WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

Stacjonarne Studia Doktoranckie
Biochemiczno-Biofizyczne

Rafał Szelenberger

Poszukiwanie w płytkach krwi molekularnych markerów określających predyspozycje człowieka do wystąpienia Ostrych Zespołów Wieńcowych

Praca doktorska

wykonana w Katedrze Biochemii Ogólnej
Instytutu Biochemii

Promotor:

- Prof. dr hab. Joanna Saluk-Bijak

Promotor pomocniczy:

- Dr n. med. Michał Kacprzak

The examination of blood platelet molecular markers associated with the human predisposition to Acute Coronary Syndrome development

Łódź, 2022

1. SPIS TREŚCI

Pragnę złożyć serdeczne podziękowania dla Pani Profesor Joanny Saluk-Bijak,
za nieocenioną pomoc, opiekę merytoryczną, cierpliwość, motywację
oraz istotny wkład w przygotowywanie pracy doktorskiej.

Dziękuję Panu Doktorowi Michałowi Kacprzakowi, za wsparcie merytoryczne,
istotną pomoc oraz zaangażowanie w realizację niniejszej pracy doktorskiej.

Dziękuję Panu Profesorowi Michałowi Bijakowi, za wszelką pomoc, przekazaną wiedzę
oraz szansę, która umożliwiła dalsze rozwijanie moich zawodowych pasji.

Dziękuję wszystkim Kolegom, Koleżankom oraz Pracownikom
Instytutu Biochemii, za stworzenie wspaniałej atmosfery.

Dziękuję moim najbliższym Przyjaciołom, w szczególności Mateuszowi oraz Angeli,
za niezłomną wiarę, wsparcie, motywację oraz pomoc.

Szczególne podziękowania chciałbym złożyć całej mojej Rodzinie, przede wszystkim mojej Żonie Jagodzie, za anielską cierpliwość, nigdy nie gasnącą wiarę w moje możliwości, za dobre słowo w chwilach sukcesu oraz za motywację, wsparcie i pomoc w chwilach zwątpienia.

2. ŹRÓDŁA FINANSOWANIA BADAŃ PROWADZONYCH W RAMACH PRACY DOKTORSKIEJ

Projekt „Diamentowy Grant” finansowany przez Ministerstwo Edukacji i Nauki nr DI2016 022046 w latach 2017 – 2021; nt.: „Płytkowe mikroRNA jako marker predykcyjny Ostrego Zespołu Wieńcowego”; Kierownik: mgr Rafał Szelenberger; Opiekun: prof. dr hab. Joanna Saluk-Bijak.

Dotacja celowa przyznawana na działalność związaną z prowadzeniem badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich w roku 2019.

3. WSPÓŁPRACA

Rozprawę doktorską zrealizowano we współpracy z Kliniką Kardiologii Interwencyjnej Katedry Kardiologii Interwencyjnej i Elektrokardiologii Centralnego Szpitala Klinicznego Uniwersytetu Medycznego w Łodzi, przy współudziale dr. n. med. Michała Kacprzaka, pełniącego w niniejszej rozprawie doktorskiej rolę promotora pomocniczego.

Analiza statystyczna została wykonana przy współpracy z dr. n. med. Michałem Sewerynem Karbownikiem z Zakładu Farmakologii i Toksykologii Katedry Farmakologii Ogólnej, Klinicznej i Toksykologii Uniwersytetu Medycznego w Łodzi.

4. UCZESTNICTWO W PROJEKTACH KRAJOWYCH I MIĘDZYNARODOWYCH

- KIEROWNIK

1) Projekt „Diamentowy Grant" finansowany ze środków MEiN pt.: „*Płytkowe mikroRNA jako marker predykcyjny Ostrego Zespołu Wieńcowego*"
2) Projekt „PRELUDIUM 17" finansowany ze środków NCN pt.: „*Identyfikacja zmian w profilu ekspresji mRNA oraz białek płytek krwi, jako potencjalnych markerów molekularnych zwiększonego ryzyka wystąpienia udaru niedokrwiennego mózgu*"

- WYKONAWCA

1) „SZAFIR" finansowany ze środków NCBiR pt.: „*Nowoczesne metody zabezpieczania miejsca zdarzenia w środowisku CBRN z wykorzystaniem nanotechnik, AR i inżynierii odwróconej*"
2) Horyzont 2020 pt.: „*Deep Learning Powered Holographic Microscopy for Biothreat Detection on Field*"

5. DOROBEK NAUKOWY WCHODZĄCY W SKŁAD ROZPRAWY DOKTORSKIEJ

Niniejsza rozprawa doktorska obejmuje dwie publikacje przeglądowe oraz trzy publikacje eksperymentalne:

- PUBLIKACJE PRZEGLĄDOWE:

1) **Rafal Szelenberger**, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska. *Blood platelet surface receptor genetic variation and risk of thrombotic episodes*. Clinica Chimica Acta, 496: 84-92, 2019.
   Punkty MEiN = 100, IF = 2,615
2) **Rafal Szelenberger**, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak. *Plasma MicroRNA as a novel diagnostic*. Clinica Chimica Acta, 499: 98-107, 2019.
   Punkty MEiN = 100, IF = 2,615

- PUBLIKACJE EKSPERYMENTALNE:

1) **Rafał Szelenberger**, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak. *Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome*. Cells, 10(12): 3526, 2021.
   Punkty MEiN = 140, IF = 6,600
2) **Rafał Szelenberger**, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak. *Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12*. Biology, 11(5): 644, 2022.
   Punkty MEiN = 100, IF = 5,079

3) **Rafał Szelenberger**, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak. *Variations in blood platelet proteome and transcriptome revealed altered expression of Transgelin-2 in Acute Coronary Syndrome patients*. International Journal of Molecular Sciences, 23(11): 6340, 2022.
Punkty MEiN = 140, IF = 5,924

Suma punktów MEiN = 580, łączny IF = 22,833

6. DOROBEK NAUKOWY NIEWCHODZĄCY W SKŁAD ROZPRAWY DOKTORSKIEJ

- PUBLIKACJE PRZEGLĄDOWE

1) Michal Bijak, Joanna Saluk, **Rafal Szelenberger**, Pawel Nowak. *Popular naturally occurring antioxidants as potential anticoagulant drugs*. Chemico-Biological Interactions, 257: 35-45, 2016.
Punkty MEiN = 100 (z dnia 31.07.2019), IF = 3,143
2) **Rafał Szelenberger**, Joanna Kostka, Joanna Saluk-Bijak, Elżbieta Miller. *Pharmacological Interventions and Rehabilitation Approach for Enhancing Brain Self-repair and Stroke Recovery*. Current Neuropharmacology, 18(1): 51-64, 2020.
Punkty MEiN = 140, IF = 7,390
3) **Rafal Szelenberger**, Joanna Saluk-Bijak, Michal Bijak. *Ischemic Stroke among the Symptoms Caused by the COVID-19 Infection*. Journal of Clinical Medicine, 9(9): 2688, 2020.
Punkty MEiN = 140, IF = 4,242
4) Lidia Wlodarczyk, **Rafal Szelenberger**, Natalia Cichon, Joanna Saluk-Bijak, Michal Bijak, Elzbieta Miller. *Biomarkers of Angiogenesis and Neuroplasticity as Promising Clinical Tools for Stroke Recovery Evaluation*. International Journal of Molecular Sciences, 22(8): 3949, 2021.
Punkty MEiN = 140, IF = 5,924

- PUBLIKACJE EKSPERYMENTALNE

1) Michal Bijak, **Rafal Szelenberger**, Joanna Saluk, Pawel Nowak. *Flavonolignans inhibit ADP induced blood platelets activation and aggregation in whole blood*. International Journal of Biological Macromolecules, 95: 682-688, 2017.
Punkty MEiN = 100 (z dnia 31.07.2019), IF = 3,909
2) Michal Bijak, **Rafal Szelenberger**, Angela Dziedzic, Joanna Saluk-Bijak. *Inhibitory Effect of Flavonolignans on the P2Y12 Pathway in Blood Platelets*. Molecules, 23(2): 374, 2018.
Punkty MEiN = 100 (z dnia 31.07.2019) , IF = 3,060
3) Angela Dziedzic, **Rafal Szelenberger**, Michal Kacprzak, Piotr Czarny, Ewelina Synowiec, Joanna Saluk-Bijak, Tomasz Sliwinski, Marzenna Zielinska, Michal Bijak. *Variations in the Gene Expression Profile in Atherosclerotic Patients with Non-Fatal ACS: A Preliminary Study*. International Journal of Molecular Sciences, 23(9): 5017, 2022.
Punkty MEiN = 140, IF = 5,924

Sumaryczny dorobek naukowy: Punkty MEiN = 1440, łączny IF = 56,425

- DONIESIENIA KONFERENCYJNE

1) Michal Bijak, **Rafal Szelenberger**, Joanna Saluk. *Flavonolignans as a potential inhibitors of platelet activation by ADP*. International Cardiovascular Research Meeting, 2016, Bydgoszcz.
2) **Rafał Szelenberger**, Michał Bijak, Michał Kacprzak. *Płytkowe miRNA jako potencjalne markery Ostrych Zespołów Wieńcowych*. Interdyscyplinarna Konferencja Naukowa TYGIEL, 2018, Lublin.
3) **Rafał Szelenberger**, Michał Kacprzak, Michał Bijak, Joanna Saluk-Bijak. *Płytkowe mikroRNA jako potencjalny marker diagnostyczny w ocenie czynnościowych zaburzeń hemostazy*. Badania Młodych Naukowców, Wiedza-Inspiracja-Pasja, 2018, Łódź.
4) **Rafał Szelenberger**, Paulina Wigner, Michał Kacprzak, Michał Bijak, Joanna Saluk-Bijak. *Single nucleotide polymorphisms in genes encoding TPH1 and TPH2 increases risk of Acute Coronary Syndrome*. III Congress BIO, 2018, Gdańsk.
5) **Rafał Szelenberger**, Paulina Wigner, Michał Kacprzak, Michał Bijak, Joanna Saluk-Bijak. *Analiza wpływu polimorfizmów genów SOD2, CAT i GPX4 na ryzyko zachorowalności pacjentów z Ostrym Zespołem Wieńcowym*. BIOOPEN, 2019, Łódź.
6) **Rafał Szelenberger**, Ewelina Synowiec, Michał Kacprzak, Paulina Operacz, Joanna Saluk-Bijak, Michał Bijak. *The Increased Level of Platelet's P2Y12 Receptor in Acute Coronary Syndromes*. The 44th FEBS Congress, 2019, Kraków.
7) **Rafał Szelenberger**, Ewelina Synowiec, Michał Kacprzak, Paulina Operacz, Joanna Saluk-Bijak, Michał Bijak. *Zwiększony poziom płytkowej glikoproteiny IIb/IIIa w Ostrym Zespole Wieńcowym*. National Scientific Conference „Understand the Science", 2019, Łódź.
8) **Rafał Szelenberger**, Joanna Saluk-Bijak, Michał Kacprzak, Michał Bijak. *Increased Level of Fibrinogen Chains in the Proteome of Blood Platelet in Acute Coronary Syndrome Patients*. National Scientific Conference „Science and Young Researchers" IV Edition, 2020, Łódź – Online.
9) **Rafał Szelenberger**, Michał Kacprzak, Michał Bijak, Joanna Saluk-Bijak. *Nadekspresja transgeliny-2 w płytkach krwi pacjentów z Ostrym Zespołem Wieńcowym.* BIOOPEN, 2021, Łódź – Online.
10) **Rafał Szelenberger**, Michał Kacprzak, Joanna Saluk-Bijak, Marzenna Zielińska, Michał Bijak. *Potential Platelet's miRNA Biomarkers of Acute Coronary Syndromes*.
    Young Scientists' Forum, 2021, Lovran – Online.
11) **Rafał Szelenberger**, Michał Kacprzak, Joanna Saluk-Bijak, Marzenna Zielińska, Michał Bijak. *Potential Platelet's miRNA Biomarkers of Acute Coronary Syndromes*. The 45th FEBS Congress, 2021, Lubljana - Online.

7. WSTĘP

Ostre zespoły wieńcowe (OZW) należą do grupy chorób kardiologicznych charakteryzujących się szerokim spektrum objawowym sięgającym od dolegliwości bólowych w klatce piersiowej przy niewielkich wysiłkach do zatrzymania akcji serca, spowodowanych częściowym lub całkowitym zablokowaniem przepływu krwi w naczyniach wieńcowych [1]. OZW są schorzeniem wieloczynnikowym o szerokiej etiologii. W zależności od obrazu klinicznego, powtarzanych zapisów elektrokardiograficznych (EKG) oraz wyników oznaczeń biochemicznych markerów martwicy mięśnia sercowego, możemy wyróżnić trzy główne typy OZW: zawał mięśnia sercowego z uniesieniem odcinka ST (ST-elevated myocardial infarction, STEMI), zawał mięśnia sercowego bez uniesienia odcinka ST (non-ST-elevated myocardial infarction, NSTEMI) oraz niestabilną dławicę piersiową (unstable angina, UA). W przebiegu zawału serca obserwowany jest wzrost markerów biochemicznych uszkodzenia mięśnia serca przy jednoczesnym występowaniu typowych zmian w zapisie EKG (uniesienie odcinka ST w przypadku STEMI) lub ich braku (w przypadku NSTEMI). U pacjentów z UA, pomimo występowania objawów niedokrwiennych, nie obserwuje się wzrostu markerów biochemicznych martwicy mięśnia serca [2].

Mechanizmem leżącym u podstaw patofizjologii OZW jest miażdżyca naczyń wieńcowych, rozwijająca się pod wpływem czynników patologicznych, prowadzących do czynnościowej i morfologicznej dysfunkcji śródbłonka naczyń krwionośnych. Nadmierna synteza oraz sekrecja związków pro-zapalnych, pro-zakrzepowych oraz stymulujących wazokonstrykcję intensywnie wpływa na zmiany mikrośrodowiska naczyniowego, prowadząc do zaburzenia równowagi hemostatycznej. Mechanizmy pierwotnej dysfunkcji śródbłonka, zależne od typowych czynników ryzyka, prowadzące do rozwoju i progresji blaszki miażdżycowej, są stosunkowo dobrze poznane. Uszkodzenie śródbłonka powoduje odsłonięcie białek adhezyjnych, które indukują aktywację i adhezję leukocytów oraz płytek krwi do ściany naczynia. Pobudzone płytki krwi wydzielają aktywne biologicznie cząsteczki, które poprzez stymulację interakcji międzykomórkowych, uczestniczą w propagacji stanu zapalnego. Nasilające się wewnątrznaczyniowe procesy patologiczne stymulują rekrutację i infiltrację monocytów oraz przenikanie lipidów z krwiobiegu do przestrzeni podśródbłonkowej, jak również indukują proces transformacji monocytów w makrofagi. Nadmierna akumulacja cholesterolu sprzyja jego utlenieniu, a powstająca silnie toksyczna pochodna (oksycholesterol) ulega fagocytozie przez zrekrutowane makrofagi. Wzmożona aktywacja układu odpornościowego oraz układu hemostazy przyczynia się do nadmiernej sekrecji komórkowych czynników pro-zapalnych oraz aktywacji płytek krwi, wywołując samonapędzającą się kaskadę procesów związanych ze wzmocnieniem stanu zapalnego oraz indukowaniem potencjału pro-zakrzepowego krwi. Makrofagi przepełnione zabsorbowanymi lipidami przekształcają się w komórki piankowate, które tracą zdolność do fagocytozy oksycholesterolu, co stymuluje aktywację szlaków apoptotycznych, sekrecję czynników pro-zapalnych oraz związków

wpływających na destabilizację blaszki miażdżycowej. Na powierzchni uszkodzonej blaszki aktywowana jest kaskada krzepnięcia, a powstający skrzep ogranicza przepływ krwi w świetle naczynia krwionośnego. Destabilizacja integralności blaszki miażdżycowej powoduje natomiast uwolnienie martwiczego rdzenia lipidowego mogącego stanowić przyczynę okluzji naczynia. Blokowanie przepływu krwi w tętnicach wieńcowych prowadzi do powstawania powikłań zakrzepowo-zatorowych [3].

Pomimo wieloczynnikowej etiologii OZW, fundamentalną rolę w ich patofizjologii można przypisać płytkom krwi, ze względu na ich zdolność do tworzenia czopu płytkowego, inicjacji mechanizmów pro-zakrzepowych i propagacji miażdżycy naczyń [4]. Płytki krwi charakteryzują się unikatową budową morfologiczną determinującą ich szczególną reaktywność. W błonie komórkowej płytek zakotwiczone są receptory powierzchniowe odpowiedzialne za indukcję przekaźnictwa sygnałowego „outside-in", istotnego dla stanu czynnościowego komórki. Aktywacja płytek wpływa na hemostazę dwutorowo. Zewnątrzkomórkowo, stymuluje osoczową kaskadę krzepnięcia poprzez m.in. ekspozycję aminofosfolipidów na powierzchni błony komórkowej. Wewnątrzkomórkowo, prowadzi do reorganizacji cytoszkieletu i zmiany kształtu płytek, ekspozycji oraz zmiany konformacji receptorów powierzchniowych, degranulacji ziarnistości i sekrecji związków czynnych oraz indukcji agregacji [5]. Występujące w dużej liczbie kopii receptory powierzchniowe różnego typu stanowią kluczowy element warunkujący prawidłowe funkcjonowanie płytek krwi. Znaczenie płytkowych receptorów powierzchniowych zostało przede wszystkim wykazane w badaniach związanych z identyfikacją podłoża patofizjologii skaz krwotocznych, w których wykryte anomalie były m.in. wynikiem nieprawidłowości strukturalnych receptorów płytek krwi [6–8]. Co więcej, badania wskazują również, że występowanie zmian w sekwencji genów dla receptorów glikoproteinowych (GP) (m.in. GP Ib, GP IIIa) obecnych na powierzchni płytek krwi może być skorelowane z ryzykiem wystąpienia zawału mięśnia sercowego [9–12]. Badania te były przeprowadzone jednak na niejednorodnych grupach pacjentów i tylko na wcześniej zidentyfikowanych wariantach polimorficznych. Analiza zmian w sekwencji genów dla płytkowego receptora aktywowanego przez proteazę-1 (protease-activated receptor-1, PAR-1) [13] oraz receptorów purinergicznych P2Y1 [14] czy P2Y12 [15] również wykazała różnice w poziomie aktywacji płytek krwi przez specyficznych agonistów w zależności od genetycznego wariantu receptora.

Obecność receptorów powierzchniowych znajdujących się na płytkach krwi determinuje ich udział w procesach hemostazy, w tym fundamentalną rolę w tworzeniu się czopu płytkowego. Wśród głównych receptorów uczestniczących w procesie aktywacji płytek należy wymienić kompleks GP Ib-IX-V, integrynę αIIbβ3 oraz receptory metabotropowe PAR-1 i P2Y12 [16].

GP Ib-IX-V jest receptorem składającym się z czterech podjednostek: GPIbα, GPIbβ, GPIX oraz GPV. Występuje on wyłącznie w płytkach krwi oraz megakariocytach i jest odpowiedzialny za adhezję płytek do białek macierzy podśródbłonkowej, co stanowi pierwszy

etap odpowiedzi płytek krwi na uszkodzenie śródbłonka naczyń [17,18]. Każda z podjednostek receptora jest białkiem przezbłonowym, natomiast ich domeny zewnątrzkomórkowe charakteryzują się występowaniem licznych powtórzeń leucyny [18]. W przypadku receptora GP Ib-IX-V, wykryte zaburzenia strukturalne były związane z nieprawidłową ekspresją płytkowego receptora dla czynnika von Willebranda (vWF), czyli podjednostki GPIb i pozwoliły na zidentyfikowanie syndromu Bernarda-Souliera będącego genetycznie uwarunkowanym defektem czynnościowym płytek krwi związanym z ich nieprawidłową aktywacją na szlaku sygnalizacyjnym indukowanym przez vWF [6]. W związku z występowaniem zaburzeń genetycznych zasocjowanych z funkcjonalnością receptora GP Ib-IX-V, podjęto próby identyfikacji polimorfizmów pojedynczego nukleotydu (single nucleotide polymorphism, SNP) w genach kodujących podjednostki GP Ib, IX i V w celu określenia ich potencjalnej roli w występowaniu ryzyka chorób sercowo-naczyniowych. Polimorfizm T/C w sekwencji Kozaka genu kodującego GPIbα u pacjentów z udarem niedokrwiennym mózgu wykazał, iż obecność chociaż jednej kopii allelu C zwiększała ryzyko wystąpienia tego epizodu niedokrwiennego. Co więcej, nosicielami allelu C byli najczęściej pacjenci, u których nie określono etiologii choroby [19]. Występowanie związku pomiędzy obecnością wariantów polimorficznych w sekwencji płytkowej GPIbα a zwiększonym ryzykiem zachorowalności na zawał mięśnia sercowego został również wykazany w pracy Ozeloa i wsp. [9].

Integryna αIIbβ3 inaczej określana jako GP IIb/IIIa, jest najliczniej występującym powierzchniowym receptorem płytek krwi (nawet do ~80 000 kopii receptora na pojedynczą komórkę) [20]. W niepobudzonych płytkach receptor wykazuje minimalną zdolność do wiązania się z vWF oraz osoczowym fibrynogenem. Jednakże, transdukcja sygnału komórkowego prowadzącego do pobudzenia płytek krwi powoduje zmiany konformacyjne w strukturze GP IIb/IIIa, które wpływają na zwiększenie powinowactwa receptora do cząsteczki ligandu (sygnalizacja „inside-out"), przez co stymulują płytki krwi do tworzenia agregatów [7]. W praktyce klinicznej, GP IIb/IIIa jest uznanym celem w leczeniu przeciwpłytkowym. W zależności od stanu klinicznego pacjenta, występowania chorób współistniejących oraz zdiagnozowanego typu OZW, do leczenia mogą być włączone takie inhibitory GP IIb/IIIa jak: abcyksymab, eptifibatyd oraz tirofiban [21,22]. Integryna αIIbβ3 pełni kluczową rolę w procesie tworzenia się czopu płytkowego. Zjawisko to potwierdza występowanie dobrze scharakteryzowanego zaburzenia genetycznego, określanego jako trombastenia Glanzmanna, które będąc wrodzoną, autosomalnie recesywną anomalią związaną z występowaniem mutacji w genach kodujących podjednostki αIIb oraz β3, prowadzi do braku agregacji płytek krwi w odpowiedzi na ich stymulację [7,8]. Ponadto, badania molekularne prowadzone na genach kodujących każdą z podjednostek GP IIb/IIIa wykazały, iż jest to receptor polimorficzny, w którym zidentyfikowano występowanie dwóch głównych wariantów genetycznych (PlA1 oraz PlA2) modulujących ryzyko wystąpienia epizodów niedokrwiennych. W badaniach Weissa i wsp. stwierdzono, iż obecność allelu PlA2 jest zasocjowana

z zakrzepicą naczyń wieńcowych [10]. Co więcej, w pracy Ardissino i wsp. zwiększone ryzyko zawału mięśnia sercowego prezentowali pacjenci będący heterozygotami PlA1/PlA2 [11]. Podobne wyniki zostały uzyskane przez Bojesena i wsp. [12]. Występowanie korelacji pomiędzy obecnością wariantów polimorficznych a zwiększonym ryzykiem rozwoju zawału mięśnia sercowego podkreśla istotny udział aberracji molekularnych w patofizjologii OZW.

Receptor PAR-1 należy do rodziny przezbłonowych receptorów sprzężonych z białkami G (G protein-coupled receptor, GPCR). Ich aktywność biologiczna jest uwarunkowana proteolizą N-końca, która pozwala na ekspozycję sekwencji aminokwasowej umożliwiającej przyłączenie się ligandu i przekazanie sygnału za pośrednictwem białka G do wnętrza komórki. Najlepiej scharakteryzowanym aktywatorem receptorów PAR jest trombina, która odgrywa również kluczową rolę w formowaniu się skrzepu [23]. W badaniach molekularnych dotyczących analizy wpływu SNP na receptor PAR-1 wykazano, iż obecność allelu T dla intronowego SNP 14A/T była powiązana z obniżoną ekspresją powierzchniową receptora i zmniejszoną odpowiedzią płytek względem zastosowanego agonisty [13]. Pacjenci z STEMI będący nosicielami chociaż jednego allelu T mieli również znacząco zmniejszone ryzyko wystąpienia epizodów niedokrwiennych [24].

Przezbłonowym receptorem GPCR obecnym na powierzchni płytek krwi jest również receptor P2Y12, którego głównym agonistą jest adenozyno-5'-difosforan (ADP). Jego rola w procesie hemostazy polega na amplifikacji odpowiedzi płytek na czynniki aktywujące, poprzez wzmocnienie i stabilizację tworzących się agregatów oraz zwiększenie aktywności pro-zakrzepowej płytek krwi [25]. Receptor P2Y12 jest głównym celem leczenia farmakologicznego. Zgodnie z wytycznymi Europejskiego Towarzystwa Kardiologicznego (dotyczącymi postępowania u pacjentów z NSTEMI z roku 2020 oraz pacjentów z STEMI z 2017 roku), podanie inhibitora P2Y12 wraz z aspiryną stanowi złoty standard w leczeniu pacjentów z OZW, co wskazuje na istotną rolę tego receptora dla funkcjonalności płytek krwi [1,22]. W przypadku badań genetycznych skupiających się na określeniu wpływu zidentyfikowanych SNP na aktywność receptora P2Y12 i zdolności do modulacji ryzyka wystąpienia powikłań zakrzepowo-zatorowych, występuje rozbieżność w uzyskanych wynikach. W badaniach Zoheira i wsp. allel C polimorfizmu T744C występował istotnie częściej u pacjentów z przebytym OZW. Co więcej, nosiciele allelu C wykazywali zwiększoną czynność płytek krwi w odpowiedzi na stymulację ADP [15]. Z drugiej strony, w badaniach Angiolillo i wsp. SNP T744C nie wpływał istotnie na funkcjonowanie i proces aktywacji płytek krwi [26]. Sprzeczne wyniki uzyskano również w badaniach Cavallari i wsp. oraz Schetterta i wsp., w których analizie poddane zostały dwa zidentyfikowane haplotypy (H1 oraz H2) [27,28]. Występowanie rozbieżności w wynikach analiz polimorficznych receptora P2Y12 wskazuje na potrzebę poszukiwania molekularnych predyktorów określających ryzyko wystąpienia OZW w innych – genetycznych, proteomicznych, czy metabolomicznych obszarach badań nad płytkami krwi.

Oprócz oceny objawów podmiotowych, takich jak utrzymujący się ból w klatce piersiowej, podstawowym narzędziem diagnostycznym w ocenie rozpoznania klinicznego OZW jest 12-odprowadzeniowy elektrokardiogram pozwalający na wykrycie m.in. charakterystycznych zaburzeń obejmujących odcinek ST i załamek T oraz biomarkery pozwalające na właściwe rozpoznanie choroby oraz na kwalifikację konkretnego typu OZW wśród pacjentów. Złotym standardem w diagnostyce OZW są oznaczenia stężenia troponin sercowych T lub I (hs-cTn T oraz hs-cTn I), które wskazują na uszkodzenie kardiomiocytów. Zastosowanie oceny dodatkowych biomarkerów takich jak stężenie: D-dimerów, glukozy, białka C reaktywnego (c-reactive protein, CRP), peptydu natriuretycznego typu B (B-type natriuretic peptide, BNP), kopeptyny; wielkość przesączania kłębuszkowego (estimated glomerular filtration rate, eGFR); czy aktywność izoformy sercowej kinazy kreatynowej (creatine kinase myocardial band, CK-MB), pozwala na dostarczenie szerszego panelu informacji, w tym danych rokowniczych pomocnych w ocenie ryzyka wystąpienia punktów końcowych, takich jak zgon [1]. Aktualnie, wciąż jednak prowadzone są badania mające na celu wykrycie nowych, wysokoczułych biomarkerów, które pozwoliłyby na uwiarygodnienie i poszerzenie dotychczasowej diagnostyki pacjentów.

W 2021 roku, Yang i wsp. przeprowadzili meta-analizę, której celem było określenie związku pomiędzy stężeniem interleukiny 6 (IL-6) a ciężkością przebiegu OZW oraz częstością występowania poważnych, niepożądanych zdarzeń sercowo-naczyniowych. Uzyskane wyniki wykazały, iż osoczowe stężenie IL-6 jest istotnie podwyższone wśród pacjentów z ciężkim przebiegiem OZW w porównaniu do pacjentów z lekkim przebiegiem choroby [29]. Stężenie osoczowej IL-6 zostało również oznaczone wśród prawie 5000 pacjentów uczestniczących w badaniu klinicznym SOLID-TIMI 52 (Stabilization of Plaque Using Darapladib-Thrombolysis in Myocardial Infarction 52), którzy zostali poddani odległej obserwacji przez okres 2,5 roku. Uzyskane wyniki wskazywały, iż pacjenci, u których zdiagnozowano wystąpienie niepożądanych zdarzeń sercowo-naczyniowych charakteryzowali się znacząco podwyższonym stężeniem IL-6 w osoczu. Zastosowanie modelowania statystycznego pozwalającego na dopasowanie parametrów socjodemograficznych oraz klinicznych pacjentów do poziomu IL-6, umożliwiło oszacowanie wartości odzwierciedlających zwiększone ryzyko występowania niepożądanych punktów końcowych. Pacjenci z najwyższym stężeniem IL-6 posiadali o 57% większe ryzyko wystąpienia poważnych powikłań sercowo-naczyniowych oraz 2-krotnie zwiększone ryzyko śmierci z powodu chorób układu krążenia i niewydolności serca [30].

Białko wiążące kwasy tłuszczowe typu sercowego (heart fatty acid-binding protein, H-FABP) jest jednym z aktualnie intensywnie badanych potencjalnych biomarkerów uszkodzenia mięśnia sercowego. H-FABP jest małym białkiem należącym do rodziny białek wiążących kwasy tłuszczowe. Występuje w formie rozpuszczalnej w cytoplazmie kardiomiocytów, które w wyniku uszkodzenia natychmiastowo uwalniają H-FABP do krwiobiegu. We wczesnej diagnostyce pacjentów stężenie H-FABP może być nawet

100-krotnie wyższe od stężenia troponin sercowych [31]. W badaniu Ruzgara i wsp. wśród pacjentów ze zdiagnozowanym NSTEMI, H-FABP wykazywał większą czułość niż hs-cTn T w pierwszych 6 godzinach od wystąpienia objawów. Niestety, wraz z upływem czasu, poziom H-FABP ulegał znaczącemu obniżeniu, co istotnie wpływało na spadek jego czułości w porównaniu do hs-cTn T [32]. Podobne wyniki zostały zaprezentowane przez Cavusa i wsp., według których H-FABP wykazywał największą czułość w kilku pierwszych godzinach od wystąpienia OZW, w zestawieniu z CK-MB, mioglobiną oraz hs-cTn T [33].

Według danych literaturowych, wciąż podejmowane są liczne badania mające na celu weryfikację wielu innych cząsteczek o potencjale biomarkera OZW, wśród których wskazywane są cząsteczki charakterystyczne dla uszkodzenia kardiomiocytów, mediatory pro-zapalne, jak również niekodujące fragmenty RNA [34,35].

Biomarkery OZW testowane w laboratoriach badawczych oraz aktualnie stosowane w praktyce klinicznej skupiają się na diagnostyce pacjentów, u których wystąpił i trwa epizod niedokrwienny, umożliwiając tym samym monitorowanie dynamiki niedokrwienia mięśnia sercowego oraz określenie potencjalnych powikłań. Zgodnie z aktualnym stanem wiedzy, nie istnieją specyficzne i czułe biomarkery, które pozwoliłyby na określenie predyspozycji pacjentów do rozwinięcia OZW, przed wystąpieniem objawów niedokrwienia. Opracowanie biomarkerów prognostycznych umożliwiłoby wdrożenie odpowiedniego leczenia profilaktycznego, zmniejszając tym samym ryzyko wystąpienia OZW.

W ostatnich dwóch dekadach, obiektem intensywnych prac badawczych, skupiających się na wyjaśnieniu molekularnego podłoża patofizjologii różnych chorób, stały się mikroRNA (miRNA). MiRNA są to krótkie, niekodujące cząsteczki RNA, które pełnią funkcję potranskrypcyjnych regulatorów ekspresji genów [36]. W organizmie człowieka wykryto 2654 dojrzałe sekwencje miRNA oraz 1917 prekursorów o charakterystycznej strukturze „spinki do włosów" [37], które poprzez parowanie się z sekwencjami mRNA regulują ekspresję ponad 60% genów [36]. Cechą charakterystyczną miRNA jest ich wyjątkowa odporność na szkodliwe warunki takie jak działanie endogennych RNaz, zmiany pH, długie przechowywanie, wielokrotne cykle rozmrażania i zamrażania, a nawet gotowanie. Dzięki wysokiej stabilności w krążeniu, odporności na czynniki szkodliwe oraz możliwości zastosowania nieinwazyjnych metod poboru próbek, miRNA stanowią idealny obszar do poszukiwania potencjalnych biomarkerów [38].

W przypadku chorób układu krążenia wykazano, iż ekspresja osoczowego miR-126, miR-17, miR-92a, miR-155 i miR-145 była istotnie obniżona u pacjentów z chorobą niedokrwienną serca, w odniesieniu do prawidłowej kontroli [39]. Zredukowany poziom osoczowego miR-155 został również potwierdzony w pracach Diehla i wsp. [40] oraz Webera i wsp. [41]. W badaniach przeprowadzonych przez Corstena i wsp. ekspresja miR-208b i miR-499 była istotnie podwyższona u pacjentów ze zdiagnozowanym zawałem mięśnia sercowego, w porównaniu do grupy kontrolnej bez chorób układu krążenia [42]. Istotny statystycznie wzrost ekspresji miR-1, miR-133a, miR-133b i miR-499-5p został

również wykryty w osoczu pacjentów ze STEMI, w porównaniu do zdrowych ochotników [43]. Podwyższony poziom miR-1, miR-133a, miR-208b oraz miR-499-5p w osoczu pacjentów ze STEMI potwierdzono w pracy Gidlöfa i wsp., co wskazuje na wysoką specyficzność zmiennej ekspresji wybranych cząsteczek osoczowego miRNA wśród pacjentów ze zdiagnozowanym OZW [44]. Analiza zmian w profilu ekspresji dla wybranych miRNA została również przeprowadzona w płytkach krwi. W badaniu Stratza i wsp. wykazano, iż płytkowy profil miRNA mierzony przez 10 dni co 24 godziny u zdrowych mężczyzn, wykazywał wysoce stabilną ekspresję przez cały czas trwania badania, sugerując tym samym, iż płytki krwi mogą stanowić idealny obszar poszukiwań biomarkerów opartych na miRNA [45]. Doniesienia literaturowe potwierdzają również występowanie zmian w ekspresji niektórych płytkowych miRNA skorelowanych z reaktywnością płytek krwi stymulowanych epinefryną [46]. Jednakże, brak jest danych dotyczących analizy porównawczej pełnego profilowania płytkowego miRNA u pacjentów z OZW w odniesieniu do zdrowych ochotników.

Niewystarczające dane literaturowe odnoszące się do identyfikacji oraz wyjaśnienia molekularnego mechanizmu zaburzeń funkcjonowania płytek krwi w OZW wskazują na nie w pełni poznaną rolę tych elementów morfotycznych w patofizjologii chorób krążenia.

Charakterystyczna budowa płytek, na którą składają się liczne receptory powierzchniowe, brak jądra komórkowego, dynamicznie zmieniający się cytoszkielet, duża liczba ziarnistości oraz mitochondriów warunkujących aktywny metabolizm, pochodzący z megakariocytów transkryptom wraz z szerokim zasobem cząsteczek miRNA oraz przekazany częściowo proteom potwierdzają, iż fizjologia płytek krwi jest znacznie bardziej skomplikowana niż początkowo sądzono, co podkreśla ich aktywny udział w wielu mechanizmach patofizjologii schorzeń sercowo-naczyniowych. Scharakteryzowanie na poziomie molekularnym czynnościowych zaburzeń płytek krwi mogłoby pomóc w lepszym poznaniu podłoża patologicznej aktywacji tych komórek zaangażowanych w powstawanie powikłań zakrzepowo-zatorowych.

W niniejszej pracy doktorskiej przeprowadzona została analiza przesiewowa płytkowego profilu miRNA wraz z analizą bioinformatyczną oraz modelowaniem statystycznym mającym na celu identyfikację oraz ocenę zmian w ekspresji miRNA, które mogłyby posłużyć jako potencjalne prognostyczne biomarkery OZW. Analizie porównawczej poddano również płytkowy proteom oraz ekspresję wybranych transkryptów, co za pomocą fluorescencyjnej elektroforezy dwukierunkowej (two-dimensional fluorescence difference gel electrophoresis, 2D-DIGE), nano-chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (nanoscale liquid chromatography coupled to tandem mass spectrometry, nanoLC-MS/MS) oraz ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym (quantitative polymerase chain reaction, qPCR), pozwoliło na wykrycie i identyfikację białek oraz mRNA wykazujących zaburzoną ekspresję u pacjentów z OZW, względem grupy kontrolnej. W celu rozszerzenia aktualnego stanu wiedzy dotyczącego nadreaktywności płytek krwi w OZW, szczególną uwagę zwrócono na zaburzenia

ekspresji wybranych powierzchniowych receptorów płytkowych na poziomie mRNA oraz białka. Wykonano również modelowanie statystyczne mające na celu wykorzystanie wykrytych zmian w ocenie ich roli jako potencjalnego prognostycznego biomarkera OZW.

8. CEL PRACY

W niniejszej rozprawie doktorskiej wyróżnione zostały dwa główne cele:

1) Określenie zaburzeń w transkryptomie i proteomie płytek krwi, które mogłyby stanowić przyczynę ich wzmożonej aktywności pro-zakrzepowej w OZW.
2) Identyfikacja cząsteczek o potencjale wysokoczułych molekularnych markerów płytkowych, określających predyspozycje do wystąpienia OZW.

9. MATERIAŁ I METODY

- POZYSKANIE MATERIAŁU DO BADAŃ

Materiał badawczy stanowiła krew pełna uzyskana od pacjentów z koronarograficznie potwierdzonym OZW oraz od zdrowych dawców, u których nie stwierdzono występowania zaburzeń ze strony układu sercowo-naczyniowego.

Krew od pacjentów była pobierana z żyły łokciowej, do probówko-strzykawek z zastosowaniem systemu S-Monovette (Sarstedt, Nümbrecht, Niemcy) z antykoagulantem CPDA-1 (citrate phosphate dextrose adenine), możliwie szybko, po wykonaniu niezbędnych czynności oraz podaniu leków mających na celu zabezpieczenie pacjenta po wystąpieniu incydentu OZW. Do badania włączeni zostali pacjenci hospitalizowani w Klinice Kardiologii Interwencyjnej Katedry Kardiologii Interwencyjnej i Elektrokardiologii Centralnego Szpitala Klinicznego Uniwersytetu Medycznego w Łodzi. Charakterystyka kliniczna pacjentów została szczegółowo przedstawiona we wszystkich publikacjach eksperymentalnych wchodzących w skład niniejszej rozprawy doktorskiej. Pacjenci zakwalifikowani do udziału w badaniach nie przekraczali 65 roku życia, nie zdiagnozowano u nich: choroby nowotworowej, cukrzycy, zaburzeń tkanki łącznej oraz niedoczynności tarczycy; wykazywali prawidłową czynność nerek, nie byli uzależnieni od narkotyków i alkoholu, a ich wskaźnik BMI określający stosunek masy ciała do wzrostu podniesionego do kwadratu nie przekraczał 35. Zastosowanie wyżej wymienionych ograniczeń w rekrutacji grupy badanej miało na celu wyselekcjonowanie pacjentów, u których przebyte OZW mogły potencjalnie mieć podłoże genetyczne, co stanowiło przedmiot niniejszej pracy, w zakresie poznania molekularnych mechanizmów odpowiedzialnych za wzmożoną aktywację płytek krwi w OZW oraz opracowania wysokoczułych biomarkerów molekularnych wskazujących na predyspozycje do wystąpienia OZW. Ponadto, krew obwodową pobierano do analiz możliwie szybko po ustabilizowaniu stanu pacjenta od wystąpienia incydentu niedokrwienia, w celu ograniczenia wpływu

zastosowanej terapii przeciwpłytkowej/przeciwzakrzepowej/przeciwzapalnej na oznaczane parametry.

Grupę kontrolną stanowili dawcy, u których nie zdiagnozowano występowania chorób układu krążenia. Grupa kontrolna była homologiczna względem grupy badanej co do liczebności, płci oraz wieku. Przed włączeniem do badania u dawców oznaczono parametry pozwalające określić ogólny stan zdrowia, w tym: morfologię, stężenie glukozy, kreatyniny, CRP, hormonu tyreotropowego (thyroid-stimulating hormone, TSH), cholesterolu całkowitego, trójglicerydów, frakcji HDL (high-density lipoprotein) i LDL (low-density lipoprotein), aktywność aminotrasferazy asparaginianowej (aspartate aminotransferase, AST) i aminotransferazy alaninowej (alanine aminotransferase, ALT), całkowity poziom immunoglobulin IgG i IgM oraz parametry układu krzepnięcia. Dawcy zakwalifikowani do badań nie przyjmowali żadnych leków oraz nie wykazywali objawów infekcji przez co najmniej 2 tygodnie przed pobraniem krwi. Krew obwodowa była pobierana na czczo, z żyły łokciowej, do probówek BD Vacutainer® (Becton Dickinson, New Jersey, Stany Zjednoczone) zawierających antykoagulant ACD-A (acid citrate dextrose – solution A) w Centrum Diagnostyki Laboratoryjnej w Łodzi.

Zakwalifikowani pacjenci oraz ochotnicy złożyli pisemną zgodę na uczestnictwo w badaniach. Wszystkie badania wykonano zgodnie z Deklaracją Helsińską oraz zostały one zatwierdzone przez Komisję ds. Etyki Badań Naukowych Uniwersytetu Łódzkiego (23/KBNN-UŁ/I/2017).

- METODY

- **Izolacja płytek krwi metodą wirowania różnicowego z zastosowaniem separacji magnetycznej**

Krew pełna została poddana wirowaniu różnicowemu, a uzyskane w procesie wirowania osocze bogatopłytkowe poddano następnie inkubacji z kuleczkami magnetycznymi w celu usunięcia zanieczyszczeń erytrocytarno-leukocytarnych na kolumnie separacyjnej (Miltenyi Biotech, Bergisch Gladbach, Niemcy). Wybór metody izolacji płytek krwi podyktowany był potrzebą osiągnięcia jak najlepszej wydajności, co było szczególnie istotne dla dalszych analiz genetycznych, w których konieczne było uzyskanie odpowiednio wysokiego stężenia kwasów nukleinowych pochodzenia płytkowego (*Publikacja eksperymentalna nr 1-3*).

- **Izolacja płytek krwi metodą filtracji żelowej z zastosowaniem złoża Sepharose 2B-BSA**

Filtracja żelowa na złożu Sepharose 2B-BSA, jako jedna z najmniej inwazyjnych metod preparatywnych, została użyta w celu wyizolowania płytek krwi do analizy powierzchniowej dystrybucji receptora P2Y12 metodą mikroskopii konfokalnej. Wybór metody miał na celu

zminimalizowanie na etapie preparatyki ryzyka aktywacji płytek poddawanych następnie obrazowaniu mikroskopowemu, kosztem wydajności metody (*Publikacja eksperymentalna nr 2*).

- **Izolacja całkowitego RNA z płytek krwi**

Całkowite RNA wyizolowano z płytek krwi za pomocą komercyjnie dostępnego zestawu ISOLATE II RNA Mini Kit (Bioline, Londyn, Wielka Brytania) wykorzystującego tiocyjanian guanidyny (zapewniający lizę komórek) oraz membranę krzemionkową wiążącą izolowane RNA (*Publikacja eksperymentalna nr 1-3*).

- **Synteza cDNA z zastosowaniem matrycy mRNA**

Synteza komplementarnej nici DNA (complementary DNA, cDNA) na matrycy wyizolowanego mRNA została przeprowadzona z zastosowaniem komercyjnie dostępnego zestawu Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone) (*Publikacja eksperymentalna nr 2 oraz 3*).

- **Synteza mikro-cDNA z zastosowaniem matrycy miRNA**

W celu otrzymania mikro-cDNA na matrycy miRNA zastosowano komercyjnie dostępny zestaw TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone) (*Publikacja eksperymentalna nr 1*).

- **Analiza porównawcza profilu miRNA w płytkach krwi metodą mikromacierzy**

Przesiewowa analiza profilu miRNA w płytkach krwi została wykonana na mikromacierzy SurePrint G3 Human miRNA (Agilent, Santa Clara, Kalifornia, Stany Zjednoczone). Całkowite RNA zostało spulowane w obrębie grupy OZW oraz grupy kontrolnej w celu analizy porównawczej ekspresji cząsteczek miRNA (*Publikacja eksperymentalna nr 1*).

- **Pomiar ekspresji płytkowego miRNA oraz mRNA metodą qPCR**

Pomiar ekspresji wybranych cząsteczek miRNA lub transkryptów mRNA pochodzących z płytek krwi został wykonany w próbkach uzyskanych od każdego pacjenta oraz dawcy za pomocą metody qPCR w celu:

  - Walidacji cząsteczek miRNA wykazujących zmienną ekspresję pomiędzy badanymi grupami w analizie mikromacierzy. W przeprowadzonej analizie qPCR wykorzystano sondy TaqMan™ Advanced miRNA Assay (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone) (*Publikacja eksperymentalna nr 1*).
  - Porównania ekspresji mRNA dla genów kodujących wybrane receptory powierzchniowe płytek krwi. W przeprowadzonej analizie qPCR wykorzystano sondy TaqMan™ Gene Expression Assay (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone) (*Publikacja eksperymentalna nr 2*).

- Analizy zaburzeń w ekspresji mRNA dla genów kodujących białka płytek krwi, których stężenie w OZW i grupie kontrolnej wykazywało w analizie 2D-DIGE różnicę na poziomie krotności zmiany wynoszącej >1,5. W przeprowadzonej analizie qPCR wykorzystano sondy TaqMan™ Gene Expression Assay (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone) (*Publikacja eksperymentalna nr 3*).

- **Izolacja frakcji białkowej z płytek krwi**

Osad płytek krwi otrzymany metodą wirowania różnicowego zawieszono w buforze lizującym. W celu oznaczenia stężenia białka w przygotowanych próbkach, wykorzystano komercyjnie dostępny zestaw RCDC™ Protein Assay (BioRad Laboratories, Hercules, Kalifornia, Stany Zjednoczone) (*Publikacja eksperymentalna nr 2 oraz 3*).

- **Pomiar stężenia białka dla wybranych receptorów powierzchniowych płytek krwi metodą ELISA**

Oznaczenie stężenia białka zostało wykonane dla wybranych receptorów powierzchniowych płytek krwi wykazujących zmiany ekspresji na poziomie mRNA pomiędzy grupą OZW a grupą kontrolną. W tym celu wykorzystano komercyjnie dostępne zestawy: Human P2RY12 ELISA Kit, Human ITGB3 ELISA Kit oraz Human CD42 ELISA Kit (FineTest, Wuhan, Chiny) (*Publikacja eksperymentalna nr 2*).

- **Oznaczenie powierzchniowej ekspresji płytkowego receptora P2Y12 za pomocą metody cytometrii przepływowej**

Pomiar powierzchniowej ekspresji płytkowego receptora P2Y12 został wykonany w krwi pełnej za pomocą metody cytometrii przepływowej w układzie znakowania dwubarwnikowego, stosując w celu identyfikacji płytek krwi przeciwciała Anti-Human CD61 (Beckton Dickinson, Franklin Lakes, New Jersey, Stany Zjednoczone) sprzężone z barwnikiem fluorescencyjnym BD Horizon Brilliant™ Blue 515 (BB515) oraz przeciwciała skierowane przeciwko receptorowi P2Y12 Anti-Human P2Y12 (BioLegend, San Diego, Kalifornia, Stany Zjednoczone) sprzężone z fluorochromem fikoerytryną (PE). Powierzchniową ekspresję receptora P2Y12 określono na podstawie intensywności świecenia obiektów P2Y12-pozytywnych w populacji komórek CD61-pozytywnych (*Publikacja eksperymentalna nr 2*).

- **Wizualizacja dystrybucji receptora P2Y12 metodą mikroskopii konfokalnej**

Wizualizację receptora P2Y12 w spoczynkowych płytkach krwi, wyizolowanych za pomocą złoża Sepharose 2B-BSA, wykonano za pomocą mikroskopii konfokalnej z zastosowaniem przeciwciał PE Anti-Human P2Y12 (BioLegend, San Diego, Kalifornia, Stany Zjednoczone) oraz przeciwciał Anti-Human CD61 wyznakowanych kompleksem białek perydyna-chlorofil sprzężonych z cyjaniną 5.5 (PerCP-Cy5.5) (Beckton Dickinson, Franklin

Lakes, New Jersey, Stany Zjednoczone). Płytki krwi utrwalono w 1% roztworze CellFix (Beckton Dickinson, Franklin Lakes, New Jersey, Stany Zjednoczone) oraz umieszczono na szkiełku podstawowym wraz z żelem CyGEL™ (Biostatus, Leicestershire, Wielka Brytania) umożliwiającym ich immobilizację przed obrazowaniem (*Publikacja eksperymentalna nr 2*).

- **Analiza porównawcza proteomu płytek krwi metodą 2D-DIGE**

Rozdział proteomu płytek krwi metodą 2D-DIGE został wykonany na zbiorczych próbkach spulowanych w obrębie grupy badanej oraz grupy kontrolnej. Aby zapewnić wysoką jakość rozdziału elektroforetycznego, frakcję białkową poddano selektywnej precypitacji w celu usunięcia zanieczyszczeń takich jak detergenty jonowe oraz związki fenolowe, przy użyciu komercyjnego zestawu ReadyPrep™ 2-D Cleanup Kit (BioRad Laboratories, Hercules, Kalifornia, Stany Zjednoczone). Oczyszczone próbki wyznakowano fluorochromami zawartymi w zestawie DyeAgnostics Refraction-2D QPLEX Kit (NH DyeAGNOSTICS, Halle, Niemcy). Próbkę OZW wyznakowano barwnikiem G-Dye300, natomiast próbkę kontrolną barwnikiem G-Dye200. W pierwszym etapie analizy 2D-DIGE wykonano ogniskowanie izoelektryczne na 11 cm pasku IPG o szerokim zakresie pH 3-10 (BioRad Laboratories, Hercules, Kalifornia, Stany Zjednoczone). Następnie, po zastosowaniu buforów Equillibration Buffer I i II (BioRad Laboratories, Hercules, Kalifornia, Stany Zjednoczone) przeprowadzono dalszy rozdział elektroforetyczny białek pod względem ich mas cząsteczkowych z wykorzystaniem komercyjnie dostępnego żelu Criterion™ TGX Precast Gel 4-20% (BioRad Laboratories, Hercules, Kalifornia, Stany Zjednoczone). Wizualizację spotów białkowych oraz pomiar intensywności świecenia zastosowanych fluorochromów w uzyskanych elektroforegramach wykonano przy użyciu skanera Typhoon FLA 950 GE (GE Healthcare, Chicago, IL, Stany Zjednoczone) oraz oprogramowania ImageMaster 2D Platinum 7.0 (GE Healthcare, Chicago, IL, USA) w Łódzkim Parku Naukowo-Technologicznym Bionanopark. Do dalszej analizy wyselekcjonowano spoty białkowe, których wartość krotności zmiany wynosiła >1,5 (*Publikacja eksperymentalna nr 3*).

- **Identyfikacja białek płytkowych metodą nanoLC-MS/MS**

W celu identyfikacji różnicowych plamek białkowych w OZW oraz grupie kontrolnej, wykonano analizę jakościową z zastosowaniem metody nanoLC-MS/MS. Wybrane spoty zostały wycięte z żelu, a znajdujące się w nich białka poddano trawieniu w trypsynie. Identyfikacja białek została wykonana za pomocą spektrometrii mas typu ESI-MS/MS poprzedzonej rozdziałem chromatograficznym przy udziale techniki chromatografii cieczowej nanoLC-MS dedykowanej do rozdziału śladowych ilości analitów, głównie w analizach proteomicznych. Analiza nanoLC-MS/MS została wykonana w Łódzkim Parku Naukowo-Technologicznym Bionanopark (*Publikacja eksperymentalna nr 3*).

- **Oznaczenie stężenia fibrynogenu w osoczu metodą ELISA**

Stężenie fibrynogenu w osoczu pacjentów z OZW oraz dawców z grupy kontrolnej zostało oznaczone za pomocą komercyjnie dostępnego zestawu Human FG(Fibrinogen) ELISA Kit (Elabscience, Houston, Teksas, Stany Zjednoczone) (*Publikacja eksperymentalna nr 3*).

- **Rozdział elektroforetyczny białek płytek krwi wraz z immunodetekcją transgeliny-2 metodą Western Blot**

Elektroforezę proteomu płytek krwi przeprowadzono w warunkach denaturujących z zastosowaniem 3% poliakrylamidowego żelu zagęszczającego oraz 12% poliakrylamidowego żelu rozdzielającego. Rozdzielone elektroforetycznie białka zostały następnie poddane transferowi na membranę Immobilon®-P PVDF (Merck, Darmstadt, Niemcy), a proces migracji białek z żelu na membranę został zweryfikowany za pomocą barwienia odczynnikiem Ponceau S (Merck, Darmstadt, Niemcy). Immunodetekcję transgeliny-2 na membranie przeprowadzono z zastosowaniem pierwszorzędowych, króliczych przeciwciał poliklonalnych Anti-TAGLN2 (Abcam, Cambridge, Wielka Brytania) oraz drugorzędowych przeciwciał Anti-Rabbit IgG (Santa Cruz Biotechnology, Dallas, Teksas, Stany Zjednoczone). Wizualizację białek przeprowadzono z zastosowaniem kliszy fotograficznej przy użyciu komercyjnego zestawu SuperSignal™ West Pico PLUS (Thermofisher Scientific, Waltham, Massachusetts, Stany Zjednoczone), a półilościowa analiza stężenia białka została wykonana za pomocą analizy densytometrycznej (*Publikacja eksperymentalna nr 3*).

- **Analiza statystyczna**

1) Publikacja eksperymentalna nr 1:

Analiza statystyczna wyników uzyskanych z profilowania płytkowych cząsteczek miRNA metodą mikromacierzy została wykonana za pomocą testu t-Studenta z zastosowaniem poprawki FDR Benjamini-Hochberga. W przypadku danych uzyskanych w wyniku walidacji poziomu ekspresji wyselekcjonowanych miRNA metodą qPCR, różnice w ekspresji były analizowane za pomocą testu t-Studenta. W celu lepszego dopasowania wartości przedstawiających parametry biochemiczne grupy badanej oraz kontrolnej do rozkładu normalnego, wykonano transformację Boxa-Coxa.

W celu weryfikacji, czy wykryte zmiany ekspresji cząsteczek miRNA w płytkach krwi są różnicujące dla OZW względem kontroli, skonstruowano wieloczynnikowy model regresji logistycznej z następczą analizą krzywych ROC (receiver operating characteristic, ROC), która ilustruje efektywność modelu predykcyjnego poprzez wykreślenie krzywej charakteryzującej jakość klasyfikatorów oraz umożliwia wyliczenie pola pod wykresem krzywej ROC (area under curve, AUC), umożliwiając tym samym ocenę poprawności predyktora oraz określając specyficzność i czułość modelu diagnostycznego. W obliczeniach statystycznych zastosowano 95% przedział ufności, a punkt odcięcia został zaproponowany na podstawie maksymalizacji

wskaźnika Youdena. Współliniowość predyktorów, będąca cechą niepożądaną, została testowana za pomocą eksploracyjnej analizy czynnikowej (exploratory factor analysis, EFA) z rotacją czynników Varimax i analizy macierzy korelacji r Pearsona. Predyktory zostały wybrane na podstawie wstecznej analizy krokowej i klinicznej istotności zastosowanych parametrów, a do porównania modeli wykorzystano kryteria informacyjne oraz statystyki pseudo-R^2 (Cox-Snell oraz Ngelkerke). Jako wewnętrzną technikę walidacji modelu zastosowano 10-krotną walidację krzyżową.

2) Publikacja eksperymentalna nr 2:

W pierwszym etapie analizy statystycznej, z pomocą testu Shapiro-Wilka, weryfikacji poddano normalność rozkładu danych uzyskanych z pomiaru ekspresji mRNA i miRNA metodą qPCR oraz stężenia białka metodą ELISA. Rozkład skośny prezentowały wartości określające stężenie białka, które w celu obniżenia odchylenia od normalności zostały poddane transformacji logarytmicznej przed dalszą analizą. Różnice w ekspresji miRNA, mRNA i stężenia białka między pacjentami z OZW a grupą kontrolną oceniono za pomocą testu t-Studenta. W przypadku nierównej wariancji, zastosowano test t-Welcha. Korelację pomiędzy analizowanymi parametrami (ekspresja miRNA i mRNA oraz stężenie białka) testowano za pomocą technik ogólnego modelu liniowego. Ocenę potrzeby redukcji wymiarowości pomiędzy analizowanymi danymi przeprowadzono z wykorzystaniem kryterium Kaisera-Meyera-Olkina oraz testu χ^2 Barletta. Model diagnostyczny służący do różnicowania pacjentów z OZW od dawców został opracowany przy użyciu wieloczynnikowej regresji logistycznej z uwzględnieniem zmiennych wejściowych nisko ze sobą skorelowanych. Następnie, został on poddany wewnętrznej 10-krotnej walidacji krzyżowej oraz zilustrowany za pomocą analizy krzywej ROC.

3) Publikacja eksperymentalna nr 3:

W pierwszym etapie analizy statystycznej wykorzystano test Shapiro-Wilka służący do oceny normalności rozkładu zmiennych. Dla zmiennych wykazujących rozkład Gaussa zastosowano test t-Studenta, natomiast w przypadku wyników prezentujących rozkład skośny, zastosowano test U Manna-Whitneya.

10. WYNIKI I DYSKUSJA

Spis publikacji eksperymentalnych wchodzących w skład rozprawy doktorskiej:

- **Publikacja eksperymentalna nr 1:** Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak. *Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome*. Cells, 10(12): 3526, 2021.
- **Publikacja eksperymentalna nr 2:** Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak. *Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12*. Biology, 11(5): 644, 2021.
- **Publikacja eksperymentalna nr 3:** Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak. *Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients*. International Journal of Molecular Sciences, 23(11): 6340, 2022.

W pierwszym etapie badań wykonanych w ramach niniejszej pracy doktorskiej przeprowadzona została analiza porównawcza płytkowego profilu miRNA metodą mikromacierzy. Wyniki badań przesiewowych wskazały 107 cząsteczek miRNA, które wykazywały istotną statystycznie różnicę w ekspresji pomiędzy badanymi grupami. Następnie, w celu wyselekcjonowania cząsteczek miRNA wykazujących największą krotność zmian w ekspresji pomiędzy grupą badaną a grupą kontrolną, zastosowano filtrowanie danych, które pozwoliło na odrzucenie wyników najmniej różnicujących grupy (odrzucono wyniki posiadające mniejszą krotność zmiany niż 2). Uzyskano w ten sposób 91 cząsteczek miRNA o wyraźnie zmienionej ekspresji w OZW względem grupy kontrolnej. W celu określenia potencjalnych konsekwencji zmiany ekspresji analizowanych miRNA, przy użyciu 6 baz danych (miRDB, microRNAorg, PITA, PICTAR, TARBASE oraz TARGETSCAN) zidentyfikowano docelowe cząsteczki mRNA interferujące z wykrytymi miRNA.

W następnym etapie badań, przeprowadzono szeroką analizę bioinformatyczną, która miała na celu wyselekcjonowanie spośród wszystkich wykrytych docelowych cząsteczek mRNA, tylko te sekwencje, które mogą w istotny sposób wpływać na funkcje płytek krwi. Sekwencje te zostały wybrane na podstawie ich kluczowej roli w szlakach sygnałowych odpowiedzialnych za aktywację płytek krwi oraz na podstawie ontologii genów, która pozwala określić ich funkcję biologiczną na poziomie molekularnym (np. transdukcja sygnałów w szlakach biochemicznych) oraz określić ich lokalizację względem struktur komórkowych,

w których docelowy mRNA pełni swoją funkcję (np. błona komórkowa). Na podstawie przeprowadzonej analizy bioinformatycznej wyselekcjonowano 15 cząsteczek miRNA, których zaburzona ekspresja pomiędzy grupą OZW a grupą kontrolną została poddana walidacji metodą qPCR (**Publikacja 1, Rycina 2**). Wyniki uzyskane w ramach przeprowadzonej analizy zostały przedstawione w Tabeli 1.

Tabela 1. Porównanie ekspresji płytkowych miRNA u pacjentów z OZW względem grupy kontrolnej. Dane przedstawione w tabeli zawierają uśrednioną wartość -ΔCt (oznaczającą względną ekspresję danego miRNA w odniesieniu do zastosowanych cząsteczek referencyjnych: cel-miR-39-3p oraz hsa-miR-191-5p).

miRNA	**OZW (-ΔCt)**		**Kontrola (-ΔCt)**		**Krotność zmiany (95% CI)**	**Wartość *p****
	Średnia	**SEM**	**Średnia**	**SEM**		
hsa-miR-223-3p	-0,76	0,19	-1,84	0,23	2,11 (1,40-3,19)	0,0004
hsa-miR-142-3p	-1,31	0,28	-3,67	0,42	5,16 (2,59-10,28)	<0,0001
hsa-miR-126-3p	-1,78	0,35	-2,66	0,29	1,84 (0,99-3,45)	0,056
hsa-miR-21-5p	-2,19	0,20	-3,66	0,36	2,77 (1,57-4,87)	0,0005
hsa-miR-107	-6,54	0,25	-8,11	0,27	2,96 (1,78-4,91)	<0,0001
hsa-miR-28-5p	-10,43	0,23	-10,59	0,44	1,12 (0,56-2,21)	0,75
hsa-miR-221-3p	-0,50	0,22	-1,33	0,30	1,78 (1,07-2,98)	0,027
hsa-miR-98-5p	-3,83	0,36	-4,67	0,58	1,80 (0,70-4,59)	0,22
hsa-let-7f-5p	-3,67	0,43	-3,04	0,31	0,65 (0,31-1,34)	0,23
hsa-let-7d-5p	-2,61	0,25	-2,93	0,34	1,25 (0,70-2,23)	0,45
hsa-let-7g-5p	-3,27	0,24	-3,23	0,24	0,97 (0,61-1,55)	0,91
hsa-miR-146a-5p	-2,32	0,21	-3,02	0,31	1,62 (0,97-2,72)	0,067
hsa-miR-301a-3p	-7,54	0,37	-10,01	0,87	5,52 (1,49-20,42)	0,011
hsa-miR-130b-3p	-9,00	0,44	-11,04	0,48	4,11 (1,68-10,09)	0,0023
hsa-miR-338-3p	-7,55	0,42	-9,66	0,40	4,31 (1,94-9,58)	0,0004

** Analizę statystyczną przeprowadzono przy użyciu testu t-Studenta.*

Na podstawie wyników otrzymanych z analizy qPCR przeprowadzono proces modelowania potencjalnego biomarkera. Opracowanie wielowymiarowego modelu diagnostycznego wykorzystującego tylko analizowane cząsteczki miRNA nie było możliwe w tym przypadku, ze względu na ich wysoką, wzajemną korelację (**Publikacja 1, Suplement 2**). W związku z brakiem możliwości wykorzystania więcej niż jednej cząsteczki miRNA w procesie modelowania potencjalnego biomarkera, w analizie krzywych ROC wykorzystano również wartości określające poziom AST, które na podstawie analizy macierzy korelacji r Pearsona mogły zostać uwzględnione w projektowanym modelu. Wybrany na podstawie istotności statystycznych oraz krotności zmiany hsa-miR-142-3p pozwalał na osiągnięcie 64% czułości i 76% specyficzności w różnicowaniu pacjentów z OZW od dawców kontrolnych (AUC (95% CI) = 0.75 (0,65-0,84); $p < 0,0001$). Krzywa ROC opracowana dla modelu bazującego na samym AST wykazywała 70% czułość i 86% specyficzność (AUC (95% CI) = 0,83 (0,75-0,91); $p < 0,0001$) (**Publikacja 1, Rycina 5A**). Model wieloczynnikowy (hsa-miR-142-3p + AST), wykonany w celu poprawy jakości klasyfikacji przeanalizowanych zmiennych, wykazywał natomiast 82% czułość oraz 88% specyficzność (AUC (95% CI) = 0,91 (0,85-0,97); $p < 0,0001$) w różnicowaniu grupy badanej od kontrolnej (**Publikacja 1, Rycina 5B**). Lepsze dopasowanie wieloczynnikowego modelu diagnostycznego w różnicowaniu badanych grup (niż modelu opartego wyłącznie na AST) potwierdzają niższe wartości przedstawione z analizy kryteriów informacyjnych oraz zwiększone wartości współczynników determinacji pseudo-R^2 (**Publikacja 1, Tabela 2**). Ponadto, czułość oraz specyficzność uzyskanego modelu była minimalnie gorsza w procedurze walidacyjnej, która wskazała na brak nadmiernego dopasowania modelu do analizowanych próbek (**Publikacja 1, Rycina 5**).

