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IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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„Ocena wpływu polimorfizmów genów ABCB1 i CYP2C19 na metabolizm klopidogrelu u pacjentów z ostrym zespołem wieńcowym.”

Rozprawa na stopień doktora nauk medycznych

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Spis treści

Spis skrótów użytych w pracy

ASA - kwas acetylosalicylowy (ang. acetylsalicylic acid)

GoF – wzmocnienie funkcji (ang. gain-of-function)_

HPR - wysoka reaktywność płytek krwi (ang. high platelet reactivity)

HLPC - wysokosprawna chromatografia cieczowa (ang. high-performance liquid chromatography

LoF - osłabienie funkcji (ang. loss-of-function)

LPR - niska reaktywność płytek krwi (ang. low platelet reactivity)

MBP- 2-bromo-3'- metoksyacetofenon

NSTEMI - zawał serca bez przetrwałego uniesienia odcinka ST (ang. non-ST-elevation myocardial infarction)

OZW – ostry zespół wieńcowy

PCI - przezskórna interwencja wieńcowa (ang. percutaneous coronary intervention)

P-GP - glikoproteina P-GP

PPP - osocze ubogopłytkowe (ang. platelet poor plasma)

PRP - osocze bogatopłytkowe (ang. platelet rich plasma)

STEMI - zawał serca z przetrwałym uniesieniem odcinka ST (ang. ST-elevation myocardial infarction)

Wykaz publikacji stanowiących rozprawę doktorską

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2. Wójcik T, Szymkiewicz P, Ściborski K, et al. Original and generic clopidogrel: A comparison of antiplatelet effects and active metabolite concentrations in patients without polymorphisms in the ABCB1 gene and the allele variants CYPC19*2 and *3. Adv Clin Exp Med. 2021;30(5):485–489. doi:10.17219/acem/133811
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Streszczenie

Najczęstszą przyczyną zgonów w Polsce są choroby układu krążenia. Spośród nich najczęstszą przyczyną bezpośrednią jest zawał mięśnia sercowego, a następnie rozwijająca się w konsekwencji niewydolność serca. Złotym standardem leczenia ostrych zespołów wieńcowych jest przezskórna interwencja wieńcowa, a następnie terapia przeciwpłytkowa (zazwyczaj przez pierwsze 12 miesięcy podwójna, następnie pojedyncza). Obok od lat stosowanego kwasu acetylosalicylowego będącym inhibitorem cyklooksygenazy-1, stosowane są 3 leki przeciwpłytkowe jak: klopidogrel, prasugrel i tikagrelor, będące inhibitorami receptora P2Y12. Pomimo szerokiego stosowania nowszych leków przeciwpłytkowych (tikagrelor i prasugrel) w leczeniu ostrych zespołów wieńcowych, wciąż wielu pacjentów jest leczonych znanym od lat klopidogrelem, głównie ze względu na konieczność stosowania dodatkowej terapii przeciwkrzepliwej, przy której wymieniowe wcześniej inhibitory receptora P2Y12 są przeciwwskazane. Wiele lat doświadczeń w zastosowaniu klopidogrelu obok jego niezaprzeczalnych korzyści, wykazały również jego ograniczenia, wiążące się głównie z formą pro-leku, którym faktycznie jest klopidogrel, a tym samym koniecznością jego metabolizacji do formy aktywnej w szeregu skomplikowanych przemian biochemicznych przez enzymy cytochromu P450 (m.in. CYP2C19). Ponadto lek po zażyciu podlega w jelicie absorbcji, której skuteczność regulowana jest aktywnością p-glikoproteiny (P-GP). Wobec powyższego, stwierdzane u ludzi polimorfizmy genów kodujących funkcjonowanie białka ABCB1 i CYP2C19, mogą przyczyniać się do nieadekwatnej odpowiedzi płytek krwi na działanie leku czyli tzw. zwiększonej reaktywności płytek pomimo leczenia klopidogrelem, co niesie ryzyko istotnych powikłań. W przedstawionej pracy zbadaliśmy dystrybucję polimorfizmów genów w populacji pacjentów z Dolnego Śląska leczonych z powodu ostrego zespołu wieńcowego, wykazując że częstość występowania polimorfizmu genu ABCB1 w populacji jest duża, częstość występowania polimorfizmu typu CYPC19*2 w populacji jest względnie duża oraz, że częstość występowania polimorfizmu typu CYPC19*3 w populacji jest bardzo niska. Potwierdziliśmy, że przyjmowanie form generycznych klopidogrelu nie różnią sie w stosunku do preparatu oryginalnego, zarówno pod względem osiągniętych stężeń aktywnego metabolitu klopidogrelu, ani także jego działania antyagregacyjnego. Finalnie udowodniliśmy, że obecność badanych polimorfizmów genów nie wpływa na stężenie aktywnego metabolitu klopidogrelu we krwi pacjentów z zawałem z- i bez uniesienia odcinka ST oraz na działanie przeciwpłytkowe leku.

Summary

Cardiovascular diseases are the leading cause of mortality in Poland. Acute coronary syndromes (ACS) constitute a common reason for hospital admission. Percutaneous coronary intervention together with dual antiplatelet therapy can be seen as a gold standard in the treatment of ACS. Besides acetylsalicylic acid – a well-established cyclooxygenase-1 inhibitor – also P2Y12 platelet receptor antagonists are employed including: prasugrel, ticagrelor and clopidogrel. While newer antiplatelet agents (namely prasugrel and ticagrelor) are preferred in the therapy of ACS, a proportion of patients present with significant contraindications to their use such as the need for parallel antithrombotic treatment. In such cases clopidogrel might be an antiplatelet of choice.Years of clinical experience with the use of clopidogrel have defined its undisputable advantages but also pointed at its limitations related to being a pro-drug which requires complex conversion to the active metabolite. Metabolic changes of clopidogrel take place in liver in several oxidative steps involving cytochrome P450 and enzymes coded by CYPC19 gene. Moreover, clopidogel is absorbed in intestines which is regulated by p-glycoprotein (P-GP) coded by ABCB1 gene. Hence, genetical polymorphisms within these genes (coding transport proteins of digestive tract and several oxidative enzymes) might contribute to inadequate platelets inhibition in response to clopidogrel. This could potentially lead to disastrous complications in patients undergoing percutaneous coronary interventions. In the current dissertation, the prevalence of gene polymorphisms involved in clopidogrel absorption and metabolism in the population of patients from Lower Silesia treated for ACS was analysed. The ABCB1 gene polymorphism was highly prevalent, type CYPC19*2 was quite prevalent and type CYPC19*3 was very rare. The generic formulations of clopidogrel were as effective as the original one both in terms of the concentration of active metabolite and the level of antiplatelet activity. Finally, we showed that the presence of investigated genetic variations does not influence the serum concentration of active metabolite nor the antiplatelet activity in patients with myocardial infarction (with and without ST elevation).

1. Wstęp

Pomimo dostępności coraz doskonalszych metod leczniczych, wciąż najczęstszą przyczyną zgonów w Polsce (46 %), pozostają od wielu lat szeroko pojęte choroby układu krążenia [1]. Zgodnie z rejestrem PL-ACS, czyli Ogólnopolskiego Rejestru Ostrych Zespołów Wieńcowych, około 45 tys. pacjentów rocznie doznaje zawału mięśnia sercowego bez- lub z uniesieniem odcinka ST (NSTEMI i STEMI). Oba rodzaje zawału, będące epizodami ostrego zespołu wieńcowego (OZW), obarczonego są znaczną śmiertelnością w okresie 6-miesięcznym od wystąpienia incydentu, ale stanowią również bezpośrednią przyczynę rozwoju niewydolności serca. Najskuteczniejszą metodą leczenia OZW jest przezskórna interwencja wieńcowa (PCI), po której konieczne jest czasowe (zazwyczaj 12 miesięczne) zablokowanie funkcji endogennych płytek krwi przy pomocy leków przeciwpłytkowych. Przez ostatnie kilkadziesiąt lat koronne miejsce w terapii przeciwpłytkowej zajmował kwas acetylosalicylowy (ASA), będący inhibitorem cyklooksygenazy-1. Kolejne lata rozwoju kardiologii interwencyjnej a w szczególności zabiegi endowaskularne połączone z implantacją stentów, wykazały konieczność zastosowania tzw. podwójnej terapii przeciwpłytkowej [2], opierającej na różnych mechanizmach hamowania aktywności płytek krwi. Przez długi czas wytyczne ekspertów dotyczące leczenia pacjentów z ostrym zespołem wieńcowym wysoko pozycjonowały zastosowanie klopidogrelu obok wymienionego wcześniej ASA. Niemniej jednak, nowsze randomizowane badania kliniczne (TRITON-TIMI 38 oraz PLATO), przyniosły rezultaty faworyzujące użycie w jego miejsce prasugrelu lub tikagreloru [3-4].

Opisane leczenie nie jest jednak optymalne dla wszystkich grup pacjentów leczonych inwazyjnie. We współczesnej kardiologii duży odsetek stanowią chorzy ze współistniejącym napadowym lub przetrwałym migotaniem przedsionków [5] lub po przebytej zatorowości płucnej czy zakrzepicy żył głębokich, którzy wymagają leczenia przeciwkrzepliwego. Z racji wyższego ryzyka powikłań krwotocznych, połączenie tego rodzaju terapii z prasugrelem i tikagrelorem nie jest zalecane. Tym, samym ich miejsce w dalszym ciągu zajmuje znany od lat klopidogrel.

1.1 Klopidorgel

Lek z grupy inhibitorów receptora P2Y12 jako drugi obok ASA, stosowany jest w celu minimalizacji ryzyka powikłań zatorowo-zakrzepowych, w szczególności zakrzepicy w stencie oraz ponownemu zawałowi serca. Wiele lat doświadczeń w zastosowaniu klopidogrelu (badanie CURE i CLARITY TIMI 28) obok jego niezaprzeczalnych korzyści wykazały również jego ograniczenia [6-7]. Wiążą się one głównie z formą pro-leku, którym faktycznie jest klopidogrel, a tym samym koniecznością jego metabolizacji do formy aktywnej w szeregu skomplikowanych przemian biochemicznych przez enzymy cytochromu P450 (m.in. *CYP2C19*) [8]. Ponadto lek po zażyciu podlega w jelicie absorbcji, której skuteczność regulowana jest aktywnością p-glikoproteiny (P-

GP). Uważa się, że polimorfizm genów kodujących funkcjonowanie białka *ABCB1* i *CYP2C19* może przyczyniać się do nieadekwatnej odpowiedzi płytek krwi na działanie leku czyli tzw. zwiększonej reaktywności płytek (HPR), pomimo leczenia klopidogrelem, co niesie ryzyko istotnych powikłań. [8] Miedzy innymi te ograniczenia były podstawą poszukiwań skuteczniejszej terapii przeciwpłytkowej.

1.2 Prasugrel i tikagleror

Nowe inhibitory receptora P2Y12 (prasugrel i tikagrelor) przyniosły dodatkowe korzyści w leczeniu pacjentów z OZW, między innymi poprzez szybsze zablokowanie funkcji płytek krwi, do którego dążymy u chorych ze świeżym zawałem serca [9-10].

Prasugrel, podobnie jak klopidogrel, jest prolekiem podlegającym jednak szybkiemu wchłanianiu z przewodu pokarmowego, ponadto w odróżnieniu od klopidogrelu, jego metabolizm jest jednoetapowy. Bardziej efektywny proces wchłaniania i utleniania prasugrelu, doprowadza do efektywniejszej i wyraźnie szybszej inhibicji płytkowej (już po 30 minutach).

Tikagrelor, kolejny z nowych inhibitorów receptora P2Y12, jest bezpośrednim i odwracalnym blokerem wymienionego receptora. Lek dla swego działanie nie wymaga przemian metabolicznych i jest aktywny bezpośrednio po wchłonięciu z przewodu pokarmowego.

Pomimo tego, że leki te znalazły już swoje ugruntowane miejsce w zaleceniach terapeutycznych, przyczyniły się również do większej liczby powikłań krwotocznych [11] i z tego względu ich zastosowanie ma w pewnych grupach pacjentów istotne ograniczenia.

2. Genetyka

2.1 Wchłanianie klopidogrelu

W pierwszym etapie przemian klopidogrelu, pro-lek musi zostać wchłonięty z przewodu pokarmowego. Ilość substancji ostatecznie wchłoniętej do organizmu, zależy od aktywności glikoproteiny P-GP obecnej w jelitach. Białko to, będące pompą zależną od ATP, zaliczane do grupy MDR1 czyli białek odporności wielolekowej 1, usuwającego znaczną liczbę ksenobiotyków dostających się do organizmu. Wśród tych substancji znajduje się klopidogrel.. Podobnie jak dla enzymów metabolizujących klopidogrel, również w przypadku ABCB1 występują polimorfizmy genowe mogące zmieniać jego aktywność . Mutacja w locus 3435 C> T prowadzi do wzmożonej aktywność P-GP i zmniejszenia absorpcji klopidogrelu z przewodu pokarmowego poprzez jego zwiększony wyrzut zwrotny do światła jelita. Takie zjawisko występuje zarówno w formie hetero i homozygotycznej genu ABCB1.

