UNIWERSYTET MEDYCZNY WE WROCŁAWIU
WYDZIAŁ LEKARSKI

lek. Maciej Bladowski

Biotransformacja tlenku azotu i ocena funkcji płytek krwi u pacjentów w ostrej fazie niekardiogennego udaru niedokrwiennego mózgu

---

ROZPRAWA DOKTORSKA

PROMOTOR: prof. dr hab. n. med. Adrian Doroszko

WROCŁAW 2022

PODZIĘKOWANIA

Chciałbym wyrazić ogromną wdzięczność Panu Profesorowi Adrianowi Doroszko za pomoc w rozwiązywaniu wydawałoby się nierozwiązywalnych problemów, za wsparcie naukowe na każdym kroku realizacji rozprawy doktorskiej oraz po prostu za bycie dobrym człowiekiem.

Składam serdeczne podziękowania Pani magister Ewie Szahidewicz-Krupskiej za skrupulatność, elastyczność i ogromną ilość poświęconej pracy w część laboratoryjną i organizacyjną badania.

Pracę dedykuję mojej cierpliwej i kochanej Żonie, bez której wsparcia rozprawa ta nie zostałaby ukończona.

SPIS TREŚCI

1. WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD CYKLU PRAC:

1.1. **Maciej Bladowski**, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity Volume 2020
DOI: https://doi.org/10.1155/2020/2979260. Epub 2020 Aug 28
IF: 6.543
pkt MEiN: 100.000

1.2. **Maciej Bladowski**, Ewa Szahidewicz-Krupska , Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar, Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955
DOI: https://doi.org/10.3390/antiox11050955. Epub 2022 May 12
***IF* 2020: 6.313**
pkt MEiN: 100.000

Suma IF = 12.856
Suma pkt MEiN = 200.000

2. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY

Zachorowalność na udar niedokrwienny mózgu wśród mieszkańców Europy wynosi obecnie w przybliżeniu 1 milion/rok, z prognozowanym dalszym wzrostem o około 3% w ciągu najbliższych 3 dekad, mimo stosowania coraz skuteczniejszych metod profilaktyki chorób układu sercowo-naczyniowego. Sam udar niedokrwienny mózgu można podzielić na kilka podtypów, w oparciu o patogenezę materiału zakrzepowego powodującego blokadę przepływu krwi w łożysku krążenia mózgowego. Kardiogenny (pierwotnie związany z powstawaniem skrzepliny w sercu) udar niedokrwienny mózgu odpowiada za około 20-30% ogółu udarów. Najczęstsza przyczyną jego wystąpienia jest migotanie przedsionków, gdzie sprawdzoną strategią terapeutyczną stosowaną zarówno w prewencji pierwotnej, jak i wtórnej jest leczenie przeciwkrzepliwe. Niekardiogenny udar mózgu stanowi pozostałe 70-80% przypadków, a rutynowo stosowaną metodą prewencji wtórnej jego wystąpienia o udokumentowanej skuteczności jest leczenie przeciwpłytkowe. Jednakże zastosowanie leków przeciwpłytkowych u niewyselekcjonowanej grupy pacjentów w ramach prewencji pierwotnej niekardiogennego udaru nie przynosi oczekiwanej korzyści pod postacią zmniejszenia ryzyka wystąpienia incydentu niedokrwienia mózgu. W związku z tym mechanizmy patofizjologiczne wpływające na funkcję płytek krwi u pacjentów z niekardiogennym udarem niedokrwiennym mózgu wymagają szczególnie dokładnego poznania, celem znalezienia nowych strategii profilaktycznych i terapeutycznych w tej grupie chorych.

Dysfunkcja śródbłonka wyrażona obniżoną biodostępnością tlenku azotu (NO), oprócz aktywacji zapalnej, angiogennej i wazokonstrykcji, odgrywa istotną rolę w patogenezie chorób układu sercowo-naczyniowego. W licznych badaniach udowodniono wpływ obniżonej biodostępności NO na rozwój nadciśnienia tętniczego, tworzenie blaszki miażdżycowej i jej destabilizację oraz na wzrost aktywacji, adhezji i zdolności do agregacji płytek krwi. Z kolei obniżanie ryzyka sercowo-naczyniowego, poprzez odpowiednią terapię nadciśnienia tętniczego, cukrzycy, dyslipidemii, czy zaprzestanie palenia papierosów, wiąże się z obserwowanym wzrostem biodostępności tlenku azotu i z poprawą funkcji śródbłonka. Nowsze dane sugerują, że nie tylko śródbłonek, ale również płytki krwi potrafią syntetyzować NO, samodzielnie regulując w ten sposób swoją funkcję. W zależności od ekspresji syntetazy tlenku azotu (NOS) wykazano istnienie dwóch odrębnych subpopulacji płytek: NOS-pozytywnych i NOS-negatywnych. Według niektórych autorów płytki NOS-negatywne są odpowiedzialne za adhezję do dysfunkcjonalnego śródbłonka oraz inicjowanie tworzenia zakrzepu, podczas gdy

rolą NOS-pozytywnych jest ograniczanie agregacji. Wpływ zaburzeń biotransformacji tlenku azotu w płytkach krwi może zatem mieć wpływ na wzrost częstości występowania incydentów zakrzepowo-zatorowych, w tym także w łożysku naczyń mózgowych. Może to mieć bardzo poważne implikacje kliniczne, wobec czego stało się przedmiotem prowadzonych przeze mnie badań w ramach pracy doktorskiej.

Zaburzenia wewnątrzpłytkowej biotransformacji tlenku azotu u osób z niekardiogennym udarem niedokrwiennym mózgu mogą być odpowiedzialne za inicjację incydentu niedokrwiennego w ośrodkowym układzie nerwowym. Weryfikacja tej hipotezy była głównym tematem niniejszej rozprawy, stanowiącej próbę przełożenia odkrycia z zakresu przedklinicznych nauk podstawowych na grunt kliniczny. Znalezienie różnic w płytkowej i osoczowej ekspresji szlaku tlenku azotu może znacząco przyczynić się do zrozumienia przyczyny nieskuteczności leczenia przeciwpłytkowego w prewencji pierwotnej udaru niedokrwiennego mózgu. Wyselekcjonowanie pacjentów z wysokim ryzykiem sercowo-naczyniowym lub z nadmiarem płytkowych inhibitorów NOS i w efekcie zmniejszoną płytkową syntezą NO mogłoby przynieść korzyści w leczeniu przeciwpłytkowym stosowanym w ramach profilaktyki pierwotnej u tych chorych. Z kolei ograniczenie stosowania terapii przeciwpłytkowej u pacjentów z dużym odsetkiem NOS-pozytywnych płytek mogłoby wpłynąć na wzrost bezpieczeństwa prowadzonej terapii poprzez redukcję ryzyka krwawień.

Ze względu na złożoność mechanizmów wpływających na biotransformację tlenku azotu w płytkach krwi i osoczu, w pracy poglądowej pt. *„Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside”* dokonałem przeglądu, interpretacji i podsumowania aktualnego stanu wiedzy na ten temat u pacjentów z udarem niedokrwiennym mózgu. Praca ta stanowi jeden z niewielu dostępnych w literaturze przekrojowych zbiorów informacji dotyczących dokładnej roli tlenku azotu w utrzymaniu homeostazy wewnątrznaczyniowej u pacjentów z objawowym niedokrwieniem w łożysku naczyń mózgowych. Artykuł ten systematyzuje dostępną wiedzę na temat roli biotransformacji tlenku azotu w płytkach krwi i osoczu oraz jej wpływu na wystąpienie i przebieg udaru niedokrwiennego mózgu, a także buduje uzasadnienie dla badań, których wyniki zaprezentowano w pracy oryginalnej stanowiącej drugi manuskrypt niniejszej dysertacji.

Obecnie wyróżniamy trzy główne izoformy syntetazy tlenku azotu (NOS): neuronalną (nNOS), indukowalną (iNOS) i śródbłonkową=endotelialną (eNOS). W standardowych warunkach produkują one tlenek azotu mający wpływ między innymi na prawidłowe

funkcjonowanie synaps nerwowych, właściwości cytotoksycznych neutrofilów, czy wazodylatację zależną od śródbłonka. Jednakże w warunkach niestabilnego potencjału redoks (uszkodzenie niedokrwienno-reperfuzyjne lub hipoksja-reoksygenacja) przy niedoborze tlenu dochodzi do dysfunkcji NOS (ang. *NOS uncoupling*), która zamiast NO, syntezuje reaktywne formy tlenu (ROS), głownie nadtlenoazotyny ($ONOO^-$). Przykładem takiej sytuacji jest niedokrwienie mózgu, podczas którego dochodzi do rozprzężenia mitochondrialnej fosforylacji oksydacyjnej, która w połączeniu z kaskadą reakcji zapalnych, prowadzi do powstawania nadtlenoazotynów. Związki te - jako skrajnie niestabilne - powodują niekontrolowane nitrowanie i S-nitrozylację wielu białek kluczowych dla homeostazy układu sercowo-naczyniowego. Prowadzi to również do zwiększenia ekspresji i aktywacji indukowalnej syntetazy tlenku azotu, która dodatkowo amplifikuje produkcję nadtlenoazotynów, zmniejsza biodostępność NO i tym samym upośledza wazodylatacyjną funkcję śródbłonka, a promuje reakcję zapalną w ścianie naczynia i progresję aterogenezy poprzez promowanie depozycji utlenionego LDL w ścianie naczynia.

W badaniach na zwierzętach wykazano, że po indukcji niedokrwienia poprzez zakleszczenie tętnicy środkowej mózgu, osoczowa biodostępność NO wytwarzanego przez nNOS i eNOS rośnie w przeciągu pierwszych 30 minut, a następnie stopniowo spada w ciągu kolejnych 4 godzin. Po tym czasie następuje kolejny wzrost stężenia osoczowego NO trwający do 7 dni. Tlenek azotu, wytwarzany przez eNOS w ciągu pierwszych godzin niedokrwienia OUN, powoduje rozszerzenie naczyń i wzrost przepływu krwi przez obszar półcienia ischemicznego (tzw. penumbrę ischemiczną). Natomiast NO produkowane przez nNOS w uszkodzonych neuronach doprowadza do przyspieszenia wewnątrzkomórkowej kaskady apoptozy prowadząc do śmierci neuronu. Makrofagi obecne w mózgu i napływające do OUN oraz neutrofile syntezują tlenek azotu dzięki iNOS, który wykorzystywany jest do produkcji reaktywnych form tlenu i do dalszej stymulacji apoptozy. Opisywany drugi szczyt produkcji NO jest więc prawdopodobnie związany z aktywnością iNOS, który oprócz NO dodatkowo nadprodukuje ROS. Ponadto udowodniono, że wzrost stężenia metabolitów NO w pierwszych dwóch dniach od wystąpienia udaru niedokrwiennego mózgu jest korzystny dla pacjentów i wiąże się z niższą punktacją w skali National Institutes of Health Stroke Scale (NIHSS) ocenianą po 3 miesiącach. Natomiast kolejny wzrost stężenia metabolitów NO, obserwowany pomiędzy 2. a 7. dniem od początku objawów neurologicznych, wiąże się ze wzrostem rozmiaru ogniska niedokrwiennego, ocenionego za pomocą rezonansu magnetycznego w 7. dniu, w porównaniu do badania wyjściowego z 1. dnia obserwacji.

Osoczowe stężenie L-Argininy (substrat dla syntetazy tlenku azotu) początkowo maleje w ciągu pierwszych sześciu godzin od początku udaru niedokrwiennego, a następnie rośnie przez kolejne 24 godziny w modelach eksperymentalnych. Niskie stężenie L-Argininy w pierwszej dobie od początku objawów deficytów neurologicznych jest związane z większą objętością obszaru niedokrwienia OUN i ostatecznie z gorszym rokowaniem u pacjentów po przebytym udarze niedokrwiennym mózgu. W badaniach przeprowadzonych na zwierzętach, podawanie L-Argininy tuż po wywołaniu okluzji tętnicy środkowej mózgu spowodowało mniejszą objętość obszaru niedokrwienia. ADMA jest najsilniejszym inhibitorem kompetycyjnym NOS. Wzrost jego osoczowego stężenia jest predyktorem wystąpienia chorób sercowo-naczyniowych, a jego podwyższone stężenie jest również obserwowane w pierwszych godzinach po incydencie niedokrwiennym w mózgu.

Wielu autorów wykazało, że tlenek azotu hamuje adhezję i agregację płytek krwi, a obecność NOS w płytkach krwi determinuje ich pro- lub przeciw-agregacyjną funkcję. Ponadto w nielicznych dostępnych pracach wykazano związek pomiędzy obniżonym płytkowym stężeniem tlenku azotu (PDNO, ang. *platelet-derived nitric oxide*) a wiekiem, nadciśnieniem tętniczym, dyslipidemią i paleniem papierosów. Jednak do tej pory nie oceniano związku pomiędzy obniżonym PDNO, a rozwojem i przebiegiem udaru niedokrwiennego mózgu. W badaniach na zwierzętach wykazano pozytywny wpływ leków przeciwpłytkowych na wzrost stężenia osoczowego NO i jednocześnie spadek aktywności iNOS po okluzji tętnicy środkowej mózgu. Jednakże, czy leki przeciwpłytkowe mają działanie antyagregacyjne również poprzez zmianę homeostazy tlenku azotu w płytkach krwi, nie było do tej pory badane.

Druga praca wchodząca w skład niniejszej rozprawy doktorskiej nosi tytuł *„Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study”* i jest to pierwsze oryginalne doniesienie kliniczne opisujące dynamikę zmian ekspresji osi biotransformacji tlenku azotu w płytkach krwi osób w ostrej fazie niekardiogennego udaru niedokrwiennego mózgu, w porównaniu do zdrowej grupy kontrolnej.

Biorąc pod uwagę złożoność procesów biotransformacji tlenku azotu opisanych w pierwszej pracy, część eksperymentalną projektu zaplanowano tak, aby podjąć próbę falsyfikacji hipotezy zerowej o braku różnic pomiędzy ekspresją metabolitów biotransformacji NO w osoczu i w płytkach krwi ich funkcji u pacjentów w ostrej fazie niekardiogennego udaru niedokrwiennego mózgu, w porównaniu do grupy kontrolnej bez ostrego incydentu niedokrwiennego w obszarze OUN. Po analizie 418 przypadków chorych z podejrzeniem udaru niedokrwiennego mózgu, do grupy badanej ostatecznie włączono 40 pacjentów. Kryterium

włączenia było wystąpienie początku zaburzeń neurologicznych poniżej 24 godzin przed przyjęciem do szpitala, potwierdzenie rozpoznania udaru mózgu w badaniu neurologicznym i/lub w badaniu tomografii komputerowej głowy oraz możliwość wyrażenia świadomej zgody. Głównymi kryteriami wykluczenia były: udar krwotoczny, migotanie przedsionków, stan zapalny, aktywna choroba nowotworowa, przewlekła choroba nerek, przewlekła choroba autoimmunologiczna, stosowanie leków przeciwkrzepliwych lub przeciwpłytkowych, zastosowanie trombolizy lub mechanicznej trombektomii. Grupę kontrolną stanowiło 39 osób dopasowanych pod względem demograficznym, chorób współistniejących (ze szczególnym uwzględnieniem chorób sercowo-naczyniowych) oraz stosowanej farmakoterapii do grupy badanej. U pacjentów z grupy badanej rozpoczęto podawanie kwasu acetylosalicylowego (ASA) w pierwszej dobie hospitalizacji, zgodnie z wytycznymi oraz przeprowadzano badanie neurologiczne, a następnie zabezpieczano krew celem oceny biotransformacji tlenku azotu w płytkach krwi i osoczu oraz wykonywano pomiar parametrów agregacji płytek krwi trzykrotnie: I. w ciągu pierwszych 24 godzin od początku objawów deficytów neurologicznych, następnie II. w 3. dobie i oraz III. 7 dobie udaru niedokrwiennego mózgu. W grupie kontrolnej badania przeprowadzono jednokrotnie, bez podawania ASA.

W 1. i 3. dniu obserwacji stwierdzono w grupie badanej wyższe stężenie L-Argininy w płytkach krwi i niższe stężenie L-Argininy w osoczu, w porównaniu do grupy kontrolnej. Stężenie inhibitorów syntetazy tlenku azotu (asymetrycznej dimetyloargininy - ADMA i symetrycznej dimetyloargininy - SDMA) w płytkach krwi były stabilnie podwyższone w grupie badanej od 1. do 7. dnia obserwacji. Agregacja płytek krwi zależna od kwasu arachidonowego była ujemnie skorelowana z płytkową biodostępnością tlenku azotu ($\frac{L-Arginina\ (płytki)}{ADMA\ (płytki)}$) w 3. i 7. dniu, a dodatnio z płytkową biodostępnością kompetycyjnego inhibitora NOS ($\frac{ADMA\ (płytki)}{ADMA\ (osocze)}$) w 7. dniu obserwacji. Agregacja płytek krwi zależna od kolagenu była ujemnie skorelowana z płytkową biodostępnością tlenku azotu i dodatnie z płytkową biodostępnością kompetycyjnego inhibitora NOS w 7. dniu obserwacji. ADP-zależna agregacja była dodatnie skorelowana z płytkową biodostępnością kompetycyjnego inhibitora NOS jedynie w 1. dniu obserwacji.

Opisany wpływ stężenia niektórych metabolitów biotransformacji tlenku azotu na agregację płytek krwi, świadczy o istnieniu złożonej roli jaką odgrywa NO w patogenezie i przebiegu niekardiogennego udaru niedokrwiennego mózgu. Pacjenci w grupie badanej charakteryzowali się stabilnie podwyższonym stężeniem ADMA i SDMA w płytkach krwi, które może być odpowiedzialne u nich za zwiększoną podatność płytek na agregację. Natomiast podawanie kwasu acetylosalicylowego w tej grupie spowodowało wzrost stężenia płytkowej

L-Argininy i płytkowej biodostępności tlenku azotu, co sugeruje, że ASA może wykazywać działanie przeciwpłytkowe nie tylko w mechanizmie cyklooksygenazo-zależnym, ale również NO-zależnym.

Podsumowując, wykazano, że osoby z niekardiogennym udarem niedokrwiennym mózgu charakteryzują się wyższym stężeniem wewnątrzpłytkowych inhibitorów syntetazy tlenku azotu w porównaniu z osobami zdrowymi, co może być przyczyną występowania u nich upośledzonego wytwarzania płytkopochodnego tlenku azotu i tym samym sprzyjać zwiększonej agregacji trombocytów w trakcie ostrej fazy niedokrwienia. Uzyskane wyniki stanowić mogą podstawę do kolejnych badań nad wpływem obniżonej płytkowej biodostępności tlenku azotu na wzrost ryzyka sercowo-naczyniowego oraz nad możliwością wyselekcjonowania grupy pacjentów mogących odnieść zysk z leczenia przeciwpłytkowego w prewencji pierwotnej udaru niedokrwiennego mózgu, a zatem personalizacji terapii. Praca ta stanowi tym samym przykład wykorzystania medycyny translacyjnej, przenosząc wyniki badań z zakresu nauk podstawowych na grunt kliniczny.

3. PRACA NR 1:

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2020, Article ID 2979260, 18 pages
https://doi.org/10.1155/2020/2979260

Review Article

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside

Maciej Bladowski, Jakub Gawrys, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, and Adrian Doroszko

Department of Internal Medicine, Hypertension and Clinical Oncology, Faculty of Medicine, Wroclaw Medical University, Poland

Correspondence should be addressed to Adrian Doroszko; adrian.doroszko@umed.wroc.pl

Received 25 June 2020; Accepted 27 July 2020; Published 28 August 2020

Ischemic stroke remains the fifth cause of death, as reported worldwide annually. Endothelial dysfunction (ED) manifesting with lower nitric oxide (NO) bioavailability leads to increased vascular tone, inflammation, and platelet activation and remains among the major contributors to cardiovascular diseases (CVD). Moreover, temporal fluctuations in the NO bioavailability during ischemic stroke point to its key role in the cerebral blood flow (CBF) regulation, and some data suggest that they may be responsible for the maintenance of CBF within the ischemic penumbra in order to reduce infarct size. Several years ago, the inhibitory role of the platelet NO production on a thrombus formation has been discovered, which initiated the era of extensive studies on the platelet-derived nitric oxide (PDNO) as a platelet negative feedback regulator. Very recently, Radziwon-Balicka et al. discovered two subpopulations of human platelets, based on the expression of the endothelial nitric oxide synthase (eNOS-positive or eNOS-negative platelets, respectively). The e-NOS-negative ones fail to produce NO, which attenuates their cyclic guanosine monophosphate (cGMP) signaling pathway and—as result—promotes adhesion and aggregation while the e-NOS-positive ones limit thrombus formation. Asymmetric dimethylarginine (ADMA), a competitive NOS inhibitor, is an independent cardiovascular risk factor, and its expression alongside with the enzymes responsible for its synthesis and degradation was recently shown also in platelets. Overproduction of ADMA in this compartment may increase platelet activation and cause endothelial damage, additionally to that induced by its plasma pool. All the recent discoveries of diverse eNOS expression in platelets and its role in regulation of thrombus formation together with studies on the NOS inhibitors have opened a new chapter in translational medicine investigating the onset of acute cardiovascular events of ischemic origin. This translative review briefly summarizes the role of platelets and NO biotransformation in the pathogenesis and clinical course of ischemic stroke.

1. Ischemic Stroke: Its Burden and Classification

Cardiovascular disease (CVD) remains the main cause of morbidity and mortality, as reported worldwide annually. In spite of constant progress in diagnostic and therapeutic strategies, according to the recent data, there were estimated 72.72 million cases of CVD and 17.8 million CVD deaths in the world population. Stroke was the fifth cause of death globally with the morbidity reaching approximately 7.750 million and mortality 2.750 million in 2017 [1]. Ischemic stroke is the most common type of acute cerebrovascular event, responsible for 81% of all the stroke cases [2]. The thromboembolic event is a common denominator of all the subtypes of ischemic stroke. Large artery atherosclerosis (LAA) is the causative event in 17-34% of ischemic strokes and is characterized by activation of platelets along with thrombus formation on atherosclerotic plaque in extra/intracranial arteries (ruptured atherosclerotic plaque accompanied with a cascade of thromboinflammation). Small vessel occlusion/lacunar stroke (SVO) is diagnosed in 20.5-29.0% of cases, and it proceeds from lipohyalinosis (vessel wall thickening induced to the greatest extent by hypertension). Further ischemic stroke subtypes include cardioembolic

(16-25.6%) which is predominantly generated by the atrial fibrillation (AF), then a stroke of unusual/other etiology (1.7-6%) and of unknown/undetermined etiology (14.2-29%) [3–6] (Figure 1).

Each of the noncardioembolic stroke subtypes is characterized by partially different pathophysiology, recurrence rate, magnitude of positive response to antiplatelet therapy, and survival rate (being relatively better for SVO than LAA strokes) [5, 7, 8]. Despite of the heterogeneous origin of particular subtypes of ischemic stroke, there are some uniform/-common mechanisms, mostly related to increased activation of platelet-derived hemostasis. Hence, some common therapeutic strategies may reveal to be effective both in the treatment of an acute phase and in the primary and secondary stroke prevention.

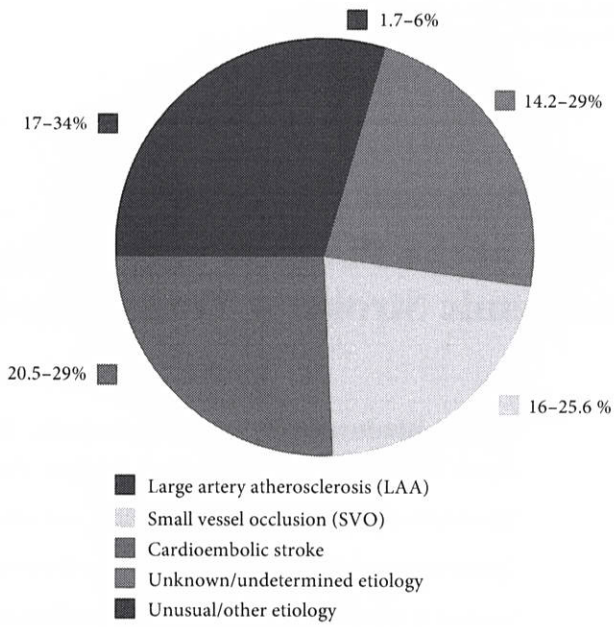


Figure 1: Incidence of each stroke subtype.

2. Characteristics of the Cerebral Vascular Bed and Pathophysiology of Cerebral Ischemia-Reperfusion Injury

Cerebral arteries with their curvatures and bifurcations are characterized by a plaque-prone development anatomy. Contrary to the coronaries, carotid and cerebral vessels are subjected to high shear stress, which protects from atherosclerotic plaque enlargement but on the other hand also predisposes to intraplaque hemorrhage and plaque rupture [9]. Nevertheless, when hypercholesterolemia appears, endothelial dysfunction is promoted, limiting the positive action of physiological shear stress, and plaque formation is observed [10]. Some data suggest that low shear stress may change the expression of genes for inflammatory proteins leading to the origin of atherosclerosis-related inflammation [11, 12]. At high shear flow rates, as found in carotid/cerebral arteries or moderately stenosed vessels, the initial capture of circulating platelets to the endothelium is mediated by the von Willebrand factor (vWF) at the vascular wall without other stimulating factors [13]. It is suggested that the plasma level of vWF—to some extent—is a marker of endothelial cell damage and it predicts the onset and progression of atherosclerotic lesions in patients with hypertension. Hypotensive therapy, by non-drug-specific reducing endothelial damage and vWF expression, contributes to inhibition of both thrombus and atherosclerosis formation pointing thus at its protective role in the primary and secondary prevention of ischemic stroke [14].