Przeprowadzona w niniejszej pracy doktorskiej analiza EFA z rotacją czynników Varimax, wraz z macierzą korelacji r Pearsona miała na celu identyfikację potencjalnych zależności (co do występowania i funkcjonalności) pomiędzy wyselekcjonowanymi 15 cząsteczkami miRNA. Wynik przeprowadzonej analizy pozwolił na rozdzielenie badanych cząsteczek miRNA na dwie niepowiązane ze sobą grupy. W Grupie 1, znajdowały się głównie miRNA zdolne do rozróżniania pacjentów z OZW od zdrowych dawców (hsa-miR-301a-3p, hsa-miR-142-3p, hsa-miR-338-3p, hsa-miR-130b-3p, hsa-miR-107, hsa-miR-21-5p, hsa-miR-223-3p, hsa-miR-126-3p). Grupa 2 składała się głównie z cząsteczek miRNA, które nie wykazały zdolności do różnicowania porównywanych grup (hsa-let-7d-5p, hsa-let-7f-5p, hsa-let-7g-5p, hsa-miR-28-5p, hsa-miR-98-5p, hsa-miR-221-3p, hsa-miR-146a-5p) (**Publikacja 1, Rycina 4**). Założenia analizy EFA z rotacją Varimax sugerują brak występowania wzajemnej zależności pomiędzy analizowanymi czynnikami. W związku z tym, można postawić hipotezę, która zakłada, iż ekspresja cząsteczek miRNA znajdujących się w Grupie 1 oraz cząsteczek miRNA znajdujących się w Grupie 2, może wpływać niezależnie na stymulowanie różnych od siebie funkcji biologicznych. W celu określenia tych funkcji, za pomocą bazy DAVID [47] przeprowadzona została analiza porównawcza ontologii genów dla

docelowych mRNA zdolnych do oddziaływania z cząsteczkami miRNA zawartymi w Grupie 1 oraz w Grupie 2 (**Publikacja 1, Suplement 3**). Dane uzyskane z analizy bioinformatycznej obu grup wskazują, iż cząsteczki miRNA wchodzące w skład Grupy 1, zdolne do rozróżniania pacjentów z OZW od dawców, wykazują asocjację z 7-krotnie większą liczbą genów odpowiedzialnych za regulację aktywności GTPaz i fosfolipazy C, z ponad 6-krotnie większą liczbą genów powiązanych z odpowiedzią na hipoksję, z 5-krotnie większą liczbą genów związanych z regulacją chemotaksji i przekaźnictwa sygnałowego kinazy 3-fosfatydyloinozytolu (Phosphatidylinositol 3-kinase, PI3K), z 2-krotnie większą liczbą genów odpowiedzialnych za odpowiedź organizmu na działanie leków i ogólną transdukcję sygnałów w szlakach komórkowych oraz regulację biosyntezy tlenku azotu, w porównaniu z Grupą 2. Bioinformatyczna analiza funkcjonalnych lokalizacji dla produktów zgrupowanych genów, wskazała przewagę ich aktywności zasocjowanych z cytoplazmą, błoną komórkową, kompleksem integryn oraz cytoszkieletem w Grupie 1, natomiast w Grupie 2 - z włóknami kolagenowymi, filopodiami i lamellipodiami (**Publikacja 1, Suplement 3**).

W kolejnym etapie analizy bioinformatycznej, dla 8 istotnie statystycznych miRNA wytypowano 43 docelowe sekwencje mRNA (obecne w płytkowym transkryptomie), które uczestniczą w szlakach molekularnych istotnych dla funkcjonalności płytek krwi (np. szlak sygnalizacyjny PI3K-Akt) **(Publikacja 1, Tabela 3).** Następnie, spośród wyodrębnionych transkryptów, na podstawie dostępnych danych literaturowych, wybrano 28 sekwencji mRNA, których białkowe produkty zostały eksperymentalnie potwierdzone w proteomie płytek krwi [48]. W celu określenia potencjalnych zaburzeń w płytkowym interaktomie, przy użyciu bazy danych STRING [49], przeprowadzona została komputerowa wizualizacja interakcji białko-białko, która pozwoliła na wyodrębnienie 3 grup białek związanych z funkcjonalnością płytek krwi (**Publikacja 1, Rycina 6**). W Grupie 1 wyróżniono 5 białek (COL6A3, COL1A2, ITGA2, ITGB3, P2Y12) biorących istotny udział w transdukcji sygnałów w szlakach receptorowych, kluczowych dla adhezji i aktywacji płytek krwi. Białka zawarte w Grupie 2 (AKT3, MAP2K1, RAP1B, YWHAQ, BCL2L11, CDKN1A, THEM4 oraz CHUK) wykazują silne powiązanie ze szlakiem sygnałowym PI3K-Akt, który w końcowym etapie indukuje zmiany konformacyjne receptora GP IIb/IIIa umożliwiając jego aktywację determinującą przyłączenie ligandu. W przypadku białek znajdujących się w grupie 3 (ITPR1, INSR, PLCB1, PRKG1, PIKFYVE, RDX, ARHGEF12, MYLK, PPP1R12A) wykazano, iż są one kluczowe dla skurczu mięśni gładkich, wywołanych zmianą wewnątrzkomórkowego stężenia jonów Ca^{2+} oraz dla regulacji cytoszkieletu aktynowego.

Podsumowując, wybrane cząsteczki miRNA wykazują dużą zmienność pomiędzy pacjentami z OZW a grupą kontrolną, jednakże w związku z ich wzajemną, istotną korelacją, nie powinny być używane razem w modelu diagnostycznym. Wyniki uzyskane z przeprowadzonego eksperymentu wskazują, iż hsa-miR-142-3p wraz z AST posiada istotny, wysoki współczynnik czułości i specyficzności w różnicowaniu porównywanych grup, co sugeruje, iż wytypowane predyktory, mogłyby w przyszłości posłużyć jako składowe

wieloczynnikowego modelu biomarkera określającego predyspozycje człowieka do wystąpienia OZW. Ponadto, dane uzyskane z analiz bioinformatycznych pozwoliły wskazać sekwencje mRNA docelowe dla cząsteczek miRNA różnicujących OZW. Regulacja ekspresji tych transkryptów może zatem stanowić potencjalny element prewencji/terapii OZW.

W następnym etapie badań przeprowadzona została analiza porównawcza poziomu ekspresji płytkowych receptorów powierzchniowych u pacjentów z OZW oraz w grupie kontrolnej, zarówno na poziomie mRNA jak i cząsteczek białka. Pomiar ekspresji wykonany metodą qPCR dla 5 genów (*P2Y12, GP1BB, ITGA2B, ITGB3* oraz *F2R*) kodujących 4 podstawowe receptory powierzchniowe, wykazał istotną statystycznie nadekspresję 4 z 5 analizowanych transkryptów mRNA (**Publikacja 2, Rycina 1**), a uzyskane wyniki zostały przedstawione w Tabeli 2.

Tabela 2. Zmiana ekspresji cząsteczek płytkowego mRNA dla genów kodujących wybrane receptory powierzchniowe w grupie pacjentów z OZW względem kontroli.

Gen	Krotność zmiany (FC)	Wartość *p**
P2Y12	2,44 ↑	< 0,0001
GP1BB	2,93 ↑	< 0,0001
ITGA2B	3,77 ↑	< 0,0001
ITGB3	3,43 ↑	< 0,0001
F2R	-	0,074

* *Analizę statystyczną przeprowadzono przy użyciu testu t-Studenta lub w przypadku nierównych wariancji – testem t-Welcha.*

Dla cząsteczek mRNA wykazujących istotną statystycznie zmianę poziomu ekspresji w grupie badanej względem grupy kontrolnej, przeprowadzony został pomiar stężenia produktu białkowego w płytkach krwi metodą immunoenzymatyczną ELISA. Ze względu na heterodimeryczną budowę receptora GP IIb/IIIa, w którego skład wchodzą podjednostki: GP IIIa (kodowana przez gen *ITGB3*) oraz GP IIb (kodowana przez gen *ITGA2B*), występujące w równowadze molowej, stężenie białka określono tylko dla jednej z podjednostek – GP IIIa. Analiza porównawcza wykazała istotny statystycznie wzrost stężenia receptora GP IIIa (843,5 (676,8 – 1134) pg/mL w grupie badanej *vs.* 592,6 (403,3 – 732,7) pg/mL w grupie kontrolnej, $p < 0,0001$, FC = 1,61 (1,34 – 1,95)) oraz receptora P2Y12 (149 (108,1 – 200,9) ng/mL w grupie badanej *vs.* 82,66 (67,2 – 105,3) ng/mL w grupie kontrolnej, $p < 0,0001$, FC = 1,78 (1,49 – 2,11)). W przypadku receptora GP Ib nie wykazano różnic istotnych statystycznie (75,29 (51,09 – 117,3) ng/mL w grupie badanej *vs.* 65,46 (49,75 – 111,1) ng/mL w grupie kontrolnej, $p > 0,99$) (**Publikacja 2, Rycina 2**).

Zwiększone stężenie GP IIIa w płytkach krwi pacjentów z OZW może wskazywać na wzrost ich zdolności do syntezy obu podjednostek receptora GP IIb/IIIa. Fenomen ten został potwierdzony w badaniach Kieffera i wsp. dzięki zastosowaniu znaczników radioaktywnych [50]. Zdolność płytek krwi do nadmiernego generowania podjednostek receptora GP IIb/IIIa w OZW ujawnia pro-zakrzepowy fenotyp tych komórek i może stanowić hipotetyczną przyczynę występowania zjawiska ich nadreaktywności w patogenezie epizodów niedokrwiennych. Wskazują na to również badania Yakushkina i wsp., w których wykazano, iż zwiększona ekspresja receptora GP IIb/IIIa była powiązana z silniejszą agregacją płytek krwi [51].

W przypadku receptora P2Y12, jego zwiększona ekspresja na poziomie mRNA oraz białka została w literaturze potwierdzona u pacjentów ze zdiagnozowaną cukrzycą typu 2 oraz wśród pacjentów z wtórnie postępującym stwardnieniem rozsianym. Jest to istotne w przypadku tych jednostek chorobowych, gdyż cechują się one wyższym ryzykiem występowania powikłań zakrzepowo-zatorowych [52,53].

W dalszych badaniach skupiono się na szerszej analizie receptora P2Y12, gdyż stanowi on główny cel leczenia w terapii przeciwpłytkowej pacjentów z OZW. Poziom ekspresji transkryptów oraz stężenie białka dla receptora P2Y12 oznaczane były w preparatach lizatów płytek krwi. Natomiast, kolejne etapy badań dotyczyły oceny ekspozycji P2Y12 na powierzchni płytek krwi metodą cytometrii przepływowej oraz mikroskopii konfokalnej. Analiza cytometryczna wykonana w krwi pełnej bez użycia agonistów komórkowych, wykazała istotny statystycznie wzrost powierzchniowej ekspresji receptora P2Y12 w OZW względem grupy kontrolnej (18% ± 2,95% w grupie badanej *vs*. 8 ± 1,26% w grupie kontrolnej, $p < 0{,}001$) (**Publikacja 2, Rycina 3**). Wyniki te zostały potwierdzone poprzez wizualizację za pomocą mikroskopii konfokalnej. Co więcej, w przypadku OZW dystrybucja receptorów obserwowana w obrazie mikroskopowym wykazywała tendencję do formowania się klastrów na powierzchni płytek krwi (**Publikacja 2, Rycina 4**).

W celu identyfikacji potencjalnego podłoża molekularnego odpowiedzialnego za nadekspresję receptora P2Y12 u pacjentów z OZW, oceniono poziom dwóch płytkowych miRNA (hsa-miR-223-3p oraz hsa-miR-126-3p), które posiadają zdolność do asocjacji z transkryptem dla receptora P2Y12 [54]. Wyniki otrzymane z analizy qPCR wykazały istotny statystycznie wzrost ekspresji hsa-miR-223-3p ($p < 0{,}0001$) w grupie badanej względem grupy kontrolnej oraz brak różnic w ekspresji hsa-miR-126-3p ($p = 0{,}099$) (**Publikacja 2, Rycina 5**).

Dostępne dane literaturowe odnoszące się do ekspresji płytkowego hsa-miR-223-3p nie są jednoznaczne. Istotny statystycznie wzrost ekspresji miR-223 został potwierdzony w płytkach krwi pacjentów ze STEMI w porównaniu do zdrowych ochotników [55] oraz w krwi pełnej pacjentów z ostrym zawałem mięśnia sercowego [56]. Z drugiej strony, w badaniach przeprowadzonych przez Shi i wsp. oraz Zhanga i wsp. poziom płytkowego [57] oraz osoczowego [58] hsa-miR-223-3p był znacząco obniżony u pacjentów po zawale serca, którzy otrzymywali farmakologiczny inhibitor receptora P2Y12. Co więcej, symultaniczna nadekspresja płytkowego transkryptu dla P2Y12 oraz hsa-miR-223-3p została po raz pierwszy

zaprezentowana w niniejszej pracy doktorskiej, a jej przyczyna pozostaje niejasna. Analiza statystyczna otrzymanych wyników nie wykazała istotnej korelacji pomiędzy ekspresją receptora P2Y12 oraz hsa-miR-223-3p, co może sugerować, iż cząsteczka ta nie jest głównym regulatorem ekspresji tego receptora. Potencjalnym wyjaśnieniem jednocześnie zwiększonej ekspresji receptora P2Y12 oraz jego ujemnego regulatora - cząsteczki hsa-miR-223-3p, może być reakcja organizmu na wystąpienie stanu patologicznego, zmierzająca do przywrócenia homeostazy poprzez obniżenie nadreaktywnego stanu płytek krwi spowodowanego nadmierną aktywnością receptora P2Y12.

W przypadku hsa-miR-126-3p, brak jest danych literaturowych dotyczących poziomu ekspresji tej cząsteczki w płytkach krwi pacjentów z OZW, natomiast wyniki badań odnoszące się do osocza nie są jednoznaczne. W pracy Liu i wsp. wykazano, iż osoczowa ekspresja miR-126 była obniżona u pacjentów z OZW, u których reaktywność płytek (mierzona za pomocą tromboelastografii) w trakcie leczenia klopidogrelem (nieodwracalnym inhibitorem receptora P2Y12) była istotnie większa niż w porównywanej grupie kontrolnej, którą stanowili pacjenci prezentujący obniżoną reaktywność płytek krwi w trakcie leczenia [59]. Z drugiej strony, u pacjentów z ostrym zawałem mięśnia sercowego oraz wykonaną przezskórną interwencją wieńcową wykazano wzrost poziomu krążącego hsa-miR-126 [60]. W celu określenia roli hsa-miR-126-3p w występowaniu zaburzeń zakrzepowo-zatorowych konieczne jest przeprowadzenie większej ilości badań, których wyniki stworzą szansę do dyskusji.

Przeprowadzone w ramach niniejszej pracy doktorskiej badania miały na celu nie tylko wykrycie zmian na poziomie molekularnym, które mogą stanowić przyczynę wzmożonej aktywności pro-zakrzepowej płytek krwi, ale również ocenę ich użyteczność w konstruowaniu diagnostycznego modelu wieloczynnikowej regresji logistycznej z analizą krzywej ROC. Predyktorami wykorzystanymi w analizie statystycznej były wyniki uzyskane z pomiarów ekspresji receptora P2Y12 na poziomie mRNA i stężenia białka receptorowego oraz ekspresji płytkowego hsa-miR-223-3p. Model zilustrowano za pomocą krzywej ROC wraz z wewnętrzną, 10-krotną walidacją krzyżową. Przeprowadzona analiza wykazała, iż uzyskany model osiągnął 97% czułość oraz 74% specyficzność w odróżnianiu pacjentów z OZW od dawców bez zaburzeń sercowo-naczyniowych (AUC (95% CI) = 0,93 (0,87-0,99), $p < 0,0001$) (**Publikacja 2, Suplement 1**).

W znakomitej większości prac, powierzchniowe receptory płytek krwi stanowią przedmiot badań wyłącznie z perspektywy oceny farmakologicznej, jako główne cele terapeutyczne dla nowo testowanych leków przeciwpłytkowych. Istniejąca nisza, związana z brakiem analizy molekularnych zaburzeń występujących w powierzchniowych receptorach płytkowych, które mogą prowadzić do wystąpienia epizodów niedokrwiennych, wskazuje ważny kierunek badań w celu lepszego zrozumienia patofizjologii powikłań zakrzepowo-zatorowych.

Różnice ilościowe powierzchniowych receptorów płytek krwi pomiędzy OZW a grupą kontrolną stały się istotną przesłanką do wykonania pełnej analizy zmian w proteomie płytek krwi. W ostatnim etapie badań przeprowadzona została przesiewowa analiza płytkowego proteomu za pomocą metody 2D-DIGE oraz identyfikacja białek wykazujących zmienną ekspresję pomiędzy porównywanymi grupami, z wykorzystaniem metody nanoLC-MS/MS. Celem badania było odnalezienie oraz zidentyfikowanie sygnatur białkowych, których zmienna ekspresja mogłyby potencjalnie stanowić jeden z elementów molekularnego mechanizmu nadreaktywności płytek krwi w OZW. Uzyskane wyniki wskazały 6 białek, których stężenie było znacząco różne w porównywanych grupach. Stężenie winkuliny i apolipoproteiny A1 było obniżone u pacjentów z OZW względem zdrowych dawców, natomiast poziom łańcucha β fibrynogenu, łańcucha γ fibrynogenu, łańcucha β tubuliny oraz transgeliny-2 był zwiększony u pacjentów z OZW względem grupy kontrolnej (**Publikacja 3, Tabela 1**). Dla sekwencji kodujących wszystkie zidentyfikowane białka wykonana została analiza ekspresji genów na poziomie mRNA. Otrzymane wyniki wykazały brak płytkowych transkryptów dla genów kodujących apolipoproteinę A1 (*APOA1*) oraz łańcuchy fibrynogenu (*FGB, FGG*). Istotną statystycznie nadekspresję w poziomie mRNA w OZW zaobserwowano dla winkuliny (*VCL*, $p = 0{,}0032$) oraz transgeliny-2 (*TAGLN2*, $p < 0{,}0001$), natomiast brak różnic w ekspresji wykazano dla łańcucha β tubuliny (*TUBB1*, $p = 0{,}5520$) (**Publikacja 3, Rycina 3**).

Winkulina należy do rodziny białek wiążących aktynę, stanowiąc łącznik pomiędzy receptorami integrynowymi a włóknami cytoszkieletu. Jej rola w płytkach krwi nie została jednakże dotychczas jednoznacznie określona. Badania przeprowadzone na mysim modelu z delecją genu *VCL* wykazały, że defekt ten nie wpływa na czynnościową odpowiedź płytek krwi, co mogłoby sugerować, iż winkulina nie odgrywa istotnej roli w procesie adhezji, aktywacji i agregacji płytek krwi [61]. Brak jest natomiast badań określających rolę płytkowej winkuliny u człowieka. Z tego względu na tę chwilę nie jest możliwa interpretacja wyników uzyskanych w niniejszej pracy, pod kątem potencjalnej roli winkuliny w patofizjologii OZW. Warto jednak zaznaczyć, że zaobserwowany w grupie pacjentów z OZW wzrost ekspresji płytkowego transkryptu dla genu kodującego winkulinę i jednocześnie obniżone stężenie produktu białkowego w tych komórkach, może sugerować wpływ modyfikacji potranskrypcyjnych indukowanych między innymi przez miRNA. Dostępne dane literaturowe wskazują, iż ekspresja genu *VCL* może być regulowana przy udziale cząsteczki hsa-miR-21-5p [62], której istotnie podwyższony poziom odnotowano w OZW w prezentowanej pracy doktorskiej (**Publikacja 1, Rycina 2**).

Istotnie podwyższona w płytkach krwi pacjentów z OZW była ekspresja transgeliny-2, zarówno na poziomie mRNA, jak i stężenia produktu białkowego. Sugeruje się, iż transgelina-2 jest białkiem aktywnie zaangażowanym w fizjologiczną czynność płytek krwi. Z tego względu wyniki uzyskane w ramach przeprowadzonej analizy 2D-DIGE zostały poddane dalszej walidacji metodą Western Blot, która potwierdziła ponad 2-krotny wzrost stężenia tego białka w płytkach krwi u pacjentów z OZW względem grupy kontrolnej

(p = 0,0007) (**Publikacja 3, Rycina 5**). Trudno jest jednoznacznie określić jakie może być znaczenie zwiększonego stężenia płytkowego transgeliny-2 w OZW, z pewnością jednak warto jest rozważyć ten fakt w dalszych badaniach prowadzonych pod kątem poszukiwania molekularnych przyczyn pro-zakrzepowego fenotypu płytek krwi. W tym względzie funkcja transgeliny-2 może wiązać się z jej udziałem w procesie polimeryzacji włókien aktynowych cytoszkieletu [63], które odgrywają istotną rolę w remodelingu płytek krwi, który towarzyszy ich aktywacji. Według danych z baz UniProt oraz GeneCards, transgelina-2 może uczestniczyć w procesach degranulacji i agregacji płytek krwi, w których dochodzi do reorganizacji cytoszkieletu [64,65]. Co więcej, wykazano udział transgeliny-2 w inhibicji ekspresji metaloproteinazy macierzy zewnątrzkomórkowej 9 (metallo-matrix proteinase 9, MMP9) [66], której aktywność w płytkach krwi wpływała na zmniejszenie stopnia ich agregacji w odpowiedzi na stymulację agonistami, takimi jak trombina, kwas arachidonowy czy ADP [67]. Można zatem sugerować, iż zwiększone stężenie transgeliny-2 w OZW może przyczyniać się do zjawiska płytkowej nadaktywności stanowiącej istotny patomechanizm tego schorzenia.

Podsumowując, wyniki uzyskane w ramach analiz prowadzonych w niniejszej pracy doktorskiej, pozwoliły na opracowanie dwóch modeli diagnostycznych, wykazujących wysoką specyficzność oraz czułość w rozróżnianiu pacjentów z OZW od zdrowych ochotników. Ponadto, wykryte zmiany na poziomie transkryptomicznym oraz proteomicznym, dostarczyły nowych informacji umożliwiających lepsze zrozumienie zaburzonej fizjologii płytek krwi w OZW.

11. WNIOSKI

Wyniki zaprezentowane w niniejszej pracy doktorskiej pozwalają sformułować następujące wnioski:

1) **Zmiany proteomiczne oraz transkryptomiczne (na poziomie mRNA oraz miRNA) zidentyfikowane w płytkach krwi wskazują na molekularne podłoże ich wzmożonej aktywności pro-zakrzepowej w OZW**
   - Zidentyfikowane cząsteczki miRNA (hsa-miR-223-3p; hsa-miR-142-3p; hsa-miR-21-5p; hsa-miR-107; hsa-miR-221-3p; hsa-miR-301a-3p; hsa-miR-130b-3p; hsa-miR-338-3p) wykazujące zaburzenia ekspresji w OZW, są regulatorami ekspresji genów, których produkty zaangażowane są w kluczowe szlaki sygnałowe płytek krwi.
   - Płytki krwi u pacjentów z OZW wykazują wzrost ekspresji (na poziomie mRNA oraz białka) receptorów powierzchniowych dla ADP i fibrynogenu.
   - Analiza molekularna wykazała wzrost ekspresji (na poziomie mRNA oraz białka) transgeliny-2, która uczestnicząc w reorganizacji cytoszkieletu może oddziaływać na proces degranulacji oraz agregacji płytek.

2) **Wykryte zaburzenia w ekspresji płytkowych cząsteczek miRNA pomiędzy badanymi grupami wykazują zdolność do różnicowania pacjentów z OZW od zdrowych dawców**
   - Włączenie cząsteczek miRNA do modelowania diagnostycznego opartego na krzywej ROC umożliwia zwiększenie czułości oraz specyficzności opracowywanego modelu w różnicowaniu porównywanych grup.
   - Predyktory wyselekcjonowane do modelu diagnostycznego (hsa-miR-142-3p oraz AST) wybrane na podstawie istotności statystycznych, krotności zmiany oraz niskiego współczynnika korelacji wykazują 82% czułość oraz 88% specyficzność w różnicowaniu porównywanych grup.
   - Krzywa ROC wykonana dla modelu opartego na ekspresji cząsteczki hsa-miR-223-3p oraz ekspresji receptora P2Y12 na poziomie mRNA i białka w płytkach krwi wykazała 97% czułość oraz 74% specyficzność w różnicowaniu OZW względem kontroli.

12. STRESZCZENIE

Ostre zespoły wieńcowe (OZW) należą do grupy schorzeń kardiologicznych cechujących się zaburzeniami w krążeniu wieńcowym spowodowanymi przez nagłe lub narastające zmniejszenie drożności tętnicy wieńcowej.

Według danych przedstawionych przez Światową Organizację Zdrowia, szacuje się, iż 32% wszystkich zgonów na Świecie jest spowodowane wystąpieniem chorób sercowo-naczyniowych, w szczególności zawału mięśnia sercowego oraz udaru mózgu. Za jedną z głównych przyczyn patofizjologii OZW uznaje się patologiczną aktywację płytek krwi, prowadzącą do indukowania zmian zakrzepowych w obrębie tętnic wieńcowych.

Celem niniejszej rozprawy doktorskiej było określenie zaburzeń w transkryptomie i proteomie płytek krwi, które mogłyby stanowić molekularne podłoże ich wzmożonej aktywności pro-zakrzepowej w OZW oraz identyfikacja cząsteczek o potencjale wysokoczułych molekularnych markerów płytkowych, określających predyspozycje człowieka do wystąpienia OZW.

Przeprowadzone badania pozwoliły na zidentyfikowanie 8 cząsteczek miRNA (hsa-miR-223-3p, hsa-miR-142-3p, hsa-miR-21-5p, hsa-miR-107, hsa-miR-221-3p, hsa-miR-301a-3p, hsa-miR-130b-3p, hsa-miR-338-3p), których poziom ekspresji różnicował pacjentów z OZW od grupy kontrolnej. Modelowanie statystyczne, przeprowadzone w celu identyfikacji potencjalnego biomarkera OZW, pozwoliło wytypować cząsteczkę hsa-miR-142-3p wraz z AST do łączonego modelu diagnostycznego, który różnicował porównywane grupy z 82% czułością oraz 88% specyficznością (podczas gdy model bazujący na samej wartości AST pozwala na osiągnięcie 70% czułości i 86% specyficzności).

Co więcej, w niniejszej pracy doktorskiej wykazano, iż wśród pacjentów z OZW obserwowany jest istotnie statystyczny wzrost ekspresji (na poziomie mRNA oraz białka) receptorów powierzchniowych dla ADP (P2Y12) i fibrynogenu (GP IIb/IIIa). Krzywa ROC wykonana dla modelu opartego na ekspresji receptora P2Y12 na poziomie mRNA oraz białka wraz z ekspresją hsa-miR-223-3p wykazała 97% czułość oraz 74% specyficzność w różnicowaniu pacjentów z OZW od grupy kontrolnej.

Wyniki uzyskane z przesiewowej analizy płytkowego proteomu, a następnie ilościowej oceny transkryptów dla zidentyfikowanych białek różnicujących, wskazały zwiększoną ekspresję transgeliny-2 jako potencjalną przyczynę wzmożonej aktywności pro-zakrzepowej płytek krwi w OZW.

Podsumowując, zmiany proteomiczne oraz transkryptomiczne (na poziomie mRNA oraz miRNA) zidentyfikowane w płytkach krwi wskazują na molekularne podłoże ich wzmożonej aktywności pro-zakrzepowej w OZW. Ponadto, wykryte zaburzenia w ekspresji płytkowych cząsteczek miRNA pomiędzy badanymi grupami wykazują zdolność do różnicowania pacjentów z OZW od zdrowych dawców.

13. SUMMARY

Acute coronary syndromes (ACS) refers to a group of cardiological disorders characterized by disturbances in the coronary circulation caused by a sudden or progressive occlusion of the coronary artery lumen. According to the data presented by the World Health Organization, it is estimated that 32% of all deaths worldwide are caused by cardiovascular diseases, with an emphasis on myocardial infarction and stroke. A crucial role in the pathophysiology of ACS is performed by the pathological activation of blood platelets, which lead to the induction of thrombotic alterations within the coronary arteries.

The main goal of this doctoral dissertation was to determine the transcriptome and proteome disorders of blood platelets, which could constitute the molecular basis of their augmented pro-thrombotic activity in ACS, and to identify molecules with the high sensitivity potential that could determine human predisposition to the occurrence of ACS.

The performed analysis allowed to identify 8 miRNA molecules (hsa-miR-223-3p, hsa-miR-142-3p, hsa-miR-21-5p, hsa-miR-107, hsa-miR-221-3p, hsa-miR-301a-3p, hsa-miR-130b-3p, hsa-miR-338-3p), which expression level differentiated ACS patients from control group. Statistical modeling carried out for the selection of ACS potential biomarker allowed to designate hsa-miR-142-3p along with AST to a combined diagnostic model that differentiated the compared groups with 82% sensitivity and 88% specificity (while the model based on AST only achieved 70% sensitivity and 86% specificity).

Moreover, in the presented doctoral dissertation, results showed a statistically significant elevation in the expression (on the mRNA and protein level) of blood platelet surface receptors for ADP (P2Y12) and fibrinogen (GP IIb/IIIa) in the ACS group. The ROC curve performed for the model based on the expression of the P2Y12 receptor at the mRNA and protein level along with the expression of hsa-miR-223-3p presented 97% sensitivity and 74% specificity in differentiating ACS from the control group.

Results obtained from the screening platelet proteome analysis followed by the quantification of transcripts for the identified differentially expressed proteins showed an increased expression of transgelin-2 as a potential cause of augmented platelet pro-thrombotic activity in ACS.

To conclude, proteomic and transcriptomic changes (at the mRNA and miRNA levels) identified in blood platelets, indicate the molecular basis of their increased pro-thrombotic activity in ACS. Moreover, detected alterations in the expression of platelet miRNAs compared between studied groups showed the ability to differentiate patients with ACS from healthy donors.

14. BIBLIOGRAFIA

1. Collet, J.-P.; Thiele, H.; Barbato, E.; Barthélémy, O.; Bauersachs, J.; Bhatt, D.L.; Dendale, P.; Dorobantu, M.; Edvardsen, T.; Folliguet, T.; i wsp. *2020 ESC Guidelines for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation*. Eur Heart J **2021**, 42(14), 1289–1367, doi:10.1093/eurheartj/ehaa575.

2. Smith, J.N.; Negrelli, J.M.; Manek, M.B.; Hawes, E.M.; Viera, A.J. *Diagnosis and Management of Acute Coronary Syndrome: An Evidence-Based Update*. J Am Board Fam Med **2015**, 28(2), 283–293, doi:10.3122/jabfm.2015.02.140189.

3. Santos-Gallego, C.G.; Picatoste, B.; Badimón, J.J. *Pathophysiology of Acute Coronary Syndrome*. Curr Atheroscler Rep **2014**, 16(4), 401, doi:10.1007/s11883-014-0401-9.

4. Stakos, D.A.; Tziakas, D.N.; Stellos, K. *Mechanisms of platelet activation in acute coronary syndromes*. Curr Vasc Pharmacol **2012**, 10(5), 578–588, doi:10.2174/157016112801784477.

5. Ghoshal, K.; Bhattacharyya, M. *Overview of Platelet Physiology: Its Hemostatic and Nonhemostatic Role in Disease Pathogenesis*. Sci World J **2014**, 2014, 781857, doi:10.1155/2014/781857.

6. Cox, K.; Price, V.; Kahr, W.H.A. *Inherited platelet disorders: a clinical approach to diagnosis and management*. Expert Rev Hematol **2011**, 4(4), 455–472, doi:10.1586/ehm.11.41.

7. French, D.L.; Seligsohn, U. *Platelet Glycoprotein IIb/IIIa Receptors and Glanzmann's Thrombasthenia*. Arterioscler Thromb Vasc Biol **2000**, 20(3), 607–610, doi:10.1161/01.ATV.20.3.607.

8. Nurden, A.T.; Fiore, M.; Nurden, P.; Pillois, X. *Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models*. Blood **2011**, 118(23), 5996–6005, doi:10.1182/blood-2011-07-365635.

9. Ozelo, M.C.; Origa, A.F.; Aranha, F.J.P.; Mansur, A.P.; Annichino-Bizzacchi, J.M.; Costa, F.F.; Pollak, E.S.; Arruda, V.R. *Platelet glycoprotein Ibalpha polymorphisms modulate the risk for myocardial infarction*. Thromb Haemost **2004**, 92(2), 384–386, doi:10.1160/TH03-12-0744.

10. Weiss, E.J.; Bray, P.F.; Tayback, M.; Schulman, S.P.; Kickler, T.S.; Becker, L.C.; Weiss, J.L.; Gerstenblith, G.; Goldschmidt-Clermont, P.J. *A Polymorphism of a Platelet Glycoprotein Receptor as an Inherited Risk Factor for Coronary Thrombosis*. N Engl J Med **1996**, 334(17), 1090–1094, doi:10.1056/NEJM199604253341703.

11. Ardissino, D.; Mannucci, P.M.; Merlini, P.A.; Duca, F.; Fetiveau, R.; Tagliabue, L.; Tubaro, M.; Galvani, M.; Ottani, F.; Ferrario, M.; i wsp. *Prothrombotic Genetic Risk Factors in Young Survivors of Myocardial Infarction*. Blood **1999**, 94(1), 46–51, doi:10.1182/blood.V94.1.46.413a27_46_51.

12. Bojesen, S.E.; Juul, K.; Schnohr, P.; Tybjaerg-Hansen, A.; Nordestgaard, B.G.; *Copenhagen City Heart Study Platelet glycoprotein IIb/IIIa Pl(A2)/Pl(A2) homozygosity associated with risk of ischemic cardiovascular disease and myocardial infarction in young men: The Copenhagen City Heart Study*. J Am Coll Cardiol **2003**, 42(4), 661–667, doi:10.1016/s0735-1097(03)00781-2.

13. Dupont, A.; Fontana, P.; Bachelot-Loza, C.; Reny, J.-L.; Bièche, I.; Desvard, F.; Aiach, M.; Gaussem, P. *An intronic polymorphism in the PAR-1 gene is associated with platelet receptor density and the response to SFLLRN*. Blood **2003**, 101(5), 1833–1840, doi:10.1182/blood-2002-07-2149.

14. Hetherington, S.L.; Singh, R.K.; Lodwick, D.; Thompson, J.R.; Goodall, A.H.; Samani, N.J. *Dimorphism in the P2Y1 ADP Receptor Gene Is Associated With Increased Platelet Activation Response to ADP*. Arterioscler Thromb Vasc Biol **2005**, 25,(1) 252–257, doi:10.1161/01.ATV.0000148708.44691.27.

15. Zoheir, N.; Abd Elhamid, S.; Abulata, N.; El Sobky, M.; Khafagy, D.; Mostafa, A. *P2Y12 receptor gene polymorphism and antiplatelet effect of clopidogrel in patients with coronary artery disease after coronary stenting*. Blood Coagul Fibrinolysis **2013**, 24(5), 525–531, doi:10.1097/MBC.0b013e32835e98bf.

16. Saboor, M.; Ayub, Q.; Ilyas, S.; Moinuddin. *Platelet Receptors; an instrumental of platelet physiology*. Pak J Med Sci **2013**, 29(3), 891–896, doi:10.12669/pjms.293.3497.

17. Berndt, M.C.; Shen, Y.; Dopheide, S.M.; Gardiner, E.E.; Andrews, R.K. *The vascular biology of the glycoprotein Ib-IX-V Complex*. Thromb Haemost **2001**, 86(1), 178–188.

18. Li, R.; Emsley, J. *The organizing principle of platelet glycoprotein Ib-IX-V complex*. J Thromb Haemost **2013**, 11(4), 605–614, doi:10.1111/jth.12144.

19. Esen, F.I.; Hancer, V.S.; Küçükkaya, R.D.; Yeşilot, N.; Coban, O.; Bahar, S.; Tuncay, R. *Glycoprotein Ib-alpha Kozak polymorphism in ischemic stroke*. Neurol Res **2012**, 34(1), 68–71, doi:10.1179/1743132811Y.0000000061.

20. Wagner, CL.; Mascelli, MA.; Neblock, DS.; Weisman, HF.; Coller, BS.; Jordan, RE. *Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets*. Blood **1996**, 88(3), 907–914, doi:10.1182/blood.V88.3.907.907.

21. Ostrowska, M.; Kubica, J.; Adamski, P.; Kubica, A.; Eyileten, C.; Postula, M.; Toma, A.; Hengstenberg, C.; Siller-Matula, J.M. *Stratified Approaches to Antiplatelet Therapies Based on Platelet Reactivity Testing*. Front Cardiovascr Med **2019**, 6, 176,

doi:10.3389/fcvm.2019.00176.

22. Ibanez, B.; James, S.; Agewall, S.; Antunes, M.J.; Bucciarelli-Ducci, C.; Bueno, H.; Caforio, A.L.P.; Crea, F.; Goudevenos, J.A.; Halvorsen, S.; i wsp. *2017 ESC Guidelines for the Management*

of Acute Myocardial Infarction in Patients Presenting with ST-Segment Elevation: The Task Force for the Management of Acute Myocardial Infarction in Patients Presenting with ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J **2018**, 39(2), 119–177, doi:10.1093/eurheartj/ehx393.

23. Nieman, M.T. *Protease-activated receptors in hemostasis*. Blood **2016**, 128(2), 169–177, doi:10.1182/blood-2015-11-636472.

24. Zhang, J.-H.; Wang, J.; Tang, X.-F.; Yao, Y.; Zhang, Y.; Ma, Y.-L.; Xu, B.; Gao, R.-L.; Song, L.; Gao, Z.; i wsp. *Effect of platelet receptor gene polymorphisms on outcomes in ST-Elevation myocardial infarction patients after percutaneous coronary intervention*. Platelets **2016**, 27(1), 75–79, doi:10.3109/09537104.2015.1034096.

25. Cattaneo, M. *P2Y12 receptors: structure and function*. J Thromb Haemost **2015**, 13, S10–S16, doi:10.1111/jth.12952.

26. Angiolillo, D.J.; Fernandez-Ortiz, A.; Bernardo, E.; Ramírez, C.; Cavallari, U.; Trabetti, E.; Sabaté, M.; Jimenez-Quevedo, P.; Hernández, R.; Moreno, R.; i wsp. *Lack of association between the P2Y12 receptor gene polymorphism and platelet response to clopidogrel in patients with coronary artery disease*. Thromb Res **2005**, 116(6), 491–497, doi:10.1016/j.thromres.2005.03.001.

27. Cavallari, U.; Trabetti, E.; Malerba, G.; Biscuola, M.; Girelli, D.; Olivieri, O.; Martinelli, N.; Angiolillo, D.J.; Corrocher, R.; Pignatti, P.F. *Gene sequence variations of the platelet P2Y12 receptor are associated with coronary artery disease*. BMC Med Genet **2007**, 8, 59, doi:10.1186/1471-2350-8-59.

28. Schettert, I.T.; Pereira, A.C.; Lopes, N.H.; Hueb, W.A.; Krieger, J.E. *Association between platelet P2Y12 haplotype and risk of cardiovascular events in chronic coronary disease*. Thromb Res **2006**, 118(6), 679–683, doi:10.1016/j.thromres.2005.11.009.

29. Yang, C.; Deng, Z.; Li, J.; Ren, Z.; Liu, F. *Meta-analysis of the relationship between interleukin-6 levels and the prognosis and severity of acute coronary syndrome*. Clinics (Sao Paulo) **2021**, 76, 2690, doi:10.6061/clinics/2021/e2690.

30. Fanola, C.L.; Morrow, D.A.; Cannon, C.P.; Jarolim, P.; Lukas, M.A.; Bode, C.; Hochman, J.S.; Goodrich, E.L.; Braunwald, E.; O'Donoghue, M.L. *Interleukin-6 and the Risk of Adverse Outcomes in Patients After an Acute Coronary Syndrome: Observations From the SOLID-TIMI 52 (Stabilization of Plaque Using Darapladib—Thrombolysis in Myocardial Infarction 52) Trial*. J Am Heart Assoc **2017**, 6(10), 005637, doi:10.1161/JAHA.117.005637.

31. Goel, H.; Melot, J.; Krinock, M.D.; Kumar, A.; Nadar, S.K.; Lip, G.Y.H. *Heart-type fatty acid-binding protein: an overlooked cardiac biomarker*. Ann Med **2020**, 52(8), 444–461, doi:10.1080/07853890.2020.1800075.

32. Ruzgar, O.; Bilge, A.K.; Bugra, Z.; Umman, S.; Yilmaz, E.; Ozben, B.; Umman, B.; Meric, M. *The use of human heart-type fatty acid-binding protein as an early diagnostic biochemical marker of myocardial necrosis in patients with acute coronary syndrome, and its comparison with troponin-T and creatine kinase-myocardial band*. Heart Vessels **2006**, 21(5), 309–314, doi:10.1007/s00380-006-0908-2.

33. Cavus, U.; Coskun, F.; Yavuz, B.; Ciftci, O.; Sahiner, L.; Aksoy, H.; Deniz, A.; Ozakin, E.; Aytemir, K.; Tokgozoglu, L.; i wsp. *Heart-type, fatty-acid binding protein can be a diagnostic marker in acute coronary syndromes*. J Natl Med Assoc **2006**, 98(7), 1067–1070.

34. Wang, X-Y.; Zhang, F.; Zhang, C.; Zheng, L-R.; Yang, J. *The Biomarkers for Acute Myocardial Infarction and Heart Failure*. Biomed Res Int **2020**, 2020, 2018035, doi:10.1155/2020/2018035.

35. Gilardi, E.; Iacomini, P.; Marsiliani, D.; De Marco, G.; Covino, M. *Biomarkers in the prediction and management of acute coronary syndrome.* Res Rep Clin Cardiol **2013**, 2014(5), 21–31.

36. Quévillon Huberdeau, M.; Simard, M.J. *A guide to microRNA-mediated gene silencing*. FEBS J **2019**, 286(4), 642–652, doi:10.1111/febs.14666.

37. Kozomara, A.; Birgaoanu, M.; Griffiths-Jones, S. *miRBase: from microRNA sequences to function.* Nucleic Acids Res **2019**, 47(D1), D155–D162, doi:10.1093/nar/gky1141.

38. Tijsen, A.J.; Pinto, Y.M.; Creemers, E.E. *Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases*. Am J Physiol Heart Circ Physiol **2012**, 303(9), H1085-1095, doi:10.1152/ajpheart.00191.2012.

39. Fichtlscherer, S.; De Rosa, S.; Fox, H.; Schwietz, T.; Fischer, A.; Liebetrau, C.; Weber, M.; Hamm, C.W.; Röxe, T.; Müller-Ardogan, M.; i wsp. *Circulating MicroRNAs in Patients with Coronary Artery Disease*. Circ Res **2010**, 107(5), 677–684, doi:10.1161/CIRCRESAHA.109.215566.

40. Diehl, P.; Fricke, A.; Sander, L.; Stamm, J.; Bassler, N.; Htun, N.; Ziemann, M.; Helbing, T.; El-Osta, A.; Jowett, J.B.M.; i wsp. *Microparticles: major transport vehicles for distinct microRNAs in circulation*. Cardiovasc Res **2012**, 93(4), 633–644, doi:10.1093/cvr/cvs007.

41. Weber, M.; Baker, M.B.; Patel, R.S.; Quyyumi, A.A.; Bao, G.; Searles, C.D. *MicroRNA Expression Profile in CAD Patients and the Impact of ACEI/ARB*. Cardiol Res Pract **2011**, 2011, 532915, doi:10.4061/2011/532915.

42. Corsten, M.F.; Dennert, R.; Jochems, S.; Kuznetsova, T.; Devaux, Y.; Hofstra, L.; Wagner, D.R.; Staessen, J.A.; Heymans, S.; Schroen, B. *Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease.* Circ Cardiovasc Genet **2010**, 3(6), 499–506, doi:10.1161/CIRCGENETICS.110.957415.

43. D'Alessandra, Y.; Devanna, P.; Limana, F.; Straino, S.; Di Carlo, A.; Brambilla, P.G.; Rubino, M.; Carena, M.C.; Spazzafumo, L.; De Simone, M.; i wsp. *Circulating microRNAs are new and sensitive biomarkers of myocardial infarction.* Eur Heart J **2010**, 31(22), 2765–2773, doi:10.1093/eurheartj/ehq167.

44. Gidlöf, O.; Andersson, P.; van der Pals, J.; Götberg, M.; Erlinge, D. *Cardiospecific MicroRNA Plasma Levels Correlate with Troponin and Cardiac Function in Patients with ST Elevation Myocardial Infarction, Are Selectively Dependent on Renal Elimination, and Can Be Detected in Urine Samples*. Cardiology **2011**, 118(4), 217–226, doi:10.1159/000328869.

45. Stratz, C.; Nührenberg, T.G.; Binder, H.; Valina, C.M.; Trenk, D.; Hochholzer, W.; Neumann, F.J.; Fiebich, B.L. *Micro-array profiling exhibits remarkable intra-individual stability of human platelet micro-RNA*. Thromb Haemost **2012**, 107(4), 634–641, doi:10.1160/TH11-10-0742.

46. Nagalla, S.; Shaw, C.; Kong, X.; Kondkar, A.A.; Edelstein, L.C.; Ma, L.; Chen, J.; McKnight, G.S.; López, J.A.; Yang, L.; i wsp. *Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity*. Blood **2011**, 117(19), 5189–5197, doi:10.1182/blood-2010-09-299719.

47. Sherman, B.T.; Hao, M.; Qiu, J.; Jiao, X.; Baseler, M.W.; Lane, H.C.; Imamichi, T.; Chang, W. *DAVID: A Web Server for Functional Enrichment Analysis and Functional Annotation of Gene Lists (2021 Update).* Nucleic Acids Res **2022**, gkac194, doi:10.1093/nar/gkac194.

48. Huang, J.; Swieringa, F.; Solari, F.A.; Provenzale, I.; Grassi, L.; De Simone, I.; Baaten, C.C.F.M.J.; Cavill, R.; Sickmann, A.; Frontini, M.; i wsp. *Assessment of a complete and classified platelet proteome from genome-wide transcripts of human platelets and megakaryocytes covering platelet functions*. Sci Rep **2021**, 11, 12358, doi:10.1038/s41598-021-91661-x.

49. Szklarczyk, D.; Gable, A.L.; Nastou, K.C.; Lyon, D.; Kirsch, R.; Pyysalo, S.; Doncheva, N.T.; Legeay, M.; Fang, T.; Bork, P.; i wsp. *The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets.* Nucleic Acids Res **2021**, 49(D1), D605–D612, doi:10.1093/nar/gkaa1074.

50. Kieffer, N.; Guichard, J.; Farcet, J.P.; Vainchenker, W.; Breton-Gorius, J. *Biosynthesis of major platelet proteins in human blood platelets*. Eur J Biochem **1987**, 164(1), 189–195, doi:10.1111/j.1432-1033.1987.tb11010.x.

51. Yakushkin, V.V.; Zyuryaev, I.T.; Khaspekova, S.G.; Sirotkina, O.V.; Ruda, M.YA.; Mazurov, A.V. *Glycoprotein IIb-IIIa content and platelet aggregation in healthy volunteers and patients with acute coronary syndrome*. Platelets **2011**, 22(4), 243–251, doi:10.3109/09537104.2010.547959.

52. Hu, L.; Chang, L.; Zhang, Y.; Zhai, L.; Zhang, S.; Qi, Z.; Yan, H.; Yan, Y.; Luo, X.; Zhang, S.; i wsp. *Platelets Express Activated P2Y12 Receptor in Patients With Diabetes Mellitus*. Circulation **2017**, 136(9), 817–833, doi:10.1161/CIRCULATIONAHA.116.026995.

53. Dziedzic, A.; Miller, E.; Saluk-Bijak, J.; Niwald, M.; Bijak, M. *The Molecular Aspects of Disturbed Platelet Activation through ADP/P2Y12 Pathway in Multiple Sclerosis*. Int J Mol Sci **2021**, 22(12), 6572, doi:10.3390/ijms22126572.

54. Landry, P.; Plante, I.; Ouellet, D.L.; Perron, M.P.; Rousseau, G.; Provost, P. *Existence of a MicroRNA Pathway in Anucleate Platelets*. Nat Struct Mol Biol **2009**, 16(9), 961–966, doi:10.1038/nsmb.1651.

55. Li, S.; Guo, L.Z.; Kim, M.H.; Han, J.-Y.; Serebruany, V. *Platelet microRNA for predicting acute myocardial infarction*. J Thromb Thrombolysis **2017**, 44(4), 556–564, doi:10.1007/s11239-017-1537-6.

56. Hromadka, M.; Motovska, Z.; Hlinomaz, O.; Kala, P.; Tousek, F.; Jarkovsky, J.; Beranova, M.; Jansky, P.; Svoboda, M.; Krepelkova, I.; i wsp. *MiR-126-3p and MiR-223-3p as Biomarkers for Prediction of Thrombotic Risk in Patients with Acute Myocardial Infarction and Primary Angioplasty*. J Pers Med **2021**, 11(6), 508, doi:10.3390/jpm11060508.

57. Shi, R.; Ge, L.; Zhou, X.; Ji, W.-J.; Lu, R.-Y.; Zhang, Y.-Y.; Zeng, S.; Liu, X.; Zhao, J.-H.; Zhang, W.-C.; i wsp. *Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity*. Thromb Res **2013**, 131(6), 508–513, doi:10.1016/j.thromres.2013.02.015.

58. Zhang, Y.-Y.; Zhou, X.; Ji, W.-J.; Shi, R.; Lu, R.-Y.; Li, J.-L.; Yang, G.-H.; Luo, T.; Zhang, J.-Q.; Zhao, J.-H.; i wsp. *Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome*. J Thromb Thrombolysis **2014**, 38(1), 65–72, doi:10.1007/s11239-013-1022-9.

59. Liu, J.; Qin, L.; Wang, Z.; Peng, L.; Liu, J.; Wang, X.; Du, R.; Zou, Y.; Wu, Y.; Yin, T. *Platelet-derived miRNAs as determinants of the antiplatelet response in clopidogrel-treated patients with ACS*. Thromb Res **2020**, 186, 71–74, doi:10.1016/j.thromres.2019.12.016.

60. Kazimierczyk, E.; Eljaszewicz, A.; Kazimierczyk, R.; Tynecka, M.; Zembko, P.; Tarasiuk, E.; Kaminski, K.; Sobkowicz, B.; Moniuszko, M.; Tycinska, A. *Altered microRNA dynamics in acute coronary syndrome*. Adv Interv Cardiol **2020**, 16(3), 287–293, doi:10.5114/aic.2020.99263.

61. Mitsios, J.V.; Prevost, N.; Kasirer-Friede, A.; Gutierrez, E.; Groisman, A.; Abrams, C.S.; Wang, Y.; Litvinov, R.I.; Zemljic-Harpf, A.; Ross, R.S.; i wsp. *What is vinculin needed for in platelets?* J Thromb Haemost **2010**, 8(10), 2294–2304, doi:10.1111/j.1538-7836.2010.03998.x.

62. Major, J.L.; Bagchi, R.A.; Pires da Silva, J. *Application of MicroRNA Database Mining in Biomarker Discovery and Identification of Therapeutic Targets for Complex Disease*. Methods Protoc **2020**, 4(1), 5, doi:10.3390/mps4010005.

63. Na, B.-R.; Kim, H.-R.; Piragyte, I.; Oh, H.-M.; Kwon, M.-S.; Akber, U.; Lee, H.-S.; Park, D.-S.; Song, W.K.; Park, Z.-Y.; i wsp. *TAGLN2 regulates T Cell activation by stabilizing the actin cytoskeleton at the immunological synapse*. J Cell Biol **2015**, 209(1), 143–162, doi:10.1083/jcb.201407130.

64. Stelzer, G.; Rosen, N.; Plaschkes, I.; Zimmerman, S.; Twik, M.; Fishilevich, S.; Stein, T.I.; Nudel, R.; Lieder, I.; Mazor, Y.; i wsp. *The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses*. Current Protocols in Bioinformatics **2016**, 54, 1.30.1-1.30.33, doi:10.1002/cpbi.5.

65. UniProt Consortium *UniProt: A Worldwide Hub of Protein Knowledge*. Nucleic Acids Res **2019**, 47(D1), D506–D515, doi:10.1093/nar/gky1049.

66. Assinder, S.J.; Stanton, J.-A.L.; Prasad, P.D. *Transgelin: an actin-binding protein and tumour suppressor*. Int J Biochem Cell Biol **2009**, 41(3), 482–486, doi:10.1016/j.biocel.2008.02.011.

67. Sheu, J.R.; Fong, T.H.; Liu, C.M.; Shen, M.Y.; Chen, T.L.; Chang, Y.; Lu, M.S.; Hsiao, G. *Expression of matrix metalloproteinase-9 in human platelets: regulation of platelet activation in in vitro and in vivo studies*. Br J Pharmacol **2004**, 143(1), 193–201, doi:10.1038/sj.bjp.0705917.

PUBLIKACJE

Clinica Chimica Acta 496 (2019) 84–92

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Review

Blood platelet surface receptor genetic variation and risk of thrombotic episodes

Rafal Szelenberger[a,*], Michal Kacprzak[b], Michal Bijak[a], Joanna Saluk-Bijak[a], Marzenna Zielinska[b]

[a] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland
[b] Intensive Cardiac Therapy Clinic, Medical University of Lodz, Pomorska 251, 91-213 Lodz, Poland

ARTICLE INFO

Keywords:
Cardiovascular disease
Acute coronary syndrome
Platelet surface receptors
Polymorphism

ABSTRACT

Haemostasis is a set of processes whose main task is to prevent blood loss by creating barriers in damaged vessels. Because of the large number of platelet surface receptors and their many agonists, platelets can be activated in normal and pathologic states leading to thromboembolic complications. Although age, blood pressure, LDL and HDL, diabetes, lack of physical activity, obesity and stress are well established risk factors, recent work has shown that platelet receptor polymorphisms also impact platelet function. The most common polymorphisms include 14A/T (PAR-1), 139C/T, 744T/C, 52G/T, i-ins801A (P2Y12), 1622A/G, −5T/C (GPIbα) 1565C/T (GPIIb/IIIa) and 807C/T (GPIa/IIa). This review examines the influence of these polymorphisms on cardiovascular disease including myocardial infarction, deep venous thromboembolism and acute coronary syndromes. Elucidation of these genetic variations will facilitate our understanding of the complex molecular mechanisms involved with physiologic and pathophysiologic platelet activation and clot formation.

1. Introduction

Blood platelets are the smallest, un-nucleated morphotic elements of the blood that play a key role in maintaining normal haemostasis, i.e. the physiological balance between the processes of pro- and anti-coagulation. Platelets are formed from fragments of the bone marrow's megakaryocytic cytoplasm. They have a discoidal shape and quantity ranging from 140 to 440*10^9/L. The vitality of the platelet counts lasts from 8 to 12 days. They are removed from the bloodstream by the reticuloendothelial system in the spleen in the phagocytosis process [1].

Platelets are surrounded by a cell membrane that forms two layers of lipids in which proteins are distributed. Phospholipids, such as phosphatidylserine, phosphatidylethanolamine and others, dominate among the lipids. Some proteins passing through both layers of the cell membrane are linked to oligosaccharides, thus forming glycoproteins, which are receptors for many of the factors that activate or inhibit platelet function [2].

The platelet cytoskeleton is responsible for maintaining the shape of the platelet and its changes during activation. The primary ingredients of the cytoskeleton are alpha and beta tubulin, actin polymers, and proteins mediating their association. In the construction of blood platelets, we can also distinguish intracellular structures such as: an open canalicular system (OCS); a dense tubular system for storage of calcium ions; enzymes directing the transformation of arachidonic acid; single mitochondria; glycogen particles; peroxisomes, and some Golgi's apparatus. Numerous specific granules dispersed in the cytoplasm include: α-granules, containing platelet factor 4; fibrinogen; von Willebrand factor (vWF) or B-thromboglobulin, and various dense granules in which ADP, ATP, serotonin, and catecholamines are stored [1,2].

Platelets play a pivotal role in primary and secondary haemostasis. In primary haemostasis, the first stage is known as adhesion and sees the indirect binding of GP Ib/IX/V and IIb/IIIa glycoprotein to the damaged vessel by vWF factor and other platelet glycoproteins. These bind with the collagen in the subendothelial matrix. Releasing the biologically active substances from a platelet's granules causes recruitment of further platelets, thereby enhancing their activation and aggregation process [2]. Specialised glycoprotein receptors provide platelets for adhesion to the proteins, which are exposed in areas of vascular damage. The process of adhesion and/or the interaction of soluble agonists with receptors on the platelet activates the platelet integrin receptors to fibrinogen, and then the platelets are able to aggregate together. This aggregation creates a platelet plug that seals the breach in the vessel wall and prevents from excess blood loss [3]. Activated platelets then facilitate secondary haemostasis, the formation of a fibrin clot, by carrying coagulation factors and providing a catalytic surface for the major interactions of the coagulation cascade [4,5]. Activation of the blood platelets is a multi-step process in which various responses occur: shape change; adherence to vessel walls; secretion of

* Corresponding author.
E-mail address: rafal.szelenberger@unilodz.eu (R. Szelenberger).

https://doi.org/10.1016/j.cca.2019.06.020
Received 27 April 2019; Received in revised form 19 June 2019
Available online 22 June 2019

biologically active compounds from granules; aggregation; expression of P-selectin; phosphorylation of specific proteins; exposure of anionic phospholipids on the extracellular surface of the platelet membrane, and release of microparticles rich in pro-coagulant activity. Morphological changes are accompanied by biochemical pathways: the enzymatic cascade of arachidonic acid, the change in concentration of cAMP and cGMP, activation of kinases and phosphorylation of proteins, the formation of reactive oxygen species (ROS) and the transformation of phosphatidylinositols [6,7]. Despite the absence of the nucleus, platelet activation is very complicated, and is associated with signal transduction through a number of surface receptors in the cell membrane of platelets combined with elements of enzymatic signal transduction chains [1].

Because of the large number of specific membrane receptors, blood platelets are highly reactive cells, readily activated by many physiological and unphysiological agonists [1]. Signalling pathways via specific receptors are dependent on the type of agonist, but always lead to physiological responses expressed as platelet activation. Platelet activation mediated by the complex series of intracellular processes involved in haemostasis, thrombosis, and inflammation is the most critical risk factor in cardiovascular system disturbance, and is associated with the occurrence of thromboembolic complications [8]. Thromboembolic complications lead to acute ischaemic coronary syndromes, stroke, and deep vein thrombosis, are a cause of death or chronic conditions that limit quality of life and generate high therapy and care costs.

2. The role of platelets in acute coronary syndromes

Acute coronary syndrome (ACS) is any group of clinical symptoms compatible with acute myocardial ischemia. ACS includes unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) [9]. It is well known that acute coronary syndromes with different clinical manifestations have a common pathophysiology, which is associated with coronary artery thrombosis [10]. Platelets are known to play a fundamental role in acute coronary syndromes. Platelets can form pathogenic, occlusive intracoronary thrombus, leading to acute ischaemic events. Platelet adhesion and aggregate formation are critical events that occur in acute coronary syndromes [11].

In the last few years, researchers have described the presence of activated platelets in systemic circulation in various cardiovascular disease states, particularly acute coronary syndromes [12]. Patients with acute coronary syndromes have increased activity and aggregation of blood platelets inside coronary circulation, resulting in partial or complete obstruction of the coronary artery [13]. Platelets contribute to acute thrombosis via a multi-step mechanism. The first step is adhesion to the endothelium. The constituents of the exposed subendothelium then interact, including collagen, vWF, fibronectin, and specific platelet surface membrane receptors. Thus, the platelets overcome the high blood-shear forces and attach themselves to the target endothelium site. After attachment, the platelets present an activation process with a specific conformational change that induces the onset of multiple internal signalling networks. Hyper-reactive platelets accelerate the formation of an intracoronary thrombus, leading to a cascade of clinical events [14].

A recent study evaluated the association between the hyper-reactivity of platelets to ADP and outcomes in patients with stable cardiovascular disease [15]. However, platelets don't only play a role in the formation of coronary artery thrombosis – many types of research indicate that platelets could also play an essential role in the initiation and propagation of atherosclerosis, potentially through interaction of activated platelets with endothelial cells and leukocytes, or by stimulating inflammation through the release of various mediators [16]. Atherosclerotic lesions rely on the creation of atherosclerotic plaque in medium or large arteries, which form a surface for the formation of thrombi. This process leads to a reduction in the lumen of the blood vessels, resulting in partial or complete obstruction. The generation of atherosclerotic plaque can be an effect of hypercholesterolaemia, diabetes, smoking and hypertension. These factors can cause dysfunction of the endothelium, the disrupted functioning of which plays a major role in the process of atherosclerosis. All complicated interactions between the endothelium, inflammatory cells and platelets, contribute to the pathogenesis of ACS. Ruptures of atherosclerotic plaque could be the cause of two-thirds of all cases of ACS. This process is dependent on many factors, such as lipid content of the plaque, blood flow, the prothrombotic and antithrombotic balance in patients, the stability of the atherosclerotic plaque and intensity of the inflammation [9]. Activated platelets release an arsenal of potent inflammatory and mitogenic substances into the local microenvironment, thereby altering the chemotactic, adhesive and proteolytic properties of endothelial cells. These platelet-induced alterations of the endothelial phenotype support chemotaxis, adhesion and the transmigration of monocytes to the site of inflammation. Different mediators, such as adhesion proteins (e.g. fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb-IIIa), growth factors (e.g. PDGF, TGF-β, EGF, bFGF), chemokines (e.g. RANTES, platelet factor 4 [PF4], CXC chemokine ligand 4 [CXCL4]), epithelial neutrophil-activating protein 78 (ENA-78; CXCL5), cytokine-like factors (e.g. IL-1β, CD40L, β-thromboglobulin) and coagulation factors (e.g. factors V, XI, PAI-1, plasminogen, protein S) are released from dense granules, α-granules, lysosomes, canalicular system, and the cytosol of activated platelets. These proteins act in a concerted and fine-regulated manner, influencing a wide variety of biological functions, including cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis – all of which accelerate inflammatory processes and cell recruitment [17].

3. G Protein-coupled receptors in blood platelets

G Protein-Coupled Receptors (GPCR) constitute the largest family of proteins in the human genome responsible for signal transmission through the lipid bilayer to the effector sites in cells. Comparative analysis of GPCR receptor sequences has led to their division into five main families: Rhodopsin, Glutamate, Adhesion, Frizzled/Taste2 and Secretin [18].

GPCR receptor sequences can be activated by a very chemically diverse group of ligands, including amines, lipids, peptides, ions, nucleotides and proteases. Because of their ability to specifically interact with various functionally different heterotrimeric guanine nucleotide-binding proteins (G proteins), agonist-activated GPCRs can induce different signalling pathways to change their cellular function.

The Gq/G11 family of G proteins couple receptors to the β isoforms of phospholipase C (PLC), of which the β2 and β3 isoforms are particularly present in platelets. Activation of PLC results in the formation of IP3 and diacylglycerol, leading to elevation of free cytoplasmic Ca^{2+} and activation of protein kinase C (PKC), respectively. Although most cells in the mammalian organism express both Gq and G11, platelets are an exception to that, containing only Gq. So far, no physiological significance for the lack of G11 in platelets has been reported. G13, a member of the G12/G13 family, has been shown to regulate several signalling pathways of which the Rho/Rho-kinase-mediated pathway is the most well established. Activated G13 binds and activates a subgroup of Rho-specific guanine nucleotide exchange factors. Gi2, the main member of the Gi family expressed on platelet-coupled receptors in an inhibitory fashion to adenylyl cyclase. In addition, Gi-type G proteins are a significant source of βγ complexes, which are released upon G-protein activation and can regulate a variety of channels or enzymes, including adenylyl cyclase and phosphatidylinositol 3-kinases (PI3K). The latter enzyme produces phosphatidylinositol-3,4,5-trisphosphate, which activates a variety of downstream effectors including the serine/threonine kinase Akt/protein kinase B (PKB) [19].

The GPCR family comprises the most extensive class of

pharmaceutically relevant target molecules, with > 30% of the total drugs on the market targeting GPCRs. The great versatility of the G protein-mediated signalling system could explain why it is the primary mediator of the second phase of platelet activation during thrombosis and haemostasis, which requires the fast, coordinated action of a variety of diffusible mediators to activate platelets and recruit them into the growing thrombus [19].

