Wydział Lekarski
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Uniwersytet Medyczny w Białymstoku

Klinika Ginekologii i Ginekologii Onkologicznej

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Ocena przydatności miRNA i sfingolipidów w surowicy, jako biomarkerów we wczesnej diagnostyce cukrzycy ciążowej

Rozprawa doktorska w oparciu o cykl publikacji naukowych
w dziedzinie nauk medycznych i nauk o zdrowiu
w dyscyplinie nauki medyczne

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Składam serdeczne podziękowania Promotorowi pracy,
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Spis treści

1 Wykaz publikacji stanowiących rozprawę doktorską

Prace przeglądowe:

Juchnicka I, Kuzmicki M. Influence of MiRNAs in gestational diabetes mellitus development. Ginekol Pol. 2021;92(8):579-582. doi: 10.5603/GP.a2021.0121. MNiSW: 40 IF: 1,232.

Juchnicka I, Kuźmicki M, Szamatowicz J. Ceramides and Sphingosino-1-Phosphate in Obesity. Front Endocrinol (Lausanne). 2021 May 13;12:635995. doi: 10.3389/fendo.2021.635995. MNiSW: 100 IF: 5,555.

Prace oryginalne:

Juchnicka I, Kuźmicki M, Niemira M, Bielska A, Sidorkiewicz I, Zbucka-Krętowska M, Krętowski AJ and Szamatowicz J (2022) miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus. Front. Endocrinol. 13:839344. doi: 10.3389/fendo.2022.839344. MNiSW: 100 IF: 5,555.

Juchnicka I, Kuźmicki M, Zabielski P, Krętowski A, Błachnio-Zabielska A, Szamatowicz J. Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes. J Clin Med. 2022 Jan 13;11(2):384. doi: 10.3390/jcm11020384. MNiSW: 140 IF: 4,242.

2 Zestawienie publikacji doktoranta

Rodzaj publikacji	Liczba	Impact Factor	Punktacja MNiSW
Prace włączone do rozprawy doktorskiej	4	16,584	380
Prace, które nie zostały włączone do rozprawy doktorskiej	1	4,242	140
Streszczenia zjazdowe	1	-	-
Razem	**6**	**20,826**	**520**

3 Wprowadzenie

3.1 Rola insulinooporności w rozwoju cukrzycy ciążowej

W czasie ciąży w organizmie kobiety zachodzi szereg zmian, których celem jest przystosowanie przemiany materii ciężarnej dla potrzeb rozwijającego się płodu. Fizjologicznym zjawiskiem jest narastająca insulinooporność. Związana jest ona z wydzielaniem hormonów diabetogennych, takich jak laktogen łożyskowy, łożyskowy hormon wzrostu, prolaktyna, progesteron i kortyzol (1). Dodatkowo produkcja mediatorów stanu zapalnego przez łożysko obniża wrażliwość na insulinę (2). W konsekwencji dochodzi do odkładania się tkanki tłuszczowej i rozwoju insulinooporności. Już w ubiegłym wieku odkryto, że mechanizmem kompensacyjnym dla narastającej insulinooporności jest hiperplazja oraz hipertrofia wysp trzustkowym (3). Za mechanizmy adaptacyjne odpowiadają czynniki zewnątrzkomórkowe (laktogen, serotonina) oraz wewnątrzkomórkowe (mTOR, HNF-4α, FOXM1) (4). U kobiet, u których nie ma wystarczającej kompensacji dla narastającej insulinooporności dochodzi do hiperglikemii.

3.2 Cukrzyca ciążowa (GDM – *Gestational Diabetes Mellitus*)

Cukrzyca ciążowa stanowi jedno z najczęstszych zaburzeń metabolicznych w okresie ciąży. Według najnowszego raportu IDF (International Diabetes Federation) 21,1 miliona żywych urodzeń w ostatnim roku było dotkniętych jedną z form hiperglikemii, z czego 80,3% stanowiły zachorowania na GDM (5). Według WHO (*World Health Organization*) (6) cukrzycę ciążową definiujemy jako nietolerancję węglowodanów objawiającą się hiperglikemią po raz pierwszy zdiagnozowaną w trakcie ciąży. Warto podkreślić, że definicja ta nie określa trymestru, w którym należy zdiagnozować GDM, jak np. wg ADA (*American Diabetes Association*), które wyklucza 1 trymestr jako okres do diagnozy GDM (7). W Polsce zaleca się, aby każda kobieta w ciąży miała wykonane badanie w kierunku GDM pomiędzy 24 a 28 tygodniem ciąży. Jednakże, badanie należy przeprowadzić już w pierwszym trymestrze, jeśli u pacjentki występują czynniki ryzyka, takie jak nadwaga lub otyłość, wiek powyżej 35 roku życia, w wywiadzie GDM w poprzednich ciążach, zachorowania na cukrzycę w rodzinie, zgony wewnątrzmaciczne, urodzenie dziecka z wadą rozwojową lub wagą powyżej 4000g, wielorództwo, nadciśnienie tętnicze, zespół policystycznych jajników (8). Niezdiagnozowana

i nieleczona hiperglikemia w okresie ciąży może doprowadzić do groźnych powikłań ze strony matki i dziecka.

Nadmiar glukozy, która przechodzi przez łożysko na zasadzie dyfuzji, a także zwiększona dostępność aminokwasów i wolnych kwasów tłuszczowych, może przyczyniać się do nadmiernej stymulacji wysp trzustkowych, wzmożonego wydzielania insuliny, a w konsekwencji do przyspieszonego wzrastania płodu (9). Powikłania występujące okołoporodowo to preeklampsja, nadciśnienie tętnicze, poród przedwczesny, poród zabiegowy, uraz okołoporodowy, LGA (*large for gestational age*), dystocja barkowa, oraz zaburzenia metaboliczne (hipoglikemia i hiperbilirubinemia) u noworodka. Najczęstszym powikłaniem jest makrosomia płodu, która wiąże się ze zwiększoną częstością porodu drogą cięcia cesarskiego (10). Ponadto, GDM rzutuje na rozwój dziecka, przyczyniając się do rozwoju otyłości, zespołu metabolicznego, upośledzonej tolerancji glukozy, cukrzycy oraz chorób układu sercowo-naczyniowego (11,12).

Według rekomendacji Polskiego Towarzystwa Ginekologów i Położników oraz Polskiego Towarzystwa Diabetologicznego, pacjentki z GDM, w okresie 6 - 12 tygodni po porodzie, powinny ponownie wykonać doustny test tolerancji glukozy (OGTT- oral glucose tolerance test) a wyniki należy interpretować w oparciu o zakresy referencyjne stosowane dla populacji ogólnej (8,13). W większości przypadków, wynik OGTT jest prawidłowy, jednakże, kobiety które w ciąży rozwinęły GDM, znacznie częściej w kolejnej ciąży mają nawrót GDM (14). Należy również nadmienić, że GDM może inicjować u kobiety powikłania oddalone w czasie. Badania wykazują, że kobiety, które miały cukrzycę ciążową w wywiadzie, mają 7-krotnie zwiększone ryzyko rozwoju cukrzycy typu 2 w ciągu następnych 10 lat w porównaniu ze zdrowymi kobietami (15). Ponadto znacznie wzrasta również ryzyko rozwinięcia zespołu metabolicznego i zaburzeń sercowo-naczyniowych (16,17).

Obecnie diagnostyka GDM jest jednostopniowa, oparta na doustnym teście tolerancji glukozy (OGTT) z użyciem 75 g glukozy rozpuszczonej w 250 - 300 ml wody, wykonywanym między 24 a 28 tygodniem ciąży. Po wprowadzeniu nowych kryteriów diagnostycznych przez Światową Organizację Zdrowia WHO (2013 rok), w oparciu o rekomendacje IADPSG (2010 rok) (*International Association of the Diabetes and Pregnancy Study Groups*) opracowanych na podstawie wyników badania HAPO (*Hyperglycemia and Adverse Cycle Outcome*), częstość przypadków GDM znacznie wzrosła (18). Jednakże w dostępnych metaanalizach skupiających się na porównaniach kryteriów diagnostycznych oraz ich stosunkiem do występujących powikłań, zauważalna jest zależność: im bardziej restrykcyjne kryteria, tym mniejsze ryzyko wystąpienia powikłań (19–22).

Czas oznaczenia	Stężenie glukozy w osoczu [mg/d]
Na czczo	92 – 125
1 h	≤180
2h	153 – 199

Tabela 1. Kryteria diagnostyczne GDM (IADPSG).

Zakresy referencyjne obowiązujące w ciąży charakteryzują się mniejszymi wartościami, na czczo, jak i po obciążeniu glukozą, w porównaniu do norm obejmujących populację ogólną. Wynika to ze zmiany dystrybucji glukozy w organizmie oraz jej zwiększonego zużycia przez tkanki, a także z dyfuzji glukozy przez łożysko celem zaspokojenia płodowego zapotrzebowania energetycznego. Obserwujemy również zwiększone magazynowanie glikogenu tkankowego oraz spadek glukoneogenezy (23).

Istotnym czynnikiem w prewencji GDM jest identyfikacja markerów wskazujących wysokie ryzyko rozwinięcia choroby przed wzrostem glikemii. W literaturze mamy szereg adipokin oraz białek, które badacze typują na potencjalne biomarkery, Są to między innymi iryzyna, betatrofina, adiponektyna, omentyna, visfatyna, leptyna, FABP4, RBP4, fetuina A, SHBG (24–27). Sugeruje się również użyteczność łatwych do oznaczenia parametrów biochemicznych. W ostatnim czasie opublikowano wyniki badań z wykorzystaniem narzędzi bioinformatycznych oceniających 74 zmienne i ich użyteczność do wczesnej diagnostyki GDM (28). Badacze sugerują, że stężenie lipoproteiny(a) może być związane z rozwojem GDM. Dodatkowo wskazuje się całkowitą tyroksynę (TT4) oraz trójjodotyroninę (T3) jako czynniki wymagające dalszych badań nad ich związkiem z rozwojem GDM.

Podstawowym leczeniem zdiagnozowanej GDM jest zmiana stylu życia oraz wprowadzenie odpowiedniej diety (29). W sytuacji, gdy nie otrzymujemy pożądanych rezultatów w postaci prawidłowych stężeń glukozy na czczo i po posiłku należy wdrożyć intensywna insulinoterapię (30). Jednakże, jak podaje literatura, mimo wprowadzonego leczenia nie jesteśmy w stanie całkowicie uchronić dorosłe osoby urodzone z ciąż powikłanych GDM przed rozwojem zaburzeń metabolicznych (31).

3.3 miRNA

MikroRNA (miRNA) to grupa niekodujących cząsteczek RNA składająca się z ok 19-25 nukleotydów, które odgrywają kluczową rolę w potranskrypcyjnej regulacji ekspresji genów (32). Powstawanie miRNA zachodzi w kilku etapach. Pierwszym z nich jest utworzenie pri-miRNA, o strukturze spinki do włosów, przez polimerazy II i III RNA. Pri-miRNA jest przetwarzany w jądrze przez enzym RNazy III Drosha, a w cytoplazmie przez enzym Dicer, powstaje 19-25 nukleotydowy dwuniciowy dupleks miRNA. Jedna z nici ulega degradacji, natomiast druga uczestniczy w powstawaniu kompleksu wyciszającego RISC-miRNA, który może oddziaływać na docelowy fragment mRNA. Warto podkreślić, że miRNA może przyłączyć się do docelowego mRNA nie będąc z nim komplementarnym, w konsekwencji duża część ludzkiego genomu jest potencjalnym celem miRNA (33). Konsekwencją przyłączenia jest zahamowanie procesu translacji lub degradacja komplementarnego RNA (34). MiRNA może pełnić rolę protektorów, np. w odpowiedzi organizmu na stres lub może mieć wpływ na procesy patologiczne (33).

Ostatnie lata przyniosły szereg doniesień o zmianach w ekspresji miRNA w różnych chorobach, w tym zaburzeniach metabolicznych, wydzielania insuliny i rozwoju komórek wysp trzustkowych (35,36). Najnowsze badania eksperymentalne i kliniczne dowodzą, że za długofalowymi konsekwencjami GDM u potomstwa może odpowiadać nie tylko cukrzyca ciążowa, ale także czynniki genetyczne. Podkreślana jest rola programowania płodowego tj. epigenetyczne zmiany w płodowym DNA (odmienny model metylacji) lub zmiany w ekspresji genów, które regulują najważniejsze szlaki sygnałowe. Zaburzenia te mogą być efektem zaburzeń funkcji supresorowej mikroRNA (37,38).

Ze względu na fakt, że miRNA są oporne na działanie RNazy i pozostają stabilne w tkankach i płynach ustrojowych, mogą być potencjalnie specyficznymi wskaźnikami zaburzeń metabolicznych (39). Zmiany ekspresji miRNA we krwi podczas ciąży powikłanej hiperglikemią były przedmiotem wielu badań, jednak nie są one jednoznaczne. Wysunęliśmy tezę, że zmiany zachodzące w profilu miRNA występują wcześniej niż zmiany stężenia glukozy, a ich określenie może przyczynić się do opracowania klinicznie użytecznych biomarkerów i wczesnego wdrożenia profilaktyki.

3.4 Sfingolipidy

Sfingolipidy są przedstawicielami bioaktywnych lipidów. Syntetyzowane *de novo* w retikulum endoplazmatycznym, a następnie w aparacie Golgiego, w wyniku kumulacji aminokwasu, zazwyczaj seryny, z kwasem tłuszczowym, zazwyczaj kwasem palmitynowym. Reakcja jest aktywowana przez CoA i katalizowana przez transferazę serynowo palmitynową (SPT). W wyniku kolejnych reakcji otrzymujemy ceramid, który jest w metabolicznym centrum przemian sfingolipidów. Głównymi bioaktywnymi sfingolipidami są sfingozyno-1-fosforan (S1P), sfingozyna oraz duża grupa ceramidów (40). Sfingolipidy zaangażowane są w szlaki sygnałowe związane z apoptozą, stanem zapalnym, reakcją immunologiczną, różnicowaniem i proliferacją komórek (41).

Dowiedziono, że akumulacja ceramidów w mięśniach szkieletowych i w wątrobie hamuje insulinowy szlak transmisji sygnałów, przyczyniając się w ten sposób do indukcji insulinooporności (42–45). Ponadto, oprócz ceramidów, udział S1P jest istotny w rozwoju chorób metabolicznych. Wykazano wyższe stężenie S1P w osoczu osób otyłych oraz dodatnią korelację z ilością tkanki tłuszczowej, oraz narastającą insulinoopornością (46). Należy jednak mieć na uwadze, że zmiany w stężeniu S1P są dynamiczne i zależą od łożyska naczyniowego, z którego jest pobrany materiał do badań oraz od rodzaju transportera, z którym jest związany S1P. Ponadto zależy również od rodzaju materiału (surowica czy osocze), gdyż S1P jest uwalniany do krwiobiegu z komórek krwi, a także od płci i wieku (47,48). W ostatnich latach dowiedziono, odmienny profil sfingolipidowy po porodzie u kobiet z rozpoznaniem GDM, które w następnych latach rozwinęły cukrzycę typu 2 (49,50). Tym istotniejsze i uzasadnione wydaje się poszerzenie wiedzy na temat profilu sfingolipidów w trakcie ciąży oraz poszukiwanie zmian zachodzących w ciążach powikłanych cukrzycą ciążową.

4 Omówienie prac składających się na rozprawę doktorską

4.1 Cel pracy

Do głównych wyzwań stojącym przed współczesną medycyną zajmującą się opieką nad kobietą ciężarną i jej dzieckiem jest zmniejszenie liczby zachorowań na GDM. Jednym ze sposobów może być wprowadzenie wczesnej diagnostyki, wyprzedzającej wzrost glikemii kobiet ciężarnych. Prowadzone przeze mnie badania mogą przyczynić się do udoskonalenia identyfikacji grupy wysokiego ryzyka i dzięki wprowadzeniu zmian w stylu życia, utrzymania prawidłowej tolerancji glukozy podczas ciąży i zminimalizowania powikłań u matki i dziecka.

Cele szczegółowe:

1. Zbadanie stężenia ceramidów i ich metabolitów w surowicy ciężarnych w pierwszym i drugim trymestrze ciąży oraz w grupie kontrolnej z oceną czy mogą stanowić wczesne biomarkery cukrzycy ciążowej.
2. Określenie profilu ekspresji miRNA w surowicy kobiet ciężarnych w pierwszym trymestrze ciąży z oceną czy mogą stanowić wczesne biomarkery cukrzycy ciążowej.
3. Analiza korelacji pomiędzy oznaczanymi parametrami a wskaźnikami insulinooporności.

4.2 Materiał i metody

Badanie miało charakter prospektywny, grupy badane zostały wyłonione spośród 525 pacjentek. W ramach badania każda pacjentka miała przewidziane 4 wizyty: w I trymestrze ciąży (9-12 tydzień), w II trymestrze (24-26 tydzień), w III trymestrze (34-37 tydzień) oraz 3 miesiące po porodzie. Dane kliniczne zebrano na podstawie ankiety wypełnianej na każdej wizycie, karty ciąży oraz badań laboratoryjnych takich jak: morfologia krwi, glikemia, profil lipidowy, TSH oraz badanie ogólne moczu.

Badaniem stężenia sfingolipidów w surowicy objęto 172 kobiety. Do grupy badanej (GDM) włączono kobiety, które w pierwszym trymestrze miały prawidłową tolerancję glukozy natomiast w drugim trymestrze rozwinęły cukrzycę ciążową (n=53). Grupę odniesienia (NGT) stanowiły kobiety z prawidłową tolerancją glukozy przez całą ciążę (n=82). Dodatkowo do badań włączono kobiety nie będące w ciąży (grupa kontrolna) (n=37). Stężenie sfingolipidów u kobiet ciężarnych oznaczono w surowicy w pierwszym i drugim trymestrze ciąży i jednokrotnie u kobiet niebędących w ciąży.

Ekspresję krążących miRNA oznaczono w surowicy 48 kobiet ciężarnych będących w I trymestrze ciąży. Grupę badaną (GDM) stanowiły pacjentki, które w drugim trymestrze rozwinęły cukrzycę ciążową (n=24), natomiast grupą kontrolną (NGT) były kobiety z prawidłową tolerancją glukozy przez całą ciążę (n=24).