Zmienność w obrębie ABCB1 wskazywana była jako przyczyna HPR oraz zwiększonej ilości zawałów serca, udarów czy zgonu wśród chorych z OZW [12]. Trzeba jednak zaznaczyć, że obserwacje te nie są powtarzalne [13-14]. Co więcej dwie metaanalizy nie wykazały istotnego wpływu polimorfizmu genu ABCB1 na międzyosobniczą odpowiedź na klopidogrel [15-16].

2.1.1 Allele właściwego (GA- „good absorbers") i osłabinego wchłaniania (PA - "poor absorbers"

Na cele obecnego cyklu przyjęto, że osobniki będące homozygotami dzikimi (CC), pozbawionymi mutacji 3435C>T, które cechuje właściwe wchłaniania klopidogrelu oznaczane będą jako GA. Do grupy obniżonego wchłaniania (PA) zaliczono osobniki heterozygotyczne (CT) oraz homozygoty mutacyjne (TT) genu ABCB1.

2.2 Metabolizm klopidogrelu

W skład cytochromu P-450 wchodzą enzymy, uczestniczące w wątrobowych przemianach metabolicznych wielu stosowanych leków: m.in. leków antydepresyjnych, benzodiazepin, inhibitorów pompy protonowej czy klopidogrelu. W tej grupie znajdują się monooksygenazy CYP1A2, CYP2C19 i CYP2B6, które są zaangażowane w pierwszym etapie metabolizmu klopidogrelu. Produktem ich działania jest 2-oksyklopidogrel, który w drodze docelowej do transformacji do aktywnego metabolitu musi przejść kolejne utlenianie z użyciem białek CYP2C9, CYP2B6, CYP3A4, CYP3A5 i CYP2C19. Wśród genów kodujących paletę CYP występują różne osobnicze odrębności genetyczne, spośród których najlepiej poznano polimorfizm genów CYP2C19, CYP3A4/5, CYP1A2. Samego białka CYP2C19 zarejestrowano 25 polimorfizmów [17].

Odmienność genetyczna wpływać może na indywidualną skuteczność leczenia klopidogrelem. Poszczególne allele, które negatywnie wpływają na metabolizm omawianego leku, a co za tym idzie, na potencjalnie większą reaktywność płytek krwi w wynikach badań laboratoryjnych (HPR), przydzielone zostały do grupy tzw. LoF (ang. loss-of-function). Odwrotnie natomiast, allele, które wzmagają działanie klopidogrelu poprze nasilenie agregacji płytek, zostały przydzielone do grupy GoF (ang. gain-of-function).

2.2.1 Allele wzmacniające (GoF- „gain-of-function") i osłabiające funkcję (LoF - "loss-of-function")

U podłoża upośledzenia metabolizmu klopidogrelu leży błąd w transkrypcji materiału genetycznego w mechanizmie tzw. przesunięcia ramki odczytu (ang. frame Shift), nieprawidłowym splicingu (składaniu genów) i mutacji lub zbyt wczesnemu występowaniu

kodonu „stop”. Do alleli powodujących osłabienie metabolizmu klopidogrelu (LoF) należą allele CYP2C19 *2-*8. Najczęstszym wariantem występującym w Europie jest CYP2C19*2 (około 25 % populacji), natomiast wariant *3 identyfikowano średnio z częstością 1-7%. Allele *4-*8 należą do rzadkości, z przynależnością poniżej 1% [17]. Wobec powyższego w cyklu publikacji skupiliśmy się na analizie wariantów *2-*3.

Jak wykazały wcześniejsze obserwacje, pacjenci posiadający jeden z wymienionych wariantów genetycznych mogą być narażeni na gorsze wyniki leczenia w mechanizmie HPR, poprzez redukcję ilości aktywnego metabolitu klopdiogrelu w osoczu [18]. U chorych poddawanych leczeniu z powodu zawału serca, przy współistnieniu wariacji genetycznej, w dalszej obserwacji stwierdzano istotny wzrost ryzyka wystąpienia kolejnego zawału serca, głównie poprzez mechanizm zakrzepicy w stencie, udar mózgu i ostatecznie zgon. [19-20]. Z drugiej jednak strony indywidualizowana terapia przeciwpłytkowa, uwzględniająca obecność polimorfizmów genetycznych nie wpływała na poprawę rokowania wskazując, na inne mechanizmy HPR i świetle tych obserwacji rutynowe oznaczenia genetyczne i terapia personalna nie są zalecane w aktualnych wytycznych [21].

Obecność u ludzi jednego z alleli CYP2C19 *17-*21 powoduje wzmożony metabolizm klopidogrelu (GoF) co przekłada się wyższe stężenie aktywnej formy leku we krwi i niską reaktywność płytek krwi (LPR). Najszerzej rozpowszechnionym i najlepiej poznanym jest allel *17. Częstość jego występowania w populacji kaukaskiej jest szacowana na 41% [22]. U pacjentów posiadających ten wariant genetyczny obserwowano silną inhibicję płytek i wysokie stężenie aktywnego metabolitu klopidogrelu w osoczu [23]. Niestety obecność tego allelu nie zabezpieczała pacjentów przed kolejnymi incydentami niedokrwiennymi, zwiększając natomiast występowanie ilości krwawień. [22]

2.3 Oznaczenia genetyczne

Wszystkie przedstawione w cyklu prace, w których analizowano występowanie polimorfizmów genów wpływających na metabolizm i wchłanianie klopidogrelu bazowały na identycznej metodologii. Jej dokładny opis przedstawiony jest w poszczególnych publikacjach. Badania przeprowadzono w Zakładzie Technik Molekularnych Uniwersytetu Medycznego we Wrocławiu.

Przybliżając po krótce sposób oznaczeń genetycznych: w celu ich przeprowadzenia pobierano krew pełną do próbówek z EDTA. Materiał genetyczny izolowano z krwi przy użyciu zestawu High Pure PCR Template Preparation Kit (Roche Diagnostics, Warszawa, Polska). W tym etapie, celem pozyskania oczyszczonego materiału genetycznego, przeprowadzano lizę i degradację białek komórkowych, wirowanie lizatu na minikolumience zawierającej membranę krzemionkową,

wiążącej DNA oraz finalnie odzyskania oczyszczonego DNA poprzez dodanie buforu elucyjnego. Następnym krokiem postępowania laboratoryjnego była amplifikacja DNA metodą PCR (polymerase chain reaction, PCR). Reakcję amplifikacji DNA przeprowadzono przy użyciu 4 par specyficznych starterów odpowiednio dla: CYP2C19*17 (-3402C>T), CYP2C19*3, ABCB1 (C3435C>T), CYP2C19*2 i zestawu Multiplex PCR Kit (Qiagen).

Oznaczanie polimorfizmów typu SNP (single nucleotide polymorphism – polimorfizm pojedynczego nukleotydu) genów CYP2C19 (CYPC19*2, *3) i ABCB1 przeprowadzano z wykorzystaniem techniki minisekwencjonowania. W tym celu zastosowano zestaw SNaPshot Multiplex Kit (AB Applied Biosystems, Thermo Scientific, Gdańsk, Polska) Reakcję minisekwencjonowania przeprowadzano z udziałem starterów zaprojektowanych w ten sposób, aby hybrydyzowały one do matrycy kończąc rekację przed oznaczanym miejscem polimorficznym. W reakcji brały udział terminatory znakowane fluorescencyjnie. Po enzymatycznym oczyszczeniu produktu minisekwencjonowania próbkę poddawano analizie z detekcją produktów techniką elektroforezy kapilarnej. Z użyciem sekwenatora 3130 Genetic Analyzer (Applied Biosystems, Thermo Scientific, Gdańsk, Polska), znaczniki fluorescencyjne dobudowane do starterów podczas reakcji minisekwencjonowania wzbudzane do swiecenia były przy użycia światła laserowego. Wyniki analizowano z wykorzystaniem programu GeneMapper ID v.3.2.

Do oznaczenia polimorfizmu CYP2C19*17 (locus -3402 C>T) zastosowano technikę RFLP (restriction fragment length polymorphism - analiza długości fragmentów restrykcyjnych). W metodzie tej powielony materiał genetyczny poddawano inkubacji z restryktazami, czyli enzymami specyficznie tnącymi nici DNA. Produkty trawienia materiału genetycznego, warunkowane wystąpieniem mutacji w zakresie badanego polimorfizmu, identyfikowano metodą elektroforezy.

3. Oznaczenie agregacji płytek krwi

We wszystkich pracach włączonych do cyklu, w których oceniano skuteczność antyagregacyjną klopidogrelu zastosowano jednolita metodologię uzyskania materiału biologicznego i oznaczeń aktywności płytek krwi.

Szczegółowy opis metodologii znajduje się w poszczególnych pracach. Pokrótce, żylne próbki krwi były pobierana między 2-4 dobą od wystąpienia OZW do dwóch próbówek z dodatkiem cytrynianu sodu. Krew wirowano w temperaturze pokojowej do uzyskania osocza bogatopłytkowego (PRP). Następnie przeprowadzano kolejne wirowanie celem pozyskania osocza ubogopłytkowego (PPP). Właściwa ocena reaktywności płytek była wykonana do 2 godzin od czasu pobrania, metodą agregacji optycznej wg Borna przy użyciu jednokanałowego agregometru świetlnego produkcji Chrono Log 560CA (Chrono-log, Havertown, PA, USA).

Zasadą tej metody jest ciągły pomiar zmian transmisji światła padającego na stale mieszane osocze PRP. Jako wzór referencyjny służyła próbka z PPP o przepuszczalności światła 100%. Następnie badane próbki z PRP (przepuszczalność świetlna 0%) były umieszczane w aparacie i pobudzane za pomocą roztworu ADP o stężeniu 5 µmol/l oraz10 µmol/l.

Celem tego pomiaru była ocena zwiększenia przepuszczalności światła widzialnego, gdyż pod wpływem wyżej wymienionych agonistów dochodzi do wytworzenia agregatów płytkowych i zmiany gęstości optycznej roztworów. Wynik badania przedstawiono jako odsetek maksymalnej agregacji płytek krwi w ciągu 6 minut obserwacji, wyrażający skuteczność farmakologicznej inhibicji płytkowej.

4. Oznaczenie aktywnego metabolitu klopidogrelu

Ocena stężenia aktywnego metabolitu klopidogrelu w krwi pacjentów jest trudna technicznie ze względu na dużą niestabilność badanej substancji i jej krótki czas półtrwania. Do chwili obecnej jedynie kilka metod z użyciem wysokosprawnej chromatografii cieczowej (ang. high-performance liquid chromatography – HLPC) okazało się wystarczająco czułych dla przeprowadzania tego rodzaju analiz [24]. W pracy doktorskiej zastosowałem metodę oznaczeń aktywnego metabolitu klopidogrelu Karaźniewicz-Łady [25], zmodyfikowaną w Katedrze i Zakładzie Biochemii Lekarskiej Uniwersytetu Medycznego we Wrocławiu. Jej podstawą jest połączenie możliwości fizycznego rozdzielania chromatografii cieczowej z możliwościami analizy masowej spektrometrii masowej i określana jest skrótem LC-MS/MS.

Wszyscy pacjenci poddani działaniu badanego leku, zarówno w formie oryginalnej jak i generycznej otrzymywali preparaty zawierające klopidogrel w postaci soli wodorosiarczanu. Analiza stężenia leku przeprowadzona była w trzecim dniu stosowania w dawce podtrzymującej. Kluczowym elementem analizy było ustabilizowanie niestabilnego aktywnego metabolitu za pomoca 2-bromo-3'- metoksyacetofenonu (MPB) ze względu na istotne zmniejszenie jego stężenia obserwowane już po 10 minutach od uzyskanie próbki krwi. Dokładny opis zastosowanej metody analitycznej przedstawiony został w poszczególnych pracach cyklu.

5. Przesłanki do prowadzenia badań i cele pracy

Rzeczywistość pokazuje, że nadal wielu pacjentów, zarówno w Polsce jak i na Świecie, w przypadku OZW i zabiegu angioplastyki z wszczepieniem stentu, leczonych jest z użyciem klopidogrelu. Przez lata swojej obecności na rynku, klopidogrel stał się lekiem powszechnie dostępnym poprzez produkcję wielu preparatów generycznych.

Istniejące przesłanki dotyczące wpływu polimorfizmu genów ABCB1 i CYPC19 na działanie klopidogrelu w dużym stopniu dotyczą populacji młodych i zdrowych [19,26].