Amisten et al. [20] detected in human platelets mRNA of 28 GPCR genes. Of the 28 verified GPCRs mRNA expressed in platelets, 12 genes could be quantified. The expression level of evaluated genes were normalized to P2Y1, whose expression is defined as 1, and fold-change was converted into percentages. The thrombin receptor PAR1 (1865 ± 178%) was the most abundant GPCR mRNA expressed on platelets, followed by the ADP receptor P2Y12 (459 ± 88%), succinate receptor 1 (257 ± 48%) and the ADP receptor P2Y1 (100%; P2Y1 was chosen as calibrator).

Thrombin is one of the strongest in-vivo platelet agonists. Platelet activation occurs at a much lower concentration of thrombin than is needed for the fibrinogen to fibrin conversion. Thrombin-activated platelets change their shape, secrete the contents of their granules and finally aggregate [21]. Receptors of the Protease-Activated Receptor (PAR) family are responsible for platelets' response to thrombin. Thrombin's attachment with PAR's N-terminus causes its cleavage, thus this terminus binds permanently with the second loop of PAR. PAR1 and PAR4 are present on the human platelets' surface receptors. PAR1 activation occurs at lower concentrations of thrombin than is required in the case of receptor PAR4. Therefore, platelet activation is responsible mainly for PAR1, whereas PAR4 only has ancillary functions. The number of copies of the PAR1 receptor on the blood platelets' surface ranges from 1500 to 2000. Thrombin cleaves the N-terminal extracellular domain of PAR to expose a new N-terminus, which binds to the central extracellular loop of the same receptor, causing its activation and initiating the intracellular signalling events. The PAR1 receptor is associated with three G proteins; G13, Gq and Gi. Thus, activation of the PAR1 receptor can result in a platelet response, dependent on a variety of intracellular signal transduction pathways [22,23].

For the PAR1 receptor, three polymorphisms are described: 1426C/T, 506I/D and 14A/T. In contrast to 14A/T polymorphism, which is related with PAR1 expression level, studies show that 1426C/T and 506I/D polymorphism are not associated with PAR1 phenotype. Analysis of the platelets' PAR1 genotype according to the PAR1 phenotype shows that the polymorphism 14A/T is associated with platelet response to the thrombin receptor activating peptide – SFLLRN (TRAP). Homozygous carriers of the 14A allele have a significantly augmented level of PAR1 than heterozygous carriers. Volunteers with two copies of 14A allele also had a higher expression level of P-selectin than heterozygous. It has been suggested that the phenotype effect of 14A/T polymorphism of the PAR1 receptor could be related with intron splicing, because of its location in intervening sequence (IVS), 14 nucleotides above the exon 2 start site (Table 1) [24].

Zhang et al. [25] showed that polymorphism 14A/T could significantly contribute to the risk of ischaemic events in Chinese patients with ST-elevation myocardial infarction. Patients with at least one T allele had a meaningfully lower risk of ischaemic disease than homozygous carriers of the 14A allele. The Zhang et al. study showed that reduced expression of PAR1 results in decreased platelet reactivity, thus decreasing the risk of ischaemic disease in patients with T allele [25,26].

Table 1
Phenotype effects of 14A/T polymorphism of PAR-1 receptor in platelets in 100 male volunteers.

Genotype	PAR-1 levels in platelets	Platelet secretory response to SFLLRN	P-selectin expression on the platelet surface
AA	1297 ± 186	11,548 ± 1735	11,548 ± 1735
AT/TT	1164 ± 203	10,160 ± 2117	10,530 ± 2078
p value	*0.013*	*0.001*	*0.011*

The ability of ADP to promote platelet aggregation has been recognized for nearly half a century. Platelets release dense granules that contain the nucleotide adenosine diphosphate which activates other platelets, ADP is also passively released from damaged erythrocytes and endothelial cells. Platelet activation by ADP is mediated by two G protein-coupled receptors, P2Y1 and P2Y12. Whereas P2Y1 couples to Gq, P2Y12 couples to the Gi type of G proteins. Studies using receptor agonists have suggested that activation of both receptors is required for a full response by platelets to ADP. P2Y1 is the pathway that initiates platelet activation, while P2Y12 plays a role in strongly amplifying the activation process. The P2Y1 receptor initiates platelet shape change and ADP-induced aggregation through the mobilisation of internal calcium stores, and the P2Y12 receptor, which is coupled to adenylyl cyclase inhibition, is essential for a full aggregation response to ADP and the stabilisation of aggregates [19]. This amplifies not only platelet aggregation but also expands the other functional consequences of activation, including granule release and platelet pro-coagulant activity. Deletion of either P2Y1 or P2Y12 in mice prolongs their bleeding time and impairs platelet responses, not only to ADP, but also to thrombin and TXA2, particularly in low concentrations [26]. Platelet responses to thrombin and TXA2 in low and intermediate concentrations are reduced in the absence of ADP receptors. Since platelet TXA2 receptors do not couple directly to Gi family members, platelet aggregation induced by TXA2 requires the secretion of ADP to inhibit adenylyl cyclase. Both P2Y1 and P2Y12 are involved in ADP-induced platelet pro-coagulant activity. Over the past decade, ADP receptors on the platelet membrane have also become a target for antithrombotic strategies with thienopyridines. Two existing thienopyridine compounds, Ticlopidine and Clopidogrel, have irreversibly inhibited P2Y12 and have been shown to have clinically useful antiplatelet activity for the secondary prevention of cardiovascular events [26].

In healthy donors, four genetic polymorphisms of P2Y12 receptor have been described, including three SNPs (139C/T, 744 T/C, 52G/T), and one nucleotide insertion in the receptor's gene (i-ins801A). This establishes two haplotypes: H1, with C in position 139, T in position 744, G in position 52 and a lack of i-ins801A; and H2, with T in position 139, C in position 744, T in position 52 and the presence of the i-ins801A. The frequencies of those haplotypes were 86% for H1 and 14% for H2 [26,27]. Fontana et al. [27] presented a study in the healthy subjects of which ADP-induced platelet aggregation was associated with an H2 haplotype of the P2Y12 receptor gene (OR = 3.3 [1.1–10.4]). In contrast, in a study conducted by Shu-Jun et al. on a Han Chinese population, the association between haplotypes and the presence of cerebral infarction was not significant ($p > .05$), but the H2 allele frequency was meaningfully higher in patients with cerebral infarction (14.5%), than in the healthy controls (8.6%). The authors suggested that the results obtained may be linked with H2 haplotype distribution, which varies depending on the race of the subjects: 8.6% in the Han Chinese population, 13.8% a in Caucasian population, and 12.8% in a Mexican population (Fig. 1) [28].

Zoheir et al. had a similar result, in which carriers of the C allele in 744 T/C were associated with augmented platelet activation in response to ADP. This suggests that there is a positive correlation between P2Y12 receptor and platelet reactivity [29]. Further, although the 744 T/C polymorphism of the P2Y12 receptor gene has been associated with enhanced platelet aggregation in healthy volunteers, it has not shown any influence on clopidogrel response assessed by ADP [30]. The 744 T/C polymorphism of the P2Y12 receptor gene also does not modulate platelet response to Clopidogrel, either in the early or long-term phases of treatment. Several P2Y12 polymorphisms were found by Hetherington et al. [31], but none of them influenced the reactivity of platelets. In addition, according to Fontana's study, Hetherington et al. did not see differences between haplotypes. The reason for the

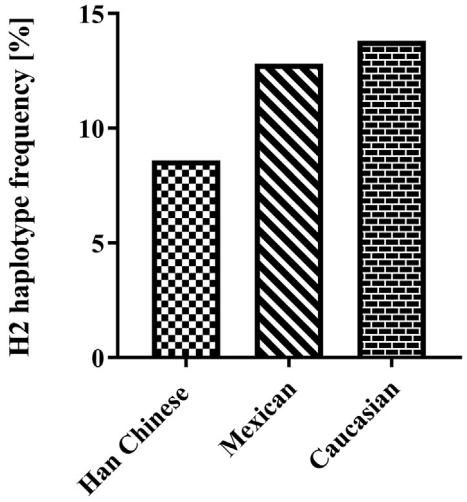


Fig. 1. Haplotype H2 frequency in different Han Chinese, Mexican and Caucasian population [28].

divergences in the results are unclear, although the difference in the age of the volunteers is suspected (Fontana's et al. volunteers had lower age) [31]. In addition, Zhang et al. [25] showed that 52G/T polymorphism is associated with bleeding events (OR = 2.71 [1.298–5.659]). The possible link between the H2 haplotype and augmented platelet aggregation in response to ADP remains unclear. Fontana et al. [27] suggested that the H2 haplotype increases the number of P2Y12 receptors on the platelet surface, and as such higher responsiveness to ADP can occur. They overrule the role of amino acid substitution and splice variants, because of the valid exon 1 to exon 2 junctions revealed by cDNA analysis.

P2Y1 gene variation 1622A/G has also been associated with platelet responsiveness to various ADP concentrations (0.1–10 μM). The response was 130% higher in GG homozygotes than in AA homozygotes when platelets were stimulated with 0.1 μM ADP. Increased platelet response for ADP affects the functioning of the GPIIb-IIIa, which is important in fibrinogen binding. 1622A/G is a silent variation which means, that this SNP is not affecting on the amino acid sequence in the genome and don't have an influence on receptor structure. The molecular mechanism explanation of 1622A/G SNP and platelet response to ADP remains unclear. Hetherington et al. suggested that this polymorphism may affect P2Y1 expression and play a potential role in regulating the promoter region [31]. However, in Fontana et al. [32], performed on 98 healthy Caucasian men, the 1622A/G polymorphism of the P2Y1 gene was only weakly associated with maximal ADP-induced aggregation in univariate analysis, and only if GG homozygotes were pooled with AG heterozygotes. In the same study, none of the P2Y12 variations were associated with platelet responsiveness to ADP. Additionally, Galic et al. [33] presented a study suggesting that 18C/T SNP of the P2Y12 gene may be an independent predictor of pharmacological response to Clopidogrel.

4. Platelet membrane glycoproteins

The initial interaction of platelets with the extracellular matrix involves the platelet vWF receptor GPIb/IX/V. During the next stage of platelet activation, a platelet plug forms through the recruitment of additional platelets from the circulation, and their integrin GPIIb/IIIa-mediated aggregation. GP Ib/IX/V complex, which is critical to platelet adhesion, especially in the initial events that tether the platelets to the subendothelial matrix, binds to vWF. This adhesive bond has a rapid dissociation rate, resulting in platelet translocation on the vessel wall [34]. vWF is multimeric glycoprotein found in 1924 by Erik von Willebrand. In humans, vWF contain 2813 amino acids with 741 amino acids pro-polypeptide and 22 amino acids signal peptide. The transformation of vWF into active form requires a cleavage of pro-polypeptide, resulting in formation of mature vWF containing 2050 amino acids. Analysis of the amino acid sequence shows that vWF has many active domains responsible for its cleavage and functions. For platelets interaction with the extracellular matrix, A1 domain is essential. It contains a binding site for GPIbα, a subunit of GP Ib/IX/V complex. The A2 domain contains the cleavage site and A3 domain contain a binding site for collagen. Very important role in haemostasis is also played by C1 domain, which is a binding site for GP IIb/IIIa (Fig. 2) [35].

Despite the fact that vWF is synthesized and stored in endothelium cells, platelets and megakariocytes, studies showed that most of the circulating vWF has endothelial origin, and platelet/megakaryocyte derived vWF constitute approximately 15%. Under physiological conditions, vWF do not interact with GPIbα. However, high shear forces in bloodstream results in immobilization of vWF and exposition of A1 domain, allowing to receptor-ligand interaction. What is more, binding the vWF with GPIbα results in exposition of GP IIb/IIIa which binds fibrinogen, thus increasing aggregation process [36]. Evidence indicating the significance of the functions performed by vWF is von Willebrand Disease (vWD), which is responsible for bleeding disorders caused by absence of vWF [37]. Above evidences shows that GP Ib/IX/V is essential in platelet adhesion to activate endothelial cells. Furthermore, GP Ib/IX/V is important in leukocyte recruitment to sites of vascular injury, and in thrombin-mediated platelet activation and coagulation, including microparticle formation. Recently, significant discoveries about the signalling pathways that regulate GP Ib/IX/V have been made; these pathways could be potential targets for antithrombotic drug development [38]. Kieffer et al. [39] described the biosynthesis of GPIb in blood platelets.

The GP Ib/IX/V complex plays an essential role in the initial phase of platelet-vessel wall interaction, which leads to the activation of integrin GP IIb/IIIa and contributes to GP IIb/IIIa-dependent platelet adhesion, spreading and aggregation [38].

Recently, a T/C polymorphism was identified in the Kozak sequence of glycoprotein Ibα gene at position −5 from the initiator ATG codons [40]. Kozak consensus sequence was originally described by Marilyn Kozak. His study revealed that all functional AUG triplets, known as start codons, are preceded by purine in position −3 and after AUG. Current state of knowledge shows that Kozak sequence is necessary for efficient initiation of translation process, because the recognition of this sequence by ribosomes constitute protein translational start site. Amino acid chain is identified by the following notation: (GCC)GCCAGCC**A UGG** [41]. The presence of the -5C allele increases the surface expression of GP Ib/IX/V complex, and it has been suggested that higher receptor levels might increase the adhesiveness of the platelets and confer the risk of thrombosis. Esen et al. [40] suggest that GPIbα T/C polymorphism might increase the risk of ischaemic stroke, especially in those with an undetermined aetiology. Blood samples were taken from 231 patients following their first ischaemic stroke event, and from 220 healthy volunteers. After analysis, 156 patients were TT homozygous, 70 patients were heterozygous, and only five patients carried the CC genotype. In the ischaemic stroke group, at least one C allele was highly presented (32.5%), compared to the control group (23%) (OR = 0.61 [0.4–0.93]). Esen et al. compared TT genotypes with heterozygous carriers and the results were important (OR = 0.58 [0.37–0.89]). Data from the Vienna Stroke Registry shows that carriers of the CC genotype have a 3.5-fold higher risk of stroke, compared with TT homozygous and heterozygous. Esen et al. could not confirm this information because of the low number of CC genotype carriers. The homozygous TT Kozak genotype could be a significant factor in recognition of coronary artery disease completed by myocardial infarction. On the other hand, Croft et al. [42] postulate that Kozak sequence polymorphism of the GPIba gene is not a major risk factor for myocardial infarction (OR = 1.03 [0.78–1.36]).

Platelet integrin GPIIb/IIIa (αIIbβ3), also known as the platelet

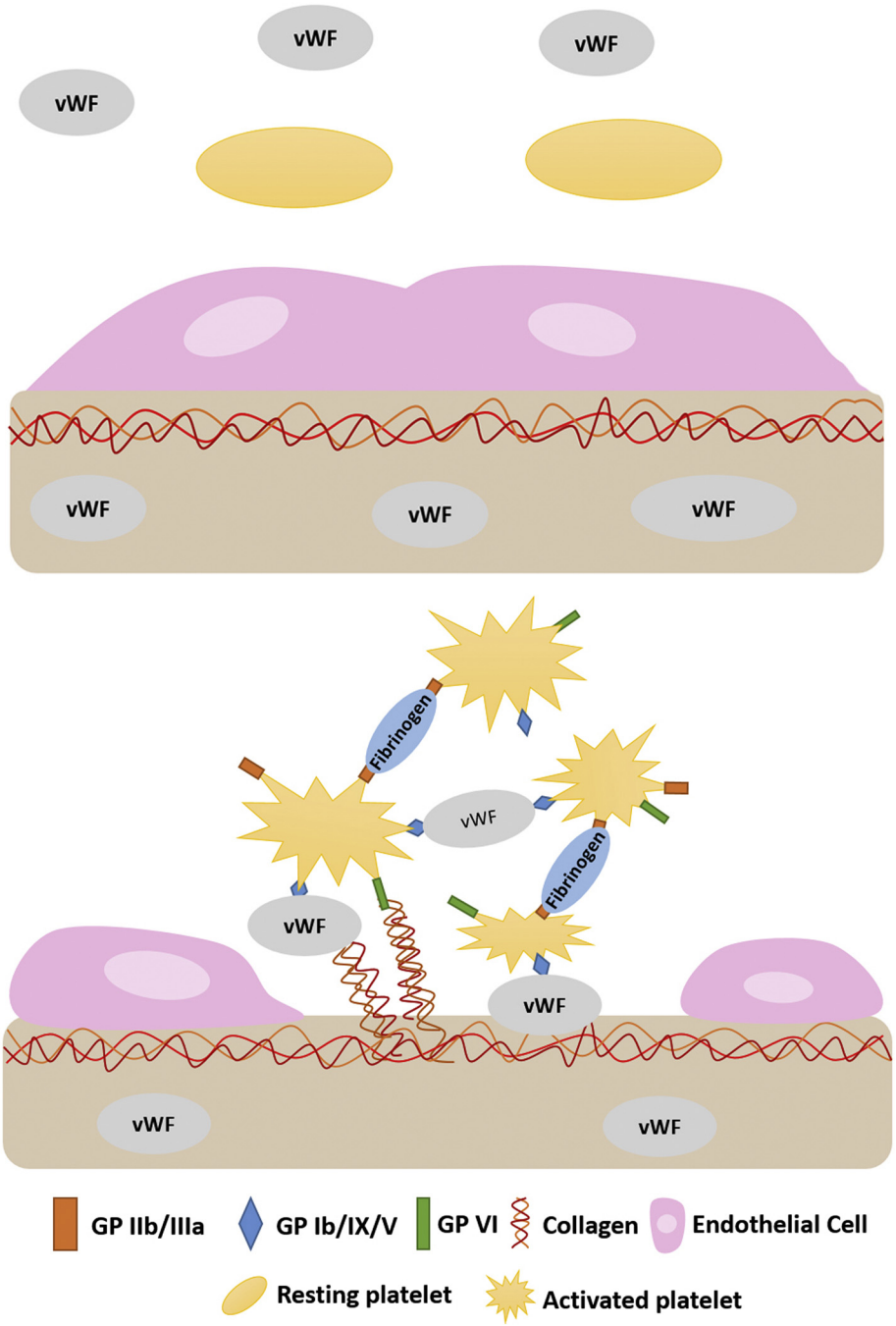


Fig. 2. The role of vWF in haemostasis. Vessel damage caused exposition of collagen and vWF contained in subendothelial matrix. Unique vWF domains bind with collagen (by A3 domain), GPIb/IX/V (by A1 domain) and GP IIb/IIIa (by C1 domain), resulting in blood platelets adhesion, shape change during activation, exposition of platelet surface receptors and aggregation.

fibrinogen receptor, is the major integrin on platelets and plays a crucial role in platelet accumulation on the activated endothelium – platelets spread and form a surface for the recruitment of additional platelets via fibrinogen bridges between two αIIbβ3 receptors. The complex is formed via the calcium-dependent association of subunits GPIIb and GPIIIa. GPIIb/IIIa is able to take two conformations; active and inactive. Only the active form is capable of binding protein ligands. Integrin activation is carried out by exposing the ligand molecule binding sites. A common feature of the ligands that bind to many integrins is the presence of the Arg-Gly-Asp (RGD) amino acid sequence, which was originally identified as the cell-binding domain of fibronectin. This RGD sequence exists in each of the four adhesive proteins that bind to GPIIb/IIIa: fibronectin, vWf, vitronectin, and fibrinogen. Integrin αIIbβ3 also reacts with the KQAGDV (Lys-Gln-Ala-Gly-Asp-Val) sequence, that is found only in the C-terminus of the fibrinogen γ chain [43]. GPIIb/IIIa is the most abundant integrin receptor on the platelet membrane, ranging from 50,000–80,000 copies per platelet. This glycoprotein is essential to platelet protein interactions in plasma and the extracellular matrix during both haemostasis and thrombosis. The GPIIb/IIIa receptor is a target for several antiplatelet drugs, including Abciximab, Eptifibatide, and Tirofiban. These antagonists block the IIb/IIIa receptors and prevent fibrinogen binding to this receptor, thereby blocking the final common pathway of platelet aggregation. They also block the 'outside-in' signalling that occurs during platelet aggregation and causes additional amplification of platelet activation. Defects in glycoprotein IIb/IIIa cause Glanzmann's

thrombasthenia. This is an extremely rare coagulopathy caused by a point mutation at position 119, in which the Asp residue is replaced by Tyr residue in GPIIIa glycoprotein [44]. As a result, fibrinogen bridging of platelets to other platelets cannot occur, and the bleeding time is significantly prolonged. In platelets, the presence of mRNA for both integrin subunits αIIb and β3 has been observed. This indicates that this receptor might be synthesized in the platelets [45]. During storage of platelets, the expression on surface GPIIb/IIIa increases after seven days by 13.4%, and after ten days by 41.9%. The total concentration of GPIIIa after seven days of storage is doubled, and after ten days is increased four-fold [46].

Glycoprotein IIb/IIIa receptors are polymorphic, with the two most common variants being PlA1 and PlA2 (by haematologists often called HPA-1a and HPA-1b respectively). These are caused by a single-point mutation of cytosine to thymidine in exon 2 of the GPIIIa gene, resulting in the substitution of proline (PlA1/HPA-1a) for leucine (PlA2/HPA-1b) at position 33, which results in a single nucleotide 1565C/T transition in the GPIIIa gene and may be associated with thrombotic cardiovascular complications [47]. PlA2 alleles might increase the risk of acute coronary syndromes. The association of the Leu33Pro polymorphism with the incidence of myocardial infarction was first reported by Marian et al. [48]. The strong association between the PlA2 polymorphism of the glycoprotein IIIa gene and acute coronary thrombosis was observed by Weiss et al. [49]. The relation between PIA2 and coronary events was higher in patients younger than 60 when their first coronary event appeared. Carriers of the PIA2 allele are more exposed to coronary heart disease than PIA1 homozygous. Weiss et al. examined 139 people and found that this association was most influential in patients who had unstable angina, or myocardial infarction, before the age of 60 (68 patients), in whom OR = 6.2 (1.8–22.4) compared to all ages OR = 2.8 (1.2–6.4). Adrissino et al. [50] showed that HPA-1b polymorphism is an influence on the higher risk of developing myocardial infarction at a young age. The odds ratio (1.89 [1.13–3.18]) in this study shows that among 200 patients, 54 had the HPA-1a/HPA-1b genotype compared to the control group, in which 33 volunteers had the same genotype. HPA-1b homozygosity is associated with a three- and four-times higher risk of ischaemic cardiovascular disease and myocardial infarction in young men [51]. Additionally, in a Scandinavian population polymorphism HPA-1b was significantly associated with an increased risk of myocardial infarction. Grove et al. [52] showed that the PIA2 allele was more frequent in patients who underwent myocardial infraction (187/529) than in patients who did not undergo MI (138/490) (OR = 1.4 [1.1–1.8]). PlA2 homozygosity was also associated with an inadequate response to aspirin therapy [53]. Kucharska-Newton et al. [54] also reported that polymorphism of the GPIIIa glycoprotein could be predisposed to an increased risk of atherosclerotic plaque rupture, and could be associated with changes in the structure of atherosclerotic plaques. Khatami et al. created a protein model of a normal and polymorphic human β3 chain. The model showed changes in protein structure, and N-terminal structure in a polymorphic protein that was disrupted [55]. However, there have been no demonstrations that can explain how 1565C/T SNP affects the phenotype of platelets. The only reasonable explanation for the increased risk of cardiovascular diseases was an augmented expression of P-selectin on the platelet surface in a patient with Leu33Pro substitution [54]. Due to the many sites at which the β3 chain was present, there is also evidence that 1565C/T polymorphism is associated with Autism Spectrum Disorders (ASD). Schuch et al. showed that proline substitution in the 33rd amino acid sequence could be related to aggression in ASD-diagnosed patients [OR = 2.931 (1.138–7.546)], and that if combined with other SNPs has an impact on echolalia and epilepsy incidents [56]. A study performed by Verdoia et al. [57] shows that PlA2 polymorphism of GPIIIa does not influence the prevalence and extent of angiographically-defined coronary artery disease in the general population, although it does play a role in younger patients. Due to the wide range of reports regarding the effect of Leu33Pro polymorphism and its impact on cardiovascular diseases, it is difficult to determine its unequivocal role in the pathogenesis of CVD.

The glycoprotein Ia/IIa complex, often called integrin α2β1, plays a pivotal role in collagen binding, which is an essential molecule involved in adhesion. Glycoprotein Ia/IIa consists of two subunits, α and β, whose mutual conformation creates the N-terminal extracellular globular head. This is a surface that binds ligands. The α subunits are composed of a seven-bladed β-propeller at the N-terminus [58]. The α2 subunit contains an exceptional domain (I domain), known as MIDAS (metal ion-dependent adhesion site), that binds Mg^{2+}/Mn^{2+} cations. Structural studies show that upon ligand binding, MIDAS and adjacent sides lead to a conformational change of the unique I-domain, from a closed to open condition. Experiments with soluble collagen and specific antibodies have shown that α2β1 can exist in multiple activation conditions. The evolutionarily-conserved unique β-cytoplasmic tail has two characteristic motifs, which recognize sequences for phosphotyrosine binding proteins (PTB). One of these, Talin, is known as a crucial integrin activator. As a result of Talin binding, the salt bridge between the two cytoplasmic integrin tails is disrupted. This consequently leads to a conformational change that shows the α2-I domain and β-A domain. Recent works show that Talin needs Kindlin, the second motif, to work properly. Kindlin binds to the β1-cytoplasmic tail, and despite normal expression of Talin, results in a lack of platelet adhesion to collagen [59]. The quantity of this integrin on platelet surfaces is highly variable, but its density is well known to be associated with 807C/T silent polymorphism (Table 2). In a study conducted by Kunicki et al. [60] the glycoprotein Ia/IIa level was differed significantly depending on genotype. Carriers of TT alleles have a higher density of GP Ia/IIa on platelet surfaces than CC homozygous carriers. This study also showed that the density level of GP Ia/IIa is inherited (the children of the patients in Kunicki's study also had augmented GPIa/IIa density on their platelets' surfaces). Due to the lack of changes in the amino acid sequence of the encoded glycoprotein, the explanation for this phenomenon remains unclear. Kunicki et al. suggested the potential role of other polymorphisms within the α2 gene that could have an impact on the promoter region. The relation between silent polymorphisms and other genetic mutations within the same gene region has been established for different inherited disorders, such as Gaucher disease. On the other hand, silent polymorphisms can affect mRNA stability, resulting in disproportions in their level. There are many works that shows the impact of 807C/T SNP on thrombotic episodes. Carlsson et al. reported results of their study conducted on 227 stroke diagnosed patients, proving that presence of T allele in 807C/T polymorphism may constitute an inherited risk factor of stroke in patients younger than 50 years old [OR = 3.02 (1.20–6.61)] [61]. Santoso et al. [62] postulate that 807C/T polymorphism is associated with the development of myocardial infarction and another coronary artery disease in a younger patient (in patients younger than 49 years old, OR = 2.61 (1.26–5.41) $p = .009$). In a study conducted by Reiner et al. [63] 807C/T SNP was related to augmented risk of ischaemic stroke in women. An increased density of GPIa/IIa might also have an impact on risk factors for cardiovascular diseases and platelet-dependent thromboembolic complications.

Dupont et al. [24] also describe differences in the timing of aggregation, at 10 s higher in the carriers of two C alleles (68 ± 16 s),

Table 2
The quantity of GPIa/IIa receptors on platelet surfaces, by genotype [24].

Genotype	Density
C/C	2810 +/− 756
C/T	3747 +/− 541
T/T	4434 +/− 449
p value	*< 0.001*

than in the carriers of two T alleles (58 ± 16 s). Bargahi et al. [58] showed that despite the influence of GPIa/IIa density caused by 807C/T, this SNP is not related to a higher risk of deep venous thromboembolism.

5. The link between genome-wide association study and cardiovascular disease

A genome-wide association study (GWAS) is an approach to the analysis of genotype frequencies in different diseases in a large population. It depends on fast scanning of the human genome to find an association between genetic variation and a particular disease. Discovery of new mutations is useful in explaining the molecular mechanisms of diseases that have not yet been thoroughly investigated [64].

The first reported association between SNPs and cardiovascular disease in a GWAS came from McPherson et al. [65]. In that study, the interval on chromosome 9p21 was scanned to indicate variations in the genome that contribute to the development of cardiovascular disease. They found two SNPs (rs10757274 and rs2383206) associated with cardiovascular disease. McPherson et al. suggested the potential role of these SNPs in promoting atherosclerotic plaque, thrombogenesis and augmented tendency of atherosclerotic plaque to rupturing [65]. This finding motivated other scientists to discover new genetic disorders having an impact on cardiovascular disease. In 2013, CARDIoGRAMplusC4D Consortium et al. [66] reported a study in which 63,746 patients with coronary artery disease and 130,681 controls participated. The authors of this study identified 15 statistically significant, new risk alleles associated with cardiovascular diseases. In 2016, Stitziel et al. [67] published the first exome-wide association study. The scientists identified novel mutations which had not previously been associated with coronary artery disease, in a study in which > 72,000 patients and 120,000 controls participated. Stitzel et al. found an association between protection from coronary artery disease and triglyceride levels which was lower in SNP carriers.

The number of SNPs and other genetic variations linked to cardiovascular disease is still growing. Kessler et al. [64] showed almost 60 different SNPs that have an impact on circulatory system disorders. GWAS is a rapidly growing field that is receiving more and more interest from scientists. All of the information obtained from GWAS has allowed researchers to find many future, promising, therapeutic targets. Discovering new variations in the human genome associated with cardiovascular disease can help with our understanding of the molecular process of pathological haemostasis.

6. Conclusions

Cardiovascular disease is one of the most common causes of death in the world. Increasing morbidity among young people without typical risk factors is a disturbing phenomenon, despite growing awareness of the need to live a healthy lifestyle, with proper physical activity and diet. This study shows that some people have an increased predisposition to cardiovascular disease, associated with changes in the human genome (Table 3) (Fig. 3). Many studies have shown that polymorphisms of platelet receptors affect the receptor's expression, density, reactivity and functioning, leading to increasing platelet aggregation and activation resulting in the formation of blood clots in vessels. On the other hand, according to the GWAS central database (https://www.gwascentral.org/), the SNPs described by us have been examined in cardiovascular disease, but unfortunately did not show statistically significant results (Table 4).

Explaining why the polymorphisms described in this work have not been included in large-scale GWAS research remains unclear. We are not sure whether the problem lies with small or poorly matched populations. In a study conducted by Matarin et al. patients were collected from The Ischaemic Stroke Genetics Study and were not excluded for typical cardiovascular disease risk factors (obesity, diabetes, smoking etc), but on the basis of diagnosed diseases (such as Alzheimer's, autism and Parkinson's), thus reducing the impact of genetic factors on the occurrence of ischaemic events [68]. Furthermore, cases were collected from 5 medical centres all over the United States on both Caucasian and African Americans, indicating large diversity in the population. Allele frequency dependent on ethnicity may be a reason for the variable results. We also did not find any data on patients who did not survive their ischaemic events. Perhaps the SNPs listed by us had a large impact on this? Besides the fact that unknown point mutations found in the coding regions of the described genes might refer to stroke or other significant changes in gene expression elements, GWAS studies focuses on more high-performance candidate genes. Characterized by low effect size, GWAS studies identify common variants, rarely showing specific variants in a gene, which results in some genetic variants remaining hidden. GWAS studies are able to nominate candidate genes for complex diseases, but usually don't identify causative alleles. These causative variants could contribute to general disease receptivity at a single locus. Massive populations and genetic variants evaluated in GWAS studies are burdened with a higher risk of false positives or negative results. Another interpretation of the variable results is linked with variation in the link between disequilibrium structures in the studied populations. The presence of causative alleles close to a particular locus could signal the association of a selected locus with the occurrence of cardiovascular disease. This is why reproducing the obtained results is important to confirming the results [69,70]. On the other hand, for various reasons epidemiological studies could contain unique populations, for instance, a particular socioeconomic stratum, or the presence of only one sex. Using such a rigorous selection of patient types could affect the ability to generalize results from experiments linked with genetic association. To conclude, the experimental works described by us indicate the ambiguous association of the described SNPs with the frequency of occurrence of various disease entities, and their possible phenotypic effect. The influence of platelet surface receptors' polymorphisms on the presence of cardiovascular disease remains unclear, but they could be the basis for their selection in more precise, future studies. If the polymorphisms presented in this study are inherited risk factors for myocardial infarction and other complications in the circulatory system, in the future we could predict the chance of these diseases occurring in young people.

Table 3
Main platelet receptors polymorphisms, with odds ratio.

Receptor	Polymorphism	Population	Cases	Controls	OR	*p value*	Ref.
P2Y12	H2 haplotype	98	–	–	3.3 (1.1–10.4)	Data not shown	[27]
Glycoprotein Ibα	-5 T/C (major allele)	451	231	220	0.61 (0.4–0.93)	0.03	[37]
Glycoprotein IIb/IIIa	PIA2 (all ages)	139	71	68	2.8 (1.2–6.4)	Data not shown	[45]
	PIA2 (under 60)	78	42	36	6.2 (1.8–22.4)	Data not shown	[45]
Glycoprotein Ia/IIa	807C/T	223	91	132	2.61 (1.26–5.41)	0.009	[58]

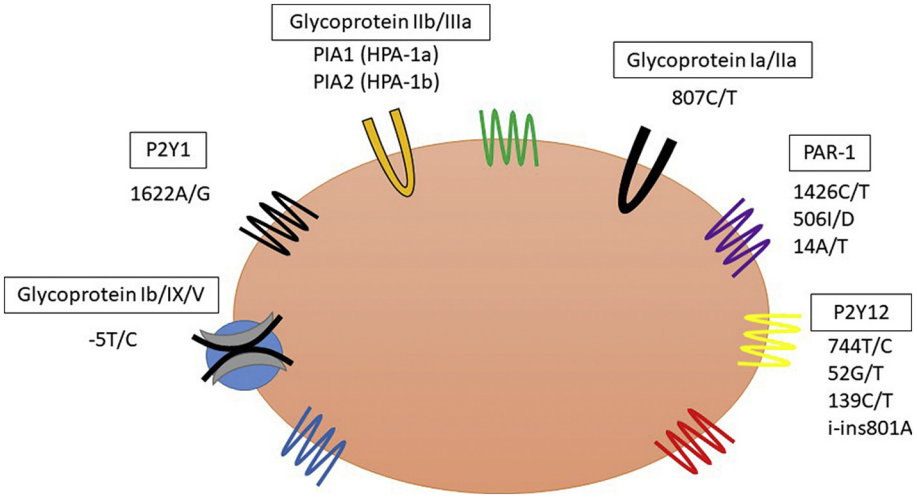


Fig. 3. Distribution of single nucleotide polymorphisms of platelet surface receptors.

Table 4
SNPs listed in the GWAS database and their association with cardiovascular disease.

Receptor	Polymorphism	Gene	Association	*p value*
PAR-1	1426C/T	F2R	Ischaemic Stroke	0.39
			Serum cholesterol	0.46
	506I/D		Lack of association with cardiovascular disease	–
	14A/T		Lack of association with cardiovascular disease	–
P2Y12	744T/C	P2YR12	Lack of association with cardiovascular disease	–
	52G/T		Lack of association with cardiovascular disease	–
P2Y1	1622A/G	P2YR1	Ischaemic stroke	0.056
			Serum cholesterol	0.267
Glycoprotein Ibα	-5T/C	GP1BA	Ischaemic Stroke	0.619
Glycoprotein Ia/IIa	807C/T	ITGA2	Ischaemic Stroke	0.546

Financial support

This work was supported by Grant 506/1136 from the University of Lodz, Poland.

Declaration of competing interests

The authors declare no conflict of interest.

References

[1] J. Sikora, B. Kostka, Blood platelets as pharmacological model, Post. Biol. Kom. 32 (3) (2005) 561–569.
[2] E. Pleban, Haemostasis – issue always up to date, Pediatr. Med. Rod. 11 (2) (2015) 166–176.
[3] K. Broos, H.B. Feys, S.F. De Meyer, K. Vanhoorelbeke, H. Deckmyn, Platelets at work in primary hemostasis, Blood Rev. 25 (4) (2011) 155–167, https://doi.org/10.1016/j.blre.2011.03.002.
[4] M. Hoffman, D.M. Monroe, Coagulation 2006: a modern view of hemostasis, Hematol. Oncol. Clin. North Am. 21 (1) (2007) 1–11, https://doi.org/10.1016/j.hoc.2006.11.004.
[5] M. McMichael, New models of hemostasis, Top. Companion. Anim. Med. 27 (2) (2012) 40–45, https://doi.org/10.1053/j.tcam.2012.07.005.
[6] U. Kralisz, Platelets – endothelial cells interactions in inflammation. Part 1. Adhesive receptors of platelets, endothelial cells and microparticles in hemostasis and inflammation, Post. Biol. Kom. 35 (1) (2008) 45–60.
[7] U. Kralisz, Platelets – endothelial cells interactions in inflammation. Part 2. Platelet-derived mediators and regulatory pathways of signal transduction in endothelial cells in inflammation, Post. Biol. Kom. 35 (1) (2008) 61–78.
[8] C. Patrono, B. Coller, G.A. FitzGerald, J. Hirsh, G. Roth, Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy, Chest 126 (3) (2004) 234–264, https://doi.org/10.1378/chest.126.3_suppl.234S.
[9] A. Kumar, Acute coronary syndromes: diagnosis and management, part I, Mayo Clin. Proc. 84 (10) (2009) 917–938, https://doi.org/10.1016/S0025-6196(11)60509-0.
[10] D.D. Yun, J.S. Alpert, JS, acute coronary syndromes, Cardiology. 88 (3) (1997) 223–237, https://doi.org/10.1159/000177335.
[11] D. A Stakos, D. N Tziakas, K. Stellos, Mechanisms of platelet activation in acute coronary syndromes, Curr. Vasc. Pharmacol. 10 (5) (2012) 578–588, https://doi.org/10.2174/157016112801784477.
[12] S. Massberg, C. Schulz, M. Gawaz, Role of platelets in the pathophysiology of acute coronary syndrome, Semin. Vasc. Med. 3 (2) (2003) 147–162, https://doi.org/10.1055/s-2003-40673.
[13] A. Beresiewicz, Pathophysiology of atherosclerosis and ischemic heart disease, Centrum Medyczne Ksztalcenia Podyplomowego, Warsaw, Poland 2011 (2011) 164–178.
[14] E.H. Elsenberg, J.W. van Werkum, R.M. van de Wal, C.A. Zomer, H.J. Bouman, F.W. Verheugt, J.M. ten Berg, C.M. Hackeng, The influence of clinical characteristics, laboratory and inflammatory markers on 'high on-treatment platelet reactivity' as measured with different platelet function tests, Thromb. Haemost. 102 (4) (2009) 719–727, https://doi.org/10.1160/TH09-05-0285.
[15] K. Kottke-Marchant, Importance of platelets and platelet response in acute coronary syndromes, Cleve, Clin. J. Med. 76 (Suppl. 1) (2009) 2–7, https://doi.org/10.3949/ccjm.76.s1.01.
[16] M.D. Linden, D.E. Jackson, Platelets: pleiotropic roles in atherogenesis and atherothrombosis, Int. J. Biochem. Cell Biol. 42 (11) (2010) 1762–1766, https://doi.org/10.1016/j.biocel.2010.07.012.
[17] M. Gawaz, H. Langer, A.E. May, Platelets in inflammation and atherogenesis, J. Clin. Invest. 115 (12) (2005) 3378–3384, https://doi.org/10.1172/JCI27196.
[18] B. Trzaskowski, D. Latek, S. Yuan, U. Goshdastider, A. Debinski, S. Filipek, Action of molecular switches in GPCRs – theoretical and experimental studies, Curr. Med. Chem. 19 (8) (2012) 1090–1109, https://doi.org/10.2174/092986712799320556.
[19] S. Offermanns, Activation of platelet function through G protein-coupled receptors, Circ. Res. 99 (12) (2006) 1293–1304, https://doi.org/10.1161/01.RES.0000251742.71301.16.
[20] S. Amisten, O.O. Braun, A. Bengtsson, D. Erlinge, Gene expression profiling for the identification of G-protein coupled receptors in human platelets, Thromb. Res. 122 (1) (2008) 47–57, https://doi.org/10.1016/j.thromres.2007.08.014.
[21] E.G. Lapetina, The signal transduction induced by thrombin in human platelets, FEBS Lett. 268 (2) (1990) 400–404, https://doi.org/10.1016/0014-5793(90)81293-W.
[22] S. Brass, Cardiovascular biology. Platelets and proteases, Nature 413 (6851) (2001) 26–27, https://doi.org/10.1038/35092647.
[23] L. Brass, Thrombin and platelet activation, Chest 124 (3 Suppl) (2003) 18–25, https://doi.org/10.1378/chest.124.3_suppl.18S.
[24] A. Dupont, P. Fontana, C. Bachelot-Loza, J.L. Reny, I. Bieche, F. Desvard, M. Aiach, P. Gaussem, An intronic polymorphism in the PAR-1 gene is associated with platelet receptor density and the response to SFLLRN, Blood 101 (5) (2003) 1833–1840, https://doi.org/10.1182/blood-2002-07-2149.
[25] J.H. Zhang, J. Wang, X.F. Tang, Y. Yao, Y. Zhang, Y.L. Ma, B. Xu, R.L. Gao, L. Song, Z. Gao, J. Chen, Y. Wu, Y.J. Yang, X.M. Meng, J.Q. Yuan, Effect of platelet receptor gene polymorphisms on outcomes in ST-elevation myocardial infarction patients after percutaneous coronary intervention, Platelets 27 (1) (2016) 75–79, https://doi.org/10.3109/09537104.2015.1034096.
[26] D. Woulfe, J. Yang, L. Brass, ADP and platelets: the end of the beginning, J. Clin. Invest. 107 (12) (2001) 1503–1505, https://doi.org/10.1172/JCI13361.
[27] P. Fontana, A. Dupont, S. Gandrille, C. Bachelot-Loza, J.L. Reny, M. Aiach, Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene

sequence variations in healthy subjects, Circulation 108 (8) (2003) 989–995, https://doi.org/10.1161/01.CIR.0000085073.69189.88.
[28] S.J. Lu, X.S. Zhou, Q. Zheng, H.L. Chen, Y.L. Geng, Platelet membrane receptor P2Y12 H1/H2 polymorphism is highly associated with cerebral infarction: a case-control study, Neuropsychiatr. Dis. Treat. 14 (2018) 2225–2231, https://doi.org/10.2147/NDT.S171213.
[29] N. Zoheir, S.A. Elhamid, N. Abulata, M.E. Sobky, D. Khafagy, A. Mostafa, P2Y12 receptor gene polymorphism and antiplatelet effect of clopidogrel in patients with coronary artery disease after coronary stenting, Blood Coagul. Fibrinolysis 24 (5) (2013) 525–531, https://doi.org/10.1097/MBC.0b013e32835e98bf.
[30] T. Cuisset, C. Frere, J. Quilici, P.E. Morange, N. Saut, M. Lambert, L. Camoin, I.J. Vague, J.L. Bonnet, M.C. Alessi, Role of the T744C polymorphism of the P2Y12 gene on platelet response to a 600-mg loading dose of clopidogrel in 597 patients with non-ST-segment elevation acute coronary syndrome, Thromb. Res. 120 (6) (2007) 893–899, https://doi.org/10.1016/j.thromres.2007.01.012.
[31] S.L. Hetherington, R.K. Singh, D. Lodwick, J.R. Thompson, A.H. Goodall, N.J. Samani, Dimorphism in the P2Y1 ADP receptor gene is associated with increased platelet activation response to ADP, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 252–257, https://doi.org/10.1161/01.ATV.0000148708.44691.27.
[32] P. Fontana, V. Remones, J.L. Reny, M. Aiach, P. Gaussem, P2Y1 gene polymorphism and ADP-induced platelet response, J. Thromb. Haemost. 3 (10) (2005) 2349–2350, https://doi.org/10.1111/j.1538-7836.2005.01483.x.
[33] E. Galic, L. Vrbanic, S. Kapitanovic, T. Catela Ivkovic, D. Petro, I. Vukovic, R.S. Bsharat, Z. Milicevic, A. Vcev, J. Mirat, P2RY12 gene polymorphisms and effect of clopidogrel on platelet aggregation, Coll. Antropol. 37 (2) (2013) 491–498.
[34] Z.S. Kaplan, S.P. Jackson, The role of platelets in atherothrombosis, Hematology. Am. Soc. Hematol. Educ. Program. 2011 (2011) 51–61, https://doi.org/10.1182/asheducation-2011.1.51.
[35] F. Peyvandi, I. Gragiola, L. Baronciani, Role of von Willebrand factor in the haemostasis, Blood Transfus. 9 (Suppl. 2) (2011) 3–8, https://doi.org/10.2450/2011.002S.
[36] S. Kanaji, S.A. Fahs, Q. Shi, S.L. Haberichter, R.R. Montgomery, Contribution of platelet versus endothelial vWF to platelet adhesion and hemostasis, J. Thromb. Haemost. 10 (8) (2012) 1646–1652, https://doi.org/10.1111/j.1538-7836.2012.04797.x.
[37] G. Castaman, S. Linari, Diagnosis and treatment of von Willebrand disease and rare bleeding disorders, J. Clin. Med. 6 (4) (2017), https://doi.org/10.3390/jcm6040045 pii: E45.
[38] L.K. Jennings, Role of platelets in atherothrombosis, Am. J. Cardiol. 103 (3 Suppl) (2009) 4–10, https://doi.org/10.1016/j.amjcard.2008.11.017.
[39] N. Kieffer, J. Guichard, J.P. Farcet, W. Vainchenker, J. Berton-Gorius, Biosynthesis of major platelet proteins in human blood platelets, Eur. J. Biochem. 164 (1) (1987) 189–195, https://doi.org/10.1111/j.1432-1033.1987.tb11010.x.
[40] F.I. Esen, V.S. Hancer, R.D. Kucukkaya, N. Yesilot, O. Coban, S. Bahar, R. Tuncay, Glycoprotein Ib-alpha Kozak polymorphism in ischemic stroke, Neurol. Res. 34 (1) (2012) 68–71, https://doi.org/10.1179/1743132811Y.0000000061.
[41] M. Kozak, At leat six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells, J. Mol. Biol. 196 (4) (1987) 947–950, https://doi.org/10.1016/0022-2836(87)90418-9.
[42] S.A. Croft, K.K. Hampton, M.E. Daly, R.P. Steeds, K.S. Channer, N.J. Samani, Kozak sequence polymorphism in the platelet GPIbalpha gene is not associated with risk of myocardial infarction, Blood 95 (6) (2000) 2183–2184.
[43] H. Ni, J. Freedman, Platelets in hemostasis and thrombosis: role of integrins and their ligands, Transfus. Apher. Sci. 28 (3) (2003) 257–264, https://doi.org/10.1016/S1473-0502(03)00044-2.
[44] A.T. Nurden, M. Fiore, P. Nurden, X. Pillois, Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models, Blood. 118 (23) (2011) 5996–6005, https://doi.org/10.1182/blood-2011-07-365635.
[45] L. Fink, H. Holschermann, G. Kwapiszewska, J.P. Muyal, B. Lengemann, R.M. Bohle, S. Santoso, Characterization of platelet-specific mRNA by real-time PCR after laser-assisted microdissection, Thromb. Haemost. 90 (4) (2003) 749–756, https://doi.org/10.1160/TH03-02-0095.
[46] J.N. Thon, D.V. Devine, Translation of glycoprotein IIIa in stored blood platelets, Transfusion. 47 (12) (2007) 2260–2270, https://doi.org/10.1111/j.1537-2995.2007.01455.x.
[47] G. Galasso, G. Santulli, F. Piscione, R. De Rosa, V. Trimarco, R. Piccolo, S. Cassese, G. Iaccarino, B. Trimarco, M. Chiariello, The GPIIIA PlA2 polymorphism is associated with an increased risk of cardiovascular adverse events, BMC Cardiovasc. Disord. 10 (41) (2010) 1–7, https://doi.org/10.1186/1471-2261-10-41.
[48] A.J. Marian, R. Brugada, N.S. Kleiman, Platelet glycoprotein IIIa PlA polymorphism and myocardial infarction, N. Engl. J. Med. 335 (14) (1996) 1071–1072, https://doi.org/10.1056/NEJM199610033351418.
[49] E.J. Weiss, P.F. Bray, M. Tayback, S.P. Schulman, T.S. Kickler, L.C. Becker, J.L. Weiss, G. Gerstenblith, P.J. Goldschmidt-Clermont, A polymorphism of a platelet glycoprotein receptor as an inherited risk factor for coronary thrombosis, N. Engl. J. Med. 334 (17) (1996) 1090–1094, https://doi.org/10.1056/NEJM199604253341703.
[50] D. Ardissino, P.M. Mannucci, P.A. Merlini, F. Duca, R. Fetiveau, L. Tagliabue, M. Turbaro, M. Galvani, F. Ottani, M. Ferrariom, J. Corral, M. Margaglione, Prothrombotic genetic risk factors in young survivors of myocardial infarction, Blood 94 (1) (1999) 46–51.
[51] S.E. Bojesen, K. Juul, P. Schnohr, A. Tybjaerg-Hansen, B.G. Nordestgaard, Platelet glycoprotein IIb/IIIa Pl(A2)/Pl(A2) homozygosity associated with risk of ischemic cardiovascular disease and myocardial infarction in young men: the Copenhagen City heart study, J. Am. Coll. Cardiol. 42 (4) (2003) 661–667, https://doi.org/10.1016/S0735-1097(03)00781-2.
[52] E.L. Grove, T.F. Orntoft, J.F. Lassen, H.K. Jensen, S.D. Kristensen, The platelet polymorphism PlA2 is a genetic risk factor for myocardial infarction, J. Intern. Med. 255 (6) (2004) 637–644, https://doi.org/10.1111/j.1365-2796.2004.01327.x.
[53] E. Papp, V. Havasi, J. Bene, K. Komlosi, L. Czopf, E. Magyar, C. Feher, G. Feher, B. Horvath, Z. Marton, T. Alexy, T. Habon, L. Szabo, K. Toth, B. Melegh, Glycoprotein IIIA gene (PlA) polymorphism and aspirin resistance: is there any correlation? Ann. Pharmacother. 39 (6) (2005) 1013–1018, https://doi.org/10.1345/aph.1E227.
[54] A.M. Kucharska-Newton, K.L. Monda, S. Campbell, P.T. Bradshaw, L.E. Wagenknecht, E. Boerwinkle, B.A. Wasserman, G. Heiss, Association of the platelet GPIIb/IIIa polymorphism with atherosclerotic plaque morphology: the Atherosclerosis Risk in Communities (ARIC) Study, Atherosclerosis 216 (1) (2011) 151–156, https://doi.org/10.1016/j.atherosclerosis.2011.01.038.
[55] M. Khatami, M.M. Heidari, S. Soheilyfar, Common rs5918 (PlA1/A2) polymorphism in the ITGB3 gene and risk of coronary artery disease, Arch. Med. Sci. Atheroscler. Dis. 1 (1) (2016) 9–15, https://doi.org/10.5114/amsad.2016.59587.
[56] J.B. Schuch, D. Muller, R.G. Endres, C.A. Bosa, D. Longo, L. Schuler-Faccini, J. Ranzan, M.M. Becker, R. dos Santos Riesgo, T. Roman, The role of β3 integrin gene variants in Autism Spectrum Disorders – Diagnosis and symptomatology, Gene. 553 (1) (2014) 24–30, https://doi.org/10.1016/j.gene.2014.09.058.
[57] M. Verdoia, E. Cassetti, A. Schaffer, G. Di Giovine, G. De Luca, Platelet glycoprotein IIIa Leu33Pro gene polymorphism and coronary artery disease: A meta-analysis of cohort studies, Platelets. 26 (6) (2015) 1–6, https://doi.org/10.3109/09537104.2014.948839.
[58] N. Bargahi, S. Ghorbian, A.A.P. Zonouzi, A.A. Zonouzi, AP, genetic susceptibility to deep venous thromboembolism: the roles of inherited thrombophilia polymorphisms, Blood Coagul. Fibrinolysis 27 (3) (2016) 308–312, https://doi.org/10.1097/MBC.0000000000000430.
[59] B.P. Nuyttens, T. Thijs, H. Deckmyn, K. Bross, Platelet adhesion to collagen, Thromb. Res. 127 (Suppl. 2) (2011) 26–29, https://doi.org/10.1016/S0049-3848(10)70151-1.
[60] T.J. Kunicki, M. Kritzik, D.S. Annis, D.J. Nugent, Hereditary variation in platelet integrin α2β1 density is associated with two silent polymorphisms in the α2 gene coding sequence, Blood 89 (6) (1997) 1939–1943.
[61] L.E. Carlsson, S. Santoso, C. Spitzer, C. Kessler, A. Greinacher, The α2 gene coding sequence T807/A873 of the platelet collagen receptor integrin α2β1 might be a genetic risk factor for the development of stroke in younger patients, Blood 93 (11) (1999) 3583–3586.
[62] S. Santoso, T.J. Kunicki, H. Kroll, W. Haberbosch, A. Gardemann, Association of the platelet glycoprotein Ia C807T gene polymorphism with nonfatal myocardial infarction in younger patients, Blood 93 (8) (1999) 2449–2453.
[63] A.P. Reiner, P.N. Kumar, S.M. Schwartz, W.T. Longstreth, R.M. Pearce, F.R. Rosendaal, B.M. Psaty, D.S. Siscovick, Genetic variants of platelet glycoprotein receptors and risk of stroke in young women, Stroke 31 (7) (2000) 1628–1633.
[64] T. Kessler, B. Vilne, H. Schunkert, The impact of genome-wide association studies on the pathophysiology and therapy of cardiovascular disease, EMBO Mol. Med. 8 (7) (2016) 688–701, https://doi.org/10.1161/01.STR.31.7.1628.
[65] R. McPherson, A. Pertsemlidis, N. Kavaslar, A. Stewart, R. Roberts, D.R. Cox, D.A. Hinds, L.A. Pennacchio, A. Tybjaerg-Hansen, A.R. Folsom, E. Boerwinkle, H.H. Hobbs, J.C. Cohen, A common allele on chromosome 9 associated with coronary heart disease, Science. 316 (5830) (2007) 1488–1491, https://doi.org/10.1126/science.1142447.
[66] CARDIoGRAMplusC4D Consortium, P, S. Deloukas, Kanoni, et al, large-scale association analysis identifies new risk loci for coronary artery disease, Nat. Genet. 45 (1) (2013) 25–33, https://doi.org/10.1038/ng.2480.
[67] Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators, N.O. Stitziel, K.E. Stirrups, N.G. Masca, et al., Coding variation in ANGPTL4, LPL, and SVEP1 and risk of coronary disease, N. Engl. J. Med. 374 (12) (2016) 1134–1144, https://doi.org/10.1056/NEJMoa1507652.
[68] M. Matarin, M. BrowN, S. Scholz, J. Simon-Sanchez, H.C. Fung, D. Hernandez, J.R. Gibbs, F.W. De Vrieze, C. Crews, A. Britton, C.D. Langefeld, T.G. Brott, R.D. Brown Jr., B.B. Worrall, M. Frankel, S. Silliman, L.D. Case, A. Singleton, J.A. Hardy, S.S. Rich, J.F. Meschia, A genome-wide genotyping study in patients with ischaemic stroke, Lancet Neurol. 6 (5) (2007) 414–420, https://doi.org/10.1016/S1474-4422(07)70081-9.
[69] J.M. Maguire, A. Thakkinstian, J. Sturm, C. Levi, L. Lincz, M. Parsons, S. Whyte, J. Attia, Polymorphisms in platelet glycoprotein 1bα and factor VII and risk of ischemic stroke, Stroke. 39 (6) (2008) 1710–1716, https://doi.org/10.1161/strokeaha.107.507228.
[70] P.M. Mannucci, L.A. Lotta, F. Peyvandi, Genome-wide association studies in myocardial infarction and coronary artery disease, J. The. Univ. Heart. Ctr. 5 (3) (2010) 116–121.

Clinica Chimica Acta 499 (2019) 98–107

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Review

Plasma MicroRNA as a novel diagnostic

Rafal Szelenberger[a,*], Michal Kacprzak[b], Joanna Saluk-Bijak[a], Marzenna Zielinska[b], Michal Bijak[a]

[a] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland
[b] Intensive Cardiac Therapy Clinic, Medical University of Lodz, Pomorska 251, 91-213 Lodz, Poland

ARTICLE INFO

Keywords:
MicroRNAs
Plasma
Non-invasive
Biomarkers
Diagnosis

ABSTRACT

MicroRNAs (miRNAs) are small, single-stranded, endogenous, non-coding RNAs necessary for proper gene expression. Their mechanism of action controls translation by base-pairing with target messenger RNA (mRNAs) thus leading to translation blockage or mRNA degradation. Many studies have shown that miRNAs play pivotal roles in cancer, cardiovascular disease and neurodegenerative disorders. The lack of blood-derived biomarkers and those markers of poor specificity and sensitivity significantly impact the ability to diagnose in general and at early disease stage specifically. As such, new, non-invasive and quantifiable biomarkers are needed. As post-transcriptional regulators of gene expression, miRNAs have been confirmed to be notably stable in cells, tissues and body fluids. These and other advantages make miRNAs ideal candidates as potential biomarkers and early experimental findings support this finding. This review examines the use of miRNAs as biomarkers in cancer, neurodegenerative, cardiovascular and liver disease and viral infection.

1. Introduction

MicroRNAs (miRNAs) are a group of small, endogenous, non-coding RNAs consisting of 21-23 nucleotides. Mature miRNAs act as post-transcriptional regulators of gene expression, by base-pairing with mRNA molecules in the 3′ untranslated region (3′-UTR), controlling the translation process of the target mRNA. Depending on the degree of complementarity of the miRNAs and the target mRNA, this leads to translation blockage, or less frequently, mRNA degradation [1]. Genes encoding miRNAs have their own regulatory sequences and promoters that control their expression, but some miRNAs genes are composed in clusters, and are regulated with other members of the same cluster. MiRNAs genes are mostly distant from the gene they interfere with, which suggests that they are from different, independent transcription units [1,2].

Over the last 20 years, miRNAs has become the object of intense research, due to its potential participation in the development of various diseases [3]. At this time, over 1,900 different miRNAs molecules have been identified in human cells, which all control the expression of different genes [4]. It has been proved that miRNAs can modulate up to 60% of protein-encoding genes affecting the cellular cycle, differentiation, proliferation and apoptosis [3,5].

Biosynthesis of miRNAs is a complex, multi-step process. The first step is a nuclear phase, in which miRNAs are transcribed to long pri-miRNA by RNA polymerases II and III. Pri-miRNA consists of a double-stranded core (~30 base pairs), two flanking unstructured single-

Abbreviations: 3′-UTR, 3′Untranslated Region; *ABC*, Activated B-cell like; *AC*, Adenocarcinoma; *ACE*, Angiotestin-converting Enzyme; *ACLF*, Acute-on-Chronic Liver Failure; *AD*, Alzheimer's Disease; *AIDS*, Acquired Immune Deficiency Syndrome; *ALT*, Alanine Transaminase; *AS*, Aortic Stenosis; *AST*, Aspartate Transaminase; *BACE1*, Beta-side Amyloid Precursor Protein Cleaving Enzyme 1; *B-CLL*, B-Cell Lymphoma 2; *CA19-9*, Carbohydrate Antigen 19-9; *CAD*, Coronary Artery Disease; *CEA*, Carcinoembryonic Antigen; *CHC*, Chronic Hepatitis C; *CK*, Creatinine Kinase; *CK-19*, Cytokeratin 19; *DCM*, Dilated Cardiomyopathy; *DGCR8*, Di George Syndrome Critical Region 8; *DLBCL*, Diffuse Large B Cell Lymphoma; *ds-miRNA*, Double-stranded miRNA; *DVT*, Deep Vein Thrombosis; *GCB*, Germinal Center B cell-like; *HIV*, Human Immunodeficiency Virus; *ICM*, Ischemic Cardiomyopathy; *IL8*, Interleukin 8; *INR*, International Normalized Ratio; *IPA*, Ingenuity Pathway Analysis; *MI*, Myocardial Infarction; *miRNA*, MicroRNA; *MRI*, Magnetic Resonance Imaging; *mRNA*, Messenger RNA; *mTOR*, Mammalian Target of Rapamycin; *NAFLD*, Non-Alcoholic Fatty-Liver Disease; *NGS*, Next-Generation Sequencing; *NSCLC*, Non-Small Cell Lung Cancers; *NSTEMI*, Non-ST-Elevation Myocardial Infarction; *PACT*, Protein Activator of PKR; *PD*, Parkinson's Disease; *PDK1*, Pyruvate Dehydrogenase Kinase 1; *PE*, Pulmonary Embolism; *PET-CT*, Positron Emission Tomography-Computed Tomography; *PSA*, Prostate Specific Antigen; *PT*, Prothrombin Time; *PTEN*, Phosphatase and Tensin Homolog; *qRT-PCR*, Quantitative Real-Time PCR; *RISC*, RNA-Induced Silencing Complex; *SCC*, Squamous Cell Carcinoma; *sncRNA*, Small Non-Coding RNA; *STAT3*, Signal Transducer and Activator of Transcription 3; *STEMI*, ST-Elevation Myocardial Infarction; *TGF-β*, Transforming Growth Factor β; *TRBP*, Trans-Activation Response RNA-Binding Protein; *VTE*, Venous Thromboembolism.

* Corresponding author.
E-mail address: rafal.szelenberger@unilodz.eu (R. Szelenberger).

https://doi.org/10.1016/j.cca.2019.09.005
Received 28 June 2019; Received in revised form 3 September 2019; Accepted 5 September 2019
Available online 06 September 2019
0009-8981/

stranded tails, a terminal loop, and several thousand nucleotides. In the next step, pri-miRNA molecules are cleaved into shorter precursor particles, consisting of ~70 nucleotides, pre-miRNA, that have a characteristic hairpin-shaped structure. Pre-miRNAs are processed by the Microprocessor complex, which includes the RNase III enzyme Drosha, and the Di George syndrome critical region 8 (DGCR8), double stranded-RNA binding protein [5]. After enzymatic treatment the pre-miRNAs have ~70 nucleotides in length, and are ready to be exported to the cytoplasm by the Exportin-5 protein [6]. In the cytoplasm, a complex consisting of the RNase III enzyme Dicer, protein activator of PKR (PACT) and trans-activation response RNA-binding protein (TRBP) process the pre-miRNAs from long to short, double-stranded duplexes. In the next step of the miRNAs' biosynthesis, short, double-stranded miRNAs (ds-miRNAs) inosculate with RNA-induced Silencing Complex (RISC) [5,6]. RISC belongs to a family of heterogeneous complexes that play a major role in gene silencing. Several of its mechanisms (repression of translation, mRNA degradation, heterochromatin formation and DNA elimination), mean that the construction of its complexes can be quite diverse. However, there are two invariable themes in every RISC complex: a core, composed of an AGO protein from the Argonaute family, responsible for binding to the small non-coding RNAs (sncRNAs); and sncRNAs, the function of which is to lead the RISC complex to the target mRNA. Argonaute proteins not only bind the sncRNAs, but also position them into a proper configuration that simplifies target recognition [7]. The way in which the RISC complex interacts with the target mRNA is determined by the AGO proteins, of which four are distinguished in human cells. miRNAs only bind to an Ago2, resulting in cleavage of target mRNA [6]. Furthermore, AGO unwinds the ds-miRNAs, leaving only one strand – a guide strand, which constitutes the mature miRNA molecule [5]. In gene silencing, the extent of the complementarity between the target mRNA and miRNAs determines the end result of the silencing. This will be either inhibition of translation, or mRNA cleavage [6].

MiRNAs particles play a pivotal role in the regulation of biological processes, which creates the opportunity for their use as predictive markers in different diseases. One of the more general groups of diseases in which miRNA sequences are used to identify and diagnose patients, is cancer. Diversity in miRNAs expression levels in cancer cells shows specific changes, pointing to the idea that miRNAs regulation could be linked with genetic mutations, such as deletion or insertion. It suggests the potential role of microRNA as oncogenes, leading to transformation of normal cells into cancerous cells, or suppressor genes, which are responsible for the genetic stability of the cell and for the inhibition of cell division and tumor development [8].

It is now thought that the miRNA expression profile in tumor cells helps with diagnosis and monitoring of patient responses to the type of therapy used. A study by Iorio et al. showed that the expression profile of just 15 miRNAs allowed for the correct distinguishing of 76 samples of breast cancer from 10 normal tissue samples. Furthermore, varied miRNAs expression was also found to be a marker for important histopathological features, such as metastasis ability, angiogenesis and the stage of cancer [9]. In Rosenfeld et al.'s study, scientists linked 48 miRNAs sequences to 22 types of tumor with an accuracy of 90% over 253 samples [10]. Based only on expression of 3 miRNAs (miR-21, miR-155 and miR-221), it is possible to divide Diffuse Large B Cell Lymphoma (DLBCL) into two classes: germinal center B cell-like (GCB), and activated B cell-like (ABC) [11].

