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Poszukiwanie mechanizmów regulacji biodostępności tlenku azotu w erytrocytach i ich wpływu na funkcję śródbłonka naczyniowego u osób z cukrzycą typu 2

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Spis treści

1. LISTA PUBLIKACJI WCHODZĄCYCH W SKŁAD CYKLU PRAC:

1.1. **Damian Gajecki,** Jakub Gawryś, Ewa Szahidewicz-Krupska, Adrian Doroszko.

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2. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY

Cukrzyca typu 2 nierozerwalnie związana jest z co najmniej wysokim ryzykiem sercowo-naczyniowym. Należy zauważyć, że zapadalność na cukrzycę przyjęła już w ubiegłym wieku rangę epidemii, a choroby sercowo-naczyniowe stanowią obecnie kluczową przyczynę śmiertelności osób w krajach rozwiniętych. Z tego względu poznawanie patomechanizmów inicjujących powikłania cukrzycy na poziomie molekularnym, we wczesnych stadiach jej zaawansowania, jest niezmierne istotne ze względu na możliwość wdrożenia działań profilaktycznych, niwelujących zarówno śmiertelność sercowo-naczyniową, jak i opóźniających wystąpienie klinicznie jawnych powikłań, stanowiących istotną przyczynę niepełnosprawności.

Dysfunkcja śródbłonka naczyniowego od dziesięcioleci pozostaje uznawana za jeden z najistotniejszych etapów inicjujących oraz propagujących aterogenezę we wczesnych jej stadiach, jeszcze przed pojawieniem się nieodwracalnych zmian strukturalnych w ścianie naczynia, a jej rozwój w konsekwencji zespołu metabolicznego jest zjawiskiem powszechnie wiadomym. Tym niemniej mechanizmy prowadzące do zaburzenia funkcji śródbłonka, w tym prowadzące do spadku biodostępności tlenku azotu (II) (NO), pozostają nadal przedmiotem badań i dyskusji. Głównym źródłem tlenku azotu w naczyniach jest syntaza tlenku azotu (NOS). W układzie sercowo-naczyniowym, występuje ona w izoformie śródbłonkowej (eNOS) oraz indukowalnej (iNOS) i odpowiada za przekształcenie L-argininy w tlenek azotu oraz L-cytrulinę. Stres oksydacyjny, powstający m.in. wskutek reakcji zapalnej i wtórnie nasilonej peroksydacji lipidów, stanowi istotny element patogenezy molekularnej powikłań cukrzycy i może prowadzić do nasilonej degradacji tlenku azotu (NO). Towarzysząca reakcja zapalna prowadzi do zakłócenia optymalnego przebiegu fosforylacji oksydacyjnej w mitochondriach i utleniania cząsteczek LDL do oksydowanych LDL (oxLDL). Rozprzężenie łańcucha oddechowego (obejmujące m.in. zmianę funkcji katalitycznej oksydaz ksantynowej (XOD) i NADPH (NOX)), poza destabilizacją metabolizmu energetycznego komórek śródbłonka, skutkuje także stresem fosforylacyjnym. Prowadzi on do modyfikacji post-translacyjnych wielu białek enzymatycznych i strukturalnych (fosforylacja), wskutek czego zmianie ulega ich funkcja (katalityczna, strukturalna lub czynnościowa) bądź podatność na proteolizę. Leży to u podstawy patogenezy wielu schorzeń układu sercowo-naczyniowego na poziomie molekularnym, jak choćby dysfunkcji skurczowej mięśnia sercowego w odpowiedzi na zmiany tkankowego potencjału redox w przebiegu uszkodzenia niedokrwienno-reperfuzyjnego lub hipoksji-reoksygenacji. OxLDL są z kolei bezpośrednim źródłem aniono-

rodników ponadtlenkowych, nasilających dalsze zmiatanie tlenku azotu, z następczą syntezą nadtlenoazotynów ($ONOO^-$), powodując z jednej strony spadek jego biodostępności w śródbłonku i dysfunkcję wazodylatacyjną, z drugiej zaś eskaluje stres nitrozacyjny. Skutkuje to modyfikacjami post-translacyjnymi wielu białek (nitrowanie i S-nitrozylacja), prowadząc w efekcie do dalszego poważnego zachwiania homeostazy układu sercowo-naczyniowego. Co istotne, nadtlenoazotyny aktywują dodatkowo jądrowy czynnik transkrypcyjny κB (NF-κB), promując w efekcie dalszą ekspresję białek związanych z reakcją zapalną, w tym także indukowalnej syntazy tlenku azotu (iNOS), która charakteryzuje się szybszą kinetyką katalizy, co wiąże się z dalszym wzrostem wytwarzania reaktywnych form tlenu i promocją stresu nitrozacyjnego. Skutkiem tego jest utlenianie tetrahydrobiopteryny (kofaktora dla syntazy tlenku azotu) oraz czynnościowy niedobór L-argininy prowadzące w efekcie do „rozprzęgania" podjednostek NOS. Kaskada opisanych zdarzeń przekształca zatem najważniejszy czynnik wazodylatacyjny w toksyczne pochodne, które nie pełnią funkcji regulatora przepływu mikronaczyniowego, a jednocześnie uszkadzają śródbłonek i nasilają powikłania naczyniowe.

Co istotne, zwiększona synteza kompetycyjnych inhibitorów syntazy tlenku azotu, może także prowadzić do wystąpienia dysfunkcji śródbłonka i promować rozwój powikłań sercowo-naczyniowych. Wiadomym jest, że do najistotniejszych inhibitorów NOS zaliczamy asymetryczną dimetyloargininę (ADMA), zaś współtworzony z nią „symetryczny" enancjomer SDMA (symetryczna dimetyloarginina) cechuje się znacznie niższą zdolnością inhibicji eNOS. Cząsteczki te powstają między innymi wskutek nasilonej proteolizy białek bogatych w metylowe pochodne argininy (m.in. histonów) w reakcji katalizowanej przez metylotransferazę argininową (PRMT). Taki mechanizm został wykazany jako sprawczy w zakresie rozwoju dysfunkcji śródbłonka u osób ze schorzeniami limfo- i mieloproliferacyjnymi, gdzie nasilona liza komórek jest źródłem zarówno kwasu moczowego, jak i ADMA. Wpływ na wewnątrzkomórkowe stężenie ADMA, SDMA oraz L-argininy mają również białka transportowe błony komórkowej, które regulują gradient ich stężeń pomiędzy kompartmentami separowanymi przez błonę komórkową. Wśród nich najistotniejsze z perspektywy omawianych zjawisk są kationowe transportery aminokwasów (CATs). Poprzez regulowanie przezbłonowych różnic stężeń aminokwasów, wpływają one na procesy metaboliczne przebiegające z ich wykorzystaniem, a nasilenie ekspresji białek transportowych w błonie komórkowej zmienia się w odpowiedzi na zapotrzebowanie na poszczególne aminokwasy.

Badania dotyczące osi biotransformacji tlenku azotu przez wiele lat skupione były niemal wyłącznie na kompartmencie osoczowym, który pozostaje w bezpośredniej łączności z biał-

kami efektorowymi komórek śródbłonka i mięśni gładkich ściany naczyniowej. Niewiele doniesień naukowych traktuje natomiast o roli erytrocytów w metabolizmie tlenku azotu. Należy zauważyć, że erytrocyty stanowią około 40% objętości krwi krążącej i są najliczniejszymi elementami morfotycznymi. Z tego powodu mogą one być niedocenianym, aczkolwiek niepomijalnym elementem omawianych procesów biochemicznych. Przez wiele lat uważane były one za bezjądrzaste komórki o zmniejszonym metabolizmie, którym atrybuowano rolę wyłącznie transportera gazów oddechowych. Doniesienia ostatnich lat znacząco zmieniają ten paradygmat myślenia wskazując, że ich rola w utrzymaniu homeostazy naczyniowej jest o wiele bardziej złożona. Dzięki temu, że otacza je półprzepuszczalna błona komórkowa mogą stanowić one środowisko procesów (pato)fizjologicznych, podobnych do tych zachodzących w osoczu, podlegającym jednak większej regulacji. Tworzą one pośrednio także układ buforowy i transportujący wiele biologicznie aktywnych mikrocząsteczek, w tym L-argininy. Wprawdzie istnieją już doniesienia potwierdzające ich znaczenie w parakrynnej regulacji funkcji śródbłonka, jednak ich rola w aspekcie regulacji biodostępności tlenku azotu na poziomie interakcji ze śródbłonkiem w warunkach (pato)fizjologicznych pozostaje w dużej mierze nieodkryta.

Z powodów wspomnianych powyżej, zaburzenia metabolizmu tlenku azotu w erytrocytach spowodowane cukrzycą mogą być niezmiernie ciekawym zagadnieniem badawczym z molekularnego punktu widzenia. Nasilona zaawansowana glikacja białek, stres oksydacyjny i reakcja zapalna z towarzyszącym zwiększonym obrotem komórek, mogą w istotny sposób wpływać na wyżej wskazane procesy regulacji homeostazy układu sercowo-naczyniowego. Ich znajomość ma ogromne znacznie w ujęciu praktycznym, gdyż ingerencja w ich przebieg może w znaczący sposób przyczynić się do modulacji tempa rozwoju powikłań, a zatem i ryzyka sercowo-naczyniowego w przebiegu schorzeń stanowiących, z epidemiologicznego punktu widzenia, jedno z największych wyzwań współczesnej medycyny. Jak powszechnie wiadomo powikłania mikronaczyniowe w cukrzycy stanowią istotny problem kliniczny. Warto zauważyć, że to właśnie obszar naczyń małego kalibru, gdzie wazoreaktywność jest kluczowa dla perfuzji, stanowi najważniejsze miejsce oddziaływania tlenku azotu. Z tego powodu zaburzenia relaksacji naczyniowej, szczególnie u pacjentów z cukrzycą, nierozerwalnie wiążą się ze spadkiem przepływu i w konsekwencji redukcją dostępności tlenu w dystalnych obszarach tkankowych, prowadząc do wzrostu ryzyka wystąpienia lokalnej kwasicy mleczanowej. Podkreśla to, jak bardzo istotnym celem terapeutycznym mogą być erytrocyty patrząc z perspektywy możliwości zapobiegania i leczenia powikłań mikronaczyniowych cukrzycy. Z

tej przyczyny poznanie patomechanizmów regulacji metabolizmu tlenku azotu w tych właśnie elementach morfotycznych krwi wydaje się być w pełni zasadne.

Dlatego też nadrzędnym celem niniejszej pracy jest poszukiwanie osi biotranformacji tlenku azotu i ocena poszczególnych jej elementów wewnątrz erytrocytów u pacjentów, którzy rozwinęli cukrzycę typu 2, ale jeszcze bez istotnych klinicznie powikłań mikro- i makronaczyniowych. Dodatkowym celem jest powiazanie powyższych procesów z wybranymi aspektami funkcji śródbłonka naczyniowego oraz przedstawienie roli erytrocytów w regulacji biodostępności tlenku azotu. Ponadto w przedstawionej dysertacji omówiono funkcję krwinek czerwonych jako regulatorów przepływu w mikrokrążeniu, zwłaszcza w obszarach zmniejszonej dostępności tlenu, ze szczególnym uwzględnieniem roli tlenku azotu (NO).

Dotychczas niewiele prac opisywało znaczenie metabolizmu tlenku azotu w erytrocytach. Wiedza dotycząca mechanizmów jego regulacji jest niepełna, brak jest również publikacji systematyzującej dotychczas przeprowadzone badania w tym zakresie. Z tego powodu w pracy przeglądowej zatytułowanej „*Role of Erythrocytes in Nitric Oxide Metabolism and Paracrine Regulation of Endothelial Function",* dokonałem podsumowania aktualnej wiedzy dotyczącej metabolizmu tlenku azotu w krwinkach czerwonych oraz ich znaczenia w regulacji przepływu naczyniowego.

Poprzednie publikacje potwierdziły, że erytrocyty zdolne są do transportu aminokwasów które z łatwością uwalniane są w miejscach zwiększonego ich zapotrzebowania. Udokumentowano także wewnątrzkomórkową ekspresję aktywnej śródbłonkowej izoformy syntazy tlenku azotu (eNOS) oraz występowanie kationowych transporterów aminokwasów w błonie komórkowej. W krwinkach czerwonych potwierdzono obecność CAT1, CAT2a oraz CAT2b, stwierdzając jednocześnie, że CAT1 wykazuje najwyższe powinowactwo do L-argininy.

Regulacja transportu przezbłonowego w erytrocytach odbywa się między innymi na poziomie po-transkrypcyjnym poprzez interakcje $mRNA_{CAT}$ z microRNA (miR-122) będącym negatywnym regulatorem translacji. Dodatkowo na ekspresję CATs wpływają także zmiany potencjału błony komórkowej oraz stres oksydacyjny. CATs w krwinkach czerwonych wykazują również powinowactwo do ADMA i SDMA, co wskazuje na ich wielokierunkowy wpływ na metabolizm tlenku azotu oraz definiuje je jako potencjalnie interesujący cel terapeutyczny w leczeniu chorób układu sercowo-naczyniowego.

Badania wykazały, że z uwagi na niewielki metabolizm peptydów w dojrzałych erytrocytach, transportują one przede wszystkim ADMA pobrane z osocza. Jednakże potwierdzono,

że krwinki czerwone zdolne są do syntezy ADMA z białek nie-hemowych, z wykorzystaniem proteasomu oraz proteaz, co może mieć szczególne znaczenie, zważywszy na fakt, że liza jadra komórkowego (bogatego w histony) jest integralnym elementem procesu erytropoezy. Ponadto dowiedziono, że degradacja białek i związana z tym zwiększona synteza ADMA, nasilana jest przez aktywację proteasomu w wyniku stresu oksydacyjnego. Potwierdzono także, że erytrocyty wykazują ekspresję dimetyloarginino-dimetyloamino-hydrolazy (DDAH), która jest zdolna do wewnątrzerytrocytarnej degradacji ADMA do dimetyloaminy i L-cytruliny, wpływając tym samym na relację inhibitor kompetycyjny-substrat dla NOS.

L-arginina trafiająca do wnętrza erytrocytu stanowi substrat dla jednego z dwóch konkurujących szlaków przemian. Jest ona przekształcana przez NOS do tlenku azotu lub przez arginazę do L-ornityny i mocznika, bowiem ekspresję białek katalitycznych cyklu mocznikowego wykazano także w erytrocytach. Jak już wspomniano wcześniej, w wyniku stresu oksydacyjnego dochodzi do „rozprzęgania" syntazy tlenku azotu oraz wzrostu stężenia nadtlenoazotynów ($ONOO^-$). Znaczenie tego procesu u pacjentów z cukrzycą zostało udowodnione przez inkubację pobranych od nich erytrocytów ze związkami redukującymi $ONOO^-$, co skutkowało przywróceniem upośledzonej wcześniej funkcji śródbłonka naczyniowego. Dodatkowo, oddziaływanie nadtlenoazotynami na erytrocyty pobrane od zdrowych osób wiązało się ze wzrostem aktywności arginazy i związanym z tym prawdopodobieństwem wystąpienia wazodylatacyjnej dysfunkcji śródbłonka. W efekcie - mimo, że erytrocyty posiadają sprawne systemy antyoksydacyjne - stają się one niewystarczające u pacjentów z chorobami układu sercowo-naczyniowego związanymi z nadmierną produkcją reaktywnych form tlenu. Wykazano, że zmniejszenie aktywności arginazy (z równoczesną aktywacją szlaku eNOS) ma znaczenie kardioprotekcyjne w reperfuzyjnym uszkodzeniu mięśnia sercowego, a także wykazuje pozytywny efekt u pacjentów z nadciśnieniem tętniczym oraz miażdżycą naczyń. Powszechnie uznany ochronny efekt żeńskich hormonów płciowych może także być częściowo zależny od tlenku azotu, jako że dowiedziono zwiększenia jego syntezy pod wpływem estrogenów.

Tlenek azotu powstający w erytrocytach w przeciągu milisekund reaguje z utlenowaną formą hemoglobiny, tworząc methemoglobinę oraz azotany. Doniesienia ostatnich lat potwierdzają jednak, że hemoglobina, poza możliwością zmiatania tlenku azotu, wykazuje także zdolność do jego wiązania i tworzenia stabilnych związków przejściowych zawierających inkorporowany azot. Są one następnie uwalniane w obszarach hipoksji lub zmniejszonego przepływu krwi. Proces ten zależny jest od konformacji allosterycznej hemoglobiny, która reguluje jej powinowactwo do tlenku azotu. W krążeniu płucnym hemoglobina przyłącza 2-3

atomy tlenu i zmienia swoją konformację ze struktury T do R. Umożliwia to szybsze przyłączenie NO i uformowanie czasowego połączenia Hb(II)NO. NO jest następnie przekazywany na resztę cysteinową w pozycji 93, czego skutkiem jest wytworzenie S-nitrozohemoglobiny, która wykazuje większą stabilność. Powinowactwo tioli do tlenku azotu zmienia się wraz ze zmianą konformacji hemoglobiny, do którego dochodzi w mikrokrążeniu tkankowym. W obszarze o zmniejszonym ciśnieniu parcjalnym tlenu dochodzi do zmiany struktury cząsteczki - przejścia w formę T oraz uwolnienia tlenku azotu do osocza.

Ponadto wykazano, że hemoglobina może dostarczać tlenku azotu także poprzez swoje właściwości redukcyjne. W obszarze hipoksji methemoglobina zdolna jest do przekształcania azotanów (III) i odtwarzania puli tlenku azotu. Biorąc pod uwagę fakt, że erytrocyty są największym wewnątrznaczyniowym magazynem azotanów (III), mechanizm ich redukcji może wykazywać większe znaczenie niż przemiany nitrozotioli.

Tlenek azotu może dodatkowo powstawać dzięki erytrocytarnej oksydoreduktazie ksantynowej (XOR), dla której substratem są azotany. Wykazano, że jej znaczenie pozostaje niewielkie w warunkach fizjologicznych, natomiast ilość syntezowanego tym szlakiem tlenku azotu istotnie wzrasta w przypadku kwasicy. Poza spadkiem prężności tlenu innymi stymulantami wywołującymi wydzielanie tlenku azotu z erytrocytów do mikrokrążenia są siły ścinające działające na erytrocyty podczas przepływania przez zwężone naczynia krwionośne. Aktywacja mechanoreceptorów skutkuje wydzielaniem NO oraz aktywacją syntazy tlenku azotu. Potwierdzono, że tlenek azotu poprzez oddziaływanie na cytoszkielet krwinki czerwonej reguluje jej zdolność do odkształcania, co zapobiega rozpadowi erytrocytów podczas ich przepływania przez zwężone naczynia krwionośne.

Mechanizm wydzielania tlenku azotu przez błonę erytrocytarną nie jest do końca poznany. Niektóre prace wskazują, że krwinki czerwone posiadają domeny błonowe, które ułatwiają jego transport do osocza. Zawierają one białko prążka 3 przenoszące aniony, które reaguje z hemoglobiną podczas zmiany jej konformacji i ułatwia przekazanie tlenku azotu na błonę komórkową. Dodatkowo w tym obszarze zakotwiczone są białka ułatwiające przezbłonowy transport NO, a strefa ta „otoczona" jest przez methemoglobinę, która zapobiega jego utlenieniu. Inne badania donoszą, że tlenek azotu transportowany jest do osocza w formie swoich stabilnych metabolitów. Wskazuje się tutaj na S-nitrozoglutation, S-nitrozo-L-cysteinę lub nitrozylowaną pochodną izomerazy disulfidowej białek.

Erytrocyty wpływają także na funkcję śródbłonka poprzez wydzielanie do osocza ATP, które reaguję z receptorami błonowymi (P2Y) komórek mięśni gładkich, prowadząc do ich

relaksacji. Jest to możliwe dzięki błonowej paneksynie 1, która wydziela ATP w odpowiedzi na hipoksję, siły ścinające czy depolaryzację, poprzez formowanie kanałów błonowych. Wykazano także, że tikagrelor stosowany jako lek przeciwpłytkowy, odpowiada za zwiększenie wydzielania ATP z erytrocytów, co może wyjaśniać jego skuteczniejsze działanie w porównaniu do innych leków z tej samej grupy, będących pochodnymi tienopirydyny. Ponadto w badaniach eksperymentalnych wykazano wpływ erytrocytarnego tlenku azotu na regulację systemowego ciśnienia tętniczego oraz potwierdzono, że zaburzenia reologii erytrocytarnej mogą stanowić przyczynę dławicy mikronaczyniowej.

