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Analiza profilu mikroRNA i identyfikacja potencjalnych biomarkerów diagnostycznych w surowicy u pacjentów ze stabilną chorobą niedokrwienną serca jako powikłanie cukrzycy typu 2

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prof. dr hab. Adamowi Krętowskiemu *za poświęcony czas, życzliwą atmosferę oraz cenne wskazówki merytoryczne.*

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Spis treści

Zestawienie publikacji

Rodzaj	Liczba publikacji	IF	MNiSW
Prace przeglądowe włączone do rozprawy	1	6,208	140
Prace oryginalne włączone do rozprawy	1	6,055	100
Prace nie włączone do rozprawy	11	61,847	1200
Streszczenia zjazdowe	14	-	-
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Rozprawa doktorska

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1. Artykuły stanowiące cykl prac włączonych do rozprawy doktorskiej

Nazwa czasopisma	Tytuł artykułu	Impact Factor na 2021 rok	Data publikacji	Rodzaj publikacji
Frontiers in Endocrinology	*Serum miRNA profile in diabetic patients with ischemic heart disease as a promising non-invasive biomarker.*	6,055	2022.05.18	praca oryginalna
International Journal of Molecular Sciences	*Recent highlights of research on miRNAs as early potential biomarkers for cardiovascular complications of type 2 diabetes mellitus.*	6,208	2021.03.19	praca przeglądowa

2. Wykaz skrótów

3′ UTR – *3′ untranslated region*, region 3′, niepodlegający translacji
ADORA1 – *adenosine A1 receptor*, receptor adenozynowy A1
Ago – białko argonaute
APLNR – *apelin receptor*, receptor apeliny
AUC – *area under the curve*, pole pod krzywą
BMI – *body mass index*, wskaźnik masy ciała
C3aR1 – *complement C3a receptor 1*, receptor 1 składnika dopełniacza 3a
CCR5 – *C-C motif chemokine receptor 5*, receptor C-C chemokin typu 5
CXCL12 – *C-X-C motif chemokine ligand 12*, chemokinowy (z motywem C-X-C) ligand typu 12
CXCL6 – *C-X-C motif chemokine ligand 6*, chemokinowy (z motywem C-X-C) ligand typu 6
ELISA – *enzyme-linked immunosorbent assay*, metoda immunoenzymatyczna
FC – *fold change*, krotność zmiany
FDR – *false discovery rate*, spodziewany odsetek wyników fałszywie dodatnich
GO – *gene ontology*, ontologia genów
HbA1c – *glycated hemoglobin*, hemoglobina glikowana
HDL – *high-density lipoprotein*, lipoproteiny o wysokiej gęstości
IHD – *ischemic heart disease*, choroba niedokrwienna mięśnia sercowego
IPA – *Ingenuity Pathway Analysis*, program Ingenuity Pathway Analysis
KEGG – *Kyoto Encyclopedia of Genes and Genomes*, encyklopedia genów i genomów
LDL – *low-density lipoprotein*, lipoproteiny o niskiej gęstości
MIF – *macrophage migration inhibitory factor*, czynnik hamujący migrację makrofagów
miRNA – microRNA, mikroRNA
mRNA – *messenger RNA*, matrycowe RNA
pri-miRNA – *primary transcripts miRNA*, pierwotny transkrypt miRNA
pre-miRNA – *precursor miRNA*, prekursorowe miRNA
RISC – *RNA induced silencing complex*, kompleks wyciszający
RNA – *ribonucleic acid*, kwas rybonukleinowy
ROC – *receiver operating characteristic curve*, krzywa charakterystyki
T2DM – *type 2 diabetes mellitus*, cukrzyca typu 2
TG – *triglyceride*, trójglicerydy
RT-qPCR – *real-time polymerase chain reaction*, reakcja łańcuchowa polimerazy w czasie rzeczywistym

3. Wstęp

Cukrzyca typu 2 (T2DM) należy do chorób cywilizacyjnych i jest najczęściej występującym zaburzeniem metabolicznym [1, 2]. Charakteryzuje się współistniejącymi defektami w wydzielaniu insuliny oraz insulinoopornością, czyli obniżeniem lub brakiem wrażliwości tkanek obwodowych na działanie insuliny [3–5]. Według najnowszych ustaleń Międzynarodowej Federacji Diabetologicznej w 2021 roku 537 mln osób cierpiało na cukrzycę i przewiduje się, że ta liczba wzrośnie nawet do 783 milionów w 2045 roku [6]. Początki choroby często są bezobjawowe, a długotrwała ekspozycja na wysokie stężenie glukozy we krwi dramatycznie zwiększa ryzyko wystąpienia dodatkowych powikłań. Najczęstszym przewlekłym powikłaniem cukrzycy jest choroba niedokrwienna serca, inaczej zwana chorobą wieńcową (IHD – *ischemic heart disease*). Schorzenie to stanowi obecnie główną przyczynę śmiertelności na świecie i według szacunków większość zgonów wśród pacjentów z cukrzycą następuje na skutek rozwinięcia się tej choroby [7, 8]. Choroba niedokrwienna serca wynika z zaburzenia równowagi między podażą krwi a zapotrzebowaniem na tlen w komórkach mięśnia sercowego, spowodowanego różnym stopniem niedrożności tętnic wieńcowych. Hiperglikemia, która towarzyszy pacjentom z cukrzycą, ma bardzo negatywny wpływ na stan naczyń krwionośnych i prowadzi do uszkodzeń w ich przekroju, co sprzyja tworzeniu się blaszek miażdżycowych. Cukrzyca, nasilając procesy zapalne, przyspiesza tworzenie się blaszki miażdżycowej, która zwęża średnicę naczyń wieńcowych. Blaszka miażdżycowa składa się głównie z osadów wapnia, makrofagów, złogów tłuszczowych i włóknika. Zwężając światło naczyń utrudnia ona przepływ krwi wraz z substancjami odżywczymi do serca i prowadzi do rozwoju IHD [9, 10].

Powikłania cukrzycy, takie jak IHD, rozwijają się znacznie wcześniej, zanim zostaną zdiagnozowane [11, 12]. Pomimo szybkiego postępu w badaniach układu sercowo-naczyniowego nie ma niezawodnego narzędzia do wczesnej diagnozy i identyfikacji osób zagrożonych jej rozwojem. Metody takie jak echokardiografia czy koronarografia mogą jedynie zdiagnozować chorobę na późniejszym etapie [13]. Istnieje pilna potrzeba wytypowania biomarkerów, które pomogłyby przewidzieć indywidualne ryzyko rozwoju niedokrwienia serca. Czułe i specyficzne biomarkery mogłyby pomóc w opracowaniu lepszych podejść terapeutycznych, zapewnić skuteczniejsze monitorowanie postępu choroby i umożliwić wczesne rozpoznanie dysfunkcji serca.

Badania naukowe wskazują, że mediatory zapalne, takie jak CXCL12 i MIF, odgrywają istotną rolę w patologii IHD [14, 15]. MIF ulega silnej ekspresji w makrofagach i komórkach śródbłonka na różnych etapach rozwoju blaszki miażdżycowej [16]. Podwyższony poziom MIF w osoczu może służyć jako wczesny biomarker ostrego niedokrwienia mięśnia sercowego i może być czynnikiem ryzyka przyszłych zdarzeń wieńcowych u pacjentów z IHD i T2DM. Wykazano, że stężenie CXCL12 w osoczu jest dobrym predyktorem pojawienia się choroby wieńcowej [17]. Stężenie MIF i CXCL12 w surowicy jest jednak charakterystyczne nie tylko dla choroby niedokrwiennej serca, ale także dla innych stanów zapalnych [15, 16]. Do diagnostyki choroby wieńcowej należy wytypować bardziej specyficzne biomarkery, które byłyby w stanie wykryć chorobę na wczesnym, bezobjawowym etapie.

Krążące mikroRNA (miRNA) pochodzące z surowicy mogą służyć jako nowe, potencjalne biomarkery do wczesnej diagnozy IHD oraz mogą pomóc w identyfikacji osób z predyspozycjami do jej rozwoju. MiRNA to krótkie (17-25 nukleotydów), jednoniciowe, niekodujące, endogenne RNA, które odgrywają zasadniczą rolę w regulacji ekspresji genów. Uczestniczą w kluczowych procesach biologicznych, takich jak proliferacja, różnicowanie, angiogeneza, onkogeneza i apoptoza komórek [18–20]. Proces powstawania miRNA jest złożony z wielu etapów rozpoczynających się w jądrze komórkowym, a kończących się w cytoplazmie. Geny kodujące miRNA są transkrybowane przez polimerazę II do pri-miRNA. Pri-miRNA jest przetwarzany na pre-miRNA przy udziale rybonukleazy Drosha. Następnie pre-miRNA jest transportowany przez eksportynę-5 z jądra do cytoplazmy komórki. Endonukleaza Dicer rozszczepia pre-miRNA na krótkie dupleksy miRNA, które są później rozwijane przez nieznaną helikazę. Dojrzała nić miRNA wiąże się z białkiem Ago, tworząc kompleks. MiRNA może regulować ekspresję genów targetowych (transkryptów docelowych) poprzez przyłączenie się do regionów 3′UTR mRNA. Efekt regulacyjny jest określany przez stopień komplementarności pomiędzy sekwencją miRNA a regionem 3′UTR docelowego mRNA. Gdy nie ma pełnej komplementarności, najczęściej dochodzi do represji translacji. Kiedy komplementarność nukleotydowa jest wysoka, dochodzi do degradacji transkryptu, natomiast częściowa komplementarność powoduje zahamowanie translacji [21, 22]. Analizy bioinformatyczne przewidują, że niemal dwie trzecie genów człowieka może być regulowana przez działanie miRNA [23]. Co więcej, jedna cząsteczka miRNA

może regulować setki genów, a jeden gen może być regulowany przez dziesiątki miRNA [24].

Zmiany w ekspresji miRNA są związane z rozwojem różnych chorób, takich jak nowotwory, choroby sercowo-naczyniowe czy zaburzenia metaboliczne. Uważa się, że profilowanie ekspresji miRNA może posłużyć jako narzędzie diagnostyczne do wczesnego wykrycia chorób, oceny stopnia ich zaawansowania oraz do zastosowania spersonalizowanych podejść terapeutycznych [25–27]. Cząsteczki miRNA mają bowiem wiele cech, jakie powinien posiadać idealny biomarker. Wyróżniają się przede wszystkim wysoką stabilnością swojej struktury od momentu pobrania materiału. Są odporne na działanie enzymów (np. RNAzy), cykle rozmrażania i zamrażania oraz zmiany pH, co jest szczególnie ważne z punktu widzenia diagnostyki. Dodatkowym atutem, jaki posiadają te krótkie niekodujące fragmenty RNA, jest ich obecność w różnych płynach biologicznych, takich jak krew, osocze, surowica, ślina, płyn mózgowo-rdzeniowy, mocz, mleko a nawet łzy, co zapewnia łatwy i bardzo mało inwazyjny sposób ich pobierania. Ponadto, można relatywnie łatwo analizować poziom ich ekspresji za pomocą niedrogich, specyficznych i czułych testów. Wszystkie te cechy w połączeniu ze specyficznością tkankową i mechanizmami leżącymi u podłoża wielu chorób oraz korelacja profilu miRNA z odpowiednimi genami powodują, że miRNA stanowią obiecujący przedmiot badań.

W badaniach nad krążącymi miRNA wykazano już ich ogromny potencjał w diagnostyce innych niż IHD powikłań T2DM, które zagrażają zdrowiu i życiu pacjentów. Do przewlekłych zaburzeń o charakterze makroangiopatii, które obejmują uszkodzenia dużych i średnich naczyń krwionośnych, można zaliczyć również chorobę tętnic obwodowych oraz choroby naczyniowo-mózgowe, w tym udar mózgu. Mikroangiopatie wynikają z uszkodzenia naczyń włosowatych oraz drobnych tętnic i żył. W przypadku powikłań cukrzycy typu 2 najczęściej zalicza się tu retinopatię cukrzycową, nefropatię cukrzycową i neuropatię cukrzycową [28–30]. Najnowsze badania dowodzą, że miRNA ma duży potencjał, aby być biomarkerem również do diagnozowania tych powikłań. Dokładny i uporządkowany przegląd najnowszego piśmiennictwa z zakresu zastosowania miRNA w diagnostyce przewlekłych naczyniowych powikłań cukrzycy znajduje się w pracy przeglądowej:

Bielska, A.; Niemira, M.; Kretowski, A. Recent Highlights of Research on MiRNAs as Early Potential Biomarkers for Cardiovascular Complications of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* 2021, *22* (6), 3153. https://doi.org/10.3390/ijms22063153.

4. Cele pracy

Pomimo licznych doniesień na temat badań miRNA w chorobach układu krążenia i w cukrzycy nie wytypowano do tej pory czułych i specyficznych biomarkerów, które właściwie klasyfikowałyby pacjentów z T2DM i IHD oraz pacjentów z T2DM, ale bez IHD. Głównym celem przeprowadzonych badań była identyfikacja charakterystycznego profilu ekspresji miRNA w surowicy u pacjentów z T2DM i IHD oraz określenie przydatności diagnostycznej poprzez opracowanie modelu regresji logistycznej z użyciem zidentyfikowanych miRNA o zróżnicowanej ekspresji. Dodatkowym celem było określenie biologicznej funkcji tych miRNA poprzez identyfikację genów docelowych potencjalnie regulowanych przez wskazane cząsteczki miRNA oraz określenie ich biologicznej roli.

5. Materiał i metody

5.1 Podstawowa charakterystyka pacjentów

Ponad 600 pacjentom z Kliniki Kardiologii Inwazyjnej Uniwersytetu Medycznego w Białymstoku w latach 2002-2004 zlecono zabieg koronarografii celem diagnostyki IHD. Podstawą zlecenia badania były wskazania oparte na całokształcie obrazu klinicznego (m.in. dolegliwości dławicowe, wynik echokardiograficznej próby dobutaminowej, dysfunkcja skurczowa lewej komory serca z frakcją wyrzutową <35%). Badanie zostało wykonane na angiografie DFP60A firmy Toshiba. Zabieg był wykonany z dostępu przez tętnicę udową, a u pacjentów z przeciwskazaniami przez tętnicę ramienną. Większość pacjentów podczas koronarografii miała podawany kontrast jonowy, w niektórych przypadkach, np. u osób z cukrzycą, zastosowano kontrast niejonowy. Za zmianę miażdżycową istotną angiograficznie uznawano zwężenie światła tętnicy wieńcowej równe lub wyższe 70%, a w przypadku pnia lewej tętnicy wieńcowej zwężenie równe lub wyższe 50%. Dodatkowo pacjentom wykonano doustny test obciążenia glukozą w celu diagnostyki T2DM. Cukrzycę potwierdzono zgodnie z wytycznymi Polskiego Towarzystwa Diabetologicznego [31]. Wyodrębniono dwie

podgrupy pacjentów z cukrzycą typu 2: z chorobą niedokrwienną serca (T2DM IHD; n = 24) i bez tego powikłania (T2DM; n = 20). Wyodrębniono również grupę osób z chorobą niedokrwienną serca bez cukrzycy (IHD; n = 9) oraz grupę kontrolną bez cukrzycy, bez stanu przedcukrzycowego i bez choroby niedokrwiennej serca (n = 16). Kryteria wyłączenia z badania obejmowały: cukrzycę typu 1, utajoną autoimmunologiczną cukrzycę dorosłych, inne powikłania cukrzycy (retinopatia, neuropatia, nefropatia, choroba tętnic obwodowych, udar mózgu, choroba naczyniowo-mózgowa), przebyty zawał serca, przezskórną angioplastykę wieńcową, inne stany zapalne (reumatoidalne zapalenie stawów, twardzina układowa), nowotwory, zakażenie ludzkim wirusem niedoboru odporności lub wirusem zapalenia wątroby typu C, niedawno przebyte zabiegi operacyjne, spożywanie alkoholu oraz palenie tytoniu. Do dalszej analizy zakwalifikowano 69 badanych. Próbki krwi od pacjentów po całkowitym wykrzepieniu odwirowano przez 10 minut przy prędkości 3000 obrotów/minutę. Zebrano surowicę i przechowywano ją w temperaturze -80°C. Badanie uzyskało zgodę Komisji Bioetycznej Uniwersytetu Medycznego w Białymstoku (numery zgód: R-I-002/583/2019, APK.002.35.2021) i zostało przeprowadzone zgodnie z zasadami Deklaracji Helsińskiej.

5.2 Izolacja miRNA

Zestaw miRNeasy Serum/Plasma Advanced Kit (Qiagen, Niemcy) został użyty do ekstrakcji RNA przy użyciu 200 µL surowicy od jednego pacjenta, zgodnie z instrukcją producenta. Stężenie miRNA mierzono za pomocą zestawu Qubit microRNA Assay Kit (Invitrogen, California, CA, Stany Zjednoczone) przy użyciu fluorometru Qubit 3.0.

5.3 Detekcja profilu miRNA

Do profilowania ekspresji miRNA przy użyciu platformy nCounter przygotowano łącznie 69 próbek zgodnie z zaleceniami producenta (NanoString Technologies, Stany Zjednoczone). Jako materiał wejściowy wykorzystano 3 ng wyizolowanego miRNA. Unikalne sondy poddano ligacji na 3′ końcu każdego dojrzałego miRNA, zapewniając identyfikator dla każdej cząsteczki miRNA w próbce. Sondy oznaczone są 4 kolorami w 6 różnych kombinacjach. Po znakowaniu odbyła się całonocna hybrydyzacja w temperaturze 65°C do sond reporterowych i wychwytujących, które pozwalały na

utworzenie kompleksu sond z targetem. Po hybrydyzacji próbki były umieszczone w nCounter Prep Station w celu automatycznego oczyszczenia i unieruchomienia kompleksów sonda-target na specjalnej kasecie w celu zebrania danych. Każda próbka była skanowana na analizatorze cyfrowym nCounter (NanoString Technologies, Stany Zjednoczone) w celu zliczenia poszczególnych fluorescencyjnych barkodów i określenia ilości oznaczanych cząsteczek miRNA obecnych w każdej próbce. Surowe dane były analizowane za pomocą oprogramowania nSolver 4.0 (NanoString Technologies, Stany Zjednoczone).

5.4 Pomiar stężenia MIF i CXCL12 w surowicy

Stężenie białek MIF i CXCL12 w surowicy pacjentów oznaczono w podwójnych powtórzeniach metodą immunoenzymatyczną (ELISA) (Quantikine Human M-CSF Immunoassay; R&D systems, Abingdon, Wielka Brytania), zgodnie z zaleceniami producenta.

5.5 Walidacja wyników

Izolaty miRNA z surowicy od 22 pacjentów z grupy T2DM i od 26 z grupy T2DM IHD zostały poddane reakcji odwrotnej transkrypcji przy użyciu zestawu miRCURY LNA RT Kit (Qiagen, Niemcy), zgodnie z instrukcją producenta, na termocyklerze Proflex (Thermo Fisher Scientific, Waltham, Stany Zjednoczone). Następnie przeprowadzono reakcję RT-qPCR z użyciem specyficznych starterów i zestawu miRCURY LNA SYBR Green PCR Kit (Qiagen, Niemcy). Eksperyment był przeprowadzony na płytkach qPCR w dwóch powtórzeniach na aparacie LightCycler 480 Real-Time PCR System (Roche, Szwajcaria). Przy użyciu narzędzia NormFinder [32] wybrano 2 miRNA: miR-103a-3p i miR-199b-5p jako endogenne, referencyjne miRNA. Obliczono wydajność reakcji dla każdej pary starterów poprzez przygotowanie serii rozcieńczeń matrycy. Względne poziomy ekspresji badanych miRNA obliczono za pomocą metody delta-delta Ct ($2^{-\Delta\Delta Ct}$). Ct oznacza cykl progowy, w którym fluorescencja przekracza poziom tła [33].

5.6. Analiza statystyczna

Na podstawie podobnych wcześniejszych eksperymentów i danych pilotażowych z NanoString nCounter miRNA Expression Assay obliczono minimalną liczbę próbek w grupie w celu wykrycia 1,5-krotnej różnicy w relatywnym poziomie ekspresji miRNA pomiędzy badanymi grupami [34]. Zastosowano pakiet R RNASeqPower [35] wykorzystując dane statystyczne obejmujące uzyskane zliczenia i współczynniki zmienności dla poszczególnych grup. Oszacowano, że do uzyskania 80% mocy testu potrzebnych jest 12 próbek na grupę, natomiast do uzyskania 90% mocy testu 16 próbek. Prezentowane grupy składały się z 20 próbek w grupie T2DM i 24 w grupie T2DM IHD, co pozwoliło na uzyskanie ponad 90% mocy testu przy p=0,05. Analizę statystyczną przeprowadzono przy użyciu programu STATISTICA w wersji 13.1 (StatSoft, Tulsa, Oklahoma). Test Shapiro-Wilka wykazał, że badane parametry nie miały rozkładu normalnego. W celu zbadania statystycznej różnicy w parametrach klinicznych pomiędzy grupami wykonano test ANOVA Kruskala-Wallisa. Ekspresję miRNA analizowano przy użyciu oprogramowania nSolver 4.0 (NanoString Technologies, Stany Zjednoczone), normalizując dane względem kontroli pozytywnych procesu ligacji. Progowa wartość zliczeń została ustawiona na 20. Wartości p zostały skorygowane przy użyciu korekty FDR dla wielokrotnych porównań. Poziom istotności statystycznej ustalono na FDR < 0,05. W celu wskazania potencjalnych transkryptów docelowych oraz ścieżek sygnałowych, w których są one zaangażowane, wykorzystano program IPA (Qiagen, Niemcy). Do wygenerowania sieci interakcji białko-białko użyto internetowej bazy STRING (http://string-db.org), programu Cytoscape w wersji 3.7.2 (http://cytoscape.org/), wraz z dodatkiem cytoHubba. Przeprowadzono również analizę funkcjonalną przy użyciu internetowych baz danych KEGG (Kyoto Encyclopedia of Genes and Genomes); g:Profiler (https://biit.cs.ut.ee/gprofiler/gos) oraz Metascape (https://metascape.org). Współczynnik korelacji rang Spearmana (r) został wyznaczony w celu oszacowania współzależności pomiędzy zidentyfikowanymi miRNA a parametrami klinicznymi. Przyjęto, że $r > 0,8$ wskazuje na silną, a $r > 0,3$ na umiarkowaną korelację. Poziom istotności statystycznej ustalono na $p < 0,05$. Do oceny wartości diagnostycznej miRNA zastosowano analizę ROC. Pole powierzchni pod krzywą zostało obliczone dla każdego badanego miRNA i badanych białek. Model regresji logistycznej dla połączonych miRNA został stworzony przy użyciu oprogramowania Weka 3.8.6 (The University of Waikato, Hamilton, Nowa Zelandia).