The MiR-15a and miR-16-1 genes are well-known miRNA sequences, located in chromosome 13 (13q14) where deletion often occurs. A lack of these genes or highly reduced gene expression is often observed in patients with B-cell Chronic Lymphocytic Leukemia (B-CLL), because of a disproportion in the level of the B-cell Lymphoma 2 protein (BCL-2), which has antiapoptotic properties and protects tumors from death. In a normal, physiological state, miR-15a and miR-16-1 act as suppressors of tumor development. Low concentrations of miR-15 and miR-16 have been also seen in patients with mantle cell lymphoma, prostate cancer and multiple myeloma [12]. Another example of disproportion in the expression level of microRNAs is miR-21, the overexpression of which has been confirmed in lung, prostate, breast, colon, head and neck, stomach, esophagus and pancreatic tumors. miR-21 leads to augmented proliferation and growth of tumors and a decreased response to apoptosis [13]. Clustered miRNAs sequences also play an important role in cancer development. The miR-17-92 cluster is well known as an oncogene, because of its overexpression in lung cancer, malignant lymphoma and many other types of cancer [14]. This cluster is located in chromosome 13 (13q31), which is known to be amplified. High expression of miR 17-92 contributes to accelerated and more aggressive tumor development, especially malignant tumors [15].

Fast-growing progress in technology and science has allowed scientists to identify 200 miRNAs expressed in cardiomyocytes. Because miRNAs mostly have tissue-specific expression, scientists have decided to use them in diagnosis of tissue-specific diseases. In the case of the heart's miRNAs, many participate in pathogenesis of cardiac hypertrophy, heart failure and ischemia [16]. In a study conducted by Ikeda et al. miRNAs were collected from the ventricular myocardium of 67 donors. Out of 428 miRNAs measured (of which 13 miRNAs sequences had differential expression in aortic stenosis (AS)), 8 miRNAs had differential expressions in ischemic cardiomyopathy (ICM) and dilated cardiomyopathy (DCM). MiR-19a and miR-19b were downregulated 2 to 2.7 times in DCM and AS. Furthermore, miR-214 was 2 to 2.8 times higher in all three disorders (DCM, ICM and AS) [17].

miR-1 and miR-133a, which are responsible for direct cardiomyocyte differentiation from stem cells and cardiac development, have been shown to have a very important role in the development of heart disease. Dysregulation of miR-1 and miR-133a can lead to pathological hypertrophy, and to ventricular septal defects and DCM. Inhibition of miR-1 in adults causes augmented regulation of twinfilin-1, an actin-binding protein that regulates cytoskeleton, inducing hypertrophy [16,18].

2. Plasma microRNA as a potential diagnostic biomarker

Advancing scientific progress has allowed scientists to study miRNAs with greater precision. Nowadays, miRNAs are considered as potentially useful diseases biomarkers. In the past few years, scientists have focused on the profile of miRNAs in plasma, due to its remarkably high stability in body fluids and resistance to endogenous RNase activity, a wide range of pH, multiple freeze-thaw cycles and prolonged storage times. They are also easy to obtain through non-invasive methods, are highly sensitive to early detection and have high specificity to different disease entities [19,20].

It is well known that expression levels of certain miRNAs are altered in many disease states. About 10% of all human miRNAs particles can be found in plasma, and the differences in expression of miRNAs can be observed in various stages of disease development. Many genome-wide associated studies show that miRNAs genes are often located in fragile sites of cancer-associated genomic regions, suggesting its potential roles in tumorigenesis [21].

2.1. Use of plasma microRNAs in diagnosis of cancers

According to data from the World Health Organization, prostate cancer is the most common malignancy among adult men, with almost 1.3 million cases per year. The occurrence of prostate cancer correlates with their age and race [22]. The first-line test in the diagnosis of prostate cancer is measurement of the prostate-specific antigen level (PSA). Because of the lack of specificity for early detection of prostate malignancies, and high rates of excessive treatment and diagnosis linked with PSA testing, there is an urgent clinical need for better diagnostic tools that will help in diagnosis and prediction of prostate cancer. At this time, the gold standard in diagnosis of prostate cancer is

prostate biopsy, but it is burdened with some limitations that can generate false negatives. Randomly collected samples can indicate an abnormal biopsy result and require repeated biopsies with more advanced equipment (i.e. MRI). In recent years, scientists have developed several molecular assays that provide new possibilities for the non-invasive diagnosis of prostate cancer [23]. A study conducted by Matin et al. investigated the plasma levels of cancer-associated miRNAs. Of 372 cancer-associated miRNAs, Matin et al. showed dysregulation of 11 in patient samples in comparison to healthy controls, indicating that 4 have potential meaning: miR-4289; miR-326; miR-152-3p and miR-98-5p. Based on these 4 miRNAs, a diagnostic test was performed in patients diagnosed with prostate cancer in comparison to healthy control. In most cases, the sensitivity and specificity of the testing exceeded the accuracy of the PSA test. The direct mechanism of action of these miRNAs remains unclear, despite the fact that circulating miRNAs are associated with cell-to-cell communication and play an important role in processes such as proliferation, metastasis and angiogenesis [24].

Bai et al. conducted a study in which they investigated plasma miR-19a in patients diagnosed with esophageal squamous cell carcinoma (ESCC), one of the most aggressive malignancies in the world. Imaging exams and biopsies are the gold standard in the diagnosis of esophageal cancer and significantly improve the detection of these malignancies, but are still very invasive or demand radiation. A less dangerous method of patient diagnosis is the use of traditional tumor markers, cytokeratin 19 (CK-19) and Cyfra21-1. However, their sensitivity leaves a lot to be desired, and so the search for less invasive and more sensitive methods of diagnosing esophageal cancer became the basis for Bai et al.'s research. MiR-19a is a member of the miR-17-92 cluster, which is highly expressed in ESCC. The data obtained by Bai et al. showed that in comparison to healthy controls, the plasma concentration of miR-19a in diagnosed patients was significantly augmented ($p < 0.001$). They also investigated the potential role of miR-19a as a diagnostic marker for ESCC. In comparison to Cyfra21-1, miR-19a was more sensitive and specific. The best results were obtained with a combination of miR-19a and Cyfra21-1, which achieved a sensitivity of 92.37%. The most important discovery in the study by Bai et al. was a decrease in the level of plasma miR-19a after surgical removal of cancer ($p < 0.001$). This suggests that miR-19a could be secreted by ESCC cells and could be an important indicator in the evaluation of therapeutic effects [21].

Lymphoid neoplasms are a type of cancer caused by transformation of normal lymphoid cells. Diagnosis is based on biopsy and PET-CT (positron emission tomography-computed tomography) imaging. Due to the high percentage of false-positive diagnoses (over 20%), patients are being unnecessarily exposed to radiation, having unnecessary biopsies and surgical investigations, and are having to settle the high financial costs of their treatment and diagnosis. Thus, as with other cancers, new, less invasive and more sensitive and specific tests are always being sought [25]. Lawrie et al. reported that miR-155, miR-210 and miR-21 had different expression in DLBCL patients in comparison to healthy controls, suggesting the potential role of these 3 miRNAs as biomarkers of lymphoma. Furthermore, they have been found that miR-21 has an anti-proliferative effect on various cancers [26]. Feng et al. additionally reported five plasma miRNAs that were significantly dysregulated in DLBCL, in comparison to healthy volunteers: miR-15a ($p = 0.0001$); miR-16-1 ($p = 0.0003$); miR-21 ($p = 0.0049$); miR-29c ($p = 0.0020$), and miR-155 ($p = 0.0023$) [27]. In a study conducted by Khare et al., 10 dysregulated plasma miRNAs in DLBCL patients were identified. In contrast to Lawrie's and Feng's studies, Khare et al. examined all miRs present in the plasma, not only those that were overexpressed. This method opens the possibility of discovering more significant miRNAs associated with DLBCL. Khare et al. reported increased plasma levels of miR-532-5p and miR-124, and down-regulated levels of miR-141, miR-425, miR-145, miR-345, miR-197, miR-424, miR-122 and miR-128. In their study, Ingenuity Pathway Analysis (IPA), was used to define mRNAs targeted by these selected miRs and to determine in which biological processes they are involved. They found up-regulation in different signaling pathways (STAT3, IL8, p13k/AKT and TGF-B), and potential down-regulation of p53 and PTEN pathways, which are very important in proliferation, differentiation and tumorigenesis. Khare et al. also identified DLBCL patients as having augmented levels of miR-25, miR-26b, miR-30d, miR-182, miR-186, miR-30a, miR-140 and miR-125a in comparison to a control group, and down-regulated levels of miR-23a, miR-93, miR-122 and miR-144. IPA analysis of selected miRNAs in both groups found that the cAMP-mediated pathway and p53 pathway can be downregulated, such as with PTEN expression [25].

Lung cancer is the most common and deadliest type of cancer, causing over 1.7 million deaths worldwide every year [22]. Nearly 90% of lung cancers are non-small cell lung cancers (NSCLC) that consist of squamous cell carcinoma (SCC) and adenocarcinoma (AC). The primary risk factor of NSCLC development is tobacco smoking. Unfortunately, for most patients the prognosis is bleak, resulting in an approximately 14% chance of a 5-year survival rate. This high mortality rate is caused by late diagnosis, when the cancer is already at an advanced stage. Therefore, the development of sensitive, specific biomarkers that can precisely diagnose the early stages of lung cancer are essential. Many studies have looked at the same lung cancer-related miRNAs [28]. For example, Zhang et al. showed upregulation of plasma miR-145, miR-20a, miR-21 and miR-223 in NSCLC patients in comparison to a healthy control group [29]. Gao et al. reported upregulation of miR-324-3p and downregulation of miR-1285 in stage I lung cancer [30]. Zaporozhchenko et al. discovered significant upregulation of miR-19b and miR-21, and downregulation of miR-25 and miR-183 with $p < 0.05$ in all cases. Furthermore, of all the miRs that have been tested, miR-19b showed the most significant diagnostic value in lung cancer patients [31]. Leng et al. investigated a whole plasma miRNAs profile showing dysregulation in the expression level of not only the same miRNAs discovered previously, but also new miRs that had not previously been linked with lung cancer. Leng et al. then developed a diagnostic panel of four-plasma miRNAs (miR-126, miR-145, miR-210 and miR-205-5p), which showed 91.56% sensitivity and 96.23% specificity in a development cohort and 91.18% sensitivity and 96.67% specificity in validation cohorts. The same research team suggested that the role of particular miRs consists in their use as diagnostic panels in tumor development. miR-145 acts as an inhibitor of lung cancer cell invasion and migration by targeting PDK1 on the mTOR signaling pathway. MiR-210 is linked with regulation of the hypoxic response of tumor cells. The expression of miR-126 and miR-205-5p varies in different types of cancer that constitute them as a significant biomarker in the diagnosis and prognosis of various other cancers, not only NSCLC [28].

Early detection of colorectal cancer gives a very good prognosis for survival. Unfortunately, as with other types of cancer a lack of efficient, practical tools for diagnosis causes colorectal cancer of the deadliest malignancies. Current methods are quite reliable, although unpleasant for patients and cost-ineffective and unprofitable, and reliant on novel biomarkers that can help diagnosing and identifying colorectal cancer. Some studies have shown that miRNAs can be good biomarkers for colorectal cancer [32]. Kanaan et al. developed a diagnostic panel of 8 plasma miRNAs (miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532, miR-532-3p, miR-652) that shows differences in their expression levels in patients with colorectal cancer, in comparison to healthy controls [33]. Wang et al. conducted a study identifying the dysregulation of miR-409-3p, miR-7 and miR-93 in colorectal cancer patients, in comparison to healthy persons [34]. Vychytilova-Faltejskova et al. reported a study in which patients with colon cancer were distinguished from healthy volunteers on the basis of a miRNA-panel consisting of miR-23a-3p, miR-27a-3p, miR-142-5p and miR-376c-3p. In comparison to currently-used diagnostic tests, this miRNA-signature testing has achieved stunning results. In 168 patients diagnosed with colorectal cancer, CEA testing identified 79 colon cancer patients, thus achieving 47% efficiency. In comparison, Ca19-9 testing identified only 46 colon cancer patients, for 27% efficiency. Use of the plasma-miRNA-

based panel then identified 149 cancer cases, thus showing 89% efficiency. The subsequent combination of CEA, CA19-9 and miRs-panel reached the highest diagnostic efficiency, at a level of 96% [35]. Wikberg et al. conducted a study with patients who had donated blood, even as far back as 20 years previously, before receiving a colorectal cancer diagnosis. Comparing the plasma concentration of particular miRNAs in diagnosed patients, healthy controls and pre-diagnostic samples from the same cases they investigated 4 miRs that differed between all groups (miR-21, miR-25, miR-18a and miR-22). A diagnostic 4-plasma-miRNA-based panel achieved 67% sensitivity and 90% specificity, and was selected on the basis of previously reported studies linked with cancer, with the most significant diagnostic value being miR-21 [32,36–40].

2.2. Use of plasma microRNA in diagnosis of cardiovascular disturbances

Coronary Artery Disease (CAD) is caused by an inflammatory process associated with atherosclerosis. Formation of plaques, their growth and acute rupture lead to blockage of blood flow in coronary arteries, causing damage to the heart muscle. The unexpected and sudden onset of the symptoms of CAD make it difficult to predict. That's why new, molecular biomarkers are needed for faster prediction and diagnosis of this disease. Fichtlscherer et al. conducted the very first study showing differences in circulating miRNAs in patients with angiographically confirmed CAD, in comparison to healthy controls. They showed that levels of miR-126, miR-17, miR-92a, miR-155 and miR-145 were significantly lower in CAD patients [41]. Furthermore Diehl et al. and Weber et al. showed that levels of miR-155 in CAD patients are significantly reduced, which could indicate the high affinity of miR-155 to CAD [42,43]. Further analysis showed that patients using angiotensin-converting enzyme (ACE) inhibitors also resulted in downregulation of miR-155, which might explain this reduction [43].

Many studies have focused on the association of dysregulation of miRNAs and development of Myocardial Infarction (MI). In a study conducted by Corsten et al., circulating miR-208b and miR-499 saw 1,500-fold and 90-fold increases respectively, for patients with MI in comparison to a control group with no cardiac disease [44]. In their work, Ai et al. showed significant upregulation of miR-1 in plasma in MI patients, compared to healthy volunteers [45]. Almost the same results were confirmed by D'Alessandra's research team, which found augmented levels of miR-1, miR-133a, miR-133b and miR-499-5p in the plasma of patients with ST-elevation myocardial infarction (STEMI), in comparison to healthy controls [46].

In a study conducted by Gidlof et al. the expression of miR-1 was 300 times greater, miR-133a 70 times greater, miR-208b 3,000 times greater, and miR-499-5p 250 times greater (all with $p < 0.01$) in patients with STEMI, compared to healthy controls [47]. Kuwabara et al. also showed augmented levels of miR-1 and miR-133a in patients with Acute Coronary Syndromes in comparison to controls without any circulatory disorders [48]. In a study by Olivieri et al., miR-499-5p saw an 80-fold increase in patients with Non-ST-elevation Myocardial Infarction (NSTEMI), in comparison to a healthy control group. Additionally, miR-21, miR-133a, miR-1 and miR-423-5p were significantly increased, but not as much as miR-499-5p [49]. Wang et al. demonstrated in a study that miR-1, miR-133a and miR-499 were meaningfully augmented in the plasma of patients after MI incidents, in comparison to a healthy group [50]. As can be seen, many studies shows dysregulation of the same plasma's miRNAs in detection of MI. The large number of repeatable results indicate that miR-1, miR-133a, miR-133b, miR-499, miR-499-5p and miR-208b could be potential biomarkers for MI. Other studies have shown that miR-208b was not always detectable, but apart from this, Wang et al. suggest that miR-208b could be an ideal candidate for an MI biomarker, as it showed the highest sensitivity and specificity, being detectable in over 90% of MI cases [48–50].

Currently, protein-based biomarkers are gold standard in diagnosis of MI. High sensitive troponins and creatinine kinase (together with CK-MB isoenzyme) are main protein-based biomarkers used to detect myocardial necrosis. Troponins are a group of regulatory proteins located in skeletal, cardiac muscle fibers and thin filaments. For myocardial injury, only cardiac troponins T and I are used, because they differ from skeletal form, in comparison to troponin C which is identical with skeletal form [51]. Troponins are released into the bloodstream 4 to 12 hours after myocardial necrosis occur, reaching peak at 12-48 hours from first symptoms onset. Unfortunately, troponin elevation may occur in other conditions, not always linked with myocardial necrosis (i.e. sepsis, acute pulmonary embolism, atrial fibrillation, heart failure) [52]. Creatinine Kinase (CK), together with CK-MB isoenzyme, was firstly presented as a cardiac biomarker in 1979. Because its composition of two subunits M – muscle and B – brain, there are three different isoenzymes: CK-BB – largely presented in brain and internal organs, MB – heart and skeletal specific, widely used for the diagnosis of myocardial necrosis, and CK-MM – skeletal muscle isoform [53]. Beside the fact of usefulness of CK-MB in diagnosis of myocardial necrosis, its specificity is lower to cardiac muscle than troponins, and cannot be used alone, without any other protein-based biomarker. Furthermore, elevation of CK-MB may be present in renal disease or muscular injury [52].

Li et al. conducted a study in which miR-1, -133a, -208b and -499 were compared to troponin T to determine which biomarker is more sensitive and specific. Results showed, that troponin T was more specific and sensitive than panel of four miRNAs [54]. Similar results were obtained from Li et al. where miR-1 was compared to troponin T, also showing lower specificity and sensitivity than troponin T [55]. However, combination of miRNAs and troponins could give higher sensitivity and specificity for myocardial infarction patients.

Stroke is the second most common cause of death, and the third main cause of temporary or permanent disability of patients due to a lack of oxygen supply to brain cells following blockage of the blood flow in the cerebral arteries. Stroke causes not only death and disability but is also a leading cause of depression and dementia [56,57]. In a study conducted by Sepramaniam et al., statistically significant dysregulation in the expression of miRNAs in plasma in stroke cases was found in 105 miRNA particles. In their experiments, they selected 32 miRNAs that can distinguish stroke subtypes during the acute phase of stroke. Subsequently, five plasma miRs (miR-125b-2; miR-27a; miR-422a; miR-488 and miR-627) were found to be potential biomarkers for diagnosis of stroke, because of the consistency of the alteration in blood from stroke patients, independent of age, stroke severity or the presence of metabolic complications [58]. Long et al. reported that miR-30a and miR-126 levels in plasma were significantly down-regulated in patients diagnosed with stroke until 24 weeks. Surprisingly, miRNA levels in plasma returned to normal 48 weeks after the symptoms began [59].

Venous Thromboembolism (VTE) is a common cardiovascular disorder that includes deep vein thrombosis (DVT) and acute pulmonary embolism (APE), and is initiated with formation of a blood clot in a deep vein, usually in the lower leg. It is estimated that 60-70% of patients with symptomatic VTE can go on to develop DVT. Sometimes, the blood clot is dislodged and travels through the blood vessels to the lungs, where it limits blood supply, thus causing APE, third most common cause of death by cardiovascular disorder, after ischemia and stroke [60,61]. Because VTE can have different clinical manifestations, diagnosis can be incorrect and/or nonspecific, especially for DVT that in many cases is silent and do not give any symptoms. Challenging diagnosis and a lack of sensitive and specific testing has led to a high mortality rate and poor prognosis for those who experience VTE [61]. However, VTE and its complications are preventable. Proper administration of antithrombotic drugs can meaningfully reduce the risk of death (from 25% to 1.5%). Despite this, VTE remains very dangerous and is an often overlooked problem. Starikova et al. conducted a study in which, for the very first time, dysregulated miRNAs were selected for their potential role as biomarkers in VTE cases, compared with healthy controls. Of 742 miRNAs, the expression of only 9 were seen to be

meaningfully different between VTE patients and healthy volunteers [60].

Advanced PE shows a high mortality rate, in which 25% of patients with the initial clinical symptoms will die. However, rapidly-spreading knowledge of miRNAs' functions has allowed scientists to determine potential biomarkers for cardiovascular system disturbances. Wang et al. investigated the utility of plasma miR-134 as a potential biomarker for APEs. They found that in comparison to healthy controls, the miR-134 level was significantly higher in patients with diagnosed acute and chronic pulmonary embolism ($p < 0.05$) [61]. Similar research were made by Xiao et al. In their study, the level of miR-134 in plasma was significantly higher in patients with APE in comparison to healthy controls. Despite both studies having small study and control groups, the fact that the same results were obtained by both research teams suggests that miR-134 has great potential as a biomarker of pulmonary embolism (PE) [62]. In a study conducted by Wang et al., the research team showed that levels of miR-27a and miR-27b in plasma were significant in patients with diagnosed APE, in comparison to healthy volunteers [63].

2.3. Use of plasma miRNAs in diagnosis of neurological disorders

Parkinson's Disease (PD) is a neurodegenerative disease that results in difficulties with proper movement, coordination and balance [64]. It is a highly complex and heterogeneous disease of the central nervous system, discovered in 1817 by James Parkinson [65]. The main histopathological hallmark of PD are fibril inclusions called Lewy Bodies, mainly consisting of alpha-synuclein. In genetically determined PD, with mutation in alpha-synuclein gene, incorrectly build protein is stored in nerve cells, forcing cells to enter the apoptosis pathway [66]. In this cell loss, along with the duration of the disease and the advancement of its development, 50-70% of neurons are lost compared to healthy controls [67,68]. The complexity of PD has caused the current standard in diagnosis and prognosis to be based on subjective clinical assessment of motor features, and imaging exams. Finding measurable, specific and sensitive molecular biomarkers would help in early diagnosis of this disease, and thus to extension of the lifetime of, and an augmented quality of life, for sick patients [64]. Khoo et al.'s study identified a diagnostic panel consisting of miR-1826, miR-450b-3p, miR-626 and miR-505, which showed higher dysregulation in PD patients compared to healthy volunteers. The diagnostic panel achieved 91% in sensitivity, 100% in specificity and 100% in positive predicted value [64]. Furthermore, miR-1826 has also been found to be increased in the plasma of multiple sclerosis patients [69]. In a study by Cardo et al., only miR-331-5p was found to be significant in patients' plasma [70]. Later, Li et al. showed the dysregulation of miR-124-3p and miR-137-3p [71]. Ding et al. conducted a study with a larger population, assessing 5 serum miRNAs (miR-195, miR-185, miR-15b, miR-221 and miR-181a) for their use as potential biomarkers in diagnosis of PD, in comparison to healthy controls [72]. Interestingly, all of these studies showed different results, which would require confirmation and further analysis in a much larger population.

Alzheimer's Disease (AD) constitutes the most common neurological disorder characterized by memory loss, intellectual disability, psychiatric symptoms and disorientation, caused by neuronal death in the hippocampus. The exact cause of Alzheimer's disease is not fully known and there is non currently available treatments that can stop or reverse its progression [73–75]. This very complex disease is diagnosed on the basis of brain imaging tests, neurological exams and mental status assessments [74]. Geekiyanage et al. conducted a study in which miR-137, miR-181c, miR-9 and miR-29a/b in plasma were downregulated in AD patients, compared to controls [76]. In Kiko et al. work plasma miR-34a and miR-146a were significantly reduced in AD patients [77]. Further, Bhatnagar et al. showed that miR-34c is meaningfully increased in AD patients' plasma, in comparison to a normal elderly control. They also suggest that because of the association of miR-34c with oxidative defense signaling and cellular survival, overexpression of miR-34c can lead to overall weakening of cell survival and stress defense [78]. Sorensen et al. conducted a study in which they detected 308 different miRNAs in the blood, with significant up-regulation of miR-590-5p and miR-142-5p and downregulation of miR-194-5p in AD-diagnosed patients, compared to healthy volunteers [79]. In a study conducted by Nagaraj et al., previously reported miRNAs linked with AD (miR-502-3p, miR-103a-3p, miR-1260a, miR-200a-3p, miR-142-3p, miR-301a-3p [80–84]), as well as 9 novel miRNAs (miR-320a, miR-18a-5p, miR-33a-5p, miR-30b-5p, miR-483-5p, miR-320b, miR-320c, miR-151-5p, miR-486-5p), were identified and suggested as the best candidates for future biomarkers of AD. Results from analyses showed 3 to 4 fold increased plasma's levels of miR-200a-3p in patients diagnosed with AD. Previous experimental work showed the same result in mononuclear cells from blood. This phenomenon might suggest that miR-200a-3p is secreted by mononuclear blood cells. Nagaraj's research team also found that transcripts regulated by miR-200a-3p are located in the 3'UTR region of BACE1, one of the major enzymes in the amyloidogenic proteolysis of amyloid precursor protein. Thus, this miRNA could be associated with the regulation pathway of BACE1 in the brain and blood cells, which may then suggest that miR-200a-3p contributes to development of AD [85].

2.4. Use of plasma miRNA in diagnosis of liver diseases

According to the data from the American Liver Foundation, around 88,000 people (70% men) die of alcoholic liver disease every year in the United States. Furthermore, over 1 million people in the US live with Hepatitis B, with 43,000 new diagnoses every year. It is estimated that 2.7-3.9 million individuals in the United States are infected with hepatitis C, of which 75% are unaware of their condition. Fatty liver disease is the most common liver disorder, and has been diagnosed in 100 million people in the United States alone [86]. Due to the wide range of varying factors affecting the promotion of liver inflammation (such as alcohol abuse, viral infections, bacterial infections, toxins, poor diet), miRNAs play an important role in regulating the process of fibrogenesis [87].

Zhang et al. conducted a study evaluating the potential role of miR-122 as a biomarker, which is highly specific to the liver, is associated with differentiation, development and homeostasis of hepatocytes, and regulates lipid metabolism [88–90]. They found that the miR-122 concentration in patients with chronic HBV infections was significantly higher than in a healthy control group ($p < 0.001$). They also found that the level of miR-122 in plasma correlates with ALT activity (Spearman $r = 0.896$, $p < 0.001$). Furthermore, they suggested that miR-122 can be used as a potential biomarker for liver injury, on the basis of results obtained from a ROC curve analysis (AUC for miR-122 was 0.989) [88]. Bihrer et al. conducted a study in which serum miR-122 was increased in patients diagnosed with Chronic Hepatitis C (CHC) [91]. Bala et al. found that levels of miR-122 and miR-155 are also increased in alcoholic liver disease. MiR-155 is well known to be associated with liver fibrosis, mediates cellular growth, and be involved in inflammatory and immune diseases [92–94]. Their experiment was performed on alcohol-fed mice, and showed a significant increase of miR-122 and miR-155 in serum [92]. In a study conducted by Cermelli et al., four miRNAs were evaluated as a potential biomarker for CHC. The results showed that in 18 patients diagnosed with CHC, miR-122 saw a 10.8-fold increase ($p < 0.0001$) and miR-16 a 3-fold increase ($p = 0.0002$) over the control group. Moreover, miR-34a exceeded the detection threshold in CHC patients, while the level of miR-21 was not significant. The same experiments were performed on an independent group of 35 HCV-infected patients. The results showed that the level of miR-122 was 7.9 times higher ($p < 0.0001$), and miR-16 6.3 times higher ($p < 0.0001$) in comparison to healthy controls. MiR-34 levels also exceeded the detection threshold, while miR-21 remained insignificant. The results that Cermelli et al. obtained from experiments on

CHC patients were very promising, and so the same research team evaluated the potential role of these four miRNAs in 34 patients diagnosed with Non-Alcoholic Fatty-Liver Disease (NAFLD). Cermelli et al. found that the level of miR-122 increased 7.2 times ($p < 0.0001$), and the level of miR-16 5.5 times ($p < 0.0001$) in NAFLD patients, in comparison to healthy volunteers. MiR-34 exceeded the detection threshold and miR-21 was not significant [95]. In order to confirm the results obtained by Cermelli et al., Salvoza et al. conducted a study which showed the upregulation of miR-34a and miR-122 in NAFLD serum patients [96]. Similar results for miR-122 in CHC patients were obtained by Butt et al. The expression level measured on 123 treatment-naïve CHC patients showed significant up-regulation of serum miR-122, compared to healthy volunteers ($p = 1.52*10^{-14}$). Furthermore, miR-122 was positively correlated with ALT, AST, INR, PT, albumin level and necroinflammation, suggesting its potential role as a biomarker for liver issues. Butt et al. also showed that serum miR-122 has great diagnostic potential (AUC of 0.954 under ROC curve) [97]. The positive correlation between miR-122 and ALT/AST, and significant upregulation in CHC patients, were both confirmed by El-Hefny et al. [98] In the case of hepatitis B, Ji et al. conducted a study in which serum miR-122 was significantly higher ($p < 0.001$) than in the control group. Furthermore, the expression of miR-223, which is important in regulation of hematopoietic and embryonic stem cell differentiation and regulation of hepatocyte apoptosis, was also meaningfully increased in hepatitis B patients ($p < 0.01$) [99,100]. Roderburg et al. focused on finding potentially useful circulating miRNAs in patients with liver fibrosis/cirrhosis. The results obtained from their experimental work showed significant downregulation of miR-29 in plasma [101]. Zheng et al. then found that serum miR-21, miR-486-5p, miR-130a, miR-192, miR-148a, miR-143, miR-200a, miR-194 and miR-122 were all upregulated in HBV-related Acute-on-Chronic Liver Failure (ACLF) patients, in comparison to healthy controls ($p < 0.05$) [102].

2.5. Use of plasma miRNA in HIV infected patients

The human immunodeficiency virus (HIV) is part of the Lentivirus group (of the family *Retroviridae*), which is characterized by long incubation periods. The ongoing weakening of the organism results in the creation of favorable conditions for various infections and cancer diseases [103]. The highest modern standard for assessing the development of AIDS is the number of circulating CD4+ T cells in peripheral blood and viral loads, but these predictors are not always highly detectable. As such, discovering new biomarkers for AIDS is necessary for the early detection, monitoring and therapy of the disease [104].

Munshi et al. conducted a study in which the plasma levels of miR-150 and miR-146b-5p were evaluated for their potential use as biomarkers of HIV infection. The results showed that plasma miR-150 and miR-146b-5p were significantly augmented in patients, compared to healthy controls. Interestingly, the expression of miR-150 and miR-146b-5p in peripheral blood mononuclear cells was significantly downregulated (0.51 +/- 0.08 ($p < 0.05$) [104]. Qi et al. conducted a study in which they created a diagnostic panel of miRNAs. Their results showed that the combination of miR-29a, miR-233, miR-27a, miR-151-3p and miR-19b demonstrates high sensitivity and specificity in the detection of HIV-1 infection. Moreover, the same miRNAs were meaningfully associated with CD4+ T cell count, suggesting their potential role in progression monitoring [105]. In a study conducted by Reynoso et al., the levels of two miRNAs were altered in HIV patients and healthy controls. Expression of plasma miR-29b-3p and miR-33a-5p were then seen to be significantly downregulated ($p < 0.05$) [106].

3. Application of miRNA profiling techniques in clinical practice

Plasma miRNAs are very interesting candidates for clinical laboratories, because of their remarkabely high stability in body fluids and easy-to-use in clinical routine. On the other hand, miRNA tests has some serious limitations that hinder their introduction into clinical practice. The origin of circulating miRNAs is not fully understood, but it is likely that it is derived from all body tissues, including white blood cells and blood platelets. The highest changes in expression patterns of particular miRNAs are usually observed in body tissues. In circulating miRNAs expression profiles, changes may be subtle. That is why, the knowledge about the expression profiles of miRNAs in various tissues is essential for developing tests based on plasma or serum patterns [107]. What is more, in comparison to protein-based biomarkers, quantification of miRNAs need appropriate standardization, which is actually the biggest limitation. At this moment, there is no one optimal strategy about normalization. In many studies exogenous oligonucleotides (added in established concentration) are used as reference gene i.e. cel-miRNA-39, -54 and -268 obtained from *Caenorhabditis elegans*. In other studies, authors use the mean of all the expressed miRNAs or stable small RNAs (i.e. RNU24, RNU6B) for normalization. Unfortunately, there is no one accepted normalization strategy use for quantification of miRNA [108].

For many clinical laboratories, detection technology is a serious limitation. Microarrays were one of the first methods used for miRNAs gene expression profiling. Based on nucleic acid hybridization, microarrays provide quantification of large numbers of miRNAs simultaneously. In contrast to other techniques, microarrays are best used for comparative analysis between two groups (i.e. patients vs. healthy controls) [109]. Big advantage of microarray technique is large number of data obtained from single experiments. On the other hand, there is a possibility of cross-hybrydization during microarray, resulting in different expression profiles. That is why, results from microarrays can differ from other miRNA profiling techniques [110].

Quantitative Real-Time PCR (QRT-PCR) is one of the main technique used for diagnostic tests like viral load testing, or gene expression profiling. Because of a small amount of miRNAs, the amplification process during PCR reaction results in the precision and sensitivity of QRT-PCR, wchich shows even subtle changes in gene expression. Furthermore, existing commercial kits are very simple, fast and ready-to-use. Besides the fact that QRT-PCR is excellent technique used for measurement of particular miRNAs showing the most blanced specificity, reproducibility, sensitivity and accuracy, it is not proper for discovery of novel miRNAs patterns [110].

Next-generation Sequencing, also known as NGS, is modern technique that provides opportunities to quantify known sequences of genes and discover potentially novel genes or transcripts simultaneously. Furthermore, NGS allows to analyze different samples in a single experiment. Those advantages shows that NGS could be a gold standard in clinical practice for profiling miRNA gene expression. Unfortunately, in comparison to qRT-PCR, NGS is pricy platform, which consume a lot of time to perform it properly [110].

Rapidly evolving field in research technology provide many measurement platforms to determine miRNAs gene expression. Any of available platforms show different sensitivity, reproducibility, specificity an accuracy, causing difficulty in choosing the right method to perform the experiment. Mestdagh et al. conducted a miRNA quality control study, in which all common platforms are compared to each other. To evaluate the accuracy of the studied platforms, authors compared the expression of samples expressed in two various samples, A – 100% Universal Human miRNA Reference RNA, and B – 100% Human Brain RNA mixed in the 4:1 ratio (sample C) and 1:4 (sample D). Expression of particular miRNAs should be exactly 3-fold higher (in C sample) or lower (in D sample). The highest accuracy was shown in platform that presented the lowest fold change difference between observed expression. Reproducibility was assessed by means of duplicated A-D samples. The highest reproducibility was shown by microarray. Detection rate and sensitivity was evaluated on the basis of detected replicates, in which qRT-PCR showed the best results. Further, authors determine the specificity of analyzed platform by measuring gene expression of miRNAs showing differences in nucleotide sequence (vary from one to four nucleotides). On the basis of signal intensity, the

Table 1
Summary of changes in miRNA expression profile in various disease entities.

Disease	miRNA	Fold change ($2^{-\Delta\Delta Ct}$)	Direction of change	P value	Ref.
Prostate cancer	miR-4289	2.15[a]	Upregulation	< 0.0004	[24]
	miR-326	2.3[a]		< 0.0004	
	miR-98-5p	2.8[a]		0.0012	
	miR-152-3p	3.5[a]		0.0016	
Diffuse large B-cell lymphoma	miR-155	5.24	Upregulation	0.009	[26]
	miR-210	4.15		0.02	
	miR-21	2.56		0.04	
Non-small cell lung cancer	miR-145	21.67	Upregulation	0.0002	[29]
	miR-20a	13.39		0.00009	
	miR-21	6.15		0.00037	
	miR-223	2.64		0.00148	
Lung cancer	miR-21	1.36	Upregulation	0.0441	[31]
	miR-19b	2.38		< 0.0001	
Colorectal adenomas	miR-15b	242	Upregulation	0.0009	[33]
	miR-17	16		0.0252	
	miR-142-3p	66		0.0031	
	miR-195	18		0.0173	
	miR-331	33		0.0172	
	miR-532	50		0.0151	
	miR-532-3p	21		0.0173	
	miR-625	84		0.0172	
Colorectal cancer	miR-409-3p	4.5	Upregulation	0.0024	[34]
	miR-7	0.2	Downregulation	< 0.0001	[34]
	miR-93	0.4		0.00022	
	miR-23a-3p	2.05	Upregulation	< 0.0001	[35]
	miR-27a-3p	1.69		< 0.0001	
	miR-142-5p	2.4		< 0.0001	
	miR-376c-3p	1.95		< 0.0001	
Acute pulmonary embolism	miR-134	25.39	Upregulation	0.047	[58]
Stroke	miR-125b-2	1.795	Upregulation	0.007	[58]
	miR-27a	3.745		0.002	
	miR-422a	1.755		0.002	
	miR-488	2.124		0.006	
	miR-627	3.992		0.003	
Coronary artery disease	miR-126	0.37[a]	Downregulation	< 0.001	[41]
	miR-17	0.27[a]		< 0.001	
	miR-92a	0.46[a]		< 0.001	
	miR-155	0.48		< 0.001	
	miR-145	0.49[a]		< 0.001	
Cardiovascular diseases	miR-208b	1500	Upregulation	< 0.005	[44]
	miR-499	90		< 0.001	
Myocardial infarction	miR-1	15[a]	Upregulation	< 0.01	[46]
	miR-133a	140[a]		< 0.01	
	miR-133b	57[a]		< 0.01	
	miR-499-5p	105[a]		< 0.01	
	miR-1	300	Upregulation	< 0.01	[47]
	miR-133a	70		< 0.01	
	miR-208b	3000		< 0.001	
	miR-499-5p	250		< 0.01	
	miR-499-5p	80	Upregulation	< 0.05	[49]
Parkinson's disease	miR-331-5p	21[a]	Upregulation	0.001	[70]
	miR-195	1.65	Upregulation	0.000261	[72]
	miR-185	0.41	Downregulation	< 0.0001	
	miR-15b	0.27		< 0.0001	
	miR-221	0.34		< 0.0001	
	miR-181a	0.35		< 0.0001	
Hepatitis C	miR-122 (1st set)	10.8	Upregulation	< 0.0001	[95]
	miR-16 (1st set)	3		0.0002	
	miR-122 (2nd set)	7.9		< 0.0001	
	miR-16 (2nd set)	6.3		< 0.0001	
HIV	miR-29a	4[a]	Upregulation	< 0.01	[105]
	miR-27a	16[a]		< 0.01	
	miR-151-3p	5.65[a]		< 0.01	
	miR-19b	11.31[a]		< 0.01	

[a] Estimated value based on the figures, and relative expression data, given by the studies.

specificity was evaluated. The best results were achieved by qRT-PCR [111].

The choice of the right platform should depend on the effect that we expect after the experiment. Many factors (i.e. RNA concentration) may affect the obtained result. In our opinion, qRT-PCR should be used in clinical laboratories, because it shows the highest sensitivity, detection rate and specificity. Microarrays and NGS should be use in experimental laboratories that focus on finding differences in miRNA expression in various diseases.

4. Conclusion

Many studies from last decade have shown that miRNAs - small, non-coding RNAs that are able to regulate gene expression, cell growth, tissue differentiation, cell proliferation or apoptosis, are differentially expressed in various disease entities. Furthermore, it has been shown that many miRNAs are secreted from cells to body fluids changing the expression patterns in response to disease state. Also, high stability of circulating miRNAs to harmful conditions means that miRNAs are considered to be a potential biomarkers. All studies cited above, shows very promising result for plasma miRNAs as a biomarker (Table 1). Unfortunately, replication of similar results obtained for particular miRNA in various diseases is a common phenomenon, affecting negatively on the specificity of future tests based on those miRNAs. Various studies have shown that miR-27a is differentially expressed in many diseases entities, including APE, Stroke, HIV and Colorectal Cancer. MiR-21 is differentially expressed in DLBCL, NSCLC, CAD and ACLF. Furthermore, miR-19b has also been significant in the diagnosis of Lung Cancer and HIV. The lack of specificity to a particular disease could suggest its use in many other diagnostic panels, with a wide spectrum of activity. Besides this, many studies have shown expression of various miRNAs in the same disease, while more and more experiments, large-scale validation studies and clinical trials are bringing us closer to finding the ideal biomarker and changing the actual state-of-the-art in diagnostics. At this moment, development of an suitable test requires finding several miRNAs, preferably showing differential expression in this particular disease (or related to it) and constructing appropriate diagnostic panel. Very promising studies have been performed by Vychytilova-Faltejskova et al. in which combinations of specific biomarkers in a miRs-panel reached the highest diagnostic efficiency – 96% in patients with Colorectal Cancer ($p < 0.0001$). Promising results were also obtained by Ding et al. in which four downregulated miRNAs were very significant in diagnosis of Parkinson's Disease ($p < 0.0001$). In one study, Cermelli et al. performed two independent experiments on different populations, which showed repeatable results in diagnosis of Hepatitis C ($p < 0.001$).

All of the works described above have shown how great an impact miRNAs will have in future diagnosis. Finding new, better biomarkers that could easily replace the current, not highly-specific nor sensitive biomarkers is a rapidly evolving field in molecular biology. Many studies have already shown very promising results, highlighting the potential of plasma miRNAs in the diagnosis of various diseases entities. On the other hand, methodological and technical limitations, the high cost of experiments and low populations are major impediments to many scientists. In spite of this, we have a hope that in the next few decades, scientists from all over the world will overcome these limitations to perform large-scale studies.

Financial support

This work was supported by Grant DI2016 022046 from the Ministry of Science and Higher Education in Poland

Declaration of Competing Interest

The authors declare no conflict of interest.

References

[1] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell. 116 (2) (2004) 281–297, https://doi.org/10.1016/S0092-8674(04)00045-5.
[2] V. Ambros, The function of animal miRNA, Nature. 431 (7006) (2004) 350–355, https://doi.org/10.1038/nature02871.
[3] M.Q. Huberdeau, M.J. Simard, A guide to microRNA-mediated gene silencing, FEBS J. 286 (4) (2018) 642–652, https://doi.org/10.1111/febs.14666.
[4] http://www.mirbase.org/summary.shtml?org=hsa – 13.02.2019
[5] C. Catalanotto, C. Cogoni, G. Zardo, MicroRNA in Control of gene expression: an overview of nuclear functions, Int. J. Mol. Sci. 17 (10) (2016), https://doi.org/10.3390/ijms17101712 pii: E1712.
[6] K.M. Nelson, G.J. Weiss, MicroRNAs and cancer: past, present, and potential future, Mol. Cancer Ther. 7 (12) (2008) 3655–3660, https://doi.org/10.1158/1535-7163.MCT-08-0586.
[7] A.J. Pratt, I.J. MacRae, The RNA-induced silencing complex: A versatile gene-silencing machine, J. Biol. Chem. 284 (27) (2009) 17897–17901, https://doi.org/10.1074/jbc.R900012200.
[8] W.C.S Cho, OncomiRs: the discovery and progress of microRNAs in cancers, Mol. Cancer 6 (60) (2007) 1–7, https://doi.org/10.1186/1476-4598-6-60.
[9] M.V. Iorio, M. Ferracin, C.G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J.P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Neci, G.A. Calin, P. Querzoli, M. Negrini, C.M. Croce, MicroRNA gene expression deregulation in human breast cancer, Cancer Res. 65 (16) (2005) 7065–7070, https://doi.org/10.1158/0008-5472.CAN-05-1783.
[10] N. Rosenfeld, R. Aharonov, E. Meiri, S. Rosenwald, Y. Spector, M. Zepeniuk, H. Benjamin, N. Shabes, S. Tabak, A. Levy, D. Lebanony, Y. Goren, E. Silberschein, N. Targan, A. Ben-Ari, S. Gilad, N. Sion-Vardy, A. Tobar, M. Feinmesser, O. Kharenko, O. Nativ, D. Nass, M. Perelman, A. Yosepovich, B. Shalmon, S. Polak-Charcon, E. Fridman, A. Avniel, I. Bentwich, Z. Bentwich, D. Cohen, A. Chajut, I. Barshack, MicroRNAs accurately identify cancer tissue origin, Nat. Biotechnol. 26 (4) (2008) 462–469, https://doi.org/10.1038/nbt1392.
[11] C.H. Lawrie, S. Soneji, T. Marafioti, C.D. Cooper, S. Palazzo, J.C. Paterson, H. Cattan, T. Enver, R. Mager, J. Boultwood, J.S. Wainscoat, C.S.R. Hatton, MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma, Int. J. Cancer 121 (5) (2007) 1156–1161, https://doi.org/10.1002/ijc.22800.
[12] A. Cimmino, G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. Liu, T.J. Kipps, M. Negrini, C.M. Croce, miR15 and miR-16 induce apoptosis by targeting BCL2, Proc. Natl. Acad. Sci. U. S. A. 102 (39) (2005) 13944–13949, https://doi.org/10.1073/pnas.0506654102.
[13] A. Bahreyni, M. Rezaei, A. Bahrami, M. Khazaei, H. Fiuji, M. Ryzhikov, G.A. Ferns, A. Avan, S.M. Hassanian, Diagnostic, prognostic, and therapeutic potency of microRNA 21 in the pathogenesis of colon cancer, current status and prospective, J. Cell. Physiol. 234 (6) (2018) 8075–8081, https://doi.org/10.1002/jcp.27580.
[14] E. Mogilyansky, I. Rigoutsos, The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease, Cell Death Differ. 20 (12) (2013) 1603–1614, https://doi.org/10.1038/cdd.2013.125.
[15] P. Jozwiak, A. Lipinska, RNA interference as a potential tool for diagnosis and therapy of some human diseases, Postepy Hig. Med. Dosw. 64 (2010) 504–512.
[16] G.W. Dorn, MicroRNAs in Cardiac Disease, Transl. Res. 157 (4) (2011) 226–235, https://doi.org/10.1016/j.trsl.2010.12.013.
[17] S. Ikeda, S.W. Kong, J. Lu, E. Bisping, H. Zhang, P.D. Allen, T.R. Golub, B. Pieske, W.T. Pu, Altered microRNA expression in human heart disease, Physiol. Genomics 31 (3) (2007) 367–373, https://doi.org/10.1152/physiolgenomics.00144.2007.
[18] Q. Li, X.W. Song, J. Zou, G.K. Wang, E. Kremneva, X.Q. Li, N. Zhu, T. Sun, P. Lappalainen, W.J. Yuan, Y.W. Qin, Q. Jing, Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy, J. Cell Sci. 123 (14) (2010) 2444–2452, https://doi.org/10.1242/jcs.067165.
[19] A.J. Tijsen, Y.M. Pinto, E.E. Creemers, Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases, Am. J. Physiol. Heart Circ. Physiol. 303 (9) (2012) H1085–H1095, https://doi.org/10.1152/ajpheart.00191.2012.
[20] M.H. Sohel, Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges, Achieve. Life. Sci. 10 (2) (2016) 175–186, https://doi.org/10.1016/j.als.2016.11.007.
[21] Y. Bai, H. Lin, Z. Fang, Q. Luo, Y. Fang, Y. Su, Q. Hu, H. Duan, F. Chen, Z.Y. Zhang, Plasma microRNA-19a as a potential biomarker for esophageal squamous cell carcinoma diagnosis and prognosis, Biomark. Med 11 (5) (2017) 431–441, https://doi.org/10.2217/bmm-2016-0286.
[22] https://www.who.int/en/news-room/fact-sheets/detail/cancer - 20.10.2018
[23] S. McGrath, D. Christidis, M. Perera, S.K. Hong, T. Manning, I. Vela, N. Lawrentschuk, Prostate cancer biomarkers: Are we hitting the mark? Prostate. Int. 4 (4) (2016) 130–135, https://doi.org/10.1016/j.prnil.2016.07.002.
[24] F. Matin, V. Jeet, L. Moya, L.A. Selth, S. Chambers, Australian prostate cancer bioresource, in: J.A. Clements, J. Batra (Eds.), A Plasma Biomarker Panel of Four MicroRNAs for the Diagnosis of Prostate Cancer, Sci. Rep, 8(1) 2018, p. 6653, , https://doi.org/10.1038/s41598-018-24424-w.
[25] D. Khare, N. Goldschmidt, A. Bardugo, D. Gur-Wahnon, I.Z. Ben-Dov, B. Avni, Plasma microRNA profiling: Exploring better biomarkers for lymphoma surveillance, PLoS One 12 (11) (2017) e0187722, , https://doi.org/10.1371/journal.pone.0187722.
[26] C.H. Lawrie, S. Gal, H.M. Dunlop, B. Pushkaran, A.P. Liggins, K. Pulford, A.H. Banham, F. Pezella, J. Boultwood, J.S. Wainscoat, C.S.R. Hatton, A.L. Harris, Detection of elevated levels of tumor-associated microRNAs in serum of patients with diffuse large B-cell lymphoma, Br. J. Hematol. 141 (5) (2008) 672–675, https://doi.org/10.1111/j.1365-2141.2008.07077.x.
[27] C. Feng, D.X. Zhu, H.J. Dong, Z.J. Zhou, Y.H. Wang, L. Liu, L. Fan, K.R. Miao, P. Liu, W. Xu, J.Y. Li, Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma, Ann. Hematol. 91 (4) (2012) 553–559, https://doi.org/10.1007/s00277-011-1350-9.
[28] Q. Leng, Y. Lin, F. Jiang, C.J. Lee, M. Zhan, H. Fang, Y. Want, F. Jiang, A plasma miRNA signature for lung cancer early detection, Oncotarget 8 (67) (2017) 111902–111911, https://doi.org/10.18632/oncotarget.22950.

[29] H. Zhang, F. Mao, T. Shen, Q. Luo, Z. Ding, L. Qian, J. Huang, Plasma miR-145, miR-20a, miR-21 and miR-223 as novel biomarkers for screening early-stage non-small cell lung cancer, Oncol. Lett. 13 (2) (2016) 669–676, https://doi.org/10.3892/ol.2016.5462.
[30] X. Gao, Y. Wang, H. Zhao, F. Wei, X. Zhang, Y. Su, C. Wang, H. Li, X. Ren, Plasma miR-324-3p and miR-1285 as diagnostic and prognostic biomarkers for early stage lung squamous cell carcinoma, Oncotarget 7 (37) (2016) 59664–59675, https://doi.org/10.18632/oncotarget.11198.
[31] I.A. Zaporozhchenko, E.S. Morozkin, T.E. Skvortsova, A.A. Ponomaryova, E.Y. Rykova, N.V. Cherdyntseva, E.S. Polovnikov, O.A. Pashkobskaya, E.A. Pokushalov, V.V. Vlassov, P.P. Laktinov, Plasma miR-19b and miR-183 as potential biomarkers of lung cancer, PLoS One 11 (10) (2016) e0165261, https://doi.org/10.1371/journal.pone.0165261.
[32] M.L. Wikberg, R. Myte, R. Palmqvist, B. van Guelpen, I. Ljuslinder, Plasma miRNA can detect colorectal cancer, but how early ? Cancer. Med. 7 (5) (2018) 1697–1705, https://doi.org/10.1002/cam4.1398.
[33] Z. Kanaan, H. Roberts, M.R. Eichenberger, A. Billeter, G. Ocheretner, J. Pan, S. Rai, J. Jorden, A. Williford, S. Galandiuk, A plasma microRNA panel for detection of colorectal adenomas: a step toward more precise screening for colorectal cancer, Ann. Surg. 258 (3) (2013) 400–408, https://doi.org/10.1097/SLA.0b013e3182a15bcc.
[34] S. Wang, J. Xiang, Z. Li, S. Lu, J. Hu, X. Gao, L. Yu, L. Wang, J. Wang, Y. Wu, Z. Chen, H. Zhu, A plasma microRNA panel for early detection of colorectal cancer, Int. J. Cancer 136 (1) (2015) 152–161, https://doi.org/10.1002/ijc.28136.
[35] P. Vychytilova-Faltejskova, L. Radova, M. Sachlova, Z. Kosarova, K. Slaba, P. Fabian, T. Grolich, V. Prochazka, Z. Kala, M. Svovoda, I. Kriss, R. Vyzula, O. Slaby, Serum-based microRNA signatures in early diagnosis and prognosis prediction of colon cancer, Carcinogenesis. 37 (10) (2016) 941–950, https://doi.org/10.1093/carcin/bgw078.
[36] C.R. Lima, C.C. Gomes, M.F. Santos, Role of MicroRNAs in endocrine cancer metastasis, Mol. Cell. Endocrinol. 456 (2017) 62–75, https://doi.org/10.1016/j.mce.2017.03.015.
[37] D. Sekar, R. Krishnan, K. Thirugnanasambantham, B. Rajasekaran, V.I. Islam, P. Sekar, Significance of microRNA 21 in gastric cancer, Clin. Res. Hepatol. Gastroenterol. 40 (5) (2016) 538–545, https://doi.org/10.1016/j.clinre.2016.02.010.
[38] W. Jinling, S. Sijing, Z. Jie, W. Guinian, Prognostic value of circulating microRNA-21 for breast cancer: a systematic review and meta-analysis, Artif. Cells. Nanomed. Biotechnol. 45 (6) (2016) 1–6, https://doi.org/10.1080/21691401.2016.1216856.
[39] A. Bahrami, A. Aledavood, K. Anvari, S.M. Hassanian, M. Maftouh, A. Yaghobzade, O. Salarzaee, S.S. Sales, A. Avan, The prognostic and therapeutic application of microRNAs in breast cancer: Tissue and circulating microRNAs, J. Cell. Physiol. 233 (2) (2017) 774–786, https://doi.org/10.1002/jcp.25813.
[40] Y.H. Feng, C.J. Tsao, Emerging role of microRNA-21 in cancer, Biomed. Rep. 5 (4) (2016) 395–402, https://doi.org/10.3892/br.2016.747.
[41] S. Fichtlscherer, S. De Rosa, H. Fox, T. Schwietz, A. Fischer, C. Liebetrau, M. Weber, C.W. Hamm, T. Roxe, M. Muller-Ardogan, A. Bonauer, A.M. Zeiher, S. Dimmeler, Circulating MicroRNAs in Patients with Coronary Artery Disease, Circ. Res. 107 (5) (2010) 677–684, https://doi.org/10.1161/CIRCRESAHA.109.215566.
[42] P. Diehl, A. Fricke, L. Sander, J. Stamm, N. Bassler, N. Htun, M. Ziemann, T. Helbing, A. El-Osta, J.B.M. Jowett, K. Peter, Microparticles: major transport vehicles for distinct microRNAs in circulation, Cardiovasc. Res. 93 (4) (2012) 633–644, https://doi.org/10.1093/cvr/cvs007.
[43] M. Weber, M.B. Baker, R.S. Patel, A.A. Quyyumi, G. Bao, C.D. Searles, MicroRNA expression profile in CAD patients and the impact of ACEI/ARB, Cardiol. Res. Pract. 532915 (2011), https://doi.org/10.4061/2011/532915.
[44] M.F. Corsten, R. Dennert, S. Jochems, T. Kuznetsova, Y. Devaux, L. Hofstra, D.R. Wagner, J.A. Staessen, S. Heymans, B. Schroen, Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease, Circ. Cardiovasc. Genet. 3 (6) (2010) 499–506, https://doi.org/10.1161/CIRCGENETICS.110.957415.
[45] J. Ai, R. Zhang, Y. Li, J. Pu, Y. Lu, J. Jiao, K. Li, B. Yu, Z. Li, R. Wang, L. Wang, Q. Li, N. Wang, H. Shan, Z. Li, B. Yang, Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction, Biochem. Biophys. Res. Commun. 391 (1) (2010) 73–77, https://doi.org/10.1016/j.bbrc.2009.11.005.
[46] Y. D'Alessandra, P. Devanna, F. Limana, S. Straino, A. Di Carlo, P.G. Brambilla, M. Rubino, M.C. Carena, L. Spazzafumo, M. De Simon, B. Micheli, P. Biglioli, F. Achilli, F. Martelli, S. Maggiolini, G. Marenzi, G. Pompilio, M.C. Capogrossi, Circulating microRNAs are new and sensitive biomarkers of myocardial infarction, Eur. Heart J. 31 (22) (2010) 2765–2773, https://doi.org/10.1093/eurheartj/ehq167.
[47] O. Gidlof, A. Andersson, J. van der Pals, M. Gotberg, D. Erlinge, Cardiospecific microRNA plasma levels correlate with troponin and cardiac function in patients with ST elevation myocardial infarction, are selectively dependent on renal elimination, and can be detected in urine samples, Cardiology. 118 (2011) 217–226, https://doi.org/10.1159/000328869.
[48] Y. Kuwabara, K. Ono, T. Horie, H. Nishi, K. Nagao, M. Kinoshita, S. Watanabe, O. Baba, Y. Kojima, S. Shizuta, M. Imai, T. Tamura, T. Kita, T. Kimura, Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage, Circ. Cardiovasc. Genet. 4 (4) (2011) 446–454, https://doi.org/10.1161/CIRCGENETICS.110.958975.
[49] F. Olivieri, R. Antonicelli, M. Lorenzi, Y. D'Alessandra, R. Lazzarini, G. Santini, L. Spazzafumo, R. Lisa, L. La Sala, R. Galezzi, R. Recchioni, R. Testa, G. Pompilio, M.C. Capogrossi, A.D. Procopio, Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction, Int. J. Cardiol. 167 (2) (2012) 531–536, https://doi.org/10.1016/j.ijcard.2012.01.075.
[50] G.K. Wang, J.Q. Zhu, J.T. Zhang, Q. Li, Y. Li, J. He, Y.W. Qin, Q. Jing, Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans, Eur. Heart J. 31 (6) (2010) 659–666, https://doi.org/10.1093/eurheartj/ehq013.
[51] S. Mythili, N. Malathi, Diagnostic markers of acute myocardial infarction, Biomed. Rep. 3 (2015) 743–748, https://doi.org/10.3892/br.2015.500.
[52] J. Howie-Esquivel, M. White, Biomarkers in Acute Cardiovascular Disease, J. Cardiovasc. Nurs. 23 (2) (2008) 124–131, https://doi.org/10.1097/01.jcn.0000305072.49613.92.
[53] H. Al-Hadi, K.A. Fox, Cardiac markers in the early diagnosis and management of patients with acute coronary syndrome, Sultan. Quaboos. Univ. Med. J 9 (3) (2009) 231–246.
[54] Y.Q. Li, M.F. Zhang, H.Y. Wen, C.L. Hu, R. Liu, H.Y. Wei, C.M. Ai, G. Wang, X.X. Liao, X. Li, Comparing the diagnostic values of circulating microRNAs and cardiac troponin T in patients with acute myocardial infarction, Clinics. 68 (1) (2013) 75–80, https://doi.org/10.6061/clinics/2013(01)OA12.
[55] L.M. Li, W.B. Cai, Q. Ye, J.M. Liu, X. Li, X.X. Liao, Comparison of plasma microRNA-1 and cardiac troponin T in early diagnosis of patients with acute myocardial infarction, World J Emerg Med 5 (3) (2014) 182–186, https://doi.org/10.5847/whem.j.issn.1920-8642.2014.03.004.
[56] W. Johnson, O. Onuma, M. Owolabi, S. Sachdev, Stroke: a global response is needed, Bull. World Health Organ. 94 (9) (2016), https://doi.org/10.2471/BLT.16.181636 634-634A.
[57] M. Vijayan, P.H. Reddy, Peripheral biomarkers of stroke: focus on circulatory microRNAs, Biochim. Biophys. Acta 1862 (10) (2016) 1984–1993, https://doi.org/10.1016/j.bbadis.2016.08.003.
[58] S. Sepramaniam, J.R. Tan, K.S. Tan, D.A. DeSilva, S. Tavintharan, F.P. Woon, C.W. Wang, F.L. Yong, D.S. Karolina, P. Kaur, F.J. Liu, K.Y. Lim, A. Armugam, K. Jeyaseelan, Circulating MicroRNAs as Biomarkers of Acute Stroke, Int. J. Mol. Sci. 15 (1) (2014) 1418–1432, https://doi.org/10.3390/ijms15011418.
[59] G. Long, F. Wang, H. Li, Z. Yin, C. Sandip, Y. Lou, Y. Wang, C. Chen, D.W. Wang, Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans, BMC Neurol. 13 (2013) 178, https://doi.org/10.1186/1471-2377-13-178.
[60] I. Starikova, S. Jamaly, A. Sorrentino, T. Blondal, N. Latysheva, M. Sovershaev, J.B. Hansen, Differential expression of plasma miRNAs in patients with unprovoked venous thromboembolism and healthy control individuals, Thromb. Res. 136 (3) (2015) 566–572, https://doi.org/10.1016/j.thromres.2015.07.005.
[61] Y. Wang, Z. Zhao, Y. Zhang, X. Jiang, J. Duo, Plasma miRNA-134 is a diagnostic biomarker for acute pulmonary embolism in middle and high altitude areas, Biomed. Res. 28 (12) (2017) 5494–5498.
[62] J. Xiao, Z.C. Jing, P.T. Ellinor, D. Liang, H. Zhang, Y. Liu, X. Chen, L. Pan, R. Lyon, Y. Liu, L.Y. Peng, X. Liang, Y. Sun, L.M. Popescu, G. Condorelli, Y.H. Chen, MicroRNA-134 as a potential plasma biomarker for the diagnosis of acute pulmonary embolism, J. Transl. Med. 9 (2011) 159, https://doi.org/10.1186/1479-5876-9-159.
[63] Q. Wang, J. Ma, Z. Jiang, F. Wu, J. Ping, L. Ming, Diagnostic value of circulating microRNA-27a/b in patients with acute pulmonary embolism, Int. Angiol. 37 (1) (2018) 19–25, https://doi.org/10.23736/S0392-9590.17.03877-9.
[64] S.K. Khoo, D. Petillo, U.J. Kang, J.H. Resau, B. Berryhill, J. Linder, L. Forsgren, L.A. Neuman, A.C. Tan, Plasma-based circulating MicroRNA biomarkers for Parkinson's disease, J. Park. Dis. 2 (4) (2012) 321–331, https://doi.org/10.3233/JPD-012144.
[65] K.E. Zeuner, E. Schaffer, F. Hopfner, N. Bruggemann, D. Berg, Progress of pharmacological approaches in Parkinson's disease, Clin. Pharmacol. Ther. 105 (5) (2019) 1106–1120, https://doi.org/10.1002/cpt.1374.
[66] K. Wakabayashi, K. Tanji, S. Odagiri, Y. Miki, F. Mori, H. Takahashi, The Lewy Body in Parkinson's Disease and Related Neurodegenerative Disorders, Mol. Neurobiol. 47 (2013) 495–508, https://doi.org/10.1007/s12035-012-8280-y.
[67] C.A. Davie, A review of Parkinson's disease, Br. Med. Bull. 86 (2008) 109–127, https://doi.org/10.1093/bmb/ldn013.
[68] A.E. Roser, L.C. Gomes, J. Schunemann, F. Maass, P. Lingor, Circulating miRNAs as diagnostic Biomarkers for Parkinson's Disease, Front. Neurosci. 12 (2018) 625, https://doi.org/10.3389/fnins.2018.00625.
[69] S.R. Siegel, J. Mackenzie, G. Chaplin, N.G. Jablonski, L. Griffiths, Circulating microRNAs involved in multiple sclerosis, Mol. Biol. Rep. 39 (5) (2012) 6219–6225, https://doi.org/10.1007/s11033-011-1441-7.
[70] L.F. Cardo, E. Coto, L. De Mena, R. Ribacoba, G. Moris, M. Menedez, V. Alvarez, Profile of microRNAs in the plasma of Parkinson's disease patients and healthy controls, J. Neurol. 260 (5) (2013) 1420–1422, https://doi.org/10.1007/s00415-013- 6900-8.
[71] N. Li, X. Pan, J. Zhang, A. Ma, S. Yang, J. Ma, A. Xie, Plasma levels of miR-137 and miR-124 are associated with Parkinson's disease but not with Parkinson's disease with depression, Neurol. Sci. 38 (5) (2017) 761–767, https://doi.org/10.1007/s10072-017-2841-9.
[72] H. Ding, Z. Huang, M. Chen, C. Wang, X. Chen, J. Chen, J. Zhang, Identification of a panel of five serum miRNAs as a biomarker for Parkinson's disease, Parkinsonism Relat. Disord. 22 (2016) 68–73, https://doi.org/10.1016/j.parkreldis.2015.11.014.
[73] R.A. Robinson, B. Amin, P.C. Guest, Multiplexing biomarkers methods, proteomics and consideration for Alzheimer's disease, Adv. Exp. Med. Biol. 974 (2017) 21–48, https://doi.org/10.1007/978-3-319-52479-5_2.
[74] M. Zendjabil, Circulating microRNAs as novel biomarkers of Alzheimer's disease,