3. Platelets as the Common Denominator of the Acute Ischemic Events and Pleiotropic Drug Target

Several years ago, Htun et al. have shown that patients with ischemic stroke or transient ischemic attack (TIA) were characterized by significantly increased P-selectin (CD62P) expression in platelets and circulating platelet-leukocyte aggregate concentration. Interestingly, other authors discovered that patients with the LAA infarction elicit higher platelet-leukocyte aggregate formation, when compared with the SVO group [15–17]. Differences in the CD62P concentration between stroke patients and controls returned to normal after 90 days of observation or gradually with implementation of antiplatelet treatment (stronger correlation with clopidogrel than with acetylsalicylic acid (ASA), but no association with warfarin treatment). Noteworthily, such treatment had no effect on normalization of the circulating platelet-leukocyte aggregate level in those patients [18, 19]. Initialization of thromboinflammation in ischemic stroke can be explained by the elevated platelet expression of the CD40 ligand (CD40L) in activated platelets, which, by triggering the expression of adhesive molecules, such as P-selectin, E-selectin, and ICAM-1, leads to formation of platelet-leukocyte aggregates [20, 21]. Moreover, Ishikawa et al. observed that, after induction of middle cerebral artery occlusion (MCAO) in the CD40-deficient rats, impaired platelet and leukocyte adhesion occurred leading to smaller brain infarct size in comparison to the control group [22]. Additionally, Jiang et al. demonstrated similar results in male rats treated with CD40 antagonist infusion before reperfusion of the occluded middle cerebral artery [23]. In human studies, high concentration of CD40 is associated with poor outcome at 3 months after ischemic stroke [15]. Moreover, leukocytes (especially regulatory T lymphocytes) have significant function in thromboinflammation during ischemic stroke by promoting ICAM-1 expression on platelets and endothelia which facilitates adhesion of granulocytes and platelets to the vessel wall [24, 25]. Hyperaggregable leukocytes, monocytes, and endothelia tend to activate platelets by platelet-activating factor secretion in the time of cerebral ischemia [26]. The PAF function is not only to activate adhesion of platelets and leukocytes (mostly neutrophils) to the damaged endothelium, but it also causes tissue edema through the increase of the vascular permeability in the peripheral tissues, increases secretion of granule-based

enzymes in platelets, and enhances superoxide and arachidonate metabolism in neutrophils generating neurotoxicity leading to brain damage after ischemic stroke [27, 28].

Platelet secretion of thromboxane A2, adenosine diphosphate (ADP), matrix metalloproteinase-9 (MMP-9), and other platelet-derived soluble mediators promote thrombus formation in a positive feedback loop [29]. ADP is one of the most prevalent platelet activators under physiological condition. It also plays a significant role in cardiovascular disease development. Puurunen et al. in the prospective study of Framingham population identified platelet hyperreactivity to ADP to be associated with myocardial infarction and ischemic stroke incidence [30]. What is more, persistent elevation of platelet aggregation in response to ADP at three months after ischemic stroke is connected with more than threefold increased recurrence of stroke. Interestingly, cross-incubation of control platelets with plasma from stroke patients resulted in activation of platelets measured by the raised basal platelet calcium level and release of serotonin from platelets. These results, accompanied with the study by Dougherty et al. suggesting that ASA and dipyridamole treatment have no effect on platelet hyperreactivity to ADP, suggest that the lower threshold of platelet activation in ischemic stroke patients may be predominantly associated with the presence of plasmatic factors rather than with platelet functional disturbances [19, 31]. Recently, an increasing number of studies suggest that nitric oxide deficiency and nitric oxide synthase inhibitors can be one of the factors responsible for greater platelet aggregation in ischemic stroke patients.

4. The Role of Nitric Oxide Synthase and of Nitric Oxide in Ischemic Stroke

Endothelial vasodilative dysfunction, identified by decreased NO bioavailability, is a well-known risk factor for ischemic stroke. Changes in the nitric oxide concentration during the course of cerebral infarction can also be used as an important prognostic tool for ischemic stroke outcome. To date, three major isoforms of the NOS are described in the literature: neuronal constructive (nNOS), inducible (iNOS) and endothelial constitutive (eNOS). Each catalyzes the reaction of NO production, and in the catalytic cycle, the Fe^{3+}+NO complex is the final intermediate from which in normal circumstances NO easily dissociates [32, 33]. However, nitric oxide overproduction autoinhibits the catalytic site of the NOS by reduction of iron to the stable Fe^{2+}+NO complex [34]. In the presence of oxygen, the enzymatic inactive Fe^{2+}+NO bond generates nitrate (reactive nitrogen species) and ferric ion, making the catalytic site of NOS again available for NO production. The described above oxygen dependency of NOS action plays a crucial negative role in ischemia and hypoperfusion [35]. Nitric oxide synthase produces not only NO and nitrates but also reactive oxygen species. Comparing to inducible NOS, eNOS and nNOS are responsible for higher production of superoxide, which is considered to be involved in atherosclerosis and recruitment of additional platelets to the sites of injury. On the other hand, iNOS and nNOS are more inclined than endothelial NOS to producing reactive nitrogen species (RNS) in which the undesirable role is to destabilize structure and function of proteins, leading to impaired catalytic activity of enzymes and even to cell apoptosis [36, 37].

After induction of middle cerebral artery occlusion, increased NO plasma concentration is observed for up to 30 minutes with its subsequent reduction in the following hours [38, 39]. After a gradual decrease, the level of NO and peroxynitrite (especially after reperfusion) increased again after 4 hours, reaching a maximum at 46 hours and lasting for up to seven days [40, 41]. The described above fluctuation of nitric oxide concentration is probably associated with different NOS subtype activities. The activity of eNOS and nNOS increases at the same time as nitric oxide concentration within the first minutes after induction of MCAO and significantly reduces thereafter [39]. The expression of iNOS is detected in the brain at 12-70 hours following cerebral ischemia and lasts up to 7 days, while the brain myeloperoxidase activity (a marker of neutrophil infiltration) is observed only after 4 hours, significantly increases at 22 h, and then decreases. These observations suggest that the initial increased level of NO after ischemia is connected with endothelial and neuronal nitric oxide production. While NO production by eNOS and nNOS slowly decreases, brain infiltration by neutrophils and their NO production by iNOS are responsible for the fluctuation of the NO bioavailability after ischemic stroke [40–42] Figure 2.

Dobrucki et al. observed lower concentration of NO before induction of ischemic stroke in spontaneously hypertensive rats (SHR) and higher concentration of O^{2-} release (connected with higher peroxynitrite production) after induction of middle cerebral artery occlusion leading to larger infarct size in SHR as compared to the control group [43]. Serrano-Ponz et al. found similar results in human studies. Those authors identified an increase in nitric oxide metabolites from day 1 to day 2 to be beneficial for the ischemic stroke patients as measured by the National Institutes of Health Stroke Scale (NIHSS) at day 7 and at 3 months and measured by the modified Rankin Scale at 3 months, while a steep increase of nitric oxide metabolite concentration from day 2 to day 7 was associated with a multiple increase in infarct volume [44]. According to Taffi et al., the high nitric oxide plasma level 30 days after cerebral infarct is associated with poor outcome in nonlacunar stroke, since a 10-unit increase in NO concentration predicts a 1-point reduction in the NIHSS score. Better outcome in patients with lacunar stroke is probably connected with higher concentration of NO in the first 24 hours after cerebral infarction and lower concentration of peroxynitrite [45].

At the molecular level and in animal model-based studies, during the first few hours after cerebral ischemia, nitric oxide production by eNOS is improving cerebral blood flow (CBF) within the ischemic penumbra (area of brain tissue surrounding the infarct that is at risk of infarction) in order to reduce infarct size and volume [46, 47]. It is documented that both eNOS-deficient mice and administration of eNOS inhibitors to rats provoke a decrease in absolute CBF in animals (up to 25–35% of the control level) [48, 49]. The activity of nNOS throughout 2 hours after reperfusion of MCAO is

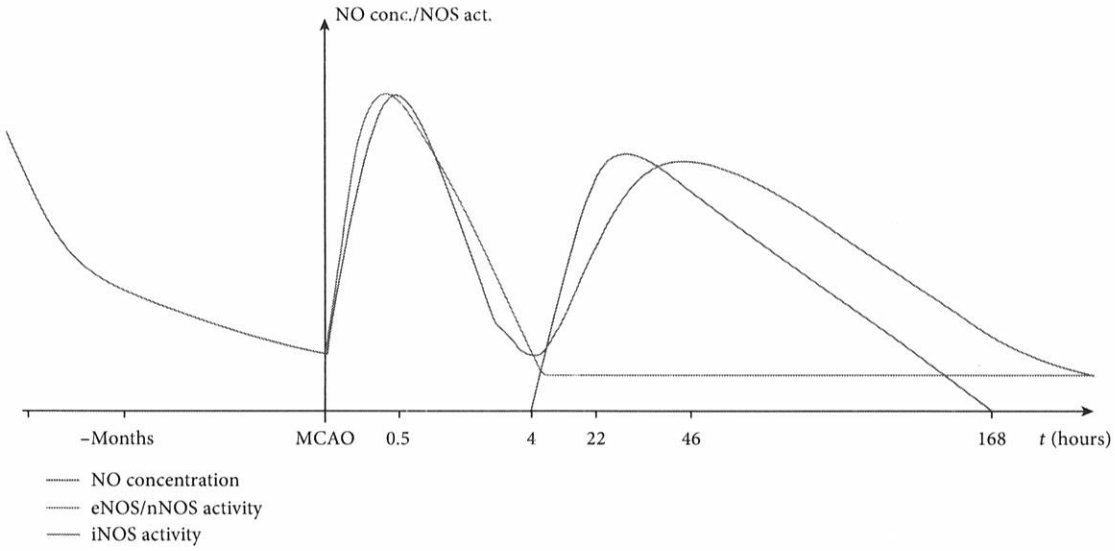


FIGURE 2: Concentration of nitric oxide and activation of NOS isoforms during the course of ischemic stroke.

also enhanced. However, in nNOS-deficient mice, CBF is significantly higher after reperfusion, which suggests the adverse effect of nNOS activation during the course of ischemic stroke [50]. Zeng et al. found that hypoxic or ischemic brain injury during early reperfusion is associated with the generation of NO from nNOS which activates the early c-Jun N-terminal kinase 1/2—a signaling pathway involved in neuronal death [51]. Stagliano et al. proved that immediate administration of a specific inhibitor for nNOS (3-bromo-7-nitroindazole) after induction of common carotid artery thrombosis in rats accelerated sensorimotor recovery [52]. While some of the authors postulate that nonspecific inhibitors of eNOS and nNOS (L-N^G-nitroarginine methyl ester (L-NAME)) reduce infarct size [53], others suggest that its biological function is partly dependent on simultaneous fluctuating N-methyl-D-aspartate (NMDA) concentration. NMDA in normal conditions is an activating neurotransmitter, but during ischemia, it is liberated from damaged neurons and has further neurotoxic activity. Globus et al. observed that brain lesions induced by NMDA was not affected by L-NAME administration. The reason for such a correlation is not fully understood. Activation of the NMDA receptor by neurotransmitters released from damaged neurons in the ischemic or penumbral area leads to NO overproduction and an increase in CBF in order to support the enhanced metabolic demand of the excited neurons. On the one hand, NO plays a role in the intracellular cascade of events leading to cell death following NMDA receptor activation; on the other hand, NO ensures adequate blood supply especially to the penumbral area. Probably, the final outcome of nitric oxide influence depends on the balance between these two processes [54, 55].

Nitric oxide produced by iNOS in the microglia (brain-based macrophages) may also lead to neuronal damage associated with the neurotoxicity mediated by NMDA receptors [56]. However, the main source of NO from iNOS during ischemic stroke originates from neutrophils. As mentioned before, infiltration of brain tissue by those phagocytes increases gradually during the first few days in the course of cerebral ischemia and reperfusion. Nitric oxide derived from neutrophils' iNOS is used in the inflammation process by peroxynitrite formation and in stimulation of neural apoptosis [57]. Garcia-Bonilla et al. discovered that after MCAO, iNOS-deficient mice engrafted with iNOS-positive bone marrow cells exhibited larger infarcts compared to iNOS-deficient mice autotransplanted with iNOS-deficient blood marrow cells. This study confirms that leukocytes play a significant role in the neuronal damage in ischemic stroke patients [58].

5. Pharmacological Approach: Nitric Oxide Donors and NOS Inhibitors

Some authors suggested that antiplatelet drugs affect nitric oxide biotransformation. According to Serebruany et al., modified-release dipyridamole and aspirin similarly increased primary diminished plasma eNOS activity in post-stroke patients in comparison to the control group [59]. In Gelosa et al.'s study, ticagrelor given in the early phase after permanent MCAO in rats significantly attenuated chemotaxis of leukocytes and reduced expression of iNOS [60]. Zhao et al. showed no association between acetylsalicylic acid (ASA), clopidogrel, or dipyridamole administration and NO metabolites together with cyclic guanosine monophosphate (cGMP) levels in patients with prior ischemic stroke and in the control group [61].

Each NOS subtype plays different roles during ischemic stroke which is demonstrated in diverse effects observed by use of particular NOS inhibitors. Pretreatment with statins or Rho-kinase inhibitors improve cerebral blood flow in the ischemic area and penumbra, decrease cerebral infarct volume, and improve neurological function after MCAO by increasing eNOS activation.in mice [62, 63] Nevertheless, inhibition of eNOS by L-N-(1-iminoethyl)ornithine (L-NIO) before MCAO elevates iNOS expression and exacerbates brain damage [64]. Aminoguanidine (iNOS inhibitor)

administration at 6 and 12 h after reperfusion in mice reduces NO concentration only in the penumbral region and lessens infarct size. Different iNOS inhibitors were studied by Armengou et al., in which N-(3-(aminomethyl)benzyl) acetamidine (1400W) administrated at the onset of ischemia and at 8-hour intervals for 3 days after MCAO resulted in a 55% reduction of infarct size, as measured 72 hours after induction of cerebral ischemia [65]. The protective role of iNOS inhibition in the thromboinflammation process is the most probably connected with decreased leukocyte activity. Matsuo et al. confirmed the essential role of neutrophils in ischemic stroke by documenting smaller infarct size in the neutropenic animals after reperfusion [66]. From a practical clinical perspective, edaravone, which directly enhances NO production, is recommended by Japanese guidelines for neuroprotection in ischemic stroke patients within 24 hours of onset [67]. This drug is commonly used in amyotrophic lateral sclerosis and exerts also a neuroprotective effect in reperfusion injury by reducing levels of superoxide increasing NO production and decreasing nNOS expression in cerebral neurons [68]. According to the Acute Infarction Study Group, administration of this substance < 72 h after ischemic stroke and through 14 days was connected with significant improvement in functional outcome evaluated by the modified Rankin Scale [69]. What is more, in Feng et al.'s systemic review on the edaravone influence on patients with acute ischemic stroke, the use of the drug was associated with neurological improvement in the intervention group compared with the control group (RR = 1.99) [70].

The plasma level of L-arginine (a substrate for NOS) is decreased in patients following the ischemic stroke and subsequently rises between 6 and 24 hours after the event. According to Armengou et al., plasma L-arginine concentrations are negatively correlated with the infarct volume and are significantly lower in patients with early neurologic deterioration as well as in those with poor outcome [65]. In Morikawa et al.'s study, administration of L-arginine 5 minutes after MCAO reduced infarct volume in rats measured 24 hours after vessel occlusion [47]. Lower levels of free radical production leading to smaller infarct size were also documented in Mason et al.'s and Zhao et al.'s animal studies of direct nitric oxide donor (diethanolamine nitric oxide and ZJM-289 novel NO-releasing derivative of 3-n-butylphthalide) administration during reperfusion [71, 72]. Although in human studies the transdermal glyceryl trinitrate (GTN) administration < 48 h and <5 days after cerebral ischemia did not improve functional outcome for ischemic stroke patients, in Woodhouse et al.'s analysis, transdermal GTN was safe and correlated with better functional outcome and with fewer deaths when administered within 6 hours of stroke onset. Significant beneficial effects were also achieved in disability (Barthel Index), quality of life, cognition, and mood [73–75]. Willmot et al. in their meta-analysis of preclinical studies confirmed the time-dependent effect of NO donors and L-arginine administration, specifying that early treatment (within 60 minutes) of ischemia was associated with the highest outcomes in comparison to neutral ones in those studies assessing treatment up to 48 hours following induction of ischemic stroke [76].

In human cells, NOS converts L-arginine to L-citrulline with a concomitant synthesis of NO, while asymmetric dimethylarginine (ADMA) is the most potent competitive inhibitor of this reaction. ADMA could play a crucial role in the CVD development as its higher plasma concentrations are significantly associated with cardiovascular risk factors, such as intima-media thickness of the carotid artery [77], hypertension [78], and diabetes mellitus types 1 and 2 [79, 80]. What is more, Ercan et al. showed that the plasma ADMA level measured during the first 24 hours in the group of patients after acute ischemic stroke was significantly higher than that in the control group [81]. Petrova et al. documented earlier reduction of plasma ADMA concentration in stroke patients after thrombolysis in comparison to the no-reperfusion group [82]. On the other hand, only symmetric dimethylarginine (with no effect on NO production) was a predictor of mortality in patients after acute ischemic stroke during 7.4 years of follow-up, while no correlation for ADMA was noted [83]. It seems that the intracellular compartment could be more significant in pathophysiology of CVD than its plasma level. Masuda et al. found the endothelial concentration of ADMA to be up to 10-fold higher than in plasma, while Yokoro et al. showed that protein arginine N-methyltransferase 1 (PRMT1) and dimethylarginine dimethylaminohydrolase-1 (DDAH-1)—enzymes responsible for the biotransformation of ADMA—are expressed also in erythrocytes, leukocytes, and platelets. Those authors also suggested that the side effect of protein methylation (a protective mechanism against highly reactive oxygen-derived free radicals) can lead to ADMA overproduction, which in consequence lowers cellular NO production, can cause endothelial damage, and can increase platelet activation and aggregation [84–87]. However, to date, there is no study conducted analyzing the association between nitric oxide biotransformation (including ADMA, PRMT1, and DDAH-1) and human platelets of ischemic stroke patients.

6. Platelet-Derived Nitric Oxide (PDNO)

A vast majority of studies documented the association between decreased endothelial NOS expression and clinical disorders predisposing to stroke, such as diabetes mellitus, atherosclerosis, hypertension, and cigarette smoking in patients [88–91]. Radomski et al. were the first authors to describe the inhibitory role of platelets' NO production on a thrombus formation. It has been found that L-arginine administration increases platelet NO formation leading to cGMP synthesis and protein kinase G (PKG) activation in thrombocytes' cytosol which in consequence inhibits thrombus formation. The described NO/cGMP/PKG pathway's antiaggregatory properties in platelets depend on provoking Ca^{2+} sequestration and inhibiting platelet degranulation [92] by

(1) refilling intraplatelet Ca^{2+} stores by promoting sarcoplasmic reticulum adenosine triphosphatase (ATPase), decreasing intracellular Ca^{2+} levels, and inhibiting influx of Ca^{2+} [93]

(2) inhibition of the inositol 1,4,5-trisphosphate-stimulated Ca^{2+} release from the sarcoplasmic reticulum [94]

(3) attenuating the TxA_2 receptor function by its phosphorylation [95]

(4) phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which enables VASP binding to the platelet cytoskeleton leading to inhibition of proaggregatory glycoprotein IIb/IIIa (GPIIb/IIIa) activation [96]

Without PKG action, cGMP also prevents platelet activation by inhibition of phosphodiesterase type 3 which increases intracellular cyclic adenosine monophosphate (cAMP; potent antiaggregation factor) [97] and inhibits phosphoinositide 3-kinase leading to GPIIb/IIIa fibrinogen receptor inactivation [98]. Nitric oxide blocks thrombus formation also on cGMP-independent mechanisms. The nitrosylation of the N-ethylmaleimide-sensitive factor inhibits aggregation by down-regulating alpha granule secretion and GPIIb/IIIa activation [99, 100]. What is more, irreversible nitration of platelet proteins by peroxynitrites results in inhibition of platelet adhesion to fibrinogen and decreased aggregation [101, 102].

Moreover, many other authors also showed that upon activation, platelets produce NO which inhibits adhesion and aggregation [92, 103–106]. Williams et al. demonstrated that high shear stress alone is sufficient to increase NO production in platelets leading to reduction of thrombus generation under blood flow. Moreover, authors described that reduction in thrombus formation (at a shear rate of $1000\,s^{-1}$) was abolished in the presence of L-NAME (the NO inhibitor), while at venous levels of shear rate ($100\,s^{-1}$), this substance had no effect on platelet activation and aggregation [107]. Cozzi et al. showed that platelet deposition is inversely related to platelet NO production and that intracytoplasmic Ca^{2+} elevation triggers platelet NO formation. Those results can suggest that the increase in intraplatelet Ca^{2+} concentration enhances the NO production which, in turn, limits thrombus size [108].

Some of the authors suggest that the generation of nitric oxide by resting platelets is constant (and is not elevated by L-arginine administration) [92, 103, 109]. Li et al. showed that basal production of NO by platelets activates cGMP-dependent protein kinase G (PKG) and enhances vWF-induced activation of platelets, which promotes rather than inhibits thrombus formation [110]. Those results can lead to the conclusion that platelet responses to NO and cGMP are both pro- and antiaggregative. However, in a study by Radziwon-Balicka et al., incubation with L-arginine inhibited platelet aggregation (by generation of NO) regardless of the platelet-activating stimulus concentration [111]. As a result from these studies, nitric oxide may serve as a platelet negative feedback regulator alone and only additional reaction of nitric oxide with superoxide anion can promote enhanced thrombus formation [112, 113].

7. Expression of the Nitric Oxide Synthase in Platelets

For many years, there was controversy whether platelets express their own nitric oxide synthase producing PDNO. Some of the authors suggested that contamination platelet samples by leukocytes account for suspected platelet NO production [114]. However, in a study on platelet subpopulations, Radziwon-Balicka et al. achieved high purity of their isolations (<2 leukocytes/100 000 platelets) and still detected significant nitric oxide production. Salvemini and colleagues showed that leukocyte contamination of >1% can inhibit aggregation via a NO-dependent mechanism, while in Radziwon-Balicka et al.'s study, leukocyte contamination was less than 0.002%. Finally, the authors concluded that this low leukocyte concentration in samples cannot account for detected NO production in the analyzed probes [112, 115–117].

In human megakaryocytes, both endothelial and inducible nitric oxide synthase isoforms are detected [118]. But whether platelets have the capacity to synthesize iNOS remains uncertain [119]. There is an interesting hypothesis that iNOS detected in platelet sample could derivate from leukocyte contamination. However, in Radziwon-Balicka et al.'s study, in the leukocyte-free probe samples (<2 leukocytes/100 000 platelets) the iNOS-selective antagonist 1400W was unable to reverse the antiaggregating effect of L-arginine. According to this study, the presence of iNOS in platelets is improbable [112].

Some authors also postulated that platelets do not contain eNOS and that this NOS isoform exists only in endothelial cells. However, Radziwon-Balicka et al. ultimately confirmed the presence of eNOS in triton-resistant platelet caveolae by a more specific identification method (fluorescence-activated cell sorting, while others used mass spectrometry) [112, 114, 120]. Both in animal and human studies, the endothelial nitric oxide synthase isoform is proved to produce PDNO [112, 121, 122]. Freedman et al. demonstrated that bleeding time was significantly decreased in eNOS-deficient versus wild-type mice. What is more, the bleeding time in thrombocytopenic eNOS-deficient mice transfused with eNOS-deficient platelets was significantly decreased compared with the same breed of mice transfused with wild-type platelets [121]. Moreover, Morrell et al. showed that the infusion of platelets from eNOS-deficient mice to animals with normal expression of eNOS resulted in increased granule exocytosis and stimulation of aggregation [99]. Riba et al. documented that vWF connection with platelet Gp Ib not only stimulates adhesion and aggregation but also activates platelet eNOS (measured by the increase in cGMP formation) with the presence of ADP and TxA_2. Interaction between collagen and platelet glycoprotein VI (GPVI) receptor activated platelet eNOS (with costimulation by ADP and TXA_2) only partially [123, 124]. Freedman et al. in a different study showed that inhibition of platelet eNOS increased P-selectin expression on the platelet surface after stimulation with ADP [106]. P-selectin is essential for leukocyte-platelet complex formation, and inhibition of platelet eNOS enhances the formation of those aggregates (especially monocyte-platelet aggregates) [125, 126].

8. Subpopulations of Platelets

Initially, the diversity among platelet size and density was attributed to the platelet aging processes. The large-dense platelets were identified as young thrombocytes recently released into the streaming blood, whereas the small and

low-density ones were postulated to represent older subpopulation. Nevertheless, studies by the Thompson et al. and Penington et al. have demonstrated that platelet size heterogeneity depends rather on platelet production from the different three ploidy classes of megakaryocytes (differ in their organelle content concentration) [127, 128]. Large-dense platelets contain a greater amount of glycogen, orthophosphate, and ADP and are characterized by upregulated glycolysis, glycogenolysis, and protein synthesis than small and low-density ones [129]. Large-dense thrombocytes aggregate more (due to higher ADP release and lower ADPase activity), require higher amount of prostacyclin concentration to inhibit aggregation, and adhere stronger to collagen (due to higher expression of the membrane GPIa/IIa receptors) than small and low-density ones [130–132]. Simultaneously, other authors showed that small and low-density platelets have an enhanced intracellular Ca^{2+} response to thrombin, which provokes them to a greater aggregation in response to these stimuli. Moreover, small and low-density platelets comparing to large-dense ones contain lower levels of the phosphorylated form of vasodilator-stimulated proteins, which can be the consequence of their weaker response to antiaggregative NO stimulation [133–135]. Although large-dense thrombocytes have higher aggregation and adhesion ability, small and low-density platelets react greater on thrombogenic stimuli with lower autoinhibitory response to NO.

In Kiliçli-Camur et al.'s study, high mean platelet volume (MPV) (associated with platelets' large and dense subpopulation) was increased during acute myocardial infarction and in the first subsequent weeks. What is more, patients with coronary artery disease (CAD) and elevated MPV had greater risk of acute myocardial infarct in comparison to those with a lower MPV, regardless of the extent of the coronary lesions [136]. Many studies also connected high MPV with ischemic stroke. In Butterworth and Bath's study, MPV was significantly higher in the ischemic stroke group than in the controls. Additionally, in stroke subgroup analysis, MPV was associated with cortical stroke but not with lacunar stroke [137]. Moreover, in Özkan et al.'s study, high MPV was associated with acute ischemic stroke only in patients with noncardioembolic stroke (with sinus rhythm and without heart failure or left atrial enlargement) [138]. More importantly, high MPV predicts also the risk of a second stroke up to 4 years before the acute event (11% increase of the relative risk of stroke for each femtoliter of MPV increase) and unfavorable outcome after cerebral infarction (death or dependency at 3 months follow-up) [137, 139]. These results underline the influence of platelets in CVD development and additionally suggest that MPV could be another risk factor for CVD development and progression.