6. Wyniki

6.1 Podstawowa charakterystyka pacjentów

Badane grupy składały się z 24 pacjentów z cukrzycą typu 2 i chorobą niedokrwienną serca (T2DM IHD) i 20 pacjentów z cukrzycą typu 2 bez tego powikłania (T2DM). Dziewięciu pacjentów cierpiało tylko na chorobę niedokrwienną serca (IHD). Szesnastu pacjentów stanowiło grupę kontrolną bez cukrzycy, z prawidłową tolerancją glukozy i ujemnym wynikiem koronarografii. W grupach T2DM IHD jak i T2DM nie stwierdzono istotnych różnic w parametrach, takich jak czas trwania cukrzycy, płytki krwi, fibrynogen, BMI, poziom HbA1c, poziom glukozy na czczo, ciśnienie tętnicze, stężenie cholesterolu, stężenie TG, LDL i HDL. Charakterystykę kliniczną pacjentów włączonych do tego badania podsumowano w tabeli 1. Badani w obu grupach chorych na cukrzycę byli leczeni standardowymi schematami i przyjmowali doustne leki hipoglikemizujące. Pacjenci byli leczeni pochodnymi biguanidu (42% T2DM i 50% T2DM IHD, $p > 0,05$) oraz pochodnymi sulfonylomocznika (58% T2DM i 68% T2DM IHD, $p > 0,05$).

6.2 Poziom krążących miRNA

Próbki wyizolowanego RNA z surowicy od wszystkich badanych grup zostały poddane analizie ekspresji 798 miRNA przy użyciu platformy nCounter. Analiza wykazała 14 cząsteczek miRNA różniących się poziomem ekspresji pomiędzy grupą T2DM IHD a T2DM. Wszystkie miRNA miały wyższą ekspresję w grupie pacjentów T2DM IHD w porównaniu do grupy T2DM *($|FC| \geq 1.5$, $FDR \leq 0.05$)*. Między profilem miRNA pacjentów z T2DM IHD a grupą bez T2DM i bez IHD znaleziono 155 miRNA ze zmienionym poziomem ekspresji. Dodatkowo wykazano 155 miRNA różniących się ekspresją pomiędzy grupą T2DM i grupą bez T2DM i bez IHD. Analizując profile miRNA pomiędzy grupą T2DM IHD i grupą IHD wskazano aż 221 miRNA o zmienionej ekspresji. Wszystkie te porównania pozwoliły na wytypowanie 6 miRNA o zmienionej ekspresji charakterystycznych tylko dla pacjentów z cukrzycą i chorobą niedokrwienną serca (tabela 2). Dane uzyskane za pomocą platformy nCounter zostały zdeponowane w bazie Gene Expression Omnibus (GEO) pod numerem GSE185845.

6.3 Analiza funkcjonalna

Analiza w programie IPA wykazała 489 potencjalnych genów targetowych, których ekspresja regulowana jest przez 6 wyodrębnionych miRNA. Do zwizualizowania połączeń pomiędzy testowanymi miRNA i genami targetowymi wykorzystano program Cytoscape w wersji 3.9.0 (rycina 1). Narzędzie IPA umożliwiło identyfikację 36 zmienionych kanonicznych ścieżek sygnalizacyjnych, gdzie główne z nich zostały zaprezentowane na rycinie 2. Wykorzystanie bazy STRING i programu Cytoscape z rozszerzeniem cytoHubba posłużyło do wizualizacji oddziaływań białko-białko, potencjalnych genów targetowych i do identyfikacji 10 kluczowych genów (ang. *hub genes*), które w sieciach mają najwięcej połączeń z innymi genami (rycina 2). STRING jest internetową bazą danych znanych i przewidywanych interakcji białko-białko opartych m.in. na najnowszych przeglądach literaturowych oraz informacjach z innych baz danych (KEGG, Pfam i InterPro). Analiza ontologii genów GO oraz ścieżek KEGG zidentyfikowała ścieżki i procesy, w jakie zaangażowane są badane geny targetowe. Ontologia genów składa się z głównych domen: procesów biologicznych, funkcji molekularnych i elementów komórkowych. Powyższe analizy wskazały terminy takie jak: rozwój układu krążenia, śmierć komórkowa, sygnalizacja pomiędzy komórkami, wiązanie enzymów, wiązanie jonów wapnia, wiązanie czynnika transkrypcyjnego, szlaki związane z chorobami układu krążenia i rozwojem cukrzycy, aktywacja płytek krwi, lipidy i miażdżyca, szlaki metaboliczne (rycina 5).

6.4 Poziom stężenia MIF i CXCL12 w surowicy

Nie zaobserwowano istotnych statystycznie różnic pomiędzy koncentracją białek MIF a CXCL12 w surowicy w badanych grupach ($p > 0,05$).

6.5 Korelacja miRNA z danymi klinicznymi

Korelacja rang Spearmana została wykorzystana do oceny związku badanych miRNA z parametrami klinicznymi (rycina 6). Analiza wykazała umiarkowaną korelację pomiędzy poziomem fibrynogenu a miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p i miR-615-3p ($r = 0,38$; $r = 0,40$; $r = 0,41$; $r = 0,36$ odpowiednio; $p < 0,05$). Liczba płytek krwi była ujemnie skorelowana z miR-548b-3p ($r = -0,4$; $p < 0,05$). Bardzo silna

pozytywna korelacja została wskazana pomiędzy wszystkimi badanymi miRNA. Najsilniejsza z nich wystąpiła pomiędzy miR-5196-3p+miR-6732-3p i miR-3147 (r = 0,81; p < 0,001). Nie znaleziono znaczących korelacji między poziomami MIF i CXCL12 a poziomami miRNA.

6.6 Ocena wartości diagnostycznej badanych miRNA

Przeprowadzono analizę krzywej ROC w celu sprawdzenia przydatności diagnostycznej badanych miRNA (rycina 7). Wartości AUC były wyższe niż 0,800 w przypadku miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p i miR-615-3p. Wynik AUC dla miR-548b-3p był również wysoki (AUC = 0,779). Wartości AUC dla MIF i CXCL12 były mniejsze niż 0,500 (AUC = 0,5 określa granicę przydatności diagnostycznej testu).

6.7 Model regresji logistycznej

W celu zbadania możliwego wzrost wartości diagnostycznej, przy jednoczesnym uwzględnieniu kilku miRNA, opracowano modele regresji logistycznej przy użyciu oprogramowania Weka 3.8.6 (The University of Waikato, Hamilton, Nowa Zelandia) [36]. Parametry modeli i standardowe miary jakości zestawiono w tabeli 3. Najwyższe AUC uzyskano dla kombinacji miR-3147 i miR-615-3p (AUC = 0,935). Model ten miał wyższą wartość diagnostyczną w porównaniu do najwyższego AUC dla miRNA stosowanego oddzielnie.

6.8 Walidacja wyników

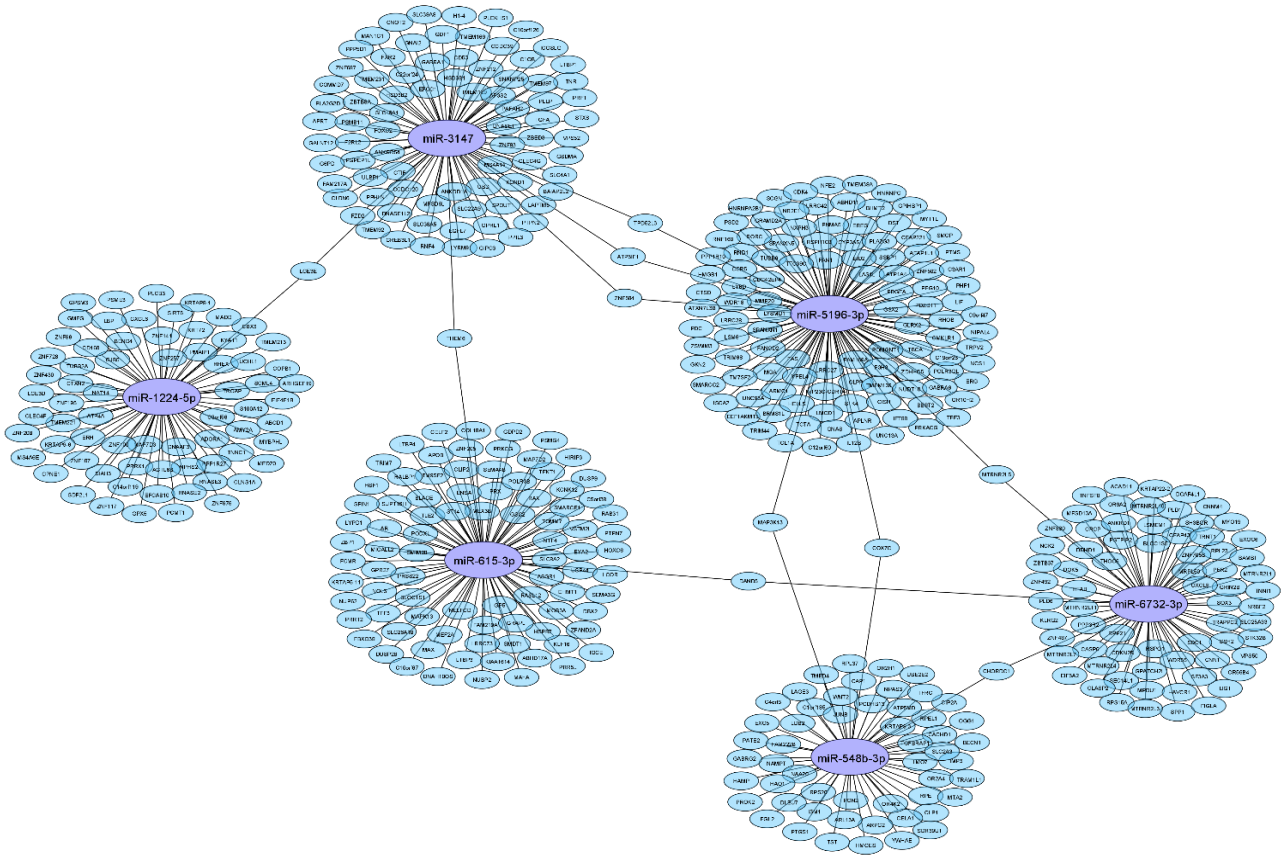
Do walidacji wyników uzyskanych przy użyciu platformy nCounter zastosowano reakcję łańcuchowej polimerazy z analizą ilości produktu w czasie rzeczywistym (real-time qPCR). Do walidacji wybrano miRNA o najbardziej zmienionej ekspresji i najwyższym AUC: miR-615-3p oraz miR-3147. Walidację przeprowadzono w dwóch grupach: 22 pacjentów z T2DM i 26 pacjentów z T2DM IHD. Wyniki uzyskane z użyciem platformy nCounter zostały potwierdzone techniką RT-qPCR. Oba miRNA miały podwyższoną ekspresję u pacjentów T2DM IHD w porównaniu z pacjentami z T2DM bez choroby wieńcowej (rycina 8).

Tabela 1. Podstawowa charakterystyka pacjentów. [a] istotnie statystycznie różne od grupy kontrolnej; [b] istotnie statystycznie różne od grupy IHD * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001;

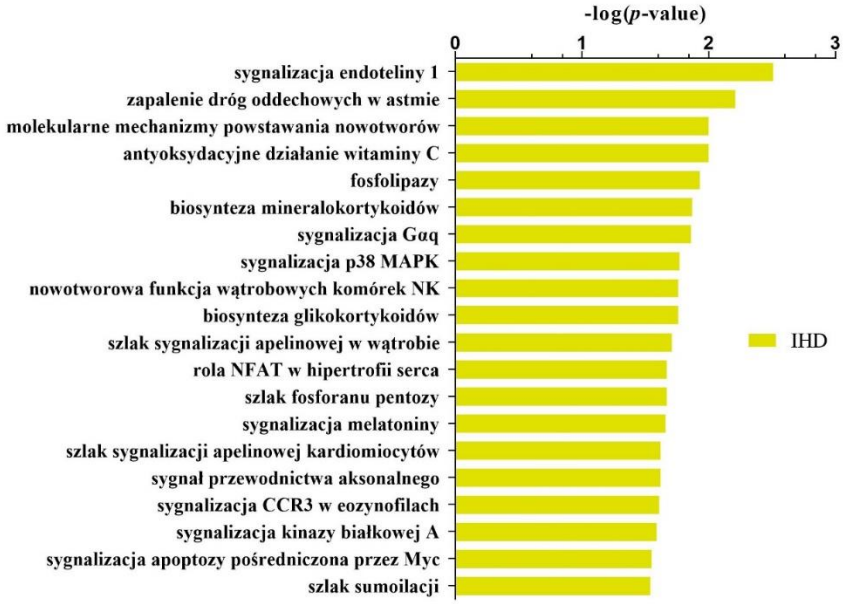
	pacjenci (*n* = 16) grupa kontrolna	**pacjenci (*n* = 9) IHD**	**pacjenci (*n* = 20) T2DM**	**pacjenci (*n* = 24) T2DM IHD**
	mediana (Q1-Q3)	mediana (Q1-Q3)	mediana (Q1-Q3)	mediana (Q1-Q3)
wiek [lata]	52,32 (49,88 - 55,91)	54,85 (50,57 - 61,20)	57,50 (52,33 - 65,17)	58,33 (53,4 - 64,58)
czas trwania T2DM [lata]	-	-	7,00 (4,00 -10,00)	5,00 (2,00-10,00)
BMI [kg/m^2]	24,62 (22,19 - 25,66)	27,73 (26,47 - 30,39)[a*]	32,19 (28,63 - 36,41)[a****]	31,24 (27,68 - 33,20)[a***]
płytki krwi [10^3/μL]	227,50 (163,00 - 268,00)	221,00 (195,00 - 248,00)	222,00 (197,00 - 243,00)	225,00 (186,00 - 322,00)
fibrynogen [mg/dL]	333,50 (272,00 - 352,00)	362,00 (296,00 - 413,00)	352,00 (315,00 - 381,00)	416,00 (354,00 - 482,00)[a**]
leukocyty [10^3/μL]	6,45 (5,49 - 7,30)	7,51 (5,60 - 8,90)	7,16 (5,40 -8,47)	7,32 (6,38 - 9,30)
glukoza na czczo [mg/dL]	89,00 (85,00 - 91,00)	90,00 (84,00 - 100,00)	122,00 (118,00 - 165,00)[a****,b***]	129,00 (104,00 - 176,00)[a***,b**]
HbA1c [%]	5,50 (5,50 - 5,65)	5,50 (5,30 - 5,70)	6,20 (5,70 - 7,50)[a*,b*]	7,60 (6,20 - 8,80)[a***,b***]
skurczowe ciśnienie tętnicze [mmHg]	120,00 (115,00 - 130)	120,00 (110,00 - 140,00)	140,00 (120,00 – 155,00)[a*]	140,00 (130,00 – 155,00)[a***b*]
rozkurczowe ciśnienie tętnicze [mmHg]	80,00 (70,00 - 80,00)	80,00 (80,00 - 100,00)	80,00 (80,00 - 90,00)	85,00 (80,00 - 90,00)
cholesterol [mg/dL]	194,00 (162,00 - 209,00)	176,00 (163,00 - 196,00)	198,50 (172,00 - 224,00)	179,50 (138,00 - 211,00)
TG [mg/dL]	109,00 (86,00 - 153,00)	134,00 (109,00 - 183,00)	124,00 (90,00 - 202,00)	179,00 (109,00 - 206,00)
LDL [mg/dL]	112,00 (92,00 - 132,00)	106,00 (102,00 - 117,00)	118,50 (102,00 - 135,00)	102,00 (66,00 - 133,00)
HDL [mg/dL]	49,00 (40,00- 62,00)	35,00 (33,00 - 45,00)	46,00 (41,00 - 57,00)[b*]	39,00 (34,00 - 50,00)

Tabela 2. MiRNA o podwyższonej ekspresji w grupie T2DM IHD w porównaniu do grupy T2DM.

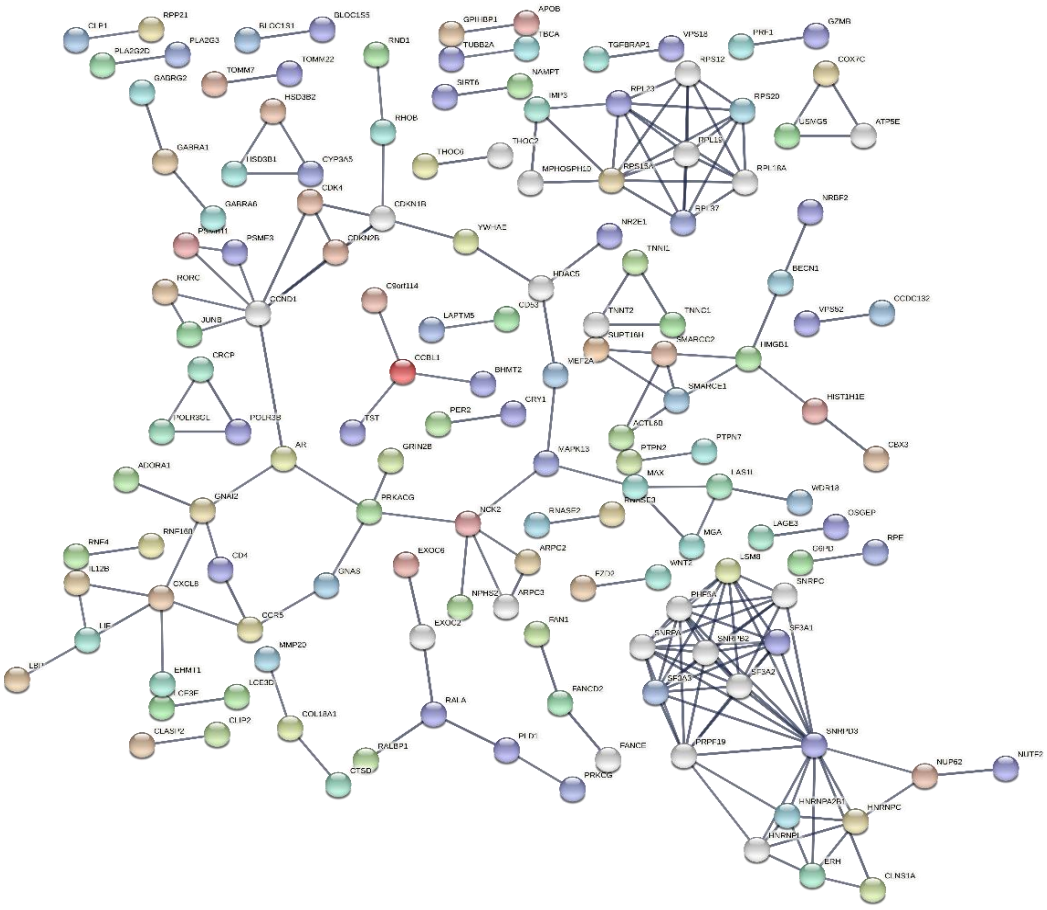
miRNA	**FC**	***p*-value**	**FDR**
miR-615-3p	2,45	0,00000497	0,00
miR-3147	2,35	0,00000200	0,00
miR-1224-5p	1,68	0,00001305	0,00
miR-5196-3p + miR-6732-3p	1,56	0,00040774	0,01
miR-548b-3p	1,55	0,0002042	0,01



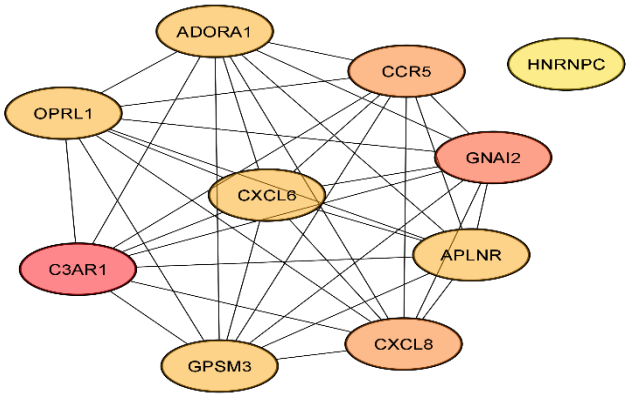
Rycina 1. Sieć powiązań pomiędzy wytypowanymi miRNA i ich potencjalnymi genami targetowymi.



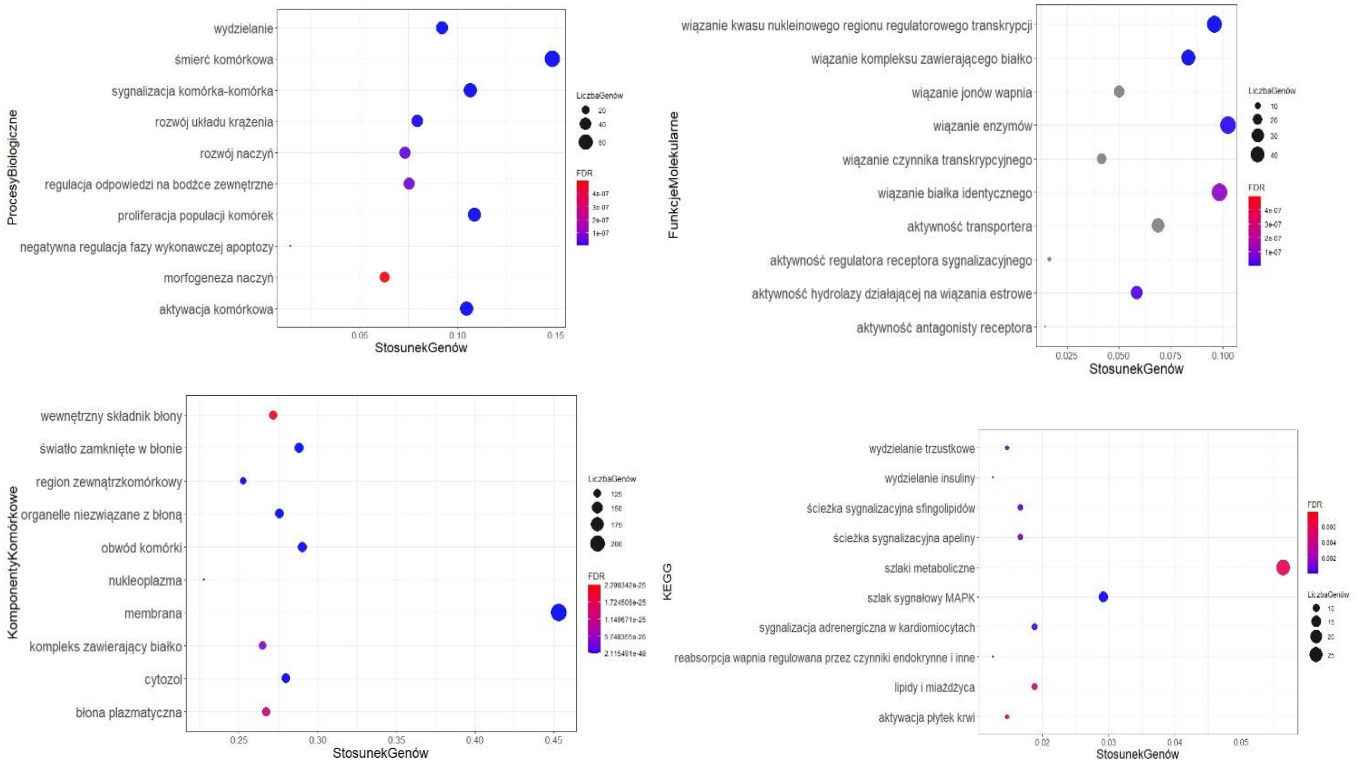
Rycina 2. Najważniejsze ścieżki kanoniczne istotnie zmienione u pacjentów T2DM IHD w porównaniu do grupy T2DM.