Clin. Chim. Acta 484 (2018) 99–104, https://doi.org/10.1016/j.cca.2018.05.039.
[75] A. Sanabria-Castro, I. Alvarado-Echeverria, C. Monge-Bonilla, Molecular Pathogenesis of Alzheimer's Disease: An Update, Ann. Neurosci. 24 (1) (2017) 46–54, https://doi.org/10.1159/000464422.
[76] H. Geekiyanage, G.A. Jicha, P.T. Nelson, C. Chan, Blood serum miRNA: non-invasive biomarkers for Alzheimer's disease, Exp. Neurol. 235 (2) (2012) 491–496, https://doi.org/10.1016/j.expneurol.2011.11.026.
[77] T. Kiko, K. Nakagawa, T. Tsuduki, K. Furukawa, H. Arai, T. Miyazawa, MicroRNA in plasma and cerebrospinal fluid as potential markers for Alzheimer's disease, J. Alzhiemers. Dis. 39 (2) (2014) 253–259, https://doi.org/10.3233/JAD-130932.
[78] S. Bhatnagar, H. Chertkow, H.M. Schipper, Z. Yuan, V. Shetty, S. Jenkins, T. Jones, E. Wang, Increased microRNA-34c abundance in Alzheimer's disease circulating blood plasma, Front. Mol. Neurosci. 7 (2014) 2, https://doi.org/10.3389/fnmol.2014.00002.
[79] S.S. Sorensen, A.B. Nygaard, T. Christensen, miRNA expression profiles in cerebrospinal fluid and blood of patients with Alzheimer's Disease and other types of dementia – an exploratory study, Transl. Neurodegener. 5 (2016) 6, https://doi.org/10.1186/s40035-016-0053-5.
[80] P. Kumar, Z. Dezso, C. MacKenzie, J. Oestreicher, S. Agoulnik, M. Byrne, F. Bernier, M. Yanagimachi, K. Aoshima, Y. Oda, Circulating miRNA biomarkers for Alzheimer's disease, PLoS One 8 (7) (2013) e69807, , https://doi.org/10.1371/journal.pone.0069807.
[81] P. Leidinger, C. Backes, S. Deutscher, K. Schmitt, S.C. Mueller, K. Frese, J. Haas, K. Ruprecht, F. Paul, C. Stahler, C.J.G. Lang, B. Meder, T. Bartfai, E. Meese, A. Keller, A blood based 12-miRNA signature of Alzheimer disease patients, Genome Biol. 14 (7) (2013) R78, https://doi.org/10.1186/gb-2013-14-7-r78.
[82] J.I. Satoh, Y. Kino, S. Niida, MicroRNA-Seq Data Analysis Pipeline to Identify Blood Biomarkers for Alzheimer's Disease from Public Data, Biomark. Insights 10 (2015) 21, https://doi.org/10.4137/BMI.S25132.
[83] H.M. Schipper, O.C. Maes, H.M. Chertkow, E. Wang, MicroRNA Expression in Alzheimer Blood Mononuclear Cells, Gene. Regul. Syst. Bio. 1 (2007) 263–274.
[84] P. Lau, K. Bossers, R. Janky, E. Salta, C.S. Frigerio, S. Barbash, R. Rothman, A.S.R. Sierksma, A. Thathiah, D. Greenberg, A.S. Papadopoulou, T. Achsel, T. Ayoubi, H. Soreq, J. Verhaagen, D.F. Swaab, S. Aerts, B. De Strooper, Alteration of the microRNA network during the progression of Alzheimer's disease, EMBO. Mol. Med. 5 (10) (2013) 1613–1634, https://doi.org/10.1002/emmm.201201974.
[85] S. Nagaraj, K. Laskowksa-Kaszub, K.J. Debski, J. Wojsiat, M. Dabrowski, T. Gabryelewicz, J. Kuznicki, U. Wojda, Profile of 6 microRNA in blood plasma distinguish early stage Alzheimer's disease patients from non-demented subjects, Oncotarget 8 (10) (2017) 16122–16143, https://doi.org/10.18632/oncotarget.15109.
[86] https://liverfoundation.org/liver-disease-statistics/#non-alcoholic-fatty-liver-disease-non-alcoholic-steato-hepatitis – 13.02.2019
[87] S. Roy, F. Benz, T. Luedde, C. Roderburg, The role of miRNAs in the regulation of inflammatory processes during hepatofibrogenesis, Hepatobiliary. Surg. Nutr. 4 (1) (2015) 24–33, https://doi.org/10.3978/j.issn.2304-3881.2015.01.05.
[88] Y. Zhang, Y. Jia, R. Zheng, Y. Guo, Y. Wang, H. Guo, M. Fei, S. Sun, Plasma MicroRNA-122 as a Biomarker for Viral-, Alcohol- and Chemical-Related Hepatic Diseases, Clin. Chem. 56 (12) (2010) 1830–1838, https://doi.org/10.1373/clinchem.2010.147850.
[89] S. Bandiera, S. Pfeffer, T.F. Baumert, M.B. Zeisel, miR-122 – A key factor and therapeutic target in liver disease, J. Hepatol. 62 (2) (2014) 448–457, https://doi.org/10.1016/j.jhep.2014.10.004.
[90] J. Hu, Y. Xu, J. Hao, S. Wang, C. Li, S. Meng, MiR-22 in hepatic function and liver diseases, Protein Cell. 3 (5) (2012) 364–371, https://doi.org/10.1007/s13238-012-2036-3.
[91] V. Bihrer, M. Friedrich-Rust, B. Kronenberger, N. Forestier, J. Haupenthal, Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection, Am. J. Gastroenterol. 106 (9) (2011) 1663–1669, https://doi.org/10.1038/ajg.2011.161.
[92] S. Bala, J. Petrasek, S. Mundkur, D. Catalano, I. Levin, J. Ward, H. Alao, K. Kodys, G. Szabo, Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced and inflammatory liver diseases, Hepatology. 56 (5) (2012) 1946–1957, https://doi.org/10.1002/hep.25873.
[93] D. Blaya, B. Aguilar-Bravo, F. Hao, S. Cascuberta-Serra, M. Coll, Expression of microRNA-155 in inflammatory cells modulates liver injury, Hepatology. 68 (2) (2018) 691–706, https://doi.org/10.1002/hep.29833.
[94] S. Bala, Y. Tilahun, O. Taha, H. Alao, K. Kodys, D. Catalano, G. Szabo, Increased microRNA-155 expression in the serum and peripheral monocytes in chronic HCV infection, J. Transl. Med. 10 (1) (2012) 151, https://doi.org/10.1186/1479-5876-10-151.
[95] S. Cermelli, A. Ruggieri, J.A. Marrero, G.N. Ioannou, L. Beretta, Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease, PLoS One 6 (8) (2011) e23937, , https://doi.org/10.1371/journal.pone.0023937.
[96] N.C. Salvoza, D.C. Klinzing, J. Gopez-Cervantes, M.O. Baclig, Association of Circulating Serum miR-34a and miR-122 with Dyslipidemia among Patients with Non-Alcoholic Fatty Liver Disease, PLoS One 11 (4) (2016) e0153497, , https://doi.org/10.1371/journal.pone.0153497.
[97] A.M. Butt, A.J. Raja, S. Siddique, J.S. Khan, M. Shahid, G. Tayyab, Z. Minhas, M. Umar, M. Idrees, Y. Tong, Parallel expression profiling of hepatic and serum microRNA-122 associated with clinical features and treatment responses in chronic hepatitis C patients, Sci. Rep. 6 (2016) 21510, https://doi.org/10.1038/srep21510.
[98] M. El-Hefny, S. Fouad, T. Hussein, R. Abdel-Hameed, H. Effat, H. Mohamed, A.H.A. Wahab, Circulating microRNAs as predictive biomarkers for liver disease progression of chronic hepatitis C (genotype-4) Egyptian patients, J. Med. Virol. 91 (1) (2019) 93–101, https://doi.org/10.1002/jmv.25294.
[99] F. Ji, B. Yang, X. Peng, H. Ding, H. You, P. Tien, Circulating microRNAs in hepatitis B virus-infected patients, J. Viral Hepat. 18 (7) (2011) e242–e251, https://doi.org/10.1111/j.1365-2893.2011.01443.x.
[100] D. Ye, T. Zhang, G. Lou, Y. Liu, Role of miR-223 in the pathophysiology of liver diseases, Exp. Mol. Med. 50 (9) (2018) 128, https://doi.org/10.1038/s12276-018-0153-7.
[101] C. Roderburg, G.W. Urban, K. Bettermann, M. Vucur, H. Zimmermann, S. Schmidt, J. Janssen, C. Koppe, P. Knolle, M. Castoldi, F. Tacke, C. Trautwein, T. Luedde, Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis, Hepatology. 53 (1) (2011) 209–218, https://doi.org/10.1002/hep.23922.
[102] Q.F. Zheng, J.Y. Zhang, J.S. Wu, Y. Zhang, M. Liu, L. Bai, J.Y. Zhang, J. Zhao, Y. Chen, Z.P. Duan, S.J. Zjeng, Upregulation of miRNA-130a Represents Good prognosis in patients with hbv-related acute-on-chronic liver failure: a prospective study, Medicine (Baltimore) 95 (6) (2016) e2639, https://doi.org/10.1097/MD.0000000000002639.
[103] German Advisory Committee Blood (Arbeitskreis Blut), Subgroup 'assessment of pathogens transmissible by blood, Human Immunodeficiency Virus (HIV), Transfus. Med. Hemother. 43 (3) (2016) 203–222, https://doi.org/10.1159/000445852.
[104] S.U. Munshi, H. Panda, P. Holla, B.B. Rewari, S. Jameel, MicroRNA-150 Is a Potential Biomarker of HIV/AIDS Disease Progression and Therapy, PLoS One 9 (5) (2014) e95920, , https://doi.org/10.1371/journal.pone.0095920.
[105] Y. Qi, H. Hu, G. Guo, P. Xu, Z. Shi, X. Huan, Z. Zhu, M. Zhou, L. Cui, MicroRNA profiling in plasma of HIV-1 infected patients: potential markers of infection and immune status, J. Public. Health. Emerg. 1 (2017) 65, https://doi.org/10.21037/jphe.2017.05.11.
[106] R. Reynoso, N. Laufer, M. Hackl, S. Skalicky, R. Monteforte, G. Turk, M. Carobene, J. Quarleri, P. Cahn, R. Werner, H. Stoiber, R. Grillari-Voglauer, J. Grillari, MicroRNAs differentially present in the plasma of HIV elite controllers reduce HIV infection in vitro, Sci. Rep. 4 (2014) 5915, https://doi.org/10.1038/srep05915.
[107] N. Ludwig, P. Leidinger, K. Becker, C. Backes, T. Fehlmann, C. Pallasch, S. Rheinheimer, B. Meder, C. Stahler, E. Meese, A. Keller, Distribution of miRNA expression across human tissues, Nucleic Acids Res. 44 (8) (2016) 3865–3877, https://doi.org/10.1093/narlgkw116.
[108] M. Faraldi, M. Gomarasca, V. Sansoni, S. Perego, G. Banfi, G. Lombardi, Normalization strategies differently affect circulating miRNA profile associated with the training status, Sci. Rep. 9 (2019) 1584, https://doi.org/10.1038/s41598-019-38505-x.
[109] C.C. Pritchard, H.H. Cheng, M. Tewari, MicroRNA profiling: approaches and considerations, Nat. Rev. Genet. 13 (5) (2012) 358–369, https://doi.org/10.1038/nrg3198.
[110] A. Kappel, A. Keller, miRNA assays in the clinical laboratory: workflow, detection technologies and automation aspects, Clin. Chem. Lab. Med. 55 (5) (2017) 636–647, https://doi.org/10.1515/cclm02016-0467.
[111] P. Mestdagh, N. Hartmann, L. Baeriswyl, D. Andreasen, N. Bernard, C. Chen, D. Cheo, P. D'Andrade, M. DeMayo, L. Dennis, S. Derveaux, Y. Feng, S.F.-S.B. Gerstmayer, J. Gouffon, C. Grimley, E. Lader, K.Y. Lee, S. Luo, P. Mouritzen, A. Narayanan, S. Patel, S. Peiffer, S. Ruberg, G. Schroth, D. Schuster, J.M. Shaffer, E.J. Shelton, S. Sliveria, U. Ulmanella, V. Veeramachaneni, F. Staedtler, T. Peters, T. Guettouche, L. Wong, J. Vandesompele, Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study, Nat. Methods 11 (8) (2014) 809–815.

cells

MDPI

Article

Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome

Rafał Szelenberger [1,2,*], Michał Seweryn Karbownik [3], Michał Kacprzak [4], Karina Maciak [1], Michał Bijak [2], Marzenna Zielińska [4], Piotr Czarny [5], Tomasz Śliwiński [6] and Joanna Saluk-Bijak [1]

1 Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; karina.maciak@edu.uni.lodz.pl (K.M.); joanna.saluk@biol.uni.lodz.pl (J.S.-B.)
2 Biohazard Prevention Centre, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; michal.bijak@biol.uni.lodz.pl
3 Department of Pharmacology and Toxicology, Medical University of Lodz, 90-725 Lodz, Poland; michal.karbownik@umed.lodz.pl
4 Department of Interventional Cardiology, Medical University of Lodz, 91-213 Lodz, Poland; michal.kacprzak@umed.lodz.pl (M.K.); marzenna.zielinska@umed.lodz.pl (M.Z.)
5 Department of Medical Biochemistry, Medical University of Lodz, 92-215 Lodz, Poland; piotr.czarny@umed.lodz.pl
6 Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; tomasz.sliwinski@biol.uni.lodz.pl
* Correspondence: rafal.szelenberger@edu.uni.lodz.pl or rafal.szelenberger@biol.uni.lodz.pl

Citation: Szelenberger, R.; Karbownik, M.S.; Kacprzak, M.; Maciak, K.; Bijak, M.; Zielińska, M.; Czarny, P.; Śliwiński, T.; Saluk-Bijak, J. Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome. *Cells* **2021**, *10*, 3526. https://doi.org/10.3390/cells10123526

Academic Editor: Yasuhito Ishigaki

Received: 2 November 2021
Accepted: 11 December 2021
Published: 14 December 2021

Abstract: Transcriptome analysis constitutes one of the major methods of elucidation of the genetic basis underlying the pathogenesis of various diseases. The post-transcriptional regulation of gene expression is mainly provided by microRNAs. Their remarkable stability in biological fluids and their high sensitivity to disease alteration indicates their potential role as biomarkers. Given the high mortality and morbidity of cardiovascular diseases, novel predictive biomarkers are sorely needed. Our study focuses for the first time on assessing potential biomarkers of acute coronary syndrome (ACS) based on the microRNA profiles of platelets. The study showed the overexpression of eight platelet microRNAs in ACS (miR-142-3p; miR-107; miR-338-3p, miR-223-3p, miR-21-5p, miR-130b-3p, miR-301a-3p, miR-221-3p) associated with platelet reactivity and functionality. Our results show that the combined model based on miR-142-3p and aspartate transaminase reached 82% sensitivity and 88% specificity in the differentiation of the studied groups. Furthermore, the analyzed miRNAs were shown to cluster into two orthogonal groups, regulated by two different biological factors. Bioinformatic analysis demonstrated that one group of microRNAs may be associated with the physiological processes of platelets, whereas the other group may be linked to platelet–vascular environment interactions. This analysis paves the way towards a better understanding of the role of platelet microRNAs in ACS pathophysiology and better modeling of the risk of ACS.

Keywords: blood platelets; acute coronary syndrome; microRNA; biomarker; prognostic modeling

1. Introduction

According to the World Health Organization, cardiovascular diseases (CVDs) are the leading cause of death worldwide, representing 32% of all global deaths. Epidemiological studies have demonstrated that 85% of all deaths caused by CVDs were associated with stroke and acute coronary syndrome (ACS) [1]. ACS refers to a wide spectrum of clinical disorders that range from cardiac arrest and cardiogenic shock, with disturbed hemodynamics or electrical instability, to symptomless manifestations at the time of onset. The individual course of ACS hinders the initial diagnosis, thus inhibiting the therapeutic effect of the applied therapy [2]. In the early assessment of symptom presentation, 12-lead

electrocardiography and the cardiac troponin level are still considered the gold standard in diagnosis and constitute a strong foundation in the ACS triage [2,3]. ACS biomarkers in current clinical usage are focused on the diagnosis of patients during ischemic events, thus enabling monitoring of the dynamics of myocardial ischemia [2]. However, there are no biomarkers that can be used to evaluate the potential risk of ACS development before ischemia occurs. The establishment of prognostic biomarkers would enable the implementation of preventive treatment, thus reducing the risk of ACS.

Increased interest, technological progress, and a rapidly expanding publication base have focused on novel, potential biomarkers during the last two decades, suggesting microRNAs (miRNAs) as biological molecules associated with the development of pathological conditions in various disease entities [4,5]. MiRNAs are a class of short, non-coding RNAs consisting of approximately 18–25 nucleotides. Mature miRNA sequences serve as a post-transcriptional regulator of gene expression through base-pairing with target mRNAs in the 3′-UTR region [6]. It has been suggested that about 60% of human protein-encoding genes are controlled by miRNAs, thus indicating their association with many biological processes, including proliferation, differentiation, apoptosis, and the cellular cycle [4]. The primary public repository of miRNA sequences and annotations, miRBase, currently reports that the human genome contains 2654 mature sequences and 1917 annotated hairpin precursors [7]. The screening analysis of miRNA profiles allows for the discovery of altered expression patterns, of which the identification may contribute to a better understanding of the basis of various human diseases. In 2008, Chen et al. showed that miRNAs derived from different tissues are contained in large amounts in serum with remarkable stability to harmful conditions, including a wide range of pH, repeated freeze–thaw cycles, boiling, RNAse activity, and long-term storage at room temperature. Furthermore, the presence of miRNAs was also found in blood cells [8], thus indicating the possibility of the rapid, accurate, and specific analysis of the miRNome in a non-invasive manner, emphasizing the potential role of miRNAs as biomarkers in clinical practice.

Human blood platelets play a central role in the hemostasis process, ensuring vascular stability and preventing blood loss due to vessel injury [9]. Furthermore, blood platelets constitute a major implication in thrombosis, leading to the formation of pathogenic blood clots that reduce blood flow in the vessel lumen [10]. In addition to the fact that human blood platelets are anucleate, studies have showed that they possess the transcriptome machinery necessary for maintaining biological processes [11]. It was reported that blood platelets express a vast amount of miRNAs that control their functions and reactivity [12]. Moreover, platelets contain all necessary protein enzymes delivered from megakaryocytes to convert pre-miRNA to mature sequences [13], thus constituting an excellent area for miRNA evaluation.

The main goal of our study was to perform a screening analysis of the platelet miRNome between patients diagnosed with acute coronary syndromes (ACS) and donors without any cardiovascular system disturbances via microarray technology to identify a possible alteration that could be used as a potential prognostic biomarker of ACS.

2. Materials and Methods

2.1. Chemicals

Phosphate-buffered saline (PBS) tablets were purchased from Biosigma (Venice, Italy); NaCl, NaHCO3, citric acid, sodium citrate, and NaH2PO4 were purchased from POCh (Gliwice, Poland); bovine serum albumin (BSA), Tris, NH4HCO3, glucose, KCL, and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) were purchased from Merck (Darmstadt, Germany).

2.2. Clinical Characterization of Study and Control Group

Whole blood samples were obtained from 55 patients with coronarographically confirmed ACS. However, due to the strong influence of risk factors such as impaired glycemia, high fibrinogen levels, and a highly elevated lipid profile, 5 patients were rejected from the

study. Thus, 50 patients were enrolled in the study. All patients were hospitalized, diagnosed, treated, and selected for our study in the Department of Interventional Cardiology at the Medical University of Lodz, according to the current guidelines of the European Society of Cardiology. Blood samples were collected between 7:00 and 8:00 a.m. from the ulnar vein via the S-Monovette system (Sarstedt, Numbrecht, Germany) with CPDA-1 as a stabilizer. Blood was drawn from the patients immediately after the necessary examinations and treatments to ensure the patient's safety. Patients were eligible to enroll in the study if they were under 65 years old, were not diagnosed with cancer, diabetes mellitus, or hypothyroidism, had normal kidney function, BMI < 35, a lack of connective tissue disorders, and were not addicted to narcotics and alcohol. Healthy donors were eligible for the study after medical tests which included morphology, blood levels of glucose, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), thyroid-stimulating hormone (TSH), and triglyceride testing. Healthy volunteers were free from any illness and were free from any medications administrated at least 2 weeks before blood collection. The study was approved by the Committee of the Ethics of Research in Human Experimentation at the University of Lodz, with resolution number 23/KBNN-UŁ/I/2017. All qualified subjects signed a written informed consent form before entering the study. All procedures performed in this study were carried out according to the Helsinki Declaration for Human Research. All clinical parameters of patients and controls are included in Table 1.

Table 1. Clinical characteristics of the ACS patients and healthy volunteers.

Clinical Parameter	Study Group	Control Group	References
Age	54 (46–59.25)	50 (40.75–57.5)	-
Male	43	40	-
Female	7	10	-
Erythrocytes ($10^6/\mu L$)	4.57 (4.283–4.988)	4.95 (4.545–5.27)	4.2–6.1
Leukocytes ($10^3/\mu L$)	8.6 (7.333–9.648)	6.165 (4.828–7.935)	4–11
Blood platelets ($10^3/\mu L$)	251.5 (201.8–281.8)	250 (215–299.8)	150–400
Glucose (mmol/l)	6 (5.365–6.535)	5.055 (4.745–5.563)	4.1–5.1
Creatinine (μmol/l)	82.5 (74.5–91)	74.26 (68.51–86.85)	64–104
GFR ($ml/min/1.73m^2$)	95.2 (79.13–101)	86.33 (79.93–99.01)	>60
Cholesterol (mmol/l)	5.645 (4.475–6.81)	4.8 (4.075–5.303)	3–5
HDL (mmol/l)	1.145 (1–1.333)	1.350 (1.120–1.810)	>1
LDL (mmol/l)	3.21 (2.373–4.41)	2.780 (2.220–3.238)	-
Triglycerides (mmol/l)	1.805 (1.155–2.815)	1.195 (0.938–1.688)	<1.7
AST (U/l)	32.00 (25.75–43.65)	19.55 (16.30–25.85)	0–50
ALT (U/l)	29 (21–39)	20.85 (14.73–33.35)	0–50
TSH (mIU/l)	1.8 (1.183–2.75)	2.055 (1.413–2.863)	0.27–4.20
BMI	<35	<35	<35

Clinical parameters are presented as a median and 1st–3rd quartile of 25th–75th percentile. Abbreviations: ALT—alanine transaminase; AST—aspartate transaminase; BMI—body mass index. GFR—glomerular filtration rate; HDL—high-density lipoprotein; LDL—low-density lipoprotein; TSH—thyroid-stimulating hormone; UA—unstable angina.

2.3. Blood Platelet Isolation

Blood platelets were isolated from 17 mL of fresh, whole-blood samples. Instantly after transport, tubes were centrifuged at 1200 rpm at room temperature for 15 min. To obtain the pure samples of platelets, three-quarters of platelet-rich plasma (PRP) was shifted to a fresh collection tube. Further, a magnetic separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) was performed. Firstly, PRP was incubated with superparamagnetic microbeads,

labeled with anti-CD45 and anti-CD235a, to deplete the leukocytes and erythrocytes, respectively. Positive separation with anti-CD61 microbeads was not recommended by the manufacturer, because of the high possibility of platelet activation. Secondly, the MS Columns responsible for the magnetic separation of labeled cells were washed 3 times with 500 µL of buffer (PBS, 0.5% BSA, 2 mM Citrate) and PRP was applied into the MS Column. Unlabeled, cleaned PRP, which passed through to the new, fresh tube, was then centrifuged at 1400 rpm at room temperature for 15 min to obtain a blood platelet pellet. Isolated blood platelets were washed three times with modified Tyrode's buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4) to remove potential contamination by plasma. In the final step, each platelet pellet was suspended in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) to preserve the integrity of the RNA. After the collection, samples were stored at −80 °C for further analysis.

2.4. RNA Isolation and Synthesis of Complementary DNA (cDNA)

The extraction of RNA from previously collected and purified platelet samples was performed using a commercially available Isolate II RNA Mini Kit (Bioline, London, UK). Because of the density of the RNAlater Solution (Thermo Fisher Scientific, Waltham, MA, USA) used during platelet isolation, an equal volume of ice-cold PBS was added and centrifuged at 5000 rpm for 5 min in 4 °C immediately before the RNA extraction to remove the RNAlater. All steps were performed according to the manufacturer's protocols. After RNA isolation, RNA integrity was examined in TapeStation 2200 using High-Sensitivity RNA ScreenTape (Agilent, Santa Clara, CA, USA). Study and control samples achieved ~8 points on the 1–10 RINe scale. Subsequently, eluted RNA was reverse-transcribed to cDNA using a Taqman™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with cel-miR-39-3p (Thermo Fisher Scientific, Waltham, MA, USA) as an exogenous control. The final concentration of exogenous control in each sample was 10 pM. Undiluted, transcribed cDNA was stored at −80 °C before real-time quantitative PCR (RT-qPCR) analysis.

2.5. Microarray Analysis

Microarray analysis was performed using an Agilent miRNA Microarray SurePrint G3 Human miRNA r21 Array Kit (Agilent, Santa Clara, CA, USA), which ensures a high-throughput system with optimal sequence discrimination and assessment of sensitivity and specificity. The applied microarray slide contained 2549 human miRNAs sourced in the miRBase Database Release 21.0. Unique SurePrint inkjet technology provided a synthesis of 40 to 60-mer oligonucleotides anchored directly on the slide with an array. The microarray contained approximately 15,000 printed probes, randomly placed to ensure the availability of all miRNAs to the tested sample. To perform an experiment, total RNA samples stored at −80 °C were transferred in dry ice to the Institute of Biology in Jan Kochanowski University in Kielce, Poland. The protocol consisted of several steps, including preparing spike-in solutions for the generation of fluorescently labeled miRNAs; and sample dephosphorylation, denaturation, ligation, and drying were carried out. After all steps were performed, microarray slides were put into the SureHyb chamber cover and inserted into the oven's rotating rack. Samples were hybridized at 55 °C for 24 h and 20 rpm. Microarray slides were then washed and scanned. To avoid possible complications after washing, slides were immediately put into the ozone-barrier slide cover. Slides were scanned in Agilent G2565C using Feature Extraction software (FE Version 10.10.1.1) with grid number 070156_D_F_20141006. Subsequently, detailed bioinformatic analysis was performed in GeneSpring GX software (Agilent, Santa Clara, CA, USA). All stages were performed according to the manufacturer's protocol.

2.6. Validation of Selected miRNAs with Real-Time PCR

RT-qPCR analysis was performed for the selected miRNAs: hsa-miR-223-3p (Assay ID: 477983_mir), hsa-miR-142-3p (477910_mir), hsa_miR-126-3p (477887_mir), hsa-miR-

21-5p (477975_mir), hsa-miR-107 (478254_mir), hsa-miR-28-5p (478000_mir), hsa-miR-221-3p (477981_mir), hsa-miR-98-5p (478590_mir), hsa-let-7f-5p (478578_mir), hsa-let-7d-5p 478439_mir), hsa-let-7g-5p (478580_mir), hsa-miR-146a-5p (478399_mir), hsa-miR-301a-3p (477815_mir), hsa-miR-130b-3p (477840_mir), hsa-miR-338-3p (478037_mir), hsa-miR-191-5p (477952_mir), cel-miR-39-3p (Sequence 5′ to 3′: (RNA)-Phos-UCACCGGGUGUAAAUC-AGCUUG). Taqman™ Advanced miRNA Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Taqman™ Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), Molecular-Grade Water (Thermo Fisher Scientific, Waltham, MA, USA), and 10-times diluted cDNA of the studied samples were used to quantify each miRNA molecule. To evaluate the quality of cDNA synthesis, the Ct value for an exogenous control was determined and compared in all analyzed samples. For normalization of the expression level, the mean value of endogenous (hsa-miR-191-5p) and exogenous (cel-miR-39-3p) controls was calculated and used as a reference for the ΔCt calculation. RT-qPCR analysis was performed using a CFX96TM Real-time PCR Detection System Thermal Cycler (Bio Rad Laboratories Inc., Hercules, CA, USA). The conditions set for the analysis consisted of polymerase activation (20 s, 95 °C) followed by denaturation (3 s, 95 °C), and extension (30 s, 60 °C) in 40 cycles. The expression of miRNAs was presented as −ΔCt. The difference in miRNA expression between ACS patients and healthy controls was tested with Student's *t*-test. The fold-change of miRNA expression, together with its 95% confidence intervals (95%CI), was based on the $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. Screening of Platelet miRNome with Microarrays

To obtain the most reproducible and statistically significant findings and to exclude false-positive or false-negative results, quality control analysis was performed in study and control groups. Data were normalized using a percentile shift algorithm, without baseline transformation. Statistical analysis was performed using the moderated *t*-test with Benjamini–Hochberg FDR correction with a *p*-value cut-off of 0.05. The obtained results showed that 107 miRNA molecules had significantly altered expression in ACS patients compared to the control group. In the next step, data were filtered by fold-change value with cut-off of 2. After filtration, we found that 91 miRNAs had significantly different expression between the groups (Supplementary Figure S1, "The heatmap of miRNAs expression in microarray analysis"). Obtained results are presented in a volcano plot (Figure 1). To assess the biological functions (BFs) of the analyzed miRNAs, six databases responsible for finding target genes were used (miRDB, microRNAorg, PITA, PICTAR, TARBASE, and TARGETSCAN). To provide the most reproducible results of the predicted targets, we considered only entities supported by all of the abovementioned target prediction databases. All genes associated with particular miRNAs were studied in DAVID Bioinformatics Resources 6.8 [14]. miRNAs and genes were selected based on the OMIM Database [15], KEGG Pathways [16], Gene Ontology [14], and available publications. For validation of the obtained results by RT-qPCR, we selected 15 miRNAs presenting altered expression in ACS patients compared to healthy volunteers and demonstrating an association with platelet function.

3.2. Validation of Blood Platelet miRNA by RT-qPCR

The results showed that of the 15 selected miRNAs from the microarray analysis, the expression of eight miRNAs was significantly augmented in ACS patients compared to healthy controls. Overexpression was found for hsa-miR-142-3p ($p < 0.0001$); hsa-miR-107 ($p < 0.0001$); hsa-miR-338-3p ($p = 0.0004$), hsa-miR-223-3p ($p = 0.0004$), hsa-miR-21-5p ($p = 0.0005$), hsa-miR-130b-3p ($p = 0.0023$), hsa-miR-301a-3p ($p = 0.011$), and hsa-miR-221-3p ($p = 0.027$). The mean values of −ΔCt, SEMs, and fold-changes are presented in Figure 2.

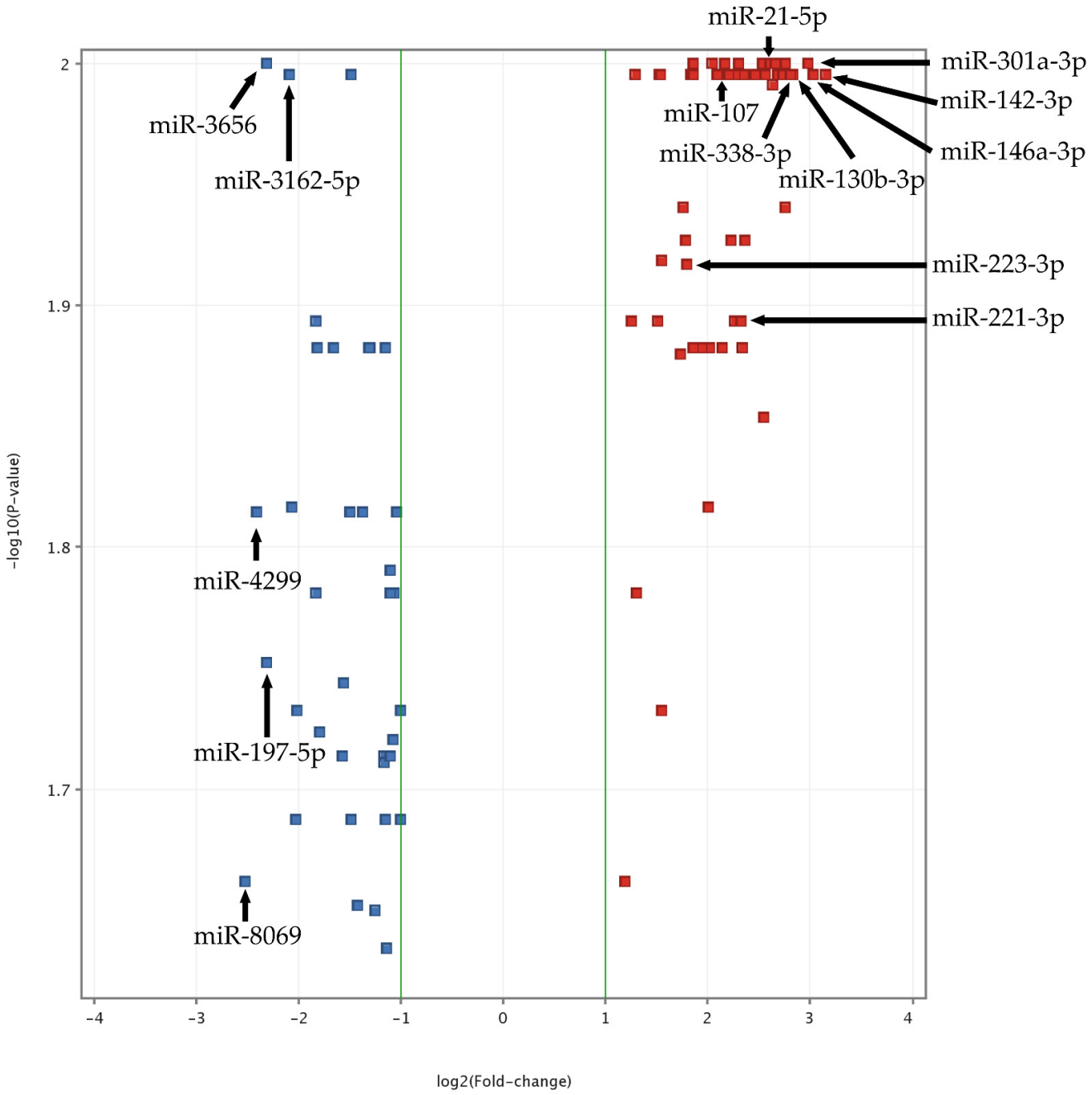


Figure 1. Volcano plot of ACS vs. Control miRNA microarray analysis. The data for all miRNAs are plotted as $\log_2$(Fold-change) vs. the $-\log_{10}$(*p*-value). A fold-change threshold is presented as green lines. The 5 most upregulated (miR-301a-3p, miR-142-3p, miR-146a-3p, miR-130b-3p, miR-338-3p) and downregulated (miR-8069, miR-4299, miR-3656, miR-197-5p, miR-3162-5p) miRNAs, selected based on fold-change values, are indicated by black arrows. Furthermore, statistically significant miRNAs, validated by qRT-PCR, are also presented (miR-142-3p, miR-107, miR-338-3p, miR-223-3p, miR-21-5p, miR-130b-3p, miR-301a-3p, and miR-221-3p). The altered expressed miRNAs between studied groups with the highest significance (the lowest *p*-value) are located at the top of the plot. MiRNAs demonstrating large fold-change values are plotted outside of the vertical threshold lines. A greater distance from the center indicates a greater fold-change. Red square points on the plot represent upregulated miRNAs, whereas blue square points on plots represent downregulated miRNAs.

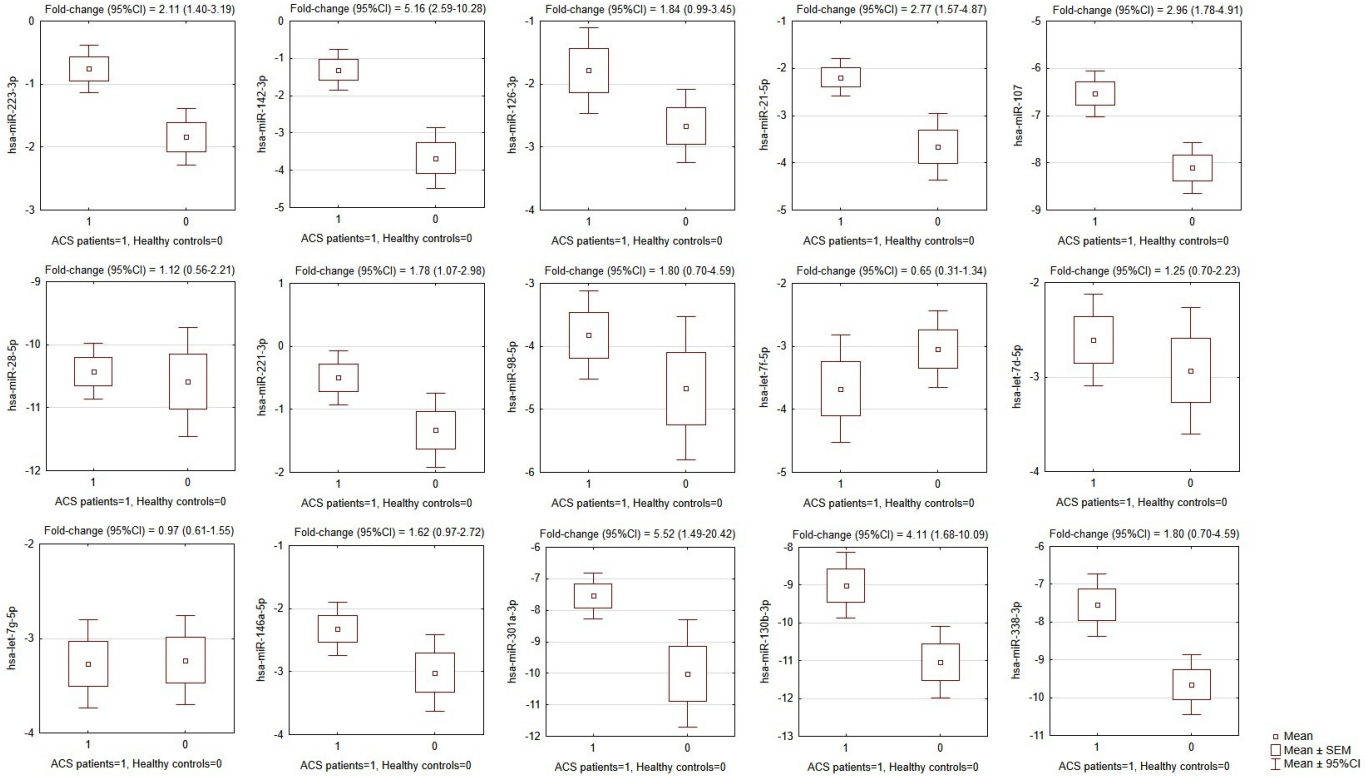


Figure 2. Comparison of miRNA expression in platelets of ACS patients and matched healthy controls. OY axes depict −ΔCt values for each given miRNA, presented graphically as the mean, SEM, and 95% CI of expression estimate. Above individual box-plots, the fold-change (95% CI) of miRNA expression in ACS patients in relation to its expression in healthy controls is presented. ACS—acute coronary syndrome, SEM—standard error of mean, CI—confidence interval.

3.3. Statistical Analysis and Modeling of Potential Biomarkers

Biochemical parameters were transformed via the Box–Cox procedure to maintain the normal distribution of the parameters. Receiver operating characteristic (ROC) curves were used to illustrate model performance to differentiate ACS patients from healthy controls and areas under the ROC curves (AUC) with 95%CI values were estimated. Cut-off points were proposed based on the maximization of Youden's index. The colinearity of miRNAs was tested with the use of exploratory factor analysis (EFA) and Pearson's *r* correlation matrix analysis. The number of factors in EFA was established based on the scree-plot analysis and the eigenvalue-more-than-one criterion. The factor differentiation in EFA was achieved by means of raw varimax factor rotation. Binary logistic regression was used to build a multivariate model to differentiate ACS patients from healthy controls. Predictors were selected based on the step-wise procedure and with the help of Pearson's *r* correlation matrix analysis. The models were evaluated with the use of Akaike and Bayesian information criteria, as well as Cox–Snell and Nagelkerke's pseudo-R^2. To assess the goodness-of-fit, the Hosmer–Lemeshow chi-squared test was performed. Ten-fold cross validation was used as an internal model validation technique. *p*-values below 0.05 were considered statistically significant. The analysis was performed using STATISTICA 13.3 Software (StatSoft; Tulsa, OK, USA).

Although some miRNAs were able to significantly differentiate ACS patients from healthy controls (Figures 2 and 3), the development of a multivariate model for that purpose with the use of miRNAs only was not possible due to their high colinearity. Two orthogonal factors yielded by EFA were able to explain as much as 63.0% of the total variance and all the miRNAs that well-differentiated patients from controls (miR-301a-3p, miR-142-3p, miR-338-3p, miR-130b-3p, miR-107, miR-21-5p, miR-223-3p, and miR-126-3p) substantially loaded the main factor (Figure 4). On the other hand, miRNAs weakly differ-

entiating patients from controls loaded the second factor, with some of them (miR-98-5p, let-7f-5p, let-7d-5p, miR-28-5p) negligibly loading the main one (Figure 4). The Pearson's *r* correlation matrix with all the tested miRNAs is presented in Supplementary Figure S2, "The correlation matrix with all the tested miRNAs".

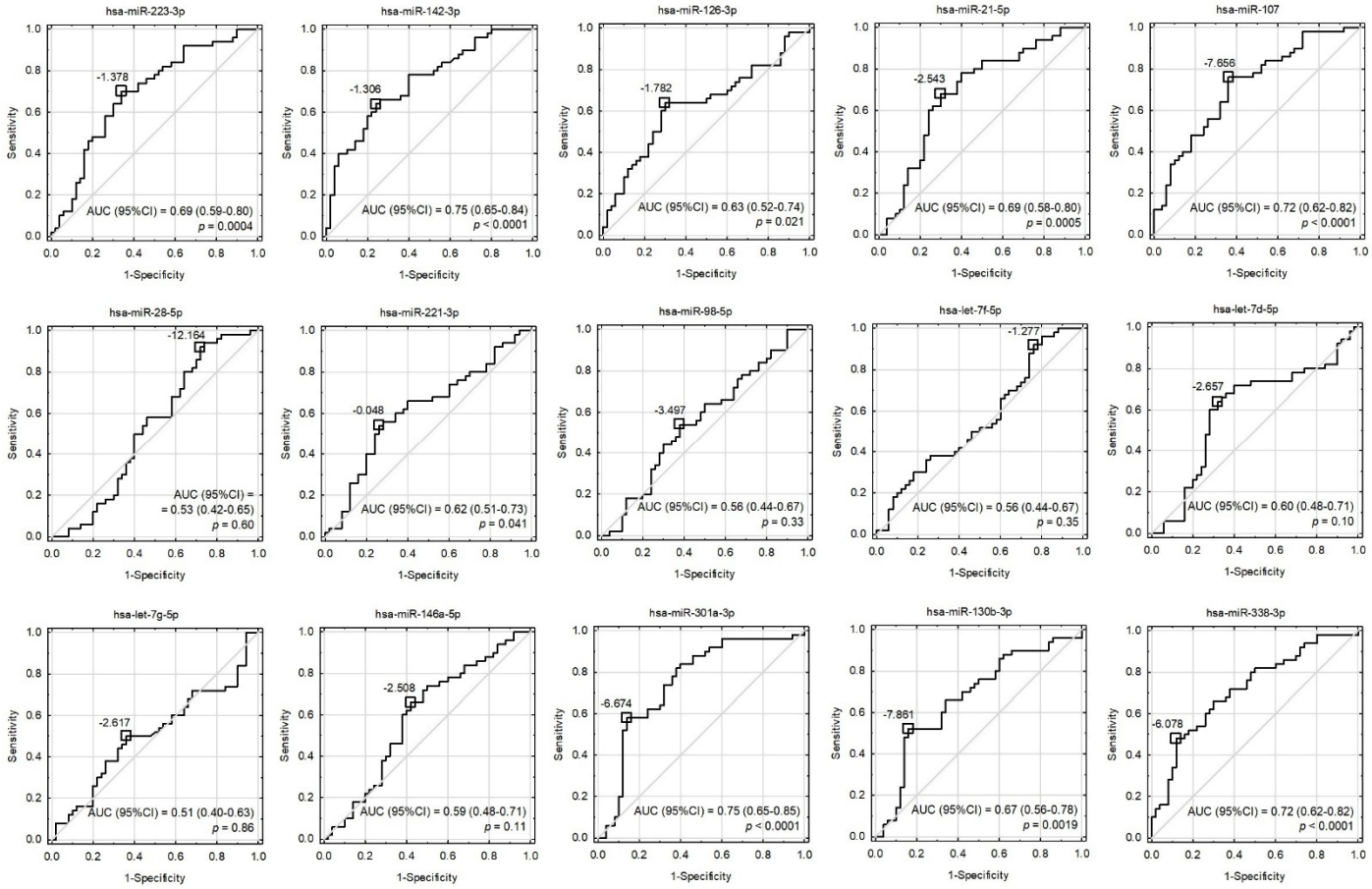


Figure 3. Receiver operating characteristic (ROC) curves for univariate models involving all the tested miRNAs. Cut-off points were proposed based on the maximization of Youden's index and their values were presented as $-\Delta$Ct. The area under the ROC curves (AUC), their 95% confidence intervals (95%CI), and the tests for AUC differences from 0.5 were displayed.

To test whether the evaluated miRNAs could contribute to the better differentiation of patients from controls performed using classical biochemical parameters, the binary logistic regression model was constructed with the use of a step-wise procedure. MiR-142-3p, together with AST, were selected for this model as their correlation was negligible (Pearson's $r = 0.067$) and both significantly differentiated patients from controls. According to the information criteria and analysis of the coefficients of determination, the model enriched with miR-142-3p performed substantially better in comparison to the model based on AST only (Figure 5 and Table 2). The multivariate model was parsimonious enough not to be prone to overfitting, as evaluated by the validation sample. The model exhibited acceptable goodness-of-fit; however, as Hosmer–Lemeshow statistics presented with borderline *p*-values, this requires further testing and development.

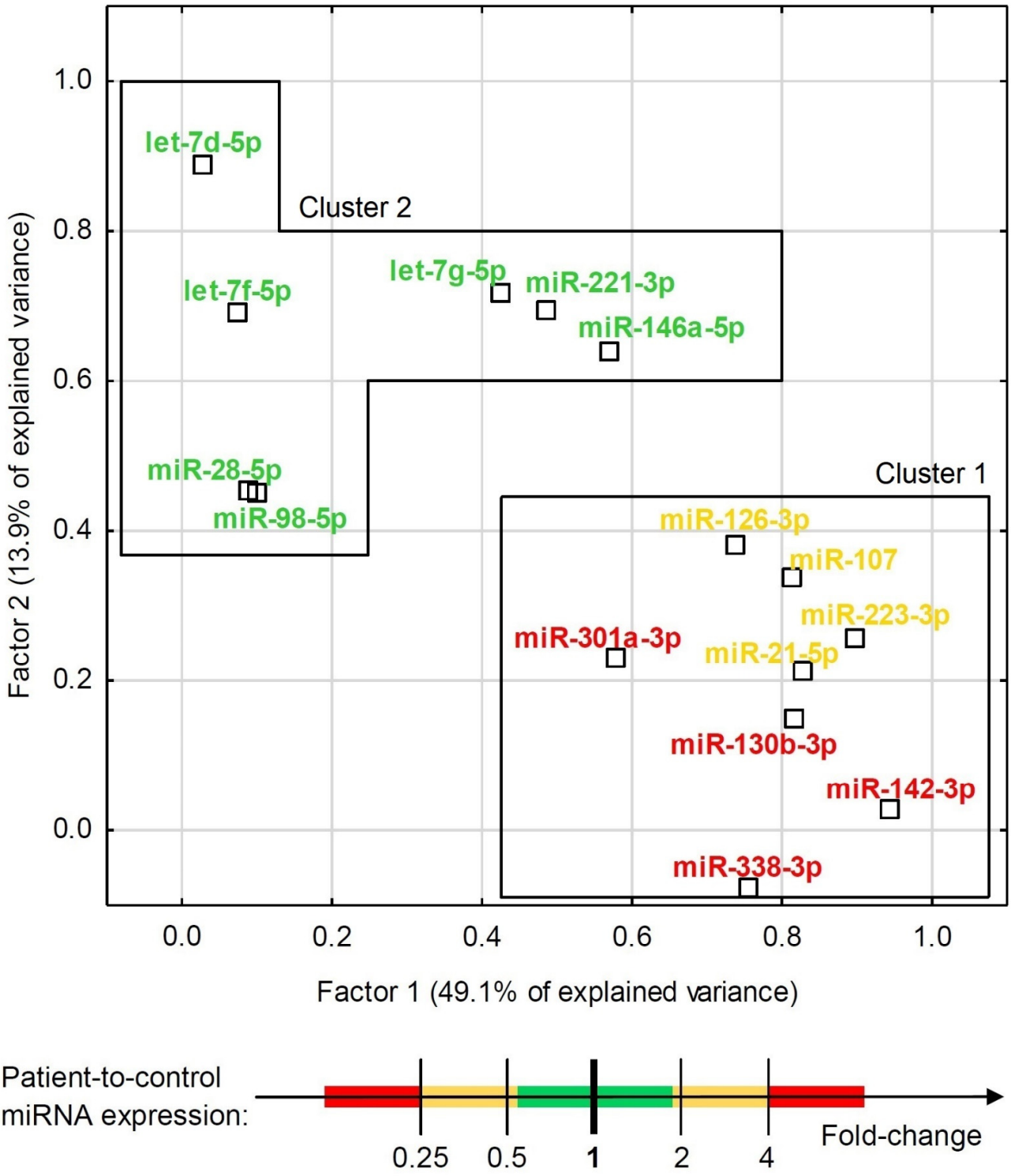


Figure 4. Exploratory factor analysis with factor loadings for expression of all the tested miRNAs in the combined group of ACS patients and healthy controls. The factor differentiation was achieved via raw varimax factor rotation. Two factors accounted for 63.0% of the total variance. MiRNAs are indicated with colors that represent their relative ACS patient-to-control expression.

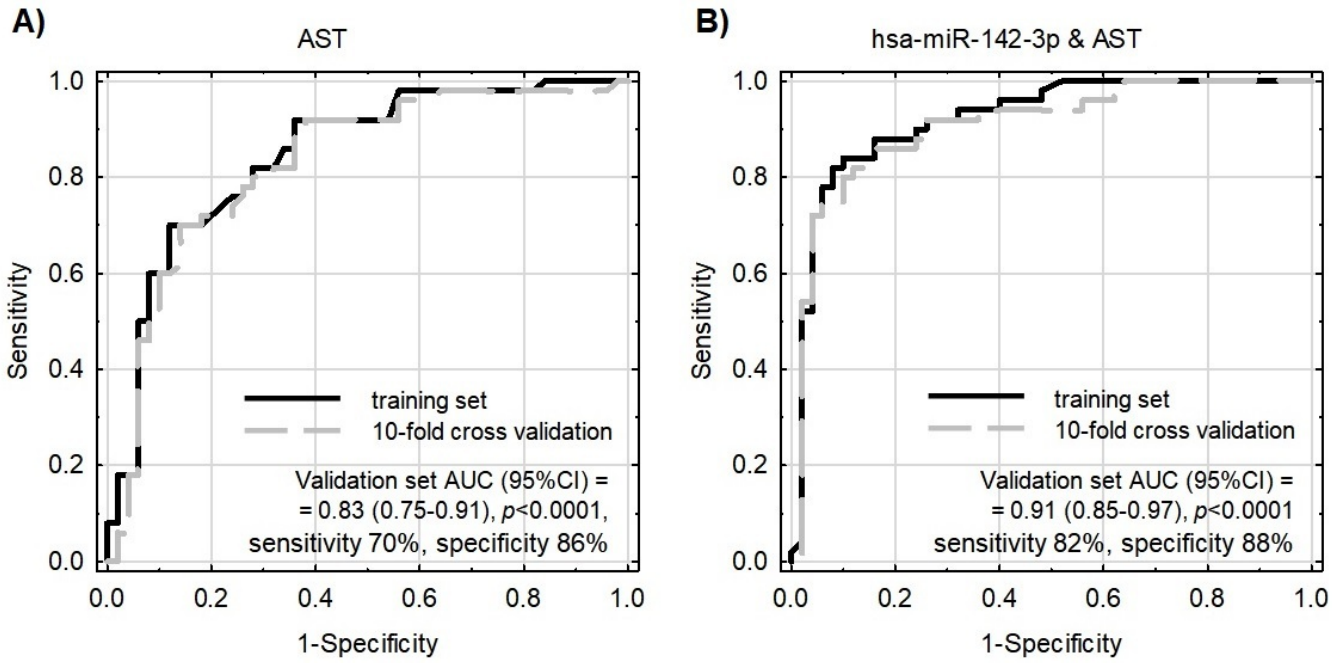


Figure 5. Receiver operating characteristic (ROC) curves for models differentiating ACS patients from healthy controls. Model internal validation was performed with a 10-fold cross-validation technique. (**A**) ROC for the model based on AST only. (**B**) ROC for the model based on miR-142-3p together with AST.

Table 2. Characteristics of the binary logistic regression models differentiating ACS patients from healthy controls based on AST only and miR-142-3p together with AST.

Model Characteristics	Model Based on AST	Model Based on miR-142-3p and AST
	Odds ratio (95%CI), *p*-value	
AST (Box–Cox transformed*100) [1]	2.08 (1.54–2.79), $p < 0.0001$	2.57 (1.73–3.81), $p < 0.0001$
miR-142-3p (−ΔCt)	N/A	1.91 (1.37–2.67), $p = 0.0001$
	Coefficients of determination:	
Cox–Snell R^2	32.5%	48.2%
Nagelkerke R^2	43.4%	64.2%
	Information criteria:	
AIC	103.3	78.9
BIC	108.5	86.7
	Goodness-of-fit:	
Hosmer–Lemeshow χ^2(df), *p*-value	6.60 (8), $p = 0.58$	14.84 (8), $p = 0.062$

AST—aspartate transaminase, N/A—not applicable, AIC—Akaike information criterion, BIC—Bayesian information criterion; [1] Box–Cox $\lambda = -0.9071$.

3.4. Bioinformatic Analysis of Potential mRNA Targets and Protein–Protein Interactions

To identify the biological significance of tested miRNAs, the bioinformatic analysis focused on the comparison of gene ontology, searching for predictive mRNA targets, and the evaluation of protein–protein interactions (PPIs) was carried out. In the first stage, target genes for statistically significant miRNA molecules were found in GeneSpring Software. To obtain the most reproducible results, the predictive targets of which the presence was confirmed in all studied databases were selected. Furthermore, based on the DAVID Bioinformatic Resources 6.8 [14] and KEGG Pathways [16], signaling pathways and gene ontology for mRNA-miRNA targets were evaluated. To avoid an overabundance

of information unrelated to the manuscript topic, only pathways and genes associated with the physiology of platelets were selected. All mRNA targets for particular miRNAs are included in Table 3.

Table 3. Summary of predictive mRNA-miRNA targets associated with the central biological functions of blood platelets.

MicroRNA	mRNA Targets [1]	Kegg Pathway	Associated Genes
miR-223-3p	46	PI3K-Akt signaling pathway	*AKT3, CREB1 *, CHUK, FGF2 *, ITGA2, PDGFRA **
		Platelet activation	*AKT3, RASGRP1 *, ITGA2, P2RY12*
miR-142-3p	41	Platelet activation	*AKT3, RASGRP1 *, ARHGEF12, COL24A1 *, ITGA2, MYLK, PLCB1, PPP1R12A*
		Regulation of actin cytoskeleton	*ARHGEF12, FGF2 *, ITGA2, MYLK, PPP1R12A*
		Focal adhesion	*AKT3, COL24A1 *, ITGA2, MYLK, PPP1R12A*
		Vascular smooth muscle contraction	*ARHGEF12, MYLK, PLCB1, PPP1R12A*
miR-21-5p	58	Platelet activation	*AKT3, RAP1B, RASGRP1 *, ARHGEF12, COL3A1, COL24A1 *, ITPR1, ITGA2, ITGB3, PIK3R1, PLCB1, PRKG1*
		PI3K-Akt signaling pathway	*AKT3, PHLPP2 *, COL3A1, COL24A1 *, CHUK, FGF2 *, ITGA2, ITGB3, PIK3R1, PDGFRA *,*
miR-107	92	PI3K-Akt signaling pathway	*AKT3, BCL2L11 *, PHLPP2 *, CREB1 *, CHRM2 *, COL3A1, COL6A3, COL24A1 *, FGF2 *, INSR, ITGA2, MAP2K1, PIK3R1, VEGFA **
		Platelet activation	*AKT3, COL3A1, COL24A1 *, ITPR1, ITGA2, MYLK, PIK3R1, PLCB1, PPP1R12A*
		Focal adhesion	*AKT3, COL3A1, COL6A3, COL24A1 *, ITGA2, MAP2K1, MYLK, PIK3R1, PPP1R12A, VEGFA **
		cAMP signaling pathway	*AKT3, ADRB2 *, CREB1*, CHRM2 *, GRIN2A *, MAP2K1, PIK3R1, PPP1R12A*
		Regulation of actin cytoskeleton	*CHRM2 *, FGF2 *, ITGA2, MAP2K1, MYLK, PIK3R1, PPP1R12A*
		Calcium signaling pathway	*HTR2A, ADRB2 *, CHRM2 *, GRIN2A *, ITPR1, MYLK, PLCB1*
		Circadian entrainment	*CREB1 *, GRIN2A *, ITPR1, PLCB1, PRKG1*
		Phosphatidylinositol signaling system	*ITPR1, PIK3R1, PLCB1*
		Chemokine signaling pathway	*AKT3, MAP2K1, PIK3R1, PLCB1, PF4V1*
miR-221-3p	71	PI3K-Akt signaling pathway	*AKT3, BCL2L11 *, PHLPP2 *, CREB1 *, CHRM2 *, ITGA2, ITGB3, MAP2K1, PIK3R1, PDGFRA *, THEM4, YWHAQ*
		Regulation of actin cytoskeleton	*CHRM2 *, ITGA2, ITGB3, MAP2K1, PIKFYVE, PIK3R1, PDGFRA *, RDX*
		Platelet activation	*AKT3, RAP1B, RASGRP1 *, ITGA2, ITGB3, PIK3R1, PLCB1, PRKG1*
		Focal adhesion	*AKT3, RAP1B, ITGA2, ITGB3, MAP2K1, PIK3R1, PDGFRA **
		cAMP signaling pathway	*AKT3, RAP1B, CREB1 *, CHRM2 *, MAP2K1, PIK3R1*

Table 3. *Cont.*

MicroRNA	mRNA Targets [1]	Kegg Pathway	Associated Genes
miR-301a-3p	91	PI3K-Akt signaling pathway	*AKT3, BCL2L11, PHLPP2 *, CREB1 *, CHRM2 *, COL1A2, COL6A3, CHUK, CDKN1A, INSR, MAP2K1, PIK3R1, PDGFRA **
		Platelet activation	*AKT3, ARHGEF12, COL1A2, ITPR1, PIK3R1, PLCB1, PRKG1, PLAU **
		cAMP signaling pathway	*AKT3, RAPGEF4 *, ADRB1 *, CREB1 *, CHRM2 *, MAP2K1, PIK3R1*
		Focal adhesion	*AKT3, COL1A2, COL6A3, MAP2K1, PIK3R1, PDGFRA **
		Regulation of actin skeleton	*ARHGEF12, CHRM2 *, MAP2K1, PIKFYVE, PIK3R1, PDGFRA *, RDX*
		Vascular smooth muscle contraction	*ARHGEF12, ITPR1, MAP2K1, PLCB1, PRKG1*
		Calcium signaling pathway	*ADRB1 *, CHRM2 *, ITPR1, PLCB1, PDGFRA **
		Phosphatidylinositol signaling system	*ITPR1, PIKFYVE, PIK3R1, PLCB1*
		Arachidonic acid metabolism	*PTGES3*
miR-130b-3p	93	PI3K-Akt signaling pathway	*AKT3, BCL2L11, PHLPP2 *, CREB1 *, CHRM2 *, COL1A2, COL6A3, CHUK, CDKN1A, INSR, MAP2K1, PDGFRA **
		Platelet activation	*AKT3, RAP1B, ARHGEF12, COL1A2, ITPR1, MYLK, PLCB1, PRKG1*
		Regulation of actin cytoskeleton	*ARHGEF12, CHRM2 *, MAP2K1, MYLK, PIKFYVE, PDGFRA *, RDX*
		cAMP signaling pathway	*AKT3, RAP1B, RAPGEF4 *, ADRB1 *, CREB1 *, CHRM2 *, MAP2K1*
		Vascular smooth muscle contraction	*ARHGEF12M ITPR1, MAP2K1, MYLK, PLCB1, PRKG1*
		Complement and coagulation cascade	*F3 *, C1S, C5, PLAU**
		Arachidonic acid metabolism	*PTGES3*
miR-338-3p	58	PI3K-Akt signaling pathway	*AKT3, PHLPP2 *, CREB1 *, CHRM2 *, COL6A3, FGF2 *, ITGB3, PDGFRA **
		Regulation of actin cytoskeleton	*ARHGEF12, CHRM2 *, FGF2 *, ITGB3, PDGFRA *, RDX*
		Platelet activation	*AKT3, ARHGEF12, ITPR1, ITGB3, PRKG1*
		Calcium signaling pathway	*ADRB2 *, CHRM2 *, GRIN2A *, ITPR1, PDGFRA **
		cAMP signaling pathway	*AKT3, ADRB2 *, CREB1 *, CHRM2 *, GRIN2A **
		Focal adhesion	*AKT3, COL6A3, ITGB3, PDGFRA **

[1] Numbers presented in rows show the overall number of mRNA targets found in the 6 databases: miRDB, microRNAorg, PITA, PICTAR, TARBASE, and TARGETSCAN in GeneSpring software. * The presence of mRNA transcripts was confirmed in blood platelets, without protein products [17]. Underlined—The presence of protein was confirmed in blood platelets, without mRNA transcripts [17].

The obtained results showed that all statistically significant miRNAs may interact with 43 mRNA transcripts present in blood platelets. To test whether crucial interactions between proteins associated with platelet functions are present, protein–protein interaction (PPI) analysis was performed. Of 43 predicted mRNA transcripts, we selected 28 proteins, which were experimentally confirmed in the platelet proteome. A visualization of the PPI network is presented in Figure 6.

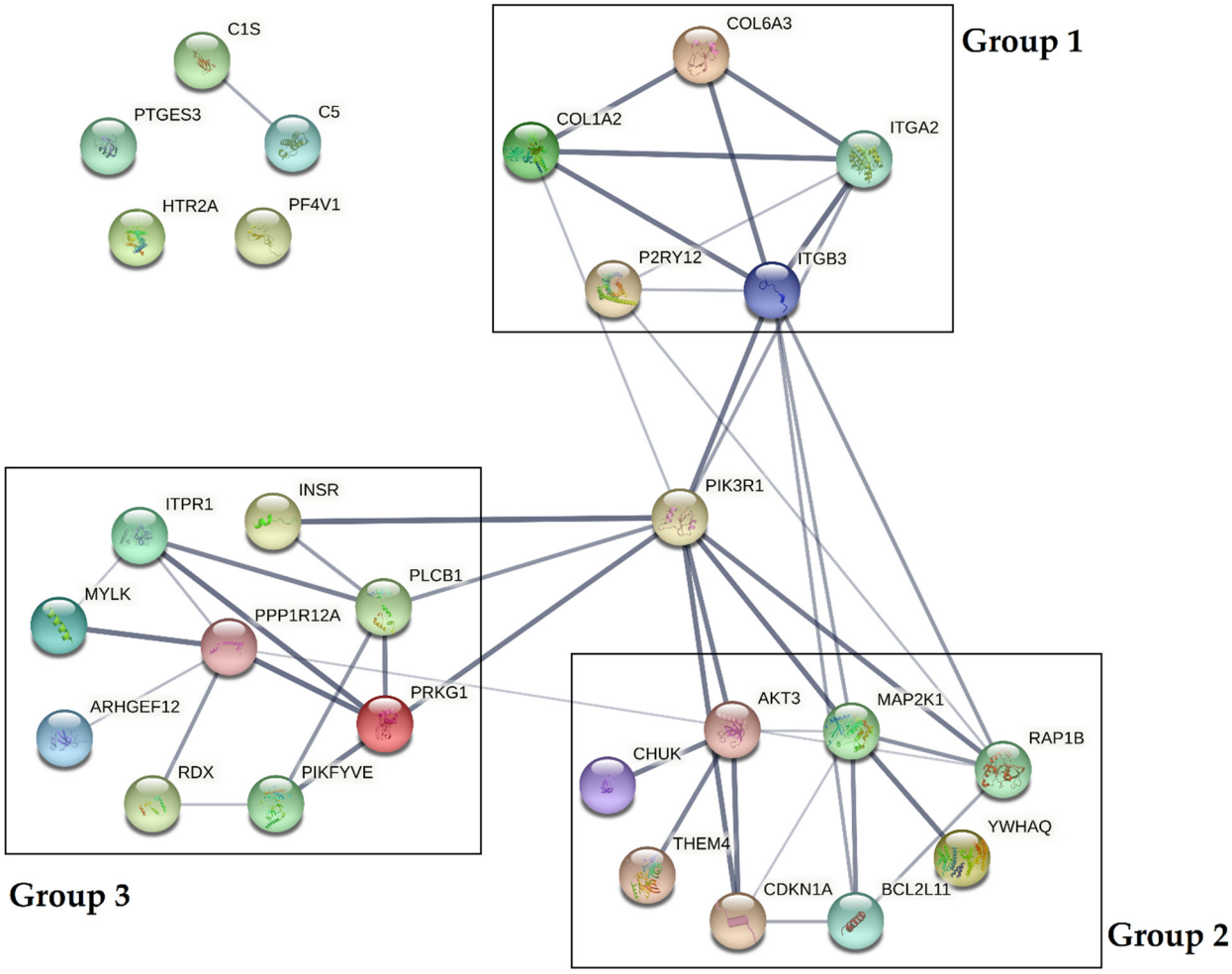


Figure 6. Protein–protein interaction (PPI) analysis of selected proteins present in the platelet proteome. PPIs were determined using the STRING database [18]. The PPI network is presented as lines between nodes, the thickness of which indicates the strength of the interaction.