Doniesienia omawiające powyższe zagadnienie u pacjentów z ostrym zespołem wieńcowym nie są spójne i pełne [27,28], co szczególnie dotyczy genu ABCB1. Choć negatywny wpływ polimorfizmu CYPC19 na przebieg kliniczny pacjentów z OZW został stwierdzony w dużych badaniach randomizowanych, w tym w badaniu TRITON-TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction 38), to brakującym elementem w pełnej ocenie pozostaje w tych obserwacjach brak analizy różnic w stężeniu aktywnego metabolitu klopidogrelu oraz agregacji płytek krwi w zależności od profilu genetycznego.

W cyklu prac składających się na pracę doktorska postawione przeze mnie cele dotyczyły:

- Oceny częstości występowania polimorfizmu genów ABCB1 i CYPC19 w badanej populacji;
- Oceny wpływu polimorfizmu genów ABCB1 i CYPC19 na skuteczność terapii klopidogrelem u pacjentów z ostrym zespołem wieńcowym
- Określenie potencjalnych różnic w stężeniu aktywnej formy klopidogrelu u pacjentów z różnymi typami ostrych zespołów wieńcowych
- Określenie potencjalnych różnic stężenia aktywnej formy klopidogrelu po zastosowaniu preparatów generycznych klopidogrelu w porównaniu z jego formą oryginalną

6. Cykl prac

W pracy pod tytułem „**Distribution of polymorphisms in the CYP2C19 and ABCB1 genes among patients with acute coronary syndrome in Lower Silesian population”** analizie poddano dystrybucję polimorfizmów genów w populacji pacjentów z OZW pochodzących z terenu Dolnego Śląska. Do badania włączono 199 pacjentów z ostrym zespołem wieńcowym, którzy hospitalizowani byli w Uniwersyteckim Szpitalu Klinicznym we Wrocławiu.

Badanie wykazało powszechność występowania polimorfizmu genu ABCB1 w analizowanej grupie, z blisko 58% odsetkiem heterozygot mutacyjnych oraz 26% homozygot mutacyjnych. Łącznie pacjenci o profilu zaburzonego wchłaniania klopidogrelu stanowili 84% całej grupy badanej. Ponadto u 26% pacjentów stwierdzono nosicielstwo, co najmniej, jednego allelu mutacyjnego CYPC19*2, a tym samym potencjalnie obniżoną zdolność metabolizmu klopidogrelu. Biorąc pod uwagę częstość występowania allelu CYPC19*17, którą oszacowano u około 45% pacjentów, odsetek osób określanych jako słabo metabolizujący klopidogrel równy był 18%. Łączne występowanie profilu genetycznego warunkujące obniżone wchłanianie leku oraz jego osłabiony metabolizm sięgnęło 14.6%. Wyniki te pozostają w zgodzie z rezultatami analiz

wykonanych przez innych autorów u pacjentów rasy kaukaskiej. Istotnym, nowatorskim elementem przedstawionej pracy jest fakt udowodnienia, po raz pierwszy w populacji polskiej, bardzo niskiej penetracji polimorfizmu CYPC19*3. Według wiedzy autorów analizy takiej do tej pory w Polsce nie przeprowadzono.

Praca „**Original and generic clopidogrel: A comparison of antiplatelet effects and active metabolite concentrations in patients without polymorphisms in the ABCB1 gene and the allele variants CYPC19*2 and *3**" została zaplanowana jako analiza potencjalnych różnic pomiędzy stosowanymi powszechnie w Polsce preparatami klopidogrelu. Analizowano różnice w stężeniu aktywnego metabolitu klopidogrelu oraz jego działania przeciwpłytkowego. Badaną grupę stanowiło 22 zdrowych ochotników. Celem eliminacji wpływu polimorfizmu badanych genów na wyniki badania wszyscy poddani analizie pozbawieni byli alleli mutacyjnych genów ABCB1 oraz CYPC19. Jak wykazało badanie dwa najczęściej stosowane preparaty generyczne klopidogrelu nie odbiegały od jego oryginalnej formy pod względem osiągniętego we krwi stężenia aktywnego metabolitu (12,7 pg/µL vs 13,0 pg/µL vs 14,4 pg/µL) oraz zdolności hamowania agregacji płytkowej, analizowanej w oparciu o transmisję światła widzialnego (34,5% vs 35,0% vs 36,6%). Uzyskane rezultaty wykazały brak różnic działania różnych preparatów klopidogrelu. Tym samym zasadną okazała się planowana w kolejnej pracy analiza wpływu polimorfizmu ABCB1 oraz CYPC19 na działanie klopidogrelu (stosowanego w postaci różnych preparatów) w większej grupie pacjentów z ostrym zespołem wieńcowym

Praca **„The influence of acute coronary syndrome on levels of clopidogrel active metabolite and platelet inhibition in patients with and without CYP2C19*2(681 G>A),*3(636 G>A) and ABCB1(C3435C> T) gene polymorphisms**" jest zamknięciem cyklu publikacji . Zaplanowano przeprowadzenie badania wpływu wyszczególnionych polimorfizmów genów, na działanie przeciwpłytkowe klopidogrelu u pacjentów z zawałem mięśnia sercowego z uniesieniem oraz bez uniesienia odcinka ST pozostających w klasie Killip I-III . Wykazano, że działanie leku u pacjentów ze STEMi lub NSTEMI, pozostaje niezmienne w stosunku do grupy kontrolnej ze stabilną chorobą wieńcową. Obserwacja ta dotyczyła zarówno grupy pacjentów pozbawionych polimorfizmów genów ABCB1 oraz CYPC19 jak i grupy z co najmniej jednym allelem mutacyjnym. Wartości, zarówno stężenia aktywnego metabolitu klopidogrelu jak i agregacji płytek krwi, pozostawały na podobnym poziomie, również w grupie potencjalnie najbardziej narażonej, w której obserwowano równoczesne występowanie genetycznego podłoża zaburzonego wchłaniania klopidogrelu i jego metabolizmu.

Opisywane w innych badaniach, przeprowadzonych na populacjach młodych, zdrowych ochotników, różnice w działaniu klopidogrelu, w zależności od występowania polimorfizmu genów

ABCB1 i CYPC19 tym samym nie potwierdzały się w populacji starszej z obciążeniami współistniejącymi. Wydaje się, że oddziaływanie polimorfizmu genetycznego na badane parametry nie jest addytywny i może być stłumiony w tej populacji przez występowanie innych czynników fenotypowych znanych z wpływu na skuteczność działania klopidogrelu (min. niewydolność nerek, palenie tytoniu, cukrzyca, stosowanie innych leków itp.). Tym samym badane różnice genetyczne okazały się nie być istotnym czynnikiem ingerującym w efekty leczenia przeciwpłytkowego u chorych z ostrym zespołem wieńcowym.

6.1 Publikacja nr 1

Original papers

Distribution of polymorphisms in the *CYP2C19* and *ABCB1* genes among patients with acute coronary syndrome in Lower Silesian population

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Abstract

Background. Dual antiplatelet therapy (DAPT) with aspirin and clopidogrel administered to treat patients with acute coronary syndrome (ACS) is still being used. However, despite the proven efficacy of this treatment regimen, thromboembolic complications have been observed in some individuals. The reason for this phenomenon is linked to the so-called increased responsiveness of platelets despite high platelet resistance (HPR). A significant role in HPR is attributed to genetically determined differences in the absorption and activation of clopidogrel.

Objectives. The aim of the study was to assess the incidence of polymorphisms of the *ABCB1* and *CYPC19* genes that encode proteins involved in the absorption and metabolism of clopidogrel.

Material and methods. The analysis was performed in 199 consecutive patients from Lower Silesian voivodeship (Poland) who underwent coronary angioplasty with stenting for ACS. The single nucleotide polymorphism of the *CYP2C19* and *ABCB1* genes was performed using a mini sequencing or restriction fragment length polymorphism method.

Results. The results of this study revealed the high incidence of patients who may be unresponsive to antiplatelet treatment due to genetic causes. The CYPC19*2 allele in the form of homozygote or mutation heterozygote appeared in 26.1% of the study population. ABCB1 (C3435C> T) polymorphism was associated with 84% of patients. The total incidence of allelic disorders of low drug absorption and metabolism reached 14.6%.

Conclusions. The data obtained should prompt clinicians to use more recent antiplatelet agents (ticagrelor or prasugrel) first, instead of clopidogrel.

Key words: *ABCB1*, clopidogrel, Lower Silesia, polymorphism, *CYP2C9*

Cite as
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Introduction

Dual antiplatel therapy (DAPT) with aspirin and clopidogrel has until recently been the gold standard for the treatment of acute coronary syndrome (ACS) patients. Nevertheless, despite the proven efficacy of this treatment regimen, thromboembolic complications continue to be observed in some patients.[1,2] The reason for this phenomenon is associated with a so-called sustained increase in platelets activity, in spite of DAPT. This phenomenon may be due to an impaired aspirin response, but it is mainly associated with an ineffective treatment with clopidogrel.

A number of factors contribute to increased platelet reactivity. The lack of expected effects of DAPT is most likely due to non-compliance to treatment recommendations. However, the complexity of genetically conditioned absorption and activation of clopidogrel is also important. The action of clopidogrel is conditioned by the efficacy of the pro-drug conversion into its active form. This process largely depends on intestinal absorption and complex hepatic metabolism. The first step of clopidogrel activation takes place in the gastrointestinal tract. The *ABCB1* gene expression product, P-GP (glycoprotein), plays a key role here. It is a membrane protein of the gastrointestinal tract, which acts to regulate xenobiotics concentrations in the human body, including clopidogrel. Spot mutations in the *ABCB1* gene type C3435C> T, resulting in the emergence of mutation homozygotes (TT) or mutation heterozygotes (CT) in place of proper homozygotes (CC), imply (TT) increased P-GP activity. This activity causes an excessive rejection of clopidogrel, hindering its attainment of adequate plasma concentrations. The polymorphism of this gene has a negative influence on the clinical effects of clopidogrel treatment and may cause high platelet resistance (HPR). An even greater association with individual differences in response to clopidogrel treatment is attributed to cytochrome P450. Its proper functioning allows for the final conversion of clopidogrel to its active metabolite.

Genetic studies are particularly difficult due to the numerous polymorphisms in the genes encoding CYP. CYP2C19, CYP3A4/5 and CYP1A2 are the most well-studied genetic variants. CYP2C19 alleles of type *2–*8 are these that impair the metabolism of clopidogrel. It was observed that having even 1 allele of CYP2C19*2 was associated with worse prognosis in a long-term follow-up of myocardial infarction with respect to the incidence of subsequent myocardial infarction, stroke and death. There have also been more cases of stent thrombosis in these patients.[3,4] The effect of the allele *17 in laboratory conditions is expressed by lower values for maximum platelet aggregation, better platelet inhibition and higher plasma concentrations of the active metabolite.[5] The clinical effect of increased transcriptional activity of this polymorphism was associated with more bleeding. There have been no significant benefits noticed in terms of ischemic events and stent thrombosis.[6]

In our research work, we evaluated the prevalence of polymorphism of major absorption genes (*ABCB1*) and clopidogrel metabolism (CYPC19 *2, *3, *17) in patients treated with coronary angioplasty with stent implantation due to ACS.

Material and method

A total of 199 patients (133 males and 63 females), mean age 65.2 ±11.9 years, randomized to the prospective study, were hospitalized for acute myocardial infarction, including unstable angina, with non ST-elevation myocardial infarction (NSTEMI), and ST-elevation myocardial infarction (ST-elevation MI). All patients engaged in the research were treated according to European Society of Cardiology (ESC) recommendations using coronary angioplasty with stent implantation. The study was approved by the appropriate research ethics committee, and all patients included in the study gave written informed consent. The basic demographic data of patients are presented in Table 1.

Table 1. Demographics

Study group	199
Sex, n (%) male female	 133 (66.8) 66 (33.2)
Age [years]	65.2 ±11.9
Caucasian race, n (%)	199 (100)
Residents of Lower Silesia, n (%)	183 (91.9)
STE-ACS, n (%)	110 (55.3)
NSTE-ACS/UA, n (%)	89 (44.7)
BMI [kg/m^2]	27.7 ±4.0
Diabetes, n (%)	40 (20.1)
Hypertension, n (%) 1st grade 2nd grade 3rd grade	 33 (16.6) 68 (34.1) 37 (18.6)
Serum creatinine concentration [mg/dL]	1.09 ±0.26
eGFR [mL/min/m^2]	70.69 ±18.81
Serum cholesterol [mg/dL]	203.74 ±48.76
LDL serum [mg/dL]	128.52 ±41.08
Smokers, n (%)	69 (34.6)
PLT [1,000/µL]	238.68 ±56.62
MPV [fL]	11.52 ±1.40
Hb [g/dL]	14.42 ±1.76

STE-ACS – ST-elevation acute coronary syndrome; NSTE-ACS – non ST-elevation acute coronary syndrome; UA – unstable angina; BMI – body mass index; eGFR – estimated glomerular filtration; LDL – low-density lipoprotein; PLT – platelet count; MPV – mean platelet volume; Hb – hemoglobin level.