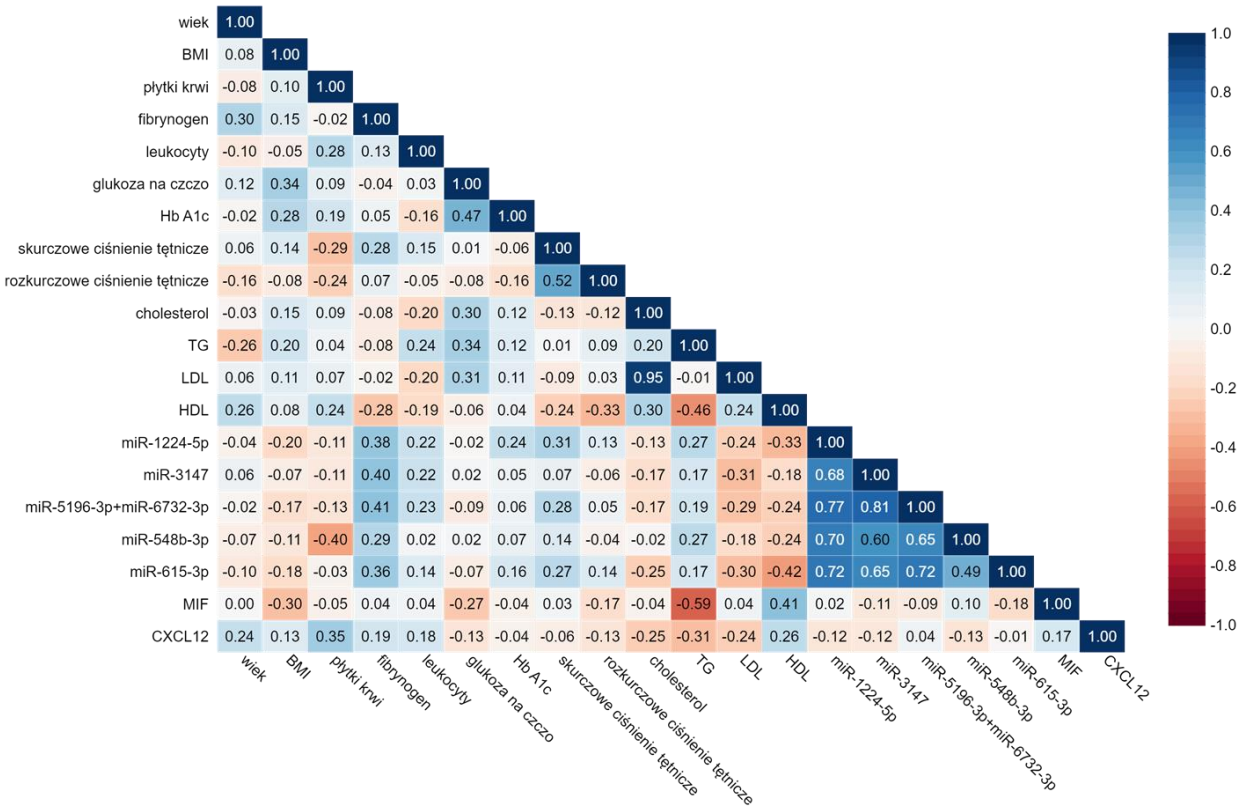
Rycina 3. Sieć interakcji białko-białko genów docelowych dla testowanych miRNA. Za wskaźnik interakcji obrano wartość 0,9 wskazując na wysokie prawdopodobieństwo, że asocjacja jest prawdziwa. W celu zwiększenia przejrzystości rycina pozbawiona jest genów, które nie wykazywały interakcji z żadnym innym genem z analizowanej grupy.



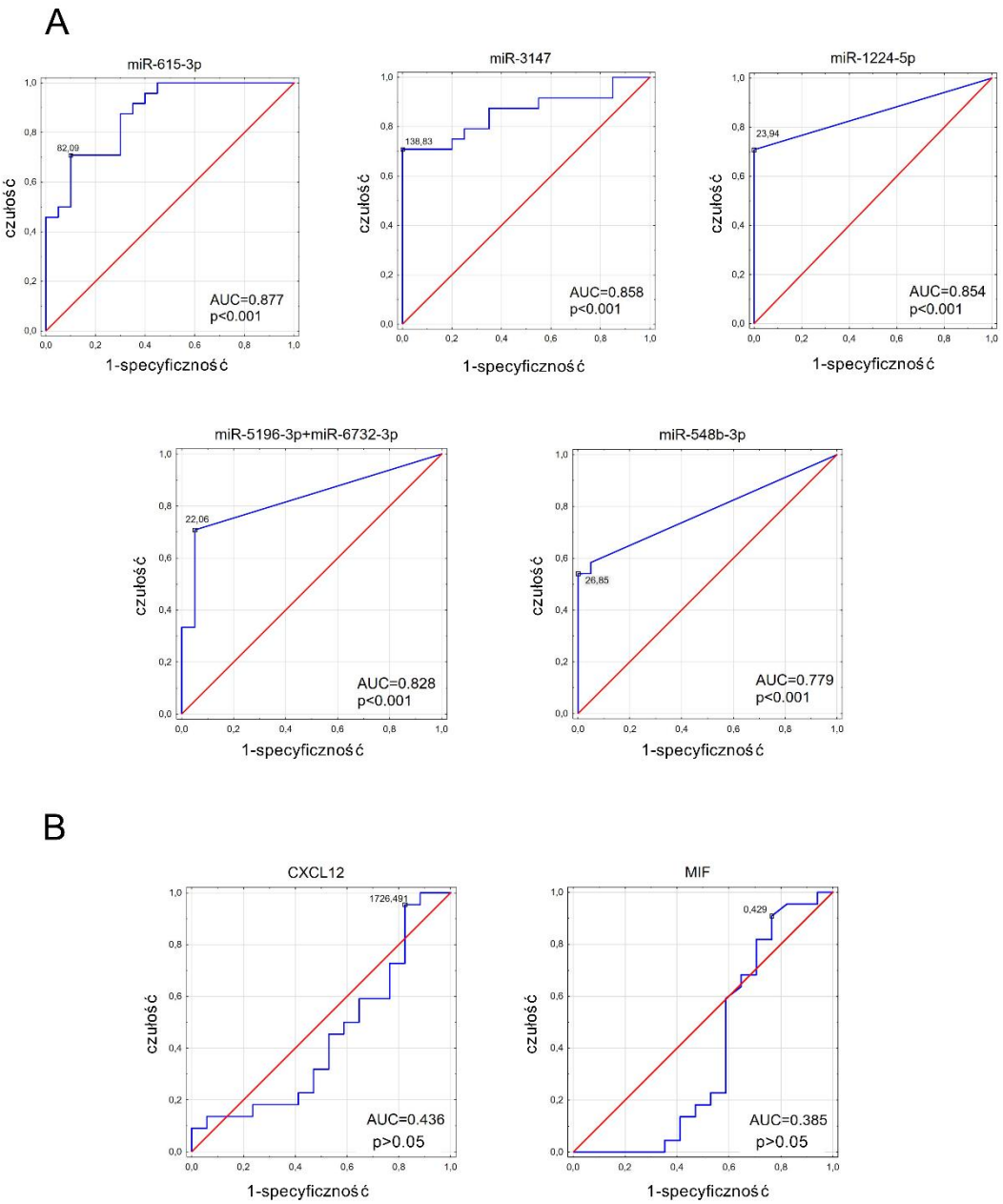
Rycina 4. Sieć połączeń 10 kluczowych genów (ang. *hub genes*).



Rycina 5. Analiza ontologii genów targetowych istotnie zmienionych miRNA. Wykres punktowy przedstawia 10 najistotniejszych kategorii w procesach biologicznych, funkcjach molekularnych, komponentach komórkowych i ścieżkach KEGG związanych z chorobami układu krążenia i cukrzycą. Kolory węzłów zilustrowano od czerwonego do niebieskiego w kolejności malejącej wartości *p*-value skorygowanej przez FDR. Rozmiary węzłów są przedstawione w zależności od liczby genów zaangażowanych w daną kategorię. Oś *x* przedstawia stosunek genów, a oś *y* terminy ontologii genów lub ścieżek KEGG.



Rycina 6. Korelacja rang Spearmana pomiędzy badanymi miRNA, białkami i parametrami klinicznymi. Kolor niebieski oznacza korelację dodatnią, a czerwony ujemną.

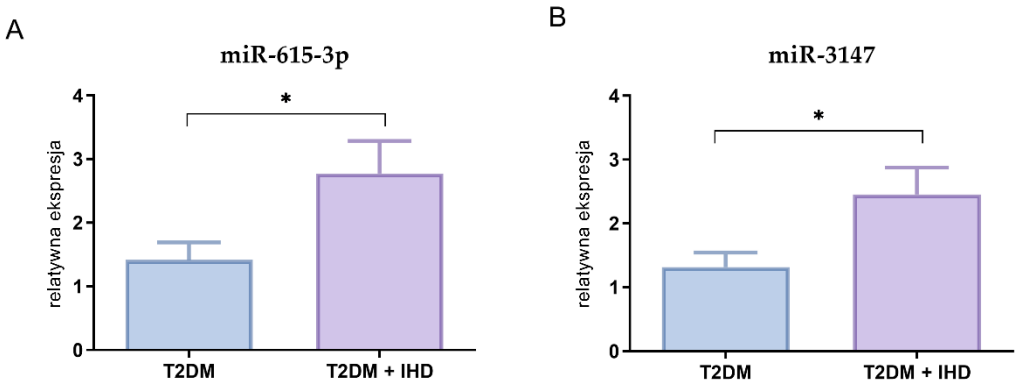


Rycina 7. Wyniki analizy ROC przeprowadzonej w celu oceny wartości wybranych miRNA (A) i białek (B) jako diagnostycznych biomarkerów.

Tabela 3. Zestawienie podstawowych parametrów modelu regresji logistycznej. PD – prawdziwie dodatnie, FU – fałszywie ujemne.

model	PD	FU	precyzja	AUC	stała regresji	współczynnik
x1 = miR-3147	0,864	0,147	0,866	**0,935**	28,56	x1 = -9,03
x2 = miR-615-3p						x2 = -6,87

x1 = miR-548b-3p x2 = miR-615-3p	0,841	0,166	0,841	0,929	20,3	x1= -7,06 x2= -7,22
x1 = miR-3147 x2 = 548b-3p x3 = 615-3p	0,886	0,12	0,887	0,927	26,78	x1 = -6,49 x2 = -3,09 x3 = -6,54
x1 = 1224-5p x2 = 615-3p	0,795	0,204	0,797	0,906	20,68	x1 = -8,78 x2 = -5,87
x1 = miR-3147 x2 = miR-615-3p x3 = 1224-5p x4 = 548b-3p x5 = 5196-3p+miR-6732-3p	0,795	0,212	0,795	0,881	28	x1 = -6,69 x2 = -6,47 x3 = -2,06 x4 = -2,27 x5 = 0,55



Rycina 8. Względna ekspresja miR-615-3p (A) i miR-3147 (B) w surowicy w grupie pacjentów z T2DM i z T2DM IHD. Każdy słupek przedstawia średnią geometryczną ± błąd standardowy średniej stosunku ekspresji miRNA i miRNA referencyjnego (miR-103a-3p i miR-199b-5p). Zastosowano test U Manna-Whitneya; *p < 0,05.

7. Dyskusja

Cukrzyca typu 2 jest złożonym zaburzeniem metabolicznym, w które zaangażowanych jest wiele genów i ścieżek sygnałowych. Liczne badania donoszą, że T2DM jest niewątpliwym czynnikiem ryzyka pojawienia się IHD oraz innych powikłań naczyniowych [37]. Opracowanie nowych biomarkerów, które identyfikowałyby osoby z cukrzycą narażone na rozwój choroby wieńcowej, mogłoby usprawnić diagnostykę takich pacjentów zanim pojawią się pierwsze objawy niedokrwienia serca. Niekodujące cząsteczki miRNA pochodzące z płynów ustrojowych mogą pełnić rolę takich biomarkerów. Istnieją badania potwierdzające ważną rolę miRNA w rozwoju powikłań cukrzycy [38–40]. Wykazano, że miRNA biorą udział w podziałach komórkowych, w różnicowaniu komórek, apoptozie, angiogenezie, regulacji szlaków metabolicznych i zapalnych [41]. Te małe niekodujące cząsteczki RNA mogą również regulować procesy wydzielania insuliny, różnicowania komórek β wysp trzustkowych oraz metabolizmu glukozy i lipidów [40].

W celu odkrycia unikalnego profilu miRNA u pacjentów T2DM IHD użyto innowacyjnej platformy nCounter firmy NanoString, która daje możliwość dokładnego profilowania dużej liczby miRNA w jednej reakcji. Główną zaletą wybranej metody jest jej wysoka czułość i specyficzność dzięki częściowemu zautomatyzowaniu procesu oraz ominięciu etapu amplifikacji, który może generować błędy [42, 43]. Było to pierwsze badanie przy użyciu technologii NanoString poszukujące nowych, nieinwazyjnych biomarkerów do wczesnego wykrywania IHD u pacjentów z T2DM.

Prezentowane badania wskazały 6 cząsteczek miRNA, które miały zwiększoną ekspresję w grupie pacjentów T2DM IHD. Spośród badanych miRNA, miR-615-3p i miR-3147 miały najwyższe FC i AUC (odpowiednio FC = 2,45 i 2,35; AUC = 0,877 i 0,858). MiR-615-3p było już wcześniej uwzględniane w badaniach jako biomarker ostrego zawału mięśnia sercowego [44]. Najnowsze opracowanie wskazuje, że nadekspresja tego miRNA działa łagodząco na uszkodzenia w linii kardiomiocytów spowodowane stresem oksydacyjnym, który ma istotne znaczenie w chorobie wieńcowej. W badaniu nie uwzględniano jednak wpływu cukrzycy [45]. MiR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p i miR-548b-3p nigdy wcześniej nie były opisywane w kontekście T2DM czy chorób układu krążenia. Może świadczyć to o ich wysokim potencjale do pełnienia roli nowych, specyficznych biomarkerów choroby niedokrwiennej serca u pacjentów z T2DM.

Dzięki analizie przeprowadzonej w programie IPA przedstawiono sieć zależności pomiędzy miRNA i określonymi genami oraz wyznaczono regulowane przez nie 489 genów targetowych. Niektóre ze wskazanych genów kluczowych, takie jak C3aR1, CCR5, CXCL6, ADORA1 czy APLNR, są silnie powiązane z procesami dotyczącymi zaburzeń sercowo-naczyniowych i z cukrzycą [46–53].

Wyniki wykazały, że jedną z najważniejszych zmienionych ścieżek kanonicznych u pacjentów z grupy T2DM IHD była ścieżka związana z sygnalizacją endoteliny-1. Endotelina-1 jest białkiem o właściwościach wazokonstrykcyjnych i ma istotny udział w patogenezie nadciśnienia tętniczego, miażdżycy, przerostu naczyń i cukrzycy [54]. U pacjentów z T2DM występuje zwiększona aktywność wazokonstrykcyjna indukowana przez endotelinę-1 [55]. Udowodniono również, że p38 MPAK pełni kluczową rolę w proliferacji i apoptozie kardiomiocytów oraz uczestniczy w regulacji przerostu serca [56]. Biosynteza mineralokortykoidów oraz glikokortykoidów należą do kolejnych ścieżek kanoniczych, które mogą być zaangażowane w rozwój choroby niedokrwiennej serca, szczególnie na etapie rozwoju miażdżycy [57, 58]. Kolejną wskazaną w analizie ścieżką kanoniczną jest szlak pentozofosforanowy, który jest istotnym szlakiem dla metabolizmu glukozy i uczestniczy w patogenezie T2DM [59, 60]. Analiza wytypowała również szlak sygnalizacji apelinowej kardiomiocytów jako jedną z głównych zmienionych ścieżek kanonicznych w porównywanych grupach. Apelina jest białkiem biorącym udział w regulacji ciśnienia tętniczego, uczestniczy w procesie angiogenezy i ma znaczenie kardioprotekcyjne. Dodatkowo uważa się, że apelina wydzielana przez tkankę tłuszczową może przyczyniać się do zaburzeń związanych z otyłością i cukrzycą. Ma również wpływ na takie procesy jak wychwyt glukozy, lipolizę czy oksydację kwasów tłuszczowych [53]. Warto dodać, że gen kodujący receptor apeliny był wskazany jako jeden z genów kluczowych w analizie genów targetowych. Zidentyfikowane ścieżki kanoniczne odgrywają istotną rolę w mechanizmach prowadzących do rozwoju chorób sercowo-naczyniowych. Pozwala to przypuszczać, że wyznaczone w tym badaniu miRNA odgrywają istotną rolę w regulacji genów związanych z chorobami układu sercowo-naczyniowego.

Warto podkreślić, że nie znaleziono statystycznie istotnej korelacji pomiędzy poziomem miRNA a parametrami klinicznymi, takimi jak wiek, BMI, leukocyty, glukoza na czczo, HbA1c, rozkurczowe ciśnienie krwi, poziom cholesterolu, trójglicerydów czy LDL. Wynikać to może z faktu, że parametry te nie są specyficzne tylko dla choroby wieńcowej. Jednakże wykazano dodatnią korelację pomiędzy poziomem fibrynogenu a

miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p oraz miR-615-3p. Wymienione miRNA nie były wcześniej opisywane jako związane z tym białkiem. W badaniach epidemiologicznych i klinicznych podwyższony poziom fibrynogenu we krwi jest niezależnym czynnikiem ryzyka chorób sercowo-naczyniowych [61, 62]. Udowodniono również, że miRNA mogą regulować produkcję fibrynogenu [63]. Zaobserwowano umiarkowaną negatywną korelację między miR-548b-3p a poziomem płytek krwi. Związek miRNA z płytkami krwi w rozwoju choroby niedokrwiennej serca został wcześniej udowodniony i opisany w licznych pracach [64–67]. Silna lub umiarkowana dodatnia korelacja pomiędzy poziomami wszystkich miRNA może sugerować, że należą one do grupy miRNA, w której wszystkie zaangażowane są w deregulację mechanizmów odpowiedzialnych za powikłania cukrzycy.

Krzywa ROC ilustruje zależność pomiędzy czułością i swoistością diagnostyczną oraz opisuje wartość diagnostyczną badanych parametrów [68]. W przeprowadzonych badaniach najwyższe wartości AUC zaobserwowano dla miR-615-3p i miR-3147. Ponadto inne badane miRNA (miR-1224-5p, miR-5196-3p, miR-6732-3p oraz miR-548b-3p) również wykazały wysoką wartość diagnostyczną. W wielu przypadkach większą moc diagnostyczną można uzyskać w przypadku badania panelu miRNA w porównaniu do pojedynczej cząsteczki miRNA [69, 70]. Stworzony model regresji logistycznej składający się z panelu miR-3147 i miR-615-3p wykazał większą dokładność diagnostyczną niż miRNA badane osobno. Zastosowanie panelu diagnostycznego miR-615-3p i miR-3147 przy identyfikowaniu pacjentów z T2DM IHD może być korzystne jako wsparcie diagnostyczne. Panel zwiększa precyzyjność i wiarygodność testu, przez co może przyczynić się do podejmowania szybkich i trafnych decyzji dotyczących postępowania z pacjentem. Wartości AUC dla MIF i CXCL12 były niższe niż 0,500, co dyskwalifikuje te dwa białka jako dobre narzędzie diagnostyczne pacjentów z cukrzycą z IHD. Należy uwzględnić fakt, że cukrzyca jest również stanem zapalnym, a nieswoiste parametry zapalne, takie jak MIF czy CXCL12, mogą być podwyższone zarówno u pacjentów z chorobą wieńcową, jak i z cukrzycą bez powikłań naczyniowo-sercowych.

W celu wytworzenia klasyfikującego modelu diagnostycznego możliwego do stosowania w praktyce, do walidacji oceny poziomu ekspresji miR-615-3p i miR-3147 zastosowano technikę RT-qPCR. Analiza wykazała znacząco zwiększoną ekspresję miR-615-3p i miR-3147 w surowicy pacjentów T2DM IHD, co potwierdza wiarygodność wyników uzyskanych za pomocą platformy nCounter.

Podsumowując, prezentowane badania wskazały profil miRNA, który odróżnia grupę pacjentów T2DM IHD od pacjentów T2DM. Wyniki nasuwają wniosek, że wytypowane miRNA mogłoby być bardzo dobrym narzędziem do diagnozowania niedokrwienia serca wśród pacjentów z T2DM lub mogłoby wskazać pacjentów predysponujących do rozwoju tego powikłania. Dzięki wykryciu choroby na początkowym jej etapie możliwe stałoby się wdrożenie wczesnego leczenia i podjęcie działań zapobiegających rozwojowi choroby. Oznaczanie profilu miRNA jest obiecującym narzędziem w diagnostyce również innych powikłań T2DM. Niezbędna jest jednak kontynuacja badań na większych grupach pacjentów oraz walidacja otrzymanych wyników innymi, wystandaryzowanymi metodami, aby móc rzetelnie wskazać na biomarkery innych powikłań cukrzycy zarówno mikro-, jak i makronaczyniowych. W celu uniknięcia błędnych wyników, badacze nieustannie powinni dążyć do normalizacji i standaryzacji pobierania i przechowywania próbek, izolacji oraz dalszej detekcji miRNA. Do tej pory najwięcej prac skupiało się na określeniu diagnostycznej roli cząsteczek miRNA w surowicy, jednak w dalszych badaniach warto skoncentrować uwagę na miRNA z jeszcze łatwiej dostępnych materiałów, takich jak ślina, łzy czy mocz.