Data obtained from the analysis demonstrated that PPIs could be divided into three separate groups, with a central role of phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1). Based on the KEGG Pathways, proteins included in group 1 (COL6A3, COL1A2, ITGA2, ITGB3, P2RY12) were shown to influence the extracellular matrix receptor interaction pathways crucial for platelet adhesion, activation, and aggregation. Proteins included in group 2 (AKT3, MAP2K1, RAP1B, YWHAQ, BCL2L11, CDKN1A, THEM4, CHUK) were shown to affect the PI3K-Akt signaling pathway, and proteins in group 3 (ITPR1, INSR, PLCB1, PRKG1, PIKFYVE, RDX, ARHGEF12, MYLK, PPP1R12A) were shown to be associated with vascular smooth muscle contraction, the calcium signaling pathway during platelet activation, and the regulation of the actin cytoskeleton. Furthermore, three proteins (PTGS3, HTR2A, PF4V1) were not shown to be associated with the studied interactome [16].

The final step of the bioinformatic analysis was focused on the comparison of biological processes and cellular components of predictive mRNA targets of miRNAs included in cluster 1 via Gene Ontology analysis (Supplementary Figures S3 and S4). Data obtained from the DAVID Database [14] revealed significant differences in the mRNA targets of miRNAs in clusters 1 and 2, indicating its multiple roles in the regulation of physiological processes associated with the activation of blood platelets. To identify the most significant potential mRNA targets for miRNAs included in cluster 1 and cluster 2, we decided to verify whether the predictive mRNA targets between both clusters coincided. Furthermore,

we tested whether there were differences in the linked targets between clusters and whether particular transcripts were specific to any of the clusters. Based on the obtained results, we selected *ITPR1, PLCB1,* and *PIK3R1* as potential targets of significant miRNAs, that could be used to differentiate between the studied groups.

4. Discussion

Our study showed that blood platelets from ACS patients had significantly upregulated expression of eight validated miRNAs: miR-142-3p; miR-107; miR-338-3p, miR-223-3p, miR-21-5p, miR-130b-3p, miR-301a-3p, and miR-221-3p. The presence of all analyzed miRNAs in blood platelets was confirmed in several studies, emphasizing their platelet origin [12,19–21]. Due to the complex and multifactorial character of ACS, it is necessary to better understand the molecular mechanisms of increased platelet activity in various areas of disease pathogenesis. The analysis of the platelet miRNA profile in our study was performed with patients presenting the least possible influence of typical risk factors, allowing for the identification and selection of altered expressed miRNA molecules, which could be potentially used to determine the human genetic predisposition to ACS. Furthermore, based on the available literature, we discussed the potential mechanisms of action of eight significant miRNAs to better understand the genetic basis of thrombus formation in blood vessels.

MiR-223-3p was shown to be one of the most abundant miRNAs in blood platelets with a relatively high $-\Delta Ct$ value. Landry et al. demonstrated that miR-223 was associated with the Ago2 protein and formed a complex with the potential capability to regulate the expression of the P2Y12 receptor, one of the main targets for the pharmacological treatment of patients with thrombotic events. The presence of *P2RY12* mRNA linked with Ago2-miR-223 in blood platelets may suggest the potential influence for de novo protein synthesis, thus influencing the organization of platelet surface receptors through modification of their concentration [12]. Several studies showed that the level of miR-223 was associated with the response of antiplatelet therapy administrated to patients, thus emphasizing its substantial role in the regulation of platelet reactivity [22,23]. Data presented in the mentioned studies showed the opposite results to ours, demonstrating a significantly downregulated level of miR-223 in patients with CVDs. Similarly, there are also other studies presenting the overexpression of miR-223 in CVDs [22–24]. The variability of results in studies may arise from the close linkage between the expression of miR-223 and the administration of pharmacotherapy, which influences its level. In our study, blood samples were collected immediately after necessary medical examinations, to avoid potential interactions of medicaments. The overexpression of miR-223 may be thus associated with the short exposition of antiplatelet agents, which could not fully affect the phenotypic changes of platelets. A possible explanation of the increased expression of platelet miR-223 may also be associated with an adaptive reaction to the ischemic event that increases the miR-223 level to inhibit the excessive activation of platelets and P2Y12 receptors.

The disturbed thrombopoiesis and altered functioning of platelets may be triggered by the incorrect regulation of the differentiation of hematopoietic cells. In a study conducted by Chapnik et al., a model of miR-142-knockout mice showed an impaired maturation of megakaryocytes and proplatelet formation, which resulted in thrombocytopenia. Despite disturbed platelet biogenesis, nullified miR-142 mice demonstrated a markedly immature organization of megakaryocytes' actin and tubulin filaments, the main component of megakaryocytes' and platelets' cytoskeleton [24]. Reorganization of the platelet cytoskeleton and the rotation of actin filaments may trigger the release of molecules consisting mostly of an inactive form, i.e., integrin $\alpha 2b\beta 3$, which is the main receptor for platelet aggregation. Excessive exposition of fibrinogen and vWF binding sites in the mentioned receptor may induce pathogenic thrombosis [25]. Our study showed that of the selected miRNAs, miR-142-3p is one of the most overexpressed in ACS patients compared to healthy controls (FC = 5.16 (95%CI: 2.59–10.28); $p < 0.0001$), both in microarray and RT-qPCR analysis. The

role of miR-142-3p overexpression remains unclear; however, the possible mechanism may be analogous to that described by Chapnik et al. [25], indicating that an augmented level of miR-142-3p relates to the disturbed organization of the platelet cytoskeleton, and may cause the uncontrollable formation of actin filaments, including lamelli- and filopodia, which are responsible for recruiting circulating cells during adhesion. Furthermore, stimulated blood platelets may potentially increase the concentration and release of has-miR-142-3p to induce interaction with endothelial cells with the simultaneous stimulation of apoptosis [26].

Moreover, blood platelets play an important role in the pathogenesis of CVDs by disturbing the circadian rhythm. This role may be associated with the genetic background of cardiovascular complications through the diurnal variation in the frequency of ischemic events [27]. Based on bioinformatic analysis, Nagalla et al. showed that miR-107 targets *CLOCK* gene transcripts, thus significantly reducing its expression [20]. Furthermore, Ohkura et al. demonstrated in a mouse model that circadian fluctuation is associated with hemostatic activity. Disturbed circadian rhythm resulted in continuously augmented hemostatic activity in mice. Interestingly, *CLOCK*-mutant mice presented a significantly diminished number of platelets with simultaneous hyperaggregability [28]. The results of our study showed that the expression of platelet miR-107 in ACS patients was nearly three-fold higher compared to the control group ($p < 0.0001$), suggesting that overexpression of miR-107 in blood platelets may be linked with impaired circadian rhythm, and may influence the molecular background of the hyperactivity of blood platelets in CVDs, including ACS.

Highly expressed miRNAs in platelets also include miR-221-3p [29], as was demonstrated in our study. A recent study showed that miR-221-3p is involved in the pathogenesis of primary immune thrombocytopenia (ITP) and is differentially expressed before and after treatment with thrombopoietin receptor agonists (TPO-RAs), indicating its value as a predictive marker of the platelet response to treatment with TPO-RA [30]. Our results showed significant overexpression of miR-221-3p in the blood platelets of ACS patients. Similarly, Ward et al. have reported the significant upregulation of platelet miR-221-3p in patients with ST-segment elevation (STEMI) compared to non-ST-segment elevation (NSTEMI) myocardial infarction. Moreover, miR-221-3p was uniquely associated with platelets and leukocytes in patients with STEMI; however, in NSTEMI patients, miR-221-3p has been revealed to be downregulated in platelets and upregulated in plasma subcomponents [29]. Furthermore, miR-221-3p has been reported as one of the most upregulated circulating miRNAs in patients with acute myocardial infarction (AMI), with a fold change of four and high potential as a prominent predictive biomarker for AMI with AUC = 0.881 (95% CI: 0.774–0.987; $p = 0.002$) [31]. The possible mechanism of action of miR-221-3p in regulating platelet function remains unclear; however, its positive correlation with cardiac troponin in a study by Coskunpinar et al. suggests its high potential of being a biomarker of ACS [31].

Furthermore, miR-21-5p has recently been proposed as a valuable platelet-associated candidate for a novel diagnostic and prognostic biomarker of CVDs, including stroke and pulmonary embolism [32]. Microarray analysis demonstrated that miR-21-5p is one of the most expressed miRNAs in human platelets, cardiac fibroblasts (CFs), endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and cardiomyocytes (CMCs) [32]. Upregulation of this miRNA has been associated with atherosclerosis; thus, its possible application as a diagnostic biomarker for AMI, as well as stroke and pulmonary embolism, has been suggested. MiR-21-5p demonstrated a comparable tendency to that of plasma cardiac troponin I (cTnC) during the early phase of myocardial infarction with a peak at 4 h after the onset of symptoms. Furthermore, plasma miR-21 levels exhibited a significant correlation with cardiac troponin and creatine kinase, which serve as current clinically established markers [33,34]. Moreover, Cenzis et al. reported a significant upregulation of plasma miR-21 in the hypertension cohort compared to healthy controls and an association with systolic and diastolic blood pressure [35]. The direct role of miR-21-5p in platelet function remains unclear; however, based on the KEGG Pathways [16], mRNA targets of

miR-21-5p are strongly associated with platelet activation, the PI3K-Akt signaling pathway, focal adhesion, and the regulation of the actin cytoskeleton by affecting e.g., *AKT3, RAP1B, RASGRP1, ITGA2, ITGB3, PIK3R1, PLCB1, THBS2, PDGFRA* genes. Furthermore, the OMIM Database showed an association between miR-21-5p targets and susceptibility to myocardial infarction [15].

In the case of miR-301a-3p, miR-130b-3p, and miR-338-3p, there is a lack of reported information concerning the possible mechanism of action in blood platelets and their role in thrombosis. Our microarray analysis showed that miR-301a-3p, miR-130b-3p, and miR-338-3p were substantially upregulated in ACS patients. RTq-PCR confirmed these results, demonstrating 5.52-fold, 4.11-fold, and 4.31-fold augmented expression within ACS patients, respectively. To assess their molecular function that could influence thrombosis, we found possible mRNA targets using GeneSpring software, and based on the Gene Ontology Database in DAVID [14] we compared the biological processes linked with targeted mRNA. Our analysis showed that transcripts targeted by miR-301a-3p, miR-130b-3p, and miR-338-3p in the case of thrombosis are responsible, e.g., for the signal transduction process, the regulation of GTPase activity, platelet activation, phosphatidylinositol phosphorylation, response to hypoxia, and wound healing. However, to confirm their association with these mentioned processes, further detailed analysis is required.

The influence of miRNAs on the post-transcriptional regulation of gene expression may have a great impact on altering physiological processes to pathological conditions. To assess the biological significance of those alterations, mRNA targets for statistically significant miRNAs were found, and the potential PPI network analysis of transcripts encoded by these genes was performed (Table 3, Figure 6). Our results showed that all analyzed proteins could be clustered in three separate groups, associated with several signaling pathways. Group 1 consisted of COL6A3, COL1A2, ITGA2, ITGB3, and P2RY12, which may influence the extracellular matrix receptor interaction pathways crucial for platelet adhesion and activation. Collagens (COL6A3 and COL1A2) are key activators of glycoprotein (GP) VI, and GP Ia/IIa (ITGA2 is an alias for GP Ia), and are considered to be prothrombotic factors [36]. Their presence at the mRNA and protein level was confirmed in a study by Huang et al. [17]. Collagens I–IV support the adhesion of platelets in high shear forces, thus inducing platelet aggregation in small arteries; however, collagens type VI–VIII are less reactive due to the lower shear rates [37]. The presence of collagens and their receptors is crucial for the proper adhesion of platelets to the endothelium. ITGB3 (GP IIIa) is one of the two subunits that form the most crucial platelet integrin, GP IIb/IIIa, which plays a key role in the aggregation process. Signal transduction in the activation of blood platelets from various receptors leads to changes in the conformation of GP IIb/IIIa, which augment its affinity for fibrinogen [16,38]. P2RY12 is one of the most important platelet surface receptors and constitutes the main target of antiplatelet therapy [2]. Activation by adenosine diphosphate (ADP) causes the inhibition of adenylyl cyclase, which decreases the level of cyclic adenosine monophosphate (cAMP) and leads to platelet aggregation. P2RY12 also influences the PI3K-Akt signaling pathway, thus providing the conformational changes of GP IIb/IIIa [39]. Disturbances in the expression of molecules associated with the extracellular matrix interaction pathways may result in the alteration of platelet activity, thus enhancing their impact on thrombosis. Proteins included in group 2 (AKT3, MAP2K1, RAP1B, YWHAQ, BCL2L11, CDKN1A, THEM4, and CHUK) present a strong association with the PI3K-Akt signaling pathway. Phosphatidylinositol 3-kinases (PI3Ks) are enzymes that phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). Furthermore, PIP3 links with protein kinase B (Akt) which induces signal transduction to platelet activation via von Willebrand factor receptor GP Ib-IX-V, collagen-associated GP VI, and GPCRs (i.e., P2Y12). Signal transduction occurs downstream by Rap1b to interact with talin and kindlin, two proteins responsible for the conformational changes of GP IIb/IIIa [40]. The occurrence of disorders affecting any of the factors involved in the complex PI3K-Akt signaling pathway may lead to platelet dysfunction during physiological hemostasis and/or pathological thrombosis. Group

3, consisting of ITPR1, INSR, PLCB1, PRKG1, PIKFYVE, RDX, ARHGEF12, MYLK, and PPP1R12A proteins, was shown to interact with the vascular smooth muscle contraction calcium signaling pathway during platelet activation, and to participate in regulation of actin cytoskeleton. Changes in the calcium levels during platelet activation may lead to the activation of actin–myosin interaction, Rap1 and PI3K-Akt pathway signal transduction, thromboxane A2 (THX2) synthesis, granule secretion, and phospholipase (PL) activity, ensuring the occurrence of processes crucial for platelet functions [41]. VSMCs are the most abundant type of arterial wall cells. Through vasodilatation and vasoconstriction, VSMCs maintain vascular homeostasis and physiological interactions with morphotic elements. Dysregulation in the functioning of VSMCs may lead to the pathogenesis of various cardiovascular diseases, which may directly or indirectly influence ACS [42]. Furthermore, the disturbed regulation of the actin cytoskeleton in blood platelets may affect the process of shape change and the secretion of biologically active molecules stored in granules [42]. All proteins included in the three groups may directly or indirectly interact with PI3KR1, potentially affecting all crucial pathways in platelet activation. The confirmation of our bioinformatics analysis requires further detailed studies, focused not only on the miRNA levels but also on other molecular factors with the ability to regulate post-transcriptional gene expression.

One of the major findings in our study was accomplished through EFA analysis, which demonstrated that the studied miRNAs could be classified into two unrelated clusters: cluster 1, represented by miRNAs significantly differentiating ACS patients from healthy donors, and cluster 2, which consisted of miRNAs which did not contribute to the differentiation of ACS patients from controls, among others. Furthermore, the EFA analysis suggested that there is no interplay between the expression of miRNAs of both cluster groups and the groups may provide independent BFs (Figure 2). To determine these differences, we performed a comparative Gene Ontology analysis of mRNA transcripts controlled by the analyzed miRNAs, with the determination of their BFs. Thus, we identified and summed up the number of genes responsible for performing a specific BF and compared the number of genes between both groups using the DAVID Database [14]. Our results showed that mRNAs targeted by miRNAs included in cluster 1, capable of differentiating ACS patients from healthy donors, presented a seven-fold higher number of transcripts for genes associated with the positive regulation of GTPase activity and positive regulation of PLC activity, over a six-fold elevated number of genes linked with the response to hypoxia, a nearly five-fold higher number of genes related to the regulation of chemotaxis and signaling of phosphatidylinositol 3-kinase, and a more than two-times increased number of genes associated with signal transduction, response to drugs, and the regulation of nitric oxide biosynthesis (Supplementary Figure S3, "Heatmap with Gene Ontology: biological function analysis". Moreover, we studied cellular components (CCs) in the Gene Ontology overview to identify the potential location relative to cellular structures in which a particular gene performs its function. CC analysis showed that in cluster 1, a strikingly higher number of genes is associated with cytosol, the plasma membrane, integrin complex, and cytoskeleton; however, in cluster 2, a significantly elevated number of genes was associated with the endoplasmic reticulum lumen, collagen trimers, proteinaceous extracellular matrix, filopodia, and lamellipodia (Supplementary Figure S4, "Heatmap with Gene Ontology: cellular compartment analysis"). Our analysis demonstrated that the miRNAs that are able to differentiate ACS patients from healthy donors, included in cluster 1, regulate genes associated with the physiology of blood platelets, including their activation in various signaling pathways, shape change, response to pathological conditions, and medications, and overall signal transduction. However, miRNAs in cluster 2 that do not contribute to distinguishing between the study and control group may be associated with the interaction of platelets in the vascular environment without significantly interfering with platelets' physiological processes.

Using the DAVID database, we assigned genes regulated by the analyzed miRNAs to those BFs for which the difference in the number of genes was from two-fold to seven-fold

(Supplementary Figure S3). Furthermore, genes belonging to the predictive transcriptome-proteome profile of blood platelets were selected [17]. Subsequent comparative analysis of the number of selected genes associated with particular BFs allowed the selection of mRNA transcripts showing the greatest differences in frequency between cluster 1 and cluster 2. We decided to select *ITPR1*, *PLCB1*, and *PIK3R1* transcripts as targets for miRNAs in Cluster 1.

In the platelet activation pathway, a variety of factors are required for proper cellular responses, including adhesion, degranulation, and the expression of coagulant activity [43]. One of the central steps in these processes is the elevation of calcium ions (Ca^{2+}) which may be initiated by soluble agonists such as thrombin, ADP, and TXA2. The further stimulation of receptors that bind to G proteins of GPCRs leads to the activation of PLCβ, encoded by *PLCB1* [43]. In platelets, PLCβ, together with PLCγ2, releases inositol 1,4,5-triphosphate (IP3), which mediates calcium mobilization, acting on the ion channel of inositol 1,4,5-triphosphate receptor type 1 (IP3R1), encoded by the *ITPR1* gene [44,45]. IP3 is generated through PLC-mediated hydrolysis of PIP2 and initiates the release of calcium ions from the platelet-dense tubular system. PLCβ mediates this process after the stimulation of GPCRs, whereas PLCγ hydrolyzes PIP2 after the stimulation of GP VI by collagen [46]. PI3K enhances the activation of PLCγ and thus increases the hydrolysis of PIP2 mediated by GP VI [46]. Calcium transporters are very sensitive to oxygen deficiency, and hypoxia has been shown to modulate *IP3R1* gene expression differently [47]. The *PIK3R1* gene is essentially important in the context of our bioinformatic analysis, as it encodes a protein of which a critical role in cardiovascular physiology and pathology has been widely proposed [48]. Furthermore, our PPI analysis showed its central role in the studied interactome. Lurong et al. suggested that PI3K contributes to mediating a thrombin-stimulated elevation of the cytosolic calcium level in platelets, mediated through PLCγ [46]. PI3K enzymes support platelet activation and thrombosis, thus serving as promising candidates for the prevention of thrombus formation [48].

Due to the fact that statistically significant overexpressed miRNAs are strongly correlated with each other (EFA analysis and Supplementary Material Figure S2) and gathered in cluster 1, the construction of a multivariate model composed only of miRNAs would be incorrect and fail during testing. For this reason, we decided to verify miR-142-3p, which presents the best sensitivity and specificity in differentiating studied groups (sensitivity: 64%, specificity: 76%, AUC = 0.75 (0.65–0.84); $p < 0.0001$) in combination with non-correlated AST. AST is a member of the aminotransferases, a family of enzymes responsible for maintaining the metabolism of amino acids by transferring amino groups. Studies showed that the AST level starts to increase in post-ACS patients 6–8 h after the ischemic event occurs, and remains increased up to 36 h. Research over the last two decades has showed widely varying results concerning its usage as a biomarker of ACS, with gradually reduced epidemiological interest, especially in the presence of more sensitive biomarkers, including troponins [49]. However, the role of AST as a predictive marker in combination with transcriptomic factors, including miRNAs, was not evaluated until the present report. Our data demonstrated that AST levels were not correlated with miRNA levels and had promising potential in the diagnostic model (AUC = 0.83). However, the addition of miR-142-3p to the AST-based model showed substantially improved quality of patient–control differentiation (AUC = 0.91). The advantage of the combined model (miR-142-3p and AST) is that it is highly accurate in differentiating ACS patients from control donors, which is indicated by pseudo-R2 values (Table 3). Moreover, the combined model better describes our result, which can be proven by the decreased values of information criteria, and a well-fitted validation set, which simultaneously indicate a very important role of platelet miR-142-3p as a potential biomarker of ACS predisposition.

Given the study design, it was impossible to avoid certain study limitations. First, blood platelets contain a very small amount of nucleic acids compared to other blood cells. Despite taking great caution to avoid possible contaminations of samples, we cannot fully exclude the presence of non-platelet-origin miRNAs in the screening analysis. Furthermore,

due to the high correlations of selected miRNAs, we could not fully evaluate their potential role as biomarkers for ACS prediction. Further studies including a larger number of miRNAs and a larger population are required to create a functional model that differentiates patients from controls. Moreover, there is currently no standardized, documented validation model for measuring miRNA expression by means of RT-qPCR. Due to the use of different endogenous and/or exogenous controls, results compared between studies may not be fully accurate. We are fully aware that using the term "prognostic biomarker" in our study should also contain results from miRNA profiling in the population of patients before ischemia occurs; however, the genetic alterations associated with disturbed transcriptomic processes demonstrated in our study may indicate a molecular background of augmented platelet reactivity, which may be linked with induced thrombosis, which results in ACS.

5. Conclusions

To conclude, the results of our study showed a significantly increased expression of miR-142-3p; miR-107; miR-338-3p, miR-223-3p, miR-21-5p, miR-130b-3p, miR-301a-3p, and miR-221-3p in the group of ACS patients compared to healthy volunteers. Furthermore, the analyzed platelet miRNAs appear to constitute two unrelated clusters, responsible for distinct BFs. Bioinformatic analysis showed that miRNAs in cluster 1 may be associated with the physiological processes of blood platelets, and miRNAs in cluster 2 may be linked with platelet–vascular environment interactions. Our analysis based on the functional annotations and signaling pathways revealed that *ITPR1*, *PLCB1*, and *PIK3R1* may constitute predictive targets of miRNAs in cluster 1. Furthermore, PPI analysis showed that proteins, of which the expression may be regulated by statistically significant miRNAs, form three groups associated with the extracellular matrix receptor interaction pathways crucial for platelet adhesion, activation, and aggregation (group 1); the PI3K-Akt signaling pathway (group 2); and vascular smooth muscle contraction, the calcium signaling pathway during platelet activation, and the regulation of actin cytoskeleton (group 3). Moreover, based on the ROC curves, we showed that miR-142-3p presents a high potential to distinguish ACS patients from healthy donors; however, in combination with AST, the combined model was able to distinguish ACS patients from controls with 82% sensitivity, 88% specificity with AUC = 0.91 (0.85–0.97); $p < 0.0001$. The data obtained in our study show that miR-142-3p has a great potential to be used as a prognostic biomarker of ACS predisposition and/or to improve the quality of existing biomarkers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10123526/s1, Supplementary Figure S1: The heatmap of miRNAs expression in microarray analysis; Supplementary Figure S2: The correlation matrix with all the tested miRNAs; Supplementary Figure S3: Heatmap with Gene Ontology: biological function analysis; Supplementary Figure S4: Heatmap with Gene Ontology: cellular compartment analysis.

Author Contributions: Conceptualization, R.S., M.S.K., M.K., M.B. and J.S.-B.; methodology, R.S., M.S.K., M.B. and J.S.-B.; validation, R.S., M.S.K. and K.M.; formal analysis, R.S. and M.S.K.; investigation, R.S., K.M. and P.C.; resources, R.S., M.K., M.B., M.Z., T.Ś. and J.S.-B.; data curation, R.S. and M.S.K.; writing—original draft preparation, R.S.; M.S.K.; K.M. and J.S.-B.; writing—review and editing, R.S., M.S.K., M.K., P.C. and J.S.-B.; visualization, R.S. and M.S.K.; supervision, M.K., M.B., M.Z. and J.S.-B.; project administration, R.S., M.B. and J.S.-B.; funding acquisition, R.S., M.B. and J.S.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Education and Science, grant number DI2016 022046.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of the University of Lodz, Poland, with Resolution No.23/KBNN-UŁ/I/2017. All participants enrolled in the study have written informed consent.

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

Data Availability Statement: All data obtained from this study are included in the manuscript.

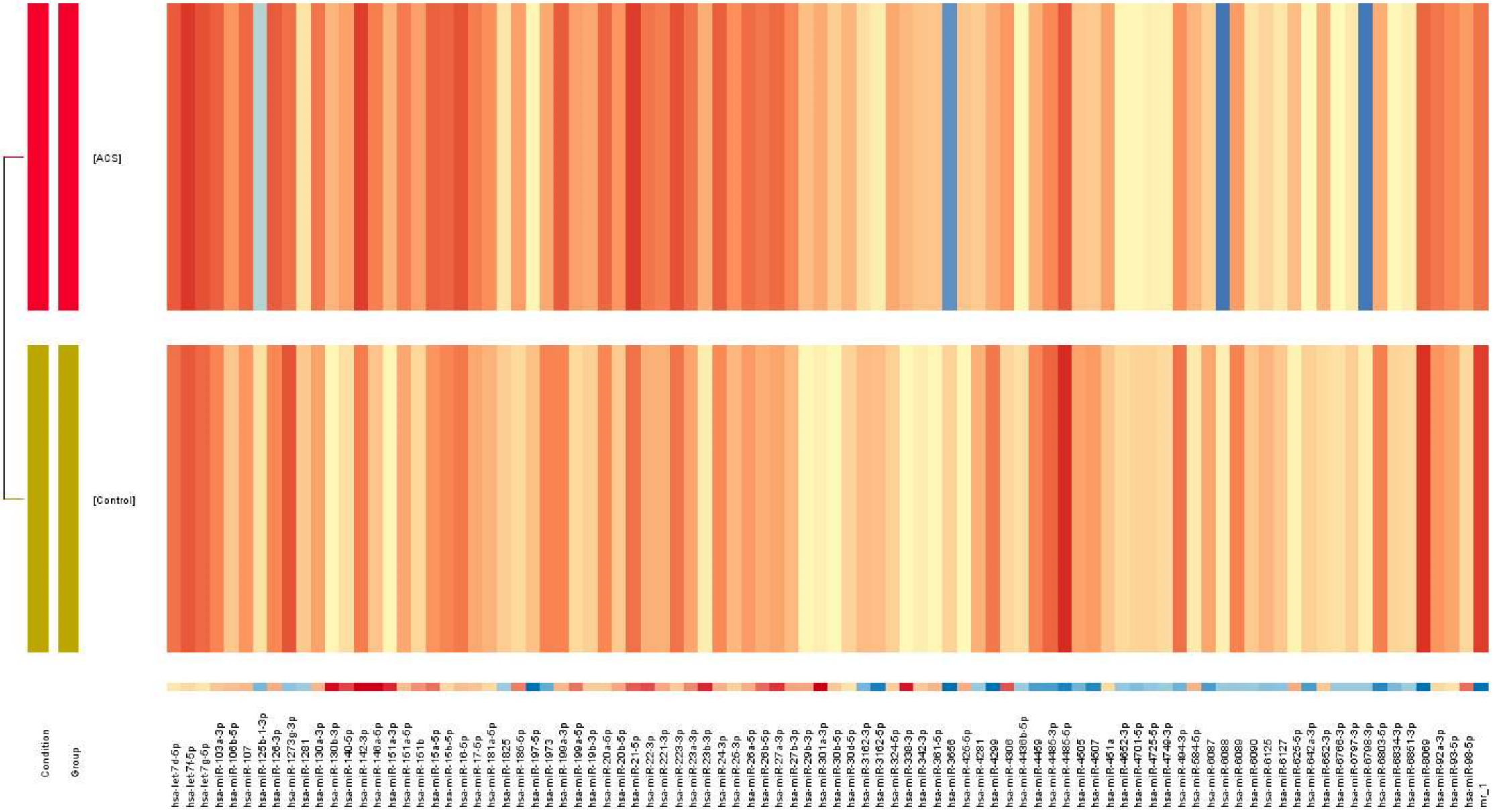
Conflicts of Interest: The authors declare no conflict of interest.

References

1. WHO. *Cardiovascular Diseases (CVDs)*; Fact Sheets; WHO: Geneva, Switzerland, 2021.
2. Collet, J.-P.; Thiele, H.; Barbato, E.; Barthélémy, O.; Bauersachs, J.; Bhatt, D.L.; Dendale, P.; Dorobantu, M.; Edvardsen, T.; Folliguet, T.; et al. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *Eur. Heart J.* **2021**, *42*, 1289–1367. [CrossRef] [PubMed]
3. Kumar, A.; Cannon, C.P. Acute coronary syndromes: Diagnosis and management, part I. *Mayo Clin. Proc.* **2009**, *84*, 917–938. [CrossRef] [PubMed]
4. Quévillon Huberdeau, M.; Simard, M.J. A guide to microRNA-mediated gene silencing. *FEBS J.* **2019**, *286*, 642–652. [CrossRef] [PubMed]
5. Li, Y.; Kowdley, K.V. MicroRNAs in Common Human Diseases. *Genom. Proteom. Bioinf.* **2012**, *10*, 246–253. [CrossRef] [PubMed]
6. Huang, H.-Y.; Lin, Y.-C.-D.; Li, J.; Huang, K.-Y.; Shrestha, S.; Hong, H.-C.; Tang, Y.; Chen, Y.-G.; Jin, C.-N.; Yu, Y.; et al. miRTarBase 2020: Updates to the experimentally validated microRNA–target interaction database. *Nucleic Acids Res.* **2020**, *48*, D148–D154. [CrossRef] [PubMed]
7. Kozomara, A.; Birgaoanu, M.; Griffiths-Jones, S. miRBase: From microRNA sequences to function. *Nucleic Acids Res.* **2019**, *47*, D155–D162. [CrossRef]
8. Chen, X.; Ba, Y.; Ma, L.; Cai, X.; Yin, Y.; Wang, K.; Guo, J.; Zhang, Y.; Chen, J.; Guo, X.; et al. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* **2008**, *18*, 997–1006. [CrossRef]
9. Ghoshal, K.; Bhattacharyya, M. Overview of platelet physiology: Its hemostatic and nonhemostatic role in disease pathogenesis. *Sci. World J.* **2014**, *2014*, 781857. [CrossRef]
10. Koupenova, M.; Kehrel, B.E.; Corkrey, H.A.; Freedman, J.E. Thrombosis and platelets: An update. *Eur. Heart J.* **2016**, *38*, 785–791. [CrossRef]
11. Zimmerman, G.A.; Weyrich, A.S. Signal-dependent protein synthesis by activated platelets: New pathways to altered phenotype and function. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, s17–s24. [CrossRef]
12. Landry, P.; Plante, I.; Ouellet, D.L.; Perron, M.P.; Rousseau, G.; Provost, P. Existence of a microRNA pathway in anucleate platelets. *Nat. Struct. Mol. Biol.* **2009**, *16*, 961–966. [CrossRef] [PubMed]
13. Czajka, P.; Fitas, A.; Jakubik, D.; Eyileten, C.; Gasecka, A.; Wicik, Z.; Siller-Matula, J.M.; Filipiak, K.J.; Postula, M. MicroRNA as Potential Biomarkers of Platelet Function on Antiplatelet Therapy: A Review. *Front. Physiol.* **2021**, *12*, 498. [CrossRef] [PubMed]
14. Huang, D.W.; Sherman, B.T.; Lempicki, R.A. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **2009**, *37*, 1–13. [CrossRef] [PubMed]
15. Amberger, J.S.; Bocchini, C.A.; Scott, A.F.; Hamosh, A. OMIM.org: Leveraging knowledge across phenotype-gene relationships. *Nucleic Acids Res.* **2019**, *47*, D1038–D1043. [CrossRef]
16. Qiu, Y.-Q. KEGG Pathway Database. In *Encyclopedia of Systems Biology*; Dubitzky, W., Wolkenhauer, O., Cho, K.-H., Yokota, H., Eds.; Springer: New York, NY, USA, 2013; pp. 1068–1069. [CrossRef]
17. Huang, J.; Swieringa, F.; Solari, F.A.; Provenzale, I.; Grassi, L.; De Simone, I.; Baaten, C.C.F.M.J.; Cavill, R.; Sickmann, A.; Frontini, M.; et al. Assessment of a complete and classified platelet proteome from genome-wide transcripts of human platelets and megakaryocytes covering platelet functions. *Sci. Rep.* **2021**, *11*, 12358. [CrossRef] [PubMed]
18. Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; et al. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* **2019**, *47*, D607–D613. [CrossRef]
19. Osman, A.; Fälker, K. Characterization of human platelet microRNA by quantitative PCR coupled with an annotation network for predicted target genes. *Platelets* **2011**, *22*, 433–441. [CrossRef]
20. Nagalla, S.; Shaw, C.; Kong, X.; Kondkar, A.A.; Edelstein, L.C.; Ma, L.; Chen, J.; McKnight, G.S.; López, J.A.; Yang, L.; et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. *Blood* **2011**, *117*, 5189–5197. [CrossRef]
21. Plé, H.; Landry, P.; Benham, A.; Coarfa, C.; Gunaratne, P.H.; Provost, P. The repertoire and features of human platelet microRNAs. *PLoS ONE* **2012**, *7*, e50746. [CrossRef]
22. Shi, R.; Ge, L.; Zhou, X.; Ji, W.-J.; Lu, R.-Y.; Zhang, Y.-Y.; Zeng, S.; Liu, X.; Zhao, J.-H.; Zhang, W.-C.; et al. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. *Thromb. Res.* **2013**, *131*, 508–513. [CrossRef]
23. Zhang, Y.Y.; Zhou, X.; Ji, W.J.; Shi, R.; Lu, R.Y.; Li, J.L.; Yang, G.H.; Luo, T.; Zhang, J.Q.; Zhao, J.H.; et al. Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome. *J. Thromb. Thrombolysis* **2014**, *38*, 65–72. [CrossRef] [PubMed]
24. Chapnik, E.; Rivkin, N.; Mildner, A.; Beck, G.; Pasvolsky, R.; Metzl-Raz, E.; Birger, Y.; Amir, G.; Tirosh, I.; Porat, Z.; et al. miR-142 orchestrates a network of actin cytoskeleton regulators during megakaryopoiesis. *eLife* **2014**, *3*, e01964. [CrossRef]
25. Shin, E.-K.; Park, H.; Noh, J.-Y.; Lim, K.-M.; Chung, J.-H. Platelet Shape Changes and Cytoskeleton Dynamics as Novel Therapeutic Targets for Anti-Thrombotic Drugs. *Biomol. Ther.* **2017**, *25*, 223–230. [CrossRef] [PubMed]
26. Bao, H.; Yao, Q.P.; Huang, K.; Chen, X.H.; Han, Y.; Jiang, Z.L.; Gao, L.Z.; Qi, Y.X. Platelet-derived miR-142-3p induces apoptosis of endothelial cells in hypertension. *Cell. Mol. Biol.* **2017**, *63*, 3–9. [CrossRef] [PubMed]

27. Peckova, M.; Fahrenbruch, C.E.; Cobb, L.A.; Hallstrom, A.P. Circadian variations in the occurrence of cardiac arrests: Initial and repeat episodes. *Circulation* **1998**, *98*, 31–39. [CrossRef]
28. Ohkura, N.; Oishi, K.; Sudo, T.; Hayashi, H.; Shikata, K.; Ishida, N.; Matsuda, J.; Horie, S. CLOCK regulates circadian platelet activity. *Thromb. Res.* **2009**, *123*, 523–527. [CrossRef]
29. Ward, J.A.; Esa, N.; Pidikiti, R.; Freedman, J.E.; Keaney, J.F.; Tanriverdi, K.; Vitseva, O.; Ambros, V.; Lee, R.; McManus, D.D. Circulating Cell and Plasma microRNA Profiles Differ between Non-ST-Segment and ST-Segment-Elevation Myocardial Infarction. *Fam. Med. Med. Sci. Res.* **2013**, *2*, 108. [CrossRef]
30. Garabet, L.; Ghanima, W.; Rangberg, A.; Teruel-Montoya, R.; Martinez, C.; Lozano, M.L.; Nystrand, C.F.; Bussel, J.B.; Sandset, P.M.; Jonassen, C.M. Circulating microRNAs in patients with immune thrombocytopenia before and after treatment with thrombopoietin-receptor agonists. *Platelets* **2020**, *31*, 198–205. [CrossRef]
31. Coskunpinar, E.; Cakmak, H.A.; Kalkan, A.K.; Tiryakioglu, N.O.; Erturk, M.; Ongen, Z. Circulating miR-221-3p as a novel marker for early prediction of acute myocardial infarction. *Gene* **2016**, *591*, 90–96. [CrossRef]
32. Pordzik, J.; Pisarz, K.; De Rosa, S.; Jones, A.D.; Eyileten, C.; Indolfi, C.; Malek, L.; Postula, M. The Potential Role of Platelet-Related microRNAs in the Development of Cardiovascular Events in High-Risk Populations, Including Diabetic Patients: A Review. *Front. Endocrinol.* **2018**, *9*, 74. [CrossRef]
33. Zhang, Y.; Liu, Y.J.; Liu, T.; Zhang, H.; Yang, S.J. Plasma microRNA-21 is a potential diagnostic biomarker of acute myocardial infarction. *Eur. Rev. Med. Pharmacol. Sci.* **2016**, *20*, 323–329.
34. Liu, X.; Dong, Y.; Chen, S.; Zhang, G.; Zhang, M.; Gong, Y.; Li, X. Circulating MicroRNA-146a and MicroRNA-21 Predict Left Ventricular Remodeling after ST-Elevation Myocardial Infarction. *Cardiology* **2015**, *132*, 233–241. [CrossRef]
35. Cengiz, M.; Yavuzer, S.; Kılıçkıran Avcı, B.; Yürüyen, M.; Yavuzer, H.; Dikici, S.A.; Karataş, Ö.F.; Özen, M.; Uzun, H.; Öngen, Z. Circulating miR-21 and eNOS in subclinical atherosclerosis in patients with hypertension. *Clin. Exp. Hypertens.* **2015**, *37*, 643–649. [CrossRef]
36. Bergmeier, W.; Hynes, R.O. Extracellular matrix proteins in hemostasis and thrombosis. *Cold Spring Harb. Perspect. Biol.* **2012**, *4*, a005132. [CrossRef] [PubMed]
37. Farndale, R.W.; Sixma, J.J.; Barnes, M.J.; De Groot, P.G. The role of collagen in thrombosis and hemostasis. *J. Thromb. Haemost.* **2004**, *2*, 561–573. [CrossRef]
38. Fullard, J.F. The role of the platelet glycoprotein IIb/IIIa in thrombosis and haemostasis. *Curr. Pharm. Des.* **2004**, *10*, 1567–1576. [CrossRef]
39. Zhang, Y.; Zhang, S.; Ding, Z. Role of P2Y(12) Receptor in Thrombosis. *Adv. Exp. Med. Biol.* **2017**, *906*, 307–324. [CrossRef] [PubMed]
40. Guidetti, G.F.; Canobbio, I.; Torti, M. PI3K/Akt in platelet integrin signaling and implications in thrombosis. *Adv. Biol. Regul.* **2015**, *59*, 36–52. [CrossRef]
41. Li, Z.; Delaney, M.K.; O'Brien, K.A.; Du, X. Signaling During Platelet Adhesion and Activation. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 2341–2349. [CrossRef]
42. Zhu, Y.; Qu, J.; He, L.; Zhang, F.; Zhou, Z.; Yang, S.; Zhou, Y. Calcium in Vascular Smooth Muscle Cell Elasticity and Adhesion: Novel Insights Into the Mechanism of Action. *Front. Physiol.* **2019**, *10*, 852. [CrossRef]
43. Pleines, I.; Elvers, M.; Strehl, A.; Pozgajova, M.; Varga-Szabo, D.; May, F.; Chrostek-Grashoff, A.; Brakebusch, C.; Nieswandt, B. Rac1 is essential for phospholipase C-γ2 activation in platelets. *Pflüg. Archiv-Eur. J. Physiol.* **2009**, *457*, 1173–1185. [CrossRef]
44. Jones, C.I.; Bray, S.; Garner, S.F.; Stephens, J.; de Bono, B.; Angenent, W.G.J.; Bentley, D.; Burns, P.; Coffey, A.; Deloukas, P.; et al. A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. *Blood* **2009**, *114*, 1405–1416. [CrossRef] [PubMed]
45. Wright, J.R.; Amisten, S.; Goodall, A.H.; Mahaut-Smith, M.P. Transcriptomic analysis of the ion channelome of human platelets and megakaryocytic cell lines. *Thromb. Haemost.* **2016**, *116*, 272–284. [CrossRef] [PubMed]
46. Lian, L.; Wang, Y.; Draznin, J.; Eslin, D.; Bennett, J.S.; Poncz, M.; Wu, D.; Abrams, C.S. The relative role of PLCbeta and PI3Kgamma in platelet activation. *Blood* **2005**, *106*, 110–117. [CrossRef]
47. Jurkovicova, D.; Sedlakova, B.; Lacinova, L.; Kopacek, J.; Sulova, Z.; Sedlak, J.; Krizanova, O. Hypoxia differently modulates gene expression of inositol 1,4,5-trisphosphate receptors in mouse kidney and HEK 293 cell line. *Ann. N. Y. Acad. Sci.* **2008**, *1148*, 421–427. [CrossRef] [PubMed]
48. Durrant, T.N.; Hers, I. PI3K inhibitors in thrombosis and cardiovascular disease. *Clin. Transl. Med.* **2020**, *9*, 8. [CrossRef] [PubMed]
49. Ndrepepa, G. Aspartate aminotransferase and cardiovascular disease—A narrative review. *J. Lab. Precis. Med.* **2021**, *6*, 8725. [CrossRef]

Figure S1. The heatmap of miRNAs expression in microarray analysis



Supplementary Figure S2. The correlation matrix with all the tested miRNAs

Pearson's r	hsa-miR-301a-3p	hsa-miR-142-3p	hsa-miR-338-3p	hsa-miR-130b-3p	hsa-miR-107	hsa-miR-21-5p	hsa-miR-223-3p	hsa-miR-126-3p	hsa-miR-98-5p	hsa-miR-221-3p	hsa-miR-146a-5p	hsa-let-7f-5p	hsa-let-7d-5p	hsa-miR-28-5p	hsa-let-7g-5p	
hsa-miR-301a-3p		0,475	0,402	0,484	0,545	0,451	0,512	0,442	0,169	0,379	0,430	0,186	0,281	0,119	0,364	MiRNAs with fold-change >4
hsa-miR-142-3p	0,475		0,668	0,728	0,808	0,793	0,854	0,676	0,164	0,450	0,567	0,072	0,068	0,091	0,425	
hsa-miR-338-3p	0,402	0,668		0,603	0,570	0,498	0,615	0,453	0,082	0,243	0,278	0,269	-0,009	0,014	0,255	
hsa-miR-130b-3p	0,484	0,728	0,603		0,704	0,604	0,749	0,603	0,245	0,441	0,514	0,135	0,208	0,207	0,419	
hsa-miR-107	0,545	0,808	0,570	0,704		0,639	0,806	0,677	0,311	0,582	0,664	0,229	0,391	0,268	0,500	MiRNAs with fold-change of 1.8-4
hsa-miR-21-5p	0,451	0,793	0,498	0,604	0,639		0,801	0,651	0,159	0,624	0,647	0,223	0,075	0,162	0,588	
hsa-miR-223-3p	0,512	0,854	0,615	0,749	0,806	0,801		0,780	0,257	0,602	0,658	0,250	0,258	0,214	0,511	
hsa-miR-126-3p	0,442	0,676	0,453	0,603	0,677	0,651	0,780		0,134	0,624	0,681	0,258	0,354	0,249	0,589	
hsa-miR-98-5p	0,169	0,164	0,082	0,245	0,311	0,159	0,257	0,134		0,116	0,144	0,362	0,383	0,145	0,300	MiRNAs with fold-change <1.8
hsa-miR-221-3p	0,379	0,450	0,243	0,441	0,582	0,624	0,602	0,624	0,116		0,750	0,439	0,568	0,315	0,769	
hsa-miR-146a-5p	0,430	0,567	0,278	0,514	0,664	0,647	0,658	0,681	0,144	0,750		0,385	0,601	0,343	0,645	
hsa-let-7f-5p	0,186	0,072	0,269	0,135	0,229	0,223	0,250	0,258	0,362	0,439	0,385		0,499	0,138	0,528	
hsa-let-7d-5p	0,281	0,068	-0,009	0,208	0,391	0,075	0,258	0,354	0,383	0,568	0,601	0,499		0,359	0,573	
hsa-miR-28-5p	0,119	0,091	0,014	0,207	0,268	0,162	0,214	0,249	0,145	0,315	0,343	0,138	0,359		0,153	
hsa-let-7g-5p	0,364	0,425	0,255	0,419	0,500	0,588	0,511	0,589	0,300	0,769	0,645	0,528	0,573	0,153		
	MiRNAs with fold-change >4				MiRNAs with fold-change of 1.8-4				MiRNAs with fold-change <1.8							

The matrix presents the values of Pearson's r correlation coefficients for all pairs of expressions of the tested miRNAs. The matrix cells are colored according to the Pearson's r values. The most top-left set of four miRNAs depicts these with extreme expression fold-change (higher than 4 – corresponding to red-colored miRNAs in Figure 4). The next top-left set of four miRNAs depicts these with intermediate expression fold-change (1.8-4 – corresponding to yellow-colored miRNAs in Figure 4). The rest of miRNAs presented in the bottom and right part of the matrix depicts these with expression fold-change lower than 1.8 (corresponding to green-colored miRNAs in Figure 4). The Pearson's r matrix informs that miRNAs that substantially differentiate ACS patients from healthy controls are generally more correlated with each other than miRNAs not differing between ACS patients and controls. This corresponds to the exploratory factor analysis presented in Figure 4.

Supplementary Figure S3. Heatmap with Gene Ontology: Biological Functions analysis. Numbers in rows presents number of genes associated with particular function.

Biological Function	Cluster 1	Cluster 2
signal transduction	102	47
positive regulation of transcription from RNA polymerase II promoter	67	36
positive regulation of GTPase activity	53	7
positive regulation of cell migration	40	33
response to drug	35	17
extracellular matrix organization	33	40
aging	29	31
protein phosphorylation	28	42
negative regulation of gene expression	24	0
phosphatidylinositol phosphorylation	23	6
positive regulation of smooth muscle cell proliferation	22	5
positive regulation of ERK1 and ERK2 cascade	22	4
MAPK cascade	22	4
response to hypoxia	19	3
activation of MAPK activity	19	0
cerebral cortex development	18	17
regulation of phosphatidylinositol 3-kinase signaling	18	4
platelet activation	16	15
collagen catabolic process	15	26
phosphatidylinositol-mediated signaling	15	3
positive regulation of phospholipase C activity	15	2
peptidyl-tyrosine phosphorylation	14	3
positive regulation of positive chemotaxis	14	3
positive regulation of nitric oxide biosynthetic process	13	6
cell adhesion	13	0
protein stabilization	12	6
chemotaxis	12	0
response to cold	11	18
positive regulation of MAP kinase activity	11	9

positive regulation of cell proliferation	11	5
cell adhesion mediated by integrin	11	2
cellular response to estradiol stimulus	10	2
nervous system development	10	0
positive regulation of gene expression	10	0
positive regulation of protein complex assembly	9	2
leukocyte chemotaxis	9	0
phosphatidylinositol-3-phosphate biosynthetic process	9	0
positive regulation of protein kinase B signaling	9	0
cell-matrix adhesion	9	0
temperature homeostasis	8	12
cellular response to insulin-like growth factor stimulus	8	2
sphingolipid biosynthetic process	8	0
insulin-like growth factor receptor signaling pathway	8	0
G-protein coupled acetylcholine receptor signaling pathway	8	0
positive regulation of protein phosphorylation	7	12
positive regulation of angiogenesis	7	3
positive regulation of developmental growth	6	6
release of sequestered calcium ion into cytosol	6	3
positive regulation of peptidyl-tyrosine phosphorylation	6	3
positive regulation of cell adhesion	6	2
response to muscle activity	6	2
negative regulation of protein phosphorylation	6	0
integrin-mediated signaling pathway	6	0
positive regulation of leukocyte migration	6	0
cellular glucose homeostasis	6	0
skeletal system development	4	8
response to superoxide	4	8
fatty acid oxidation	4	2
regulation of immunoglobulin secretion	4	0
glutamate receptor signaling pathway	4	0
regulation of smooth muscle contraction	4	0

cellular response to amino acid stimulus	3	12
positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	3	0
positive regulation of endothelial cell migration	3	0
positive regulation of cell division	3	0
aorta smooth muscle tissue morphogenesis	2	10
positive regulation of glycolytic process	2	4
smooth muscle contraction	2	4
positive regulation of lipophagy	2	0
inositol phosphate biosynthetic process	2	0
regulation of phosphatidylinositol 3-kinase activity	2	0
regulation of establishment of endothelial barrier	2	0
positive regulation of protein kinase C signaling	2	0
tube formation	2	0
phosphatidylinositol metabolic process	2	0
sleep	2	0
cell migration involved in sprouting angiogenesis	2	0
positive regulation of blood vessel endothelial cell migration	2	0
positive regulation of focal adhesion assembly	2	0

Supplementary Figure S4. Heatmap with Gene Ontology: Cellular Compartment analysis. Numbers in rows presents number of genes associated with particular function.

Cellular Compartment	Cluster 1	Cluster 2
cytoplasm	72	46
membrane	71	13
extracellular region	58	68
extracellular space	51	43
cytosol	48	0
plasma membrane	43	16
Golgi apparatus	42	34
proteinaceous extracellular matrix	23	36
collagen trimer	21	38
extracellular matrix	20	14
external side of plasma membrane	20	0
receptor complex	16	27
cytoskeleton	15	4
intracellular	15	0
membrane raft	14	3
nuclear euchromatin	12	2
endoplasmic reticulum lumen	11	40
integral component of plasma membrane	10	9
extrinsic component of membrane	9	0
early endosome	8	16
endoplasmic reticulum	8	0
focal adhesion	5	0
lamellipodium	4	8
integrin complex	4	0
filopodium	3	6
cell-cell junction	3	3
intrinsic component of plasma membrane	2	2
CD40 receptor complex	2	0
collagen type IV trimer	0	8
organelle membrane	0	6
collagen type VI trimer	0	4
endosome membrane	0	3

biology

MDPI

Article

Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients—Emphasis on P2Y12

Rafał Szelenberger [1,2,*], Michał Seweryn Karbownik [3], Michał Kacprzak [4], Ewelina Synowiec [5], Sylwia Michlewska [6], Michał Bijak [2], Marzenna Zielińska [4], Alina Olender [7] and Joanna Saluk-Bijak [1]

1 Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; joanna.saluk@biol.uni.lodz.pl
2 Biohazard Prevention Centre, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; michal.bijak@biol.uni.lodz.pl
3 Department of Pharmacology and Toxicology, Medical University of Lodz, 90-752 Lodz, Poland; michal.karbownik@umed.lodz.pl
4 Department of Interventional Cardiology, Medical University of Lodz, 91-213 Lodz, Poland; michal.kacprzak@umed.lodz.pl (M.K.); marzenna.zielinska@umed.lodz.pl (M.Z.)
5 Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; ewelina.synowiec@biol.uni.lodz.pl
6 Laboratory of Microscopic Imaging and Specialized Biological Techniques, Faculty of Biology and Environmental Protection, University of Lodz, 90-237 Lodz, Poland; sylwia.michlewska@biol.uni.lodz.pl
7 Chair and Department of Medical Microbiology, Medical University of Lublin, 20-093 Lublin, Poland; alina.olender@umlub.pl
* Correspondence: rafal.szelenberger@edu.uni.lodz.pl

Simple Summary: Acute Coronary Syndrome is a disease of the circulatory system characterized by the partial or complete blockage of coronary arteries. In thrombosis, a major role is played by platelets—the smallest, anucleate, morphotic elements in the bloodstream. Platelets are involved in the process of hemostasis, which ensures the continuity of the blood vessel by forming a clot that prevents blood loss. Unique structures of blood platelets ensure their high reactivity in the vascular microenvironment due to the interaction with many biologically active molecules, which is recognized by the surface receptors. The research carried out in the manuscript is aimed at detecting potential changes at the molecular level in platelet surface receptors that could constitute the potential importance of the occurrence of Acute Coronary Syndrome. The obtained results indicate that the P2Y12 receptor, which is the main target of antiplatelet therapy, is expressed more frequently among patients. In addition, we have shown that at the genetic level, quantitative changes also occur in the case of other receptors that are important in the activation of platelets. The following manuscript suggests the potential mechanisms responsible for the differences between patients and healthy donors due to a better understanding of the molecular causes of Acute Coronary Syndrome pathogenesis.

Abstract: The pathological conditions caused by blood platelet activation constitute a fundamental core in the pathogenesis of Acute Coronary Syndrome (ACS). The hyperactivity of platelets in ACS is well-documented, but there is still little research into the molecular basis of phenotypic changes in platelet functionality. To expand the knowledge of this phenomenon, we analyzed the disturbances in the expression of several key platelet receptors and the aspect of regulating potential abnormalities. Platelet surface receptors are responsible for maintaining the hemostatic balance, platelet interaction with immune cells, and support of the coagulation cascade leading to occlusion of the vessel lumen. Due to their prominent role, platelet receptors constitute a major target in pharmacological treatment. Our work aimed to identify the molecular alteration of platelet surface receptors, which showed augmented mRNA expression of P2Y12, GP1BB, ITGA2B, and ITGB3 and increased protein concentrations of P2Y12 and GP IIb/IIIa in ACS. The upregulation of the P2Y12 level was also confirmed by confocal and cytometric visualization. Furthermore, we evaluated the expression of two microRNAs: miR-223-3p and miR-126-3p, which were suggested to regulate

check for updates

Citation: Szelenberger, R.; Karbownik, M.S.; Kacprzak, M.; Synowiec, E.; Michlewska, S.; Bijak, M.; Zielińska, M.; Olender, A.; Saluk-Bijak, J. Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients—Emphasis on P2Y12. *Biology* **2022**, *11*, 644. https://doi.org/10.3390/biology11050644

Academic Editors: Waltraud C. Schrottmaier and Marion Mussbacher

Received: 18 March 2022
Accepted: 20 April 2022
Published: 22 April 2022

platelet P2Y12 expression. Results of our study present new insight into the molecular background of ACS.

Keywords: blood platelets; acute coronary syndrome; surface receptors; miRNA; P2Y12; GP IIb/IIIa

1. Introduction

Acute Coronary Syndrome (ACS) describes the myocardial ischemic events arising from partial or complete blockage of coronary arteries. ACS can be divided into the three different clinical manifestations characterized by the occurrence of the various symptoms, including ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina (UA) [1]. The major role in the pathogenesis of ACS is performed by blood platelets, the smallest morphotic elements that play a fundamental role in ensuring the hemostasis process. The physiological functioning of blood platelets is strictly controlled and begins with the adhesion to the injured vessel wall, which is accomplished with the formation of a blood clot that prevents blood loss. However, under certain pathological conditions, platelets may spontaneously form pathogenic aggregates, which constitute a fundamental factor in the development of cardiovascular diseases, including ACS [2]. Complex processes responsible for the platelet plug formation require a series of events including initiation, stimulation, and thrombus stabilization. All phases determine the dynamics of clot formation and are coordinated by a vast amount of platelet surface receptors [3]. The conditions prevailing in the vascular environment are extremely important for platelets. The surface-receptor-dependent adhesion process induces intracellular signal transduction, which leads to the activation of platelets, shape change, release of biologically active compounds from granules, and aggregation [4]. Activation of platelet pathways leads to recruitment of cells from the bloodstream; induces accumulation; enhances secondary aggregation induced by ADP; and provides overall interaction of platelets with leukocytes, endothelium, and coagulation factors [5]. Multitude platelet surface receptors ensure all necessary abilities of platelets to carry out hemostasis, thus emphasizing their important role in the proper functioning of these cells [3–5].

Structural and functional changes in the surface receptors of blood platelets may therefore play a pivotal role in the pathogenesis of ACS. Platelet hyperreactivity, the extension of blood procoagulative potential, development of the inflammatory processes, and increased interaction between platelet activators and their receptors constitute a complicated network of mutual relationships. One of the major targets for decreasing platelets reactivity by pharmacological blockade is the P2Y12 receptor. According to the novel guidelines of The European Society of Cardiology, the gold standard therapy for patients diagnosed with ACS include aspirin and a potent P2Y12 receptor inhibitor, with a recommendation for ticagrelor or prasugrel [6]. However, because of the various clinical presentations and characteristics of patients, the presence of comorbidities, comedication, and procedural aspects, the inhibitors of glycoprotein (GP) IIb/IIIa also constitute an effective antiplatelet therapy [6]. Besides the key role of the platelet surface receptors in the pathogenesis and prevention of ACS, studies related to their activity are largely reduced to testing new antiplatelet drugs. Our study aims to determine the molecular basis of the observed functional changes in blood platelets in ACS. The research hypothesis in this study assumes that a hyperreactivity of blood platelets in ACS may be caused by the altered expression of platelet surface receptors. In this study, we found that the expression on the mRNA level of major platelet surface receptors (P2Y12, ITGA2B, ITGB3, GP1BB) was significantly elevated in ACS patients. Furthermore, the concentrations of two main pharmacological targets, P2Y12 and subunit of GP IIb/IIIa, were significantly increased in the study group. Additionally, to evaluate the potential regulatory mechanism of overexpressed P2Y12 at the mRNA level, the expression levels of two human blood platelet microRNAs (miRNAs), miR-223-3p and -126-3p, were determined. Moreover, our study for the first time presents

the cytometric and confocal visualization of altered expression of platelet P2Y12 receptor between ACS patients and controls, thus providing novel data that demonstrate observable characteristics that differentiate the study group from healthy donors.

2. Materials and Methods

2.1. Chemicals

Glucose, KCL, Thiourea, Urea, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Bovine Serum Albumin (BSA), Tris, and NH_4HCO_3 were obtained from Sigma-Aldrich (Saint Louis, MO, USA). NaCl, $NaHCO_3$, Citric acid, Sodium Citrate, and NaH_2PO_4 were obtained from POCh (Gliwice, Poland). PBS tablets were obtained from Biosigma (Venice, Italy).

2.2. Blood Collection

Blood samples from the study group were taken from 35 patients of both sexes, aged 30–65 years old from the Department of Interventional Cardiology of the Medical University of Lodz with angiographically documented episodes of ACS. Blood was drawn from the patients immediately after the necessary examinations and treatments to ensure the patient's safety. Patients received a one-time dose of antiplatelet drugs based on clinical characteristics such as age, sex, race, clinical picture, procedural aspects, administered drugs, and possible comorbidities. ACS patients registered in the study were under 65 years old; had normal kidney function; were not addicted to alcohol and narcotics; and in the medical history, had never been diagnosed with connective tissue disorders, hyperthyroidism, diabetes mellitus, and/or cancer. Furthermore, enrolled subjects had BMI < 35. Patients enrolled in the study had not been previously diagnosed with any cardiovascular diseases and did not receive any antiplatelet drugs before ACS occurred. The control group consisted of donors without any problems with the circulatory system. All volunteers that qualified for the study before inclusion were subjected to the following medical tests: morphology, creatinine, TSH (Thyroid-Stimulating Hormone), coagulation, CRP (C-Reactive Protein), IgG (Immunoglobulin G), and IgM titers, ALT (Alanine Transaminase), AST (Aspartate Transaminase), and levels of glucose; LDL (Low-Density Lipoprotein), HDL (High-Density Lipoprotein), cholesterol, and triglycerides. Donors enrolled in the study were free from any diseases and did not administer any medications at least 2 weeks before the blood draw. All clinical and demographical characteristics of patients and donors are included in Table 1. The study was approved by the Committee of the Ethics of Research in Human Experimentation at the University of Lodz with resolution number 23/KBNN-UŁ/I/2017. All enrolled patients and controls signed an informed consent form before entering the study. All procedures were performed according to the Helsinki Declaration for Human Research.

Table 1. Sociodemographic, anthropometric, and blood biochemical characteristics of ACS patients and healthy controls.

Parameter	ACS (n = 35)	Control (n = 35)	Reference Range	p-Value
	Median (1st–3rd Quartiles) or Number (Frequency)			
Sociodemographic and Anthropometric Characteristics				
Age (years)	50 (45–61)	48 (41–57)	-	0.545
Sex (male)	29 (83%)	28 (80%)	-	0.999
BMI (kg/m^2)	30 (27–33)	29 (28–32)	<35	0.876

Table 1. *Cont.*

Parameter	ACS (*n* = 35)	Control (*n* = 35)	Reference Range	*p*-Value
	Median (1st–3rd Quartiles) or Number (Frequency)			
Blood biochemical characteristics				
Leukocytes ($10^3/\mu L$)	8.60 (7.10–9.80)	5.98 (4.83–7.98)	4–11	<0.001
Erythrocytes ($10^6/\mu L$)	4.47 (4.25–4.95)	5.07 (4.61–5.30)	4.2–6.1	0.0064
Blood platelets ($10^3/\mu L$)	260 (202–287)	249 (208–292)	150–400	0.946
Glucose (mmol/L)	6.00 (5.31–6.35)	4.99 (4.76–5.57)	4.1–5.5	<0.001
Creatinine (μmol/L)	81.0 (72.8–90.0)	76.0 (69.9–87.5)	64–104	0.240
GFR ($mL/min/1.73\ m^2$)	96.7 (81.0–104.3)	91.5 (81.7–103.1)	>60	
AST (U/I)	34 (26–38)	19 (17–25)	<50	<0.001
ALT (U/I)	29 (21–42)	22 (22–38)	<50	0.120
Total cholesterol (mmol/L)	5.12 (4.36–5.96)	4.93 (4.37–5.42)	3–5	0.296
LDL (mmol/L)	3.07 (2.57–4.20)	2.84 (2.35–3.36)	-	0.085
HDL (mmol/L)	1.15 (1.02–1.33)	1.29 (1.12–1.67)	>1	0.009
Triglycerides (mmol/L)	1.67 (1.01–2.78)	1.24 (0.97–1.79)	<1.7	0.015
TSH (μIU/mL)	1.71 (1.16–2.53)	1.93 (1.31–2.61)	0.27–4.20	0.463

Clinical parameters are presented as a median and 1st–3rd quartile of 25th–75th percentile. Abbreviations: ALT—alanine transaminase; AST—aspartate transaminase; BMI—body mass index; GFR—glomerular filtration rate; HDL—high-density lipoprotein; LDL—low-density lipoprotein; TSH—thyroid-stimulating hormone.

2.3. Blood Platelet Isolation and Purification

The whole blood samples were collected by the S-Monovette® CPDA1 system (Sarstedt, Numbrecht, Germany) and centrifuged (1200 rpm, 12 min, 37 °C). The top layer of platelet-rich plasma (PRP) was transported to fresh tubes. To avoid leukocyte and erythrocyte contamination, MACS® magnetic cell separation was used. Obtained PRP was purified using superparamagnetic particles conjugated with specific antibodies: anti-CD45 and anti-CD235a (Miltenyi Biotech, Bergisch Gladbach, Germany). Further, PRP with antibodies was applied on the MS Column (Miltenyi Biotech, Bergisch Gladbach, Germany) and on the manual separator (Miltenyi Biotech, Bergisch Gladbach, Germany). Antibodies linked with leukocytes and erythrocytes were retained in the magnetic area of the MS Column and purified PRP flowed to a fresh tube. All steps were performed according to the manufacturer's protocol, except for the wash buffer components, which were changed to minimize the possibility of platelet activation. For washing steps, a modified buffer containing PBS, 2-mM Citrate, and 0.5% BSA was used. Platelets were isolated from purified PRP by density centrifugation (1400 rpm, 15 min). Isolated platelets were washed 2 times with modified Tyrode's Buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4), and suspended in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) to stabilize mRNA. Samples were stored at −80 °C.

