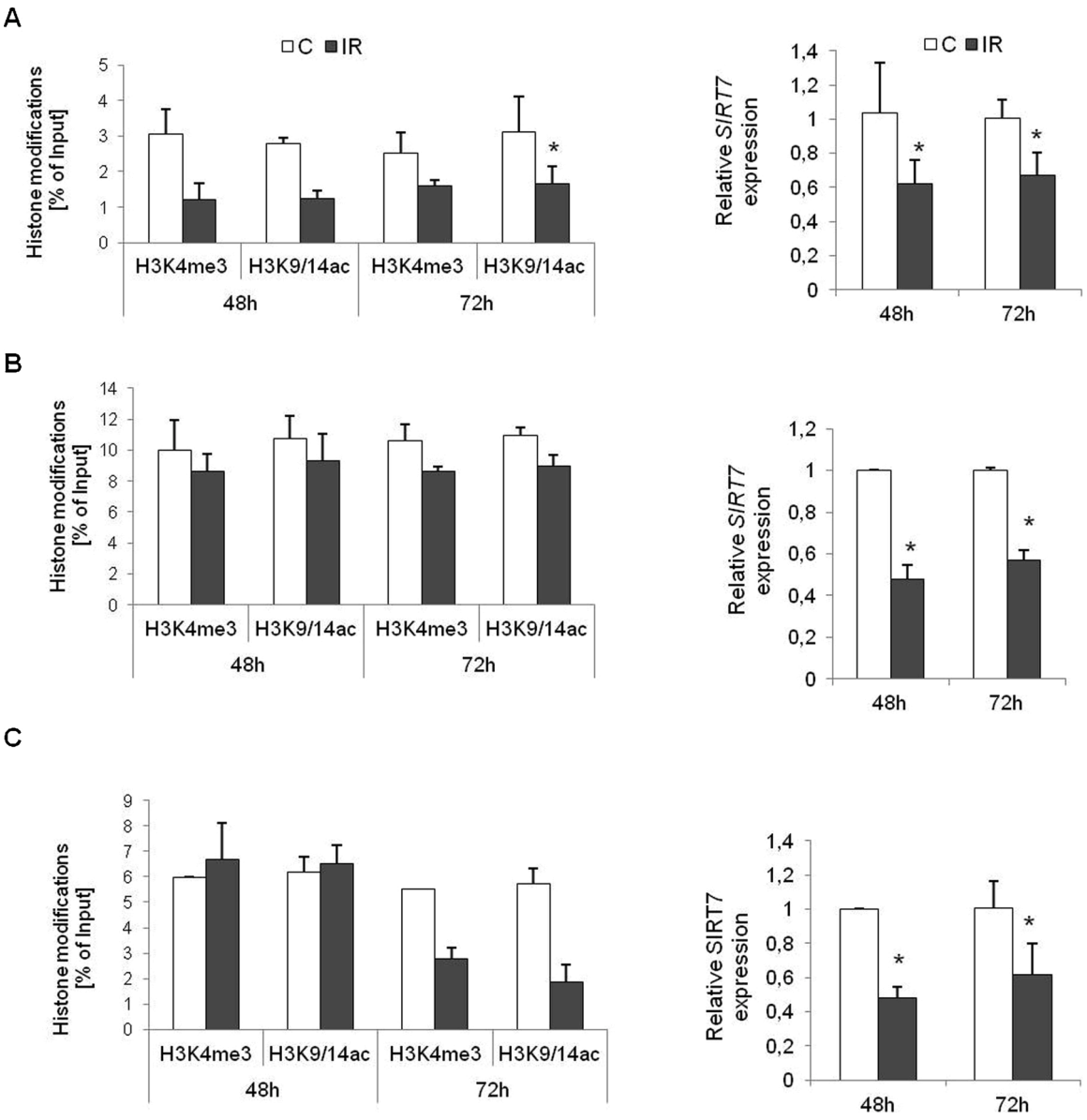

Fig. 2. Global epigenetic changes in cultured cells. Controls (C) and insulin-resistant cells (IR). **A** – 3T3-L1 adipocytes, **B** – Human SAT-derived adipocytes, **C** – Human VAT-derived adipocytes.

induction (48 h: p = 0.0262, 72 h: p = 0.0201) as well as in VAT adipocytes (48 h: p = 0.0137, 72 h: p = 0.0118).

4. Discussion

Epigenetic modifications are well-known factors influencing metabolic disorders like IR or type 2 diabetes. Epigenetic changes represent mechanisms that regulate cell response to various environmental and cellular factors. Insulin sensitivity is regulated by various transcription factors inducing or repressing the expression of numerous insulin-sensitizing genes [1819].

In this paper, we demonstrated that changes in histone modifications are an essential component of IR in adipose tissue, both subcutaneous and visceral. Furthermore, by inducing IR in experimental cells, we presented the timeline of epigenome changes during IR induction. The IR was induced in experimental cells: 3T3-L1 adipocytes and human adipocytes collected from SAT and VAT by palmitic acid, which is one of the most common methods used by numerous researchers (Pinel et al., 2018; Shinjo et al., 2017). We successfully induced IR both in 3T3-L1 and human adipocytes, which was confirmed by glucose uptake assay. At both time points (48 h and 72 h), the cells were IR. We demonstrated that most of the epigenetic changes began to differ as soon as 72 h following IR induction at the global and site-specific level.

We found a decrease in global histone methylation (H3K4me3) and acetylation (H3K9/14ac) in IR patients. What is more, both analyzed markers negatively correlated with HOMA; however, only the SAT correlation was statistically significant. Analyzing the cell model, we found the same changes in histone methylation and acetylation; however, similar to adipose tissue, the reduction was not statistically significant. Other reports indicate dysregulation in histone modifications in IR and type 2 diabetes, mainly related to dysregulation of histone-modified enzymes like HDAC (histone deacetylases) (Kaiser and James, 2004; Noh et al., 2009; Sathishkumar et al., 2016). Moreover, the HDAC family inhibitors stand as promising treatment agents against IR and type 2 diabetes (Sun and Zhou, 2008). Our study, both *in vivo* using adipose tissue biopsies and *in vitro* (experimental cells), did not reveal any changes in histone deacetylase gene expression (*HDAC1* and *HDAC3*). However, we found lower expression of *SIRT 7* in both fat

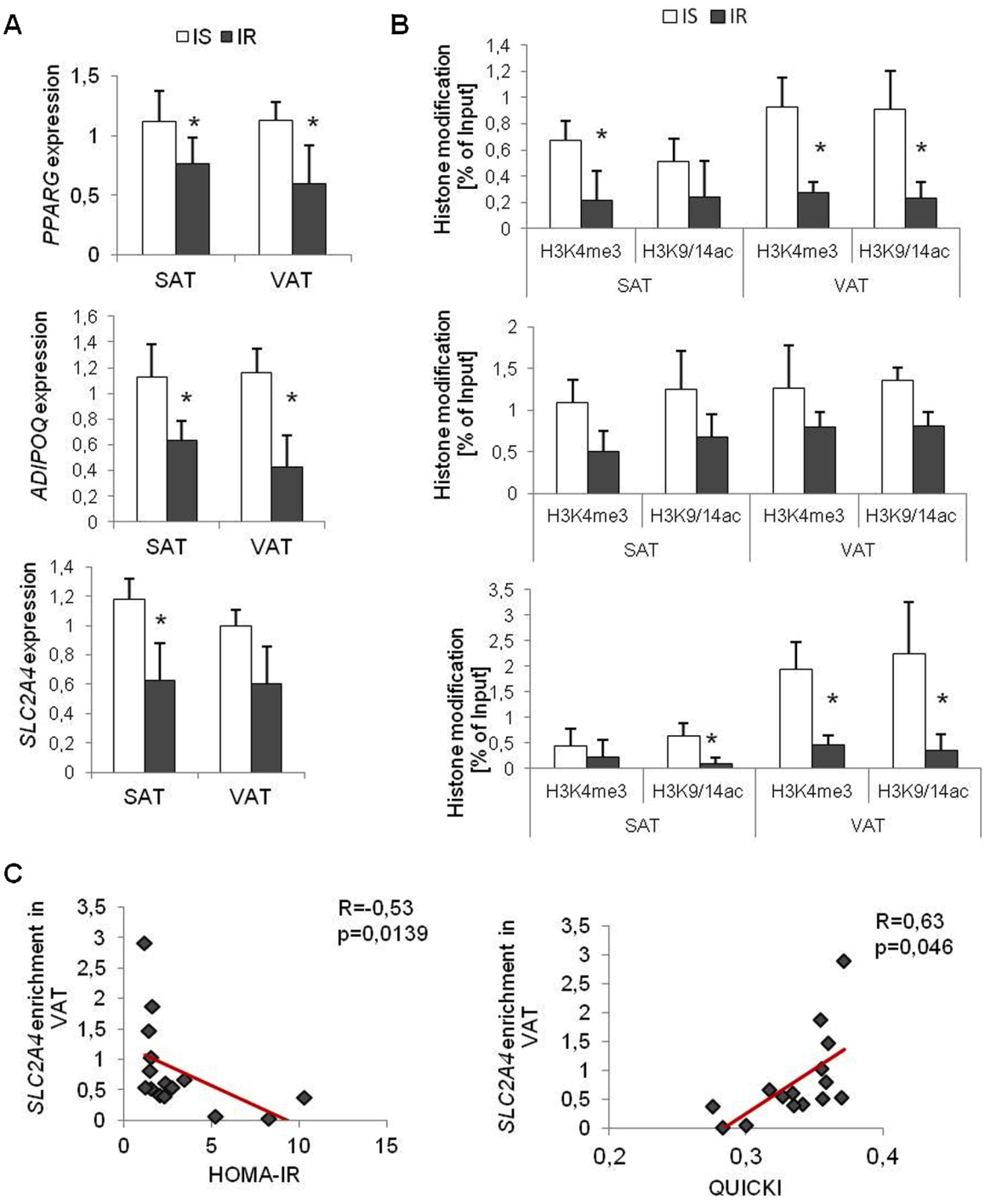

Fig. 3. Site-specific epigenetic changes in VAT and SAT fat depots of IS and IR patients. A – gene expression, B – site-specific H3K4me3 and H3K9/14ac enrichment (% of input), **C** – correlation between H3K9/14ac enrichment of *SLC2A4* promoter in VAT and IR (HOMA-IR, QUICKI).