Genetic research

In order to identify genetic polymorphism identification, genetic material was extracted from 200 µL of whole blood

samples of each patient using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Warszawa, Poland). Using the ability of the DNA to bind under certain conditions with silica, centrifugation of the lysate was carried out in a mini column containing the silica membrane, which was then rinsed twice with washing buffer. Finally, the membrane was given a mini column elution buffer to recover the purified DNA. Amplified polymerase chain reaction (PCR) was the next step with the use of 4 pairs of specific primers, respectively for: CYP2C19*17 (-3402C> T), CYP2C19*3, *ABCB1* (C3435C> T) and CYP2C19 *2, as well as Multiplex PCR Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Single-nucleotide (DNA) polymorphism of the CYP2C19*2, *3 and *ABCB1* genes was performed using a mini-sequencing technique, a PCR modification. The SNaPshot Multiplex Kit (Applied Biosystems – Thermo Fisher Scientific, Gdańsk, Poland) was used for the analysis according to the manufacturer's instructions. Purification of the duplicated genetic material was carried out with alkaline phosphatase digestion and exonuclease to eliminate primers and deoxynucleotides that were not consumed in PCR. The mini-sequencing reaction was performed with specific primers designed to hybridize to the template, ending before the designated polymorphic site. Dideoxynucleotide triphosphates (ddNTP), or fluorescent labeled terminators, were involved in the reaction. Product detection was performed with capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems – Thermo Fisher Scientific). The results were analyzed with the use of the GeneMapper ID v. 3.2 program (Applied Biosystems, Foster City, USA) against the internal GeneScan™ LIZ 120 standard. To assess the polymorphism of CYP2C17*17 (locus -3402 C>T), a restriction fragment length polymorphism (RFLP) technique was used.

Division into absorption and metabolic phenotypes

The study population was divided into the group of "good absorbers" (GA), which included the wild-type homozygote (CC), which lacked the C3435C> T spot mutation in the *ABCB1* gene, and the "poor absorbers" group (PA), which included the mutation heterozygotes (CT) and mutation homozygotes (TT) of the *ABCB1* gene.

Alleles that negatively affect clopidogrel metabolism (CYP2C *2, *3), and thus contribute to higher platelet reactivity in laboratory tests, have been assigned to the so-called loss-of-function (LoF) group. In contrast, alleles promoting the effects of clopidogrel (CYP2C *17), thus decreasing platelet aggregation, were given GoF (gain-of-function) name. For the purposes of the present work, patients were eligible for 2 metabolic groups, due to the polymorphism and the onset of the LoF or GoF allele:

– poor metabolizers (PM) – *2 or *3 carriers/non-*17 carriers – individuals with at least 1 LoF allele and no GoF allele;

– not poor metabolizers (NPM) – without the presence of any LoF allele (i.e., *2 and *3) and simultaneously having at least 1 GoF allele (i.e., *17) or patients without LoF (i.e., *2 and *3) and GoF (i.e., *17) or carriers simultaneously with both opposing alleles (*2 or *3 and simultaneously *17).

Patients with both PA and PM (PA + PM) phenotype were also isolated from the study group. The rest of the patients were referred to as not PA + PM.

Results

The basic demographic data of the study population is presented in Table 1. The data is typical of the population with ischemic heart disease.

Genetic research

The incidence of *ABCB1* polymorphism in the study group is shown in Fig. 1. The largest percentage of subjects were heterozygous (57.9%). The minority of the patients were identified as wild-type homozygotes (CC). The incidence of CYP2C19 *2 gene polymorphism is shown in Fig. 2. Most of the variants studied were patients with alleles coding for normal protein activity, but 26.1% of them had at least 1 mutation. No CYP2C19 *3 allele was observed in the study population to reduce the activity of clopidogrel metabolism. A relatively high percentage of heterozygotes and wild-type homozygotes in the CYP2C19*17 allele were found in the study group (Fig. 3).

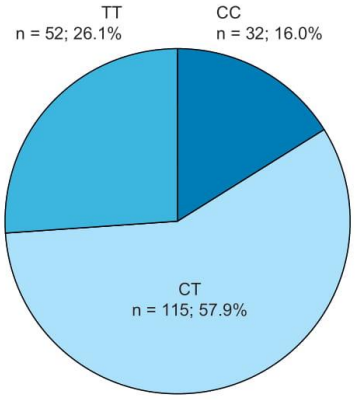

Fig. 1. Frequency of polymorphism of the *ABCB1* gene

CC – wild-type homozygote; CT – mutation heterozygote; TT – mutation homozygote.

Division into absorption and metabolic phenotypes

The incidence of clopidogrel GA and PA patients in the study population is shown in Fig. 4. The majority of the group were PA. Figure 5 shows the percentages

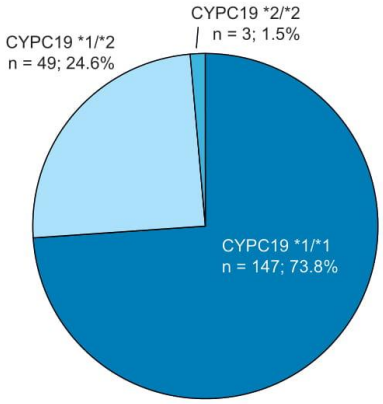

Fig. 2. Frequency of CYPC19*2 polymorphism

*1 – allele of normal CYP2C19 protein function; * 2 – CYP2C19 protein allelic dysfunction; *1/*1 – wild-type homozygote; *1/2 – mutation heterozygote; *2 – mutation homozygote.

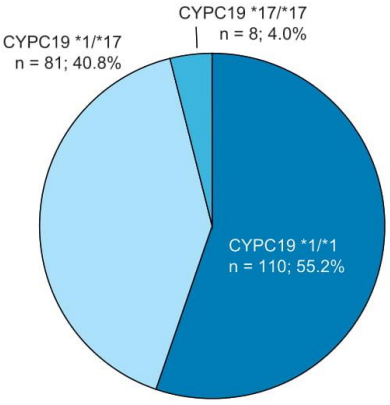

Fig. 3. Frequency of CYPC19 *17 polymorphism

*1 – allele of the normal function of the CYP2C19 protein, *17 – allele improving the function of the CYP2C19 protein, *1/*1 – wild-type homozygote, *17 – mutation heterozygote.

of individual metabolic phenotype in the study population. The relatively high proportion of patients (18%) was found to be PM patients. The simutaneous incidence of PA and PM phenotypes is shown in Fig. 6. As many as 29 patients in the study group were members of this group.

Discussion

In this work, genetic testing was performed in a homogeneous ethnic group of patients with ACS. An important element of the study was the high homogeneity of the study group. Each of the 199 categorized patients was Caucasian, and 92% of the study group were residents of Lower Silesia.

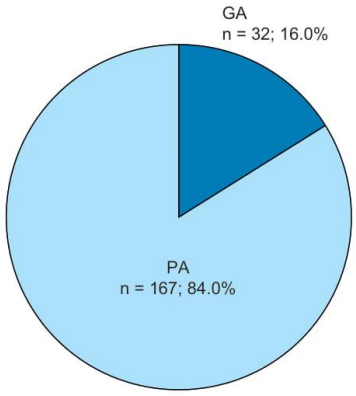

Fig. 4. Division into absorption phenotypes

PA – reduced absorption of clopidogrel; GA – good absorption of clopidogrel.

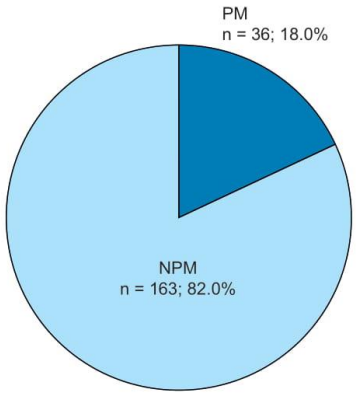

Fig. 5. Division into metabolic phenotypes

PM – poor metabolizers; NPM – not poor metabolizers.

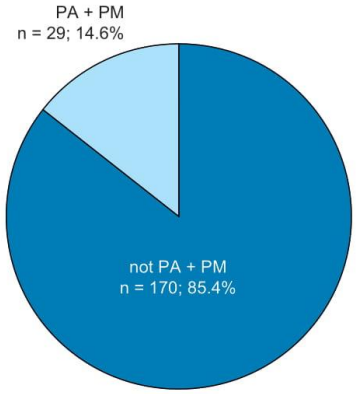

Fig. 6. Incidence of poor phenotype absorption and poor metabolism

PA + PM – reduced absorption plus poor metabolism.

The occurrence of *ABCB1* homozygous mutation polymorphism was demonstrated in 26.1% of the subjects (n = 52), which is comparable to the 27% of the carriers of the modified gene present within the large TRITON-TIMI 38 study, based on a population of nearly 3,000 patients.[7] Analyses concerning the variability of polymorphisms in various ethnic groups have revealed the prevalence of mutation homozygotes in the indigenous population of South America (27%), among Caucasians (20%) and Africans (13%).[8] The distribution of polymorphisms in *ABCB1* genes in the present study (CC 16.0%, CT 57.9%, TT 26.1%) is almost identical to that observed in large, randomized, multicenter studies. The obtained data correlates well with the analysis of genotypes of healthy volunteers in West Pomerania[9] and are slightly different with regards to wild-type homozygotes and heterozygotes among ACS patients in the population of Warszawa (CC 28%, CT 51%, TT 21%). However, the homozygous mutations percentage is very alike in different groups, as demonstrated in the study by Śpiewak et al.[10]

The incidence of particular polymorphisms of the CYP2C19 variant depends on the ethnic group under consideration. The differences apply to each of the alleles being analyzed.

One of the dysfunctional alleles, CYP2C19*2, has been found in the East Asian population in the amount of up to 55%. Among Africans, this polymorphism was observed in 40% of the subjects, and in the Caucasian population in Europe, the prevalence of CYP2C19 *2 was reported in 30% of the subjects.[11] In France, Collet et al. analyzed 259 patients with ACS episode. In their work, the wild-type homozygotes group of CYP2C19*1/*1 variant embraced 72% of the subjects, heterozygote *1/*2 25%, and mutation homozygote *2/*2 – only 2%.[4] Almost identical results were obtained in German and Portuguese populations, and only slightly different results – in Hungarian population.[12–14] As far as we know, the research on the prevalence of this polymorphism in the Polish population was conducted only once in a study by Malek et al.[15] The authors reported that wild-type homozygotes appear in 80% of the population, heterozygotes in 18% and mutation homozygotes in 2%. Our study has shown 73.9% of wild-type homozygotes, 24,6% of heterozygotes and 1,5% of mutation homozygotes; therefore, the data confirms the predecessors' observations.

The CYP2C19 *3 allele frequencies tend to vary across human populations. Most often this polymorphism is observed in East Asia. The incidence of *1/*3 genotype was reported by Jeong et al. at the level of 11%.[16] In Africa and Europe, this allele is much less frequent and sporadically exceeds 1%. In the Italian population, this percentage was reported at 1.6%[17] and in Russian population at 0.3%.[18] In our group of 188 patients, there was no single CYP2C19*3 mutation. To the best of our knowledge, our study is the first work which investigates the incidence of the CYP2C19*3 allele in the Polish population. The results confirm poor inspection of the discussed allele in this part of Europe.

Recently, CYP2C19*17 allele has been the most frequently reported polymorphism. In Europe, the heterozygous form of CYP2C19*17 allelic variant can be found in 31.7% of the population on average and the mutation homozygous – in 4.2%. Thus, the differences concern populations all over Europe. In Germany, the incidence of at least 1 allele *17 was reported at the frequency of 41%, in France 20% and in Sweden 18%.[5,6,11] In Poland, Kurzawski et al. analyzed the distribution of these polymorphisms in a group of 125 patients and found allele *17 in 27.2% of patients.[20] Widespread dissemination of the allele polymorphism in Africa is rarely reported in literature, but some reports indicate a frequency similar to that of the Caucasian population.[21] The incidence of the allele *17 in the Japanese population is estimated at 1.3%[22] and Chinese at 4%.[5] In our study on CYP2C19*17 polymorphisms, a higher incidence of this allele (40.8% heterozygous individuals and 4% homozygotes) was found, compared to the study by Kurzawski et al.[9] Nevertheless, when results obtained in this study are compared with those from a large group of over 1,500 German patients, one will find them to be very similar.[6] They also correspond to the results of studies conducted and published in Poland by Kubica et al.[23]

In the study, the prevalence of the phenotype absorption and metabolism was assessed for the first time in the Polish population. As many as 14.6% of patients showed a cumulative occurrence of the polymorphism of the investigated genes which condition the malabsorption of clopidogrel and its impaired metabolism. The use of clopidogrel in this group of patients may pose a particular risk of achieving an inadequate effect of the drug and, hence, the risk of thromboembolic complications with all clinical implications.