2.4. Total RNA Isolation and Synthesis of Complementary DNA

Isolation of total RNA from previously collected and purified blood platelet samples was performed using Isolate II RNA Mini Kit (Bioline, London, England). In the first step, RNAlater was removed from the sample by adding ice-cold PBS with a 1:1 ratio and centrifuged (5000 rpm, 5 min, 4 °C). Further steps were performed following the manufacturer's protocol. Total RNA samples were treated with DNase I to digest potential DNA contamination. In the next step, total RNA was reverse transcribed to obtain cDNA with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the included protocol. Obtained cDNA was suspended in nuclease-free

water and stored at −80 °C in the form of a cDNA library until use. To prepare a cDNA template for miRNA detection, TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. To the total RNA samples, cel-miR-39-3p (Thermo Fisher Scientific, Waltham, MA, USA) was added as an exogenous control. The final concentration of cel-miR-39-3p in each sample was 10 pM. All steps were performed according to the manufacturer's protocol.

2.5. Isolation of Protein Fraction from Platelets

Isolated and purified platelet samples (obtained from 2 mL of PRP) were washed 3 times with modified Tyrode's Buffer to avoid plasma contamination. The cell pellet was suspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris) and mixed until dissolved. Prepared samples were stored at −32 °C for subsequent analysis.

2.6. Gene Expression and miRNA Analysis by the Real-Time Quantitative PCR (RT-qPCR) Technique

For the following genes: P2Y12 (Hs01881698_s1), ITGB3 (Hs01001469_m1), ITGA2B (Hs00166246_m1), GP1BB (Hs02579226_s1), F2R (Hs05045041_s1), and 18S rRNA (Hs99999901_s1) expression analysis was performed using RT-qPCR with TaqMan Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA). As a reference gene, the expression of 18S rRNA was used. The RT-qPCR thermal cycling conditions were as follows: polymerase activation (95 °C, 10 min), 40 cycles of denaturation (95 °C, 15 s) and extension (60 °C, 1 min). For miRNA analysis, the following TaqMan™ Advanced miRNA Assays (Thermo Fisher Scientific, Waltham, MA, USA) were used: hsa-miR-223-3p (477983_mir), hsa-miR-126-3p (477887_mir), hsa-miR-191-5p (477952_mir), and cel-miR-39-3p (478293_mir). The RT-qPCR thermal cycling conditions were set according to the TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and were as follows: polymerase activation (95 °C, 20 s), 40 cycles of denaturation (95 °C, 3 s) and extension (60 °C, 30 s). As a reference miRNA, the expression of endogenous miR-191-5p and exogenous cel-miR-39-3p was used. Exogenous control was synthesized by Thermo Fisher Scientific with the following sequence (5′ to 3′) (RNA)-Phos-UCACCGGGUGUAAAUCAGCUUG. Data normalization was performed on the mean value of endo- and exogenous reference controls for the ΔCt calculation. For miRNA quantification, cDNA samples were diluted 10 times for analysis.

2.7. Determination of Protein Concentration Using ELISA Technique

Based on the results obtained from gene expression analysis, P2Y12, GPIIIa, and GPIb were selected for the determination of its concentration by ELISA technique (FineTest, Wuhan, China). All steps were performed according to the manufacturer's protocol. Absorbance was measured at 450 nm and protein concentration was calculated from the standard curve.

2.8. Determination of P2Y12 Platelet Surface Expression by Flow Cytometry Method

Measurement of P2Y12 receptor was performed in whole blood samples from ACS patients and healthy controls using the flow cytometry method. In the first stage of sample preparation, erythrocytes were lysed by BD FACS Lysing Solution (Beckton Dickinson, San Diego, CA, USA). Further, samples were incubated for 30 min in the dark, at 24 °C, with specific murine monoclonal antibodies: BB515 Anti-Human CD61 (Beckton Dickinson, San Diego, CA, USA), PE Anti-Human P2Y12 (BioLegend, San Diego, CA, USA). Next, samples were fixed with 1% CellFix (Beckton Dickinson, San Diego, CA, USA), and centrifuged (6200 rpm, 12 min, 24 °C). After centrifugation, the supernatant was discarded and the obtained pellet was dissolved in 800 µL of 0.9% NaCl. Subsequently, samples were immediately analyzed using CUBE6 flow cytometry with CyView Software v.1.5.5.8 (Partec, Görlitz, Germany). During all performed measurements, the fluorescence of 15,000 events was recorded every time. Gates for used fluorochromes were set based on the fluorescence signal of unstained probes. FSC parameters were used to identify platelet microparticles

(FSC below 10^2) and platelet aggregates (FSC above 10^3). Surface expression of the P2Y12 receptor on CD61-positive cells was assessed by the fluorescence intensity.

2.9. Visualization of P2Y12 Receptor via Confocal Microscopy

Visualization of the P2Y12 receptor level on the human blood platelets was performed via confocal microscopy. Due to maintaining platelets in a resting state, Sepharose 2B-BSA gel was used to isolate pure platelets without any potentially aggressive methods that could activate them before imaging. To avoid any possibility of false-positive or false-negative results, no inhibitory substances were added during platelet isolation. In the first stage, Sepharose 2B-BSA gel was washed to discard the ethanol layer and suspended in the modified Tyrode's buffer. Next, the gel was packed in the column and PRP was put on the top of the column. The platelet fraction was collected in a fresh 2-mL tube. Further, 20 µL of suspended platelets were transferred to the fresh tube containing 6 µL of antihuman P2Y12 antibody conjugated with PE fluorochrome (BioLegend, San Diego, CA, USA) and 4 µL of antihuman CD61 antibody conjugated with PerCP-Cy5.5 (BD Biosciences, San Jose, CA, USA), and incubated in darkness for 30 min. Furthermore, 20 µL of 1% CellFix (Beckton Dickinson, San Diego, CA, USA) was added and incubated for 1 h at 37 °C. To reduce the background glow in the confocal microscope, samples were centrifuged (5000 rpm, 5 min, 24 °C) and the supernatant was discarded. Samples were suspended in 30 µL of PBS for analysis and deposited on glass slides with CyGEL™ (Biostatus, Leicestershire, UK) to immobilize platelets before imaging. For microscopic imaging, the confocal laser scanning microscopy platform TVS SP8 (Leica Microsystems, Wetzlar, Germany) with the objective 100×/1.40 (HC APO CS2, Leica Microsystems, Wetzlar, Germany) was used. Samples were imaged with the following wavelength values of excitation and emission: 489 and 500–550 nm for PE; 565 and 540–620 nm for PerCP-Cy5.5. Leica Application Suite X (LAS X, Leica Microsystems, Wetzlar, Germany) was used for cell imaging. Visualization of the P2Y12 receptor was performed in the Laboratory of Microscopic Imaging and Specialized Biological Techniques in the Faculty of Biology and Environmental Protection at the University of Lodz.

2.10. Statistical Analysis

mRNA and miRNA expressions were presented as $-\Delta Ct$, whereas protein concentrations were log-transformed to bring their distribution closer to normal. The difference in miRNA, mRNA expression, and protein concentration between ACS patients and healthy controls was evaluated with the use of Student's *t*-test or, in case of unequal variance, Welch's *t*-test. The correlation between miRNA, mRNA, and protein expression/concentration was tested with general linear modeling techniques. To assess the relevance of dimensionality reduction between the measurands of interest, Kaiser–Meyer–Olkin (KMO) statistics and the Bartlett's $\chi 2$ test were used. The model to differentiate ACS patients from healthy controls was built with the use of multivariate logistic regression and was internally validated with a 10-fold cross-validation procedure. The model was further illustrated with the receiver operating characteristic (ROC) curve (data included in the Supplementary Material Table S1 and Figure S1). *p*-values below 0.05 were considered statistically significant. The analysis was performed using STATISTICA 13.3 Software (StatSoft; Tulsa, OK, USA).

3. Results

3.1. Gene Expression Analysis of Selected Platelet Surface Receptors

In our comparative analysis, we demonstrated a significant increase in the mRNA levels of P2Y12, GP1BB, ITGA2B, and ITGB3 genes ($p < 0.0001$) in the ACS group, compared with healthy volunteers. For the statistically significant results, alterations are presented as a fold change with 95% CI. For the abovementioned genes, the expression was a 2.44-fold (1.73–3.42), 2.93-fold (2.04–4.22), 3.77-fold (2.77–5.14), and 3.43-fold (2.56–4.59) increase in the ACS group compared with healthy donors, respectively (Figure 1). The expression at

the mRNA level of F2R was not statistically significant; however, the upregulation was shifted towards the ACS group.

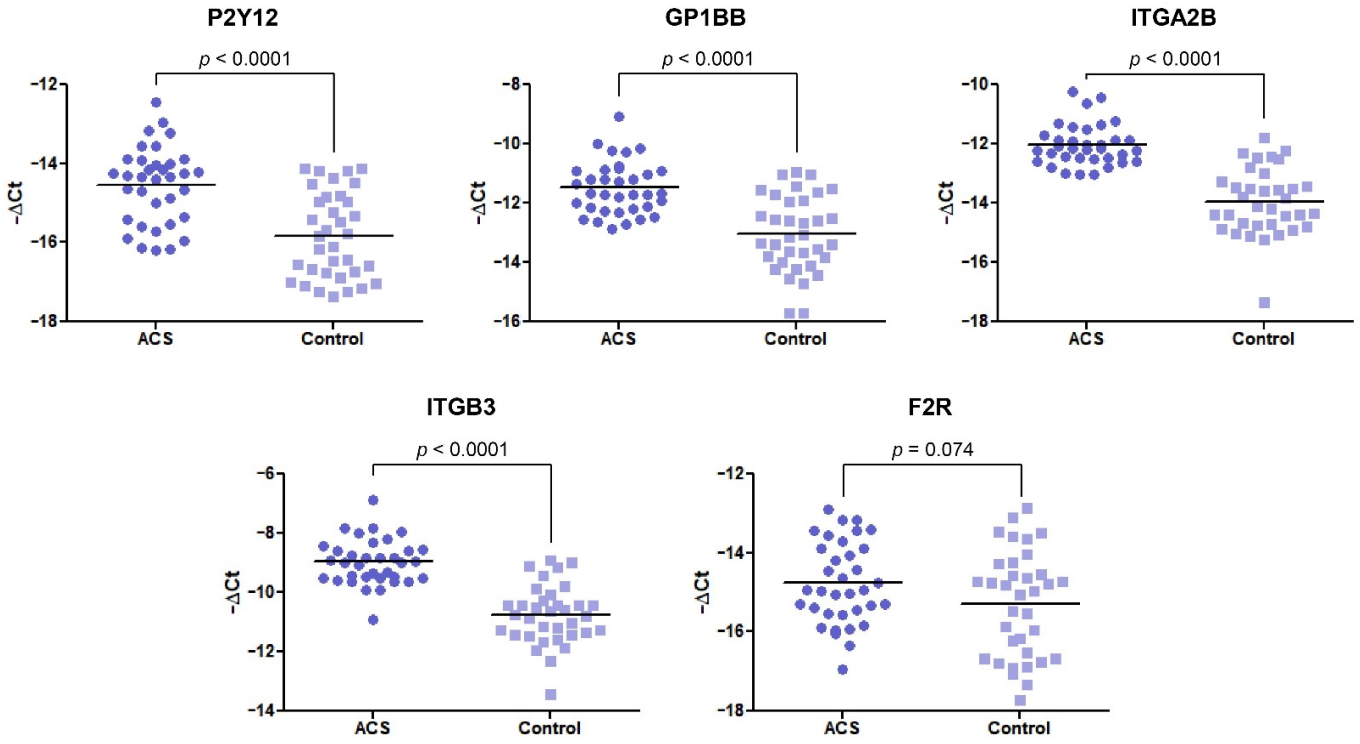


Figure 1. The mRNA expression levels of P2Y12, GP1BB, ITGA2B, ITGB3, and F2R in platelets of ACS patients compared with the control group. The relative expression of the selected genes was illustrated with −ΔCt value. 18S rRNA was used as a reference gene. Data are plotted as individual values with horizontal bars presenting the mean. The *p*-values reported above the graphs represent the results of the Student's *t*-test, or, in cases of unequal variance, Welch's *t*-test. F2R was not statistically significant with $p = 0.074$.

3.2. Determination of Protein Concentration by ELISA

Based on the results obtained from the expression analysis of genes encoding platelet surface receptors, we selected 3 proteins—P2Y12, GPIIIa, and GPIb—for concentration measurement in the study and control group. We decided to determine the concentration of only one GPIIb/IIIa subunit. The GPIIIa remains in molar equilibrium with the GPIIb subunit and its amount is the determinant of the creation of functional GPIIb/IIIa.

Our comparative analysis showed an increased concentration of P2Y12 and GPIIIa in blood platelet lysates of ACS patients in comparison with healthy donors, with fold-changes (95%CI) = 1.78 (1.49–2.11) and 1.61 (1.34–1.95), respectively. The median value (with 25% and 75% percentile) of concentration for P2Y12 were 149 ng/mL (108.1–200.9) in ACS patients compared with 82.66 ng/mL (67.20–105.3) in the control group ($p < 0.0001$). The concentration of GPIIIa in ACS patients was 843.5 pg/mL (676.8–1134) compared with 592.6 pg/mL (403.3–732.7) in the control group ($p < 0.0001$). The concentration of GPIb did not show any significant differences between studied groups (ACS patients, 75.29 ng/mL (51.09–117.3); control group, 65.46 ng/mL (49.75–111.1); $p > 0.99$) (Figure 2). Despite the significant results of the two major platelet receptors, our further attention was focused on the P2Y12 receptor due to its primary role in the antiplatelet therapy assessed by actual ESC guidelines [6].

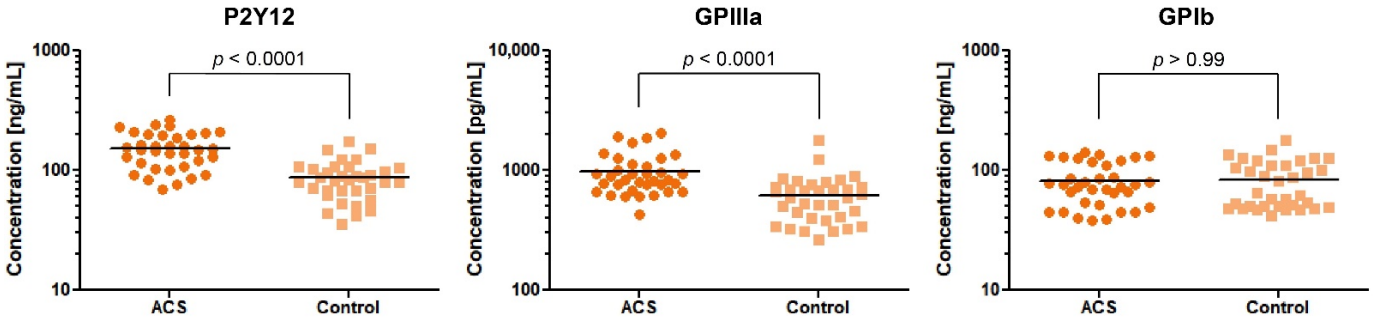


Figure 2. Levels of P2Y12, GPIIIa, and GPIb proteins in blood platelet lysates from ACS patients compared with the control group. Data are plotted as individual values with horizontal bars presenting the mean concentration. The *p*-values reported above the graphs represent the results of *t*-tests following log-transformation.

3.3. Platelet Surface Expression of P2Y12 by Flow Cytometry

The expression level of surface P2Y12 in nonstimulated samples from ACS patients was significantly higher compared with the control group. The mean percentage of P2Y12 in CD61-positive, nonstimulated objects for the ACS group was approximately 18% (±2.95) vs. 8% (±1.26) in the control group (Figure 3).

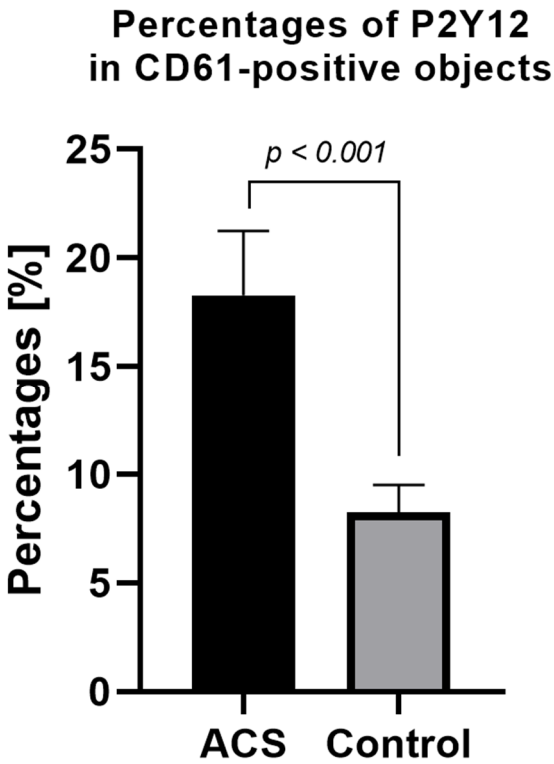


Figure 3. Platelet surface P2Y12 expression measured by flow cytometry method using PE Anti-Human P2Y12. Results presented in the figure show the percentage of P2Y12-positive targets in the population of CD61-positive subjects. Data are presented as a column bar graph with mean ± SD.

3.4. Visualization of Blood Platelets P2Y12 Receptor

Blood platelet samples from ACS patients and the control group were also analyzed by confocal microscopy using fluorescent-labeled antibodies. In the obtained images, the stronger fluorochrome emission for P2Y12 can be observed in ACS patients (Figure 4). Furthermore, receptors showed the tendency to be formed in clusters on the surface of blood platelets.

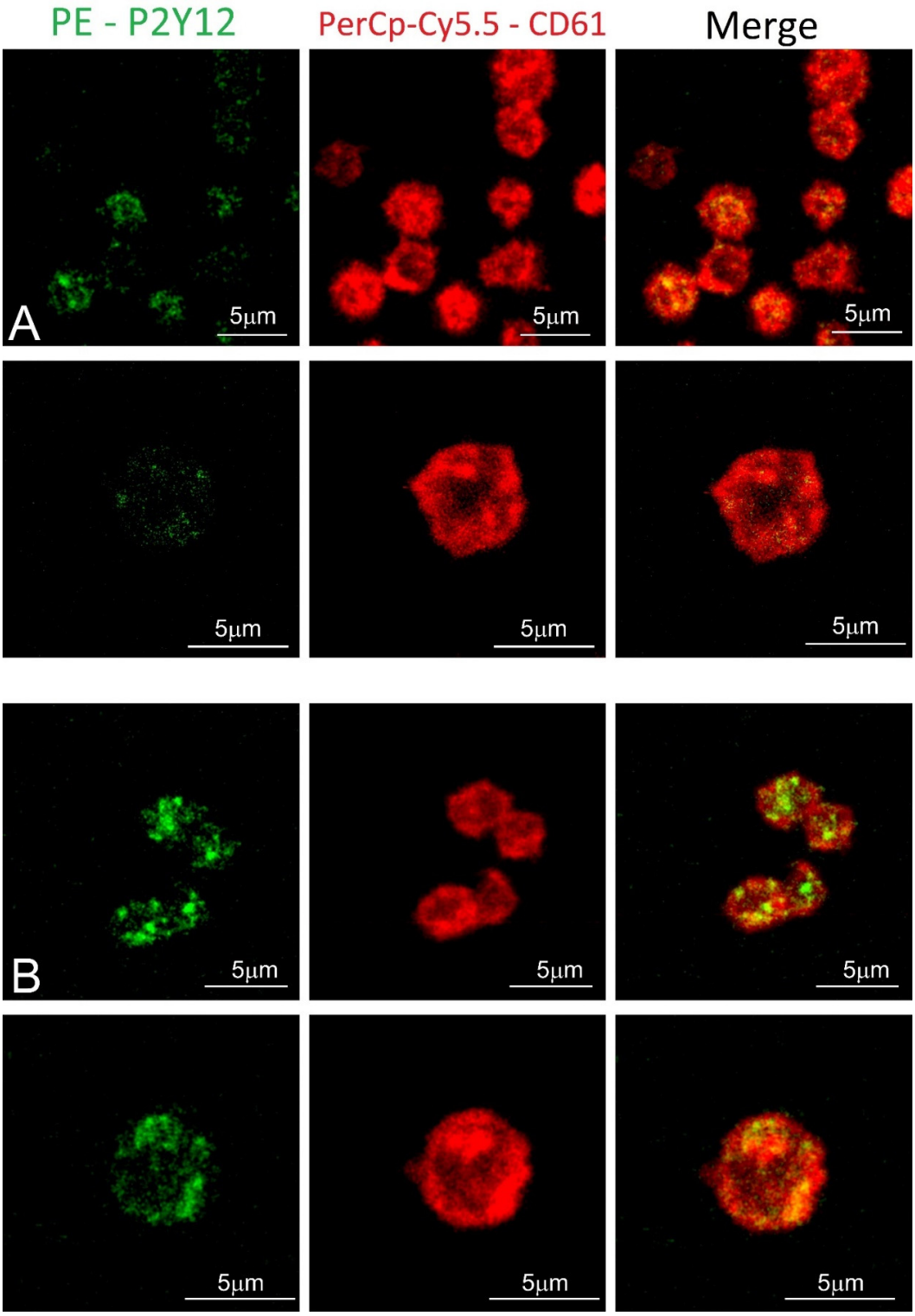


Figure 4. Confocal microscopy of fixed platelets. Samples were labeled with the platelet marker CD61-PerCP-Cy5.5 and with P2Y12-PE. Control and study platelets were deposited on the glass slides with CyGEL™. (**A**) blood platelets obtained from control donor; (**B**) blood platelets obtained from ACS patient.

3.5. Elevated Expression of miR-223-3p and miR-126-3p in Blood Platelets of ACS Patients

Data obtained from the RT-qPCR showed significant alteration in the expression of miR-223-3p. We found that miR-223-3p was 3.48-fold (2.28–5.33) increased in ACS patients

vs. healthy volunteers. In the case of miR-126-3p, we did not observe any statistical differences (Figure 5).

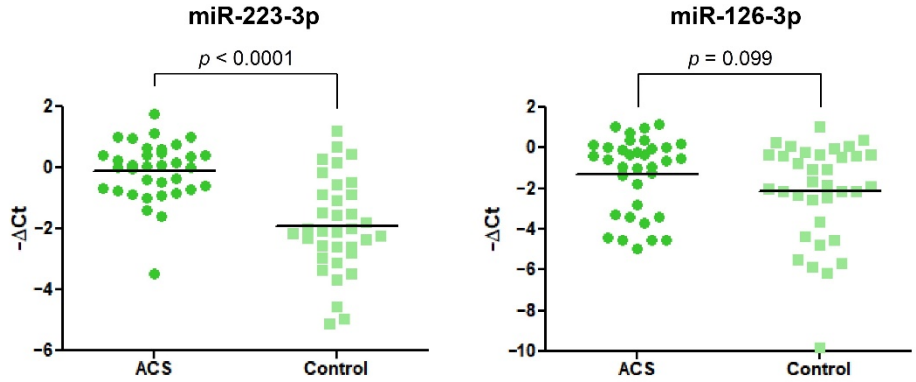


Figure 5. The relative expression of miR-223-3p and miR-126-3p in blood platelets of the study and control group. Data were calculated using a −ΔCt value with the means of both miR-191-3p and cel-miR-39-3p as reference miRNAs. Data are plotted as individual values with horizontal bars presenting the mean. The *p*-values reported above the graphs represent the results of Student's *t*-tests or, in cases of unequal variance, Welch's *t*-test.

3.6. Protein-mRNA-miRNA Correlation in ACS Patients and Healthy Controls

The analysis of correlations between the statistically significant upregulated expressions of miR-223-3p, P2Y12 mRNA, and P2Y12 proteins was performed using a general linear modeling technique by adjusting for the group (ACS patient vs. healthy control), to account for its possibly confounding effect on the correlation. In this analysis, none of the pairs of measurands were significantly correlated: β = 0.05 (95%CI −0.20 to 0.30), $p = 0.68$ for miR-223 and P2Y12 mRNA pair; β = −0.05 (95%CI −0.28 to 0.18), $p = 0.66$ for miR-223 and P2Y12 protein pair; β = 0.10 (95%CI −0.13 to 0.32), $p = 0.38$ for P2Y12 mRNA and P2Y12 protein pair. Further, the group did not significantly differentiate the extent of any of these correlations as assessed in the interaction models: $p = 0.50$, $p = 0.48$, and $p = 0.92$ for miR-223 and P2Y12 pair, miR-223 and P2Y12 pair, and P2YR12 and P2Y12 pair, respectively. As a result, no dimensionality reduction between the tested measurands was warranted, as further confirmed by the KMO statistics of 0.490 and the Bartlett's $\chi 2$ test result: $\chi 2(3) = 0.87$, $p = 0.83$. On the other hand, miR-223, P2Y12, and P2Y12 proteins could be validly included in the model construction to differentiate ACS patients from healthy controls.

4. Discussion

Human blood platelets are small, anucleate cells with a discoidal shape that freely circulate in blood vessels [7]. Their ultrastructure provides unique functional and behavioral properties. As a fragment of larger, precursor cells–megakaryocytes, blood platelets are surrounded by a phospholipid bilayer in which a massive number of surface receptors are anchored. The arrangement of phospholipids present in the inner layer is asymmetrical, thus providing the stability of the cell membrane. Surface receptors are mainly responsible for the activation of blood platelets on various pathways and for triggering the release of bioactive molecules contained in their α- and dense granules. Unleashed factors maintain not only the coagulation process but also inflammation, atherosclerosis, angiogenesis, tumorigenesis, bacterial interaction, and wound repair [4,8]. Due to the increased state of knowledge, suggesting that platelet dysfunction and related genetic disorders can have a decisive importance in ischemic events, we carried out a comparative analysis of gene expression and protein concentration of blood platelet surface receptors.

In the first stage, the expression of the selected platelet surface receptors on the mRNA level was evaluated. Results from RT-qPCR showed that P2Y12, GP1BB, ITGA2B, and ITGB3 mRNA transcripts were significantly upregulated in ACS patients ($p < 0.0001$) pre-

senting 2.44-fold, 2.93-fold, 3.77-fold, and 3.43-fold elevation, respectively. Besides the fact that individual values and medians indicated the growing tendency in the ACS group, the statistical analysis showed no differences in the relative expression of F2R between studied groups (Figure 1). Furthermore, for receptors showing an increase in relative expression, the measurement of P2Y12, GPIb, and GPIIIa concentration in lysates obtained from blood platelets was performed. Results obtained from ELISA tests showed significant upregulation in the concentration of P2Y12 ($p < 0.0001$) and GPIIIa ($p < 0.0001$) (Figure 2). The augmented expression and concentration of GPIIIa may be associated with the increased synthesis of both GPIIb and GPIIIa subunits in blood platelets. Despite the lack of nuclei, de novo biosynthesis of proteins in human platelets has been confirmed in several studies [9–12]. Kieffer et al. [13], using radioactive labels, showed that circulating platelets are able to synthesize major membrane glycoproteins, including GPIIb and IIIa. The increased generation and presence of GP IIb/IIIa receptors may have a great impact on the hyperresponsiveness of blood platelets in ACS. In a study conducted by Yakushkin et al., the increased expression of GP IIb/IIIa was associated with a higher aggregation response [14], emphasizing that excessive exhibition of GP IIb/IIIa receptors for fibrinogen is an important factor of exaggerated aggregation of blood platelets that intensifies the process of thrombosis. The screening analysis of GP- IIb/IIIa and its subunits concentration could help in identifying a human predisposition to developing thromboembolic complications.

Further analysis performed in our study was focused on the P2Y12 receptor, especially because of its very important role in the pharmacotherapy of ACS patients. P2Y12 is a purinergic receptor for ADP that belongs to G-protein-coupled receptors (GPCRs) family. Its major role in human platelets is to inhibit the adenylyl cyclase, which is a key feature in the enhancement of platelet activation and stabilization of forming aggregates [15]. In a study conducted by Hu et al., the expression of human platelet P2Y12 on the mRNA and protein level was 4-fold higher in Type 2 Diabetes Mellitus (T2DM) than in healthy donors [16]. Obtained results were confirmed in the rats model that developed T2DM spontaneously in comparison with wild, Wistar rats. Furthermore, the authors did not observe statistically significant differences in F2R mRNA level either [16]. The statistically significant upregulation of mRNA and protein levels of P2Y12 were also observed in patients with secondary-progressive multiple sclerosis, which presents an increased risk of ischemic events such as stroke or myocardial infarction [17]. Unfortunately, there is a lack of studies presenting differences in protein concentration or gene expression of platelet P2Y12 in cardiovascular diseases. The possible explanation for the increased mRNAs expression may be associated with the role of splicing in blood platelets. Studies show that the transformation of blood platelets from the quiescent to the active state may stimulate the processing of the pre-mRNAs found in platelets into mature mRNAs via the functional spliceosome [18,19]. This phenomenon could explain the increased expression of mRNAs and proteins in the ACS group. However, as demonstrated by Nassa et al., in the case of P2Y12 receptor and GP IIb/IIIa subunits, there are no differences in expression depending on the state of the platelets (quiescent or activated), which may suggest that splicing does not participate in the mechanism of P2Y12 overexpression [20]. However, the lack of experimental data confirming the assumed hypothesis should be considered a limitation in our manuscript, and the explanation of this mechanism requires further research.

To confirm the augmented surface expression of P2Y12 in resting platelets, we decided to use confocal microscopy visualization. In the obtained images, stronger emission of P2Y12 PE fluorochrome can be observed in the ACS group. Interestingly, results showed that P2Y12 in the ACS group is more frequently observed in large clusters than being distracted in the cell membrane (Figure 4); however, the understanding of this phenomenon remains unclear. A possible explanation may be associated with the protein synthesis capacity of platelets, especially in the presence of an increased amount of transcripts; nonetheless, it needs further, detailed analysis. Results obtained from confocal microscopy were also confirmed in the flow cytometry, which, on the basis of fluorescent intensities, showed a significant increase in the surface expression of P2Y12 in ACS patients (Figure 3). Our data

for the first time demonstrate the alteration in the expression of platelet surface receptors on the mRNA and protein level in patients with ACS compared with healthy controls.

To evaluate the potential mechanism associated with an increased quantity of mRNA transcripts for P2Y12, we decided to measure the expression of two miRNAs (miR-223-3p and miR-126-3p), which were shown to be associated with the P2Y12 receptor. Both miR-223 and -126 are some of the most abundant miRNAs stored in blood platelets [21]. In a study conducted by Landry et al., miR-223 was shown to regulate the expression of P2Y12 in the megakaryocytes, thus suggesting a concept in which miR-223 may regulate the expression of P2Y12 in blood platelets [22]. Our results showed that miR-223-3p was significantly upregulated in ACS patients compared with healthy controls (Figure 5). Similar results were shown in a study conducted by Li et al., where the level of platelet miR-223 was significantly elevated in STEMI patients compared with healthy controls [23]. Furthermore, Hromadka et al. demonstrated that the level of miR-223 in whole blood samples of Acute Myocardial Infarction (AMI) patients was significantly elevated [24]. Upregulation of miR-223 was also shown in the serum of AMI patients compared with healthy donors [25]. These findings are important due to the fact that miR-223 was shown to be exclusive for blood platelets [26]. However, there are also studies staying contrary to our and the abovementioned results. In studies conducted by Shi et al. and Zhang et al., the level of platelet and serum miR-223-3p was shown to be significantly decreased in post-myocardial infarction patients that received clopidogrel and presented a low response to used medicament, suggesting the potential role of miR-223 as a biomarker for pharmacotherapy monitoring [27,28]. These findings suggest that downregulation of miR-223 may be associated with the reduced post-transcriptional regulation of P2Y12 expression or be involved in the mechanisms of clopidogrel resistivity. In contrast, Leireseder et al. showed that miR-223-deficient mice did not differ in terms of platelet number, lifespan, volume, and level of platelet surface receptors from wild-type mice, suggesting that miR-223 did not affect the functionality of P2Y12 and is not associated with the ADP-induced aggregation. However, their theory may be controversial since bioinformatic analysis did not show a specific binding site for miR-223 in mice platelets [29]. Unfortunately, there is no clear explanation of simultaneous overexpression of P2Y12 and miR-223. Statistical analysis showed no correlation between expression of miR-223-3p, P2Y12 mRNA transcript, and P2Y12 protein concentration, which may suggest that miR-223-3p is not the key regulator of P2Y12 expression and finding the possible molecular mechanisms of action requires further detailed studies. However, a possible explanation for the parallel increase in the expression of miR-223-3p and P2Y12 may be the organism's reaction to the appearance of a pathological condition, which, in order to restore homeostasis and reduce the expression of an excessively stimulated platelet receptor, increases the concentration of associated miRNA. Furthermore, blood samples of patients enrolled in our study were collected relatively fast after ACS incidence, where the effect of antiplatelet therapy may not be reflected on the platelet miRNA profile. In studies presenting downregulation of miR-223 in blood platelets, patients received antiplatelet agents from 1 to 5 days before blood collection, which might trigger significant changes at the molecular level of platelets and megakaryocytes. Furthermore, the results demonstrated by Shi et al. and Zhang et al. were not compared with healthy controls [27,28].

Our study also showed no significant alteration in the level of platelet miR-126-3p in ACS patients compared with healthy volunteers (Figure 5). In the current state of the art, there is great controversy regarding miR-126-3p expression. Several studies showed that circulating miR-126 is downregulated in patients with cardiovascular diseases in comparison with control subjects [30–32]. Liu et al. showed that the expression of plasma miR-126 is decreased in ACS patients and its level is associated with platelet reactivity during clopidogrel treatment [33]. On the other hand, Zampetaki et al. showed that the level of miR-126 was positively correlated with the incident of myocardial infarction [26]. Furthermore, Kazimierczyk et al. demonstrated that AMI patients with percutaneous coronary intervention also had elevated levels of circulating miR-126 [34]. The potential

mechanism of miR-126 and P2Y12 receptor association was studied by Kaudewitz et al., where the application of antagomir-126-3p in a mice model caused the reduced expression of P2Y12 [35]. In a physiological aspect of this model, a reduced amount of miR-126-3p will be associated with the reduced expression of P2Y12. Taking into consideration the opposite direction of alteration in our study (despite the lack of statistical significance), increased expression of miR-126-3p could be linked with increased expression of P2Y12 at the mRNA level, which was shown in our study. In a study conducted by Garcia et al., the functionality of miR-126-3p was assessed on a platelet-like structure (PLS) derived from human hematopoietic stem cells. Results showed that PLS transfected with miR-126-3p had a 30% increased expression of P-selectin after thrombin stimulation. Furthermore, overexpression of miR-126-3p resulted in the downregulation of PLXBN2 expression, thus affecting RhoGTPase activity and actin dynamics regulation, demonstrating that overexpression of miR-126-3p may be associated with the increased reactivity of blood platelets [36]. Discrepancies in the results of cited studies may arise from the time of miRNAs expression measurements and time of blood sample collection after the ACS incident. Results mainly indicate that the downregulation of miR-126 expression is caused by antiplatelet therapy. Furthermore, there is a lack of studies that measure the level of miR-126-3p in blood platelets of patients with cardiovascular diseases. Moreover, it is suggested that miR-126 mainly originates in endothelial cells [37]; thus, its level in serum may not reflect the augmented expression in blood platelets. Altogether, miR-126-3p seems to be not very specific for differentiating ACS patients from healthy controls, and its possible role as a biomarker should be tested on the larger population.

Our study aimed to indicate the important role of the molecular basis underlying the observed functional changes in platelet surface receptors, especially P2Y12, in the pathogenesis of myocardial ischemia, with a possible finding of the potential biomarker. In a vast number of studies, the role of platelet surface receptors is examined from the perspective of novel antiplatelet drug introduction in clinical usage. However, there is a gap in molecular studies of changes in surface receptors, which may lead to the development of thrombosis. The possible hypothesis that could explain the hyperreactivity of platelets associated with the augmented expression of the P2Y12 receptor concerns the constitutive activity of GPCRs. The two-state model establishes that GPCRs existing in the active or inactive state remain in equilibrium. However, in particular conditions, the effector system and G-protein activity may be upregulated and cause spontaneous isomerization of GPCRs, thus changing from the inactive to the active state, in the absence of agonists [38]. In a study conducted by Zhang et al., transgenic mice expressing constitutively active chimeric P2Y12 showed an increased reactivity of blood platelets, shortened bleeding time, faster and more stable formation of thrombus, decreased level of platelet cyclic adenosine monophosphate (cAMP), and constitutive phosphorylation of Akt without agonists [39]. The constitutive activity of GPCRs with the simultaneous elevation of P2Y12 concentration may be the main factor of hyperreactivity of blood platelets in ACS patients, and early detection of this disorder could help in the identification of human predisposition to ischemic events.

5. Conclusions

To conclude, our manuscript presents an attempt to determine the molecular alterations of platelet surface receptors in ACS patients compared with healthy controls, by assessing changes in the expression level of transcripts, protein concentrations, as well as visualizing the arrangement of the platelet surface receptor P2Y12 by confocal microscopy. RTq-PCR analysis showed that mRNAs of P2Y12, GP1BB, ITGA2B, and ITGB3 were overexpressed in the ACS group, such as miR-223-3p. Furthermore, augmented protein concentration from blood platelet lysates was also found for P2Y12 and GPIIIa in ACS patients. What is more, confocal microscopy and flow cytometry show an elevated surface expression of P2Y12 in blood platelets of ACS patients in comparison with healthy donors. The results of our study may suggest the possible role of P2Y12 overexpression in the increased activity of blood platelets and in the pathogenesis of ACS; however, to fully

understand this complex association between the overstimulated platelets and the level of their surface receptors, further molecular studies on larger populations are required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology11050644/s1, Figure S1: Receiver operating characteristic curve for the multivariate logistic regression model differentiating ACS patients from healthy controls; Table S1: Characteristics of the logistic regression model differentiating ACS patients from healthy controls based on miR-223-3p as well as mRNA and protein levels of P2Y12.

Author Contributions: Conceptualization, R.S., M.S.K., M.B. and J.S.-B.; methodology, R.S., M.S.K., M.B., J.S.-B. and S.M.; formal analysis, R.S., M.S.K., M.B. and J.S.-B.; investigation, R.S., E.S. and S.M.; resources, M.K. and M.Z.; data curation, R.S.; writing—original draft preparation, R.S., M.S.K. and J.S.-B.; writing—review and editing, R.S., M.S.K. and J.S.-B.; visualization, R.S., M.S.K., E.S. and S.M.; supervision, M.B., M.K., M.Z., A.O. and J.S.-B.; project administration, R.S., M.B., M.K. and J.S.-B.; funding acquisition, R.S., M.B. and J.S.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Education and Science, grant number DI2016 022046.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of the University of Lodz, Poland, with Resolution No.23/KBNN-UŁ/I/2017. All participants enrolled in the study provided written informed consent.

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

Data Availability Statement: All data obtained from this study are included in the manuscript.

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

References

1. Smith, J.N.; Negrelli, J.M.; Manek, M.B.; Hawes, E.M.; Viera, A.J. Diagnosis and Management of Acute Coronary Syndrome: An Evidence-Based Update. *J. Am. Board Fam. Med.* **2015**, *28*, 283–293. [CrossRef] [PubMed]
2. Stakos, D.A.; Tziakas, D.N.; Stellos, K. Mechanisms of Platelet Activation in Acute Coronary Syndromes. *Curr. Vasc. Pharm.* **2012**, *10*, 578–588. [CrossRef] [PubMed]
3. Rivera, J.; Lozano, M.L.; Navarro-Núñez, L.; Vicente, V. Platelet Receptors and Signaling in the Dynamics of Thrombus Formation. *Haematologica* **2009**, *94*, 700–711. [CrossRef] [PubMed]
4. Wachowicz, B.; Morel, A.; Miller, E.; Saluk, J. The Physiology of Blood Platelets and Changes of Their Biological Activities in Multiple Sclerosis. *Acta Neurobiol. Exp.* **2017**, *76*, 269–281. [CrossRef]
5. Saboor, M.; Ayub, Q.; Samina Ilyas, M. Platelet Receptors: An Instrumental of Platelet Physiology. *Pak. J. Med. Sci.* **2013**, *29*, 891–896. [CrossRef]
6. Collet, J.-P.; Thiele, H.; Barbato, E.; Barthélémy, O.; Bauersachs, J.; Bhatt, D.L.; Dendale, P.; Dorobantu, M.; Edvardsen, T.; Folliguet, T.; et al. 2020 ESC Guidelines for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation. *Eur. Heart J.* **2021**, *42*, 1289–1367. [CrossRef]
7. Thon, J.N.; Italiano, J.E. Platelets: Production, Morphology and Ultrastructure. *Handb. Exp. Pharm.* **2012**, *210*, 3–22. [CrossRef]
8. Ghoshal, K.; Bhattacharyya, M. Overview of Platelet Physiology: Its Hemostatic and Nonhemostatic Role in Disease Pathogenesis. *Sci. World J.* **2014**, *2014*, 781857. [CrossRef]
9. Booyse, F.; Rafelson, M.E. In Vitro Incorporation of Amino-Acids into the Contractile Protein of Human Blood Platelets. *Nature* **1967**, *215*, 283–284. [CrossRef]
10. Lindemann, S.; Tolley, N.D.; Dixon, D.A.; McIntyre, T.M.; Prescott, S.M.; Zimmerman, G.A.; Weyrich, A.S. Activated Platelets Mediate Inflammatory Signaling by Regulated Interleukin 1β Synthesis. *J. Cell Biol.* **2001**, *154*, 485–490. [CrossRef]
11. Weyrich, A.S.; Dixon, D.A.; Pabla, R.; Elstad, M.R.; McIntyre, T.M.; Prescott, S.M.; Zimmerman, G.A. Signal-Dependent Translation of a Regulatory Protein, Bcl-3, in Activated Human Platelets. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5556–5561. [CrossRef] [PubMed]
12. Brogren, H.; Karlsson, L.; Andersson, M.; Wang, L.; Erlinge, D.; Jern, S. Platelets Synthesize Large Amounts of Active Plasminogen Activator Inhibitor 1. *Blood* **2004**, *104*, 3943–3948. [CrossRef] [PubMed]
13. Kieffer, N.; Guichard, J.; Farcet, J.P.; Vainchenker, W.; Breton-Gorius, J. Biosynthesis of Major Platelet Proteins in Human Blood Platelets. *Eur. J. Biochem.* **1987**, *164*, 189–195. [CrossRef] [PubMed]
14. Yakushkin, V.V.; Zyuryaev, I.T.; Khaspekova, S.G.; Sirotkina, O.V.; Ruda, M.Y.A.; Mazurov, A.V. Glycoprotein IIb-IIIa Content and Platelet Aggregation in Healthy Volunteers and Patients with Acute Coronary Syndrome. *Platelets* **2011**, *22*, 243–251. [CrossRef] [PubMed]
15. Cattaneo, M. P2Y12 Receptors: Structure and Function. *J. Thromb. Haemost.* **2015**, *13*, S10–S16. [CrossRef]

16. Hu, L.; Chang, L.; Zhang, Y.; Zhai, L.; Zhang, S.; Qi, Z.; Yan, H.; Yan, Y.; Luo, X.; Zhang, S.; et al. Platelets Express Activated P2Y12 Receptor in Patients with Diabetes Mellitus. *Circulation* **2017**, *136*, 817–833. [CrossRef]
17. Dziedzic, A.; Miller, E.; Saluk-Bijak, J.; Niwald, M.; Bijak, M. The Molecular Aspects of Disturbed Platelet Activation through ADP/P2Y12 Pathway in Multiple Sclerosis. *Int. J. Mol. Sci.* **2021**, *22*, 6572. [CrossRef]
18. Denis, M.M.; Tolley, N.D.; Bunting, M.; Schwertz, H.; Jiang, H.; Lindemann, S.; Yost, C.C.; Rubner, F.J.; Albertine, K.H.; Swoboda, K.J.; et al. Escaping the Nuclear Confines: Signal-Dependent Pre-MRNA Splicing in Anucleate Platelets. *Cell* **2005**, *122*, 379–391. [CrossRef]
19. Schwertz, H.; Tolley, N.D.; Foulks, J.M.; Denis, M.M.; Risenmay, B.W.; Buerke, M.; Tilley, R.E.; Rondina, M.T.; Harris, E.M.; Kraiss, L.W.; et al. Signal-Dependent Splicing of Tissue Factor Pre-MRNA Modulates the Thrombogenecity of Human Platelets. *J. Exp. Med.* **2006**, *203*, 2433–2440. [CrossRef]
20. Nassa, G.; Giurato, G.; Cimmino, G.; Rizzo, F.; Ravo, M.; Salvati, A.; Nyman, T.A.; Zhu, Y.; Vesterlund, M.; Lehtiö, J.; et al. Splicing of Platelet Resident Pre-MRNAs upon Activation by Physiological Stimuli Results in Functionally Relevant Proteome Modifications. *Sci. Rep.* **2018**, *8*, 498. [CrossRef]
21. Nagalla, S.; Shaw, C.; Kong, X.; Kondkar, A.A.; Edelstein, L.C.; Ma, L.; Chen, J.; McKnight, G.S.; López, J.A.; Yang, L.; et al. Platelet MicroRNA-MRNA Coexpression Profiles Correlate with Platelet Reactivity. *Blood* **2011**, *117*, 5189–5197. [CrossRef] [PubMed]
22. Landry, P.; Plante, I.; Ouellet, D.L.; Perron, M.P.; Rousseau, G.; Provost, P. Existence of a MicroRNA Pathway in Anucleate Platelets. *Nat. Struct. Mol. Biol.* **2009**, *16*, 961–966. [CrossRef] [PubMed]
23. Li, S.; Guo, L.Z.; Kim, M.H.; Han, J.-Y.; Serebruany, V. Platelet MicroRNA for Predicting Acute Myocardial Infarction. *J. Thromb. Thrombolysis* **2017**, *44*, 556–564. [CrossRef] [PubMed]
24. Hromadka, M.; Motovska, Z.; Hlinomaz, O.; Kala, P.; Tousek, F.; Jarkovsky, J.; Beranova, M.; Jansky, P.; Svoboda, M.; Krepelkova, I.; et al. MiR-126-3p and MiR-223-3p as Biomarkers for Prediction of Thrombotic Risk in Patients with Acute Myocardial Infarction and Primary Angioplasty. *J. Pers. Med.* **2021**, *11*, 508. [CrossRef]
25. Li, C.; Fang, Z.; Jiang, T.; Zhang, Q.; Liu, C.; Zhang, C.; Xiang, Y. Serum MicroRNAs Profile from Genome-Wide Serves as a Fingerprint for Diagnosis of Acute Myocardial Infarction and Angina Pectoris. *BMC Med. Genom.* **2013**, *6*, 16. [CrossRef]
26. Zampetaki, A.; Willeit, P.; Tilling, L.; Drozdov, I.; Prokopi, M.; Renard, J.-M.; Mayr, A.; Weger, S.; Schett, G.; Shah, A.; et al. Prospective Study on Circulating MicroRNAs and Risk of Myocardial Infarction. *J. Am. Coll. Cardiol.* **2012**, *60*, 290–299. [CrossRef]
27. Shi, R.; Ge, L.; Zhou, X.; Ji, W.-J.; Lu, R.-Y.; Zhang, Y.-Y.; Zeng, S.; Liu, X.; Zhao, J.-H.; Zhang, W.-C.; et al. Decreased Platelet MiR-223 Expression Is Associated with High on-Clopidogrel Platelet Reactivity. *Thromb. Res.* **2013**, *131*, 508–513. [CrossRef]
28. Zhang, Y.-Y.; Zhou, X.; Ji, W.-J.; Shi, R.; Lu, R.-Y.; Li, J.-L.; Yang, G.-H.; Luo, T.; Zhang, J.-Q.; Zhao, J.-H.; et al. Decreased Circulating MicroRNA-223 Level Predicts High on-Treatment Platelet Reactivity in Patients with Troponin-Negative Non-ST Elevation Acute Coronary Syndrome. *J. Thromb. Thrombolysis* **2014**, *38*, 65–72. [CrossRef]
29. Leierseder, S.; Petzold, T.; Zhang, L.; Loyer, X.; Massberg, S.; Engelhardt, S. MiR-223 Is Dispensable for Platelet Production and Function in Mice. *Thromb. Haemost.* **2013**, *110*, 1207–1214. [CrossRef]
30. Wang, X.; Lian, Y.; Wen, X.; Guo, J.; Wang, Z.; Jiang, S.; Hu, Y. Expression of MiR-126 and Its Potential Function in Coronary Artery Disease. *Afr. Health Sci.* **2017**, *17*, 474–480. [CrossRef]
31. Long, G.; Wang, F.; Duan, Q.; Chen, F.; Yang, S.; Gong, W.; Wang, Y.; Chen, C.; Wang, D.W. Human Circulating MicroRNA-1 and MicroRNA-126 as Potential Novel Indicators for Acute Myocardial Infarction. *Int. J. Biol. Sci.* **2012**, *8*, 811–818. [CrossRef] [PubMed]
32. Hsu, A.; Chen, S.-J.; Chang, Y.-S.; Chen, H.-C.; Chu, P.-H. Systemic Approach to Identify Serum MicroRNAs as Potential Biomarkers for Acute Myocardial Infarction. *Biomed. Res. Int.* **2014**, *2014*, 418628. [CrossRef] [PubMed]
33. Liu, J.; Qin, L.; Wang, Z.; Peng, L.; Liu, J.; Wang, X.; Du, R.; Zou, Y.; Wu, Y.; Yin, T. Platelet-Derived MiRNAs as Determinants of the Antiplatelet Response in Clopidogrel-Treated Patients with ACS. *Thromb. Res.* **2020**, *186*, 71–74. [CrossRef] [PubMed]
34. Kazimierczyk, E.; Eljaszewicz, A.; Kazimierczyk, R.; Tynecka, M.; Zembko, P.; Tarasiuk, E.; Kaminski, K.; Sobkowicz, B.; Moniuszko, M.; Tycinska, A. Altered MicroRNA Dynamics in Acute Coronary Syndrome. *Adv. Interv. Cardiol.* **2020**, *16*, 287–293. [CrossRef] [PubMed]
35. Kaudewitz, D.; Skroblin, P.; Bender, L.H.; Barwari, T.; Willeit, P.; Pechlaner, R.; Sunderland, N.P.; Willeit, K.; Morton, A.C.; Armstrong, P.C.; et al. Association of MicroRNAs and YRNAs With Platelet Function. *Circ. Res.* **2016**, *118*, 420–432. [CrossRef] [PubMed]
36. Garcia, A.; Dunoyer-Geindre, S.; Zapilko, V.; Nolli, S.; Reny, J.-L.; Fontana, P. Functional Validation of MicroRNA-126-3p as a Platelet Reactivity Regulator Using Human Haematopoietic Stem Cells. *Thromb. Haemost.* **2019**, *119*, 254–263. [CrossRef]
37. Wahl, P.; Wehmeier, U.F.; Jansen, F.J.; Kilian, Y.; Bloch, W.; Werner, N.; Mester, J.; Hilberg, T. Acute Effects of Different Exercise Protocols on the Circulating Vascular MicroRNAs -16, -21, and -126 in Trained Subjects. *Front. Physiol.* **2016**, *7*, 643. [CrossRef]
38. Seifert, R.; Wenzel-Seifert, K. Constitutive Activity of G-Protein-Coupled Receptors: Cause of Disease and Common Property of Wild-Type Receptors. *Naunyn-Schmiedeberg's Arch. Pharm.* **2002**, *366*, 381–416. [CrossRef]
39. Zhang, Y.; Ye, J.; Hu, L.; Zhang, S.; Zhang, S.H.; Li, Y.; Kunapuli, S.P.; Ding, Z. Increased Platelet Activation and Thrombosis in Transgenic Mice Expressing Constitutively Active P2Y12 Receptor. *J. Thromb. Haemost.* **2012**, *10*, 2149–2157. [CrossRef]

biology

MDPI

Supplementary Material S1: Biomarker modeling for differentiating ACS patients from healthy controls

As platelets of ACS patients were found to overexpress miR-223-3p as well as P2Y12 mRNA and P2Y12 protein, and the three measurands were negligibly correlated between each other, all of them could be included as predictors in the model differentiating ACS patients from healthy controls. Such a multivariate logistic model was constructed and internally validated with 10-fold cross-validation. The model appeared accurate and not prone to overfitting as it presented favorable goodness-of-fit statistics and performed well even in the validation set reaching high sensitivity with satisfactory specificity (Table S1, Figure S1). The obtained model reached 97% of sensitivity and 74% of specificity to differentiate ACS patients from donors.

Table S1. Characteristics of the logistic regression model differentiating ACS patients from healthy controls based on miR-223-3p as well as mRNA and protein levels of P2Y12. Likelihood-ratio $\chi2(3) = 59.65$, $p < 0.0001$. Hosmer-Lemeshow goodness-of-fit $\chi2(8) = 2.47$, $p = 0.96$.

Predictor	Wald $\chi2$	Odds ratio [a]		*P*-value
		Point estimate	95% CI	
miR-223-3p	**9.69**	3.52	1.59–7.78	0.0019
P2Y12 mRNA	4.91	3.08	1.14–8.31	0.027
P2Y12 protein	10.44	16.85	3.04–93.53	0.0012

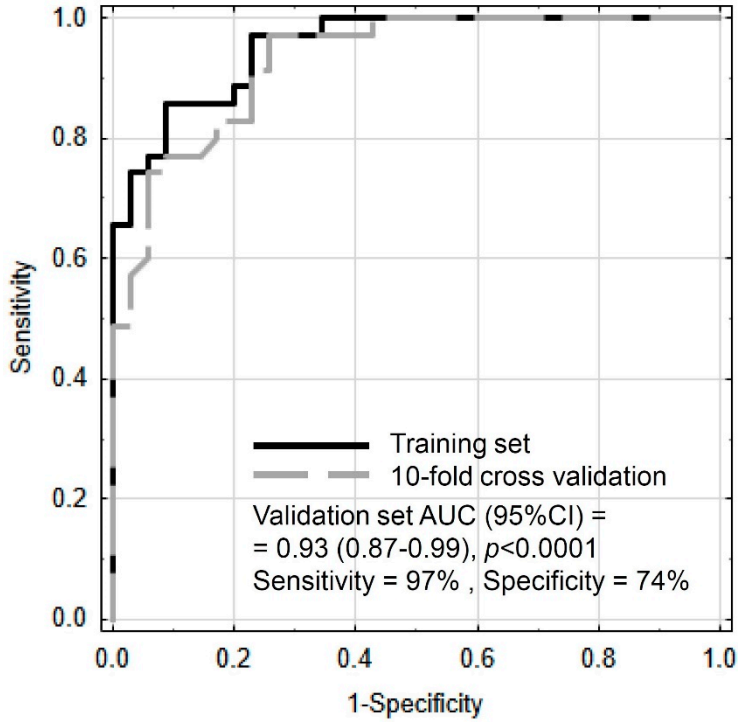


Figure S1. Receiver operating characteristic curve for the multivariate logistic regression model differentiating ACS patients from healthy controls. The model was based on protein and mRNA levels of P2Y12 as well as miR-223-3p. The sensitivity and specificity of the model were proposed based on the maximization of Youden's index. Model internal validation was performed with a 10-fold cross-validation technique.

International Journal of
Molecular Sciences

MDPI

Article

Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients

Rafał Szelenberger [1,2,*], Paweł Jóźwiak [3], Michał Kacprzak [4], Michał Bijak [2], Marzenna Zielińska [4], Alina Olender [5] and Joanna Saluk-Bijak [1]

[1] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; joanna.saluk@biol.uni.lodz.pl
[2] Biohazard Prevention Centre, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; michal.bijak@biol.uni.lodz.pl
[3] Department of Cytobiochemistry, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; pawel.jozwiak@biol.uni.lodz.pl
[4] Department of Interventional Cardiology, Medical University of Lodz, 91-213 Lodz, Poland; michal.kacprzak@umed.lodz.pl (M.K.); marzenna.zielinska@umed.lodz.pl (M.Z.)
[5] Chair and Department of Medical Microbiology, Medical University of Lublin, 20-093 Lublin, Poland; alinaolender@umlub.pl
* Correspondence: rafal.szelenberger@edu.uni.lodz.pl

Abstract: Proteomic analyses based on mass spectrometry provide a powerful tool for the simultaneous identification of proteins and their signatures. Disorders detection at the molecular level delivers an immense impact for a better understanding of the pathogenesis and etiology of various diseases. Acute coronary syndrome (ACS) refers to a group of heart diseases generally associated with rupture of an atherosclerotic plaque and partial or complete thrombotic obstruction of the blood flow in the infarct-related coronary artery. The essential role in the pathogenesis of ACS is related to the abnormal, pathological activation of blood platelets. The multifactorial and complex character of ACS indicates the need to explain the molecular mechanisms responsible for thrombosis. In our study, we performed screening and comparative analysis of platelet proteome from ACS patients and healthy donors. Two-dimensional fluorescence difference gel electrophoresis and nanoscale liquid chromatography coupled to tandem mass spectrometry showed altered expressions of six proteins (i.e., vinculin, transgelin-2, fibrinogen β and γ chains, apolipoprotein a1, and tubulin β), with the overlapping increased expression at the mRNA level for transgelin-2. Dysregulation in protein expression identified in our study may be associated with an increased risk of thrombotic events, correlated with a higher aggregability of blood platelets and induced shape change, thus explaining the phenomenon of the hyperreactivity of blood platelets in ACS.

Keywords: blood platelets; proteome; transcriptome; acute coronary syndrome; transgelin-2

Citation: Szelenberger, R.; Jóźwiak, P.; Kacprzak, M.; Bijak, M.; Zielińska, M.; Olender, A.; Saluk-Bijak, J. Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients. *Int. J. Mol. Sci.* **2022**, *23*, 6340. https://doi.org/10.3390/ijms23116340

Academic Editor: Isabella Russo

Received: 10 May 2022
Accepted: 4 June 2022
Published: 6 June 2022

1. Introduction

Mass spectrometry-based proteomic analysis constitutes a powerful tool that allows parallel identification of proteins from the cells, tissues, and biological fluids. Current applications cover and facilitate the evaluation of alterations in protein expression, structure, and function. A wide range of applications allows for assessing specific protein signatures, novel biomarkers for disease diagnosis, and progression evaluation as well as new drug targets. Modern prognostic diagnostics depend on finding the biochemical, cellular, or genetic alteration by which pathological conditions in the human body can be recognized early and prevent the disease state from occurring. Recent advances in molecular techniques, technologies, and bioinformatics opened new possibilities to study genomics, proteomics, metabolomics, and imaging. These techniques have a tremendous bearing on having a

better understanding of disrupted cellular mechanisms during an ongoing pathological state. The etiology of the disease has a great impact on the selection of proper methodology, which may offer better prognostic, diagnostic, and therapeutic value [1,2].

According to the data presented by the World Health Organization (WHO), acute coronary syndrome (ACS) and stroke constitute 85% of all cardiovascular diseases and are the most common causes of death globally, taking 17.9 million lives each year. Although most cardiovascular diseases can be prevented through appropriate behavior and lifestyle and high morbidity and mortality rates emphasize their variable, unpredictable, nonlinear, and clinically silent character [3].

One of the key roles in the thrombosis and pathogenesis of ACS is played by blood platelets, which are the smallest and anucleate morphotic elements. Under physiological conditions, platelets freely circulate in the bloodstream without stimulating pivotal interactions with the vascular microenvironment. However, in the result of vessel injury, platelets induce the hemostasis process which includes complex interplay among all elements of the circulatory system [4]. In the first stage of primary hemostasis, platelets adhere to the damaged vessel wall and interact with the subendothelial matrix proteins. To ensure the stable contact between platelets and the injured endothelium, via surface receptors, platelets bind with the collagen and von Willebrand Factor (vWF). Initial binding is vouched by the glycoprotein (GP) Ib-IX-V, which recruits blood platelets from circulation, reduces their velocity, and allows them to induce the interaction in multiple directions (i.e., by stimulating interaction with leukocytes, endothelium, and other platelets). Further, platelets bind with damaged endothelium by other surface receptors, including GP VI and GP Ia/IIa, thus enhancing the stability of the mutual connection and initiating platelet activation [5]. The signal transduction from the agonists released by platelets causes terrific changes in their physiology. Stimulated platelets change their shape, form numerous pseudopods, expose more receptors on their surface, and release from the α- and dense granules a vast amount of biologically active molecules that enhance their activation, aggregation, and stimulate the development of inflammation [4]. An ongoing process of platelet activation promotes the conformational changes in the structure of the most potent platelet receptor—GP IIb/IIIa, which causes the formation of fibrinogen bridges between activated platelets. During the aggregation process, the platelet response is continuously amplified to induce the growth of the forming thrombus. The multifactorial hemostatic response caused by excessive activation of blood platelets, in the final result induces the occlusion of the vessels and limits the blood flow, causing thrombotic complications such as ACS [6]. ACS patients were shown to present persistent platelet hyperreactivity [7], which may be strongly associated with the pathological formation of blood clots in coronary arteries. Thus, a better understanding of the potential alterations on the molecular level may be crucial for a faster inclusion of preventive approaches and/or assessing the potential risk of ACS development that could reduce its morbidity and mortality.

Because of the complex, multifactorial character of ACS, the search for potentially important alterations should be performed at various levels to identify possible interactions or mechanisms associated with the ischemia. The role of blood platelets in the pathogenesis of thrombosis is well established; however, their unique properties (i.e., lack of nucleus and transcription process, which significantly limits the pool of presented proteins; easy, non-invasive method to collect samples) show that platelet proteome could be an ideal area for the discovering new targets that could be linked with the ACS development. The main objective of our study was to find and identify, through the proteomic comparative analysis, dysregulated platelet proteins along with their expression at the mRNA level that could provide new insights into the molecular mechanism responsible for hyperreactivity and spontaneous activation of platelets associated with ACS.

2. Results

2.1. Analysis of Fluorescence Intensity in Two-Dimensional Fluorescence Difference Gel Electrophoresis

To determine the differences in platelet proteome between patients diagnosed with ACS and the control group, clinical material was first analyzed using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). To avoid any unexpected mechanical and technical problems, two gels were prepared simultaneously as a separate analysis. Scanned gels for both groups are presented in Figure 1. For each spot, the range ratio value, which corresponded to the differences in fluorescent intensity between the study and control group, was assessed. For spots S1–S6, the range ratios were 3.05223, 1.72152, 1.99851, 2.38866, 2.12995, and 1.94427, respectively. Furthermore, spots S1 and S4 were downregulated, while other spots were upregulated.