depots of IR patients and lower expression of *SIRT1* in SAT of IR patients. Similarly, in artificially induced IR in the cell model, the *SIRT7* expression was decreased. Sirtuins belong to the deacetylase family that is involved in histone and nonhistone protein deacetylation (Strycharz et al., 2018, p. 1). Furthermore, this group of enzymes is considered a biosensor of metabolic status regulating mitochondria biogenesis, inflammation, and many other cell homeostasis components, mainly by PCD1/PPARG complex (Gerhart-Hines et al., 2007). What is more, it has been shown that reduction in the SIRT family is related to the pathogenesis of type 2 diabetic complications (Strycharz et al., 2018, p. 1). In the present study, we showed a strong positive correlation between the expression of *SIRT1* and *SIRT7* and the expression of the main insulin-related genes, including *INSR, PPARG,* or *SLC2A4*, that supports the critical role of the SIRT family in energy metabolism regulation in adipocytes. The other strong prove might be a positive correlation between the expression of *SIRT1* and *SIRT7* with the expression of lipids metabolism genes. Dysregulation of the lipids metabolism is also a critical part of insulin resistance. What is more, the expression of *SIRT1* positively correlated with the expression of *HDAC1* and *HDAC2*, on the other hand, *SIRT7* positively correlated with the expression of *DNMT1,* which might suggest complex cooperation of numerous epigenetically regulated genes in energy metabolism regulation and metabolic disorders development.

The next critical point that might indicate the role of the SIRT genes

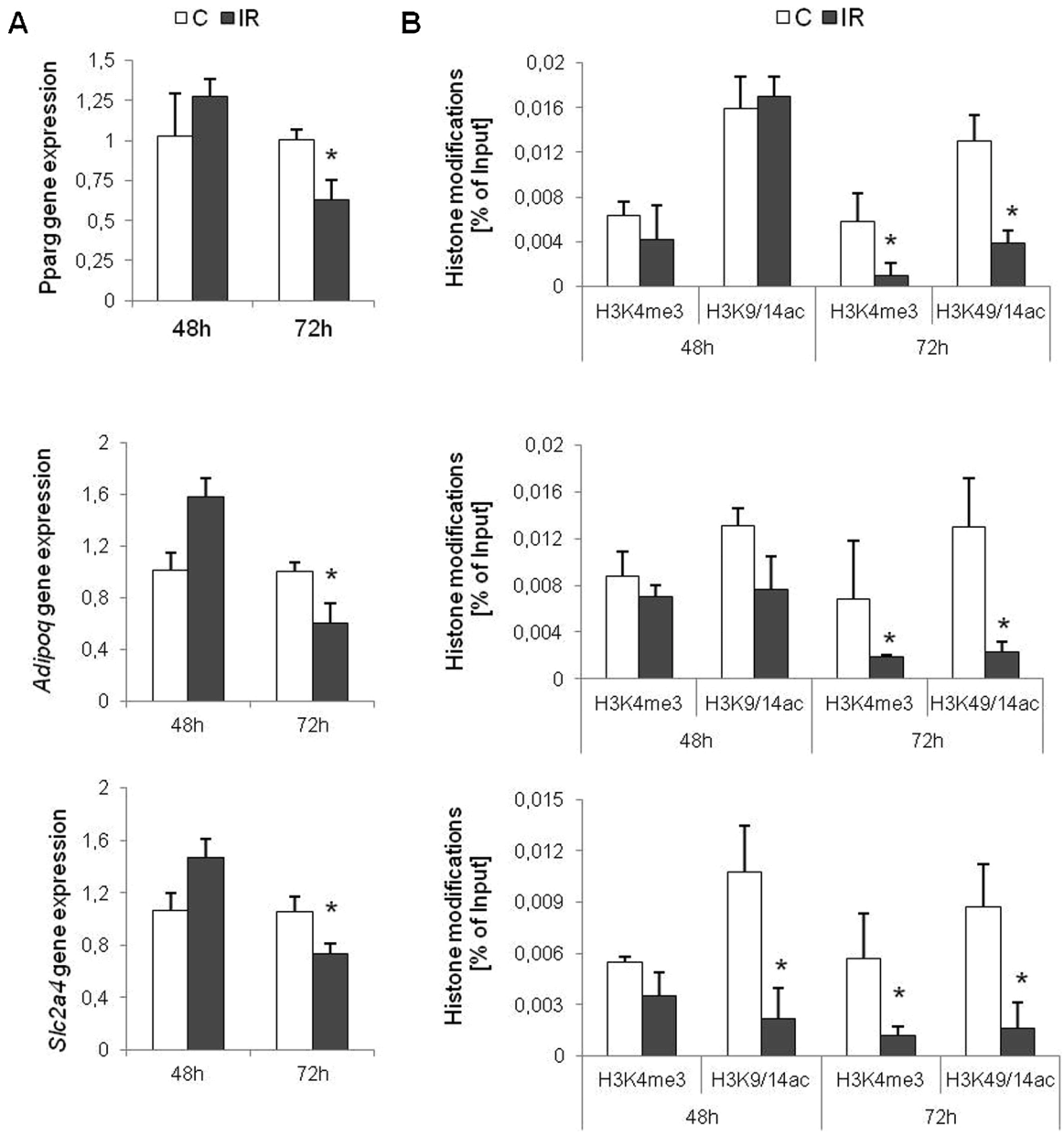

Fig. 4. Site-specific epigenetic changes of insulin-sensitizing genes in 3T3-L1 adipocytes. Controls (C) and experimentally-induced insulin resistance (IR). A – gene expression, B – site-specific H3K4me3 and H3K9/14ac enrichment (% of input).

family in IR pathogenesis is the correlation between the expression of *SIRT1* and *SIRT7* and IR (assessed based on HOMA-IR and QUICKI) in both fat depots. The above-presented results prove the important role of the SIRT family in glucose metabolism. Indeed, it has been previously shown that at the hyperglycemic condition, the expression of *SIRT1* drops. On the other hand, treatment by metformin attenuated the reduction of *SIRT1* (Arunachalam et al., 2014).

Analyzing the epigenetic modification timeline in IR, we observed no significant changes at the global level of histone modifications in experimental cells after IR induction. This suggests that globally, the epigenome changes are relatively minor.

We also considered the site-specific epigenetic modification at the promoter and TSS site of primary insulin-related genes. First, we analyzed the expression rate of numerous genes in adipose tissue biopsies and experimental cells. Several genes were differently expressed in IR adipocytes, including *PPARG, SLC2A4, ADIPOQ,* and *INSR*. However, the last one only in adipose tissue biopsies, not in experimental cells, which means that depletion in *INSR* expression might be developed later during IR progression. The number of genes downregulated in our study is consistent with other studies (Kim et al., 2015; Kubota et al., 1999; Małodobra-Mazur et al., 2019). It is worth mentioning that in experimental cells obtained from SAT, only *SLC2A4* was expressed at a lower rate in IR cells after 48 h of IR induction. The expression of other analyzed genes did not differ compared to control cells at this time point of analysis. This might suggest that the first stage of IR pathogenesis is related to glucose transport failure. However, in VAT-derived adipocytes, all analyzed genes were downregulated in IR cells at both time points after 48 h and 72 h of IR induction. We have shown recently that visceral fat is more metabolically active and more prone to develop metabolic disorders, including IR, probably due to higher inflammatory markers (Małodobra-Mazur et al., 2020). In the 3T3-L1 cell, the reduced expression of mentioned genes was similar to SAT adipocytes; the reduction was seen only at the second time point. For that reason, it needs to be taken into account that using 3T3-L1 as an adipocytes model does not provide an accurate representation of adipocytes metabolism or is more similar to SAT rather than VAT.