The reported variability in polymorphism frequency, with confirmation of increased platelet reactivity despite clopidogrel administration, is a serious clinical problem. The presence of these polymorphisms is an independent and contributing factor to HPR and thromboembolic complications following ACS. This data, confirming a relatively high level of penetration in the area of clopidogrel polymorphisms, should prompt Polish clinicians to recommend firstly the most recent antiplatelet drugs (ticagrelor or prasugrel), in spite of significantly higher costs of such pharmacotherapy for their patients.

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6.2 Publikacja nr 2

Original papers

Original and generic clopidogrel: A comparison of antiplatelet effects and active metabolite concentrations in patients without polymorphisms in the *ABCB1* gene and the allele variants CYPC19*2 and *3

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Ticagrelor and prasugrel are widely used as antiplatelet therapy after coronary angioplasty. However, there is a group of patients with indications for clopidogrel treatment. This population includes patients with chronic or acute coronary syndrome who are treated invasively and have contraindications to the use of novel antiplatelet drugs due to antithrombotic treatment (particularly with non-vitamin K antagonist oral anticoagulants). A wide range of generic forms of clopidogrel are available on the market. However, it is unclear whether they are as effective as the originator drug.

Objectives. In the current study, we aimed to assess the concentrations of the active metabolite of clopidogrel and its effect on platelet aggregation inhibition in patients receiving the originator drug in comparison with those receiving generic clopidogrel.

Materials and methods. We enrolled 22 healthy individuals without polymorphisms in the *ABCB1* gene and the allele variants *CYPC19*2* and *CYPC19*3*. All participants received a loading dose of clopidogrel (600 mg), followed by a maintenance dose of 75 mg for the next 3 days. On day 3, blood samples were obtained 1 h after drug administration to assess active metabolite concentrations using liquid chromatography with tandem mass spectrometry. In each participant, platelet aggregation was assessed with light transmission aggregometry after 5-µmol/L and 10-µmol/L adenosine diphosphate (ADP) stimulation. Assays were performed for the originator clopidogrel and 2 different generic groups.

Results. The mean ± standard deviation (SD) concentrations of active clopidogrel did not differ between the originator drug and 2 generic products with clopidogrel (12.7±5 pg/µL compared to 13.0 ±4 pg/µL compared to 14.4 ±4 pg/µL). Platelet aggregation inhibition after stimulation with 5 µmol/L and 10 µmol/L ADP was similar for all preparations.

Conclusions. In comparison with original clopidogrel, the use of its generic form does not affect the blood concentrations of the active metabolite or its antiplatelet effect.

Key words: *ABCB1*, clopidogrel active metabolite, *CYPC19*2*, *CYPC19*3*, clopidogrel bioequivalence

Introduction

Although ticagrelor and prasugrel are increasingly widely used as antiplatelet therapy after coronary angioplasty, there is a large group of patients with indications for clopidogrel treatment. This population includes primarily symptomatic patients with chronic coronary syndrome and acute coronary syndrome (ACS), who are treated invasively, and who have contraindications to the use of novel antiplatelet drugs due to concomitant atrial fibrillation and antithrombotic treatment (particularly with non-vitamin K antagonist oral anticoagulants).[1,2] Several generic forms of clopidogrel are available on the market. However, it is unclear whether they are as effective in daily clinical practice as the originator drug. It has been shown that agreement between platelet function measurements is relatively poor in patients receiving original and generic clopidogrel bisulfate forms.[3] Thus, physicians may be cautious when routinely introducing generic clopidogrel bisulfate. On the other hand, risks of mortality, bleeding and drug discontinuation were not different between Plavix and generics.[4] The available evidence is therefore limited and does provide sufficient data on differences in efficacy or safety between branded and generic products.

Clopidogrel is a prodrug metabolized to its active form through complex biochemical processes in the liver.[5,6] Its absorption is regulated by glycoprotein P, a transport protein encoded by the *ABCB1* gene. Eight-five percent of the absorbed drug is transformed by carboxyl esterases into a major but inactive clopidogrel metabolite – a carboxylic acid derivative. Only 15% of the absorbed clopidogrel is transformed by cytochrome P450 (CYP) isoenzymes (*CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP3A4*) into a thiol metabolite, which is responsible for blocking adenosine diphosphate (ADP) binding to the platelet $P2Y_{12}$ receptor and ADP-induced platelet aggregation.

Objectives

In the current study, we aimed to assess the concentrations of clopidogrel active metabolite as well as its effect on platelet aggregation inhibition, in patients receiving the originator drug in comparison with those receiving generic clopidogrel. Active metabolite generation following clopidogrel administration is diminished by limited intestinal absorption (which may be influenced by the *ABCB1* gene polymorphism), as well as by functional variability in the activity of the CYP isoenzymes (which is influenced by single nucleotide polymorphisms (SNPs) in genes encoding the CYP isoenzymes).[7] Therefore, to exclude genetic variability that might affect drug concentration and activity, participants were assessed for the presence of the most common genetic polymorphisms that reduce the absorption (*ABCB1*) and activation (*CYPC19*2* and *CYPC19*3*) of clopidogrel.

Materials and methods

Study population

We enrolled 22 healthy, non-smoking participants, who provided written informed consent to be included in the study. The study protocol was approved by the bioethics committee of Wroclaw Medical University, Poland. None of the participants were carriers of polymorphisms in the *ABCB1* gene or the allele variants *CYPC19*2* or *CYPC19*3*. The study protocol was approved by the bioethics committee of Wroclaw Medical University, and it was also in line with the Helsinki Declaration.

Genetic studies

To identify genetic polymorphism, genetic material was extracted from 200 µL of whole-blood samples of each patient, using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Warszawa, Poland). Using the ability of the DNA to bind with silica under certain conditions, the lysate was centrifuged in a mini-column containing the silica membrane, which was then rinsed twice with a washing buffer. Finally, a mini-column elution buffer was applied to the membrane to recover the purified DNA. Next, amplified polymerase chain reaction (PCR) was carried out with the use of 3 pairs of specific primers for *CYP2C19*3*, *ABCB1* (C3435C> T) and *CYP2C19*2*, as well as a Multiplex PCR Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A single nucleotide polymorphism of *CYP2C19*2*, *CYP2C19*3* and *ABCB1* was genotyped using a mini-sequencing technique, which is a modification of PCR. The SNaPshot Multiplex Kit (Applied Biosystems, Foster City, USA) was used for the analysis, according to the manufacturer's instructions. The mini-sequencing reaction was performed with specific primers designed to hybridize to the template, ending before the designated polymorphic site. Dideoxynucleotide triphosphates, or fluorescent-labeled terminators, were involved in the reaction. Product detection was performed with capillary electrophoresis, using a 3130 Genetic Analyzer (Applied Biosystems). The results were analyzed with the use of the GeneMapper ID v. 3.2 program (Applied Biosystems) against the internal GeneScan™ LIZ 120 standard.

Clopidogrel administration, blood collection and plasma preparation

At baseline, all participants received a loading dose of original clopidogrel (600 mg), followed by a maintenance dose of 75 mg for the next 3 days. On day 3, blood samples of 7.5 mL were drawn into collection tubes containing ethylenediaminetetraacetic potassium salt, to assess the concentrations of the drug active metabolite in patients' plasma. Samples were obtained 1 h after drug administration (C1h), taking into account the high active

metabolite concentration reported in previous pharmacokinetic studies.[7] Due to the irreversible nature of the receptor modification, subsequent clopidogrel preparations were analyzed after 1 week, which ensured a natural restoration of the platelet pool. In this crossover study, assays were performed separately for the originator drug – Plavix (Sanofi-Aventis, Paris, France) (P) and 2 different generic forms – Areplex (Adamed, Pieńków, Poland) (A) and Egitromb (Egis Pharmaceuticals, Budapest, Hungary) (E). Each subject received generic products and the reference product. All drugs contained clopidogrel bisulfate as the active substance.

Sample preparation

To stabilize the active clopidogrel metabolite, 2-bromo-3'-methoxyacetophenone (MPB) was added to the blood sample immediately after collection, in accordance with the procedure described by Takahashi et al.[8]

The MPB-derivatized clopidogrel active metabolite hydrochloride and [13C6]-clopidogrel carboxylic acid (internal standard) were purchased from Alsachim (Illkirch-Graffenstaden, France). Clopidogrel and clopidogrel carboxylic acid were obtained from the Pharmaceutical Research Institute (Warszawa, Poland). Liquid chromatography mass spectrometry (LC-MS) grade water, methanol, and acetonitrile were obtained from J.T. Baker (Deventer, the Netherlands). The formic acid (purity ≥98%), trichloroacetic acid (purity ≥99.5%) and MPB were purchased from Sigma-Aldrich (Poznań, Poland), while leucine-enkephalin was sourced from Waters (Warszawa, Poland).

Plasma concentrations of clopidogrel active metabolite hydrochloride, clopidogrel and clopidogrel carboxylic acid were quantified using stable-isotope dilution LC-MS, according to a modified method adapted from Karaźniewicz-Łada et al.[7] Briefly, a volume of 100 µL of either plasma sample or internal standard was combined with 20 µL of internal standard solution (500 pg/µL). Then, 400 µL of acetonitrile was added and vortexed for 5 min at 1100 rpm. After additional centrifugation, the supernatant was analyzed using LC with tandem MS (LC-MS/MS).

Liquid chromatography – tandem mass spectrometry

The LC-MS/MS analysis was conducted using the nanoACQUITY UPLC system, combined with a Xevo G2 QT of mass spectrometer (Waters). The analyzed compounds were separated in the HSS C18 column with membrane inline filter (Waters). The column temperature was set at 45°C. Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisted of 0.1% formic acid in acetonitrile with an increasing gradient. The total run time of the method was 4 min, with a flow rate of 45 µL/min.

Mass spectra for the analyzed compounds were acquired in positive ion mode electrospray ionization. Data acquisition was performed with MassLynx Software (Waters), using the characteristic precursor and product ions. A quantitative analysis was also performed using QuanLynx software (Waters). We considered a range of 80–125% as an acceptance interval criterion for the clopidogrel mean concentration ratio for each tested product. For the measurement of clopidogrel active metabolite, a previously known measurement method was used, which was subject to detailed validation.[7]

Platelet aggregation

For each participant, platelet aggregation was assessed on day 3 of treatment with each preparation. Blood samples were obtained from a venous cannula into 2 tubes containing 0.109 mol/L of trisodium citrate, and centrifuged at room temperature (800 × g for 15 min) to collect platelet-rich plasma. The sample was re-centrifuged at 2400 × g for 15 min, and platelet-poor plasma was collected. Platelet reactivity was assessed within 2 h of collection, with light transmission aggregometry as developed by Born,[9] using a single-channel Chrono Log 560CA lumi-aggregometer (Chrono-Log, Haverton, USA). A platelet-poor plasma sample with 100% light transmission was used as a reference. Next, consecutive platelet-rich plasma samples (light transmission, 0%) were placed in cuvettes and stimulated with adenosine diphosphate (ADP; 5 µmol/L and 10 µmol/L). The results were expressed as percentage of the maximum platelet aggregation (MPA) within 6 min. Calculations and platelet aggregation curves were performed using the dedicated Agro-Link software (Chrono-Log). All reagents and laboratory equipment were purchased from Biogenet (Piaseczno, Polska). They were stored and used according to the manufacturer's instructions.

Cut-off MPA values higher than 46% and 67% were used to identify the lack of response to stimulation with 5 µmol/L and 10 µmol/L ADP, respectively.

Statistical analysis

The statistical analyses were performed using the STATISTICA v. 9.0 PL program (StatSoft Inc., Tulsa, USA). The variance homogeneity of each quantitative variable was determined using Levene's test. To compare the quantitative variables between groups, a one-way analysis of variance (ANOVA) was used. All hypotheses were verified at the statistical significance level $p \leq 0.05$.

Results

Demographic and clinical features of participants are presented in Table 1. The genetic analysis revealed that none of the participants were carriers of genetic polymorphisms responsible for reduced clopidogrel absorption and metabolism.

Table 1. Study population characteristics

Parameter	Study cohort
Age [years]	32 ±6.5
Sex (F/M)	8/14
Diabetes	0
Hypertension	0
Smoking	0
Hemoglobin [g/dL]	12.3 ±1.3
White blood cells [10^3/μL]	7.3 ±0.4
Creatinine [mg/dL]	0.9 ±0.4
Creatinine clearance [mL/min]	106.7 ±11.2
Body mass [kg]	62.7 ±16.3
BMI	24.6 ±4.2

SI conversion factors: to convert creatinine to μmol/L, multiply by 88.4. F – female; M – male; BMI – body mass index. Data are presented as mean ± standard deviation (SD).