9. Clinical Importance of PDNO and Expression of eNOS in Platelet Subpopulation

Radziwon-Balicka et al. identified in humans the thrombocyte subpopulations based on the presence of endothelial nitric oxide synthase (eNOS-positive or eNOS-negative platelets). Thrombocytes that are eNOS-negative constitute about 20% of total human platelet population and fail to produce NO, which attenuates their cGMP signaling pathway and—as result—promotes adhesion and formation of larger aggregates. The authors postulate that the role of e-NOS-negative platelets in thrombogenesis is probably to initiate adhesion and aggregation (the seed platelet hypothesis), while e-NOS-positive ones limit thrombus formation through NO production [112]. In the presence of vascular injury, eNOS-negative thrombocytes are the first to adhere to exposed collagen and/or to the von Willebrand factor. Thanks to the absence of endogenous NO generation, a quicker activation of integrin $\alpha IIb\beta 3$ appears, alongside with stabilization of initial rolling and adhesion of platelets [140]. Further recruitment of eNOS-positive platelets to a site of injury and formation of a greater aggregate is supported by matrix metalloproteinase secreted by eNOS-negative thrombocytes. Following aggregation, the eNOS-positive platelets form the bulk of a thrombus due to their greater thromboxane generation in comparison to the eNOS-negative thrombocytes. Finally, the limitation of aggregate size is achieved through nitric oxide generation by eNOS-positive platelets, when their number in the thrombus overbalances eNOS-negative ones [112].

Some data suggest that platelet-derived nitric oxide (PDNO) might be connected with development of cardiovascular disorders, including ischemic stroke [91, 141]. Ikeda et al. showed a negative correlation between PDNO and age, mean arterial pressure, total cholesterol, and LDL-cholesterol level. What is more, the PDNO release was also significantly decreased in long-term smokers [142]. Queen et al. has demonstrated that platelet nitric oxide synthase activity at baseline was lower in diabetic patients than in control subjects, while the platelet nitric oxide generation stimulated by beta-adrenoceptors attenuated in the course of diabetes [143]. Another study postulates that lower PDNO production was an independent predictor for acute coronary syndrome with odds ratio reaching 4.0 [144]. Laufs et al. showed the influence of PDNO on the course of ischemic stroke pointing out that statin-related improvement in the outcome is mediated by the increase in the eNOS expression in the thrombocytes and aorta [145]. Therefore, changes in the eNOS-negative to eNOS-positive platelet ratio might result in modification of the risk and outcome of acute ischemic cardiovascular events such as ischemic stroke or acute coronary syndrome [112].

On the other hand, there is still some controversy about association between nitric oxide and ischemic stroke. Platelets are characterized by the expression of several activation pathways. Noteworthily, Taka et al. showed that the NO donor and the NO synthase inhibitor did not affect shear-induced platelet reactivity or vasodilatation in stroke-prone spontaneously hypertensive rats [146]. Interestingly, Lafrati et al. found that eNOS-deficient animals showed a prolongation of time to occlusion, which was explained by the compensatory mechanism. Although eNOS-deficient mice had increased platelet recruitment, simultaneously they had also enhanced fibrinolysis due to lack of NO-dependent inhibition of Weibel-Palade body release (containing tissue plasminogen activator) from the endothelium [147]. What is

more, results from other studies show that a cumulative effect of NO on ischemic stroke could cause harm; as in animals treated at reperfusion with the nonselective NOS inhibitor, the infarct volume was significantly almost twofold decreased [148]. Manickam et al. suggests also that inhibition of peroxynitrite and other ROS production by superoxide dismutase rather than nitric oxide itself protects against ischemia/reperfusion injury in the brain [149]. Hence, the Janus-faced action of NO in stroke requires further precise studies.

10. Prevention and Treatment of Ischemic Noncardioembolic Stroke: A Translational Focus on Platelets in the Shade of Current Guidelines and Trials in Cardiovascular Medicine and Neurology from Bench to Bedside

Specific therapeutic strategy for ischemic stroke is thrombolytic therapy (alteplase treatment), in which efficacy has been clearly shown especially when performed within the therapeutic time frame (up to 4.5 hours from the onset of stroke symptoms and in particular cases, if the risk-benefit ratio approves its implementation, within 6 hours) [150]. Additionally, every patient eligible for mechanical thrombectomy (complementary treatment option to alteplase infusion) should have previous thrombolysis performed (depending on inclusion/exclusion criteria). According to the American Heart Association/American Stroke Association (ASA/AHA) 2019 guidelines, intravenous aspirin should not be administered within 90 minutes after the start of i.v. alteplase treatment because it increases risk of symptomatic intracranial hemorrhage more than twofold without any positive effect on functional outcome within 3 months of observation. The safety and efficacy of i.v. glycoprotein IIb/IIIa inhibitors administered after alteplase infusion or thrombectomy is uncertain [151–154].

Intravenous administration of tirofiban is the most commonly used antiplatelet therapy following rescue angioplasty with or without stenting after myocardial infarction. In acute ischemic stroke, tirofiban has been reported to facilitate further recanalization if primary mechanical thrombectomy failed and the highest benefit was achieved in LAA ischemic stroke subtype. Thus, tirofiban can be an interesting adjuvant therapy after unsuccessful thrombolysis/thrombectomy [155–157]. However, recent guidelines for the early management of patients with acute ischemic stroke recommends consideration of antiplatelet/antithrombotic therapy < 24 hours after treatment with i.v. alteplase only if the patient has concomitant conditions for which such treatment given in the absence of i.v. alteplase is known to provide substantial benefit or withholding such treatment is known to cause substantial risk. This recommendation is based only on a single-center retrospective analysis, which found no increased risk of hemorrhage with early initiation of antiplatelet or anticoagulant therapy after i.v. alteplase or endovascular treatment compared with initiation > 24 hours after ischemic stroke [154, 158]. Those recommendations prevent clinicians from wide usage of tirofiban in ischemic stroke patients after unsuccessful thrombolysis.

Approximately 8% of patients with ischemic stroke are admitted to the hospital in the time window allowing thrombolysis procedure [159]. For the remaining, about 90% of the only available current treatment option is secondary prevention of ischemic stroke. According to AHA/ASA 2019 guidelines for stroke prevention, administration of acetylsalicylic acid (160-300 mg/24 h) is recommended in patients with acute ischemic stroke within 24 to 48 hours after onset of disease or >24 hours after alteplase treatment with lifetime continuation of such antiplatelet treatment [154]. In Chen et al.'s meta-analysis, early use of ASA (<48 h) in acute ischemic stroke decreased the risk of recurrent stroke or death in a hospital with a nonsignificant increase in hemorrhagic stroke or hemorrhagic transformation of the original infarct [160]. In animal studies, the high-dose ASA therapy in temporary induced ischemia significantly reduced infarct size compared to placebo, in humans corresponding dosage would account for 19 grams of ASA with probably unfavorable benefit/risk ratio (higher hemorrhage risk) [161]. What is more, patients with diagnosed minor noncardioembolic ischemic stroke (NIHSS score ≤ 3) or at high-risk transient ischemic attack (TIA) ($ABCD_2$ (Age, Blood Pressure, Clinical Features, Duration, Diabetes) score ≥ 4) who did not receive thrombolysis should be treated with dual antiplatelet therapy (ASA and clopidogrel) started within 24 hours after symptom onset and continued for 21 days [162]. Finally, ASA alone is significantly reducing the 6-week risk of recurrent ischemic stroke by about 60% and disabling or fatal ischemic stroke by about 70% (with the greatest benefit in patients with TIA or minor stroke) [163]. However, according to the CAST study, the number needed to treat for ASA to prevent one stroke within one year is 100 patients [164].

Acetylsalicylic acid is a drug of choice in the secondary prevention of ischemic stroke. In case of intolerance, it can be replaced by clopidogrel 75 mg daily according to 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases [165, 166]. The meta-analysis by Paciaroni et al. even postulates clopidogrel to be a better choice in the secondary prevention of ischemic stroke due to the significant risk reduction for major adverse cardiovascular and cerebrovascular events, any ischemic or hemorrhagic stroke, and recurrent ischemic stroke in patients who received clopidogrel versus ASA. The risk of bleeding was also lower for clopidogrel in comparison to acetylsalicylic acid [167]. However, 2019 updated guidelines for the early management of patients with acute ischemic stroke suggest that increasing the dose of acetylsalicylic acid or switching to an alternative antiplatelet agent in patients who have a noncardioembolic ischemic stroke while taking ASA is still not well established [154]. There are only few indications for dual ASA and clopidogrel therapy mainly due to high risk of life-threatening hemorrhages. Dual antiplatelet therapy is indicated in minor noncardioembolic ischemic stroke or with high-risk TIA (as described before), after myocardial infarct or after carotid artery stenting [154, 166, 168]. The benefit of single antiplatelet therapy for preventing stroke in asymptomatic patients with carotid artery stenosis > 50% is not proven to be beneficial in randomized control trials. However, optimal medical treatment with acetylsalicylic acid or clopidogrel is recommended for the majority of those

Table 1: Effect of different antiplatelet drug treatments on outcome in ischemic stroke.

	Mechanism of action	Primary prevention of ischemic stroke		Acute phase of ischemic stroke		Secondary prevention of ischemic stroke	
		Animal studies	Human studies	Animal studies	Human studies	Animal studies	Human studies
Acetylsalicylic acid	Cyclooxygenase inhibitor	Beneficial [182]	Neutral (beneficial after artery stenting) [166]	Beneficial [161]	Beneficial in TIA and minor stroke (NIHSS ≤ 3) [183]	Beneficial [184]	Beneficial [154]
Clopidogrel	Inhibitor of P2Y12 receptor	Beneficial [185]	Neutral (beneficial after artery stenting) [166]	Beneficial [186]	Beneficial in TIA and minor stroke (NIHSS ≤ 3) [183]	Beneficial [185]	Beneficial [154]
Prasugrel	Inhibitor of P2Y12 receptor	Beneficial [187]	Neutral (beneficial after ACS) [188]	Beneficial [186]	Harmful [154]	Beneficial [189]	Harmful [190]
Ticagrelor	Inhibitor of P2Y12 receptor	Beneficial [191]	Neutral (better prevention with higher hemorrhage incidence) [192]	Beneficial [191]	Harmful [154]	No data found	Neutral (better prevention with higher hemorrhage incidence) [193]
Cangrelor	Inhibitor of P2Y12 receptor	Neutral [188]	No data found	Beneficial [186]	Harmful [154]	No data found	Beneficial in stroke prevention in the perioperative period [194]
Vorapaxar	PAR-1 antagonist	No data found	Harmful [195]	No data found	Harmful [169]	No data found	Harmful/neutral [196, 197]
Tirofiban	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [172]	Beneficial [157]	Beneficial [198]	Uncertain [200]
Abciximab	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [201]	Uncertain [154]	Beneficial [198]	Harmful [199]
Eptifibatide	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [201]	Beneficial [151]	Beneficial [198]	Uncertain [200]
Anfibatide	GPIb blocker	Beneficial [202]	No data found	Beneficial [172]	No data found	No data found	No data found
Caplacizumab	Anti-vWF antibody, blocker of platelet GPI-vWF adhesion	Beneficial [173]	No data found	Beneficial [173]	No data found	No data found	No data found
ADAMTS13	Recombinant human enzyme transforming vWF to smaller, less active forms	Beneficial [175]	No data found	Beneficial [203]	No data found	No data found	No data found
rHA-infestin-4	XIIa inhibitor	Beneficial [176]	No data found	Beneficial [204]	No data found	No data found	No data found
Revacept	Competitive blocker of platelet	Beneficial [177]	Beneficial [205]	Beneficial [206]	No data available (ongoing study of	No data found	No data found

Table 1: Continued.

	Mechanism of action	Primary prevention of ischemic stroke		Acute phase of ischemic stroke		Secondary prevention of ischemic stroke	
		Animal studies	Human studies	Animal studies	Human studies	Animal studies	Human studies
	GPVI adhesion to vWF				patients with stable coronary artery disease undergoing elective PCI)		
F-0401	Dihydropyridine calcium antagonist with PAF antagonistic action	Beneficial [179]	No data found	Beneficial [207]	Beneficial (in the study of human astrocytoma and neuroblastoma cells) [207]	No data found	No data found
BN 50739	PAF antagonist	Beneficial [180]	No data found	Beneficial [209]	No data found	No data found	No data found
Inclacumab	P-selectin neutralizing antibody	No data found	No data found	No data found	No data available (beneficial in non-ST-segment elevation myocardial infarction) [181]	No data found	No data found

patients in the primary prevention of ischemic stroke to reduce the risk of stroke and other cardiovascular events, as these patients are also at twice the risk for myocardial infarct. In symptomatic extracranial carotid stenosis, antiplatelet monotherapy is always recommended [166]. Newer drugs from the same class as clopidogrel (inhibitors of P2Y12 receptor: prasugrel, ticagrelor, and cangrelor) are not beneficial in the acute phase of ischemic stroke. Vorapaxar, protease-activated receptor-1 antagonist (PAR-1), treatment during acute myocardial infarct is proved to be beneficial in clinical tests; its use in acute ischemic stroke is harmful leading to a greater hemorrhagic transformation [154, 169]. Another antiplatelet drug, abciximab (glycoprotein IIb/IIIa inhibitor) as medical treatment for the secondary prevention of ischemic stroke, is potentially harmful and should not be used, while efficacy of eptifibatide is not well established yet [154, 170].

Even though the main function of antiplatelet agents is to inhibit platelet-platelet aggregation, in case of penumbral protection, platelet-endothelium adhesion and platelet-leukocyte aggregation are similarly important. Anfibatide by inhibiting adhesive properties of platelets (blocker of platelet glycoprotein receptor Ib) significantly reduces infarct size, increases the number of intact neuronal cells, and improves neurobehavioral function by reducing postischemic blood-brain barrier damage, leukocyte migration, and microthrombus formation [171, 172]. Caplacizumab (humanized anti-vWF antibody) by binding to vWF inhibits platelet adhesion to the vessel wall (platelet GPIb—vascular vWF interaction blockade). This drug in Momi et al.'s study both prevented middle carotid artery thrombosis and reduced brain damage without provoking hemorrhage by inducing reperfusion when given before or up to 15 minutes after complete artery occlusion. Tirofiban (GPIIb/IIIa blocker) prevented thrombosis but did not induce reperfusion and caused striking brain hemorrhage [173, 174]. The activity of vWF is regulated by a disintegrin-like and metalloprotease with thrombospondin type I repeats-13 (ADAMTS13) that transforms vWF to smaller, less active forms. According to Zhao et al., infusion of a high dose of recombinant human ADAMTS13 into a wild-type mouse immediately before reperfusion reduces infarct size and improves functional outcome without producing cerebral hemorrhage pointing thus at ADAMTS13 to be a useful potential therapeutic target in ischemic stroke [175]. Another potential drug connected with GPIb interactions is specific factor XIIa inhibitor rHA-infestin-4. This substance completely inhibits occlusive arterial thrombus formation in mice and rats while leaving hemostasis fully intact [176]. The revacept (GPVI-Fc fusion protein) blocks competitively binding of vWF to collagen and GPVI-mediated platelet adhesion. Goebel et al. showed that this medication prevents thrombus formation after endothelial injury and, if applied immediately before reperfusion in mice with ischemic stroke, significantly improves functional outcome and decreases cerebral infarct size [177]. de Brito Toscano et al. showed that a platelet-activating factor (PAF) receptor-deficient mouse had a smaller brain-infarcted area in comparison to the control group [178]. Furthermore, pretreatment with F-0401, dihydropyridine calcium antagonist with PAF antagonistic action, prevents the occurrence of brain edema, disruption of the blood-brain barrier, and neuronal damage caused by cerebral ischemia [179]. Pretreatment with PAF antagonist (BN 50739) before induction of focal cortical lesions in anesthetized rats improved the penumbral cerebral blood flow and reduced edema and the progression of neuronal damage [180]. Inclacumab (a potent and selective P-selectin-neutralizing antibody) appears to reduce myocardial damage after percutaneous coronary intervention (PCI) in patients with non-ST-segment elevation myocardial infarction. However, there is no study conducted analyzing effects of inclacumab on ischemic stroke [181] (Table 1).

Despite the numerous antiplatelet drugs implemented in clinical practice and large body of evidence for their effectiveness in particular clinical scenarios, the precise and disease-

dedicated therapy remains a difficult task for clinicians. Further studies, based more and more on translational medicine, are required to combine pathophysiological knowledge and the basics of pharmacotherapy with data from epidemiological clinical trials in order to formulate optimal recommendations.

11. Clinical Implications and Future Directions

In summary, there is a large body of evidence on the important role of nitric oxide in the pathophysiology of thrombogenesis in patients at high and very high cardiovascular risk. However, there are scarcely no studies separating the contribution of endothelial and platelet-derived NO in the onset and clinical course of particular cardiovascular events of atherothrombotic origin. The study discovering the eNOS-negative and eNOS-positive subpopulations of platelets constitutes a milestone that may change the paradigm stating that decreased endothelial NO bioavailability and endothelial dysfunction itself may promote the onset of acute ischemic events. What is more, the quantitative analysis accompanied with the verification of some activation-related platelet features might become a useful tool as a prognostic biomarker for ischemic stroke and thromboinflammation. Furthermore, the role of ADMA in platelets, which elevated the plasma level, is a well-known cardiovascular risk factor and requires further studies. A detailed analysis of the platelet ADMA biotransformation (including its synthesis with PRMT, transmembrane translocation with CAT, and degradation by the DDAH) should provide some new important data on this issue. Future experimental studies using the selective iNOS and nNOS inhibitors or antiplatelet agents blocking the GPI and GPVI receptors in the management of brain ischemia-reperfusion injury are required to clarify the Janus-faced action of nitric oxide in stroke.

Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgments

The present manuscript and publishing costs were covered by the Wrocław Medical University, according to the records in the simple system with the number ST-A210.17.057.

References

[1] "Global Burden of Disease Study 2017 (GBD 2017)," in *Institute for Health Metrics and Evaluation, University of Washington*, Seattle, WA, 2018http://ghdx.healthdata.org/gbd-results-tool. Accessed June 18,2020.

[2] R. V. Krishnamurthi, S. Barker-Collo, V. Parag et al., "Stroke Incidence by Major Pathological Type and Ischemic Subtypes in the Auckland Regional Community Stroke Studies: Changes Between 2002 and 2011," *Stroke*, vol. 49, no. 1, pp. 3–10, 2018.

[3] P. K. Yip, J. S. Jeng, T. K. Lee et al., "Subtypes of ischemic stroke. A hospital-based stroke registry in Taiwan (SCAN-IV)," *Stroke*, vol. 28, no. 12, pp. 2507–2512, 1997.

[4] A. J. Grau, C. Weimar, F. Buggle et al., "Risk Factors, Outcome, and treatment in subtypes of ischemic stroke: the German stroke Data Bank," *Stroke*, vol. 32, no. 11, pp. 2559–2566, 2001.

[5] D. Kim, S.-H. Lee, B. J. Kim et al., "Secondary prevention by stroke subtype: a nationwide follow-up study in 46 108 patients after acute ischaemic stroke," *European Heart Journal*, vol. 34, no. 35, pp. 2760–2767, 2013.

[6] D. Mozaffarian, E. J. Benjamin, A. S. Go et al., "Executive Summary: Heart Disease and Stroke Statistics—2016 Update," *Circulation*, vol. 133, no. 4, pp. 447–454, 2016.

[7] J. K. Lovett, A. J. Coull, and P. M. Rothwell, "Early risk of recurrence by subtype of ischemic stroke in population-based incidence studies," *Neurology*, vol. 62, no. 4, pp. 569–573, 2004.

[8] J. Serena, T. Segura, J. Roquer, M. García-Gil, and J. Castillo, "The ARTICO study: identification of patients at high risk of vascular recurrence after a first non-cardioembolic stroke," *BMC Neurology*, vol. 15, no. 1, 2015.

[9] P. Eshtehardi and Z. Teng, "Protective or destructive: High wall shear stress and atherosclerosis," *Atherosclerosis*, vol. 251, pp. 501–503, 2016.

[10] K. C. Koskinas, Y. S. Chatzizisis, M. I. Papafaklis et al., "Synergistic effect of local endothelial shear stress and systemic hypercholesterolemia on coronary atherosclerotic plaque progression and composition in pigs," *International Journal of Cardiology*, vol. 169, no. 6, pp. 394–401, 2013.

[11] A. Stanek, A. Gadowska-Cicha, K. Gawron et al., "Role of nitric oxide in physiology and pathology of the gastrointestinal tract," *Mini-Reviews in Medicinal Chemistry*, vol. 8, no. 14, pp. 1549–1560, 2008.

[12] F. Vozzi, J. Campolo, L. Cozzi et al., "Computing of low shear stress-driven endothelial gene network involved in early stages of atherosclerotic process," *BioMed research international*, vol. 2018, Article ID 5359830, 12 pages, 2018.

[13] B. J. Fredrickson, J. F. Dong, L. V. McIntire, and J. A. López, "Shear-dependent rolling on Von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex," *Blood*, vol. 92, no. 10, pp. 3684–3693, 1998.

[14] A. D. Blann and M. A. Waite, "Von Willebrand factor and soluble E-selectin in hypertension: influence of treatment and value in predicting the progression of atherosclerosis," *Coronary Artery Disease*, vol. 7, no. 2, pp. 143–148, 1996.

[15] N.-W. Tsai, W.-N. Chang, C.-F. Shaw et al., "Levels and value of platelet activation markers in different subtypes of acute non-cardio-embolic ischemic stroke," *Thrombosis research*, vol. 124, no. 2, pp. 213–218, 2009.

[16] P. Htun, S. Fateh-Moghadam, B. Tomandl et al., "Course of platelet activation and platelet-leukocyte interaction in cerebrovascular ischemia," *Stroke*, vol. 37, no. 9, pp. 2283–2287, 2006.

[17] T. Ishikawa, M. Shimizu, S. Kohara, S. Takizawa, Y. Kitagawa, and S. Takagi, "Appearance of WBC-platelet complex in acute ischemic stroke, predominantly in atherothrombotic infarction," *Journal of atherosclerosis and thrombosis*, vol. 19, no. 5, pp. 494–501, 2012.

[18] H.-K. Yip, S.-S. Chen, J. S. Liu et al., "Serial changes in platelet activation in patients after ischemic stroke: role of pharmacodynamic modulation," *Stroke*, vol. 35, no. 7, pp. 1683–1687, 2004.

[19] J. H. Dougherty Jr., D. E. Levy, and B. B. Weksler, "PLATELET ACTIVATION IN ACUTE CEREBRAL ISCHÆMIA: Serial Measurements of Platelet Function in Cerebrovascular Disease," *Lancet*, vol. 309, pp. 8016821–8016824, 1977.

[20] T. Oberheiden, X. D. Nguyen, M. Fatar et al., "Platelet and monocyte activation in acute ischemic stroke–is there a correlation with stroke etiology?," *Clinical and Applied Thrombosis/Hemostasis*, vol. 18, no. 1, pp. 87–91, 2011.

[21] V. Henn, J. R. Slupsky, M. Grafe et al., "Cd40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells," *Nature*, vol. 391, no. 6667, pp. 591–594, 1998.

[22] M. Ishikawa, T. Vowinkel, K. Y. Stokes et al., "CD40/CD40 ligand signaling in mouse cerebral microvasculature after focal ischemia/reperfusion," *Circulation*, vol. 111, no. 13, pp. 1690–1696, 2005.

[23] R.-H. Jiang, X.-Q. Xu, C.-J. Wu et al., "The CD40/CD40L system regulates rat cerebral microvasculature after focal ischemia/reperfusion via the mTOR/S6K signaling pathway," *Neurological Research*, vol. 40, no. 9, pp. 717–723, 2018.

[24] C. Kleinschnitz, P. Kraft, A. Dreykluft et al., "Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature," *Blood*, vol. 121, no. 4, pp. 679–691, 2013.

[25] P. J. Lindsberg, O. Carpén, A. Paetau, M. L. Karjalainen-Lindsberg, and M. Kaste, "Endothelial ICAM-1 expression associated with inflammatory cell response in human ischemic stroke," *Circulation*, vol. 94, no. 5, pp. 939–945, 1996.

[26] S. Uchiyama, M. Yamazaki, and S. Maruyama, "Role of platelet-activating factor in aggregation of leukocytes and platelets in cerebral ischemia," *Lipids*, vol. 26, no. 12, pp. 1247–1249, 1991.

[27] G. A. Zimmerman, T. M. McIntyre, M. Mehra, and S. M. Prescott, "Endothelial cell-associated platelet-activating factor: a novel mechanism for signaling intercellular adhesion," *Journal of Cell Biology*, vol. 110, no. 2, pp. 529–540, 1990.

[28] P. J. Lindsberg, J. M. Hallenbeck, and G. Feuerstein, "Platelet-activating factor in stroke and brain injury," *Annals of Neurology*, vol. 30, pp. 117–129, 1991.

[29] G. Sawicki, E. Salas, J. Murat, H. Miszta-Lane, and M. W. Radomski, "Release of gelatinase A during platelet activation mediates aggregation," *Nature*, vol. 386, no. 6625, pp. 616–619, 1997.

[30] M. K. Puurunen, S.-J. Hwang, M. G. Larson et al., "ADP platelet hyperreactivity predicts cardiovascular disease in the FHS (Framingham Heart Study)," *Journal of the American Heart Association*, vol. 7, no. 5, article e008522, 2018.

[31] R. Joseph, K. M. Welch, S. B. Oster, S. Grunfeld, and G. D'Andrea, "A plasmatic factor may cause platelet activation in acute ischemic stroke," *Circulation research*, vol. 65, no. 6, pp. 1679–1687, 1989.

[32] S. Boggs, L. Huang, and D. J. Stuehr, "Formation and reactions of the hemedioxygen intermediate in the first and second steps of nitric oxide synthesis as studied by stopped-flow spectroscopy under single-turnover conditions," *Biochemistry*, vol. 39, no. 9, pp. 2332–2339, 2000.

[33] A. Stanek, B. Fazeli, S. Bartuś, and E. Sutkowska, "The role of endothelium in physiological and pathological states: new data," *BioMed Research International*, vol. 2018, Article ID 1098039, 3 pages, 2018.

[34] J. Santolini, S. Adak, C. M. Curran, and D. J. Stuehr, "A kinetic simulation model that describes catalysis and regulation in nitric-oxide synthase," *Journal of Biological Chemistry*, vol. 276, no. 2, pp. 1233–1243, 2001.

[35] H. M. Abu-Soud, D. L. Rousseau, and D. J. Stuehr, "Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension," *Journal of Biological Chemistry*, vol. 271, no. 51, pp. 32515–32518, 1996.

[36] W. K. Alderton, C. E. Cooper, and R. G. Knowles, "Nitric oxide synthases : structure, function and inhibition," *Biochemical Journal*, vol. 357, no. 3, pp. 593–615, 2001.

[37] Y. Xia, L. J. Roman, B. S. Masters, and J. L. Zweier, "Inducible nitric-oxide synthase generates superoxide from the reductase domain," *Journal of Biological Chemistry*, vol. 273, no. 35, pp. 22635–22639, 1998.