8. Wnioski

1. Podwyższona ekspresja sześciu miRNA pochodzących z surowicy (miR-615-3p, miR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p i miR-548b-3p) stanowi specyficzny profil charakterystyczny tylko dla grupy pacjentów T2DM IHD.
2. Cząsteczki miR-615-3p i miR-3147 miały najbardziej podwyższoną ekspresję w surowicy pacjentów T2DM IHD w porównaniu do pacjentów T2DM bez IHD.
3. Analizy bioinformatyczne wykazały, że wytypowane miRNA oraz geny przez nie regulowane uczestniczą w ścieżkach i procesach prowadzących do dysfunkcji układu krążenia i rozwoju IHD u pacjentów z T2DM.
4. Wszystkie wytypowane cząsteczki miRNA mają wysoką czułość oraz wysoką swoistość i potencjalnie mogą służyć jako nowe, nieinwazyjne biomarkery do wczesnego wykrywania IHD u pacjentów z T2DM.
5. Klasyfikujący model diagnostyczny, utworzony z użyciem modelu regresji logistycznej na podstawie poziomu ekspresji tylko dwóch cząsteczek miRNA, miR-615-3p oraz miR-3147, różnicuje pacjentów T2DM z IHD od pacjentów T2DM, ale bez IHD, z wyższą swoistością i czułością niż każde miRNA ze

zróżnicowaną ekspresją, analizowane pojedynczo. Zastosowanie zestawu składającego się jedynie z dwóch biomarkerów miRNA jest ogromną szansą na stworzenie nieinwazyjnego testu diagnostycznego do szybkiej i efektywnej diagnostyki powikłań sercowo-naczyniowych u pacjentów z T2DM. Jest to szczególnie korzystne w aspekcie prowadzenia diagnostyki w badaniach przesiewowych oraz wdrożenia skutecznego leczenia na bardzo wczesnym etapie choroby.

9. Publikacja przeglądowa

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Review

Recent Highlights of Research on miRNAs as Early Potential Biomarkers for Cardiovascular Complications of Type 2 Diabetes Mellitus

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Abstract: Type 2 diabetes mellitus (T2DM) and its complications pose a serious threat to the life and health of patients around the world. The most dangerous complications of this disease are vascular complications. Microvascular complications of T2DM include retinopathy, nephropathy, and neuropathy. In turn, macrovascular complications include coronary artery disease, peripheral artery disease, and cerebrovascular disease. The currently used diagnostic methods do not ensure detection of the disease at an early stage, and they also do not predict the risk of developing specific complications. MicroRNAs (miRNAs) are small, endogenous, noncoding molecules that are involved in key processes, such as cell proliferation, differentiation, and apoptosis. Recent research has assigned them an important role as potential biomarkers for detecting complications related to diabetes. We suggest that utilizing miRNAs can be a routine approach for early diagnosis and prognosis of diseases and may enable the development of better therapeutic approaches. In this paper, we conduct a review of the latest reports demonstrating the usefulness of miRNAs as biomarkers in the vascular complications of T2DM.

Keywords: diabetes; cardiovascular; complications; miRNA; biomarker

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1. Introduction

Diabetes mellitus (DM) is a group of chronic endocrine and metabolic disorders characterized by defects in insulin production, secretion, and signaling that are insufficient to maintain the right blood glucose level [1,2]. This serious disease affects increasing numbers of people every year. According to the International Diabetes Federation, 463 million people suffered from diabetes in 2019, and the prognosis suggests that this number will increase to 700 million in 2045 [3]. Type 2 diabetes mellitus (T2DM) is the most common type of diabetes. This complex disease is characterized by insulin resistance when cells are not able to respond properly to a normal level of insulin and a progressive loss of β-cell function [1]. Diabetes can lead to acute or chronic complications (Table 1). Many patients do not have any symptoms of T2DM for a long time, and prolonged exposure to high blood sugar levels dramatically increases the risk of additional complications [4]. Due to numerous clinical consequences associated with diabetes, this chronic disease is linked with shorter life expectancy [5]. Notably, long-term hyperglycemia can result in chronic micro- and macro-vascular complications, and related to them, the diabetic foot, nephropathy, neuropathy, and, finally, heart attack and stroke [6–10].

Table 1. Main complication of diabetes mellitus.

Complication of Diabetes			
Acute	**Chronic**		
diabetic ketoacidosis	**Vascular**		**Nonvascular**
nonketotic hyperosmolar coma	**Microvascular**	**Macrovascular**	sexual dysfunctions
hypoglycemia diabetic coma	retinopathy nephropathy neuropathy	coronary artery disease peripheral artery disease cerebrovascular disease including ischemic stroke	skin complications

Recent research has shown that cardiovascular diseases are the leading reasons for morbidity and mortality in diabetes [11,12]. The hyperglycemia intensifies and promotes the glycation of proteins, which is a nonenzymatic attachment of sugars to the free amino groups of proteins that can accelerate the occurrence of serious cardiovascular problems.

Nowadays, there is no reliable tool for prompt identification of the patient at risk of developing T2DM and its cardiovascular complications. Most of the currently used methods can only identify the disease at a later stage. These statements suggest that there is an urgent need to identify early specific biomarkers, which would help to predict individual risk of development of diabetes and its complications.

Numerous studies have established that miRNA has tremendous potential to serve as a tool for improving the diagnosis of T2DM, indicate cardiovascular complications at an early stage, or identify patients with a predisposition to develop them. MiRNAs are small (17–25 nucleotides), endogenous, noncoding, single-stranded RNAs, which have a variety of important regulatory effects in cells. MiRNAs play a pivotal role in the regulation of gene expression. They participate in important processes, including cell proliferation, differentiation, and adhesion [13,14]. The control of the expression of targeted genes is achieved by interacting with the 3′ untranslated region (3′UTR) of its target messenger RNA (mRNA). The complementary degree between the miRNA sequence and 3′UTR of its target mRNA determines the regulatory effect of miRNA [15]. MiRNAs are derived from partially complementary primary RNA transcripts (pri-miRNA) produced mainly by RNA polymerase II in the nucleus (Figure 1). To form a precursor of miRNAs (pre-miRNAs), the stem–loop structure of pri-miRNA is cleaved by Drosha and then transported by Exportin 5 to the cytoplasm. At this moment, pre-miRNA is about 70 nucleotides in length. In the next step, a miRNA-specific nuclease Dicer splits it into double-stranded miRNA. In the last step of this process, protein AGO2, which is a member of RISC (RNA-included silencing complex), is involved. One of the strands of miRNA is removed, and the remaining strand is bound to AGO2. This complex targets the 3′ UTR region of mRNA. Association of miRNA with its target mRNA can result in mRNA cleavage, translational repression, or mRNA deadenylation [16,17].

Dysregulation of the expression of miRNAs is associated with the development of various types of cancer, cardiovascular diseases, lung diseases, autoimmune disease, or metabolic disorders. Expression of miRNA is tissue-specific, which allows for identification of its origin [18,19]. MiRNAs have many features of an ideal biomarker. They are stable in biofluids even after a long time after collection and freeze–thaw cycles [20]. The undoubted advantage of these small particles is the availability of material for research. MiRNAs can be collected and detected in a minimally invasive way in easily accessed biofluids, such as serum, plasma, blood, tears, urine, or saliva [14,21]. Despite their numerous benefits, it is also worth paying attention to the limitations of miRNAs as potential biomarkers for various diseases. To avoid misleading results, researchers should strive for normalization and standardization of sample collection, storage, isolation method, and further detection of miRNAs. Interestingly, the level of circulating miRNAs is confounded by age, gender, or some medications [22]. It has been proven that statins decrease circulating miR-122 levels, and heparin (used in medicine as an anticoagulant) influences the PCR reaction

during the quantification process [23]. Moreover, some miRNAs are not specific to one disease only [24].

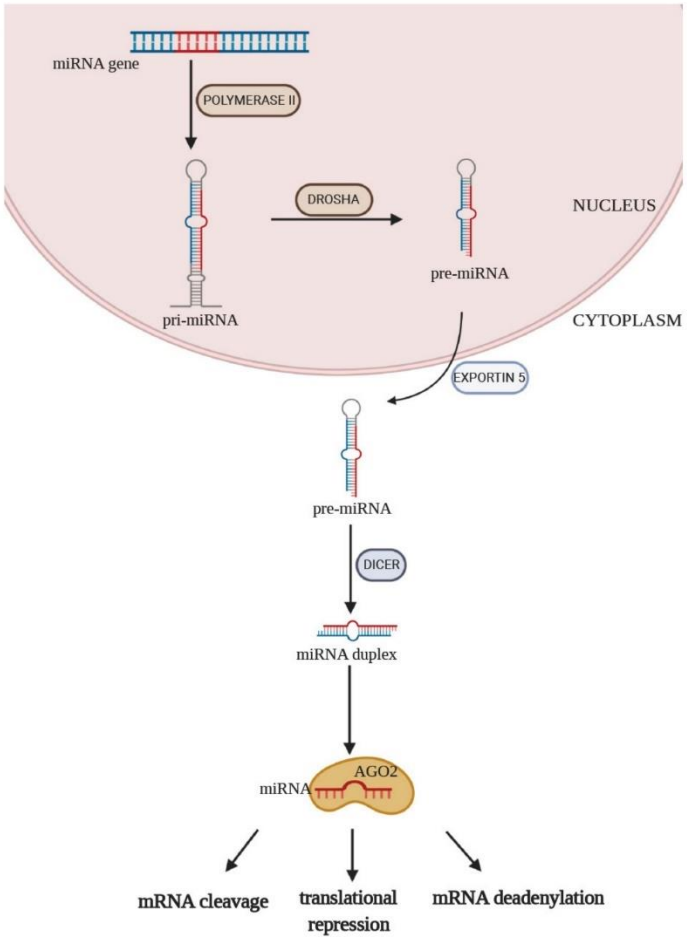

Figure 1. MiRNA biosynthesis and functions. Process of the biosynthesis of miRNA begins in the nucleus. MiRNA genes are transcribed by polymerase II to primary RNA (pri-miRNA). Pri-miRNA is processed to pre-miRNA with the participation of the ribonuclease Drosha. Subsequently, pre-miRNA is transported via Exportin 5 from the nucleus to the cytoplasm of the cell. Dicer is an endonuclease that cleaves pre-miRNAs into short miRNA duplexes, which are later unwound by an unknown helicase. The mature miRNA strand binds to an Argonaute (Ago) protein, forming a complex.

Altered expression of miRNAs can be used as a disease biomarker as well as to understand the pathogenesis. The measurement of the expression levels of miRNA is best carried out with a specific and sensitive assay that allows for the collection of exact data in a short amount of time [25]. Currently, the most commonly used methods for detecting miRNA expression include quantitative reverse transcription PCR (RT-qPCR), different types of microarrays, and next-generation RNA sequencing [21,24]. Identifying the potential targets of miRNAs is crucial for understanding miRNA function. Lately, multiple bioinformatic tools have been developed so that scientists are able to predict

possible regulated genes by miRNAs and their functions or their implication in signal pathways [26]. In addition, knowledge that abnormal miRNA expression is associated with many diseases allows for its use as potential therapeutic targets. Manipulation of their expression is used as a new form of clinical treatment [27]. All bioinformatic tools should be validated by scientists to discover novel biomarkers.

Currently, according to the database miRBase (www.mirbase.org, version 22), more than 2600 human mature miRNAs have been identified [28]. Interestingly, it is believed that they are collectively able to regulate about 30% of all genes in the human genome [29]. Bioinformatics prediction suggests that one miRNA could target more than a hundred mRNAs. Conversely, if a single mRNA had complementary sequences for more than one miRNA, it could be regulated by many different miRNAs (Figure 2) [30,31].

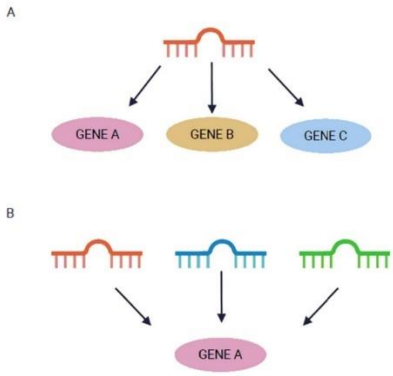

Figure 2. MiRNA binding to target mRNA. A single miRNA can target many mRNAs (**A**). Many miRNAs can target one mRNA (**B**).

Over the last years, several studies on diabetes have indicated specific miRNAs as putative biomarkers for predicting the occurrence of the disease. Interestingly, the dysfunctions of pancreatic β cells composed of the abnormal insulin secretion, and thus impaired glucose tolerance, can occur even 10 years before clinical diagnosis [29,30]. In the prediabetic and diabetic states, expression of miRNAs is altered in organs, but also in biofluids. Many studies have shown that miRNA expression, such as that of miR-375, miR-7, and miR-184, is associated with dysfunction of pancreatic β cells. These miRNAs can play an important role in processes such as proliferation or regulation of crucial pathways [32–34]. We know that miRNAs contribute to the regulation of processes related to the proper functioning of pancreatic cells. In addition, many studies argue that these small molecules can serve as a biomarker in detecting T2DM. MiR-126 is one of the most known and the most studied miRNAs in diabetes and its complications. It plays an essential role in endothelial protection and angiogenesis. Interestingly, serum miR-126 was proposed as a biomarker for prediabetes and T2DM [35]. We have previously shown in our study that serum miR-491-5p, miR-1307-3p, and miR-298 can serve as a biomarker for the progression of diabetes. These miRNAs have high diagnostic value for the prediction of T2DM, and we indicated that their expression is dysregulated before T2DM development [36]. MiR-23a seems to be a valuable marker for the early detection of T2DM [37]. Moreover, Pescador et al. found that a three-miRNA panel (miR-15b, miR-138, and miR-376a) is significant for predicting diabetes and obesity [38]. Other interesting findings show that serum-derived miR-486, miR-146b, and miR-15b can also play an important role in predicting the risk of developing DM in obese children. Oerlemans et al. showed in their research that some miRNAs can improve the diagnostic value of currently used biomarkers for acute coronary syndrome identification. MiR-1, miR-499, and miR-21 manifested the significantly

increased diagnostic value in combination with hs-troponin T (hs-TnT) (for a combination area under the curve (AUC) = 0.94, whereas hs-TnT alone reached AUC = 0.89) [39].

Levels of certain circulating miRNAs might also be predictive for long-term diabetes complications [25]. The most common complications of DM are vascular pathologies. They are considered as serious manifestations of the disease, and there is an urgent need to understand the pathophysiology of their appearance. This can help with accurate and quick diagnostics and the development of better therapeutic approaches. MiRNAs are the key regulators of cardiovascular system development and maintenance. Altered expression of miRNA in cardiovascular diseases such as arrhythmias, hypertension, myocardial infarction, coronary artery disease, or vascular inflammation is observed [40,41]. In this review, the newest reports regarding the role of miRNA in the vascular complication of T2DM are summarized. In brief, data from a PubMed search were used.

2. Vascular Complications of T2DM

T2DM is undoubtedly the cause of numerous vascular diseases, affecting almost all types and sizes of blood vessels [42]. Diabetes vascular complications are divided into microvascular diseases, such as retinopathy (DR), nephropathy (DNP), and neuropathy (DN), and macrovascular diseases, such as coronary artery disease (CAD), peripheral artery disease (PAD), and cerebrovascular diseases including stroke [6,43].

2.1. Microvascular Complications

2.1.1. Diabetic Retinopathy (DR)

In DR, two main stages can be distinguished: non-proliferative diabetic retinopathy (NPDR), which usually has no symptoms, and proliferative diabetic retinopathy (PDR) [44], which is the result of damage to the small blood vessels and neurons of the retina in diabetic patients and can lead to blindness [45–47]. It is considered that DR might be the most common microvascular complication of diabetes. The risk of developing this disease, as well as other microvascular complications of diabetes, depends on the duration and severity of hyperglycemia [48]. Unfortunately, changes that lead to retinopathy can occur even seven years before the diagnosis of diabetes [49]. Recent studies highlight that miRNA is a very promising tool for the early detection of changes, leading to the development of this pathological state that can help in the prevention of loss of vision in patients with diabetes.

The latest research has shown altered expression of circulating miRNAs in diabetic patients with retinopathy compared to healthy controls and compared to diabetic patients without DR. It was demonstrated that dysregulation of many miRNAs can influence retina cells. Recent studies showing the potential of miRNA as biomarkers for DR are summarized in Table 2.

Table 2. Latest reports concerning miRNAs in biofluids of human patients with DR.

miRNA	Sample Type	Expression in Research Group vs. Controls	Number of Cases	Method	Significant Findings	Year of Publication	References
miR-126	serum	down in T2DM patients compared with control group	*n* = 186; 100 T2DM (14 without complications, 26 with macrovascular complications, 17 DN, 24 DNP, 19 DR), 86 IGT	qPCR	serum miR-126 expression might serve as a potential biomarker for DR	2016	[50]
	serum	down	*n* = 184; 125 DM (44 NDR, 42 NPDR, 39 PDR) and 59 HC	qPCR	high values of AUC in ROC analysis determine miR-126 as a good diagnostic biomarker that differentiates PDR patients from HC	2017	[51]
miR-200b	serum	down	*n* = 508; 255 DR, 253 HC	qPCR	miR-200b targets *VEGFA* gene	2017	[52]
miR-93	plasma	up	*n* = 267; 140 T2DM (75 DR, 65 NDR) 127 HC	qPCR	levels of plasma miR-122 might serve as DR biomarkers	2017	[53]
miR-21	plasma	up	*n* = 304; 65 NDR, 73 NPDR, 51 PDR, 115 HC	qPCR	elevated miR-21 expression can be used to identify occurrence and stage of DR	2017	[54]
miR-221	serum	up (progressively upregulated in NDR, NPDR, and PDR)	*n* = 134; (33 HC, 37 NDR, 34 NPDR, 30 PDR)	qPCR	miR-221 might serve as a biomarker for progression and occurrence of DR	2018	[55]
let-7a- 5p miR-28-3p miR-novel-chr5_15976	serum	up	screening phase: 9 (3 T2DM NDR, 3 T2DM DR, 3 HC); training phase: 20 (10 T2DM NDR, 10 T2DM DR); validation phase: 79 (29 T2DM-DR, 50 T2DM NDR)	RNASeq, qPCR	this miRNA signature may serve as a biomarker for DR; better than single miRNA	2018	[56]
miR-423	serum	down in PDR	*n* = 69; (22 HC, 10 T2DM NDR, 22 NPDR, 15PDR)	qPCR	miR-423 may serve as a biomarker for DR; is correlated with *VEGF*, *NO*, and *eNOS* expression	2019	[57]
miR-122	serum	up in T2DM NDR and T2DM with NPDRdown in T2DM PDR	*n* = 40; (10 of HC, 10 of T2DM NDR, 10 of T2DM with NPDR, 10 of T2DM with PDR	qPCR	levels of miR-122 in serum of T2DM patients might determine occurrence and progression of DR	2019	[58]
miR-29b, miR-200b	plasma	down	*n* = 206; 186 T2DM (91 NDR, 46 NPDR, 49PDR), 20 HC	qPCR	downregulation of miR-29b is associated with progression of DR	2019	[59]
miR-4448, miR-338-3p, miR-190a-5p, miR-485-5p, miR-9-5p	serum	down: miR-4448, miR-338-3p, miR-485-5p, and miR-9-5pup: miR-190a-5p	*n* = 21; 10 NPDR, 11 NDR	RNASeq	these miRNAs might serve as good potential biomarkers for DR with high AUC value (0.909)	2019	[60]
miR-3197, miR-2116-5p	serum	up	*n* = 90; 42 NPDR, 3 PDR, 45 NDR	microarray, qPCR	high diagnostic value of these 2 miRNAs can indicate patients with DR; *NOTCH2* as a possible target gene of miR-2116-5p	2020	[61]

Table 2. *Cont.*

miRNA	Sample Type	Expression in Research Group vs. Controls	Number of Cases	Method	Significant Findings	Year of Publication	References
miR-320a	plasma	down	*n* = 170; 60 HC, 48 DM without DR, 62 DR	qPCR	DR can be identified by plasma miR-320a measurement; *TSC1* and *CDK6* are possible target genes for this miRNA	2020	[62]
let-7b, miR320b, miR-762, miR-4488	aqueous humor, plasma, vitreous	miRNA let-7b—up in aqueous and vitreous, down—plasma miR-320b—up in aqueous, vitreous, and plasma miR-762 and miR-4488—up in vitreous; up in PDR, down in NPDR in aqueous; down in PDR and up in NPDR in plasma	*n* = 27; 11 HC, 16 DM: 5 T1DM PDR, 7 T2DM PDR and 4T2DM NPDR	microarray, qPCR	this miRNA signature may contribute to the diagnostic tests or therapeutic approaches for the DR	2020	[63]

AUC: area under curve; DR: diabetic retinopathy; DN: diabetic neuropathy; DNP: diabetic nephropathy; eNOS: endothelial nitric oxide synthase; HC: healthy controls; IGT: impaired glucose tolerance; NDR: no diabetic retinopathy; NO: nitric oxide; NPDR: non-proliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; qPCR: quantitative polymerase chain reaction; RNASeq: RNA sequencing; ROC: receiver operating characteristic curve; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; VEGF: vascular endothelial growth factor.

