Uniwersytet Jagielloński
Collegium Medicum

Magdalena Kopytek

Czynniki modulujące progresję stenozy aortalnej – związki z zapaleniem, aktywacją układu krzepnięcia/fibrynolizy i kalcyfikacją

Factors modulating the progression of aortic stenosis - association with inflammation, activation of the coagulation/fibrinolysis system and calcification

Praca doktorska

Promotor: dr hab. n. med. Joanna Natorska

Pracę wykonano w Zakładzie Chorób Zatorowo-Zakrzepowych
Kierownik Prof. dr hab. n. med. Anetta Undas

Kraków, rok 2023

Pragnę złożyć wyrazy głębokiej wdzięczności
Mojej Pani Promotor, dr hab. n. med. Joannie Natorskiej
za ukierunkowanie mojego rozwoju naukowego,
wsparcie, cierpliwość, nieocenioną pomoc i przekazaną wiedzę.

Gorące podziękowania składam Prof. dr hab. n. med. Anetcie Undas,
za umożliwienie realizacji mojej pracy doktorskiej,
wiarę w moje możliwości i motywację do pracy.

Panu dr hab. n. med. Michałowi Ząbczykowi
za nieocenioną pomoc, wsparcie i przekazaną mi wiedzę.

Pani dr hab. n. med. Ewie Wypasek
i zespołowi Pracowni Biologii Molekularnej
za wszelką okazaną pomoc i życzliwość.

Pracę dedykuję mojemu mężowi.
W obliczu wszystkich wyzwań i trudności nigdy we mnie nie wątpiłeś i zawsze wspierałeś
w podejmowanych decyzjach życiowych, w tym realizacji mojej działalności naukowej.

Spis treści

1. WSTĘP

Niniejsza rozprawa doktorska pt. „Czynniki modulujące progresję stenozy aortalnej – związki z zapaleniem, aktywacją układu krzepnięcia/fibrynolizy i kalcyfikacją", powstała w oparciu o cykl pięciu artykułów opublikowanych w międzynarodowych czasopismach naukowych indeksowanych w bazie PubMed oraz znajdujących się na liście Journal Citation Reports (Thomson Reuters).

Na pracę doktorską składają się następujące artykuły:

1. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J. *Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes.* Cardiovasc Diabetol. 2020; 19: 92. doi: 10.1186/s12933-020-01068-7. (współczynnik oddziaływania [*impact factor*, **IF**] = **9,951**; liczba punktów według wykazu czasopism naukowych Ministra Nauki i Szkolnictwa Wyższego z dnia 21 grudnia 2021 r. [**MNiSW**] = **140 pkt.)**
2. Kopytek M, Mazur P, Ząbczyk M, Undas A, Natorska J. *Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification.* Diabetologia. 2021; 64: 2562-2574. doi: 10.1007/s00125-021-05545-w. **(IF=10,460; MNiSW=140 pkt.)**
3. Kopytek M, Ząbczyk M, Mazur P, Siudut J, Natorska J. *Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis*. Pol Arch Intern Med. 2022; 132: 16372. doi: 10.20452/pamw.16372. **(IF=5,218; MNiSW=140 pkt.)**
4. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023; 12: 1402. doi.org/10.3390/cells12101402. **(IF=7,666; MNiSW=140 pkt.)**
5. Natorska J, Kopytek M, Undas A. Aortic valvular stenosis: *Novel therapeutic strategies.* Eur J Clin Invest. 2021; 51: e13527. doi: 10.1111/eci.13527. **(IF=5,722; MNiSW=100 pkt.)**.

Łączna wartość **IF** czasopism, w których ukazały się składowe cyklu, wyniosła **39,017** według *Thomson Reuters Journal Citation Reports*. Teksty artykułów zamieszczone są w niniejszej rozprawie w wersjach autorskich, będących w pełni zgodnymi z wersjami ostatecznymi, które ukazały się drukiem w czasopismach.

2. WYKAZ SKRÓTÓW

AGE, *advanced glycation end products,* końcowe produkty zaawansowanej glikacji

Apo(a), *apolipoprotein(a)*, apolipoproteina(a)

AS, *aortic stenosis,* stenoza aortalna

AVA, *aortic valve area,* pole powierzchni ujścia zastawki

BAY 11–7082, *nuclear factor kappa B inhibitor,* inhibitor jądrowego czynnika kappa B

BMI, *body-mass index,* wskaźnik masy ciała

BMP, *bone morphogenetic proteins,* białka morfogenetyczne kości

CI, *confidence interval,* przedział ufności

CLT, *clot lysis time,* czas lizy skrzepu

CRP, *C-reactive protein,* białko C-reaktywne

DMT2, *type 2 diabetes mellitus,* cukrzyca typu 2

F1+2, *prothrombin fragments 1+2,* fragmenty F1+2 protrombiny

FII, *prothrombin,* protrombina

FVIIa-AT, *active factor VIIa–antithrombin complex,* kompleks czynnika VII aktywnego i antytrombiny

FXa, *active factor X,* aktywny czynnik X

HbA_{1c}, *glycated hemoglobin,* hemoglobina glikowana

IL-6, *interleukin 6,* interleukina 6

IQR, *interquartile range,* rozstęp międzykwartylowy

LDL, *low-density lipoprotein,* lipoproteiny o niskiej gęstości

Lp(a), *lipoprotein(a),* lipoproteina(a)

NAC, *N-acetyl-L-cysteine,* N-acetylo-L-cysteina

NF-κB, *nuclear factor kappa B,* jądrowy czynnik kappa B

OxLDL, *oxidized low-density lipoprotein,* utlenione lipoproteiny o niskiej gęstości

OxPL, *oxidized phospholipids,* utlenione fosfolipidy

PAI-1, *plasminogen activator inhibitor 1,* inhibitor aktywatora plazminogenu 1

PCR, *polymerase chain reaction,* łańcuchowa reakcja polimerazy

PG_{max}, *maximal transvalvular pressure gradient,* maksymalny gradient ciśnienia przez zastawkę

PG_{mean}, *mean transvalvular pressure gradient,* średni gradient ciśnienia przez zastawkę

RAGE, *receptor for advanced glycation end products,* receptor dla końcowcyh produktów zaawansowanej glikacji

RELA, *gene symbol for NF-κB,* symbol genu dla NF-κB

ROS, *reactive oxygen species,* reaktywne formy tlenu

SERPINE1, *gene encoding PAI-1,* gen kodujący PAI-1

sRAGE, *soluble RAGE,* rozpuszczalna izoforma RAGE

TAFI, *thrombin activatable fibrinolysis inhibitor,* inhibitor fibrynolizy aktywowany trombiną

TF, *tissue factor,* czynnik tkankowy

TGF-β, *transforming growth factor β,* transformujący czynnik wzrostu β

TM5275, *PAI-1 activity inhibitor,* inhibitor aktywności PAI-1

TNF-α, *tumor necrosis factor α,* czynnik martwicy nowotworów α

tPA, *tissue plasminogen activator,* tkankowy aktywator plazminogenu

uPA, *urokinase-type plasminogen activator,* urokinazowy aktywator plazminogenu

V_{max}, *peak transvalvular velocity,* szczytowa prędkość przepływu przez zastawkę

VICs, *valve interstitial cells,* komórki śródmiąższowe zastawki aortalnej

3. PODSUMOWANIE PRACY DOKTORSKIEJ

3.1. WPROWADZENIE

Stenoza aortalna (*aortic stenosis*, AS) jest związana ze zmniejszeniem powierzchni ujścia zastawki aortalnej (*aortic valve area*, AVA) i ruchliwości jej płatków w wyniku gromadzenia się hydroksyapatytu wapnia. Konsekwencją tej wady jest upośledzona zdolność wyrzutu krwi z lewej komory serca do aorty. AS jest najczęstszą nabytą wadą zastawkową serca w krajach zachodnich, bez możliwości leczenia farmakologicznego [1]. Częstość występowania ciężkiej postaci AS u pacjentów w wieku powyżej 65 lat wynosi 3,4% [1]. Szacuje się, że do 2030 roku na całym świecie istotna hemodynamicznie AS zostanie zdiagnozowana u około 4,5 miliona ludzi [2]. Wymiana zastawki aortalnej, chirurgiczna lub przezskórna, jest jedynym sposobem leczenia AS.

Przez wiele lat uważano, że patogeneza AS jest tożsama z procesem powstawania blaszki miażdżycowej. Koncepcja ta była poparta badaniami epidemiologicznymi wskazującymi, że występowanie AS jest związane z czynnikami ryzyka sercowo-naczyniowego, takimi jak zaawansowany wiek [3], hipercholesterolemia, cukrzyca, nadciśnienie tętnicze [4], płeć męska oraz palenie papierosów [5]. Obecnie wiadomo, że procesy te różnią się, głównie ze względu na obecność z zastawkach aortalnych komórek śródmiąższowych zastawki (*valve interstitial cells,* VICs), będących miofibroblastami [6]. VICs są źródłem wielu cytokin prozapalnych, przeciwzapalnych, czynników chemotaktycznych oraz proteinaz, które w sposób czynny modulują procesy zachodzące podczas rozwoju i progresji AS [6,7].

Początkowym etapem wapnienia zastawki aortalnej jest uszkodzenie śródbłonka w wyniku obciążenia mechanicznego i działania sił ścinających (ang. *shear stress*) [8-10]. W uszkodzonych zastawkach gromadzą się utlenione lipoproteiny o małej gęstości (*oxidized low-density lipoprotein*, OxLDL), a ich fagocytoza przez naciekające makrofagi prowadzi do tworzenia się komórek piankowatych [8,11-13]. Wapnienie postępuje, gdy w warunkach patologicznych dochodzi do chronicznej aktywacji komórek VICs, stanowiących główną populację komórek płatków zastawek aortalnych, które ostatecznie różnicują się w komórki osteoblastopodobne [7]. Aktywowane komórki VICs reagują na typowe mediatory osteogenne, takie jak transformujący czynnik wzrostu β (*transforming growth factor β*, TGF-β) i białka morfogenetyczne kości (*bone morphogenetic proteins*, BMP) [12,14]. Po aktywacji VICs zmieniają swój fenotyp i charakteryzują się ekspresją specyficznych markerów, takich jak α-aktyna dla miofibroblastów (różnicowanie chondrogenne) oraz fosfataza alkaliczna,

osteopontyna, sialoproteina kostna i BMP-2 oraz BMP-4 dla fibroblastów osteoblastopodobnych (różnicowanie osteogenne) [7]. Regulacja wapnienia zastawki aortalnej podlega kontroli jądrowego czynnika transkrypcyjnego kappa B (*nuclear factor kappa B*, NF-κB), będącego jednocześnie głównym modulatorem odpowiedzi zapalnej. NF-κB jest aktywowany między innymi przez czynnik martwicy nowotworów α (*tumor necrosis factor α*, TNF-α), który jest wydzielany przez monocyty i makrofagi, co w konsekwencji prowadzi do wapnienia zastawek poprzez stymulację BMP-2 [12,14].

Jednym z czynników modulujących AS jest występowania cukrzycy typu 2 (*type 2 diabetes mellitus*, DMT2). Liczne badania wykazały, że częstość DMT2 jest wyższa wśród pacjentów z AS niż w populacji ogólnej i waha się między 11,4% a 15,8% [15-17]. Culler i wsp. [15] pokazali, że w Stanach Zjednoczonych w latach 2009-2015 częstość występowania DMT2 u pacjentów z AS wzrosła z 19,7% do 31,6%. Podobne wyniki odnotowano w populacji hiszpańskiej podczas 15-letniej obserwacji [17]. Ponadto zauważono, że DM zwiększa ryzyko wapnienia zastawek i przyspiesza progresję AS [18]. W dużym badaniu kohortowym obejmującym 1,12 mln osób obserwowanych średnio przez 13 lat, występowanie DM wiązało się z 49% wyższym ryzykiem rozwoju AS [19]. Jednak niewiele wiadomo na temat mechanizmów leżących u podstaw wpływu hiperglikemii na stan zapalny i kalcyfikację zastawek aortalnych u chorych z AS. Nasz Zespół zaobserwował zwiększoną zastawkową ekspresję białka C-reaktywnego (*C-reactive protein*, CRP) i jego mRNA u pacjentów z AS i współistniejącą DMT2 w porównaniu z osobami bez DMT2, a obszary immunopozytywne dla CRP dodatnio korelowały z mRNA dla czynnika tkankowego (*tissue factor*, TF) [20]. Dane te wskazują, że towarzysząca DMT2 u chorych z AS prowadzi do nasilonego stanu zapalnego w stenotycznych zastawkach i jest związana ze zwiększoną ekspresją czynników krzepnięcia.

U pacjentów z DM istotną rolę odgrywa stres oksydacyjny i związane z tym nagromadzenie reaktywnych form tlenu (ROS), glukozy czy związków karbonylowych, które prowadzi do zaburzeń w metabolizmie komórkowym i sprzyja powstawaniu końcowych produktów zaawansowanej glikacji (*advanced glycation end products*, AGE) [21]. AGEs są heterogenną grupą cząsteczek, które powstają w wyniku nieenzymatycznej modyfikacji białek przez cukry redukujące, przeważnie glukozę. Do białek szczególnie poddatnych na działanie AGEs należą kolagen, hemoglobina czy albuminy osocza [22-24]. Warto zauważyć, że wraz ze wzrostem liczby wiązań krzyżowych obecnych w AGEs, wzrasta ich odporność na proteolizę, co utrudnia ich usuwanie i prowadzi do ich akumulacji w organizmie [21]. AGEs zmieniają funkcję tkanek i ich właściwości mechaniczne poprzez sieciowanie białek [22-24],

a poprzez wiązanie się z receptorem RAGE (*receptor for advanced glycation end products)* na powierzchni komórek, są zdolne do modulowania wielu procesów wewnątrzkomórkowych [25]. Zaobserwowano, że AGEs wpływają na rozwój i progresję miażdżycy przez sieciowanie białek strukturalnych, prowadząc do pogrubienia błony podstawnej naczyń krwionośnych [11], zatrzymania lipoprotein w ścianach naczyń [17] i indukcję stresu oksydacyjnego [26]. Pomimo licznych doniesień o udziale AGEs i RAGE w patogenezie chorób sercowo-naczyniowych niewiele wiadomo o mechanizmach, za pomocą których związany z hiperglikemią wzrost AGEs i RAGE wpływa na stan zapalny i wapnienie płatków zastawek aortalnych. W związku z tym, postanowiliśmy zbadać u pacjentów z ciężką AS i współistniejącą DMT2, poziom długoterminowych wskaźników glikemii takich jak fruktozamina i hemoglobina glikowana (*glycated hemoglobin*, HbA_{1c}) we krwi obwodowej, jak i akumulację AGEs i RAGE w stenotycznych zastawach.

Najlepiej poznanym procesm związanym z rozwojem i progresją AS jest akumulacja w obrębie płatków zastawek lipidów i lipoprotein, takich jak LDL, OxLDL, utlenione fosfolipidy (*oxidized phospholipids*, OxPLs) oraz lipoproteina(a) (*lipoprotein(a)*, Lp(a)) [11,27-30]. Lp(a) jest głównym nośnikiem fosfolipidów i ich utlenionych form [31,32]. Co istotne, Lp(a) wykazuje właściwości proaterogenne i prozakrzepowe, dzięki domenie podobnej do LDL – apolipoproteinie B100 (apoB100) i glikoproteinie zwanej apolipoproteiną(a) (apo(a)) [31,32]. Apo(a) jest białkiem wysoce homologicznym z plazminogenem, zawiera powtórzone kopie sekwencji podobnej do sekwencji plazminogenu kringle IV (KIV), kringle V (KV) i domeny proteazy plazminogenu [33]. Konkurencja między apo(a) i plazminogenem o miejsca wiązania sprawia, że Lp(a) jest w stanie hamować fibrynolizę i promować krzepnięcie [33-35]. Nasz Zespół zaobserwował, że stopień zawansowania AS jest związany z zastawkową ekspresją TF, którego największą ekspresję stwierdzono w okolicy złogów tłuszczowo-wapniowych, czemu towarzyszyła duża infiltracja makrofagów [36]. Ponadto, stwierdzono dodatnią korelację między ekspresją TF *in loco* a poziomem cholesterolu LDL i TF we krwi obwodowej u pacjentów z ciężką AS [36]. Wykazano również, że pacjenci z ciężką AS charakteryzują się hipofibrynolizą wyrażoną jako wydłużony czas lizy skrzepu (*clot lysis time*, CLT) oraz zwiększony poziom inhibitora aktywatora plazminogenu typu 1 (*plasminogen activator inhibitor 1*, PAI-1) we krwi krążącej [37]. Zarówno CLT jak i osoczowy poziom PAI-1 korelowały ze stopniem zwapnienia zastawek i progresją choroby mierzoną echokardiograficznie [37]. Poszukując czynników, które mogą upośledzać fibrynolizę u pacjentów z ciężką AS, nasz Zespół zwrócił uwagę na stres oksydacyjny, który jest jedną ze składowych procesu zapalnego toczącego się

w stenotycznych zastawkach. Oceniono wydajność fibrynolityczną osocza oraz oksydacyjną modyfikację białek i lipidów osocza, wyrażoną jako karbonylacja białek (*protein carbonylation*, PC) i peroksydacja lipidów (*thiobarbituric acid reactive substances*, TBARS) [38]. Zarówno poziom PC, jak i TBARS wykazywały związek z progresją choroby mierzoną echokardiograficznie [38]. Ponadto, poziomy PC i TBARS były dodatnio skorelowane z czasem lizy skrzepów i poziomem PAI-1 [38]. Dane te sugerują, że nasilony stres oksydacyjny przyczynia się do upośledzenia fibrynolizy w AS i może być związany z progresją choroby. W kolejnym badaniu wykazaliśmy, że u pacjentów z izolowaną ciężką AS, OxLDL i Lp(a) są lepszymi niż LDL predyktorami hipofibrynolizy u pacjentów z AS [39]. Lp(a) jest głównym nośnikiem OxPL we krwi obwodowej [31,32], a Kamstrup i wsp. [40] wykazali, że podwyższone poziomy OxPL w surowicy krwi są związane ze zwiększonym ryzykiem AS. Na tej podstawie, postanowiliśmy zbadać u pacjentów z ciężką AS czy poziom OxPL w surowicy krwi ma związek z nasileniem wady zastawkowej i hipofibrynolizą.

Niewyjaśnionym jak dotąd zjawiskiem jest obserwowany wcześniej podwyższony poziom ekspresji PAI-1 w zastawkach stenotycznych oraz we krwi obwodowej pacjentów z ciężką AS [37,38]. Jedna z prac naszego Zespołu sugerowała, że mastocyty infiltrujące zastwki mogą być zaangażowane w lokalne zaburzenia fibrynolizy i przyczyniać się do odkładania fibryny oraz kolagenu na powierzchni zastawki [13]. Jednak ostatnie obserwacje skłoniły nas do zbadania czy to VICs nie są głównym źródłem PAI-1 w zastawkach stenotycznych.

3.2. CELE

Celem niniejszej pracy było badanie mechanizmów związanych z działaniem czynników modulujących progresję AS, tj. hiperglikemii i nasilonego stresu oksydacyjnego ze szczególnym uwzględnieniem ich potencjalnych związków z zapaleniem, aktywacją układu krzepnięcia, hipofibrynolizą i kalcyfikacją u pacjentów z ciężką AS.

Cztery oryginalne hipotezy badawcze pracy doktorskiej były następujące:

Hipoteza 1: Zwiększona akumulacja AGEs u pacjentów z AS i współistniejącą DMT2 jest związana ze zwiększoną produkcją ROS i wapnieniem zastawki.

Hipoteza 2: DMT2 współistniejąca z AS wiąże się ze zwiększoną ekspresją NF-κB, aktywacją układu krzepnięcia *in loco* oraz nasilonym wapnieniem zastawki.

