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Uszkodzenie śródbłonka w przebiegu chorób układowych tkanki łącznej

Praca doktorska

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Z podziękowaniem dla
Rodziców, Rodzeństwa, Piotra i Przyjaciół
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Spis treści

1. Nota informacyjna

Praca doktorska *Uszkodzenie śródbłonka w przebiegu chorób układowych tkanki łącznej*, opiera się na cyklu trzech oryginalnych artykułów opublikowanych w czasopismach o zasięgu międzynarodowym znajdujących się w bazie PubMed:

1. Renata Pacholczak, Stanisława Bazan-Socha, Teresa Iwaniec, Lech Zaręba, Stan Kielczewski, Jerzy A. Walocha, Jacek Musiał, Jerzy Dropiński. *Endothelial dysfunction in patients with granulomatosis with polyangiitis: a case-control study.* Rheumatology International 2018 Aug;38(8):1521-1530 (IF=1,95; MNiSW=20),
2. Renata Pacholczak, Stanisława Bazan-Socha, Teresa Iwaniec, Lech Zaręba, Stan Kielczewski, Jerzy A. Walocha, Jacek Musiał, Jerzy Dropiński. *Endothelial dysfunction in patients with eosinophilic granulomatosis with polyangiitis: a case-control study.* Clinical Rheumatololy 2018 Aug 21 [Epub ahead of print] (IF=2,14; MNiSW=20),
3. Renata Pacholczak, Piotr Kuszmiersz, Stanisława Bazan-Socha, Joanna Kosałka, Teresa Iwaniec, Lech Zaręba, Stan Kielczewski, Anna Rams, Jerzy A. Walocha, Jacek Musiał, Jerzy Dropiński. *Endothelial dysfunction in patients with systemic sclerosis*. Advances in allergology and dermatology 2019 (IF=1,47; MNiSW=15).

Współczynnik IF (Impact Factor) dla wyżej wymienionych prac wyniósł 5,56; zaś MNiSW (wskaźnik Ministerstwa Nauki i Szkolnictwa Wyższego) – 55. Praca finansowana była z dotacji na utrzymanie potencjału badawczego (projekty statutowe, K/ZDS/007897), dotacji na rozwój młodych naukowców (K/DSC/004605) w Uniwersytecie Jagiellońskim Collegium Medicum przyznanych dla autorki oraz z Narodowego Centrum Nauki (DEC-2013/09/B/NZ5/00758 dla dr hab. Stanisławy Bazan-Sochy).

2. Wykaz skrótów

ANCOVA- *ang. analysis of covariance,* analiza kowariancji

BMI- *ang. body-mass index,* index masy ciała

BVAS- *ang. Birmingham Vasculitis Activity Score,* punktacja aktywności zapalenia naczyń według Birmingham

CRP- *ang. c-reactive protein,* stężenie białka C-reaktywnego

EGPA- *ang. eosinophilic granulomatosis with polyangiitis,* eozynofilowa ziarniniakowatość z zapaleniem naczyń

ELISA- *ang. enzyme-linked immunosorbent assay,* test immunoenzymatyczny

FMD%- *ang. flow-mediated dilatation,* pomiar poszerzenia naczynia związanego ze zwiększeniem przepływu

GPA- *ang. granulomatosis with polyangiitis*, ziarniniakowatość z zapaleniem naczyń

HDL- *ang. high-density lipoprotein,* lipoproteina wysokiej gęstości

IL-6- interleukina 6

CI- *ang. confidence interval,* przedział ufności

IMT- *ang. intima-media thickness,* ocena grubości kompleksu intima-media tętnicy szyjnej wspólnej

LDL- *ang. low-density lipoprotein,* lipoproteina niskiej gęstości

VCAM-1- *ang. vascular cell adhesion molecule,* cząsteczka adhezji komórkowej naczyń

VDI- *ang. Vascular Damage Index,* wskaźnik uszkodzenia naczyń

3. Podsumowanie pracy doktorskiej w języku polskim

3.1. Wstęp

Choroby układowe tkanki łącznej stanowią grupę ogólnoustrojowych schorzeń o podłożu autoimmunologicznym do których zaliczamy przede wszystkim tocznia rumieniowatego układowego, zapalenie skórno-mięśniowe, twardzinę układową, reumatoidalne zapalenie stawów oraz układowe zapalenia naczyń. Charakterystyczną cechą tych schorzeń jest zajęcie procesem zapalnym wielu układów i narządów, a we krwi obecność przeciwciał przeciwko różnym składnikom jądra komórkowego i cytoplazmy, często też przeciw natywnemu DNA. Rokowanie, które kiedyś było niepomyślne, w ostatnich latach bardzo się poprawiło, co wiązane jest z szeroko stosowanym leczeniem immunosupresyjnym. Nadal jednak, ze względu na wielonarządowy charakter schorzenia, śmiertelność jest wysoka, a miażdżyca stanowi najczęstszą przyczynę zgonu wśród pacjentów z wieloletnim przebiegiem tych chorób, pomimo optymalnego leczenia immunosupresyjnego (1,2).

Dokładny mechanizm rozwoju miażdżycy w wymienionych jednostkach chorobowych pozostaje niewyjaśniony. Klasyczne czynniki ryzyka miażdżycy nie wydają się występować w tej grupie chorych na tyle często, aby wytłumaczyć tak szybką manifestację chorób sercowo-naczyniowych. Mimo, iż powszechnie stosowane leczenie immunosupresyjne w chorobach układowych hamuje proces zapalny, to są doniesienia, iż może być ono bezpośrednim czynnikiem inicjującym uszkodzenie śródbłonka (3,4).

Śródbłonek odgrywa istotną rolę w utrzymywaniu homeostazy naczyniowej, a jego dysfunkcja prowadzi do upośledzenia rozkurczu naczyń, inicjowania kaskady zapalno-zakrzepowej i stanowi istotny, niezależny czynnik ryzyka chorób sercowo-naczyniowych (5). U osób z hipercholesterolemią oraz u palaczy dysfunkcję śródbłonka stwierdza się jeszcze przed powstaniem blaszek miażdżycowych (6). Stąd potrzeba jak najwcześniejszego wykrywania nawet bardzo subtelnych zaburzeń jego funkcji, a monitorowanie funkcji śródbłonka może być pomocne w ocenie skuteczności terapii chorób układu krążenia.

W literaturze istnieją podobne badania, w szczególności w grupie pacjentów z toczniem rumieniowym układowym (7,8), twardziną układową (9,10), zespołem antyfosfolipidowym (11,12), jednak były one przeprowadzane na małych, niereprezentatywnych grupach, a wyniki poszczególnych badań często pozostają sprzeczne. U pacjentów z ziarniniakowatością z zapaleniem naczyń (dawniej Wegenera, *ang. granulomatosis with polyangitis,* GPA) oraz z eozynofilową ziarniniakowatością

z zapaleniem naczyń (dawniej zespół Churg-Strauss'a, ang. *eosinophilic granulomatosis with polyangitis*, EGPA) podobne badania nie były dotychczas publikowane.

3.2. Cel pracy i pytania badawcze

Celem badania jest ocena funkcji śródbłonka i ryzyka rozwoju miażdżycy za pomocą badań obrazowych i laboratoryjnych u pacjentów z wybranymi chorobami tkanki łącznej: GPA, EGPA, twardzina układowa oraz znalezienie potencjalnych przyczyn stwierdzonych nieprawidłowości.

W ramach niniejszego badania zostały sformułowane następujące pytania badawcze:

- Czy u pacjentów z chorobami układowymi tkanki łącznej uszkodzenie śródbłonka jest bardziej nasilone niż w grupie zdrowych pacjentów?
- Co może być przyczyną obserwowanych nieprawidłowości?
- Czy dynamika rozwoju oraz zaawansowanie miażdżycy wykazują różnice w poszczególnych schorzeniach autoimmunologicznych w zależności od przebiegu klinicznego oraz obecności, typu i miana autoprzeciwciał?
- Czy stosowane leczenie immunosupresyjne jest dodatkowym czynnikiem wykazującym negatywny wpływ na układ sercowo-naczyniowy?
- Czy poziom rozpuszczalnych molekuł adhezyjnych, jako laboratoryjnych markerów zaburzenia funkcji śródbłonka, koreluje z zaawansowaniem zmian miażdżycowych ocenianych za pomocą ultrasonografii?

3.3. Metody zbierania danych i analizy

Niniejsze badanie kliniczno-kontrolne zostało przeprowadzone po uzyskaniu zgody Komisji Bioetycznej UJ CM (numer zgody: KBET/79/B/2013 z dnia 9 maja 2013r). Uczestnictwo w badaniu było dobrowolne i każdy uczestnik otrzymał opis i cele badania oraz wyraził pisemną zgodę.

Badaniem zostali objęci losowo wybrani pacjenci hospitalizowani w Oddziale Alergii i Immunologii Kliniki Chorób Wewnętrznych Szpitala Uniwersyteckiego w Krakowie w latach 2014-2018.

Do badania włączono 44 pacjentów z GPA, 30 pacjentów z EGPA oraz 42 pacjentów z twardziną układową. Rozpoznanie zostało postawione na podstawie kryteriów American College of Rheumatology (13). Wśród pacjentów z GPA zakwalifikowano zarówno chorych

z zaostrzeniem choroby oraz pozostających w stadium choroby przewlekłej (*ang. persistent disease*), natomiast w EGPA oraz twardzinie układowej pacjenci byli w stadium remisji. Zaostrzenie w GPA zostało zdefiniowano jako nowy objaw według skali BVAS (*ang. Birmingham Vasculitis Activity Score,* punktacja aktywności zapalenia naczyń według Birmingham) (14). Choroba przewlekła jako utrzymujący się przez 1 do 3 miesięcy objaw/objawy charakterystyczne dla choroby w stadium zaostrzenia, natomiast remisja jako całkowity brak aktywnych objawów choroby podstawowej lub nowych objawów oraz utrzymana, stała dawka leków immunosupresyjnych (15). Wśród pacjentów z GPA, ze stałymi objawami utrzymującymi się przez ponad 3 miesiące, dodatkowo oceniono VDI (*ang. Vascular Damage Index,* wskaźnik uszkodzenia naczyń) (16). Pacjenci z zastoinową niewydolnością serca, niestabilną dusznicą bolesną, niekontrolowanym nadciśnieniem, niewydolnością wątroby i będący w trakcie leczenia onkologicznego, zostali zdyskwalifikowani z udziału w badaniu.

Do grupy kontrolnej włączono 58 zdrowych osób (dobranych pod względem płci, wieku, BMI [*ang.body-mass index*, indeks masy ciała] oraz chorób współistniejących do poszczególnych grup badawczych), bez klinicznych cech miażdżycy będących pracownikami szpitala lub krewnymi. Kryteria wyłączenia były takie same jak w grupie badawczej.

U wszystkich osób włączonych do badania wykonano podstawowe badania laboratoryjne: morfologia krwi obwodowej, poziom elektrolitów (sód, potas, magnez), cholesterolu całkowitego, trójglicerydów, LDL (*ang.low-density lipoprotein,* lipoproteina niskiej gęstości), HDL (*ang.high-density lipoprotein,* lipoproteina wysokiej gęstości), glukozy, stężenie białka C-reaktywnego (*ang. c-reactive protein,* CRP) w analizatorze VITROS 250 Johnson&Johnson oraz stężenie interlukiny 6 (IL-6) metodą ELISA (*ang. enzyme-linked immunosorbent assay,* test immunoenzymatyczny**,** R&D Systems, Minneapolis, MN, USA). Ponadto u pacjentów z grupy badawczej oznaczono stężenie przeciwciał specyficznych dla danej jednostki chorobowej metodą ELISA (EUROIMMUN, Lübeck, Germany).

U wszystkich osób oceniono również stężenie markerów uszkodzenia śródbłonka tj.: cząsteczka adhezji komórkowej naczyń (*ang. vascular cell adhesion molecule,* VCAM-1) oraz rozpuszczalna trombomodulina, z wykorzystaniem metody ELISA (R&D Systems, Minneapolis, MN, USA).

Każdy pacjent miał wykonane rutynowe badanie echokardiograficzne przy użyciu ultrasonografu (Sequoia 512, Mountainview, Ca, USA). W celu eliminacji zmienności

pomiaru wszystkie badania wykonano przez dwie osoby, a analizowane wyniki stanowiły średnią z uzyskanych pomiarów. Dodatkowo wykonano następujące pomiary:

- Pomiar poszerzenia naczynia związanego ze zwiększeniem przepływu (*ang. Flow-mediated dilatation*, FMD) przeprowadzono w oparciu o zmodyfikowaną metodę Celermayera (17). Badanie wykonano w wyciszonym i zaciemnionym pomieszczeniu, a badani przebywali przed każdym pomiarem przez 10 minut w pozycji leżącej. Wymiar strzałkowy obwodowego odcinka tętnicy ramiennej monitorowano na przekroju poprzecznym w prezentacji M, z zastosowaniem głowicy linearnej o częstotliwości 10 MHz. Następnie na przedramię badanej kończyny nałożono sfigmomanometr napompowany do ciśnienia przewyższającego ciśnienie skurczowe (200 mmHg). Ucisk był utrzymany przez 5 minut. FMD wyrażono jako odsetek maksymalnego wzrostu średnicy tętnicy ramiennej po zdjęciu mankietu w stosunku do wartości zmierzonej w spoczynku (FMD%).
- Ocena grubości kompeksu intima-media tętnicy szyjnej wspólnej (*ang. Intima-media thickness*, IMT). Pomiar wykonano na ścianie tylnej i przedniej tętnicy szyjnej wspólnej, dystalnie do jej bifurkacji. Zanotowany wynik stanowił średnią z pomiarów prawej i lewej tętnicy.
- Ocena sztywności aorty (tylko u pacjentów z GPA). Aorta wstępująca została zmierzona w projekcji przymostkowej długiej w trybie M-mode około 3 cm powyżej zastawki aorty. Pomiar średnicy aorty w fazie skurczu (ASD) i rozkurczu (ADD) został oszacowany w trakcie 5 kolejno następujących po sobie cykli pracy serca, a zarejestrowany wynik stanowił średnią z pomiarów. Wymiar skurczowy aorty został wykonany w czasie pełnego otwarcia zastawki aorty, natomiast wymiar rozkurczowy w czasie jej zamknięcia. Na podstawie otrzymanych wyników oceniono sztywność aorty według wzoru: odkształcenie aorty (%) = 100 x (ASD-ADD)/ADD (18).

Opracowanie statystyczne wyników

Otrzymane wyniki opracowano statystycznie z wykorzystaniem programu STATSTICA 12.5. Porównywano grupy badawczą i kontrolną. Dla zmiennych ciągłych policzono wszystkie istotne dla badania parametry statystyczne oraz zbadano normalność rozkładu za pomocą testu Shapiro–Wilka. W przypadku braku normalności, aby móc wykorzystać metody t-testów czy też analizy wariancji i analizy kowariancji wyniki zostały zlogarytmowane z użyciem logarytmu naturalnego. Porównanie parametrów lub rozkładów cech w grupach badanych wykonano za pomocą t-testu lub jego odpowiednika

nieparametrycznego jakim jest test Mann-Whitey'a. W przypadku stwierdzenia różnic w więcej niż dwóch grupach wykorzystano analizę wariancji lub jej nieparametryczny odpowiednik jakim jest test Kruskalla-Wallisa oraz połączone z tymi analizami testy post-hoc lub testy wielokrotnych porównań. Zmienne jakościowe zostały przedstawione jako liczba i procent całości, a następnie porównane z wykorzystaniem testu $\chi 2$. Aby wyeliminować wpływ zmiennych, które mogą ewentualnie zakłócać obraz różnic tj.: wiek, BMI, płeć, choroby współistniejące (nadciśnienie tętnicze, cukrzyca, hipercholesterolemia) dla parametrów FMD%, IMT, sztywność aorty, VCAM-1 i trombomodulina przeprowadzono analizę kowariancji (ANCOVA). Dla określenia siły i kierunku związku pomiędzy cechami ciągłymi wykorzystano zarówno współczynnik korelacji liniowej Pearsona jak i współczynnik korelacji Spearmana. W przypadku silnych związków ujawnionych przez współczynnik korelacji Pearsona związki opisano z wykorzystaniem regresji liniowej z uwzględnieniem zmiennych zakłócających (wiek, BMI, płeć, choroby współistniejące: nadciśnienie tętnicze, cukrzyca, hipercholesterolemia). W celu określenia niezależnych czynników warunkujących FMD zbudowano model regresji wielorakiej lub regresji prostej. Wyniki uznawano za statystycznie istotne przy wartości p poniżej 0.05.

3.4. Wyniki

Artykuł nr. 1

Endothelial dysfunction in patients with granulomatosis with polyangiitis: a case-control study

Uszkodzenie śródbłonka u pacjentów z ziarniniakowatością z zapaleniem naczyń: badanie kliniczno-kontrolne

W badaniu wzięło udział 44 pacjentów z GPA (21 mężczyzn oraz 23 kobiety) oraz 53 osoby z grupy kontrolnej (22 mężczyzn oraz 31 kobiet) dopasowane do grupy badanej względem wcześniej określonych kryteriów.

Wyniki:

U pacjentów z GPA stwierdzono 15.9% wyższe stężenie VCAM-1 ($p=0.01$) oraz 50.9% wyższe stężenie trombomoduliny ($p<0.001$) we krwi obwodowej w porównaniu do zdrowych uczestników badania. Po standaryzacji do potencjalnych czynników zakłócających,

w analizie ANCOVA, jedynie stężenie trombomoduliny pozostało istotnie wyższe u pacjentów z GPA. Laboratoryjne wykładniki funkcji śródbłonka pozytywnie korelowały z markerami stanu zapalnego: CRP (β=0.18 [95% CI:0.08-0.28] oraz β=0.28 [95% CI:0.27-0.29], odpowiednio dla VCAM-1 i trombomoduliny) oraz IL-6 (β=0.27 [95% CI:0.15-0.39], oraz β=0.4 [95% CI:0.27-0.53], odpowiednio dla VCAM-1 i trombomoduliny), a także parametrami funkcji nerek oraz stężeniem przeciwciał anty-PR3.