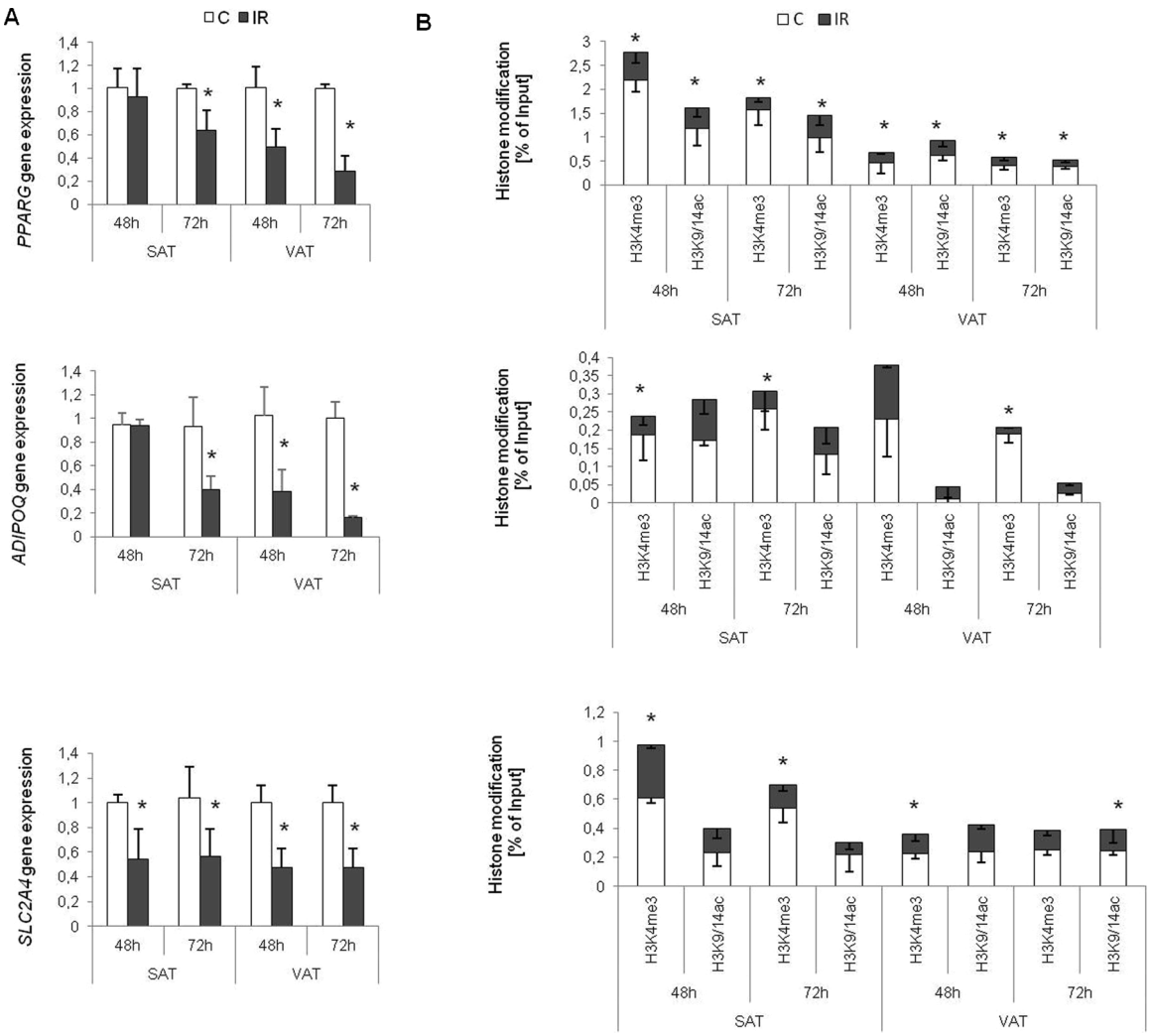

Fig. 5. Site-specific epigenetic changes of insulin-sensitizing genes in human adipocytes (SAT and VAT). Controls (C) and experimentally-induced insulin resistance (IR). A – gene expression, B – site-specific H3K4me3 and H3K9/14ac enrichment (% of input).

We also looked at the site-specific changes of insulin-related genes, especially those that show aberrant expression rates. We have displayed that both H3K4me3 and H3K9/14ac histone markers were lower in adipose tissue of IR patients, the enrichment of both markers in VAT was lower for *SLC2A4* and *PPARG* genes. What is more, the H3K9/14ac at *SLC2A4* promoter negatively correlated with BMI and IR ratios. Furthermore, the reduction in H3K4me3 enrichment of analyzed genes was detected in adipose tissue samples of IR patients (*in vivo*) and in experimental insulin resistant cells at both times points (*in vitro*). The above results link these histone marks with obesity and obesity-related disorders, including IR, which is consistent with other results (Castellano-Castillo et al., 2019). It has been shown that the decrease in *SLC2A4* expression is mediated by HDAC5, deacetylase belonging to class II (McGee et al., 2008). The *SLC2A4* expression is regulated by MEF2 (myocyte enhancer factor) and other transcription factors. Thus deacetylation of promoter sites might decrease transcription factors availability, which results in downregulation of *SLC2A4* expression (Thai et al., 1998). In our study, we did not find any dysregulation in the expression rate of main HDACn; however, there was a significant depletion in SIRT genes; what is more, *SIRT1* and *SIRT7* positively correlated with *SLC2A4* gene expression in both fat depots.

PPARG is one of the most important transcription factors regulating insulin sensitivity. The reduction rate of *PPARG* expression in IR has been shown by numerous researchers. We found lower histone H3 acetylation and methylation of the *PPARG* gene based on *in vivo* and *in vitro* studies in both fat depots and at both time points. It has been reported that HDAC5 is related to a dysregulation in *PPARG* expression and action with coreceptor PGC1 (Gerhart-Hines et al., 2007). As stated above, there was no correlation between *PPARG* expression or epigenetic modification and *HDAC* expression, but with *SIRT1* and *SIRT7*. What is more, epigenetic changes and their effect on *PPARG* expression were observed in all experimental models, including human adipose tissue and experimental cells, which proves the critical role of *PPARG* in insulin signaling regulation.

Contrary to *SLC2A4* and *PPARG,* there were no statistical differences in the enrichment of analyzed histone marks at the *ADIPOQ* gene in adipose tissue of insulin resistant patients. The observed reduction in enrichment both for H3K4me3 and H3K9/14ac was not statistically significant. On the other hand, in experimentally induced insulin resistant 3T3-L1 adipocytes there was a significant reduction in H3K4me3 enrichment, similar to SAT-derived adipocytes. There are a few available data describing the influence of histone marks on *ADIPOQ* expression and its link with IR (Qiao et al., 2006; Sakurai et al., 2009), however, most of the available data concern 3T3-L1 adipocytes. In the present study, we have shown the reduced enrichment of H3K4me3 at *ADIPOQ* promoter of human SAT-derived adipocytes with induced insulin resistance. Subcutaneous adipose tissue is the major fat depot of adiponectin synthesis and secretion (Małodobra-Mazur et al., 2020), contrary to VAT depot. Due to the overall lower synthesis and secretion of adiponectin by VAT, the enrichment of histone modifications detected

in visceral tissue is less significant.

However, the presented work has many methodological limitations. Due to the crossling in 1% PFA, it was not possible to perform additional analyzes at the protein level (eg. Western Blot) to confirm the results of gene expression. Despite the attempts made, it was not possible to obtain representative results. Secondly, limitations in the amount of available adipose tissue biopsies collected from patients prevented the examination of more than three chromatin immunoprecipitation experiments. For this reason, it has been limited to the most widely studied and best-known modifications of the histones (H3K4me3 and H3K9/14ac, and the rabbit IgG negative control). It would be possible to analyze additional histone marks like H4K36me2 or H3K27me2 using the cell model solely, however, the study would not be comprehensive, and conclusions drawn based on the obtained results not supported by *in vivo* studies.

To summarize, epigenetic factors are important components in IR pathogenesis. Based on obtained results, the PPARG and its epigenetic modification appear to be of the greatest importance. Furthermore, it has been shown that in VAT the insulin resistance and associated molecular aberration develop earlier than in SAT, which explains the fact that VAT is associated with a higher risk of metabolic disorders. Additionally, an extremely important fact is the observed participation of deacetylases from the SIRT family (*SIRT 1* and *SIRT7*) and their role in insulin action and IR pathogenesis.

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Institutional review board statement

The study protocol was approved by the Ethics Committee Board of Wroclaw Medical University. Informed consent was obtained from all individuals involved in the study.

Informed consent statement

Informed consent was obtained from all individuals involved in the study.

Data availability statement

Data are available on request from the corresponding author.

CRediT authorship contribution statement

Małgorzata Małodobra-Mazur: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Aneta Cierzniak:** Validation, Investigation, Data curation. **Aneta Myszczyszyn:** Resources. **Krzysztof Kaliszewski:** Resources. **Tadeusz Dobosz:** Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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The Preliminary Evaluation of Epigenetic Modifications Regulating the Expression of *IL10* in Insulin-Resistant Adipocytes

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Abstract: A higher level of *IL10* expression in obesity and insulin resistance was observed in both human and mouse WAT. In our research, we analyzed the influence of insulin resistance on epigenetic modification within the promoter region *IL10* gene and the potential influence of these modifications on its expression. Studies were performed using two cell models for the analysis: human, preadipocytes derived from adipose (visceral and subcutaneous) tissues and murine 3T3-L1 fibroblasts. We demonstrated a significant increase in the *IL10* expression level, *IL10* promoter region methylation, and histone 3 epigenetic modifications: H3K4me and H3K9/14ac, in insulin resistance cells (IR) from SAT cell culture. In IR cells from VAT cell culture, we observed decreased *IL10* expression with a simultaneous increase of *IL10* promoter region methylation. In IR cells from 3T3L1 cell culture, we observed the increased expression of *IL10* as well as the decreased levels of methylation in the *IL10* promoter region and histone methylation (H3K4me) and acetylation (H3K9/14ac). The presented analyses suggest a potential impact of epigenetic modifications on gene expression and a potential mutual influence of epigenetic modifications on each other or the activation of specific epigenetic regulation at a different stage of the development of insulin resistance in cells.

Keywords: *IL10* expression; insulin resistance; epigenetic modifications

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1. Introduction

The excessive accumulation of adipose tissue is associated with environmental, metabolic, psychological, endocrine and genetic factors. The process of excessive adipogenesis causes changes in metabolism and endocrine function, which can lead to an increased release of hormones, fatty acids and proinflammatory factors that contribute to metabolic disorders. Obesity is considered to be the strongest risk factor predisposing patients to insulin resistance [1,2]. One of the pathomechanisms that could explain the relationship between obesity and insulin resistance is chronic inflammation. Obesity is characterized by altered levels of circulating cytokines, adipose tissue macrophage accumulation, and inflammation state [3,4]. Studies conducted on both murine and human tissues indicate an increased accumulation of proinflammatory factors and cells in white adipose tissue (WAT) [5–7].

Interleukin-10 (IL-10) is considered an anti-inflammatory cytokine that inhibits the production of such proinflammatory factors as TNF-α, IL-2, IL-3, IL-6. IL-10 is produced mainly by macrophages, dendritic cells, B lymphocytes, and T lymphocytes [8,9]. Moreover, it is also secreted by the mature adipocyte fraction of human WAT [10,11].

A higher level of *IL10* expression was observed in obesity and insulin resistance subjects [10–12]. What is more the current scientific reports suggest that IL-10 could have a protective effect in the obesity-related development of insulin resistance in some tissue, including skeletal muscle [13–15]. This protective effect of IL-10 was associated with a reduction in local cytokine expression in skeletal muscle and macrophage levels

in mice [13]. The exact mechanism of the influence of IL-10 on the improvement of cell insulin sensitivity is at the moment unclear, however, it is suggested that it is directly related to IL-10 anti-inflammatory activity manifested by promoting an anti-inflammatory phenotype of macrophage [16]. On the other hand, Lumeng et al. suggested that the therapeutic effect of IL-10 is associated with the protection of adipocytes against the physiological effects of TNF-α causing insulin desensitization observed in obesity [17]. Given the potential therapeutic value of this cytokine, it is important to research the mechanism of the expression of this gene in insulin resistance and obesity.