Praca zatytułowana *"A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus"* jest pierwszym oryginalnym doniesieniem, w którym dokonano kompleksowej oceny funkcji śródbłonka naczyniowego i metabolitów szlaku przemian tlenku azotu w erytrocytach oraz osoczu osób z cukrzycą typu 2, we wczesnej fazie choroby. Do badania zostało wstępnie zakwalifikowanych 100 osób. Spośród nich wyłoniono ostatecznie 35-osobową grupę badaną, którą stanowiły osoby w wieku 35-80 lat ze świeżo rozpoznaną cukrzycą typu 2, u których nie występowały powikłania naczyniowe. Grupę kontrolną utworzyło 45 zdrowych ochotników, którzy zostali dopasowani demograficznie do grupy badanej, po wcześniejszym wykluczeniu u nich zaburzeń metabolizmu glukozy.

W obu grupach przeprowadzono oznaczenia parametrów osi biotransformacji tlenku azotu w osoczu oraz w izolowanych erytrocytach. Wyliczono stosunki stężeń poszczególnych metabolitów względem siebie oraz pomiędzy kompartymentem osoczowym i erytrocytarnym, co umożliwiło dokładniejszą ocenę kierunku przemian metabolicznych oraz transportu przezbłonowego. Otrzymane wyniki porównano z parametrami odpowiedzi naczyniowej na zastosowanie bodźca termicznego i niedokrwiennego podczas badan śródbłonka metodami Laser Doppler i EndoPAT.

Poza niższym stężeniem L-cytruliny nie stwierdzono innych istotnych statystycznie różnic w stężeniach metabolitów w kompartmencie erytrocytarnym między grupą badaną, a kontrolną. Wskazuje to, że erytrocyty pozostają buforem, który zachowuje względnie prawidłowy metabolizm tlenku azotu w początkowej fazie cukrzycy typu 2. Globalna biodostępność tlenku azotu została porównana między kompartmentem osoczowym i erytrocytarnym, przy użyciu stosunku L-arginina/ADMA. Wykazano istotnie większą biodostępność NO w kompartymencie erytrocytarnym, co dodatkowo podkreśla istotność tych komórek w utrzymaniu funkcji śródbłonka naczyniowego u osób z cukrzycą.

Istotne różnice w stężeniach metabolitów osi biotransformacji NO zostały natomiast stwierdzone w kompartmencie osoczowym. Wykazano znacząco niższe stężenie L-argininy, która włączana jest do szlaku przemian arginazy, na co wskazuje zwiększony stosunek L-ornityny do L-argininy w osoczu. Jak już wcześniej wspomniano ma to związek ze zwiększonym stresem oksydacyjnym oraz rozprzęganiem NOS, co zostało już uprzednio potwierdzone, jako jeden z patomechanizmów prowadzących do dysfunkcji śródbłonka u pacjentów z cukrzycą. Porównując stężenia argininy pomiędzy kompartmentami, potwierdzono także nasilony transport L-argininy do wnętrza erytrocytów, co stanowić może mechanizm adaptacyjny, mający utrzymać odpowiedni poziom syntezy tlenku azotu, pomimo nasilonego stresu oksydacyjnego.

Co więcej, stwierdzono znacznie obniżony osoczowy stosunek argininy do ADMA w grupie badanej, co wskazuje na zmniejszoną aktywność NOS. Biorąc pod uwagę równoczesne porównywalne stężenia ADMA oraz podwyższone stężenie dimetyloargininy (DMA-produkt rozpadu ADMA), należy stwierdzić, że w grupie badanej nasilony jest proces osoczowej przemiany metyloamin. Oznaczenia metabolitów osi biotransformacji tlenku azotu nie wskazywały pośrednio na upośledzoną funkcję DDAH w grupie badanej, co można wyjaśnić wczesnym etapem cukrzycy. Pozostaje to w zgodzie z badaniami, w których stwierdzono, że spadek aktywności DDAH, a co za tym idzie wzrost stężenia ADMA u cukrzyków, koreluje z powikłaniami makro-naczyniowymi, których nie stwierdzaliśmy w naszej grupie badanej.

Ocena funkcji śródbłonka naczyniowego u osób z cukrzycą o krótkim czasie trwania wykazała spadek reaktywności mikrokrążenia w odpowiedzi na bodziec cieplny, czego nie zaobserwowano w odpowiedzi na niedokrwienie. Różnica ta spowodowana jest odmiennymi szlakami przekaźnikowymi aktywowanymi w zależności od rodzaju działającego bodźca. Potwierdzono, że odpowiedź termiczna jest w większym stopniu zależna od tlenku azotu, dlatego metody oparte o ten mechanizm szybciej wykazują nieprawidłowości w funkcji śródbłonka. Zwiększony wskaźnik wzmocnienia (AI – *augmentation index*) w tej grupie potwierdza natomiast zwiększoną sztywność naczyń, która pojawia się u osób z cukrzycą już w początkowej fazie choroby.

Podsumowując, wykazano znaczne różnice w stężeniach metabolitów osi przemian tlenku azotu, dotyczące głownie kompartmentu osoczowego. Jednocześnie metabolizm tlenku azotu pozostaje w dużej mierze nienaruszony w erytrocytach pacjentów z cukrzycą typu 2. Co za tym idzie, biodostępność NO jest istotnie wyższa wewnątrz krwinek czerwonych. Badania przedstawione w niniejszej rozprawie mogą translacyjnie nieść za sobą dość istotne implika-

cje kliniczne, bowiem poznanie dokładnych molekularnych aspektów przemian tlenku azotu w erytrocytach może pozwolić na opracowanie inhibitorów arginazy, leków przeciwdziałających rozprzęganiu NOS lub redukujących stres oksydacyjny. Dodatkowo zastosowanie przepływomierza laserowego wraz z oceną odpowiedzi na bodziec termiczny może być istotnym narzędziem w ocenie wczesnych powikłań cukrzycy, pozwalając na pełniejszą stratyfikację ryzyka i formułowanie racjonalnych, spersonalizowanych przesłanek do eskalacji terapii w grupie osób bez jawnych klinicznie powikłań narządowych, lecz z dużym ryzykiem ich szybkiego wystąpienia.

3. PRACA NR 1:

Role of Erythrocytes in Nitric Oxide Metabolism and Paracrine Regulation of Endothelial Function

antioxidants

Review

Role of Erythrocytes in Nitric Oxide Metabolism and Paracrine Regulation of Endothelial Function

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Abstract: Emerging studies provide new data shedding some light on the complex and pivotal role of red blood cells (RBCs) in nitric oxide (NO) metabolism and paracrine regulation of endothelial function. NO is involved in the regulation of vasodilatation, platelet aggregation, inflammation, hypoxic adaptation, and oxidative stress. Even though tremendous knowledge about NO metabolism has been collected, the exact RBCs' status still requires evaluation. This paper summarizes the actual knowledge regarding the role of erythrocytes as a mobile depot of amino acids necessary for NO biotransformation. Moreover, the complex regulation of RBCs' translocases is presented with a particular focus on cationic amino acid transporters (CATs) responsible for the NO substrates and derivatives transport. The main part demonstrates the intraerythrocytic metabolism of L-arginine with its regulation by reactive oxygen species and arginase activity. Additionally, the process of nitrite and nitrate turnover was demonstrated to be another stable source of NO, with its reduction by xanthine oxidoreductase or hemoglobin. Additional function of hemoglobin in NO synthesis and its subsequent stabilization in steady intermediates is also discussed. Furthermore, RBCs regulate the vascular tone by releasing ATP, inducing smooth muscle cell relaxation, and decreasing platelet aggregation. Erythrocytes and intraerythrocytic NO metabolism are also responsible for the maintenance of normotension. Hence, RBCs became a promising new therapeutic target in restoring NO homeostasis in cardiovascular disorders.

Keywords: red blood cells; nitric oxide; nitrates; nitrites; hemoglobin; endothelium; nitrosylation

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1. Human Erythrocytes as the Storage Pool of Amino Acids

Erythrocytes (red blood cells, RBCs) are the most numerous formed elements in human blood. Over the last several decades they have been considered to be responsible for gas exchange, as they transport oxygen and partly carbon dioxide. Even though RBCs are enucleated and lack numerous organelles, they consist of up to 2650 proteins, with at least 41 membrane transporters [1]. Hence, recently, a more complex function of RBCs has been postulated. It was demonstrated that RBCs play an important role in the transport of amino acids. In a vast majority of cases, amino acids' concentrations in RBCs and plasma are relatively equal. Nevertheless, cationic amino acids, including L-citrulline, L-lysine, L-histidine and L-arginine (L-Arg), dominate in plasma, whereas L-ornithine—in the erythrocyte compartment [2].

Studies by Thorn et al. [3] confirmed that RBCs are capable of exchanging up to 15–17% of the total erythrocyte pool of amino acids with plasma, without subsequent alteration of the cellular osmotic balance. Moreover, in some in vitro studies, extended incubation of RBCs in plasma did not result in further intercompartmental exchange. It suggests that RBCs are the storage that could easily supply amino acids to deficient tissues as they circulate through capillaries and act as an inter-organ transporter.

2. RBC—The Importance of Transmembrane Translocases

So far, seven different amino acid transmembrane transport systems have been identified in erythrocytes. Four of them are based on facilitated diffusion (y+, y+L, L, T), and three constitute secondary active transporters (ASC, Gly, N) [3]. Interestingly, none of these systems have been found to transport threonine or methionine. The first characterized in RBCs was the y+L transport system, which binds cationic and neutral amino acids (leucine, lysine) and exhibits Na^+-dependence. L transport is sodium-independent and transfers neutral amino acids, while the T transport system binds to tryptophan. Since not all of them are pivotal for regulating NO metabolism, in this review, only the main ones are discussed. As far as the literature is concerned, the most-studied systems in human erythrocytes are cationic amino acid transporters (CATs), presenting high structural homology to the classical amino acid transport system y+. CATs maintain the Na^+- and pH-independent transport of cationic amino acids (CAAs), determined by transmembrane amino acids' gradient. They are sensitive to trans-stimulation and saturable with amino acids plasma concentration. CATs are relatively selective for CAAs, including L-arginine, L-lysine, and L-ornithine. The family of CATs includes CAT1, CAT2a, CAT 2b, CAT3, and CAT4, where the first three have been demonstrated to transport CAAs through the erythrocyte membrane. CAT1 is characterized by the highest quantitative L-arginine transport [4]. CATs regulate the transmembrane CAAs arrangement, thereby controlling the intracellular metabolic processes. It has been proven that CATs undergo adaptive regulation based on the molecules' availability. As the depletion of amino acids occurs, the protein membrane density increases in order to supply the CAAs more efficiently (adaptive de-repression). On the contrary, along with CAAs abundance, CATs express adaptive repression [5–7]. So far, this mechanism has been demonstrated in numerous human and animal cells. However, its exact role in erythrocyte transmembrane transport requires further confirmation.

Noteworthy, microRNAs (miRNAs) seem to be another CAT-1-regulating factor. The small noncoding RNAs consist of 21–25 nucleotides and act at the post-transcriptional level as negative regulators of mRNA expression. miRNAs bind to the target mRNA and cause translational repression or cleave mRNA sequences. Bhattacharyya et al. [8] reported that CAT-1 mRNA is regulated with miR-122. It is consistent with a recent study, which has demonstrated higher expression of miR-122 with subsequent decreased expression of CAT-1 among patients with hypertension and endothelial dysfunction [9]. Additionally, miR-122 level was proven to be positively correlated with markers of myocardial damage [10]. Furthermore, the use of miR-122 inhibitors seems to be a promising therapeutic target, as they reverse endothelial dysfunction [11]. Interestingly, miRNA-dependent CAT-1 down-regulation is revised in case of amino acid starvation [8,12]. Contrary, the activity of CAT-1 is increased in cell stress, however, the pathomechanism is poorly understood [8,13,14].

Additionally, transmembrane polarization modulates CAT activity—hyperpolarization induces L-arginine cellular influx and increases erythrocyte L-arginine concentration.

3. Transport of the Selected Nitric Oxide Metabolic Pathway Intermediates between Erythrocytes and Plasma

The CATs-dependent concentration of L-Arginine regulates nitric oxide (NO) synthesis, as erythrocytes have been shown to express the two subtypes of nitric oxide synthase—endothelial and inducible (eNOS and iNOS, respectively) [15]. Furthermore, CATs also influence NO synthase by transporting L-arginine derivates, including asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). ADMA is a competitive inhibitor of nitric oxide synthase, and simultaneously with its enantiomer, is formed from methylarginine-rich proteins, such as histones during their degradation. Strobel et al. [4] pointed out that CAT1 manages intracellular ADMA influx at physiological concentrations. Although both molecules compete for CATs at physiological concentration, L-arginine is characterized by greater affinity to the translocases. Hence, the influx of L-Arg to RBCs is accompanied by simultaneous inhibition of ADMA and SDMA influx [4].

The literature is inconsistent in terms of generation and storage of ADMA in RBCs. Davids et al. [16] demonstrated that RBCs transport ADMA, which had been previously incorporated from plasma. ADMA concentration stays in equilibrium between the intra- and extracellular compartments and is rapidly interchangeable. Studies with protease and the proteasome inhibitors have proven that RBCs are able to produce ADMA by enzymatic proteolysis of methylated proteins by the 20S proteasome [17]. The 20S proteasome generates peptides consisting of seven to nine amino acids in length, which might be subsequently cleaved by proteases. Mature RBCs are characterized by minimal turnover of proteins, and oxidative damage persistently leads to proteasome activation and increased ADMA generation. Even though hemoglobin is the major protein source, some studies indicate the low degradation rate of hemoglobin due to lower susceptibility to enzymatic degradation [16,18]. Nevertheless, other proteins were proven to be the targets for 20S proteasome and to become a source of intra-RBCs ADMA.

No consensus has been made regarding the ADMA degradation pathway. David et al. [16] postulate that ADMA is subsequently transferred out of RBCs, since no degradation of ADMA in RBCs has been observed. As a result, dimethylarginine dimethylaminohydrolase (DDAH) may not be present in RBCs. Contrary to that, Yokoro et al. [19] confirmed that DDAH-1 and protein-arginine ethyl transferase (PMRT) are expressed in red blood cells. Similarly, Kang et al. [20] demonstrated the expression of DDAH and its activity in RBCs. Therefore, RBCs seem to transport free ADMA taken mostly from plasma. Nevertheless, the lysis of RBCs during oxidative stress makes RBCs the potential ADMA generators.

CAT-dependent L-Arg transport is complex and is also regulated by hormones. Progesterone was found to inhibit transmembrane transfer via both phosphorylated protein kinase Cα (PKCα) and extracellular signal-regulated kinases (ERK1/2). On the contrary, estrogens were proven to increase L-Arg influx by modulating the constitutive ERK 1/2 signaling pathways and display protective properties [21].

Other studies reported that thyroid hormones cause upregulation of CATs, as they participate in cardiovascular abnormalities observed in the course of thyroid disorders. Thyroxine or triiodothyronine activate the membrane αvβ3 integrin receptor and transduce the signal through the stimulation of Phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPKs), and the intracellular calcium-dependent signaling pathways. Finally, it leads to increased mRNA expression of L-arginine transporters [22]. Even though RBCs contain abundant ERK1/2, the mentioned processes were presented in the endothelium, and further studies are needed to prove their presence and importance in erythrocytes.

4. Intraerythrocytic Metabolism of L-Arg and Its Regulation

Once L-arginine is moved to the erythrocyte compartment, it might be metabolized by arginase 1 or nitric oxide synthase (NOS), leading to NO synthesis. The exact mechanism regulating the proportion of entrance to one of these two competing metabolic pathways is unknown. However, it was shown that there is a negative correlation between arginase activity and NO synthesis. Arginase is supposed to be an important NOS regulator.

Emerging data reveal that peroxynitrite ($ONOO^-$) might be a key player in modulating NO^- bioavailability, as it enhances arginase activity in red blood cells. Enhanced arginase activity causes inadequate substrate availability and leads to NOS uncoupling [23]. NOS loses its ability to convert L-arginine to L-citrulline. Nevertheless, NOS remains capable of transferring an electron from NADH and donating it to oxygen, leading to superoxide (O^{2-}) production. NO can react with superoxide, forming peroxynitrite [24]. $ONOO^-$ itself can also directly lead to NOS uncoupling by dissolving dimeric NOS conformation [25]. Excessive reactive oxygen species' (ROS) formation decreases NO bioavailability in different ways. First of all, ROS decrease NO production by an increase in arginase activity, leading to a lack of L-Arg, while NOS uncoupling reduces the reaction rate. Secondly, ROS enhance NO degradation, by a reaction with ROS, resulting in peroxynitrite. In line with these studies, the role of $ONOO^-$ in endothelial dysfunction in diabetes mellitus

patients was investigated. It was demonstrated that incubation of RBCs obtained from diabetic subjects with peroxynitrite scavenger (FeTTPS) completely reverses RBCs-induced endothelial dysfunction [26,27]. Additionally, RBCs from healthy subjects were treated with peroxynitrite, leading to increased arginase activity.

Even though RBCs possess antioxidative and redox systems able to maintain intracellular homeostasis and to prevent oxidative damage, these mechanisms may be insufficient in patients with cardiovascular diseases and extensive ROS production [28]. Peroxynitrate and arginase activity may have great importance in myocardial ischemia-reperfusion injury, since arginase inhibition might exert a cardioprotective effect [29]. Moreover, some studies show that arginase plays an important role in pathogenesis of endothelial dysfunction in hypertension, atherosclerosis, obesity, and in the course of ageing [24,30].

The exact mechanisms underlying the regulation of arginase with $ONOO^-$ in RBCs remain unclear. However, Rho-kinase-dependent increase of argininase expression by $ONOO^-$ seems to be essential [31,32]. Alternative modifications of arginase like S-nitrosylation, glycosylation, or phosphorylation may play an important role, but this hypothesis requires further investigation [33]. Kim et al. [34] proved that alternative splicing may also have an impact on arginase activity. Addition of 24 nucleotides in gene exon 3 cause incorporation of eight amino acids (Val–Thr–Gln–Asn–Phe–Leu–Ile–Leu) in a hydrophobic-polar-hydrophobic sequence, leading to structural changes and reducing enzymatic activity up to 50% [32,34].

Interestingly, estrogens were found to increase NO production in RBCs by inducing eNOS phosphorylation via interaction with estrogen receptors (ERs) and activation of the PI-3 kinase/AKT pathway. The distribution and function of ERs (estrogen receptor ERα and ERβ) are different in premenopausal women and men. In premenopausal women, ERα are mostly localized on the RBCs membrane, which is related with increased PI-3 kinase/AKT-dependent eNOS phosphorylation. On the contrary, in men, ERα are mainly identified in the cytoplasm. It might be indicative of the role of red blood cells in NO-mediated, sex-dependent differences in cardiovascular risk [35,36].

5. RBCs as a Source of NO. Mechanisms Underlying Homeostasis of Its Bioavailability

As NO is a highly reactive molecule with an intravascular half-life of two milliseconds [37], for a long time it was considered to exert only an autocrine, local effect. NO reacts extremely fast ($6–8 \times 10^8$ $M^{-1}s^{-1}$) with oxyhemoglobin (oxyHb), forming methemoglobin (metHb) and nitrate (NO_3^-)-metabolic end products [38]. The oxidation of NO to NO_3^- is protective against respiratory poisoning and nitrosative stress, although in pathological conditions, this mechanism reduces NO availability. For that reason, RBCs were initially considered to be NO scavengers, rather than a NO source. However, several studies revealed that RBCs are not only capable of producing NO but also able to store and transport NO to distal parts of the cardiovascular system (Figure 1).

Hemoglobin does not scavenge NO so easily, as it is surrounded with the cellular membrane, characterized by low permeability for endothelial NO [39]. Moreover, it was demonstrated that a pressure gradient created by the blood flow pushes RBCs to the center of the vascular lumen, forming a cell-free zone near the vascular wall [40]. In addition, it protects NO from scavenging by the cell-free hemoglobin released during hemolysis and makes downstream NO transport possible [39,41,42]. Furthermore, hemoglobin is a NO scavenger under normoxic conditions, but otherwise it manifests reductase activity [43].

Stamler et al. [44] revealed that NO is capable of binding to hemoglobin (Hb). However, this reaction depends on the Hb conformation and the magnitude of Hb oxygenation.