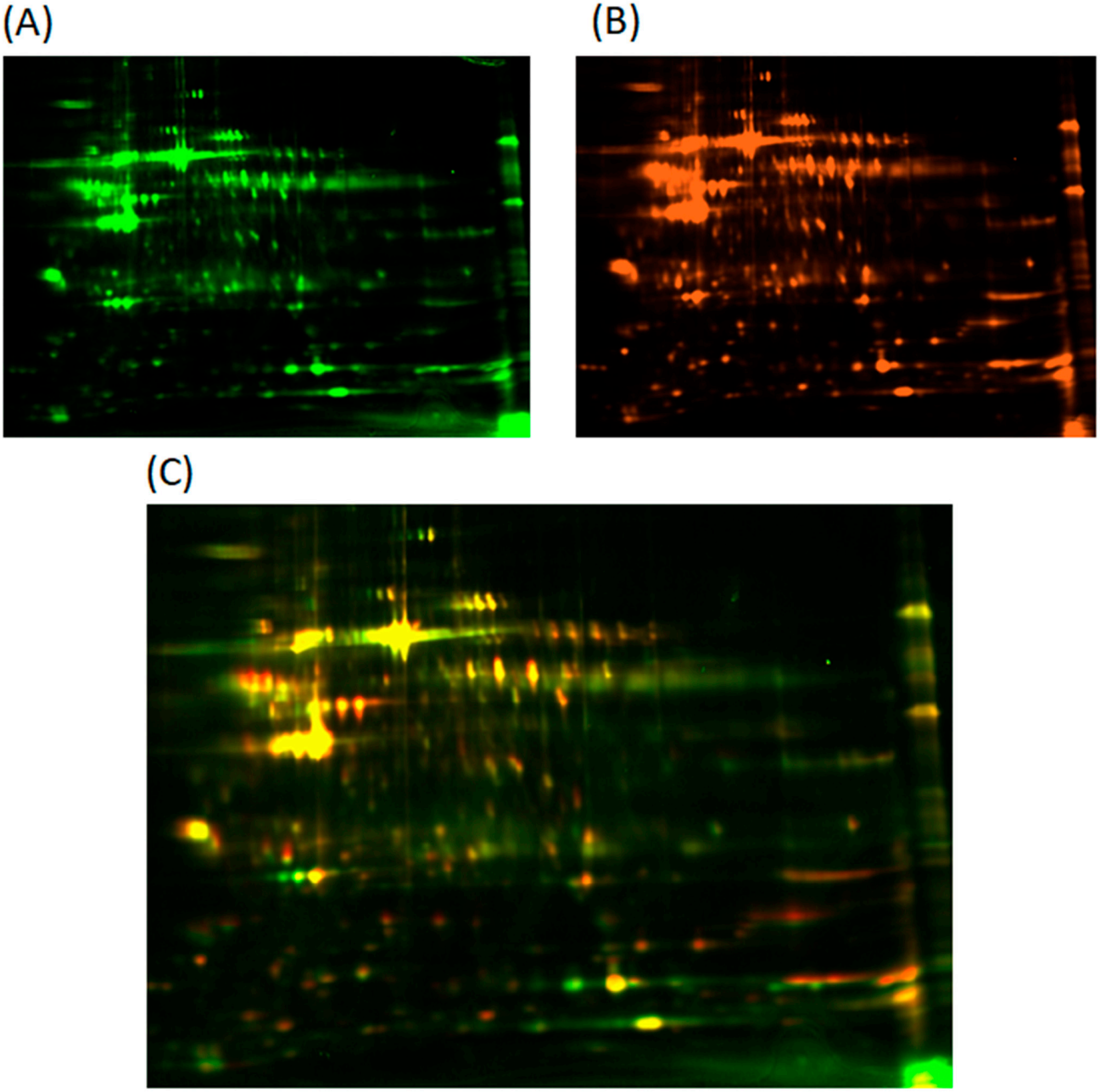


Figure 1. Fluorescently labeled electrophoretic gels of blood platelets proteome obtained from the control group (**A**) and ACS patients (**B**). Merge spots (**C**) represent the comparison between proteomes.

2.2. Identification of Altered Expressed Proteins via Nanoscale Liquid Chromatography Coupled to Tandem Mass Spectrometry

To identify the protein features, nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) was carried out for six (S1–S6) chosen spots. Differentially expressed proteins were divided into four functional groups: actin-binding proteins (S1: vinculin; S5: transgelin-2), cytoskeleton (S3: tubulin β chain), lipid metabolism (S4: apolipoprotein a1), and blood clot formation (S2: fibrinogen β chain; S6: fibrinogen γ chain). Altered expressed proteins are visualized in Figure 2 and listed in Table 1.

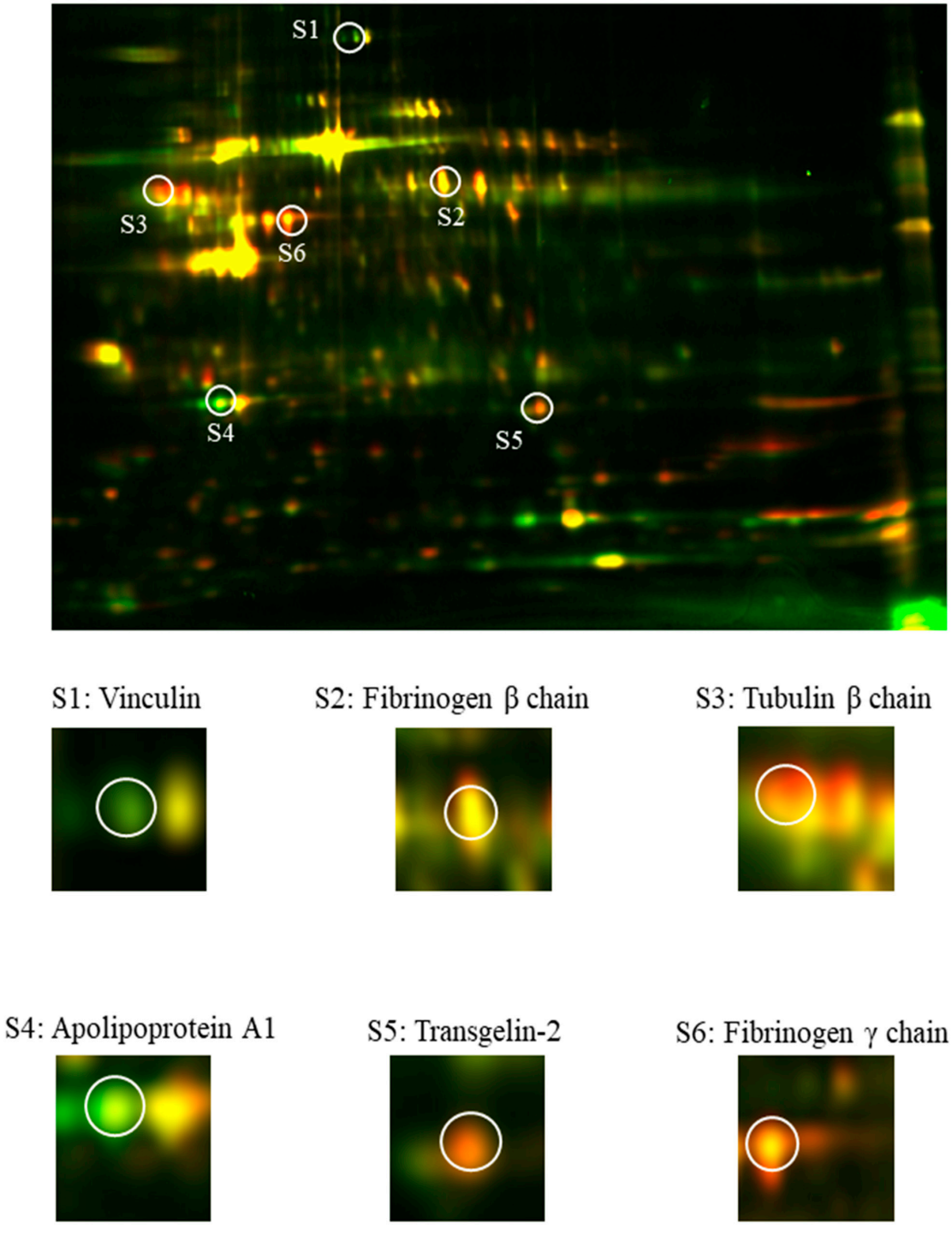


Figure 2. Identification of selected protein spots from blood platelets of ACS patients via nanoscale liquid chromatography coupled to tandem mass spectrometry.

Table 1. List of identified proteins with altered expression in ACS patients in comparison to healthy donors by nanoscale liquid chromatography coupled to tandem mass spectrometry.

Spot Number	Protein	Molecular Weight (kDa)	pI	Ratio Range (RR)	Scores	# Peptides
S1	Vinculin	123.7	5.4	3.05223 (Downregulated)	767.1	16
S2	Fibrinogen β chain	55.9	9.3	1.72152 (Upregulated)	556.3	11
S3	Tubulin β chain	49.6	4.6	1.99851 (Upregulated)	202.4	12.2
S4	Apolipoprotein A1	30.8	5.5	2.38866 (Downregulated)	670.3	16
S5	Transgelin-2	22.4	9.3	2.12995 (Upregulated)	131.3	3
S6	Fibrinogen γ chain	51.5	5.3	1.94427 (Upregulated)	492.5	11

Peptides—number of peptides.

2.3. mRNAs Expression Levels in Blood Platelets

Our comparative analysis showed that of the six analyzed genes encoding apolipoprotein A1 (*APOA1*), vinculin (*VCL*), transgelin-2 (*TAGLN2*), tubulin β-1 chain (*TUBB1*), fibrinogen β chain (*FGB*), and fibrinogen γ chain (*FGG*), three did not show expression in blood platelets (i.e., APOA1, FGB, and FGG), two were significantly upregulated in ACS patients (i.e., VCL and TAGLN2), and one did not differ between studied groups (i.e., TUBB1) (Figure 3). The lack of expression was associated with no observed formation of product and did not exceed the threshold during analysis.

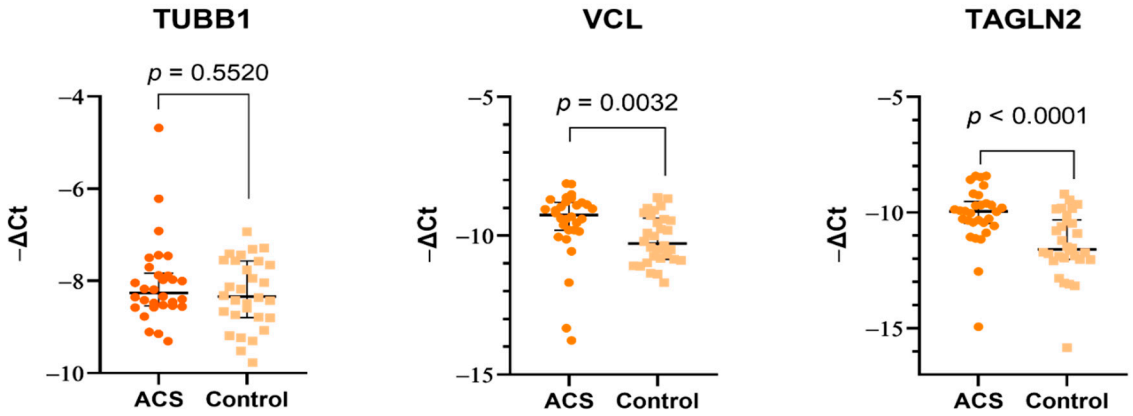


Figure 3. The mRNA expression levels of TUBB1, VCL, and TAGLN2 in blood platelets of ACS patients and healthy donors. The relative expression was assessed by the −ΔCt value. As a reference gene, 18S rRNA was used. Data are plotted as individual values with horizontal bars representing medians and interquartile range. *p*-Values were calculated using the Mann–Whitney U test.

2.4. The Concentration of Fibrinogen in Plasma

To verify that the augmented level of fibrinogen is exclusive to ACS blood platelets, the measurement of fibrinogen concentration was performed on the plasma samples. Obtained results from the enzyme-linked immunosorbent assay (ELISA) experiment showed that in the plasma of ACS diagnosed patients, the fibrinogen level was significantly increased in comparison to the control group: 3.968 ± 0.404 g/L in the study group vs. 3.601 ± 0.525 g/L in the control group ($p = 0.018$) (Figure 4).

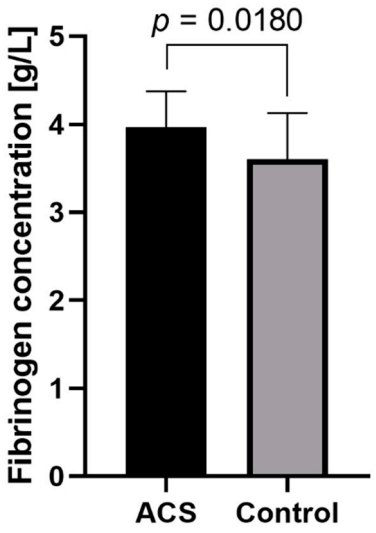


Figure 4. The comparison of plasma fibrinogen levels in ACS and control groups. Data presented in the graph show the mean concentration ± SD. Obtained results passed the Shapiro–Wilk's test, and the unpaired Student's *t*-test was used to calculate the differences.

2.5. Validation of Transgelin-2 Concentration in Blood Platelets by Western Blot

Densitometric analysis of the bands corresponding to the transgelin-2 from blood platelets showed statistically significant augmented expression in ACS patients with a median of 1.851 (1.286–2.903) compared to healthy volunteers with a median of 0.885 (0.215–1.835) (p = 0.0007; range ratio = 2.091) (Figure 5).

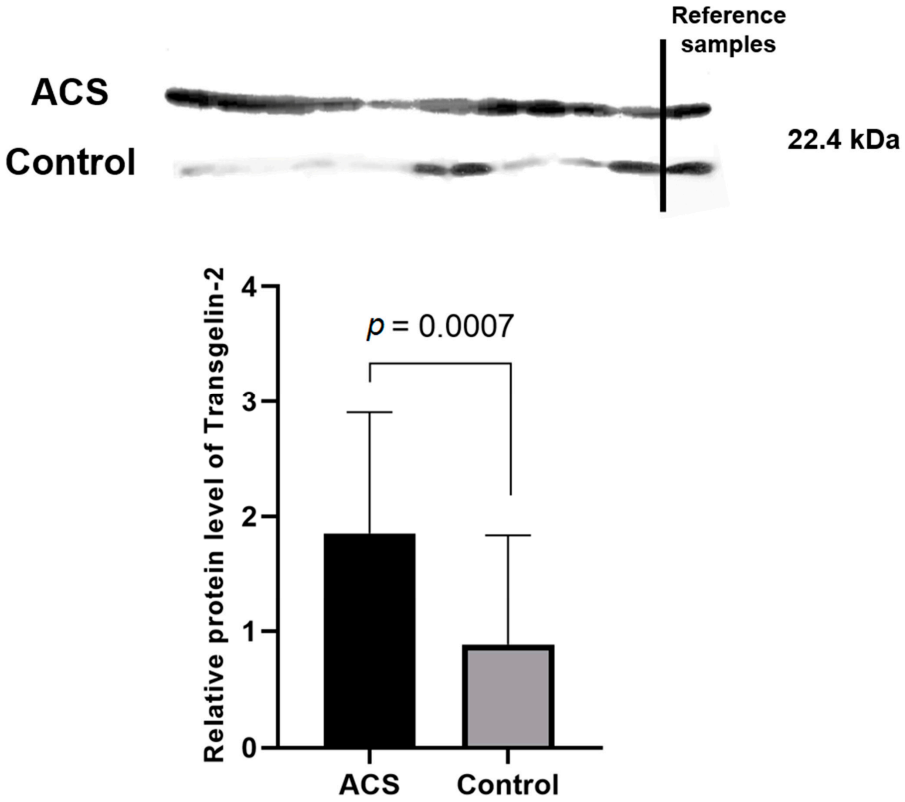


Figure 5. Expression of transgelin-2 protein in blood platelets of ACS patients and control subjects. The integrated optical density (IOD) was analyzed by densitometry and normalized by the protein concentration and reference sample. The graph demonstrates the median value from IODs with an interquartile range (25–75%). Data were calculated using the Mann–Whitney U test.

3. Discussion

The pathophysiology of coronary arteries thrombosis is complex and includes not only the classic risk factor, such as age, gender, dyslipidemia, arterial hypertension, diabetes, lack of physical activity, or smoking, but also an interplay between many genes that by regulating biochemical pathways, modify the risk of developing arterial thrombosis [8]. The role of blood platelets in thrombosis is well known [9]; however, the phenomenon of hyperreactivity of platelets and its undesirable interactions with other types of cells may lead to various, life-threatening complications including ACS. The participation of blood platelets in vasoconstriction, microembolization, and atherosclerotic plaque progression contributes to inherent persistent platelet hyperreactivity, resulting in the augmented risk of ischemic events [7,10]. The main goal of our study was to find and identify overlapping alterations in the proteome and transcriptome of blood platelets that could help in a better understanding of the molecular mechanisms of thrombosis, platelet behavior, and contributes to the development of new approaches for evaluating the risk of ACS occurring.

Our comparative analysis showed the presence of six differentially expressed proteins (i.e., vinculin, tubulin β chain, fibrinogen β and γ chains, apolipoprotein A1, and transgelin-2) in ACS patients in comparison to donors without any cardiovascular system disturbances. Vinculin is a membrane-cytoskeletal protein that serves as a linker between membrane-associated proteins and the actin cytoskeleton, thus regulating cell adhesion and cell–cell interactions [11]. To evaluate the role of vinculin in blood platelets, Mitsios et al. performed a study in which platelet functions were studied in vinculin knockout mice. Surprisingly, the results showed that vinculin-deficient platelets did not reveal any disturbances and functioned normally [12]. Our 2D-DIGE analysis showed an over three-fold decrease in the concentration of vinculin (Table 1). A similar result was obtained by Lopez-Ferre et al. in a study in which proteomic analysis of ACS patients showed a significantly downregulated expression of vinculin [13]. Although the concentration of vinculin in our study was reduced, the expression on the mRNA level of VCL was significantly augmented. A possible explanation may be associated with the post-transcriptional modifications and the role of microRNA (miR). In our previously published study [14], we showed that blood platelet possesses an elevated level of hsa-miR-21-5p, which was shown to target VCL transcripts [15]. The various directions of changes in proteome and transcriptome, and the lack of a direct effect for platelets in the mice model suggest that the role of vinculin may not be crucial for thrombosis. Moreover, the decreased concentration of vinculin should be associated with the lower platelet response to adhesion and cell–cell interactions, which seems to be contrary to the nature of ACS pathogenesis.

The major components of the platelet cytoskeleton are microtubules, which play an essential role in maintaining cell shape and its changes and participating in intracellular transport. The most important components of microtubules are two isoforms of tubulin protein: α-tubulin, the specific role of which in blood platelets is not fully understood, and β tubulin, which is more abundant and essential for microtubule and platelet formation. Their movement from the center to the cell cortex initiates the creation of pseudopods, which have elongations that lead to the formation of proplatelets. Furthermore, microtubules are essential for maintaining the discoid and/or spherical shape of blood platelets [16]. A study performed on β1-tubulin-deficient mice showed a prolonged bleeding time, weaker response to thrombin, and a lack of discoid shape [17], thus supporting the important role of microtubules in platelet function. Unfortunately, there are no studies concerning the overexpression of tubulins and their potential role in the dysregulation of platelet function. The increased concentration of tubulin β chain (Table 1) was not reflected at the mRNA level (Figure 3), which suggests that molecular alterations may arise directly in megakaryocytes. However, it requires further detailed research focus not only on platelets but also on their precursor cells.

Preventing blood loss by ensuring the continuity of the blood vessel following vascular injury is the most important process in hemostasis. The activation of the coagulation cascade and blood platelets leads to the enzymatic conversion of fibrinogen to insoluble

fibrin, the main component of blood clots. Fibrinogen is a homodimeric glycoprotein of an acute phase, composed of two sets of three different polypeptide chains (i.e., Aα, Bβ, and Yγ) linked by 29 disulfide bridges. Fibrin deposition tethers the circulating cells enhancing the primary platelet plug and extending a fibrin network, eventually forming a clot, preventing blood extravasation or in pathological conditions leading to a significant reduction in the vascular lumen or even complete vessel occlusion [18]. Studies have showed that elevated levels of plasma fibrinogen are associated with an increased risk of thrombotic events in patients and correlated with higher aggregability of blood platelets [19–21]. In 2015, Appiah et al. performed a large prospective study in which the level of the plasma fibrinogen γ chain was positively associated with the occurrence of coronary artery disease, ischemic stroke, heart failure, peripheral artery disease, and even death (caused by cardiovascular diseases) [22]. Data combined from 53 prospective studies showed that evaluations of fibrinogen levels in male patients with an intermediate risk of cardiovascular diseases could significantly increase the efficiency of predicting the disease and help to prevent one event for every 400–500 people screened over 10 years [23]. Our comparative analysis showed a statistically significant upregulation in spots identified as either a fibrinogen β and γ chain in blood platelets samples from ACS patients (Table 1). Fibrinogen is synthesized in the liver and released into the bloodstream in concentrations ranging from 1.5 to 4 g/L [24]. Due to the assessment of the possible molecular disorders associated with the ability of platelets to synthesize the fibrinogen, the levels of mRNA transcripts for FGB and FGG were evaluated. The results showed that blood platelets lacked fibrinogen transcripts, which suggests that its intraplatelet augmented level is not associated with possible synthesis in platelets. Further, the level of plasma fibrinogen was assessed between the studied groups. Received data showed a statistically significant increase in plasma fibrinogen concentration in patients with ACS (Figure 4). Despite the differences between the studied groups, the mean values fit within the physiological range of fibrinogen concentration. Unfortunately, the obtained results enable to pinpoint the direct cause of the increased fibrinogen levels. The overexpression in the 2D-DIGE analysis may be caused by the presence of plasma fibrinogen residues, as platelets were isolated from plasma. On contrary, the elevated concentration of plasma fibrinogen may be linked with the process of releasing the platelet granule content in the response to their activation. The increased reactivity of blood platelets may also be associated with the elevated level of fibrinogen receptor, GP IIb/IIIa, on the blood platelets' surface [7]. Augmented exposition of GP IIb/IIIa to the higher fibrinogen level could result in the effective, stronger, and more willing activation of blood platelets in ACS patients. Due to the lack of the possibility of a precise explanation of the obtained results, further detailed studies are necessary to determine the fibrinogen level without the potential background from both sides: platelets and plasma. Especially, because of platelets' ability to take up proteins from plasma or other cells [25].

Apolipoprotein A1 is a crucial protein that constitutes a functional and structural component of high-density lipoprotein (HDL) [26]. Studies showed that HDL and apolipoprotein A1 possess an ability to block vWF self-association resulting in the formation of shorter fibers. In patients with hyperadhesive vWF disorders, apolipoprotein A1 was significantly reduced in comparison to healthy controls, thus suggesting the vWF-specific antithrombotic property of apolipoprotein A1 and HDL. The antithrombotic capacity of HDL-apolipoprotein A1, however, affected platelet adhesion only to the reduced formation of vWF fibers, thus suggesting that they are not influencing the platelet activity directly [27]. In our study, HDL and apolipoprotein A1 levels were significantly decreased in ACS patients (Tables 1 and 2) which, according to Chung et al.'s study, may be implicated with the decreased level of antithrombotic and antiadhesive properties, thus increasing risk of thrombosis. A similar result was obtained in the Cevik et al. study, in which apolipoprotein A1 was downregulated in acute ischemic stroke patients [28].

The most interesting and important finding in our study was the significant elevation in the proteomic and transcriptomic levels of Transgelin-2. Transgelin-2 is a small ~22 kDa

protein that belongs to the actin-associated protein group and is one of three known transgelin isoforms. The specific function of transgelin-2 in platelets has not yet been determined; however, available studies have showed that the principle role of transgelin-2 is linked with actin polymerization [29], which in blood platelets plays a very important role in shape change, morphogenesis, migration, and apoptosis [30,31]. Moreover, data from the UniProt and GeneCards databases indicate that transgelin-2 can be involved in processes of platelet degranulation, platelet activation, signaling, aggregation, and response to elevated platelet cytosolic Ca^{2+} levels [32,33]. The C-terminal calponin-like repeated region and the N-terminal single calponin-homolog domain, which is a potential Ca^{2+} binding site, can be distinguished in the structure of transgelins. Furthermore, transgelins can also act as suppressors for the expression of metallo-matrix proteinase 9 (MMP-9) [34]. In a study conducted by Sheu et al., activated MMP-9 significantly reduced platelet aggregation induced by thrombin, arachidonic acid, adenosine diphosphate, U46619, and collagen, suggesting that this phenomenon may be associated with the inhibition of Ca^{2+} mobilization [35]. The suppression of MMP-9 may affect blood platelets to exhibit a poorer mechanism of natural inhibition, which may result in their hyperreactivity. Furthermore, the ability of transgelin-2 to bind F- and G-actin resulted in specific localization in the actin structures such as podosomes or stress fibers [36]. It was suggested by Poulter et al. that platelet podosome-related structures may be crucial for the platelet–platelet interactions [37]. The results of our study showed for the first time the altered expression of transgelin-2 in blood platelets on the proteome (Table 1, Figure 5) and transcriptome levels (Figure 3). The 2D-DIGE and Western blot analyses showed an over two-fold increase in the concentration of transgelin-2 in the blood platelets of ACS patients in comparison to healthy donors ($p = 0.0007$). The expression at the mRNA level also significantly increased in the study group. We speculate that the increased amount of transgelin-2 may be associated with the stimulation of changes in the shape of blood platelets by regulating actin polymerization and the formation of structures, such as lamellipodia or pseudopodia, which may contribute to a spontaneous reaction of platelet adhesion, activation, aggregation, and/or translocation of receptors responsible for interactions with other types of cells.

Similar studies have been carried out over the last 10 years; however, different proteins were identified [14,28,38–40]. Our study had a big advantage due to the use of fluorescent labeling, which shows the most sensitive detection and is the most suitable for use with mass spectrometry. Moreover, the usage of only one gel limits the need for replicates because of the co-migration of two different protein samples as a single spot [41]. In addition to a few overlapping results, our study provides new insights into the molecular changes in the proteome and transcriptome of blood platelets. For the first time, we showed an altered expression of transgelin-2, which may contribute to the processes responsible for the degranulation and shape change of platelets. The role of transgelin-2 in platelets remains unclear; however, it seems to have a potential impact on thrombosis and may partially explain the molecular disorders responsible for the hyperreactivity of blood platelets. Finding new measurable indicators of pathological, unwanted activation of blood platelets may help in identifying new targets for future therapy, monitoring the medical condition, and in assessing the probability of the future incidence of ACS in potential patients.

4. Materials and Methods

4.1. Chemicals

KCl, glucose, urea, thiourea, 2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (HEPES), 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), Tris, bovine serum albumin (BSA), $K_3[Fe(Cn)_6]$, iodoacetamide, trypsin from bovine pancreas (sequencing grade), and NH_4HCO_3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaCl, NaH_2PO_4, $NaHCO_3$, trifluoroacetic acid optima (TFA), and citrate were purchased from POCh (Gliwice, Poland). Na_2SO_3 was purchased from Alfa Aesar (Haverhill, MA, USA). Acetonitrile (AcN) LC-MS was purchased from Chempur (Piekary Slaskie,

Poland). Dithiothreitol (DTT) was purchased from GE Healthcare (Chicago, IL, USA). PBS tablets were purchased from Biosigma (Venice, Italy).

4.2. Characteristics of Patients and Donors

Blood samples were drawn from 30 patients admitted to the Department of Interventional Cardiology of the Medical University of Lodz via the S-Monovette system (Sarstedt, Numbrecht, Germany) with CPDA-1 anticoagulant. The ACS incident was confirmed angiographically. The inclusion and exclusion criteria were the same as previously described [14,42]. The control group was homologous to the study group in terms of number, age, and sex. Blood samples from the control group were collected by BD Vacutainer® (Becton Dickinson, Franklin Lakes, NJ, USA)probes with ACD-A as an anticoagulant in the Laboratory Diagnostics Centre in Lodz. All donors qualified for the study were subjected to the following blood tests: morphology, creatinine, coagulation, TSH (thyroid-stimulating hormone), IgG and IgM titers (immunoglobulin G and M), CRP (C-reactive protein), AST (aspartate transaminase), ALT (alanine transaminase), triglycerides, total cholesterol, HDL, LDL (low-density lipoprotein), and glucose. All registered participants in the control group did not administer any medications for at least 2 weeks before blood collection and were free from any illness. Furthermore, patients and donors were not previously diagnosed with any cardiovascular diseases. The study was approved by the Committee of Ethics of Research in Human Experimentation at the University of Lodz with resolution number 23/KBNN-UŁ/I/2017. Patients and volunteers enrolled in the study signed an informed consent form before inclusion. All procedures were carried out according to the Helsinki Declaration for Human Research. Clinical characteristic of patients and donors is included in Table 2.

Table 2. Characteristics of ACS patients and healthy donors.

Parameter	ACS (n = 30)	Control (n = 30)	Reference Range	p-Value
	Median (1st–3rd Quartiles) or Number (Frequency)			
Age (years)	50 (45–61)	49 (41–57)	-	0.594
Sex (male)	25	25	-	-
BMI (kg/m^2)	30 (26–31)	29 (26–32)	<35	0.874
Leukocytes (10^3/µL)	8.42 (7.00–9.65)	6.00 (4.83–7.73)	4–11	<0.001
Erythrocytes (10^6/µL)	4.42 (4.18–4.95)	5.03 (4.53–5.33)	4.2–6.1	0.011
Blood platelets (10^3/µL)	246 (200–281)	251 (217–300)	150–400	0.478
Glucose (mmol/L)	6.00 (5.31–6.28)	4.96 (4.73–5.46)	4.1–5.5	<0.001
Creatinine (µmol/L)	81.0 (71.7–88.7)	75.6 (69.6–86.8)	64–104	0.291
GFR (ml/min/1.73 m^2)	96.8 (80.3–104.3)	93.4 (81.6–104.2)	>60	0.921
AST (U/I)	34 (25–38)	19 (16–24)	<50	<0.001
ALT (U/I)	28 (19–39)	20 (14–34)	<50	0.116
Total cholesterol (mmol/L)	4.90 (4.21–5.69)	4.93 (4.42–5.31)	3–5	0.783
LDL (mmol/L)	2.86 (2.52–4.04)	2.83 (2.45–3.26)	-	0.326
HDL (mmol/L)	1.16 (0.99–1.31)	1.28 (1.12–1.71)	> 1	0.006
Triglycerides (mmol/L)	1.61 (1.00–2.75)	1.23 (0.95–1.65)	<1.7	0.049
TSH (µIU/mL)	1.53 (1.00–2.58)	1.98 (1.34–2.66)	0.27–4.20	0.322

All parameters are presented as the median and 1st–3rd quartile of the 25th–75th percentile. ALT—alanine transaminase; AST—aspartate transaminase; BMI—body mass index; GFR—glomerular filtration rate; HDL—high-density lipoprotein; LDL—low-density lipoprotein; TSH—thyroid-stimulating hormone.

4.3. Blood Platelet Isolation

All obtained blood samples were centrifuged immediately after transport (1200 rpm, 15 min, room temperature). Three-quarters of the platelet-rich plasma's top layer was transferred to a fresh tube. To prepare pure blood platelet samples, we performed negative magnetic separation. Platelet-rich plasma was incubated with MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) conjugated with anti-CD45 (to avoid contamination by leukocytes) and anti-CD235a (to avoid contamination by erythrocytes). Positive separa-

tion with anti-CD61 was not recommended as a method for obtaining quiescent platelets. After cell labeling, MS columns were washed three times with 500 µL of modified wash buffer (PBS, 2 mM citrate, 0.5% BSA). Further, platelet-rich plasma was passed through the column. The labeled leukocytes and erythrocytes were retained in the column and pure platelets passed through to the new, fresh tube. After magnetic separation, purified platelet-rich plasma was centrifuged (1400 rpm, 15 min, room temperature) to obtain a blood platelet pellet. Isolated platelets were washed three times with modified Tyrode's Buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, and 5.6 mM glucose, pH 7.4) to avoid contamination by plasma and suspended in the lysis buffer intended for 2D-DIGE (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris) and in RNAlater (Invitrogen, Carlsbad, CA, USA) for the determination of mRNA expression levels. Prior to analysis, samples were stored at −80 °C.

4.4. Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE)

The 2D-DIGE was performed on the pooled samples from the study and control group. In the first stage, the protein concentration in the platelet samples was determined by the RCDC™ Protein Assay (BioRad Laboratories, Hercules, CA, USA). Samples were diluted to obtain 500 µg of proteins in the final volume of 100 µL and purified from ionic contaminants by ReadyPrep™ 2-D Cleanup Kit (BioRad Laboratories, Hercules, CA, USA). The protein concentration was determined one more time by RCDC™ Protein Assay after a clean-up procedure to prepare the appropriate dilution of samples required for fluorescent labeling by G-Dye200 and G-Dye300 markers from DyeAgnostics Refraction-2D QPLEX kit (NH DyeAGNOSTICS, Halle, Germany). After labeling, isoelectric focusing (IEF) of samples was performed on 11 cm IPG strips of a 3–10 pH gradient (BioRad Laboratories, Hercules, CA, USA) using PROTEAN i12 IEF Cell (BioRad Laboratories, Hercules, CA, USA). All IEF parameters were set according to the manufacturer's protocol. Further, IPG strips were balanced in the Equilibration Buffer 1 (BioRad Laboratories, Hercules, CA, USA), containing dithiothreitol (DTT) for the reduction of proteins in the strip, and in Equilibration Buffer 2 (BioRad Laboratories, Hercules, CA, USA), containing iodoacetamide for alkylation of proteins in the strip. A second dimension separation was performed using Criterion™ TGX Precast Gels 4–20% (BioRad Laboratories, Hercules, CA, USA). All parameters for separation were set according to the manufacturer's protocol. The fluorescently stained gel was scanned using a Typhoon FLA 950 GE scanner (GE Healthcare, Chicago, IL, USA) (G-Dye300 was used for samples from ACS patients, and G-Dye200 was used for control samples). Furthermore, a bioinformatics analysis of the obtained images was carried out using ImageMaster 2D Platinum 7.0 software (GE Healthcare, Chicago, IL, USA). Changes in protein expression were assessed based on a ratio of 1.5.

4.5. Nanoscale Liquid Chromatography Coupled to Tandem Mass Spectrometry (nanoLC-MS/MS)

Protein spots cut from a gel were decolorized with $K_3[Fe(Cn)_6]$/100 mM Na_2SO_3, rinsed with 100 mM NH_4HCO_3, shrunk with acetonitrile (AcN), and dried in a vacuum centrifuge. Next, samples were reduced with 10 mM DTT/100 mM NH_4HCO_3 for 45 min at 60 °C and alkylated in 55 mM iodoacetamide/100 mM NH_4HCO_3 for 30 min at room temperature in darkness. Proteins were digested overnight in a buffer containing 0.1 µg/µL trypsin at 37 °C with gentle shaking (150 rpm). Extraction of peptides from the gel was carried out in a solution of 50 mM NH_4HCO_3 and 2.5% TFA in 50% AcN by incubating the extracts in the ultrasonic bath. The extract was completely dried in a vacuum centrifuge and the samples were desalted using ZipTip C18 tips (Merck Millipore, Billerica, MA, USA). Peptides were suspended in a 0.1% TFA solution. Identification of digested proteins was performed using electrospray ionization tandem mass spectrometry (ESI-MS/MS) preceded by nanoLC-MS chromatographic separation with the application of the following conditions: gradient 4–99%; mass range 50–2000 m/z; composition of mobile phase: (A) 100% H_2O; 0.05% TFA; (B) 80% AcN, 0.04% TFA. For high-resolution analysis, Acclaim™ PepMap™ and Acclaim PepMap RSLC™ columns were used (Trap column: Acclaim

PepMap100 C18: particle size—5 µm; diameter—0.1 mm; length—20 mm and pore size—100 A; Analytic column Acclaim PepMap RSLC C18: particle size—2 um, diameter—0.05 mm, length—15 cm, pore size 100 A (Thermofisher, Waltham, MA, USA)). Proteins were identified on the MASCOT server (Swiss-Prot database) using ProteinScape software (Bruker, Billerica, MA, USA).

4.6. Determination of mRNA Expression Level of Identified Proteins in Platelets

The total RNA samples were isolated from purified blood platelets using the commercial kit ISOLATE II RNA Mini Kit (Bioline, London, UK). All steps were performed according to the manufacturer's protocol. To avoid and remove genomic DNA contamination of samples, DNase I was used. The RNA samples were further transcribed into complementary DNA (cDNA) using Maxima First Strand cDNA Synthesis Kit (Thermofisher, Waltham, MA, USA) according to the attached instruction. The expression on the mRNA level was determined by Real-Time PCR Detection System Thermal Cycler CFX96TM (Bio Rad Laboratories Inc., Hercules, CA, USA) using TaqMan® Gene Expression Assays and TaqMan® Universal Master Mix, no. UNG. The following TaqMan® Assays were used: 18S rRNA (Hs_99999901_s1), APOA1 (Hs_0098500_g1), VCL (Hs_00419715_m1), TAGLN2 (Hs_00761239_s1), TUBB1 (Hs_00917771_g1), FGB (Hs_00170586_m1), and FGG (Hs_00241037_m1). The relative expression levels of mRNA for all studied genes were calculated using $-\Delta$Ct.

4.7. Determination of Plasma Fibrinogen Concentration

The concentration of plasma fibrinogen in the study and control group was assessed by ELISA kit (Elabscience, Houston, TX, USA). Plasma samples were obtained after centrifugation of whole blood samples according to the manufacturer's instructions. The concentration of fibrinogen was calculated by comparing the absorbance values of the samples to the standard curve.

4.8. Western Blot Analysis of Transgelin-2

Protein samples were first separated by the 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30 µg of protein mixture was applied onto the lane) and electrotransferred onto the Immobilon-P membrane (Millipore, Bedford, MA, USA). To assess the quality of the transfer, Ponceau S staining was performed. Obtained blots were incubated with the Anti-TAGLN2 antibody (Abcam, Cambridge, UK) for 2 h at room temperature with the 1:3000 dilution. Further, blots were washed with Tris-buffered saline buffer (TBS) containing 0.1% Tween-20 and incubated with the anti-rabbit IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Visualization of the proteins was performed on the X-ray film by the SuperSignal™ West Pico PLUS (Thermofisher, Waltham, MA, USA). Densitometric analysis of the protein bands on blots was carried out by Gel-Pro Analyzer Software 3.0 (Media Cybernetics Inc., Bethesda, MD, USA). To avoid any possible changes in the protein concentration, data were normalized by the IOD of total proteins after Ponceau S staining and also by the reference sample applied on all blots. As a reference sample, the protein mixture obtained from one patient was used and loaded onto each performed blot. The result of TAGLN2 is shown as a ratio of the IOD of the bands which corresponds to a relative protein level.

4.9. Statistical Analysis

All statistical analyses were performed in STATISTICA 13.3 software (StatSoft; Tulsa, OK, USA). The normality of distribution was assessed by the Shapiro–Wilk test. Depending on the Gaussian distribution, the Mann–Whitney U test or unpaired Student's *t*-test was performed. A p-value < 0.05 was considered statistically significant. The range ratio was calculated as the ratio between the means or medians between studied groups.

Author Contributions: Conceptualization, R.S., P.J., M.B. and J.S.-B.; methodology, R.S., P.J., M.B. and J.S.-B.; validation, R.S.; formal analysis, R.S. and P.J.; investigation, R.S., P.J., M.B. and J.S.-B.; resources, R.S., M.K., M.B., M.Z. and J.S.-B.; data curation, R.S.; writing—original draft preparation, R.S. and J.S.-B.; visualization, R.S.; supervision, M.K, M.B., M.Z., A.O. and J.S.-B.; project administration, R.S., M.B. and J.S.-B.; funding acquisition, R.S., M.B. and J.S.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Lodz (grant number: B1911000002114.02).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of the University of Lodz, Poland, with Resolution No. 23/KBNN-UŁ/I/2017. All participants enrolled in the study provided written informed consent.

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

Data Availability Statement: All data obtained from this study are included in the manuscript.

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

References

1. Nair, M.; Sandhu, S.S.; Sharma, A.K. Prognostic and Predictive Biomarkers in Cancer. *Curr. Cancer Drug Targets* **2014**, *14*, 477–504. [CrossRef]
2. Letunica, N.; Van Den Helm, S.; McCafferty, C.; Swaney, E.; Cai, T.; Attard, C.; Karlaftis, V.; Monagle, P.; Ignjatovic, V. Proteomics in Thrombosis and Hemostasis. *Thromb. Haemost.* **2021**. [CrossRef] [PubMed]
3. World Health Organization. Available online: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (accessed on 1 May 2022).
4. Hou, Y.; Carrim, N.; Wang, Y.; Gallant, R.C.; Marshall, A.; Ni, H. Platelets in Hemostasis and Thrombosis: Novel Mechanisms of Fibrinogen-Independent Platelet Aggregation and Fibronectin-Mediated Protein Wave of Hemostasis. *J. Biomed. Res.* **2015**, *29*, 437–444. [CrossRef]
5. Stakos, D.A.; Tzikas, D.N.; Konstantinos, S. Mechanisms of platelet activation in acute coronary syndromes. *Curr. Vasc. Pharm.* **2012**, *10*, 578–588. [CrossRef] [PubMed]
6. Massberg, S.; Schulz, C.; Gawaz, M. Role of platelets in the pathophysiology of acute coronary syndrome. *Semin. Vasc. Med.* **2003**, *3*, 147–162. [CrossRef]
7. Merlini, P.A.; Bauer, K.A.; Oltrona, L.; Ardissino, D.; Cattaneo, M.; Belli, C.; Mannucci, P.M.; Rosenberg, R.D. Persistent Activation of Coagulation Mechanism in Unstable Angina and Myocardial Infarction. *Circulation* **1994**, *90*, 61–68. [CrossRef] [PubMed]
8. Bray, P.F. Platelet Hyperreactivity: Predictive and Intrinsic Properties. *Hematol. Oncol. Clin. N. Am.* **2007**, *21*, 633–645. [CrossRef]
9. Koupenova, M.; Kehrel, B.E.; Corkrey, H.A.; Freedman, J.E. Thrombosis and Platelets: An Update. *Eur. Heart J.* **2017**, *38*, 785–791. [CrossRef] [PubMed]
10. Braunwald, E.; Angiolillo, D.; Bates, E.; Berger, P.B.; Bhatt, D.; Cannon, C.P.; Furman, M.I.; Gurbel, P.; Michelson, A.D.; Peterson, E.; et al. The Problem of Persistent Platelet Activation in Acute Coronary Syndromes and Following Percutaneous Coronary Intervention. *Clin. Cardiol.* **2008**, *31*, I17–I20. [CrossRef]
11. Zemljic-Harpf, A.; Manso, A.M.; Ross, R.S. Vinculin and Talin: Focus on the Myocardium. *J. Investig. Med.* **2009**, *57*, 849–855. [CrossRef] [PubMed]
12. Mitsios, J.V.; Prevost, N.; Kasirer-Friede, A.; Gutierrez, E.; Groisman, A.; Abrams, C.S.; Wang, Y.; Litvinov, R.I.; Zemljic-Harpf, A.; Ross, R.S.; et al. What Is Vinculin Needed for in Platelets? *J. Thromb. Haemost.* **2010**, *8*, 2294–2304. [CrossRef]
13. López-Farré, A.J.; Zamorano-Leon, J.J.; Azcona, L.; Modrego, J.; Mateos-Cáceres, P.J.; González-Armengol, J.; Villarroel, P.; Moreno-Herrero, R.; Rodríguez-Sierra, P.; Segura, A.; et al. Proteomic Changes Related to "Bewildered" Circulating Platelets in the Acute Coronary Syndrome. *Proteomics* **2011**, *11*, 3335–3348. [CrossRef]
14. Szelenberger, R.; Karbownik, M.S.; Kacprzak, M.; Maciak, K.; Bijak, M.; Zielińska, M.; Czarny, P.; Śliwiński, T.; Saluk-Bijak, J. Screening Analysis of Platelet MiRNA Profile Revealed MiR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome. *Cells* **2021**, *10*, 3526. [CrossRef] [PubMed]
15. Major, J.L.; Bagchi, R.A.; Pires da Silva, J. Application of MicroRNA Database Mining in Biomarker Discovery and Identification of Therapeutic Targets for Complex Disease. *Methods Protoc.* **2020**, *4*, 5. [CrossRef]
16. Cuenca-Zamora, E.J.; Ferrer-Marín, F.; Rivera, J.; Teruel-Montoya, R. Tubulin in Platelets: When the Shape Matters. *Int. J. Mol. Sci.* **2019**, *20*, 3484. [CrossRef] [PubMed]
17. Schwer, H.D.; Lecine, P.; Tiwari, S.; Italiano, J.E.; Hartwig, J.H.; Shivdasani, R.A. A Lineage-Restricted and Divergent β-Tubulin Isoform Is Essential for the Biogenesis, Structure and Function of Blood Platelets. *Curr. Biol.* **2001**, *11*, 579–586. [CrossRef]
18. Kattula, S.; Byrnes, J.R.; Wolberg, A.S. Fibrinogen and Fibrin in Hemostasis and Thrombosis. *Arter. Thromb. Vasc. Biol.* **2017**, *37*, e13–e21. [CrossRef]

19. Wilhelmsen, L.; Svärdsudd, K.; Korsan-Bengtsen, K.; Larsson, B.; Welin, L.; Tibblin, G. Fibrinogen as a Risk Factor for Stroke and Myocardial Infarction. *N. Engl. J. Med.* **1984**, *311*, 501–505. [CrossRef] [PubMed]
20. Meade, T.W.; Vickers, M.V.; Thompson, S.G.; Seghatchian, M.J. The Effect of Physiological Levels of Fibrinogen on Platelet Aggregation. *Thromb. Res.* **1985**, *38*, 527–534. [CrossRef]
21. Fibrinogen Studies Collaboration; Danesh, J.; Lewington, S.; Thompson, S.G.; Lowe, G.D.O.; Collins, R.; Kostis, J.B.; Wilson, A.C.; Folsom, A.R.; Wu, K.; et al. Plasma Fibrinogen Level and the Risk of Major Cardiovascular Diseases and Nonvascular Mortality: An Individual Participant Meta-Analysis. *JAMA* **2005**, *294*, 1799–1809. [CrossRef]
22. Appiah, D.; Schreiner, P.J.; MacLehose, R.F.; Folsom, A.R. Association of Plasma Gamma Prime (γ′) Fibrinogen with Incident Cardiovascular Disease: The Atherosclerosis Risk in Communities (ARIC) Study. *Arter. Thromb. Vasc. Biol.* **2015**, *35*, 2700–2706. [CrossRef]
23. Emerging Risk Factors Collaboration; Kaptoge, S.; Di Angelantonio, E.; Pennells, L.; Wood, A.M.; White, I.R.; Gao, P.; Walker, M.; Thompson, A.; Sarwar, N.; et al. C-Reactive Protein, Fibrinogen, and Cardiovascular Disease Prediction. *N. Engl. J. Med.* **2012**, *367*, 1310–1320. [CrossRef]
24. Vilar, R.; Fish, R.J.; Casini, A.; Neerman-Arbez, M. Fibrin(Ogen) in Human Disease: Both Friend and Foe. *Haematologica* **2020**, *105*, 284–296. [CrossRef]
25. Senzel, L.; Gnatenko, D.V.; Bahou, W.F. The Platelet Proteome. *Curr. Opin. Hematol.* **2009**, *16*, 329–333. [CrossRef] [PubMed]
26. Mangaraj, M.; Nanda, R.; Panda, S. Apolipoprotein A-I: A Molecule of Diverse Function. *Indian J. Clin. Biochem.* **2016**, *31*, 253–259. [CrossRef] [PubMed]
27. Chung, D.W.; Chen, J.; Ling, M.; Fu, X.; Blevins, T.; Parsons, S.; Le, J.; Harris, J.; Martin, T.R.; Konkle, B.A.; et al. High-Density Lipoprotein Modulates Thrombosis by Preventing von Willebrand Factor Self-Association and Subsequent Platelet Adhesion. *Blood* **2016**, *127*, 637–645. [CrossRef]
28. Cevik, O.; Baykal, A.T.; Sener, A. Platelets Proteomic Profiles of Acute Ischemic Stroke Patients. *PLoS ONE* **2016**, *11*, e0158287. [CrossRef]
29. Yin, L.-M.; Ulloa, L.; Yang, Y.-Q. Transgelin-2: Biochemical and Clinical Implications in Cancer and Asthma. *Trends. Biochem. Sci.* **2019**, *44*, 885–896. [CrossRef]
30. Bearer, E.L.; Prakash, J.M.; Li, Z. Actin Dynamics in Platelets. *Int. Rev. Cytol.* **2002**, *217*, 137–182. [CrossRef] [PubMed]
31. Dvorakova, M.; Nenutil, R.; Bouchal, P. Transgelins, Cytoskeletal Proteins Implicated in Different Aspects of Cancer Development. *Expert. Rev. Proteom.* **2014**, *11*, 149–165. [CrossRef]
32. Stelzer, G.; Rosen, N.; Plaschkes, I.; Zimmerman, S.; Twik, M.; Fishilevich, S.; Stein, T.I.; Nudel, R.; Lieder, I.; Mazor, Y.; et al. The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. *Curr. Protoc. Bioinform.* **2016**, *54*, 1.30.1–1.30.33. [CrossRef]
33. UniProt Consortium UniProt: A Worldwide Hub of Protein Knowledge. *Nucleic. Acids Res.* **2019**, *47*, D506–D515. [CrossRef]
34. Assinder, S.J.; Stanton, J.-A.L.; Prasad, P.D. Transgelin: An Actin-Binding Protein and Tumour Suppressor. *Int. J. Biochem. Cell. Biol.* **2009**, *41*, 482–486. [CrossRef]
35. Sheu, J.R.; Fong, T.H.; Liu, C.M.; Shen, M.Y.; Chen, T.L.; Chang, Y.; Lu, M.S.; Hsiao, G. Expression of Matrix Metalloproteinase-9 in Human Platelets: Regulation of Platelet Activation in in Vitro and in Vivo Studies. *Br. J. Pharm.* **2004**, *143*, 193–201. [CrossRef] [PubMed]
36. Jo, S.; Kim, H.-R.; Mun, Y.; Jun, C.-D. Transgelin-2 in Immunity: Its Implication in Cell Therapy. *J. Leukoc. Biol.* **2018**, *104*, 903–910. [CrossRef] [PubMed]
37. Poulter, N.S.; Pollitt, A.Y.; Davies, A.; Malinova, D.; Nash, G.B.; Hannon, M.J.; Pikramenou, Z.; Rappoport, J.Z.; Hartwig, J.H.; Owen, D.M.; et al. Platelet Actin Nodules Are Podosome-like Structures Dependent on Wiskott-Aldrich Syndrome Protein and ARP2/3 Complex. *Nat. Commun.* **2015**, *6*, 7254. [CrossRef]
38. Banfi, C.; Brioschi, M.; Marenzi, G.; De Metrio, M.; Camera, M.; Mussoni, L.; Tremoli, E. Proteome of Platelets in Patients with Coronary Artery Disease. *Exp. Hematol.* **2010**, *38*, 341–350. [CrossRef] [PubMed]
39. Parguiña, A.F.; Fernández Parguiña, A.; Grigorian-Shamajian, L.; Agra, R.M.; Teijeira-Fernández, E.; Rosa, I.; Alonso, J.; Viñuela-Roldán, J.E.; Seoane, A.; González-Juanatey, J.R.; et al. Proteins Involved in Platelet Signaling Are Differentially Regulated in Acute Coronary Syndrome: A Proteomic Study. *PLoS ONE* **2010**, *5*, e13404. [CrossRef]
40. Parguiña, A.F.; Grigorian-Shamagian, L.; Agra, R.M.; López-Otero, D.; Rosa, I.; Alonso, J.; Teijeira-Fernández, E.; González-Juanatey, J.R.; García, Á. Variations in Platelet Proteins Associated with ST-Elevation Myocardial Infarction: Novel Clues on Pathways Underlying Platelet Activation in Acute Coronary Syndromes. *Arter. Thromb. Vasc. Biol.* **2011**, *31*, 2957–2964. [CrossRef]
41. Chevalier, F. Standard Dyes for Total Protein Staining in Gel-Based Proteomic Analysis. *Materials* **2010**, *3*, 4784–4792. [CrossRef]
42. Szelenberger, R.; Karbownik, M.S.; Kacprzak, M.; Synowiec, E.; Michlewska, S.; Bijak, M.; Zielińska, M.; Olender, A.; Saluk-Bijak, J. Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients—Emphasis on P2Y12. *Biology* **2022**, *11*, 644. [CrossRef] [PubMed]

OŚWIADCZENIA WSPÓŁAUTORÓW

Mgr Rafał Szelenberger
Katedra Biochemii Ogólnej
Uniwersytet Łódzki

Łódź, 10.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska *„Blood platelet surface receptor genetic variation and risk of thrombotic episodes"* Clinica Chimica Acta, 496: 84-92, 2019, mój udział wynosił 40% i obejmował pomoc w opracowaniu koncepcji pracy przeglądowej, zebranie danych literaturowych, przygotowanie wstępnej wersji manuskryptu, stworzenie tabel oraz rycin, przygotowanie finalnej wersji manuskryptu oraz udział w przygotowywaniu odpowiedzi dla recenzentów.

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak *„Plasma MicroRNA as a novel diagnostic"* Clinica Chimica Acta, 499: 98-107, 2019, mój udział wynosił 50% i obejmował pomoc w opracowaniu koncepcji pracy przeglądowej, zebranie danych literaturowych, przygotowanie wstępnej wersji manuskryptu, stworzenie tabeli podsumowującej występowanie zmian w profilu ekspresji cząsteczek miRNA w omawianych jednostkach chorobowych, przygotowanie finalnej wersji manuskryptu oraz przygotowywanie odpowiedzi dla recenzentów.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 45% i obejmował pomoc w opracowaniu koncepcji oraz sformułowaniu hipotezy badawczej, przygotowanie wersji podstawowej abstraktu, wstępu, opisu metod i wyników oraz dyskusji, wykonanie następujących metod badawczych: izolacja płytek krwi, izolacja całkowitego RNA (wraz z mikroRNA) oraz synteza mikro-cDNA, analiza mikromacierzy, walidacja uzyskanych wyników metodą ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym, wykonanie analizy bioinformatycznej, przygotowanie części rycin (Rycina 1, Rycina 6) oraz tabel (Tabela 1, Tabela 3), współudział w opracowaniu recenzji oraz przygotowaniu finalnej wersji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 40% i obejmował pomoc w opracowaniu koncepcji oraz sformułowaniu hipotezy badawczej, przygotowanie wersji podstawowej abstraktu, wstępu, opisu metod i wyników oraz dyskusji, wykonanie następujących metod badawczych: izolacja płytek krwi, izolacja frakcji białkowej płytek krwi, określenie stężenia białka dla receptorów powierzchniowych wykazujących zmienną ekspresję (na poziomie mRNA) pomiędzy analizowanymi

grupami metodą immunoenzymatyczną ELISA, ocena powierzchniowej ekspresji płytkowego receptora P2Y12 metodą cytometrii przepływowej, pomoc w izolacji całkowitego RNA i w syntezie cDNA oraz pomoc w analizie ekspresji genów wyselekcjonowanych receptorów powierzchniowych płytek krwi na poziomie mRNA, przygotowanie Ryciny 3 oraz Tabeli 1, współudział w opracowaniu recenzji oraz przygotowaniu finalnej wersji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 40% i obejmował pomoc w opracowaniu koncepcji oraz sformułowaniu hipotezy badawczej, przygotowanie wersji podstawowej abstraktu, wstępu, opisu metod i wyników oraz dyskusji, wykonanie następujących metod badawczych: izolacja płytek krwi, izolacja frakcji białkowej płytek krwi, analiza przesiewowa płytkowego proteomu metodą dwukierunkowej elektroforezy fluorescencyjnej, określenie stężenia osoczowego fibrynogenu metodą ELISA oraz współudział w analizie proteomicznej wykonywanej metodą Western Blot, opracowanie statystyczne wyników, przygotowanie wszystkich rycin oraz tabel, współudział w opracowaniu recenzji oraz przygotowaniu finalnej wersji manuskryptu.

Szelenberger

Rafał Szelenberger

Prof. dr hab. Joanna Saluk-Bijak
Katedra Biochemii Ogólnej
Uniwersytet Łódzki

Łódź, 10.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska *„Blood platelet surface receptor genetic variation and risk of thrombotic episodes"* Clinica Chimica Acta, 496: 84-92, 2019, mój udział wynosił 15% i obejmował przygotowywanie pierwszego rozdziału pracy, pomoc i nadzór w przygotowywaniu pozostałych rozdziałów oraz recenzję manuskryptu.

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak *„Plasma MicroRNA as a novel diagnostic"* Clinica Chimica Acta, 499: 98-107, 2019, mój udział wynosił 15% i obejmował opracowanie koncepcji pracy przeglądowej oraz pomoc w zakresie przygotowywania pracy poprzez nadzór, edycję tekstu oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 10% i obejmował opracowanie koncepcji oraz sformułowanie hipotezy badawczej, pomoc w zakresie interpretacji wyników uzyskanych z analizy porównawczej profilu mikroRNA płytek krwi metodą mikromacierzy i metodą ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym oraz nadzór, edycję tekstu i pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 10% i obejmował opracowanie koncepcji oraz sformułowanie hipotezy badawczej, pomoc w interpretacji wyników uzyskanych z pomiarów stężenia białka wyselekcjonowanych receptorów powierzchniowych płytek krwi i oceny powierzchniowej ekspresji receptora P2Y12 metodą cytometrii przepływowej i mikroskopii konfokalnej oraz pomoc w przygotowywaniu pracy poprzez nadzór, edycję tekstu i recenzję manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 10% i obejmował przygotowanie opisu charakterystyki klinicznej pacjentów i dawców oraz pomoc w przygotowywaniu pracy doświadczalnej poprzez nadzór i edycję pozostałych rozdziałów manuskryptu i pomoc w jego recenzji.

Joanna Saluk-Bijak

Dr Michał Kacprzak
Klinika Kardiologii Interwencyjnej
Uniwersytet Medyczny w Łodzi

Łódź, 10.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska *„Blood platelet surface receptor genetic variation and risk of thrombotic episodes"* Clinica Chimica Acta, 496: 84-92, 2019, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowania rozdziału dotyczącego roli płytek krwi w Ostrym Zespole Wieńcowym.

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak *„Plasma MicroRNA as a novel diagnostic"* Clinica Chimica Acta, 499: 98-107, 2019, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania pracy przeglądowej poprzez nadzór i edycję tekstu manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną oraz pomoc w opracowaniu charakterystyki klinicznej pacjentów.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną, pomoc w opracowaniu charakterystyki klinicznej pacjentów, oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną, pomoc w opracowaniu charakterystyki klinicznej pacjentów, oraz pomoc w recenzji manuskryptu.

Michał Kacprzak

dr n. med. Michał Kacprzak
specjalista KARDIOLOG
2374582

Dr Michał Seweryn Karbownik
Zakład Farmakologii i Toksykologii
Uniwersytet Medyczny w Łodzi

Łódź, 25.05.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 15% i obejmował wykonanie analiz statystycznych i interpretację uzyskanych wyników, udział w przygotowywaniu manuskryptu poprzez stworzenie rozdziału dotyczącego analizy statystycznej i modelowania potencjalnego biomarkera, oraz stworzenie części rycin (Rycina 2, Rycina 3, Rycina 4, Rycina 5) oraz tabel (Tabela 2) zaprezentowanych w manuskrypcie.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology 11(5): 644, 2022, mój udział wynosił 10% i obejmował wykonanie analiz statystycznych i interpretację uzyskanych wyników, udział w przygotowywaniu manuskryptu poprzez stworzenie rozdziału dotyczącego analizy statystycznej i modelowania potencjalnego biomarkera, oraz stworzenie części rycin (Rycina 1, Rycina 2, Rycina 5) zaprezentowanych w manuskrypcie.

Michał Seweryn Karbownik

Dr hab. Michał Bijak, prof. UŁ
Centrum Zapobiegania Zagrożeniom Biologicznym
Uniwersytet Łódzki

Łódź, 10.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska *„Blood platelet surface receptor genetic variation and risk of thrombotic episodes"* Clinica Chimica Acta, 496: 84-92, 2019, mój udział wynosił 25% i obejmował wyselekcjonowanie polimorfizmów pojedynczego nukleotydu opisanych w pracy przeglądowej oraz pomoc w przygotowaniu pierwotnej wersji manuskryptu oraz jego recenzji.

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak *„Plasma MicroRNA as a novel diagnostic"* Clinica Chimica Acta, 499: 98-107, 2019, mój udział wynosił 15% i obejmował pomoc w zakresie przygotowywania pracy przeglądowej poprzez nadzór, edycję tekstu oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w zakresie interpretacji wyników uzyskanych z analizy porównawczej profilu mikroRNA płytek krwi metodą mikromacierzy oraz metodą ilościowej reakcji łańcuchowej polimerazy w czasie rzeczywistym.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 5% i obejmował wyselekcjonowanie receptorów powierzchniowych płytek krwi, które zostały poddane analizie proteomicznej i transkryptomicznej oraz stworzenie protokołu eksperymentalnego umożliwiającego ocenę powierzchniowej ekspresji płytkowego receptora P2Y12 metodą cytometrii przepływowej.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 10% i obejmował pomoc w zakresie interpretacji wyników uzyskanych z analizy dwukierunkowej elektroforezy fluorescencyjnej i nano-chromatografii cieczowej sprzężonej z tandemową spektrometrią mas.

Michał Bijak

Prof. dr hab. Marzenna Zielińska
Klinika Kardiologii Interwencyjnej
Uniwersytet Medyczny w Łodzi

Łódź, 10.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Michal Bijak, Joanna Saluk-Bijak, Marzenna Zielinska *„Blood platelet surface receptor genetic variation and risk of thrombotic episodes"* Clinica Chimica Acta, 496: 84-92, 2019, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania publikacji poprzez nadzór i edycję tekstu oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafal Szelenberger, Michal Kacprzak, Joanna Saluk-Bijak, Marzenna Zielinska, Michal Bijak *„Plasma MicroRNA as a novel diagnostic"* Clinica Chimica Acta, 499: 98-107, 2019, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania pracy przeglądowej poprzez nadzór i edycję tekstu manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną oraz nadzór i edycję tekstu.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 10% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez rekrutację pacjentów stanowiących grupę badaną oraz pomoc merytoryczną w opisie roli płytek krwi w Ostrym Zespole Wieńcowym.

Marzenna Zielińska

KIEROWNIK
Kliniki Kardiologii Interwencyjnej
Centralnego Szpitala Klinicznego
Uniwersytetu Medycznego w Łodzi
prof. dr hab. n. med. Marzenna Zielińska

Prof. dr hab. Alina Olender

Katedra i Zakład Mikrobiologii Lekarskiej

Uniwersytet Medyczny w Lublinie

Lublin, 08.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology 11(5): 644, 2022, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania publikacji poprzez nadzór i edycję tekstu oraz pomoc w recenzji manuskryptu.

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences 23(11): 6340, 2022, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania publikacji poprzez nadzór i edycję tekstu oraz pomoc w recenzji manuskryptu.

Alina Olender

KIEROWNIK
Katedry i Zakładu Mikrobiologii Lekarskiej
Uniwersytetu Medycznego w Lublinie
Prof. dr hab. n. med. Alina M. Olender

Dr Paweł Jóźwiak
Katedra Cytobiochemii
Uniwersytet Łódzki

Łódź, 07.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Paweł Jóźwiak, Michał Kacprzak, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Variations in Blood Platelet Proteome and Transcriptome Revealed Altered Expression of Transgelin-2 in Acute Coronary Syndrome Patients"* International Journal of Molecular Sciences, 23(11): 6340, 2022, mój udział wynosił 15% i obejmował pomoc w wykonaniu analizy proteomicznej metodą Western Blot oraz pomoc w interpretacji uzyskanych wyników.

Paweł Jóźwiak

Dr Ewelina Synowiec
Katedra Genetyki Molekularnej
Uniwersytet Łódzki

Łódź, 08.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak „*Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 10% i obejmował pomoc w zakresie wykonywania oraz interpretacji wyników dotyczących analizy ekspresji genów wyselekcjonowanych receptorów powierzchniowych płytek krwi na poziomie mRNA.

Ewelina Synowiec

Mgr Karina Maciak
Katedra Biochemii Ogólnej
Uniwersytet Łódzki

Łódź, 06.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w wykonaniu analiz bioinformatycznych i interpretacji uzyskanych wyników.

Maciak K.

Karina Maciak

Dr Piotr Czarny
Zakład Biochemii Medycznej
Uniwersytet Medyczny w Łodzi

Łódź, 07.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w wykonaniu analizy porównawczej profilu mikroRNA płytek krwi metodą mikromacierzy.

Piotr Czarny

Dr Sylwia Michlewska

Pracownia Obrazowania Mikroskopowego
i Specjalistycznych Technik Biologicznych

Uniwersytet Łódzki

Łódź, 09.06.2022

Oświadczenie o udziale w publikacjach

Oświadczam, iż w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Ewelina Synowiec, Sylwia Michlewska, Michał Bijak, Marzenna Zielińska, Alina Olender, Joanna Saluk-Bijak *„Dysregulation in the Expression of Platelet Surface Receptors in Acute Coronary Syndrome Patients – Emphasis on P2Y12"* Biology, 11(5): 644, 2022, mój udział wynosił 5% i obejmował pomoc w zakresie analizy i interpretacji wyników dotyczących wizualizacji receptora P2Y12 na płytkach krwi metodą mikroskopii konfokalnej.

Sylwia Michlewska

Prof. dr hab. Tomasz Śliwiński
Katedra Genetyki Molekularnej
Uniwersytet Łódzki

Łódź, 30.05.2022

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy: Rafał Szelenberger, Michał Seweryn Karbownik, Michał Kacprzak, Karina Maciak, Michał Bijak, Marzenna Zielińska, Piotr Czarny, Tomasz Śliwiński, Joanna Saluk-Bijak *„Screening Analysis of Platelet miRNA Profile Revealed miR-142-3p as a Potential Biomarker in Modeling the Risk of Acute Coronary Syndrome"* Cells, 10(12): 3526, 2021, mój udział wynosił 5% i obejmował pomoc w zakresie przygotowywania pracy doświadczalnej poprzez nadzór i edycję tekstu manuskryptu.

Prof. dr hab. Tomasz Śliwiński