Obesity and insulin resistance are characterized by severe long-term metabolic changes, based on alteration in gene expression, which occur throughout life due to external factors (high-fat diet, sedentary lifestyle, stress). Therefore, it is highly appropriate to look for the ground for these epigenetic regulation changes, both at the DNA and histone levels [18]. DNA methylation is a significant epigenetic modification; it is mostly associated with transcriptional repression. The histone modification effect on gene expression is directly related to the location of methylation and acetylation within the histone. These epigenetic regulation modifications can be both silencing and transcription stimulating.

In our research, we analyzed the influence of insulin resistance on epigenetic modification (both at the DNA and histone levels) within the promoter region of the gene encoding IL-10 (*IL10*) as well as the potential influence of these modifications on *IL10* expression. Studies were performed using two cell models for the analysis: human preadipocyte, obtained from the previously collected adipose tissues (visceral and subcutaneous), and murine 3T3-L1 cell line. The use of adipocytes in the study is essential in the analysis of potential relationships between obesity and insulin resistance. Adipose cells are the most representative in this type of analysis because WAT has the ability to self-produce IL-10.

2. Materials and Methods

The research protocols and all procedures were approved by the Ethical Review Board of Wroclaw Medical University (approval No. KB-124/2017).

2.1. Human and Mouse Cell Culture

Human preadipocytes were extracted from both subcutaneous and visceral adipose tissues obtained from three healthy subjects with BMI 20–25 kg/m^2 and proper insulin sensitivity, aged between 40–60 years old. All enrolled patients were men. Adipose tissues were collected during abdominal surgery in the Department of General, Minimally Invasive and Endocrine Surgery of Wroclaw Medical University, following a written agreement. Informed consent was obtained from all subjects involved in the study. Each patient completed a questionnaire regarding metabolic diseases (type 2 diabetes, hypertension, sclerosis) and medications. The mean age of patients was 45.

Adipose tissue biopsies were transported to the Molecular Technique Unit in PBS enriched with PI (protein inhibitors, BioShop, Burlington, ON, Canada). Each tissue (SAT and VAT) was treated according to the following procedure. In the laboratory, tissue was cleaned of blood vessels and cut with scissors. The fragmented tissue was completely digested with collagenase (Sigma-Aldrich, St. Louis, MO, USA) (1 mg/mL) with the addition of BSA (Bovine Serum Albumin, Sigma-Aldrich, St. Louis, MO, USA) (10 mg/mL). After centrifugation (2000 rpm, 5 min), the supernatant was discarded and the cell pellet was washed twice with ice-cold PBS and then once with DMEM/F12 (50:50, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Corning, New York, NY, USA) supplemented with antibiotics (50 µg/mL of streptomycin and 50 U/mL of penicillin, Corning, New York, NY, USA). The cells were suspended in DMEM/F12 enriched with 10% FCS (Fetal Calf Serum, Sigma-Aldrich, St. Louis, MO, USA) and antibiotics after the final centrifugation,

Commercial mouse fibroblasts of the 3T3-L1 (ATCC, CRL-3242™, University Blvd. Manassas, VA, USA) cell line were used for mouse cell culture.

Mouse cell line and VAT/SAT primary cultures have been tested and found free of Mycoplasma.

2.2. Human and Mouse Cell Line Culture, Differentiation and Insulin Resistance Induction

Both types of cells were cultured in a humidified incubator at 37 °C and 5% CO_2 (Heracell 240i incubator, Thermo Scientific, Waltham, MA, USA).

The human cell line was cultured with medium contained DMEM/F12 (Corning, New York, NY, USA), 10% FCS (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics (penicillin, 50 U/mL; streptomycin, 50 µg/mL, Corning, New York, NY, USA) until it became 100% confluent.

The 3T3-L1cell line was cultured with medium contained DMEM (Dulbecco's Modified Eagle Medium, Corning, New York, NY, USA), 10% FCS (Sigma-Aldrich, St. Louis, MO, USA), and antibiotics (penicillin, 50 U/mL; streptomycin, 50 µg/mL, Corning, New York, NY, USA) until it became 100% confluent.

The human and 3T3-L1 cell cultures were differentiated after the achievement of 100% confluence. The human cells were cultured in the differentiation medium containing 10% FCS (Sigma-Aldrich, St. Louis, MO, USA), DMEM/F12, antibiotics (penicillin, 50 U/mL; streptomycin, 50 µg/mL, Corning, New York, NY, USA), dexamethasone (390 ng/mL, Sigma-Aldrich, St. Louis, MO, USA), 3-isobutyl-1-methylxanthine (115 µg/mL, Sigma-Aldrich, St. Louis, MO, USA), human transferrin (10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA), insulin (10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) and pioglitazone hydrochloride (0.1 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) for three days. Next, the medium was changed to DMEM/F12 with antibiotics, 10% FCS, human transferrin (10 µg/mL), and insulin (10 µg/mL). After three more days, the medium was changed to DMEM/F12 with antibiotics, 10% FCS, and further cultured for additional two days to achieve the fully mature phenotype. All three sets of human adipocytes (VAT and SAT derived cells) were run in two independent experiments.

The 3T3-L1 cells were cultured in the differentiation medium containing DMEM, 10% FBS (Corning, New York, NY, USA), antibiotics (penicillin, 50 U/mL; streptomycin, 50 µg/mL, Corning, New York, NY, USA), 3-isobutyl-1-methylxanthine (115 µg/mL, Sigma-Aldrich, St. Louis, MO, USA), dexamethasone (390 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) and insulin (10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) for three days. Next, the medium was changed to DMEM with antibiotics, 10% FBS, and insulin (10 µg/mL). After three more days, the medium was changed to DMEM with antibiotics, 10% FBS, and further cultured for additional two days to achieve the fully mature phenotype. The cell culture was done in three independent experiments.

The process of preadipocyte differentiation into mature adipocytes has previously been optimized and published [19,20]. The progress of the adipogenesis process was controlled by lipids accumulation measurement and morphological changes. The cells were recorded using Olympus IX81 before differentiation and at the end of adipogenesis (Figure S1). Lipids accumulation was measured using Oil Red O stain (Sigma-Aldrich, St. Louis, MO, USA) (Figure S2). The cells were incubated with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, with 60% isopropanol (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, and with the previously prepared Oil Red O solution for 30 min. All incubations were performed at room temperature. The accumulated Oil Red O in the cells was extracted with 100% isopropanol and the absorbance was measured at 492 nm.

2.3. Insulin Resistance Induction

When the adipose cells were completely differentiated, insulin resistance was induced by 0.5 mM palmitic acid (16:0) (Sigma-Aldrich, St. Louis, MO, USA). Insulin resistance was stimulated for 48 h and 72 h using palmitic acid. After each time point, the glucose uptake test was performed to assess the insulin resistance state of the mature adipocytes. Glucose uptake was analyzed using Glo-Glucose Uptake (Promega, Madison, WI, USA) according to the manufacturer's protocol. Luminescence was read using a Victor3 1420 Multilabel Counter.

2.4. DNA and RNA Isolation

DNA from the cells from the cell cultures was isolated using the phenol:chloroform method (Sigma-Aldrich, St. Louis, MO, USA). Total RNA from the cells from the cell cultures was isolated through the trizol method (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Reverse Transcription Reaction and Gene Expression Level

Reverse transcription was performed using isolated total RNA (200 ng) and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Gene expression was done using real-time PCR based on an SYBR Green assay (Applied Biosystems, Waltham, MA, USA). Primers to *IL10* and β-actin (*ACTB*) were manually designed to flank two mRNA exons (Table 1). The specificity of primers was checked using Primer-BLAST; secondary structures were analyzed using OligoAnalyzer. Prior to real-time PCR, the efficiency of the primers was analyzed using the standard curve method. Specificity was checked based on the denaturation curve. Only primers characterized by efficiency values higher than $R^2 \geq 0.92$ were used for gene expression studies. A relative gene expression level, normalized to the housekeeping gene β-actin, was calculated using the delta-delta Ct (ΔΔCt) model.

Table 1. The primer pairs used in a study for analysis of gene expression, site-specific DNA methylation (meDIP) and site-specific histone modifications (ChIP).

Organism	Application	Gene	Forward Sequence	Reverse Sequence	Product Length (Base Pair)	Amount of CpG Sites *
Human	Gene expression	*IL10*	GGACTTTAAGGGTTACCTGG	CTGGGTCTTGGTTCTCAGC	95	-
Human	Gene expression	*ACTB*	GAGAAGATGACCCAGATCA	TAGCACAGCCTGGATAGCAA	72	-
Human	meDIP/ChIP	*IL10*	ACTGCTCTGTTGCCTGGTC	GTCTTCACTCTGCTGAAGG	144	4
3T3L1	Gene expression	*IL10*	TAAGGGTTACTTGGGTTGCC	CGCATCCTGAGGGTCTTCA	144	-
3T3L1	Gene expression	*ACTB*	CCCAGATCATGTTTGAGACC	CTGGATGGCTACGTACATG	53	-
3T3L1	meDIP/CHIP	*IL10*	CTTGCTCTTGCACTACCAAAG	TCCTCATGCCAGTCAGTAAG	108	2

* The CpG sites are regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5′ → 3′ direction. Cytosines in these sites can be methylated.

2.6. Site-Specific DNA Methylation of Cells from Cell Cultures

The DNA methylation of the cells from the cell cultures was analyzed using MagMeDIP qPCR Kit (Diagenode, Denville, NJ, USA) according to the manufacturer's protocol. After precipitation and DNA extraction, DNA concentration was measured, using Pico488 dsDNA quantification reagent (Lumiprobe, Cockeysville, MD, USA). The standard curve was prepared from human DNA quantified by Quantifiler™ Duo DNA Quantification Kit (Thermo Fisher, Waltham, MA, USA). The global DNA methylation was quantified as the percentage of DNA immunoprecipitated using C-me antibodies (Diagenode, Denville, NJ, USA) to the input amount of DNA, based on the concentration results. The site-specific DNA methylation was analyzed in Real-Time PCR, using Fast SYBR Green Master Mix (Thermo Fisher, Waltham, MA, USA). The primer sequences are presented in Table 1. The promoter region of the *IL10* gene was the target of primers. The calculation of % of the input was done according to the manufacturer's protocol. The following formula was used:

$$\% \text{ of recovery} = 2^{(\text{CtInput} - 3.32 - \text{CtCme})} * 100,$$

where, CtInput—Ct value of 10% Input; CtCme—Ct value of precipitated DNA using Cme antibody.