We did not find any significant differences in the C1h concentrations of active clopidogrel between different preparations, specifically 12.7 ±5 pg/μL in group P, compared to 13.0 ±4 pg/μL in group A and 14.4 ±4 pg/μL in group E. This data is presented in Fig. 1.

Mean platelet aggregation inhibition values were similar for all drugs, without any significant differences, both after stimulation with 5 μmol/L ADP and 10 μmol/L ADP. Aggregation data are shown in Table 2.

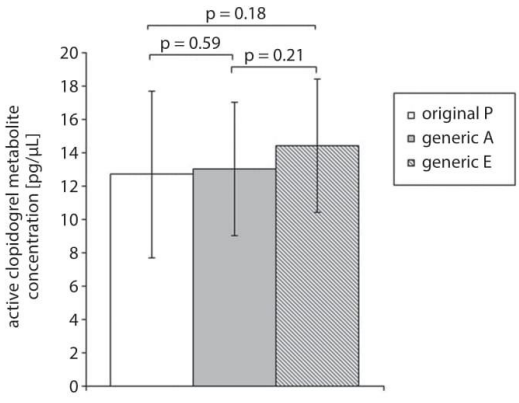

Fig. 1. Concentrations of clopidogrel active metabolite for original and generic drug

Table 2. Platelet aggregation values after ADP stimulation

Platelet aggregation	P	A	E
5-μmol/L ADP	34.5 ±9	35.0 ±7	36.6 ±8
10-μmol/L ADP	40.7 ±9	40.0 ±8	37.1 ±8

Data are presented as mean ± standard deviation (SD). ADP – adenosine diphosphate.

Discussion

Multiple lines of evidence suggest that insufficient active metabolite generation is the primary reason for variability in clopidogrel response and the lack of response where a negligible antiplatelet effect of clopidogrel is observed. High platelet reactivity to clopidogrel has been found to be associated with a significantly higher incidence of ischemic recurrence in patients undergoing percutaneous coronary intervention with stent implantation.[1,2,5] Therefore, the equal efficacy of generic drugs to that of the originator remains an important issue.

The cut-off values for defining clopidogrel non-responsiveness using aggregometry are often arbitrary.[10] The most important studies focusing on platelet activity used various concentrations of the $P2Y_{12}$ receptor inhibitor. The most common agonist in the largest studies was 5 μmol/L and 20 μmol/L ADP. In a group of 1069 patients with established chronic coronary syndrome undergoing percutaneous coronary intervention, Breet et al.[10] showed that an MPA of 42.9% or higher for 5 μmol/L ADP and of 64.5% or higher for 20 μmol/L ADP was associated with an increased risk of death, myocardial infarction, stent thrombosis, and ischemic stroke during a one-year follow-up. Gurbel et al.[11] reported a higher risk of cardiovascular events in a two-years follow-up in patients with an MPA higher than 46% and 59%, respectively. Cuisset et al.[12] revealed an elevated risk of stent thrombosis for an aggregation cut-off value higher than 67%. In 2010, Bonello et al.[13] published a consensus statement in which they proposed an ADP (5 μmol/L)-induced MPA of 46% as a cut-off value to identify high platelet reactivity. In our study, we accepted the recommended threshold of 46% for MPA induced by 5 μmol/L ADP, while for 10 μmol/L ADP, the cut-off value of more than 67% was used to identify an inadequate response to clopidogrel, as in the study by Cuisset et al.[12]

The quantitative assessment of the active form of clopidogrel in blood is a complex process, particularly due to the short half-life of the drug. High-performance liquid chromatography assays are not sensitive enough to measure clopidogrel levels in biological fluids after oral administration of therapeutic doses. The LC-MS/MS has been increasingly used because it can analyze test samples regardless of their purity in biological substances and measure drug concentrations in blood with high sensitivity and selectivity.[14,15] In our study, we used modified methods that allow for the stabilization of the active metabolite in blood.[8] The time of blood sampling, 1 h after drug administration, was chosen based on previous clopidogrel pharmacokinetic studies.[7] We decided to test each clopidogrel product after 1 week of treatment discontinuation, as it has been proven that complete recovery of platelet function can be seen 7 days after the last clopidogrel dose.[16]

We showed that the use of generic forms of clopidogrel does not significantly affect the blood concentrations of the active metabolite in healthy individuals with

the absence of the most common genetic polymorphisms (*ABCB1*, *CYPC19*2* and *CYPC19*3*). Moreover, none of our participants showed a lack of response to antiplatelet treatment, expressed as a low rate of platelet aggregation inhibition. Therefore, the original clopidogrel and the tested generic forms can be considered equivalent.

Our findings are in line with previous studies that revealed similar efficacy of the original and generic forms of clopidogrel in patients with ACS and chronic coronary syndrome.[17,18] However, other studies have reported contradictory results.[19,20] An Italian study evaluated 1579 patients with ACS and found that a significantly higher proportion of patients treated with clopidogrel base had high platelet reactivity when compared with original clopidogrel. However, it is important to note that clopidogrel base is a generic preparation that differs from original clopidogrel, which is formulated using clopidogrel bisulfate. In contrast, in our study, both generic and original clopidogrel contained the same salt.

Limitations

Our study has several limitations. First, the study group was relatively small, and we did not assess the presence of other polymorphisms and other variables that might have affected drug concentrations in blood. However, our results indicate that the generic preparations of clopidogrel bisulfate tested have similar efficacy to original clopidogrel and thus may be used in clinical practice.

Conclusions

In comparison with original clopidogrel, the use of its generic form does not affect the blood concentrations of the active metabolite or its antiplatelet effect.

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6.3 Publikacja nr 3

ADVANCES IN INTERVENTIONAL CARDIOLOGY

The influence of acute coronary syndrome on the levels of clopidogrel active metabolite and platelet inhibition in patients with and without CYP2C19 and ABCB1 gene polymorphisms

Type

Original paper

Keywords

ABCB1, acute coronary syndrome, CYP2C19, active clopidogrel metabolite

Abstract

Introduction
Although ticagrelor and prasugrel remain the standard of antiplatelet treatment in acute coronary syndrome (ACS), numerous patients still present with indications for clopidogrel use.

Aim
We aimed to assess the levels of clopidogrel active metabolite and to evaluate the effect of the drug on platelet inhibition in patients with ACS as compared with those with stable coronary disease. Patients were assessed for the presence of the most common genetic polymorphisms that reduce the absorption (ABCB1) and activation (CYP2C19*2 and CYP2C19*3) of clopidogrel to exclude the effect of genetic variability on drug concentrations and activity.

Material and methods
This single-center, open-label, prospective study included 199 patients hospitalized due to ST-segment elevation myocardial infarction (STEMI) or non-STEMI (NSTEMI) in Killip class I-III, who underwent percutaneous coronary intervention. The control group included 22 patients with stable coronary artery disease.

Results
The mean (SD) levels of active clopidogrel were 17.1 (12.3) ng/ml in controls and 16.4 (12.0) ng/ml in the whole study group (P <0.68). No differences were noted in clopidogrel levels between patients with STEMI and NSTEMI (mean [SD], 17.6 [2.3] ng/ml and 15.1 [11.5] ng/ml; P <0.45) or between STEMI and NSTEMI groups and controls (P <0.38 and P <0.61, respectively). No effect of ABCB1 and CYP2C19 polymorphism was observed in the study subgroups.

Conclusions
We showed that ACS does not affect the levels of clopidogrel active metabolite or platelet inhibition in patients in Killip class I-III with or without CYP2C19 or ABCB1 gene polymorphisms.

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ADVANCES IN INTERVENTIONAL CARDIOLOGY

The influence of acute coronary syndrome on the levels of clopidogrel active metabolite and platelet inhibition in patients with and without CYP2C19*2(681 G>A),*3(636 G>A) and ABCB1(C3435C> T) gene polymorphisms

Short title: Clopidogrel active metabolite in acute coronary syndrome

ABSTRACT

Introduction. Although ticagrelor and prasugrel remain the standard of antiplatelet treatment in acute coronary syndrome (ACS), numerous patients still present with indications for clopidogrel use.

Aim. We aimed to assess the levels of clopidogrel active metabolite and to evaluate the effect of the drug on platelet inhibition in patients with ACS as compared with those with stable coronary disease. Patients were assessed for the presence of the most common genetic polymorphisms that reduce the absorption (*ABCB1*) and activation (*CYP2C19*2* and *CYP2C19*3*) of clopidogrel to exclude the effect of genetic variability on drug concentrations and activity.

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subgroups.

Conclusions. We showed that ACS does not affect the levels of clopidogrel active metabolite or platelet inhibition in patients in Killip class I-III with or without *CYP2C19* or *ABCB1* gene polymorphisms.

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Key words

active clopidogrel metabolite, acute coronary syndrome, *ABCB1*, *CYP2C19*

Introduction

Although ticagrelor and prasugrel have become the standard of antiplatelet treatment in acute coronary syndromes (ACSs), a large group of patients still present with indications for the use of clopidogrel. In particular, this refers to patients with stable coronary artery disease who are treated invasively as well as patients with ACS who have contraindications to the use of novel antiplatelet drugs (eg, due to a high risk of bleeding associated with antithrombotic treatment) [1-3].

Clopidogrel is a prodrug that is metabolized to its active form by cytochrome p450 via complex biochemical processes that occur in the liver. The absorption of clopidogrel is regulated by glycoprotein P [4]. Only the active metabolite of clopidogrel can block the $P2Y_{12}$ receptor, thus leading to inhibition of platelet activation and aggregation. It is believed that the clinical status of patients may affect clopidogrel metabolism [5,6], which, in turn, may influence the antiplatelet effect of its active metabolite.

In the current study, we aimed to assess the levels of clopidogrel active metabolite with liquid chromatography – tandem mass spectrometry (LC-MS/MS) and to evaluate the effect of the drug on platelet inhibition in patients with ST-segment elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI). To exclude genetic variability that might have affected the drug concentration and activity, patients were also assessed for the presence of the most common genetic polymorphisms that reduce the absorption (*ABCB1*) and activation (*CYP2C19*2* and *CYP2C19*3*) of clopidogrel.

Material and Methods

Study population

This single-center, open-label, prospective study included 199 patients hospitalized due to

NSTEMI (n=91) or STEMI (n=108), who underwent percutaneous coronary intervention (PCI) with drug-eluting stent implantation. Patients with cardiogenic shock and those with Killip class IV were excluded. All patients provided written informed consent to participate in the study, and the study was approved by the local ethics committee.

The control group included 22 patients with stable coronary artery disease treated with PCI. All participants of the control group were tested to exclude the presence of *ABCB1* as well as *CYP2C19*2* and *CYP2C19*3* gene polymorphisms.

All patients provided written informed consent to participate in the study, and the study was approved by the Wroclaw Medical University Ethics Committee.

Clopidogrel administration, blood collection, and plasma preparation

All participants received a loading dose of clopidogrel containing clopidogrel bisulfate as the active substance (600 mg), followed by a maintenance dose of 75 mg for the next 3 days. On day 3, blood samples were obtained 1 hour after drug administration to assess the concentrations of the active metabolite.

Sample preparation

The method used to assess clopidogrel active metabolite was described in our previous study [7]. Briefly, to stabilize the active clopidogrel metabolite, immediately after blood collection 2-bromo-30-methoxyacetophenone (MPB) was added to the sample. The MPB-derivatized clopidogrel active metabolite hydrochloride and [13C6]-(±)-clopidogrel carboxylic acid (internal standard) were purchased from Alsachim (Illkirch-Graffenstaden, France). Clopidogrel and clopidogrel carboxylic acid were obtained from Pharmaceutical Research Institute (Warsaw, Poland). Liquid chromatography – mass spectrometry (LC-MS) grade water, methanol, and

acetonitrile were obtained from J.T. Baker (Deventer, the Netherlands). The formic acid (purity ≥98%), trichloroacetic acid (purity ≥99.5%), and MPB were purchased from Sigma-Aldrich (Poznań, Poland), while leucine-enkephalin, from Waters (Warsaw, Poland). The serum concentrations of clopidogrel active metabolite hydrochloride, clopidogrel, and clopidogrel carboxylic acid were quantified by stable-isotope dilution LC-MS, according to a modified method adapted from Karaźniewicz-Łady et al [4]. After additional centrifugation, the supernatant was analyzed using LC-MS/MS.