Rezk et al. in their research manifested a potential diagnostic value of serum miR-126 for DR. In the group of patients with T2DM with DR, they observed significant under-expression of miR-126 compared to diabetic patients without this complication [50]. These results were confirmed in a study by Qin et al., who also suggest that miR-126 might be a suitable candidate for early diagnosis of PDR. The expression of this miRNA was lower in T2DM patients than in the control group. However, the authors did not describe whether patients had T1DM or T2DM [51]. In 2017, Li et al. published a report in which they claimed that miR-200b might target the *VEGFA* gene. Downregulation of serum miR-200b was connected with higher expression of the *VEGFA* gene in the group of patients with DR [52]. The next interesting study indicates a key role of miR-93 in the progression of DR. Elevated plasma miR-93 levels appear to be associated with this complication and can potentially serve as a biomarker. The authors indicate its good diagnostic value in DR using receiver operating characteristic curves (ROCs), where the AUC for miR-93 was 0.866 [53]. About 190 patients with diabetes took a part in the study, which indicated that plasma miR-21 expression was increased in the group of T2DM with DR. ROC analysis for this miRNA showed high AUC values for distinguishing DR patients, indicating the good potential of this miRNA in the diagnosis of this state [54]. The newest work of Liu et al. in the form of a preliminary study indicated that serum-derived miR-221 could be a good potential marker for DR and also for its progression [55]. This study showed upregulation of expression in DR patients and high diagnostic efficiency of miR-221 (AUC = 0.89), which suggest that this miRNA has potential value as a biomarker with more effectiveness than serum angiotensin II (ANG II) and vascular endothelial growth factor (VEGF) (AUC = 0.888 and AUC = 0.785, respectively). In recent studies, it has been suggested that signatures of few miRNAs might be a better predictor for DR than single miRNA. Recent research indicated that serum miRNA signatures consisting of let-7a- 5p, miR-28-3p, and miR-novel-chr5_15976 have good diagnostic value and may serve as a diagnostic biomarker for DR. The calculated AUC of every single miRNA was less than 0.8. Interestingly, a combination of these three miRNAs in one profile showed a significantly higher value of AUC = 0.937 in recognizing diabetic patients with and without DR. Furthermore, the authors of [56] indicated the great utility of this miRNA profile in distinguishing patients in the early stage of the disease from controls without DR. Blum et al. described plasma miR-423 as an important factor that might be involved in PDR. Expression of this miRNA was significantly lower in patients with this disease compared to NPDR, T2DM patients without PDR, and healthy controls. The authors highlight miR-423 correlation with *VEGF*, *NO* (nitric oxide), and *eNOS* (endothelial nitric oxide synthase) expression. This may suggest that changes in the expression of this miRNA are involved in the deterioration of endothelial dysfunction and accelerated atherosclerosis [57]. Pastukh et al. indicated that levels of serum miR-122 are correlated with the severity of DR in T2DM patients. The highest levels of this miRNA were noted in the group of NPDR and the lowest in PDR in comparison to healthy controls and to T2DM patients without retinopathy. This study shed light on the role of miR-122 in DR, but these data should be validated on a larger sample size [58]. Another work indicated that plasma levels of miR-200b were lower in patients with PDR in comparison to patients without this complication [59]. These studies reinforce the importance of further work on miR-200b. RNA sequencing proved that levels of circulating miRNAs from serum might be used as noninvasive biomarkers for early detection of DR. In a recent study, circulating miR-4448, miR-338-3p, miR-485-5p, and miR-9-5p were downregulated, and miR-190a-5p was upregulated in serum samples of DR compared to NDR T2DM patients. Furthermore, bioinformatics validation confirmed that these miRNAs regulate 55 target genes that are mainly connected with the regulation of vascularization processes. Interestingly, miR-9-5p may regulate 41 genes from these targets, which shows the great potential of this miRNA in further analysis [60]. A recent study described miR-3197 and miR-2116-5p as good potential biomarkers for DR. Furthermore, this paper presents notch homolog 2 (*NOTCH2*) as a possible target gene of miR-2116-5p. This gene can be expressed in the retina and regulate *VEGF* gene. ROC analysis showed that the combination of these two miRNAs

provides a high AUC score (AUC = 0.972) [61]. Elevated plasma miR-320a expression can differentiate a DR group from diabetic patients without this condition. Interestingly its targeted genes are involved in biological processes significant for DR development. This important report demonstrated that miR-320a plays an important role in the pathogenesis of this complication of T2DM [62]. Smit-McBride et al. conducted comprehensive studies on the miRNA profile in aqueous humor, plasma, and vitreous. They found four miRNAs differently expressed in patients with DR compared to healthy controls. Signatures of let-7b, miR320b, miR-762, and miR-4488 might serve as potential biomarkers for DR prognosis and diagnosis [63].

Some studies show changes in miRNAs levels in the cells of the eye tissues. This may be a clue for future indications of biomarkers based on circulating miRNAs. Retinal pigment epithelium is the pigmented cell layer between the neural retina and the choroid, and it is required for proper vision [64]. Many researchers claim that modification of this layer is involved in the pathophysiology of DR. Shao et al. indicated that miR-451a acts as a regulator of retinal pigment epithelium (RPE) function. It might play a pivotal role in the regulation of proliferation and migration in RPE cells [65]. Another study proves that miR-142-5p levels were downregulated in high-glucose-treated human retinal endothelial cells. As a direct target of this miRNA, insulin-like growth factor 1 (*IGF1*) was identified. The authors of this article claim that miR-142-5p participates in the progression of DR [66].

2.1.2. Diabetic Nephropathy (DNP)

Late complications of T2DM can also be observed in the kidneys. DNP is characterized by albuminuria and chronic loss of renal function as a result of microvascular failure due to prolonged hypoglycemia [67]. The leading cause of kidney failure is diabetic kidney disease. It is considered as a major kidney-related complication of both types of diabetes mellitus (T1DM and T2DM) and as leading causes of end-stage renal disease (ESRD) [68]. Reports state that miRNAs play a role in the regulation of processes related to kidney failures, such as fibrosis, podocyte apoptosis, mesangial cells proliferation, extracellular matrix accumulation, inflammation, and oxidative damage [69]. Microalbuminuria may be an indicator of early DNP, and macroalbuminuria is related to the progression of this disease [70]. It is crucial to avoid the serious consequences of this disease. MiRNAs seems to be a good prognostic tool for the detection of diabetic kidney failure [71]. One of the most remarkable studies indicated that the level of miR-29a in patient urine can be a predictive biomarker for DNP. The level of this miRNA was significantly upregulated in patients with albuminuria compared to normoalbuminuric individuals [72]. Levels of other studied miRNAs (miR-29b and miR-29c) did not show a significant difference between tested groups. Subsequent studies showing the potential of miRNA as biomarkers for DNP are summarized in Table 3.

Table 3. Latest reports concerning miRNAs in biofluids of human patients with DNP.

miRNA	Sample Type	Expression in Research Group vs. Controls	Number of Cases	Method	Significant Findings	Year of Publication	References
miR-192, miR-194, miR-215	urinary EVs	up in microalbuminuria patients	*n* = 90; 80 T2DM (30 normoalbuminuric, 30 microalbuminuric, 20 macroalbuminuric) 10 HC	qPCR	miR-192 has the highest diagnostic value (AUC = 0.802); miR-192 and miR-215 levels are positively correlated with TGF-β1 levels	2016	[73]
miR-192	serum	down	*n* = 591; 464 T2DM (157 normal albuminuria, 159 microalbuminuria, 148 large albuminuria), 127 HC	qPCR	lower level of miR-192 is connected with the decrease in urine albumin ratio; miR-192 has potential for DNP diagnosis	2016	[74]
miR-196a	urine	up	*n* = 209 T2DM DNP	qPCR	miR-196a is a good candidate for a noninvasive marker for the progression of renal fibrosis in DN patients	2018	[75]
miR-192, miR-377	whole blood	miR-377—up, miR-192—down	*n* = 85; 55 T2DM (30 without DN, 15 microalbuminuric, 10 macroalbuminuric), 30 HC	qPCR	both miRNAs can serve as a potential biomarker for DNP and are correlated with DNP risk factors	2018	[76]
miR-1246, miR-642a-3p, let-7c-5p, miR-1255b-5p, let-7i-3p, miR-5010-5p, miR-150-3p, miR-4449	serum exosomes	up	*n* = 74; 18 HC, 33 DM without DNP, 23 DNP	RNASeq	presented miRNAs are correlated with the albuminuria degree and might be helpful for diagnosis of DNP	2019	[77]
miR-499a	serum	down	*n* = 180; 90 T2DM with ESRD; 90 T2DM without ESRD	qPCR	altered expressions of miR-499a are possibly involved in DNP, and its level is correlated with serum MALAT1	2018	[78]

AUC: area under curve; DNP: diabetic nephropathy; EVs: extracellular vesicles; ESRD: end-stage renal disease; HC: healthy controls; qPCR: quantitative polymerase chain reaction; RNASeq: RNA sequencing; T2DM: type 2 diabetes mellitus.

A previous study on miR-192 showed that extracellular vesicles (microvesicles and exosomes) isolated from urine could help with the diagnosis of initial stages of DNP. This study showed that levels of miR-192 from urinary extracellular vesicles in T2DM patients with different degrees of albuminuria are increased in the microalbuminuric group compared with the normoalbuminuric subjects and decreased in the macroalbuminuric group. Calculations of the AUC for miR-192 showed a high diagnostic value (AUC = 0.802) in discriminating the normoalbuminuric from the microalbuminuric group [73]. A larger study including 591 patients showed that serum levels of miR-192 in patients of the large albuminuria group were lower (the lowest) than in the microalbuminuria and control groups. It was also lower in the microalbuminuria group than in the healthy control group. This study showed that lower levels of miR-192 were connected with the decrease in the urine albumin ratio and nephritic fibrosis in DNP patients [74]. In a study with over 200 patients with DNP, the authors found that increased miR-196a in urine is associated with progression of renal abnormalities and might be a good prognostic biomarker for renal fibrosis in the patients with DNP [75]. Circulating miR-192 and also miR-377 from blood can serve as potential biomarkers for early detection of DNP. MiR-377 showed increased expression and miR-192 decreased expression in T2DM patients with and without DNP compared with healthy controls. Furthermore, miR-377 and miR-192 were directly associated with albuminuria and showed the ability to distinguish patients with microalbuminuria/macroalbuminuria from those without this state. In discriminating the normoalbuminuric group from the microalbuminuric/macroalbuminuric groups, the AUC was 0.71 for miR-377 and 0.70 for miR-192. Due to the relatively small number of subjects (n = 85), further studies taking into account the usefulness of these miRNAs as potential biomarkers for DNP detection are recommended [76]. Kim et al. identified a unique profile of circulating exosomal miRNAs, which may serve as an indicator of DNP in patients with DM [77]. RNA sequencing showed upregulation of serum exosomal miR-1246, miR-642a-3p, let-7c-5p, miR-1255b-5p, let-7i-3p, miR-5010-5p, and miR-150-3p in a group of diabetic patients compared to healthy controls. MiR-4449 was overexpressed in patients with DNP in comparison to a group of patients with DM without this pathological condition. These miRNAs are promising biomarkers for the diagnosis of DNP, and further exploration is needed. The study includes 180 patients with T2DM (90 with ESRD and 90 euglycemic diabetes individuals) and showed that changes in expression of miR-499a are possibly involved in ESRD pathogenesis. The level of miR-499a was decreased in patients with ESRD compared to diabetic individuals without this condition. This difference did not show statistical significance but showed significant correlation with serum MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) and with clinical findings [78]. MALAT1 is a lncRNA (long noncoding RNA) that is able to regulate genes involved in the cycle of cells and is associated with many types of cancers and with microvascular complications of diabetes in mice. Levels of serum MALAT1 were significantly higher in diabetic patients with ESRD than those in the control group. These findings might highlight the importance of further studies on miR-499a. Due to the bioinformatic examination, we are able to observe the relationships between miRNAs and target genes. Assmann et al. in their systematic review and bioinformatic analysis showed six dysregulated miRNAs (miR-21-5p, miR-29a-3p, miR-126-43 3p, miR-192-5p, miR-214-3p, and miR-342-3p) in DNP individuals in comparison to controls. This study showed that these miRNAs with their targeted genes are involved in processes such as apoptosis, fibrosis, and extracellular matrix accumulation, which are important in pathways of known relevance for DNP pathogenesis [79]. The possibility of linking the miRNA–gene signaling pathways and the identified regulatory network of key miRNA–genes has indicated the crucial role of miR-29 and miR-200 in DNP. Such findings may provide a new idea for further studies focusing on these miRNAs as potential biomarkers [80]. An interesting meta-analysis of profiling studies on humans and animal models of diabetic nephropathy identified three upregulated miRNAs (miR-21-5p, miR-146a-5p, and miR-10a-5p) and two with lower expression (miR-25-3p and miR-26a-5p) compared to controls [81]. These miRNAs required further attention in future studies.

2.1.3. Diabetic Neuropathy (DN)

The cause of DN is a high concentration of glucose in the blood, which results in the formation of glycation end-products that cause changes in the nerves (such as demyelination) or the nerve fibers themselves. It is associated with sensory neuronal damages and neuropathic pain. It can increase the risk of infections, foot ulcers, and nontraumatic amputation. Two main types of neuropathy are distinguished: that involving the peripheral sensorimotor and that involving the autonomic nervous system [82–84]. Many studies report that miRNAs are extremely important in the nervous system as a regulator of gene expression. MiRNAs have long been known to play an essential role in processes such as neurogenesis, neuron survival, dendritic outgrowth, and spine formation [85]. Changes in miRNA levels might be relevant to disorders such as DN [86]. In comparison with other microvascular complications, there are not many studies in the literature considering miRNAs as a potential biomarker for DN. Most of the research is carried out on animal models. The topic of miRNA in DN undoubtedly requires further investigation based on the material from diabetic patients suffering from this condition.

It has been observed that diabetes in rats is responsible for reduced expression of miR-146a in static nerves. The changes in its expression are related to the inflammatory responses that intensify nerve damage [87]. In another study, miR-146a showed downregulation in sciatic nerve tissue in mice. Furthermore, the authors claimed that treatment with miR-146a mimics elevated miR-146a levels in plasma and reduces the neuropathy [88]. An interesting study on mice models showed that miR-190a-5p is involved in DN through targeting *SLC17A6* (solute carrier family 17 member 6) [89]. In this study, the authors observed decreasing level of miR-190a-5p and at the same time increased expression of *SLC17A6* in the spinal tissue of mice with neuropathy compared to controls. *SLC17A6* plays a key role in synaptic transmission in the nervous system. Such findings can provide a new perspective for the treatment of this disease. Further research on circulating miR-190a-5p is needed to determine the possibility of this biomarker for DN in humans. Zhang et al. suggest that miR-25 might be a good diagnostic and therapeutic target for DN. Their study gave pieces of evidence for reduced miR-25 expression on sciatic nerves in diabetic mice [90].

The abovementioned miR-146a was also tested in human samples. Research based on sequencing claims that polymorphisms in miR-146a and miR-128a gene are associated with DN [91]. In white blood cell fraction, expression of miR-21, miR-146a, and miR-155 is altered in patients with peripheral neuropathies of different origins [92]. Ciccacci et al. in their study showed that miR-499a polymorphism might be associated with DN tendency [93]. Polymorphism studies indicated the crucial role of these miRNAs in the development of DN.

MiR-199a-3p is considered a pro-angiogenetic factor [94]. Li et al. indicated elevated expression of plasma miR-199a-3p in patients with T2DM and elevated expression of this miRNA from lower limb skin tissues in a group of patients with DN compared to a control group. Furthermore, the research shows that overexpression of this miRNA inhibits the expression of strepin E2 (extracellular serine protease inhibitor E2). It was observed that miR-199a-3p is associated with the progression of DN by lowering the level of strepin E2. The authors also suggest that regarding miR-199a-3p, further research should focus on the potential role of this miRNA as a biomarker for the detection of DN, but also as a new therapeutic target for the treatment of this chronic complication [95]. Another study indicated increased miR-199a-3p levels in the group of patients observed in ectosomes of T2DM patients compared to healthy controls [96]. Another interesting study used weight correlation network analysis and identified genes and signaling pathways related to DN. For miR-377, miR-216a, and miR-217 associated with T2DM, targeted genes were predicted, which should be considered as putative biomarkers for progression of the disease [97].

Poorly controlled diabetes, prolonged hyperglycemia, and damaged nerves can lead to diabetic foot and diabetic foot ulcers [45]. Healing of the foot wound is important in the treatment of foot ulcers and the prevention of lower limb amputation. Researchers have stated that endothelial progenitor cells from bone marrow have a crucial role in

angiogenesis and functions of vascular endothelial cells. Reduced levels of these cells in foot lesions are observed [98]. Gao et al. proved that miR-155 is overexpressed in endothelial progenitor cells (EPCs) from patients with diabetic foot ulcers. Moreover, the expression of this miRNA was also elevated in high glucose-induced EPCs from healthy people. MiR-155 takes part in the regulation of crucial signaling pathways connected with angiogenesis, proliferation, and wound healing [99]. Other remarkable findings pointed out a crucial role of miR-203. Liu et al. proved that this miRNA in the human skin tissue sample can serve as a new biomarker for early diagnosis for the severity of diabetic foot ulcers. As miR-203 can play a pivotal role in diabetic wound healing, further research should be carried out taking into account this miRNA for easier detection of DN [100].

Undoubtedly, there is a lack of studies that examine miRNAs in biofluids associated with DN in humans. There is a need for further investigation to emphasize specific circulating miRNAs as potential biomarkers for DN and therapeutic targets for this pathological condition.

2.2. Macrovascular Complications

Atherosclerosis is the main mechanism responsible for the appearance of macrovascular complications. Atherosclerosis is the result of chronic inflammation and damage to the arterial walls. This disease leads to a narrowing lumen of arteries in the whole body. The cause of the narrowing is atherosclerotic plaque, mainly made of deposits of calcium and fatty lipids, which grows from the artery wall, causing a decline in blood flow and resulting in hypoxia of the organs [101,102]. Diabetes accelerates all atherosclerotic lesions by intensifying inflammatory processes, as a result of which the plaque increases. The plaque gradually narrows the diameter of the coronary vessels. These pathological changes in diabetic patients might result in coronary artery disease (CAD), myocardial infarction (MI), peripheral artery disease (PAD), and cerebrovascular disease [42,102].

2.2.1. Coronary Artery Disease (CAD) and Myocardial Infarction (MI)

It is considered that cardiovascular disease and especially CAD are the leading reasons for morbidity and mortality in DM patients [11,12]. CAD typically occurs when a coronary artery develops atherosclerosis [103]. A different degree of artery obstruction is the main cause of imbalance between blood supply and oxygen demand in myocardial cells, which is a characteristic feature of this disease. This condition is also related to reduced availability of nutrients and insufficient removal of metabolic products [104]. It often leads to heart muscle damage and MI [105]. In most cases, MI is caused by an acute clot closing the lumen of the coronary artery supplying blood to the heart. MI can cause the cardiac remodeling and the development of chronic heart failure and is considered as the leading cause of death [106].

It is well understood that miRNAs have a huge impact on cardiovascular biology. Differences in miRNAs expression have been described in many cardiac cases, including CAD and MI [107,108]. MiRNA also seems to be usable in predicting cardiological complications of diabetes. A recent, well-designed study examined the miRNA profiles in diabetic patients suffering from heart disease and in DM patients free from complications. Preferably, such results should be compared to miRNA levels in healthy people, but also in people with heart disease without T2DM.

Kumar et al. identified two plasma miRNAs that can have a pivotal role in the early prediction of CAD. The authors claimed that that miR-133b and miR-21 have different expression in patients with CAD in comparison to healthy controls. MiR-133b showed underexpression and miR-21 overexpression in the studied groups [109]. This work claimed that the group of T2DM patients with CAD did not show any significant differences in the expression of the studied miRNAs. Luo et al. first suggested that plasma miR-30c might be a potential novel biomarker for diagnosis, treatment, and prognosis of CAD in T2DM patients. Their study showed that the levels of circulating miR-30c were remarkably lower in groups of patients with T2DM and T2DM with CAD in comparison with CAD patients and the

control group. Diabetic patients with CAD showed the lowest level of miR-30c. Patients from the CAD T2DM group showed a significant negative correlation between circulating miR-30c levels and the degree of coronary lesion severity ($r = -0.7817$, $p < 0.0001$). The authors also postulated that miR-30c takes part in the regulation of plasminogen activator inhibitor 1–vitronetin interactions. The ROC analysis showed high values of the AUC for miR-30c as a diagnostic biomarker for CAD, T2DM, and T2DM, with CAD showing AUC values close to 0.9, which can distinguish these groups from healthy controls. However, the ROC analysis showed that miR-30c can differentiate CAD T2DM patients from diabetic patients without complications only with an AUC of 0.685 [110]. Seleem et al. indicated that serum miR-342 and miR-450 might be important indicators of CAD in T2DM patients [111]. It was found that miR-342 was significantly overexpressed in T2DM, CAD, and T2DM with CAD groups when compared with the control group. In the T2DM with CAD group, the expression was higher than in the CAD group. MiR-450 showed significantly lower expression in the studied group compared to controls. In the group of T2DM with CAD, the expression was significantly lower than in the CAD group. Additionally, according to chi-squared analysis of the T2DM group and the T2DM with CAD groups, these miRNAs demonstrated potential as bioindicators for CAD as a complication of T2DM. MiR-342 reached an AUC of 0.781, whereas miR-450 had an AUC of 0.824. A new, interesting study displayed that serum miR-1 and miR-21 have a crucial role in the diagnosis of CAD. Serum miR-1 levels were significantly lower in patients with T2DM when compared to the healthy control group. Moreover, the authors found out that the expression was the lowest in the group of T2DM patients with heart failure. In contrast, serum miR-21 levels were higher in T2DM patients compared to healthy individuals. The expression was highest in the group of diabetic patients with heart failure. ROC analysis showed that miR-21 might be a novel biomarker in the prediction of heart failure in diabetes patients [112]. These two miRNAs were previously described as related to heart hypertrophy in hypertensive patients. This proves that expression profiles in patients with hypertension differ to those in patients in healthy control groups [113]. Plasma miR-126 and miR-210 expressions differ in patients with diabetes and CAD to those of healthy controls. MiR-210 showed reduced levels in diabetes but also in diabetes with CAD. In contrast, levels of miR-126 were higher in T2DM patients and increased further in those with diabetes with CAD. Both miRNAs presented high diagnostic values in distinguishing T2DM patients with and without CAD from healthy controls. These findings suggest that these miRNAs might serve as potential markers for CAD as a complication of T2DM [114]. It is worth considering that miR-126 was also tested for different diabetes complications (DR and DNP) mentioned in this review. To determine the exact specificity of this miRNA for diabetes complications, further studies need to be conducted on a larger group of subjects. Interestingly, miR-126 and miR-26a from circulating microparticles are considered as associated with risk of CAD. Levels of these miRNAs were significantly lower in T2DM patients than in healthy controls. Decreased expression levels of miR-26a and miR-126 are related to the coexistence of the CAD [115]. Diabetic cardiomyopathy is diagnosed when ventricular myocardial dysfunction develops in patients with diabetes, even if CAD or hypertension has not developed. It is primarily caused by metabolic disorders in diabetes due to insulin deficiency. Accumulation of lipids in the myocardium is considered a hallmark of diabetic cardiomyopathy. Another study suggests that serum miR-1 and miR-133a might be potential biomarkers for early diagnosis of diabetic cardiomyopathy [116]. A good direction for future research might be considering miR-483-3p as a potential prognostic biomarker for cardiovascular diseases in T2DM patients. This miRNA is a crucial regulator of endothelial integrity. Furthermore, the authors claimed that miR-483-3p might be a potential therapeutic target [117].