W eksperymentach grupy zostały dopasowane pod względem przedciążowego BMI, wieku pacjentki oraz wieku ciążowego. W pierwszym trymestrze ciąży wszystkie pacjentki biorące udział w badaniu miały prawidłową tolerancję glukozy. Do badań nie rekrutowano pacjentek, u których w poprzednich ciążach zdiagnozowano cukrzycę ciążową, rodzinnie obciążonych występowaniem cukrzycy oraz palących papierosy. Kobiety, u których doszło w trakcie obecnej ciąży do powikłań takich jak: cholestaza, preeklampsja, nadciśnienie tętnicze lub obecny przewlekły stan zapalny zostały wykluczone z badania. Wszystkie pacjentki wyraziły świadomą, pisemną zgodę na udział w badaniu. Badanie zostało przeprowadzone zgodnie z zasadami Deklaracji Helsińskiej oraz zatwierdzone przez Komisję Bioetyczną Uniwersytetu Medycznego w Białymstoku (nr zgody: R-I-002/176/2018).

4.2.1 Pobieranie materiału

Krew żylna do badań została pobrana na czczo (po nocnym odpoczynku i 8-10 godzin po ostatnim posiłku) w pierwszym trymestrze ciąży oraz na czczo, po 30, 60 i 120 minutach w czasie testu OGTT wykonywanego w drugim trymestrze ciąży. Materiał został pobrany system aspiracyjno próżniowym S-Monovette (SARSTEDT, Niemcy). Do oznaczenia stężenia glukozy użyto S-Monovette z EDTA jako antykoagulantem i fluorkiem sodu o stężeniu 1,0 mg/ml pełnej krwi jako inhibitora glikolizy. Oznaczenie glukozy zostało wykonane do 60 minut od pobrania. W celu zbadania stężenia insuliny pobrano 2,7 ml krwi żylnej z aktywatorem krzepnięcia (S-Monovette). Natomiast, do przeprowadzenia badań nad ekspresją miRNA oraz stężeń krążących sfingolipidów w surowicy, pobrano 4,9 ml krwi żylnej z aktywatorem koagulacji (S-Monovette). Po pełnym wykrzepieniu (30 minut po pobraniu krwi) próbki zostały zwirowane przy obrotach 4000rpm w temperaturze pokojowej przez 10 minut. Następnie surowica została przeniesiona do probówek Eppendorfa wolnych od DNaz i RNaz i zamrożona w -80 ° C, do czasu wykonania analiz.

4.2.2 Metodyka

Oznaczenia biochemiczne w krwi (glukoza, profil lipidowy) przeprowadzono z użyciem metod enzymatycznych na aparacie Cobas c111(Roche Diagnostics Ltd., Szwajcaria). Stężenie insuliny w surowicy zostało oznaczone metodą immunoradiometryczna (DiaSource) z wykorzystaniem licznika gamma Wallac Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Finlandia). Na podstawie uzyskanych wyników stężenia glukozy i insuliny wyliczone zostały wskaźniki oceniające insulinooporność oraz insulinowrażliwość:

- HOMA-IR *(homeostasis model assessment of insulin resistance)*

$$\text{HOMA-IR} = \frac{stęż.glukozy\ na\ czczo\left[\frac{mmol}{l}\right] x\ stęż.insuliny\ na\ czczo\ [\frac{mU}{l}]}{22{,}5}$$

- HOMA-β (*homeostasis model assessment of β cell function)*

$$\text{HOMA-}\beta\ [\%] = \frac{20\ x\ stęż.insuliny\ na\ czczo\ [\frac{mU}{l}]}{stęż.glukozy\ na\ czczo\left[\frac{mmol}{l}\right] - 3{,}5}$$

- ISI_{OGTT} *(insulin sensitivity index)*

$$ISI_{OGTT} = \frac{10000}{\sqrt{\begin{array}{c}\left(stęż\ glukozy\ na\ czczo\left[\frac{mmol}{l}\right] x\ stęż\ insulina\ na\ czczo\left[\frac{mU}{l}\right]\right) x \\ (średnie\ stęż.glukozy\ podczas\ OGTT\left[\frac{mmol}{l}\right]) x (\ średnie\ stęż.insuliny\ podczas\ OGTT\ \left[\frac{mU}{l}\right])\end{array}}}$$

Stężenie ceramidów i ich metabolitów (sfinganina - SPA, sfingozyna - Sph, sfingozyno-1-fosforan – S1P) w surowicy, oznaczone zostało przy użyciu ultrawysokosprawnej chromatografii cieczowej połączonej ze spektrometrem masowym typu potrójny kwadrupol (UHPLC/MS/MS) według procedury dokładnie opisanej w publikacji „*Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes*".

MikroRNA wyizolowano z surowicy kobiet ciężarnych, pobranej w pierwszym trymestrze ciąży, z użyciem zestawu miRNeasy Serum/Plasma Advanced Kit (Qiagen), gdzie izolacja odbywa się metodą separacji na krzemionkowej membranie znajdującej się w specjalnej kolumnie. Do badań nad profilem miRNA w surowicy pacjentek wykorzystano technologię NanoString tzw. *digital color-coded barcode technology*, pozwalającą na jednoczasowe bezpośrednie oznaczenie 800 różnych miRNA w próbce. Do analizy

wykorzystywane są dwie sondy (ok 50 nukleotydowe) na 1 miRNA. Na końcu 5' umieszczony jest zestaw 6 znakowanych fluorescencyjnie tzw."barkodów", natomiast na końcu 3' znajduje się "sonda wychwytująca" z biotyną. Ze względu na zastosowaną procedurę pomija się etapy syntezy i amplifikacji cDNA, co pozwala na zmniejszenie prawdopodobieństwa błędu laboratoryjnego. Pierwszym etapem analizy jest hybrydyzacja poszczególnych miRNA ze specyficznymi sondami, kolejnym jest oczyszczenie i umieszczenie zhybrydyzowanej próbki na standardowej płytce. Ostatni etap to odczyt wyników na skanerze NanoString nCounter. Zastosowana metoda pozwala określić liczbę kopii miRNA w każdej próbce. Walidacja otrzymanych wyników została przeprowadzona metodą RT-PCR. Pierwszym etapem walidacji było przepisanie sekwencji miRNA na cDNA, do tego użyto zestawu miRCURY LNA RT Kit (Qiagen). W kolejnym kroku przeprowadzono RT-PCR z zastosowaniem miRCURY LNA SYBR Green PCR Kit (Qiagen) i specyficznych starterów dla każdego z analizowanych miRNA. Reakcje zostały przeprowadzone w termocyklerze LightCycler 480 (Roche Diagnostics Ltd., Szwajcaria). Wszystkie próbki były oznaczane w duplikatach.

Analizę statystyczną wykonano z użyciem programu Statistica 13.3 firmy StatSoft. Dane opisowe zostały przedstawione jako mediana i kwartyle. Porównania pomiędzy dwiema grupami zostały wykonane przy pomocy testu nieparametrycznego U Manna-Whitneya, natomiast pomiędzy trzema grupami testem Kruskala-Wallisa. Powiązania pomiędzy zmiennymi oceniono testem korelacji Spearmana. Zmiany stężeń sfingolipidów pomiędzy trymestrem pierwszym a drugim oceniono testem rang Wilcoxona. Profil ekspresji miRNA analizowano dodatkowo z użyciem programu nSolver 4.0. Dane zostały znormalizowane w odniesieniu do 100 cząsteczek o najwyższej ekspresji. Wyniki walidacji metodą RT-PCR zostały przeliczone na podstawie metody $\Delta\Delta CT$. W dalszym etapie dane zostały przeanalizowane w programie Statistica testami opisanymi powyżej. Przy użyciu krzywej ROC oceniono użyteczność wybranych parametrów jako biomarkerów cukrzycy ciążowej. We wszystkich testach przyjęto poziom istotności $\alpha=5\%$.

4.3 Wyniki dotyczące sfingolipidów

W pierwszy trymestrze ciąży całkowite stężenie ceramidów było istotnie wyższe u pacjentek w ciąży w porównaniu do grupy kontrolnej (3419,3 [3101,9-3824,9] vs. 3023,7 [2738,7-3225,9] ng/ml, p < 0,0001). Pacjentki ciężarne wykazywały również wyższe stężenie C16:0-Cer (183,4 [149,5-232,9] vs. 135,7 [118,6-156,6] ng/ml, p=0,0002), C18:1-Cer (21,6 [17,3-24,4] vs. 16,3 [14,3-18,5] ng/ml, p = 0,0003), C22:0-Cer (280,9 [250,3-324,1] vs. 215,5 [198,3-240,4] ng/ml, p<0,0001), C24:1-Cer (280,4 [239,1-320,3] vs. 219,9 [200,8-251,5] ng/ml, p<0,0001) oraz C24:0-Cer (2260,7 [2027,5-2613,6] vs. 1941,6 [1819,1-2304,9] ng/ml p=0,002). W porównaniu z grupą kontrolną kobiety ciężarne charakteryzowały się znacznie niższymi stężeniami SPA (13,4 [9,8-19,7] vs. 37,5 [34,2-43,8], p<0,0001) oraz S1P (328,3 [250,8-387,4] vs. 400,9 [357,2-436,8], p<0,0001).

Porównanie stężeń ceramidów oraz ich metabolitów w pierwszym trymestrze ciąży w grupie GDM oraz NGT wykazało wyższe stężenie C18:1-Cer w surowicy pacjentek u których w drugim trymestrze zdiagnozowano cukrzyce ciążową (GDM 24,16 [20,1-29,5] vs. NGT 20,72 [16,6-23,7], p=0,01). Skonstruowanie krzywej ROC pozwoliło na oszacowanie użytecznego stężenia C18:1-Cer jako markera wystąpienia GDM, proponowany punkt odcięcia to 23,87 ng/ml (AUC = 0,702, 95% CI 0,552-0,852, p = 0,008) .

Analizując wyniki całej populacji zaobserwowano istotnie (p<0,05) ujemną korelacje pomiędzy SPA a stężeniem insuliny (R= -0,33), wskaźnikiem HOMA-IR (R= -0,3) oraz HOMA-β (R= -0,38). Dodatnia korelacja została wykazana między stężeniem C22:0-Cer a stężeniem insuliny (R= 0,3), HOMA-IR (R= 0,3) oraz HOMA- β (R= 0,31), a także między stężeniem C24:0-Cer a stężeniem insuliny (R= 0,27) oraz wskaźnikami HOMA-IR (R= 0,23) i HOMA-β (R= 0,32). Ponadto C18:1-Cer i C24:1-Cer korelowało ze stężeniem insuliny (odpowiednio, R= 0,21; R= 0,22) oraz wskaźnikiem HOMA-β (odpowiednio, R= 0,21; R= 0,29).

Porównanie stężeń w grupie GDM w pierwszym oraz drugim trymestrze ciąży wykazało znaczny wzrost stężenia C16:0-Cer (p<0,05) oraz C18:0-Cer (p<0,05) a także spadek C18:1-Cer (p<0,05) i C24:1-Cer (p<0,05). Natomiast w grupie NGT w trakcie ciąży doszło do istotnego wzrostu C18:1-Cer (p<0,05) i C24:0-Cer (p<0,001).

4.4 Wyniki dotyczące miRNA

Metoda Nanostring pozwoliła na wytypowanie 4 cząsteczek miRNA, miR-16-5p (p=0,07), miR-142-3p (p=0,02), miR-144-3p (p=0,003) oraz miR-320e (p=0,02), których ekspresja znacznie różniła się pomiędzy grupami GDM a NGT. Mimo braku istotności statystycznej, miR-16-5p zostało włączone do dalszych badań, gdyż po dokładnej analizie biorącej pod uwagę również odchylenia standardowe, krotność zmian, FDR (*false discovery rate*) oraz ilość zliczeń postanowiliśmy poddać je walidacji. Walidacja potwierdziła istotnie wyższą ekspresję miR-16-5p (p<0,0001), miR-142-3p (p=0,001) oraz miR-144-3p (p=0,003) w surowicy kobiet, które w drugim trymestrze rozwinęły GDM.

Następnie zbadano korelacje pomiędzy walidowanymi miRNA a wskaźnikami oceniającymi odpowiedź na insulinę. miR-16-5p dodatnio korelowało z HOMA-IR (R=0,36, p<0,05). Zaobserwowano również ujemną zależność pomiędzy ISI_{OGTT} a miR-16-5p a (R=-0,34, p<0,05) oraz miR-144-3p (R=-0,33, p<0,05).

4.5 Wnioski

1. Okres ciąży związany jest z istotnym wzrostem w surowicy stężenia C16:0-Cer, C18:1-Cer, C22:0-Cer, C24:1-Cer oraz C24:0-Cer.
2. Oznaczenie stężenia C18:1-Cer w pierwszym trymestrze ciąży może stanowić nowy marker rozwoju cukrzycy ciążowej.
3. Zmiany w stężeniach C18:1-Cer, C22:0-Cer, C24:0-Cer oraz C24:1-Cer mogą być powiązane z narastającą insulinoopornością w ciąży.
4. Profil miRNA w pierwszym trymestrze ciąży jest różny u pacjentek, które w trakcie ciąży rozwijają cukrzycę ciążową w porównaniu do zdrowych ciężarnych.
5. miR-16-5p, miR-142-3p oraz miR-144-3p mogą potencjalnie służyć jako markery wczesnej diagnostyki GDM.

5 Publikacje stanowiące rozprawę doktorską

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Influence of MiRNAs in gestational diabetes mellitus development

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ABSTRACT

Gestational Diabetes Mellitus (GDM) is a metabolic disorder that is considered a prediabetes state. According to the International Diabetes Federation every year an increase in the number of women diagnosed with gestational diabetes is being noticed. It is known that GDM can cause many complications during pregnancy and labor. What is more, women with GDM history and their offspring are at risk of developing diabetes in the future. A new factor in the pathogenesis of GDM is epigenetics, which is described as changes in gene expression without directly modifying the DNA sequence. One of its regulating mechanisms is based on microRNA (miRNA). A small non-coding RNA sequence that has an influence on protein formation by suppressing gene expression. A better understanding of the miRNA's function could potentially lead to their usage as potential new biomarkers or treatment targets. In this article we review the most significant miRNA molecules in gestational diabetes.

Key words: gestational diabetes mellitus; GDM; miRNA; epigenetics

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INTRODUCTION

Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) is a metabolic disorder which is characterized by carbohydrate intolerance first recognized during pregnancy. Despite many years of experimental studies, the pathogenesis of GDM remains unclear. Increased insulin secretion and progressive insulin resistance are physiological phenomena during pregnancy. This occurs due to adipose tissue growth and elevated levels of insulin antagonists such as progesterone, estrogen, prolactin and placental lactogen [1]. Normally, there is an increase of insulin secretion by the pancreatic β-cells to sustain normoglycemia. However, in GDM insufficient insulin compensation is being observed [1, 2]. Risk factors of developing GDM include previous GDM history, maternal obesity or overweight, older age, family history of diabetes mellitus, previous child macrosomia, fetal death or stillbirth history. Currently, an increase in the incidence of GDM is being noted, especially in developed countries. The International Diabetes Federation estimated that almost one in six births are affected by GDM [3, 4].

More than 40 years ago, O'Sullivan JB created the first diagnostic criteria [5] that have been improved over the years. Currently, GDM diagnosis is based on Oral Glucose Tolerance Test (OGTT) with 75 g of glucose dissolved in 300ml of water measured between the 24th and the 28th week of pregnancy. The implementation of these criteria by World Health Organization (WHO) and International Association of the Diabetes and Pregnancy Study Groups (IADPSG) was based on the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study [6] (Tab. 1).

Epigenetics

Epigenetics is a rapidly growing field of science. The term includes any changes in the gene activity without modification in the DNA sequence. It was used for the first

Table 1. Cut-off values for diagnosing gestational diabetes mellitus according to International Association of the Diabetes and Pregnancy Study Groups [6]

International Association of Diabetes and Pregnancy Study Group	
Fasting glucose [mmol/L]	≥ 5.1
1-hour glucose [mmol/L]	≥ 10
2-hour glucose [mmol/L]	≥ 8.5

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time in 1939 in Waddington's paper [7]. For many years now, scientists have been interested in the mechanism of epigenetics and have studied this phenomenon extensively. Furthermore, the development of diagnostic techniques will allow for a more accurate analysis of changes that are not related with changes in the gene sequence. Epigenetic mechanisms operate through the regulation of gene expression as a result of chemical modification of DNA or proteins related to DNA. Thus far, the best known of the mentioned processes are DNA methylation, histone alteration, chromatin modification and a mechanism that uses non-coding RNA sequences (microRNA) [8]. This article will concentrate on the role of microRNA (miRNA) as an epigenetic mechanism in GDM development.

MicroRNA

MiRNA was discovered in 1993 by Rosalind C. Lee, Rhonda L. Feinbaum and Victor Ambros [9] and from that time the state of knowledge has significantly expanded. MiRNA expression occurs in several stages.

The first pathway starts with the DNA transcription catalyzed by RNA polymerase II and III which then forms harpin structure primary-miRNA (pri-miRNA). The pri-miRNA is processed in the nucleus by the RNase III Drosha enzyme into the pre-miRNA. Subsequently, it continues in the cytoplasm with the use of the Dicer enzyme resulting in 19–22 double-stranded miRNA nucleotides. In the next stage the RNA-induced silencing complex (RISC) is being formed. One of the strands degrades and the second takes part in gene transcription regulation.

There is a second pathway — The Mirtron Pathway of miRNA formation. During the splicing process the pri-miRNA is being created and then it follows the same pattern as stated above [10].

For many years it was considered that non-coding sequences of RNA had no significant role. Whereas nowadays it have been proved that that one mRNA may contain many binding locations for different miRNAs and that one miRNA can affect several different genes [10, 11]. The RISC-miRNA complex can interact with the target mRNA, without perfect homology and consequently, inhibit the translation process or lead to the degradation of complementary RNA [12]. The main role of microRNA is the regulation of post-transcriptional gene expression through the mechanisms of mRNA cleavage or deadenylation, leading to the down-regulation of gene expression by [13]. On the other hand, miRNA can possibly promote protein expression. MiRNA can play a role in enhancing the organism's response to stress and can have an impact on many pathological processes [14]. Furthermore, it is known that miRNA can play a role in autocrine or paracrine regulations and it is present in every kind of human fluid.

MIRNAS IN GESTATIONAL DIABETES MELLITUS

One of the first papers assuming the usefulness of a miRNAs in diagnosis of GDM was Zhao C. et al. [15] article. They suggest three miRNAs as an early serum biomarker of GDM, hsa-miR29a, hsa-miR222 and has-miR132 whose level was significantly decreased in GDM compared to the control group. Hsa-miR29a is known as a regulating factor for hepatic gluconeogenesis and a stimulator for insulin secretion by the pancreatic β cells contributing to preventing diabetes development [15, 16]. Meta-analysis conducted by Zhu H. [17] has confirmed the presence of miR-29a and miR-132 in the blood of T2 diabetes patients, whereas upregulation of miR-222 has been noted in the adipose tissue of GDM patients. It is known that estrogen receptor α (Erα) is a target for miR-222, which, when activated, leads to an increase in estrogen concentration and consequently, the inhibition of GLUT4 transporter. That phenomenon leads to estrogen induced insulin resistance [18].