2.7. Global and Site-Specific Histone Modifications

The cultured cells were treated with 1% formaldehyde (FA, Sigma-Aldrich, St. Louis, MO, USA) with the addition of PI (BioShop, Burlington, ON, Canada) and PMSF (phenylmethylsulfonyl fluoride, Sigma-Aldrich, St. Louis, MO, USA) (1 mM) for ten minutes at 37 °C. After ten minutes, the reaction was stopped by Glycine. Next, the cells were washed three times with PBS with PI and PMSF. The cells were scraped and suspended in 300 µL of

SDS Lysis Buffer supplemented with PI and PMSF and sonicated ((30 s ON, 45 s OFF) × 15). The next steps of the procedure were carried out according to the manufacturer's protocol of the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore, Burlington, MA, USA).

The H3K4me3, H3K9/14ac, and IgG rabbit antibodies were purchased from Diagenode (Denville, NJ, USA)and added at the precipitation stage according to the manufacturer's protocol.

After decross-link by adding 20 µL of 5 M NaCl, DNA was extracted as described in Section 2.3.

The ChIP-Real-Time PCR was done as described in Section 2.5. Primers for real-time PCR were designed manually (Table 1). The promoter region of the *IL10* gene was the target of primers. The results were calculated as the percentage of input (% of input) according to the following formula:

$$\% \text{ of Input} = 100 \times 2^{(\text{CtAI-CtIP})}$$

where CtAI—Ct value of adjusted Input (calculated as Ct value—6.644), CtIP—Ct value of IP samples.

The results of the sonication process optimization are presented in Figure S3.

2.8. Statistical Analysis

Statistical analysis was performed using STATISTICA 13.1 (TIBCO Software Inc, Palo Alto, CA, USA) and Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA). The differences between the studied groups were performed using Student's T-Test. The correlation between numerical values was made using the Pearson correlation coefficient. Statistical significance was set at $p < 0.05$.

Statistical analysis was performed based on the results obtained from three replicate experiments for the mouse culture and the results obtained from three different human cell cultures.

3. Results

The analyses were performed at two time points, 48 and 72 h after the induction of insulin resistance by palmitic acid (16:0). In the experimental cells after culturing with 0.5 mM of palmitic acid (16:0), the insulin resistance state was developed, confirmed by the glucose uptake test. After 10 min insulin stimulation (1 µM), no increase in insulin-stimulated glucose uptake by those cells was observed. Control cells remained insulin-sensitive; insulin caused at least a twofold increase in glucose uptake compared to basal glucose utilization. The results have been shown by us in the recent publication [21].

3.1. IL10 Expression Level

In the human VAT-derived adipocytes, we observed a 1.5-fold decrease in *IL10* expression in insulin-resistant cells (IR), compared to control cells ($p = 0.169$) after 48 h after IR induction, and a 2.5-fold decrease after 72 h ($p = 0.002$) (Figure 1A).

In the human SAT-derived adipocytes, we observed a 3.5-fold increase in *IL10* expression in IR cells, compared to control cells after 48 h after insulin resistance induction ($p = 0.011$), and a 1.5-fold increase after 72 h ($p = 0.137$) (Figure 1A).

In 3T3-L1 IR cells, similar to the SAT-derived IR adipocytes, we observed approximately a 4.5-fold increase of the *IL10* expression level in IR cells, compared to control cells after 48 h after induction insulin resistance ($p = 0.014$), and more than a twofold increase after 72 h ($p = 0.001$) (Figure 2A).

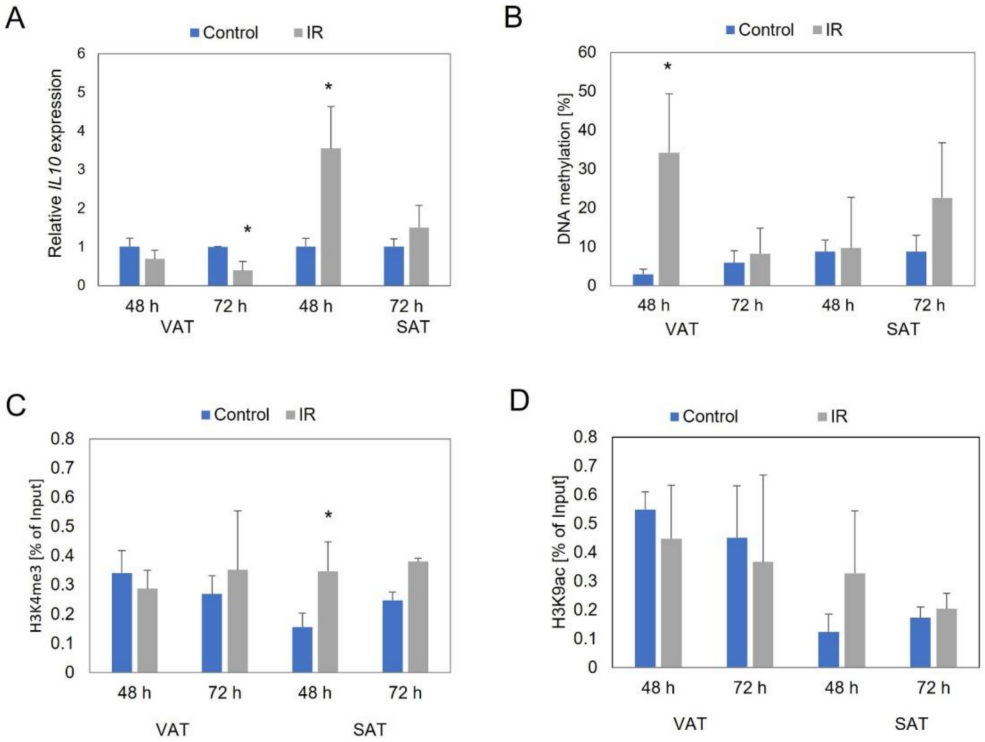

Figure 1. The *IL10* expression (**A**), DNA methylation (**B**), histone methylation (**C**), and histone acetylation level (**D**) in visceral (VAT) and subcutaneous (SAT) derived control adipocytes and insulin resistant adipocytes (IR) after 48 and 72 h after insulin resistance induction. * $p < 0.05$.

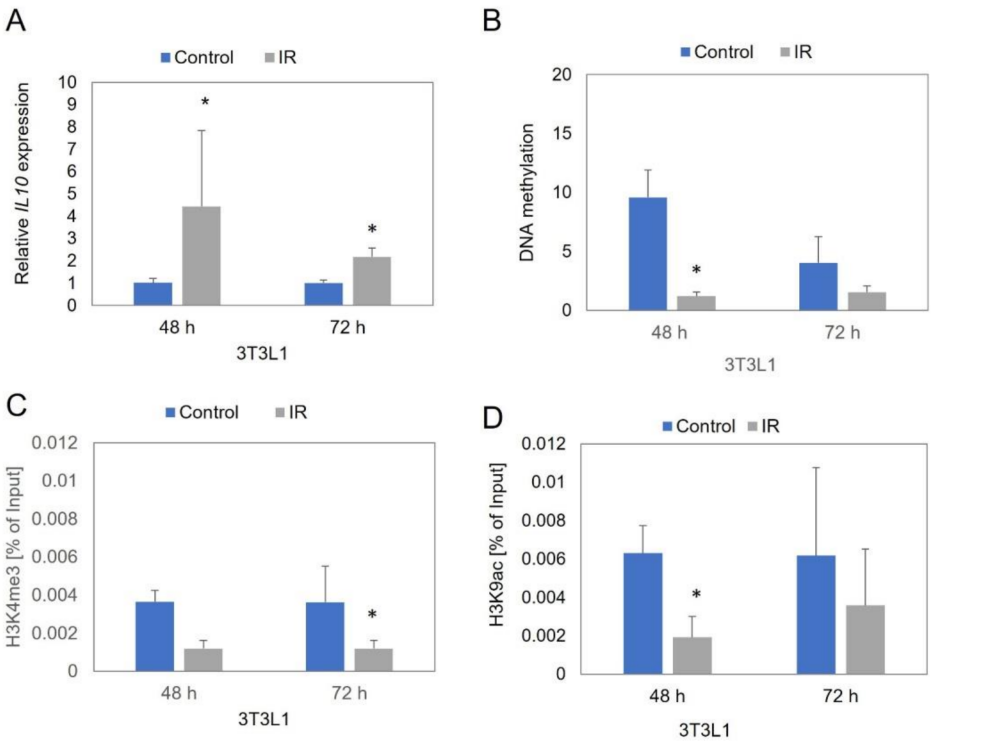

Figure 2. The *IL10* expression (**A**), DNA methylation (**B**), histone methylation (**C**), and histone acetylation level (**D**) in control 3T3L1 adipocytes and in insulin resistant (IR) adipocytes after 48 and 72 h after insulin resistance induction. * $p < 0.05$.

3.2. IL10 Promoter Region Methylation Level

The site-specific DNA methylation analyses showed in both the VAT and SAT human adipocytes an increase in the *IL10* promoter region methylation level (assessment based on % of the input). In the VAT samples, the *IL10* promoter was almost twelve times more highly methylated in IR cells, compared to control cells ($p = 0.006$) after 48 h of IR induction and a slight increase in *IL10* promoter methylation after 72 h (Figure 1B). In the SAT-derived adipocytes, we noticed a slight increase in *IL10* promoter methylation after 48 h in IR cells, compared to control cells, and a 2.5-fold increase after 72 h ($p = 0110$) (Figure 1B).