Hipoteza 3: Podwyższony poziom OxPL w surowicy krwi jest nowym czynnikiem determinującym hipofibrynolizę u pacjentów z ciężką AS.

Hipoteza 4: U pacjentów z ciężką AS akumulacja lipidów w obrębie stenotycznych zastawek prowadzi do nadekspresji PAI-1, co przyczynia się do hipofibrynolizy.

3.3. PACJENCI

Do badania włączono pacjentów obu płci, w wieku od 18 do 85 lat z ciężką AS rozpoznaną na podstawie echokardiografii przezklatkowej, zdefiniowanej jako średni gradient ciśnienia przez zastawkę (*mean transvalvular pressure gradient*, PG_{mean}) ≥40 mm Hg oraz AVA ≤1 cm^2. Ze względu na rekrutację większości pacjentów przed rokiem 2021 w **publikacji 1**, **2** i **4** jako kryterium ciężkiej AS nie stosowano wskaźnika szczytowej prędkości (*peak transvalvular velocity*, V_{max}), który został włączony do wytycznych Europejskiego Towarzystwa Kardiologicznego w roku 2021 [41]. Fakt ten został zaadresowany w odpowiednich sekcjach poszczególnych publikacji cyklu. Rekrutacja pacjentów odbywała się w Klinice Chirurgii Serca, Naczyń i Transplantologii w Szpitalu im. Jana Pawła II w Krakowie.

Dane dotyczące historii medycznej, aktualnego leczenia i danych demograficznych zebrano za pomocą standardowego kwestionariusza. DMT2 była zdiagnozowana na podstawie stężenia glukozy ≥7,0 mmol/l (126 mg/dl) w surowicy krwi, które było wykonane na czczo i dwukrotne powtórzone, oraz na podstawie poziomu HbA_{1c} ≥6,5% (48 mmol/mol) lub doustnego testu tolerancji glukozy, którego wynik był w zakresie od 140 mg/dl (7,8 mmol/l) do 199 mg/dl (11,0 mmol/l) według kryteriów Amerykańskiego Stowarzyszenia Diabetyków [42]. Nadciśnienie tętnicze rozpoznano na podstawie wywiadu medycznego (ciśnienie krwi >140/90 mmHg) lub leczenia hipotensyjnego przed przyjęciem do szpitala. Hipercholesterolemię rozpoznano na podstawie dokumentacji medycznej, terapii obniżającej poziom cholesterolu lub jako poziom cholesterolu całkowitego równy lub większy niż 5,0 mmol/l.

Kryteria wykluczenia obejmowały miażdżycową chorobę naczyń wymagającą rewaskularyzacji, ostrą infekcję, w tym infekcyjne zapalenie wsierdzia, reumatyczną AS, przewlekłą chorobę nerek, konieczność jednoczesnej operacji zastawek (wymiana zastawki mitralnej), przezskórną interwencję wieńcową, niedawny (<3 miesięcy) ostry zespół wieńcowy lub incydent mózgowo-naczyniowy, rozpoznany nowotwór złośliwy, ciążę i brak zgody na udział w badaniu. W **publiacji 3** i **4** kryteria wyłączenia były dodatkowo rozszerzone o występowanie DMT2, ze wzlędu na nasilenie procesów zapalnych obserwowanych w stenotycznych płatkach zastawki.

Anatomia zastawek została potwierdzona śródoperacyjnie przez kardiochirurga, a pacjenci z zastawką dwupłatkową i wymagający interwencji z powodu poszerzenia aorty wstępującej zostali wykluczeni z badania. Rozpoznanie miażdżycy opierało się na

udokumentowanym angiograficznie zwężeniu tętnicy wieńcowej większym niż 20% średnicy i tacy pacjenci zostali wykluczeni z badania, aby uniknąć istotnego wpływu miażdżycy na uzyskane wyniki [43].

Wszyscy pacjenci wyrazili pisemną świadomą zgodę na udział w badaniu. Badanie uzyskało pozytywną opinię Komisji Bioetycznej (102/KBL/OIL/2016 dla **publikacji 1, 2, 3** i **4** oraz 53/KBL/OIL/2022 dla **publikacji 3**). Protokół badania był zgodny z wytycznymi Deklaracji Helsińskiej wraz z jej późniejszymi poprawkami.

3.4. METODY

Krew pobierano na czczo z żyły łokciowej z wykorzystaniem systemu próżniowego z antykoagulantami: cytrynianem sodowym (3,2% w stosunku 9:1), wersenianem sodowym oraz do probówki bez antykoagulantu w celu uzyskania surowicy krwi. Rutynowe badania laboratoryjne obejmowały morfologię krwi, stężenia: glukozy, kreatyniny, cholesterolu całkowitego, cholesterolu LDL, cholesterolu HDL, triglicerydów, CRP oraz fibrynogenu. Krew odpowiednio odwirowano i przechowywano w temperaturze -80 °C do czasu kolejnych analiz. W **publikacji 1 i 2** oznaczono dodatkowo poziom HbA_{1c} oraz fruktozaminy. W **publikacji 3** dodatkowo oznaczono stężenie Lp(a), aktywność plazminogenu i α_2-antyplazminy. Poziom Lp(a) powyżej 50 mg/dl przyjęto za czynnik ryzyka wystąpienia AS według kryteriów Europejskiego Towarzystwa Badań nad Miażdżycą [44].

Testy immunoenzymatyczne (ELISA) wykonano w celu oceny ilościowej wybranych markerów w osoczu lub surowicy krwi u pacjentów z AS.

Pobrane podczas operacji wymiany zastawki, stenotyczne zastawki aortalne służyły jako materiał do barwień immunofluorescencyjnych i histochemicznych w celu wykrycia specyficznych antygenów w skrawkach tkanki oraz do badań *in vitro* (hodowle *in vitro* komórek VICs wraz z badaniem ekspresji genów na poziomie mRNA za pomocą łańcuchowej reakcji polimerazy - PCR).

Ogólną sprawność układu fibrynolitycznego w osoczu cytrynianowym oznaczono turbidymetrycznie i wyrażono jako CLT, stosując jako aktywator krzepnięcia 0,5 U/ml egzogenną ludzką trombinę oraz egzogenny rekombinowany tkankowy aktywator plazminogenu (tPA). Ponadto, zastosowano modyfikację metody CLT do analizy supernatantów pochodzących z komórek VICs. Metoda ta została specjalnie opracowana przez Doktorantkę do tego celu, a szczegóły przedstawiono w **publikacji 4**.

Szczegółową metodykę wraz z opisem zastosowanych analiz statystycznych przedstawiono w **publikacjach 1-4**.

Publikacja 5 jest pracą przeglądową, w której Doktorantka jest autorką wiodącą, co świadczy o uznaniu przez Redaktora czasopisma Jej ekspertyzy z zakresu stenozy aortalnej.

3.5. PODSUMOWANIE WYNIKÓW

Artykuł nr 1

Kopytek M, et al. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol. 2020; 19: 92.

Celem pracy było zbadanie czy dochodzi do akumulacji AGEs/RAGE w aortalnych zastawkach stenotycznych oraz czy akmuluacja ta nasila procesy związane z progresją wady.

Do badania włączono 126 chorych z rozpoznaną ciężką AS w tym 50 pacjentów z współistniejąca DMT2. Mediana czasu od rozpoznania DMT2 wyniosła 11 lat (rozstęp międzykwartylowy [IQR] 7-18 lat).

Pacjenci z AS nie różnili się od pacjentów z AS i współistniejąca DMT2 (AS-DM) pod względem czynników ryzyka sercowo-naczyniowego czy demograficznych, za wyjątkiem wyższego wskaźnika masy ciała (*body-mass index*, BMI) w grupie AS-DM ($p<0,001$). Chorzy AS-DM charakteryzowali się o 44,2% wyższym stężeniem glukozy, 25,9 % wyższym poziomem HbA_{1c} i 16,4% wyższym poziomem fruktozaminy w porównaniu do pacjentów z AS bez DMT2 (wszystkie $p<0,0001$). W grupie AS-DM 24 pacjentów (48%) miało źle kontrolowaną DMT2, definiowaną jako HbA_{1c} >7%.

Analiza ekspresji *in loco* wykazała nasiloną akumulację AGEs w stenotycznych zastawkach (6,6-krotny wzrost; $p<0,001$) i 12-krotny wzrost AGEs w surowicy ($p<0,0001$) pacjentów AS-DM w porównaniu do pacjentów z izolowaną AS. Podobnie, chorzy AS-DM mieli 1,8-krotnie wyższą ekspresję RAGE w stenotycznych zastawkach ($p<0,001$) i 1,3-krotnie wyższy poziom rozpuszczalnej izoformy RAGE (*soluble RAGE,* sRAGE) w surowicy krwi ($p<0,0001$) w porównaniu do pacjentów bez DMT2. Ponadto, zastawki pozyskane od pacjentów z towarzyszącą DMT2 w porównaniu z zastawkami pacjentów bez DMT2 charakteryzowały się zwiększoną ekspresją ROS ($p<0,0001$) i tendencją do zwiększonej ekspresji interleukiny 6 (IL-6; $p=0,062$).

Zastawkowa akumulacja AGEs korelowała z poziomem HbA_{1c} i stężeniem fruktozaminy w surowicy krwi oraz z ciężkością choroby wyrażoną jako AVA i PG_{mean}. Ekspresja RAGE korelowała wyłącznie z poziomem HbA_{1c}. Nie stwierdzono związków pomiędzy zastawkową ekspresją RAGE, ROS lub IL-6 a parametrami echokardiograficznymi.

W grupie AS-DM stężenie AGEs dodatnio korelowało z sRAGE, a HbA_{1c} z fruktozaminą. W tej grupie stężenie AGEs w surowicy krwi korelowało także z poziomem

HbA_{1c} i fruktozaminą, podczas gdy poziomy sRAGE tylko z HbA_{1c}. AGEs korelowały z ciężkością wady wyrażoną jako AVA, PG_{mean} i PG_{max} (maksymalny gradient ciśnienia przez zastawkę), natomiast sRAGE z AVA i PG_{max}.

Warto podkreślić, że pacjenci ze słabo kontrolowaną DMT2 charakteryzowali się zwiększoną zastawkową akumulacją AGEs (23,3% vs. 10,8%; p<0,0001) i RAGE (8,1% vs. 5,7%; p=0,015) oraz nasiloną ciężkością choroby wyrażoną jako PG_{mean} (61 mmHg vs. 44 mmHg; p=0,003), PG_{max} (95 mmHg vs. 64 mmHg; p<0,0001) i AVA (0,65 cm^2 vs 0,85 cm^2; p<0,0001) w porównaniu do pacjentów z dobrze kontrolowaną DMT2.

Artykuł nr 2

Kopytek M, et al. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021; 64: 2562-2574.

Celem pracy było zbadanie czy DMT2 współistniejąca z AS wiąże się ze zwiększoną ekspresją NF-κB i białek układu krzepnięcia *in loco* oraz nasilonym wapnieniem zastawki.

Do badania włączono 150 pacjentów z rozpoznaną ciężką AS w tym 50 pacjentów z współistniejącą DMT2. Mediana czasu od rozpoznania DMT2 wyniosła 11 lat ([IQR] 7-18 lat).

Pacjenci z AS nie różnili się od pacjentów AS-DM pod względem czynników ryzyka sercowo-naczyniowego czy demograficznych, za wyjątkiem nieco wyższego wskaźnika BMI w grupie AS-DM (p=0,049). Chorzy z grupy AS-DM charakteryzowali się o 41,5% wyższym stężeniem glukozy (p<0,0001), 23,6% wyższym poziomem HbA_{1c} (p<0,0001) i 17,5% wyższym poziomem fruktozaminy (p=0,007) w porównaniu do pacjentów z izolowaną AS. W grupie AS-DM 36 pacjentów (72%) miało źle kontrolowaną DMT2, definiowaną jako $HbA_{1c} \geq 6,5\%$.

Zastawki pobrane od chorych z AS i towarzyszącą DMT2 w porównaniu z zastawkami od pacjentów bez DMT2 charakteryzowały się zwiększoną ekspresją *in loco* NF-κB (+92%; p=0,001), BMP-2 (+148%; p<0,001), protrombiny (FII, +113%; p<0,001) i FXa (aktywny czynnik X, +66%; p<0,001). Słabo kontrolowana DMT2 była związana z najsilniejszą ekspresją badanych markerów. Ekspresja NF-κB korelowała z zastawkową ilością BMP-2, FII i FXa w obu grupach. Dodatkowo u pacjentów z DMT2 zastawkowa ekspresja BMP-2 korelowała z ekspresją FII i FXa.

Ponadto, u chorych z grupy AS-DM zastawkowa ekspresja NF-κB dodatnio korelowała zarówno z poziomem glukozy, jak i z parametrami długoterminowej kontroli

glikemii, tj. HbA_{1c} i fruktozaminą. Analogicznie, zastawkowa ekspresja BMP-2 korelowała z poziomami HbA_{1c} i fruktozaminy. W tej grupie chorych obserwowano także asocjacje pomiędzy ekspresją NF-κB, BMP-2, FII i FXa a ciężkością AS wyrażoną jako AVA i PG_{max}. Z kolei u pacjentów z izolowaną AS odnotowano jedynie korelacje pomiędzy NF-κB i BMP-2 a AVA.

Chorzy z grupy AS-DM w porównaniu z pacjentami z AS mieli o 59% wyższe stężenie kompleksu czynnika VII aktywnego i antytrombiny w osoczu krwi (FVIIa-AT; $p<0,0001$), ale nie poziom TF lub fragmentów F1+2 protrombiny (F1+2; obydwa $p>0,05$). Tylko u pacjentów z DMT2, stężenie FVIIa-AT dodatnio korelowało z poziomem fruktozaminy, a TF z HbA_{1c} i fruktozaminą. Nie stwierdzono związków pomiędzy poziomami FVIIa-AT, TF lub F1+2 a parametrami echokardiograficznymi.

Badania *in vitro* z wykorzystaniem VICs stymulowanycyh glukozą wykazały, że hiperglikemia zwiększa zarówno ekspresję NF-κB (+56%; $p<0,001$), jak i BMP-2 (+52%; $p<0,001$). Analiza ekspresji mRNA w komórkach VICs potwierdziła, że wysokie stężenia glukozy powodują 7-krotny wzrost ekspresji *RELA* (symbol genu NF-κB, podjednostka p65). Eksperymenty mechanistyczne z użyciem inhibitora stresu oksydacyjnego (N-acetylo-L-cysteina; NAC) lub NF-κB (BAY 11-7082) pokazały, że ekspresja NF-κB była hamowana przez oba inhibitory (-29% i -31%, obydwa $p<0,01$; odpowiednio). Podobny efekt zaobserwowano dla ekspresji BMP-2 po zastosowaniu wysokiego stężenia glukozy w połączeniu z inhibitorem ROS (-31%; $p<0,01$) lub NF-κB (-33 %; $p<0,01$) w hodowlach komórek VICs. Analiza PCR potwierdziła te obserwacje na poziomie mRNA (*RELA*).

Artykuł nr 3

Kopytek M, et al. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022; 132: 16372.

Celem badania było określenie czy u pacjentów z ciężką AS poziom Lp(a) >50 mg/dl jest związany z akumulacją OxPL w zastawce i ich podwyższonym stężeniem w surowicy krwi, oraz czy akumulacja OxPL wiąże się z hipofibrynolizą i/lub ciężkością wady.

Badanie objęło 70 chorych z rozpoznaną izolowaną ciężką AS, w tym 50 pacjentów z Lp(a) ≥50 mg/dl oraz 20 pacjentów z Lp(a) <50 mg/dl. Mediana stężeń OxPL wynosiła odpowiednio w badanych grupach 0,62 μg/ml i 0,46 μg/ml.

Pacjenci z Lp(a) ≥50 mg/dl nie różnili się od pacjentów z Lp(a) <50 mg/dl pod względem czynników demograficznych i ryzyka sercowo-naczyniowego, stosowanych leków czy parametrów laboratoryjnych. Pacjenci z AS i wysokim poziomem Lp(a) mieli o 10%

wyższy V_{max} (p<0,001), 13% wyższy PG_{mean} (p=0,04), 15% wyższy PG_{max} (p<0,001) i o 11% niższy AVA (p=0,003) w porównaniu z pacjentami z niskim poziomem Lp(a).

Analiza *in loco* wykazała obecność OxPL we wszystkich badanych zastawkach aortalnych. Co istotne, pacjenci z Lp(a) ≥50 mg/dl mieli o 29% większą zastawkową ekspresję OxPL (p<0,001) i o 35% wyższe stężenie w surowicy krwi (p=0,03) w porównaniu z pacjentami z Lp(a) <50 mg/dl. Ponadto, ekspresja OxPL korelowała dodatnio z poziomem OxPL oraz ze stężeniem Lp(a) w surowicy krwi.

Pacjentów z wysokim stężeniem Lp(a) charakteryzowało o 11% wydłużone CLT (p<0,001) oraz o 38% i 12% wyższe poziomy PAI-1 (p=0,003) i TAFI (p=0,007) w porównaniu do pacjentów z niskim stężeniem Lp(a). Nie stwierdzono różnic w poziomach tPA lub aktywności plazminogenu i α_2-antyplazminy pomiędzy badanymi grupami. Wyłącznie u chorych ze stężeniem Lp(a) ≥50 mg/dl poziom OxPL w surowicy krwi korelował z CLT, stężeniem PAI-1 i TAFI. Ponadto, poziomy OxPL w surowicy krwi korelowały z ciężkością wady mierzoną jako V_{max} i PG_{mean} oraz słabiej z AVA.

Analiza jednoczynnikowej regresji liniowej wykazała, iż stężenia OxPL, Lp(a), PAI-1, TAFI, V_{max}, PG_{mean}, PG_{max} i wiek, ale nie aktywność plazminogenu były związane z CLT u pacjentów z Lp(a) ≥50 mg/dl. Co ciekawe, poziom OxPL (β=9,17; 95% przedział ufności [CI] 6,12-12,22) silniej determinował CLT niż stężenie Lp(a) (β=0,14; 95% CI 0,09-0,19). Natomiast analiza metodą wieloczynnikowej regresji liniowej wykazała, iż stężenie OxPL było niezależnym predyktorem CLT (β=7,87; 95% CI 4,71-11,03) u chorych z Lp(a) ≥50 mg/dl.

Artykuł nr 4

Kopytek M, et al. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023; 12: 1402.

Celem pracy było zbadanie czy u pacjentów z ciężką AS nagromadzenie się lipidów w obrębie stenotycznych zastawek prowadzi do nadekspresji PAI-1, która nasila procesy związane z hipofibrynolizą i progresją wady.

Do badania włączono 75 pacjentów z rozpoznaną ciężką objawową AS. Pacjenci z AS nieleczeni statynami (n=18) charakteryzowali się o 39% zwiększonym stężeniem cholesterolu LDL (p=0,0013) w porównaniu z chorymi przyjującymi statyny.