Zhu Y. et al. [19], reported a potential biomarker role of miR-16-5p, miR-17-5p, miR-19a-3p, miR-19b-3p and 20a-5p which were upregulated in the plasma taken from the GDM patients between 16–19th weeks of pregnancy. Cao L.Y. et al. [20], checked those miRNAs in plasma samples of women between 24th–28th weeks of pregnancy when GDM is usually diagnosed. Results confirmed a significant upregulation of miR-16-5p, miR-17-5p and 20a-5p. Moreover, authors proved a positive correlation of those miRNAs and HOMA-IR, one of the indicators of GDM. Data describes the role of miR-16-5p and miR-17-5p in pathogenesis of T2 diabetes. Target genes for miR-16-5p were reported as downregulated genes in T2 diabetes. The miR-17-5p is involved in cell proliferation and is upregulated especially in samples from diabetic patients with vascular complications. So far, there is no data about the role of miR-20a-5p in diabetes development [19, 20]. Surprisingly, Carmen Pheiffer [21] did not show an increased expression of miR-16-5p and 17-5p in his paper. Only miR-20a-5p was upregulated. The difference between those results may be caused by race because Zhu and Cao [19] examined Asian women, whereas Carmen conducted his research in South Africa. MiRNA occurrence is sensitive and can depend on many factors such as BMI, race, nutrition and even sex of the fetus.

More recently, Sebastiani G. et al. [22], reported a miR-330 upregulation in GDM plasma. They found a correlation between miR-330 level and caesarean section rate and pregnancy complications (fetal macrosomia, polyhydramnios and maternal hydronephrosis). Authors suggested that high levels of examined miRNA may predispose to a more severe diabetic phenotype. Proof of miR-330 involvment in GDM pathophysiology are the target genes, CDC42 and E2F1. Both are associated with insulin resistance. CDC42 im-

paired insulin release whereas E2F1 reduced beta-cells proliferation [23, 24].

During pregnancy, an additional source of miRNA is the placenta. Nair S. at al. [25], reported that has-miR-125a-3p and has-miR224-5p were upregulated in chorionic villi and skeletal muscle tissue in GDM. Those RNAs are involved in CD40 and Glypican 4 expression which are associated with body fat composition and insulin resistance (correlation with HOMA-IR). What is more, a glypican 4 has an affinity to insulin receptors and increases insulin signaling [26]. Increased miR-125a-3p level was also described in the liver and the adipose tissue in a diabetic rat model as a molecule involved in insulin resistance development. The target for miR-125a-3p is PI3K, a crucial kinase in PI3K/AKT pathway leading to an increased glucose uptake in skeletal muscle [27].

Human Molecular Genetics published a paper indicating long term effects in the adult offspring of women with GDM. Individuals exposed to maternal diabetes have an increased miR-15a and miR-15b expression in skeletal muscle. These miRNAs may alter the expression of proteins important in insulin signaling pathways and decrease insulin receptors development causing impaired glucose tolerance or even diabetes in the offspring of diabetic women [28]. It testifies that epigenetics could potentially prove a potent diagnostic tool and a treatment option being a chance for a better care for people suffering from GDM and its complications.

SUMMARY

Due to the fact that miRNAs are resistant to RNase and remain stable in tissues and body fluids, even after multiple freeze-throw cycles, changes in their expression may be both sensitive and specific indicators of metabolic disorders like gestational diabetes mellitus (GDM). Furthermore, miRNAs can be collected from peripheral blood, thus rendering miRNAs an easy to collect, minimally invasive diagnostic biomarker [17]. However, changes in the miRNA expression in the blood during hyperglycemia complicated pregnancy have so far been ambiguous and further research needs to be done to create a GDM prediction miRNA profile.

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Ceramides and Sphingosino-1-Phosphate in Obesity

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Obesity is a growing worldwide problem, especially in developed countries. This disease adversely affects the quality of life and notably contributes to the development of type 2 diabetes, metabolic syndrome, and cardiovascular disorders. It is characterised by excessive lipids accumulation in the subcutaneous and visceral adipose tissue. Considering the secretory function of adipose tissue, this leads to impaired adipokines and cytokines release. Changes in adipose tissue metabolism result in chronic inflammation, pancreatic islets dysfunction and peripheral insulin resistance. In addition to saturating various adipocytes, excess lipids are deposited into non-adipose peripheral tissues, which disturbs cell metabolism and causes a harmful effect known as lipotoxicity. Fatty acids are metabolised into bioactive lipids such as ceramides, from which sphingolipids are formed. Ceramides and sphingosine-1-phosphate (S1P) are involved in intracellular signalling, cell proliferation, migration, and apoptosis. Studies demonstrate that bioactive lipids have a crucial role in regulating insulin signalling pathways, glucose homeostasis and β cell death. Data suggests that ceramides may have an opposite cellular effect than S1P; however, the role of S1P remains controversial. This review summarises the available data on ceramide and sphingolipid metabolism and their role in obesity.

Keywords: ceramides, sphingolipids, S1P, obesity, adipose tissue

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INTRODUCTION

Obesity is an increasingly common phenomenon. The global prevalence of obesity has almost tripled in the last 40 years (1). Obesity is a risk factor for several serious chronic diseases. It contributes to developing certain types of cancer, cardiovascular complications, insulin resistance, metabolic syndrome, type 2 diabetes, asthma, hepatic and renal dysfunction, infertility and sleep disturbances (2).

There are no doubts about the harmfulness of obesity, but, at the same time, the power of adipose tissue is becoming clear. Adipose tissue is the largest endocrine organ built from diverse types of cells. The primary cells are adipocytes. In addition to adipocytes, there are pre-adipocytes, mesenchymal cells, fibroblasts, endothelial cells and immune cells (3). There are two basic types of adipose tissue. The dominant is white adipose tissue (WAT). Due to the richness of the cells from which it is produced, WAT performs many different functions. First, it serves as a vast energy store that regulates fatty acid homeostasis. During excessive food intake, free fatty acids (FFA) accumulate in WAT as triacylglycerols (TAG). Another function of WAT is the secretion of adipokines,

including adiponectin, leptin, resistin, apelin visfatin and cytokines like tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) (4, 5). There are two types of WAT. The subcutaneous adipose tissue (SAT) is located under the dermal layer. Visceral adipose tissue (VAT) is located around the internal organs. The two classes have similar morphological structures, but the most important aspect is their metabolic diversity (5).

In obesity, the phenomena of hypertrophy (increased adipocyte size) and hyperplasia (increased adipocyte number) are present. Hypertrophy is harmful and associated with the reduced release of adiponectin, increased release of pro-inflammatory cytokine and fatty acid, impaired insulin sensitivity, hypoxia, and immune cell activation. In contrast, hyperplasia has the opposite effect (6). Adipocytes are overloaded and lose their lipid storage capacity. They can store an excessive amount of fat and energy. Still, in the case of unnecessarily high food intake, a release of FFA from the adipocytes occurs which then is being stored in non-adipose tissue. This release has an adverse effect on the human body, called lipotoxicity (7).

Brown adipose tissue (BAT) is located supraclavicularly and paravertebrally. BAT controls the body's temperature by activating an uncoupling protein 1 (UCP1) located in the mitochondrial membrane. The UCP1 is stimulated by exposure to cold and immediately uses energy and conducts heat (6, 8). A meal rich in carbohydrates and essential macronutrients is also a stimulus to UCP1 for thermogenesis (9).

Additionally, a tissue that is a combination of both is described as a beige, so-called browning adipose tissue. It could emerge *de novo* from the progenitor cells or SAT under the influence of stimuli such as cold or by the activation of the β3-adrenergic receptor, for example, by catecholamines. Beige adipocytes contain UCP1 in ten-fold lower concentration than brown tissue. Thanks to brown and beige adipocytes' unique ability to generate energy and thus consume glucose and triglycerides, their protective effect against obesity is recognised (8, 10). The beneficial influence of browning WAT and energy expenditure may be considered one of the therapeutic goals in obesity treatment.

This paper aims to review the current state of knowledge on ceramides and S1P and their link to obesity. Considering the rapid development in this field of science, a summary of the available data could prove valuable.

CERAMIDES

Ceramides generally contain the sphingoid 18 carbon chain base with a 14 to 30 carbon length fatty acyl chain. They can be modified to produce more complex sphingolipids like sphingomyelin, galactosylceramide, glucosylceramide, ganglioside and globoside (11). As the primary components of the plasmatic membrane, ceramides have an impact on cell membrane properties. The potential for their redistribution within the membrane leads to a change in its activity and response to enzymes (12). When substrates for synthesis are provided in excess, ceramides may accumulate in tissues. There are three biosynthesis pathways leading to sphingolipid formation that have ceramides as their metabolic hub.

SPHINGOLIPIDS SYNTHESIS

Sphingolipids are a diverse lipid class built of an amino alcohol, sphingosine or dihydrosfingosine (sphinganine) as an N-acylated backbone. Due to the modification of this basic structure, the identification of a family of numerous sphingolipids such as ceramides, sphingomyelins, glycolipids, and gangliosides is possible. Structural variety is followed by a variety of multiple biological functions (11, 13). The synthesis of sphingolipids depends on many metabolic compounds exogenously delivered or transferred from sphingolipid turnover. There are three biosynthesis pathways (**Figure 1**).

1. The *de novo* pathway is placed in the endoplasmic reticulum (ER) and begins with the condensation of palmitoyl coenzyme A (CoA) and L-serine. Although palmitoyl-CoA and serine are preferred in this reaction, stearate or myristate and alanine or glycine can also be used. An enzyme mutation could cause the substrate shift (13). For example, alanine is used as a substrate in the serine palmitoyltransferase (SPT) mutation. As a result of the reaction, neurotoxic deoxysphingolipids are formed (14). Under normal conditions, the reaction generates 3-ketosphinganine by SPT. Subsequently, 3-ketosphinganine reductase is responsible for reducing 3-ketosphinganine to sphinganine, which is acetylated to dihydroceramide by ceramide synthase (CerS1-6). Dihydroceramide is oxidised by desaturase, which results in the formation of the ceramide. Ceramide synthase occurs in six isoforms in mammals, each of which creates a ceramide with a particular acyl chain length (C14:0-C30:0) (15). The specific location of those enzymes remained unclear. However, data indicates the ER as the primary site of CerS occurrence. Other localisations of CerS are mitochondria and the nucleus (16).
2. The *salvage pathway* is part of the second biosynthesis route. The ceramide is deacylated by ceramidases to produce sphingosine, which is phosphorylated by sphingosine kinases (SphK) to sphingosine-1-phosphate (S1P). As a result of further changes catalysed by S1P lyase, S1P is transformed into fatty aldehydes and ethanolamine phosphate, which become substrates for the cascade of enzymatic reactions from which fatty acyl-CoA is obtained. Another possible transformation of S1P is dephosphorylation by S1P phosphatase leading back to sphingosine and then by ceramide synthase to ceramide (17).
3. The *Sphingomyelin pathway* takes place in the Golgi apparatus. Through the action of sphingomyelin synthase out of a ceramide the sphingomyelin (SM) is formed. Afterwards SM is transported to the plasma membrane. In the plasma membrane, the reaction is reversible, with SM transforming back to ceramide using sphingomyelinase. Then, ceramides can be deacylated by ceramidase to

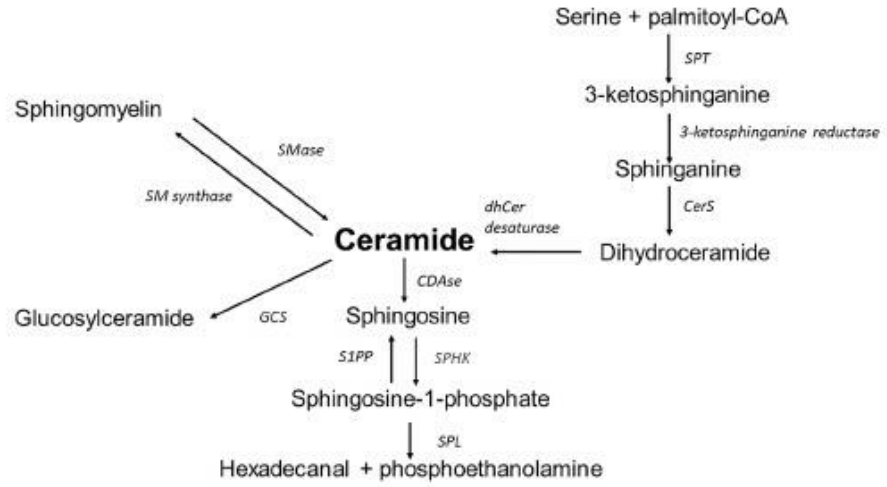

FIGURE 1 | Overview of sphingolipid metabolism; SPT, serine palmitoyltransferase; CerS, ceramide synthase; dhCer desaturase, dihydroceramide desaturase; S1PP, sphingosine-1-phosphate phosphatase; SPHK, sphingosine kinase; SPL, sphingosine-1-phosphate lyase; CDase, ceramidase; SMase, sphingomyelinase; SM synthase, sphingomyelinase synthase; GCS, glucosyl-ceramide synthase.

sphingosine, which can be phosphorylated by SphK to S1P. SM can be transported to the lysosome from the plasma membrane, where the cascade of reactions is the same; it progresses from SM through ceramide to sphingosine (18).

CERAMIDES IN SKELETAL MUSCLES

In skeletal muscles, which play an important role in glucose uptake, the accumulation of the ceramides is strongly related with insulin resistance and diabetes (19). Elevated total ceramides content disturbs the insulin pathway mainly at the level of kinase B (Akt) through the activation of protein phosphatase 2A (PPA2) which keeps Akt unphosphorylated, thereby inhibiting further steps of the pathway. Consequently, the glucose transporter type 4 (GLUT4) translocation to the plasma membrane is impaired and the muscles are not efficient in glucose uptake (20).

Data demonstrated that decreasing ceramide concentration in skeletal muscles eliminates the deleterious effect and improves glucose tolerance. Inhibition of SPT by myriocin treatment prevents ceramide-induced glucose intolerance and insulin resistance by enabling Akt phosphorylation (21). Moreover, research focused on the ceramide transport from the ER to the Golgi apparatus through the ceramide transporter (CERT) showed that CERT overexpression decreased ceramide accumulation in muscles thus improving insulin signalling (22).

CERAMIDES IN ADIPOSE TISSUE

The content of ceramides in obesity is different in every type of investigated adipose tissue. VAT shows a strong positive correlation with metabolic diseases and cardiovascular complications (23). Studies have shown that VAT shows a greater ability to change tissue metabolism than SAT. The proximity of internal organs enables VAT to modulate their metabolism easily. Data showed elevated levels of C14-Cer, C16-Cer and C18:1-Cer in the obese non-diabetic group compared to the lean non-diabetic group. Interestingly, further growth of C18-Cer and C24:1Cer was proven in the third obese diabetic group. This may indicate the direct participation of those components in the development of diabetes (24). The accumulation of ceramides in VAT was also observed in the metabolic syndrome (25). In both experiments, total ceramide concentration was increased in the VAT of obese subjects compared to lean non-diabetic patients. The enhanced ability to gather ceramides in VAT adipocytes may cause a weak insulin response, reduced lipogenesis and fewer lipid droplets than SAT. Visceral obesity positively correlates with glucose level, insulin resistance, TAG and cholesterol concentration (26).

However, the total ceramide content was also measured in the SAT. It proved to be elevated in lean patients compared to obese patients (27) and obese with metabolic syndrome (25). Another study reports a decreased total ceramide level in lean, healthy patients compared to obese patients (24). The ambiguity of the results is due to the difference in the location of the collected tissues. The SAT was taken from the abdomen (25, 27) and the sternum (24). Researchers suggest that excessive food intake leads to hyperplasia in lower body SAT and hypertrophy in upper body SAT (26). Such a high variability within one tissue prompts further research to deepen the understanding of the pathomechanisms occurring in it and the factors that may modulate its glucose and lipid metabolism.

It is considered that accumulation of ceramides in the adipose tissue negatively affects the inhibitory effect of insulin on

hormone-sensitive lipase (HSL) activity. Under physiological conditions HSL stimulates lipolysis but postprandial insulin release has a known HSL inhibitory effect. It has been demonstrated that in obesity accompanied by insulin resistance the accumulated ceramides influence insulin causing a decreased HSL inhibition. Consequently, resulting in an increased FFA concentration in the plasma (28, 29).

In the WAT isolated from obese humans and rodents, the CerS6 was significantly increased, and the correlation between BMI, hyperglycaemia and body fat content was favourable compared to lean subjects. An experiment with knockdown CerS6 mice was performed, demonstrating a reduced concentration of C16:0 in WAT, BAT and liver compared to control mice. The concentration in skeletal muscles was at the same level. However, most relevant was that in mice with a deletion of CerS6 despite being HFD-fed, a reduced body mass and body fat content, improved glucose metabolism, reduced adipocyte size and decreased leptin concentration were observed. Research revealed that deletion of CerS6 in BAT appears to be crucial in improving glucose homeostasis by increasing mitochondrial β-oxidation (30, 31). These data are strong evidence that C16:0, a product of CerS6, is a significant factor in the development of obesity and its related complications.

Data demonstrated that sensitising tissues to insulin is possible by inhibiting *de novo* ceramides synthesis using myriocin, which blocks SPT activity. A study was conducted on mice in which insulin resistance was induced by HFD. After *in vivo* myriocin treatment, a decreased Cer and diacylglycerol (DAG) concentrations in VAT and SAT were measured. Furthermore, a strong correlation between total ceramide content in AT and adiponectin secretion (negative) and TNFα levels (positive) was observed (28).

LIPOTOXICITY IN OBESITY

Lipotoxicity is caused by excessive nutrient intake and increased lipid levels in the bloodstream. This process leads to defective lipid oxidation, increased ceramide formation and accumulation of bioactive lipids in organs and tissues. Lipotoxicity has a substantial impact on pancreatic β-cells by impairing glucose-stimulated insulin secretion (32). Most significant is that ceramides contribute to β-cell apoptosis by releasing cytochrome c from the mitochondria and activating the apoptotic cascade in the lipotoxicity process (33). It has been shown that palmitate harms the insulin promoter and blocks insulin gene expression in rat pancreatic islets. The entire process is accompanied by *de novo* production of ceramides (34). However, fumonisin B1, a ceramide synthetase inhibitor, may be able to stop the harmful effects of palmitic acid and ceramides (32). Inhibition of ceramide synthesis prevents the harmful effects of palmitate on insulin gene expression (34).