W analizie podgrup pacjentów z GPA, osoby z nadciśnieniem tętniczym oraz z niewydolnością nerek miały wyższe stężenie VCAM-1 oraz trombomoduliny. Pozostałe schorzenia towarzyszące oraz faza choroby (zaostrzenie/choroba przewlekła) nie miały wpływu na laboratoryjne parametry uszkodzenia śródbłonka.

W badaniach ultrasonograficznych pacjenci z GPA mieli o 48.9% niższą wartość FMD% w porównaniu do grupy kontrolnej, również po standaryzacji do potencjalnych czynników zakłócających ($p<0.001$). FMD% negatywnie korelowało ze stężeniem leukocytów (β=-0.24 [95% CI:-0.32 to -0.15]), CRP (β=-0.17 [95% CI:-0.27 to -0.07]), IL-6 (β=-0.29 [95% CI: -0.39 to -0.19]), kreatyniny (β=-0.2 [95% CI:-0.3 to -0.1]), paleniem papierosów (‘paczkolata’ β=-0.33 [95%CI:-0.44 to -0.12)], długością trwania choroby (β=-0.18 [95% CI:-0.32 to -0.04]). W modelu regresji krokowej czynnikami, które niezależnie wpływały na wartość FMD była obecność cukrzycy, paczkolata, stężenie mocznika we krwi, VCAM-1 oraz wartość IMT. W analizie podgrup FMD% było niższe u pacjentów z towarzyszącym nadciśnieniem tętniczym, cukrzycą t.2, palaczy tytoniu, leczonych statynami oraz azatiopryną.

Sztywność aorty oraz wartość IMT były porównywalne w obu badanych grupach. IMT pozytywnie korelowało z wartością FMD% (β=-0.12 [95% CI:-0.22 to -0.02]), stężeniem kreatyniny (β=0.18 [95%CI: 0.08 to 0.28]), długością leczenia sterydami (β=0.19 [95% CI: 0.03 to 0.35]), długością trwania choroby (β=0.27 [95% CI: 0.14 to 0.41]). Pacjenci z GPA oraz z towarzyszącą przewlekłą niewydolnością nerek mieli wyższą wartość IMT.

Artykuł nr. 2

Endothelial dysfunction in patients with eosinophilic granulomatosis with polyangiitis: a case-control study

Uszkodzenie śródbłonka u pacjentów z eozynofilową ziarniniakowatością z zapaleniem naczyń: badanie kliniczno-kontrolne

W badaniu wzięło udział 30 pacjentów z eozynofilową ziarniniakowatością z zapaleniem naczyń (EGPA, 10 mężczyzn oraz 20 kobiet) będących w remisji choroby oraz 58 zdrowych osób dopasowanych względem wieku, płci i BMI do grupy badanej.

Wyniki:

Stężenie VCAM-1 było o 20% wyże u pacjentów z EGPA, natomiast stężenie trombomoduliny o 41.9% (oba parametry $p<0.001$). Po standaryzacji do potencjalnych czynników zakłócających oba parametry zachowały różnicę istotną statystycznie z $p<0.001$. Zarówno stężenie VCAM-1 jak i trombomoduliny negatywnie korelowało z długością choroby (β=-0.66 [95% CI:-0.9 to -0.41], β=-0.43 [95% CI:-0.79 to -0.07] odpowiednio dla VCAM-1 i trombomoduliny) oraz pozytywnie z mocznikiem (β=0.49 [95% CI:0.21-0.77], β=0.39 [95% CI:0.04-0.74] odpowiednio dla VCAM-1 i trombomoduliny) i kreatyniną (β=0.36 [95% CI:0.07-0.65], β=0.69 [95% CI:0.41-0.97] odpowiednio dla VCAM-1 i trombomoduliny). Ponadto stężenie trombomoduliny pozytywnie korelowało ze stężeniem parametrów stanu zapalnego (Il-6, CRP, leukocyty). Choroby współistniejące, leki internistyczne oraz stosowane leczenie immunosupresyjne nie miały wpływu na laboratoryjne wykładniki funkcji śródbłonka.

Pacjenci z EGPA mieli o 38.8% niższą wartość FMD% od grupy kontrolnej ($p<0.001$ również po standaryzacji do potencjalnych czynników zakłócających). FMD% negatywnie korelowało ze stężeniem CRP (β=-0.5 [95% CI:-0.75 to -0.25]) i nie wykazano korelacji z innymi markerami stanu zapalnego. W modelu regresji prostej wykazano, iż stężenie IL-6, trombomoduliny oraz paczkolata mogą wpływać na niższą wartość FMD%. W analizie podgrup niższe FMD% zaobserwowano jedynie wśród pacjentów z polineuropatią. Wartość IMT była porównywalna w obu badanych grupach i nie korelowała istotnie z innymi parametrami.

Artykuł nr. 3

Endothelial dysfunction in patients with systemic sclerosis

Uszkodzenie śródbłonka u pacjentów z twardziną

Do badania zakwalifikowano 42 pacjentów z twardziną układową (7 mężczyzn i 35 kobiet) z czego większość stanowiły osoby z postacią uogólnioną (69%) oraz 36 zdrowych osób (11 mężczyzn i 25 kobiet) dopasowanych względem wcześniej określonych czynników.

Wyniki:

Stężenie trombomoduliny i VCAM-1 było podobne w obu badanych grupach, jednak po standaryzacji do czynników zakłócających w analizie ANCOVA stężenie trombomoduliny było istotnie wyższe u pacjentów z twardziną układową ($p=0.03$). Stężenie trombomoduliny korelowało ze stężeniem mocznika ($\beta=0.84$ [95% CI: 0.53-1.15]) i kreatyniny ($\beta=0.69$ [95% CI: 0.44-0.93]), a także CRP ($\beta=0.57$ [95% CI: 0.31 to 0.83]), natomiast stężenie VCAM-1 jedynie ze stężeniem kreatyniny ($\beta=0.67$ [95% CI: 0.39-0.95]).

Pacjenci z twardziną układową mieli o 45% niższą wartość FMD% oraz 13% wyższą wartość IMT niż osoby z grupy kontrolnej ($p<0.01$, również po standaryzacji do czynników zakłócających). W modelu regresji prostej wartość FMD% była determinowana głównie przez wiek ($\beta=-0.57$ [95% CI:-0.72 to -0.43]) oraz CRP ($\beta=-0.38$ [95% CI:-0.55 to -0.22]). Wartość IMT korelowała z wiekiem ($\beta=0.64$ [95% CI:0.52 to 0.67]), wskaźnikiem filtracji kłębuszkowej eGFR ($\beta=-0.34$ [95%CI:-0.5 to -0.18]) oraz stężeniem trombomoduliny ($\beta=0.45$ [95% CI: 0.13 to 0.76]), a w analizie podgrup była wyższa u pacjentów z towarzyszącym nadciśnieniem tętniczym.

Pacjenci z postacią uogólnioną i ograniczoną choroby mieli podobne wartości laboratoryjnych i ultrasonograficznych parametrów uszkodzenia śródbłonka. Analiza podgrup względem chorób współistniejących i przyjmowanych leków również nie wykazała istotnych różnic.

3.5. Podsumowanie

Zarówno pacjenci z układowymi zapaleniami naczyń (GPA oraz EGPA) jak i osoby z twardziną układową prezentują zaburzenie funkcji śródbłonka wyrażone przez podwyższone stężenie laboratoryjnych wskaźników uszkodzenia śródbłonka tj. trombomodulina i VCAM-1 oraz obniżoną wartość FMD% i podwyższoną IMT.

Wspólnym mechanizmem obserwowanych zaburzeń jest przewlekły proces zapalny towarzyszący każdej z układowych chorób tkanki łącznej. Przewlekła choroba nerek, która jest konsekwencją przede wszystkim zapaleń naczyń (w twardzinie występuje głównie jako twardzinowy przełom nerkowy) również prowadzi do uszkodzenia śródbłonka w sposób pośredni przez nadciśnienie tętnicze i wzrost sztywności naczyń. W niniejszym badaniu staraliśmy się wyeliminować wpływ tradycyjnych czynników ryzyka chorób sercowo-naczyniowego przez standaryzację do wieku, płci, BMI oraz chorób współistniejących (cukrzyca, nadciśnienie tętnicze i hipercholesterolemia), jednak wyniki regresji krokowej i liniowej świadczą o ich niezaprzeczalnym wpływie na śródbłonek. Ponadto palenie tytoniu pozostaje istotnym czynnikiem uszkadzającym śródbłonek i to zarówno w grupie badanej jak i kontrolnej. Patofizjologiczne czynniki danej choroby, czyli wpływ przeciwciał w układowych zapaleniach naczyń czy zespół poreperfuzyjny w twardzinie układowej, są innymi czynnikami ryzyka uszkodzenia śródbłonka w chorobach autoimmunologicznych. W każdej z badanych grup stosowane było leczenie immunosupresyjne, które może powodować uszkodzenie komórek śródbłonka w sposób bezpośredni (cyklofosfamid) lub pośredni (glikokortykosterydy), poprzez rozwój otyłości, nadciśnienia tętniczego i dyslipidemii.

Wykonane w pracy badania laboratoryjne w chwili obecnej są jedynie narzędziem stosowanym w pracach naukowych ze względu na koszty oznaczeń i brak zakresów referencyjnych. Pomiar FMD% i IMT może być jednak przydatną metodą do zastosowania w codziennej praktyce klinicznej. Sztywność aorty badana u pacjentów z GPA jest mało wiarygodnym badaniem i wymaga dalszej weryfikacji. Wyniki ocenianych badań laboratoryjnych i ultrasonograficznych korelują ze sobą pozytywnie i niejako się uzupełniają, gdyż podwyższone stężenie molekuł adhezyjnych świadczy o zaistniałym uszkodzeniu śródbłonka, a badania ultrasonograficzne są wyrazem zaburzeń funkcjonalnych.

W związku z obserwowanymi zmianami, pacjenci z chorobami układowymi mogą odnieść dużą korzyść z monitorowania funkcji śródbłonka oraz wdrażania strategii profilaktycznych mających na celu zmniejszenie ryzyka sercowo-naczyniowego. Farmakologiczna ochrona śródbłonka może być zapewniona poprzez podaż statyn, aspiryny oraz pośrednio poprzez właściwe leczenie immunosupresyjne, które hamują proces zapalny i zapobiegają powikłaniom choroby podstawowej. Może ono być jednak bronią obosieczną, gdyż jak podają dane z literatury, również lekki immunosupresyjne mogą prowadzić do uszkodzenia śródbłonka. Niniejsza hipoteza wymaga jednak dalszej weryfikacji. U pacjentów z twardziną układową, również inne leki, takie jak inhibitory fosfodiesterazy-5, analogi

prostacykliny oraz inhibitory receptora dla endoteliny, zarejestrowane w leczeniu owrzodzeń palców, ciężkiego objawu Raynaud'a oraz nadciśnienia płucnego, mogą wykazywać dodatkowy, korzystny wpływ na czynność śródbłonka. Mechanizm ich działania polega na relaksacji mięśni gładkich naczyń z następową wazodylatacją.

Powyższa, farmakologiczna strategia profilaktyczna, w połączeniu z zastosowaniem prezentowanych metod diagnostycznych mogą być rozważone w codziennej praktyce klinicznej w celu wczesnego wykrycia zburzeń czynności śródbłonka i zapobieganiu powikłaniom sercowo-naczyniowym u pacjentów z układowymi chorobami tkanki łącznej.

4. Podsumowanie pracy doktorskiej w języku angielskim

4.1. Introduction

Connective tissue diseases are a group of generalized disorders with autoimmunological background, such as: systemic lupus erythematosus, dermatomiositis, systemic sclerosis, rheumatoid arthritis and systemic vasculitis. The common hallmark of these diseases is inflammation of different organs and presence in the blood antibodies directed against cell nucleus, cytoplasm or DNA. A prognosis which used to be unfavorable, has improved recently thank to more efficient diagnostic methods and introduced immunosuppressive treatment which literally suppresses the immune system. However, mortality rate is still high due to inflammation of many organs and cardiovascular complications are of major importance in spite of proper immunosupressive treatment (1,2).

The exact mechanism by which atherosclerosis is promoted in the abovementioned diseases remains unclear. It might be associated with a chronic inflammation, dyslipidaemia, presence of autoantibodies and endothelial dysfunction. However, the classical atherosclerotic risk factors do not seem to be present in these patients frequently enough to explain such an early manifestation of ischemic heart disease. Moreover, introduced immunosuppressive treatment not only suppresses chronic inflammation but also act as an additional trigger for endothelial injury (3,4).

The endothelium plays a key role in maintaining a vascular homeostasis and its dysfunction disturbs vasorelaxation, initiate thrombus formation and it is an independent cardiovascular risk factor (5). Endothelial disturbances are detectable before atherosclerosis plaque formation in smokers and in hypercholesterolemia (6). Therefore, detection of the preclinical stage of atherosclerosis is of major importance and monitoring of endothelial function might be helpful in the treatment effectiveness assessment of cardiovascular system diseases.

In the literature there are studies where endothelial function has been assessed especially in patients with systemic lupus erythematosus (7,8), systemic sclerosis (9,10) and antiphospholipid syndrome (11,12). However, these researches were performed on a small, unrepresentative groups of patients and its outcomes produced a contradictory results. In patients with granulomatosis with polyangitis (GPA) and eosinophilic granulomatosis with polyangitis (EGPA) similar studies have hitherto not been published.

4.2. Aim of the study and research' questions

The aim of the study was to assess endothelial function and cardiovascular risk development in patients with selected systemic connective tissue diseases: GPA, EGPA, systemic sclerosis and to find an explanation of observed abnormalities.

Therefore, the following research' questions have been established:

- Do the patients with systemic connective tissue diseases present with endothelial dysfunction? Is atherosclerosis more pronounced in these patients than in asymptomatic, disease-free individuals?
- What is a possible explanation of observed anomalies?
- Does a development, dynamics and advancement of atherosclerosis vary between connective tissue diseases and depends on a course of the disease, exacerbation rate and a presence and titre of autoantibodies?
- Has an introduced immunosuppressive treatment a negative impact on the cardiovascular system?
- Does endothelial dysfunction detected by ultrasound-based imagining techniques correlate with a level of adhesive molecules?

4.3.Methods

A case-control observational study was carried out with approval of the Bioethics Committee of Jagiellonian University Medical College (9^{th} May 2013, number of protocol: KBET/79/B/2013). All participants received a detailed, brief of the methodology and safety protocols for the study and provided written consent for their participation.

The patients were recruited from the population of patients at the Department of Allergy and Clinical Immunology at the University Hospital in Cracow in the period between 2014 and 2018.

The case group constituted 44 patients with GPA, 30 patients with EGPA and 42 patients with systemic sclerosis. Each patient had a current diagnosis based on the criteria of the American College of Rheumatology (13). We analyzed GPA patients with the disease flare or those, who were symptomatic and diagnosed with persistent disease. EGPA and systemic sclerosis patients were in remission. Disease activity was measured using the Birmingham Vasculitis Activity Score (BVAS) (14). Disease flare was defined as a presence of new symptoms (major or minor item in BVAS). Persistent disease was defined as a presence of 1 or more persistent symptoms attributed to active disease for more than 1 month but less than 3 months. Remission was defined as an absence of disease activity

attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy (15). For symptoms which occurred in patients since the onset of GPA and were present for more than 3 months we used Vascular Damage Index (VDI) (16). Patients with congestive heart failure, coronary heart disease, uncontrolled hypertension, liver failure, and cancer were excluded from the study.

The control group consist of 58 individuals (matched to patients by gender, age, BMI, and smoking habit, as well as comorbidities, including hypertension, hypercholesterolemia, and diabetes mellitus). The control group was enrolled from the hospital personnel and relatives. They were selected according to matching criteria. Exclusion criteria were the same as in the case group.

All participants had measured basic laboratory parameters: blood cell count, electrolytes (sodium, potassium and magnesium), triglycerides, cholesterol (LDL, HDL), glucose, C-reactive protein (CRP, in analyzer VITROS 250 Johnson&Johnson) and Interleukin 6 (Il-6) using standardized ELISA method (R&D Systems, Minneapolis, MN, USA). Disease-specific antibodies were assessed in the case group (EUROIMMUN, Lübeck, Germany).

Every participant were assessed for the presence and concentration of laboratory parameters of endothelial injury. Vascular cell adhesion molecule (VCAM-1), and soluble thrombomodulin levels were measured using standardized ELISA method (R&D Systems, Minneapolis, MN, USA).

The transthoracic echocardiogram was performed on each subject using standard methods using the Siemens Acuson Sequoia. Examinations were conducted by two independent ultrasonography experts and considered parameters constituted a mean of three subsequent measurements. Some additional measurements were also performed:

- Flow-mediated dilatation (FMD) of the brachial artery was measured using Celermayer' method (17). It was performed in a darkened, quiet room with subjects resting in a supine position for 10 minutes. A baseline sagittal diameter (D1) of a distal part of the brachial artery was measured in the M-presentation by using a 10 MHz linear array ultrasonic transducer placed 2-3 cm proximal to the arterial bifurcation.A sphygmomanometer cuff was then placed on the forearm below the elbow and inflated to a pressure of 200 mmHg for 5 minutes and released. One minute after releasing the cuff, the brachial artery diameter was measured again (D2) at the same point. FMD was defined as the increase of brachial artery diameter after

deflation of the cuff and was expressed as a percentage of the baseline diameter (FMD %= [(D2-D1)/D1] x 100%).