Badanie histochemiczne wykazało o blisko 20% wyższą akumulację lipidów w zastawkach stenotycznych w porównaniu do zastawek aortalnych pochodzących od dawców autopsyjnych dopasowanych pod względem wieku. Z kolei barwienie

immunofluorescencyjne wykazało nasiloną eskpresję PAI-1 w stenotycznych zastawkach (24,6±4,1% powierzchni immunopozytywnej płatka zastawki), podczas gdy w zastawkach kontrolnych nie obserwowano ekspresji PAI-1. Ekspresja PAI-1 prezentowała skondensowany wzór fluorescencji, obserwowany zarówno w warstwie włóknistej jak i gąbczastej. Co ciekawe, podwójne barwienie ujawniło, że dochodzi w 84% do ko-ekspresji NF-κB i PAI-1 w stenotycznych zastawkach. Ponadto, ekspresja *in loco* PAI-1 była dodatnio skorelowana z akumulacją lipidów i ciężkością AS mierzoną jako PG_{mean}. Wykazano także ekspresję *in loco* plazminogenu (16,6 ±3,9%), α_2-antyplazminy (12,2 ±4,1%) i tPA (8,4 ±3,6%) w warstwie podśródbłonkowej i włóknistej w stenotycznych, ale nie w kontrolnych zastawkach. Ekspresję D-dimeru obserwowanow warstwie włóknistej i częściowo w warstwie gąbczastej wyłącznie w stenotycznych zastawkach, a wzór fluorecencji był rozproszony. Obecność D-dimeru w zastawkach stenotycznych świadczy o lokalnie zachodzącej fibrynolizie.

Badania *in vitro* wykazały, że niezależnie od zastosowanych warunków hodowli, komórki VICs wykazywały stałą ekspresję PAI-1 (100% komórek). Nie stwierdzono ekspresji tPA i α_2-antyplazminy w VICs hodowanych w medium kontrolnym lub prokalcyfikacyjnym. Obserwowano słabą ekspresję plazminogenu w VICs hodowanych w medium prokalcyfikacyjnym (12±2% komórek pozytywnych). Po stymulacji prozapalnej za pomocą TNF-α lub LDL zaobserwowano wzrost eskpresji plazminogenu (18±3% i 21±3% pozytywnych VICs) i α_2-antyplazminy (15±2% i 17±3% pozytywnych VICs), podczas gdy ekspresja tPA była śladowa (<10% pozytywnych VICs). Po zastosowaniu inhibitora TM5275, który hamuje zdolność do tworzenia kompleksów pomiędzy PAI-1 i tPA, ekspresja tPA w komórkach VICs wciąż była na bardzo niskim poziomie. Analiza poziomu antygenu PAI-1 w supernatantach komórek VICs wykazała, że stymulacja LDL zwiększyła sekrecję PAI-1 o 32% (p=0,0005) w porównaniu z VICs hodowanymi w medium prokalcyfikacyjnym. Podobnie, stymulacja TNF-α zwiększyła stężenie PAI-1 w supernatantach o 25% (p=0,011) w porównaniu z VICs hodowanymi w medium prokalcyfikacyjnym.

Eksperymenty mechanistyczne ujawniły, że ekspresja PAI-1 w komórkach VICs stymulowanych LDL w obecności inhibitora NF-κB – BAY 11-7082 była silnie tłumiona (około 80% komórek), a poziom PAI-1 w supernatantach komórek VICs obniżył się o 22% (p=0,0025) w porównaniu z VICs hodowanymi z samym LDL. Z kolei, inhibitor aktywności PAI-1 – TM5275 nie zmniejszył ekspresji PAI-1 w hodowlach VICs, ani poziomu antygenu PAI-1 w supernatantach (p=0,99) w porównaniu z komórkami VICs hodowanymi z samym LDL.

Ocena wpływu supernatantów z hodowli VICs na zdolność fibrynoliotyczną osocza pozbawionego PAI-1 wykazała, że stymulacja VICs za pomocą LDL wydłuża CLT o 20% ($p<0,001$) w porównaniu z medium prokalcyfikacyjnym. Natomiast zastosowanie inhibitorów BAY 11-7082 lub TM5275 w hodowli VICs skróciło CLT odpowiednio o 12% i 15% w porównaniu z VICs hodowanymi z samym LDL (obydwa $p<0,001$).

Analiza ekspresji genów w komórkach VICs wykazała, że zastosowanie medium prokalcyfikacyjnego spowodowało 1,3-krotny wzrost ekspresji *SERPINE1* w porównaniu z pożywką kontrolną. Stymulacja prozapalna spowodowała około 2,5-krotny wzrost ekspresji *SERPINE1* w komórkach VICs w porównaniu z medium kontrolnym, podczas gdy inhibicja szlaku NF-κB obniżyła ekspresję *SERPINE1* około 2-krotnie w porównaniu z VICs stymulowanymi samym TNF-α lub LDL. Zastosowanie inhibitora TM5275 nie obniżyło ekspresji *SERPINE1* w porównaniu z VICs hodowanymi z samym LDL.

Artykuł nr 5

Natorska J, Kopytek M, Undas A. Aortic valvular stenosis: Novel therapeutic strategies. Eur J Clin Invest. 2021; 51: e13527.

Ostatni artykuł z cyklu jest pracą przeglądową przygotowaną na zaproszenie Edytora czasopisma *The European Journal of Clinical Investigation*, co świadczy o docenieniu mojego wkładu w wiedzę dotyczącą patomechanizmów prowadzących do rozwoju i progresji AS. Praca ta jest próbą zestawienia i usystematyzowania aktualnej wiedzy na temat szlaków molekularnych zaangażowanych w rozwój i progresję choroby i ich potencjalnych implikacji terapeutycznych w niechirurgicznym leczeniu AS, a także podsumowaniem badań naszego Zespołu w tym obszarze.

3.6. DYSKUSJA

W chorobach takich jak DM, stres oksydacyjny przyczynia się do powstawania AGEs, co odgrywa kluczową rolę w uszkodzeniu tkanek [45,46]. Sugerowano, że zwiększona glikacja białek zastawkowych związana z nagromadzeniem AGEs przyczynia się do szybszej progresji AS [47-49]. W **publikacji 1** wykazaliśmy, że pacjenci z AS i współistniejącą DMT2 charakteryzowali się zwiększoną zastawkową akumulacją AGEs, RAGE i ROS oraz wyższymi poziomami AGEs i sRAGE w surowicy krwi w porównaniu z pacjentami z AS bez DMT2. Ponadto, AGEs i RAGE/sRAGE korelowały z ciężkością AS. Nasze wyniki potwierdziły obserwacje pochodzące z badań przeprowadzonych na mysim i króliczym modelu AS, gdzie gromadzenie się AGEs i RAGE w zastawkach prowadziło do degeneracji płatków aortalnych i osteoblastycznego różnicowania się VICs [50,51] oraz do nasilonego stresu oksydacyjnego i nadekspresji NF-κB [51]. Warto zauważyć, że mediowana przez RAGE aktywacja NF-κB jest zaangażowana w syntezę cytokin prozapalnych i TF przez monocyty/makrofagi [52], co jest zgodne z naszymi wcześniejszymi obserwacjami, że u pacjentów z ciężką AS i współistniejącą DMT2 zastawkowa ekspresja CRP dodatnio korelowała z zastawkową ekspresją mRNA dla TF [20]. Udowodniono również, że gromadzenie się AGEs w macierzy pozakomórkowej naczyń prowadzi do zmian strukturalnych i czynnościowych kolagenu oraz późniejszych powikłań sercowo-naczyniowych [53]. Dlatego przypuszczamy, że stopień degeneracji płatków zastawki aortalnej u pacjentów z współistniejącą DMT2 jest wynikiem związanego z akumulacją AGEs usieciowania zastawkowego kolagenu, co prowadzi do nasilonego stanu zapalnego, aktywacji układu krzepnięcia i w konsekwencji do wapnienia płatków zastawki. Pokazaliśmy również, że wskaźniki długoterminowej kontroli glikemii (HbA_{1c} i fruktozamina), ale nie stężenie glukozy, korelują z poziomem AGEs we krwi krążącej i co ważne, w stenotycznych zastawkach aortalnych. Ponadto, słabo kontrolowana DMT2 była związana z największą ekspresją *in loco* AGEs i RAGE oraz nasileniem wady u pacjentów z AS. Obserwacja ta może tłumaczyć brak związku między progresją AS a zespołem metabolicznym lub DM podczas 3-letniej obserwacji prowadzonej przez Testuz i wsp. [54], w której Autorzy stwierdzili brak powyższej asocjacji wyłącznie na podstawie oceny poziomu glukozy na czczo. Nasze badanie sugeruje, że u pacjentów z AS i współistniejącą DMT2 akumulacja AGEs i RAGE może mieć wpływ na tempo progresji wady, a utrzymywanie HbA_{1c} i/lub fruktozaminy w zakresie norm referencyjnych może spowolnić tempo choroby.

Kontynuując wątek wpływu hiperglikemii na wapnienie zastawek, w **publikacji 2** pokazaliśmy, że pacjenci z ciężką AS i współistniejącą DMT2 w porównaniu z osobami bez

DMT2, wykazują zwiększoną zastawkową ekspresję NF-κB w połączeniu z nasiloną ekspresją *in loco* BMP-2. Co ważne, najwyższą zastawkową ekspresję NF-κB i BMP-2 zaobserwowano u osób z słabo kontrolowaną DMT2. Warto zauważyć, że u chorych z DMT2 ekspresja NF-κB korelowała także z ciężkością AS. Eksperymenty mechanistyczne przeprowadzone na VICs potwierdziły, że wysokie stężenia glukozy generują stan zapalny poprzez sygnalizację za pośrednictwem szlaku transkrypcyjnego NF-κB, co prowadzi do późniejszego wapnienia VICs. Wykazaliśmy również, że hamowanie ROS zapobiega wapnieniu komórek VICs. Obserwacje te po raz pierwszy pokazały, że ludzkie VICs podobnie jak VICs wyizolowane ze świńskich zastawek ulegają różnicowaniu osteogennemu poprzez ścieżkę sygnalizacyjną NF-κB [55] oraz że ekspresja BMP-2 w VICs jest ściśle kontrolowana przez NF-κB. Jako pierwsi wykazaliśmy, że pacjenci z AS i współistniejącą DMT2 w porównaniu z pacjentami bez DMT2, charakteryzują się znacznie wyższą zastawkową ekspresją FII i FXa, która korelowała z BMP-2 i ciężkością choroby, a słabo kontrolowana DMT2 była związana z najwyższą ekspresją *in loco* markerów krzepnięcia i z istotnie wyższymi stężeniami TF i FVIIa-AT w osoczu krwi. Warto zwrócić uwagę na fakt, że Breyne i wsp. [56] wykazali kolokalizację TF i trombiny z osteopontyną w zastawkach aortalnych pobranych od pacjentów z ciężką AS, co sugeruje, że aktywacja układu krzepnięcia jest związana z procesem wapnienia zastawek. Nasz Zespół potwierdził tę hipotezę w badaniach *in vitro* poprzez obserwację, że prozapalna stymulacja VICs prowadzi do nasilonej ekspresji TF, trombiny, FVII i FX/FXa zarówno na poziomie białka, jak i mRNA, uwalniania zwiększonych ilości cytokin prozapalnych, metaloproteinaz oraz markerów wapnienia w porównaniu z komórkami niestymulowanymi [57]. W związku z naszą obserwacją, iż hiperglikemia jest czynnikiem prozapalnym dla VICs, wydaje się, że źle kontrolowana DMT2 jest związana ze stanem prozakrzepowym, który może wpływać na progresję AS. Co istotne tylko HbA_{1c} i fruktozamina, ale nie poziom glukozy we krwi korelowały z markerami zapalenia i wapnienia zastawek. Dane te są zgodne z wynikami przedstawionymi w **publikacji 1** i potwierdzają nasza hipotezę, że utrzymywanie parametrów długoterminowej kontroli glikemii w zakresie wartości referencyjnych może mieć kluczowe znaczenie w opóźnianiu progresji AS u pacjentów z towarzyszącą DMT2.

Podsumowując wpływ hiperglikemii na progresję AS zaproponowaliśmy następujący mechanizm: hiperglikemia nasila stres oksydacyjny i prowadzi do zwiększonej akumulacji AGEs i RAGE oraz wzmożonego usieciowania zastawkowego kolagenu, co nasila stan zapalny i aktywację układu krzepnięcia w obrębie płatków zastawki. Procesy te prowadzą do zwiększonej produkcji BMP-2 i szybszego wapnienia płatków zastawki aortalnej.

Rola procesów oksydacji lipoprotein w patogenezie AS była wielokrotnie badana [58-61]. Badania histologiczne wykazały, że OxLDL, Lp(a) i OxPL promują osteogenne różnicowanie i wapnienie zastawek, co skutkuje rozwojem i progresją AS [29,30,58,61,62]. Z kolei analiza proteomiczna białek osocza ujawniła, że pacjenci z ciężką AS mają zwiększony poziom białek związanych z układem krzepnięcia, stresem oksydacyjnym oraz upośledzonym transportem cholesterolu w porównaniu do pacjentów z niedomykalnością zastawki aortalnej [63]. Nie jest jednak jasne, czy zaburzona hemostaza, zwłaszcza hipofibrynoliza, są związane ze stanem prooksydacyjnym w AS. Jak wspomniano we Wprowadzeniu, nasz Zespół wykazał, że zwiększony stres oksydacyjny był silnym niezależnym predyktorem CLT, silniejszym niż fibrynogen, i korelował z ciężkością AS [38]. Innymi czynnikami wpływającymi istotnie na fibrynolizę u pacjentów z izolowaną ciężką AS były OxLDL i Lp(a) [39]. Biorąc pod uwagę, że Lp(a) jest preferencyjnym nośnikiem OxPL [32] i jest w stanie hamować fibrynolizę [34], zbadaliśmy czy pacjenci z AS i wysokim stężeniem Lp(a) definiowanym jako poziom ≥50 mg/dl wykazują wyższe poziomy OxPL w stenotycznych zastawkach aortalnych i czy OxPL są związane z hipofibrynolizą u pacjentów z ciężką AS.

W **publikacji 3** wykazaliśmy po raz pierwszy, że u pacjentów z Lp(a) ≥50 mg/dl obserwuje się podwyższone poziomy OxPL, które silniej niż Lp(a) predykują hipofibrynolizę. Warto zauważyć, że odnotowaliśmy także silne asocjacje między ciężkością AS a stężeniami OxPL w surowicy krwi, co jest zgodne z wcześniejszymi doniesieniami [40,59]. Ponieważ OxPL mają zdolność aktywacji szlaku NF-κB [64], w naszej ocenie mogą one być jednym z czynników nasilających lokalny stan zapalny, a obecność Lp(a) towarzysząca wysokim stężeniom OxPL może jednocześnie upośledzać lizę depozytów fibryny w płatkach zastawek. Zatem, wpływ wysokich stężeń Lp(a) na rozwój/progresję AS może być związany z obecnością podwyższonych poziomów OxPL.

W **publikacji 4** pokazaliśmy, jako pierwsi, że w obrębie stenotycznych zastawek aortalnych zachodzi proces fibrynolizy, o czym świadczy obecność D-dimeru, produktu degradacji fibryny. Ponadto, wykazaliśmy, że ekspresja PAI-1 w obrębie stenotycznych zastawek aortalnych jest podwyższona w stosunku do zastawek kontrolnych pozyskanych od dawców autopsyjnych. Podobny wzorzec ekspresji PAI-1 odnotowano w fibroblastach raka płuc [65]. Zaobserwowaliśmy również, że zastawkowa nadekspresja PAI-1 korelowała z akumulacją lipidów, ciężkością wady i kolokalizowała z NF-κB. Ekspresja genu *SERPINE1* kodującego PAI-1 jest kontrolowana przez szlak NF-κB [66], a na sekrecję PAI-1 istotny wpływ mają cytokiny prozapalne takie jak TNF-α i interleukina-1β (IL-1β) [67], których

obecność w obrębie stenotycznych zastawek aortalnych została udowodniona [68,69]. Ponadto, zwiększone siły ścinające również może stymulować komórki śródbłonka do uwalniania PAI-1 [70], przyczyniając się do systemowej hipofibrynolizy. Alexopoulos i wsp. [71] zaobserwowali, że PAI-1 był silnie zaangażowany w patogenezę miażdżycy, którą cechuje podobny do AS patomechanizm. Eksperymenty mechanistyczne opisane w **publikacji 4** wykazały, że zastosowanie inhibitora NF-κB hamowało ekspresję PAI-1 zarówno na poziomie białka, jak i mRNA w VICs oraz promowało fibrynolizę. Z drugiej strony, hamowanie aktywności PAI-1 przez TM5275 nie wpłynęło na ekspresję *SERPINE1* i poziom antygenu PAI-1, ale zgodnie z oczekiwaniami sprzyjało fibrynolizie. Dlatego podejrzewamy, że indukowana przez LDL nadekspresja PAI-1 przyczynia się do hipofibrynolizy w AS. Jako pierwsi wykazaliśmy także słabą ekspresję α_2-antyplazminy, zarówno w stenotycznych zastawkach aortalnych, jak i w hodowlach VICs. Ekspresja α_2-antyplazminy w VICs była bardzo niska, nawet po stymulacji prozapalnej. Podobnie niską ekspresję *in loco* wykazywały plazminogen i tPA, natomiast w hodowlach VICs obserwowano śladową ekspresję obu białek, co sugeruje, że mogą być one uwalniane przez komórki śródbłonka zastawek, a nie syntetyzowane przez komórki VICs lub dostarczane do zastawki wraz z krwią. Pozostaje zatem do ustalenia, za pomocą metod ilościowych, takich jak PCR czy proteomika, czy VICs mają zdolność syntezy α_2-antyplazminy, plazminogenu i tPA. Z drugiej strony, Kochtebane i wsp. [72] zidentyfikowali niewielkie ilości plazminogenu, urokinazowego aktywatora plazminogenu (*urokinase-type plasminogen activator*, uPA), tPA i PAI-1 we wszystkich trzech warstwach zastawek aortalnych oraz w hodowlach miofibroblastów, jednak z dużą zmiennością między zastawkami. Co ciekawe, uPA było jedynym białkiem o aktywności enzymatycznej w lizatach miofibroblastów [72]. Jednak ekspresja wolnego tPA w miofibroblastach nie została potwierdzona metodą Western blot, a tPA pokazano wyłącznie jako kompleks z PAI-1 [72]. Nasze eksperymenty *in vitro*, wykazały znikome ilości wolnego tPA w hodowlach VICs, nawet po zastosowaniu inhibitora TM5275, który zmniejsza zdolność tworzenia kompleksów PAI-1–tPA, ujawniając wolny tPA i przekształcając PAI-1 w jego nieaktywną formę [73]. Zatem nasze dane sugerują, że VICs mają ograniczoną zdolność syntezy tPA. Podsumowując, powyższe obserwacje mogą mieć istotne implikacje kliniczne związane z hamowaniem szlaku NF-κB i promowaniem fibrynolizy, jako potencjalnego celu terapeutycznego. Obecnie, inhibitory szlaku NF-κB są szeroko testowane w badaniach klinicznych pod kątem interwencji terapeutycznej [74].

W **publikacji 5** przedstawiliśmy aktualny stan wiedzy na temat szlaków molekularnych i ich potencjalnych implikacji terapeutycznych w leczeniu AS. Szczegółowe

podsumowanie głównych celów farmakologicznych i ich skutków w badaniach klinicznych i modelach przedklinicznych AS jest zaprezentowane w rozdziale **Persepektywy**.

3.7. WNIOSKI

Wnioski wysunięte z przedstawionej dysertacji są następujące:

a. Źle kontrolowana DMT2 prowadzi do akumulacji AGEs w zastawkach aortalnych, przez co nasila lokalny stres oksydacyjny i zapalenie, co w konsekwencji może prowadzić do szybszej progresji AS.

b. Współistniejąca DMT2 u pacjentów z AS nasila lokalny stan zapalny, aktywację układu krzepnięcia oraz syntezę mediatorów wapnienia zastawki poprzez nasiloną aktywację *in loco* szlaku NF-κB.

c. U pacjentów z AS i współistniejącą DMT2 utrzymywanie HbA_{1c} i fruktozaminy w zakresie norm referencyjnych może spowolnić tempo progresji AS.

d. U pacjentów z ciężką AS i stężeniem Lp(a) ≥50 mg/dl obserwuje się podwyższone poziomy OxPL, które mają silniejszy wpływ na hipofibrynolizę niż Lp(a).

e. U pacjentów z ciężką AS akumulacja lipidów prowadzi do nadekspresji PAI-1, wynikającej z nadmiernej aktywacji szlaku NF-κB w zastawkach aortalnych, co przyczynia się do hipofibrynolizy.