Recently the promotion of lipotoxicity was indicated by activation of SphK2 in β-cells. The excess of palmitic acid present in obesity predisposes to redistribution of SphK2 from the nucleus to the cytoplasm; this signal to relocate is responsible for β-cell lipotoxicity. The lack of SphK2 ameliorates insulin secretion by protecting β-cells against apoptosis (35).

Lipotoxicity impairs the proper functioning of the liver, kidneys, and muscles, including cardiomyocytes. High levels of saturated fatty acids result in mitochondrial membrane superoxide and reactive oxygen species (ROS) production, causing oxidative stress with a reduced antioxidant response (36). Lipotoxicity leads to ER stress, which plays an essential role in insulin resistance and cell death. Interestingly, increased ER stress in the hypothalamus modulates the sympathetic response of BAT, leading to reduced thermogenesis and weight gain (37). Lipotoxicity is a destructive process that can contribute to the development of metabolic disorders.

SPHINGOLIPIDS AND ADIPONECTIN

Adiponectin controls lipid metabolism and glucose homeostasis by increasing glucose consumption in skeletal muscles. Adiponectin works with two receptors, AdipoR1 and AdipoR2. AdipoR1/2 have ceramidase activity by binding and hydrolysing the ceramide to FFA and sphingosine, substrates in S1P production (38). As a result, ceramide levels are reduced, and glucose utilisation and tissue insulin sensitivity are improved. Data confirms that this binding leads to lipid oxidation, mitochondrial biogenesis, and anti-apoptotic modifications. Lack of those receptors may be the reason for metabolic dysfunction (38, 39). It proves that adiponectin receptors may be crucial in bioactive lipid balance.

A study conducted on mice showed that increased concentrations of circulating adiponectin negatively correlates with ceramide levels. Moreover, it enhances insulin sensitivity caused by the fibroblast growth factor (FGF21), which stimulates adiponectin secretion. FGF21 treatment in mice showed an increased adiponectin secretion, reducing the accumulation of ceramides in tissues prevents lipotoxicity. In obese and diabetic mice, the FGF21 reduced blood glucose concentration and improved insulin sensitivity. However, adiponectin knockout mice showed no positive changes after FGF21 stimulation (40). Noteworthy is that the ablation of SPT also increased the release of FGF21 and improved metabolism (41).

SPHINGOSINE-1-PHOSPHATE

Ceramides are the primary source of *de novo* S1P synthesis through a process of sphingosine diacylation. Diacylation is catalysed by two isoenzymes, SphK1 (located in the cytoplasm) and SphK2 (located in the nucleus, mitochondria, and ER), both widely expressed in human tissues. S1P is a bioactive lipid that takes part in numerous cellular processes such as angiogenesis, cell growth, apoptosis and inflammation by binding to $S1P_{1-5}$ receptors (42, 43). S1P has anti-apoptotic properties, enhances insulin sensitivity and reduces immune response (44) (**Figure 2**). The study conducted on HFD mice demonstrated a positive influence of the S1P analogue on insulin signalling and reduced leukocyte accumulation in adipose tissue (50). On the other hand,

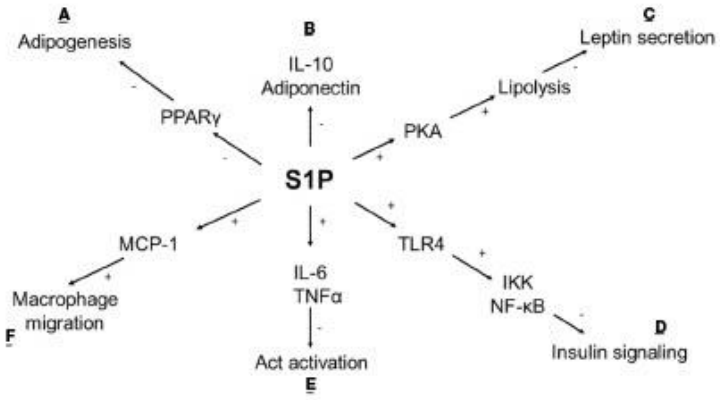

FIGURE 2 | Metabolic effect of S1P in adipocyte. **(A)** S1P inhibits adipogenesis by affecting the expression of transcriptional factor PPARγ (45); **(B)** S1P reduces the anti-inflammatory response (IL-10) and adiponectin synthesis which is accompanied by **(E)** an increased proinflammatory cytokine levels (IL-6, TNFα) lead to the activation of PP2A leaving Akt dephosphorylated and inactive (46); **(C)** In cAMP/PKA dependent pathway S1P stimulates lipolysis and inhibits insulin-mediated leptin synthesis (47); **(D)** S1P intensifies TLR4 activation cause impaired insulin signalling *via* IKK- NF-κB axis (48); **(F)** S1P stimulates secretion of chemokine MCP-1 leads to increased macrophage migration (49); (+) activation, (-) inhibition, TNFα -tumor necrosis factor, IL-6, interleukin 6; IL-10, interleukin 10; Act, protein kinase 3; TLR4, Toll like receptor 4; IKK, inhibitor kappa kinase; NF-κB, nuclear factor κB; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor gamma; MCP-1, monocyte chemoattractant protein-1.

an increased level of S1P is observed in the SAT of obese diabetic patients, and a negative effect on insulin signalling is confirmed (24, 51, 52). The difference may depend on a non-specific affinity of S1P to the S1P receptor. It was surprising that in-vitro S1P interacts with CerS2 by a motif located in CerS2, which is similar to the S1P receptor causing the inhibition of CerS2. This could explain the antagonistic effect of S1P on ceramides (53).

Another critical point is the tissue of action, the type of SphK isoenzymes and their expression. In muscles, S1P leads to Akt activation, responsible for improved insulin response by increasing glucose uptake and glycogen synthesis (54). By contrast, in adipose tissue, S1P inhibits Akt activation after insulin stimulation (49). In both tissues, the increased expression of SphK1 was observed. No data indicate the participation of SphK2 in the impaired insulin response of the described tissues. The SphK1 and SphK2 effect on pancreatic β cell activity is believed to be antagonistic. Saturated fatty acids stimulate the SphK1/S1P axis by inhibiting lipotoxicity-induced β cell apoptosis (55).

In contrast, the SphK2 under the lipotoxic condition passes to the cytoplasm, promoting the apoptosis of β-cells and leading to impaired glucose homeostasis (35, 56). Surprisingly, the positive role of SphK1 and SphK2 was observed after exposure to high glucose levels. This resulted in increased S1P production and elevated insulin synthesis and secretion, leading to reduced serum glucose level (57).

Another controversy is the influence of S1P on inflammatory processes. In obesity a chronic inflammation state is present. As an endocrine organ, adipose tissue secretes adipokines and chemokines such as pro-inflammatory cytokines. HFD results in an accumulation of DAG and ceramides in the adipose tissue and, simultaneously, leads to an increased SphK1 expression and conversion of ceramide to S1P. S1P promotes pro-inflammatory cytokine expression (TNFα, Il-6) and secretion in adipose tissue Studies have shown that the SphK1 deficiency in DIO mice resulted in enhanced adipogenesis and anti-inflammatory cytokine expression (Il-10). Further, glucose tolerance and insulin sensitivity in muscle and adipose tissue were improved (49).

In contrast, endogenous S1P has a protective impact on β-cells against cytokine-induced apoptosis in rat islets (58). The difference in the S1P action is determined by the protein with which S1P is combined. In the bloodstream, S1P is transported by albumin (~35%) or apolipoprotein M (apoM) combined with HDL cholesterol (~65%) (42). Albumin is a protein which binds many hydrophobic compounds in the bloodstream whereas apoM/HDL remains specific and probably is critical in biological response. The S1P/apoM/HDL complex reveals an anti-inflammatory effect on endothelial cells and helps to maintain vascular integrity, which is the aim of vascular disease treatment (59).

CONCLUSIONS

Obesity is increasingly a global problem. The basis of complications related to obesity is adipose tissue overgrowth and accumulation of bioactive lipids. Their role seems to be crucial in insulin resistance, diabetes, hypertension and dyslipidaemia development (60).

Treatment that reduces sphingolipid levels in the bloodstream is a promising method in fighting obesity and other related diseases (61, 62). Nevertheless, continuous identification of the mechanisms controlled by bioactive lipids is essential. The cognition of modulation of immune response, thermogenesis, glucose, and lipid homeostasis by sphingolipids will be crucial in the upcoming years.

AUTHOR CONTRIBUTIONS

IJ wrote the draft of the manuscript. MK reviewed and edited. JS reviewed and edited. All authors contributed to the article and approved the submitted version.

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miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus

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Introduction: Circulating miRNAs are important mediators in epigenetic changes. These non-coding molecules regulate post-transcriptional gene expression by binding to mRNA. As a result, they influence the development of many diseases, such as gestational diabetes mellitus (GDM). Therefore, this study investigates the changes in the miRNA profile in GDM patients before hyperglycemia appears.

Materials and Methods: The study group consisted of 24 patients with GDM, and the control group was 24 normoglycemic pregnant women who were matched for body mass index (BMI), age, and gestational age. GDM was diagnosed with an oral glucose tolerance test between the 24th and 26th weeks of pregnancy. The study had a prospective design, and serum for analysis was obtained in the first trimester of pregnancy. Circulating miRNAs were measured using the NanoString quantitative assay platform. Validation with real time-polymerase chain reaction (RT-PCR) was performed on the same group of patients. Mann-Whitney U-test and Spearman correlation were done to assess the significance of the results.

Results: Among the 800 miRNAs, 221 miRNAs were not detected, and 439 were close to background noise. The remaining miRNAs were carefully investigated for their average counts, fold changes, p-values, and false discovery rate (FDR) scores. We selected four miRNAs for further validation: miR-16-5p, miR-142-3p, miR-144-3p, and miR-320e, which showed the most prominent changes between the studied groups. The validation showed up-regulation of miR-16-5p ($p<0.0001$), miR-142-3p ($p=0.001$), and miR-144-3p ($p=0.003$).

Conclusion: We present changes in miRNA profile in the serum of GDM women, which may indicate significance in the pathophysiology of GDM. These findings emphasize the role of miRNAs as a predictive factor that could potentially be useful in early diagnosis.

Keywords: gestational diabetes, miR-16-5p, miR-142-3p, miR-144-3p, epigenetics, serum profiling, biomarkers, miRNA

INTRODUCTION

Gestational Diabetes Mellitus (GDM) is one of the leading diseases during pregnancy. According to the newest edition of the International Diabetes Federation (IDF) Diabetes Atlas, GDM affected nearly 17 million live births in the last year (1). Extensive hormonal changes during pregnancy are one of the reasons for increased insulin resistance. For instant, the hyperestrogenemic state observed during pregnancy contributes to alterations in insulin sensitivity. Estrogen may bind directly to insulin or its receptors, making them unavailable for insulin (2). Furthermore, human placental lactogen (hPL) decreases maternal insulin sensitivity in order to provide the fetus with sufficient nutrition (3). When the insulin release is insufficient and a glucose-lowering response is not achieved, the risk of GDM development is high (4). Meta-analysis showed that the most relevant risk factors for GDM are high BMI and thyroid disease (5). Another risk factors are increased fasting glycemia in the first trimester of pregnancy, abdominal obesity, family history of diabetes mellitus, genetic factors, environmental factors including lifestyle and diet, comorbidities like polycystic ovary syndrome (PCOS) (6, 7). Combinations of several risk factors more confidently indicate women at high risk of developing GDM (8). Considering, that utility of risk factors, such as i.e first-trimester fasting blood glucose concertation is limited (9), it is essential to search for the most ideal non-invasive biomarker for early GDM detection or even a predisposition to develop GDM.

MiRNAs are a group of non-encoding RNA molecules of 19-22 nucleotides that play a key role in the regulation of post-transcriptional gene expression (10, 11). Notably, one miRNA has the ability to bind with many genes by recognizing the not-necessarily complementary sequence at the end of the 3'-untranslated region (3'UTR) of the target mRNA (12). In this way, endogenous miRNAs control the expression of many genes and influence the processes that take place in cells, such as cell metabolism, proliferation, DNA repair, and apoptosis. Furthermore, data suggest that extracellular miRNAs act as modulators during physiological and pathological processes by transferring information between cells (13). Depending on which gene that the miRNA impacts, it can be either a stimulator or a suppressor of a pathological state (14).

MiRNA is detectable in various biological fluids, such as blood, urine, tears, saliva, and cerebrospinal, amniotic, or synovial fluid (15). In contrast to other RNA molecules, an important feature of miRNA is their stability and resistance to external factors, such as RNAse (16). This is due to the form in which they occur in biofluids. MiRNA forms complexes with lipoproteins or proteins (17). Moreover, the protective effect may be a result of their encasement inside membrane structures like exosomes, microparticles, or apoptotic bodies (17, 18). It has also been shown that repeated cycles of freezing and thawing do not cause significant changes in miRNA content in the serum (19). These mechanisms and non-invasive collection mean that circulating miRNAs have good potential as a biomarker.

In recent years, there have been a number of reports on changes in miRNA expression in various diseases, including metabolic disorders. One of the ultimate purposes of most of the studies is finding miRNAs that could help with identifying pathological processes, estimate the success of a patient's response to therapy (20), or support the identification of high-risk groups (21). Zhao et al. were some of the first to describe changes in the sera of pregnant women with GDM (22). Since that time, many scientists have focused on changes in miRNA expression in GDM, but the available data are not consistent. Thus, the purpose of this study was to compare the miRNA expression profile in a group of patients in the first trimester of pregnancy and GDM diagnosed in the second trimester of pregnancy with that of a healthy control group. Then, based on these results, we sought to identify potential biomarkers of early GDM diagnosis.

MATERIALS AND METHODS

Study Population

Project included four meetings, in the first trimester (9-12 week), in the second (24-26 week), in the third trimester (34-37 week) and three months after delivery. During the first trimester of pregnancy, fasting venous blood samples were collected into S-Monovette Gel Clotting Activator tubes (Sarstedt, Numbrecht, Germany). After complete clotting and centrifugation, the serum to be used for miRNA analysis was separated, transferred into DNase- and RNase-free tubes (Eppendorf, Hamburg, Germany), and stored at -80°C until they were assayed. To diagnose GDM all patients underwent a 75g oral glucose tolerance test (OGTT) in the second trimester, between 24^{th} and 27^{th} weeks of pregnancy. GDM was diagnosed according to the World Health Organization (WHO) criteria (23). In the experiment the serum from the first trimester was examined while both groups revealed normoglycemia. The study group (GDM) (n=24) and control group with normal glucose tolerance (NGT) (n=24) were carefully matched for pre-pregnancy body mass index (BMI), age, and gestational age. Women with the history of GDM, stillbirth, childbirth with congenital anomalies, pregnancy-induced hypertension, preeclampsia, cholestasis, premature delivery, acute or chronic inflammation, multiple pregnancy, pre-existing glucose intolerance, and active smokers were excluded from the study. Written informed consent was obtained from each patient, and the study was approved by the local ethics committee (Medical University of Bialystok).

Biochemical Methods

Plasma glucose concentrations were measured using an enzymatic method with hexokinase (Cobas C11, Roche Diagnostics Ltd, Switzerland), and the serum insulin level was evaluated by an immunoradiometric method (DiaSource Europe SA, Belgium) using a Wallac Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Life Science, Turku, Finland). Glycated hemoglobin (HbA1c) was assayed by high-performance liquid chromatography (Bio-Rad D-10, Bio-Rad Laboratories, Hercules, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function were calculated for all women in each trimester

of pregnancy. Moreover, in the second trimester, insulin sensitivity was measured using the OGTT insulin sensitivity index of Matsuda and DeFronzo (ISI_{OGTT}).

miRNA Isolation

MiRNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) by following the manufacturer's protocol. The isolation method is based on the innovative spin-column separation method with a silica membrane. The use of this kit allows us to obtain miRNA of high quality and purity, which is necessary for the subsequent stages of the experiment. The content of miRNA in extracted samples was checked with a fluorometer (Qubit 3.0, Thermo Fisher Scientific, Waltham, USA).

Nanostring Analysis

For miRNA profiling, we used NanoString technology with a digital color-coded barcode for direct and multiplex marking of target sequences of 800 miRNAs. The method uses about 50 nucleotide probes per 1 miRNA. At the 5' end, a set of 6 fluorescently labeled "barcodes" is placed, and at the 3' end, a "capture probe" with biotin is placed. One set allows for simultaneous determination of 800 miRNAs in 12 samples.

Due to the procedure used, the cDNA synthesis and amplification stages were omitted, which allows us to reduce the probability of laboratory error. The results were read out on a NanoString nCounter scanner. The first stage of the analysis was the hybridization of individual miRNAs with specific probes, and the next was the purification and placing of hybridized samples on a specially standardized plate. The last stage was reading of the obtained results. The method allowed for the exact number of miRNA copies to be specified in each sample.

RT-PCR Validation

Validation of the results was carried out on the same group of patients (24 women in the NGT control group and 24 in GDM study group). To validate the results, the real-time PCR method was used. In the first step, reverse transcription was performed to transcribe miRNA to cDNA using the miRCURY LNA RT Kit (Qiagen, Germany) in accordance with the manufacturer's procedure on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, USA). Subsequently, we performed RT PCR reaction using the miRCURY LNA SYBR Green PCR Kit (Qiagen, Germany) and specific primers for each of the analyzed miRNAs (Qiagen, Germany) on a LightCycler 480 thermal cycler (Roche Diagnostics Ltd, Switzerland). Expression of circulating miRNAs was evaluated using miR-103a-3p as an endogenous control gene. All samples were assayed in duplicate, and the comparative Ct method was used to calculate the relative changes in gene expression.

Data Analysis

Analysis of raw miRNA data obtained using NanoString technology was performed in nSolver software version 4.0. Data were normalized by the average geometric mean of the top 100 probes detected. The miRNAs' expression values in RT-PCT validation were calculated based on the ΔΔCT method. The differences in miRNA expressions between groups were calculated by the Mann-Whitney U test using Statistica 13 for Microsoft Software (StatSoft Inc., Tulsa, USA). The relationships between variables were tested using the Spearman rank correlation coefficient. Results were considered statistically significant with p-value less than 0.05.