[38] T. Malinski, F. Bailey, Z. G. Zhang, and M. Chopp, "Nitric oxide measured by a porphyrinic Micro Sensor in rat brain after transient middle cerebral artery occlusion," *Journal of Cerebral Blood Flow & Metabolism*, vol. 13, no. 3, pp. 355–358, 1993.

[39] A. Kader, V. I. Frazzini, R. A. Solomon, and R. R. Trifiletti, "Nitric oxide production during focal cerebral ischemia in rats," *Stroke*, vol. 24, no. 11, pp. 1709–1716, 1993.

[40] M. Niwa, S. Inao, M. Takayasu et al., "Time course of expression of three nitric oxide synthase isoforms after transient middle cerebral artery occlusion in rats," *Neurologia Medico-Chirurgica*, vol. 41, no. 2, pp. 63–73, 2001.

[41] M. S. M. Tabuchi, M. Ikeda, and T. Tomita, "Concurrent formation of peroxynitrite with the expression of inducible nitric oxide synthase in the brain during middle cerebral artery occlusion and reperfusion in rats," *Brain Research*, vol. 951, no. 1, pp. 113–120, 2002.

[42] M. Udayabanu, D. Kumaran, R. U. Nair et al., "Nitric oxide associated with iNOS expression inhibits acetylcholinesterase activity and induces memory impairment during acute hypobaric hypoxia," *Brain Research*, vol. 1230, pp. 138–149, 2008.

[43] L. W. Dobrucki, L. Kalinowski, W. Uracz, and T. Malinski, "The protective role of nitric oxide in the brain ischemia," *Journal of Physiology and Pharmacology*, vol. 51, 4 Part 1, pp. 695–703, 2000.

[44] M. Serrano-Ponz, C. Rodrigo-Gasqué, E. Siles, E. Martínez-Lara, L. Ochoa-Callejero, and A. Martínez, "Temporal profiles of blood pressure, circulating nitric oxide, and adrenomedullin as predictors of clinical outcome in acute ischemic stroke patients," *Molecular medicine reports*, vol. 13, no. 5, pp. 3724–3734, 2016.

[45] R. Taffi, L. Nanetti, L. Mazzanti et al., "Plasma levels of nitric oxide and stroke outcome," *Journal of Neurology*, vol. 255, no. 1, pp. 94–98, 2008.

[46] E. Morikawa, Z. Huang, and M. A. Moskowitz, "L-arginine decreases infarct size caused by middle cerebral arterial occlusion in SHR," *American Journalof Physiology*, vol. 263, no. 5, pp. H1632–H1635, 1992.

[47] E. Morikawa, M. A. Moskowitz, Z. Huang, T. Yoshida, K. Irikura, and T. Dalkara, "L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat," *Stroke*, vol. 25, no. 2, pp. 429–435, 1994.

[48] D. N. Atochin, I. T. Demchenko, J. Astern, A. E. Boso, C. A. Piantadosi, and P. L. Huang, "Contributions of endothelial and neuronal nitric oxide synthases to cerebrovascular responses to hyperoxia," *Journal of Cerebral Blood Flow & Metabolism*, vol. 23, no. 10, pp. 1219–1226, 2003.

[49] J. Ma, W. Meng, C. Ayata, P. L. Huang, M. C. Fishman, and M. A. Moskowitz, "L-NNA-sensitive regional cerebral blood flow augmentation during hypercapnia in type III NOS mutant mice," *Journal of Physiology-Heart and Circulatory Physiology*, vol. 271, 4 Part 2, pp. H1717–H1719, 1996.

[50] Y. Ito, T. Ohkubo, Y. Asano et al., "Nitric oxide production during cerebral ischemia and reperfusion in eNOS- and nNOS-knockout mice," *Current neurovascular research*, vol. 7, no. 1, pp. 23–31, 2010.

[51] X. W. Zeng, M. W. Li, J. Pan et al., "Activation of c-Jun N-terminal kinase 1/2 regulated by nitric oxide is associated with neuronal survival in hippocampal neurons in a rat model of ischemia," *Chinese Medical Journal*, vol. 124, no. 20, pp. 3367–3372, 2011.

[52] N. E. Stagliano, W. D. Dietrich, R. Prado, E. J. Green, and R. Busto, "The role of nitric oxide in the pathophysiology of thromboembolic stroke in the rat," *Brain Research*, vol. 759, no. 1, pp. 32–40, 1997.

[53] M. T. Mohammadi, "Overproduction of nitric oxide intensifies brain infarction and cerebrovascular damage through reduction of claudin-5 and ZO-1 expression in striatum of ischemic brain," *Pathology - Research and Practice*, vol. 212, no. 11, pp. 959–964, 2016.

[54] M. Y. Globus, R. Prado, J. Sanchez-Ramos et al., "A dual role for nitric oxide in NMDA-mediated toxicity in vivo," *Journal of Cerebral Blood Flow & Metabolism*, vol. 15, no. 6, pp. 904–913, 1995.

[55] V. L. Dawson, T. M. Dawson, D. A. Bartley, G. R. Uhl, and S. N. Snyder, "Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures," *Journal of Neuroscience*, vol. 13, no. 6, pp. 2651–2661, 1993.

[56] I. Paakkari and P. Lindsberg, "Nitric oxide in the central nervous system," *Annals of medicine*, vol. 27, no. 3, pp. 369–377, 1995.

[57] G. C. Brown, "Nitric oxide and neuronal death," *Nitric Oxide*, vol. 23, no. 3, pp. 153–165, 2010.

[58] L. Garcia-Bonilla, J. M. Moore, G. Racchumi et al., "Inducible nitric oxide synthase in neutrophils and endothelium contributes to ischemic brain injury in mice," *The Journal of Immunology*, vol. 193, no. 5, pp. 2531–2537, 2014.

[59] V. Serebruany, Y. Sani, C. Eisert, A. Schevchuck, A. Fong, and D. Hanley, "Effects of Aggrenox and aspirin on plasma endothelial nitric oxide synthase and oxidised low-density lipoproteins in patients after ischaemic stroke. The AGgrenox versus Aspirin Therapy Evaluation (AGATE) Biomarker Substudy," *Thrombosis and haemostasis*, vol. 105, no. 1, pp. 81–87, 2011.

[60] P. Gelosa, D. Lecca, M. Fumagalli et al., "Microglia is a key player in the reduction of stroke damage promoted by the new antithrombotic agent ticagrelor," *Journal of Cerebral Blood Flow & Metabolism*, vol. 34, no. 6, pp. 979–988, 2014.

[61] L. Zhao, L. Gray, J. Leonardi-Bee, C. S. Weaver, S. Heptinstall, and P. M. W. Bath, "Effect of aspirin, clopidogrel and dipyridamole on soluble markers of vascular function in normal volunteers and patients with prior ischaemic stroke," *Platelets*, vol. 17, no. 2, pp. 100–104, 2006.

[62] M. Endres, U. Laufs, Z. Huang et al., "Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase," *Proceedings of the National Academy of Sciences*, vol. 95, no. 15, pp. 8880–8885, 1998.

[63] H. K. Shin, S. Salomone, E. M. Potts et al., "Rho-kinase inhibition acutely augments blood flow in focal cerebral ischemia via endothelial mechanisms," *Journal of Cerebral Blood Flow & Metabolism*, vol. 27, no. 5, pp. 998–1009, 2007.

[64] R. Greco, C. Demartini, A. M. Zanaboni, F. Blandini, D. Amantea, and C. Tassorelli, "Endothelial nitric oxide synthase inhibition triggers inflammatory responses in the brain of male rats exposed to ischemia-reperfusion injury," *Journal of Neuroscience Research*, vol. 96, no. 1, pp. 151–159, 2018.

[65] A. Armengou, O. Hurtado, R. Leira et al., "L-Arginine levels in blood as a marker of nitric oxide-mediated brain damage in acute stroke: a clinical and experimental study," *Journal of Cerebral Blood Flow & Metabolism*, vol. 23, no. 8, pp. 978–984, 2003.

[66] Y. Matsuo, H. Onodera, Y. Shiga et al., "Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat," *Effects of neutrophil depletion. Stroke*, vol. 25, no. 7, pp. 1469–1475, 1994.

[67] Y. Shinohara and T. Yamaguchi, "Outline of the Japanese Guidelines for the Management of Stroke 2004 and subsequent revision," *International Journal of Stroke*, vol. 3, no. 1, pp. 55–62, 2008.

[68] H. Kawasaki, Y. Ito, C. Kitabayashi et al., "Effects of edaravone on nitric oxide, hydroxyl radicals and neuronal nitric oxide synthase during cerebral ischemia and reperfusion in mice," *Journal of Stroke and Cerebrovascular Diseases*, vol. 29, no. 3, article 104531, 2020.

[69] Edaravone Acute Infarction Study Group, "Effect of anovel free radical scavenger, edaravone (MCI-186), on acute brain infarction. Randomized, placebo-controlled,double-blind study at multicenters," *Cerebrovascular Diseases*, vol. 15, no. 3, pp. 222–229, 2003.

[70] S. Feng, Q. Yang, M. Liu et al., "Edaravone for acute ischaemic stroke," *Cochrane Database of Systematic Reviews*, no. 12, article CD007230, 2011.

[71] R. B. Mason, R. M. Pluta, S. Walbridge, D. A. Wink, E. H. Oldfield, and R. J. Boock, "Production of reactive oxygen species after reperfusion in vitro and in vivo: protective effect of nitric oxide," *Journal of neurosurgery*, vol. 93, no. 1, pp. 99–107, 2000.

[72] Q. Zhao, C. Zhang, X. Wang, L. Chen, H. Ji, and Y. Zhang, "(S)-ZJM-289, a nitric oxide-releasing derivative of 3-*n*-butylphthalide, protects against ischemic neuronal injury by attenuating mitochondrial dysfunction and associated cell death," *Neurochemistry international*, vol. 60, no. 2, pp. 134–144, 2012.

[73] E. C. Sandset, J. P. Appleton, E. Berge et al., "ENOS trial investigator.Associations between change in blood pressure and functional outcome, early events and death: results from the efficacy of nitric oxide in stroke trial," *Journal of Hypertension*, vol. 37, no. 10, pp. 2104–2109, 2019.

[74] P. M. Bath, R. Pathansali, R. Iddenden, and F. J. Bath, "The effect of transdermal glyceryl trinitrate, a nitric oxide donor, on blood pressure and platelet function in acute stroke," *Cerebrovascular Diseases*, vol. 11, no. 3, pp. 265–272, 2001.

[75] L. Woodhouse, P. Scutt, K. Krishnan et al., "ENOS Investigators Effect of hyperacute administration (within 6 hours) of transdermal glyceryl trinitrate, a nitric oxide donor, on outcome after stroke: subgroup analysis of the Efficacy of Nitric Oxide in Stroke (ENOS) trial," *Stroke*, vol. 46, no. 11, pp. 3194–3201, 2015.

[76] M. Willmot, L. Gray, C. Gibson, S. Murphy, and P. M. W. Bath, "A systematic review of nitric oxide donors and L-arginine in experimental stroke; effects on infarct size and cerebral blood flow," *Nitric Oxide*, vol. 12, no. 3, pp. 141–149, 2005.

[77] H. Miyazaki, H. Matsuoka, J. P. Cooke et al., "Endogenous nitric oxide synthase Inhibitor," *Circulation*, vol. 99, no. 9, pp. 1141–1146, 1999.

[78] D. Wang, S. Strandgaard, J. Iversen, and C. S. Wilcox, "Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 296, no. 2, pp. R195–R200, 2009.

[79] A. E. Altinova, M. Arslan, A. Sepici-Dincel, M. Akturk, N. Altan, and F. B. Toruner, "Uncomplicated type 1 diabetes is associated with increased asymmetric dimethylarginine concentrations," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 5, pp. 1881–1885, 2007.

[80] F. Abbasi, T. Asagmi, J. P. Cooke et al., "Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus," *American Journal of Cardiology*, vol. 88, no. 10, pp. 1201–1203, 2001.

[81] M. Ercan, S. Mungan, I. Güzel et al., "Serum asymmetric dimethylarginine and nitric oxide levels in Turkish patients with acute ischemic stroke," *Advances in Clinical and Experimental Medicine*, vol. 28, no. 5, pp. 693–698, 2019.

[82] J. J. Petrova, V. E. Manolov, V. G. Vasilev, D. K. Zlatareva, and K. N. Tzatchev, "ADMA–a possible marker for early therapeutic outcome in acute stroke," *Clinical Laboratory*, vol. 61, no. 11, pp. 1653–1658, 2015.

[83] F. Schulze, A. M. Carter, E. Schwedhelm et al., "Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke," *Atherosclerosis*, vol. 208, no. 2, pp. 518–523, 2010.

[84] H. Masuda, M. Goto, S. Tamaoki, and H. Azuma, "Accelerated intimal hyperplasia and increased endogenous inhibitors for NO synthesis in rabbits with alloxan-induced hyperglycaemia," *British journal of pharmacology*, vol. 126, no. 1, pp. 211–218, 1999.

[85] M. Yokoro, M. Suzuki, K. Murota et al., "Asymmetric dimethylarginine, an endogenous NOS inhibitor, is actively metabolized in rat erythrocytes," *Bioscience, biotechnology, and biochemistry*, vol. 76, no. 7, pp. 1334–1342, 2012.

[86] D.-J. Jiang, S.-J. Jia, J. Yan, Z. Zhou, Q. Yuan, and Y.-J. Li, "Involvement of DDAH/ADMA/NOS pathway in nicotine-induced endothelial dysfunction," *Biochemical and biophysical research communications*, vol. 349, no. 2, pp. 683–693, 2006.

[87] T. Brunini, M. Moss, M. Siqueira et al., "Inhibition of L-arginine Transport in platelets by asymmetric dimethylarginine and N-monomethyl-l-arginine: effects of arterial hypertension," *Clinical and experimental pharmacology and physiology*, vol. 31, no. 10, pp. 738–740, 2004.

[88] B. S. Oemar, M. R. Tschudi, N. Godoy, V. Brovkovich, T. Malinski, and T. F. Lüscher, "Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis," *Circulation*, vol. 97, no. 25, pp. 2494–2498, 1998.

[89] Z. He, Y. Chen, C. Hou, W. He, and P. Chen, "Cigarette smoke extract changes expression of endothelial nitric oxide synthase (eNOS) and p16(INK4a) and is related to endothelial progenitor cell dysfunction," *Medical Science Monitor*, vol. 23, pp. 3224–3231, 2017.

[90] M. Settergren, F. Böhm, R. E. Malmström, K. M. Channon, and J. Pernow, "l-Arginine and tetrahydrobiopterin protects against ischemia/reperfusion- induced endothelial dysfunction in patients with type 2 diabetes mellitus and coronary artery disease," *Atherosclerosis*, vol. 204, no. 1, pp. 73–78, 2009.

[91] A. Camilletti, N. Moretti, G. Giacchetti et al., "Decreased nitric oxide levels and increased calcium content in platelets of hypertensive patients," *American Journal of Hypertension*, vol. 14, no. 4, pp. 382–386, 2001.

[92] R. Murray, D. Granner, P. Mayes, and V. Rodwell, *Biochemia Harpera*, PZWL, Warszawa, 2004.

[93] M. W. RADOMSKI, R. M. J. PALMER, and S. MONCADA, "An L-arginine/nitric oxide pathway present in human platelets regulates aggregation," *Proceedings of the National Academy of Sciences*, vol. 87, no. 13, pp. 5193–5197, 1990.

[94] E. S. Trepakova, R. A. Cohen, and V. M. Bolotina, "Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase-dependent refilling of Ca^{2+} stores," *Circulation research*, vol. 84, no. 2, pp. 201–209, 1999.

[95] J. Schlossmann, A. Ammendola, K. Ashman et al., "Regulation of intracellular calcium by a signalling complex of IRAG, IP_3 receptor and cGMP kinase Iβ," *Nature*, vol. 404, no. 6774, pp. 197–201, 2000.

[96] G. R. Wang, Y. Zhu, P. V. Halushka, T. M. Lincoln, and M. E. Mendelsohn, "Mechanism of platelet inhibition by nitric oxide: *in vivo* phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase," *Proceedings of the National Academy of Sciences*, vol. 95, no. 9, pp. 4888–4893, 1998.

[97] K. Horstrup, B. Jablonka, P. Honig-Liedl, M. Just, K. Kochsiek, and U. Walter, "Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition," *European journal of biochemistry*, vol. 225, pp. 21–27, 1994, 1.

[98] D. H. Maurice and R. J. Haslam, "Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP," *Molecular pharmacology*, vol. 37, no. 5, pp. 671–681, 1990.

[99] J. Zhang, S. J. Shattil, M. C. Cunningham, and S. E. Rittenhouse, "Phosphoinositide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relativeactivation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIb-beta3 integrin," *Journal of Biological Chemistry*, vol. 271, pp. 6265–6272, 1996.

[100] C. N. Morrell, K. Matsushita, K. Chiles et al., "Regulation of platelet granule exocytosis by S-nitrosylation," *Proceedings of the National Academy of Sciences*, vol. 102, no. 10, pp. 3782–3787, 2005.

[101] G. M. Walsh, D. Leane, N. Moran et al., "S-Nitrosylation of platelet $_{\alpha IIb}\beta 3$As revealed by Raman Spectroscopy," *Biochemistry*, vol. 46, no. 21, pp. 6429–6436, 2007.

[102] S. Y. Low, M. Sabetkar, and K. R. Bruckdorfer, "The role of protein nitration in the inhibition of platelet activation by peroxynitrite," *FEBS Letters*, vol. 511, no. 1-3, pp. 59–64, 2002.

[103] S. Marcondes, M. H. M. Cardoso, R. P. Morganti et al., "Cyclic GMP-independent mechanisms contribute to the

inhibition of platelet adhesion by nitric oxide donor: a role for -actinin nitration," *Proceedings of the National Academy of Sciences*, vol. 103, no. 9, pp. 3434–3439, 2006.

[104] T. Malinski, M. W. Radomski, Z. Taha, and S. Moncada, "Direct electrochemical measurement of nitric oxide released from human platelets," *Biochemical and biophysical research communications*, vol. 194, no. 2, pp. 960–965, 1993.

[105] M. W. Radomski, R. M. J. Palmer, and S. Moncada, "The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium," *Biochemical and biophysical research communications*, vol. 148, no. 3, pp. 1482–1489, 1987.

[106] M. W. Radomski, R. M. J. Palmer, and S. Moncada, "Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets," *British journal of pharmacology*, vol. 92, no. 1, pp. 181–187, 1987.

[107] J. E. Freedman, J. Loscalzo, M. R. Barnard, C. Alpert, J. F. Keaney, and A. D. Michelson, "Nitric oxide released from activated platelets inhibits platelet recruitment," *The Journal of clinical investigation*, vol. 100, no. 2, pp. 350–356, 1997.

[108] R. H. Williams and M. U. Nollert, "Platelet-derived NO slows thrombus growth on a collagen type III surface," *Thrombosis Journal*, vol. 2, no. 1, 2004.

[109] M. R. Cozzi, G. Guglielmini, M. Battiston et al., "Visualization of nitric oxide production by individual platelets during adhesion in flowing blood," *Blood*, vol. 125, no. 4, pp. 697–705, 2015.

[110] Q. Zhou, G. R. Hellermann, and L. P. Solomonson, "Nitric oxide release from resting human platelets," *Thrombosis Research*, vol. 77, no. 1, pp. 87–96, 1995.

[111] Z. Li, X. Xi, M. Gu et al., "A Stimulatory Role for cGMP-Dependent Protein Kinase in Platelet Activation," *Cell*, vol. 112, no. 1, pp. 77–86, 2003.

[112] A. Radziwon-Balicka, C. Moncada dela Rosa, B. Zielnik, A. Doroszko, and P. Jurasz, "Temporal and pharmacological characterization of angiostatin release and generation by human platelets: implications for endothelial cell migration," *PLoS One*, vol. 8, no. 3, article e59281, 2013.

[113] A. Radziwon-Balicka, G. Lesyk, V. Back et al., "Differential eNOS-signalling by platelet subpopulations regulates adhesion and aggregation," *Cardiovascular Research*, vol. 113, no. 14, pp. 1719–1731, 2017.

[114] S. Gambaryan, J. Geiger, U. R. Schwarz et al., "Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase," *Blood*, vol. 103, no. 7, pp. 2593–2600, 2004.

[115] S. Gambaryan, A. Kobsar, S. Hartmann et al., "NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity," *Journal of Thrombosis and Haemostasis*, vol. 6, no. 8, pp. 1376–1384, 2008.

[116] L. B. Valdez and A. Boveris, "Nitric oxide and superoxide radical production by human mononuclear leukocytes," *Antioxidants and Redox Signaling*, vol. 3, no. 3, pp. 505–513, 2004.

[117] A. F. A. Wykretowicz, A. Szczpanik, and H. Wysocki, "Dipyridamole inhibits hydroxylamine augmented nitric oxide (NO) production by activated polymorphonuclear neutrophils through an adenosine-independent mechanism," *Physiological Research*, vol. 53, no. 6, pp. 645–652, 2004.

[118] D. Salvemini, G. de Nucci, R. J. Gryglewski, and J. R. Vane, "Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor," *Proceedings of the National Academy of Sciences*, vol. 86, no. 16, pp. 6328–6332, 1989.

[119] T. Wallerath, I. Gath, W. E. Aulitzky, J. S. Pollock, H. Kleinert, and U. Forstermann, "Identification of the NO synthase isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets," *Thrombosis and haemostasis*, vol. 77, no. 1, pp. 163–167, 1997.

[120] J. L. Mehta, L. Y. Chen, B. C. Kone, P. Mehta, and P. Turner, "Identification of constitutive and inducible forms of nitric oxide synthase in human platelets," *The Journal of laboratory and clinical medicine*, vol. 125, no. 3, pp. 370–377, 1995.

[121] A. Böhmer, S. Gambaryan, and D. Tsikas, "Human blood platelets lack nitric oxide synthase activity," *Platelets*, vol. 266, pp. 583–588, 2015.

[122] J. E. Freedman, R. Sauter, E. M. Battinelli et al., "Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene," *Circulation research*, vol. 84, no. 12, pp. 1416–1421, 1999.

[123] R. van Haperen, C. Cheng, B. M. E. Mees et al., "Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice," *The American journal of pathology*, vol. 163, no. 4, pp. 1677–1686, 2003.

[124] R. Riba, M. Sharifi, R. W. Farndale, and K. M. Naseem, "Regulation of platelet guanylyl cyclase by collagen: evidence that glycoprotein-VI mediates platelet nitric oxide synthesis in response to collagen," *Thromb Haemost*, vol. 94, pp. 395–403, 2005.

[125] R. Riba, N. G. Oberprieler, W. Roberts, and K. M. Naseem, "Von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a GPIb-dependent mechanism," *Journal Thromb Haemost*, vol. 12, pp. 2638–2644, 2006.

[126] A. W. Chung, A. Radomski, D. Alonso-Escolano et al., "Platelet-leukocyte aggregation induced by PAR agonists: regulation by nitric oxide and matrix metalloproteinases," *British Journal of Clinical Pharmacology*, vol. 143, no. 7, pp. 845–855, 2004.

[127] E. Gkaliagkousi, V. Corrigall, J. Ritter, and A. Ferro, "Nitric oxide regulates the formation of circulating monocyte-platelet aggregates in healthy subjects," *British Journal of Clinical Pharmacology*, vol. 61, pp. 612–646, 2005.

[128] C. B. Thompson, D. G. Love, P. G. Quinn, and C. R. Valeri, "Platelet size does not correlate with platelet age," *Blood*, vol. 62, no. 2, pp. 487–494, 1983.

[129] D. G. Penington, K. Streatfield, and A. E. Roxburgh, "Megakaryocytes and the heterogeneity of circulating platelets," *British Journal of Haematology*, vol. 34, no. 4, pp. 639–653, 1976.

[130] S. Karpatkin and A. Charmatz, "Heterogeneity of human platelets. *I. Metabolic and kinetic evidence suggestive of young and old platelets*," *Journal of Clinical Investigation*, vol. 48, no. 6, pp. 1073–1082, 1969.

[131] S. Karpatkin, "Heterogeneity of human platelets. *II. Functional evidence suggestive of young and old platelets*," *The Journal of clinical investigation*, vol. 48, no. 6, pp. 1083–1087, 1969.

[132] R. Polanowska-Grabowska, S. Raha, and A. R. L. Gear, "Adhesion efficiency, platelet density and size," *British Journal of Haematology*, vol. 82, no. 4, pp. 715–720, 1992.

[133] J. A. Jakubowski, B. Alder, C. B. Thompson, C. R. Valeri, and D. Deykin, "Influence of platelet volume on the ability of prostacyclin to inhibit platelet aggregation and the release

reaction," *The Journal of laboratory and clinical medicine*, vol. 105, no. 2, pp. 271–276, 1985.

[134] C. Opper, G. Schuessler, M. Kuschel et al., "Analysis of GTP-binding proteins, phosphoproteins, and cytosolic calcium in functional heterogeneous human blood platelet subpopulations," *Biochemical pharmacology*, vol. 54, no. 9, pp. 1027–1035, 1997.

[135] C. Opper, C. Fett, B. Capito, S. Raha, and W. Wesemann, "Plasma membrane properties in heterogeneous human blood platelet subfractions modulate the cellular response at the second messenger level," *Thrombosis research*, vol. 72, no. 1, pp. 39–47, 1993.

[136] C. Opper, E. Schrumpf, A. R. L. Gear, and W. Wesemann, "Involvement of guanylate cyclase and phosphodiesterases in the functional heterogeneity of human blood platelet subpopulations," *Thrombosis research*, vol. 80, no. 6, pp. 461–470, 1995.

[137] N. Kiliçli-Camur, R. Demirtunç, C. Konuralp, A. Eskiser, and Y. Başaran, "Could mean platelet volume be a predictive marker for acute myocardial infarction?," *Medical science monitor*, vol. 11, no. 8, pp. CR387–CR392, 2005.

[138] R. J. Butterworth and P. M. Bath, "The relationship between mean platelet volume, stroke subtype and clinical outcome," *Platelets*, vol. 9, no. 6, pp. 359–6410.1080/09537109876429, 1998.

[139] B. Özkan, O. Z. Arik, M. Y. Gözükara et al., "Mean platelet volume is related with ischemic stroke in patients with sinus rhythm," *Blood Coagul Fibrinolysis*, vol. 27, no. 5, pp. 490–493, 2016.

[140] P. Bath, C. Algert, N. Chapman, and for the PROGRESS Collaborative Group, "Association of mean platelet volume with risk of stroke among 3134 individuals with history of cerebrovascular disease," *Stroke*, vol. 35, no. 3, pp. 622–626, 2004.