3.8. OGRANICZENIA

Liczba włączonych do badań pacjentów w poszczególnych podgrupach była ograniczona, zwłaszcza tych z dobrze i źle kontrolowaną DMT2. Niemniej jednak, badania te miały odpowiednią moc i reprezentują typowych pacjentów z objawową ciężką AS spotykanych w praktyce klinicznej. Zastawkowa ekspresja poszczególnych czynników została określona półilościowo. Dlatego też, w ramach grantu PRELUDIUM pt. „Wpływ lipoprotein na fibrynolizę i plejotropowe działanie inhibitorów proproteinowej konwertazy subtylizyny/keksyny typu 9 (PCSK9) w stenozie aortalnej - powiązania ze stanem zapalnym i hemostazą", którego jestem kierownikiem zaplanowano analizę proteomiczną, która pozwoli jednoznacznie określić skład ilościowy i jakościowy proteomu zastawki, zwłaszcza białek związanych z hemostazą. Ponadto, poziomy badanych markerów w osoczu i surowicy krwi były mierzone jednorazowo podczas włączenia pacjentów do badania, dlatego mogą nie ujawniać pewnych zależności. W **publikacji 1** należy zwrócić uwagę na fakt, że większość pacjentów z AS i współistniejąca DMT2 otrzymywała metforminę, która ma zdolność zmniejszania akumulacji AGEs [75]. Z kolei występowanie nadciśnienia tętniczego czy stosowanie leków hipotensyjnych może wpływać na stężenie sRAGE w osoczu krwi [76]. Ponadto, prawie połowa pacjentów z DMT2 miała dobrze kontrolowaną DM, co mogło skutkować nieujawnieniem pewnych zależności. W **publikacji 2** i **4** można rozważyć modyfikację warunków hodowli VICs, takie jak stężenie stymulantów i inhibitorów lub różne czasy inkubacji, w celu dokładniejszego zbadania działania wybranych czynników na VICs. W **publikacji 4** nie oceniono ekspresji uPA, a hipofibrynolizę w supernatantach komórek VICs oceniono na podstawie CLT, który jest modelem zależnym od tPA. Warto zauważyć, że badania przeprowadzono na osobach z zastawkami trójpłatkowymi i z ciężką wysokogradientową postacią AS, zatem naszych wyników nie można bezpośrednio ekstrapolować na pacjentów z łagodną lub umiarkowaną AS, jak również z dwupłatkową zastawką aortalną. Ponadto, ciężkość AS w **publikacji 1, 2 i 4** mierzono jako gradienty ciśnień przez zastawkę i AVA, a nie jako V_{max}, zgodnie z obecnymi wytycznymi Europejskiego Towarzystwa Kardiologicznego [41].

3.9. PERSPEKTYWY

Obecnie, standardem postępowania u pacjentów z ciężką objawową AS pozostaje wymiana stenotycznej zastawki aortalnej metodą chirurgiczną lub przezskórną. Niestety wciąż nie ma dostępnego leczenia farmakologicznego, które zapobiegałoby lub opóźniało progresję tej choroby. Ponadto udowodniono, że terapie obniżające poziom lipidów takie jak statyny nie wywierają korzystnego wpływu na hamowanie progresji AS [77,78]. Warto zauważyć, że wtórna analiza danych z badania ASTRONOMER (the Aortic Stenosis Progression Observation: Measuring the Effects of Rosuvastatin) wykazała, że podwyższone poziomy Lp(a) i OxPL były związane z szybszą progresją choroby u pacjentów z AS [79]. Terapie skierowane na obniżenie poziomu Lp(a) i/lub LDL takie jak: inhibitory konwertazy białkowej subtylizyny/keksyny typu 9 (PCSK9), inhibitory białka przenoszącego estry cholesterylu (CETP), antysensowne oligonukleotydy ukierunkowane na apo(a) oraz kombinacja symwastatyna/ezetymib są obecnie testowane jako środki hamujące wapnienie zastawek u pacjentów z AS [**publikacja 5**]. Coraz więcej danych wskazuje również, że terapie ukierunkowane na obniżenie poziomu OxPL mogą mieć w przyszłości znaczenie w opóźnianiu progresji AS [**publikacja 3**,27,28,40]. Warto podkreślić, iż terapie hipolipemizujące są obecnie najbardziej obiecującym obszarem badawczym w zakresie potencjalnego zahamowania rozwoju/progresji AS we wczesnych stadiach choroby [**publikacja 5**].

Ścisła kontrola parametrów długoterminowej kontroli glikemii, takich jak poziom HbA_{1c} i/lub fruktozaminy oraz leczenie lekami przeciwcukrzycowymi są uznawane za korzystne dla pacjentów z AS i współistniejącą DMT2 [**publikacja 1,2**,80,81]. Obecnie leki ukierunkowane na hamowanie osi AGEs-RAGE, takie jak pioglitazon lub alagebrium (ALT-711), nowe leki przeciwhiperglikemiczne, a mianowicie agoniści receptora glukagonopodobnego peptydu-1 (GLP-1RA; liraglutyd, luraglutyd i semaglutyd) oraz inhibitory kotransportera sodowo-glukozowego-2 (SGLT2; empagliflozyna, kanagliflozyna, dapagliflozyna i ertugliflozyna) wymagają badań, aby ocenić ich znaczenie w opóźnieniu progresji AS [80,81]. Pomimo faktu, że hamowanie osi AGEs-RAGE wydaje się być obiecującym celem terapeutycznym, nie ma danych potwierdzających, czy hamowanie tej osi ma działanie kardioprotekcyjne u pacjentów z DMT2. W przyszłości spersonalizowane leczenie farmakologiczne ukierunkowane na rozpoznanie chorób współistniejących może opóźnić progresję AS lub zminimalizować ryzyko powikłań.

Z kolei, ostatnie badania *in vitro* i *in vivo* wskazują, że doustne antykoagulanty niebędące antagonistami witaminy K (NOAC) mogą wpływać nie tylko na układ krzepnięcia,

ale również na zapalenie i przebudowę zastawek, przez co mogą służyć jako strategia terapeutyczna w celu zahamowania progresji AS przy jednoczesnym korzystnym wpływie na układ sercowo-naczyniowy, przynajmniej u pacjentów z AS i wskazaniem do leczenia przeciwzakrzepowego [57,82]. Co ciekawe, nasze najnowsze dane [**publikacja 2 i 4]** wskazują, że inhibitory szlaku NF-κB również mogą być potencjalnym celem teraputycznym w leczeniu AS, poprzez hamowanie zapalenia i promowanie fibrynolizy. Wprowadzenie terapii farmakologicznej opóźniającej progresję AS zrewolucjonizowałoby leczenie AS.

4. STRESZCZENIE W JĘZYKU POLSKIM

Wprowadzenie

Stenoza aortalna (AS) jest najczęstszą przyczyną nabytej wady zastawkowej serca u osób powyżej 65 roku życia, bez dostępnego leczenia farmakologicznego. Patomechanizm AS jest procesem złożonym i ściśle regulowanym, związanym z aktywacją wielu szlaków na poziomie molekularnym, komórkowym i tkankowym. Liczne badania wskazują, że podwyższony poziom lipoprotein, oksydowanych fosfolipidów (OxPL) lub towarzysząca cukrzyca typu 2 (DMT2) mogą przyspieszyć rozwój AS. Jednak, mechanizmy leżące u podstaw wpływu tych czynników na stan zapalny, układ krzepnięcia i fibrynolizy oraz kalcyfikację zastawek aortalnych nie zostały w pełni poznane.

Cel badania

Zbadanie mechanizmów związanych z działaniem czynników modulujących progresję AS, tj. hiperglikemii i nasilonego stresu oksydacyjnego ze szczególnym uwzględnieniem ich potencjalnych związków z zapaleniem, aktywacją układu krzepnięcia, hipofibrynolizą i kalcyfikacją u pacjentów z ciężką AS.

Metody i Wyniki

W **Publikacji 1** przebadano 76 pacjentów z ciężką AS (bez DMT2) i 50 pacjentów z AS i DMT2 (AS-DM). Zastawkową ekspresję końcowych produktów zaawansowanej glikacji (AGEs) i receptora dla AGEs (RAGE) oceniano immunofluorescencyjnie. Poziomy AGEs i rozpuszczalnej izoformy RAGE (sRAGE) w surowicy krwi oceniano za pomocą testów ELISA. Pacjenci AS-DM mieli zwiększoną akumulację AGEs i RAGE w obrębie stenotycznych zastawek aortalnych oraz w surowicy krwi w porównaniu z pacjentami bez DMT2. Ponadto, w grupie AS-DM ekspresja AGEs i RAGE, oraz ich poziom w surowicy krwi korelowały z poziomem hemoglobiny glikowanej (HbA_{1c}). Co istotne, u pacjentów AS-DM zastawkowa ekspresja AGEs i poziom AGEs w surowicy krwi były związane z ciężkością choroby.

W **Publikacji 2** oceniono 100 pacjentów z AS (bez DMT2) i 50 pacjentów z AS i DMT2 (AS-DM). Zastawkową ekspresję jądrowego czynnika kappa B (NF-κB), białka morfogenetycznego kości-2 (BMP-2), protrombiny (FII) i aktywnego czynnika X (FXa) oceniono immunofluorescencyjnie. Ekspresję NF-κB i BMP-2 w hodowlach pierwotnych komórek interstycjalnych zastawki (VICs) po stymulacji glukozą oceniono na poziomie białka i mRNA (*RELA*). W badaniach mechanistycznych zastosowano inhibitor reaktywnych form tlenu – ROS (NAC) lub inhibitor szlaku NF-κB (BAY 11-7082). W zastawkach z grupy

AS-DM obserwowano zwiększoną ekspresję NF-κB, BMP-2 i białek układu krzepnięcia w porównaniu do zastawek z grupy bez DM. Zastawkowa ekspresja NF-κB i BMP-2 dodatnio korelowała z ekspresją FII i FXa. Tylko u chorych z DM, zastawkowa ekspresja NF-κB była związana z poziomem HbA_{1c} i fruktozaminy we krwi oraz ciężkością AS. Eksperymenty *in vitro* wykazały, że wysokie stężenie glukozy zwiększyło ekspresję NF-κB i BMP-2, podczas gdy zastosowanie inhibitorów ROS (NAC) lub NF-κB (BAY 11–7082) znacząco je obniżyło. Analiza ekspresji mRNA (*RELA*) w komórkach VICs potwierdziła te wyniki.

W **Publikacji 3** oceniono 50 pacjentów z AS i stężeniem lipoproteiny(a) [Lp(a)] ≥50 mg/dl oraz 20 pacjentów z AS i Lp(a) <50 mg/dl. Zastawkową ekspresję OxPL oceniano za pomocą immunofluorescencji. Stężenie OxPL, antygenu inhibitora aktywatora plazminogenu typu 1 (PAI-1) i inhibitora fibrynolizy aktywowanego trombiną (TAFI) oceniono za pomocą testów ELISA. Czas lizy skrzepu (CLT) w osoczu wyznaczono turbidymetrycznie. Pacjenci z AS i Lp(a) ≥50 mg/dl mieli zwiększoną ekspresję OxPL w zastawkach i wyższe stężenie OxPL w surowicy krwi w porównaniu do pacjentów z AS i Lp(a) <50 mg/dl. Zastawkowa ekspresja OxPL korelowała ze stężeniami OxPL i Lp(a) w surowicy krwi. Tylko u pacjentów z Lp(a) ≥50 mg/dl, stężenia OxPL w surowicy korelowały z CLT, stężeniami PAI-1 i TAFI w osoczu, oraz ciężkością AS. Analiza regresji wieloczynnikowej wykazała, że wyższe poziomy OxPL determinowały wydłużone CLT u pacjentów z AS i Lp(a) ≥50 mg/dl.

W **Publikacji 4** przebadano 75 pacjentów z ciężką AS. Zastawkową akumulację lipidów oceniono histochemicznie, zaś ekspresję PAI-1 i NF-κB metodą immunofluorescencji. Zastawki pobrane od dawców autopsyjnych stanowiły kontrolę. Ekspresję PAI-1 w komórkach VICs hodowanych w różnych warunkach oceniono na poziomie białka i mRNA (*SERPINE1*). W badaniach mechanistycznych zastosowano inhibitor aktywności PAI-1 (TM5275) lub szlaku NF-κB (BAY 11-7082). Supernatanty hodowli VICs dodano do osocza pozbawionego PAI-1 i oznaczono CLT zmodyfikowaną metodą. W supernatantach oznaczono także poziom antygenu PAI-1 za pomocą testu ELISA. Wyłącznie w stenotycznych zastawkach aortalnych stwierdzono ekspresję PAI-1, która korelowała z akumulacją lipidów, ekspresją NF-κB oraz ciężkością AS. *In vitro*, VICs wykazywały wysoką ekspresję PAI-1. Stymulacja LDL zwiększyła poziom PAI-1 w supernatantach VICs i wydłużyła CLT. Hamowanie aktywności PAI-1 skróciło CLT, natomiast hamowanie szlaku NF-κB zmniejszyło ekspresję PAI-1 w VICs, poziom jego antygenu w supernatantach oraz skróciło CLT. Analiza ekspresji mRNA (*SERPINE1*) w komórkach VICs potwierdziła te wyniki.

W **Publikacji 5** omówione zostały najnowsze potencjalne strategie terapeutyczne hamujące progresję AS.

Wnioski

U pacjentów z AS i towarzyszącą DMT2 źle kontrolowana cukrzyca prowadzi do nadmiernej akumulacji AGEs w zastawkach aortalnych, przez co nasila lokalny stres oksydacyjny i zapalenie oraz syntezę mediatorów wapnienia zastawki, co w konsekwencji może prowadzić do szybszej progresji AS. Hiperglikemia niekorzystnie wpływa na procesy związane z progresją AS poprzez nasiloną aktywację *in loco* szlaku NF-κB. Nasze badanie sugeruje, że u pacjentów z AS i współistniejącą DMT2 utrzymywanie HbA_{1c} i fruktozaminy w zakresie norm referencyjnych może spowolnić tempo progresji AS. Pacjentów z ciężką AS charakteryzuje hipofibrynoliza. Wysokie stężenia Lp(a) w surowicy krwi wiążą się z podwyższonym poziomem OxPL, które determinują hipofibrynolizę silniej niż Lp(a). Ponadto, nadekspresja PAI-1 w obrębie stenotycznych zastawek spowodowana akumulacją lipidów, jest mediowana nadmierną aktywacją szlaku NF-κB i przynajmniej częściowo jest odpowiedzialna za hipofibrynolizę. Zatem, nasze wyniki sugerują, że szlak NF-κB może być potencjalnym celem terapeutycznym w AS.

5. STRESZCZENIE W JĘZYKU ANGIELSKIM (ABSTRACT)

Background

Aortic stenosis (AS) is the most common cause of acquired valvular heart disease in individuals over 65 years of age, with no available pharmacological treatment. The pathomechanism of AS is a complex and tightly regulated process involving the activation of multiple molecular, cellular, and tissue pathways. Numerous studies indicate that elevated levels of lipoproteins, oxidized phospholipids (OxPL), or coexisting type 2 diabetes (DMT2) may accelerate the development of AS. However, the underlying mechanisms by which these factors influence inflammation, coagulation and fibrinolysis pathways, and aortic valve calcification have not been fully elucidated.

Aims

To investigate the mechanisms involving factors favouring AS progression, such as hyperglycemia and increased oxidative stress, with particular emphasis on their potential associations with inflammation, coagulation activation, hypofibrinolysis, and calcification in patients with severe AS.

Methods and Results

In **Publication 1**, 76 patients with severe AS (without DMT2) and 50 patients with AS and DMT2 (AS-DM) were studied. Valvular expression of advanced glycation end products (AGEs) and receptor for AGEs (RAGE) was evaluated by immunofluorescence. Levels of AGEs and soluble isoform of RAGE (sRAGE) in serum were assessed using ELISA tests. AS-DM patients exhibited increased accumulation of AGEs and RAGE within stenotic aortic valves and in serum compared to patients without DMT2. Furthermore, in the AS-DM group, the expression of AGEs and RAGE, as well as their serum levels, correlated with glycosylated hemoglobin (HbA_{1c}) levels. Importantly, in AS-DM patients, valvular expression of AGEs and serum AGE levels were associated with disease severity.

Publication 2 evaluated 100 patients with AS (without DMT2) and 50 patients with AS and DMT2 (AS-DM). Valvular expression of nuclear factor kappa B (NF-κB), bone morphogenetic protein-2 (BMP-2), prothrombin (FII), and activated factor X (FXa) was assessed by immunofluorescence. The expression of NF-κB and BMP-2 in valve interstitial cells (VICs) *in vitro* cultures stimulated with glucose was evaluated at the protein and mRNA (*RELA*) levels. Mechanistic studies were performed using a reactive oxygen species inhibitor (NAC) or NF-κB pathway inhibitor (BAY 11-7082). Increased expression of NF-κB, BMP-2, and coagulation proteins was observed in AS-DM valves compared to those without DM.

Valvular expression of NF-κB and BMP-2 positively correlated with FII and FXa expression. Only in patients with DM, valvular NF-κB expression was associated with HbA_{1c} and blood fructosamine levels, as well as AS severity. *In vitro* experiments demonstrated that high glucose concentrations increased NF-κB and BMP-2 expression, while the use of ROS (NAC) or NF-κB (BAY 11–7082) inhibitors significantly reduced their expression. Analysis of mRNA (*RELA*) expression in VICs confirmed these results.

In **Publication 3**, 50 patients with AS and lipoprotein(a) [Lp(a)] concentration ≥50 mg/dl were evaluated, along with 20 patients with AS and Lp(a) <50 mg/dl. Valvular OxPL expression was assessed using immunofluorescence. The levels of OxPL, plasminogen activator inhibitor type 1 (PAI-1) antigen, and thrombin-activated fibrinolysis inhibitor (TAFI) were evaluated using ELISA tests. Clot lysis time (CLT) in plasma was determined turbidimetrically. AS patients with Lp(a) ≥50 mg/dl had increased OxPL valvular expression and higher serum levels of OxPL compared to patients with AS and Lp(a) <50 mg/dl. Valvular OxPL expression correlated with serum OxPL and Lp(a) levels. Only in patients with Lp(a) ≥50 mg/dl, serum OxPL levels correlated with CLT, PAI-1 and TAFI concentrations, as well as the severity of AS. Multifactorial regression analysis showed that higher levels of OxPL were associated with prolonged CLT in patients with AS and Lp(a) ≥50 mg/dl.

In **Publication 4**, 75 patients with severe AS were examined. Valvular lipid accumulation was assessed histochemically, while the expression of PAI-1 and NF-κB was evaluated using immunofluorescence. Valves obtained from autopsy donors served as controls. The expression of PAI-1 in VICs cultured under different conditions was assessed at the protein and mRNA (*SERPINE1*) levels. Mechanistic studies were performed using an inhibitor of PAI-1 activity (TM5275) or an NF-κB pathway inhibitor (BAY 11-7082). VICs culture supernatants were added to PAI-1 – depleted plasma, and CLT was measured using a modified method. The level of PAI-1 antigen in the supernatants was determined using an ELISA test. Valvular expression of PAI-1, which correlated with lipid accumulation, NF-κB expression, and the severity of AS, was observed only in stenotic aortic valves. *In vitro*, VICs showed high PAI-1 expression. LDL stimulation increased the level of PAI-1 in VICs supernatants and prolonged CLT. Inhibition of PAI-1 activity shortened CLT, while inhibition of the NF-κB pathway reduced PAI-1 expression in VICs, the level of its antigen in supernatants, and shortened CLT. Analysis of mRNA expression (*SERPINE1*) in VICs confirmed these results.