There are not many studies demonstrating differences in miRNA profiles in human suffering from diabetes and macrovascular complications and those free from complications. However, many works describe the important role of miRNA in the development of diseases such as CAD, MI, and hypertension without a T2DM group. MiRNA such as miR-21, miR-155, miR-126, miR-146a/b, miR-143/145, miR-223, and miR-221 are most

frequently described in hypertension and atherosclerotic [118]. The presented miRNAs were also indicated as significant in patients with diabetes. Research showed that miR-223 is associated with the severity of CAD. The AUC for this miRNA reached 0.933, which indicates a promising ability to differentiate CAD cases from healthy controls. Expression of this miRNA was higher in the group of CAD individuals than in the control group. The level of miR-223 increased with increasing severity of the disease. This shows evidence that this miRNA could be a good potential biomarker in the assessment of CAD. However, the authors did not separately analyze the group of patients with diabetes [119]. Interestingly, it has been proven that miR-223 is upregulated in the blood of patients with T2DM [120].

Wang et al. in a meta-analysis study showed that the level of serum miR-133a-3p was upregulated in samples of patients with CAD. Additionally, this study suggested that miR-122-5p might be a valuable biomarker for this disease [121]. This study confirmed the earlier report about the importance of this miRNA in the pathology of the cardiovascular system [122]. MiR-122-5p was also previously described to have a crucial role in atherosclerosis and CAD [123,124]. These reports suggest that miR-122 and miR-133a should be considered as potential biomarkers for cardiovascular events and should be also tested for diabetes complications. Serum exosomal miR-1915-3p, miR-4507, and miR-3656 might be novel diagnostic tools for early-stage acute myocardial infarction [125]. The expression of these miRNAs was significantly lower than in the stable CAD sample exosomes. ROC analysis indicated that miR-1915-3p and miR-3656 had the highest diagnostic value (AUC > 0.77), which suggests that this miRNA can be a predictive tool for acute MI. Expression of miR-21, miR-155, and miR-221 in peripheral blood mononuclear cells (PBMCs) was significantly different among patients with CAD and controls. MiR-21 showed overexpression, whereas miR-221 and miR-155 were downregulated in the studied group [126]. It is worth noting that these miRNAs have a crucial role in the pathogenesis of diabetes and were also indicated as potential biomarkers in microvascular complications as well as in T2DM patients with CAD [55,92,127,128]. Changes in these miRNAs seem to be typical for vascular inflammation, and to use them as potential biomarkers, more studies with larger cohorts are necessary. Serum miR-584-5p was recently considered as a potential biomarker for CAD. It is observed that in patients with CAD, expression of this miRNA is lower than in patients without this disease. However, the groups were relatively small, and the authors of the study did not identify a group with T2DM [129]. A recent study from 2020 reports that the combination of four plasma miRNAs (let-7i-5p, miR-32–3p, miR-3149, and miR-26a-5p) has good diagnostic value (AUC = 0.837) for distinguishing patients with severe CAD from controls [130]. While the mentioned studies did not include patients suffering from diabetes, further research should be directed to these miRNAs as potential biomarkers, indicating a group of diabetics with a predisposition to developing specific macrovascular complications.

2.2.2. Peripheral Artery Disease (PAD)

Peripheral artery disease is considered a group of diseases affecting the peripheral arteries that lead to narrowing or blockage of the large arteries, except for coronary arteries, aortic arch, and brain arteries [131,132]. PAD plays a role as a predictor of MI, stroke, and death due to vascular abnormalities [133]. A big challenge for the diagnosis of this condition is the fact that a lot of patients are living without symptoms, causing slow progression of the disease [134]. Diabetic patients may remain asymptomatic due to the often associated neuropathies [131]. The circulating miRNA has been connected with PAD in a number of studies [135–137]. Numerous pieces of evidence shows that miRNAs are involved in the regulation of many key processes connected with the pathogenesis of PAD, such as angiogenesis, inflammation, and endothelial function [138]. Signorelli at al. in their pilot study indicated serum miR-130a, miR-27b, and miR-210 as potential good biomarkers for PAD. The expression of this miRNA was significantly increased in PAD patients versus healthy controls. In this case, about 37% of PAD group had diabetes [139]. There is also research focused on miRNA as a potential marker for the advanced stage of PAD, which is

critical limb ischemia. Plasma miR-4739 levels are significantly elevated in the group of T2DM patients with critical limb ischemia in comparison to T2DM individuals without this complication [140]. Similarly, serum miR-323b-5p seems to have a great diagnostic value in distinguishing patients with critical limb ischemia in T2DM groups [141]. Both miR-4739 and miR-323b-5p have not been previously studied in the context of PAD. Studies are showing an enormous role of miRNA in the pathogenesis of PAD. Most of them are based on animal (miR-93, miR-92a, miR-503, and miR-100), cell cultures (miR-221, miR-126, and miR-1), or human tissue studies (miR-15a, miR-126, miR-223, miR-28-3p, miR-21, and miR-503) [142]. There is a need to select fluid biomarkers for humans. More research is required to find the right, reliable biomarker for the early diagnosis of PAD in people with diabetes. The current state of knowledge allows scientists to focus their efforts on intensified research on larger groups of patients.

2.2.3. Cerebral Vascular Disease and Stroke

Particular attention should be paid to the search for markers in body fluids that can help indicate predisposition to developing cerebral complications among T2DM patients. Cerebral vascular disease including stroke is categorized as a macrovascular complication of diabetes. Unfortunately, such complications are more severe and require more attention during the treatment of patients with diabetes in comparison to patients without it. Individuals with DM are more likely to develop cerebrovascular disease and strokes [143]. The main reason for these complications is atherosclerosis [143,144]. Cerebrovascular disease refers to medical conditions in which blood flow to specific parts of the brain is impaired. Vascular diseases often lead to a stroke. These changes are not, unlike microangiopathy, due to a disease associated with diabetes only and might also occur without any relation to diabetes. However, the coexistence of diabetes significantly increases the course of the atherogenic process and worsens the prognosis. [145]. Stroke was determined to be the second leading cause of death after CAD in 2015 [146]. There are two main types of stroke: ischemic and hemorrhagic. An ischemic stroke occurs when an artery that supplies the brain with blood is blocked. The most common cause is the enlargement of atherosclerotic plaque, which leads to obstructed blood flow to the brain. Hemorrhagic stroke is a consequence of the rupture of the cerebral artery wall and bleeding outside the vessel [147,148]. Diabetes is a risk factor for both types of strokes, and patients with diabetes are at a much higher risk of stroke than those without it [149–152].

Comparatively, like in other diabetes complications, miRNA plays a crucial role in risk factors of the cerebral dysfunctions. As of 2009, circulating miRNAs began to be viewed as potential stroke biomarkers [153]. In a recent meta-analysis conducted in 2020, the authors pointed out the crucial role of miR-320b and miR-320d in the pathogenesis of stroke [154]. Interestingly, these two miRNAs were previously described as biomarkers related to diabetes [155,156]. MiR-320b was also described as a potential indicator of carotid atherosclerosis [157]. Many studies present miR-21 as a biomarker in different types of strokes. However, this particle seems to be typical not only of strokes, but also of many diseases, including DM and its other complications [54,158].

Plasma and platelet miRNAs also play a crucial role in the development of complications related to diabetes. In their work from 2014, the authors of [159] indicated significant downregulation of platelet and plasma miR-223 and miR-146a in patients with T2DM and ischemic stroke or only with T2DM compared to HC. This expression was not downregulated in patients with ischemic stroke only. This study suggests that platelet and plasma miR-233 and miR-146a could be specific markers for T2DM with or without ischemic stroke. However, this study should be repeated on a larger number of patients. MiR-223 from blood was previously described as a marker for acute ischemic stroke. In a study by Wang et al., the authors compared patients with ischemic stroke with healthy controls, where 39.2% of patients had diabetes. The level of this miRNA was increased in the group with ischemic stroke compared to controls. The authors of [160] also suggest that IGF-1 might be a new target for miR-223. Long et al. investigated the expression of miR-223

from PBMCs in patients with cerebral infarction. This study also had a group of patients with T2DM and stroke. Expression of this miRNA was lower in patients with T2DM than in HC. There was no significant difference between the expression of this miRNA when comparing patients with cerebral infarction or cerebral infarction with T2DM to healthy controls [161]. One of the better-designed studies shows that plasma and platelet levels of miR-223 and miR-144 may serve as biomarkers associated with ischemic stroke in T2DM patients. The miR-144 levels were significantly higher in T2DM patients with ischemic stroke than in healthy controls and the T2DM group. In contrast, levels of miR-223 were lower in the group of patients with T2DM and ischemic stroke. It is suggested that these altered expressions of this miRNA increased susceptibility to ischemic stroke in T2DM [162]. Serum miR-503 could be a good biomarker for ischemic stroke in diabetic patients. In recent research, the authors of [163] observed overexpression of this miRNA in a group of DM patients with ischemic stroke when compared to nondiabetic patients with stroke, DM patients, and healthy controls.

3. Conclusions

To avoid long-term vascular complications associated with T2DM, scientists must pay attention to early diagnosis. MiRNAs play an extremely important role in many processes leading to the development of T2DM and related complications. These molecules have substantial potential as biomarkers for vascular complication of T2DM, as evident in the growing body of research data, mainly due to their high specificity and sensitivity. Recent studies show their identification mostly in serum; however, in further studies, it is worth noting the examination of miRNA signatures in other body fluids, such as saliva or urine. Almost all of the work mentioned above requires continuation and further research on a much larger group of people, as well as validation of the results by other, standardized methods. However, it is clear that, despite some limitations, the identification of new miRNAs offers a promising perspective for future functional research related to the development of complications in T2DM patients.

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Serum miRNA Profile in Diabetic Patients With Ischemic Heart Disease as a Promising Non-Invasive Biomarker

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The increasing morbidity and mortality of type 2 diabetic mellitus (T2DM) patients with ischemic heart disease (IHD) highlight an urgent need to identify early biomarkers, which would help to predict individual risk of development of IHD. Here, we postulate that circulating serum-derived micro RNAs (miRNAs) may serve as potential biomarkers for early IHD diagnosis and support the identification of diabetic individuals with a predisposition to undergo IHD. We obtained serum samples from T2DM patients either with IHD or IHD-free and analysed the expression levels of 798 miRNAs using the NanoString nCounter technology platform. The prediction of the putative miRNAs targets was performed using the Ingenuity Pathway Analysis (IPA) software. Gene Ontology (GO) analysis was used to identify the biological function and signalling pathways associated with miRNA target genes. Hub genes of protein-protein interaction (PPI) network were identified by STRING database and Cytotoscape tool. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic value of identified miRNAs. Real-time quantitative polymerase chain reaction (qRT-PCR) was used for nCounter platform data validation. Our data showed that six miRNAs (miR-615-3p, miR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p, and miR-548b-3p) were significantly upregulated in T2DM IHD patients compared to T2DM patients without IHD. Further analysis indicated that 489 putative target genes mainly affected the endothelin-1 signalling pathway, glucocorticoid biosynthesis, and apelin cardiomyocyte signalling pathway. All tested miRNAs showed high diagnostic value (AUC = 0.779 - 0.877). Taken together, our research suggests that circulating miRNAs might have a crucial role in the development of IHD in diabetic patients and may be used as a potential biomarker for early diagnosis.

Keywords: miRNA, ischemic heart disease, diabetes, miRNA profiling, biomarker

INTRODUCTION

Diabetes mellitus is a group of endocrine and metabolic disorders characterised by insufficient insulin secretion to maintain the right blood glucose level. According to the International Diabetes Federation, 463 million people had diabetes in 2019, and this number is expected to rise in the following year's (1). Several forms of diabetes are distinguished; however, type 2 diabetes mellitus (T2DM) is the most common and accounts for over 90% of cases (2). T2DM is characterised by cell resistance to the normal concentration of insulin circulating in the blood. With the progression of the disease, pancreatic β-cell may also become dysfunctional and inevitably stop producing insulin (3). The most common long-term complications of diabetes is ischemic heart disease (IHD) (4, 5), and it is well known as the leading cause of morbidity and mortality in diabetes (6). IHD results from an imbalance between blood supply and oxygen demand in myocardial cells caused by different degrees of coronary artery obstruction, and occurs when atherosclerosis develops in the coronary artery.

Ischemia is related to inadequate oxygen supply and reduced availability of nutrients, and incomplete removal of metabolic products (7). Patients with T2DM have a higher risk of developing IHD and mortality following IHD than healthy ones (8).

Diabetes complications, such as heart diseases, develop much earlier before diagnosing (9). To prevent the progression of the disease, special attention should be paid to its early detection. Despite the rapid progress in cardiovascular research, there is no reliable tool for prompt diagnosis and identification of people at risk of developing IHD. Coronary angiography remains the gold standard in the diagnosis of IHD. Unfortunately, it is an invasive medical procedure that uses contrast dye to detect blockages in coronary arteries at x-ray pictures (10). Additionally, this method can only diagnose the disease at a later stage. Therefore, early and noninvasive detection of this condition at the initial state or diagnosis of diabetic patients with a predisposition to IHD is crucial.

Recent research shows that inflammatory mediators like chemokines CXCL12 and macrophage migration-inhibitory factor (MIF) play an essential role in the pathology of IHD. In humans, MIF is abundantly produced by various cells in different stages of plaque development (11, 12). Elevated levels of MIF in plasma can serve as an early biomarker for acute myocardial ischemia and can be risk factor for future coronary events in IHD patients with T2DM (13, 14). It has been shown that CXCL12 levels in plasma are better predictors of IHD outcomes than traditional risk factors (15, 16). Unfortunately, the serum concentration of MIF and CXCL12 seems to be characteristic not only for ischemic heart disease but also for different inflammatory states raising the need for more selective markers for IHD diagnosis (17).

A promising tool for understanding the pathogenesis of IHD is miRNAs (18). MiRNAs are small (17-25 nucleotides) non-coding RNAs that play an essential role in regulating gene expression. MiRNAs control the expression of target genes by base pairing to the 3' untranslated regions (3' UTRs) of mRNA and inducing repression of the target mRNA. This effect can occur by transcript destabilisation or inhibition of translation (19). Bioinformatics predictions indicate that one miRNA could target more than a hundred mRNAs (20). MiRNAs participate in critical biological processes such as proliferation, differentiation, and apoptosis of cells (21). Abnormal expression of miRNAs is associated with various diseases, such as cancer, cardiovascular diseases or metabolic disorders. It is commonly known that levels of specific circulating miRNAs might be predictive for long-term diabetes complications (22). MiRNAs may be a helpful biomarker because of their stability in biofluids even after prolonged collection and several freezing-thaw cycles (23). Moreover, those small particles can be easily collected and measured with specific, sensitive assays (22).

MiRNAs such as miR-92a, miR-503, and miR-126 might control and regulate angiogenesis, crucial for the repair of myocardial cells after ischemic injury (24, 25). MiR-155 and miR-342-5p are involved in vascular inflammation by modulation of inflammatory response and atherosclerosis progression (26). MiR-125b and miR-205 can regulate vascular calcification that contributes to atherosclerosis (27, 28). Decreased level of miR-126 is known as a predictor of diabetes, and it also occurs in patients with IHD (21). In diabetes, there is an established connection between miR-223 and activation of platelets, the latter of which significantly contributes to the development of cardiac disease (29). However, despite evident progress, our understanding of the regulation and function of specific miRNAs in IHD is still limited.

In the present study we investigated the differential expression of IHD-associated miRNAs in the serum samples from T2DM patients with and without IHD using the nCounter platform, a novel technique offering a high level of precision and sensitivity without amplification reaction (30).

MATERIALS AND METHODS

Baseline Characteristics of the Patients

To diagnose IHD in over 600 patients who participated in the cohort study conducted in the Department of Invasive Cardiology, Medical University of Bialystok, coronary angiography was performed. This procedure has distinguished two subsets of T2DM patients – with IHD (n = 24) and without IHD (n = 20), IHD group (n = 9) and the control group without diabetes or prediabetes and IHD (n = 16). T2DM was confirmed according to the Diabetes Poland criteria (31). Exclusion criteria for this study included: type 1 diabetes mellitus, latent autoimmune diabetes of adults, other T2DM complications (retinopathy, neuropathy, nephropathy, peripheral artery disease, stroke, cerebrovascular disease), previous myocardial infraction, percutaneous coronary intervention, other inflammatory states (rheumatoid arthritis, systemic sclerosis), cancers, human immunodeficiency virus (HIV) or hepatitis C virus (HCV) infection, recent surgery, alcohol consumption, and smoking. A group of 69 individuals was qualified for further analysis. Serum samples were collected, centrifuged and stored at −80°C. This study was approved by the Bioethics Committee of

the Medical University of Bialystok, Poland (approval numbers: R-I-002/583/2019 and APK.002.35.2021) and was performed according to the principles of the Declaration of Helsinki.

MiRNAs Isolation

The miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) was used for RNA extraction (smaller than 1000 nucleotides) using 200 µL of serum aliquots from one patient according to the manufacturer's instructions. The miRNA concentration was measured by The Qubit microRNA Assay Kit (Invitrogen, California, CA, United States) with the Qubit 3.0 Fluorometer.

Detection of miRNAs Profile

A total of 69 samples were prepared for nCounter miRNA expression profiling according to the manufacturer's recommendations (NanoString Technologies, USA). A three ng of isolated microRNA were used as input material. Unique DNA tags were ligated onto the 3′ end of each mature miRNA, providing an identifier for each miRNA in the sample. Tagging was performed in the ligation reaction followed by an overnight hybridization (65°C) to nCounter Reporter and Capture probes that allowed complex sequence-specific probes with targets. After hybridization, samples were placed into the nCounter Prep Station for automated sample purification and target/probe complexes immobilization on the cartridge for data collection. Each sample was scanned for 555 FOV (fields of view) on the nCounter Digital Analyzer (NanoString Technologies, USA) to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. NanoString raw data were analysed with nSolver software (NanoString Technologies, USA).

Measurement of MIF and CXCL12 in Serum

The concentration of MIF and CXCL12 in serum was determined in duplicate samples by enzyme-linked immunosorbent assay (ELISA) (Quantikine Human M-CSF Immunoassay; R&D systems, Abingdon, United Kingdom), according to the manufacturer's recommendations.

Validation of the NanoString Results by Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

The serum of 22 T2DM and 26 T2DM IHD miRNA samples were reverse-transcribed using a miRCURY LNA RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, on a Proflex thermal cycler (Thermo Fisher Scientific, Waltham, USA). Subsequently, the qRT-PCR reaction was performed using specific primers and a miRCURY LNA SYBR Green PCR Kit (Qiagen, Germany). The samples were run on the qPCR plates in duplicate on the LightCycler 480 Real-Time PCR System (Roche, Switzerland). Based on the NormFinder (32), miR-103a-3p and miR-199b-5p were used as endogenous reference miRNAs. The primers efficiency for targets and reference miRNAs has been checked. After calculating the qPCR efficiency, relative expression levels of miRNAs were calculated using the delta-delta *Ct* ($2^{-\Delta\Delta Ct}$) method. *Ct* is the threshold cycle which is a point when fluorescence reading surpasses a set baseline. This method calculates samples' relative fold expression, using a reference miRNA as the normalizer (33).

Statistical Analysis

Based on similar previous experiments and pilot data on NanoString nCounter miRNA Expression Assay we have calculated the minimal number of samples per experimental group (T2DM IHD or T2DM) in order to detect 1.5 fold differences in relative miRNA expression level between groups at the true positives detection powers of 80% and 90% under p=0.05 (34). We have used RNASeqPower *R* package (35) applying the statistics data covering obtained counts and coefficients of variations per different groups. We have estimated that to obtain 80% power we would need 12 samples, whereas to obtain high power of 90% we would need 16 samples per group. Finally our groups consisted of 20 samples in T2DM and 24 in IHD T2DM group thus allowing for more than 90% power under p=0.05. Statistical analyses were performed using STATISTICA version 13.1 (StatSoft, Tulsa, Oklahoma). Preliminary statistical analysis (Shapiro–Wilk test) revealed that the studied parameters did not follow a normal distribution. The ANOVA Kruskal-Wallis test followed by Dunn's test was performed to examine the difference in clinical parameters between the groups. miRNAs were tested for differential expression using nSolver 4.0 Analysis software (NanoString), including normalization using the positive ligation controls. The threshold count value was set to 20. The p-value was adjusted using the False Discovery Rate (FDR) correction for multiple comparisons limited to 0.05. Ingenuity Pathway Analysis (QIAGEN Inc.) was performed to generate a list of predicted targeted genes for studied miRNAs and identify canonical pathways. To find the highly connected hub genes of six miRNAs in the protein-protein network (PPI) STRING database (http://string-db.org) (36), Cytoscape version 3.9.0 (http://cytoscape.org/) (37) and its plugin (cytoHubba) were applied. GO and functional annotation clustering were carried out using the KEGG (Kyoto Encyclopedia of Genes and Genomes) Enrichment Analysis; g:Profiler (https://biit.cs.ut.ee/gprofiler/gos), and Metascape (https://metascape.org) online databases. GO analysis permits linking a list of genes to specific functional annotations categorized into functional groups (38). The Revigo web-based tool (http://revigo.irb.hr/) was used to summarise and visualise lists of Gene Ontology. The Spearman rank-order correlation coefficient (r) was determined to estimate the correlation between the identified miRNAs and clinical parameters. It was assumed that $r > 0.8$ indicates a strong correlation, and $r > 0.3$ shows a moderate correlation. The statistical significance level was set at $p < 0.05$. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic value of miRNAs, and for each miRNA, the area under the curve (AUC) was calculated. The logistic regression model was created using Weka software to assess the diagnostic values of multi-miRNAs assays. MiRNAs for analysis were selected using attribute selection (evaluator: Info Gain, search: Ranker). MiRNAs with the highest ranks were used for further modelling. Logistic regression, random tree, J48 tree, and naive bayes classification algorithms with 10-fold cross-validation were

used to select the combination of miRNAs with the highest diagnostic value.

RESULTS

Patient Baseline Characteristic

The study groups consisted of 44 patients with T2DM; 24 of them were diagnosed with IHD. Nine patients suffered only from IHD, without T2DM. Sixteen patients were control group with normal glucose tolerance and negative coronarography results. IHD and IHD-free groups did not show significant differences in clinical parameters such as duration of diabetes, platelets, fibrinogen, body mass index (BMI), glucose level, glycated haemoglobin (HbA1c) level, blood pressure, cholesterol, triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The clinical characteristics of patients enrolled in this study are summarized in **Table 1**. Patients in both diabetes groups had been treated with standard regimens and took oral hypoglycaemic medications. Patients with T2DM had been treated with derivatives of biguanide (42% of T2DM and 50% of T2DM IHD group, $p > 0.05$) and, sulfonylurea derivatives (58% of T2DM and 68% of T2DM IHD group, $p > 0.05$).