- The intima-media thickness (IMT) of the carotid artery was measured by ultrasound with a 10 MHz linear transducer. The thickness of the anterior and posterior walls of common carotid arteries were measured bilaterally in the longitudinal projection immediately proximal to their bifurcation. The mean value of the right and left thicknesses was used in further analysis.
- Aortic stiffness (only in GPA patients). Measurements of an aortic diameter (only in GPA patients) were performed using a 4 MHz echocardiographic transducer. The aortic diameter was measured in the parasternal long axis in M-mode approximately 3 centimeters above the aortic valve. Aortic systolic diameter (ASD) was measured during a full opening of aortic valve and aortic diastolic diameter (ADD) at the peak of the QRS complex. Aortic stiffness was expressed as a percentage of ASD and ADD (Aortic stiffness %= [(ADD-ASD)/ASD] x 100%) .

Statistical analysis

The results of the were compared using STATISTICA 12.5 software. The continuous variables were all non-normally distributed according to the Shapiro-Wilk test. They are reported here as median and interquartile range and compared using the Mann-Whitney U-test. Categorical variables are reported as percentages and compared by $\chi 2$ test. Potential confounders were age, sex, BMI and comorbidities, such as arterial hypertension, diabetes mellitus, and hypercholesterolemia. To adjust for these, the results of FMD%, IMT, VCAM-1, and thrombomodulin were log-transformed and a one way covariance analysis (ANCOVA) was performed, first with an adjustment for age, sex, BMI and subsequently to comorbidities. The univariate linear regression models with adjustment for BMI, age, and sex were used to analyze associations between two selected parameters. Unconditional multivariate logistic regression was performed to calculate odds ratios (ORs) with 95% confidence intervals (CIs). The cut-off values for VCAM-1, FMD%, and thrombomodulin were determined by receiver operating characteristic (ROC) curves. Determinants of FMD% were assessed using multiple regression models or simple regression models. Results were considered statistically significant when the p value was less than 0.05.

4.4. Results

Article No. 1

Endothelial dysfunction in patients with granulomatosis with polyangiitis: a case-control study

The case group constituted 44 patients with GPA (21 men and 23 women) and the control group consist of 53 individuals (22 men and 31 women) matched to GPA patients by previously established criteria.

Results:

GPA patients had a 15.9% higher levels of VCAM-1 ($p=0.01$) and a 50.9% increased thrombomodulin concentrations ($p<0.001$) in peripheral blood, comparing to healthy individuals. However, in ANCOVA analysis we documented that only thrombomodulin levels remained higher in GPA subjects after adjustment for potential confounders ($p<0.001$). Both endothelial injury markers were related to the CRP ($\beta=0.18$ [95% CI:0.08-0.28], and $\beta=0.28$ [95% CI:0.27-0.29], VCAM-1 and thrombomodulin, respectively), IL-6 level ($\beta=0.27$ [95% CI:0.15-0.39], and $\beta=0.4$ [95% CI:0.27-0.53], VCAM-1 and thrombomodulin, respectively), kidney function parameters and anti-PR3 antibodies.

In the subgroup analysis GPA patients, those with hypertension and chronic kidney disease had higher VCAM-1 and thrombomodulin levels. Other comorbidities and phase of the disease (active/persistent) had no impact on laboratory markers of endothelial damage.

In ultrasonograpy, GPA patients had 48.9% decrease in FMD%, comparing to controls ($p<0.001$, also after adjustment for potential confounders $p<0.001$). FMD% was negatively associated with white blood cells ($\beta=-0.24$ [95% CI:-0.32 to -0.15]), CRP ($\beta=-0.17$ [95% CI:-0.27 to -0.07]), IL-6 ($\beta=-0.29$ [95% CI: -0.39 to -0.19]), the blood creatinine level ($\beta=-0.2$ [95% CI:-0.3 to -0.1]), smoking (packs/years) ($\beta=-0.33$ [95% CI:-0.44 to -0.12)], and duration of the disease ($\beta=-0.18$ [95% CI:-0.32 to -0.04]). A multiple regression model showed that various factors independently determined FMD%, including presence of diabetes mellitus, pack-years of smoking, serum urea, VCAM-1 and IMT. In the subgroups analysis among GPA patients, lower FMD% was observed in those with hypertension, diabetes, smoking currently or in the past, those treated with azathioprine and statins.

The values of aortic stiffness% and IMT were similar in both studied groups. IMT was related to FMD% ($\beta=-0.12$ [95% CI:-0.22 to -0.02]), blood creatinine level ($\beta=0.18$ [95% CI:

0.08 to 0.28]), duration of steroid treatment (β=0.19 [95% CI: 0.03 to 0.35]) duration of the disease (β=0.27 [95% CI: 0.14 to 0.41]). Patients with chronic kidney disease had higher IMT.

Article No. 2

Endothelial dysfunction in patients with eosinophilic granulomatosis with polyangiitis: a case-control study.

The case group was made up 30 patients with EGPA (20 women and 10 men) remaining in a remission and 58 healthy individuals (34 women and 24 men) matched to the EGPA group by gender, age and BMI.

Results:

Patients with EGPA had a 20% higher serum level of VCAM-1 and a 42% of thrombomodulin compared to healthy individuals (both p<0.001). In ANCOVA analysis we documented that both markers remained higher in EGPA patients also after adjustment for potential confounders (age, sex, BMI, hypercholesterolemia, hypertension, and diabetes mellitus, both p<0.001). Both endothelial injury parameters were negatively associated with the duration of the disease (β=-0.66 [95% CI:-0.9 to -0.41], β=-0.43 [95% CI:-0.79 to -0.07] VCAM-1 and thrombomodulin, respectively) and positively associated with urea (β=0.49 [95% CI:0.21-0.77], β=0.39 [95% CI:0.04-0.74] VCAM-1 and thrombomodulin, respectively) and creatinine (β=0.36 [95% CI:0.07-0.65], β=0.69 [95% CI:0.41-0.97] VCAM-1 and thrombomodulin, respectively). Level of thrombomodulin was positively related to the inflammatory markers (IL-6, CRP and white blood cell count). Comorbidities, medications and type of immunosuppressive treatment had no impact on laboratory markers of endothelial damage.

The EGPA group was characterized by a 38.8% decrease in FMD%, compared to the control group (p<0.001, also after adjustment for potential confounders). FMD% was negatively associated with CRP level (β=-0.5 [95% CI:-0.75 to -0.25]) without associations with remaining inflammatory markers. However, a simple regression models showed that serum level of IL-6, trombomodulin and pack-years of smoking may be related to lower FMD% values. In the subgroups analysis among EGPA patients, a lower FMD% was observed in those with polyneuropathy. The IMT measurement was similar in the EGPA and control groups and it did not correlate with laboratory parameters of endothelial injury.

Article No. 3

Endothelial dysfunction in patients with systemic sclerosis.

The 42-patient case group (35 women and 7 men) with systemic sclerosis and the 36-individual healthy control group (25 women and 11 men) that matched the case group in gender, age, BMI, comorbidities and smoking habit were enrolled in the study. The majority of the patients (69%, n=29) had a diffuse type of the disease.

Results:

Patients with systemic sclerosis had similar serum levels of VCAM-1 and thrombomodulin as compared to healthy individuals. However, the ANCOVA analysis performed with adjustment for potential confounders revealed that levels of thrombomodulin, but not VCAM-1, were increased in the systemic sclerosis group ($p=0.03$). Thrombomodulin correlated with creatinine ($\beta=0.84$ [95% CI: 0.53-1.15]), urea $\beta=0.69$ [95% CI: 0.44-0.93]) and CRP level ($\beta=0.57$ [95% CI: 0.31 to 0.83]) and VCAM-1 was associated only with creatinine concentration ($\beta=0.67$ [95% CI: 0.39-0.95]).

Patients with systemic sclerosis were characterized by 45% lower FMD% and 13% higher IMT, as compared to the control group ($p<0.01$, both, also after adjustment for potential confounders). In a simple regression models lower FMD% was determined by age ($\beta=-0.57$ [95% CI:-0.72 to -0.43]) and CRP ($\beta=-0.38$ [95% CI:-0.55 to -0.22]). Thicker IMT was also related to age ($\beta=0.64$ [95% CI:0.52 to 0.67]), to impaired kidney function (for eGFR $\beta=-0.34$ [95% CI:-0.5 to -0.18]), and to thrombomodulin blood levels ($\beta=0.45$ [95% CI: 0.13 to 0.76]). In a subgroup analysis IMT was higher in the patients with hypertension.

Patients with systemic or limited systemic sclerosis did not differ in laboratory and ultrasonographic parameters of endothelial injury. Similarly, disease duration, treatment mode, or type of antibodies had no impact on these variables.

4.5. Summary

Patients with systemic vasculitis (GPA and EGPA) as well as with systemic sclerosis present with endothelial dysfunction that is manifested by increased level of laboratory parameters of endothelial damage (thrombomodulin, VCAM-1), decreased value of FMD% and increased IMT.

A common mechanism of observed disturbances is a chronic inflammation process which accompanies every connective tissue disease. A chronic kidney disease which is a consequence of vasculitis (in systemic sclerosis it can be present as a renal crisis) can lead to endothelial injury indirectly by hypertension and increased stiffness of arteries. In this study we tried to eliminate an influence of traditional cardiovascular risk factors by an adjustment for age, sex, BMI and comorbidities (diabetes mellitus, hypertension, hypercholesterolemia) but the results of multivariate regression model and simple regression model confirmed undoubtedly that they influence on endothelium. Moreover, smoking habit remains an additional factor that injures endothelium in both evaluated groups. Patophysiology of the disease- an influence of antibodies in vasculitis and ischemia-reperfusion injury in systemic sclerosis- are another risk factors. In every disease immunosuppressants were administered (including steroids) which cause endothelial damage directly (cyclophosphamide) or indirectly (steroids) via obesity, hypertension and dyslipidaemia.

The laboratory tests which were used in this study are only research tools due to their costs and lack of reference ranges. Measurements of FMD% and IMT seem to be an useful method to introduce in a clinical practice. Aortic stiffness which was evaluated in GPA patients is not a reliable method and requires further evaluation. The results of laboratory and ultrasonographic tests are correlated to each other and supplement each other. Increased concentration of adhesion molecules reveals ongoing endothelial injury and ultrasonographic tests proves functional disturbances.

Due to observed anomalies, patients with systemic connective tissue diseases may benefit from monitoring of endothelial function and some preventive strategies which decrease cardiovascular risk. Pharmacologic protection of endothelium may be achieved by administration of statins, aspirin and indirectly by proper immunosuppressive treatment that decrease inflammation and prevents from complications of the disease. It can be also a double-bladed sword because it leads to endothelial injury as it was concluded in some researches. This hypothesis requires further evaluation. Among patients with systemic

sclerosis also additional medications may be introduced especially in the treatment of refractory symptoms. Phosphodiesterase type 5 enzyme inhibitors, synthetic analogs of prostacyclin, and an endothelin receptor antagonist are registered in the treatment of severe Raynaud's phenomenon, digital ulcers and pulmonary arterial hypertension. They lead to the smooth muscle relaxation with subsequent vasodilatation.

Abovementioned prophylactic strategies together with easy and accessible diagnostic tools can be considered in everyday clinical practice in order to prevent from cardiovascular complications in patients with connective tissue diseases.

5. Piśmiennictwo

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Endothelial dysfunction in patients with granulomatosis with polyangiitis: a case–control study

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Abstract

Background Granulomatosis with polyangiitis (GPA) is a rare granulomatous vasculitis affecting small- and medium-sized blood vessels. In optimally treated patients with long-standing disease, the common cause of death is atherosclerosis even in the absence of typical risk factors.

Objective To evaluate endothelial dysfunction in GPA patients.

Methods 44 patients (21 men and 23 women) diagnosed with GPA and 53 controls matched for age, sex, BMI and typical risk factors for cardiovascular diseases (22 men and 31 women) were enrolled in the study. We measured each participant's serum levels of vascular cell adhesion molecule-1 (VCAM-1), interleukin 6 (IL-6), and thrombomodulin. We also studied flow-mediated dilatation (FMD) of the brachial artery, intima-media thickness (IMT) of the common carotid artery and aortic stiffness using echocardiography.

Results Patients with GPA showed a 15.9% increase in serum levels of VCAM-1 ($p=0.01$), 66% of IL-6 ($p<0.001$) and 50.9% of thrombomodulin ($p<0.001$) compared to controls. FMD% was 48.9% lower in patients with GPA in comparison to controls ($p<0.001$), after adjustment for potential confounders, with no differences regarding IMT or aortic stiffness. FMD% was negatively associated with duration of the disease ($\beta=-0.18$ [95% CI: − 0.32 to − 0.04]), C-reactive protein ($\beta=-0.17$ [95% CI: − 0.27 to − 0.07]), IL-6 ($\beta=-0.29$ [95% CI: − 0.39 to − 0.19]), blood creatinine level ($\beta=-0.2$ [95% CI: − 0.3 to − 0.1]), and IMT ($\beta=-0.14$ (− 0.24 to − 0.04). In a multiple linear regression model, kidney function, IMT, pack-years of smoking, diabetes and level of VCAM-1 were independent predictors of lower FMD%.

Conclusion GPA is characterized by endothelial dysfunction. FMD is a useful tool for the detection of endothelial injury.

Keywords Endothelium · Atherosclerosis · Systemic vasculitis · Ultrasonography

Introduction

Endothelium plays a key role in vascular homeostasis. It acts as a barrier between tissues and circulating blood and as a signal transducer that regulates vasomotor activity [1]. Activation of endothelial cells leads to upregulation of adhesion molecules, such as P-selectin, E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (VCAM-1), resulting in attachment and migration of circulating leukocytes. Differentiation of migrated monocytes into macrophages and the subsequent uptake of lipids by these cells results in foam cell generation and fatty streak formation. Further recruitment of inflammatory cells and proliferation of smooth muscle cells leads to the development of atherosclerotic plaque [2]. Endothelial function can be determined with a noninvasive ultrasound measurement

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of flow-mediated dilatation (FMD) of a brachial artery. This is the direct measurement of the arterial endothelium's response to hyperemia (shear stress) that leads to the nitric oxide release and vasodilatation.

Granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis) is the common vasculitis with a prevalence of 3 per 100, 000 and peak incidence at the age of 50–60. Anti-neutrophil cytoplasmic antibodies (ANCA) are considered as the marker of the disease and are targeted against proteinase 3 (PR3). Its most common clinical features are granulomatous lesions of the upper and lower airways accompanied by the kidney failure [3, 4]. Cardiac involvement is infrequent in GPA, but coronary heart disease, arrhythmias, pericarditis, and nonbacterial thrombotic endocarditis can be present in these patients [5–8].

The increased morbidity from ischemic heart disease in GPA suggests that not only small vessels but also big ones are affected [9].

Atherosclerosis and its complications are one of the leading cause of death even in properly treated patients with long-standing ANCA-associated vasculitis (AAVs) [10, 11]. However, the mechanism by which atherosclerosis is promoted in these diseases is not explained by the classical atherosclerotic risk factors and remains under investigation.

The relationship between inflammation, vascular dysfunction and atherosclerosis is well-established. Premature atherosclerosis has been observed in patients with chronic inflammatory diseases such as systemic lupus erythematosus [12, 13], systemic sclerosis [14, 15] and antiphospholipid syndrome [16, 17]. There is currently a lack of reliable data on endothelial injury and development of premature atherosclerosis in the setting of vasculitis.

Previously, a few studies have analyzed the endothelial damage and progression of atherosclerosis in patients with systemic vasculitis [18–21]. Their results, however, were inconsistent and limited in significance due to the small number of subjects studied. For this reason, we sought to evaluate ultrasonographic and laboratory markers of endothelial injury in patients with GPA, which might be related to the premature and accelerated atherosclerosis and increased risk of cardiovascular events.

Methods

A case–control observational, retrospective study was carried out with approval of the Bioethics Committee of XXXX University Medical College (9th May 2013, number of protocol: KBET/79/B/2013). The patients were recruited from the population of patients at the Department of Allergy and Clinical Immunology at the University Hospital in XXXXX in the period between 2014 and 2017 who were in the disease flare or symptomatic. The control group was enrolled from the hospital personnel and relatives. They were selected according to matching criteria. All participants received a detailed brief of the methodology and safety protocols for the study and provided written consent for their participation.

Study groups

The case group constituted 44 patients with GPA—21 men and 23 women.

The control group consist of 53 individuals, 22 men and 31 women, matched to GPA patients by gender, age, body mass index (BMI) and smoking habit, as well as comorbidities, including hypertension, hypercholesterolemia, and diabetes mellitus.

Patients

Each patient had a current or previous diagnosis of GPA based on the criteria of the American College of Rheumatology [22]. We analyze only patients with the disease flare or those, who were symptomatic and diagnosed with persistent disease. Disease activity was measured using the Birmingham Vasculitis Activity Score (BVAS) [23]. Disease flare was defined as the presence of new symptoms (major or minor item in BVAS). Persistent disease was defined as the presence of one or more persistent symptoms attributed to active disease for more than 1 month but less than 3 months. For symptoms which occurred in patients since the onset of GPA and were present for more than 3 months, we used Vascular Damage Index (VDI) [24]. Patients with congestive heart failure, coronary heart disease, uncontrolled hypertension, liver failure, and cancer were excluded from the study (for details see supplementary material).

Main outcome variable

In this case–control study, we analyzed whether GPA is associated with vascular endothelial damage. We measured flow-mediated dilatation of the brachial artery, intima-media thickness of the common carotid artery, aortic stiffness as well as evaluated serum levels of thrombomodulin and VCAM-1 in GPA patients and matched control subjects.