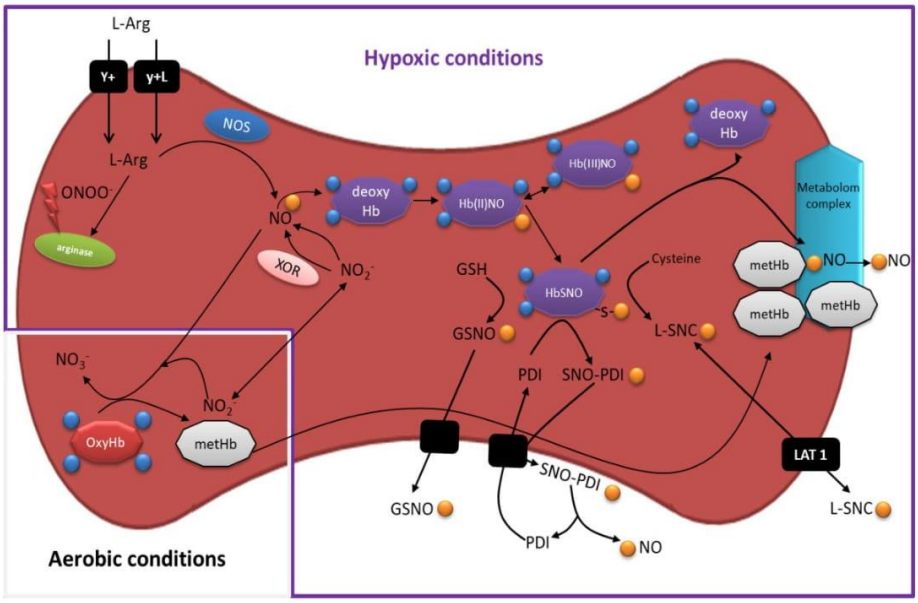

Figure 1. Nitric oxide metabolism in erythrocytes under aerobic and hypoxic conditions. In aerobic conditions, OxyHb reacts with NO, forming metHb and NO_3^--metabolic end products. However, in a hypoxic environment, RBCs become a NO producer. L-arginine is transported through the RBCs membrane by y+ and yL. Subsequently, it is incorporated into NOS or the arginase pathway, depending on the redox RBCs status. NO reacts with deoxyHb and further undergoes S-nitrosylation, forming HbSNO. NO is finally transmitted to GSH, PDI, or cysteine and, as a steady intermediate, leaves the RBCs. Alternatively, NO_2^- is reduced by XOR or deoxyHb and turned into another NO source. *Abbreviations: GSH: glutathione; GS-NO: S-nitrosoglutathione; HbSNO: S-nitroso hemoglobin; LAT 1: L-neutral amino acid transporter 1; L-SNC: S-nitroso-L-cysteine; SNO-PDI: S-nitrosylated protein disulphide isomerase; PDI: protein disulphide isomerase; XOR: xanthine oxidoreductase.*

Hemoglobin exists in two allosteric conformations, which regulate Hb's affinity to NO. The O_2-dependent Hb quaternary conformation change is caused by allosteric anionic effectors, such as chloride, 2,3-diphosphoglycerate (DPG), and inositol hexaphosphate (IHP) [45]. While RBCs pass through pulmonary arteries, Hb binds two to three oxygen molecules and favors the R structure (relaxed oxyhemoglobin) [44]. On the contrary, in capillaries, it releases oxygen and changes the conformation to the T structure (tension-deoxyhemoglobin). Stamler demonstrated that R-state Hb binds NO and forms iron nitrosyl Hb (Hb (II)NO). The highest effectiveness of Hb reduction was observed with 50% Hb saturation. The reaction of binding NO to Hb is 5.6 times faster in the R-state, while NO releasing in the T-quaternary conformation increases 9.6-fold [46].

As NO is poorly bound to Hb, it is subsequently bound by S-nitrosylation of hemoglobin to the cysteine-93 residue, forming S-nitrosohemoglobin (HbSNO). This process is dependent on Hb conformation. The thiol affinity for NO is high in the R structure and low in the T structure [47]. This hypothesis was supported by demonstrating different HbSNO concentrations in venous and arterial RBCs [47].

As it results from other studies, nitrite seems to be another stable source of NO. Plasma is abundant with nitrite originating from dietary intake, endogenous synthesis, and inhalation of NO from the polluted air [48]. Once nitrite (NO_2^-) reaches the intra-RBCs compartment, it might be oxidized or reduced depending on the dominant oxygenation level and presumably the redox state. In well-oxygenated arterial blood, nitrite (NO_2^-) reacts with oxygen, forming nitrate (NO_3^-). It was shown that under hypoxic conditions, nitrites are reduced by deoxyhemoglobin, leading to NO regeneration. Subsequently, NO reacts with deoxy-Hb once more, as well as with other molecules, or is alternatively released

to the plasma [49]. Concurrently, an alternative storage pool of NO becomes bioavailable, which might be easily mobilized when needed [50].

Contrary to the Stamler studies, Nagababu et al. [43] revealed that during the reduction of nitrates to nitrosohemoglobin, Hb(II)NO, the intermediate molecule is Hb(III)NO, which stays in equilibrium with Hb(II)NO. This discovery was of a great importance as it identified the pool of labile Hb(III)NO trapped in a hem pocket, which is not scavenged and can easily release NO under reductive conditions. To support this thesis, the authors demonstrated a relatively increased Hb(III)NO level in venous blood when compared to that of the arterial one. It may be considered as an indicator of increased nitrite reduction in capillaries, where hemoglobin becomes partially deoxygenated [43]. Keeping that in mind, nitrites (NO_2^-) might serve as a more important source of NO than S-nithrosothiols, especially in erythrocytes, being the major intravascular storage sites of nitrite in blood [51].

Other studies raise the different mechanism of nitrite conversion by xanthine oxidoreductase (XOR), located in the outer membrane of RBCs. In the study by Rathod et al. [52], the RBCs were incubated under acidic conditions with nitrate, xanthine, and a XOR or NOS inhibitor (allopurinol and L-N^G-Nitro arginine methyl ester-L-NAME, respectively). It was demonstrated that NO generation was enhanced by ~43% in the presence of xanthine, pointing thus at the major role of XOR in NO synthesis in severe acidosis. Simultaneously, no difference was noted when the pH was in a normal range.

Another study with XOR inhibitor allopurinol demonstrated that although XOR activity is low under normal physiological conditions, it becomes important in pathological conditions, especially when pH drops to 6.8. In those conditions, XOR activity is responsible for ~2/3 of nitrite-derived NO production [53].

Dejam et al. [54] presented some other aspects of the role of XOR in regulating NO bioavailability. They showed concurrent nitrite reduction in normal physiology. The authors suggested that XOR inhibition increases NO bioavailability by reducing the ROS pool, produced by XOR in hypoxic conditions, finally leading to NO scavenging.

Other researchers provided some evidence that RBCs subjected to shear stress produce NO, which may have great importance while passing through vasoconstricted or stenotic vessels with a local response in NO secretion [55]. It was shown that RBCs are able to activate NOS after being exposed to shear stress [56]. Studies with eNOS inhibitors and extracellular EDTA calcium chelation show that intracellular calcium efflux through mechanosensitive RBC membrane channels provokes the binding of the calcium-calmodulin complex to the eNOS protein and its subsequent activation [57,58]. Further studies confirmed that this process is L-arginine-dependent and is highly limited in the presence of L-NAME [59].

The passage of erythrocytes through stenotic vessels and narrow capillaries in the microcirculation depends on RBCs deformability, which is also regulated by NO. One study, using eNOS inhibitors, demonstrated significant impairment of RBC deformability, which was reversed by exposure to exogenous NO donors [60]. The deformability is at least partially mediated by soluble guanylate cyclase (sGC) activation. Cortese-Krott et al. [61] revealed the existence of an sGC/PDE5/PKG-dependent signaling pathway, fully responsive to NO. The exact mechanism of regulation of the shape of RBCs by PKG is not well-studied, but K–Cl cotransport (COT) may be involved. It was proven that COT participates in the recovery of cell volume by modulating the cytoskeleton. As cell volume sensors detect deformation, they activate COT and induce regulatory volume decrease (RVD) [62]. NO is also involved in the regulation of eryptosis. This process may be triggered by different stimuli, leading to the opening of cation channels either directly or by protein kinase C with Ca^{2+}. Erythrocyte shrinkage may be also induced by ceramide formation or by activation of some proteases [63]. NO displays a protective effect by stimulating cGMP-dependent protein kinase, resulting in a CA^{2+} influx decrease [64,65]. Additionally, NO is involved in the regulation of eryptosis via nitrosylation of eryptotic enzymes with NO to prevent RBCs lysis [65]. So far, the mechanism of NO migration from RBCs leading to induced smooth muscle cell relaxation, in order to restore blood flow and increase oxygen supply,

remains unknown. Chen et al. [66] in an experimental study showed that the amount of NO transported from erythrocytes through free diffusion appears to be insufficient. It was demonstrated that in such an experimental setting, NO concentration would be below the half maximum effective concentration (EC50) for sGC and would not induce vasorelaxation. The question arises on how RBCs can supply enough NO to evoke the response of smooth muscle cells (SMCs). Another mathematic analysis discovered that the transport of inactive NO intermediates could be more effective and lead to a subsequent release of greater NO amounts. [66] Alternatively, facilitated NO transfer with membrane-bound proteins would also maintain RBC-dependent SMCs reaction [66].

Some authors suggest that Hb undergoes transnitrosylation again and that NO is transferred to glutathione (GSH), forming S-nitrosoglutathione (GSNO). GSNO may subsequently escape from RBCs via a transporter, release NO outside the erythrocytes, thus inducing vasorelaxation [50].

Opposite results were presented by Sandmann et al. [67], who claim that L-amino acids inhibit intracellular GSNO formation and postulate that it should not be considered as a vasoactive molecule. The authors suggest that S-nitrosocysteine might be a transfer molecule of NO out of RBC by the L-amino acids transporter system. Furthermore, Pawloski et al. [68] presented an alternative way of releasing NO from HbSNO. RBCs are postulated to form the cell lipid rafts that contain enzymes necessary in NO metabolism. The major component is the anion exchange protein 1 (AE1 or band 3 protein), which was proven to be an oxygen-sensitive molecular switch, that controls RBCs properties [69]. The quaternary conformation of Hb (R/T-structure) regulates the band 3 affinity to HbSNO. Along with decreased oxygen tension, N terminus of Band-3 associates reversibly with deoxyHb and the cytoplasmic domain of band 3 drives the transition from the R- to the T-state. It is accompanied with simultaneous NO transfer to the RBC membrane and facilitates the export of NO through membrane channels. The development of this hypothesis provided the study by Gladwin [70], according to which the erythrocytes contain the membrane rafts with complete "metabolome complex"(MCx) that facilitates NO effusion. MCx, besides the AE1/band 3, includes carbonic anhydrase, Rh, and aquaporin channels, and mixed hybrids of deoxyhemoglobin, methemoglobin, and carboxyhemoglobin that could catalytically amplify nitrite reduction. Additionally, local production of metHb in lipid rafts protects NO from scavenging by oxyHb [15,70]. Interestingly, emerging studies confirmed other interesting Band-3 properties. Its pivotal role was demonstrated in regulating RBCs' glucose metabolism, cellular membrane stability, and ATP release [71]. In a hypoxic environment it promotes glycolysis over the pentose phosphate pathway, ATP-dependent vasodilatation, and increase RBCs' deformability [69]. Band-3 was also found to transfer nitrate (NO_3), nitrite (NO_2), and peroxynitrite ($OONO^-$) [72].

Alternatively, Dosier et al. [73] proved the significance of the L-neutral amino acid transporter 1 (LAT 1) in transferring S-nitroso-L-cysteine (SNC) out of the RBCs. LAT 1 is a high-affinity transport system for both cationic and neutral amino acids and exports cationic amino acids into the blood in exchange for large neutral amino acids [74]. It was confirmed that LAT 1 is expressed in the healthy RBC membrane, and LAT1 mRNA was identified in human reticulocytes. While using the LAT1-specific inhibitor (JPH-203), it was demonstrated that the blockage of LAT1 is connected with reduction in SNC efflux [73]. This mechanism could underlie the export of NO from RBCs, but its importance needs further investigation.

Another interesting theory was suggested by Kallakunta et al. [75] while analyzing the role of the protein disulphide isomerase (PDI) in NO transmembrane transport. PDI was detected in the membrane and in the cytosolic fraction of RBCs. The studies showed that RBCs acquire soluble PDI, which is released into the blood by various cells (hepatocytes, endothelial cells, leukocytes, platelet, pancreas). This protein is subsequently S-nityrosylated from SNO-Hb in cytosol and forms S-nitrosylated protein disulphide isomerase (SNO-PDI) [76]. Under normoxic conditions, SNO-PDI is transferred back through the membrane and stays attached to the external surface of RBCs. When RBCs reach a

hypoxic environment, PDI is secreted in soluble form and interacts with the endothelium, triggering NO-dependent hypoxic vasodilation [75].

6. Erythrocyte-Dependent Paracrine Regulation of the Vascular NO Bioavailability

It is well-established that RBCs release adenosine triphosphate (ATP) and contain glycolytic enzymes required for its production [77,78]. It was proved that RBCs contain the gap junction protein pannexin 1 (Panx-1), which acts as an ATP-releasing channel in response to hypoxia, shear stress, or depolarization by forming transient channels between the intracellular compartment and the extracellular milieu [79,80]. Released ATP binds to P2Y receptors on erythrocytes and endothelium cells. P2Y receptors on RBCs mediate ATP-induced ATP release and lead to further increase in plasmatic ATP concentration [81]. Emerging data demonstrate that the P2Y12 inhibitor ticagrelor also induces ATP release from RBCs. The exact mechanism underlying ATP efflux from RBCs in response to that agent is not clear. However, it might be based on membrane channels (chloride channel or Panx-1), as ATP efflux decreases in the presence of some anion channel blockers [82]. The described pathway may explain the more potent anti-aggregative effect of ticagrelor in comparison to that of other antiplatelet agents, taking into consideration that increased extracellular level of adenosine (from ATP degradation) inhibits platelet aggregation additionally by A2A receptors [83].

Interestingly, when ATP reaches P2Y receptors and Panx-1 on endothelial cells, it evokes a calcium wave that propagates along the endothelium [84]. It leads to eNOS activation with associated a NO-dependent vasodilatory response, probably mediated by Ca^{2+}-activated Cl^{-} channels and AMP-activated protein kinase (AMPK) [77,85–87]. Studies with Panx-1 inhibitors, such as carbenoxolone or probenecid, confirmed that ATP release is mostly mediated by Panx-1. Nevertheless, the inhibition was incomplete, suggesting the impact of other unknown mechanisms [88,89].

ATP released to the blood stream may also exert action by activating another subtype of purinergic receptors—P2X. In opposition to the P2Y receptors that protrude on the endothelium, P2X receptors are found on vascular smooth muscle cells (VSMCs). The direct stimulation of P2X with ATP may lead to local vasospasm [77].

Other transmembrane proteins participating in ATP transfer are the voltage dependent anion channels (VDACs). It was demonstrated that they are expressed on the RBCs' membrane and create a complex with translocator protein 2 (TSPO2), the adenine nucleotide transporter (ANT) [90]. As activating ligands bind to the complex, they provoke calcium infusion and enhance cAMP-dependent signaling. Ca^{2+} and cAMP activate protein kinases C and A, respectively, which results in VDACs phosphorylation and polymerization, leading finally to ATP release through the complex [91]. Noteworthy, Sridharan et al. [89] pointed out that VDACs are also responsible for ATP release in response to prostacyclin analogues or β-adrenergic stimulation.

Numerous papers demonstrate that RBCs adjust the blood flow by a decrease in platelet aggregation. Srihirun et al. [92] reported that RBCs are mandatory for inhibiting ADP or collagen-induced platelet aggregation by releasing NO from nitrite. Nitrite alone has no effect on platelets, while reduction in platelet aggregation was observed in the presence of RBCs. The result was limited by adding a NO scavenger (C-PTIO) which confirmed the NO-dependent characteristics of this reaction. Additionally, the antiplatelet outcome was enhanced under hypoxic conditions, demonstrating thus the presence of reductase activity of deoxygenated hemoglobin.

Furthermore, it is well-documented that endothelial eNOS, via NO synthesis, affects systemic blood pressure (BP). Leo et al. [93] shed some light on the role of RBCs in preserving normotension in an eNOS-knockout mice model.

One of the reasons leading to a higher BP profile might be CAT-1 alteration, which is a risk factor for the development of hypertension. Yang et al. [94] studied the polymorphism in the 3′UTR region of CAT-1 chromosome 13q12-q14, which leads to altered L-arginine metabolism with hypertensive presentation. CAT-1 was also proven to be at

least partially responsible for cardiovascular toxicity, with increased BP induced by cyclosporine. It was presented that cyclosporine significantly attenuates L-arginine transport and induces protein nitration, leading to the development of hypertension and accelerated atherosclerosis [95].

NO, released by RBCs to the blood stream, also becomes an autocrine agent that increases RBCs' deformability. It is essential when RBCs pass through narrow capillaries in microcirculation. The exact way of this regulation is uncertain, however, NO may act by regulating guanylate cyclase, ion transport across the RBC membrane, or S-nitrosylation of cytoskeleton spectrins [96,97]. Oxidative stress also reduces RBCs deformability, which aggravates blood flow in the microvascular bed. In line with that, the abnormalities of hemorheological properties of RBCs were demonstrated to play a pivotal role in pathogenesis of microvascular angina (MVA). Furthermore, patients with MVA demonstrate an increased oxidated-to-reduced glutathione ratio (the GSSG/GSH index), suggesting a central role of oxidative stress in MVA development [98].

7. Future Perspectives

Over the past few years, significant progress regarding the knowledge on the role of erythrocytes in nitric oxide metabolism has been made. From simple gas transporters, through nitric oxide scavengers, RBCs have become important players in nitric oxide synthesis and in the maintenance of appropriate vascular tone. Erythrocytes seem to be a promising novel therapeutic target in management of endothelial dysfunction. Development of specific arginase inhibitors (AI) may bring benefits in patients with coronary artery disease, diabetes mellitus, heart failure, hypertension, and erectile dysfunction [99–102]. Studies on animal models demonstrated that increased activity of arginase has fundamental importance in myocardial ischemia-reperfusion injury and that its inhibition mediates cardiac protection via increased NO production [103–105]. Despite many in vitro studies, further clinical trials should be conducted to provide some more evidence on the usefulness of AI in cardiovascular risk reduction. Up to date, a promising trial with an arginase inhibitor (Nω-hydroxy-nor-L-arginine) was conducted, with restoration of endothelial function in patients with type 2 diabetes [106]. Similar results were obtained in endothelial dysfunction caused by ischemia-reperfusion in patients with CAD [107]. e-NOS uncoupling and prevention of oxidative stress seem to be other interesting future therapeutic targets.