RESULTS

Characteristics of the Groups Studied

The clinical characteristics of the studied groups are presented as medians and interquartile ranges (**Tables 1**, **2**). In the 1st trimester of pregnancy, there were no significant differences between groups. Women in both groups were normoglycemic. Most patients had normal pre-pregnancy BMI (n=10 in GDM group and n=11 in NGT group had BMI >25 kg/m^2 indicating overweight). In the 2nd trimester, groups revealed significant differences in fasted and post-loaded glucose measurements (glucose at 0, 30, 60, and 120 minutes: p=0.0001, p=0.0000, p=0.0000, and p=0.001, respectively). The GDM group had a higher insulin level at 60 minutes (p=0.02), insulin level at 120 minutes (p=0.004), and HOMA-IR (p=0.02). Fasting insulin and

TABLE 1 | Clinical characteristics of groups studied in the 1st trimester.

	NGT	GDM	p-value
n	24	24	
Age (years)	28 (26-31.5)	26 (24-30.5)	0.36
Pre-pregnancy BMI (kg/m^2)	21.8 (20.0-28.2)	23.5 (21.6-26.8)	0.73
Gestational age (week)	11 (10-12)	10 (9.5-11)	0.24
Fasting glucose (mg/dl)	86 (84-88)	87.5 (85-90)	0.29
Fasting insulin (μU/ml)	10.7 (9.1-12.9)	11.3 (10.2-13.3)	0.25
HOMA-IR	2.3 (1.9-2.8)	2.5 (2.1-2.9)	0.18
HOMA-β	168.0 (145.5-187.5)	166.5 (146.9-201.0)	0.97
HbA1c (%)	5.0 (4.9-5.4)	5.0 (4.9-5.4)	0.98
Total cholesterol (mmol/l)	170 (149.5-191.5)	169.5 (156.5-186)	0.81
HDL-cholesterol (mmol/l)	81 (69.5-90.5)	73.5 (59.5-84.5)	0.19
LDL-cholesterol (mmol/l)	78.2 (64.3-91.6)	81 (64.9-95.3)	0.78
Triglycerides (mmol/l)	72 (60.5-109.5)	95.5 (68.5-118)	0.14

Data are shown as medians (interquartile range); The difference between NGT vs GDM group was compared with the Mann-Whitney U-test.

TABLE 2 | Clinical characteristics of groups studied in the 2nd trimester.

	NGT	GDM	P value
n	24	24	
Gestational age (week)	25 (25-26)	25 (25-26)	0.81
Fasting glucose (mg/dl)	82.5 (79-85)	92 (84-94)	0.0001
Glucose 30' (mg/dl)	127.5 (121-140)	158 (148-165)	< 0.0001
Glucose 60' (mg/dl)	122 (101.5-141.5)	169 (136.5-184)	< 0.0001
Glucose 120' (mg/dl)	105.5 (86-119)	125.5 (111.5-166)	0.001
Fasting insulin (μU/ml)	11.2 (8.6-13.3)	13.4 (10.1-18.2)	0.08
Insulin 30' (μU/ml)	74.2 (60.0-110.9)	80.1 (61.0-137.3)	0.62
Insulin 60' (μU/ml)	80.1 (54.3-107.2)	106.5 (74.2-174.0)	0.02
Insulin 120' (μU/ml)	56.0 (42.0-72.4)	108.8 (60.5-131.0)	0.004
HOMA-IR	2.3 (1.7-2.7)	3.0 (2.1-4.4)	0.02
HOMA-β	188.3 (168.8-282.0)	191.4 (149.4-240.3)	0.21
ISI OGTT	4.4 (3.4-5.4)	2.8 (2.1-3.9)	0.002
HbA1c (%)	4.8 (4.7-5.1)	4.9 (4.6-5.1)	0.74
Total cholesterol (mmol/l)	267.5 (203-287.5)	238 (188-257)	0.046
HDL-cholesterol (mmol/l)	96.5 (85.5-108.5)	85 (69.5-104.5)	0.13
LDL-cholesterol (mmol/l)	131 (101.4-171.2)	119.2 (85.0-140.4)	0.12
Triglycerides (mmol/l)	138 (120.5-170.5)	159 (135.5-204.5)	0.13

Data are shown as medians (interquartile range); The difference between NGT versus GDM group was compared with the Mann-Whitney U-test.

insulin after 30 minutes post-loading were also higher in the study group than in the NGT group, but the differences were insignificant. Moreover, the GDM group demonstrated lower ISI_{OGTT} (p=0.002) and lower total cholesterol (p=0.046) than the NGT group.

Nanostring Profiling

We identified 28 miRNAs with expression that was significantly altered in the GDM group compared to the NGT group (p-value p<0.05). A careful analysis was done while considering not only the p-value, but also the false discovery rate, count ranges, fold change, and standard deviation. The results pointed out miR-16-5p (p=0.07), miR-142-3p (p=0.02), miR-144-3p (p=0.003), and miR-320e (p=0.02) for further validation. Changes in expression of miR-16-5p were not significant, whereas the mean value ranges of the counts were high (GDM=1056.03 versus NGT=756.86) with a wide standard deviation. Considering the method of simultaneous determination of many miRNAs and high count number, we decided to evaluate these molecules in further analysis.

Validation of the Results

NanoString results were validated by RT-PCR. The fold change of gene expression was calculated using the ΔΔCt method, and then log transformation was used to avoid a non-normal distribution of the results. We obtain confirmation of three miRNAs: miR-16-5p (p<0.0001), miR-142-3p (p=0.001), and miR-144-3p (p=0.003), which were significantly upregulated in the GDM group. No significant difference was observed for miR-320e (p=0.16) (**Figure 1**).

ROC curve analysis was performed for significant miRNAs in the 1st trimester of pregnancy as parameters to discriminate those who are at high risk group of developing GDM in the 2nd trimester of pregnancy (**Figure 2**). The AUC for miR-16-5p was 0.868 (95% confidence interval: 0.757–0.98; p<0.0001). AUC was 0.778 (95% confidence interval: 0.644-0.913; p<0.0001) for miR-142-3p, and for miR-144-3p, AUC was 0.756 (95% confidence interval: 0.613-0.898; p=0.0004).

The relationships between prominent molecules' expressions and other variables were checked. Across the study population, 1st-trimester miR-16-5p expression correlated positively with fasting plasma glucose concentration in the 2nd trimester (R=0.56, p<0.05), plasma glucose concentration at 30 minutes post-loading (R=0.43, p<0.05), and HOMA-IR (R=0.36, p<0.05). Its expression negatively correlated with ISI_{OGTT} (R=-0.34, p<0.05). MiRNA-142-3p positively correlated with plasma glucose levels post-loading with indexes as follows: 30 minutes (R=0.35, p<0.05), 60 minutes (R=0.37, p<0.05), and 120 minutes (R=0.36, p<0.05). Furthermore, there were correlations between miR-144-3p and plasma glucose concentration at 30 minutes post-loading (R= 0.41, p<0.05) and the plasma glucose level at 60 minutes post-loading (R=0.42, p<0.05), as well as a negative correlation with ISI_{OGTT} (R=-0.33, p<0.05). Multiple regression analysis confirmed the dependences described except for the association of miR-16-5p and ISI_{OGTT}.

DISCUSSION

Researchers for many years have been trying to find the most ideal GDM biomarker. Among many significant features of the perfect indicator the most relevant is prediction value (24). In case of GDM, the diagnosis nowadays is based on OGTT performed in the second trimester of pregnancy. Considering complications during pregnancy and delivery and a high risk of long-term complications for the child and the mother the GDM a biomarker revealed before changes in the glycemia occur seems to be crucial. Yoffe et al. (25) studied women between 9th and 11th weeks of pregnancy and showed an up-regulation of the miR-223 and miR-23a in plasma of GDM women. Interesting point of view was presented by Wander et al. connection of the miR-21-3p and miR-210-3p with GDM diagnosed in overweight and

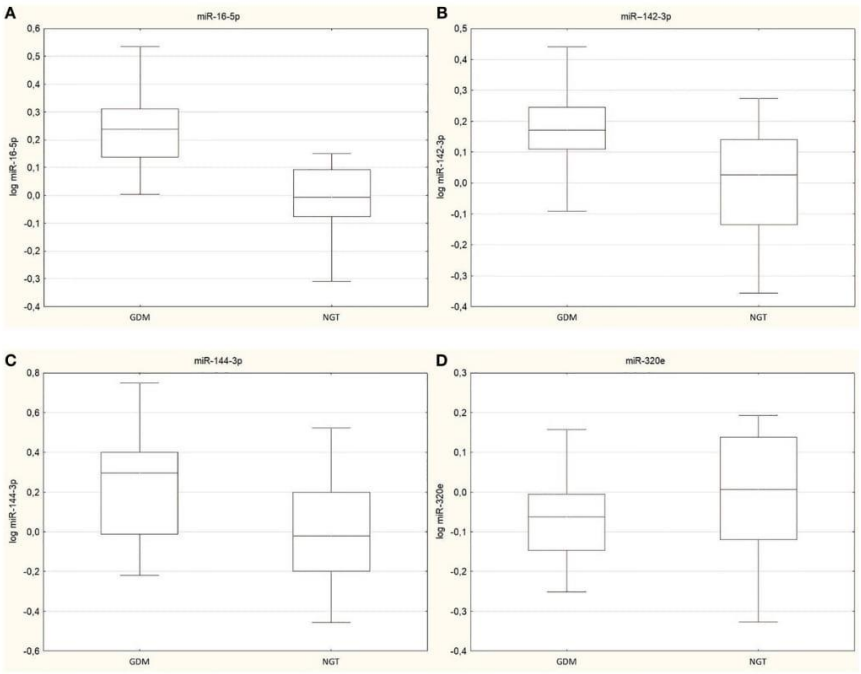

FIGURE 1 | Box plots presented changes in expression of validated miRNAs between GDM group and NGT, **(A)** miR-16-5p ($p<0.0001$), **(B)/** miR-142-3p ($p=0.001$), **(C)** miR-144-3p ($p=0.003$) and **(D)** miR-320e ($p=0.16$). Data are presented by median indicated by line in each box and interquartile range. Maximum and minimum values are represented by whiskers.

obese women (26). Lamadrid-Romero et al. (27) showed that miR-183-5p was increased in every trimester in serum collected from women diagnosed with GDM. Simultaneously, the higher expression of miR-125b-3p, miR-200b-3p and miR-1290 were observed in the first trimester of pregnancy.

Our study shows that circulating miR-16-5p is upregulated in women before the onset of GDM, which is consistent with the results obtained by other studies. Zhu et al. (28) conducted studies on women at 16-19 weeks of pregnancy and described five molecules that were upregulated in the GDM group (e.g., miR-16-5p). Other studies reported increased expression of miR-16-5p in serum at 24 -28 weeks of pregnancy (29). Our results show this difference earlier between the 9^{th} and 12^{th} weeks of pregnancy. Moreover, we observed a positive correlation with HOMA-IR, which was also described by Cao et al. (29). Apart from miR-16-5p they described an up-regulation of the miR-17-5p and miR-20a-5p which was not observed in our experiment.

Attempts were made to determine miR-16-5p in leukocytes of women with GDM, but no significant differences were observed (30, 31). It turns out that high miR-16-5p expression also persists after pregnancy and correlates with high cardiovascular risk (32). This indicates that epigenetic changes during GDM are permanent, and women with a history of GDM are predisposed to the development type 2 diabetes (T2D) or cardiovascular disease in the following years (33). Another study revealed increased expression of miR16-5p in overweight women before the 20^{th} week of pregnancy. In contrast to previously cited reports, that study was conducted on European women (34). On the other hand, Martinez-Ibarra et al. demonstrated no significant changes in miR16-5p expression in serum collected in the 2^{nd} trimester from GDM patients compared to NGT (35). A similar result was obtained by scientists from South Africa (36).

Considering that miRNAs could be related to genetic and environmental factors, Sørensen et al. proposed ethnicity as a potential explanation of differences in obtained results (34). Furthermore, they also suggested age, which is a known risk factor for GDM. The idea was supported by the correlation obtained between age and miR-16-5p expression. However, this dependence was not observed in our study.

Available data show that miR-16-5p is one of the most potent regulating molecules in the insulin-signaling pathway. Target genes for miR-16-5p encode insulin receptor substrate (IRS) proteins 1 and 2 and the insulin receptor itself (INSR) (37, 38). These proteins are crucial factors in a proper insulin signaling pathway, and their downregulation results in insulin resistance and metabolic disorders like diabetes. Additionally, miR-16-5p-targeted genes are involved in pancreatic β-cell proliferation and apoptosis (39). Target genes for miR-16-5p that are downregulated in type 2 diabetes are located in not only β-

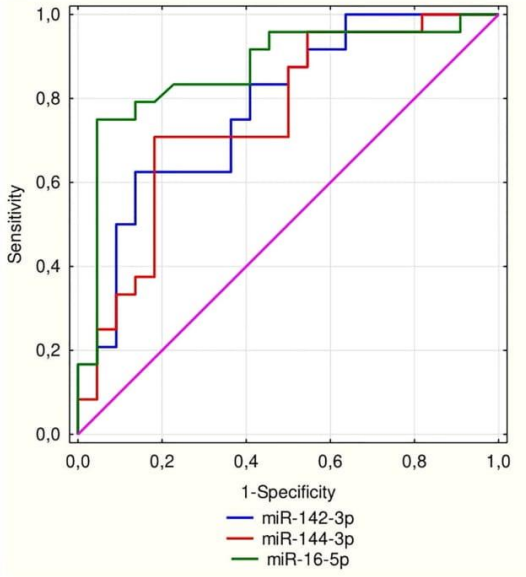

FIGURE 2 | ROC curve for miRNA 16-5p (AUC = 0.868; $p < 0.0001$), miR-142-3p (AUC = 0.778; $p < 0.0001$) and for miR-144-3p (AUC = 0.756; $p = 0.0004$).

cells on pancreatic islets, but also peripheral blood mononuclear cells (PBMCs), the liver, and skeletal muscle (40).

An experimental study on *Cmah*-null mice showed that diabetic mice have upregulated miR-16-5p (among others) and downregulated IRS1, IRS2, AKT1, and mTOR mRNA (41). As a result of these changes, the crucial pathway in insulin-signaling PI3K-Akt-mTOR is dysregulated (42). Interestingly, Lee et al. (43) demonstrated a decrease in miR-16-5p expression in insulin-resistant skeletal muscle. Moreover, their *in-vitro* study revealed that miR-16-5p is involved in autophagy through controlling Bcl-2 protein synthesis. Also, an overexpression of miR-16-5p was accompanied by decreased mTOR content. Based on these findings, the inhibition of miR-16-5p expression might be important in treatment (44).

There are two reports on miR-142-3p in GDM. However, neither of these studies considers circulating human miR-142-3p. Collares et al. (45) described nine miRNAs (e.g., miR-142-3p) that are upregulated in PBMC obtained from type 1 diabetes (T1D), T2D, and gestational diabetes mellitus. The study did not associate the molecule with a specific gene, but its involvement in diabetes in general was noticeable. A study conducted on GDM-induced mice reported an overexpression of miR-142-3p in the circulating blood and embryonic tissue of GDM mice. Data demonstrated that *in-vitro* up-regulation of miR-143-3p has a positive effect on β-cells by promoting their proliferation, as well as inhibiting apoptosis by blocking the expression of p27, Bax, and caspase-3. In addition, bioinformatic analysis indicated forkhead box protein O1 (FOXO1) as a target gene for miR-142-3p (46). FOXO1 is known as a multifunctional protein, and besides controlling glycogenolysis and gluconeogenesis, it regulates the differentiation of β-cells and promotes their apoptosis (47). This could be a self-protective effect of miR-142-3p.

Escalated expression of miR-142-3p has been described in obese adults as a parameter that is strongly associated with insulin, HOMA-IR, BMI, adiponectin, and leptin levels (48). Similar results were obtained in the case of childhood obesity, which revealed an increased concentration of miR-142-3p and a positive correlation with BMI, fat mass, adipose tissue distribution, and HOMA-IR. Interestingly, during a 3-year follow-up, upregulation in the expression of this molecule was observed solely in the serum of patients whose BMI remained stable or decreased (49). The data showed that the expression of the miR-142-3p may be sex-related.

Overexpression of miR-142-3p in the group of patients with pre-diabetes and diabetes was found only among women (50). The studies mentioned the possibility of age affecting these results because the male group was significantly younger. However, there may also be an influence from the distribution of adipose tissue according to the studies cited. In contrast to our study, Liang et al. showed a decreased expression of miR-142-3p in the serum of T2D patients, and a negative correlation with HOMA-IR was observed (51). In the present study, a positive correlation was revealed between miR142-3p and plasma glucose post-loading.

Another study has shown that miR-144-3p is upregulated in the liver, pancreas, skeletal muscle, adipose tissue, and blood of a diabetic rat model. The result was confirmed in circulating blood obtained from human T2D patients. In addition, a study of pancreatic cells cultured from rats revealed an increased level of miR-144-3p in a high glucose environment, and similar to miR-16-5p, it caused a downregulation of the expression of IRS1 (52). Moreover, the upregulation of miR-144-3p was observed in PBMCs collected from patients with T1D, T2D, and GDM (45). However, Akerman et al. (53) investigated patients with T1D and did not observe an elevation of serum miR-144-3p levels, but there was a positive correlation with islet antigen 2 antibodies (IA2A), indicating a possible relationship with the assessment of those at risk for T1D development.

Upregulated expression and a positive correlation with HOMA-IR of circulating miR-144-3p were observed in a Chinese cohort with impaired fasting glucose (IFG). Furthermore, high miR-144-3p was a predictor of T2D development (51). Interestingly, Wang et al. (54) showed an increased expression of miR-144-3p in T2D patients but solely in a Swedish population, not in patients from Iraq. Thus, this report confirms the contribution of environmental factors to epigenetic changes mentioned above. In a meta-analysis, Zhu and Leung (55) selected eight molecules as potential biomarkers of T2D, including miR-142-3p and miR-144-3p.

In summary, we found significantly upregulated expression of miR-16-5p, miR-142-3p, and miR-144-3p in the serum of patients in their 1st trimester of pregnancy who suffered from GDM diagnosed in the 2nd trimester. NanoString technology allowed us to study a wide panel of miRNA profiles. Considering

the research on miRNAs, a strong point of our experiment was the large number of patients in the studied groups. Although our findings are limited by the validation using the same group of women, our observations strongly suggest that changes taking place in the miRNA profile occur earlier than changes in glucose levels, and research on the more sensitive and specific biomarkers of GDM should be continued.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee Medical University of Bialystok. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: IJ and MK. Methodology: MN, IJ, IS and AB. Formal analysis: MZK and AK. Writing—original draft preparation: IJ and MK. Writing—review and editing: AJK and JS. Supervision: JS and AJK. All authors contributed to the article and approved the submitted version.