Publication 5 discusses the latest potential therapeutic strategies for inhibiting the progression of AS.

Conclusions

In patients with AS and concomitant poorly controlled DMT2, uncontrolled diabetes leads to excessive accumulation of AGEs in aortic valves, thereby exacerbating local oxidative stress, inflammation, and the synthesis of valvular calcification mediators, ultimately resulting in faster progression of AS. Hyperglycemia adversely affects processes related to AS progression by enhanced *in loco* activation of the NF-κB pathway. Our study suggests that maintaining HbA_{1c} and fructosamine within the reference range in patients with AS and concomitant DMT2 may retard the rate of AS progression. Patients with severe AS exhibit hypofibrinolysis. High levels of serum Lp(a) are associated with elevated levels of OxPL, and better than Lp(a) predict hypofibrinolysis. Additionally, the overexpression of PAI-1 within stenotic valves, caused by lipid accumulation, is mediated by enhanced activation of the NF-κB pathway and, at least in part, contributes to hypofibrinolysis. Therefore, our results suggest that the NF-κB pathway may be a potential therapeutic target in AS.

6. PIŚMIENNICTWO

1. Osnabrugge RL, Mylotte D, Head SJ, Van Mieghem NM, Nkomo VT, LeReun CM, Bogers AJ, Piazza N, Kappetein AP. Aortic stenosis in the elderly: disease prevalence and number of candidates for transcatheter aortic valve replacement: a meta-analysis and modeling study. J Am Coll Cardiol. 2013;62:1002-12.
2. Yutzey KE, Demer LL, Body SC, Huggins GS, Towler DA, Giachelli CM, Hofmann-Bowman MA, Mortlock DP, Rogers MB, Sadeghi MM, Aikawa E. Calcific aortic valve disease: a consensus summary from the Alliance of Investigators on Calcific Aortic Valve Disease. Arterioscler Thromb Vasc Biol. 2014;34:2387-93.
3. Lindroos M, Kupari M, Valvanne J, Strandberg T, Heikkilä J, Tilvis R. Factors associated with calcific aortic valve degeneration in the elderly. Eur Heart J. 1994;15:865-70.
4. Aronow WS, Schwartz KS, Koenigsberg M. Correlation of serum lipids, calcium, and phosphorus, diabetes mellitus and history of systemic hyper-tension with presence or absence of calcified or thickened aortic cusps or root in elderly patients. Am J Cardiol. 1987;59:998-999.
5. Stewart BF, Siscovick D, Lind BK, Gardin JM, Gottdiener JS, Smith VE, Kitzman DW, Otto CM. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol. 1997;29:630-4.
6. Akerström F, Barderas MG, Rodríguez-Padial L. Aortic stenosis: a general overview of clinical, pathophysiological and therapeutic aspects. Expert Rev Cardiovasc Ther. 2013;11:239-50.
7. Liu AC, Joag VR, Gotlieb AI. The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. Am J Pathol. 2007;171:1407-18.
8. Natorska J and Undas A. Blood coagulation and fibrinolysis in aortic valve stenosis: links with inflammation and calcification. Thromb. Haemost. 2015;114:217-227.
9. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. Circ Res. 2011;108:1392-412.
10. Pawade TA, Newby DE, Dweck MR. Calcification in Aortic Stenosis: The Skeleton Key. J Am Coll Cardiol. 2015;66:561-77.
11. Yetkin E, Waltenberger J. Molecular and cellular mechanisms of aortic stenosis. Int J Cardiol. 2009;135:4-13.
12. O'Brien KD. Pathogenesis of calcificaortic valve disease: a disease process comes of age (and a good deal more). Arterioscler ThrombVasc Biol. 2006;8:1721-1728.

13. Wypasek E, Natorska J, Grudzień G, Filip G, Sadowski J, Undas A. Mast cells in human stenotic aortic valves are associated with the severity of stenosis. Inflammation. 2013;36:449-56.
14. Nagy E, Eriksson P, Yousry M, Caidahl K, Ingelsson E, Hansson GK, Franco-Cereceda A, Bäck M. Valvular osteoclasts in calcification and aortic valve stenosis severity. Int J Cardiol. 2013;168:2264-71.
15. Culler SD, Cohen DJ, Brown PP, Kugelmass AD, Reynolds MR, Ambrose K, Schlosser ML, Simon AW, Katz MR. Trends in Aortic Valve Replacement Procedures Between 2009 and 2015: Has Transcatheter Aortic Valve Replacement Made a Difference? Ann Thorac Surg. 2018;105:1137-1143.
16. Ljungberg J, Johansson B, Engström KG, Albertsson E, Holmer P, Norberg M, Bergdahl IA, Söderberg S. Traditional Cardiovascular Risk Factors and Their Relation to Future Surgery for Valvular Heart Disease or Ascending Aortic Disease: A Case-Referent Study. J Am Heart Assoc. 2017;6:e005133.
17. López-de-Andrés A, Perez-Farinos N, de Miguel-Díez J, Hernández-Barrera V, Méndez-Bailón M, de Miguel-Yanes JM, Jiménez-García R. Impact of type 2 diabetes mellitus in the utilization and in-hospital outcomes of surgical aortic valve replacement in Spain (2001-2015). Cardiovasc Diabetol. 2018;17:135.
18. Kamalesh M, Ng C, El Masry H, Eckert G, Sawada S. Does diabetes accelerate progression of calcific aortic stenosis? Eur J Echocardiogr. 2009;10:723-5.
19. Yan AT, Koh M, Chan KK, Guo H, Alter DA, Austin PC, Tu JV, Wijeysundera HC, Ko DT. Association Between Cardiovascular Risk Factors and Aortic Stenosis: The CANHEART Aortic Stenosis Study. J Am Coll Cardiol. 2017;69:1523-1532.
20. Natorska J, Wypasek E, Grudzień G, Sobczyk D, Marek G, Filip G, Sadowski J, Undas A. Does diabetes accelerate the progression of aortic stenosis through enhanced inflammatory response within aortic valves? Inflammation. 2012;35:834-40.
21. Hecker M, Wagner AH. Role of protein carbonylation in diabetes. J Inherit Metab Dis. 2018;41:29-38.
22. Kiuchi K, Nejima J, Takano T, Ohta M, Hashimoto H. Increased serum concentrations of advanced glycation end products: a marker of coronary artery disease activity in type 2 diabetic patients. Heart. 2001;85:87-91.
23. Di Pino A, Currenti W, Urbano F, Scicali R, Piro S, Purrello F, Rabuazzo AM. High intake of dietary advanced glycation end-products is associated with increased arterial

stiffness and inflammation in subjects with type 2 diabetes. Nutr Metab Cardiovasc Dis. 2017;27:978-984.

24. Thorpe SR, Baynes JW. Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids. 2003;25:275-81.
25. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab. 2001;280:E685-94.
26. Bobrowska B, Zasada W, Surdacki A, Rakowski T, Kleczyński P, Świerszcz J, Kruszelnicka O, Rajtar-Salwa R, Arif S, Sorysz D, Dudek D, Dubiel JS. Predictors of coronary and carotid atherosclerosis in patients with severe degenerative aortic stenosis. Int J Med Sci. 2013;10:1361-6.
27. Yeang C, Wilkinson MJ, Tsimikas S. Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. Curr Opin Cardiol. 2016;31:440-50.
28. Yu B, Hafiane A, Thanassoulis G, Ott L, Filwood N, Cerruti M, Gourgas O, Shum-Tim D, Al Kindi H, de Varennes B, Alsheikh-Ali A, Genest J, Schwertani A. Lipoprotein(a) Induces Human Aortic Valve Interstitial Cell Calcification. JACC Basic Transl Sci. 2017;2:358-371.
29. Freeman RV and Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. Circulation. 2005;111:3316-3326.
30. Mohty D, Pibarot P, Després JP, Côté C, Arsenault B, Cartier A, Cosnay P, Couture C, Mathieu P. Association between plasma LDL particle size, valvular accumulation of oxidized LDL, and inflammation in patients with aortic stenosis. Arterioscler Thromb Vasc Biol. 2008;28:187-93.
31. Koschinsky ML. Novel insights into Lp(a) physiology and pathogenicity: more questions than answers? Cardiovasc Hematol Disord Drug Targets. 2006;6:267-78.
32. Anuurad E, Boffa MB, Koschinsky ML, Berglund L. Lipoprotein(a): a unique risk factor for cardiovascular disease. Clin Lab Med. 2006;26:751-72.
33. Romagnuolo R, Marcovina SM, Boffa MB, Koschinsky ML. Inhibition of plasminogen activation by apo(a): role of carboxyl-terminal lysines and identification of inhibitory domains in apo(a). J Lipid Res. 2014;55:625-34.
34. Miles LA, Fless GM, Levin EG, Scanu AM, Plow EF. A potential basis for the thrombotic risks associated with lipoprotein(a). Nature. 1989;339:301-3.
35. Marcovina SM, Koschinsky ML. Evaluation of lipoprotein(a) as a prothrombotic factor: progress from bench to bedside. Curr Opin Lipidol. 2003;14:361-366.

36. Natorska J, Marek G, Hlawaty M, Sobczyk D, Sadowski J, Tracz W, Undas A. Evidence for tissue factor expression in aortic valves in patients with aortic stenosis. Pol Arch Med Wewn. 2009;119:636-43.
37. Natorska J, Wypasek E, Grudzień G, Sadowski J, Undas A. Impaired fibrinolysis is associated with the severity of aortic stenosis in humans. J Thromb Haemost. 2013;11:733-40.
38. Siudut J, Natorska J, Wypasek E, Wiewiórka Ł, Ostrowska-Kaim E, Wiśniowska-Śmiałek S, Plens K, Legutko J, Undas A. Impaired Fibrinolysis in Patients with Isolated Aortic Stenosis is Associated with Enhanced Oxidative Stress. J Clin Med. 2020;9:2002.
39. Siudut J, Natorska J, Wypasek E, Wiewiórka Ł, Ostrowska-Kaim E, Wiśniowska-Śmiałek S, Plens K, Musialek P, Legutko J, Undas A. Apolipoproteins and lipoprotein(a) as factors modulating fibrin clot properties in patients with severe aortic stenosis. Atherosclerosis. 2022;344:49-56.
40. Kamstrup PR, Hung MY, Witztum JL, Tsimikas S, Nordestgaard BG. Oxidized Phospholipids and Risk of Calcific Aortic Valve Disease: The Copenhagen General Population Study. Arterioscler Thromb Vasc Biol. 2017;37:1570-1578.
41. Vahanian A, Beyersdorf F, Praz F, Milojevic M, Baldus S, Bauersachs J, Capodanno D, Conradi L, De Bonis M, De Paulis R, Delgado V, Freemantle N, Gilard M, Haugaa KH, Jeppsson A, Jüni P, Pierard L, Prendergast BD, Sádaba JR, Tribouilloy C, Wojakowski W; ESC/EACTS Scientific Document Group. 2021 ESC/EACTS Guidelines for the management of valvular heart disease. Eur Heart J. 2022;43:561-632.
42. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014;37(Suppl 1):S81-90.
43. Maddox TM, Stanislawski MA, Grunwald GK, Bradley SM, Ho PM, Tsai TT, Patel MR, Sandhu A, Valle J, Magid DJ, Leon B, Bhatt DL, Fihn SD, Rumsfeld JS. Nonobstructive coronary artery disease and risk of myocardial infarction. JAMA. 2014;312:1754-63.
44. Kronenberg F, Mora S, Stroes ESG, Ference BA, Arsenault BJ, Berglund L, Dweck MR, Koschinsky M, Lambert G, Mach F, McNeal CJ, Moriarty PM, Natarajan P, Nordestgaard BG, Parhofer KG, Virani SS, von Eckardstein A, Watts GF, Stock JK, Ray KK, Tokgözoğlu LS, Catapano AL. Lipoprotein(a) in atherosclerotic cardiovascular disease and aortic stenosis: a European Atherosclerosis Society consensus statement. Eur Heart J. 2022;43:3925-3946.

45. Forbes JM, Soldatos G, Thomas MC. Below the radar: advanced glycation end products that detour "around the side". Is HbA1c not an accurate enough predictor of long term progression and glycaemic control in diabetes? Clin Biochem Rev. 2005;26:123-34.
46. Nowotny K, Jung T, Höhn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules. 2015;5:194-222.
47. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107: 1058-1070.
48. Khan MS, Tabrez S, Rabbani N, Shah A. Oxidative Stress Mediated Cytotoxicity of Glycated Albumin: Comparative Analysis of Glycation by Glucose Metabolites. J Fluoresc. 2015;25:1721-6.
49. Saku K, Tahara N, Takaseya T, Otsuka H, Takagi K, Shojima T, Shintani Y, Zaima Y, Kikusaki S, Fukuda T, Oryoji A, Nishino Y, Matsui T, Kakuma T, Akiba J, Fukumoto Y, Yamagishi SI, Tanaka H. Pathological Role of Receptor for Advanced Glycation End Products in Calcified Aortic Valve Stenosis. J Am Heart Assoc. 2020;9:e015261.
50. Hofmann B, Yakobus Y, Indrasari M, Nass N, Santos AN, Kraus FB, Silber RE, Simm A. RAGE influences the development of aortic valve stenosis in mice on a high fat diet. Exp Gerontol. 2014;59:13-20.
51. Li F, Cai Z, Chen F, Shi X, Zhang Q, Chen S, Shi J, Wang DW, Dong N. Pioglitazone attenuates progression of aortic valve calcification via down-regulating receptor for advanced glycation end products. Basic Res Cardiol. 2012;107:306.
52. Lv B, Wang H, Tang Y, Fan Z, Xiao X, Chen F. High-mobility group box 1 protein induces tissue factor expression in vascular endothelial cells via activation of NF-kappaB and Egr-1. Thromb Haemost. 2009;102:352-9.
53. Mayer O, Gelžinský J, Seidlerová J, Mateřánková M, Mareš Š, Svobodová V, Trefil L, Cífková R, Filipovský J. The role of advanced glycation end products in vascular aging: which parameter is the most suitable as a biomarker? J Hum Hypertens. 2021;35:240-249.
54. Testuz A, Nguyen V, Mathieu T, Kerneis C, Arangalage D, Kubota N, Codogno I, Tubiana S, Estellat C, Cimadevilla C, Vahanian A, Messika-Zeitoun D. Influence of metabolic syndrome and diabetes on progression of calcific aortic valve stenosis. Int J Cardiol. 2017;244:248-253.
55. Gee T, Farrar E, Wang Y, Wu B, Hsu K, Zhou B, Butcher J. NFκB (Nuclear Factor κ-Light-Chain Enhancer of Activated B Cells) Activity Regulates Cell-Type-Specific and Context-Specific Susceptibility to Calcification in the Aortic Valve. Arterioscler Thromb Vasc Biol. 2020;40:638-655.

56. Breyne J, Juthier F, Corseaux D, Marechaux S, Zawadzki C, Jeanpierre E, Ung A, Ennezat PV, Susen S, Van Belle E, Le Marec H, Vincentelli A, Le Tourneau T, Jude B. Atherosclerotic-like process in aortic stenosis: activation of the tissue factor-thrombin pathway and potential role through osteopontin alteration. Atherosclerosis. 2010;213:369-76.
57. Wypasek E, Natorska J, Mazur P, Kopytek M, Gawęda B, Kapusta P, Madeja J, Iwaniec T, Kapelak B, Undas A. Effects of rivaroxaban and dabigatran on local expression of coagulation and inflammatory factors within human aortic stenotic valves. Vascul Pharmacol. 2020;130:106679.
58. Miller JD, Chu Y, Brooks RM, Richenbacher WE, Peña-Silva R, Heistad DD. Dysregulation of Antioxidant Mechanisms Contributes to Increased Oxidative Stress in Calcific Aortic Valvular Stenosis in Humans. J. Am. Coll. Cardiol. 2008;52:843-850.
59. Wada S, Sugioka K, Naruko T, Kato Y, Shibata T, Inoue T, Inaba M, Ohsawa M, Yoshiyama M, Ueda M. Relationship between oxidative stress and aortic valve stenosis in humans: An immunohistochemical study. Osaka City Med. J. 2013;59:61-67.
60. Zheng KH, Tsimikas S, Pawade T, Kroon J, Jenkins WSA, Doris MK, White AC, Timmers NKLM, Hjortnaes J, Rogers MA, Aikawa E, Arsenault BJ, Witztum JL, Newby DE, Koschinsky ML, Fayad ZA, Stroes ESG, Boekholdt SM, Dweck MR. Lipoprotein(a) and Oxidized Phospholipids Promote Valve Calcification in Patients With Aortic Stenosis. J. Am. Coll. Cardiol. 2019;73:2150-2162.
61. Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM, Pibarot P. Calcific aortic stenosis. Nat Rev Dis Primers. 2016;2:16006.
62. Capoulade R, Chan KL, Yeang C, Mathieu P, Bossé Y, Dumesnil JG, Tam JW, Teo KK, Mahmut A, Yang X, et al. Oxidized phospholipids, lipoprotein(a), and progression of calcific aortic valve stenosis. J. Am. Coll. Cardiol. 2015;66:1236-1246.
63. Mourino-Alvarez L, Baldan-Martin M, Gonzalez-Calero L, Martinez-Laborde C, Sastre-Oliva T, Moreno-Luna R, Lopez-Almodovar LF, Sanchez PL, Fernandez-Aviles F, Vivanco F, Padial LR, Akerstrom F, Alvarez-Llamas G, de la Cuesta F, Barderas MG. Patients with calcific aortic stenosis exhibit systemic molecular evidence of ischemia, enhanced coagulation, oxidative stress and impaired cholesterol transport. Int J Cardiol. 2016;225:99-106.
64. Freigang S. The regulation of inflammation by oxidized phospholipids. Eur J Immunol. 2016;46:1818-1825.

65. Robert C, Bolon I, Gazzeri S, Veyrenc S, Brambilla C, Brambilla E. Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor progression. Clin Cancer Res. 1999;5:2094-102.
66. Rahman FA, Krause MP. PAI-1, the Plasminogen System, and Skeletal Muscle. Int J Mol Sci. 2020;21:7066.
67. Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. Cardiovasc Ther. 2010;28:e72-91.
68. Kaden JJ, Dempfle CE, Grobholz R, Fischer CS, Vocke DC, Kiliç R, Sarikoç A, Piñol R, Hagl S, Lang S, Brueckmann M, Borggrefe M. Inflammatory regulation of extracellular matrix remodeling in calcific aortic valve stenosis. Cardiovasc Pathol. 2005;14:80-7.
69. Kaden JJ, Dempfle CE, Grobholz R, Tran HT, Kiliç R, Sarikoç A, Brueckmann M, Vahl C, Hagl S, Haase KK, Borggrefe M. Interleukin-1 beta promotes matrix metalloproteinase expression and cell proliferation in calcific aortic valve stenosis. Atherosclerosis. 2003;170:205-11.
70. Eren M, Painter CA, Gleaves LA, Schoenhard JA, Atkinson JB, Brown NJ, Vaughan DE. Tissue- and agonist-specific regulation of human and murine plasminogen activator inhibitor-1 promoters in transgenic mice. J Thromb Haemost. 2003;1:2389-96.
71. Alexopoulos N, Katritsis D, Raggi P. Visceral adipose tissue as a source of inflammation and promoter of atherosclerosis. Atherosclerosis. 2014;233:104-12.
72. Kochtebane N, Choqueux C, Passefort S, Nataf P, Messika-Zeitoun D, Bartagi A, Michel JB, Anglés-Cano E, Jacob MP. Plasmin induces apoptosis of aortic valvular myofibroblasts. J Pathol. 2010;221:37-48.
73. Yasui H, Suzuki Y, Sano H, Suda T, Chida K, Dan T, Miyata T, Urano T. TM5275 prolongs secreted tissue plasminogen activator retention and enhances fibrinolysis on vascular endothelial cells. Thromb Res. 2013;13:100-5.
74. Ramadass V, Vaiyapuri T, Tergaonkar V. Small Molecule NF-κB Pathway Inhibitors in Clinic. Int J Mol Sci. 2020;21:5164.
75. Ahmad S, Shahab U, Baig MH, Khan MS, Khan MS, Srivastava AK, Saeed M, Moinuddin. Inhibitory effect of metformin and pyridoxamine in the formation of early, intermediate and advanced glycation end-products. PLoS One. 2013;8:e72128.
76. Yamagishi S, Matsui T. Soluble form of a receptor for advanced glycation end products (sRAGE) as a biomarker. Front Biosci. 2010;2:1184-95.