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References

1. Bryk, A.H.; Wiśniewski, J.R.W. Quantitative Analysis of Human Red Blood Cell Proteome. *J. Proteome Res.* **2017**, *16*, 2752–2761. [CrossRef] [PubMed]
2. Agli, A.N.; Schaefer, A.; Geny, B.; Piquard, F.; Haberey, P. Erythrocytes Participate Significantly in Blood Transport of Amino Acids during the Post Absorptive State in Normal Humans. *Eur. J. Appl. Physiol. Occup. Physiol.* **1998**, *78*, 502–508. [CrossRef] [PubMed]
3. Thorn, B.; Dunstan, R.H.; Macdonald, M.M.; Borges, N.; Roberts, T.K. Evidence That Human and Equine Erythrocytes Could Have Significant Roles in the Transport and Delivery of Amino Acids to Organs and Tissues. *Amino Acids* **2020**, *52*, 711–724. [CrossRef]

4. Strobel, J.; Mieth, M.; Endreß, B.; Auge, D.; König, J.; Fromm, M.F.; Maas, R. Interaction of the Cardiovascular Risk Marker Asymmetric Dimethylarginine (ADMA) with the Human Cationic Amino Acid Transporter 1 (CAT1). *J. Mol. Cell. Cardiol.* **2012**, *53*, 392–400. [CrossRef] [PubMed]
5. Franchi Gazzola, R.; Sala, R.; Bussolati, O.; Visigalli, R.; Dall'Asta, V.; Ganapathy, V.; Gazzola, G.C. The Adaptive Regulation of Amino Acid Transport System A Is Associated to Changes in ATA2 Expression. *FEBS Lett.* **2001**, *490*, 11–14. [CrossRef]
6. Gräf, P.; Förstermann, U.; Closs, E.I. The Transport Activity of the Human Cationic Amino Acid Transporter HCAT-1 Is Downregulated by Activation of Protein Kinase C. *Br. J. Pharmacol.* **2001**, *132*, 1193–1200. [CrossRef]
7. Fernandez, J.; Bode, B.; Koromilas, A.; Alan Diehl, J.; Krukovets, I.; Snider, M.D.; Hatzoglou, M. Translation Mediated by the Internal Ribosome Entry Site of the Cat-1 MRNA Is Regulated by Glucose Availability in a PERK Kinase-Dependent Manner. *J. Biol. Chem.* **2002**, *277*, 11780–11787. [CrossRef]
8. Bhattacharyya, S.N.; Habermacher, R.; Martine, U.; Closs, E.I.; Filipowicz, W. Relief of MicroRNA-Mediated Translational Repression in Human Cells Subjected to Stress. *Cell* **2006**, *125*, 1111–1124. [CrossRef]
9. Zhang, H.-G.; Zhang, Q.-J.; Li, B.-W.; Li, L.-H.; Song, X.-H.; Xiong, C.-M.; Zou, Y.-B.; Liu, B.-Y.; Han, J.-Q.; Xiu, R.-J. The Circulating Level of MiR-122 Is a Potential Risk Factor for Endothelial Dysfunction in Young Patients with Essential Hypertension. *Hypertens. Res.* **2020**, *43*, 511–517. [CrossRef]
10. Wang, L.; Chen, H. Correlation between Serum MiR-122 and Myocardial Damage and Ventricular Function in Patients with Essential Hypertension. *J. Thorac. Dis.* **2021**, *13*, 4999–5006. [CrossRef]
11. Gaddam, R.R.; Dhuri, K.; Kim, Y.-R.; Jacobs, J.S.; Kumar, V.; Li, Q.; Irani, K.; Bahal, R.; Vikram, A. γ Peptide Nucleic Acid-Based MiR-122 Inhibition Rescues Vascular Endothelial Dysfunction in Mice Fed a High-Fat Diet. *J. Med. Chem.* **2022**, *65*, 3332–3342. [CrossRef] [PubMed]
12. Hatzoglou, M.; Fernandez, J.; Yaman, I.; Closs, E. Regulation of Cationic Amino Acid Transport: The Story of the CAT-1 Transporter. *Annu. Rev. Nutr.* **2004**, *24*, 377–399. [CrossRef]
13. Doss, J.F.; Corcoran, D.L.; Jima, D.D.; Telen, M.J.; Dave, S.S.; Chi, J.T. A Comprehensive Joint Analysis of the Long and Short RNA Transcriptomes of Human Erythrocytes. *BMC Genom.* **2015**, *16*, 952. [CrossRef] [PubMed]
14. Kabanova, S.; Kleinbongard, P.; Volkmer, J.; Andrée, B.; Kelm, M.; Jax, T.W. Gene Expression Analysis of Human Red Blood Cells. *Int. J. Med. Sci.* **2009**, *6*, 156–159. [CrossRef] [PubMed]
15. Cortese-Krott, M.M.; Kelm, M. Endothelial Nitric Oxide Synthase in Red Blood Cells: Key to a New Erythrocrine Function? *Redox Biol.* **2014**, *2*, 251–258. [CrossRef]
16. Davids, M.; van Hell, A.J.; Visser, M.; Nijveldt, R.J.; van Leeuwen, P.A.M.; Teerlink, T. Role of the Human Erythrocyte in Generation and Storage of Asymmetric Dimethylarginine. *Am. J. Physiol.-Heart Circ. Physiol.* **2012**, *302*, 1762–1770. [CrossRef]
17. Neelam, S.; Kakhniashvili, D.G.; Wilkens, S.; Levene, S.D.; Goodman, S.R. Functional 20S Proteasomes in Mature Human Red Blood Cells. *Exp. Biol. Med.* **2011**, *236*, 580–591. [CrossRef]
18. Sitar, M.E.; Kayacelebi, A.A.; Beckmann, B.; Kielstein, J.T.; Tsikas, D. Asymmetric Dimethylarginine (ADMA) in Human Blood: Effects of Extended Haemodialysis in the Critically Ill Patient with Acute Kidney Injury, Protein Binding to Human Serum Albumin and Proteolysis by Thermolysin. *Amino Acids* **2015**, *47*, 1983–1993. [CrossRef]
19. Yokoro, M.; Suzuki, M.; Murota, K.; Otsuka, C.; Yamashita, H.; Takahashi, Y.; Tsuji, H.; Kimoto, M. Asymmetric Dimethylarginine, an Endogenous NOS Inhibitor, Is Actively Metabolized in Rat Erythrocytes. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 1334–1342. [CrossRef]
20. Kang, E.S.; Cates, T.B.; Harper, D.N.; Chiang, T.M.; Myers, L.K.; Acchiardo, S.R.; Kimoto, M. An Enzyme Hydrolyzing Methylated Inhibitors of Nitric Oxide Synthase Is Present in Circulating Human Red Blood Cells. *Free Radic. Res.* **2001**, *35*, 693–707. [CrossRef]
21. Bentur, O.S.; Schwartz, D.; Chernichovski, T.; Ingbir, M.; Weinstein, T.; Chernin, G.; Schwartz, I.F. Estradiol Augments While Progesterone Inhibits Arginine Transport in Human Endothelial Cells through Modulation of Cationic Amino Acid Transporter-1. *Am. J. Physiol. Integr. Comp. Physiol.* **2015**, *309*, R421–R427. [CrossRef] [PubMed]
22. Toral, M.; Jimenez, R.; Montoro-Molina, S.; Romero, M.; Wangensteen, R.; Duarte, J.; Vargas, F. Thyroid Hormones Stimulate L-Arginine Transport in Human Endothelial Cells. *J. Endocrinol.* **2018**, *239*, 49–62. [CrossRef] [PubMed]
23. Kietadisorn, R.; Juni, R.P.; Moens, A.L. Tackling Endothelial Dysfunction by Modulating NOS Uncoupling: New Insights into Its Pathogenesis and Therapeutic Possibilities. *Am. J. Physiol. Metab.* **2012**, *302*, E481–E495. [CrossRef] [PubMed]
24. Roe, N.D.; Ren, J. Nitric Oxide Synthase Uncoupling: A Therapeutic Target in Cardiovascular Diseases. *Vascul. Pharmacol.* **2012**, *57*, 168–172. [CrossRef]
25. Zou, M.-H.; Shi, C.; Cohen, R.A. Oxidation of the Zinc-Thiolate Complex and Uncoupling of Endothelial Nitric Oxide Synthase by Peroxynitrite. *J. Clin. Investig.* **2002**, *109*, 817–826. [CrossRef]
26. Mahdi, A.; Tengbom, J.; Alvarsson, M.; Wernly, B.; Zhou, Z.; Pernow, J. Red Blood Cell Peroxynitrite Causes Endothelial Dysfunction in Type 2 Diabetes Mellitus via Arginase. *Cells* **2020**, *9*, 1712. [CrossRef]
27. Zhou, Z.; Mahdi, A.; Tratsiakovich, Y.; Zahorán, S.; Kövamees, O.; Nordin, F.; Uribe Gonzalez, A.E.; Alvarsson, M.; Östenson, C.G.; Andersson, D.C.; et al. Erythrocytes from Patients with Type 2 Diabetes Induce Endothelial Dysfunction Via Arginase I. *J. Am. Coll. Cardiol.* **2018**, *72*, 769–780. [CrossRef]
28. Pernow, J.; Mahdi, A.; Yang, J.; Zhou, Z. Red Blood Cell Dysfunction: A New Player in Cardiovascular Disease. *Cardiovasc. Res.* **2019**, *115*, 1596–1605. [CrossRef]

29. Yang, J.; Gonon, A.T.; Sjoquist, P.-O.; Lundberg, J.O.; Pernow, J. Arginase Regulates Red Blood Cell Nitric Oxide Synthase and Export of Cardioprotective Nitric Oxide Bioactivity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 15049–15054. [CrossRef]
30. Mahdi, A.; Kövamees, O.; Pernow, J. Improvement in Endothelial Function in Cardiovascular Disease-Is Arginase the Target? *Int. J. Cardiol.* **2020**, *301*, 207–214. [CrossRef]
31. Romero, M.J.; Platt, D.H.; Tawfik, H.E.; Labazi, M.; El-Remessy, A.B.; Bartoli, M.; Caldwell, R.B.; Caldwell, R.W. Diabetes-Induced Coronary Vascular Dysfunction Involves Increased Arginase Activity. *Circ. Res.* **2008**, *102*, 95–102. [CrossRef] [PubMed]
32. El-Remessy, A.B.; Tawfik, H.E.; Matragoon, S.; Pillai, B.; Caldwell, R.B.; Caldwell, R.W. Peroxynitrite Mediates Diabetes-Induced Endothelial Dysfunction: Possible Role of Rho Kinase Activation. *Exp. Diabetes Res.* **2010**, *2010*, 247861. [CrossRef] [PubMed]
33. Santhanam, L.; Lim, H.K.; Lim, H.K.; Miriel, V.; Brown, T.; Patel, M.; Balanson, S.; Ryoo, S.; Anderson, M.; Irani, K.; et al. Inducible NO Synthase–Dependent S -Nitrosylation and Activation of Arginase1 Contribute to Age-Related Endothelial Dysfunction. *Circ. Res.* **2007**, *101*, 692–702. [CrossRef] [PubMed]
34. Kim, P.S.; Iyer, R.K.; Lu, K.V.; Yu, H.; Karimi, A.; Kern, R.M.; Tai, D.K.; Cederbaum, S.D.; Grody, W.W. Expression of the Liver Form of Arginase in Erythrocytes. *Mol. Genet. Metab.* **2002**, *76*, 100–110. [CrossRef]
35. Duckles, S.P.; Miller, V.M. Hormonal Modulation of Endothelial NO Production. *Pflügers Arch.-Eur. J. Physiol.* **2010**, *459*, 841–851. [CrossRef]
36. Vona, R.; Gambardella, L.; Ortona, E.; Santulli, M.; Malorni, W.; Carè, A.; Pietraforte, D.; Straface, E. Functional Estrogen Receptors of Red Blood Cells. Do They Influence Intracellular Signaling? *Cell. Physiol. Biochem.* **2019**, *53*, 186–199. [CrossRef]
37. Liu, X.; Miller, M.J.S.; Joshi, M.S.; Sadowska-Krowicka, H.; Clark, D.A.; Lancaster, J.R. Diffusion-Limited Reaction of Free Nitric Oxide with Erythrocytes. *J. Biol. Chem.* **1998**, *273*, 18709–18713. [CrossRef]
38. Helms, C.C.; Gladwin, M.T.; Kim-Shapiro, D.B. Erythrocytes and Vascular Function: Oxygen and Nitric Oxide. *Front. Physiol.* **2018**, *9*, 125. [CrossRef]
39. Vaughn, M.W.; Huang, K.-T.; Kuo, L.; Liao, J.C. Erythrocytes Possess an Intrinsic Barrier to Nitric Oxide Consumption. *J. Biol. Chem.* **2000**, *275*, 2342–2348. [CrossRef]
40. Chebbi, R. A Two-Zone Shear-Induced Red Blood Cell Migration Model for Blood Flow in Microvessels. *Front. Phys.* **2019**, *7*, 206. [CrossRef]
41. Deonikar, P.; Kavdia, M. A Computational Model for Nitric Oxide, Nitrite and Nitrate Biotransport in the Microcirculation: Effect of Reduced Nitric Oxide Consumption by Red Blood Cells and Blood Velocity. *Microvasc. Res.* **2010**, *80*, 464–476. [CrossRef] [PubMed]
42. Liao, J.C.; Hein, T.W.; Vaughn, M.W.; Huang, K.-T.; Kuo, L. Intravascular Flow Decreases Erythrocyte Consumption of Nitric Oxide. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8757–8761. [CrossRef] [PubMed]
43. Nagababu, E.; Ramasamy, S.; Abernethy, D.R.; Rifkind, J.M. Active Nitric Oxide Produced in the Red Cell under Hypoxic Conditions by Deoxyhemoglobin-Mediated Nitrite Reduction. *J. Biol. Chem.* **2003**, *278*, 46349–46356. [CrossRef] [PubMed]
44. Stamler, J.S.; Jia, L.; Eu, J.P.; McMahon, T.J.; Demchenko, I.T.; Bonaventura, J.; Gernert, K.; Piantadosi, C.A. Blood Flow Regulation by S -Nitrosohemoglobin in the Physiological Oxygen Gradient. *Science* **1997**, *276*, 2034–2037. [CrossRef] [PubMed]
45. Fago, A.; Crumbliss, A.L.; Hendrich, M.P.; Pearce, L.L.; Peterson, J.; Henkens, R.; Bonaventura, C. Oxygen Binding to Partially Nitrosylated Hemoglobin. *Biochim. Biophys. Acta.-Proteins Proteom.* **2013**, *1834*, 1894–1900. [CrossRef] [PubMed]
46. Rifkind, J.M.; Nagababu, E.; Ramasamy, S. The Quaternary Hemoglobin Conformation Regulates the Formation of the Nitrite-Induced Bioactive Intermediate and the Dissociation of Nitric Oxide from This Intermediate. *Nitric Oxide* **2011**, *24*, 102–109. [CrossRef]
47. Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J.S. S-Nitrosohaemoglobin: A Dynamic Activity of Blood Involved in Vascular Control. *Transfus. Med. Rev.* **1997**, *11*, 82. [CrossRef]
48. Baylis, C.; Vallance, P. Measurement of Nitrite and Nitrate Levels in Plasma and Urine—What Does This Measure Tell Us about the Activity of the Endogenous Nitric Oxite System? *Curr. Opin. Nephrol. Hypertens.* **1998**, *7*, 59–62. [CrossRef]
49. Cosby, K.; Partovi, K.S.; Crawford, J.H.; Patel, R.P.; Reiter, C.D.; Martyr, S.; Yang, B.K.; Waclawiw, M.A.; Zalos, G.; Xu, X.; et al. Nitrite Reduction to Nitric Oxide by Deoxyhemoglobin Vasodilates the Human Circulation. *Nat. Med.* **2003**, *9*, 1498–1505. [CrossRef] [PubMed]
50. Schmidt, H.H.H.W.; Feelisch, M. Red Blood Cell–Derived Nitric Oxide Bioactivity and Hypoxic Vasodilation. *Circulation* **2019**, *139*, 2664–2667. [CrossRef]
51. Dejam, A. Erythrocytes Are the Major Intravascular Storage Sites of Nitrite in Human Blood. *Blood* **2005**, *106*, 734–739. [CrossRef] [PubMed]
52. Rathod, K.S.; Webb, A.J.; Lovell, M.J.; Lecomte, F.; Ahluwalia, A. Nitrite Is Reduced to Nitric Oxide by Xanthine Oxidoreductase and Nitric Oxide Synthase in the Erythrocyte Membrane in Hypoxemia. *Blood* **2006**, *108*, 1560. [CrossRef]
53. Webb, A.J.; Milsom, A.B.; Rathod, K.S.; Chu, W.L.; Qureshi, S.; Lovell, M.J.; Lecomte, F.M.J.; Perrett, D.; Raimondo, C.; Khoshbin, E.; et al. Mechanisms Underlying Erythrocyte and Endothelial Nitrite Reduction to Nitric Oxide in Hypoxia. *Circ. Res.* **2008**, *103*, 957–964. [CrossRef] [PubMed]
54. Dejam, A.; Hunter, C.J.; Tremonti, C.; Pluta, R.M.; Yuen, Y.H.; Hon, Y.; Grimes, G.; Partovi, K.; Pelletier, M.M.; Oldfield, E.H.; et al. Nitrite Infusion in Humans and Nonhuman Primates Endocrine Effects, Pharmacokinetics and Tolerance Formation. *Circulation* **2007**, *116*, 1821–1831. [CrossRef]

55. Ulker, P.; Yaras, N.; Yalcin, O.; Celik-Ozenci, C.; Johnson, P.C.; Meiselman, H.J.; Baskurt, O.K. Shear Stress Activation of Nitric Oxide Synthase and Increased Nitric Oxide Levels in Human Red Blood Cells. *Nitric Oxide* **2011**, *24*, 184–191. [CrossRef]
56. Ulker, P.; Sati, L.; Celik-Ozenci, C.; Meiselman, H.J.; Baskurt, O.K. Mechanical Stimulation of Nitric Oxide Synthesizing Mechanisms in Erythrocytes. *Biorheology* **2009**, *46*, 121–132. [CrossRef]
57. Feron, O.; Saldana, F.; Michel, J.B.; Michel, T. The Endothelial Nitric-Oxide Synthase-Caveolin Regulatory Cycle. *J. Biol. Chem.* **1998**, *273*, 3125–3128. [CrossRef]
58. Ulker, P.; Meiselman, H.J.; Baskurt, O.K. Nitric Oxide Generation in Red Blood Cells Induced by Mechanical Stress. *Clin. Hemorheol. Microcirc.* **2010**, *45*, 169–175. [CrossRef]
59. Fleming, I.; Busse, R. Molecular Mechanisms Involved in the Regulation of the Endothelial Nitric Oxide Synthase. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2003**, *284*, R1–R12. [CrossRef]
60. Bor-Kucukatay, M.; Wenby, R.B.; Meiselman, H.J.; Baskurt, O.K. Effects of Nitric Oxide on Red Blood Cell Deformability. *Am. J. Physiol.-Heart Circ. Physiol.* **2003**, *284*, H1577–H1584. [CrossRef]
61. Cortese-Krott, M.M.; Mergia, E.; Kramer, C.M.; Lückstädt, W.; Yang, J.; Wolff, G.; Panknin, C.; Bracht, T.; Sitek, B.; Pernow, J.; et al. Identification of a Soluble Guanylate Cyclase in RBCs: Preserved Activity in Patients with Coronary Artery Disease. *Redox Biol.* **2018**, *14*, 328–337. [CrossRef] [PubMed]
62. Adragna, N.C.; Fulvio, M.D.; Lauf, P.K. Regulation of K-Cl Cotransport: From Function to Genes. *J. Membr. Biol.* **2004**, *201*, 109–137. [CrossRef] [PubMed]
63. Dreischer, P.; Duszenko, M.; Stein, J.; Wieder, T. Eryptosis: Programmed Death of Nucleus-Free, Iron-Filled Blood Cells. *Cells* **2022**, *11*, 503. [CrossRef] [PubMed]
64. Brun, J.-F.; Varlet-Marie, E.; Myzia, J.; de Mauverger, E.R.; Pretorius, E. Metabolic Influences Modulating Erythrocyte Deformability and Eryptosis. *Metabolites* **2021**, *12*, 4. [CrossRef] [PubMed]
65. Föller, M.; Feil, S.; Ghoreschi, K.; Koka, S.; Gerling, A.; Thunemann, M.; Hofmann, F.; Schuler, B.; Vogel, J.; Pichler, B.; et al. Anemia and Splenomegaly in CGKI-Deficient Mice. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6771–6776. [CrossRef]
66. Chen, K.; Piknova, B.; Pittman, R.N.; Schechter, A.N.; Popel, A.S. Nitric Oxide from Nitrite Reduction by Hemoglobin in the Plasma and Erythrocytes. *Nitric Oxide* **2008**, *18*, 47–60. [CrossRef] [PubMed]
67. Sandmann, J.; Schwedhelm, K.S.; Tsikas, D. Specific Transport of S -Nitrosocysteine in Human Red Blood Cells: Implications for Formation of S -Nitrosothiols and Transport of NO Bioactivity within the Vasculature. *FEBS Lett.* **2005**, *579*, 4119–4124. [CrossRef]
68. Pawloski, J.R.; Hess, D.T.; Stamler, J.S. Export by Red Blood Cells of Nitric Oxide Bioactivity. *Nature* **2001**, *409*, 622–626. [CrossRef]
69. Chu, H.; McKenna, M.M.; Krump, N.A.; Zheng, S.; Mendelsohn, L.; Thein, S.L.; Garrett, L.J.; Bodine, D.M.; Low, P.S. Reversible Binding of Hemoglobin to Band 3 Constitutes the Molecular Switch That Mediates O_2 Regulation of Erythrocyte Properties. *Blood* **2016**, *128*, 2708–2716. [CrossRef]
70. Gladwin, M.T.; Schechter, A.N.; Kim-Shapiro, D.B.; Patel, R.P.; Hogg, N.; Shiva, S.; Cannon, R.O.; Kelm, M.; Wink, D.A.; Espey, M.G.; et al. The Emerging Biology of the Nitrite Anion. *Nat. Chem. Biol.* **2005**, *1*, 308–314. [CrossRef]
71. Zhao, Y.; Wang, X.; Wang, R.; Chen, D.; Noviana, M.; Zhu, H. Nitric Oxide Inhibits Hypoxia-induced Impairment of Human RBC Deformability through Reducing the Cross-linking of Membrane Protein Band 3. *J. Cell. Biochem.* **2019**, *120*, 305–320. [CrossRef]
72. Jennings, M.L. Cell Physiology and Molecular Mechanism of Anion Transport by Erythrocyte Band 3/AE1. *Am. J. Physiol. Physiol.* **2021**, *321*, C1028–C1059. [CrossRef] [PubMed]
73. Dosier, L.B.M.; Premkumar, V.J.; Zhu, H.; Akosman, I.; Wempe, M.F.; McMahon, T.J. Antagonists of the System L Neutral Amino Acid Transporter (LAT) Promote Endothelial Adhesivity of Human Red Blood Cells. *Thromb. Haemost.* **2017**, *117*, 1402–1411. [CrossRef] [PubMed]
74. Verrey, F.; Closs, E.I.; Wagner, C.A.; Palacin, M.; Endou, H.; Kanai, Y. CATs and HATs: The SLC7 Family of Amino Acid Transporters. *Pflügers Arch.-Eur. J. Physiol.* **2004**, *447*, 532–542. [CrossRef] [PubMed]
75. Kallakunta, V.M.; Slama-Schwok, A.; Mutus, B. Protein Disulfide Isomerase May Facilitate the Efflux of Nitrite Derived S-Nitrosothiols from Red Blood Cells. *Redox Biol.* **2013**, *1*, 373–380. [CrossRef]
76. Ramachandran, N.; Root, P.; Jiang, X.-M.; Hogg, P.J.; Mutus, B. Mechanism of Transfer of NO from Extracellular S-Nitrosothiols into the Cytosol by Cell-Surface Protein Disulfide Isomerase. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9539–9544. [CrossRef]
77. Bergfeld, G.R.; Forrester, T. Release of ATP from Human Erythrocytes in Response to a Brief Period of Hypoxia and Hypercapnia. *Cardiovasc. Res.* **1992**, *26*, 40–47. [CrossRef]
78. Ellsworth, M.L.; Forrester, T.; Ellis, C.G.; Dietrich, H.H. The Erythrocyte as a Regulator of Vascular Tone. *Am. J. Physiol. Circ. Physiol.* **1995**, *269*, H2155–H2161. [CrossRef]
79. Locovei, S.; Bao, L.; Dahl, G. Pannexin 1 in Erythrocytes: Function without a Gap. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 7655–7659. [CrossRef]
80. Ransford, G.A.; Fregien, N.; Qiu, F.; Dahl, G.; Conner, G.E.; Salathe, M. Pannexin 1 Contributes to ATP Release in Airway Epithelia. *Am. J. Respir. Cell Mol. Biol.* **2009**, *41*, 525–534. [CrossRef]
81. Di Virgilio, F.; Chiozzi, P.; Ferrari, D.; Falzoni, S.; Sanz, J.M.; Morelli, A.; Torboli, M.; Bolognesi, G.; Baricordi, O.R. Nucleotide Receptors: An Emerging Family of Regulatory Molecules in Blood Cells. *Blood* **2001**, *97*, 587–600. [CrossRef] [PubMed]
82. Öhman, J.; Kudira, R.; Albinsson, S.; Olde, B.; Erlinge, D. Ticagrelor Induces Adenosine Triphosphate Release from Human Red Blood Cells. *Biochem. Biophys. Res. Commun.* **2012**, *418*, 754–758. [CrossRef] [PubMed]