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Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes

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Abstract: We hypothesized that sphingolipids may be early biomarkers of gestational diabetes mellitus (GDM). Here, 520 women with normal fasting plasma glucose levels were recruited in the first trimester and tested with a 75 g oral glucose tolerance test in the 24th–28th week of pregnancy. Serum sphingolipids concentrations were measured in the first and the second trimester by ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC/MS/MS) in 53 patients who were diagnosed with GDM, as well as 82 pregnant women with normal glucose tolerance (NGT) and 32 non-pregnant women. In the first trimester, pregnant women showed higher concentrations of C16:0, C18:1, C22:0, C24:1, and C24:0-Cer and lower levels of sphinganine (SPA) and sphingosine-1-phosphate (S1P) compared to non-pregnant women. During pregnancy, we observed significant changes in C16:0, C18:0, C18:1, and C24:1-Cer levels in the GDM group and C18:1 and C24:0-Cer in NGT. The GDM (pre-conversion) and NGT groups in the first trimester differed solely in the levels of C18:1-Cer (AUC = 0.702 p = 0.008), also considering glycemia. Thus, C18:1-Cer revealed its potential as a GDM biomarker. Sphingolipids are known to be a modulator of insulin resistance, and our results indicate that ceramide measurements in early pregnancy may help with GDM screening.

Keywords: gestational diabetes; sphingolipids; ceramides; lipidomic; C18:1-Cer

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1. Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance first recognized during pregnancy. It is usually the result of β-cell dysfunction on a background of chronic insulin resistance [1]. Other factors affecting insulin sensitivity are maternal obesity, extensive hormones release, adipocytokine production, genetic and epigenetic changes, and novel potential omics factors [2]. Hyperinsulinemic-euglycemic clamp studies in healthy, lean women show that insulin sensitivity is reduced by 56% compared with pre-pregnancy, and basal endogenous glucose production is increased by 30% in the third trimester [3,4]. During pregnancy, we observe an increase in lipid concentration, especially in triglycerides, and, to a lesser extent, phospholipids and cholesterol. It is the result of altered maternal metabolism [5].

Insulin resistance and hyperlipidemia are important physiological processes essential during pregnancy to ensure sufficient fetal nutrition. In women with GDM, the physiological changes in insulin and lipids are excessive but also transitional and may indicate

underlying metabolic dysfunction [6,7]. As is well known, lipids perform a crucial role in the biology of the human body, not only being an energy storage and a component of cell membranes, but also acting as an agent in signaling pathways and altering the metabolism. Disturbances in lipid metabolic signaling pathways are associated with inflammation and systemic diseases such as the metabolic syndrome and hypertension [8]. Population-based lipidomic studies indicate that a number of ceramides (Cers), sphingomyelins, and lactosyloceramides are significantly downregulated years before type 2 diabetes onset, suggesting that the downregulation of sphingolipid metabolism could be partially responsible for the future onset of type 2 diabetes among women with GDM history [9]. Furse et al. showed that lipid metabolism was altered at least 10 weeks before a clinical diagnosis of GDM was made [10].

Sphingolipids are a group of biologically active lipids involved in regulation of various cellular processes including cell migration, inflammatory response, proliferation, differentiation, and apoptosis [11,12]. The central molecule in sphingolipid metabolism and the precursor for the complex sphingolipids is ceramide. Available data suggest that it is also a major contributing factor of insulin resistance in skeletal muscles and the liver [13–15]. These compounds induce insulin resistance at the level of RACα serine/threonine-protein kinase, also known as Akt or protein kinase B-PKB [16]. This compound activates protein phosphataseA2 (PPA2) and directly catalyzes PKB/Akt dephosphorylation, thus, inhibiting the activity of the insulin pathway [16]. Moreover, type 2 diabetes is often associated with chronic, moderate inflammation. Sphingosine-1-phosphate (S1P) belongs to the sphingolipid family and is a pro-inflammatory compound that increases the expression and secretion of cytokines (e.g., TNFα, IL-6, MCP-1) [17]. However, the effect of S1P on the inflammatory response has been demonstrated to be dependent on a carrier protein. The major carrier proteins for S1P are apolipoprotein M (apoM) and albumin. Most of the plasma S1P is bound to the apoM/ApoM-S1P that binds preferentially to HDL. ApoM-S1P has been shown to inhibit inflammatory responses in endothelial cells [18]. These features suggest that S1P may induce the disorders leading to GDM, but there is little literature data on this subject.

Here, we hypothesized that circulating sphingolipids may be early biomarkers of GDM development. To test this hypothesis, serum sphingolipid levels were measured in the first and the second trimester and compared between the patients with normal glucose tolerance (NGT) and GDM diagnosed between 24 and 28 weeks of pregnancy.

2. Materials and Methods

2.1. Study Population

Women (n = 520) with normal fasting plasma glucose levels (<92 mg/dL (5.1 mmol/L)) were recruited in the first trimester of pregnancy from the Gynecological Out-Patient Clinic of the Medical University of Bialystok. Women with a history of GDM, stillbirth, congenital anomalies, pregnancy-induced hypertension, multiple pregnancy, pre-existing glucose intolerance, or acute or chronic inflammation and active smokers were not included. All patients underwent a 75 g oral glucose tolerance test (OGTT) in the 24th–28th week of pregnancy and gestational diabetes was diagnosed in 53 women (GDM) according to the WHO 2013 criteria [19]. Their results were compared with the results of the carefully selected 82 pregnant women with normal glucose tolerance (NGT). We also enrolled a third control group that consisted of 37 healthy, non-pregnant women. All groups were matched for age, and pre-pregnancy BMI was calculated as weight in kilograms divided by height in meters. Written informed consent was obtained from all participants before enrolment, and the protocol was approved by the local ethics committee (Medical University of Bialystok).

2.2. Diabetic Parameters

In the 1st trimester, venous blood samples were collected in the fasting state. Serum was collected by allowing freshly drawn blood to clot, followed by centrifugation at 2000× *g* for 10 min in a refrigerated centrifuge. The resulting supernatant was collected and stored

at −80 °C until further analysis. The 75 g oral glucose tolerance test (OGTT) was performed in the 24th–28th week of pregnancy in the pregnant patients, as well as in the control group, after an overnight fast. Blood samples were collected at 0, 30, 60, and 120 min after glucose load. Plasma glucose concentration was measured via an enzymatic method with hexokinase (Cobas c111, Roche Diagnostics Ltd., Switzerland). Serum insulin levels were assayed by immunoradiometric method (DiaSource Europe SA, Belgium), and glycated hemoglobin (HbA1c) was evaluated with a high-performance liquid chromatography technique (BIO-RAD Laboratories, Germany). The following indices of insulin sensitivity and insulin secretion were calculated: (1) HOMA-IR (the homeostasis model assessment of insulin resistance) = FPG (mmol/L) × FPI (μU/mL)/22.5, HOMA-β [%] = 20 × FPI (mU/L)/FPG (mmol/L) − 3.5; and (2) the Matsuda and de Fronzo index (ISOGTT) = 10,000/$\sqrt{}$ ((FPG × FPI) × (G × I)), where FPG = fasting plasma glucose, FPI = fasting plasma insulin, G = mean glucose, and I = mean insulin during the OGTT [20]. Total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured by enzymatic methods (Cobas c111, Roche Diagnostics Ltd., Rotkreuz, Switzerland). LDL-cholesterol concentration was calculated using the Friedewald equation.

2.3. Sphingolipid Measurements

The concentration of serum sphingolipids was measured in the first and the second trimester of pregnancy using a UHPLC/MS/MS approach according to Błachnio-Zabielska et al. [21]. Briefly, an internal standard mix (17C-sphingosine, 17C-S1P, d17:1/8:0, d17:1/18:0, d17:1/18:1 (9Z), d17:1/20:0, d17:1/24:0, and d17:1/24:1 (15Z)) (Avanti Polar Lipids, Alabaster, Al), as well as an extraction mixture (isopropanol:water:ethyl acetate, 30:10:60; v:v:v), was added to each serum sample. The samples were vortexed and then centrifuged. The supernatants were transferred to new tubes and pellets were re-extracted. After centrifugation, supernatants were combined and evaporated under nitrogen. The dried samples were reconstituted in LC Solvent B (2 mM ammonium formate, 0.15% formic acid in methanol) for UHPLC/MS/MS analysis (Sciex 6500+; AB Sciex Germany GmbH, Darmstadt, Germany). The chromatographic separation was performed on a reverse-phase Zorbax SB-C8 column 2.1 × 150 mm, 1.8 μm (Agilent Technologies, Santa Clara, CA, USA) in a binary gradient using 1 mM ammonium formate with 0.1% formic acid in water as solvent A and 2 mM ammonium formate and 0.1% formic acid in methanol as solvent B at the flow rate of 0.4 mL/min. Sphingolipids concentrations were analyzed via a triple quadrupole mass spectrometer using positive ion electrospray ionization (ESI) (except for S1P, which was analyzed in negative mode) with multiple reaction monitoring (MRM) against the concentration standard curves.

2.4. Statistical Analysis

The data were analyzed by the STATISTICA 13 for Windows (StatSoft. Inc., Tulsa, OK, USA). Data were shown as medians and interquartiles. Differences between the two groups were compared by Mann–Whitney U test, differences between all three groups were compared via a Kruskal–Wallis test, and relationships between variables were tested by Spearman's correlations. A Wilcoxon signed rank test was used to compare sphingolipids levels in the 1st and 2nd trimester. A p-value less than 0.05 was considered statistically significant. Data are shown as medians with interquartile ranges.

3. Results

3.1. Characteristics of the Studied Groups

Tables 1 and 2 compare the clinical and biochemical characteristics of the study patients.

Table 1. Clinical characteristics of the groups studied in the 1st trimester.

	Control	NGT	GDM (Pre-Conversion)	*p*-Value
n	37	82	53	
Age (years)	26 (23–31)	28 (24–32)	25.5 (24–30)	0.4 ° 0.81 *
Gestational age (week)		11 (10–12)	11 (10–11)	0.19 *
Prepregnancy BMI (kg/m^2)	21.9 (20.6–23.4)	20.9 (19.8–28.5)	24.1 (21.6–26.8)	0.42 ° 0.87 *
Current BMI (kg/m^2)		24.5 (20.4–29.8)	24.8 (22.0–26.7)	0.95 *
Fasting glucose (mg/dL)	90 (86–92)	86 (84–88)	87 (84.5–89.5)	0.0005 ° 0.19 *
Fasting insulin (µU/mL)	7.5 (5.2–10.7)	11.6 (8.9–14.7)	11.3 (10.1–13.3)	<0.0001 ° 0.83 *
HOMA–IR	1.6 (1.1–2.4)	2.4 (1.8–3.2)	2.5 (2.1–2.9)	0.0002 ° 0.89 *
HOMA–β	101.1 (67.3–149)	176.9 (151.9–226.8)	173.7 (155.7–220.0)	<0.0001 ° 0.82 *
HbA1c (%)	5.2 (5.0–5.4)	5.0 (4.9–5.3)	5.1 (4.9–5.4)	0.2 ° 0.31 *
Total cholesterol (mmol/L)	166 (158–182)	174 (150–202)	172 (156.5–187)	0.64 ° 0.64 *
HDL-cholesterol (mmol/L)	86 (69.6–102.8)	73 (63–88)	72.5 (59.5–80.5)	0.01 ° 0.49 *
LDL-cholesterol (mmol/L)	64 (53–83)	78 (63.4–96.6)	87.6 (69.3–95.5)	0.03 ° 0.79 *
Triglycerides (mmol/L)	69 (59–75)	87 (65–116)	84.5 (64.5–125.5)	0.0009 ° 0.92 *

Data are shown as medians (interquartile range); ° differences between all groups were analyzed by Kruskal–Wallis test. * The difference between NGT and GDM groups was compared by Mann–Whitney U test.

Table 2. Clinical characteristics of the groups studied in the 2nd trimester.

	NGT	GDM	*p*–Value
n	82	53	
Gestational age (week)	25 (24–26)	25.5 (24–26)	0.41
Current BMI (kg/m^2)	26.17 (22.6–31.8)	27.8 (23.7–29.7)	0.76
Fasting glucose (mg/dL)	83 (80–86)	94 (89–97)	<0.0001
Glucose 30′ (mg/dL)	127 (117–139)	157.5 (139.5–166)	<0.0001
Glucose 60′ (mg/dL)	121.5 (103–139)	164 (129.5–184)	<0.0001
Glucose 120′ (mg/dL)	108 (89–121)	124 (113–157)	<0.0001
Fasting insulin (µU/mL)	11.1 (9.2–14.2)	15.9 (12.8–20.5)	<0.0001
Insulin 30′ (µU/mL)	77.9 (56.2–115.1)	89.3 (61.1–137.3)	0.18
Insulin 60′ (µU/mL)	72.3 (47.9–112.8)	108.3 (91.1–152.4)	<0.0001
Insulin 120′ (µU/mL)	56.0 (35.2–78.0)	104.8 (68.7–131)	<0.0001
HOMA–IR	2.3 (1.9–2.9)	3.6 (2.8–4.7)	<0.0001
HOMA–β	223.2 (169.8–276.1)	201.4 (154.4–244.3)	0.13
ISI OGTT	4.3 (3.3–5.4)	2.6 (2.1–3.4)	<0.0001
HbA1c (%)	4.8 (4.7–5.1)	5.0 (4.8–5.2)	0.006
Total cholesterol (mmol/L)	243 (219–276)	233 (203–257)	0.04
HDL-cholesterol (mmol/L)	86 (73–95)	79 (65–93)	0.13
LDL-cholesterol (mmol/L)	120.4 (95.6–149.2)	119.2 (86.8–134.4)	0.08
Triglycerides (mmol/L)	172 (133–196)	179 (136–219)	0.31

Data are shown as medians (interquartile range); the difference between NGT and GDM groups was compared the Mann–Whitney U test.

In the first trimester, the patients who were later diagnosed with GDM (converters) had no significant differences compared with the women with NGT (Table 1). The comparison of the three groups revealed significant differences between fasting glucose levels ($p < 0.001$), which is a consequence of a variation in norms (19). Fasting insulin ($p < 0.001$), HOMA-IR

($p < 0.001$), HOMA-β ($p < 0.001$), and triglycerides levels ($p < 0.001$) were higher in both pregnant women groups; HDL-cholesterol ($p = 0.01$) was higher in the control group.

In the second trimester, the GDM converters had significantly higher fasting and post-load glucose concentration ($p < 0.001$); higher fasting and post-load insulin concentrations ($p < 0.001$) except for insulin 30′, as well as higher HOMA-IR ($p < 0.001$) and HbA1c ($p = 0.006$). Furthermore, GDM converters had lower ISI OGTT ($p < 0.001$) and lower total cholesterol levels ($p = 0.04$) versus the NGT group (Table 2).

3.2. Sphingolipids Profile

The concentration of total serum ceramides was significantly lower in non-pregnant women versus pregnant ones ($p = 0.0006$ versus the GDM pre-conversion and $p < 0.0001$ versus the NGT group). The control group had lower levels of C16:0-Cer ($p = 0.02$ vs. GDM pre-conversion and $p = 0.0002$ vs. NGT), C18:1-Cer ($p < 0.0001$ vs. GDM pre-conversion and $p = 0.006$ vs. NGT), C22:0-Cer ($p < 0.0001$ in both comparisons), C24:1-Cer ($p = 0.0001$ vs. GDM pre-conversion and $p < 0.0001$ vs. NGT), and C24:0-Cer ($p = 0.03$ vs. GDM pre-conversion and $p = 0.003$ vs. NGT). The control group had higher sphinganine (SPA) and S1P levels ($p < 0.0001$ in both comparisons vs. GDM pre-conversion and the NGT group).

Among the pregnant women, comparisons between the GDM converter group and the NGT group using the Mann–Whitney U test revealed prominent differences in C18:1 concentration ($p = 0.01$) (Table 3, Figure 1). The diagnostic value of this ceramides species was evaluated by ROC analysis. The values of AUC and optimal cut-off value for C18:1-Cer were as follows: 0.702 confidence interval: 0.552–0.852, $p = 0.008$ (Figure 2).

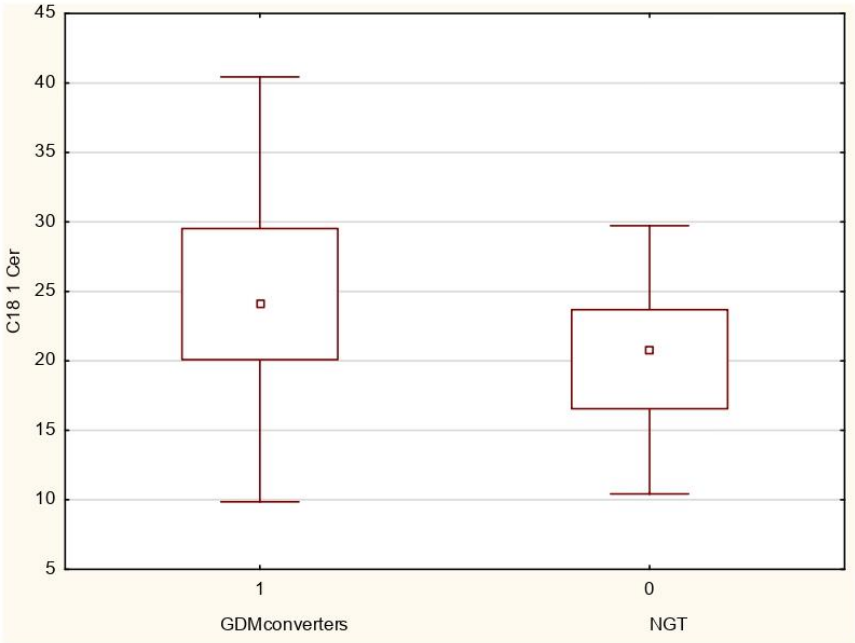

Figure 1. Serum C18:1-Cer concentrations measured in the first trimester of pregnancy. Data are shown as medians and interquartile range.