Liquid chromatography – tandem mass spectrometry

The LC-MS/MS analysis was performed using the nanoACQUITY UPLC system combined with a Xevo G2 QTof mass spectrometer (Waters, Warsaw, Poland). The analyzed compounds were separated in the HSS C18 column with membrane inline filter (Waters, Milford, Massachusetts, United States) at 45°C. Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisted of 0.1% formic acid in acetonitrile with an increasing gradient. The total run time of the method was 4 minutes, with a flow rate of 45 µl/min. Mass spectra for the analyzed compounds were acquired in positive ion mode electrospray ionization. Data acquisition was performed by means of the MassLynx Software (Waters, Warsaw, Poland), using the characteristic precursor and product ions. A quantitative analysis was performed using the QuanLynx software (Waters, Warsaw, Poland).

Platelet aggregation

Platelet aggregation was assessed on day 3 of clopidogrel treatment. Blood samples were obtained from a venous cannula into 2 tubes containing 0.109-mol/l trisodium citrate. Then, the blood was centrifuged at room temperature (800 × *g* for 15 minutes) to collect platelet-rich

plasma. The sample was recentrifuged at 2400 × *g* for 15 minutes, and platelet-poor plasma was collected. Platelet reactivity was assessed within 2 hours since collection by light transmission aggregometry developed by Born [6], using a single-channel Chrono Log 560CA lumi aggregometer (Chrono-log, Haverton, Pennsylvania, United States). A platelet-poor plasma sample with 100% light transmission was used as a reference. Consecutive platelet-rich plasma samples (light transmission, 0%) were placed in cuvettes and stimulated with adenosine diphosphate (ADP; 5 µmol/l and 10 µmol/l). The results were expressed as percentage of the maximum platelet aggregation (MPA) within 6 minutes. Calculations and platelet aggregation curves were performed using the dedicated AgroLink software (Chrono-log, Haverton, Pennsylvania, United States). All reagents and laboratory equipment were purchased from Biogenet (Piaseczno, Poland). They were stored and used according to the manufacturer's instructions. The cutoff MPA values of higher than 46% and 67% were used to identify the high on-treatment platelet reactivity (HPR) to stimulation with 5-µmol/l and 10-µmol/l ADP, respectively

Genetic studies

To identify ABCB1(rs1045642, c.3435C> T), CYP2C19*2 (rs4244285, c.681 G>A), and CYP2C19*3 (rs4986893, c.636 G>A) polymorphism, a genetic material was extracted from 200 µl of whole-blood samples of each participant, using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The lysate was centrifuged in a mini-column containing the silica membrane, which was then rinsed twice with a washing buffer. Finally, a mini-column elution buffer was applied to the membrane to recover the purified DNA. Next, polymerase chain reaction (PCR) was carried out with 3 pairs of specific primers for CYP2C19*3(636 G>A), ABCB1 (C3435C> T), and CYP2C19*2 (681 G>A) with use ~~a~~

Multiplex PCR Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. A single nucleotide polymorphism of *CYP2C19*2*, *CYP2C19*3*, and *ABCB1* was genotyped using a minisequencing technique, which is a modification of PCR. The SNaPshot Multiplex Kit (Applied Biosystems, Foster City, California, United States) was used for the analysis, according to the manufacturer's instructions. The minisequencing reaction was performed with specific forward and reverse primers designed to hybridize to the template, ending before the designated polymorphic site. Dideoxynucleotide triphosphates, or fluorescent-labeled terminators, were involved in the reaction. Product detection was performed with capillary electrophoresis, using a 3130 Genetic Analyzer (Applied Biosystems). The results were analyzed with the GeneMapper ID v. 3.2 software (Applied Biosystems) against the internal GeneScan™ LIZ 120 standard. Patients with at least one allele that negatively affects clopidogrel metabolism (*CYP2C19*2*, **3*), were assigned to the loss-of-function (LoF) group, as opposed to the normal-function group (NoF), which included patients with *CYP2C19*1*. The study population was also divided into so called "good absorbers" (the GA group homozygous for the wild-type allele at position 3435CC) and "poor absorbers" (the PA group with heterozygous [TC] and homozygous [TT] mutations of the *ABCB1* gene).

Statistical analysis

The statistical analysis was performed using the Statistica 9.0 PL program. The type of variable distribution was determined by the Shaipro-Wilk test. For the comparison of quantitative variables between two groups, depending on the distribution of the variable, the U'Mann-Whitney or T-student test were used. For more than two groups and variables with an abnormal distribution the ANOVA Rang Kruskal Wallis was used. The chi-square test was used to compare

158 the frequency of the occurrence of the qualitative variable between the groups.

159 **Results**

160 There were no significant differences in the demographic data between the study and control
161 groups (Table 1). The results of exact genotype distributions are briefly summarized in Table 2.
162 There were no *CYPC19*3* allel carriers in the entire population.
163 The median (IQR) levels of active clopidogrel in 22 controls were 16.7 (9.0-21.1) ng/ml, as
164 compared with 14.9 (6.9-21.9) ng/ml for the whole study group (P <0.59). A subgroup analysis
165 revealed no differences in the levels of active clopidogrel between patients with STEMI and
166 those with NSTEMI (median [IQR], 16.1 [7.8-23.4] ng/ml and 13.6 [9.5-19.1] ng/ml; P <0.32).
167 No differences were also noted between patients with STEMI and controls or between those with
168 NSTEMI and controls (P <0.33 and P <0.54, respectively). Moreover, the levels of active
169 clopidogrel did not differ between the NoF and LoF groups (P <0.47) or between the PA and GA
170 groups (P <0.71). The simultaneous presence of the *ABCB1* and *CYP2C19*2* polymorphisms
171 (PA+LoF) was not associated with lower drug levels in comparison with the GA+NoF group (P =
172 0.28). There were no significant differences between the individual genetic subgroups in the
173 whole study population (Figure 1 A) or separately in patients with STEMI and NSTEMI (Figure
174 1 B and 1 C).
175 Platelet inhibition, both after stimulation with 5-µmol/l and 10-µmol/l ADP, was similar in all
176 study subgroups as well as controls (Figure 2 A, B, C and Figure 3 A, B, C).
177 High on-treatment platelet reactivity was noted in 26.7% of controls, as compared with 28.1% of
178 the whole study population (P = 0.89). Moreover, there were no differences in HPR between the
179 STEMI group [29,6%] and controls (P = 0.76) or between the NSTEMI group [27,1%] and

controls ($P = 0.83$).

In a linear regression model including clinical data and genetic analysis, no relationship was found between these variables and the concentration of clopidogrel active metabolite.

Discussion

A short half-life of active clopidogrel complicates the quantitative assessment of its blood levels. To date only few high-performance liquid chromatography assays appeared sensitive enough to measure active clopidogrel levels in human plasma [8]. In our study, we used modified methods that allowed us to stabilize the clopidogrel active metabolite in blood.[7] To assess its concentrations, we decided to use LC-MS/MS because it can determine the drug concentrations in blood with high sensitivity and selectivity [9,10].

The cutoff values for defining clopidogrel nonresponsiveness using aggregometry are often arbitrary. In 2010, Bonello et al [11] published a consensus statement in which they proposed a 5-µmol/l ADP–induced MPA of 46% as a cutoff value to identify HPR. In our study, we accepted this recommended threshold for MPA induced by 5-µmol/l ADP, while for 10-µmol/l ADP, the cutoff value of more than 67% was used to identify inadequate response to clopidogrel, similarly to a study by Cuisset et al.[12]

Multiple data suggest that insufficient active metabolite generation is a significant reason for variability in clopidogrel response. High on-clopidogrel platelet reactivity has been reported to be associated with a significantly higher incidence of ischemic adverse effects in patients undergoing PCI. There is evidence suggesting that the severity of the patient's clinical status significantly affects clopidogrel metabolism, which consequently influences the concentrations and antiplatelet effects of its active metabolite. Among patients admitted to intensive care units, the number of poorly responding individuals was reported to range from 65% to 80%. [5] The

generation of clopidogrel active metabolite was markedly reduced when compared with healthy volunteers or other patient groups.[13-15]. In a study by Součková et al [6], including patients undergoing successful cardiopulmonary resuscitation who received clopidogrel because of a PCI, clopidogrel bioavailability and platelet inhibition were diminished when compared with patients in a stable clinical condition. [6]

Our findings show that in patients with lower cardiovascular burden (ie, patients with STEMI and NSTEMI but excluding those with cardiogenic shock and those with Killip class IV), ACS is not associated with changes in the blood levels of clopidogrel active metabolite or platelet aggregation in comparison with patients with stable coronary artery disease. No differences in the analyzed parameters were also shown between patients classified according to the type of ACS (STEMI vs NSTEMI).

Our data on the incidence of HPR are in line with previous studies, which reported the incidence of HPR in about 30% of the population [16,17].

Considering the available data on the possible effect of the *CYP2C19* and *ABCB1* polymorphisms on the bioavailability of clopidogrel and its antiplatelet effects, we decided to assess platelet aggregation and the levels of clopidogrel active metabolite in combination with genotyping.

Precise data on *ABCB1* and *CYP2C19* genotype distribution observed in our population (Summarized in Table 2) was published previously [7]. Results were very similar to previous observations in Caucasian population [18,19] and confirm poor inspection of the *CYP2C19* *3 polymorphism in this part of Europe.

Our study revealed no correlations between *ABCB1* and *CYP2C19*2* polymorphisms and the drug concentrations or platelet aggregation.Available data on the effect of *ABCB1* and *CYP2C19* polymorphisms on the concentrations of clopidogrel active metabolite and platelet aggregation are contradictory [20]. The *ABCB1* C3435T polymorphism has been associated with changes in

the intestinal efflux of drugs and thus their bioavailability [21]. A clinical study in patients undergoing PCI reported that peak plasma concentrations and area under the curve of clopidogrel active metabolite following the administration of a loading dose of clopidogrel were significantly lower in 3435T/T homozygotes than those in 3435C/T heterozygotes or 3435C/C (wild-type) homozygotes [22]. However, these results were not confirmed by subsequent studies for the maintenance clopidogrel doses of 75 mg or 150 mg [23,24]. Our findings are in line with the results of 2 meta-analyses showing that the *ABCB1* C3435T polymorphism is unlikely to play a major role in between-subject variability in response to clopidogrel [25,26].

The available data on the *CYP2C19* polymorphism on the blood levels of clopidogrel active metabolite were mostly derived from studies on young healthy volunteers. A major study on the effect of genes on the hepatic metabolism of clopidogrel, conducted in a group of healthy volunteers, demonstrated that the carriers of a mutant allele of the *CYP2C19* gene had lower plasma levels of clopidogrel by 32.4% and higher platelet aggregation by 25% as compared with noncarriers.[18] Similar findings were reported by Kelly et al [27], who studied 90 healthy young Chinese subjects. We lack big data in older population with high cardiovascular burden, only Xia-Quin et al showed that the genetic polymorphisms of *CYP2C19**2 and *ABCB1* affect the pharmacokinetic and pharmacodynamic responses to clopidogrel in patients with ACS [14]. More convincing data refer only to the effect of the *CYP2C19* polymorphism on the clinical course of ACS in patients receiving clopidogrel. However, there is no information on how the polymorphism affects drug concentrations and platelet aggregation. In the TRITON-TIMI 38 trial (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction 38) the carriers of *CYP2C19* reduced-function allele had an increased risk of ischemic adverse events [18]. Similarly, studies reported that *CYP2C19* reduced-function alleles were associated with worse clinical outcomes, including

cardiovascular death, myocardial infarction, and stroke [28-31]. This observation was confirmed by several meta-analyses [32-34].

Importantly, also in our studies healthy volunteers without *CYP2C19*2* and *ABCB1* polymorphisms had higher levels of clopidogrel active metabolite (P <0.001) and reduced platelet aggregation (P <0.05), as compared with ACS patients (in press). This may suggest that the effect of genetic polymorphism on drug concentrations and platelet aggregation is much more notable in younger and more homogenous populations, while other factors, such as age, diabetes, renal insufficiency, and the use of multiple medications, including other cytochrome p450-metabolized drugs [18], have a significantly greater effect on reduced bioavailability and antiplatelet effects of clopidogrel in older and more heterogeneous groups, thus masking the possible influence of genetic variability. This may partially explain why prospective randomized trials have failed to demonstrate the efficacy of personalized antiplatelet treatment based on platelet function on reducing the frequency of ischemic events [35-39].

Our study has several limitations. First, we included a relatively small cohort of participants from a limited geographic region. We have to admit that study's sample was not powered sufficiently to formulate final conclusions thus our findings need to be proved in more statistically powerful studies. Second, we did not assess other genetic polymorphisms or other factors (diabetes, smoking, drug use) that might have affected the drug concentrations. Moreover, considering that the we did not find any *CYP2C19*3* allels carriers and homozygous carriers of the *CYP2C19*2* allele constituted only 1.5% of our study population [8] it was difficult to assess the effect of this genetic variant on the analyzed parameters. Therefore, the relationship between these genotypes and exposure to the active metabolite of clopidogrel and the corresponding pharmacodynamic effects should be confirmed in larger cohorts.