[141] W. Roberts, A. Michno, A. Aburima, and K. M. Naseem, "Nitric oxide inhibits von Willebrand factor-mediated platelet adhesion and spreading through regulation of integrin $_{\alpha II}b\beta_3$ and myosin light chain," *Journal of Thrombosis and Haemostasis*, vol. 7, no. 12, pp. 2106–2115, 2009.

[142] R. A. Rabini, R. Staffolani, P. Fumelli, B. Mutus, G. Curatola, and L. Mazzanti, "Decreased nitric oxide synthase activity in platelets from IDDM and NIDDM patients," *Diabetologia*, vol. 41, no. 1, pp. 101–104, 1998.

[143] H. Ikeda, Y. Takajo, T. Murohara et al., "Platelet-derived nitric oxide and coronary risk factors," *Hypertension*, vol. 35, no. 4, pp. 904–907, 2000.

[144] L. R. Queen, Y. Ji, I. Goubareva, and A. Ferro, "Nitric oxide generation mediated by beta-adrenoceptors is impaired in platelets from patients with type 2 diabetes mellitus," *Diabetologia*, vol. 46, no. 11, pp. 1474–1482, 2003.

[145] J. E. Freedman, B. Ting, B. Hankin, J. Loscalzo, J. F. Keaney Jr., and J. A. Vita, "Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes," *Circulation*, vol. 98, no. 15, pp. 1481–1486, 1998.

[146] U. Laufs, K. Gertz, P. Huang et al., "Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice," *Stroke*, vol. 31, no. 10, pp. 2442–2449, 2000.

[147] T. Taka, Y. Ohta, J. Seki, J. C. Giddings, and J. Yamamoto, "Impaired flow-mediated vasodilation in vivo and reduced shear-induced platelet reactivity in vitro in response to nitric oxide in prothrombotic, stroke-prone spontaneously hypertensive rats," *Pathophysiology of Haemostasis and Thrombosis*, vol. 32, no. 4, pp. 184–189, 2002.

[148] M. D. Iafrati, O. Vitseva, K. Tanriverdi et al., "Compensatory mechanisms influence hemostasis in setting of eNOS deficiency," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 288, no. 4, pp. H1627–H1632, 2005.

[149] Y. Gürsoy-Ozdemir, H. Bolay, O. Saribaş, and T. Dalkara, "Role of endothelial nitric oxide generation and peroxynitrite formation in reperfusion injury after focal cerebral ischemia," *Stroke*, vol. 31, no. 8, pp. 1974–1981, 2000.

[150] D. S. Manickam, A. M. Brynskikh, J. L. Kopanic et al., "Well-defined cross-linked antioxidant nanozymes for treatment of ischemic brain injury," *Journal of controlled release*, vol. 162, no. 3, pp. 636–645, 2012.

[151] A. Członkowska and A. Kobayashi, "Expert Group of the Section of Cerebrovascular Diseases of the Polish Neurological Society.Management of acute stroke - guidelines from the Expert Group of the Section of Cerebrovascular Diseases of the Polish Neurological Society. Update 2013: thrombolysis," *Neurologia i Neurochirurgia Polska*, vol. 4, no. 47, pp. 303–309, 2013.

[152] O. Adeoye, H. Sucharew, J. Khoury et al., "Recombinant tissue-type plasminogen activator plus eptifibatide versus recombinant tissue-type plasminogen activator alone in acute ischemic Stroke," *Stroke*, vol. 46, no. 2, pp. 461–464, 2015.

[153] O. Adeoye, H. Sucharew, J. Khoury et al., "Combined approach to lysis utilizing eptifibatide and recombinant tissue-type plasminogen ac-tivator in acute ischemic stroke: Full Dose Regimen Stroke Trial," *Stroke*, vol. 46, no. 9, pp. 2529–2533, 2015.

[154] S. M. Zinkstok and Y. B. Roos, "Early administration of aspirin in patients treated with alteplase for acute ischaemic stroke: a randomised controlled trial," *Lancet*, vol. 380, no. 9843, pp. 731–737, 2012.

[155] W. J. Powers, A. A. Rabinstein, T. Ackerson et al., "Guidelines for the early management of patients with acute ischemic stroke: 2019 update to the 2018 guidelines for the early management of acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 50, no. 12, pp. e344–e418, 2019.

[156] D. H. Kang, Y. W. Kim, Y. H. Hwang, S. P. Park, Y. S. Kim, and S. K. Baik, "Instant reocclusion following mechanical thrombectomy of in situ thromboocclusion and the role of Low-Dose intra-arterial tirofiban," *Cerebrovascular Diseases*, vol. 37, no. 5, pp. 350–355, 2014.

[157] Y. W. Kim, J. M. Hong, D. G. Park et al., "Effect of intracranial atherosclerotic disease on endovascular treatment for patients with acute vertebrobasilar occlusion," *American Journal of Neuroradiology*, vol. 37, no. 11, pp. 2072–2078, 2016.

[158] J. H. Seo, H. W. Jeong, S. T. Kim, and E. G. Kim, "Adjuvant tirofiban injection through deployed Solitaire stent as a rescue technique after failed mechanical thrombectomy in acute stroke," *Neurointervention*, vol. 10, no. 1, pp. 22–27, 2015.

[159] H. G. Jeong, B. J. Kim, M. H. Yang, M. K. Han, H. J. Bae, and S. H. Lee, "Stroke outcomes with use of antithrombotics within 24 hours after recanalization treatment," *Neurology*, vol. 87, no. 10, pp. 996–1002, 2016.

[160] X. Tong, J. L. Wiltz, M. G. George et al., "A decade of improvement in door-to-needle time among acute ischemic

stroke patients, 2008 to 2017," *Circulation: Cardiovascular Quality and Outcomes*, vol. 11, no. 12, article e004981, 2018.

[161] Z. M. Chen, P. Sandercock, H. C. Pan et al., "Indications for early aspirin use in acute ischemic stroke : a combined analysis of 40 000 randomized patients from the Chinese Acute Stroke Trial and the International Stroke Trial. On Behalf of the CAST and IST Collaborative Groups," *Stroke*, vol. 31, no. 6, pp. 1240–1249, 2000.

[162] C. Berger, A. Stauder, F. Xia, C. Sommer, and S. Schwab, "Neuroprotection and glutamate attenuation by acetylsalicylic acid in temporary but not in permanent cerebral ischemia," *Experimental neurology*, vol. 210, no. 2, pp. 543–548, 2008.

[163] Y. Wang, Y. Wang, X. Zhao et al., "Clopidogrel with Aspirin in acute minor stroke or transient ischemic attack," *The New England Journal of Medicine*, vol. 369, no. 1, pp. 11–19, 2013.

[164] P. M. Rothwell, A. Algra, Z. Chen, H.-C. Diener, B. Norrving, and Z. Mehta, "Effects of aspirin on risk and severity of early recurrent stroke after transient ischaemic attack and ischaemic stroke: time-course analysis of randomised trials," *Lancet*, vol. 388, no. 10042, pp. 365–375, 2016.

[165] Z.-M. Chen, "CAST (Chinese Acute Stroke Trial) Collaborative Group (1997) CAST: randomised placebo-controlled trial of early aspirin use in 20 000 patients with acute ischaemic stroke," *Lancet*, vol. 349, no. 9066, pp. 1641–1649, 1997.

[166] CAPRIE Steering Committee and G. J. Hankey, "A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE)," *Lancet*, vol. 348, pp. 1329–1339, 1996.

[167] V. Aboyans, J. B. Ricco, M. L. E. L. Bartelink et al., "2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS)," *European Heart Journal*, vol. 39, no. 9, pp. 763–816, 2018.

[168] M. Paciaroni, B. Ince, B. Hu et al., "Benefits and risks of clopidogrel vs. aspirin monotherapy after recent ischemic stroke: a systematic review and meta-analysis," *Cardiovascular therapeutics*, vol. 2019, Article ID 1607181, 12 pages, 2019.

[169] B. Ibanez, S. James, S. Agewall et al., "2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC)," *European Heart Journal*, vol. 39, no. 2, pp. 119–177, 2018.

[170] D. A. Morrow, E. Braunwald, M. P. Bonaca et al., "Vorapaxar in the secondary prevention of atherothrombotic events," *The New England Journal of Medicine*, vol. 366, no. 15, pp. 1404–1413, 2012.

[171] H. P. Adams, M. B. Effron, J. Torner et al., "Emergency administration of abciximab for treatment of patients with acute ischemic stroke: results of an international phase III Trial:Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II)," *Stroke*, vol. 39, no. 1, pp. 87–99, 2008.

[172] C. Chen, T. Li, Y. Zhao et al., "Platelet glycoprotein receptor Ib blockade ameliorates experimental cerebral ischemia-reperfusion injury by strengthening the blood-brain barrier function and anti-thrombo-inflammatory property," *Brain, Behavior, and Immunity*, vol. 69, pp. 255–263, 2018.

[173] T.-T. Li, M.-L. Fan, S.-X. Hou et al., "A novel snake venom-derived GPIb antagonist, anfibatide, protects mice from acute experimental ischaemic stroke and reperfusion injury," *British journal of pharmacology*, vol. 172, no. 15, pp. 3904–3916, 2015.

[174] S. Momi, M. Tantucci, M. Van Roy, H. Ulrichts, G. Ricci, and P. Gresele, "Reperfusion of cerebral artery thrombosis by the GPIb–VWF blockade with the nanobody ALX-0081 reduces brain infarct size in guinea pigs," *Blood*, vol. 121, no. 25, pp. 5088–5097, 2013.

[175] D. Wu, K. Vanhoorelbeke, N. Cauwenberghs et al., "Inhibition of the von Willebrand (VWF)–collagen interaction by an antihuman VWF monoclonal antibody results in abolition of in vivo arterial platelet thrombus formation in baboons," *Blood*, vol. 99, no. 10, pp. 3623–3628, 2002.

[176] B. Q. Zhao, A. K. Chauhan, M. Canault et al., "von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke," *Blood*, vol. 114, no. 15, pp. 3329–3334, 2009.

[177] I. Hagedorn, S. Schmidbauer, I. Pleines et al., "Factor XIIa inhibitor recombinant human albumin infestin-4 abolishes occlusive arterial thrombus formation without affecting bleeding," *Circulation*, vol. 121, pp. 1510–1517, 2010.

[178] S. Goebel, Z. Li, J. Vogelmann et al., "The GPVI-Fc fusion protein revacept improves cerebral infarct volume and functional outcome in stroke," *PLoS ONE*, vol. 8, no. 7, article e66960, 2013.

[179] E. C. de Brito Toscano, B. C. Silva, E. C. G. Victoria et al., "Platelet-activating factor receptor (PAFR) plays a crucial role in experimental global cerebral ischemia and reperfusion," *Brain research bulletin*, vol. 124, pp. 55–61, 2016.

[180] T. Chen, H. Kato, H. Ban et al., "Protective effects of a novel calcium antagonist with platelet-activating factor-antagonistic action, F-0401, against ischemic brain damage," *Archives internationales de pharmacodynamie et de therapie*, vol. 327, no. 3, pp. 266–278, 1994.

[181] K. U. Frerichs, P. J. Lindsberg, J. M. Hallenbeck, and G. Feuerstein, "Platelet-activating factor and progressive brain damage following focal brain injury," *Journal of neurosurgery*, vol. 73, no. 2, pp. 223–233, 1990.

[182] J. C. Tardif, J. F. Tanguay, S. S. Wright et al., "Effects of the P-selectin antagonist inclacumab on myocardial damage after percutaneous coronary intervention for non-ST-segment elevation myocardial infarction: results of the SELECT-ACS trial," *Journal of the American College of Cardiology*, vol. 61, no. 20, pp. 2048–2055, 2013.

[183] G. Uddin, M. Hussain, C. X. Wang, K. G. Todd, and A. Shuaib, "Acetylsalicylic acid reduces perfusion deficit in ischemic injured brain in rats," *Neuroreport*, vol. 14, no. 13, pp. 1753–1755, 2003.

[184] S. C. Johnston, J. D. Easton, M. Farrant et al., "Clinical Research CollaborationNeurological Emergencies Treatment Trials Network, and the POINT Investigators, Clopidogrel and aspirin in acute ischemic stroke and high-risk TIA," *New England Journal of Medicine*, vol. 379, no. 3, pp. 215–225, 2018.

[185] M. W. Riepe, K. Kasischke, and A. Raupach, "Acetylsalicylic acid increases tolerance against hypoxic and chemical hypoxia," *Stroke*, vol. 28, no. 10, pp. 2006–2011, 1997.

[186] A. Coukell and A. Markham, "Clopidogrel," *Drugs*, vol. 54, no. 5, pp. 745–750, 1997.

[187] D. Forster and G. Reiser, "Supportive or detrimental roles of P2Y receptors in brain pathology?—The two faces of P2Y receptors in stroke and neurodegeneration detected in neural cell and in animal model studies," *Purinergic Signal*, vol. 11, no. 4, pp. 441–454, 2015.

[188] A. Tomizawa, K. Ohno, J. A. Jakubowski, M. Mizuno, and A. Sugidachi, "Prasugrel reduces ischaemic infarct volume and ameliorates neurological deficits in a non-human primate model of middle cerebral artery thrombosis," *Thrombosis research*, vol. 136, no. 6, pp. 1224–1230, 2015.

[189] J. M. Siller-Matula, J. Krumphuber, and B. Jilma, "Pharmacokinetic, pharmacodynamic and clinical profile of novel antiplatelet drugs targeting vascular diseases," *British journal of pharmacology*, vol. 159, no. 3, pp. 502–517, 2010.

[190] Y. Niitsu, A. Sugidachi, T. Ogawa et al., "Repeat oral dosing of prasugrel, a novel $P2Y_{12}$ receptor inhibitor, results in cumulative and potent antiplatelet and antithrombotic activity in several animal species," *European journal of pharmacology*, vol. 579, no. 1-3, pp. 276–282, 2008.

[191] S. D. Wiviott, E. Braunwald, C. H. McCabe et al., "Prasugrel versus Clopidogrel in Patients with Acute Coronary Syndromes," *New England Journal of Medicine*, vol. 357, no. 20, pp. 2001–2015, 2007.

[192] K. Yamauchi, T. Imai, M. Shimazawa, T. Iwama, and H. Hara, "Effects of ticagrelor in a mouse model of ischemic stroke," *Scientific reports*, vol. 7, no. 1, article 12088, 2017.

[193] S. Husted, H. Emanuelsson, S. Heptinstall, P. M. Sandset, M. Wickens, and G. Peters, "Pharmacodynamics, pharmacokinetics, and safety of the oral reversible $P2Y_{12}$ antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin," *European heart journal*, vol. 27, no. 9, pp. 1038–1047, 2006.

[194] "AstraZeneca Submits US New Drug Application For Ticagrelor BRILINTA (tm), An investigational antiplatelet agent. MedicalNews Today," November 22, 2009. http://www.medicalnewstoday.com/articles/171785.php.Accessed July 12, 2010.

[195] D. J. Angiolillo, M. S. Firstenberg, M. J. Price et al., "Bridging antiplatelet therapy with cangrelor in patients un-dergoing cardiac surgery: a randomized controlled trial," *JAMA*, vol. 307, no. 3, pp. 265–274, 2012.

[196] A. Wang, "Review of vorapaxar for the prevention of atherothrombotic events," *Expert opinion on pharmacotherapy*, vol. 16, no. 16, pp. 2509–2522, 2015.

[197] J. W. Cheng, "Impact of selective platelet inhibition in reducing cardiovascular risk - role of vorapaxar," *Vascular health and risk management*, vol. 12, pp. 263–268, 2016.

[198] A. Sharma, G. Helft, A. Garg et al., "Safety and efficacy of vorapaxar in secondary prevention of atherosclerotic disease: a meta-analysis of randomized control trials," *International Journal of Cardiology*, vol. 227, pp. 617–624, 2017.

[199] T. F. Choudhri, B. L. Hoh, H. G. Zerwes et al., "Reduced microvascular thrombosis and improved outcome in acute murine stroke by inhibiting GP IIb/IIIa receptor-mediated platelet aggregation," *The Journal of clinical investigation*, vol. 102, no. 7, pp. 1301–1310, 1998.

[200] V. L. Serebruany, A. I. Malinin, C. M. O'Connor, and P. A. Gurbel, "Effects of roxifiban on platelet aggregation and major receptor expression in patients with coronary artery disease for the Roxifiban Oral Compound Kinetics Evaluation Trial-I (ROCKET-I Platelet Substudy)," *American Heart Journal*, vol. 146, no. 1, pp. 91–98, 2003.

[201] C. Geeganage, R. Wilcox, and P. M. W. Bath, "Triple antiplatelet therapy for preventing vascular events: a systematic review and meta-analysis," *BMC medicine*, vol. 8, no. 1, 2010.

[202] B. S. Coller, J. D. Folts, S. R. Smith, L. E. Scudder, and R. Jordan, "Abolition of in vivo platelet thrombus formation in primates with monoclonal antibodies to the platelet GPIIb/IIIa receptor. correlation with bleeding time, platelet aggregation, and blockade of GPIIb/IIIa receptors," *Circulation*, vol. 80, no. 6, pp. 1766–1774, 1989.

[203] Y. Hou, H. Zhou, Y. Wang et al., "Anfibatide, a novel GPIb complex antagonist, inhibits platelet adhesion and thrombus formation *in vitro* and *in vivo* in murine models of thrombosis," *Thrombosis and haemostasis*, vol. 111, no. 2, pp. 279–289, 2014.

[204] F. Denorme, F. Langhauser, L. Desender et al., "ADAMTS13-mediated thrombolysis of t-PA-resistant occlusions in ischemic stroke in mice," *Blood*, vol. 127, no. 19, pp. 2337–2345, 2016.

[205] S. Hopp, C. Albert-Weissenberger, S. Mencl et al., "Targeting coagulation factor XII as a novel therapeutic option in brain trauma," *Annals of neurology*, vol. 79, no. 6, pp. 970–982, 2016.

[206] M. Ungerer, K. Rosport, A. Bültmann et al., "Novel antiplatelet drug revacept (dimeric glycoprotein VI-Fc) specifically and efficiently inhibited collagen-induced platelet aggregation without affecting general hemostasis in humans," *Circulation*, vol. 123, no. 17, pp. 1891–1899, 2011.

[207] A. Reimann, Z. Li, S. Goebel et al., "Combined administration of the GPVI-Fc fusion protein revacept with low-dose thrombolysis in the treatment of stroke," *Heart international*, vol. 11, no. 1, pp. e10–e16, 2016.

[208] M. Micucci, C. Carotenuto, M. Durante, S. Cosconati, and R. Budriesi, "4-Imidazo[2,1-b]thiazole-1,4-DHPs and neuroprotection: preliminary study in hits searching," *European journal of medicinal chemistry*, vol. 169, pp. 89–102, 2019.

[209] D. D. Gilboe, D. Kintner, J. H. Fitzpatrick et al., "Recovery of postischemic brain metabolism and function following treatment with a free radical scavenger and platelet-activating factor antagonists," *Journal of neurochemistry*, vol. 56, no. 1, pp. 311–319, 1991.

4. PRACA NR 2:

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

antioxidants

MDPI

Article

Changes in the Plasma and Platelet Nitric Oxide Biotransformation Metabolites during Ischemic Stroke—A Dynamic Human LC/MS Metabolomic Study

Maciej Bladowski [1], Ewa Szahidewicz-Krupska [1], Jerzy Wiśniewski [2,3], Paulina Fortuna [2], Justyna Chojdak-Łukasiewicz [4], Slawomir Budrewicz [4], Mariusz Fleszar [2] and Adrian Doroszko [1,*]

1 Department and Clinic of Internal Medicine, Hypertension and Clinical Oncology, Faculty of Medicine, Wroclaw Medical University, Borowska 213 Str., 50-556 Wroclaw, Poland; maciej.bladowski@umed.wroc.pl (M.B.); ewa.szahidewicz-krupska@umw.edu.pl (E.S.-K.)
2 Department of Medical Biochemistry, Wroclaw Medical University, Chalubinskiego 10 Str., 50-368 Wroclaw, Poland; jerzy.wisniewski@pwr.edu.pl (J.W.); paulina.fortuna@umw.edu.pl (P.F.); mariusz.fleszar@umw.edu.pl (M.F.)
3 Department of Biochemistry, Molecular Biology and Biotechnology, Wroclaw University of Science and Technology, Wyspianskiego 27, 50-370 Wroclaw, Poland
4 Department and Clinic of Neurology, Wroclaw Medical University, Borowska 213 Str., 50-556 Wroclaw, Poland; justyna.chojdak-lukasiewicz@umw.edu.pl (J.C.-Ł.); slawomir.budrewicz@umw.edu.pl (S.B.)
* Correspondence: adrian.doroszko@umw.edu.pl; Tel.: +48-71-736-4000

check for updates

Citation: Bladowski, M.; Szahidewicz-Krupska, E.; Wiśniewski, J.; Fortuna, P.; Chojdak-Łukasiewicz, J.; Budrewicz, S.; Fleszar, M.; Doroszko, A. Changes in the Plasma and Platelet Nitric Oxide Biotransformation Metabolites during Ischemic Stroke—A Dynamic Human LC/MS Metabolomic Study. *Antioxidants* **2022**, *11*, 955. https://doi.org/10.3390/antiox11050955

Received: 10 April 2022
Accepted: 10 May 2022
Published: 12 May 2022

Abstract: Despite improvement in the management of modifiable cardiovascular risk factors, ischemic stroke remains the leading cause of morbidity and mortality in the adult population. The aim of this study was to analyze the time-dependent dynamic differences in expression of the nitric oxide (NO) metabolic pathway in the platelet and plasma compartment between subjects with and without ischemic stroke. Additionally, the interplay between these parameters and platelet aggregation was investigated. A total of 418 patients in acute phase of non-cardioembolic stroke were investigated. Following the inclusion and exclusion criteria, finally 40 subjects with stroke and 39 demographically matched healthy participants were enrolled. Neurological physical examination, followed by assessment of the platelet and plasma levels of the nitric oxide synthase (NOS) inhibitors, including asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), as well as NOS substrate-L-Arginine were performed dynamically three times within the first 24-h, then on the 3rd and 7th day after the stroke onset, which was compared with the healthy control. The platelet L-Arginine concentration was significantly higher on the 1st and 3rd day of stroke, while the plasma levels were significantly lower on exact days in comparison to the control. The competitive NOS-inhibitors in platelets were stably elevated in stroke subjects, whereas no significant differences in plasma compartment were noted. The arachidonic-acid-induced platelet aggregation was negatively associated with the platelet NOS substrate bioavailability, as assessed by the $\frac{L-Arginine}{ADMA}$-ratio on the 3rd and 7th day. Subjects with non-cardioembolic ischemic stroke are characterized by elevated platelet levels of NOS inhibitors. Management of stroke results in increasing the platelet L-Arginine concentration and subsequent NO bioavailability in the platelet compartment.

Keywords: ischemic stroke; platelet nitric oxide; L-Arginine; asymmetric dimethylarginine (ADMA); acetylsalicylic acid; platelet aggregation

1. Introduction

Cardiovascular disease (CVD) is among the main causes of morbidity and mortality, as reported worldwide annually. Stroke accounts for approximately 1.1 million new incidents in the European Union each year, with estimated further increase of 3% within the next 3 decades [1]. Ischemic stroke can be divided into subtypes based on the pathomechanism

of thrombus origin. Cardioembolic stroke, predominantly caused by the thrombus formation in the left atrial appendage (LAA) during atrial fibrillation, is responsible for ca. 20%, while the non-cardioembolic stroke for 80% of all ischemic stroke cases [2,3].

Endothelial dysfunction and decreased nitric oxide (NO) bioavailability play a pivotal role in the pathogenesis of non-cardioembolic stroke. Limited NO action promotes atherosclerotic plaque formation and its rupture, as well as accelerates platelet activation, adhesion, and aggregation [4,5]. Decreased endothelial NO bioavailability leads to subsequent increase in the expression of adhesive molecules on the platelet surface, such as P-selectin (CD62P), CD40 ligand (CD40L), and ICAM-1, which promote platelet adhesion to the vascular wall and initiate thrombus formation. Activated platelets secrete thromboxane A2, adenosine diphosphate (ADP), and other platelet-derived soluble mediators, which subsequently trigger thrombus growth in a positive feedback loop [6]. Numerous studies have already confirmed that adequate management of cardiovascular risk factors, including hypertension, diabetes mellitus, dyslipidemia, as well as smoking cessation, led to limiting the ischemic stroke burden by increase in endothelial NO bioavailability and restoration of the endothelial vasodilatory function [7].

Interestingly, some new data suggests that not only endothelium, but also platelets are capable of synthesizing the nitric oxide to autoregulate own function. Radziwon-Balicka et al. have recently discovered two separate platelet subpopulations: with or without the ability to synthesize nitric oxide (eNOS-positive or eNOS-negative platelets, respectively). According to the authors, the eNOS negative platelets are the first to form a thrombus on the damaged endothelium, while the eNOS-positive platelets function is to limit the thrombus growth [8]. Furthermore, the authors hypothesize that platelet derived nitric oxide (PDNO) might be a key negative-feedback regulator of thrombus formation in numerous pathological conditions, including acute phase of ischemic stroke.

Hence, the aim of this study was to verify if alterations in the platelet or plasma NO homeostasis appear in the acute phase of stroke and whether the onset of antiplatelet management restores the nitric oxide biotransformation homeostasis. The effect of the platelet to plasma NO-balance on the platelet aggregation was investigated by analyzing the L-Arginine (L-Arg)—a substrate for NO synthesis; asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)—the competitive NOS inhibitors; then the dimethylamine (DMA)—product of ADMA degradation; citrulline—a product of both L-Arg and ADMA degradation; and ornithine—a product of L-Arg degradation. Schematic presentation of possible interactions between platelet and endothelial nitric oxide biotransformation leading to the maintenance of the NO-dependent platelet functional homeostasis is presented in Figure 1.