Baseline Levels of Circulating miRNAs

To determine the differentially expressed miRNAs (DEMs) in serum of diabetic patients with IHD (n = 24) compared to patients with T2DM (n = 20), patients with IHD (n = 9), and control group (n = 16), we performed the expression analysis of 798 unique miRNAs using the nCounter platform. nSolver 4.0 analysis indicated that 14 miRNAs were identified as differentially expressed between T2DM IHD and T2DM groups. All of them were upregulated in serum samples of T2DM patients with IHD when compared to diabetic patients without IHD ($|FC| \geq 1.5$, FDR ≤ 0.05) (**Supplementary Table 1**). Altogether 155 DEMs were found between the T2DM IHD compared to the control group (**Supplementary Table 2**). Additionally, 155 DEMs were found between T2DM and control group (**Supplementary Table 3**). Furthermore, 221 DEMs were found between T2DM IHD and IHD groups (**Supplementary Table 4**). Those comparisons allowed extract six DEMs typical only for T2DM IHD patients (**Table 2**).

Functional Enrichment Analysis in T2DM IHD Patient Samples

The IPA analysis demonstrated 489 putative target genes for six DE miRNAs (**Supplementary Table 5**). To visualise connections between tested miRNAs and target genes, Cytoscape version 3.9.0 (http://cytoscape.org/) was used. The obtained network of connections is shown in **Figure 1**.

Ingenuity core analysis identified 36 altered canonical pathways, including the endothelin-1 signalling pathway, glucocorticoid biosynthesis, and apelin cardiomyocyte signalling pathway (**Supplementary Table 6**). The top 20 deregulated canonical pathways are shown in **Figure 2**.

STRING database (36) (https://string-db.org/) in Cytoscape tool (37) and the *cytoHubba* plugin (39) were used to visualise the protein-protein interaction (PPI) network of the DE miRNA target genes and identify top 10 hub genes (**Figure 3**).

According to cyto*Hubba* plugin's MCC (The Maximal Clique Centrality) ranking, the top 10 hub genes in the PPI network were *C3AR1* (Complement C3a Receptor 1), *CCR5* (C-C Motif Chemokine Receptor 5), *APLNR* (Apelin Receptor), and *HNRNPC* (Heterogeneous Nuclear Ribonucleoprotein C) targeted by miR-5196-3p; *GNAI2* (G Protein Subunit Alpha I2) and *OPRL1* (Opioid Related Nociceptin Receptor 1) targeted by miR-3147; *CXCL8* (C-X-C Motif Chemokine Ligand 8) targeted by miR-6732-3p, *ADORA1* (Adenosine A1 Receptor), *CXCL6* (C-X-C Motif Chemokine Ligand 6), and *GPSM3* (G Protein Signaling Modulator 3) targeted by miR-1224-5p (**Figure 4**).

Gene ontology (GO) enrichment analysis identified biological pathways and processes associated with the target genes. The GO-biological process (BP) analysis identified the most significant pathways included cell death, cell activation, cell-cell

TABLE 1 | Patient baseline characteristics.

Characteristics	Patients (n = 16) controls	Patients (n = 9) IHD	Patients (n = 20) T2DM	Patients (n = 24) T2DM IHD
	median (Q1-Q3)	median (Q1-Q3)	median (Q1-Q3)	median (Q1-Q3)
Age [years]	52.32 (49.88 - 55.91)	54.85 (50.57 - 61.20)	57.50 (52.33 - 65.17)	58.33 (53.4 - 64.58)
T2DM duration [years]	–	–	7.00 (4.00 -10.00)	5.00 (2.00-10.00)
BMI [kg/m^2]	24.62 (22.19 - 25.66)	27.73 (26.47 - 30.39)^{a*}	32.19 (28.63 - 36.41)^{a****}	31.24 (27.68 - 33.20)^{a***}
Platelets [10^3/µL]	227.50 (163.00 - 268.00)	221.00 (195.00 - 248.00)	222.00 (197.00 - 243.00)	225.00 (186.00 - 322.00)
Fibrinogen [mg/dL]	333.50 (272.00 - 352.00)	362.00 (296.00 - 413.00)	352.00 (315.00 - 381.00)	416.00 (354.00 - 482.00)^{a**}
Leukocytosis [10^3/µL]	6.45 (5.49 - 7.30)	7.51 (5.60 - 8.90)	7.16 (5.40 -8.47)	7.32 (6.38 - 9.30)
Fasting glucose [mg/dL]	89.00 (85.00 - 91.00)	90.00 (84.00 - 100.00)	122.00 (118.00 - 165.00)^{a****,b***}	129.00 (104.00 - 176.00)^{a***,b**}
HbA1c [%]	5.50 (5.50 - 5.65)	5.50 (5.30 - 5.70)	6.20 (5.70 - 7.50)^{a*,b*}	7.60 (6.20 - 8.80)^{a***,b***}
Systolic blood pressure [mmHg]	120.00 (115.00 - 130)	120.00 (110.00 - 140.00)	140.00 (120.00 – 155.00)^{a*}	140.00 (130.00 – 155.00)^{a***,b*}
Diastolic blood pressure [mmHg]	80.00 (70.00 - 80.00)	80.00 (80.00 - 100.00)	80.00 (80.00 - 90.00)	85.00 (80.00 - 90.00)
Cholesterol [mg/dL]	194.00 (162.00 - 209.00)	176.00 (163.00 - 196.00)	198.50 (172.00 - 224.00)	179.50 (138.00 - 211.00)
TG [mg/dL]	109.00 (86.00 - 153.00)	134.00 (109.00 - 183.00)	124.00 (90.00 - 202.00)	179.00 (109.00 - 206.00)
LDL [mg/dL]	112.00 (92.00 - 132.00)	106.00 (102.00 - 117.00)	118.50 (102.00 - 135.00)	102.00 (66.00 - 133.00)
HDL [mg/dL]	49.00 (40.00-62.00)	35.00 (33.00 - 45.00)	46.00 (41.00 - 57.00)^{b*}	39.00 (34.00 - 50.00)

*[a]Significantly different from control group;[b]Significantly different from IHD group *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; CI, confidence interval; the p-value describes the significance of the difference between patients with T2DM without IHD compared to patients with T2DM and IHD using ANOVA Kruskal-Wallis test followed by Dunn's test.*

TABLE 2 | MiRNAs upregulated in T2DM IHD compared with T2DM patients.

miRNA	FC	*p*-value	FDR
miR-615-3p	2.45	0.00000497	0.00
miR-3147	2.35	0.00000200	0.00
miR-1224-5p	1.68	0.00001305	0.00
miR-5196-3p + miR-6732-3p	1.56	0.00040774	0.01
miR-548b-3p	1.55	0.0002042	0.01

FC, fold change; FDR, false discovery rate, adjusted p-value; FC ≥ 1.5; FDR ≤ 0.05.

signalling, secretion, cell population proliferation, circulatory system development, tube development, regulation of response to external stimulus, negative regulation of execution phase of apoptosis, and tube morphogenesis. Transcription regulatory region nucleic acid binding, protein-containing complex binding, enzyme binding, hydrolase activity, acting on ester bonds, identical protein binding, transporter activity, receptor antagonist activity, calcium ion binding, signalling receptor regulator activity, and transcription factor binding were pointed as the main important in GO-molecular function analysis. According to the GO-cellular component analysis, target genes were mainly enriched in the membrane, non-membrane-bounded organelle, membrane-enclosed lumen, cytosol, extracellular region, cell periphery, nucleoplasm protein-containing complex, plasma membrane, and an intrinsic component of membrane. KEGG pathway enrichment analysis showed pathways involved in cardiovascular diseases and diabetes development (**Figure 5**).

Levels of MIF and CXCL12 in Serum

No significant differences were observed between the serum concentration of MIF and CXCL12 in T2DM IHD and T2DM groups (p>0.05). The median values of MIF were 0.65 ng/ml in the T2DM IHD group and 0.85 ng/ml in the T2DM group. The median values of CXCL12 in the T2DM IHD and T2DM groups were 2001.54 pg/ml and 2158.37 pg/ml, respectively ($p > 0.05$). Both levels of CXCL12 were significantly different in the T2DM and T2DM IHD compared to the control group (**Supplementary Table 7**). These results indicate that neither MIF nor CXCL12 can serve as good IHD prognostic markers in T2DM patients.

Correlation of Circulating miRNAs With Clinical Data

A Spearman's rank-order regression analysis was performed to assess the relationship between miRNAs levels and clinical parameters of patients (**Figure 6**). The analysis indicated significant moderate correlation between fibrinogen and miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p, and miR-615-3p (r = 0.38; r = 0.40; r = 0.41; r = 0.36 respectively; $p < 0.05$). Platelet count was negatively correlated with miR-548b-3p (r = -0.4; $p < 0.05$). Systolic blood pressure was correlated with miR-1224 (r = 0.31; $p < 0.05$). Analysis indicated moderate correlation between HDL and miR-615-3p (r = -0.42, $p < 0.05$). Statistical analysis showed strong positive correlation between serum levels of all studied miRNAs. The strongest positive correlation was indicated between miR-5196-3p+miR-6732-3p and miR-3147 (r = 0.81; $p < 0.001$). MiR-3147 was also correlated with miR-615 and with miR-548b-3p (r = 0.65; r = 0.60 respectively; $p < 0.001$). MiR-1224-5p showed correlation with miR-548b-3p, miR-5196-3p+miR-6732-3p, miR-3147 (r = 0.70; r = 0.77; r = 0.68; $p < 0.001$). MiR-615-3p showed correlation with miR-5196-3p+miR-6732-3p and miR-548b-3p (r = 0.72; r = 0.49 respectively; $p < 0.001$). No significant correlations between MIF and CXCL12 levels and upregulated miRNAs were found.

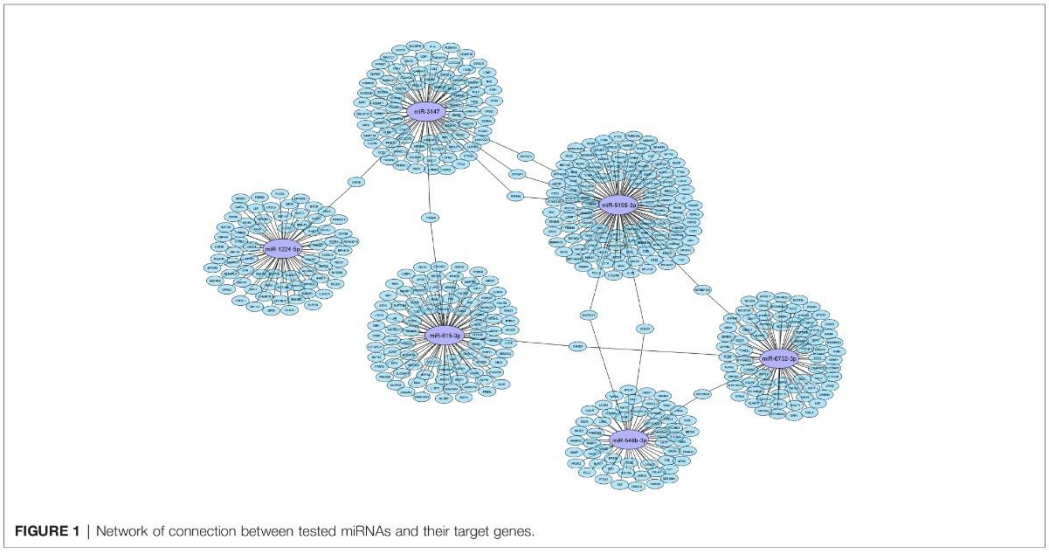

FIGURE 1 | Network of connection between tested miRNAs and their target genes.

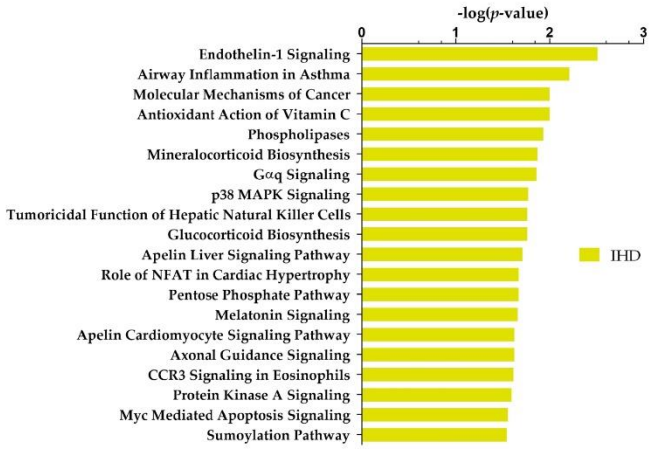

FIGURE 2 | Top 20 Ingenuity Pathway Analysis (IPA) canonical pathways most significantly changed in T2DM patients with IHD compared to individuals without IHD. The x-axis shows the -log of *p*-value calculated by the Benjamini-Hochberg (B-H) method.

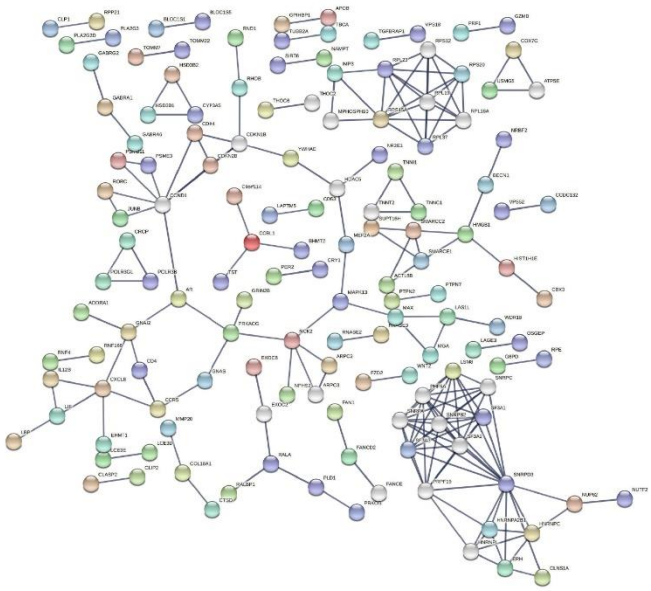

FIGURE 3 | Protein-protein interaction (PPI) network of DE miRNA's targeted genes by STRING. STRING is a database of known and predicted protein-protein interactions, including physical and functional associations based on text mining of the scientific literature, other databases, computational predictions, and knowledge transfer between organisms. The network gives information on the degree of overall connectivity across imputed genes. The interaction score was set to the highest confidence (0.9), showing a high probability of the association being true.

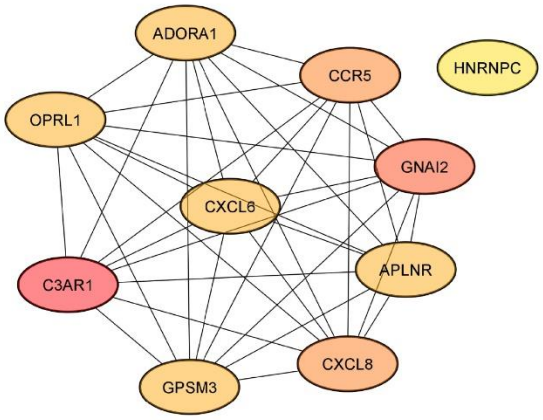

FIGURE 4 | The networks of the top 10 hub genes.

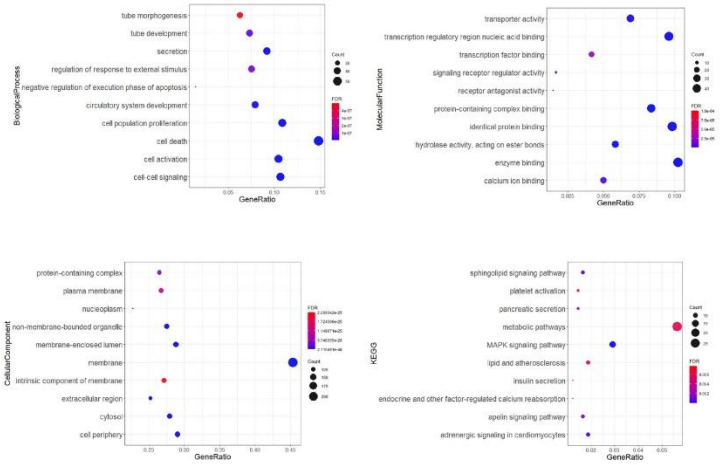

FIGURE 5 | Gene ontology (GO) enrichment analysis. Dot plots showing top 10 significantly enriched GO terms of the target genes in the biological process, molecular function, cellular components, and top 10 KEGG (Kyoto Encyclopedia of Genes and Genomes) connected with cardiovascular diseases and diabetes. The colors of the nodes are illustrated from red to blue in descending order of the FDR adjusted *p*-value. The sizes of the nodes are shown from small to large in ascending order of gene counts. The x-axis represents the gene ratio, and the y axis represents the GO or KEGG terms. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Evaluation of Diagnostic Values of Tested miRNAs

ROC curve analysis was performed to estimate the possible roles of identified miRNAs as biomarkers for IHD in diabetic patients (**Figure 7**). AUC (area under the ROC curve) for all six miRNAs reached significance comparing to AUC = 0.500 ($p < 0.001$ in all cases). The AUC values > 0.800 were found for miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p and miR-615-3p. AUC score for the miR-548b-3p was also high (AUC = 0.779). The AUC values for MIF and CXCL12 were less than

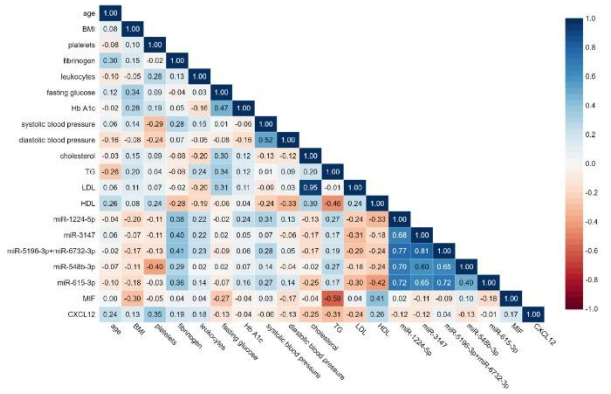

FIGURE 6 | Spearman's rank-matrix correlation between tested miRNAs, MIF, CLXC12 and clinical parameters. The blue colour indicates a positive correlation, and red indicates the opposite.

0.500 (AUC = 0.5 borderline of the diagnostic usefulness of the test).

Logistic Regression Model

To investigate the possible increase of diagnostic value by simultaneous consideration of multiple DE miRNAs, logistic regression models were developed using Weka 3.8.6 (The University of Waikato, Hamilton, New Zealand) software (40). The parameters of the models and the standard quality measures are summarized in the **Table 3**. The highest AUC was obtained for a combination of miR-3147 and miR-615-3p (AUC = 0.935). This model had a higher diagnostic value compared to the highest AUC for miRNA used separately.

Data Validation

To verify the results of the NanoString analysis, the most upregulated miRNAs from the miRNA profiling (miR-615-3p, miR-3147) were validated in the group of 22 T2DM and 26 T2DM IHD patients. In comparison with the results from the nCounter platform to qRT-PCR validation showed similarity between the expression patterns of tested miRNA (**Figure 8**).

DISCUSSION

T2DM is a complex metabolic disorder involving multiple genes that affect different signalling pathways. Diabetes is a known risk factor for IHD, and patients with higher HbA1c levels are more at risk of cardiovascular mortality (41). The development of new molecular biomarkers of identification T2DM patients with risk of IHD can improve the care of such patients. MiRNAs, as essential mediators of cell-to-cell communication, have critical roles in the epigenetic regulation of metabolic, inflammatory, and antiangiogenic pathways in diabetes-related to long term complications (42). The latest research show that miRNA can play an important role as a biomarker for diabetes and its complications (3). The potential for biofluids–derived miRNA to serve as a diagnostic tool has stimulated a wide range of studies regarding the disease-specific expression of miRNA and its stability (43). To find the unique miRNA profile in T2DM patients with IHD, we used a state-of-the-art NanoString nCounter platform that provides the opportunity to profile a large number of miRNAs that have not been previously investigated about IHD in T2DM.

In comparison to other methods of miRNA detection, the NanoString offers maximal sensitivity and specificity with high-quality data due to the elimination of amplification (44). It has been shown that this platform detects miRNAs in biofluids with sensitivity and specificity more significant than other miRNA detection methods like qPCR or microarrays (45). Moreover, this technique represents a more accessible and accurate method for everyday clinical practice because of the partially automated process that eliminates the risk of errors (46). To our best knowledge, this study is the first miRNA profiling in the serum of IHD patients using the nCounter platform, which seems to be an optimal strategy to identify novel biomarker candidates for IHD prognosis.

Our research revealed six DE miRNAs in patients with T2DM and IHD compared to diabetic individuals without IHD. No significant expression of those miRNAs was observed in the group of T2DM without IHD.

Upregulated miRNAs in the T2DM IHD group with the highest fold change and AUC value were miR-615-3p and miR-3147 (FC = 2.45 and 2.35; AUC = 0.877 and 0.858 respectively). A study by Zong et al. compared miRNA profiles in patients with acute myocardial infarction to controls. The results from RNA sequencing pointed out 96 up-, and 85 down-regulated miRNAs included miR-615-3p, which was also confirmed by qPCR

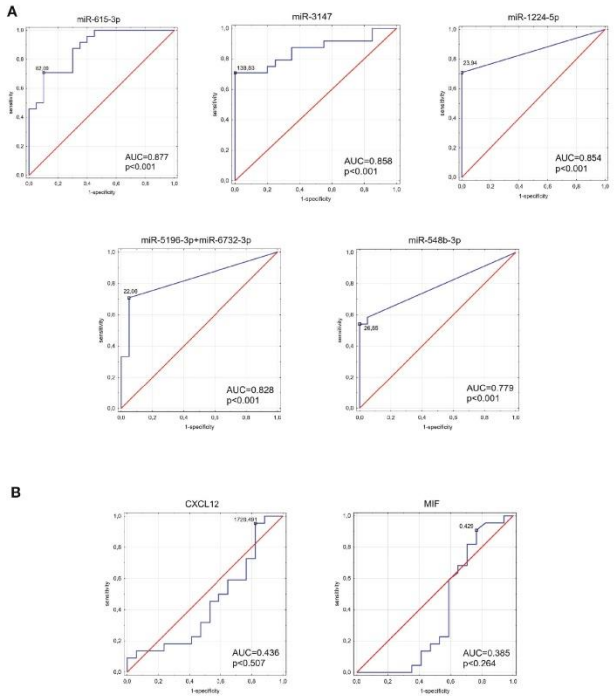

FIGURE 7 | The area under the curve (AUC) of the receiver operating characteristic (ROC) curves for tested miRNAs **(A)** and MIF and CXCL12 proteins **(B)**. ROC analysis was carried out to evaluate the diagnostic potential of selected miRNA as a predictive biomarker of type 2 diabetes compared to MIF and CXCL12 proteins.