83. Nylander, S.; Femia, E.A.; Scavone, M.; Berntsson, P.; Asztély, A.-K.; Nelander, K.; Löfgren, L.; Nilsson, R.G.; Cattaneo, M. Ticagrelor Inhibits Human Platelet Aggregation via Adenosine in Addition to P2Y 12 Antagonism. *J. Thromb. Haemost.* **2013**, *11*, 1867–1876. [CrossRef] [PubMed]
84. Kirby, B.S.; Sparks, M.A.; Lazarowski, E.R.; Domowicz, D.A.L.; Zhu, H.; McMahon, T.J. Integrative Cardiovascular Physiology and Pathophysiology: Pannexin 1 Channels Control the Hemodynamic Response to Hypoxia by Regulating O_2-Sensitive Extracellular ATP in Blood. *Am. J. Physiol.-Heart Circ. Physiol.* **2021**, *320*, H1055. [CrossRef]
85. von Kügelgen, I.; Wetter, A. Molecular Pharmacology of P2Y-Receptors. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **2000**, *362*, 310–323. [CrossRef]
86. Forsberg, E.J.; Feuerstein, G.; Shohami, E.; Pollard, H.B. Adenosine Triphosphate Stimulates Inositol Phospholipid Metabolism and Prostacyclin Formation in Adrenal Medullary Endothelial Cells by Means of P2-Purinergic Receptors. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 5630–5634. [CrossRef]
87. Burnstock, G. Purinergic Signaling in the Cardiovascular System. *Circ. Res.* **2017**, *120*, 207–228. [CrossRef]
88. Sridharan, M.; Adderley, S.P.; Bowles, E.A.; Egan, T.M.; Stephenson, A.H.; Ellsworth, M.L.; Sprague, R.S. Pannexin 1 Is the Conduit for Low Oxygen Tension-Induced ATP Release from Human Erythrocytes. *Am. J. Physiol. Circ. Physiol.* **2010**, *299*, H1146–H1152. [CrossRef]
89. Sridharan, M.; Bowles, E.A.; Richards, J.P.; Krantic, M.; Davis, K.L.; Dietrich, K.A.; Stephenson, A.H.; Ellsworth, M.L.; Sprague, R.S. Prostacyclin Receptor-Mediated ATP Release from Erythrocytes Requires the Voltage-Dependent Anion Channel. *Am. J. Physiol.-Heart Circ. Physiol.* **2012**, *302*, H553–H559. [CrossRef]
90. Bouyer, G.; Cueff, A.; Egée, S.; Kmiecik, J.; Maksimova, Y.; Glogowska, E.; Gallagher, P.G.; Thomas, S.L.Y. Erythrocyte Peripheral Type Benzodiazepine Receptor/Voltage-Dependent Anion Channels Are Upregulated by Plasmodium Falciparum. *Blood* **2011**, *118*, 2305–2312. [CrossRef]
91. Marginedas-Freixa, I.; Alvarez, C.L.; Moras, M.; Leal Denis, M.F.; Hattab, C.; Halle, F.; Bihel, F.; Mouro-Chanteloup, I.; Lefevre, S.D.; Le Van Kim, C.; et al. Human Erythrocytes Release ATP by a Novel Pathway Involving VDAC Oligomerization Independent of Pannexin-1. *Sci. Rep.* **2018**, *8*, 11384. [CrossRef] [PubMed]
92. Srihirun, S.; Sriwantana, T.; Unchern, S.; Kittikool, D.; Noulsri, E.; Pattanapanyasat, K.; Fucharoen, S.; Piknova, B.; Schechter, A.N.; Sibmooh, N. Platelet Inhibition by Nitrite Is Dependent on Erythrocytes and Deoxygenation. *PLoS ONE* **2012**, *7*, e30380. [CrossRef] [PubMed]
93. Leo, F.; Suvorava, T.; Heuser, S.K.; Li, J.; Lobue, A.; Barbarino, F.; Piragine, E.; Schneckmann, R.; Hutzler, B.; Good, M.E.; et al. Red Blood Cell and Endothelial ENOS Independently Regulate Circulating Nitric Oxide Metabolites and Blood Pressure. *Circulation* **2021**, *144*, 870–889. [CrossRef] [PubMed]
94. Yang, Z.; Venardos, K.; Jones, E.; Morris, B.J.; Chin-Dusting, J.; Kaye, D.M. Identification of a Novel Polymorphism in the 3′UTR of the L-Arginine Transporter Gene SLC7A1. *Circulation* **2007**, *115*, 1269–1274. [CrossRef] [PubMed]
95. Grupper, A.; Shashar, M.; Bahry, D.; Pri-Paz, Y.; Tur, O.B.; Levi, S.; Chernichovski, T.; Chernin, G.; Schwartz, I.F. Cyclosporine Attenuates Arginine Transport, in Human Endothelial Cells, through Modulation of Cationic Amino Acid Transporter-1. *Am. J. Nephrol.* **2013**, *37*, 612–618. [CrossRef]
96. Grau, M.; Pauly, S.; Ali, J.; Walpurgis, K.; Thevis, M.; Bloch, W.; Suhr, F. RBC-NOS-Dependent S-Nitrosylation of Cytoskeletal Proteins Improves RBC Deformability. *PLoS ONE* **2013**, *8*, e56759. [CrossRef]
97. Barbarino, F.; Wäschenbach, L.; Cavalho-Lemos, V.; Dillenberger, M.; Becker, K.; Gohlke, H.; Cortese-Krott, M.M. Targeting Spectrin Redox Switches to Regulate the Mechanoproperties of Red Blood Cells. *Biol. Chem.* **2021**, *402*, 317–331. [CrossRef]
98. Porro, B.; Eligini, S.; Veglia, F.; Lualdi, A.; Squellerio, I.; Fiorelli, S.; Giovannardi, M.; Chiorino, E.; Dalla Cia, A.; Crisci, M.; et al. Nitric Oxide Synthetic Pathway in Patients with Microvascular Angina and Its Relations with Oxidative Stress. *Oxid. Med. Cell. Longev.* **2014**, *2014*, 726539. [CrossRef]
99. Holowatz, L.A.; Kenney, W.L. Up-Regulation of Arginase Activity Contributes to Attenuated Reflex Cutaneous Vasodilatation in Hypertensive Humans. *J. Physiol.* **2007**, *581*, 863–872. [CrossRef]
100. Pernow, J.; Jung, C. Arginase as a Potential Target in the Treatment of Cardiovascular Disease: Reversal of Arginine Steal? *Cardiovasc. Res.* **2013**, *98*, 334–343. [CrossRef]
101. Quitter, F.; Figulla, H.R.; Ferrari, M.; Pernow, J.; Jung, C. Increased Arginase Levels in Heart Failure Represent a Therapeutic Target to Rescue Microvascular Perfusion. *Clin. Hemorheol. Microcirc.* **2013**, *54*, 75–85. [CrossRef] [PubMed]
102. Masuda, H. Significance of Nitric Oxide and Its Modulation Mechanisms by Endogenous Nitric Oxide Synthase Inhibitors and Arginase in the Micturition Disorders and Erectile Dysfunction. *Int. J. Urol.* **2008**, *15*, 128–134. [CrossRef] [PubMed]
103. Hein, T.W.; Zhang, C.; Wang, W.; Chang, C.-I.; Thengchaisri, N.; Kuo, L. Ischemia-reperfusion Selectively Impairs Nitric Oxide-Mediated Dilation in Coronary Arterioles: Counteracting Role of Arginase. *FASEB J.* **2003**, *17*, 2328–2330. [CrossRef]
104. Jung, C.; Gonon, A.T.; Sjoquist, P.-O.; Lundberg, J.O.; Pernow, J. Arginase Inhibition Mediates Cardioprotection during Ischaemia-Reperfusion. *Cardiovasc. Res.* **2010**, *85*, 147–154. [CrossRef] [PubMed]
105. Gonon, A.T.; Jung, C.; Katz, A.; Westerblad, H.; Shemyakin, A.; Sjöquist, P.-O.; Lundberg, J.O.; Pernow, J. Local Arginase Inhibition during Early Reperfusion Mediates Cardioprotection via Increased Nitric Oxide Production. *PLoS ONE* **2012**, *7*, e42038. [CrossRef] [PubMed]

106. Kövamees, O.; Shemyakin, A.; Checa, A.; Wheelock, C.E.; Lundberg, J.O.; Östenson, C.-G.; Pernow, J. Arginase Inhibition Improves Microvascular Endothelial Function in Patients with Type 2 Diabetes Mellitus. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 3952–3958. [CrossRef]
107. Kövamees, O.; Shemyakin, A.; Pernow, J. Effect of Arginase Inhibition on Ischemia-Reperfusion Injury in Patients with Coronary Artery Disease with and without Diabetes Mellitus. *PLoS ONE* **2014**, *9*, e103260. [CrossRef]

4. PRACA NR 2:

A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus

nutrients

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Article

A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus

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Abstract: (1) Background: Type-2-diabetes-mellitus (DM) is one the most important cardiovascular-risk-factors. Among many molecules regulating vascular tone, nitric oxide appears to be the most pivotal. Although micro- and macrovascular-abnormalities are extensively studied, the alterations in the nitric-oxide-metabolic-pathway require further investigations. Additionally, the role of erythrocytes in the vascular tone regulation has not been extensively explored. The aim of this study was to evaluate the endothelial-function and the nitric-oxide-metabolic-pathway in erythrocytes and plasma of diabetic individuals. (2) Methods: A total of 80 subjects were enrolled in this cross-sectional study, including 35 patients with DM and 45 healthy individuals. The endothelial-function was evaluated in response to different stimuli. (3) Results: In the DM group, decreased Arginine and citrulline concentrations in the plasma compartment with reduced Arginine/ADMA and ADMA/DMA-ratios were observed. Preserved nitric-oxide-metabolism in erythrocytes with reduced citrulline level and significantly higher NO-bioavailability were noted. Significant endothelial dysfunction in DM individuals was proved in response to the heat-stimulus. (4) Conclusions: DM patients at an early stage of disease show significant differences in the nitric-oxide-metabolic-pathway, which are more pronounced in the plasma compartment. Erythrocytes constitute a buffer with a higher nitric-oxide-bioavailability, less affected by the DM-related deviations. Patients at an early-stage of DM reveal endothelial-dysfunction, which could be diagnosed earlier using the laser-Doppler-flowmetry.

Keywords: erythrocyte; nitric oxide; ADMA; type 2 diabetes mellitus; endothelial dysfunction

1. Introduction

Type 2 diabetes mellitus (DM) is one of the most important health emergencies in the 21st century. The population suffering from DM is estimated for 463 million worldwide and is rapidly growing. Predictions say that the number may reach 578 million by 2030, and 700 million by 2045 [1].

DM is associated with higher relative risk of cardiovascular disorders (CVD) which is estimated between 1.6 and 2.6 and slightly higher in women [2]. Cardiovascular disease in DM patients occurs approximately 15 years earlier than in healthy subjects. It is the main cause of mortality in this group which is different based on sex-dependence. CVD mortality in men with diabetes increases 1–3 times compared to diabetes-free individuals, while the same coefficient estimated in women varies from 2 to 5 times [3]. It is not clear what plays the major role in inducing the differences, but the most important factor seems to be the attenuation of estrogens protective influence against cardiovascular complications

in DM women. It was proved that DM impairs endothelial response in women to a greater extent than among males [4]. DM diminishes antiproliferative effects of estrogen on the vascular smooth muscle cells by modifying expression, activity, and balance between the estrogen receptors (ER): ERα and ERβ. Domination of ERβ over ERα activity results in increased inflammatory profile, excessive reactive oxygen species (ROS) formation, and aggravated atherosclerotic plaque formation [5]. In that way, estrogens increase the nitric oxide (NO) bioavailability reducing NO inactivation and additionally may directly increase NO generation [6]. Furthermore, diabetic females are characterized by worse cardiovascular profile, including higher average HbA1c, LDL cholesterol, and BMI levels [4]. Catalan et al. proved that, despite the fact that men more often present carotid plaques in general, in a subgroup of newly diagnosed diabetic women, carotid atherosclerosis was more prevalent [7].

Cardiovascular risk in DM patients is highly dependent on endothelial dysfunction as well as micro- and macrovascular complications coexisting in DM. Among numerous factors, nitric oxide (NO) bioavailability and its metabolic pathway abnormalities seem to be the most crucial. Asymmetric dimethylarginine (ADMA) is a pivotal element leading to the NO synthesis disturbances by competitive inhibiting the nitric oxide synthase (NOS). Significant evidence indicates that not only ADMA concentration per se decides on endothelial function but also the decreased Arginine level and the Arginine/ADMA ratio seems to be additional and potentially more sensitive markers of endothelial dysfunction. The Arginine/ADMA ratio as an indicator of reduced global bioavailability of Arginine and NO production was postulated to be an independent CVD risk factor and to correspond with the severity of hypertension, congestive heart failure, and atherosclerosis [8,9].

Up to date, most of the studies have focused on the NO metabolism abnormalities being seen in the plasma compartment of diabetic subjects. Hardly any of them aims to evaluate the erythrocytes' role in this process. As the red blood cells (RBC) remain in constant contact with the endothelium and enable nitric oxide transport into distant hypoxic areas, their role in regulating the nitric oxide bioavailability might be underestimated. For many years erythrocytes have been considered to just eliminate the NO, which easily reacts with hemoglobin. However, recent studies pointed out that erythrocytes contain NOS and are able to produce NO. Subsequently they release it into the vasculature, regulating the blood flow in distal hypoxic regions [10]. It demonstrates that the conventional theory needs to be verified and the specific role of erythrocytes in the nitric oxide metabolism and its influence on endothelial dysfunction requires detailed evaluation.

Hence, the main goal of this study was to evaluate the nitric oxide metabolism abnormalities in plasma and erythrocytes of diabetic subjects with a close assessment of endothelial function using different tools. Additionally, it was intended to define which of the nitric oxide metabolites reflects better endothelial dysfunction at the early stages of diabetes mellitus.

2. Materials and Methods

2.1. Ethical Approval

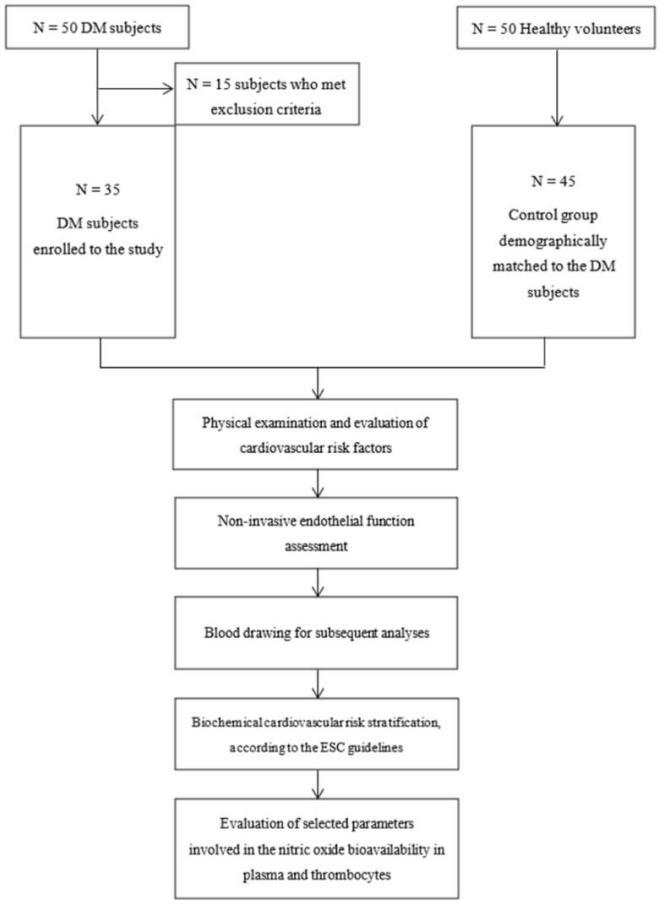
All experiments were conducted and approved in accordance with the guidelines of the Bioethics Committee at the Wroclaw Medical University (KB-155/2019) from 28 February 2019 and adhered to the principles of the Declaration of Helsinki (Seventh Revision, 64th World Medical Association meeting, Fortaleza, 2013). All of the individuals agreed to participate in the study by signing a written informed consent.

2.2. Patients

A total of 100 patients were investigated in the study. The inclusion criteria comprised diabetes mellitus diagnosed according to the American and Polish Diabetes Associations criteria, treated with oral metformin at age of 35–80 years. Subjects with the presence of diabetic complications, including microangiopathy, macrovascular diseases, past history of stroke, or myocardial infarction, as well as taking anticoagulant or antiplatelet treatment,

were excluded from this study. In order to exclude the potential variables affecting the differences between groups, we excluded subjects with concomitant hypertension. Finally, a total of 80 consecutive subjects met properly the inclusion and exclusion criteria and were enrolled to the study, including 35 patients with diabetes mellitus (female: 12, male: 23) and 45 healthy individuals qualified to the control group, respectively.

The control group was recruited from outpatient clinics in pursuance of demographic characteristics (age, sex, region), meeting the inclusion and exclusion criteria properly. All of them had previously undergone the screening for the presence of the glucose metabolism alterations, including diabetes, impaired fasting glycemia, impaired glucose tolerance, and insulin resistance. Subjects with any of these disturbances were excluded from the control group. All of the participants underwent a standard detailed physical examination (Scheme 1).



Scheme 1. A flow chart presenting the study protocol.

2.3. Blood Sample Collection

Forty-four milliliters of peripheral blood was collected using the Sarstedt S-Monovette® system (Sarstedt AG & Co., Nümbrecht, Germany). The sample (1.6 mg EDTA/mL blood) tubes within 30 min after the collection, were centrifuged at 1000× *g* for 15 min at 4 °C and stored at −80 °C until further analysis.

2.4. Erythrocytes Preparation

The erythrocytes were removed from the plasma within the 10 min following blood drowning. Erythrocytes samples were thawed on ice. Subsequently, a 10 µL of internal standards solution and a 1200 µL of cold extraction solution containing methanol, acetonitrile, and water (5:3:2) were added and vortexed (for 15 min, 1200 rpm at 4 °C). Samples were centrifuged (for 15 min, 22,000 rpm at 4 °C) and the supernatants were transferred into new microtubes. Samples were then dried at 55 °C and afterwards dissolved in 100 µL of water and vortexed (for 5 min, 1200 rpm at 25 °C). Subsequently, 50 µL of borate buffer (0.025 M $Na_2B_40_7 \times 10H_2O$, 1.77 mM NaOH, pH = 9.2), 400 µL of acetonitrile, and 10 µL of 10% benzoyl chloride (BCl) in acetonitrile were added and vortexed again (for 10 min, 1200 rpm, at 25 °C). After derivatization samples were dried at 55 °C using the Speed-Vac evaporator. Dried samples were reconstituted in 50 µL of 3% of methanol in water and centrifuged (for 10 min, 15,000 rpm, at 4 °C). Clear supernatant was transferred into chromatographic polypropylene vial with attached 200 µL insert.