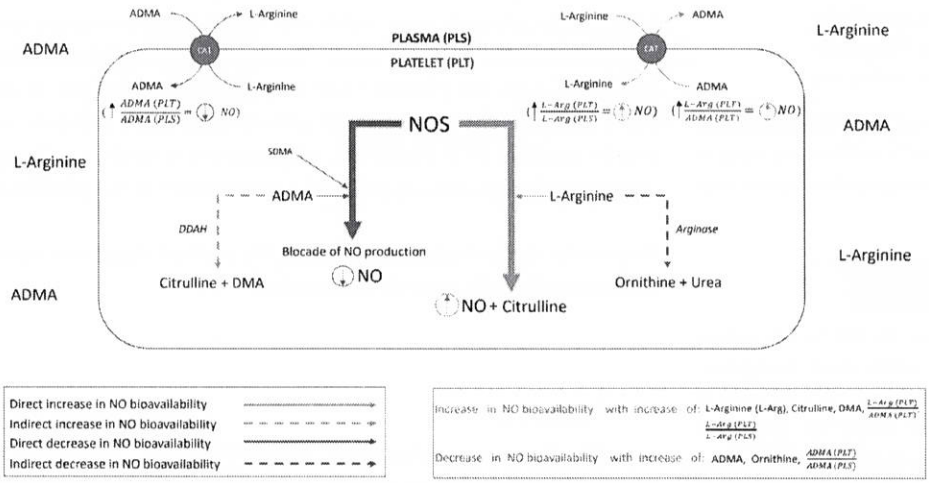


Figure 1. A schematic presentation of the nitric oxide biotransformation. *Abbreviations:* nitric oxide synthase (NOS), nitric oxide (NO), asymmetric dimethylarginine (ADMA), symmetric

dimethylarginine (SDMA), dimethylarginine dimethylaminohydrolase (DDAH, an enzyme responsible for ADMA degradation), citrulline, dimethylamine (DMA), L-Arginine (L-Arg), arginase (enzyme responsible for L-Arg degradation), ornithine, urea (product of the L-Arg degradation), cationic-amino acid transporter (CAT, transmembrane L-arginine and ADMA transporter), $\frac{L-Arg\ in\ platelets\ (PLT)}{L-Arg\ in\ plasma(PLS)}$ (comparison of nitric oxide bioavailability in platelet vs. in plasma), $\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$ – (potential for inhibition of NO production in platelet vs. in plasma), $\frac{L-Arg\ (PLT)}{ADMA\ (PLT)}$ (potential for the platelet NO synthesis).

2. Materials and Methods

2.1. Recruitment of Patients

A total of 418 patients with diagnosis of ischemic stroke, admitted to the University Clinical Hospital in Wroclaw were enrolled in this study. A total of 98 of them were disqualified due to the prolonged time between first neurological symptoms and admission to the hospital (>24 h). Subsequently, 230 patients have undergone thrombolysis and/or thrombectomy and therefore met exclusion criteria. The next 49 patients presented other exclusion criteria. Only one subject revoked informed consent to participate in the study, while none of the patients died during study observation. Finally, a total number of 40 patients at age of 29–80 years with diagnosed acute ischemic stroke were included in the study group. A flow chart presenting the recruitment of the subjects with stroke to the study is presented in Figure 2.

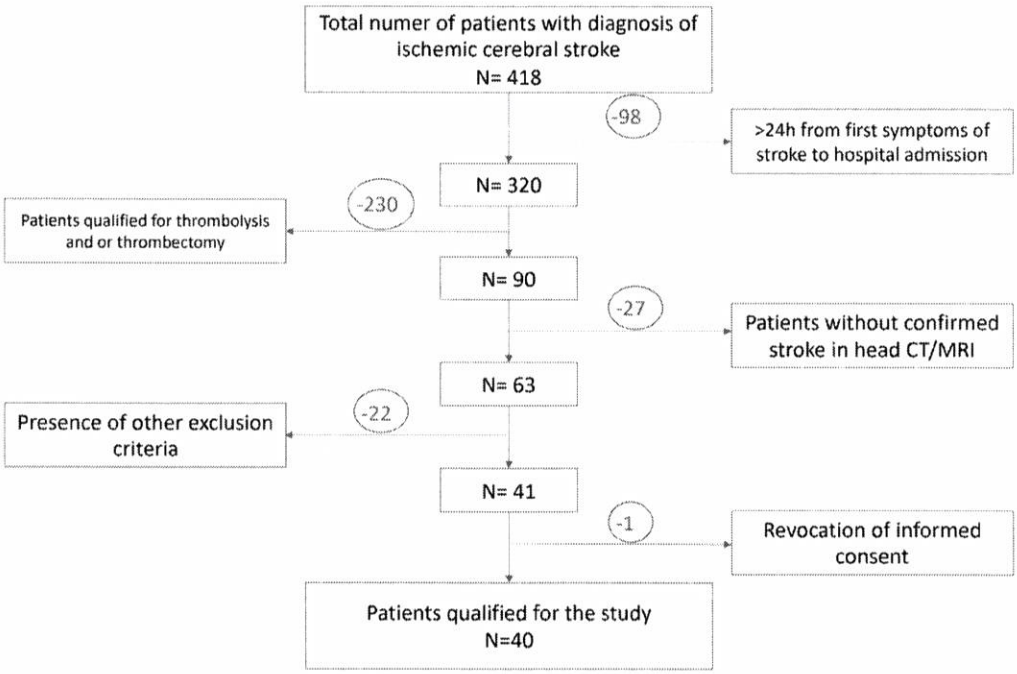


Figure 2. A flow chart of the patients selection.

1. The inclusion criteria for the study group were:
   - clinical symptoms of stroke lasting for no longer than 24 h before the hospital admission;
   - diagnosis of ischemic stroke confirmed by neurological examination and/or new cerebral ischemia visualized in the magnetic resonance imaging/computer tomography scan (simultaneously excluding the hemorrhagic stroke);
   - patient signed informed consent to participate in the study.
2. The exclusion criteria for the study group and the control group were:

- hemorrhagic stroke;
- thrombolytic treatment or thrombectomy (at present or in the past medical history);
- history of severe nervous system disease (including previous ischemic or hemorrhagic stroke, neuroinfections, autoimmune, inflammatory, or neurodegenerative diseases);
- past serious head injuries;
- atrial fibrillation (previously diagnosed or confirmed during the 72-h ECG monitoring during hospitalization);
- severe anemia (Hg < 7 g%);
- thrombocytopenia (platelets < 100,000/μL);
- ongoing therapies with drugs potentially affecting the obtained results before hospitalization (anticoagulants, antiplatelets drugs, contraceptives, hormone replacement therapy, anti-inflammatory drugs);
- current infections;
- active malignancy;
- chronic inflammatory diseases;
- chronic kidney disease (eGFR < 45 mL/min/1.73 m^2);
- incomplete medical history;
- inability to provide informed consent.

The control group comprised of 39 volunteers recruited from the hospital outpatient clinic and matched to the study group by demography, similar comorbidities, and undergoing comparable drug therapy before enrolling to the study. Comparison of comorbidities between the control group and study group is shown in Supplementary Table S1, while in the applied treatment is described in Supplementary Table S2.

2.2. Study Protocol

Patients with diagnosed non-cardioembolic ischemic stroke and initialized acetylsalicylic acid (ASA) treatment (75–150 mg) on the first day of hospitalization were enrolled to the study group after providing written informed consent. Afterwards, the study participants from the stroke group were exanimated thrice: at the time of admission, and on the 3rd and 7th day after ischemic stroke onset. The demographically matched healthy subjects formed the control group, where physical examination, blood collection, and neurological examination was performed once. Subjects from both the study group and control group had to be without any previous history of antiplatelet treatment. ASA treatment was initialized only in the study group (Figure 3).

2.3. Blood Collection

The blood for laboratory tests was collected with a single puncture of the antecubital vein, in atraumatic conditions using the S-Monovette set (S-Monovette 10 mL 9NC with tri-sodium citrate at concentration of 0.106 mol/L; S-Monovette 4.9 mL with silicate as clot activator; S-Monovette with 1.6 mg EDTA/mL of blood; Sarstedt AG & Co, Sarstedt, Germany). Whole blood collected in a tube with silicate as an activator of coagulation was centrifuged for 15 min at 1000× *g* in 45 min from its collection. Preserved in the Eppendorf tube, serum was transferred to the accredited university hospital laboratory. The tests were performed using routine certified biochemical methods.

2.4. Platelet Preparation for Liquid Chromatography–Mass Spectrometry (LC/MS) Analysis of the Nitric Oxide Metabolic Pathway

The collected whole blood to the S-Monovette tube with trisodium citrate was supplemented with prostacyclin (PGI_2) at the final target concentration of 0.16 μM and centrifuged for 20 min at 230× *g* at 21 °C to obtain the platelet-rich plasma (PRP). Subsequently, PRP was supplemented with PGI_2 (with final concentration of 0.8 μM) and centrifuged for 10 min at 1000× *g* at 21 °C. The plasma was discarded, and the platelet pellet was carefully washed three times with 1 mL of the Tyrodes HEPES buffer (134 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM Dextrose) pH 7.4.

The resulted suspension was immediately analyzed for platelet count and contamination with white blood cells and red blood cells (Sysmex device, Sysmex, Norderstedt, Germany). Samples containing platelets in amounts of 5.0×10^8 cells were preserved for subsequent LC-MS analysis. The samples were obtained by centrifugation of the suspension of known concentration for 5 min, 10,000× *g* at 4 °C, and stored at −80 °C until further analyses [9,10].

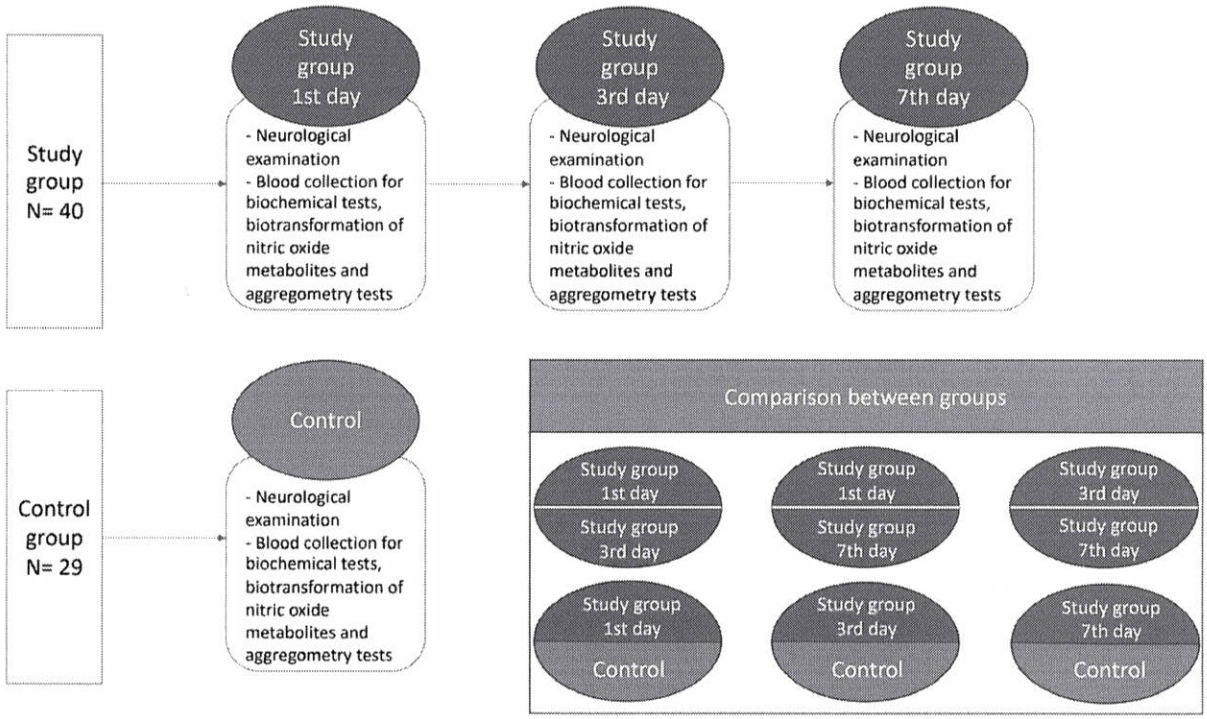


Figure 3. A flow chart of the study protocol.

2.5. Assessment of the Platelet Derived Nitric Oxide Metabolites

Previously prepared platelet samples (PRP), as described in Section 2.4, were thawed on ice. Subsequently, 10 µL of internal standard solution and 1200 µL of cold extraction solution containing methanol, acetonitrile, and water (5:3:2) were added and vortexed (15 min, 1200 rpm, 4 °C). Samples were centrifuged (15 min, 22,000× *g*, 4 °C) and clear supernatants were transferred into new microcentrifuge tubes. Samples were then dried at 50 °C.

Amino acids derivatization was performed using benzoyl chloride (BCl) reagent. Dried samples were dissolved in 100 µL of water and vortexed (5 min, 1200 rpm, 25 °C). Subsequently, 50 µL of borate buffer (0.025 M $Na_2B_4O_7 \cdot 10H_2O$, 1.77 mM NaOH, pH = 9.2), 400 µL of acetonitrile, and 10 µL of 10% BCl in acetonitrile were added and vortexed again (10 min, 1200 rpm, 25 °C). After derivatization, samples were dried at 45 °C using SpeedVac Vacuum Concentrator. Dried samples were reconstituted in 50 µL of 3% of methanol in water and centrifuged (10 min, 15,000× *g*, 4 °C). Supernatant was transferred into chromatographic polypropylene vial with attached 200 µL insert.

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed using SYNAPT G2 Si mass spectrometer coupled with Acquity I-Class UPLC system (Waters, Milford, MA, USA). MS was equipped with electrospray ionization source (ESI). The sprayer voltage, source temperature, desolvation temperature, and desolvation gas flow were set at 0.5 kV, 140 °C, 450 °C, and 900 L/h, respectively. The UPLC system was equipped with cooled sample manager; samples temperature was 8 °C and the injection volume was 2 µL. The Waters BEH Shield C18 column (1.7 µm, 2.1 × 50 mm) was heated to 60 °C. The flow rate was 0.350 mL/min, and the total time of the method was 8 min. The mobile phase solvent A was water with 0.1% formic acid (FA) and solvent B was methanol

with 0.1% FA. The following gradient method was used: 0.0 min—3% B, 2.5 min—14% B, 4.6 min—60% B, 4.8 min—90% B, and 6.1 min—3% B. Data acquisition was performed using MassLynx 4.1 software (Waters) for the following ions (m/z): 237.1239, 243.1339, 263.1090, 267.1382, 279.1457, 286.1897, 307.1770, and 314.2209 for ornithine, D6-ornithine, citrulline, D4-cytrulline, L-Arginine, D7-arginine, ADMA, SDMA, and D7-ADMA, respectively [11].

2.6. Measurement of the Plasma Nitric Oxide Metabolites

To obtain plasma, the blood was collected in tubes with EDTA as an anticoagulant (1.6 mg-EDTA/mL blood) and were centrifuged within 30 min after collection at 1000× *g* for 15 min at 4 °C and stored at −20 °C until further analysis. A total of 100 µL of plasma, 50 µL of borate buffer, and 10 µL of internal standard solution (100 µM D7-L-Arginine, 20 µM D7-ADMA, 25 µM D6-DMA, 100 µM D6-ornithine, and 50 µM D4-citrulline) were transferred into 2 mL polypropylene tubes and mixed (1 min, 1200 RPM, 25 °C). Then, 400 µL of acetonitrile and 10 µL of 10% BCl in acetonitrile were added and mixed (10 min, 1200 RPM, 25 °C). Subsequently, samples were centrifuged (7 min, 4 °C, 22,000× *g*) and 100 µL of clear supernatant was diluted four times with water, transferred to chromatographic glass vials, and analyzed. LC-MS analysis was performed using the equipment and methods described above [12].

2.7. Measurement of Platelet Aggregation

Platelet function was assessed using the impedance aggregation method in whole blood using the four-channel optical aggregometer (Chrono-log 700, Chrono-Log, Havertown, PA, USA). This method is based on multiple platelet aggregation on the electrodes and changing of the electrical resistance between their two wires. The whole blood was collected to the polypropylene tubes for 10% sodium citrate using the Sarstedt S-Monovette® (Sarstedt Ag & Co., Nümbrecht, Germany) aspiration and vacuum kit. After collection, the tubes were kept at room temperature for a maximum of 90 min before engaging the test. Three different aggregation activators were used: adenosine diphosphate (ADP), arachidonic acid (AA), and collagen. The 1:1 solution of whole blood at room temperature with 0.9% natrium chloride was placed in the test chambers. Then, a certain number of agonists, necessary to obtain appropriate concentrations (0.5 mg/mL for AA, 20 µmol for ADP, 1 µg for collagen), were added to the prepared solution. After 6 min, aggregation curves were recorded, measured, and analysed by dedicated software (Aggrolink®, Chrono-Log, PA, USA). The increase in electrical impedance was given in aggregation units.

2.8. Statistical Analysis

Statistical analysis was performed using the Statistica 13.3 StatSoft®. The presented data is expressed as an arithmetic mean with SEM or median with 1st and 4th quartile if the distribution of variables were not normal. The Mann-Whitney U-test or a student's t-test, following the Shapiro-Wilk test and Levene's test as appropriate, were used to assess the significance of differences between the mean values and ANOVA followed by Tukey's test, or a Friedman test was used when more than two groups were investigated. Spearman test was performed to assess the correlation between nitric biotransformation metabolites and platelet aggregation.

3. Results

3.1. Baseline Characteristics

The stroke subjects and healthy controls were matched with respect to the age and sex distribution. There were however differences between groups in white blood count (WBC), glucose level, mean platelet volume (MPV), potassium, and thyroid-stimulating hormone (TSH). The baseline demographic and biochemical characteristics of both groups is presented in Table 1.

Table 1. Demographic and biochemical characteristics between studied groups including cardiovascular risk stratification parameters. Results are presented as mean ± SEM if the distribution of variables were normal or median with 1st and 4th quartile if the distribution of variables were not normal.

		Stroke Group N = 40			Control Group N = 39			p
		Mean	[±SEM]	[1st–4th Quartile]	Mean	[±SEM]	[1st–4th Quartile]	
Women	**Number [%]**	18 [45%]			21 [54%]			ns
Age	**[y]**	63.45	±1.37		63.67	±1.65		ns
Hemoglobin	**[g/dL]**	14.40	±0.24		13.93	±0.32		ns
Hematocrit	**[%]**	42.6	±0.65		41.43	±0.9		
RBC	**[mln/μL]**	4.78	±0.08		4.77	±0.11		ns
WBC 1st day	**[k/μL]**	8.92	±0.49		6.74	±0.39		$p < 0.05$
WBC 3rd day	**[k/μL]**	8.82	±0.45					$p < 0.05$
WBC 7th day	**[k/μL]**	8.34	±0.68					ns
PLT	**[k/μL]**	225.79	±9.08		244.58	±9.22		ns
MPV 1st day	**[fl]**	9.83	±0.24		10.94	±0.17		$p < 0.05$
MPV 3rd day	**[fl]**	10.17	±0.30					ns
MPV 7th day	**[fl]**	10.20	±0.39					ns
hsCRP	**[mg/L]**	5.05		1.6–6.0	4.55		2.91–4.12	ns
ESR	**[mm/h]**	13.63		7.0–16.5	16.39		10.0–18.0	ns
Sodium	**[mmol/L]**	139.40	±0.41		140.19	±0.48		ns
Potassium 1st day	**[mmol/L]**	3.91	±0.06		4.14	±0.08		$p < 0.05$
Potassium 3rd day	**[mmol/L]**	3.99	±0.05					ns
Potassium 7th day	**[mmol/L]**	4.01	±0.09					ns
Glucose 1st day	**[mg/dL]**	132.97		9.8–145.0	101.12		86.0–102.0	$p < 0.05$
Glucose 3rd day	**[mg/dL]**	98.72		81.0–99.0				ns
Glucose 7th day	**[mg/dL]**	100.52		80.0–125.0				ns
Urea	**[mg/dL]**	33.08		26.0–38.0	32.20		25.5–36.0	ns
Creatinine	**[mg/dL]**	0.93		0.76–1.14	0.89		0.67–0.99	ns
Total protein	**[g/dL]**	6.51	±0.17		6.36	±0.14		ns
AST	**[IU/L]**	19.38		16.0–22.0	19.15		14.5–21.5	ns
ALT	**[IU/L]**	21.50		15.0–26.0	23.96		16.0–30.0	ns
Total bilirubin	**[mg/dL]**	0.80	±0.09		0.73	±0.07		ns
TCh	**[mg/dL]**	181.59	±8.08		203.38	±11.74		ns
HDL	**[mg/dL]**	48.18	±2.06		56.57	±3.73		ns
LDL	**[mg/dL]**	106.21	±6.86		122.81	±10.35		ns
Tg	**[mg/dL]**	128.16		91.0–142.0	128.24		87.0–163.0	ns
TSH	**[μIU/L]**	3.37		1.26–5.02	1.50		0.83–1.83	$p < 0.05$
APTT	**[s]**	27.31	±0.43		27.76	±0.64		ns
INR		0.99		0.94–1.04	1.00		0.96–1.03	ns

Abbreviations: mean platelet volume (MPV), hsCRP (high sensitivity C-reactive protein), erythrocyte sedimentation rate (ESR), total cholesterol (TCh), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (Tg), activated partial thromboplastin time (APTT), international normalized ratio (INR), ns–non significant.

3.2. The Plasma Nitric Oxide Metabolites

The plasma L-Arginine in the stroke group on the 1st and 3rd day were significantly lower than in the control group, while on the 7th day it reached a level not statistically different from the control group. Plasma ADMA and SDMA levels, together with L-Arginine to ADMA ratio in plasma ($\frac{L-Arg\ (PLS)}{ADMA\ (PLS)}$)-reflecting the NOS substrate bioavailability, were not significantly different from the control group (Figure 4a).

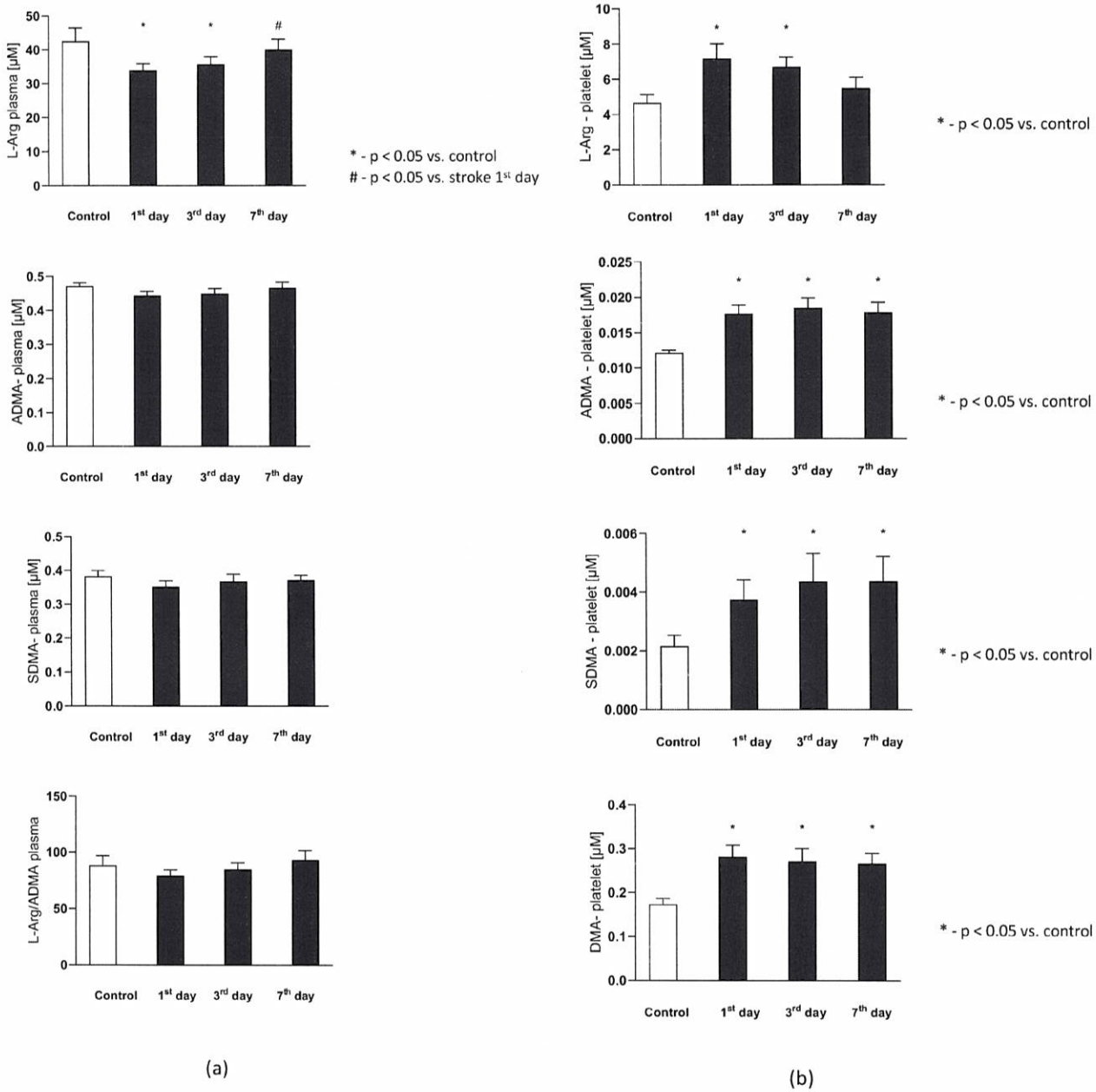
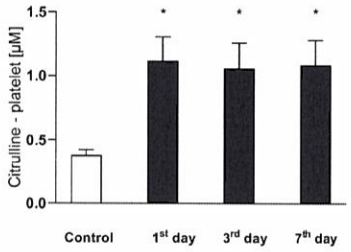
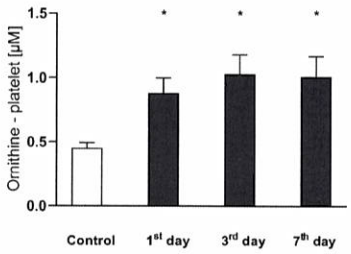


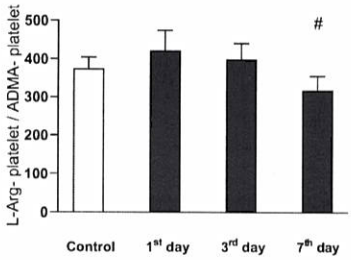
Figure 4. *Cont.*



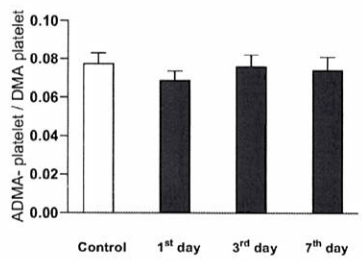
* - p < 0.05 vs. control



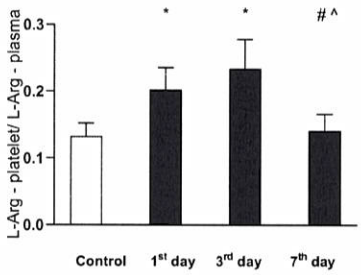
* - p < 0.05 vs. control



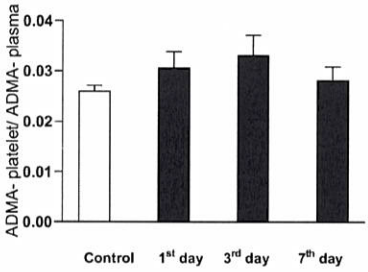
- p < 0.05 vs. stroke 1st day

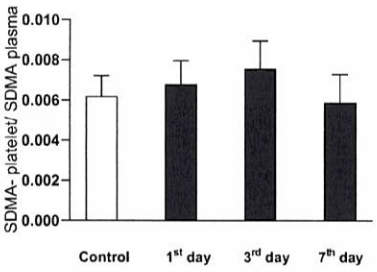


(c)



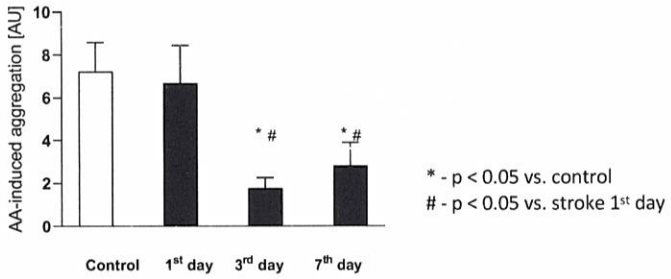
* - p < 0.05 vs. control
# - p < 0.05 vs. stroke 1st day
^ - p < 0.05 vs. stroke 3rd day

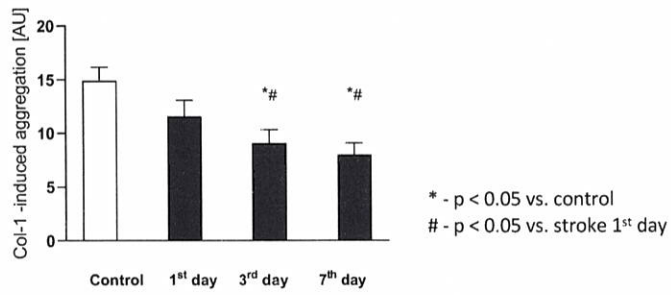


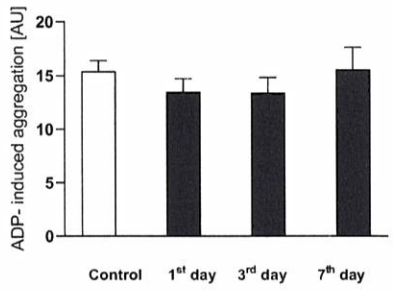


(d)

Figure 4. *Cont.*







(e)

Figure 4. (**a**) The plasma nitric oxide metabolic pathway metabolites in the control group and the study group. *Abbreviations:* Plasma L-Arginine (L-Arg-plasma), the plasma asymmetric dimethylarginine (ADMA-plasma), the plasma symmetric dimethylarginine (SDMA-plasma), L-Arginine to ADMA ratio in plasma (L-Arg/ADMA-plasma). (**b**,**c**) The intraplatelet nitric oxide metabolites in the control group and the study group. *Abbreviations:* L-Arginine in platelets (L-Arginine-platelet), asymmetric dimethylarginine in platelets (ADMA-platelet), symmetric dimethylarginine in platelets (SDMA-platelet), dimethylamine in platelets (DMA-platelet), L-Arginine to asymmetric dimethylarginine ratio in platelets (L-Arginine-platelet/ADMA-platelet), asymmetric dimethylarginine to dimethylamine ratio in platelets (ADMA-platelet/DMA-platelet). (**d**) The balance of the NO biotransformation metabolites between the platelet and plasma compartment. *Abbreviations:* L-Arginine in platelet to L-Arginine in plasma ratio (L-Arg-platelet/L-Arg-plasma), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA). (**e**) Platelet aggregation in the control group and the study group. *Abbreviations:* arachidonic acid-induced aggregation (AA-induced aggregation), collagen-1-induced aggregation (COL 1-induced aggregation), adenosine diphosphate- induced aggregation (ADP-induced aggregation), *—$p < 0.05$ vs. control, #—$p < 0.05$ vs. stroke in the first day.