Procedures

Laboratory analysis

Fasting blood samples were drawn in the morning from the antecubital vein using minimal stasis. Lipid profile, glucose, liver enzymes, urine, creatinine with eGFR, complete blood cell and platelet count were analyzed by routine laboratory techniques. C-reactive protein (CRP) was measured using

analyzer VITROS 250 Johnson & Johnson. Blood samples were drawn into serum separation tubes, centrifuged at 2000×g for 20 min at room temperature, within 2 h from sampling. The supernatant was frozen in aliquots and stored at -70 °C until analysis. Interleukin-6 (IL-6), VCAM-1, and soluble thrombomodulin were measured using standardized ELISA method (all, R&D Systems, Minneapolis, MN, USA). Anti-PR3 IgG was measured in all GPA subjects using ELISA assay (EUROIMMUN, Lübeck, Germany).

Ultrasound examinations

Ultrasound examinations were performed in a darkened, quiet, room, after at least 10 min rest in a supine position, using high-quality ultrasonograph (Sequoia 512 with a 10 MHz linear array ultrasonic transducer, MountainView, Ca, USA). Before examination, the subjects refrained from eating for at least 10 h. Examinations were conducted by two independent ultrasonography experts and considered parameters constituted a mean of three subsequent measurements. A complete transthoracic echocardiogram (TTE) was performed in every participant with estimation of ejection fraction of the left ventricle (EF) and systolic pulmonary artery pressure in accordance to standard methods [25]. Flow-mediated dilatation (FMD) of the brachial artery was measured in accordance to Celermayer' method [26]. Aortic stiffness was expressed as a percentage of aortic systolic diameter (ASD) and aortic diastolic diameter (ADD): aortic stiffness % = [(ADD − ASD)/ASD] × 100%. The intima-media thickness (IMT) of the carotid artery was also measured and in the further analysis we used a mean value of the IMT measured on the right and left common carotid artery (for details see supplementary material).

Statistical analysis

The results were compared between the case and control groups using STATISTICA 12.5 Software. Continuous variables, all non-normal distributed values (verified by the Shapiro–Wilk test), were given as median and interquartile range and compared by the Mann–Whitney U test. Categorical variables were presented as numbers (percentages) and compared by χ^2 test. Potential confounders were identified as: age, BMI, sex, and comorbidities such as arterial hypertension, diabetes mellitus and hypercholesterolemia. To adjust for these, obtained results of FMD%, IMT, aortic stiffness%, IL-6, VCAM-1, and thrombomodulin were log-transformed and a one way covariance analysis (ANCOVA) was performed, to achieve the overall p value. The univariate linear regression tests (with adjustment for aforementioned confounders) were used to analyze associations between two selected parameters. Independent determinants of FMD% were established in multiple linear regression model, built by a forward stepwise selection procedure, verified by F Snedecore's statistics, with $F > 1$. The R^2 was used as a measure of the variance. To calculate odds ratios (ORs) with 95% confidence intervals (CIs), unconditional multivariate logistic regression was performed. The cut-off values for IL-6, VCAM-1, FMD%, and thrombomodulin were determined based on receiver operating characteristic (ROC) curves. Results were considered statistically significant when the p value was less than 0.05.

Results

Characteristics of patients and controls

Demographic, clinical and laboratory characteristics of the studied subjects, including basic laboratory tests, ultrasound parameters, and cardiovascular risk factors were given in Table 1. Both groups were similar in age, sex, BMI as well as prevalence of comorbidities (hypercholesterolemia, hypertension, and diabetes mellitus), smoking habit, and family history of cardiovascular diseases. Parameters describing GPA activity, as well as current and past therapy were given in Table 2. The median duration of the disease was 4.5 years. More than half of the patients had active disease at the time of evaluation. All of them had detectable anti-PR3. Most of them were being treated with steroids currently or in the past with other immunosuppressive agents, such as: azathioprine, cyclophosphamide, methotrexate, mycophenolate mofetil and rituximab. Additionally, GPA patients were receiving statins, beta-blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists, diuretics and calcium channel blockers. Lungs were the most commonly involved organs, followed by paranasal sinuses and kidneys.

Basic laboratory tests and basic transthoracic echocardiographic parameters

As expected, GPA patients were characterized by higher inflammatory markers, such as CRP, IL-6 (reference range: 0.45–9.96 pg/ml) and white blood cells, as well as impaired kidney function and lower hemoglobin level (Table 1). Moreover, there were characterized by higher triglycerides.

In TTE GPA subjects had larger left and right ventricles and left atria, thicker posterior walls and interventricular septa, as well as lower ejection fraction and higher systolic pulmonary artery pressure.

Laboratory markers of endothelial injury

GPA patients had a 15.9% higher levels of VCAM-1 ($p = 0.01$) and a 50.9% increased thrombomodulin concentrations ($p < 0.001$) in peripheral blood, comparing to

Table 1 A summary of demographic, laboratory and echocardiographic parameters in patients with granulomatosis with polyangiitis and controls

	Patients, $n^* = 44$	Controls, $n = 53$	p value
Age (years)	59 (46–65)	48 (43–61)	0.07
Male gender, number (%)	21 (47.6)	22 (41.5)	0.67
Body mass index (kg/m2)	26.1 (24.1–29.6)	26.6 (23.9–29.1)	0.93
Basic laboratory tests			
Hemoglobin (g/dl)	12.25 (10.55–13.55)	13.7 (12.7–15)	<0.001[a]
Red blood cells (10^3/ul)	4.13 (3.7–4.5)	4.5 (4.2–4.9)	<0.001[a]
White blood cells (10^3/ul)	7.46 (5.76–10.06)	5.9 (5.03–6.96)	<0.001[a]
Platelet count (10^3/ul)	235.5 (171–287)	225 (200–275)	0.67
Total cholesterol (mmol/l)	4.7 (3.9–5.4)	4.9 (4.2–5.25)	0.58
Low-density lipoprotein (mmol/l)	2.4 (1.9–3.2)	3.1 (2.5–3.6)	0.003[a]
Triglycerides (mmol/l)	1.7 (1.2–2.1)	1.1 (0.7–1.5)	0.002[a]
Glucose (mmol/l)	5 (4.45–5.43)	4.95 (4.72–5.2)	0.95
Creatinine (mmol/l)	101.9 (72.5–240)	76.1 (68.3–90)	0.01[a]
Urea (mmol/l)	7.55 (5.8–12.7)	4.56 (3.93–5.3)	<0.001[a]
Estimated glomerular filtration rate (ml/min/1.73 m^2)	60 (26–67)	60 (60–80)	0.01[a]
Alanine transaminase (U/l)	21.5 (15–32)	22.5 (14–28)	0.72
C-reactive protein (mg/dl)	7.6 (5–19.4)	1.2 (1–2.1)	<0.001[a]
Interleukin-6 (pg/ml)	5.03 (3.02–10.5)	1.7 (1.08–2.16)	<0.001[a]
Echocardiographic parameters			
Left ventricular diastolic diameter (cm)	4.8 (4.6–5.3)	4.7 (4.5–4.9)	0.29
Left ventricular systolic diameter (cm)	3 (3–3.4)	3 (2.9–3.1)	0.049[a]
Right ventricular diameter (cm)	2.2 (2–2.3)	2.1 (1.9–2.3)	0.01[a]
Left atrial diameter (cm)	3.9 (3.7–4.1)	3.7 (3.5–3.9)	0.004[a]
Left ventricle posterior wall thickness (cm)	1.05 (0.9–1.2)	0.9 (0.8–1)	<0.001[a]
Interventricular septum thickness (cm)	1.1 (1–1.2)	0.9 (0.8–1)	<0.001[a]
Ejection fraction (%)	65 (60–68)	68 (68–70)	<0.001[a]
Pulmonary artery pressure (mmHg)	32 (30–36)	32 (26–32)	0.01[a]
Laboratory parameters of endothelial injury			
Vascular cell adhesion molecule-1 (ng/ml)	957 (749.1–1273.4)	804.6 (694.4–936.7)	0.01[a]
Thrombomodulin (ng/ml)	8.9 (5.2–1.4)	4.3 (3.9–4.7)	<0.001[a]
Ultrasound parameters of endothelial injury and atherosclerosis			
Relative increase of flow-mediated dilatation of a brachial artery	5.26 (4.08–8.01)	10.3 (8.89–12,5)	<0.001[a]
Aortic stiffness (%)	7.14 (4–9.09)	7.4 (6.25–10.34)	0.27
Median value of intima-media thickness of a common carotid artery (cm)	0.07 (0.06–0.08)	0.07 (0.06–0.08)	0.20
Other cardiovascular risk factors			
Hypertension n(%)	21 (50)	16 (30.2)	0.08
Diabetes mellitus n(%)	9 (21.43)	6 (11.3)	0.2
Hypercholesterolemia n(%)	14 (33.33)	19 (35.8)	0.68
Smoking currently n(%)	3 (7.14)	4 (7.55)	0.9
In the past n(%)	13 (30.95)	15 (28.3)	0.33
Smoking (packs/years)	0 (0–15)	0 (0–3)	0.64
Positive family history of cardiovascular diseases n(%)	9 (21.42)	7 (13.2)	0.32

[a]Categorical variables are presented as numbers (percentage), continuous variables as median and interquartile range. The results which are statistically significant are marked

healthy individuals. However, in ANCOVA analysis we documented that only thrombomodulin levels remained higher in GPA subjects after adjustment for potential confounders (age, sex, BMI, hypercholesterolemia, hypertension, and diabetes mellitus, $p < 0.001$). The VCAM-1 was similar in GPA and control groups in this analysis ($p = 0.54$).

Table 2 Clinical characteristics of the patients ($n=44$) with granulomatosis with polyangiitis

	Patients
Duration of the disease (years)	4.5 (1–9)
Active disease *n* (%)	26 (59.1)
BVAS in active disease	9 (8–10)
Persistent disease n(%)	16 (36.36)
BVAS in persistent disease	4 (3–5)
Anti-proteinase 3 antibodies (IU/ml)	20.5 (5–65)
VDI score in eligible patients	3 (0–5)
Organ involvement	
Cutaneous vasculitis *n* (%)	13 (30.95)
Granulomatous lesions in ears/hearing disturbances *n* (%)	11 (26.19)
Granulomatous lesions in larynx *n* (%)	6 (14.63)
Paranasal sinuses inflammation *n* (%)	30 (71.42)
Bone destruction of paranasal sinuses n(%)	16 (38.1)
Chronic kidney disease *n* (%)	22 (52.38)
Lungs *n* (%)	31 (73.81)
Peripheral nerves *n* (%)	10 (23.8)
Gastrointestinal system *n* (%)	1 (2.38)
Heart *n* (%)	1 (2.38)
Treatment characteristic	
Current steroids *n* (%)	37 (88.1)
Current steroids dose (mg/day of prednisone)	8 (4–20)
Systemic steroids therapy (years)	2 (0.5–5)
Immunosuppressive treatment (currently or in the past)	
Azathioprine *n* (%)	12 (28.57)
Cyclophosphamide *n* (%)	37 (88.1)
Total dose of cyclophosphamide (grams)	8.15 (3.9–19)
Methotrexate *n* (%)	5 (11.9)
Mycophenolate mofetil *n* (%)	2 (5.26)
Rituximab *n* (%)	13 (30.95)
Internal medicine medications	
Angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists *n* (%)	12 (28.57)
Statins *n* (%)	21 (51.22)
Beta-blockers *n* (%)	17 (40.48)
Diuretics *n* (%)	12 (28.57)
Calcium channel blockers *n* (%)	12 (28.57)

Categorical variables are presented as numbers (percentage), continuous variables as median and interquartile range

n number, *BVAS* Birmingham Vasculitis Activity Score, *VDI* vascular damage index

Moreover, patients with GPA had increased risk of elevated VCAM-1 (OR 5.75 [95% CI: 2–16.38], reference range: 349–991 ng/ml), and thrombomodulin (OR 6.71 [95% CI: 3.37–13.3], reference range: 2.9–5.3 ng/ml) compared to the healthy individuals (cut-off points: 1213.96 and 5.9 ng/ml, respectively). As expected, both endothelial injury markers were related to the CRP ($\beta=0.18$ [95% CI: 0.08–0.28], and $\beta=0.28$ [95% CI: 0.27–0.29], VCAM-1 and thrombomodulin, respectively) and IL-6 level ($\beta=0.27$ [95% CI: 0.15–0.39], and $\beta=0.4$ [95% CI: 0.27–0.53], VCAM-1 and thrombomodulin, respectively). Moreover, we demonstrated strong positive association between white blood cells and thrombomodulin ($\beta=0.2$ [95% CI: 0.11–0.29]). Table 3 demonstrates the most important associations of selected laboratory and echocardiographic parameters after adjustment for confounders with linear regression models. We documented positive associations between laboratory parameters of endothelial damage and kidney function, anti-PR3 level as well as interventricular septum and posterior wall thickness (see Table 3).

GPA patients with hypertension had 30. 6% higher levels of VCAM-1 (1178.3 [815.2–1600.1] vs. 893.8 [635.4–959.8] ng/ml, $p=0.02$) and 41% higher thrombomodulin levels (11.3 [7.8–17.6] vs. 5.9 [4.4–9.5] ng/ml, $p=0.01$) than the remaining GPA patients. Moreover, GPA patients with chronic kidney disease were characterized by higher thrombomodulin level (11.7 [9.4–17.6) vs. 5.4 [4.4–6.9] ng/ml, $p<0.001$) and VCAM-1 level (1258.2 [893.2–1457.7] vs. 747.4 [546.9–917.5] ng/ml, $p<0.001$). Other comorbidities had no impact on laboratory markers of endothelial damage. Patients taking statins and antihypertensive medications had increased thrombomodulin blood level (10.7 [7.5–15.2] vs. 5.6 [4.1–11.9] ng/ml, $p=0.03$, and 11.1 [7.1–16.1] vs. 5.4 [3.9–8.1] ng/ml, $p=0.01$, respectively), Moreover, those treated with antihypertensives had higher level of VCAM-1 (1089.7 [884.5–1293.1] vs. 803.4 [520.1–905.3] ng/ml, $p=0.01$).

In the subgroups analysis, patients in persistent disease or in active disease had similar results of laboratory parameters of endothelial injury (thrombomodulin 12.63 [5.99–18.86] vs. 10.18 [5.24–11.4] ng/ml, $p=0.19$, VCAM-1 1099.05 [863.94–1263.64] vs. 1065.82 [745.64–1273.38] ng/ml, $p=0.58$).

Ultrasound parameters of endothelial injury

GPA patients had 48.9% decrease in FMD% compared to controls ($p<0.001$, also after adjustment for potential confounders: age, sex, BMI, hypercholesterolemia, hypertension, diabetes mellitus $p<0.001$), and markedly higher risk of diminished FMD% defined as values below the cut-off point of 8.51 (OR 4.9 [95% CI: 2.88–8.23]).

In Table 3 are given selected associations of FMD% with other laboratory and ultrasound parameters. As presented, FMD% was negatively associated with white blood cells ($\beta=-0.24$ [95% CI: -0.32 to -0.15]), CRP ($\beta=-0.17$ [95% CI: -0.27 to -0.07]), IL-6 ($\beta=-0.29$ [95% CI: -0.39 to -0.19]) and the blood creatinine level ($\beta=-0.2$ [95% CI:-0.3 to -0.1]) in univariate linear regression models. Interestingly, FMD% was also negatively related to smoking

Table 3 Correlations of selected laboratory and echocardiographic parameters in GPA patients

	Flow-mediated dilatation% β (95% CI)	Intima-media thickness (cm) β (95% CI)	Aortic stiffness%, β (95% CI)	Vascular cell adhesion molecule-1 (ng/ml), β (95% CI)	Trombomodulin (ng/ml), β (95% CI)
White blood cells (10^3/ul)	− 0.24 (− 0.32 to − 0.15)*	0.07 (− 0.02 to 0.16)	− 0.23 (− 0.36 to − 0.1)*	0.08 (− 0.02 to 0.18)	0.2 (0.11 to 0.29)*
C-reactive protein (mg/dl)	− 0.17 (− 0.27 to − 0.07)*	− 0.03 (− 0.13 to 0.07)	− 0.19 (− 0.31 to − 0.7)*	0.18 (0.08 to 0.28)*	0.28 (0.27 to 0.29)*
Interleukin-6 (pg/ml)	− 0.29 (− 0.39 to − 0.19)*	− 0.03 (− 0.13 to 0.07)	0.08 (− 0.06 to 0.22)	0.24 (0.14 to 0.34)*	0.29 (0.2 to 0.38)*
Creatinine(mmol/l)	− 0.2 (− 0.3 to − 0.1)*	0.18 (0.08 to 0.28)*	0.27 (0.15 to 0.39)*	0.39 (0.29 to 0.49)*	0.6 (0.53 to 0.67)*
Urea(mmol/l)	− 0.06 (− 0.16 to 0.04)	0.11 (0 to 0.22)	0.11 (− 0.04 to 0.26)	0.27 (0.06 to 0.38)*	0.63 (0.55 to 0.71)*
Estimated glomerular filtration rate (ml/min/1.73 m^2)	0.05 (− 0.05 to 0.15)	− 0.12 (− 0.24 to 0)	0.00 (− 0.19 to 0.19)	− 0.23 (− 0.36 to − 0.1)*	− 0.48 (− 0.38 to − 0.58)*
Interventricular septum thickness (cm)	− 0.23 (− 0.33 to − 0.13)*	0.12 (0.01 to 0.23)*	0.01 (− 0.13 to 0.15)	0.29 (0.17 to 0.41)*	0.43 (0.33 to 0.53)*
Posterior wall thickness (cm)	− 0.29 (− 0.39 to − 0.19)*	0.16 (0.05 to 0.27)*	0.00 (− 0.14 to 0.14)	0.35 (0.24 to 0.46)*	0.5 (0.41 to 0.59)*
Flow-mediated dilatation%	–	− 0.12 (− 0.22 to − 0.02)*	0.25 (0.1 to 0.4)*	0.03 (− 0.09 to 0.15)	− 0.07 (− 0.17 to 0.03)
Intima-media thickness (cm)	− 0.14 (− 0.24 to − 0.04)*	–	0.00 (− 0.18 to 0.18)	− 0.09 (− 0.22 to 0.04)	0.05 (− 0.05 to 0.15)
Aortic stiffness%	0.2 (− 0.07 to 0.43)	0.00 (− 0.12 to 0.12)	–	0.31 (0.17 to 0.44)*	0.16 (0.03 to 0.29)*
Vascular cell adhesion molecule-1 (ng/ml)	0.02 (− 0.08 to 0.12)	− 0.08 (− 0.18 to 0.02)	0.3 (0.17 to 0.43)*	–	0.57 (0.49 to 0.65)*
Thrombomodulin (ng/ml)	− 0.09 (− 0.19 to 0.01)	0.05 (− 0.05 to 0.15)	0.19 (0.04 to 0.34)*	0.72 (0.62 to 0.82)*	–
Smoking (packs/years)	− 0.33 (− 0.44 to − 0.12)*	0.04 (− 0.02 to 0.1)	− 0.17 (− 0.47 to 0.13)	− 0.2 (− 0.47 to 0.07)	− 0.04 (− 0.3 to 0.2)
Steroids time of treatment (years)	− 0.07 (− 0.23 to 0.1)	0.19 (0.03 to 0.35)*	− 0.19 − 0.43 to 0.05	− 0.1 − 0.29 to 0.09	0.05 − 0.13 to 0.22
Duration of the disease	− 0.18 (− 0.32 to − 0.04)*	0.27 (0.14 to 0.41)*	0.13 (− 0.08 to 0.34)	− 0.1 (− 0.28 to 0.08)	0.09 (− 0.06 to 0.24)
Concentration of anti-proteinase 3 antibodies (IU/ml)	0.00 (− 0.14 to 0.14)	− 0.08 (− 0.23 to 0.07)	− 0.04 (− 0.24 to 0.16)	0.19 (0. 02 to 0.36)*	0.163 (0.001 to 0.33)*