TABLE 3 | Summary of the basic parameters and common quality measures of the models.

model	TP rate	FP rate	Precision	AUC	Intercept	Coefficients
x1 = miR-3147	0.864	0.147	0.866	0.935	28.56	a1 = -9.03
x2 = miR-615-3p						a2 = -6.87
x1 = miR-548b-3p	0.841	0.166	0.841	0.929	20.30	a1= -7.06
x2 = miR-615-3p						a2= -7.22
x1 = miR-3147	0.886	0.120	0.887	0.927	26.78	a1 = -6.49
x2 = 548b-3p						a2 = -3.09
x3 = 615-3p						a3 = -6.54
x1 = 1224-5p	0.795	0.204	0.797	0.906	20.68	a1 = -8.78
x2 = 615-3p						a2 = -5.87
x1 = miR-3147	0.795	0.212	0.795	0.881	28.00	a1 = -6.69
x2 = miR-615-3p						a2 = -6.47
x3 = 1224-5p						a3 = -2.06
x4 = 548b-3p						a4 = -2.27
x5 = 5196-3p+miR-6732-3p						a5 = 0.55

TP, true positive; FP, false positive; AUC, area under the curve of the receiver operating characteristic.

validation. In this case, the AUC for miR-615-3p was 0.688 (47). MiR-3147,miR-1224-5p, miR-5196-3p, miR-6732-3p, and miR-548b-3p have never been previously described concerning T2DM or vascular disease. Their indication in our study may evidence that they are new potential biomarkers of IHD in T2DM. These results suggest that indicated miRNAs candidates have a high probability of being specific for the T2DM IHD phenotype and could play a pivotal role in the IHD diagnosis in T2DM patients.

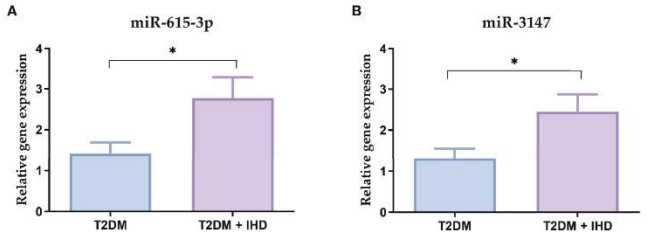

FIGURE 8 | Relative mRNA expressions of the miR-615-3p **(A)** and miR-3147 **(B)** in the serum samples in the group of 22 T2DM and 26 T2DM IHD patients. Each bar represents the mean ratio of gene expression and miR-103a-3p and miR-199b-5p ± standard error of the mean. Significance: U Mann-Whitney test; *$p < 0.05$ in comparison to control.

The IPA analysis indicated 489 molecules regulated by identified miRNAs. Further analysis linked the target genes into a PPI network and extracted ten hub genes. Some of them are connected with cardiovascular abnormalities and T2DM. *C3aR1* showed elevated expression in obese patients compared to the control group (48). Research implicates that *CCR5* plays an important role in the initiation and progression of atherosclerosis (49). *CXCL6* and *CXCL8* belong to the cytokine family connected with heart failure and T2DM (50, 51). *ADORA1* gene encodes adenosine A1 receptor and, in the heart, it is expressed in cardiomyocytes, and its activation might promote angiogenesis (52). *ADORA1* is upregulated in the epicardial adipose tissue, which may be involved in IHD pathogenesis compared with mediastinal adipose tissue (53). Adenosine signalling has a crucial role in diabetes mellitus pathophysiology due to its modulation of insulin secretion and regulation of β-cell homeostasis (54). Our analysis showed that *APLNR*, which encodes apelin receptor, is a target gene for miR-5196-3p. Apelin showed vascular effects in numerous studies and under normal conditions, it lowers blood pressure (55, 56). Recent studies have found that apelin-mediated signalling is connected to heart failure and IHD. Furthermore, apelin participates in the pathology of diabetes by playing a pivotal role in increasing glucose uptake and insulin sensitivity (57). Moreover, a meta-analysis study showed that circulating apelin levels in humans are higher in T2DM patients than in healthy controls (58). In contrast, a study by Castan-Laurell et al. has proven that apelin can regulate blood glucose levels, and elevated levels of plasma apelin might be beneficial in reducing the risk of diabetes (59). Given that most of the presented miRNAs have not been previously described and are potentially associated with this gene involved in critical processes leading to IHD, special attention should be paid to it in further studies.

Ingenuity core analysis allowed us to identify which canonical pathways are dysregulated by tested upregulated miRNAs. Our results showed that one of the most important dysregulated canonical pathways in the T2DM IHD patients was endothelin-1 signalling. Endothelin-1 is a potent vasoconstrictor and pro-inflammatory protein and is an important contributor to the pathogenesis of hypertension, atherosclerosis, hypertrophy, and diabetes (60). Influenced by risk factors for cardiovascular disorders, its expression is altered, which plays an important role in the pathology of the cardiovascular system (61). Furthermore, patients with T2DM have increased vasoconstrictor activity induced by endothelin-1 (62). It has been proven that p38 MPAK has a crucial role in myocyte proliferation and apoptosis in the heart and participates in cardiac hypertrophy regulation (63). Interestingly Liang et al. conducted a study that confirms that miR-124 inhibits macrophage cells by targeting the p38/MAPK signalling pathway in the development of atherosclerosis. Mineralocorticoid biosynthesis is another canonical pathway that might be involved in IHD, especially at the atherosclerosis development (64, 65). Similarly, glucocorticoids and their receptors are also related to blood pressure regulation, atherosclerosis, and heart failure (66). Another important dysregulated canonical pathway is the pentose phosphate pathway which is a significant pathway for glucose metabolism, is required to synthesise ribonucleotides and produces a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which is a key reductant in anabolic processes (67). This pathway participates in the T2DM pathogenesis (68). Melatonin signalling is another dysregulated canonical pathway in T2DM IHD patients. Melatonin has a considerable role in arterial blood pressure, insulin resistance, lipid and glucose metabolism, and control sleep-wake cycles. Patients with IHD have low melatonin production rates. Interestingly, this analysis also confirmed the importance of apelin signalling. Indeed, these identified canonical pathways play a significant role in the mechanisms leading to the development of cardiovascular diseases. It allows supposing that miRNAs determined by us play a relevant role in the regulation of genes associated with cardiovascular diseases and top dysregulated canonical pathways.

Nevertheless, we did not find any statistically significant correlation between the level of miRNAs and clinical parameters such as age, BMI, leucocytes, fasting glucose, HbA1c, diastolic blood pressure, cholesterol, triglycerides, or LDL level. However, those parameters are not specific only for IHD. Fibrinogen is not only an indicator of hypercoagulability

but, as an acute-phase protein, is also an indicator of inflammation. In epidemiological and clinical studies, elevated blood fibrinogen levels are an independent risk factor for cardiovascular diseases (69–71). It has been proven that miRNAs can regulate fibrinogen production (72). Interestingly, we indicated a moderate positive correlation between levels of fibrinogen and miR-1224-5p, miR-3147, miR-5196-3p+miR-6732-3p, and miR-615-3p which have not been previously described as being related to this protein. We have indicated a moderate negative correlation between miR-548b-3p and platelets. The link between miRNAs and platelet in the development of IHD has been previously proven and summed up in a review by Stojkovic et al. (73). A strong or moderate positive correlation between all miRNA's levels suggested they belong to the miRNA group, which dysregulation of mechanisms responsible for diabetic complications.

The area under the ROC curve remains a major criterion for diagnostic biomarkers. The ROC curve illustrates the relationship between diagnostic sensitivity and specificity and describes the diagnostic value of the tested parameters (74). To the best of our knowledge, there is no evidence in the literature describing the usefulness of miR-615-3p, miR-3147, (miR-1224-5p, miR-5196-3p+miR-6732-3p, miR-548b-3p) as a diagnostic tool for IHD in T2DM. The highest AUC values were observed in this study for miR-615-3p and miR-3147. Furthermore, other tested miRNA (miR-1224-5p, miR-5196-3p+miR-6732-3p, and miR-548b-3p) also showed high AUC results. The combined analysis showed that a logistic regression model consisting of miR-3147 and miR-615-3p demonstrates higher diagnostic accuracy than those miRNAs individually. These findings suggest that it would be beneficial to introduce such a panel in predicting IHD development in T2DM patients. High AUC scores for these miRNAs provide the groundwork for future confirmatory studies with a comprehensive validation in a larger cohort of patients. Additionally, AUC values for MIF and CXCL12, which play an important role in IHD, were below 0.500, disqualifying these two proteins as a good diagnostic tool for IHD diabetic patients.

MIF and CXCL12 are considered as a potential biomarker for heart diseases in patients with T2DM (75). However, one must consider that diabetes is also an inflammation state, and non-specific inflammatory parameters like MIF or CXCL12 can be elevated in patients with IHD or without it. Low AUC values for those proteins support our observations that the changes in miRNA levels could be better prognostic IHD biomarkers than the level of MIF or CXCL12 in T2DM patients.

Currently, the RT-qPCR technique, which we have used to validate results, remains the most commonly used approach for miRNA expression profiling due to its price, sensitivity, and specificity. The RT-qPCR analysis showed significantly increased expressions of miR-615-3p and miR-3147 in the serum of patients with T2DM who developed IHD, which confirms the reliability of the nCounter platform.

Presented data indicate a specific serum miRNA profile of patients with T2DM and IHD, and the different levels of selective miRNA expression might have a crucial role in further IHD diagnosis. We believe that identified circulating miRNAs might serve as new, non-invasive biomarkers for early detection of IHD in T2DM patients. However, this data should be considered preliminary. Additional studies on a larger cohort of patients are required to validate the predictive value of those miRNAs to prompt diagnosis of IHD in T2DM patients or better identification of at-risk individuals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185845

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Bioethics Committee of the Medical University of Bialystok, Poland (approval numbers: R-I-002/583/2019 and APK.002.35.2021). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, AB, MN, and AK; methodology, AB, MN, IS, ASz, and AK; formal analysis, AB, MN, IS, and WB, investigation, AB, ASz, JR, DO, KG, and MN; writing—original draft preparation, AB; writing—review and editing, AK, MN, and ASk; visualization, AB and MN; supervision, MN, SD, and AK. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

11. Streszczenie w języku polskim

Cukrzyca typu 2 (T2DM) jest najczęściej występującym zaburzeniem metabolicznym. Towarzysząca jej przewlekła hiperglikemia powoduje zaburzenia czynności i niewydolność m.in. oczu, nerek, nerwów, serca i naczyń krwionośnych. Najczęstszym i najniebezpieczniejszym powikłaniem cukrzycy jest choroba niedokrwienna serca (IHD). Obecnie nie ma narzędzia do wczesnego przewidywania rozwoju IHD u pacjentów z T2DM, a stosowane dotychczas koronarografia czy echokardiografia są metodami inwazyjnymi i mogą stwierdzić obecność choroby dopiero na zaawansowanym etapie. Wszystkie te czynniki powodują, że istnieje pilna potrzeba wytypowania nowych, nieinwazyjnych biomarkerów wykrywania IHD u pacjentów z T2DM. Krążące mikroRNA (miRNA) w surowicy mają duży potencjał, aby służyć jako marker do wczesnej diagnozy IHD i identyfikacji osób z predyspozycjami do jej rozwoju. MiRNA to krótkie, jednoniciowe, niekodujące, endogenne RNA, które odgrywają kluczową rolę w regulacji ekspresji genów, uczestniczą w procesach takich jak proliferacja, różnicowanie, angiogeneza, onkogeneza czy apoptoza komórek.

Głównym celem prezentowanych badań była analiza profilu miRNA i identyfikacja potencjalnych biomarkerów diagnostycznych w surowicy u pacjentów z IHD jako powikłanie T2DM. Wśród 69 badanych zakwalifikowanych do dalszych analiz wyodrębniono następujące podgrupy: chorych na T2DM z IHD, chorych na T2DM bez IHD, grupę pacjentów z IHD oraz grupę kontrolną bez T2DM i bez IHD. Zebrane próbki surowicy posłużyły jako materiał do oznaczenia profilu ekspresji miRNA w każdej z wybranych grup. Ekspresję miRNA profilowano przy zastosowaniu platformy nCounter firmy Nanostring. Wyniki zostały zwalidowane metodą RT-qPCR w czasie rzeczywistym. Analizę wyników przeprowadzono przy użyciu programu STATISTICA oraz oprogramowania nSolver 4.0. Do analizy funkcjonalnej wykorzystano oprogramowanie Ingenuity Pathway Analysis, bazę STRING, programy Cytoscape, g:Profiler oraz Metascape. Na podstawie przeprowadzonych analiz wskazano sześć miRNA (miR-615-3p, miR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p i miR-548b-3p) istotnie różnicujących grupy pacjentów z T2DM i IHD od pacjentów z T2DM bez IHD. Analiza funkcjonalna wykazała, że geny regulowane przez wyznaczone miRNA uczestniczą w procesach prowadzących do dysfunkcji układu krążenia. Wszystkie zidentyfikowane miRNA mogą służyć jako nowe, nieinwazyjne biomarkery do

wczesnego wykrywania IHD u pacjentów z T2DM, a klasyfikujący model diagnostyczny utworzony z użyciem regresji logistycznej opartej o miR-615-3p i miR-3147 najdokładniej odróżnia pacjentów T2DM z IHD od pacjentów bez IHD.

12. Streszczenie w języku angielskim

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder. Chronic hyperglycemia can cause dysfunction and failure of the eyes, kidneys, nerves, heart, and blood vessels. The most common and dangerous complication of diabetes is ischemic heart disease (IHD). Currently, there is no dedicated tool to predict the early development of IHD in patients with T2DM. Coronary angiography or echocardiography are invasive methods and can diagnose the disease at an advanced stage. There is an urgent need to identify new non-invasive biomarkers for detecting IHD in patients with T2DM. Circulating micro RNAs (miRNAs) from serum have great potential to serve as a marker for early diagnosis of IHD and identification of individuals with a predisposition to its development. MiRNAs are short, single-stranded, non-coding, endogenous RNAs that play a pivotal role in gene expression regulation and are involved in processes such as proliferation, differentiation, angiogenesis, oncogenesis, and cell apoptosis.

The main objective of this study was to analyse the profile of miRNAs and identify potential diagnostic biomarkers in the serum of patients with IHD as a complication of T2DM. Among the 69 subjects qualified for further analysis, the following subgroups were identified: patients with T2DM and IHD, patients with T2DM without IHD, patients with IHD, and a control group without T2DM and IHD. Collected serum samples served as material for miRNA profiling. The expression of miRNAs was profiled using Nanostring's nCounter platform. The results were validated by real-time PCR. Analysis of the results was performed using STATISTICA and nSolver 4.0 software. Ingenuity Pathway Analysis software, STRING database, Cytoscape, g:Profiler, and Metascape programs were used for functional analysis. Six miRNAs (miR-615-3p, miR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p, and miR-548b-3p) were identified as a significantly upregulated in T2DM IHD group. Functional analysis showed that genes regulated by the selected miRNAs are involved in processes leading to cardiovascular dysfunction. All identified miRNAs have high diagnostic power and may serve as novel, non-invasive biomarkers for the early detection of IHD in T2DM patients. A diagnostic classification model, logistic regression based on miR-615-3p and miR-3147, most accurately classifies T2DM patients with IHD and T2DM patients without IHD.

A logistic regression model for a panel consisting of miR-615-3p and miR-3147 significantly increases their diagnostic value (AUC= 0.935).

13. Oświadczenia współautorów

Bielska, A.; Niemira, M.; Bauer, W.; Sidorkiewicz, I.; Szałkowska, A.; Skwarska, A.; Raczkowska, J.; Ostrowski, D.; Gugała, K.; Dobrzycki, S.; Krętowski, A. Serum MiRNA Profile in Diabetic Patients With Ischemic Heart Disease as a Promising Non-Invasive Biomarker. *Front. Endocrinol.* 2022, *13*.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka – Agnieszka Bielska	opracowanie koncepcji badań, udział w planowaniu eksperymentów, przeprowadzanie eksperymentów, opracowanie i analiza wyników, przygotowanie tabel i rycin, napisanie manuskryptu, autor korespondencyjny	60%
dr n. chem. Magdalena Niemira	udział w planowaniu eksperymentów, pomoc w opracowaniu metodyki, nadzorowanie przebiegu badań, pomoc w przygotowaniu manuskryptu, nadzór merytoryczny	15%
dr n. med. Witold Bauer	wybór i analiza podstawowych parametrów pacjentów	4%
dr n. med. Iwona Sidorkiewicz	współwykonawstwo badań laboratoryjnych, pomoc w analizie wyników	5%
mgr Anna Zeller (Szałkowska)	współwykonawstwo badań laboratoryjnych	3%
dr n. chem. Anna Skwarska	pomoc w przygotowaniu manuskryptu, korekta językowa	3%
mgr Justyna Raczkowska	współwykonawstwo badań laboratoryjnych	1%
mgr Damian Ostrowski	współwykonawstwo badań laboratoryjnych	1%
lek. Kamil Gugała	zbieranie próbek do badań	1%
prof. dr hab. Sławomir Dobrzycki	konsultacja merytoryczna	2%
prof. dr hab. Adam Jacek Krętowski	konsultacja merytoryczna, ocena ostatecznej wersji manuskryptu	5%

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

Agnieszka Bielska

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

Bielska, A.; Niemira, M.; Kretowski, A. Recent Highlights of Research on MiRNAs as Early Potential Biomarkers for Cardiovascular Complications of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* 2021, *22* (6), 3153.

Imię i nazwisko współautora	Charakter udziału	Procentowy wkład
Doktorantka – Agnieszka Bielska	opracowanie koncepcji pracy, przegląd literaturowy, zebranie piśmiennictwa dotyczącego tematyki pracy, napisanie manuskryptu, przygotowanie tabel i rycin, autor korespondencyjny	60%
dr n. chem. Magdalena Niemira	konsultacja merytoryczna podczas pisania pracy, ocena ostatecznej wersji manuskryptu	30%
prof. dr hab. Adam Jacek Krętowski	konsultacja merytoryczna podczas pisania pracy, ocena ostatecznej wersji manuskryptu	10%

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Oświadczenie

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autorów: Bielska Agnieszka, Niemira Magdalena, Krętowski Adam
opublikowanej w International Journal of Molecular Science,
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- „Serum MiRNA Profile in Diabetic Patients With Ischemic Heart Disease as a Promising Non-Invasive Biomarker."

autorów: Bielska Agnieszka, Niemira Magdalena, Bauer Witold, Sidorkiewicz Iwona, Szałkowska Anna, Skwarska Anna, Raczkowska Justyna, Ostrowski Damian, Gugała Kamil, Dobrzycki Sławomir, Krętowski Adam
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Potwierdzam, iż indywidualny udział mgr Agnieszki Bielskiej w powstaniu niniejszych prac wyniósł po 60% w każdej. Jednocześnie wyrażam zgodę na wykorzystanie przez Agnieszkę Bielską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

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Oświadczenie

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autorów: Bielska Agnieszka, Niemira Magdalena, Bauer Witold, Sidorkiewicz Iwona, Szałkowska Anna, Skwarska Anna, Raczkowska Justyna, Ostrowski Damian, Gugała Kamil, Dobrzycki Sławomir, Krętowski Adam
opublikowanej w czasopiśmie Frontiers in Edocrinology
wchodzącej w skład rozprawy doktorskiej *„Analiza profilu mikro RNA i identyfikacja potencjalnych biomarkerów diagnostycznych w surowicy u pacjentów ze stabilną chorobą niedokrwienną serca jako powikłanie cukrzycy typu 2."*, wynoszący **1%** polegał na zbieraniu próbek do badań.

Potwierdzam, iż indywidualny udział mgr Agnieszki Bielskiej w powstaniu niniejszej pracy wyniósł 60%. Jednocześnie wyrażam zgodę na wykorzystanie przez Agnieszkę Bielską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Kamil Gugała
.....

Białystok, 21.12.2022

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Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

- „Serum MiRNA Profile in Diabetic Patients With Ischemic Heart Disease as a Promising Non-Invasive Biomarker."

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Potwierdzam, iż indywidualny udział mgr Agnieszki Bielskiej w powstaniu niniejszej pracy wyniósł 60%. Jednocześnie wyrażam zgodę na wykorzystanie przez Agnieszkę Bielską publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

KIEROWNIK
Kliniki Kardiologii Inwazyjnej
z OIOK i Pracownią Hemodynamiki

.....prof. dr hab. Sławomir Dobrzycki.....

14. Uchwała Komisji Etycznej

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Białystok, 19-12-2019

Uchwała nr: R-I-002/583/2019

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: „Profilowanie krążącego miRNA u pacjentów z cukrzycą typu 2 i chorobą niedokrwienną serca " przez mgr Agnieszkę Bielską wraz z zespołem badawczym z UMB.

Przewodnicząca Komisji Bioetycznej UMB

prof. dr hab. Otylia Kowal-Bielecka

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Białystok, 28.01.2021 r.

Uchwała nr: APK.002.35.2021

Na podstawie art. 29 ust. 2 i 14 ustawy dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty (t.j. Dz. U z 2020, poz. 514 ze zm.), Komisja Bioetyczna przy Uniwersytecie Medycznym 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: „Analiza profilu krążących miRNA u pacjentów z chorobą wieńcową w celu wytypowania potencjalnych biomarkerów diagnostycznych" przez mgr Agnieszkę Bielską wraz z zespołem badawczym z UMB.

Planowany okres realizacji od 28.01.2021 r. do 02.01.2022 r.

Przewodnicząca Komisji Bioetycznej przy UMB

prof. dr hab. Otylia Kowal-Bielecka

Pouczenie:
1. Odwołanie od uchwały komisji bioetycznej wyrażającej opinię może wnieść:
1) wnioskodawca;
2) kierownik podmiotu, w którym eksperyment medyczny ma być przeprowadzony;
3) komisja bioetyczna właściwa dla ośrodka, który ma uczestniczyć w wieloośrodkowym eksperymencie medycznym.
2. Odwołanie, o którym mowa w ust. 1, wnosi się za pośrednictwem komisji bioetycznej, która podjęła uchwałę, do Odwoławczej Komisji Bioetycznej w terminie 14 dni od dnia doręczenia uchwały wyrażającej opinię.

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