3.3. Intraplatelet Nitric Oxide Metabolites

The intra-platelet nitric oxide metabolites in both groups are presented in Figure 4b. Six out of eight evaluated nitric oxide metabolites in platelets were found to be significantly higher in subjects with stroke. The platelet L-Arginine on th 1st and 3rd day was greater in the study group, while on the 7th day its level decreased to the concentrations observed in the control group. The ADMA, SDMA, DMA, citrulline, and ornithine concentration on each of the testing days were elevated in the stroke group, in comparison to the healthy individuals. The platelet L-Arginine to ADMA ratio ($\frac{L-Arg\ (PLT)}{ADMA\ (PLT)}$) decreased consecutively from the 1st to 7th day in the stroke group, but it did not reach a statistical significance in comparison to the control group. Finally, the ADMA to DMA ratio in platelets ($\frac{ADMA\ (PLT)}{DMA\ (PLT)}$) from the stroke group was not significantly different from the control group on any of the analyzed days.

3.4. The Balance of the NO Biotransformation Metabolites between the Platelet and Plasma Compartment in Subjects with Stroke and in the Control Group

The balance of the NO biotransformation metabolites between the platelet and plasma compartment in both groups is presented in Figure 4d. Only the platelet to plasma of L-Arginine ratio ($\frac{L-Arg\ (PLT)}{L-Arg\ (PLS)}$) showed significant difference between groups. On the 1st and 3rd day it was higher in the stroke group, while on the 7th day it was similar as in the control group. There were no differences between groups in the platelet to plasma ADMA ($\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$) and SDMA ($\frac{SDMA\ (PLT)}{SDMA\ (PLS)}$) ratios in any of the analyzed days.

3.5. Platelet Aggregation

There were no differences in the arachidonic acid (AA), collagen-1 at 1 μg/mL concentration (Col-1), nor in the adenosine diphosphate (ADP)-induced platelet aggregation between the control group and stroke subjects on the 1st day after the stroke onset. The AA-induced aggregation significantly decreased on the 3rd and 7th day in comparison to the control group and to the stroke group on the 1st day, reflecting the beginning of ASA treatment. Similarly, Col-1-induced aggregation significantly decreased on the 3rd and 7th day in comparison to the control group and to the 1st day of stroke. The ADP-induced aggregation did not change following ASA treatment on any of the analyzed days. The platelet aggregations in both groups are presented in Figure 4e.

3.6. The Correlation between NO Biotransformation Metabolites and Platelet Aggregation

The arachidonic acid-induced aggregation was negatively correlated with the L-Arginine concentration in platelets on the 3rd day, whereas the platelet NO-bioavailability reflected by the $\frac{L-Arg\ (PLT)}{ADMA\ (PLT)}$ ratio was both on the 3rd and 7th day. The $\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$ ratio was positively associated with AA-induced aggregation only on the 7th day.

The collagen-1-induced aggregation was negatively associated with the platelet NOS substrate bioavailability reflected by the $\frac{L-Arg\ (PLT)}{L-Arg\ (PLS)}$ and $\frac{L-Arg\ (PLT)}{ADMA\ (PLT)}$ ratios on the 7th day. Interestingly, at the same time, a positive association with the NOS inhibitor bioavailability expressed as the $\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$ ratio was noted. Col-1-induced aggregation was also negatively correlated with the platelet citrulline concentration on the 3rd day, and positively with ornithine level on the 7th day.

The ADP-induced aggregation was positively correlated with the ADMA platelet to plasma ratio but only on the first day following the stroke onset. The ADP-induced aggregation was also significantly associated with the $\frac{L-Arg\ (PLT)}{L-Arg\ (PLS)}$ ratio on the 3rd day. The rest of the analyzed NO metabolites and their ratios were not correlated with the AA–, Col-1-, nor with ADP-induced aggregation. The correlations between NO biotransformation metabolites and AA-, Col-1-, and ADP- induced aggregation are shown in Supplementary Material: Table S3a–c.

4. Discussion

This is the first study to analyze the dynamic, time-dependent changes in the intraplatelet and plasma expression of selected nitric oxide metabolites during acute phase of human ischemic stroke. Furthermore, we are the first to assess the correlation between platelet aggregation and nitric oxide metabolites from these two compartments in stroke patients.

4.1. Nitric Oxide Biotransformation Metabolites in Plasma

Our study showed initially decreased plasma level of L-Arginine during ischemic stroke with its gradual increase to the control level within one week following the stroke onset. Other authors have already shown that the lower concentration of L-Arg and the longer time it takes for L-Arg to reach the control level, the greater magnitude of neurologic deterioration and the poorer outcome in ischemic stroke patients is observed [13,14]. Up to date, the overall effect of the plasma nitric oxide during the first days after stroke onset remains uncertain. Serrano-Ponz et al. documented that an increase in the plasma NO bioavailability detected within the first two days of stroke was associated with the lower National Institutes of Health Stroke Scale score (NIHSS), as assessed both on the 7th day and after 3 months following the ischemic event. Noteworthy, a steep increase in the plasma NO bioavailability detected from day 2 to day 7 was associated with an increase in infarct size and, as consequence, in the magnitude of neurological deterioration [15]. This unambiguous, Janus-like effect of the NO could be related to the source of its production. The constitutional endothelial NOS (eNOS) isoform is activated at the beginning of the ischemic stroke, facilitating vasodilatation, inhibition of platelet aggregation, and induction of angiogenesis [16]. Noteworthy, the activation of inducible NOS isoform (iNOS) is initialized a few days after the stroke onset and is considered to damage the surrounding tissue due to the participation in inflammation and unregulated peroxynitrite (NOO^-) production [17,18]. The enhanced oxidative stress during ischemic stroke could also lead to endothelial NO synthase dysfunction (eNOS uncoupling) characterized by production of superoxide instead of NO. The eNOS uncoupling could be a reason for reduced endothelial transport of L-Arginine, increased rate of L-Arginine efflux, and finally gradual increase of plasma L-Arginine [19,20], which was also detected in our study.

We have shown that the plasma ADMA and SDMA concentrations, together with the plasma L-Arginine/ADMA ratios, did not differ between the stroke and control group. In most of the studies, elevation of the NOS inhibitors concentration and decrease in the L-Arginine/ADMA ratio have been associated with endothelial dysfunction being thus well-recognized CVD risk factors [21–23]. Nevertheless, significantly increased plasma ADMA and SDMA concentration were not identified in every study on acute ischemic stroke. According to Brouns et al., the inconsistency regarding the relevance of ADMA and SDMA levels might be linked to the stroke severity. Other authors have already shown that the plasma concentrations of NOS inhibitors are associated with greater severity of stroke, as assessed by the NIHSS score [24–26]. Noteworthy, our study group consisted mostly of mild to moderately severe stroke cases with no further progression of neurological deficits.

4.2. Nitric Oxide Biotransformation Metabolites in Platelets

We have found significantly higher platelet L-Arginine concentration (substrate for endothelial NOS-eNOS) on the first and third day after the onset of stroke compared to the control group. Although it is well-known that platelet aggregation is NO-dependent, only recently Radziwon-Balicka et al. have documented that platelet can produce nitric oxide on their own. According to those authors, platelets can be divided into two groups depending on ability or inability to produce nitric oxygen (eNOS-positive or eNOS-negative platelets, respectively). The eNOS-negative platelets play a key role in a thrombus formation, while the eNOS-positive ones are responsible for limiting the thrombus growth and consecutively for its dissolving by intraplatelet NO production [8,27]. However, to our knowledge, there

was no human study conducted yet on changes in the platelet-derived nitric oxide and its metabolites during acute phase of ischemic stroke.

Our study has also shown stably and significantly elevated platelet concentration of ADMA, SDMA, DMA, citrulline, and ornithine in patients with stroke during the 7-day period of observation. The ADMA and SDMA in platelets, as NOS competitive inhibitors, are responsible for decreased platelet derived nitric oxide production. Gawrys et al. have already demonstrated that the intraplatelet ADMA concentration may promote platelet activation in diabetes mellitus [28]. However, in the study by Meirelles et al., increased ADMA level led to enhanced platelet aggregation in both hypertensive and healthy subjects [29]. Stably elevated levels of the intraplatelet NOS-inhibitors (ADMA, SDMA) and their metabolites (including the DMA, ornithine, citrulline) throughout acute stroke event suggest the presence of their increased pro-aggregatory function before the onset of ischemic stroke. This leads to the conclusion that L-Arginine and ADMA from platelets may be important in two different intervals of ischemic stroke course (ADMA before, and L-Arginine after the thrombotic event), nevertheless further studies in this matter are required.

4.3. The Balance of the NO Biotransformation Metabolites between the Platelet and Plasma Compartment

NO is a highly reactive molecule and has an ability to diffuse through a cell membrane [30]. However, the nitric oxide biotransformation metabolites must be actively transported between plasma and platelet compartment in both ways via the cationic amino acid transporters (CAT) or y (+) L system [31]. In our study, only the platelet to plasma L-Arginine ratio ($\frac{L-Arg\ (PLT)}{L-Arg\ (PLS)}$) was significantly different between the study group and control group. High platelet and low plasma L-Arginine level at the onset of ischemic stroke were followed by the platelet decrease and plasma increase of L-Arg during the 7 days of observation. Mury et al. have already demonstrated reduced plasma levels of L-Arginine, lower nitric oxide synthase activity, and compensatory increase in L-Arginine transmembrane transport from plasma to platelet compartment in patients being at high risk of thrombotic event [32]. Furthermore, Mendes Ribeiro et al. showed decreased plasma L-Arginine concentration and increased platelet capacity for L-Arginine transport in patients with heart failure or chronic kidney disease [31]. It may suggest the presence of the active NO biotransformation metabolites transport between plasma and platelet compartment also in acute phase of ischemic stroke, however direct measurement of such a transport was not conducted in our study.

Moreover, we have not observed significant changes in $\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$ and $\frac{SDMA\ (PLT)}{SDMA\ (PLS)}$ ratios in our study. Tymyios at al. demonstrated that elevated plasma ADMA level does not alter platelet NO production, while De Meirelles et al. found that plasma ADMA can decrease the intraplatelet NOS activity [29,33]. Inconsistency in the effect of the plasma ADMA concentration on platelet NO production suggests the complexity of mechanisms controlling the PDNO release. The accumulation of NOS inhibitors in thrombocytes could inhibit further plasma to platelet ADMA transport and be a trigger factor for ischemic stroke incidence, however further studies are required [34].

4.4. The Correlation between NO Biotransformation Metabolites and Platelet Aggregation

There are scarcely no studies analyzing the influence of antiplatelet treatment on the platelet NO production. Madajka et al. showed, that ASA improves platelet NO synthesis, without significant effect on the NO bioavailability in endothelial cells [35]. According to other authors, the ASA administration seems to have two different effects on the NOS activity. Kane et al. showed that chronic acetylsalicylic acid treatment and the use of other non-steroidal anti-inflammatory drugs decrease the platelet NO production by limiting the NOS-activating response to stimulation of platelet beta-adrenergic receptors (cyclooxygenase inhibition-dependent mechanism). Nevertheless acute ASA administration activates basal platelet NOS by its acetylation and thereby acts through a mechanism

independent of cyclooxygenase inhibition [36,37]. In our study platelet arachidonic acid and collagen-1—dependent aggregation was decreased on the 3rd and the 7th day after the ASA treatment onset, while the ADP-dependent aggregation was unchanged. We have also shown that during acute ASA administration arachidonic acid-induced aggregation was negatively associated with L-Arginine and the $\frac{L-Arg\ (PLT)}{ADMA\ (PLT)}$ ratio, while after 7 days platelet aggregation was positively associated with platelet ADMA bioavailability (described as high $\frac{ADMA\ (PLT)}{ADMA\ (PLS)}$ ratio) in arachidonic acid-, collagen-1 and ADP- dependent mechanism. We confirmed observations of other authors, that ASA induces the platelet NO production leading to thrombus dissolving only during acute administration, while thrombus formation in the course of chronic acetylsalicylic acid treatment is probably positively associated with ADMA in platelets to ADMA in plasma ratio. However, it is hard to distinguish between the influence of ASA treatment and the natural course of ischemic stroke disease on changes in NO biotransformation metabolites and platelet aggregation, as ASA was not administrated in the control group.

4.5. Demography and Comorbidity Differences between the Stroke and the Control Group—Possible Effect on the Results

Although the study protocol assumed that stroke subjects and healthy controls were closely matched, significant differences in comorbidities, drug treatment and biochemic results were found. Higher hypertension burden and angiotensin-converting-enzyme inhibitors (ACE-I) intake was detected in the stroke group in comparison to the control. Administration of some other hypotensive drugs (angiotensin-receptor blocker (ARB) β-blocker, dihydropyridine calcium channel blocker, thiazide/thiazide like diuretic, loop diuretic) was significantly higher only in the stroke group at discharge. Finally, the diagnosis of dyslipidaemia or use of statin was significantly higher at the discharge than on admission in the stroke group. Other authors have already shown that basal platelet concentration of L-Arginine and platelet derived NO production is diminished in presence of such cardiovascular risk factors as age, smoking, hypercholesterolemia with oxidized LDL, hypertension, diabetes mellitus and coronary artery disease. [28,38–42]. While Gryglewski et al. have shown in animal model study, that ACE-I treatment could induce thrombolysis by the increase in endothelial nitric oxide production. Moreover, statin-related improvement in the stroke outcome is documented to be mediated among others by the increase in the platelet eNOS expression [35,43,44]. Similarly increase in the L-Arginine concentration in stroke patients observed in our study could result not only from ASA but also from other drugs used during hospitalization. Nevertheless, further studies are required to verify if observed L-Arginine increase is due to the natural course of disease or whether it results from medications and from which ones.

The study group and the control group were also significantly different regarding the white blood cells count (WBC), mean platelet volume (MPV), serum glucose, potassium, and thyroid-stimulating hormone (TSH) levels. Increased WBC, together with hyperglycaemia observed in the stroke group, might be a symptom of acute stress responses involving the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in reaction to extensive brain injury. Leucocytosis is also associated with activation of coagulation cascades, thrombus formation, and is a typical finding in acute phase of ischemic stroke (thrombo-inflammation theory) [45,46]. The MPV was smaller in the stroke group, and it increased gradually in the course of disease. Although elevated MPV is a well-recognised risk factor for ischemic stroke, low MPV is a characteristic feature for patients with active thrombosis. Observed increase in MPV in the course of disease can be a response to the thrombus formation by enhanced production of antiaggregatory molecules in platelets and probably inhibition of megacariopoiesis (the greater magnitude of thrombopoiesis, the smaller platelet volume) [47–50]. Lower level of potassium in the study group corresponds to the findings of other authors who described hypokalaemia in up to $\frac{1}{4}$ of patients with ischemic stroke. One of the pathomechanisms aimed at explaining this correlation is that hypokalemia by reducing conductance hyperpolarization in potassium

channel of cells promotes formation of free radicals, which could lead to endothelial dysfunction—documented ischemic stroke risk factor [51,52]. Finally, we have found higher level of TSH in the study group in comparison to the control group. Manolis et al. have already described a positive correlation between subclinical thyroid dysfunction (subclinical hypothyroidism or subclinical hyperthyroidism) and increased cardiovascular risk. However, due to lack of randomized controlled trials, no consensus has been reached on whether treatment of dysfunction is beneficial in prevention of ischemic stroke [53].

4.6. Perspectives

There are only few studies analyzing the influence of increased NO bioavailability on the course of ischemic stroke. Saleh et al. showed in an animal model that L-Arginine administration is more effective than ASA supplementation in primary prevention of thrombotic events, which could be achieved by greater inhibition of platelet aggregation and higher reduction of the low-density lipoprotein (LDL) oxidation in comparison to ASA treatment [54,55]. Li et al. documented in an animal model of acute ischemic stroke that platelet membrane biomimetic magnetic nanocarriers with NO achieve rapid targeting to ischemic stroke lesions, encouraging the release of L-Arginine at the thrombus site leading to disruption of the local platelet aggregation and reperfusion of the ischemic penumbra [56]. ASA combination with NO-donor could be a promising drug in prevention and treatment of non-cardioembolic ischemic stroke, but further human studies should be conducted. Moreover, studies analyzing the dynamic changes in production of platelet nitric oxide correlated with NOS expression (both endothelial and inducible one), oxidative stress parameters, and ischemic stroke outcome would be of great importance for further understanding of ischemic stroke patomechanisms, as NO not only acts as inhibitor of aggregation and vasodilator but can also be transformed to the one of reactive oxygen species.

5. Conclusions

Human subjects with non-cardioembolic ischemic stroke are characterized by stably elevated platelet levels of the NOS inhibitors which could be associated with increased platelet susceptibility to aggregation. ASA treatment in acute phase of stroke results in increase in the platelet L-Arginine concentration together with higher platelet NO bioavailability. It suggests that ASA has not only the cyclooxygenase-1-, but also the nitric oxide-dependent antiplatelet function. Hence, we suggest that the platelet nitric oxide and its biotransformation metabolites play an important role in regulating the aggregation in acute phase of non-cardioembolic ischemic stroke. However, further studies on platelet nitric oxide role in pathogenesis of ischemic stroke are required, aiming at explanation if decreased platelet NO bioavailability could be considered as a risk factor and simultaneously therapeutic target in ischemic stroke.

6. Limitations

Several limitations of this study should be underlined. The first regards selection of the stroke group, due to study inclusion and exclusion criteria. Approximately 70% of patients from the initial cohort met the exclusion criteria, as treatment by thrombolysis/thrombectomy or both could affect the measurement of nitric oxide biotransformation and platelet aggregation in the natural course of the disease. Moreover, 15% of patients had to be excluded, due to the stroke severity (lack of informed consent). Finally, only 10% of patients (with mild to moderate course of disease) from over 400 ones met the inclusion criteria which could create "parapatric speciation" bias in the study group. Another limitation regards the measured molecules. As NO is highly reactive, it was not possible to measure it directly in our experimental setting. The cyclic guanosine monophosphate (cGMP), as effector of nitric oxide but also of various other metabolic pathways, was not studied either. Hence, our study is based on more stable nitric oxide biotransformation metabolites, but in consequence it makes our conclusions on NO correlation with platelet aggregation indirect. Moreover, different

permeability of the platelet membrane and laboratory separation processes could affect the inter-compartment distribution and study results. Therefore, additional experiments should be conducted in order to assess the significance of these phenomena. Finally, it is not possible to certainly distinguish between natural change in platelet function during ischemic stroke course and ASA administration effect on platelet nitric oxide production, as the control group did not receive any ASA treatment at all.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11050955/s1. Table S1. Comorbidities in the control group, stroke group on admission (diagnosed before the onset of stroke) and stroke group at the discharge diagnosed both, during hospitalizationd and before onset of stroke). Table S2. Treatment applied in the control group (chronic management), stroke group on admission (until hospitalization) and stroke group at discharge. Table S3a. The correlations between NO biotransformation metabolites and platelet aggregation. Table S3b. The correlations between NO biotransformation metabolites and platelet aggregation. Table S3c. The correlations between NO biotransformation metabolites and platelet aggregation.

Author Contributions: Conceptualization: M.B., S.B. and A.D.; methodology: J.C.-Ł., E.S.-K., J.W., P.F. and M.F.; statistical analysis: A.D. and M.B.; investigation: M.B. and J.C.-Ł.; writing: M.B.; writing—review and editing: A.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All experiments were conducted and approved in accordance with the guidelines of the local Bioethics Committee (Opinion number KB-724/2017) and adhered to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects (revised 13 November 2001, effective 13 December 2001). All participants provided their written consent to participate in the study. The written consent form had been also approved by the ethics committee.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: Data is contained within the article and supplementary material.