The resulting regression coefficients (β) were given after adjustment for age, sex, BMI, and comorbidities (hypertension, diabetes mellitus and hypercholesterolemia)

*The results which are statistically significant are marked

(packs/years) ($\beta = -0.33$ [95% CI: − 0.44 to − 0.12)], duration of the disease ($\beta = -0.18$ [95% CI: − 0.32 to − 0.04]), as well as posterior wall and interventricular septum thickness ($\beta = -0.29$ [95% CI: − 0.39 to − 0.19], $\beta = -0.23$ [95% CI: − 0.33 to − 0.13], respectively).

A multiple regression model showed that various factors independently determined FMD%, including presence of diabetes mellitus ($\beta = -0.41$ [95% CI: − 0.55 to − 0.27]), pack-years of smoking ($\beta = -0.14$ [95% CI: − 0.29 to − 0.01]), IMT ($\beta = -0.34$ [95% CI: − 0.5 to − 0.18]), serum urea ($\beta = -0.41$ [95% CI: − 0.61 to − 0.21]) or VCAM-1 ($\beta = -0.33$ [95% CI: − 0.53 to − 0.13]), (Table 4).

Among GPA patients, lower FMD% was observed in those with hypertension (5 [2.9–5.9] vs. 6.4 [4.7–8.9], $p=0.01$), diabetes (3.4 [2.9–5] vs. 6 [4.3–8.4], $p=0.01$) and smoking currently or in the past (4.3 [2.9–5] vs. 6.7 [5–0.3]). Interestingly, only those treated with azathioprine had lower FMD% (4.13 [2.9–5.13] vs. 6.7 [4.9–8.4], $p=0.01$) without influence of other immunosuppressive drugs. Patients treated with statins were also characterized by decrease in FMD% (4.5 [2.9–5.3] vs. 6.8 [5.1–9.4], $p=0.01$).

The values of aortic stiffness% and IMT were similar in both studied groups. Aortic stiffness% and IMT did not correlate with laboratory parameters of endothelial injury.

Table 4 Multiple linear regression model for a relative increase of flow-mediated dilatation of a brachial artery comparing patients and control group

	Patients		Control	
	β (95% CI)	R^2	β (95% CI)	R^2
Duration of the disease (years)	0.15 (− 0.08 to 0.38)	0.51	–	0.32
Total cholesterol level (mmol/l)	− 0.06 (− 0.19 to 0.07)		0.07 (− 0.05 to 0.19)	
Urea (mmol/l)	− 0.41 (− 0.61 to − 0.21)*		− 0.22 (− 0.36 to − 0.08)*	
Posterior wall thickness (cm)	0.03 (− 0.15 to 0.21)		− 0.41 (− 0.56 to − 0.26) *	
Intima-media thickness of a common carotid artery (cm)	− 0.34 (− 0.50 to − 0.18)*		− 0.20 (− 0.35 to − 0.05)*	
Total dose of cyclophosphamide (grams)	− 0.19 (− 0.41 to 0.03)		–	
Smoking (packs/years)	− 0.14 (− 0.29 to − 0.01)*		–	
Diabetes mellitus	− 0.41 (− 0.55 to − 0.27)*		–	
Vascular cell adhesion molecule-1 (ng/ml)	− 0.33 (− 0.53 to − 0.13)*		− 0.09 (− 0.22 to 0.04)	
Adjustment statistics	$F=2.84$, $p=0.01$		$F=4.2$, $p<0.001$	

The resulting standardized regression coefficient (β) with 95% confidence intervals (95% CI) for a factor (independent variable) indicates the increase/decrease in standard deviations (SDs) of dependent variable, when that particular factor increases with 1 SD and all other variables in the model are unchanged. The results which are statistically significant are marked *

However, IMT was related to FMD% ($\beta = -0.12$ [95% CI: − 0.22 to − 0.02]), blood creatinine level ($\beta=0.18$ [95% CI: 0.08 to 0.28]), duration of steroid treatment ($\beta=0.19$ [95% CI: 0.03 to 0.35]) duration of the disease ($\beta=0.27$ [95% CI: 0.14 to 0.41]), posterior wall thickness ($\beta=0.16$ [95% CI:0.05 to 0.27]), and interventricular septum thickness ($\beta=0.12$ [95% CI:0.01 to 0.23]) (Table 3).

In the subgroup of GPA patients with chronic kidney disease IMT was higher (0.08 [0.06–0.09] vs. 0.065 [0.055–0.075], cm $p=0.01$). GPA subjects treated with statins and with hypertension had higher IMT than remaining GPA patients (0.08 [0.07–0.09] vs. 0.06 [0.05–0.07] cm, $p=0.01$, and 0.08 [0.07–0.09] vs. 0.06 [0.06–0.07] cm, $p=0.001$, respectively). Only one patient had an atherosclerotic plaque in the left common carotid artery.

Aortic stiffness% was negatively associated with blood leukocyte count ($\beta = -0.23$ [95% CI: − 0.36 to − 0.1] and CRP level ($\beta = -0.19$ [95% CI: − 0.31 to − 0.7]. Comorbidities and medication had no impact on aortic stiffness%.

Ultrasound parameters of endothelial injury were comparable between the patients in the disease flare and in the persistent disease (FMD% 5.92 [3.35–8.89] vs. 5.86 [4.35–7.14], $p=0.76$, IMT 0.07 [0.06–0.09] vs. 0.07 [0.06–0.08] cm, $p=0.92$, aortic stiffness% 7.93 [4.56–10.53] vs. 7.69 [3.85–8.82], $p=0.4$).

Discussion

This study demonstrates that GPA patients suffer vascular endothelial damage that is manifested by increased serum levels of thrombomodulin and VCAM-1, as well as lower flow-mediated dilatation of the brachial artery. Both the laboratory markers of endothelial injury increase while FMD decreases in association with inflammatory markers, such as IL-6 and CRP. These observations suggest that the most important predictor of endothelial damage in GPA is a persistent systemic inflammatory state. However, multiple regression analysis shows that impaired FMD was also independently determined by other factors, including kidney insufficiency, diabetes as well as smoking habit.

The associations of laboratory and ultrasound parameters of endothelial damage with markers of inflammation may indicate that the process of endothelial injury is more prominent in active phase of the disease. However, comparing the subgroups in persistent disease or in active disease these parameters did not differ regardless of phase of the disease. According to Tervaert [27] atherosclerosis is accelerated in the active phase but endothelial function returns to normal when inflammation is pharmacologically controlled. Another study showed that successful immunosuppressive treatment improves endothelial function, reaffirming the key role of inflammation in pathogenesis of atherosclerosis in this disease [28]. Such thesis was also recognized in children with primary systemic vasculitis without common cardiovascular risk factors [29]. Moreover, Nienhuis et al. [21] demonstrated impaired endothelium-dependent vasodilatation in microcirculation of the hand vessels of GPA patients, even if they did not have clinical manifestations of atherosclerosis.

In our study, we did not document any significant differences in IMT between GPA patients and healthy controls. This observation stays in line with results published by De Souza et al. [30], who suggested that premature atherosclerosis in GPA patients might be postponed by use of statins or prednisolone. This contradicts other reports [21, 31] that have shown that GPA patients are characterized by higher

IMT, indicating accelerated atherosclerosis. Potential discrepancy between these results may be due to the small sample size, different treatment mode or duration of the disease in evaluated patients. Importantly in our study, we found that IMT related positively to the time course of GPA.

The reason of endothelial dysfunction in GPA remains unknown. However, it seems that it might be related to the pathogenesis of the disease and eventually its therapeutic possibilities. In our study, the level of anti-PR3 antibodies was related to markers of endothelial injury. As it has been shown, autoantibodies in AAVs activate neutrophils which then adhere to the inner vessel wall causing endothelial impairment by release of proteolytic enzymes and triggering vasculopathic cascade [32, 33]. Moreover, pro-inflammatory cytokines increased in active systemic vasculitis depress endothelium-dependent relaxation in vitro, as well as in vivo [34], while reactive oxygen species lead to oxidation of lipoproteins which are responsible for endothelial cell injury [35].

In the study by Clarke et al. [29] the level of endothelial damage biomarkers was predominantly affected by disease activity rather than by treatment. However, it has been shown that cyclophosphamide, a cytotoxic drug used to induce remission in AAVs patients, directly injures endothelial cells leading to subsequent leakage of plasma to the extravascular space [36]. Colleoni et al. [37] observed a significant drop in the level of vascular endothelial growth factor in breast cancer patients after oral administration of cyclophosphamide in small doses, which also suggests its anti-angiogenic effect. This finding was also confirmed by Folkman et al. [38], who found that systemic administration of cyclophosphamide, anthracyclines or paclitaxel, inhibits neovascularization in the mouse cornea. In cancer treatment, cyclophosphamide is used in higher doses than in AAVs, however, it should be considered as an additional factor of potential endothelial damage. In our study, we did not observe differences in parameters of endothelial injury in those patients treated vs. those not treated with cyclophosphamide or other immunosuppressive drugs, such as methotrexate, mycophenolate mofetil, and rituximab (data not shown). Only in those treated with azathioprine the FMD% value was lower. There are just a few reports describing potential role of azathioprine in endothelial cell injury [39, 40]. However, the majority of the GPA patients were also treated with steroids. Prolonged steroid therapy is associated with hypertension, diabetes mellitus and change in the lipid profile, all of which influence the risk of atherosclerosis and cardiovascular events [41].

It has been demonstrated that renal insufficiency might be related to the endothelial dysfunction in other autoimmune diseases [2] as well as in peritoneal dialysis [42]. Hypertension, one of the complications of kidney failure, is known to be implicated in increased arterial stiffness and endothelial dysfunction. Additionally, uremia has been considered as a nontraditional cardiovascular risk factor [43]. However, this relationship in GPA patients has not been described in previous reports [44]. In our study, we observed positive association of blood creatinine, as well as urea level with FMD%, VCAM-1, and thrombomodulin. GPA patients with chronic kidney disease also had increased levels of VCAM-1, thrombomodulin and IMT. This is a novel finding of our study. Urea level in GPA patients was also independent predictors of lower FMD% in a multiple linear regression model.

The lower FMD in subjects with GPA documented in our study is possibly related not only to the endothelial dysfunction, but also to the arteries wall remodeling. In the previous study the blood vessels of these patients showed an increased level of matrix metalloproteinases (markers of vascular remodeling) [31]. Moreover, we have found a negative correlation between FMD and interventricular septum and posterior wall thickness. We also demonstrated that patients with GPA are characterized by larger left and right ventricular diameters and left atrial diameter, as well as increased left ventricle posterior wall thickness and interventricular septum thickness. In our opinion, these findings are most likely related to the inflammatory process of the kidneys and lungs, the organs, most often affected by GPA. Kidney damage results in overload of the circulatory system, whereas the inflammatory process in the lungs leads to the pulmonary hypertension. Both of these processes lead to the secondary hypertrophy of the heart cavities, which we have found in our patients. Observed differences might be also related to the cardiovascular system involvement in course of GPA and vessel wall remodeling, leading to the increased stiffness of the arteries and higher afterload, as well as heart hypertrophy. These findings, however, require further investigation.

Smoking currently or in the past also influenced lower FMD in GPA patients and it was an independent predictor of lower FMD.

Described changes may lead to the increased risk of cardiovascular events, what has been previously demonstrated by Faurschou et al. [9]. In his study, patients with GPA had 1.9 (95% CI: 1.4–2.4) higher risk of cardiovascular disease. Based on this result, we may speculate that early detection of endothelial dysfunction in GPA patients may help in selecting the most suitable preventive strategy. Tervaert [27] suggested that patients with large-vessels vasculitis should be treated with aspirin (75–125 mg/day) to prevent ischemic complications. Statins should also be advised in most patients with GPA for endothelial protection. Obviously, patients benefit from optimal immunosuppressive treatment that controls inflammation and prevents from consequences of GPA. However, it is necessary to keep in mind that immunosuppressive treatment directly leads to endothelial cell injury.

Study limitation

The limited number of GPA patients decreases the power of our findings. However, our study group is one of the biggest evaluated in the literature so far in terms of endothelial dysfunction. Moreover, the GPA is a rare disease and in our opinion every report is valuable. Patients with GPA had some comorbidities (diabetes mellitus, hypertension or kidney insufficiency), which in the majority of analyzed subjects were related to the systemic complications of vasculitis and might be considered as a consequence of GPA. We attempted to eliminate these confounding variables by an adjustment for comorbidities (hypercholesterolemia, hypertension, and diabetes mellitus) during statistical analysis and recruitment of controls with similar common cardiovascular risk factors. GPA patients were younger than controls, but this difference did not reach statistical significance. Finally, patients with vasculitis were being treated with many medications, notably immunosuppressive drugs and corticosteroids. The impact of the medications used on endothelial dysfunction was beyond the scope of our study; however, decreased values of FMD% in patients on statins and antihypertensive medications seemed to be related more to comorbidities (hypercholesterolemia, hypertension) than the drugs themselves. Nevertheless, we believe that the presented results reflect true intergroup differences.

Conclusion

In summary, the patients with GPA are characterized by endothelial dysfunction, which is likely related to the chronic systemic inflammation observed in autoimmune diseases. Although large observational studies are needed to verify whether lower FMD% is associated with increased risk of cardiovascular events in GPA patients, this noninvasive and simple ultrasound test seems to represent a new tool/predictor of endothelial injury for clinical practice.

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Compliance with ethical standards

A case–control observational study was carried out with approval of the Bioethics Committee of Jagiellonian University Medical College on 9th May 2013, number of protocol: KBET/79/B/2013.

Conflict of interest Authors declare that they have no conflict of interest

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ORIGINAL ARTICLE

Endothelial dysfunction in patients with eosinophilic granulomatosis with polyangiitis

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Abstract
Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare form of vasculitis associated with asthma and eosinophilia. Endothelial dysfunction has been well documented in other types of vasculitis but not in EGPA. Thirty patients (10 men and 20 women) diagnosed with EGPA and remaining in a remission, and 58 controls (24 men and 34 women) matched for age, sex, and body mass index, were enrolled in the study. We assessed each participants for typical risk factors of cardiovascular diseases and measured serum levels of vascular cell adhesion molecule-1 (VCAM-1), interleukin 6 (IL-6), and thrombomodulin. We also measured flow-mediated dilatation (FMD) of the brachial artery and intima-media thickness (IMT) of the common carotid artery using ultrasonography. Patients with EGPA had 20% higher serum level of VCAM-1 ($p < 0.001$) and 41.9% of thrombomodulin ($p < 0.001$). They also had 38.8% lower relative increase of FMD (FMD%) ($p < 0.001$), indicating endothelial dysfunction. These differences remained significant also after adjustment for potential confounders. Laboratory and ultrasonographic parameters of endothelial injury were correlated to the markers of inflammation and impaired kidney function. Determinants of lower FMD% in a simple regression model were pack-years of smoking ($\beta = -0.3$ [95% confidence interval (CI) −0.5 to −0.1]), serum level of IL-6 ($\beta = -0.36$ [95% CI −0.62 to −0.1]), and thrombomodulin ($\beta = -0.34$ [95% CI −0.6 to −0.08]). EGPA patients are characterized by inflammatory endothelial injury that is likely related to the pathogenesis of the disease. Proper immunosuppressive treatment is the best method to prevent atherosclerosis and future cardiovascular events, the patients may also benefit from additional preventive interventions.