However, the analysis of 3T3-L1 cells showed a decrease of the *IL10* promoter region methylation level in IR cells compared to control cells. After 48 h, we observed an almost eightfold decrease of methylation in IR cells ($p = 0.000$), and more than a 2.5-fold decrease after 72 h ($p = 0.073$) (Figure 2B).

3.3. Site-Specific Histone Modifications near IL10 Gene

In the human VAT adipocytes, we did not observe statistically significant epigenetic changes within the histones at both time points. (Figure 1C,D).

We observed significant changes in the level of histone modification in human adipocytes collected from SAT. The increased methylation (H3K4me3; more than twofold increase; $p = 0.025$) and acetylation of histone 3 (H3K9/14ac; more than 2.5-fold increase; $p = 0.279$) were shown in IR cells, compared to control cells after 48 h. At the second time point (72 h), we also observed increases of these modifications in IR cells but these increases were smaller (H3K4me3: $p = 0.078$; H3K9/14ac: $p = 0.279$) (Figure 1C,D).

In 3T3-L1 IR cells, we observed a decreased histone modification level, compared to control cells both after 48 and 72 h of IR induction in experimental cells. We observed more than a threefold decrease of the methylation and acetylation level of histone 3 after 48 h (H3K4me3: $p = 0.094$; H3K9/14ac: $p = 0.003$) and a threefold decrease of methylation and almost a twofold decrease of acetylation in IR cells (H3K4me3: $p = 0.047$; H3K9/14ac: $p = 0.379$) (Figure 2C,D).

3.4. Correlation Analysis

At the first time point (48 h), in VAT derived adipocytes we observed positive correlations between *IL10* expression and H3K9/14 enrichment (R = 0.6812; $p = 0.136$). At the second time point (72 h), we observed negative correlations between *IL10* expression and the histone 3 methylation level (R = −0.6196; $p = 0.138$). We also observed a correlation between measured epigenetic modifications. A positive correlation between H3K4me3 and H3K9/14 enrichment was observed at both time points (48 h: R = 0.5735; $p = 0.137$; 72 h: R = 0.4839; $p = 0.271$). After 72 h we observed also negative correlation between *IL10* promoter region methylation and histone 3 epigenetic modifications (H3K4me3: R = −0.6372; $p = 0.124$; H3K9ac: R = −0.5252; $p = 0.181$).

After 48 h in SAT-derived adipocytes we showed positive correlations between *IL10* expression and both histone modifications (H3K4me3: R = 0.6693; $p = 0.146$; H3K9ac: R = 0.8778; $p = 0.021$). At the second time point, we showed positive correlations between *IL10* expression and histone 3 methylation level (R = 0.5371; $p = 0.272$). At the first time point we observed negative correlations between *IL10* promoter methylation level and histone methylation (H3K4me3: R = −0.9015; $p = 0.037$). After 72 h we observed positive correlation between *IL10* promoter region methylation and histone 3 methylation level (R = 0.6513; $p = 0.161$), and negative correlation between *IL10* promoter region methylation and histone 3 acetylation (R = −0.6484; $p = 0.115$).

In the case of 3T3-L1 adipocytes, at the first time point, we observed a negative correlation between *IL10* expression and the histone methylation level (R = −0.6842; $p = 0.061$), at the second time point, we observed a positive correlation between the expression level of *IL10* and histone 3 acetylation level (R = 0.8949; $p = 0.016$). After 48 h we observed positive correlations between methylation of the *IL10* promoter region and histone 3 methylation, and also histone 3 acetylation (H3K4me3: R = 0.4210; $p = 0.299$; H3K9ac: R = 0.8333;

$p = 0.010$). After 72 h we showed positive correlation between histone 3 acetylation and histone 3 methylation (R = 0.4767; $p = 0.232$). The summarizing results of correlation analysis are presented in Table 2.

Table 2. Results of correlation analysis.

SAT		***IL10* Expression**		***IL10* Promoter Region Methylation**		**H3K4me3**		**H3K9/14ac**	
		48 h	**72 h**	**48 h**	**72 h**	**48 h**	**72 h**	**48 h**	**72 h**
IL10 expression	48 h	-	-	-	-	r = 0.6693 $p = 0.146$		r = 0.8778 $p = 0.021$	-
	72 h	-	-	-	-	-	r = 0.5371 $p = 0.272$	-	-
IL10 promoter region methylation	48 h	-	-	-	-	r = −0.9015 $p = 0.037$	-	-	-
	72 h	-	-	-	-	-	r = 0.6513 $p = 0.161$	-	r = −0.6484 $p = 0.115$
H3K4me3	48 h	r = 0.6693 $p = 0.146$	-	r = −0.9015 $p = 0.037$		-	-	-	-
	72 h	r = 0.5371 $p = 0.272$	-	-	r = 0.6513 $p = 0.161$	-	-	-	-
H3K9/14ac	48 h	r = 0.8778 $p = 0.021$	-	-	-	-	-	-	-
	72 h	-	-	-	r = −0.6484 $p = 0.115$	-	-	-	-
VAT		*IL10* expression		*IL10* promoter region methylation		H3K4me3		H3K9/14ac	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
IL10 expression	48 h	-	-	-	-	-	-	r = 0.6812 $p = 0.136$	-
	72 h	-	-	-	-	-	r = −0.6196 $p = 0.138$	-	-
IL10 promoter region methylation	48 h	-	-	-	-	-	-	-	-
	72 h	-	-	-	-	-	r = −0.6372 $p = 0.124$	-	r = −0.5252 $p = 0.181$
H3K4me3	48 h	-	-	-	-	-	-	r = 0.5735 $p = 0.137$	-
	72 h	-	r = −0.6196 $p = 0.138$	-	r = −0.6372 $p = 0.124$	-	-	-	r = 0.4839 $p = 0.271$
H3K9/14ac	48 h	r = 0.6812 $p = 0.136$	-	-	-	r = 0.5735 $p = 0.137$	-	-	-
	72 h	-	-	-	r = −0.5252 $p = 0.181$	-	r = 0.4839 $p = 0.271$	-	-
3T3L1		*IL10* expression		*IL10* promoter region methylation		H3K4me3		H3K9/14ac	
		48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
IL10 expression	48 h	-	-	-	-	r = −0.6842 $p = 0.061$	-	-	-
	72 h	-	-	-	-	-	-	-	r = 0.8949 $p = 0.016$
IL10 promoter region methylation	48 h	-	-	-	-	r = 0.4210 $p = 0.299$	-	r = 0.8333 $p = 0.010$	-
	72 h	-	-	-	-	-	-	-	-
H3K4me3	48 h	r = −0.6842 $p = 0.061$	-	r = 0.4210 $p = 0.299$	-	-	-	-	-
	72 h	-	-	-	-	-	-	-	r = 0.4767 $p = 0.232$
H3K9/14ac	48 h	-	-	r = 0.8333 $p = 0.010$	-	-	-	-	-
	72 h	-	r = 0.8949 $p = 0.016$	-	-	-	r = 0.4767 $p = 0.232$	-	-

4. Discussion

Insulin resistance and obesity belong to serious epidemiological problems in the world. However, the only clinically used drug that increases the sensitivity of cells to insulin is metformin; however, long-term treatment decreases its efficiency. Given that these metabolic disorders constitute a serious problem in modern society, affecting children and adults, it is important to find effective treatments. Scientific reports indicating the effect of improving insulin sensitivity by IL-10 provide grounds for further research on this cytokine [13–15]. In the present study, we analyzed the influence of insulin resistance on epigenetic modification in the promoter region of the *IL10* gene as well as the potential influence of these modifications on *IL10* expression in adipocytes.

Studies were performed using two independent cell models for the analysis: human cells, based on mesenchymal stem cells collected from stromal fraction of white adipose tissue obtained from previously collected adipose tissues (visceral and subcutaneous), and murine cell line, based on the commercial 3T3-L1 cell line. The cells of both cultures were differentiated in the adipogenesis process into mature adipocytes. The use of adipocytes in the study is essential in the analysis of potential relationships between obesity and insulin resistance. Adipose cells are the most representative in this type of analysis, given the fact that WAT has the ability to self-produce IL-10. Cellular insulin resistance was induced with the use of palmitic acid. This is one of the most popular methods for diet-induced insulin resistance cellular models [22].

The increase in the *IL10* expression level in cells with induced insulin resistance, which we observed in the subcutaneous human cell culture in first time point (after 48 h), confirms a previous scientific report [11]. The increase in *IL10* expression is probably the result of the inflammation processes occurring during the development of insulin resistance in adipocytes. It could be a cell defense mechanism against intensifying inflammation. The developing inflammation accompanying the development of insulin resistance in cells may be a factor stimulating the increase in the expression of anti-inflammatory cytokines such as IL-10. We observed an increase of the *IL10* expression level in IR cells from the SAT cell culture at both time points of the experiment. However, the strength of these increases is different. After 48 h, we observed a 3.5-fold increase but after 72 h, it was only 1.5-fold, which probably indicates the process of expression inhibition. We only obtained statistical significance at the first time point.

Changes in the expression level at time points may be directly related to the changes in methylation levels. The increase in methylation after 48 h was insignificant. However, after 72 h, it is already two and a half times, which could explain the decreasing level of expression in these cells. This observation could confirm the transcription silencing effect of DNA methylation [23]. However, the above-mentioned hypothesis needs to be confirmed experimentally, especially since we didn't obtain the statistical significance of these results from both time points.

The initial increase in *IL10* expression at the first time point should also be referred to the histone 3 methylation analysis. After 48 h, we observed more than a double increase of H3K4me3, compared to the control, which also positively correlated with the increased expression of *IL10*. Acetylation of histone 3 in IR cells also be increased but the results didn't show a statistical significance, however we observed a strong positive correlation between *IL10* expression and H3K9/14ac with statistical significance. This observation could confirm the transcription activating effect of H3K4me3 and H3K9/14ac [24]. After 72 h, we observed a reduced increase of histone modifications, compared to the first time point, which could explain the simultaneous decrease in expression, however, this experiment should be repeated given the lack of statistical significance in the results obtained.