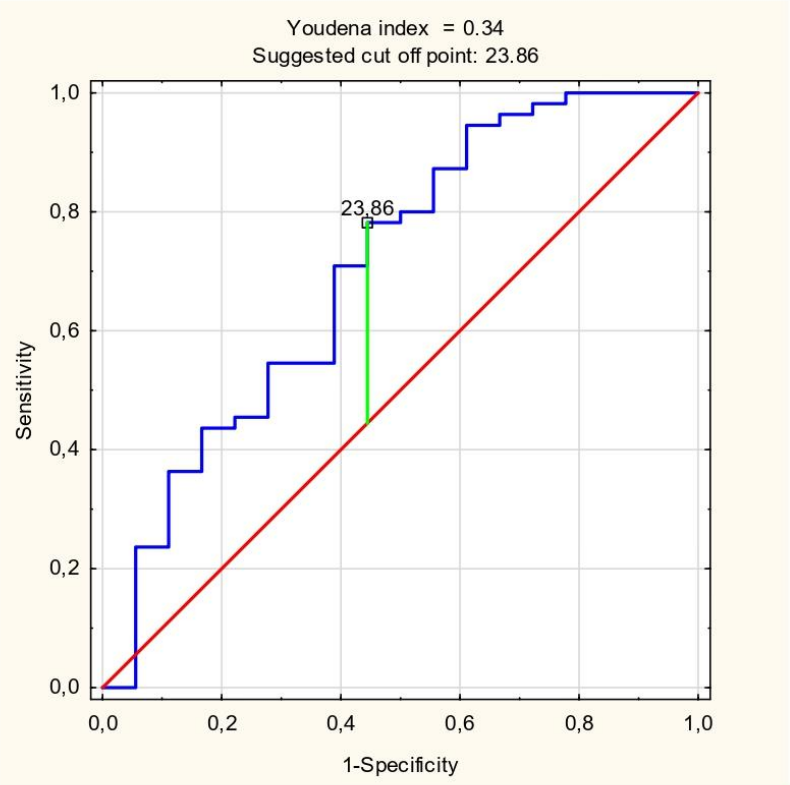

Figure 2. ROC curve for C18:1-Cer measured in the 1st trimester of pregnancy (AUC = 0.702 (95% confidence interval: 0.552–0.852; $p = 0.008$) with optimal cut-off value of 23.87 ng/mL).

Table 3. The concentration of sphingolipids in the serum of patients in the non-pregnant control group and pregnant GDM pre-conversion and NGT group in their 1st trimester.

Compound	Control Group	NGT	GDM Pre-Conversion	*p*-Value
	Me (Q1–Q3) [ng/mL]	Me (Q1–Q3) [ng/mL]	Me (Q1–Q3) [ng/mL]	
Sph	23.4 (20.6–26.4)	18.02 (13.3–31.7)	17.2 (14.5–36.9)	* $p = 0.73$ ° $p = 0.2$ ^ $p = 0.09$
SPA	37.5 (34.2–43.8)	13.89 (9.9–19.7)	10.82 (8.0–20.2)	* $p = 0.28$ ° $p < 0.0001$ ^ $p < 0.0001$
S1P	400.93 (357.2–436.8)	348.81 (251.4–403.4)	296.78 (235.3–342.4)	* $p = 0.06$ ° $p < 0.0001$ ^ $p < 0.0001$
C14:0 Cer	30.56 (26.5–34.7)	32.4 (25.6–42.9)	29.6 (23.4–36.7)	* $p = 0.22$ ° $p = 0.5$ ^ $p = 0.19$
C16:0 Cer	135.68 (118.6–156.6)	183.37 (151.3–246.6)	178.01 (129.9–201.4)	* $p = 0.49$ ° $p = 0.02$ ^ $p = 0.0002$
C18:1 Cer	16.33 (14.3–18.5)	20.72 (16.6–23.7)	24.16 (20.1–29.5)	* $p = 0.01$ ° $p < 0.0001$ ^ $p = 0.006$
C18:0 Cer	127.75 (114.3–142.2)	133.01 (108.4–160.5)	132.87 (108.1–199.6)	* $p = 0.57$ ° $p = 0.6$ ^ $p = 0.5$
C20:0 Cer	183.56 (156.4–206.7)	172.96 (130.1–194.2)	152.08 (123.6–177.9)	* $p = 0.25$ ° $p = 0.09$ ^ $p = 0.3$
C22:0 Cer	215.53 (198.3–240.4)	280.68 (243.5–317.7)	316.04 (256.0–376.7)	* $p = 0.1$ ° $p < 0.0001$ ^ $p < 0.0001$
C24:1 Cer	219.88 (200.8–251.5)	280.36 (237.9–336.5)	278.96 (241.5–320.3)	* $p = 0.92$ ° $p = 0.0001$ ^ $p < 0.0001$
C24:0 Cer	1941.61 (1819.1–2306.9)	2200.14 (2040.8–2608.7)	2356.23 (2026.8–2917.9)	* $p = 0.52$ ° $p = 0.03$ ^ $p = 0.003$
Cer Total	3023.67 (2738.7–3225.9)	3344.21 (3101.9–3682.7)	3552.17 (3007.1–3923.5)	* $p = 0.39$ ° $p = 0.0006$ ^ $p < 0.0001$

Data are presented as medians (interquartile range). Analysis was performed with the Mann–Whitney U test. * GDM vs. NGT; ° GDM vs. control; ^ NGT vs. control.

Across the study population, the SPA concentration correlated negatively with serum insulin ($R = -0.33$, $p < 0.05$), HOMA-IR ($R = -0.3$, $p < 0.05$), and HOMA-β ($R = -0.38$, $p < 0.05$). Positive correlations with serum insulin parameters were observed in C22:0-Cer with insulin ($R = 0.3$, $p < 0.05$), HOMA-IR ($R = 0.3$, $p < 0.05$), and HOMA-β ($R = 0.31$, $p < 0.05$); C24:0-Cer correlated with insulin ($R = 0.27$, $p < 0.05$), HOMA-IR ($R = 0.23$, $p < 0.05$), and HOMA-β ($R = 0.32$, $p < 0.05$). Furthermore, there were correlations between C18:1-Cer and serum insulin levels ($R = 0.21$, $p \leq 0.05$) and HOMA-β ($R = 0.21$, $p \leq 0.05$), as well as between C24:1-Cer and insulin level ($R = 0.22$, $p \leq 0.05$) and HOMA-β ($R = 0.29$, $p \leq 0.05$).

Wilcoxon analysis demonstrated progressive changes in ceramide concentration levels during pregnancy (Table 4). We compared measurements from the first trimester with measurements from the second trimester. The most relevant change was observed in

NGT C24:0-Cer in the second trimester. This appeared to be higher compared to the first ($p < 0.001$). Levels of C16:0-Cer and C18:0-Cer in the GDM group were increased while these parameters were stable in the NGT group. C24:1-Cer in the GDM group was higher in the first trimester than in the second; the NGT group had the opposite situation, but it was not significant. Moreover, the concentration of C18:1-Cer in GDM decreased during pregnancy, but it increased in NGT.

Table 4. The concentration of sphingolipids in the serum of patients from the GDM and NGT groups by trimester.

Compound	NGT				GDM Converters			
	1st Trimester		2nd Trimester		1st Trimester		2nd Trimester	
	Me	Q1–Q3	Me	Q1–Q3	Me	Q1–Q3	Me	Q1–Q3
Sph	18.02	13.3–31.7	18.7	15.3–23.7	17.2	14.5–36.9	18.07	15.4–20.8
SPA	13.89	9.9–19.7	16.14	12.6–21.5	10.82	8.0–20.2	14.71	10.9–18.5
S1P	348.81	251.4–403.4	307.53	214.1–423.3	296.78	235.3–342.4	263.73	180.9–304.2
C14:0 Cer	32.4	25.6–42.9	35.87	23.9–42.6	29.6	23.4–36.7	35.47	27.8–43.4
C16:0 Cer	183.37	151.3–246.6	184.34	133.8–234.7	178.01 *	129.9–201.4	214.38 *	178.5–250.2
C18:1 Cer	20.72 *	16.6–23.7	22.36 *	17.7–25.9	24.16 *	20.1–29.5	20.36 *	15.6–26.5
C18:0 Cer	133.01	108.4–160.5	137.56	116.9–154.6	132.87 *	108.8–199.6	168.21 *	138.4–201.0
C20:0 Cer	172.96	130.1–194.2	174.51	136.7–210.6	152.08	123.6–177.9	180.41	146.8–242.8
C22:0 Cer	280.68	243.5–317.7	296.23	248.7–347.2	316.04	256.0–376.7	278.25	237.0–336.5
C24:1 Cer	280.36	237.9–336.5	285.22	234.5–344.5	278.96 *	241.5–320.3	257.62 *	214.8–296.6
C24:0 Cer	2200.14 °	2040.8–2608.7	2612.07 °	2306.4–2936.5	2356.23	2026.8–2917.9	2545.29	2115.7–3077.4
Cer Total	3344.21	3101.9–3682.7	3731.5	3494.8–4298.8	3552.17	3007.1–3923.5	3737.46	3310.1–4454.1

This table shows changes during pregnancy. Data are presented as medians and interquartile range; * $p < 0.05$; ° $p < 0.001$. Analysis was performed with Wilcoxon test.

Comparison of sphingolipid concentrations measured in the second trimester of pregnancy showed prominent differences between the GDM and NGT group in terms of S1P ($p = 0.009$), C16:0-Cer ($p = 0.04$), C18:0-Cer ($p = 0.0002$), and C24:1-Cer ($p = 0.03$). The diagnostic value of those sphingolipids was evaluated by ROC analysis. The values of AUC and optimal cut-off value for S1P were 0.638 (95% confidence interval: 0.541–0.735; $p = 0.005$) and 304.15 ng/mL. AUC was 0.606 (95% confidence interval: 0.508–0.705; $p = 0.03$) and the cut-off value was 184.67 ng/mL for C16:0-Cer. AUC was 0.696 (95% confidence interval: 0.6–0.792; $p = 0.0001$) and the cut-off value was 155.95 ng/mL for C18:0-Cer. AUC = 0.618 (95% confidence interval: 0.519–0.718; $p = 0.02$) and the cut-off value was 272.16 ng/mL for C24:1-Cer.

4. Discussion

The data show that the main pathophysiological dysfunction in GDM is the increasing insulin resistance and insufficient insulin compensation, usually as a result of the β-cell impairment [1,22]. Sphingolipids, particularly ceramides, are potential factors contributing to diabetes [23]. Our study was designed to measure and compare the changes in circulating sphingolipids concentration in pregnancy and in GDM. It is worth mentioning that, according to O'Sullivan, Carpenter and Coustan [24] and some associations, such as the Spanish Group of Diabetes and Pregnancy (GEDE) [25], OGTT should be taken with 100 g of glucose and measured in a fasting state, 60, 120, and 180 min after overload. However, Cabrera-Fernandez et al. [26] revealed that using the GEDE criteria measurement at 180 min could be omitted. In our work, we applied criteria recommended by the International Association of Diabetes and Pregnancy Study Group (IADPSG) [24] and the International Diabetes Federation (IDF) [27], where 75 g of glucose is used, and the test lasts 120 min.

This study showed that the total concentration of circulating ceramides measured in the first trimester of pregnancy was significantly higher in pregnant patients versus healthy, non-pregnant women. The pregnant women group showed a significant increase in C16:0-Cer, C 18:1-Cer, C22:0-Cer, C24:1-Cer, and C24:0-Cer. It is known that ceramide biosynthesis is enhanced by insulin in skeletal muscles [28]. During pregnancy, insulin

resistance increases, and the same insulin concentration is higher. This might explain the increasing ceramide levels.

Elevated C16:0-Cer concentrations have been found in overweight patients and those with type 2 diabetes [29,30]. Here, we excluded the possibility of obesity influencing the results. The groups were selected so that they did not present differences in the BMI. However, we show significantly increased C16:0-Cer levels between the first trimester and the second trimester in patients who developed GDM. Raichur et al. [30] reported that the inhibition of synthesis of C16:0-Cer improved insulin resistance and decreased hyperglycemia. They also reported that C16:0-Cer is an important factor in diabetes development, and our study confirmed this theory.

Available data report an association between an increased risk of diabetes development and higher C18:0-Cer levels in plasma [31]. Similarly, in our experiment, in the GDM group this parameter increased during pregnancy and, in the second trimester, was revealed to be significantly higher than in the NGT group where it stayed at a constant level.

Other authors reported increased C18:0, C18:1, C20:0, C22:0, and C24:0-Cer in subjects with a glucose tolerance impairment phenotype [10,32]. They also reported a positive association between circulating ceramide levels and insulin-resistance parameters including disruption of β-cell function and inflammation [32]. During pregnancy, even physiological pregnancy, there is an increase in insulin resistance and a prothrombotic state. This showed pro-inflammatory features [33]. We confirmed a positive correlation between C22:0-Cer and insulin levels and HOMA-IR, as well as between C18:1, C24:0, and C24:1 with insulin concentrations and HOMA β across the entire study population. Considering the subgroups, we noted that the GDM converters group's C24:1 positively correlated with insulin concentration and HOMA β.

Khan et al. [9] recently employed artificial intelligence and demonstrated the predictive value of reduced ceramide levels in the pathophysiology of transition from GDM to type 2 diabetes. Suppression of ceramide synthesis in pancreatic islets impairs glucose-stimulated insulin secretion. This discovery suggests that any imbalance may contribute to insulin resistance. Further research into the role of ceramides and mechanisms underlying GDM and diabetes development—especially in the pathophysiology of β-cells dysfunction and insulin resistance—is required.

Early in pregnancy, fat accumulation occurs due to an increased lipid synthesis and hyperphagia; in the last trimester of pregnancy, fat accumulation halts due to decreased adipose tissue lipoprotein lipase activity [5]. Adipose tissue is an endocrine organ, and its adipocytes synthesize i.a. adiponectin. This adipokine was found to elicit broad spectrum antidiabetic action by activating ceramidase to degrade ceramides [34]. Adiponectin receptors have a homology with ceramidase enzymes. They can activate or deplete adiponectin receptors to markedly alter cellular ceramidase activity [35]. It is known that decreased levels of adiponectin during pregnancy indicates an increased risk of GDM [36].

The pregnant women in our study had lower SPA and S1P levels compared to healthy, non-pregnant women. It is known that S1P is a powerful bioactive lipid that can act intracellularly and extracellularly, and its receptors are widely expressed in the human body [37]. S1P is carried on apolipoprotein M (apoM) [8], a minor apolipoprotein on HDL [9]; it has been proposed to be responsible for many of the pleiotropic qualities of HDL, i.e., having antiapoptotic [10], anti-inflammatory [38], and vasoprotective effects [39].

Recent studies demonstrated the crucial role of S1P in insulin sensitivity and glucose homeostasis. Kurano et al. [40] provided evidence that at least a part of HDL's antidiabetic action involves apolipoprotein M (apoM) and its lipid ligand sphingosine-1-phosphate (S1P); these are two quantitatively minor components of HDL. They demonstrated that apoM/S1P protects against the development of insulin resistance in the liver, adipose tissue, and skeletal muscles by activating AKT and AMPK signaling, which are the main signaling pathways and act via S1P1 and/or S1P3 by enhancing mitochondrial functions, perhaps through the upregulation of the SIRT1 protein levels.

A prior study with an animal model showed that increased glycemia resulted in activation of sphingosine kinase isoform 2 (Sphk2) in pancreatic β-cells and prominently increased S1P. Knockdown of Sphk2 led to the abolition of insulin secretion in response to glucose [41]. Liu et al. [42] proved that S1P prevents β-cell apoptosis—thus, the authors suggested inhibition of the voltage-dependent potassium channel in pancreatic β-cells, which also induces Ca^{2+} inflow into the cell to stimulate insulin secretion.

The literature suggests elevated levels of SPA in type 2 diabetes patients versus healthy control subjects [43]. Moreover, SPA was shown to negatively correlate with insulin sensitivity [44]. In contrast to those reports, we present, here, decreased levels of SPA in the GDM group versus the control group. This may be due to the use of SPA for ceramide synthesis—it is a major precursor in the de novo synthesis pathway [45].

Interestingly, our study demonstrated that the C18:1-Cer level was significantly higher in GDM converters than in the NGT group in the first trimester. It is worth emphasizing that this was the only difference between those groups considering measured sphingolipids, as well as clinical/biochemical parameters. Thus, we suspect that C18:1-Cer may be a potential GDM biomarker. It can manifest a predisposition for disease development before glycemic changes occur. Our results demonstrate that ceramide levels are elevated in pregnancy and changes in the ceramide profile are not independent of BMI. We are aware of only one report about circulating ceramides in early pregnancy. Liu et al. [46] selected the studied groups (n = 486) from a large cohort of Chinese women (n = 22302). The differences in concentration of ceramides in early pregnancy are significant. Scientists noted that three ceramides were significantly associated with GDM development in later pregnancy. The GDM group showed an increased level of C18:0-Cer and C18:1-Cer and a decreased level of C24:0-Cer. Our results partially confirm this data. We proved only an increase of C18:1-Cer—the remining ceramides showed no significant differences in our groups. Further analysis of ceramides in the second trimester demonstrated that C18:1-Cer concentrations decreased in the GDM serum, but this increased in the NGT group. In contrast to Liu et al., we studied smaller but BMI-matched groups of varying ethnicities.

In summary, our results show differences in the metabolic signature between GDM and control pregnant group in the second trimester of pregnancy. The results emphasize the potential of C 18:1-Cer in the first trimester of pregnancy as a lipidomic biomarker of GDM development. The main limitation of this research was the low number of patients. Furthermore, we did not control for patient diet, which can impact circulating ceramides [47]. Further studies are required to validate our data and to clarify and improve the understanding of GDM pathophysiology.

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6 Streszczenie w języku polskim

Cukrzyca ciążowa (gestational diabetes mellitus – GDM) stanowi jedno z najczęstszych zaburzeń metabolicznych w okresie ciąży. Niezdiagnozowana i nieleczona GDM prowadzi do groźnych powikłań zarówno u matki, jak i u dziecka. Obecnie diagnostyka opiera się na doustnym teście tolerancji glukozy wykonywanym między 24 a 28 tygodniem ciąży wg kryteriów zaproponowanych przez IADPSG (International Association of Diabetes and Pregnancy Study Group).

Określenie zmian w profilu ekspresji miRNA oraz stężeniu poszczególnych ceramidów w surowicy w pierwszym trymestrze ciąży może przyczynić się nie tylko do lepszego poznania patogenezy GDM, ale również do identyfikacji przydatnych klinicznie biomarkerów genetycznych i biochemicznych w celu wczesnego wdrożenia profilaktyki.

Celem pracy było zbadanie stężenia ceramidów i ich metabolitów w surowicy ciężarnych w pierwszym i drugim trymestrze ciąży oraz w grupie kontrolnej z oceną czy mogą stanowić wczesne biomarkery cukrzycy ciążowej. Ponadto, w tym samym celu wykonano oznaczenie ekspresji miRNA w surowicy kobiet ciężarnych w pierwszym trymestrze ciąży. Dodatkowym celem było sprawdzenie zależności pomiędzy oznaczanymi parametrami a wskaźnikami insulinooporności.