Keywords Atherosclerosis · Endothelium · Eosinophilic granulomatosis with polyangiitis · FMD

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA, formerly named Churg-Strauss syndrome) is a rare disease with a prevalence that ranges from 10.7 to 13 cases per million [1]. Although EGPA is considered to be an anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), a significant ANCA serum concentration is found only in approximately 40% of patients, usually in the perinuclear immunofluorescence pattern (p-ANCA) [2]. Tissues affected show necrotizing vasculitis in EGPA patients who are ANCA-positive while in those without ANCAs, the vessel walls are infiltrated mainly by eosinophils [3]. Due to the lack of specificity of antibodies, diagnosis of EGPA is generally made clinically. However, a biopsy of the affected organ may reveal some typical features, such as the extravascular granulomas, small- and medium-sized vessels vasculitis, and the eosinophilic infiltrates [1]. In contrast to the other types of AAV, patients with EGPA characteristically suffer from asthma with eosinophilia, the hallmark of this disease, occurring in over 95% of individuals [4, 5]. Other symptoms include nasal polyps, lung infiltrates, and mononeuritis multiplex [6]. As

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EGPA is a systemic disease, clinical manifestation can be highly variable and patients with EGPA may also present with gastrointestinal, neurological, and cardiovascular symptoms [7–9].

In ANCA-positive AAV, vascular damage is caused by the stimulation of neutrophils by antibodies, their subsequent adhesion and migration to endothelium, release of proteolytic enzymes, and proinflammatory cytokines, leading to endothelial cell damage [3]. In the 60% of EGPA patients who are ANCA-negative, the exact pathology in unknown, but it is suspected to be related to impaired eosinophil function. A few studies have found endothelial damage and progression of atherosclerosis in AAV patients [10–13] but the significance of their results is limited by small sample size. We have found no such investigation specifically concerning patients with EGPA in the literature.

In this study, we sought to evaluate the endothelium function in subjects with EGPA. We performed a comprehensive analysis using laboratory and ultrasonographic tests. The inflammatory state was estimated by interleukin-6 (IL-6) serum level, while injury of endothelium was assessed by serum concentration of vascular cell adhesion molecule-1 (VCAM-1) and thrombomodulin. Endothelial function was determined by ultrasound measurements of flow-mediated dilatation (FMD) of a brachial artery. This method enables estimation of the endothelium's response to shear, releasing nitric oxide and dilatation. Progression of atherosclerosis was assessed by intima-media thickness (IMT) measurements of the common carotid arteries.

Methods

Participants

Our study is based on the case-control observational format. We received approval from the Bioethics Committee of Jagiellonian University Medical College. All study participants were given a thorough explanation of the methodology and safety protocol before giving written consent for their inclusion in the study.

The case group was made up of 30 patients with EGPA—20 women and 10 men. They were recruited in the period from 2014 to 2017 at the Department of Allergy and Clinical Immunology at the University Hospital in Cracow, a diagnostics and treatment center for vasculitis in southern Poland. The patients were all previously diagnosed with EGPA based on the criteria of the American College of Rheumatology [14]. The activity of the disease was measured using the Birmingham Vasculitis Activity Score (BVAS) [15]. We included only patients remaining in a remission of the disease. Remission was defined as an absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy [16]. Patients were excluded from the study if they also had angina pectoris, congestive heart failure, uncontrolled hypertension, liver failure, or cancer.

The control group was made up of 58 healthy individuals—34 women and 24 men matched to the EGPA group by gender, age, and body mass index [BMI]. Exclusion criteria were any manifestation of atherosclerosis, arterial hypertension, hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, congestive heart failure, liver injury, chronic kidney disease, smoking (current or more than 1 year in the past), and a positive family history of cardiovascular diseases. The criteria for each comorbidity were as follows: arterial hypertension, a history of hypertension (blood pressure > 140/90 mmHg) or ongoing antihypertensive therapy; hypercholesterolemia, a serum total cholesterol > 5.2 mmol/l or ongoing antihypercholesterolemic therapy; hypertriglyceridemia, a serum triglycerides > 1.7 mmol/l; diabetes mellitus, use of insulin or oral hypoglycemic agents or a fasting serum glucose > 7.0 mmol/l; congestive heart failure, left ventricular ejection fraction below 40%; liver injury, a serum alanine aminotransferase elevated more than twice above upper limit of the reference range; and chronic kidney failure, an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73 m^2.

Laboratory analysis

Fasting blood samples were drawn in the morning from the antecubital vein with minimal tourniquet use. Routine laboratory techniques were used to measure lipid profile, glucose, alanine transaminase, urine, creatinine for eGFR, and complete blood count (including eosinophilia and platelet count). C-reactive protein (CRP) was measured by the Johnson & Johnson VITROS 250. Blood samples were drawn into serum separation tubes, centrifuged at 2000×*g* for 10 min at room temperature within 2 h of collection. The supernatant was frozen in aliquots and stored at − 70 °C until analyzed. IL-6, VCAM-1, and soluble thrombomodulin levels were measured using standardized ELISA assay (R&D Systems, Minneapolis, MN, USA). Antinuclear antibodies (ANA) and ANCA were analyzed in patients by indirect immunofluorescence tests (ThermoFisher, Waltham, USA). Sera with positive ANA or ANCA were further analyzed with antigen-specific ELISA for autoantibodies against proteinase 3 (anti-PR3 IgG), myeloperoxidase (anti-MPO IgG) (EUROIMMUN, Lübeck, Germany).

Spirometry

Spirometry was performed using a Jaeger Master Screen spirometer on patients with EGPA according to the standards of the American Thoracic Society.

Ultrasound examinations

Ultrasound studies were performed in a darkened, quiet room with subjects resting in a supine position for 10 min prior and fasting for 10 h. Examinations were conducted by two independent ultrasound experts using the Siemens Acuson Sequoia 512 with a 10-MHz linear array ultrasonic transducer (MountainView, CA, USA). Both experts made three consecutive measurements of each parameter (described below). The value used for each parameter was the mean of the six measurements. In addition, a transthoracic echocardiogram (TTE) was performed for every participant with measurements of left ventricular ejection fraction (EF) and of systolic pulmonary artery pressure using standard methods [17].

Brachial artery ultrasonography

Flow-mediated dilatation (FMD) of the brachial artery was measured using Celermayer's method [18]. A baseline sagittal diameter (D1) of a distal part of the brachial artery was measured in the M-presentation by using a 10-MHz linear array ultrasonic transducer placed 2–3 cm proximal to the arterial bifurcation. A sphygmomanometer cuff was then placed on the forearm below the elbow and inflated to a pressure of 200 mmHg for 5 min and released. One minute after releasing the cuff, the brachial artery diameter was measured again (D2) at the same point. FMD was defined as the increase of brachial artery diameter after deflation of the cuff and was expressed as a percentage of the baseline diameter (FMD% = [(D2 − D1) D1] × 100%).

Intima-media thickness of the common carotid artery

The intima-media thickness (IMT) of the carotid artery was measured by ultrasound with a 10-MHz linear transducer. The thickness of the anterior and posterior walls of common carotid arteries was measured bilaterally in the longitudinal projection immediately proximal to their bifurcation. The mean value of the right and left thicknesses was used in further analysis.

Statistical analysis

The results of the EGPA and control groups were compared using STATISTICA 12.5 software. The continuous variables were all non-normally distributed according to the Shapiro-Wilk test. They are reported here as median and interquartile range and compared using the Mann-Whitney U test. Categorical variables are reported as percentages and compared by χ^2 test. Potential confounders are age, sex, BMI, and comorbidities, such as arterial hypertension, diabetes mellitus, and hypercholesterolemia. To adjust for these, the results of FMD%, IMT, VCAM-1, and thrombomodulin were log-transformed and a one-way covariance analysis (ANCOVA) was performed, first with an adjustment for age, sex, and BMI and subsequently to comorbidities. The univariate linear regression models with adjustment for BMI, age, and sex were used to analyze associations between two selected parameters. Unconditional multivariate logistic regression was performed to calculate odds ratios (ORs) with 95% confidence intervals (CIs). The cut-off values for VCAM-1, FMD%, and thrombomodulin were determined by receiver operating characteristic (ROC) curves. Determinants of FMD% were assessed using simple regression models. Results were considered statistically significant when the p value was less than 0.05.

Results

Characteristics of patients and controls

Demographic and laboratory characteristics of patients with EGPA and controls are presented in Table 1. Both groups were similar in age, sex, and BMI. Patients with EGPA had higher eosinophilia and inflammatory markers, such us white blood cells, CRP, and IL-6. They had also higher triglycerides level, while healthy individuals had increased low-density lipoprotein. The remaining basic laboratory parameters were comparable between both analyzed groups. In TTE, subjects with EGPA had lower ejection fraction; the values of the remaining parameters were similar in patients and controls (Table 1).

In Table 2, we have shown parameters determining characterization of the EGPA at the time of evaluation. The median duration of the disease was 4.5 years (range 3–8) and all the patients were in remission. Almost all of the subjects were diagnosed with asthma (96.7%, $n = 29$), with low to moderate bronchial obstruction in spirometry. Inflammation of the paranasal sinuses was found in 24 (80%) subjects and nasal polyps in 14 (46.7%). Heart involvement was documented in 66.7% ($n = 20$) of patients, 13 (43.3%) had lung infiltrates, and the same number was diagnosed with peripheral nerve damage.

Positive ANCA were detected in 53.3% of patients ($n = 16$), ANA in 33.3% ($n = 10$), and anti-MPO in 23.3% ($n = 7$), while anti-PR3 was present only in one subject.

Among patients with EGPA, 86.7% ($n = 26$) were currently being treated with corticosteroids (at a median dose equivalent to 8 mg/day of methylprednisolone, range 4–16). They also received immunosuppressive therapy in the past: azathioprine (30%, $n = 9$), cyclophosphamide (43.3%, $n = 13$), methotrexate (16.7%, $n = 5$), and mycophenolate mofetil or rituximab (both 3.3%, $n = 1$). Comorbidities in EGPA patients

Table 1 A summary of demographic, laboratory, and echocardiographic parameters in patients with eosinophilic granulomatosis with polyangiitis and controls

	Patients, $n^a = 30$	Controls, $n = 58$	p value
Age (years)	49 (44–58)	47.5 (40.5–58.5)	0.63
Male gender, number (%)	10 (33.3)	24 (41.4)	0.36
Female gender, number (%)	20 (66.6)	34 (58.8)	0.72
Body mass index (kg/m^2)	24.9 (21.2–27.5)	26.6 (23.9–29.1)	0.1
Basic laboratory tests			
Hemoglobin (g/dl)	13.4 (12.3–14.7)	13.7 (12.7–15)	0.42
Red blood cells (106/μl)	4.5 (4.1–5.0)	4.5 (4.2–4.9)	0.67
White blood cells (103/μl)	8.6 (6.96–13.1)	5.9 (5.03–6.96)	0 < 001*
Blood eosinophilia (103/μl)	0.3 (0.1–0.9)	0.1 (0.09–0.2)	0.02*
Blood platelet count (103/μl)	242 (187–327)	225 (200–275)	0.36
Total cholesterol (mmol/l)	5.4 (4.3–6)	4.9 (4.2–5.3)	0.12
Low-density lipoprotein (mmol/l)	2.5 (2.3–3.1)	3.1 (2.5–3.6)	0.02*
Triglycerides (mmol/l)	1.4 (1.1–2.1)	1.1 (0.7–1.5)	0.01*
Glucose (mmol/l)	4.7 (4.1–5.1)	4.95 (4.7–5.2)	0.07
Creatinine (mmol/l)	71.2 (59–81)	76.1 (68.3–90)	0.06
Urea (mmol/l)	5.3 (3.93–6.3)	4.6 (3.9–5.3)	0.15
Estimated glomerular filtration rate (ml/min/1.73 m^2)	81 (60–102.5)	60 (60–80)	0.03*
Alanine transaminase (IU/l)	26 (21–34)	22.5 (14–28)	0.05
C-reactive protein (mg/dl)	5 (1.7–11.1)	1.2 (1–2.1)	0 < 001*
Interleukin-6 (pg/ml)	4.5 (1.7–9.4)	1.7 (1.1–2.2)	0 < 001*
Echocardiographic parameters			
Left ventricular diastolic diameter (cm)	4.7 (4.4–4.9)	4.7 (4.5–4.9)	0.43
Left ventricular systolic diameter (cm)	3 (2.9–3.2)	3 (2.9–3.1)	0.55
Right ventricular diameter (cm)	2.1 (2–2.3)	2.1 (1.9–2.3)	0.42
Left atrial diameter (cm)	3.7 (3.4–3.8)	3.7 (3.5–3.9)	0.43
Left ventricle posterior wall thickness (cm)	1 (0.9–1)	0.9 (0.8–1)	0.19
Interventricular septum thickness (cm)	1 (0.9–1.1)	0.9 (0.8–1)	0.15
Ejection fraction (%)	62.5 (50–68)	68 (68–70)	0 < 001*
Pulmonary artery pressure (mmHg)	33 (30–36)	32 (26–32)	0.06
Laboratory parameters of endothelial injury			
Vascular cell adhesion molecule-1 (ng/ml)	966.1 (845.8–1129.5)	804.6 (694.4–936.7)	0 < 001*
Thrombomodulin (ng/ml)	6.1 (5.2–6.5)	4.3 (3.9–4.7)	0 < 001*
Ultrasound parameters of endothelial injury and atherosclerosis			
Relative increase of flow-mediated dilatation of a brachial artery (%)	6.3 (5.3–8.8)	10.3 (8.9–12.5)	0 < 001*
Median value of intima-media thickness of a common carotid artery (cm)	0.07 (0.06–0.08)	0.07 (0.06–0.08)	0.24

Categorical variables are presented as numbers (percentages) and continuous variables as median and interquartile range. The results which are statistically significant are marked *

[a] n number

included arterial hypertension (23.3%, $n = 7$), diabetes mellitus (10%, $n = 3$), and hypercholesterolemia (26.7%, $n = 8$) (Table 2). Angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists were taken by 36.7% of patients ($n = 11$), beta-blockers and diuretics by 26.7% ($n = 8$), and statins by 20% ($n = 6$), while calcium channel blockers were used in 13.3% ($n = 4$) of subjects. Ten subjects (33%) were ex-smokers.

Laboratory markers of endothelial injury

Patients with EGPA had a 20% higher serum level of VCAM-1 and a 41.9% of thrombomodulin compared to healthy individuals (both $p < 0.001$). In ANCOVA analysis, we demonstrated that both these markers remained increased in EGPA subjects also after adjustment for potential confounders (age, sex, BMI, hypercholesterolemia, hypertension, and diabetes

Table 2 Clinical characteristics of the patients ($n = 30$) with eosinophilic granulomatosis with polyangiitis

	Patients
Duration of the disease (years)	4.5 (3–8)
Antinuclear antibodies presence, n[a](%)	10 (33.3)
Anti-neutrophil cytoplasmic antibodies presence, n (%)	16 (53.3)
Anti-proteinase 3 IgG presence, n (%)	1 (3.33)
Anti-myeloperoxidase IgG presence (%)	7 (23.3)
Organ involvement	
Asthma, n (%)	29 (96.7)
Cutaneous vasculitis, n (%)	7 (23.3)
Granulomatous lesions in ears/hearing disturbances, n (%)	1 (3.3)
Nasal polyps, n (%)	14 (46.7)
Paranasal sinuses inflammation, n (%)	24 (80)
Bone destruction of paranasal sinuses, n (%)	7 (23.3)
Chronic kidney disease, n (%)	2 (6.7)
Lungs infiltrates, n (%)	13 (43.3)
Peripheral nerves damage, n (%)	13 (43.3)
Gastrointestinal symptoms, n (%)	6 (20.0)
Heart involvement, n (%)	20 (66.7)
Comorbidities	
Hypertension, n (%)	7 (23.3)
Diabetes mellitus, n (%)	3 (10.0)
Hypercholesterolemia, n (%)	8 (26.7)
Spirometry	
FEV1%[b]	77.8 (64.6–94.5)
VC%[c]	93.9 (86.9–108.3)
FEV1/VC%	69.5 (57.7–79.7)
Smoking	
Currently, n (%)	2 (6.7)
In the past, n (%)	10 (33.3)
Smoking (packs/years)	0 (0–2.5)
Treatment characteristic	
Current steroids, n (%)	26 (86.7)
Current corticosteroid dose, mg per day, recalculated to methylprednisolone	8 (4–16)
Systemic steroids therapy (years)	4 (2–6)
Immunosuppressive treatment (in the past)	
Azathioprine, n (%)	9 (30.0)
Cyclophosphamide, n (%)	13 (43.3)
Methotrexate, n (%)	5 (16.7)
Mycophenolate mofetil, n (%)	1 (3.3)
Rituximab, n (%)	1 (3.3)
Internal medicine medications	
Angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists, n (%)	11 (36.7)
Statins, n (%)	6 (20.0)
Beta-blockers, n (%)	8 (26.7)
Diuretics, n (%)	8 (26.7)
Calcium channel blockers, n (%)	4 (13.3)

Categorical variables are presented as numbers (percentages) and continuous variables as median and interquartile range

[a] n number

[b] *FEV1%* forced expiratory volume in 1 s, percentage of reference range

[c] *VC%* vital capacity, percentage of reference range

mellitus) (both, $p < 0.001$). EGPA subjects were characterized by a 2.63 [95% CI 1.13–5.17] increased risk of elevated VCAM-1 and a 4.68 [95% CI 2.3–9.5] risk of higher thrombomodulin, as compared to the controls (cut-off points 837.6 ng/ml and 5.2 ng/ml, respectively). Interestingly, both endothelial injury parameters were negatively associated with the duration of the disease ($\beta = -0.66$ [95% CI −0.9 to −0.41], $\beta = -0.43$ [95% CI −0.79 to −0.07] VCAM-1 and thrombomodulin, respectively).