77. Cowell SJ, Newby DE, Prescott RJ, Bloomfield P, Reid J, Northridge DB, Boon NA; Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression (SALTIRE) Investigators. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. N Engl J Med. 2005;352:2389-97.
78. Chan KL, Teo K, Dumesnil JG, Ni A, Tam J; ASTRONOMER Investigators. Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. Circulation. 2010;121:306-14.
79. Capoulade R, Yeang C, Chan KL, Pibarot P, Tsimikas S. Association of mild to moderate aortic valve stenosis progression with higher lipoprotein(a) and oxidized phospholipid levels: secondary analysis of a randomized clinical trial. JAMA Cardiol. 2018;3:1212-1217.
80. Mazur P, Kopytek M, Ząbczyk M, Undas A, Natorska J. Towards Personalized Therapy of Aortic Stenosis. J Pers Med. 2021;11:1292.
81. Natorska J. Diabetes mellitus as a risk factor for aortic stenosis: from new mechanisms to clinical implications. Kardiol Pol. 2021;79:1060-1067.
82. Di Lullo L, Tripepi G, Ronco C, D'Arrigo G, Barbera V, Russo D, Di Iorio BR, Uguccioni M, Paoletti E, Ravera M, Fusaro M, Bellasi A. Cardiac valve calcification and use of anticoagulants: Preliminary observation of a potentially modifiable risk factor. Int J Cardiol. 2019;278:243-249.

7. ARTYKUŁY I OŚWIADCZENIA WSPÓŁAUTORÓW

Cardiovascular Diabetology

ORIGINAL INVESTIGATION

Open Access

Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes

Magdalena Kopytek[1,2], Michał Ząbczyk[1,2], Piotr Mazur[1,2], Anetta Undas[1,2] and Joanna Natorska[1,2*]

Abstract

Background: Accumulation of advanced glycation end products (AGEs) leads to chronic glycation of proteins and tissue damage, particularly in patients with diabetes mellitus (DM). We aimed to evaluate whether increased accumulation of AGEs in patients with aortic stenosis (AS) and concomitant type 2 diabetes (DM) is associated with AS severity.

Methods: We prospectively enrolled 76 patients with severe AS (47.1% males; nonDM), aged 68 [66–72] years, and 50 age-matched DM patients with a median blood glucose level of 7.5 [5.9–9.1] mM and glycated hemoglobin (HbA1c) of 6.8 [6.3–7.8]%, scheduled for aortic valve replacement. Valvular expression of AGEs, AGEs receptor (RAGE), interleukin-6 (IL-6), and reactive oxygen species (ROS) induction were evaluated ex vivo by immunostaining and calculated as the extent of positive immunoreactive areas/total sample area. Plasma levels of AGEs and soluble RAGE (sRAGE) were assessed by ELISAs.

Results: Subjects with DM had increased valvular expression of both AGEs (6.6-fold higher, 15.53 [9.96–23.28]%) and RAGE (1.8-fold higher, 6.8 [4.9–8.45]%) compared to nonDM patients (2.05 [1.21–2.58]% and 2.4 [1.56–3.02]%, respectively; both $p < 0.001$). Plasma levels of AGEs (12-fold higher) and sRAGE (1.3-fold higher) were elevated in DM patients, compared to nonDM (both $p < 0.0001$). The percentage of valvular ROS-positive (2.28 [1.6–3.09] vs. 1.15 [0.94–1.4]%, $p < 0.0001$) but not IL-6-positive areas was higher within DM, compared to nonDM valves. In DM patients, the percentage of valvular AGEs- and RAGE-positive areas correlated with HbA1c ($r = 0.77$, $p < 0.0001$ and $r = 0.30$, $p = 0.034$). Similarly, plasma AGEs and sRAGE levels were associated with HbA1c in the DM group ($r = 0.32$, $p = 0.024$ and $r = 0.33$, $p = 0.014$, respectively). In all DM patients, we found an association between the amount of valvular AGEs and the disease severity measured as aortic valve area (AVA; $r = 0.68$, $p < 0.0001$). Additionally, in DM patients with HbA1c > 7% ($n = 24$, 48%) we found that valvular expression of AGEs correlated with mean transvalvular pressure gradient (PG_{mean}; $r = 0.45$, $p = 0.027$). Plasma AGEs levels in the whole DM group correlated with AVA ($r = -0.32$, $p = 0.02$), PG_{mean} ($r = 0.31$, $p = 0.023$), and PG_{max} ($r = 0.30$, $p = 0.03$).

Conclusions: Our study suggests that poorly-controlled diabetes leads to increased AGEs and RAGE valvular accumulation, which at least partially, might result in AS progression in DM patients.

*Correspondence: j.natorska@szpitaljp2.krakow.pl
[2] Institute of Cardiology, Jagiellonian University Medical College, 80 Pradnicka St, 31-202 Kraków, Poland
Full list of author information is available at the end of the article

Keywords: Aortic stenosis (AS), Diabetes mellitus (DM), Advanced glycation end products (AGEs), Inflammation, Oxidative stress

Background

Aortic stenosis (AS) is the most common cause of acquired valvular heart disease in the adult population, with 2–3% prevalence for individuals older than 65 years in developed countries [1]. AS is initiated as aortic valve sclerosis, associated with a mild valve thickening. Histopathologic heterogeneity of AS indicates the involvement of cell dependent mechanisms that regulate calcium load in the valve leaflets, as well as the participation of different cell types, including valvular interstitial cells (VICs), endothelial cells and cardiac leukocytes [2]. Under pathological conditions, such as inflammation or oxidative stress VICs can differentiate into myofibroblasts (causing fibrosis) and osteoblast-like cells (causing calcification) [2]. Diabetes mellitus (DM), a known cardiovascular risk factor, has also been reported as a risk factor for AS progression [3]. In patients with AS and concomitant type 2 DM reduced systemic arterial compliance and left ventricular dysfunction at the midwall level, corresponding to slightly depressed myocardial contractility have been shown [4]. Chronic hyperglycemia is a common feature of DM and has been related to vascular and inflammatory cell interactions with advanced glycation end products (AGEs) [5]. Advanced glycation is the irreversible attachment of reducing sugars to the free amino groups of proteins. Conditions such as DM rapidly accelerate AGE formation [6]. It has been shown that AGEs accumulation plays a key role in development of vascular calcification in DM patients [7–9]. AGEs, which are a heterogeneous group of molecules generated through non-enzymatic glycation and oxidation of proteins such as collagen, elastin and laminin [10–12], lipids, and nucleic acids [10] can alter tissue function and its mechanical properties [10–12]. Interestingly, it has been shown that chronic high dietary AGEs lead to increased arterial stiffness with subsequent elevation of systolic blood pressure and inflammatory activation, which may lead to the development of vascular complications in type 2 DM [11]. AGEs modulate multiple cellular processes through the cross-linking of intracellular and extracellular matrix proteins [12] or binding to their cell surface receptor (RAGE), which produces excesses in inflammatory molecule production [13, 14]. It has been shown that elevated circulating soluble RAGE (sRAGE) levels are associated with the presence of bicuspid aortic valve and associated aortopathies, independently of aortic diameter [15]. Moreover, Hofmann et al. [16] have shown in model using RAGE knock-out mice that both AGEs and RAGE are involved in the aortic leaflets calcification and subsequent AS. Low levels of both sRAGE and endogenous secretory receptor for RAGE (esRAGE), which are considered to be protective against AGEs, were shown as a very early marker of initial target organ damage in mild hypertensives [17] or a factor associated with negative coronary artery remodeling in diabetics [18]. Moreover, the concentration of sRAGE has been associated with increased arterial wall stiffening over time [19]. Despite numerous reports on AGE and RAGE involvement in the pathogenesis of cardiovascular diseases little is known about mechanisms by which hyperglycemia-related increase of AGEs and RAGE affects inflammation and calcification within aortic stenotic valves. We aimed to investigate whether in AS patients with concomitant DM, the severity of AS is associated with enhanced accumulation of both valvular and plasma AGEs and RAGE.

Methods

Study subjects

We enrolled 126 patients with isolated AS on tricuspid aortic valve (nonDM group), including 50 AS patients with concomitant type 2 DM (DM group). Patients were hospitalized between August 2016 and February 2019 in the Department of Cardiovascular Surgery and Transplantology at the John Paul II Hospital, Krakow, Poland. Severe AS was defined by a mean transvalvular gradient (PG_{mean}) ≥ 40 mmHg and/or aortic valve area (AVA) < 1 cm^2, based on transthoracic echocardiography, performed by an experienced cardiologist on a Toshiba APLIO 80 (Toshiba, Tokyo, Japan) ultrasound machine. All patients were scheduled to undergo aortic valve replacement, following the Heart Team indication. Left ventricular ejection fraction (LVEF) was routinely measured during echocardiography. The diagnosis of atherosclerosis was based on angiographically documented coronary artery stenosis > 20% diameter. Patients classified as having DM had to be diagnosed preoperatively based on fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL) on two separate occasions, HbA1c equal or exceeding 6.5% (48 mmol/mol) or oral glucose tolerance test of 140 mg/dL (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) according to The International Expert Committee [20] and only individuals receiving insulin or oral hypoglycemic agents preoperatively were included in this study. Hypercholesterolemia was diagnosed based on medical records, cholesterol-lowering therapy or total

cholesterol of 5.0 mmol/L or more. Arterial hypertension was diagnosed based on a history of hypertension (blood pressure > 140/90 mmHg) or preadmission antihypertensive treatment. Smoking was defined as the use of one or more cigarettes per day. The exclusion criteria were: angiographically documented coronary artery stenosis > 20% diameter, rheumatic AS, acute infection, diagnosed malignancy, endocarditis, chronic kidney disease, any previous heart surgeries, acute cardiovascular event in the last 3 months before inclusion, any concomitant valvular surgery and pregnancy. The valvular anatomy was confirmed intraoperatively by a cardiac surgeon, and bicuspid valve was used as an exclusion criterion. The Local Ethical Committee in Krakow approved the study and participants provided informed consent in accordance with the Declaration of Helsinki.

Laboratory analysis

At the day of valve replacement, after an overnight fast venous blood was drawn between 7:00 and 9:00 AM from the antecubital vein into citrated (9:1 of 0.106 M sodium citrate) or EDTA (both SARSTEDT, Nümbrecht, Germany) tubes. Blood was centrifuged at 2500*g* at 20 °C for 20 min and stored in aliquots at − 80 °C until analysis. Blood drawn into serum tubes was centrifuged at 1600*g* at 4 °C for 10 min and stored at − 80 °C. To determine lipid profile, glucose and creatinine in serum, routine laboratory assays were used. Serum high-sensitivity C-reactive protein (hsCRP) was determined using immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Fibrinogen was measured by the von Clauss method in citrated plasma (Instrumentation Laboratory, Bedford, MA, USA). Glycated hemoglobin HbA1c was determined using turbidimetric inhibition immunoassay TINIA in hemolysate prepared from whole blood (Roche Diagnostics, Mannheim, Germany). Serum fructosamine levels were measured using a colorimetric assay (Roche Diagnostics, Risch-Rotkreuz, Switzerland).

Enzyme-linked immunosorbent assay (ELISA)

Human advanced glycation end products (AGEs; all species with the predominance of N(6)-Carboxymethyllysine) and circulating soluble human receptor for advanced glycation end products (sRAGE) concentrations in EDTA plasma samples were assayed quantitatively using commercial ELISA kits (EIAab, Wuhan, China, no. E0263h and Boster Biological Technology, California, USA, no. EK0827, respectively) in accordance with manufacturer's instructions. Serum insulin levels were assessed using Insulin Human ELISA Kit (Invitrogen —Thermo Fisher Scientific, Waltham, MA, USA).

Aortic valves preparation

Valve samples embedded in Tissue Tec-OCT compound (Sakura, Torrance, CA, USA) were cryosectioned (4.5 µm thick sections) onto SuperFrost slides (Menzel-Glaser, Braunschweig, Germany) by a Leica CM 1520 cryostat. Transverse sections were taken from the mid and commissural areas of the leaflet and stored at − 20 °C until immunostaining.

Immunofluorescence analysis

Sections were fixed in ice-cold methanol-acetone (1:1) mixture. After endogenous peroxidase activity quenching with 3% H_2O_2 (15 min) at room temperature (RT) and blocking of unspecific background with 3% bovine albumin (BSA, Sigma Co, St. Louis, MO, USA) at RT in dark (30 min). Primary adequate antibodies against AGE (1:500; no. ab23722), tissue RAGE (1:100; no. ab3611), interleukin-6 (IL-6, 1:800; no. ab6672), and reactive oxygen species (ROS) modulator 1 (ROMO1, indirectly evaluating ROS production; 1:100; no. ab121379) (all from Abcam, Cambridge, UK) were applied overnight at 4 °C. Primary antibodies were followed by the corresponding secondary goat or mouse antibodies conjugated with fluorochrome AlexaFluor 488 or AlexaFluor 594 (Abcam, Cambridge, UK) (1:1000) at 4 °C for 1 h in dark. Double-label immunofluorescence analyses were performed using the same antibodies. The co-localization of AGE and RAGE was performed. A negative control (without primary antibody incubation) was included routinely to all staining. Images were analyzed using Olympus BX 43 microscope equipped with software (Cell Sense Standard, version 11.0.06). Per each valve 30 images were taken and the percentage of positively-stained areas was calculated as the extent of positive immunoreactive areas/total sample area as follows [21]:

$$\frac{\Sigma \ of \ immunoreactive \ areas}{Total \ section \ area}$$

The fluorescence intensity (FI) was computed as the ratio of fluorescence of positively and negatively stained areas. The investigators analyzing the data were blinded to the study groups. The ratio of valvular AGEs and sRAGE (AGEs/sRAGE) has also been calculated [22].

Statistical analysis

All statistical analysis were performed using STATISTICA Version 12.5 (StatSoft STATISTICA™, Poland) program. Categorical variables were presented as numbers and percentages and were analyzed by Pearson's χ^2 or two-tailed Fisher's exact test. Continuous variables

were expressed as mean ± standard deviation (SD) or median and interquartile range [IQR]. Normality was analyzed by the Shapiro–Wilk test. The Student's test was used for normally distributed continuous variables. Differences between groups were compared using the Mann–Whitney U test for non-normally distributed continuous variables. Associations between nonparametric variables were assessed by Spearman's tests. The multivariate analyses were performed using linear regression models adjusted for potential confounders such as age, hypertension, hypercholesterolemia, and the use of statin and/or angiotensin-converting enzyme inhibitors. P-values of < 0.05 were considered as statistically significant. The study was powered to have a 90% chance of detecting a 15% difference in valvular expression of different factors using a p-value of 0.01, based on the previous study [14]. In order to demonstrate such a difference or grater, the group size is equal to 44 patients, or more is required in each group.

Results

DM patients did not differ from the nonDM group with regard to demographic factors, but were more frequently obese (BMI > 30 kg/m^2, Table 1). More DM

Table 1 Characteristics of patients with aortic stenosis with or without concomitant diabetes mellitus (DM)

Variable	NonDM patients (n = 76)	DM patients (n = 50)	p-value
Age, years	68 [66–72]	70 [66–74]	0.18
Male, n (%)	41 (53.9)	31 (62)	0.37
BMI, kg/m^2	28.4 [26.6–31.2]	31.3 [28.7–34.5]	< 0.001
Risk factors, n (%)			
Arterial hypertension	70 (92.1)	50 (100)	0.08
Hypercholesterolemia	20 (26.3)	10 (20)	0.52
Current smoking	9 (11.8)	8 (16)	0.60
Medications, n (%)			
Beta-blockers	61 (80.3)	47 (94)	0.038
Acetylsalicylic acid	52 (68.4)	40 (80)	0.22
ACE inhibitors	53 (69.7)	45 (90)	0.008
Statins	56 (73.7)	46 (92)	0.011
Insulin	0	14 (28)	< 0.0001
Metformin	0	36 (72)	< 0.0001
Echocardiographic parameters			
Mean gradient, mmHg	48 [42–59]	52 [43–66]	0.067
Maximum gradient, mmHg	82.3 ± 14.2	89.2 ± 12.3	0.054
LVEF, %	60 [55–65]	60 [58–64]	0.39
AVA, cm^2	0.86 [0.7–0.95]	0.78 [0.6–0.8]	0.048
Laboratory investigation			
Fibrinogen, g/L	3.4 ± 0.8	3.6 ± 0.6	0.28
Creatinine, µmol/L	74 [65–89]	81 [74–100]	0.011
hsCRP, mg/L	1.2 [1.0–4.0]	1.0 [1.0–2.0]	0.34
Glucose, mmol/L	5.2 [4.9–5.6]	7.5 [5.9–9.1]	< 0.0001
HbA1c, %	5.4 [5.2–5.7]	6.8 [6.3–7.8]	< 0.0001
Insulin, µIU/mL	16.5 [13.1–19.9]	16.4 [12.3–23.4]	0.41
Fructosamine, µmol/L	225 [217–236]	262 [241–291]	< 0.0001
TC, mmol/L	4.1 [3.7–4.8]	3.8 [3.0–4.6]	0.06
LDL-C, mmol/L	2.6 [2.1–3.3]	2.3 [1.5–3.1]	0.17
HDL-C, mmol/L	1.3 [1.1–1.6]	1.2 [1.0–1.4]	0.039
Triglycerides, mmol/L	1.2 [0.9–1.8]	1.5 [1.2–2.0]	0.27

Data presented as numbers (percentages), mean ± SD or medians [interquartile range]. Categorical variables were analyzed by the Chi square test. The Mann–Whitney U or Student tests were used to compare differences between groups

AS aortic stenosis, *DM* type 2 diabetes mellitus, *BMI* body mass index, *ACE inhibitors* angiotensin converting enzyme inhibitors, *LVEF* left ventricular ejection fraction, *AVA* aortic valve area, *hsCRP* high-sensitivity C-reactive protein, *HbA1c* glycated hemoglobin, *TC* total cholesterol, *LDL-C* low density lipoprotein cholesterol, *HDL-C* high density lipoprotein cholesterol

patients used statins, beta-blockers and angiotensin converting enzyme (ACE) inhibitors compared to subjects from the nonDM group (all $p < 0.05$; Table 1). The median time of DM duration was 11 [7–18] years, and DM patients had 44.2% higher glucose, 25.9% higher HbA1c, and 16.4% higher fructosamine levels compared to nonDM subjects (all $p < 0.05$; Table 1). DM patients were also characterized by slightly poorer renal function (Table 1). No intergroup differences were found in other laboratory parameters (Table 1). Among DM patients, as many as 24 (48%) individuals had poorly controlled diabetes, defined as HbA1c > 7% (Table 2).

Valvular expression of AGEs and RAGE

The thickness of the aortic valve leaflets was greater in DM patients compared to nonDM valves (1926.32 ± 197.85 μm vs. 1298.32 ± 183.37 μm, $p = 0.017$) (Fig. 1a, b).