W badaniach nad stężeniami sfingolipidów do grupy badanej (GDM) włączono kobiety, które w pierwszym trymestrze (9-12 tydzień) miały prawidłową tolerancję glukozy natomiast w drugim trymestrze (24-26 tydzień) rozwinęły cukrzycę ciążową (n=53). Grupę odniesienia (NGT) stanowiły kobiety z prawidłową tolerancją glukozy przez całą ciążę (n=82). Dodatkowo do badań włączono kobiety nie będące w ciąży (grupa kontrolna) (n=37). Stężenie sfingolipidów u kobiet ciężarnych oznaczono w surowicy w pierwszym i drugim trymestrze i jednokrotnie u kobiet niebędących w ciąży. Ekspresję krążących miRNA oznaczono w surowicy kobiet ciężarnych w pierwszym trymestrze ciąży. Grupę badaną (GDM) stanowiły pacjentki, które w drugim trymestrze rozwinęły cukrzycę ciążową (n=24), natomiast grupą kontrolną (NGT) były kobiety z prawidłową tolerancją glukozy przez całą ciążę (n=24).

Stężenie ceramidów i ich metabolitów w surowicy oznaczone zostało przy użyciu ultrawysokosprawnej chromatografii cieczowej połączonej ze spektrometrem masowym typu potrójny kwadrupol (UHPLC/MS/MS). Profil miRNA w surowicy pacjentek został określony przy użyciu technologii NanoString, natomiast walidację otrzymanych wyników przeprowadzono metodą RT-PCR.

W surowicy kobiet ciężarnych zaobserwowano istotnie wyższe stężenie C16:0-Cer, C18:1-Cer, C22:0-Cer, C24:1-Cer i C24:0-Cer oraz niższe poziomy SPA i S1P w porównaniu do odpowiednich wartości w grupie kontrolnej. Porównanie wyników stężenie sfingolipidów w surowicy grupy badanej GDM z grupą NGT wykazało wyższe stężenie C18:1-Cer w surowicy pacjentek, u których w drugim trymestrze zdiagnozowano cukrzycę ciążową. Analizując wyniki całej badanej populacji zaobserwowano ujemną korelację pomiędzy stężeniem SPA a wskaźnikiem HOMA-IR oraz HOMA-β. Ponadto, stężenie C22:0-Cer i C24:0-Cer korelowało dodatnio ze stężeniem insuliny oraz wskaźnikami insulinooporności HOMA-IR i HOMA-β. Oprócz tego, stężenie C18:1-Cer i C24:1-Cer korelowało dodatnio ze stężeniem insuliny i wartością HOMA-β.

Analiza profilu miRNA przy użyciu metody NanoString pozwoliła wytypować 4 cząsteczki miRNA, których ekspresja znacznie różniła się u kobiet, u których zdiagnozowano GDM w porównaniu do NGT. Na podstawie tej analizy metodą RT-PCR oceniono ekspresję miR-16-5p, miR-142-3p, miR-144-3p oraz miR-320e. Walidacja wykazała istotne różnice pomiędzy grupami w ekspresji miR-16-5p, miR-142-3p i miR-144-3p. Dalsza analiza wykazała dodatnią korelację pomiędzy miR-16-5p a HOMA-IR oraz ujemną zależność pomiędzy ISIOGTT a miR-16-5p i miR-144-3p.

Na podstawie uzyskanych wyników sformułowano następujące wnioski:

1. Okres ciąży związany jest z istotnym wzrostem w surowicy stężenia C16:0-Cer, C18:1-Cer, C22:0-Cer, C24:1-Cer oraz C24:0-Cer.
2. Oznaczenie stężenia C18:1-Cer w pierwszym trymestrze ciąży może stanowić nowy marker rozwoju cukrzycy ciążowej.
3. Zmiany w stężeniach C18:1-Cer, C22:0-Cer, C24:0-Cer oraz C24:1-Cer mogą być powiązane z narastającą insulinoopornością w ciąży.
4. Profil miRNA w pierwszym trymestrze ciąży jest różny u pacjentek, które w trakcie ciąży rozwijają cukrzycę ciążową w porównaniu do zdrowych ciężarnych.
5. miR-16-5p, miR-142-3p oraz miR-144-3p mogą potencjalnie służyć jako markery wczesnej diagnostyki GDM.

7 Streszczenie w języku angielskim

Gestational diabetes mellitus (GDM) is one of the most common metabolic disorders during pregnancy. Undiagnosed and untreated GDM leads to serious complications in both the mother and the child. Currently, diagnostics are based on an oral glucose tolerance test performed between 24 and 28 weeks of pregnancy according to the criteria proposed by the IADPSG (International Association of Diabetes and Pregnancy Study Group).

Determination of changes in the miRNA expression profile and the concentration of individual ceramides in the serum in the first trimester of pregnancy may contribute not only to a better understanding of GDM pathogenesis, but also to the identification of clinically useful genetic and biochemical biomarkers for the early implementation of prophylaxis.

The aim of the study was to investigate the concentration of ceramides and their metabolites in the serum of pregnant women in the first and second trimesters of pregnancy compared to the control group, assessing whether they may constitute early biomarkers of gestational diabetes. Moreover, with the same intention, an examination of the miRNA expression profile in the serum of pregnant women in the first trimester of pregnancy was performed. An additional goal was to check the relationship between the measured parameters and the indicators of insulin resistance.

In the studies on sphingolipid concentrations, the study group (GDM) included women who had normal glucose tolerance in the first trimester (9-12 weeks) and developed gestational diabetes in the second trimester (24-26 weeks) (n = 53). The reference group (NGT) was comprised by women with normal glucose tolerance during pregnancy (n = 82). Additionally, non-pregnant women (control group) (n = 37) were included in the study. The concentration of sphingolipids in pregnant women was measured in serum in the first and second trimesters and once in non-pregnant women. Expression of circulating miRNAs was determined in the serum of pregnant women in the first trimester of pregnancy. The study group (GDM) constituted of women who developed gestational diabetes in the second trimester (n = 24), while in the control group (NGT) were women with normal glucose tolerance throughout pregnancy (n = 24).

The concentration of ceramides and their metabolites in the serum was determined using ultra-high performance liquid chromatography combined with a triple quadrupole mass spectrometer (UHPLC / MS / MS). The miRNA profile in the serum of the patients was determined using NanoString technology, and validation of obtained results was performed using the RT-PCR method.

In the serum of pregnant women, significantly higher concentrations of C16:0-Cer, C18:1-Cer, C22:0-Cer, C24:1-Cer and C24:0-Cer were observed, as well as lower levels of SPA and S1P compared to the control group. The comparison of the sphingolipid concentration in the serum of the GDM group with the NGT group showed a higher concentration of C18:1-Cer in the serum of patients diagnosed with gestational diabetes in the second trimester. Across the study population, a negative correlation was observed between the concentration of SPA and the HOMA-IR and HOMA-β. Moreover, the concentration of C22:0-Cer and C24:0-Cer positively correlated with the concentration of insulin, HOMA-IR and HOMA-β. In addition, the concentration of C18:1-Cer and C24:1-Cer positively correlated with the concentration of insulin and HOMA-β.

Analysis of the miRNA profile using the NanoString method allowed to select 4 miRNA molecules, the expression of which was significantly different in women diagnosed with GDM compared to NGT. Based on this analysis, the expression of miR-16-5p, miR-142-3p, miR-144-3p and miR-320e was assessed by RT-PCR. The validation showed significant differences between the groups in the expression of miR-16-5p, miR-142-3p and miR-144-3p. Further analysis showed a positive correlation between miR-16-5p and HOMA-IR and a negative correlation between ISIOGTT and miR-16-5p and miR-144-3p.

Based on obtained results, the following conclusions were drawn:

1. Pregnancy is associated with a significant increase in serum concentrations of C16:0-Cer, C18:1-Cer, C22:0-Cer, C24:1-Cer and C24:0-Cer.
2. The determination of C18:1-Cer concentration in the first trimester of pregnancy may be a new marker of the development of gestational diabetes.
3. Changes in the concentrations of C18:1-Cer, C22:0-Cer, C24:0-Cer and C24:1-Cer may be associated with increasing insulin resistance in pregnancy.
4. The miRNA profile during the first trimester of pregnancy is different in patients who developed gestational diabetes during pregnancy compared to healthy pregnant women.
5. miR-16-5p, miR-142-3p and miR-144-3p can potentially serve as markers for early diagnosis of GDM.

8 Piśmiennictwo

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9 Informacje o charakterze udziału współautorów i oświadczenia współautorów

Informacja o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu

Juchnicka I, Kuzmicki M. Influence of MiRNAs in gestational diabetes mellitus development. Ginekol Pol. 2021;92(8):579-582. doi: 10.5603/GP.a2021.0121. MNiSW: 40 IF: 1,232.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka- Ilona Juchnicka	Zamysł pracy, zebranie piśmiennictwa dotyczącego tematyki pracy i napisanie manuskryptu	75%
dr hab. n. med. Mariusz Kuźmicki	Konsultacja merytoryczna podczas pisania pracy oraz ocena ostatecznej wersji manuskryptu	25%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Ilony Juchnickiej.

.....Ilona Juchnicka.....
Podpis

Informacja o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu

Juchnicka I, Kuźmicki M, Szamatowicz J. Ceramides and Sphingosino-1-Phosphate in Obesity. Front Endocrinol (Lausanne). 2021 May 13;12:635995. doi: 10.3389/fendo.2021.635995. MNiSW: 100 IF: 5,555.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka- Ilona Juchnicka	Zamysł pracy, zebranie piśmiennictwa dotyczącego tematyki pracy i napisanie manuskryptu	70%
dr hab. n. med. Mariusz Kuźmicki	Konsultacja merytoryczna podczas pisania pracy oraz ocena ostatecznej wersji manuskryptu	15%
prof. dr hab. n. med Jacek Szamatowicz	Konsultacja merytoryczna podczas pisania pracy oraz ocena ostatecznej wersji manuskryptu	15%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Ilony Juchnickiej.

Ilona Juchnicka

Podpis

Informacja o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu

Juchnicka I, Kuźmicki M, Niemira M, Bielska A, Sidorkiewicz I, Zbucka-Krętowska M, Krętowski AJ and Szamatowicz J (2022) miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus. Front. Endocrinol. 13:839344. doi: 10.3389/fendo.2022.839344. MNiSW: 100 IF: 5,555.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka- Ilona Juchnicka	Planowanie badań, rekrutacja pacjentek, współtworzenie bazy danych, wykonywanie badań laboratoryjnych, analiza statystyczna otrzymanych wyników, poszukiwanie i analiza piśmiennictwa, współtworzenie manuskryptu	55%
dr hab. n. med. Mariusz Kuźmicki	Planowanie badań, współtworzenie bazy danych oraz manuskryptu	10%
dr Magdalena Niemira	Opracowanie metodyki, nadzorowanie przebiegu eksperymentu oraz konsultacja merytoryczna prowadzonych badań	10%
mgr Agnieszka Bielska	Współwykonawstwo badań laboratoryjnych	5%
dr Iwona Sidorkiewicz	Współwykonawstwo badań laboratoryjnych	5%
dr hab. n. med. Monika Zbucka-Krętowska	Konsultacja merytoryczna podczas pisania manuskryptu	5%
prof. dr hab. n. med. Adam Jacek Krętowski	Konsultacja merytoryczna podczas pisania manuskryptu, analiza i ocena manuskryptu	5%
prof. dr hab. n. med Jacek Szamatowicz	Konsultacja merytoryczna podczas pisania manuskryptu, analiza i ocena manuskryptu	5%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Ilony Juchnickiej.

.....Ilona Juchnicka.....

Podpis

Informacja o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu

Juchnicka I, Kuźmicki M, Zabielski P, Krętowski A, Błachnio-Zabielska A, Szamatowicz J. Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes. J Clin Med. 2022 Jan 13;11(2):384. doi: 10.3390/jcm11020384. MNiSW: 140 IF: 4,242.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka- Ilona Juchnicka	Planowanie badań, rekrutacja pacjentek, współtworzenie bazy danych, wykonywanie badań laboratoryjnych, analiza statystyczna otrzymanych wyników, poszukiwanie i analiza piśmiennictwa, współtworzenie manuskryptu	55%
dr hab. n. med. Mariusz Kuźmicki	Planowanie badań, współtworzenie bazy danych oraz manuskryptu	10%
dr Piotr Zabielski	Konsultacja merytoryczna podczas pisania manuskryptu, analiza i ocena manuskryptu	10%
prof. dr hab. n. med. Adam Jacek Krętowski	Konsultacja merytoryczna podczas pisania pracy	5%
prof. dr hab. n. med. Agnieszka Błachnio-Zabielska	Opracowanie metodyki, nadzorowanie przebiegu eksperymentu oraz konsultacja merytoryczna prowadzonych badań	10%
prof. dr hab. n. med Jacek Szamatowicz	Konsultacja merytoryczna podczas pisania pracy	5%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Ilony Juchnickiej.

.....Ilona Juchnicka.....

Podpis

Białystok, 31.05.2022

dr hab. n. med. Mariusz Kuźmicki

Klinika Ginekologii i Ginekologii Onkologicznej
Uniwersytet Medyczny w Białymstoku
ul. J. Kilińskiego 1
15-089 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *Juchnicka I, Kuzmicki M. Influence of MiRNAs in gestational diabetes mellitus development. Ginekol Pol. 2021;92(8):579-582. doi: 10.5603/GP.a2021.0121. Epub 2021 Jun 9.*
2. *Juchnicka I, Kuźmicki M, Szamatowicz J. Ceramides and Sphingosino-1-Phosphate in Obesity. Front Endocrinol (Lausanne). 2021 May 13;12:635995. doi: 10.3389/fendo.2021.635995.*
3. *Juchnicka I, Kuźmicki M, Niemira M, Bielska A, Sidorkiewicz I, Zbucka-Krętowska M, Krętowski AJ and Szamatowicz J (2022) miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus. Front. Endocrinol. 13:839344. doi: 10.3389/fendo.2022.839344.*
4. *Juchnicka I, Kuźmicki M, Zabielski P, Krętowski A, Błachnio-Zabielska A, Szamatowicz J. Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes. J Clin Med. 2022 Jan 13;11(2):384. doi: 10.3390/jcm11020384.*

wchodzących w skład rozprawy doktorskiej mgr Ilony Juchnickiej wynosił i polegał na:
Ad. 1 25%, ocenie manuskryptu oraz opiece merytorycznej
Ad. 2 15%, ocenie manuskryptu oraz opiece merytorycznej
Ad. 3 10%, pracy nad koncepcją badań oraz współtworzenie bazy danych i manuskryptu.
Ad. 4 10%, pracy nad koncepcją badań oraz współtworzenie bazy danych i manuskryptu.

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

M. Kuźmicki

.....

Podpis

Białystok, 31.05.2022

prof. dr hab. n. med. Jacek Szamatowicz

Klinika Ginekologii i Ginekologii Onkologicznej
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Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *Juchnicka I, Kuźmicki M, Szamatowicz J. Ceramides and Sphingosino-1-Phosphate in Obesity. Front Endocrinol (Lausanne). 2021 May 13;12:635995. doi: 10.3389/fendo.2021.635995.*
2. *Juchnicka I, Kuźmicki M, Niemira M, Bielska A, Sidorkiewicz I, Zbucka-Krętowska M, Krętowski AJ and Szamatowicz J (2022) miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus. Front. Endocrinol. 13:839344. doi: 10.3389/fendo.2022.839344.*
3. *Juchnicka I, Kuźmicki M, Zabielski P, Krętowski A, Błachnio-Zabielska A, Szamatowicz J. Serum C18:1-Cer as a Potential Biomarker for Early Detection of Gestational Diabetes. J Clin Med. 2022 Jan 13;11(2):384. doi: 10.3390/jcm11020384.*

wchodzących w skład rozprawy doktorskiej mgr Ilony Juchnickiej wyniosił Ad.1 15%, Ad. 2, Ad.3 5% i polegał na analizie i ocenie wyżej wymienionych manuskryptów oraz opiece merytorycznej.

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

.....
Podpis

Białystok, 31.05.2022

prof. dr hab. n. med. Adam Jacek Krętowski

Klinika Endokrynologii, Diabetologii i Chorób Wewnętrznych
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Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *Juchnicka I, Kuźmicki M, Niemira M, Bielska A, Sidorkiewicz I, Zbucka-Krętowska M, Krętowski AJ and Szamatowicz J (2022) miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus. Front. Endocrinol. 13:839344. doi: 10.3389/fendo.2022.839344.*
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.....
Podpis

Białystok, 12.06.2022

prof. dr hab. Agnieszka Błachnio-Zabielska

Zakład Higieny, Epidemiologii i Zaburzeń Metabolicznych
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wchodzącej w skład rozprawy doktorskiej mgr Ilony Juchnickiej, wynoszący 10%, polegał na opracowaniu metodyki, nadzorowaniu przebiegu eksperymentu oraz konsultacji merytorycznej prowadzonych badań.

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

.....
Podpis

Białystok, 31.05.2022

dr hab. n. med. Monika Zbucka- Krętowska

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Monika Zbucka-Krętowska

.....

Podpis

Białystok, 12.06.2022

Dr hab. Piotr Zabielski

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Podpis

Białystok, 31.05.2022

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Oświadczenie współautora

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Magdalena Niemira

Podpis

Białystok, 31.05.2022

dr. n. med. Iwona Sidorkiewicz

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Iwona Sidorkiewicz

Podpis

Białystok, 31.05.2022

mgr Agnieszka Bielska

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Oświadczenie współautora

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Agnieszka Bielska
.....
Podpis

10 Zgoda Komisji Bioetycznej

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Białystok, 26-04-2018

Uchwała nr: R-I-002/176/2018

Komisja Bioetyczna Uniwersytetu Medycznego w Białymstoku, po zapoznaniu się z projektem badania zgodnie z zasadami GCP/ Guidelines for Good Clinical Practice /- **w y r a ż a z g o d ę** na prowadzenie tematu badawczego: „Ocena ekspresji mikroRNA-124a w surowicy pacjentek z prawidłową tolerancją glukozy i cukrzycą ciążową" przez mgr Ilonę Juchnicką wraz z zespołem badawczym z UMB.

Przewodnicząca Komisji Bioetycznej UMB

prof. dr hab. Otylia Kowal-Bielecka