The level of thrombomodulin, but not VCAM-1, was positively related to the inflammatory markers, such as IL-6 ($\beta =$ 0.52 [95% CI 0.17–0.87]), CRP ($\beta = 0.58$ [95% CI 0.17–0.99]), and white blood cell count ($\beta = 0.39$ [95% CI 0.06–0.72]). We found positive associations between VCAM-1 and urea or creatinine blood levels ($\beta = 0.49$ [95% CI 0.21–0.77] and $\beta = 0.36$ [95% CI 0.07–0.65], respectively). We also documented similar relationships for thrombomodulin ($\beta = 0.39$ [95% CI 0.04–0.74], $\beta = 0.69$ [95% CI 0.41–0.97], urea and creatinine blood

levels, respectively). Additionally, VCAM-1 in patients with EGPA was related to the interventricular septum and posterior wall thickness ($\beta = 0.52$ [95% CI 0.29–0.75], $\beta = 0.63$ [95% CI 0.42–0.84], respectively). Comorbidities, medications, and type of immunosuppressive treatment in the past had no impact on laboratory markers of endothelial damage in the EGPA group after comparison in subgroups.

Ultrasound parameters of endothelial injury

The EGPA group was characterized by a 38.8% decrease in FMD%, compared to the control group ($p < 0.001$), also after adjustment for potential confounders (age, sex, BMI, hypercholesterolemia, hypertension, and diabetes mellitus), and higher risk of diminished FMD% defined as values below the cut-off point of 8.51 (OR 3.8 [95% CI 2.2–6.56]). FMD% was negatively associated with CRP level ($\beta = -0.5$ [95% CI −0.75 to −0.25]) without associations with remaining inflammatory markers. However, a simple regression models showed that serum level of IL-6 ($\beta = -0.36$ [95% CI −0.62 to −0.1]) and thrombomodulin ($\beta = -0.34$ [95% CI −0.6 to −0.08]) may be related to lower FMD% values. The same relationship was found for pack-years of smoking ($\beta = -0.3$ [95% CI −0.5 to −0.1]).

Among patients with EGPA, a lower FMD% was observed in those with polyneuropathy (5.7 [5.1–6.2] vs. 8.3 [6.3–9.5], $p = 0.01$) without differences to the other subgroups regarding comorbidities or clinical symptoms.

The IMT measurement was similar in the EGPA and control groups and it did not correlate with laboratory parameters of endothelial injury.

Discussion

This study is the first to find that EGPA is characterized by endothelial injury. We demonstrated that even when EGPA is in remission, increased levels of thrombomodulin and VCAM-1 are present in the blood, suggesting ongoing endothelial damage. Moreover, laboratory markers of endothelial injury were accompanied by functional abnormalities in vasodilatation of the brachial artery without acceleration of atherosclerosis reflected by IMT values. We also found a positive correlation between VCAM-1 and interventricular septum and posterior wall thickness, which indicate general stiffness not only of vessels but also of the heart structures. The evaluated parameters of endothelial damage did not differ regardless of comorbidities, medications, or immunosuppressive treatment in the past, as it has been shown in statistical analyses performed in this study.

Our findings are partly in the line with other studies regarding endothelial injury in vasculitis. Schimtt et al. [19] demonstrated that serum levels of thrombomodulin were significantly elevated only during the active stage of EGPA and correlated with disease activity. However, anti-endothelial cell antibodies which may be one of the driving mechanisms for vascular injury in AAV were detected in EGPA patients regardless of the stage of the disease in their study. Sangle et al. [12] confirmed accelerated atherosclerosis in AAV patients (seven of them were EGPA subjects) by measurements of ankle-brachial pressure index. In the other study, performed by Chironi et al. [11], subclinical atherosclerosis was detected by ultrasonic measurements of plaque in three peripheral vessels (carotid and femoral arteries and abdominal aorta; in this study 11 EGPA patients were enrolled).

Several explanations for the mechanism of endothelial injury in EGPA have been proposed. Activated eosinophils secrete diverse pharmacologically active granule components with cytotoxic action capable of inducing damage of endothelial cells [20]. Eosinophil activation can be assessed by the detection of eosinophil cationic protein (ECP), major basic protein, and eosinophil peroxidase. All may accumulate on endothelial surfaces and have a potent damaging effect, directly and indirectly via the inhibition of the cell's ability to support thrombin-dependent activation of protein C [21]. It has also been shown that ECP can bind to Hageman factor (coagulation factor XII) in vitro and cause activation of the intrinsic coagulation pathway [20]. Notably, in a retrospective study assessing the role of eosinophils in thrombosis, 56% of patients with EGPA presented in medical history with venous thrombosis, 38% with arterial thrombosis, and 4% with mixed thrombosis [22]. In a study by Kain et al. [23], over 90% of enrolled patients with autoimmune diseases had autoantibodies to lysosomal membrane protein-2 that cause injury to endothelial cells in vitro. This may explain ANCA negativity in some AAV patients. In ANCA-positive subjects, the contact of neutrophils and ANCAs with endothelium is considered to be the key event in endothelial injury. In our study, the parameters of endothelial injury did not differ in subgroups regarding presence of ANCA, ANA, and anti-MPO antibodies (data not shown).

An alternative possibility for explanation endothelial injury in EGPA is the influence of traditional cardiovascular risk factors, including smoking and comorbidities, such as arterial hypertension, diabetes mellitus, and hypercholesterolemia. Although in our study, the pack-year smoking history was an important factor affecting diminished FMD%, it had no impact on laboratory markers of endothelial injury. Moreover, in our study, no comorbidity had any effect on endothelial injury parameters. Chronic kidney failure is another factor that is thought to lead to endothelial dysfunction in EGPA as it does in other autoimmune diseases [24]. This hypothesis is partly supported in our study, reflected by the positive correlation between VCAM-1 and thrombomodulin with the serum levels of creatinine and urea. However, only 7% of EGPA patients enrolled in our study had confirmed chronic kidney disease. Moreover, the presence of this

complication can be attributed to a manifestation of EGPA in these patients and may not even be a separate comorbidity.

Another mechanism is the atherogenic effect of systemic inflammation. This is suggested in our study by the inverse associations of white blood cells, CRP, and IL-6 with laboratory parameters of endothelial damage in the overall population of enrolled patients and controls (data not shown). Because the duration of the disease was negatively related to the CRP concentration (data not shown), we concluded that a longer disease course that is well managed with corticosteroids and immunosuppressants is a less active disease in terms of acute inflammation. This may explain our results of negative associations between VCAM-1, thrombomodulin, and the duration of the disease. Other studies found that only the active phase of vasculitis accelerates atherosclerosis and immunosuppressive treatment is the best method to control not only the disease but also to prevent endothelial injury [25, 26]. Gonzalez-Juanatey et al. [27] demonstrated significant improvement of FMD% 4 weeks after the onset of steroid therapy. Interestingly, other authors have shown that immunosuppressive treatment, especially with cyclophosphamide and azathioprine, directly injures endothelial cells [28, 29]. The number of patients in our study was too small to allow reliable subgroup analysis in terms of immunosuppressive treatment. Additionally, the majority of our patients with EGPA were also treated with steroids. Prolonged steroid therapy is associated with hypertension, diabetes mellitus, and change in the lipid profile, all of which influence on the risk of cardiovascular events. This is may be partially offset by the finding that steroids also improve endothelial function [27].

Based on the aforementioned results, we conclude that immunosuppressive treatment is the best method to control acute inflammation and prevent endothelial injury. However, even in the remission phase of EGPA, patients undergo endothelial injury and would therefore benefit from additional preventive interventions such as aspirin or statins therapy.

Our study is limited by the small number of participants due to the relative rarity of the disease, not allowing for the recruitment of a sufficient number of patients. Our control group was not well matched in terms of comorbidities but we attempted to eliminate these confounding variables by an adjustment for comorbidities (hypercholesterolemia, hypertension, diabetes mellitus) during statistical analysis. FMD% and IMT are subjective measurements. To reduce the effect of inter-operator variability, we used the same two researchers to perform all the measurements and we considered mean of three subsequent measurements in a further analysis.

Conclusion

Patients with EGPA present with endothelial injury which is mainly caused by the pathogenesis of the disease: the damaging effect of eosinophils, anti-neutrophil antibodies, and inflammation. Although the process is mainly active in acute inflammation, patients in remission of the disease also present with ongoing endothelial injury. Proper immunosuppressive treatment is the best method to prevent atherosclerosis and future cardiovascular events; however, the patients may benefit from additional preventive interventions.

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Compliance with ethical standards

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8.Artykuł nr. 3

Endothelial dysfunction in patients with systemic sclerosis

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Key words: atherosclerosis; endothelium; flow-mediated dilatation; intima-media thickness; scleroderma

Abstract

Introduction: Patients with systemic sclerosis experience endothelial dysfunction and damage even in the absence of clinical manifestations.

Aim: We evaluated various methods for assessing endothelial function for their applicability to clinical practice.

Patients and methods: 42 patients (7 men and 35 women) with systemic sclerosis and 36 controls (11 men and 25 women) matched for age, sex, body mass index, smoking habit, and comorbidities were enrolled in the study. We assessed each participants for typical risk factors of cardiovascular diseases and measured serum levels of vascular cell adhesion molecule-1 (VCAM-1), and thrombomodulin together with flow-mediated dilatation (FMD) of the brachial artery and intima-media thickness (IMT) of the common carotid artery using ultrasonography.

Results: Patients with systemic sclerosis did not differ from controls in serum levels of VCAM-1 and thrombomodulin, however, the statistical analysis with adjustment for potential confounders revealed increased levels of thrombomodulin in the patients ($p=0.03$). They also had 45% lower relative increase of FMD (FMD%), and 13% higher IMT ($p<0.01$, both, also after adjustment for potential confounders). In a simple regression models lower FMD% was determined by age ($\beta=-0.57$ [95% confidence interval (CI):-0.72 to -0.43]) and C-reactive protein levels ($\beta=-0.38$[95%CI:-0.55 to -0.22]). Thicker IMT was related to age ($\beta=0.64$ [95%CI:0.52 to 0.67]), glomerular filtration rate ($\beta=-0.34$ [95%CI:-0.5 to -0.18]), and blood thrombomodulin levels ($\beta=0.45$ [95%CI: 0.13 to 0.76]).

Conclusions: Patients with systemic sclerosis present with endothelial dysfunction which may be detected using ultrasonographic methods. The exact mechanism of observed abnormalities is unknown, but it possibly is related to the chronic inflammation and ischemia-reperfusion injury.

Key words: atherosclerosis; endothelium; flow-mediated dilatation; intima-media thickness; systemic sclerosis

Introduction

Systemic sclerosis (scleroderma) is a chronic disease of the connective tissue characterized by thickening and fibrosis of the skin and internal organs. Historically, there are two major disease subsets: the diffuse type, which is associated with a high risk of pulmonary arterial hypertension, acute renal injury and diffuse skin involvement (extremities proximal to elbows and knees, chest, abdomen and back) [1]; and the limited type, related to sclerodactyly and Raynaud's phenomenon, calcinosis, esophageal dysmotility, telangiectasia and limited skin involvement (face, neck and extremities distal to elbows and knees) [1,2]. Vascular damage in both systemic sclerosis subsets is mainly microvascular and Raynaud's phenomenon is the earliest manifestation of the disease. The other clinical symptoms related to microvascular abnormalities are digital ulcerations, renal insufficiency and pulmonary arterial hypertension [3,4]. Macrovascular damage is less frequent but increased stiffness of large arteries, plaque presence and increased intima-media thickness of the common carotid artery (IMT) have also been reported [1,5,6]. Systemic sclerosis is associated with increased risk of myocardial infarct, stroke and peripheral artery disease, although exact mechanisms of these abnormalities remain unknown [7,8].

Endothelial dysfunction seems to be a key mechanism in the pathophysiology of systemic sclerosis. In many studies the function of the endothelium was measured through flow-mediated dilatation (FMD) of the brachial artery which evaluates the response to shear, the subsequent release of. nitric oxide and dilatation of the vessel [9–18]. Several studies have found elevated IMT in patients with systemic sclerosis, which is considered as an indicator of atherosclerosis [5,9,12,16,18]. These aforementioned studies, however, conflict in results and were performed on small groups of patients. Endothelial damage can also be assessed through laboratory tests. It is related to the higher expression of adhesions molecules on endothelium, such as E-selectin, endothelin-1, and vascular cell adhesion molecule-1 (VCAM-1) which might be sheared into the blood stream. Activated endothelium also releases other molecules, such as von Willebrand factor, soluble thrombomodulin, and tissue plasminogen factor, which could be the markers of procoagulant activity [19].

Aim

Taking into account the ambiguous results of previously published studies on endothelial dysfunction in systemic sclerosis, we sought to determine the validity laboratory

parameters of endothelium damage, such as: serum concentration of thrombomodulin and VCAM-1, as well as to evaluate in ultrasound FMD of the brachial artery and measure IMT of the common carotid arteries [5,9,12,16,18]. Finally, we analyzed their relationships to the clinical features of the systemic sclerosis and antibodies levels. We believe that due to the rarity of that disease every report is valuable.

Material and methods

Our study follows the case-control observational, retrospective format and received approval from the Bioethics Committee of XXXX (9th May 2013, number of protocol: KBET/79/B/2013). All study participants received an explanation of the methodology and safety protocol and gave written consent to be included in the study.

The 42-patient case group was made up of 35 women and 7 men. They were enrolled in the period from 2014 to 2017 at the XXXX, a diagnostics and treatment center for autoimmune diseases in southern Poland. These patients were all previously diagnosed with systemic sclerosis according to the criteria of the American College of Rheumatology and all evaluated for the presence of typical systemic sclerosis autoantibodies such as anti-centromere, anti-topoisomerase I (anti-Scl-70, AC-29), anti-RNA polymerase III antibodies, and anti-PM/Scl antibodies [20]. Candidates for the case group were excluded from the study if they had confirmed atherosclerosis, angina pectoris, congestive heart failure, liver failure, uncontrolled hypertension, or cancer. Other comorbidities that did not disqualify a patient but were recorded include hypertension (a history of blood pressure > 140/90 mmHg or current antihypertensive treatment), hypercholesterolemia (a serum total cholesterol > 5.2 mmol/l or ongoing antihypercholesterolemic therapy), and diabetes mellitus (use of insulin or oral hypoglycemic agents, or a fasting serum glucose > 7.0 mmol/l).

The 36-individual healthy control group was made up of 25 women and 11 men that matched the case group in gender, age, body mass index (BMI) and smoking habit. They were selected with the same exclusion criteria as the studied group and their concomitant morbidities were likewise recorded. The control group was enrolled from the hospital's personnel and their relatives.

Laboratory analysis

Hospital staff collected fasting blood samples in the morning from the antecubital vein with the least possible tourniquet use. Lipid profile, levels of glucose, alanine transaminase, urine, creatinine for glomerular filtration rate (eGFR), and complete blood count were measured using routine laboratory techniques and CRP with the Johnson & Johnson VITROS

250. We measured serum parameters specific to this study from blood samples centrifuged in separation tubes at 2,000 x g for 10 minutes at room temperature within 2 hours of collection. Standardized ELISA method (all, R&D Systems, Minneapolis, MN, USA) was used in order to measure Interleukin (IL)-6, VCAM-1, and soluble thrombomodulin levels. Antinuclear antibodies (ANA) were analyzed using indirect immunofluorescence tests (ThermoFisher, Waltham, USA) and antigen-specific ELISA for autoantibodies against topoisomerase I, centromeres, RNA polymerase III, NOR 90, PM-Scl, Ku, PDGFR, fibrillarin, and Ro-52 (EUROIMMUN, Lübeck, Germany).

Ultrasound examinations

Two independent experts performed the ultrasound studies using the Siemens Acuson Sequoia 512 with a 10 MHz linear array ultrasonic transducer (Mountain View, Ca, USA). They performed the studies in a dark, quiet room with the subjects in a supine position for 10 minutes and having fasted for 10 hours. They measured flow-mediated dilation of the brachial artery and intima-media thickness of the carotid artery. They both made three consecutive measurements of every parameter and we recorded the mean of the six measurements for analysis. The ultrasonographers then performed a transthoracic echocardiogram (TTE) on each subject using standard methods [21].

Brachial artery ultrasonography. Celemayer's method was used to measure flow-mediated dilatation (FMD) of the brachial artery [22]. We used the technique described in details in our previous papers [23,24]. "The baseline sagittal diameter (D1) of a distal part of the brachial artery was measured in the M-presentation with a 10 MHz linear array ultrasonic transducer placed 2-3 cm proximal to the arterial bifurcation. A sphygmomanometer cuff was then placed on the forearm below the elbow and inflated to 200 mmHg for 5 minutes and released. One minute after releasing the cuff, the brachial artery diameter was measured a second time (D2) at the same point. FMD is the increase of brachial artery diameter after deflation of the cuff and is expressed as a percentage of the baseline diameter (FMD %= [(D2-D1)/D1] x 100%). We recorded the mean of the FMB of both arms for analysis."

Intima-media thickness of the common carotid artery. As previously described by us "the intima-media thickness (IMT) of the carotid artery was measured by ultrasound with a 10 MHz linear transducer. The thickness of the anterior and posterior walls of the common carotid arteries was measured in the longitudinal projection immediately proximal to their

bifurcation [23,24].” We recorded the mean value of the IMT on the right and left side for analysis.

Statistical analysis

The results of the case and control groups were compared using STATISTICA 12.5 software. The continuous variables all had non-normal distribution according to the Shapiro-Wilk test. We report them here as median and interquartile ranges and compare them using the Mann-Whitney U-test. Categorical variables are given as percentages and compared by $\chi 2$ test. Age, sex, BMI, and comorbidities, such as: arterial hypertension, diabetes mellitus, and hypercholesterolemia were considered as potential confounders. Therefore, the main outcome results: FMD%, IMT, VCAM-1, and thrombomodulin were log-transformed and a one way covariance analysis (ANCOVA) was performed to adjust for them. The univariate linear regression models with adjustment for BMI, age, and sex were used to analyze associations between two selected parameters. Markers of endothelial injury were assessed using simple regression models. Results were considered significant when the p value was less than 0.05.