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

References

1. Wafa, H.A.; Wolfe, C.D.A.; Emmett, E.; Roth, G.A.; Johnson, C.O.; Wang, Y. Burden of Stroke in Europe: Thirty-Year Projections of Incidence, Prevalence, Deaths, and Disability-Adjusted Life Years. *Stroke* **2020**, *51*, 2418–2427. [CrossRef] [PubMed]
2. Tsai, C.F.; Thomas, B.; Sudlow, C.L. Epidemiology of stroke and its subtypes in Chinese vs white populations: A systematic review. *Neurology* **2013**, *81*, 264–272. [CrossRef] [PubMed]
3. Kim, D.; Lee, S.-H.; Kim, B.J.; Jung, K.-H.; Yu, K.-H.; Lee, B.-C.; Roh, J.-K.; For Korean Stroke Registry Investigators. Secondary prevention by stroke subtype: A nationwide follow-up study in 46 108 patients after acute ischaemic stroke. *Eur. Heart J.* **2013**, *34*, 2760–2767. [CrossRef] [PubMed]
4. Loscalzo, J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circ. Res.* **2001**, *88*, 756–762. [CrossRef]
5. Förstermann, U.; Xia, N.; Li, H. Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis. *Circ. Res.* **2017**, *120*, 713–735. [CrossRef]
6. Estevez, B.; Du, X. New Concepts and Mechanisms of Platelet Activation Signaling. *Physiology* **2017**, *32*, 162–177. [CrossRef]
7. Jamaluddin, M.S.; Liang, Z.; Lu, J.M.; Yao, Q.; Chen, C. Roles of cardiovascular risk factors in endothelial nitric oxide synthase regulation: An update. *Curr. Pharm. Des.* **2014**, *20*, 3563–3578. [CrossRef]
8. Radziwon-Balicka, A.; Lesyk, G.; Back, V.; Fong, T.; Loredo-Calderon, E.L.; Dong, B.; El-Sikhry, H.; El-Sherbeni, A.A.; El-Kadi, A.; Ogg, S.; et al. Differential eNOS-signalling by platelet subpopulations regulates adhesion and aggregation. *Cardiovasc. Res.* **2017**, *113*, 1719–1731. [CrossRef]
9. Radomski, M.; Moncada, S. An improved method for washing of human platelets with prostacyclin. *Thromb. Res.* **1983**, *30*, 383–389. [CrossRef]
10. Mustard, J.F.; Perry, D.W.; Ardli, N.G.; Packham, M.A. Preparation of suspension of washed platelets from humans. *Br. J. Haematol.* **1972**, *22*, 193–204. [CrossRef]

11. Fleszar, M.G.; Wiśniewski, J.; Krzystek-Korpacka, M.; Misiak, B.; Frydecka, D.; Piechowicz, J.; Lorenc-Kukuła, K.; Gamian, A. Quantitative Analysis of l-Arginine, Dimethylated Arginine Derivatives, l-Citrulline, and Dimethylamine in Human Serum Using Liquid Chromatography-Mass Spectrometric Method Mass to charge ratio MRM Multiple reaction monitoring NO Nitric oxide NOS Nitric oxide synthase OPA Ortho-phthaldialdehyde. *Chromatographia* **2018**, *81*, 911–921. [PubMed]
12. Tóth, O.; Calatzis, A.; Penz, S.; Losonczy, H.; Siess, W. Multiple electrode aggregometry: A new device to measure platelet aggregation in whole blood. *Thromb. Haemost.* **2006**, *96*, 781–788. [PubMed]
13. Rashid, P.A.; Whitehurst, A.; Lawson, N.; Bath, P.M. Plasma nitric oxide (nitrate/nitrite) levels in acute stroke and their relationship with severity and outcome. *J. Stroke Cerebrovasc. Dis.* **2003**, *12*, 82–87. [CrossRef] [PubMed]
14. Molnar, T.; Csuka, D.; Pusch, G.; Nagy, L.; Garred, P.; Illes, Z. Associations between serum L-arginine and ficolins in the early phase of acute ischemic stroke—A pilot study. *J. Stroke Cerebrovasc. Dis.* **2020**, *29*, 104951. [CrossRef] [PubMed]
15. Serrano-Ponz, M.; Rodrigo-Gasqué, C.; Siles, E.; Martínez-Lara, E.; Ochoa-Callejero, L.; Martínez, A. Temporal profiles of blood pressure, circulating nitric oxide, and adrenomedullin as predictors of clinical outcome in acute ischemic stroke patients. *Mol. Med. Rep.* **2016**, *13*, 3724–3734. [CrossRef]
16. Cui, X.; Chopp, M.; Zacharek, A.; Zhang, C.; Roberts, C.; Chen, J. Role of endothelial nitric oxide synthetase in arteriogenesis after stroke in mice. *Neuroscience* **2009**, *159*, 744–750. [CrossRef]
17. Khan, M.; Sekhon, B.; Giri, S.; Jatana, M.; Gilg, A.G.; Ayasolla, K.; Elango, C.; Singh, A.K.; Singh, I. S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. *J. Cereb. Blood Flow Metab.* **2005**, *25*, 177–192. [CrossRef]
18. Rodrigo, J.; Fernández, A.P.; Serrano, J.; Peinado, M.A.; Martínez, A. The role of free radicals in cerebral hypoxia and ischemia. *Free Radic. Biol. Med.* **2005**, *39*, 26–50. [CrossRef]
19. Łuczak, A.; Madej, M.; Kasprzyk, A.; Doroszko, A. Role of the eNOS Uncoupling and the Nitric Oxide Metabolic Pathway in the Pathogenesis of Autoimmune Rheumatic Diseases. *Oxid. Med. Cell Longev.* **2020**, *2020*, 1417981. [CrossRef]
20. Karbach, S.; Wenzel, P.; Waisman, A.; Munzel, T.; Daiber, A. eNOS uncoupling in cardiovascular diseases–the role of oxidative stress and inflammation. *Curr. Pharm. Des.* **2014**, *20*, 3579–3594. [CrossRef]
21. Böger, R.H.; Sullivan, L.M.; Schwedhelm, E.; Wang, T.J.; Maas, R.; Benjamin, E.J.; Schulze, F.; Xanthakis, V.; Benndorf, R.A.; Vasan, R.S. Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community. *Circulation* **2009**, *119*, 1592–1600. [CrossRef] [PubMed]
22. Yu, E.; Ruiz-Canela, M.; Hu, F.B.; Clish, C.B.; Corella, D.; Salas-Salvadó, J.; Hruby, A.; Fitó, M.; Liang, L.; Toledo, E.; et al. Plasma Arginine/Asymmetric Dimethylarginine Ratio and Incidence of Cardiovascular Events: A Case-Cohort Study. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 1879–1888. [CrossRef] [PubMed]
23. Schulze, F.; Carter, A.M.; Schwedhelm, E.; Ajjan, R.; Maas, R.; von Holten, R.A.; Atzler, D.; Grant, P.J.; Böger, R.H. Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke. *Atherosclerosis* **2010**, *208*, 518–523. [CrossRef] [PubMed]
24. Worthmann, H.; Chen, S.; Martens-Lobenhoffer, J.; Li, N.; Deb, M.; Tryc, A.B.; Goldbecker, A.; Dong, Q.; Kielstein, J.T.; Bode-Böger, S.M.; et al. High Plasma Dimethylarginine Levels are Associated with Adverse Clinical Outcome After Stroke. *J. Atheroscler. Thromb.* **2011**, *18*, 753–761. [CrossRef] [PubMed]
25. Rueda-Clausen, C.F.; Córdoba-Porras, A.; Bedoya, G.; Silva, F.A.; Zarruk, J.G.; López-Jaramillo, P.; Villa, L.A. Increased Plasma Levels of Total Homocysteine but Not Asymmetric Dimethylarginine in Hispanic Subjects with Ischemic Stroke FREC-VI Sub-Study. *Eur. J. Neurol.* **2012**, *19*, 417–425. [CrossRef] [PubMed]
26. Brouns, R.; Marescau, B.; Possemiers, I.; Sheorajpanday, R.; De Deyn, P.P. Dimethylarginine Levels in Cerebrospinal Fluid of Hyperacute Ischemic Stroke Patients are Associated with Stroke Severity. *Neurochem. Res.* **2009**, *34*, 1642–1649. [CrossRef]
27. Radomski, M.W.; Palmer, R.M.; Moncada, S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5193–5197. [CrossRef]
28. Gawryś, J.; Wiśniewski, J.; Szahidewicz-Krupska, E.; Gajecki, D.; Leśniewska, J.; Majda, F.; Gawryś, K.; Fortuna, P.; Mlynarz, P.; Doroszko, A. Increased Intraplatelet ADMA Level May Promote Platelet Activation in Diabetes Mellitus. *Oxid. Med. Cell Longev.* **2020**, *2020*, 6938629. [CrossRef]
29. De Meirelles, L.R.; Mendes-Ribeiro, A.C.; Santoro, M.M.; Mendes, M.A.; da Silva, M.N.; Mann, G.E.; Brunini, T.M. Inhibitory effects of endogenous L-arginine analogues on nitric oxide synthesis in platelets: Role in platelet hyperaggregability in hypertension. *Clin. Exp. Pharmacol. Physiol.* **2007**, *34*, 1267–1271. [CrossRef]
30. Möller, M.N.; Denicola, A. Diffusion of nitric oxide and oxygen in lipoproteins and membranes studied by pyrene fluorescence quenching. *Free Radic. Biol. Med.* **2018**, *128*, 137–143. [CrossRef]
31. Mendes Ribeiro, A.C.; Brunini, T.M.; Ellory, J.C.; Mann, G.E. Abnormalities in L-arginine transport and nitric oxide biosynthesis in chronic renal and heart failure. *Cardiovasc. Res.* **2001**, *49*, 697–712. [CrossRef]
32. Mury, W.V.; Brunini, T.M.; Abrantes, D.C.; Mendes, I.K.; Campos, M.B.; Mendes-Ribeiro, A.C.; Matsuura, C. Hyperaggregability and impaired nitric oxide production in platelets from postmenopausal women. *Maturitas* **2015**, *80*, 75–81. [CrossRef] [PubMed]
33. Tymvios, C.; Moore, C.; Jones, S.; Solomon, A.; Sanz-Rosa, D.; Emerson, M. Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. *Br. J. Pharmacol.* **2009**, *158*, 1735–1742. [CrossRef]
34. Cozzi, M.R.; Guglielmini, G.; Battiston, M.; Momi, S.; Lombardi, E.; Miller, E.C.; de Zanet, D.; Mazzucato, M.; Gresele, P.; de Marco, L. Visualization of nitric oxide production by individual platelets during adhesion in flowing blood. *Blood* **2015**, *125*, 697–705. [CrossRef] [PubMed]

35. Madajka, M.; Korda, M.; White, J.; Malinski, T. Effect of aspirin on constitutive nitric oxide synthase and the biovailability of NO. *Thromb. Res.* **2003**, *110*, 317–321. [CrossRef]
36. O'Kane, P.D.; Reebye, V.; Ji, Y.; Stratton, P.; Jackson, G.; Ferro, A. Aspirin and clopidogrel treatment impair nitric oxide biosynthesis by platelets. *J. Mol. Cell Cardiol.* **2008**, *45*, 223–229. [CrossRef]
37. O'Kane, P.D.; Queen, L.R.; Ji, Y.; Reebye, V.; Stratton, P.; Jackson, G.; Ferro, A. Aspirin modifies nitric oxide synthase activity in platelets: Effects of acute versus chronic aspirin treatment. *Cardiovasc. Res.* **2003**, *59*, 152–159. [CrossRef]
38. Moss, M.B.; Brunini, T.M.; Soares De Moura, R.; Novaes Malagris, L.E.; Roberts, N.B.; Ellory, J.C.; Mann, G.E.; Mendes Ribeiro, A.C. Diminished L-arginine bioavailability in hypertension. *Clin. Sci.* **2004**, *107*, 391–397. [CrossRef]
39. Chen, L.Y.; Mehta, P.; Mehta, J.L. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: Relevance of the effect of oxidized LDL on platelet function. *Circulation* **1996**, *93*, 1740–1746. [CrossRef]
40. Ikeda, H.; Takajo, Y.; Murohara, T.; Ichiki, K.; Adachi, H.; Haramaki, N.; Katoh, A.; Imaizumi, T. Platelet-derived nitric oxide and coronary risk factors. *Hypertension* **2000**, *35*, 904–907. [CrossRef]
41. Freedman, J.E.; Ting, B.; Hankin, B.; Loscalzo, J.; Keaney, J.F., Jr.; Vita, J.A. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. *Circulation* **1998**, *98*, 1481–1486. [CrossRef] [PubMed]
42. Martina, V.; Bruno, G.A.; Trucco, F.; Zumpano, E.; Tagliabue, M.; Di Bisceglie, C.; Pescarmona, G. Platelet cNOS activity is reduced in patients with IDDM and NIDD. *Thromb. Haemost.* **1998**, *79*, 520–522. [PubMed]
43. Laufs, U.; Gertz, K.; Huang, P.; Nickenig, G.; Böhm, M.; Dirnagl, U.; Endres, M. Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice. *Stroke* **2000**, *31*, 2442–2449. [CrossRef] [PubMed]
44. Gryglewski, R.J.; Święs, J.; Uracz, W.; Chlopicki, S.; Marcinkiewicz, E. Mechanisms of angiotensin-converting enzyme inhibitor induced thrombolysis in Wistar rats. *Thromb. Res.* **2003**, *110*, 323–329. [CrossRef]
45. Bi, R.; Chen, S.; Chen, S.; Peng, Q.; Jin, H.; Hu, B. The role of leukocytes in acute ischemic stroke-related thrombosis: A notable but neglected topic. *Cell Mol Life Sci.* **2021**, *78*, 6251–6264. [CrossRef]
46. Ferrari, F.; Moretti, A.; Villa, R.F. Hyperglycemia in acute ischemic stroke: Physiopathological and therapeutic complexity. *Neural Regen. Res.* **2022**, *17*, 292–299.
47. Levin, J.; Bessman, J.D. The inverse relation between platelet volume and platelet number: Abnormalities in hematologic disease and evidence that platelet size does not correlate with platelet age. *J. Lab. Clin. Med.* **1983**, *101*, 295–307.
48. Llorente-Chávez, A.; Martín-Nares, E.; Núñez-Álvarez, C.; Hernández-Molina, G. Thrombosis and thrombocytopenia in antiphospholipid syndrome: Their association with mean platelet volume and hematological ratios. *Thromb. Res.* **2021**, *203*, 12–17. [CrossRef]
49. Zheng, M.; Chen, S.; Zhu, Y.; Gu, X. Mean platelet volume: A new predictor of ischaemic stroke risk in patients with nonvalvular atrial fibrillation. *BMC Cardiovasc. Disord.* **2020**, *20*, 241. [CrossRef]
50. Gasparyan, A.Y.; Ayvazyan, L.; Mikhailidis, D.P.; Kitas, G.D. Mean platelet volume: A link between thrombosis and inflammation? *Curr. Pharm. Des.* **2011**, *17*, 47–58. [CrossRef]
51. Alam, M.N.; Uddin, M.J.; Rahman, K.M.; Ahmed, S.; Akhter, M.; Nahar, N.; Swapan, M.K.; Alam, M.M.; Sultana, N.; Hallaz, M.M.; et al. Electrolyte changes in stroke. *Mymensingh Med. J.* **2012**, *21*, 594–599. [PubMed]
52. Wang, A.; Tian, X.; Gu, H.; Zuo, Y.; Meng, X.; Chen, P.; Li, H.; Wang, Y. Electrolytes and clinical outcomes in patients with acute ischemic stroke or transient ischemic attack. *Ann. Transl. Med.* **2021**, *9*, 1069. [CrossRef] [PubMed]
53. Manolis, A.A.; Manolis, T.A.; Melita, H.; Manolis, A.S. Subclinical thyroid dysfunction and cardiovascular consequences: An alarming wake-up call? *Trends Cardiovasc. Med.* **2020**, *30*, 57–69. [CrossRef] [PubMed]
54. Saleh, A.I.; Abdel Maksoud, S.M.; El-Maraghy, S.A.; Gad, M.Z. Protective effect of L-arginine in experimentally induced myocardial ischemia: Comparison with aspirin. *J. Cardiovasc. Pharmacol. Ther.* **2011**, *16*, 53–62. [CrossRef]
55. Chen, Y.; Cui, G.; Zhao, M.; Wang, C.; Qian, K.; Morris-Natschke, S.; Lee, K.H.; Peng, S. Synthesis, nano-scale assembly, and in vivo anti-thrombotic activity of novel short peptides containing L-Arg and L-Asp or L-Glu. *Bioorg. Med. Chem.* **2008**, *16*, 5914–5925. [CrossRef]
56. Li, M.; Li, J.; Chen, J.; Liu, Y.; Cheng, X.; Yang, F.; Gu, N. Platelet Membrane Biomimetic Magnetic Nanocarriers for Targeted Delivery and in Situ Generation of Nitric Oxide in Early Ischemic Stroke. *ACS Nano* **2020**, *14*, 2024–2035. [CrossRef]

5. OŚWIACZENIA WSPÓŁAUTORÓW

Wrocław, 13.05.2022

lek. Maciej Bladowski
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz stworzeniem zasadniczej części manuskryptu wraz z rycinami.

Podpis

M Bladowski

Wrocław, 13.05.2022

dr n. med. Jakub Gawryś
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz tworzenia manuskryptu.

Podpis

Jakub Gawryś

Wrocław, 13.05.2022

lek. Damian Gajecki
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz tworzenia manuskryptu.

Podpis

D. Gajecki

Wrocław, 13.05.2022

mgr Ewa Szahidewicz-Krupska
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz tworzenia manuskryptu.

Podpis

Wrocław, 13.05.2022

lek. Anna Sawicz-Bladowska
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz tworzenia manuskryptu.

Podpis

Anna Sawicz-Bladowska

Wrocław. 13.05.2022

prof. dr hab. n. med. Adrian Doroszko
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski, Jakub Gawryś, Damian Gajecki, Ewa Szahidewicz-Krupska, Anna Sawicz-Bladowska, Adrian Doroszko.

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside.

Oxidative Medicine and Cellular Longevity; Aug 28;2020 Article ID 2979260

mój udział polegał na opracowaniu koncepcji badań, uzyskaniu finansowania, kierowania ich realizacją, analizie danych statystycznych oraz nadzorze nad pisaniem manuskryptu.

A. Do...

Podpis

Wrocław, 13.05.2022

lek. Maciej Bladowski
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na przeszukaniu bazy danych w kierunku publikacji zgodnych z założonym tematem, współpracy przy analizie zebranych danych oraz stworzeniem zasadniczej części manuskryptu wraz z rycinami.

Podpis

M Bladowski

Wrocław, 13.05.2022r.

mgr Ewa Szahidewicz-Krupska
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na współpracy przy realizacji projektu: zabezpieczenie materiału biologicznego do wykonania oznaczeń metodą LC/MS, tworzenie bazy danych oraz napisaniu części dotyczącej metodyki w wyżej wymienionym manuskrypcie.

Podpis

Wrocław, 13.05.2022

dr n. med. Jerzy Wiśniewski
Centralne Laboratorium Analizy Eksperymentalnej
Wydział Chemiczny Politechniki Wrocławskiej

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na współpracy przy realizacji projektu: wykonanie oznaczeń metodą LC/MS z wykorzystaniem zabezpieczonego materiału biologicznego, tworzeniu bazy danych oraz współpracy przy tworzeniu części dotyczącej metodyki w wyżej wymienionym manuskrypcie.

Jerzy Wiśniewski

Podpis

Wrocław, 13.05.2022

dr Paulina Fortuna
Katedra i Zakład Biochemii Lekarskiej
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na współpracy przy realizacji projektu: wykonanie oznaczeń metodą LC/MS z wykorzystaniem zabezpieczonego materiału biologicznego, tworzeniu bazy danych oraz współpracy przy tworzeniu części dotyczącej metodyki w wyżej wymienionym manuskrypcie.

Podpis

Paulina Fortuna

Wrocław, 13.05.2022

dr n. med. Justyna Chojdak-Łukasiewicz
Katedra i Klinika Neurologii
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na współpracy przy realizacji projektu: rekrutacji i badaniu pacjentów do grupy badawczej, zbieraniu i analizie statystycznej danych oraz współpracy przy tworzeniu manuskryptu

Podpis

Justyna Chojdak-Łukasiewicz

Wrocław, 13.05.2022

dr. hab. n. med. Sławomir Budrewicz, prof. nadzw. UMW
Katedra i Klinika Neurologii
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na nadzorze nad pisaniem manuskryptu.

Podpis

S. Budrewicz

Wrocław, 13.05.2022

dr Mariusz Fleszar
Katedra i Zakład Biochemii Lekarskiej
Uniwersytet Medyczny we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

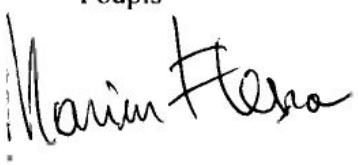
Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na współpracy przy realizacji projektu: wykonanie oznaczeń metodą LC/MS z wykorzystaniem zabezpieczonego materiału biologicznego, tworzeniu bazy danych oraz współpracy przy tworzeniu części dotyczącej metodyki w wyżej wymienionym manuskrypcie.

Podpis



Wrocław, 13.05.2022

prof. dr hab. n. med. Adrian Doroszko
Katedra i Klinika Chorób Wewnętrznych,
Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej
Uniwersytetu Medycznego we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy:

Maciej Bladowski , Ewa Szahidewicz-Krupska, Jerzy Wiśniewski, Paulina Fortuna, Justyna Chojdak-Łukasiewicz, Slawomir Budrewicz, Mariusz Fleszar i Adrian Doroszko

Changes in the plasma and platelet nitric oxide biotransformation metabolites during ischemic stroke- a dynamic human LC/MS metabolomic study

Antioxidants 2022, 11(5), 955; Article ID 1050955

mój udział polegał na opracowaniu koncepcji badań, uzyskaniu finansowania, kierowania ich realizacją, analizie danych statystycznych oraz nadzorze nad pisaniem manuskryptu.

A. Doroszko

Podpis

6. STRESZCZENIE

Udar niedokrwienny mózgu jako jedna z chorób sercowo-naczyniowych o znaczącej chorobowości i śmiertelności, niezmiennie od wielu lat pozostaje w centrum zainteresowania współczesnej medycyny. Standardową metodą prewencji wtórnej niekardiogennego (niezwiązanego z materiałem zatorowym powstałym w sercu) udaru niedokrwiennego mózgu jest terapia przeciwpłytkowa. Jej skuteczność została potwierdzona w licznych wieloośrodkowych badaniach klinicznych i stała się kanonem postępowania u tych chorych. Te same leki przeciwpłytkowe nie przynoszą jednak korzyści w prewencji pierwotnej u niewyselekcjonowanej grupy pacjentów narażonej na powikłania sercowo-naczyniowe. Ze względu na udowodniony wpływ zmniejszonej biodostępności tlenku azotu i zwiększonego stężenia asymetrycznej dimetyloargininy (ADMA- kompetycyjny inhibitor syntetazy tlenku azotu) na rozwój dysfunkcji śródbłonka naczyniowego i zwiększenia ryzyka wystąpienia chorób sercowo-naczyniowych, głównym tematem niniejszej rozprawy doktorskiej była ocena zależności pomiędzy zaburzeniami biotransformacji tlenku azotu, a funkcją płytek krwi u pacjentów w ostrej fazie niekardiogennego udaru niedokrwiennego mózgu (od < 24 godzin od początku objawów neurologicznych, do 7 doby obserwacji).

W związku z postulowaną w dotychczasowych doniesieniach kluczową rolą tlenku azotu nie tylko jako wyznacznika dysfunkcji śródbłonka, ale także jako związku regulującego aktywność płytek krwi, jego rolę w patogenezie i przebiegu udaru niedokrwiennego mózgu usystematyzowano i podsumowano w pracy poglądowej będącej częścią tego cyklu publikacji. Następnie w pracy oryginalnej oceniono wpływ metabolitów biotransformacji tlenku azotu obecnych w osoczu i w trombocytach, na agregację płytek krwi u pacjentów w ostrej fazie niekardiogennego udaru niedokrwiennego mózgu.

W przeprowadzonym badaniu stwierdzono znamiennie wyższe i utrzymujące się przez cały okres obserwacji podwyższone stężenie inhibitorów syntetazy tlenku azotu (NOS) w płytkach krwi pacjentów z udarem niedokrwiennym mózgu. W 1. i 3. dniu obserwacji stwierdzono w grupie badanej wyższe stężenie L-Argininy w płytkach krwi i niższe stężenie L-Argininy w osoczu, w porównaniu do grupy kontrolnej. Agregacja płytek krwi zależna od kwasu arachidonowego była ujemnie skorelowana z płytkową biodostępnością tlenku azotu ($\frac{L-Arginina\ (płytki)}{ADMA\ (płytki)}$) w 3. i 7. dniu obserwacji w grupie badanej. W powstałym oryginalnym manuskrypcie, oprócz opisu otrzymanych wyników oznaczeń biochemicznych i badań agregometrycznych, przedstawiono ich potencjalny wpływ na funkcję płytek krwi. Podjęto

również próbę wyjaśnienia związku osoczowych i płytkowych zaburzeń biotransformacji tlenku azotu, na wzrost ryzyka wystąpienia udaru niedokrwiennego mózgu.

Przedstawiony cykl publikacji zwraca uwagę na powiązanie pomiędzy zmniejszoną biodostępnością tlenku azotu w płytkach krwi, a ich funkcją badaną za pomocą agregometrii. Identyfikacja mechanizmu odpowiedzialnego za wzrost ryzyka wystąpienia udaru niedokrwiennego mózgu zależnego od biotransformacji tlenku azotu w płytkach krwi może w przyszłości umożliwić wyselekcjonowanie grupy chorych, która odniesie korzyści z prewencji pierwotnej lekami przeciwpłytkowymi. Ponadto uzyskane wyniki mogą być przesłanką do kontynuacji badań nad lekami zwiększającymi biodostępność tlenku azotu w płytkach krwi celem zmniejszenia ryzyka niedokrwiennych zdarzeń sercowo-naczyniowych.

7. SUMMARY

Ischemic stroke is among the most important cardiovascular disorders, due to its incidence and mortality. The antiplatelet treatment is a typical therapeutic strategy administered in the secondary prevention of noncardioembolic ischemic stroke (that is not connected with an ischemia originating from the cardiogenic thrombus). However, primary prevention with antiplatelet drugs is ineffective in a non-selected group of patients with elevated cardiovascular risk. Since the disturbances in the plasma nitric oxide bioavailability and the levels of asymmetric dimethylarginine (ADMA, a competitive inhibitor of the nitric oxide synthase) are associated with development of endothelial dysfunction and with increase of cardio-vascular risk, the main scope of this dissertation is an analysis of the influence of nitric oxide biotransformation on platelet function in patients with acute phase of noncardioembolic ischemic stroke (observation from <24 hours to the 7th day following the stroke onset).

As it has been documented in numerous studies, nitric oxide (NO) deficiency is the main reason underlying endothelial vasodilatory dysfunction. Nevertheless, the NO can also regulate the platelet function. The role of nitric oxide in the pathogenesis of ischemic stroke has been summarized in the review of literature, which is a part of this dissertation. Subsequently, the influence of plasma and platelet nitric oxide biotransformation metabolites on platelet aggregation in acute phase of non-cardioembolic stroke has been studied and the results are presented in the original article.

Stably elevated platelet levels of the nitric oxide synthase (NOS) inhibitors in patients with acute phase of ischemic stroke, higher concentration of platelet L-Arginine and simultaneously lower level of plasma L-Arginine on the 1st and 3rd day was also observed in the study group in comparison to the control group. Arachidonic acid-dependent platelet aggregation was negatively correlated with the platelet nitric oxide bioavailability ($\frac{L-Arginine\ (platelets)}{ADMA\ (platelets)}$) on the 3rd and 7th day of observation.

This dissertation emphasizes the links between lower platelet nitric oxide (PDNO) bioavailability and prothrombotic activation of thrombocytes, assessed by aggregometric tests. Identifying the mechanism responsible for higher risk of ischemic stroke in subjects with the PDNO biotransformation disturbances could help to select patients which would benefit from antiplatelet treatment in primary prevention of cardio-vascular diseases. Moreover, presented results could provide a rationale for designing drugs aiming at increasing the PDNO bioavailability in order to limit the risk for ischemic cardiovascular events.

8. ZGODA KOMISJI BIOETYCZNEJ

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 145/2020

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 133/XV R/2017 z dnia 21 grudnia 2017 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

prof. dr hab. Jacek Daroszewski (choroby wewnętrzne, endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód, prof. nadzw. (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
prof. dr hab. Leszek Szenborn, (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

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

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

„Ocena biotransfuzji tlenku azotu i funkcji płytek krwi u pacjentów w ostrej fazie udaru niedokrwiennego mózgu"

zgłoszonym przez **lek. Macieja Marka Bladowskiego** uczestnika studiów doktoranckich w Katedrze i Klinice Chorób Wewnętrznych, Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Katedrze i Klinice Chorób Wewnętrznych, Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej Uniwersytetu Medycznego we Wrocławiu pod nadzorem dr hab. Adriana Doroszko, prof. nadzw. **pod warunkiem zachowania anonimowości uzyskanych danych.**

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

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

Opinia powyższa dotyczy: projektu badawczego będącego podstawą rozprawy doktorskiej

Wrocław, dnia 30 marca 2020 r.

BW

KOMISJA BIOETYCZNA
przy Uniwersytecie Medycznym
im. Piastów Śląskich we Wrocławiu
ul. J. Mikulicza-Radeckiego 4a, 50-367 Wrocław
tel. 71 784 10 14, 71 784 17 10
e-mail: bioetyka@umw.edu.pl
http://www.umw.edu.pl/bioetyka

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