Valvular expression of AGEs and RAGE was mostly detected at the aortic site of the leaflets and presented a diffused pattern of fluorescence for AGEs, whereas RAGE expression was more condensed and located mainly on the edge of the leaflets (Fig. 1c–f). Valvular AGE expression was 6.6-fold higher in DM compared to nonDM patients (15.53 [9.96–23.28]% vs. 2.05 [1.21–2.85]%, $p < 0.001$) (Fig. 1c, d). Similarly, in the DM group we found 1.8-fold higher expression of valvular

Table 2 Comparison of AS patients with concomitant type 2 DM stratified according to glycated hemoglobin levels (HbA1c)

Variable	Patients with DM and HbA1c > 7% (n = 24)	DM and HbA1c ≤ 7% (n = 26)	p-value
Age, years	74 [70–76]	70 [66–73]	0.12
Male, n (%)	13 (54.2)	18 (69.3)	0.38
BMI, kg/m^2	30.4 [26.6–31.2]	32.2 [28.7–34.5]	0.30
Risk factors, n (%)			
Arterial hypertension	24 (100)	26 (100)	0.99
Hypercholesterolaemia	7 (29.2)	3 (11.5)	0.16
Current smoking	2 (8.3)	6 (23.1)	0.25
Medications, n (%)			
Beta-blockers	21 (87.5)	26 (100)	0.10
Acetylsalicylic acid	19 (79.2)	21 (80.8)	0.99
ACE inhibitors	22 (91.7)	23 (88.5)	0.99
Statins	21 (87.5)	25 (96.2)	0.34
Insulin	14 (58.3)	0	< 0.0001
Metformin	10 (41.7)	26 (100)	< 0.0001
Echocardiographic parameters			
Mean gradient, mmHg	61 [46–67]	44 [41–50]	0.003
Maximum gradient, mmHg	95 [72–109]	64 [58–77]	<0.00001
LVEF, %	60 [55–65]	60 [58–64]	0.98
AVA, cm^2	0.65 [0.56–0.80]	0.85 [0.8–0.9]	< 0.0001
Laboratory investigation			
Creatinine, μmol/L	91 [88–101]	79 [73–99]	0.19
hsCRP, mg/L	1.0 [1.0–2.0]	1.0 [1.0–2.0]	0.53
Glucose, mmol/L	6.8 [6.2–8.4]	6.4 [5.5–7.5]	0.21
Insulin, μIU/mL	16.2 [12.4–23.3]	16.3 [12.4–23.4]	0.89
Fructosamine, μmol/L	271 [250–304]	259 [231–266]	0.04
Valvular AGEs, %	23.3 [17.5–28.1]	10.8 [9.3–13.5]	< 0.00001
Valvular RAGE, %	8.1 [6.6–8.5]	5.7 [4.8–7.7]	0.015
Plasma AGEs, ng/mL	9.3 [8.5–12.0]	9.6 [8.6–10.4]	0.55
Plasma sRAGE, pg/mL	1977 [1596–2455]	1988 [1517–2613]	0.70

Data presented as numbers (percentages), mean ± SD or medians [interquartile range]. Categorical variables were analyzed by the Chi square test. The Mann–Whitney U or Student tests were used to compare differences between groups

AGEs advanced glycation end products, *RAGE* receptor for AGEs, for abbreviations see Table 1

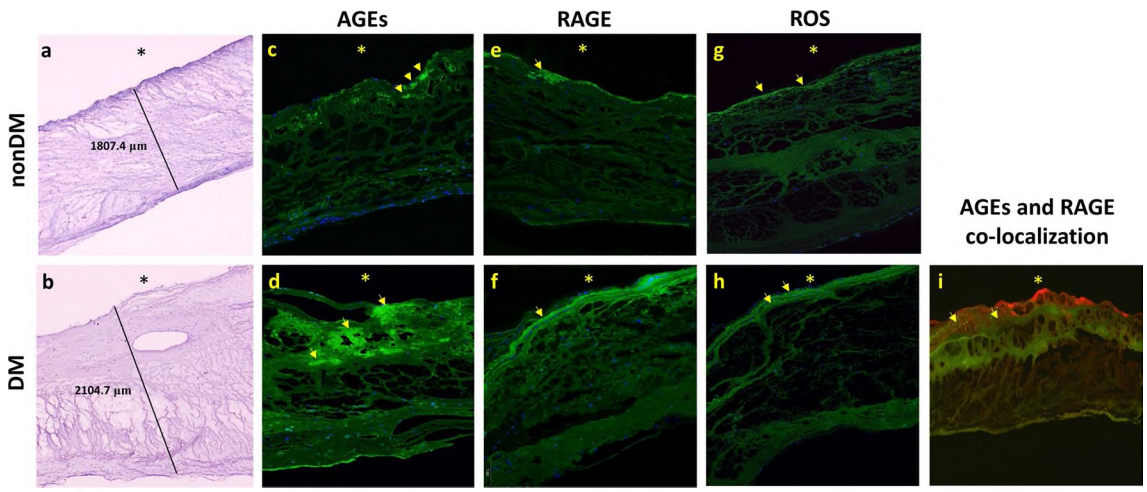


Fig. 1 Expression of AGEs and RAGE within aortic stenotic valves. Representative microphotographs of aortic stenotic valves dissected from patients with aortic stenosis (nonDM) or aortic stenosis with concomitant type 2 diabetes mellitus (DM). **a**, **b** The thickness of the leaflets stained with hematoxylin, **c**, **d** the valvular expression of advanced glycation end products (AGEs) and **e**, **f** their receptor (RAGE), **g**, **h** the expression of reactive oxygen species (ROS) modulator 1 (indirect staining for ROS), and **i** co-localization of AGEs (green) and RAGE (red). Cell nuclei are stained in blue (DAPI). *Aortic site of the leaflet. Original magnification ×40

RAGE than in the nonDM group (6.8 [4.9–8.45]% vs. 2.4 [1.56–3.02]%, $p < 0.001$) (Fig. 1e, f). Of note, AGEs and RAGE showed co-expression in 51.5% of positively stained areas (Fig. 1i). DM compared to nonDM valves were characterized by higher ROS-positive area (2.28 [1.6–3.09]% vs 1.15 [0.94–1.4]%; $p < 0.0001$; Fig. 1g, h). The valvular expression of IL-6 tended to be increased in DM compared to nonDM patients (1.57 [0.96–2.15]% vs. 1.18 [0.96–1.8]%; $p = 0.062$). There were no associations between valvular ROS or IL-6 expression and echo parameters in both patient groups.

In DM patients the amount of valvular AGEs was positively associated with HbA1c and fructosamine levels (Fig. 2a, b). The valvular RAGE correlated positively with HbA1c (Fig. 2c) but not with plasma glucose, insulin or fructosamine levels (data not shown). In DM, but not in nonDM patients, we found negative association between the amount of valvular AGEs and aortic valve area (AVA; Fig. 2d), even after adjustment for potential confounders (Table 3). Similar association was not observed for valvular RAGE (Table 3). Furthermore, in DM patients with HbA1c > 7% (n = 24, 48%), we observed 1.2-fold higher expression of AGEs compared to those with HbA1c ≤ 7% (Table 2; Fig. 2e) which correlated with PG_{mean} (Fig. 2f).

Plasma levels of AGEs and sRAGE

DM compared to nonDM patients had 12-fold higher plasma concentrations of AGEs (9.55 [8.56–10.92] vs. 0.73 [0.68–0.77] ng/mL; $p < 0.0001$) and 1.3-fold higher plasma sRAGE levels (1982 [1517–2613] vs. 858 [648–971] pg/mL; $p < 0.0001$). Of note, in the DM group plasma AGEs correlated with sRAGE levels ($r = 0.61$, $p < 0.0001$) and plasma concentrations of AGEs and sRAGE were positively associated with HbA1c levels (Fig. 3a, b). Importantly, in the DM group, plasma AGEs levels positively correlated with fructosamine (Fig. 3c). In DM, but not in nonDM patients, HbA1c was associated with fructosamine levels ($r = 0.54$, $p < 0.0001$). Associations of plasma AGEs and sRAGE levels with HbA1c or fructosamine were not observed in the nonDM group (data not shown).

Moreover, in the DM group, plasma levels of AGEs correlated positively with valvular AGEs expression ($r = 0.67$, $p = 0.037$) but valvular RAGE was not associated with sRAGE ($p > 0.05$).

There were no associations between echo parameters and HbA1c or glucose levels in the whole group of DM subjects. However, DM patients with HbA1c > 7.0% compared to those with HbA1c ≤ 7.0% were characterized by higher PG_{max} and PG_{mean} (Table 2; Fig. 4a).

In DM patients plasma levels of AGEs and sRAGE were associated with the disease severity reflected by AVA (Fig. 4b, c), even after adjustment for defined confounders (Table 3). Moreover, plasma AGEs (but not sRAGE) levels correlated positively with PG_{mean} (Fig. 4d), while PG_{max} was associated with both AGE and sRAGE plasma levels (Fig. 4e, f). However, associations of transvalvular gradients with plasma AGEs and sRAGE were no more significant after adjustment for defined confounders (Table 3).

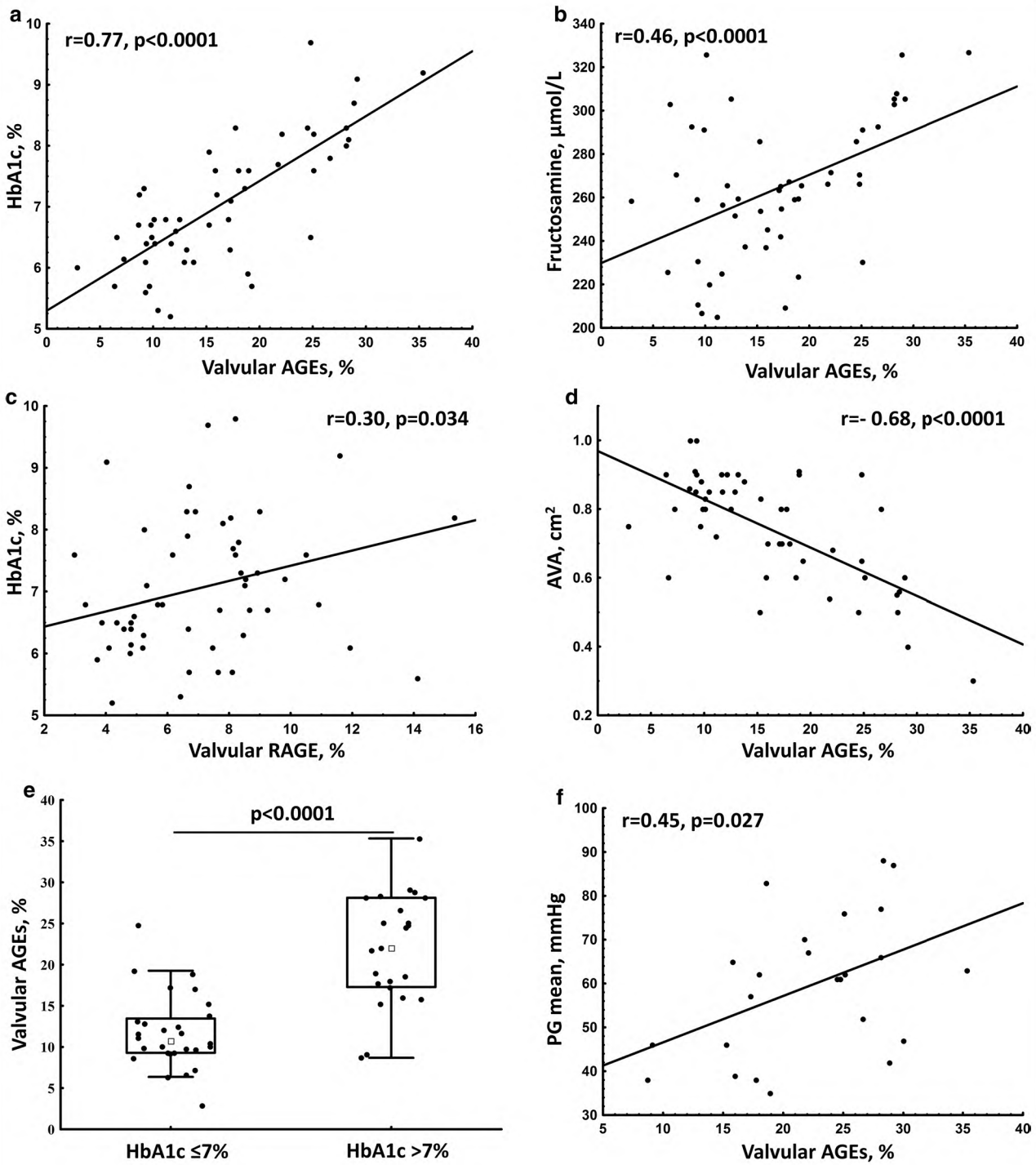


Fig. 2 Associations of valvular AGEs and RAGE with glycation markers and echocardiographic parameters in patients with AS and concomitant DM. Associations of: **a** the expression of valvular advanced glycation end products (AGEs) with glycated hemoglobin (HbA1c), **b** valvular AGEs with serum fructosamine, **c** valvular AGEs receptor (RAGE) with HbA1c, **d** valvular AGEs with aortic valve area (AVA), **e** expression of valvular AGEs stratified according to elevated HbA1c levels, and **f** valvular AGEs with mean transvalvular pressure gradient (PG)

Table 3 Multivariate associations between echocardiographic parameters and valvular or plasma AGEs and RAGE levels

Variable	AVA, estimate (95% CI)	p-value	Mean gradient, estimate (95% CI)	p-value	Maximum gradient, estimate (95% CI)	p-value
Valvular AGEs, %	−0.29 (−0.10; −0.47)	0.0035	0.24 (0.05; 0.43)	0.014	0.34 (0.15; 0.52)	0.0005
Valvular RAGE, %	−0.13 (−0.32; 0.066)	0.19	0.082 (−0.11; 0.28)	0.41	0.09 (−0.11; 0.29)	0.36
Plasma AGEs, ng/mL	−0.20 (−0.0041; −0.39)	0.045	0.13 (−0.069; 0.32)	0.20	0.04 (−0.15; 0.24)	0.66
Plasma sRAGE, pg/mL	−0.26 (−0.063; −0.45)	0.01	0.13 (−0.07; 0.33)	0.20	0.18 (−0.16; 0.38)	0.071
Valvular AGEs/sRAGE ratio	−0.19 (0.007; −0.39)	0.058	0.18 (−0.02; 0.38)	0.078	0.19 (−0.008; 0.39)	0.059

All models were adjusted for age, hypertension, hypercholesterolemia, the use of statin, and angiotensin-converting enzyme inhibitors. *CI* confidence interval; for other abbreviations see Tables 1 and 2

Ratio of valvular AGEs and plasma sRAGE levels (AGEs/sRAGE)

We found significant associations between the AGEs/sRAGE ratio and glucose levels ($r=0.36$, $p=0.077$), HbA1c ($r=0.46$, $p=0.0004$) as well as with echocardiographic parameters such as AVA ($r=-0.41$, $p=0.0033$), PG_{mean} ($r=0.32$, $p=0.019$), PG_{max} ($r=0.37$, $p=0.007$). However, after adjustment for defined confounders all estimates showed only a tendency to be significant (Table 3).

Discussion

To the best of our knowledge, this study is the first to show that: (1) patients with AS and concomitant DM compared to nonDM patients with tricuspid aortic valves, demonstrate abundant valvular accumulation of AGEs and RAGE associated with AS severity. Interestingly, poorly controlled DM was associated with the highest valvular AGEs expression; (2) the expression of valvular AGEs corresponded to AGEs level in plasma but this association held no true for valvular RAGE and sRAGE, and (3) plasma levels of AGEs and sRAGE were correlated with AVA but not with transvalvular pressure gradients.

AGEs involvement in AS

Our results showing that enhanced valvular expression of AGEs associated with AS severity in AS patients with concomitant DM are in line with studies performed in mice, in which AGEs accumulation resulted in aortic valve degeneration [16]. Previously, we have shown that diabetes is associated with increased valvular inflammation, measured by valvular CRP expression [23]. The present study provides the initial evidence for a pro-inflammatory effect of valvular AGEs accumulation. Despite the fact that AGEs are accumulated as a result of hyperglycemia, their effects can occur independently of glycemic control and their levels better predicted both DM progression and vascular calcification than HbA1c [24]. Although glycemic control is important, it is not sufficient to prevent complications of diabetes. Other factors, such as oxidative stress, may be more important mediators of advanced glycation than hyperglycemia per se in patients already receiving interventions directed to improve glycemic control [6]. On the other hand, our study provided additional data that both biomarkers of long-term glycemic control (HbA1c and fructosamine) show associations with plasma and valvular AGEs. Arguably, they may give insight into disease course in AS patients with concomitant DM, especially in those with mild-to-moderate AS. However, further studies are needed to explore this issue.

It is known that AGEs accumulation in the extracellular matrix of vessels leads to structural and functional changes of collagen and subsequent cardiovascular complications [22]. Therefore, it might be hypothesized that more severe AS observed in patients with concomitant DM is a result of AGE-related valvular collagen cross-linking leading to enhanced inflammation, oxidative stress, and calcification of the leaflets. Studying a cohort with confirmed normal-flow high-gradient AS, we observed an association between valvular AGEs expression and AVA in DM. This observation was further corroborated by correlation of valvular AGEs expression and mean transvalvular pressure gradient. Considered the fact that also plasma AGEs levels were associated with AVA, it becomes evident that AGEs have multilevel interrelation with AS severity in diabetics, even at the very late stage of the disease, where surgical intervention is inevitable.