Results

Characteristics of patients and controls

Table 1 presents characteristics of patients with systemic sclerosis and controls. As it has been shown, both groups were similar in age, sex, BMI, smoking habits and comorbidities (hypertension, diabetes mellitus and hypercholesterolemia).

Characteristics of the patients with systemic sclerosis at the time of evaluation were given in Table 2. The median duration of the disease was 4 (range: 1-7) years and the majority of the patients (69%, n=29) had a diffuse type of the disease. All of the systemic sclerosis patients had positive result of antinuclear antibodies in indirect immunofluorescence. The disease specific anti-topoisomerase I antibodies were present in half of them. Other types of antibodies, such as anti-centromere, anti-RNA polymerase III, anti-Ro-52, and anti-Ku antibodies had lower prevalence. Raynaud’s phenomenon was the most common clinical manifestation, followed by interstitial lung disease, skin lesions on the fingers, and pulmonary arterial hypertension. Half of the patients with systemic sclerosis were being treated with systemic glucocorticosteroids at the time of evaluation. They were also receiving or had received other immunosuppressive agents such as azathioprine, cyclophosphamide, methotrexate, mycophenolate mofetil, or rituximab. Among other medications, the most

common were statins, beta-blockers, angiotensin converting enzyme inhibitors or angiotensin receptor antagonists, diuretics and calcium channel blockers.

Basic laboratory tests and basic transthoracic echocardiographic parameters

Table 1 presents the results of basic laboratory tests and TTE parameters. As expected patients with systemic sclerosis had higher inflammatory markers, such as CRP and IL-6, as well as lower hemoglobin level. Surprisingly, individuals in the control group were characterized by higher total cholesterol level and creatinine concentration (in all subjects in normal range).

In TTE, patients with systemic sclerosis had slightly lower ejection fraction, thicker interventricular septum and higher systolic pulmonary artery pressure (Table 1). Moreover, those with the diffuse type of the disease were characterized by larger left atrium (3.9 [3.5-4.2] vs. 3.9 [3.8-3.9] cm, p=0.005) and left ventricle (both, diastolic diameter 4.6 [4.4-4.9] vs. 4.3 [4.2-4.6] cm, p=0.04 and systolic diameter 3 [2.9-3.1] vs. 2.9 [2.8-2.9] cm, p=0.02), thicker interventricular septum (1.1 [1-1.2] vs. 0.9 [0.8-1.1] cm, p=0.003) and thicker left ventricle posterior wall (1 [0.9-1.1] vs. 0.8 [0.9-0.9] cm, p=0.007).

Laboratory markers of endothelial injury

Patients with systemic sclerosis had similar serum levels of VCAM-1 and thrombomodulin as compared to healthy individuals (Table 3). However, the ANCOVA analysis performed with adjustment for potential confounders revealed that levels of thrombomodulin, but not VCAM-1, were increased in the systemic sclerosis group (p=0.03). Both laboratory markers of endothelial injury remained in a strong positive association between each other in patients and in controls (β=0.58 [95% confidence interval (CI): 0.4-0.76], β=0.44 [95%CI: 0.28-0.6], respectively).

In systemic sclerosis patients, we documented a strong positive association between thrombomodulin and kidney function (β=0.84 [95%CI: 0.53-1.15], β=0.69 [95%CI: 0.44-0.93], for creatinine and urea blood levels, respectively). For VCAM-1 such relation was demonstrated only for serum creatinine concentrations (β=0.67 [95%CI: 0.39-0.95]. Additionally, thrombomodulin in the patients with systemic sclerosis was related to the interventricular septum and posterior wall thickness (β=0.78 [95% CI: 0.5-1.06], β=0.83 [95% CI: 0.56-1.1], respectively), as well as CRP level (β=0.57 [95%CI: 0.31 to 0.83]).

Patients with systemic or limited disease had similar VCAM-1 and thrombomodulin levels. Likewise, comorbidities, treatment mode and type of antibodies had no impact on either laboratory markers of endothelial damage.

Ultrasound parameters of endothelial injury

As shown in Table 3 patients with systemic sclerosis were characterized by 45% lower FMD% and 13% higher IMT, as compared to the control group ($p<0.01$, both, also after adjustment for potential confounders). Moreover, both these unfavorable changed ultrasound parameters of endothelial injury remained in a mutual relationship to each other, but only in a patient group (β=-0.48 [95%CI:-0.63 to -0.32]). In a simple regression models lower FMD% was determined by age (β=-0.57 [95%CI:-0.72 to -0.43]) and CRP (β=-0.38[95%CI:-0.55 to -0.22]). Thicker IMT was also related to age (β=0.64 [95%CI:0.52 to 0.67]), to impaired kidney function (for eGFR β=-0.34 [95%CI:-0.5 to -0.18]), and to thrombomodulin blood levels (β=0.45 [95%CI: 0.13 to 0.76]).

For TTE parameters, IMT was positively associated with interventricular septum thickness (β=0.28 [95%CI: 0.07 to 0.49]), and posterior wall thickness (β= 0.24 [95%CI: 0.03 to 0.45]) in systemic sclerosis, while for FMD% such associations were not documented. IMT, but not FMD% was also related to the thrombomodulin level (β= 0.39 [95%CI: 0.11 to 0.67]) and duration of glucocorticosteroids treatment (β= 0.31 [95%CI: 0.1 to 0.52]) in the patient group.

Patients with systemic or limited systemic sclerosis did not differ in FMD% or IMT. Similarly, disease duration, treatment mode, or type of antibodies had no impact on these variables.

Finally, none of the analyzed comorbidities had any influence on FMD%, but IMT was greater in those with hypertension (0.06 [0.05-0.065] vs. 0.075 [0.065-0.085], p=0.004).

Discussion

In this study we have shown that patients with systemic sclerosis present with endothelial dysfunction which is demonstrated by decreased FMD% and increased IMT compared to well matched controls. Also, thrombomodulin level, a laboratory marker of endothelium damage, was higher in the patient group, after adjustment for potential confounders. As expected, parameters of endothelial dysfunction were determined by age, but also by variables related to the specificity of the disease, such as kidney injury and inflammatory markers. Another interesting finding of this study is that IMT is associated with

the interventricular septal and posterior wall thicknesses, and that it correlates with the thrombomodulin level. This relationship has not been reported before and allows us to speculate that endothelium damage might be followed by a remodeling of the large vessels wall, leading to increased stiffness of the arteries and higher afterload, as well as hypertrophy of the heart structures. Parameters evaluated in our study were related to the inflammatory markers, but did not depend on systemic sclerosis clinical manifestation, type of immunosuppressive treatment used currently or in the past, and type of antibodies present in the subgroups analysis.

Many previous reports have shown decreased FMD in patients with systemic sclerosis [5,9,12–14]. In some studies, however, the results were contradictory [15,16]. This discrepancy might be explained by the patient selection. In studies with negative results, more subjects presented limited systemic sclerosis, suggesting that the diffuse type is more prone to endothelial dysfunction. Our study contradicts this hypothesis because parameters of endothelial injury were comparable in subjects with limited and diffuse subtype of disease, although the number of enrolled subjects with the limited form was much smaller than those with diffuse disease.

Increased IMT is a diagnostic tool which indicates ongoing, subclinical atherosclerosis. Our results are consistent with previous reports documenting thicker IMT in systemic sclerosis [9,11]. However, Szucs et al. and Domsic et al. demonstrated that IMT was similar in patients with systemic sclerosis and controls [5,18]. Andersen et al. combined ultrasonographic and laboratory tests in order to evaluate endothelial function and have found elevated level of soluble E-selectin and VCAM-1 in patients with systemic sclerosis with inverse correlation to the FMD% [15].

The etiology of endothelial damage and atherosclerosis in systemic sclerosis might be related to multiple factors, including inflammation, traditional cardiovascular risk factors and the influence of ischemia with subsequent reperfusion. In our study, both groups were similar in terms of cardiovascular risk factors (BMI, gender, smoking habits, comorbidities). Individuals in the control group had slightly higher total cholesterol and low-density lipoprotein level. This may be due to higher prevalence of statins intake by patients with systemic sclerosis because the prevalence of hypercholesterolemia was similar in both groups. Higher creatinine levels in the controls (but within normal limits) is possibly due to greater muscle mass, although both groups had similar BMI. Traditional cardiovascular risk factors do not alone explain the accelerated atherosclerosis seen in patients with systemic sclerosis. The prevalence of hypertension, obesity, hypercholesterolemia, and diabetes were similar in

other cohort studies [7,25]. In fact, cardiovascular risk factors were less prevalent in patients with systemic sclerosis in the Australian Systemic sclerosis Cohort Study, suggesting that other factors are responsible for the observed abnormalities [8]. Mok et al. hypothesized that systemic sclerosis is an independent determinant of coronary calcification, as it is observed in the skin of affected subjects [25].

Inflammation is well-established as a factor leading to vascular dysfunction and atherosclerosis. Premature atherosclerosis has been observed in patients with chronic inflammatory diseases such as systemic lupus erythematosus, antiphospholipid syndrome or systemic vasculitides [26–30]. We also documented higher CRP and IL-6 level in the case group. Therefore, we may postulate that chronic inflammation accelerates atherosclerosis in systemic sclerosis.

Chronic endothelial damage is also caused by ischemia and reperfusion which lead to dysfunction and loss of cell integrity and tissue injury. In systemic sclerosis, tissue hypoxia together with chronic blood flow reduction related to microvascular abnormalities is a stimulus for increased expression of vascular endothelial growth factors (VEGF) and angiogenesis. VEGF also contributes to the development of fibrosis [31,32].

Timar et al. found that rosuvastatin improves FMD, corrects unfavorable changes in blood lipid profile, and decreases CRP, but does not reduce carotid atherosclerosis after a six-month treatment period in patients with systemic sclerosis [33]. In their study, Kuwana et al. conclude that statins may be beneficial in treating vascular manifestations such as Raynaud's phenomenon [34]. Therefore, statins should be advised in most patients with systemic sclerosis for endothelial protection but more clinical trials are needed to verify this hypothesis [35]. The other options in the treatment of endothelial dysfunction in systemic sclerosis are phosphodiesterase type 5 enzyme inhibitors (sildenafil, tadalafil), synthetic analogs of prostacyclin, and bosentan - an endothelin receptor antagonist. They lead to the smooth muscle relaxation with subsequent vasodilatation, and might be considered in the treatment of severe Raynaud’s phenomenon, digital ulcers and pulmonary arterial hypertension [36-38]. The patients enrolled in our study did not use abovementioned medications, but their impact on parameters of endothelial dysfunction is an interesting subject for further researches. Obviously, patients benefit most from optimal immunosuppressive treatment that controls inflammation and prevents from consequences of clinically active systemic sclerosis.

In summary, in this study we demonstrated that patients with systemic sclerosis present with endothelial dysfunction which may be detected using ultrasonographic methods.

FMD and IMT are easy tools with diagnostic and therapeutic relevance which can help elicit a group of subjects with subclinical atherosclerosis. Measurements of selected adhesion molecules blood levels might give conflicting results and should only be used in research. The exact mechanism of the observed disturbances is unknown, however, we may speculate that it is due to chronic inflammation and ischemia-reperfusion injury. Although large observational studies are needed to verify this hypothesis, it seems that proper immunosuppressive treatment might influence endothelium injury indirectly by reducing inflammatory response in systemic sclerosis subjects.

The authors declare that they have no conflict of interest.

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Authors' contribution: RP, PK, SB-S, JK,TI, LZ, SK, AR, JW, JM, JD conceived and designed the study, RP, PK, JD, JK, AR recruited the patients and analyzed clinical data, RP, and JD performed ultrasound tests, RP, PK, SB-S, JK, SK, AR, JW, JM, JD drafted the manuscript, LZ did statistical analysis and described section regarding statistics, TI performed the experiments, interpreted the laboratory data and described section regarding laboratory analysis, SK made a linguistic correction, RP, PK,SB-S, JK,TI, LZ, SK, AR, JW, JM, JD revised it critically and approved the final version.

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Tables

Table 1. A summary of demographic, laboratory and echocardiographic parameters in patients with systemic sclerosis and controls.

	Patients n^a=42	Controls n=36	p-value
Age, years	63.5 (49-70)	57 (47-65)	0.3
Male gender, number, %	7 (16.7)	11 (30.6)	0.15
Body mass index, kg/m^2	25 (23.3-27.5)	26.9 (23.7-28.7)	0.2
Hypertension, number, %	17 (40.5)	10 (27.8)	0.18
Diabetes mellitus, number, %	5 (11.9)	4 (11.1)	0.87
Hypercholesterolemia, number, %	15 (35.7)	8 (22.2)	0.18
Smoking currently, number, %	2 (4.8)	4 (11.1)	0.68
Smoking in the past, number, %	12 (28.6)	11 (30.6)	0.89
Smoking, packs/years	0 (0-5)	0 (0-3.5)	0.8
Basic laboratory tests			
High-density lipoprotein, mmol/l	1.3 (1.1-1.5)	1.4 (1.2-1.8)	0.12
Triglycerides, mmol/l	1.3 (1.1-2.1)	1.2 (0.8-1.8)	0.16
Glucose, mmol/l	4.8 (4.4-5)	5 (4.8-5.2)	0.11
Creatinine, mmol/l	67 (58-80)	74 (67-88)	0.03*
Urea, mmol/l	5.2 (4.3-6.1)	4.9 (4.2-5.5)	0.32
Alanine transaminase, U/l	23.5 (18.5-33)	23.5 (14.9- 30)	0.48
C-reactive protein, mg/dl	6.8 (5-18.7)	1.3 (1-2.6)	<0.01*
Interleukin-6, pg/ml	3.6 (2.5-10)	1.8 (1.3-2.3)	<0.01*
Echocardiographic parameters			
Left ventricular diastolic diameter, cm	4.6-(4.3-4.9)	4.7 (4.5- 4.9)	0.13
Left ventricular systolic diameter, cm	3 (2.9-3.1)	3 (2.9- 3.1)	0.56
Right ventricular diameter, cm	2.1 (1.9-2.3)	2.1 (1.9- 2.3)	1

Left atrial diameter, cm	3.7 (3.3-4)	3.7 (3.6-4)	0.76
Left ventricle posterior wall thickness**,** cm	1 (0.9-1.1)	0.9 (0.8-1)	0.14
Interventricular septal thickness, cm	1 (0.9-1.2)	0.9 (0.9-1.1)	0.04*
Ejection fraction, %	65 (60-67)	68 (67-68)	<0.01*
Systolic pulmonary artery pressure, mmHg	36 (32-42)	32 (29-34)	<0.01*

Categorical variables are presented as numbers (percentages), continuous variables as median and interquartile range. The results which are statistically significant are marked *.

Abbreviation: n[a]- number

Table 2. Clinical characteristics of the patients with systemic sclerosis (n=42).

	Patients
Duration of the disease, years	4 (1-7)
Limited disease	13 (30.9)
Diffuse disease	29 (69.1)
Antinuclear antibodies presence n[a], %	42 (100)
Anti-topoisomerase I antibodies presence n, %	18 (42.9)
Anti- PM/Scl antibodies presence n, %	5 (11.9)
Anti-centromere antibodies presence n, %	9 (21.4)
RNA polymerase III antibodies presence n, %	1 (2.4)
Anti-Ku antibodies presence n, %	3 (7.1)
Organ involvement	
Digital ulcers n, %	12 (28.6)
Abnormal nailfold capillaries n, %	9 (21.4)
Fingertip lesions n, %	17 (40.5)
Telangiectasia n, %	6 (14.3)
Raynaud's phenomenon n, %	33 (78.6)
Dysphagia n, %	13 (31)
Interstitial lung disease n, %	25 (59.5)
Treatment characteristic	
Current glucocorticosteroids n, %	20 (47.6)
Current glucocorticosteroids dose, mg per day, recalculated to methylprednisolone	2 (0-4)
Systemic glucocorticosteroids therapy, years	1 (0-3)
Immunosuppressive treatment (currently or in the past)	
Azathioprine n, %	3 (7.1)
Cyclophosphamide n, %	48 (42.9)
Methotrexate n, %	6 (14.3)
Mycophenolan mofetil n, %	8 (19)

Rituximab n, %	15 (35.7)
Internal medicine medications	
Angiotensine converting enzyme inhibitors or angiotensin receptor antagonists n, %	15 (35.7)
Statins n, %	11 (26.2)
Beta-blockers n, %	9 (22.4)
Diuretics n, %	11 (26.2)
Calcium channel blockers n, %	15 (35.7)

Categorical variables are presented as numbers (percentages), continuous variables as median and interquartile range.

Abbreviation: n[a] - number

Table 3. Parameters of endothelial injury in patients with systemic sclerosis and controls.

	Patients n[a]=42	Controls n=36	p-value
Laboratory parameters of endothelial injury			
Vascular cell adhesion molecule-1, ng/ml	834.6 (703.2-1037.3)	817.7 (714.2-921.8)	0.36
Thrombomodulin, ng/ml	4.8 (3.9-5.5)	4.4 (3.9- 4.9)	0.31
Ultrasound parameters of endothelial injury and atherosclerosis			
Relative increase of flow mediated dilatation of a brachial artery	5.5 (2.6-8.3)	10 (8.5-11.4)	<0.01*
Median value of intima-media thickness of a common carotid artery, cm	0.075 (0.06-0.08)	0.065 (0.06-0.085)	0.04*

Continuous variables are presented as median and interquartile range. The results which are statistically significant are marked *.

Abbreviation: n[a] - number

9.Oświadczenia współautorów

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oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to: koncepcja pracy, selekcja pacjentów, pomoc w wykonywanych badaniach ultrasonograficznych, przygotowanie bazy danych, analiza statystyczna, interpretacja wyników, przygotowanie manuskryptu.

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Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Renatę Pacholczak jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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