With a reduced increase of histone modifications, we also observed an increase in *IL10* promoter region methylation. A similar linkage between H3K4me3 and DNA methylation has been observed by others. It has been shown that these modifications tend to be mutually exclusive. The role in this mechanism was assigned to the DNMT3L protein. These data indicate that DNMT3L recognizes histone H3 tails that are unmethylated

at lysine 4 and induces de novo DNA methylation by the recruitment or activation of DNMT3A2 so—in this way—nonmethylated H3K4 could support DNA methylation [25]. Our observations suggest that these two epigenetic mechanisms may have a compensatory effect on each other, as shown by the strong negative correlation we observed between promoter methylation and H3K4me3 after 48 h, and between promoter methylation and H3K9/14ac after 72 h.

In the case of the visceral human cell culture, at the first time point, we observed a 1.5-fold decrease in expression of *IL10* in IR cells, and after 72 h, it was already a 2.5-fold decrease. At the same time, we observed an increase in methylation of the promoter region of *IL10* in these cells. After 48 h, the increase in IR DNA methylation was as much as twelvefold. After 72 h it was only 1.5-fold and didn't show statistical significance. The demonstrated significant increase in methylation of the promoter region of *IL10* after 48 h could directly impact a significant reduction in gene expression, which was observed after 72 h. In turn, the change in the increase in DNA methylation between these two time points may result from the changes in the level of H3K4me3 observed by us. We showed a negative correlation between the methylation of the promoter region of *IL10* and the level of H3K4me3. We observed a significant increase in DNA methylation (twelve-fold) with a simultaneous decrease in H3K3me3 after 48 h and a decreased power of the increase in DNA methylation (only 1.5-fold) with a simultaneous increase in histone methylation after 72 h. It could confirm an inverse relationship between these epigenetic modifications. However, unlike the SAT cell culture, DNA methylation decreased with increasing methylation within histone 3. In a recent study, it was observed that DNMT3L can bind to nonmethylated H3K4 but cannot bind to H3K4me3. It was suggested that H3K4 methylation can play a role in blocking de novo DNA methylation at some genomic loci [26].

Different expression profiles of *IL10* in VAT and SAT cell cultures may directly result from the metabolic differences between these two adipose tissue types. As has been shown before by us, VAT is more susceptible to developing chronic inflammation and shows an increased expression of proinflammatory factors (IL-6 and TNFα), compared to SAT [19]. The increased expression of proinflammatory factors may be the reason for the downregulation of *IL10* expression.

Observations in the mouse cell model, as well as in the SAT human cell culture, showed a strong 4.5-fold increase in *IL10* expression in IR cells 48 h after the induction of insulin resistance and twofold after 72 h. At the same time, we observed a decrease in the degree of methylation of the *IL10* promoter region in IR cells, which would explain the changes in expression observed. Methylation levels dropped eightfold at the first time point and by two and a half times at the second time point. The increase in methylation in IR cells between 48 and 72 h would explain the reduction in the level of *IL10* expression.

The analysis of histone epigenetic modifications showed decreased levels of both H3K4me3 and H3K9/14ac in IR cells at both time points. We observed a strong negative correlation between *IL10* expression and histone methylation at the first time point. On the other hand, after 72 h, we showed a strong positive correlation between *IL10* expression and histone acetylation. This observation could suggest a different effect on the transcription activity of both histone modifications in the mouse model. According to the authors' knowledge, this is the first scientific report related to the epigenetic regulation of *IL10* expression in 3T3L1 cells.

The presented study has a few limitations. First of all, the amount of human adipose tissue samples used to isolate MSC is relatively low, and in the study were used only male-derived human cells. Another limitation that should be discussed is the method of inducing insulin resistance in cells. Bearing in mind the fact that the protocol for inducing insulin resistance with palmitic acid is usually used in cell culture studies, it should be remembered that in vivo this process is much more complicated and generated by more than one factor. Furthermore, epigenetic regulation is a complex process, which gives difficulties in the interpretation of single regulation. Unfortunately, the cell culture model itself has limitations due to the lack of influence of other organs, such as the liver or pancreas,

on the development of metabolic disorders. Therefore, it would be important to check the mechanisms described in the paper in vivo with the use of adipose tissue samples.

The analyses presented in this study suggest a potential impact of epigenetic modifications on gene expression and could confirm the mutual influence of epigenetic modifications on each other or the activation of specific epigenetic regulation at a different stage of the development of insulin resistance in cells. We showed that epigenetic modifications could significantly impact changes in the expression of *IL10* in adipose tissue. What is more, we presented a different expression profile of *IL10* between the SAT and VAT while confirming the metabolic dissimilarity of the two depots of adipose tissue.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13020294/s1, Figure S1: title Differentiation of isolated human cells from visceral and subcutaneous adipose tissue into mature adipocytes. Visceral (A) and subcutaneous (C) preadipocytes at the start of differentiation. Visceral (B) and subcutaneous (D) matured adipocytes filled with lipid droplets upon reaching maturity; Figure S2: title The analysis of the amount of accumulated lipids measured using Oil Red O for subcutaneous (SAT) (A) and visceral (VAT) (B) cells compared to control (preadipocytes cells); Figure S3: title Results of electrophoresis in agarose gel after sonicated DNA. Lines: 1 – Size Marker (100 bp – 1000 bp; the brightest band: 500 bp); 2 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) × 15); 3 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) × 15); 4 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) × 20); 5 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) × 20); 6 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) × 25); 7 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) × 25).

Author Contributions: A.C.: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing—original draft preparation, Visualization. K.K.: Investigation, Resources. M.M.-M.: Conceptualization, Methodology, Software Validation, Formal analysis, Investigation, Data curation, Writing—review and editing, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The research protocols and all procedures were approved by the Ethical Review Board of Wrocław Medical University (approval No. KB-124/2017).

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

Data Availability Statement: Data are available on request from the corresponding author.

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

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Supplemental Material

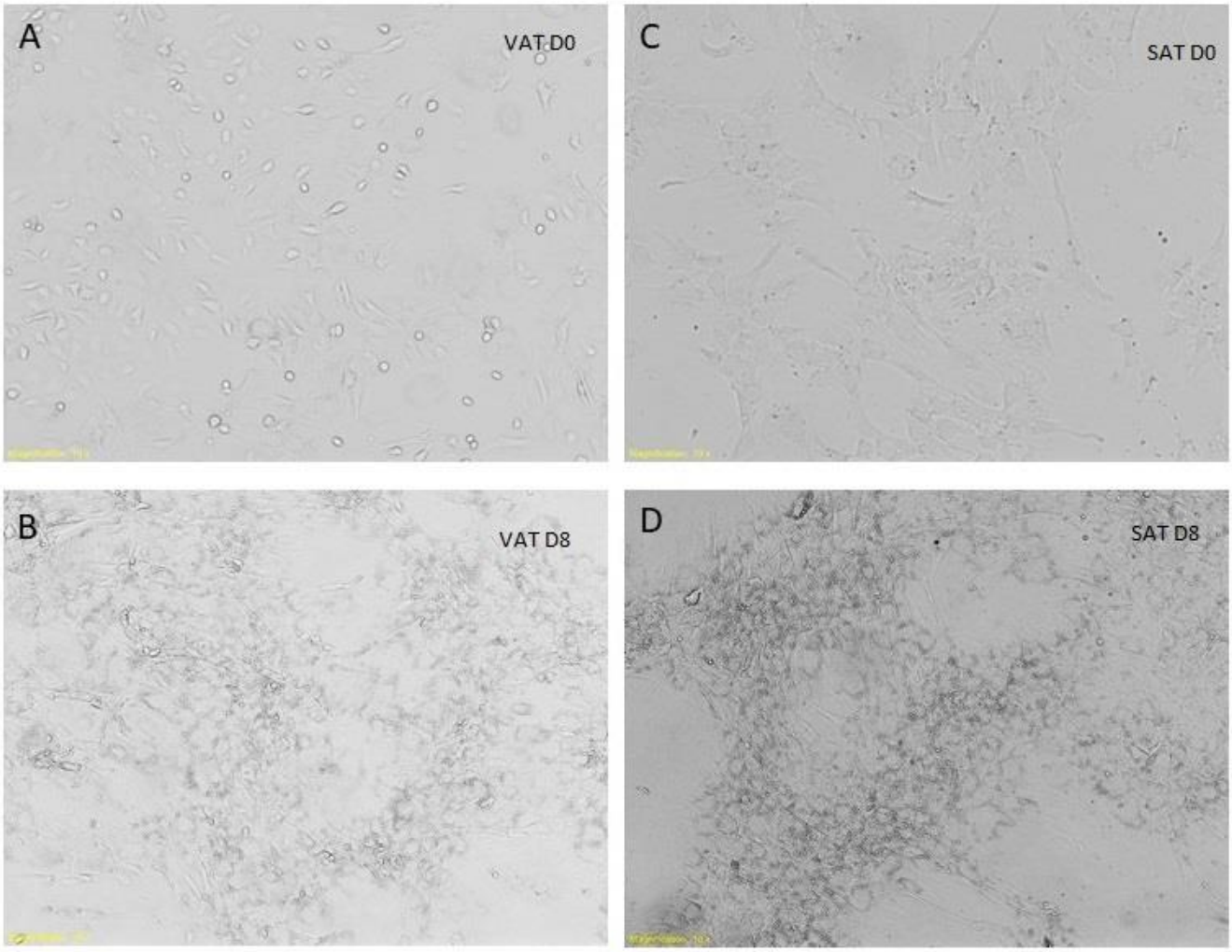

Figure S1. Differentiation of isolated human cells from visceral and subcutaneous adipose tissue into mature adipo-cytes. Visceral (A) and subcutaneous (C) preadipocytes at the start of differentiation. Visceral (B) and subcutaneous (D) matured adipocytes filled with lipid droplets upon reaching maturity.