2.5. Plasma Preparation

Plasma concentrations of metabolites involved in nitric oxide (NO) synthesis were measured according to method described by Fleszar et al. [11]. Briefly, a 100 µL of plasma, 50 µL of borate buffer, 10 µL of internal standard solution (100 µM D7-Arginine, 20 µM D7-ADMA, 25 µM D6-DMA, 100 µM D6-ornithine, and 50 µM D4-citrulline) were transferred into the 2 mL polypropylene tubes and mixed (for 1 min, 1200 rpm, at 25 °C). Then, 400 µL of acetonitrile and 10 µL of 10% BCl in acetonitrile were added and mixed (for 10 min, 1200 rpm, at 25 °C). Subsequently, samples were centrifuged (for 7 min, at 4 °C, 22,000 RCF) and 100 µL of clear supernatant was diluted four times with water, transferred to chromatographic glass vials, and analyzed.

2.6. Plasma and Erythrocytes Samples Analysis

The LC-MS/MS analysis was performed using the Acquity UPLC system (Waters, Milford, MA, USA) equipped with cooled autosampler. The sample temperature in an autosampler was 6 °C and the injection volume was 2 µL. The Waters BEH Shield C18 column (1.7 µm, 2.1 × 10 mm) was thermostatted in a column oven at 60 °C. The flow rate was 0.350 mL/min, and a total run time was 8 min. Eluents: A: water with 0.1% formic acid (FA), B: methanol with 0.1% FA. The following gradient was used: 0.0 min–3% B, 2.5 min–14% B, 4.6 min–60% B, 4.8 min–90% B, 6.1 min–3% B.

MS analysis was performed using the SYNAPT G2 Si mass spectrometer (Waters, Milford, MA, USA) equipped with electrospray ionization source (ESI) in a positive ionization mode. The spray voltage, source temperature and the de-solvation temperature were set at 0.5 kV, 140 °C, and 450 °C, respectively. Data acquisition was performed using the MassLynx software (Waters) for the following ions (m/z): 150.0919, 156.1295, 237.1239, 243.1339, 263.1090, 267.1382, 279.1457, 286.1897, 307.1770, and 314.2209 for DMA, D6-DMA, ornithine, D6-ornithine, citrulline, D4-cytrulline, Arginine, D7-Arginine, ADMA, symmetric dimethylarginine (SDMA), and D7-ADMA, respectively. Standard calibration curves were prepared using the following concentration ranges: 3 to 150 µM for ornithine, 5 to 250 µM for Arginine, 0.05 to 2.5 µM for ADMA and SDMA, 1 to 50 µM for citrulline, and 0.14 to 7 µM for DMA.

2.7. Laser Doppler

All the individuals underwent the laser Doppler flowmetry (LDF) measurements using the Perimed PeriFlux System 5000, Sweden, with a PeriFlux heating unit, performed strictly according to the manufacturer's instructions. The protocol was adapted from our previous study [12]. First, all the individuals were advised to rest for 15 min in horizontal position in a quiet laboratory at the room temperature 22 °C.

Afterward, their forearm was fixed by a vacuum pillow and the laser probe was placed on a skin, where no superficial veins were visible. The baseline flow and values were

obtained within the first 10 min with a probe heated to 33 °C. It was subsequently heated to 43 °C by the PeriFlux heating unit for the next 30 min of protocol. According to the previous studies [13–16], there are at least two independent mechanisms that are involved in the skin microvascular vasodilatation in response to local heating. First—a peak observed in laser Doppler flowmetry is caused by the fast-responding vasodilator axon reflex activated by heat-sensitive receptors on afferent nerves mediated by antidromic release of a vasodilatory neurotransmitters (calcitonin gene-related peptide (CGRP), substance P) [17]. After initial brisk increase, the plateau phase is observed, which is followed (after 10–20 min) by slowly responding vasodilator system based on nitric oxide production during prolonged local heating. In order to evaluate the change in the cutaneous blood flow, the maximum heating index (MHI) was counted. It is the ratio between areas under the curve of 10 min blood flow during maximum heating (late NO-dependent phase) to 10 min baseline flow (before heating). Using the MHI better reflects nitric-oxide dependent endothelial function, reducing the overlap influence of autonomic system.

2.8. EndoPAT

The profile of endothelial function was assessed using the EndoPAT 2000 device (Itamar Medical, Caesarea, Israel). The EndoPAT detects plethysmographic pressure changes in the fingertips, caused by the arterial pulse, where special sensors are placed and translates it to a peripheral arterial tone (PAT). After 6 min of basal register, the occlusion by sphygmomanometer cuff inflated 40 mmHg over systolic pressure was made. Occlusion measurement was done with additional 5 min of post occlusion measurement (hyperaemic period). The PAT values obtained from contralateral limb serve as a control to exclude the individual systemic changes in blood flow. The achieved data were calculated using a computerized automated algorithm provided with the device. The results were compared using the reactive hyperaemia index (RHI) and its logarithm (lnRHI), which corresponded with the endothelium-mediated changes in vascular tone after occlusion and reflected the arterial endothelial function. Additionally, the augmentation index (AI) was assessed by analyzing the two pressure peaks signals from ascending aorta generated during the cardiac cycle. The first peak was derived from the pulse wave generated by the left ventricle, where the second was the reflection from arterial walls which superimpose to the first ventricular wave. AI is defined by the difference in these pressures, expressed as a percentage of the measured pulse pressure [18]. Its value depends on the vascular stiffness and aorta elasticity. Since it was shown that the augmentation index is heart rate dependent, a more reliable indicator is the augmentation index normalized for a heart rate of 75 bpm (AI-75) [19]. The whole protocol was conducted in pursuance of the manufacturer's instructions. According to the Bonetti et al. study, the RHI cut-off value was set at RHI < 1,67 referring endothelial dysfunction [20]. All experiments were performed in an air-conditioned, quiet room with a constant air temperature of 20 °C.

2.9. Statistical Analysis

The data are presented as the mean ± SD. The differences between two continuous parameters were assessed using a Mann–Whitney U-test or a Student's *t*-test, following the Shapiro–Wilk test and Levene's test as appropriate. Additionally, Spearman's rank correlation coefficient was performed. All calculations were made with Statsoft® Statistica 13.3 software, Krakow, Poland and Graph Pad PRISM® 8.4 San Diego, CA, USA.

3. Results

3.1. Baseline Characteristics

The study diabetic subjects and healthy controls were matched with respect to the age and sex distribution. There were, however, significant differences in the waist to hip ratio (WHR), weight, body mass index, lipid and glucose metabolism (Table 1). Additionally, higher levels of high-sensitive CRP and uric acid with decreased magnesium and sodium

concentration were noted in the diabetic subjects. As compared to healthy individuals, patients suffering from DM had significantly higher HOMA-IR and lower QUICKI indexes.

Table 1. Demographic and biochemical characteristics between studied groups including cardiovascular risk stratification parameters. Results are presented as mean ± SD.

Parameter	Diabetes Group *n* = 35 (Mean ± SD)	Control Group *n* = 45 (Mean ± SD)	*p* Value
Age (year)	59.80 ± 9.00	55.42 ± 10.75	NS
Women (%)	12 (34%)	19 (42%)	NS
Weight (kg)	88.37 ± 14.49	78.16 ± 14.43	<0.05
WHR	0.96 ± 0.08	0.91 ± 0.12	<0.05
BMI (kg/m^2)	30.25 ± 4.07	25.80 ± 3.81	<0.05
WBC (k/µL)	7.12 ± 2.06	6.17 ± 1.46	NS
RBC (mln/µL)	4.71 ± 0.46	4.85 ± 0.58	NS
Hemoglobin (g/dL)	14.30 ± 1.24	14.58 ± 1.47	NS
Hematocrit (%)	42.04 ± 3.53	43.35 ± 4.68	NS
MCV (fL)	89.50 ± 4.33	89.48 ± 3.82	NS
MCH (pg)	30.47 ± 1.87	30.12 ± 1.41	NS
MCHC (g/dL)	33.99 ± 1.03	33.68 ± 1.13	NS
Platelets (k/µL)	253.27 ± 55.92	244.33 ± 55.69	NS
Glucose (mg/dL)	116 ± 39.11	93.30 ± 13.47	<0.05
HbA1 c (%)	6.14 ± 0.57	5.60 ± 0.30	<0.05
Insulin (uU/mL)	9.17 ± 5.38	6.65 ± 3.49	<0.05
Total cholesterol (mg/dL)	194.00 ± 49.90	214.38 ± 46.73	NS
LDL (mg/dL)	113.14 ± 42.24	130.57 ± 37.74	NS
HDL (mg/dL)	49.83 ± 11.70	56.98 ± 13.76	<0.05
Triglycerydes (mg/dL)	169.14 ± 108.98	134.26 ± 69.21	NS
Urea (mg/dL)	34.12 ± 6.59	33.03 ± 8.25	NS
Creatinine (mg/dL)	0.92 ± 0.15	0.96 ± 0.17	NS
eGFR (mL/min/1.73 m(2))	83.94 ± 14.49	79.26 ± 10.93	NS
Uric acid (mg/dL)	6.26 ± 1.68	5.44 ± 1.39	<0.05
Sodium (mmol/L)	139.41 ± 1.78	140.36 ± 2.42	<0.05
Potassium (mmol/L)	4.31 ± 0.28	4.22 ± 0.35	NS
Magnesium (mg/dL)	1.93 ± 0.23	2.12 ± 0.16	<0.05
Calcium (mmol/L)	9.70 ± 0.37	9.58 ± 0.38	NS
hsCRP (mg/L)	2.43 ± 2.42	1.94 ± 4.67	<0.05
TSH (ulU/mL)	1.66 ± 0.95	1.50 ± 0.67	NS
Alat (U/L)	27.53 ± 10.97	27.18 ± 12.63	NS
hsTroponin I (pg/mL)	2.31 ± 2.56	1.32 ± 1.53	NS
BNP (pg/mL)	29.16 ± 38.05	17.68 ± 15.28	NS
HOMA-IR	2.65 ± 1.56	1.58 ± 0.82	<0.05
HOMA beta	87.64 ± 119.41	90.17 ± 49.21	NS
QUICKI	0.34 ± 0.03	0.36 ± 0.03	<0.05

Abbreviations: NS: result statistically non-significant; BMI: body mass index; WHR: waist–hip ratio; WBC: white blood cells; RBC: red blood cells; MCV: mean (red blood) cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PDW: platelet distribution width; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; hsCRP: high-sensitivity C-reactive protein; TSH: thyroid-stimulating hormone; BNP: brain natriuretic peptide.

3.2. Assessment of Endothelial Function

Assessment of endothelial function by EndoPAT 2000 indicated a significantly higher augmentation index (AI) and the AI normalized for a heart rate of 75 beats/min in diabetic patients. As compared to healthy individuals, diabetics had a decreased vascular response observed in the laser Doppler flowmetry, as reflected by decreased maximum hyperaemia index (MHI). The endothelial function assessment is shown in Table 2 and Figure 1.

Table 2. Assessment of endothelial function by EndoPAT 2000 and the Laser Doppler Flowmetry.

Parameter	Diabetes (Mean ± SD)	Control (Mean ± SD)	*p* Value
RHI	2.19 ± 0.62	2.28 ± 0.59	NS
AI (%)	32.97 ± 22.50	17.76 ± 19.00	<0.05
AI-75 (%)	25.86 ± 21.18	10.45 ± 20.15	<0.05
MHI	9.17 ± 4.82	13.04 ± 7.44	<0.05

Abbreviations: NS: result statistically non-significant; RHI: reactive hyperaemia index (EndoPAT 2000); AI: augmentation index (EndoPAT 2000), AI-75 (%): augmentation index normalized for a heart rate of 75 beats/min (EndoPAT 2000), MHI: maximum hyperaemia index (Laser Doppler Flowmetry).

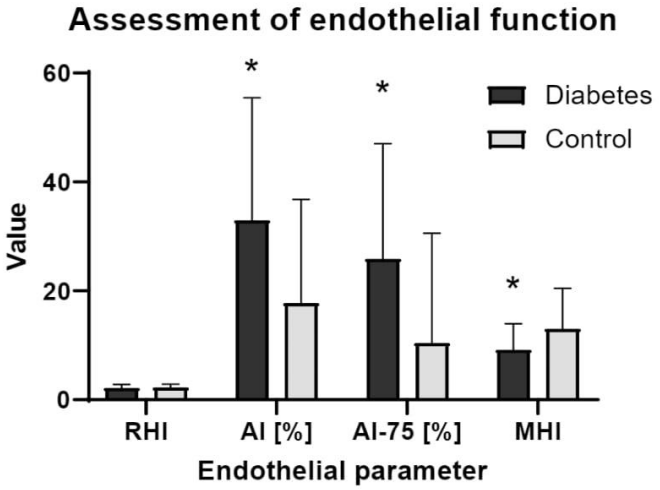
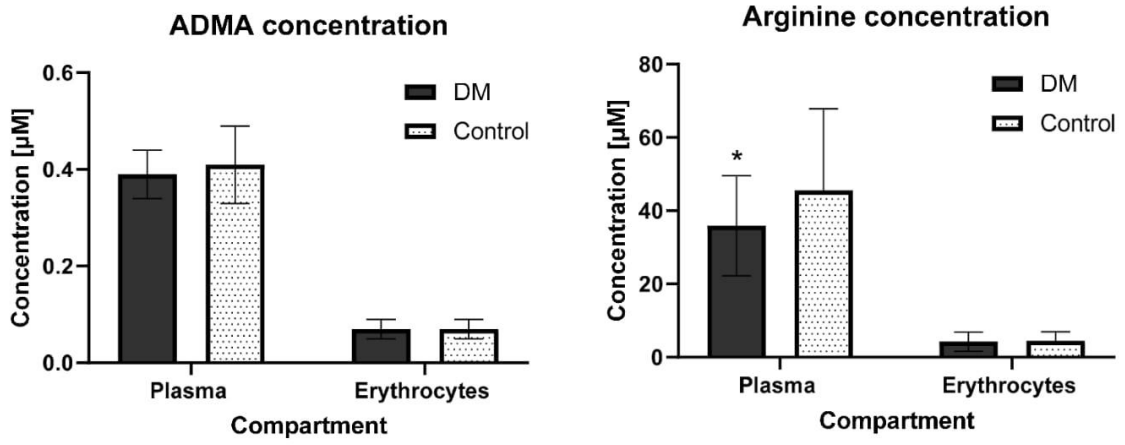


Figure 1. Assessment of endothelial function by EndoPAT 2000 and Laser Doppler Flowmetry. Abbreviations: RHI: reactive hyperaemia index (EndoPAT 2000); AI (%): augmentation index (EndoPAT 2000), AI-75 (%): augmentation index normalized for a heart rate of 75 beats/min (EndoPAT 2000), MHI: maximum hyperaemia index (Laser Doppler Flowmetry). Correlation of endothelial function with NO and biochemical metabolites in DM patients. *- $p < 0.05$ vs. control.

3.3. Parameters of the Nitric Oxide Bioavailability in Erythrocytes and Plasma

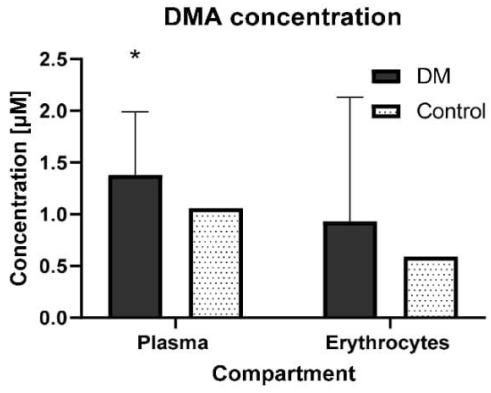
Three of the six evaluated metabolites of the nitric oxide pathway were found to be significantly different in the plasma compartment (Arginine, DMA, citrulline). One was found to be altered in the erythrocytes (citrulline), when compared to the control group. The citrulline level was decreased in both compartments among DM patients, which was more noticeable in the erythrocytes. Simultaneously, the Arginine level was reduced in plasma, with no differences in the erythrocyte levels between groups. On the contrary, the dimethylamine (DMA) concentration was increased in plasma which was consecutively not observed in red blood cells (RBCs). All of the alterated nitric oxide metabolites were found at higher concentrations in plasma in both groups.

In order to define the involvement of particular pathways in the alterations of the nitric oxide biotransformation the product to substrate ratios both, in the plasma and erythrocyte compartment in particular reactions were assessed. The study revealed significantly increased ornithine/Arginine and decreased ADMA/DMA ratio in the plasma of DM subjects. Additionally, reduced Arginine/ADMA ratio was noted in plasma with preserved proportion in erythrocytes. By comparing these ratios, higher NO bioavailability in the RBC of diabetic subjects was also identified. Futhermore, Arginine and citrulline compartmental concentration ratio were proved to be statistically different between the groups (Table 3 and Figures 2–4).



(a)

(b)



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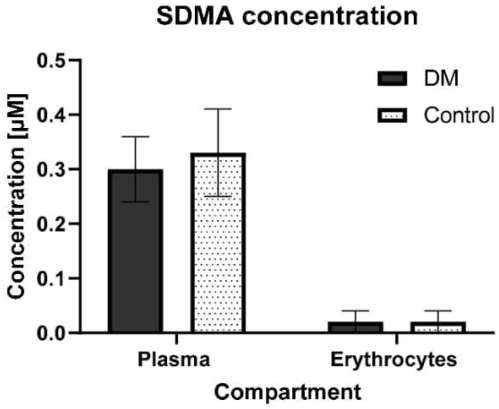

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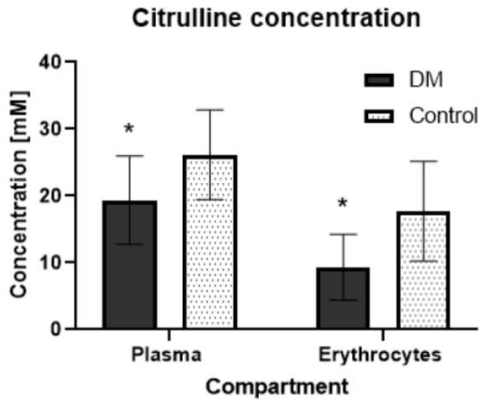

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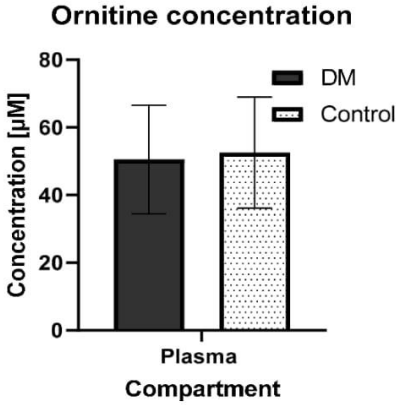
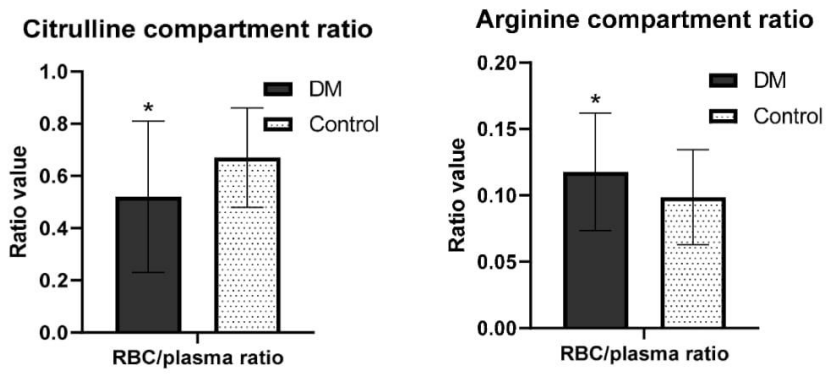


(f)

Figure 2. (**a**–**f**) Nitric oxide metabolites concentration in different compartments. *- $p < 0.05$ vs. control.



(**a**) (**b**)

Figure 3. (**a**,**b**) Citrulline and Arginine compartment ratios. *- $p < 0.05$ vs. control.

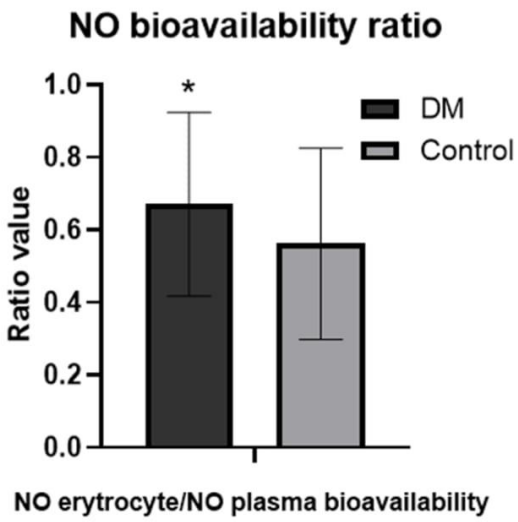


Figure 4. Nitric oxide (NO) bioavailability ratio. *- $p < 0.05$ vs. control.