Conclusions

In conclusion, our study showed that ACS is not associated with the levels of clopidogrel active metabolite or platelet inhibition in patients with Killip class I-III, and this association was not affected by the presence of *ABCB1* and *CYP2C19*2* gene polymorphisms. This suggests that clopidogrel may be safely used in this population in the presence of contraindications to prasugrel or ticagrelor treatment.

Conflict of interest: none declared

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Table 1. Demographic data.

	Control (n=22)	Study population (n=199)	p
Age, years	63.0 ± 8.2	65.5 ± 9.9	0.64
Male, n (%)	14 (63.6)	121 (60.8)	0.98
Hypertension, n (%)	16 (72.7)	155 (77.8)	0.78
Body mass index, kg/m^2	24.7 ± 5.4	27.2 ± 3.2	0.55
Smoking, n (%)	6 (27.2)	52 (26.1)	0.89
Hyperlipideamia	15 (68.1)	132 (65.8)	0.95
Previous MI, n (%)	5 (22.7)	42 (21.1)	0.91
Diabetes, n (%)	8 (36.3)	64 (32.1)	0.87
eGFR, ml/min/m^2	46.5 ± 12.6	41.3 ± 10.1	0.63

Abbreviations: eGFR – estimate glomerular filtration rate, MI – myocardial infarction

Table 2. Genotype distributions in the study population

Genotype frequency			
NA	**CYPC19*2, n (%)**		
199	**NoF**	**LoF**	
	GG	GA	AA
	49 (24.6)	147 (73,9)	3 (1,5)
199	**CYPC19*3, n (%)**		
	NoF	**LoF**	
	GG	GA	AA
	199 (100)	0 (0)	0 (0)
199	**ABCB1, n (%)**		
	GA	**PA**	
	CC	TC	TT
	32 (16)	115 (57,9)	52 (26,1)

GA – good absorbers, NA - number of individuals analyzed, NoF – normal function, LoF – loss of function, PA – poor absorbers

Figure 1
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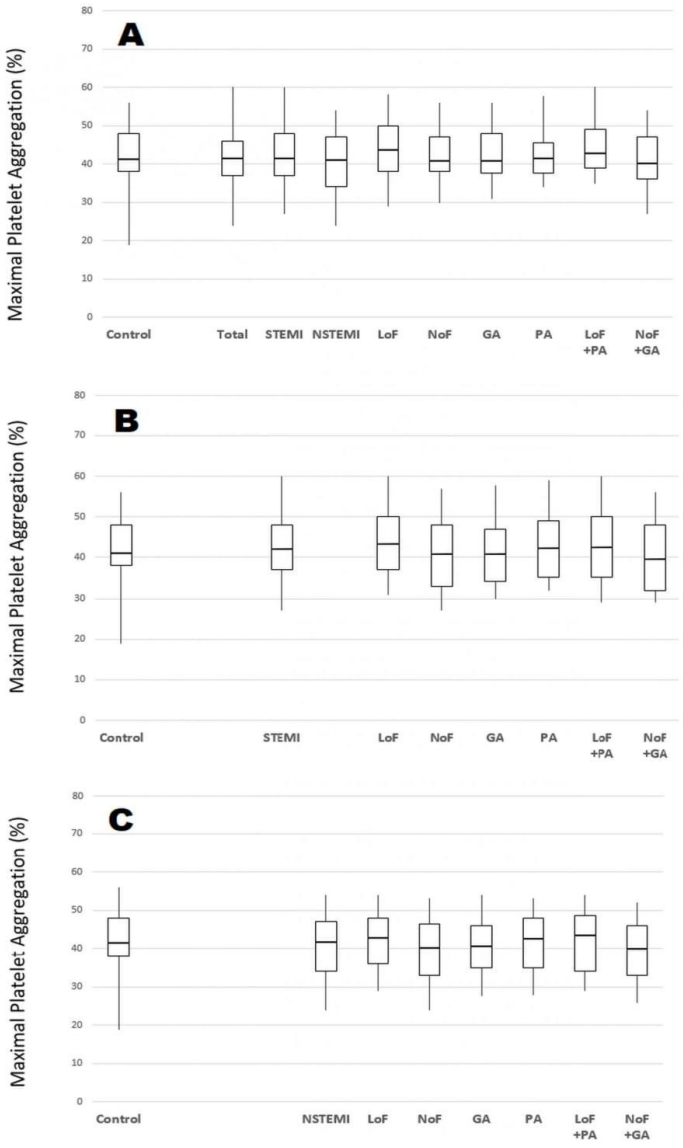

Figure 2. Maximum platelet aggregation after stimulation with 5-µmol/l adenosine diphosphate in: A – the whole study group and subgroups; B ¬– patients with ST-segment elevation myocardial infarction (STEMI) and STEMI subgroups; C – patients with non–ST-segment elevation myocardial infarction (NSTEMI) and NSTEMI subgroups.
Control – control group
GA – good absorbers
PA- poor absorbers
LoF – loss of function alleles
NoF – normal function alleles

Figure 1

Download source file (28.84 MB)

NSTEMI – non-ST-segment elevation myocardial infarction
STEMI - ST-segment elevation myocardial infarction
Total – whole population

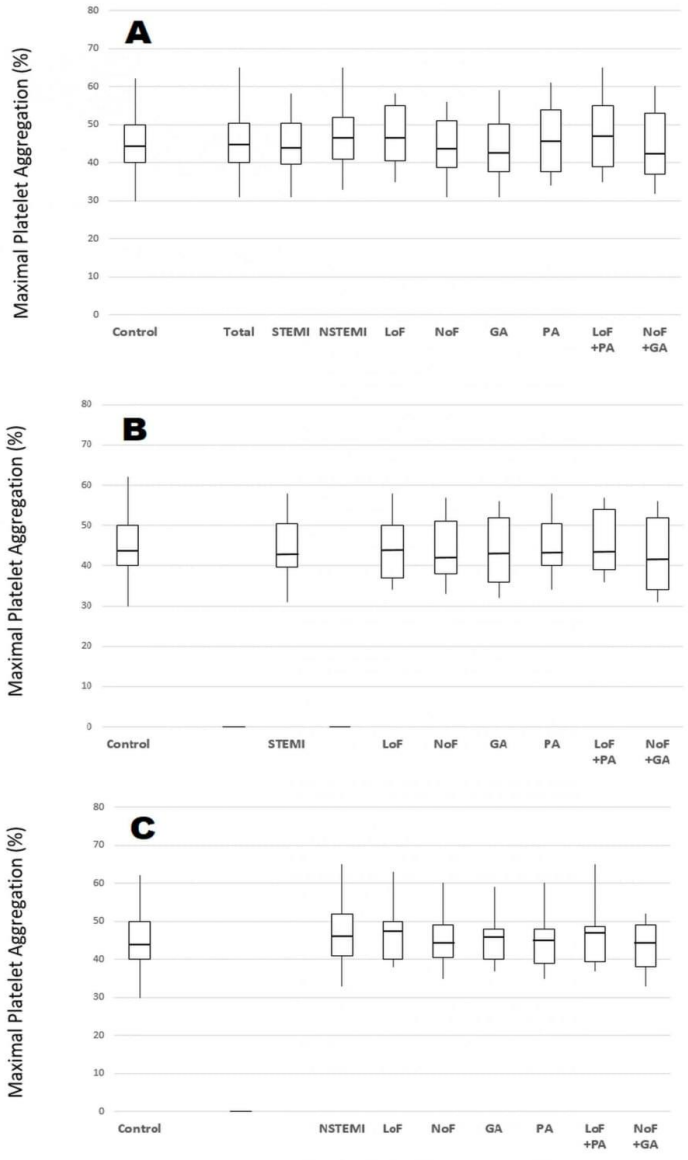

Figure 3. Maximum platelet aggregation after stimulation with 10-µmol/l adenosine diphosphate: A – the whole study group and subgroups; B ¬– patients with ST-segment elevation myocardial infarction (STEMI) and STEMI subgroups; C – patients with non–ST-segment elevation myocardial infarction (NSTEMI) and NSTEMI subgroups.
Control – control group
GA – good absorbers
PA- poor absorbers
LoF – loss of function alleles
NoF – normal function alleles

Figure 2

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NSTEMI – non-ST-segment elevation myocardial infarction
STEMI - ST-segment elevation myocardial infarction
Total – whole population

Figure 3
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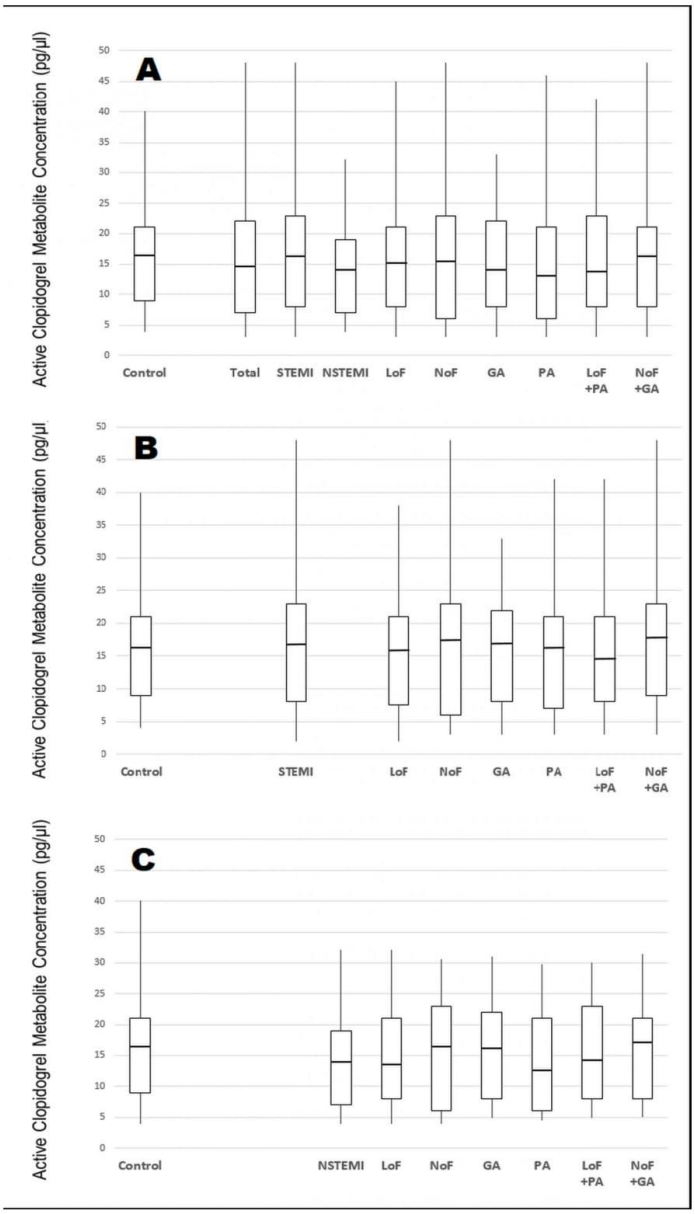

Figure 1. Levels of clopidogrel active metabolite in: A – the whole study group and subgroups; B ¬– patients with ST-segment elevation myocardial infarction (STEMI) and STEMI subgroups; C – patients with non–ST-segment elevation myocardial infarction (NSTEMI) and NSTEMI subgroups.
Control – control group
GA – good absorbers
PA- poor absorbers
LoF – loss of function alleles
NoF – normal function alleles

Figure 3

Download source file (28.84 MB)

NSTEMI – non-ST-segment elevation myocardial infarction
STEMI - ST-segment elevation myocardial infarction
Total – whole population

7. Wnioski

Na podstawie przeprowadzonych badań można stwierdzić, że:

1. Częstość występowania polimorfizmu genu ABCB1 w populacji polskiej jest duża
2. Częstość występowania polimorfizmu typu CYPC19*2 w populacji polskiej jest względnie duża
3. Częstość występowania polimorfizmu typu CYPC19*3 w populacji polskiej jest bardzo niska
4. Badane formy generyczne klopidogrelu nie różnią sie w stosunku do preparatu oryginalnego zarówno pod względem osiągniętych stężeń aktywnego metabolitu klopidogrelu, jak i jego działania antyagregacyjnego
5. Wpływ badanych polimorfizmów genów nie wpływa na stężenie aktywnego metabolitu klopidogrelu we krwi pacjentów z zawałem z- i bez uniesienia odcinka ST oraz na działanie przeciwpłytkowe leku
6. Róże rodzaje ostrego incydentu wieńcowego (STEMI i NSTEMI) nie wpływają na stężenie aktywnego metabolitu klopidogrelu we krwi pacjentów z OZW oraz na działanie przeciwpłytkowe leku.

8. Piśmiennictwo

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