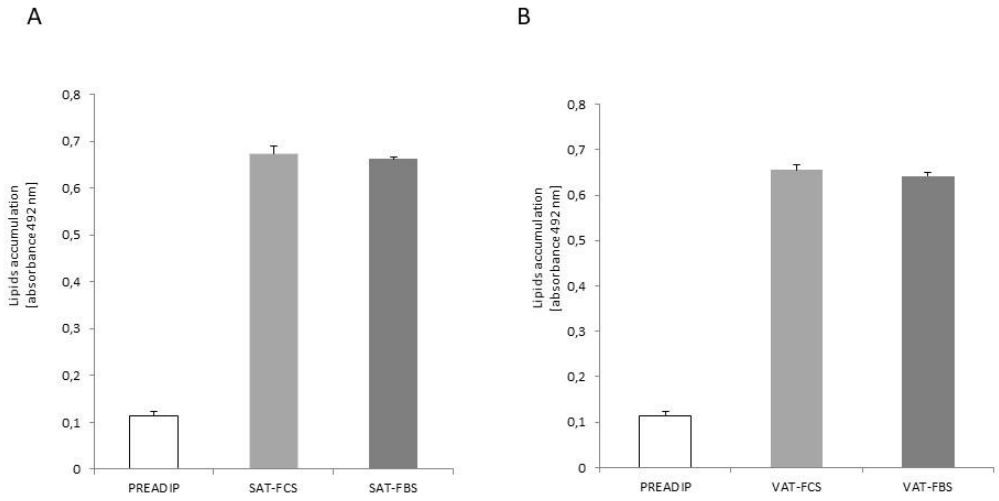

Figure S2. The analysis of the amount of accumulated lipids measured using Oil Red O for subcutaneous (SAT) (A) and visceral (VAT) (B) cells compared to control (preadipocytes cells).

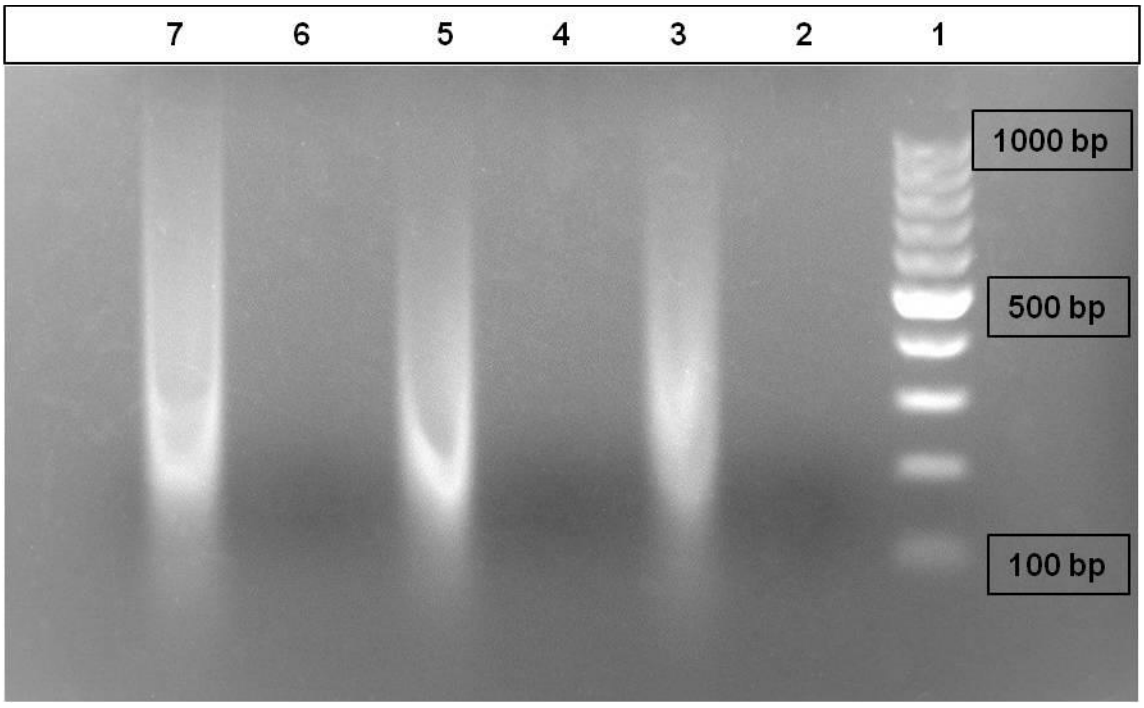

Figure S3. Results of electrophoresis in agarose gel after sonicated DNA. Lines: 1 – Size Marker (100 bp – 1000 bp; the brightest band: 500 bp); 2 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) x 15); 3 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) x 15); 4 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) x 20); 5 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) x 20); 6 – DNA isolated using MinElute PCR Purification Kit (QIAGEN) ((30s ON, 45s OFF) x 25); 7 – DNA isolated phenol:chloroform method (Sigma-Aldrich) ((30s ON, 45s OFF) x 25).

8.2. Załącznik nr 2. Oświadczenia współautorów publikacji.

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Wrocław, 25.02.2022

OŚWIADCZENIE

Oświadczam, że w pracy

1. *„DNA methylation in adipocytes from visceral and subcutaneous adipose tissue influences insulin-signaling gene expression in obese individual"* Aneta Cierzniak, Dorota Pawełka, Krzysztof Kaliszewski, Jerzy Rudnicki, Tadeusz Dobosz, **Małgorzata Małodobra-Mazur**; 2021; 45(3): 650-658 mój udział w pracy polegał na opracowaniu koncepcji badań, metodologii, współudziale w wykonywaniu badań i analizie wyników, zapewnieniu środków finansowych na realizację badań oraz nadzorze merytorycznym nad projektem.
2. *„Histone modifications influence the insulin-signaling genes and are related to insulin resistance in human adipocytes";* **Małgorzata Małodobra-Mazur**, Aneta Cierzniak, Aneta Myszczyszyn, Krzysztof Kaliszewski, Tadeusz Dobosz; 2021; 137; art.106031 [11 s.], mój udział w pracy polegał na opracowaniu koncepcji badań, metodologii, współudziale w wykonywaniu badań i analizie wyników, zapewnieniu środków finansowych na realizację badań, nadzorze merytorycznym nad projektem oraz redagowaniu publikacji.
3. *"The preliminary evaluation of epigenetic modifications regulating the expression of IL10 in insulin-resistant adipocytes"*; Aneta Cierzniak, Krzysztof Kaliszewski, **Małgorzata Małodobra-Mazur**; 2022; 13(2); art.294 [13 s.] mój udział w pracy polegał na opracowaniu koncepcji badań, metodologii, współudziale w wykonywaniu badań i analizie wyników, zapewnieniu środków finansowych na realizację badań i nadzorze merytorycznym nad projektem.

Podpis

Uniwersytet Medyczny we Wrocławiu
Katedra Medycyny Sądowej
ZAKŁAD TECHNIK MOLEKULARNYCH
p.o. KIEROWNIKA
dr n. med. Małgorzata Małodobra-Mazur

Prof. dr hab. Tadeusz Dobosz
Zakład Technik Molekularnych
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Wrocław, 25.02.2022

OŚWIADCZENIE

Oświadczam, że w pracy

1. *„DNA methylation in adipocytes from visceral and subcutaneous adipose tissue influences insulin-signaling gene expression in obese individual"* Aneta Cierzniak, Dorota Pawełka, Krzysztof Kaliszewski, Jerzy Rudnicki, **Tadeusz Dobosz**, Małgorzata Małodobra-Mazur; 2021; 45(3): 650-658 mój udział w pracy polegał na merytorycznym współredagowaniu publikacji.
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Podpis

00800 *Prof. dr hab. Tadeusz Dobosz*
DIAGNOSTA LABORATORYJNY
Specjalista Laboratoryjnej Genetyki Medycznej

dr hab. Krzysztof Kaliszewski
Klinika Chirurgii Ogólnej, Małoinwazyjnej i Endokrynologicznej
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

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Podpis

prof. dr hab. Jerzy Rudnicki
Klinika Chirurgii Ogólnej, Małoinwazyjnej i Endokrynologicznej
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Wrocław, 25.02.2022

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Podpis

KIEROWNIK KLINIKI
Chirurgii Ogólnej, Małoinwazyjnej i Endokrynologicznej
Uniwersyteckiego Szpitala Klinicznego
im. Jana Mikulicza-Radeckiego we Wrocławiu

Prof. dr hab. Jerzy Rudnicki

dr Dorota Pawełka
Katedra i Zakład Dydaktyki Chirurgicznej
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Wrocław, 25.02.2022

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Podpis

Dorota Pawełka

lek. Aneta Myszczyszyn
I Katedra i Klinika Ginekologii i Położnictwa
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Wrocław, 25.02.2022

OŚWIADCZENIE

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Podpis

8.3. Załącznik nr 3. Zgoda komisji bioetycznej.

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB –124/2017

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 78/XV R/2014 z dnia 26 listopada 2014 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

prof. dr hab. Maciej Bagłaj (chirurgia, pediatria)
prof. dr hab. Karol Bal (filozofia)
dr hab. Jacek Daroszewski (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód (duchowny)
mgr Luiza Müller (prawo)
prof. dr hab. Krystyna Orzechowska-Juzwenko (farmakologia kliniczna, choroby wewnętrzne)
prof. dr hab. Zbigniew Rudkowski (pediatria)
dr hab. Sławomir Sidorowicz (psychiatria)
Danuta Tarkowska (położnictwo)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.

„Modyfikacje epigenetyczne genów szlaku insulinowego oraz ich rola w indukcji insulinooporności związanej z otyłością"

zgłoszonym przez **dr n. med. Małgorzatę Małodobrą –Mazur** zatrudnioną w Zakładzie Technik Molekularnych Katedry Medycyny Sadowej Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Zakładzie Technik Molekularnych Katedry Medycyny Sadowej oraz I Katedrze i Klinice Chirurgii Ogólnej, Gastroenterologicznej i Endokrynologicznej Uniwersytetu Medycznego we Wrocławiu **pod warunkiem zachowania anonimowości uzyskanych danych**.

Uwaga: Badanie to zostało objęte ubezpieczeniem odpowiedzialności cywilnej Uniwersytetu Medycznego we Wrocławiu z tytułu prowadzonej działalności.

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu.

Opinia powyższa dotyczy: projektu badawczego finansowanego przez Narodowe Centrum Nauki.

Wrocław, dnia 2 marca 2017 r.

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kornafel

EZ