Table 3. The Nitric oxide pathway metabolites ratios.

Plasma Ratios	DM Group (Mean ± SD)	Control Group (Mean ± SD)	*p* Value	RBC Ratios	DM Group (Mean ± SD)	Control Group (Mean ± SD)	*p* Value	Plasma/RBC Ratios	DM Group (Mean ± SD)	Control Group (Mean ± SD)	*p* Value
Arginine/ ADMA	94.6 ± 40.3	116.5 ± 50.4	<0.05	Arginine/ ADMA	65.03 ± 40.97	62.72 ± 34.66	NS	$\frac{\text{Citrulline}_{RBC}}{\text{Citrulline}_{plasma}}$	0.52 ± 0.29	0.67 ± 0.19	<0.05
ADMA/DMA	0.37 ± 0.23	0.47 ± 0.24	<0.05	ADMA/DMA	0.06 ± 0.04	0.07 ± 0.07	NS	$\frac{\text{Arginine}_{RBC}}{\text{Arginine}_{plasma}}$	0.12 ± 0.04	0.10 ± 0.04	<0.05
Ornitine/ Arginine	1.69 ± 0.90	1.18 ± 0.59	<0.05								

Abbreviations: RBC: red blood cells.

3.4. Assessment of the Relationship between Biochemical Results and Endothelial Function

The analysis of biochemical results and endothelial function revealed a moderate correlation between AI and eGFR and BNP. A similar correlation was found between endothelial function measured using EndoPAT and ADMA concentration, ADMA/Arginine ratio, and NO bioavailability in the RBC compartment with no such dependence in the plasma of DM patients (Figure 5).

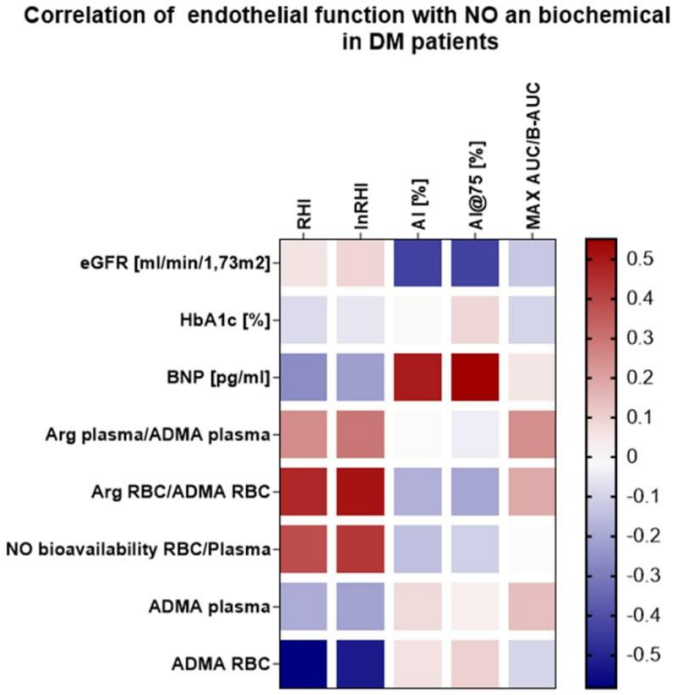


Figure 5. Correlation of endothelial function with NO and biochemical metabolites in DM patients. Abbreviations: RHI: reactive hyperaemia index (EndoPAT 2000); AI (%): augmentation index (EndoPAT 2000), AI-75 (%): augmentation index normalized for a heart rate of 75 beats/min (EndoPAT 2000), MHI: maximum hyperaemia index (Laser Doppler Flowmetry); RBC: red blood cells; eGFR: estimated glomerular filtration rate; BNP: brain natriuretic peptide; ADMA: Asymmetric dimethylarginine; Arg: L-Arginine.

4. Discussion

To our knowledge, this is the first research that evaluates the nitric oxide metabolic pathway both, in erythrocyte and plasma compartments. Furthermore, the erythrocyte nitric oxide metabolism and endothelial dysfunction in diabetic subjects were assessed. Recent findings emphasize the key role of endothelial dysfunction in diabetic patients as the first step leading to the development of vascular complications and organ damage.

Insulin resistance, a hallmark of metabolic syndrome, impairs vascular response and increases cardiovascular risk. Involvement of insulin resistance and endothelial dysfunction in pathological disorders contribute to impairment in the NO-dependent vasodilatation, cellular glucose uptake, enhancement in oxidative stress, and inflammation, leading finally to atherosclerosis. Strong association of insulin and endothelial signaling disturbances contributes inflammation, disrupting the balance between vasodilating–vasoconstrictive endothelial mechanisms as well as between the insulin-dependent PI3-K/Akt–MAPK/ERK pathways [21].

For many years erythrocytes have been considered to be a natural sink for the NO molecules because of hemoglobin abundance which easily scavenge NO. However, recent

studies give more evidence that erythrocytes' role is far more complex, and they appear to be an important source of NO. In line with the studies that confirmed the activity of NOS in erythrocytes, the research proved the existence of nitric oxide metabolites and enzymatic inhibitors in RBCs. Besides citrulline concentration, no other alterations in the nitric oxide metabolites in erythrocytes of diabetic patients were found. It indicates that erythrocytes remain a buffer which is less affected by the deviations connected with endothelial dysfunction and that the synthesis of nitric oxide in erythrocytes remain unaltered in early diabetic individuals. Nitric oxide bioavailability in different compartments was also compared using the Arginine/asymmetric dimethylarginine ratio, which is a well-established marker of global Arginine bioavailability and the NO production. The analysis revealed its significantly higher values in the RBC which points out, even more distinctly, the importance of erythrocytes in preserving NO production in DM (Figure 6).

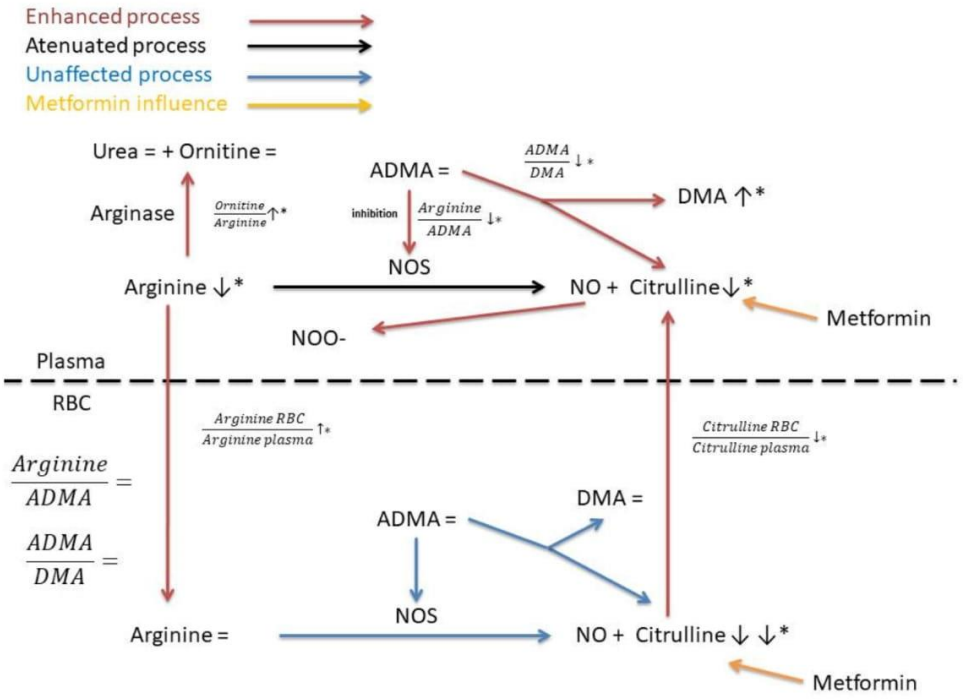


Figure 6. Nitric oxide metabolism alteration in different compartments. Abbreviations: ADMA: asymmetric dimethylarginine; DMA: dimethylamine; NOS: nitric oxide synthase; NO: nitric oxide RBC: erythrocyte SDMA: symmetric dimethylarginine. *- $p < 0.05$ vs. control.

Contrary to the RBC compartment, substantial abnormalities in the NO synthesis in the plasma of DM patients were confirmed. First of all, a significantly reduced Arginine concentration was shown. It may be caused by shunting the L-Arginine from the eNOS to the arginase pathway with its enhanced arginase degradation, as an increased ornithine to Arginine ratio in plasma was found [18,22,23]. It is noteworthy that the results are in accordance with the studies by Shemyakin et al., proving that the downregulation of arginase improves endothelial function among patients with DM and may become a promising therapeutic target [24].

Additionally, enhanced Arginine transport from plasma to erythrocytes were found as Arginine compartment ratios were significantly different. Increased transport via system y+ in human erythrocytes had already been confirmed in different diseases affecting nitric oxide transformation, such as renal failure and chronic heart diseases [25,26]. It

may indicate that the same upregulation of y+ transport occurred in the DM group as a compensatory response to maintain the NO production.

Furthermore, low level of Arginine leads to the NOS uncoupling and results in excessive reactive oxygen species (ROS) formation, which also stems from NADPH oxidase (NOX) and mitochondrial complexes reactions [27]. As a result, the NO bioavailability is reduced in different ways—by decreased production because of substrate depletion and increased elimination through reaction with reactive oxygen species.

Moreover, significantly reduced Arginine to ADMA ratio in the DM group was also revealed, pointing at reduced NOS action and a subsequently diminished NO production. As no difference in ADMA concentration per se was found nor an increased DMA level, it collectively points at higher ADMA turnover in DM patients. Current literature is inconclusive in the assessment of dimethylarginine.

Dimethylaminohydrolase (DDAH) activity in diabetics, which is related with ADMA concentration. Numerous studies suggest that the high glucose level accompanied by increased concentration of proinflammatory cytokines, such as tumour necrosis factor (TNF), results in decreased DDAH activity and consequent increased ADMA level [28–32]. Our study cohort was composed of early-stage diabetic patients treated with metformin without the micro- and macrovascular complications. The low HbA1c levels reflect good glucose control, which may progressively result in preserved DDAH activity. This observation is consistent with the Xiong et al. study, proving the correlation of ADMA level with the macroangiopathy occurrence but not with the disease duration [33]. Additionally, DM patients were treated with metformin, which is a structural analog of ADMA reducing its concentration, possibly in the DDAH-dependent manner [34–37]. Furthermore, metformin reduces the advanced glycation end-products (AGEs) concentration what additionally restores the NO bioavailability [38].

Significantly lower plasma values of the citrulline in DM patients resulting from reduced eNOS activity caused by depletion of Arginine and increased inhibition were also found. Additionally, the use of metformin reduces citrulline levels [39,40]. According to Breier, M. et al. studies, short-term therapy with metformin reduces citrulline concentration by mean of 24% which is in compliance with results of this study—a 26% depletion on average of citrulline concentration between groups [39]. The mechanism of metformin-dependent citrulline reduction is not clear. Some authors seek the mechanism in urea cycle activity changes, inhibition of mitochondrial complex I, increased urinary excretion by kidneys, or decreased gut absorption associated with gastrointestinal side effects of metformin [39,41–43]. Reduced citrulline concentration in the erythrocyte compartment, more pronounced in the DM group was also noted.

In this study, the endothelial function using the two independent methods was also evaluated. First, the vascular response was assessed using the peripheral arterial tonometry with a post-occlusive reactive hyperaemia (PORH) measured as a reactive hyperaemia index (RHI). Second, with the laser Doppler flowmetry (LDF). A diminished endothelial function was found only by using the second method.

The pathophysiology of the vascular relaxation in response to numerous stimuli (heating or occlusion) is different and may explain to some extent, why only local thermal hyperemia (LTH) indicates endothelial dysfunction in the diabetic subjects. The sensory nerves response and endothelium-derived hyperpolarizing factors have been described as the major points affecting both, the initial peak and the following increased blood flow after occlusion. Putative elements affecting PORH include the cytochrome epoxygenase metabolites and the large-conductance calcium-activated potassium channels (BKCa) in the vascular smooth muscle cells and in sensory nerves [44–46]. Hence, compared to other mechanisms regulating vascular response, the role of nitric oxide pathway in the post-occlusion response turned out not to be so crucial [47,48].

On the contrary, in the LDF response to heating stimuli, nitric oxide is responsible for vascular response in approximately 70% [49]. The first phase is mediated by a local sensory nerve axon reflex and the plateau depends mostly on the NO [45,49]. The maximum

hyperemia index (MHI) was used to eliminate the neuronal influence. It reflects the nitric oxide-dependent plateau phase and expresses the endothelial function. Applying MHI results in a higher sensitivity and explains the discrepancies between the LTH and PORH outcomes. The results of this research are in line with Faisel and Heier studies, who confirmed endothelial dysfunction in diabetes mellitus type 1 group using LDF with intact vascular reaction in peripheral arterial tonometry [50,51].

The results showed that the augmentation index (AI) in diabetics was statistically higher, strictly corresponding with the increased vascular stiffness and the reduced flexibility of the aorta among diabetic patients Additionally, the AI-75 positively correlated with BNP concentration ($r = 0.55$) and negatively with the eGFR value ($r = -0.43$). It reflects that arterial stiffness in DM patients appears at the early stage of the disease and induces the organ-mediated complications from the beginning.

The nitric oxide metabolic pathway was also compared by correlating the products' concentrations with the endothelial function results. The analysis confirmed that the endothelial function in DM patients correlated with NO bioavailability and NOS activity more in the RBC compartment than in plasma ($r = 0.38$ and $r = 0.46$). Furthermore, similar dependency was found within the erythrocyte ADMA concentration ($r = -0.58$) (Figure 2). Hence, it may be postulated that erythrocytes play an important role in compensating the endothelial dysfunction occurring in DM. Further studies are needed to evaluate their specific position in the NO metabolism and to define novel therapeutic targets to prevent the DM micro- and macrovascular complications.

5. Conclusions

Patients at an early stage of DM revealed endothelial dysfunction, which could be diagnosed earlier using the laser Doppler flowmetry. This group of subjects showed significant differences in the nitric oxide metabolism, which was more pronounced in the plasma compartment. The research proved decreased NOS activity with aggravated ADMA degradation and Arginine depletion. It indicated that erythrocytes remain a buffer less affected by the DM deviations with higher NO bioavailability than in the plasma compartment. Additionally, it was revealed that endothelial dysfunction correlates to a greater extent with abnormalities of nitric oxide pathway noted in RBC than in plasma compartment. The disclosed findings show the importance of the RBC as a NO-buffer. Hence, the RBCs play a key-role in maintenance a healthy vascular status in those with early diabetes mellitus. Additional studies in that field should be performed in order to extend the knowledge regarding the RBC NO-buffer, which may be used in the prevention or treatment of vascular complications in the diabetic group.

6. Limitations

Several limitations of this study should be addressed. The first limitation regards the measured molecules. As NO easily reacts with hemoglobin it was not possible to measure it directly and our results are mainly based on by-products which are more stable. Furthermore, different permeability of erythrocyte membrane may affect compartment distribution and study outcomes. The separation process might affect the analytes equilibrium, therefore additional experiments should be conducted to assess the potential significance of that process. In this study, subjects with newly onset of diabetes, without concomitant cardiovascular disorders, were investigated. Hence, the results of this study cannot be simply extrapolated to the whole spectrum of diabetic population.

Author Contributions: Conceptualization, A.D. and D.G.; methodology, A.D., J.W., P.F., and E.S.-K.; software, D.G. and A.D.; validation, D.G., A.D., and J.G.; formal analysis, D.G. and A.D.; investigation, D.G. and J.G.; resources, A.D.; data curation, D.G.; writing—original draft preparation, D.G.; writing—review and editing, A.D.; visualization, D.G.; supervision, A.D.; project administration, J.G. and E.S.-K.; funding acquisition, J.G. and A.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee at the Wroclaw Medical University (KB-155/2019) from 28 February 2019.

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

Data Availability Statement: The original data used to support the findings of this study are available from the corresponding author upon request.

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

Abbreviations

ADMA	asymmetric dimethylarginine
AGEs	advanced glycation end-products
AI	augmentation index
AI-75 [%]	augmentation index normalized for a heart rate of 75 beats/min
Arg	L-Arginine
BCl	benzoyl chloride
BMI	body mass index
BNP	brain natriuretic peptide
CVD	cardiovascular disorders
DDAH	Dimethylaminohydrolase
DM	Diabetes mellitus
DMA	dimethylamine
eGFR	estimated glomerular filtration rate
ER	estrogen receptors
ESC	European Society of Cardiology
HbA1c	glycated hemoglobin
HDL	high-density lipoprotein
hsCRP	high-sensitivity C-reactive protein
LDF	laser Doppler flowmetry
LDL	low-density lipoprotein
lnRHI	reactive hyperaemia index logarithm
LTH	local thermal hyperemia
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean (red blood) cell volume
MHI	maximum heating index
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
NS	result statistically non-significant
PAT	peripheral arterial tone
PDW	platelet distribution width
PORH	post-occlusive reactive hyperaemia
RBC	red blood cells
RHI	reactive hyperemia index
ROS	reactive oxygen species
SDMA	symmetric dimethylarginine
TNF	tumour necrosis factor
TSH	thyroid-stimulating hormone
WBC	white blood cells
WHR	waist–hip ratio

References

1. International Diabetes Federation. *International Diabetes IDF Diabetes Atlas*, 9th ed.; International Diabetes Federation: Brussels, Belgium, 2019.
2. Sarwar, N.; Gao, P.; Kondapally Seshasai, S.R.; Gobin, R.; Kaptoge, S.; Di Angelantonio, E.; Ingelsson, E.; Lawlor, D.A.; Selvin, E.; Stampfer, M.; et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: A collaborative meta-analysis of 102 prospective studies. *Lancet* **2010**, *375*, 2215–2222. [PubMed]
3. Lee, W.; Cheung, A.; Cape, D.; Zinman, B. Impact of Diabetes on Coronary Artery A meta-analysis of prospective studies. *Diabetes Care* **2000**, *23*, 962–968. [CrossRef]
4. Ballotari, P.; Ranieri, S.C.; Luberto, F.; Caroli, S.; Greci, M.; Rossi, P.G.; Manicardi, V. Sex Differences in Cardiovascular Mortality in Diabetics and Nondiabetic Subjects: A Population-Based Study (Italy). *Int. J. Endocrinol.* **2015**, *2015*, 1–10. [CrossRef] [PubMed]
5. Dantas, A.P.V.; Fortes, Z.B.; de Carvalho, M.H.C. Vascular Disease in Diabetic Women: Why Do They Miss the Female Protection? *Exp. Diabetes Res.* **2012**, *2012*, 1–10. [CrossRef]
6. Novensa, L.; Selent, J.; Pastor, M.; Sandberg, K.; Heras, M.; Dantas, A.P. Equine Estrogens Impair Nitric Oxide Production and Endothelial Nitric Oxide Synthase Transcription in Human Endothelial Cells Compared with the Natural 17β-Estradiol. *Hypertens* **2010**, *56*, 405–411. [CrossRef] [PubMed]
7. Catalan, M.; Herreras, Z.; Pinyol, M.; Sala-Vila, A.; Amor, A.; de Groot, E.; Gilabert, R.; Ros, E.; Ortega, E. Prevalence by sex of preclinical carotid atherosclerosis in newly diagnosed type 2 diabetes. *Nutr. Metab. Cardiovasc. Dis.* **2015**, *25*, 742–748. [CrossRef] [PubMed]
8. Yu, E.; Ruíz-Canela, M.; Hu, F.B.; Clish, C.B.; Corella, L.; Salas-Salvadó, J.; Hruby, A.; Fíto, 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]
9. Bode-Böger, S.; Scalera, F.; Ignarro, L. The l-arginine paradox: Importance of the l-arginine/asymmetrical dimethylarginine ratio. *Pharmacol. Ther.* **2007**, *114*, 295–306. [CrossRef] [PubMed]
10. Cortese-Krott, M.M.; Kelm, M. Endothelial nitric oxide synthase in red blood cells: Key to a new erythrocrine function? *Redox Biol.* **2014**, *2*, 251–258. [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**, *1*, 911–921.
12. Doroszko, A.; Szahidewicz-Krupska, E.; Janus, A.; Jakubowski, M.; Turek, A.; Ilnicka, P.; Szuba, A.; Mazur, G.; Derkacz, A. Endothelial dysfunction in young healthy men is associated with aspirin resistance. *Vasc. Pharmacol.* **2015**, *67–69*, 30–37. [CrossRef]
13. Kellogg, D.L. In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. *J. Appl. Physiol.* **2006**, *100*, 1709–1718. [CrossRef] [PubMed]
14. Farrell, D.M.; Bishop, V.S. Permissive role for nitric oxide in active thermoregulatory vasodilation in rabbit ear. *Am. J. Physiol. Circ. Physiol.* **1995**, *269*, H1613–H1618. [CrossRef]
15. Shibasaki, M.; Wilson, T.E.; Cui, J.; Crandall, C.G. Acetylcholine released from cholinergic nerves contributes to cutaneous vasodilation during heat stress. *J. Appl. Physiol.* **2002**, *93*, 1947–1951. [CrossRef]
16. Shastry, S.; Dietz, N.M.; Halliwill, J.R.; Reed, A.S.; Joyner, M.J. Effects of nitric oxide synthase inhibition on cutaneous vasodilation during body heating in humans. *J. Appl. Physiol.* **1998**, *85*, 830–834. [CrossRef]
17. Minson, C.T.; Berry, L.T.; Joyner, M.J. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J. Appl. Physiol.* **2001**, *91*, 1619–1626. [CrossRef]
18. Beleznai, T.; Feher, A.; Spielvogel, D.; Lansman, S.L.; Bagi, Z. Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. *Am. J. Physiol. Circ. Physiol.* **2011**, *300*, H777–H783. [CrossRef] [PubMed]
19. Lantelme, P.; Mestre, C.; Lievre, M.; Gressard, A.; Milon, H. Heart rate: An important confounder of pulse wave velocity assessment. *Hypertension* **2002**, *39*, 1083–1087. [CrossRef] [PubMed]
20. Bonetti, P.O.; Pumper, G.M.; Higano, S.T.; Holmes, D.R.; Kuvin, J.T.; Lerman, A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *J. Am. Coll. Cardiol.* **2004**, *44*, 2137–2141. [CrossRef]
21. Janus, A.; Szahidewicz-Krupska, E.; Mazur, G.; Doroszko, A. Insulin Resistance and Endothelial Dysfunction Constitute a Common Therapeutic Target in Cardiometabolic Disorders. *Mediat. Inflamm.* **2016**, *2016*, 1–10. [CrossRef]
22. Hein, T.W.; Omae, T.; Xu, W.; Yoshida, A.; Kuo, L. Role of Arginase in Selective Impairment of Endothelium-Dependent Nitric Oxide Synthase-Mediated Dilation of Retinal Arterioles during Early Diabetes. *Investig. Opthalmol. Vis. Sci.* **2020**, *61*, 36. [CrossRef]
23. Kashyap, S.R.; Lara, A.; Zhang, R.; Park, Y.M.; DeFronzo, R.A. Insulin Reduces Plasma Arginase Activity in Type 2 Diabetic Patients. *Diabetes Care* **2007**, *31*, 134–139. [CrossRef]
24. Shemyakin, A.; Kövamees, O.; Rafnsson, A.; Böhm, F.; Svenarud, P.; Settergren, M.; Jung, C.; Pernow, J. Arginase Inhibition Improves Endothelial Function in Patients With Coronary Artery Disease and Type 2 Diabetes Mellitus. *Circulation* **2012**, *126*, 2943–2950. [CrossRef] [PubMed]

25. Mendes Ribeiro, A.C.; Hanssen, H.; Kiessling, K.; Roberts, N.B.; Mann, G.E.; Ellory, J.C. Transport of L-arginine and the nitric oxide inhibitor N(G)-monomethyl-L-arginine in human erythrocytes in chronic renal failure. *Clin. Sci.* **1997**, *93*, 57–64. [CrossRef]
26. Hanssen, H.; Brunini, T.M.C.; Conway, M.; Banning, A.P.; Roberts, N.B.; Mann, G.E.; Ellory, J.C.; Ribeiro, A.C.M. Increased l-arginine Transport in Human Erythrocytes in Chronic Heart Failure. *Clin. Sci.* **1998**, *94*, 43–48. [CrossRef] [PubMed]
27. Zhou, Z.; Mahdi, A.; Tratsiakovich, Y.; Zahorán, S.; Kövamees, O.; Nordin, F.; Gonzalez, A.E.U.; Alvarsson, M.; Östenson, C.-G.; Andersson, D.C.; et al. Erythrocytes From Patients With Type 2 Diabetes Induce Endothelial Dysfunction Via Arginase I. *J. Am. Coll. Cardiol.* **2018**, *72*, 769–780. [CrossRef] [PubMed]
28. Yamagishi, S.-I.; Ueda, S.; Nakamura, K.; Matsui, T.; Okuda, S. Role of Asymmetric Dimethylarginine (ADMA) in Diabetic Vascular Complications. *Curr. Pharm. Des.* **2008**, *14*, 2613–2618. [CrossRef] [PubMed]
29. Murphy, R.B.; Tommasi, S.; Lewis, B.C.; Mangoni, A.A. Inhibitors of the Hydrolytic Enzyme Dimethylarginine Dimethylaminohydrolase (DDAH): Discovery, Synthesis and Development. *Molecules* **2016**, *21*, 615. [CrossRef]
30. Palm, F.; Onozato, M.L.; Luo, Z.; Wilcox, C.S. Dimethylarginine dimethylaminohydrolase (DDAH): Expression, regulation, and function in the cardiovascular and renal systems. *Am. J. Physiol. Circ. Physiol.* **2007**, *293*, H3227–H3245. [CrossRef] [PubMed]
31. Bestermann, W.H. The ADMA-Metformin Hypothesis: Linking the Cardiovascular Consequences of the Metabolic Syndrome and Type 2 Diabetes. *Cardiorenal Med.* **2011**, *1*, 211–219. [CrossRef]
32. Wadham, C.; Mangoni, A.A. Dimethylarginine dimethylaminohydrolase regulation: A novel therapeutic target in cardiovascular disease. *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 303–319. [CrossRef]
33. Xiong, Y.; Lei, M.; Fu, S.; Fu, Y. Effect of diabetic duration on serum concentrations of endogenous inhibitor of nitric oxide synthase in patients and rats with diabetes. *Life Sci.* **2005**, *77*, 149–159. [CrossRef]
34. Asagami, T.; Abbasi, F.; Stuelinger, M.; Lamendola, C.; McLaughlin, T.; Cooke, J.; Reaven, G.; Tsao, P. Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes. *Metabolism* **2002**, *51*, 843–846. [CrossRef]
35. Tsai, C.-M.; Kuo, H.-C.; Hsu, C.-N.; Huang, L.-T.; Tain, Y.-L. Metformin reduces asymmetric dimethylarginine and prevents hypertension in spontaneously hypertensive rats. *Transl. Res.* **2014**, *164*, 452–459. [CrossRef]
36. Stühlinger, M.C.; Abbasi, F.; Chu, J.W.; Lamendola, C.; McLaughlin, T.L.; Cooke, J.; Reaven, G.M.; Tsao, P.S. Relationship Between Insulin Resistance and an Endogenous Nitric Oxide Synthase Inhibitor. *JAMA* **2002**, *287*, 1420–1426. [CrossRef] [PubMed]
37. Bal, F.; Bekpinar, S.; Unlucerci, Y.; Kiraz, Z.K.; Önder, S.; Uysal, M.; Gurdol, F. Antidiabetic drug metformin is effective on the metabolism of asymmetric dimethylarginine in experimental liver injury. *Diabetes Res. Clin. Pract.* **2014**, *106*, 295–302. [CrossRef] [PubMed]
38. Andújar-Plata, P.; Pi-Sunyer, X.; Laferrère, B. Metformin effects revisited. *Diabetes Res. Clin. Pract.* **2012**, *95*, 1–9. [CrossRef] [PubMed]
39. Breier, M.; Wahl, S.; Prehn, C.; Ferrari, U.; Sacco, V.; Weise, M.; Grallert, H.; Adamski, J.; Lechner, A. Immediate reduction of serum citrulline but no change of steroid profile after initiation of metformin in individuals with type 2 diabetes. *J. Steroid Biochem. Mol. Biol.* **2017**, *174*, 114–119. [CrossRef]
40. Adam, J.; Brandmaier, S.; Leonhardt, J.; Scheerer, M.F.; Mohney, R.P.; Xu, T.; Bi, J.; Rotter, M.; Troll, M.; Chi, S.; et al. Metformin Effect on Nontargeted Metabolite Profiles in Patients With Type 2 Diabetes and in Multiple Murine Tissues. *Diabetes* **2016**, *3*, 23. [CrossRef] [PubMed]
41. Owen, M.R.; Doran, E.; Halestrap, A.P. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem. J.* **2000**, *348*, 607–614. [CrossRef]
42. Irving, B.A.; Carter, R.E.; Soop, M.; Weymiller, A.; Syed, H.; Karakelides, H.; Bhagra, S.; Short, K.; Tatpati, L.; Barazzoni, R.; et al. Effect of Insulin Sensitizer Therapy on Amino Acids and Their Metabolites. *Metabolism* **2015**, *64*, 720–728. [CrossRef]
43. Irving, B.A.; Spielmann, G. Does Citrulline Sit at the Nexus of Metformin's Pleotropic Effects on Metabolism and Mediate Its Salutatory Effects in Individuals with Type 2 Diabetes? *Diabetes* **2016**, *65*, 3537–3540. [CrossRef]
44. Lorenzo, S.; Minson, C.T. Human cutaneous reactive hyperaemia: Role of BK Ca channels and sensory nerves. *J. Physiol.* **2007**, *585*, 295–303. [CrossRef]
45. Roustit, M.; Cracowski, J.-L. Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends Pharmacol. Sci.* **2013**, *34*, 373–384. [CrossRef]
46. Cracowski, J.-L.; Gaillard-Bigot, F.; Cracowski, C.; Sors, C.; Roustit, M.; Millet, C. Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans. *J. Appl. Physiol.* **2013**, *114*, 245–251. [CrossRef] [PubMed]
47. Wong, B.; Wilkins, B.W.; Holowatz, L.A.; Minson, C.T. Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation. *J. Appl. Physiol.* **2003**, *95*, 504–510. [CrossRef]
48. Zhao, J.L.; Pergola, P.E.; Roman, L.J.; Kellogg, D.L. Bioactive nitric oxide concentration does not increase during reactive hyperemia in human skin. *J. Appl. Physiol.* **2004**, *96*, 628–632. [CrossRef]
49. Brunt, V.E.; Minson, C.T. Cutaneous thermal hyperemia: More than skin deep. *J. Appl. Physiol.* **2011**, *111*, 5–7. [CrossRef] [PubMed]
50. Khan, F.; Elhadd, T.A.; Greene, S.A.; Belch, J.J.F. Impaired Skin Microvascular Function in Children, Adolescents, and Young Adults with Type 1 Diabetes. *Diabetes Care* **2000**, *23*, 215–220. [CrossRef]
51. Heier, M.; Espeland, C.N.; Brunborg, C.; Seljeflot, I.; Margeirsdottir, H.D.; Hanssen, K.F.; Fugelseth, D.; Dahl-Jørgensen, K. Preserved endothelial function in young adults with type 1 diabetes. *PLoS ONE* **2018**, *13*, e0206523. [CrossRef] [PubMed]

5. OŚWIADCZENIA WSPÓŁAUTORÓW

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OŚWIADCZENIE

Oświadczam, że w pracy *Gajecki, Damian, Jakub Gawryś, Ewa Szahidewicz-Krupska, and Adrian Doroszko. 2022. "Role of Erythrocytes in Nitric Oxide Metabolism and Paracrine Regulation of Endothelial Function"* Antioxidants *11, no. 5: 943.* https://doi.org/10.3390/antiox11050943 mój udział polegał na stworzeniu koncepcji manuskryptu, nadzorowaniu jego powstawania oraz wprowadzeniu poprawek merytorycznych i językowych do pierwotnej wersji manuskryptu.

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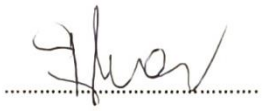

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OŚWIADCZENIE

Oświadczam, że w pracy *Gajecki, Damian, Jakub Gawryś, Jerzy Wiśniewski, Paulina Fortuna, Ewa Szahidewicz-Krupska, and Adrian Doroszko. 2021. "A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus" Nutrients 13, no. 7: 2306. https://doi.org/10.3390/nu13072306* mój udział polegał na stworzeniu koncepcji manuskryptu, nadzorowaniu jego powstawania oraz wprowadzeniu poprawek merytorycznych i językowych do pierwotnej wersji manuskryptu.

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Oświadczam, że w pracy *Gajecki, Damian, Jakub Gawryś, Jerzy Wiśniewski, Paulina Fortuna, Ewa Szahidewicz-Krupska, and Adrian Doroszko. 2021. "A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus" Nutrients 13, no. 7: 2306. https://doi.org/10.3390/nu13072306* mój udział polegał na współudziale w rekrutacji i badaniu grupy badanej i kontrolnej projektu oraz tworzeniu bazy danych klinicznych potrzebnych do analizy statystycznej.

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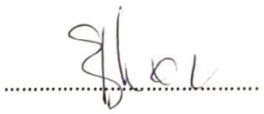

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Oświadczam, że w pracy *Gajecki, Damian, Jakub Gawryś, Jerzy Wiśniewski, Paulina Fortuna, Ewa Szahidewicz-Krupska, and Adrian Doroszko. 2021. "A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus" Nutrients 13, no. 7: 2306. https://doi.org/10.3390/nu13072306* mój udział polegał na przeprowadzeniu oznaczeń biochemicznych w materiale biologicznym zabezpieczonym od uczestników projektu.

Podpis

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OŚWIADCZENIE

Oświadczam, że w pracy *Gajecki, Damian, Jakub Gawryś, Jerzy Wiśniewski, Paulina Fortuna, Ewa Szahidewicz-Krupska, and Adrian Doroszko. 2021. "A Cross-Talk between the Erythrocyte L-Arginine/ADMA/Nitric Oxide Metabolic Pathway and the Endothelial Function in Subjects with Type 2 Diabetes Mellitus" Nutrients 13, no. 7: 2306. https://doi.org/10.3390/nu13072306* mój udział polegał na przeprowadzeniu oznaczeń biochemicznych w materiale biologicznym zabezpieczonym od uczestników projektu oraz współudziale w tworzeniu bazy danych niezbędnej do dalszych analiz statystycznych.

6. STRESZCZENIE

Cukrzyca typu 2 nierozerwalnie związana jest z co najmniej wysokim ryzykiem sercowo-naczyniowym. Spośród wielu cząsteczek regulujących funkcję śródbłonka naczyniowego największe znaczenie wydaje się mieć niezmiennie tlenek azotu (NO). Mimo, że powikłania obejmujące mikro- i makro-krążenie były dotychczas przedmiotem licznych prac, wiedza na temat roli zaburzeń osi biotransformacji tlenku azotu, w tym kontekście pozostaje niepełna.

Przez wiele lat koncentrowano się na przemianach tlenku azotu wyłącznie w kompartmencie osoczowym. Potwierdzono jednak, że szlaki biotranformacji NO wykazują swoją ekspresję także we wnętrzu krwinek czerwonych. Mając na uwadze fakt, że erytrocyty stanowią około 40% objętości krwi krążącej i są najliczniejszymi elementami morfotycznymi, mogą one być niedocenianym, a jednocześnie niepomijalnym elementem regulatorowym złożonych procesów biochemicznych. Szczególnie istotna wydaje się ich rola w zależnej od tlenku azotu regulacji perfuzji na poziomie mikrokrążenia.

Z tego powodu nadrzędnym celem niniejszej pracy jest poszukiwanie osi regulującej biodostępność NO i ocena poszczególnych jej elementów wewnątrz erytrocytów, u pacjentów, którzy rozwinęli już cukrzycę typu 2, ale nie obserwuje się u nich jeszcze istotnych klinicznie powikłań narządowych. Ponadto powiązano otrzymane oznaczenia biochemiczne z czynnościowymi parametrami odpowiedzi mikrokrążenia, które oceniono w badaniach EndoPAT i Laser Doppler wykorzystując bodźce termiczne i niedokrwienne.

Wykazano znacząco niższe stężenia L-argininy i L-cytruliny w kompartmencie osoczowym, z równoczesnym obniżeniem stosunków L-arginina/ADMA oraz ADMA/DMA w grupie pacjentów z cukrzycą. Stwierdzono także zachowany metabolizm NO w krwinkach czerwonych z obniżonym stężeniem L-cytruliny oraz znacząco wyższą biodostępnością tlenku azotu w tym kompartmencie. Ponadto obserwowano nieprawidłową odpowiedź śródbłonka w reakcji na bodziec termiczny w grupie pacjentów z cukrzycą. Przedstawione wyniki wskazują, że cukrzyca wiąże się z istotnymi zaburzeniami metabolizmu NO, co bardziej wyrażone jest w kompartmencie osoczowym. Erytrocyty mogą natomiast pozostawać buforem, który zachowuje względnie prawidłowy metabolizm NO w początkowej fazie cukrzycy typu 2.

Niniejsza dysertacja podkreśla niezwykle ważną rolę erytrocytów w utrzymaniu odpowiedniej funkcji śródbłonka naczyniowego w osób z cukrzycą typu 2 i może translacyjnie nieść za sobą dość istotne implikacje kliniczne, bowiem poznanie dokładnych molekularnych aspektów przemian tlenku azotu w erytrocytach może pozwolić na opracowanie inhibitorów arginazy, leków przeciwdziałających rozprzęganiu NOS lub redukujących stres oksydacyjny. Dodatkowo zastosowanie przepływomierza laserowego wraz z oceną odpowiedzi na bodziec termiczny może być istotnym narzędziem w ocenie wczesnych powikłań cukrzycy, pozwalając na pełniejszą stratyfikację ryzyka i formułowanie racjonalnych, spersonalizowanych przesłanek do eskalacji terapii w grupie osób bez jawnych klinicznie powikłań narządowych, lecz z dużym ryzykiem ich szybkiego wystąpienia.

7. SUMMARY

Type 2 diabetes mellitus (DM) is inseparably associated with at least high cardiovascular risk. Among numerous molecules regulating the vascular tone, the nitric oxide (NO) is considered to play a pivotal role. Even though the micro- and macrovascular abnormalities are being extensively studied, the alterations in the nitric oxide metabolic pathway still require further investigations. For many years, the studies on the NO were mostly focused on the plasma compartment. However, emerging data confirmed that nitric oxide metabolic pathway is present also in red blood cells. Taking into consideration, that erythrocytes constitute about 40% of circulating blood, they can be an underappreciated, albeit indisputable part of complex biochemical processes. Their role in the nitric oxide-dependent regulation of perfusion at the level of microcirculation seems to be of particular importance.

Therefore, the main scope of this dissertation was to evaluate the role of the erythrocytes' nitric oxide metabolism in preserving the appropriate vascular function, as well as to identify the alterations that occur at the early stage of diabetes mellitus. Additionally, the biochemical results were compared with the endothelial function parameters, obtained from EndoPAT and LaserDoppler flowmetry, in response to the thermal and ischemic stimuli.

This study has demonstrated significantly decreased L-arginine and L-citrulline concentrations in the plasma compartment with reduced Arginine/ADMA and ADMA/DMA-ratios in the *diabetes mellitus* group. Preserved nitric oxide metabolism in erythrocytes with reduced L-citrulline level and significantly higher NO-bioavailability have also been noted. What is more, an endothelial vasodilatory dysfunction in microcirculation at the early stage of diabetes mellitus has been identified and, what is noteworthy - was more pronounced in response to the thermal-stimulus.

The original paper is the first study to show that diabetes mellitus is associated with significant differences in the expression of the nitric oxide metabolic pathway, which are more pronounced in the plasma than in the erythrocyte compartment. Hence, the red blood cells could constitute a buffer with a higher nitric oxide bioavailability, less affected by the diabetes mellitus-related abnormalities.

This dissertation points at the importance of erythrocytes in preserving the appropriate endothelial function in subjects with diabetes mellitus. The identification of the mechanisms responsible for limiting the nitric oxide bioavailability may lead to the discovery of novel drug targets, limiting the burden of nitrosative-stress and in a consequence induced this way damage to microcirculation. It provides a rationale for personalized therapy aiming at minimizing the residual cardiovascular risk in diabetic subjects without clinically relevant complications but being at high risk for their development.

8. ZGODA KOMISJI BIOETYCZNEJ

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 155/2019

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:

dr hab. Jacek Daroszewski, prof. nadzw. (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 Müller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn, prof. nadzw (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.

„Poszukiwanie mechanizmów regulacji biodostępności tlenku azotu w erytrocytach i ich wpływu na funkcję śródbłonka naczyniowego u osób z cukrzyca typu 2"

zgłoszonym przez **lek. Damiana Gajeckiego** 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 Klinice Chorób Wewnętrznych, Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej Uniwersyteckiego Szpitala Klinicznego im. Jana Mikulicza-Radeckiego we Wrocławiu pod nadzorem dr. hab. Adriana Doroszko i dr Anny Szymańskiej - Chabowskiej – promotora pomocniczego **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 28 lutego 2019 r.

BW

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