Our novel observation is that poorly controlled DM was associated with the highest valvular AGEs expression, which correlated not only with AVA but also with transvalvular pressure gradients. The influence of diabetes on AS progression has been previously studied. Katz et al. [7] have shown that the metabolic syndrome and DM are associated with increased risk of valvular calcification, and AS prevalence is associated with increasing number of metabolic syndrome components. Kamalesh et al. [25] have shown an influence of diabetes on AS progression in patients with mild AS,

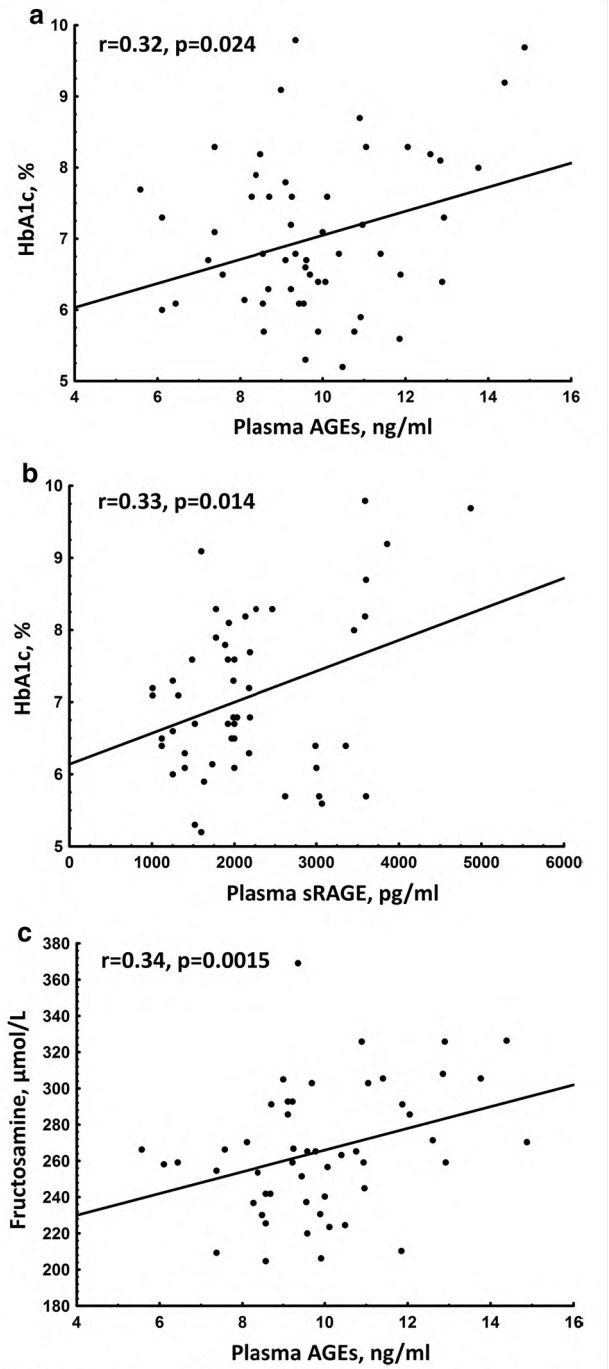


Fig. 3 Associations of plasma AGEs and RAGE with glycation markers in patients with AS and concomitant DM. Associations of: **a** plasma levels of advanced glycation end products (AGEs) with glycated hemoglobin (HbA1c), **b** plasma levels of AGE receptor (RAGE) with HbA1c, **c** plasma AGEs with serum fructosamine levels

but not in those with heavily calcified stenotic valves. They speculated that once the valve is calcified, the diabetes-driven inflammation may play less of a role in AS progression than in a valve with mild lesions. Recently, Larsson et al. in a prospective cohort study comprising > 70,000 individuals have confirmed that type 2 DM is associated with an increased risk of AS (hazard ratio: 1.34; 95% confidence interval 1.05–1.71) [26]. On the contrary, Testuz et al. [27] failed to demonstrate an association between AS progression and metabolic syndrome or diabetes during 3-years follow-up. However, the authors evaluated only levels of fasting glucose, reflecting short-term glucose dynamics. Neither the levels of HbA1c, fructosamine nor AGEs in their cohort were assessed [27], while our study showed that in AS patients with well-controlled type 2 DM an influence of hyperglycemia on AS severity is minor.

RAGE involvement in AS

Despite the role of RAGE in vascular calcification has been previously demonstrated in animal models [28, 29], our study showed that RAGE in contrast to AGEs is poorly associated with AS severity measures. Moreover, we found no associations between AGE/sRAGE ratio and AS severity after adjustment for potential confounders, while this ratio has been suggested as a more sensitive marker than single factors [22, 30]. These discrepancies may be connected with the fact that there are different species of RAGE, including the membrane RAGE, responsible for the harmful effects of AGEs and circulating RAGE isoforms which are protective against AGEs due to competing with the tissue RAGE for binding of AGEs [31]. Thus, this dual nature of RAGE, together with an increased consumption of AGE by sRAGE in subjects with impaired glucose metabolism [32] may disturb a direct associations between RAGE and parameters describing AS severity. Moreover, we showed relatively high levels of sRAGE observed in DM compared to nonDM patients, while there are studies reporting decreased, increased or unchanged sRAGE levels in DM patients compared to subjects without DM [33–35]. This difference may be explained by a presence of several confounding factors, with major impact of DM duration. It has been shown that the longer duration of DM the higher AGEs generation and AGE-stimulated increased RAGE expression [36]. Moreover, the presence of hypertension, use of antihypertensive drugs or inflammatory diseases can affect plasma sRAGE levels [36].

Study limitations

This study has several limitations. The number of study participants was limited, however, the study was adequately powered to detect intergroup differences in AGEs levels. Moreover, almost half of the DM patients had well-controlled diabetes with HbA1c < 7%. This could be a reason that we failed to show associations of valvular

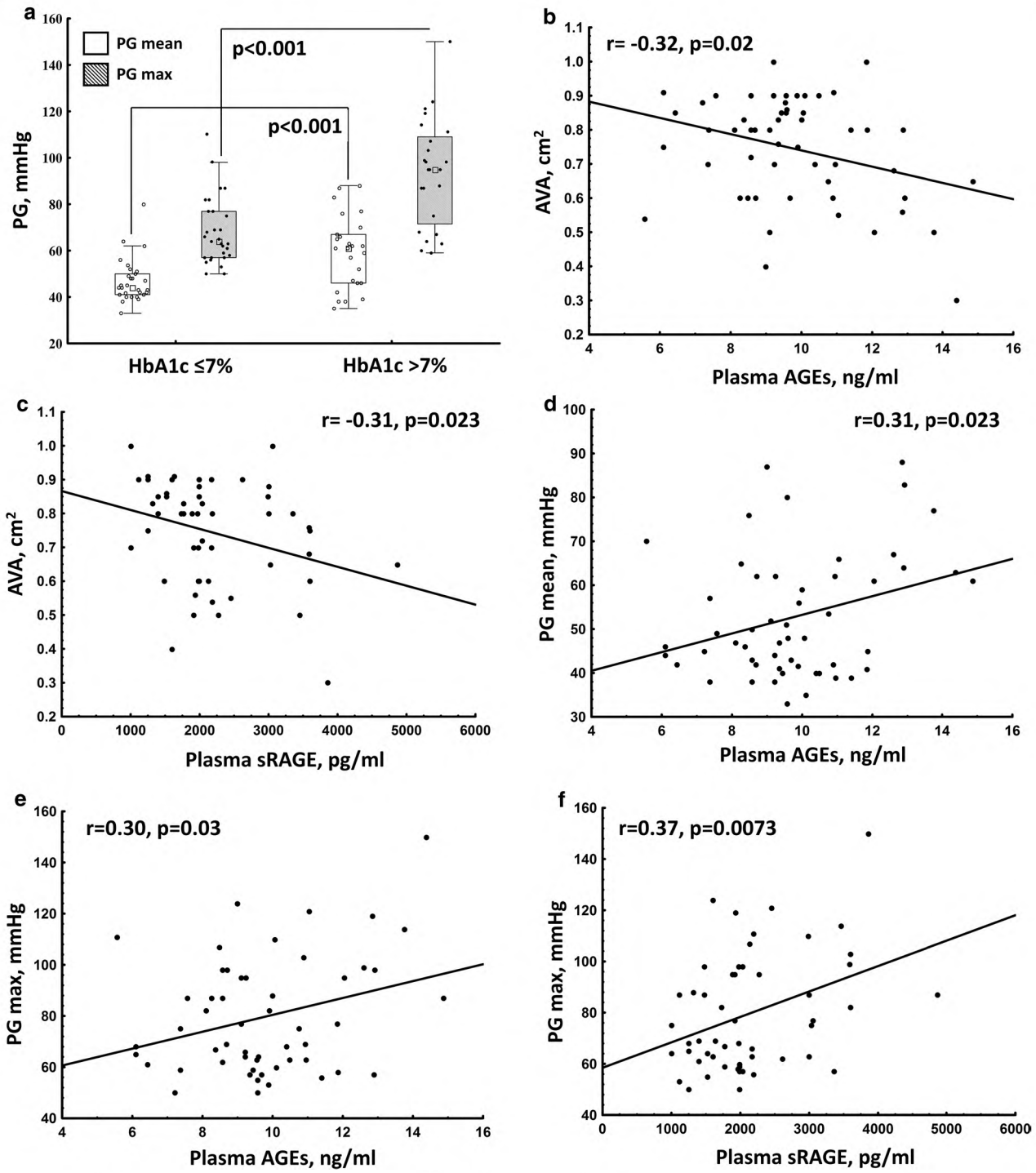


Fig. 4 Associations of plasma AGEs and RAGE with echocardiographic parameters in patients with AS and concomitant DM. Associations of: **a** mean and maximal transvalvular pressure gradients (PG) stratified according to elevated levels of glycated hemoglobin (HbA1c), **b** plasma levels of advanced glycation end products (AGEs) with aortic valve area (AVA), **c** plasma levels of AGEs receptor (RAGE) with AVA, **d** plasma levels of AGEs with mean PG or **e** maximal PG, and **f** plasma RAGE levels with maximal PG

AGEs and RAGE accumulation with valvular inflammation or oxidation. On the other hand, more sensitive markers of inflammation and oxidation, or using another type of antibodies for immunostaining, could be more informative. Second, although the analysis of valvular expression of AGEs and RAGE along with IL-6 and ROS was semiquantitative, but assessed by two independent investigators, results of such analyses should be interpreted with caution. Third, metformin has the ability to reduce AGEs accumulation [37], and it should be mentioned that most of DM patients in our cohort received this drug. AGEs levels in newly diagnosed or untreated DM patients might be higher.

Conclusions

We conclude that AGEs and RAGE accumulate within stenotic aortic valves in DM patients, and the degree of this accumulation is associated with AS severity. Moreover, we found that plasma AGE and sRAGE levels were associated with AVA, thus they may be considered as new biomarkers of the AS course in patients with concomitant type 2 DM. Further studies are urgently needed to elucidate whether more strict control of diabetes is capable of slowing AS progression in patients with mild-to-moderate valvular disease.

Abbreviations

AGEs: Advanced glycation end products; AS: Aortic stenosis; DM: Aortic stenosis with concomitant type 2 diabetes; AVA: Aortic valve area; HbA1c: Glycated hemoglobin; IL-6: Interleukin 6; $PG_{mean/max}$: Transvalvular pressure gradients mean or maximal; RAGE: AGEs receptor; ROS: Reactive oxygen species.

Acknowledgements

Not applicable.

Authors' contributions

MK and JN contributed to the work by acquisition of data, analysis and interpretation of data, as well as writing and revision of the manuscript. MK, PM, MZ, and JN contributed to the work by patients recruitment, acquisition and analysis of data. MK, JN and PM contributed to the work by performing most of laboratory testing and revision of the manuscript. MZ performed most statistical analysis. AU contributed to the conception of the study and revised the manuscript. JN made substantial contributions to conception and design of the study, analysis and interpretation of data, revision of the manuscript and final approval of the version to be published. All authors read and approved the final manuscript.

Funding

This study was supported by the grant from the Polish National Science Center (UMO-2015/19/B/NZ5/00647 to J.N).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study complied with the principles of the Good Clinical Practice International Conference on Harmonization rules and was approved by the Local Ethical Committee in Krakow. Each study participant provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] John Paul II Hospital, Kraków, Poland. [2] Institute of Cardiology, Jagiellonian University Medical College, 80 Pradnicka St, 31-202 Kraków, Poland.

Received: 10 April 2020 Accepted: 8 June 2020
Published online: 17 June 2020

References

1. Lindroos M, Kupari M, Heikkilä J, Tilvis R. Prevalence of aortic valve abnormalities in the elderly: an echocardiographic study of a random population sample. J Am Coll Cardiol. 1993;21:1220–5.
2. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. Circ Res. 2011;108:1392–412.
3. Ngo MV, Gottdiener JS, Fletcher RD, Fernicola DJ, Gersh BJ. Smoking and obesity are associated with the progression of aortic stenosis. Am J Geriatr Cardiol. 2001;10:86–90.
4. Czestkowska E, Rożanowska A, Długosz D, Bolt K, Świerszcz J, Kruszelnicka O, et al. Depressed systemic arterial compliance and impaired left ventricular midwall performance in aortic stenosis with concomitant type 2 diabetes: a retrospective cross-sectional study. Cardiovasc Diabetol. 2019;18:92.
5. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107:1058–70.
6. Forbes JM, Soldatos G, Thomas MC. Below the radar: advanced glycation end products that detour "around the side". Is HbA1c not an accurate enough predictor of long term progression and glycaemic control in diabetes? Clin Biochem Rev. 2005;26:123–34.
7. Katz R, Wong ND, Kronmal R, Takasu J, Shavelle DM, Probstfield JL, et al. Features of the metabolic syndrome and diabetes mellitus as predictors of aortic valve calcification in the Multi-Ethnic Study of Atherosclerosis. Circulation. 2006;113:2113–9.
8. Hegab Z, Gibbons S, Neyses L, Mamas MA. Role of advanced glycation end products in cardiovascular disease. World J Cardiol. 2012;4:90–102.
9. Kizer JR, Benkeser D, Arnold AM, Ix JH, Mukamal KJ, Djousse L, et al. Advanced glycation/glycoxidation endproduct carboxymethyl-lysine and incidence of coronary heart disease and stroke in older adults. Atherosclerosis. 2014;235:116–21.
10. Kiuchi K, Nejima J, Takano T, Ohta M, Hashimoto H. Increased serum concentrations of advanced glycation end products: a marker of coronary artery disease activity in type 2 diabetic patients. Heart. 2001;85:87–91.
11. Di Pino A, Currenti W, Urbano F, Scicali R, Piro S, Purrello F, et al. High intake of dietary advanced glycation end-products is associated with increased arterial stiffness and inflammation in subjects with type 2 diabetes. Nutr Metab Cardiovasc Dis. 2017;27:978–84.
12. Thorpe SR, Baynes JW. Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids. 2003;25:275–81.
13. Francis-Sedlak ME, Moya ML, Huang JJ, Lucas SA, Chandrasekharan N, Larson JC, et al. Collagen glycation alters neovascularization in vitro and in vivo. Microvasc Res. 2010;80:3–9.
14. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab. 2001;280:E685–94.
15. Branchetti E, Bavaria JE, Grau JB, Shaw RE, Poggio P, Lai EK, et al. Circulating soluble receptor for advanced glycation end product identifies patients with bicuspid aortic valve and associated aortopathies. Arterioscler Thromb Vasc Biol. 2014;34:2349–57.
16. Hofmann B, Yakobus Y, Indrasari M, Nass N, Santos AN, Kraus FB, et al. RAGE influences the development of aortic valve stenosis in mice on a high fat diet. Exp Gerontol. 2014;59:13–20.
17. Maresca AM, Guasti L, Bozzini S, Mongiardi C, Tandurella N, Corso R, et al. sRAGE and early signs of cardiac target organ damage in mild hypertensives. Cardiovasc Diabetol. 2019;18:17.

18. Du R, Zhang RY, Lu L, Shen Y, Pu LJ, Zhu ZB, et al. Increased glycated albumin and decreased esRAGE levels in serum are related to negative coronary artery remodeling in patients with type 2 diabetes: an Intravascular ultrasound study. Cardiovasc Diabetol. 2018;17:149.
19. Gelžinský J, Mayer O Jr, Seidlerová J, Mateřánková M, Mareš Š, Kordíková V, et al. Soluble receptor for advanced glycation end-products independently influences individual age-dependent increase of arterial stiffness. Hypertens Res. 2020;43:111–20.
20. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014;37(Suppl 1):S81–90.
21. Natorska J, Marek G, Hlawaty M, Sadowski J, Tracz W, Undas A. Fibrin presence within aortic valves in patients with aortic stenosis: association with in vivo thrombin generation and fibrin clot properties. Thromb Haemost. 2011;105:254–60.
22. Mayer O, Gelžinský J, Seidlerová J, et al. The role of advanced glycation end products in vascular aging: which parameter is the most suitable as a biomarker? J Hum Hypertens. 2020. https://doi.org/10.1038/s41371-020-0327-3.
23. Natorska J, Wypasek E, Grudzień G, Sobczyk D, Marek G, Filip G, et al. Does diabetes accelerate the progression of aortic stenosis through enhanced inflammatory response within aortic valves? Inflammation. 2012;35:834–40.
24. Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study. JAMA. 2003;290:2159–67.
25. Kamalesh M, Ng C, El Masry H, Eckert G, Sawada S. Does diabetes accelerate progression of calcific aortic stenosis? Eur J Echocardiogr. 2009;10:723–5.
26. Larsson SC, Wallin A, Håkansson N, Stackelberg O, Bäck M, Wolk A. Type 1 and type 2 diabetes mellitus and incidence of seven cardiovascular diseases. Int J Cardiol. 2018;262:66–70.
27. Testuz A, Nguyen V, Mathieu T, Kerneis C, Arangalage D, Kubota N, et al. Influence of metabolic syndrome and diabetes on progression of calcific aortic valve stenosis. Int J Cardiol. 2017;244:248–53.
28. Li F, Cai Z, Chen F, Shi X, Zhang Q, Chen S, et al. Pioglitazone attenuates progression of aortic valve calcification via down-regulating receptor for advanced glycation end products. Basic Res Cardiol. 2012;107:306.
29. Wang B, Cai Z, Liu B, Liu Z, Zhou X, Dong N, et al. RAGE deficiency alleviates aortic valve calcification in ApoE−/− mice via the inhibition of endoplasmic reticulum stress. Biochim Biophys Acta Mol Basis Dis. 2017;1863:781–92.
30. Prasad K. Low levels of serum soluble receptors for advanced glycation end products, biomarkers for disease state: myth or reality. Int J Angiol. 2014;23:11–6.
31. Grauen Larsen H, Marinkovic G, Nilsson PM, Nilsson J, Engstrom G, Melander O, et al. High plasma sRAGE (soluble receptor for advanced glycation end products) is associated with slower carotid intima-media thickness progression and lower risk for first-time coronary events and mortality. Arterioscler Thromb Vasc Biol. 2019;39:925–33.
32. Miranda ER, Somal VS, Mey JT, Blackburn BK, Wang E, Farabi S, et al. Circulating soluble RAGE isoforms are attenuated in obese, impaired-glucose-tolerant individuals and are associated with the development of type 2 diabetes. Am J Physiol Endocrinol Metab. 2017;313:E631–40.
33. Devangelio E, Santilli F, Formoso G, Ferroni P, Bucciarelli L, Michetti N, et al. Soluble RAGE in type 2 diabetes: association with oxidative stress. Free Radic Biol Med. 2007;43:511–8.
34. Nakamura K, Yamagishi S, Adachi H, Kurita-Nakamura Y, Matsui T, Yoshida T, et al. Elevation of soluble form of receptor for advanced glycation end products (sRAGE) in diabetic subjects with coronary artery disease. Diabetes Metab Res Rev. 2007;23:368–71.
35. Grossin N, Wautier MP, Meas T, Guillausseau PJ, Massin P, Wautier JL. Severity of diabetic microvascular complications is associated with a low soluble RAGE level. Diabetes Metab. 2008;34:392–5.
36. Yamagishi S, Matsui T. Soluble form of a receptor for advanced glycation end products (sRAGE) as a biomarker. Front Biosci. 2010;2:1184–95.
37. Ahmad S, Shahab U, Baig MH, Khan MS, Khan MS, Srivastava AK, et al. Inhibitory effect of metformin and pyridoxamine in the formation of early, intermediate and advanced glycation end-products. PLoS ONE. 2013;8:e72128.

Publisher's Note

Diabetologia (2021) 64:2562–2574
https://doi.org/10.1007/s00125-021-05545-w

ARTICLE

Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification

Magdalena Kopytek[1,2] · Piotr Mazur[2] · Michał Ząbczyk[1,2] · Anetta Undas[1,2] · Joanna Natorska[1,2]

Received: 3 February 2021 / Accepted: 26 May 2021 / Published online: 7 September 2021

Abstract

Aims/hypothesis Type 2 diabetes has been demonstrated to predispose to aortic valve calcification. We investigated whether type 2 diabetes concomitant to aortic stenosis (AS) enhances valvular inflammation and coagulation activation via upregulated expression of NF-κB, with subsequent increased expression of bone morphogenetic protein 2 (BMP-2).

Methods In this case–control study, 50 individuals with severe isolated AS and concomitant type 2 diabetes were compared with a control group of 100 individuals without diabetes. The median (IQR) duration of diabetes since diagnosis was 11 (7–18) years, and 36 (72%) individuals had HbA_{1c} ≥48 mmol/mol (≥6.5%). Stenotic aortic valves obtained during valve replacement surgery served for in loco NF-κB, BMP-2, prothrombin (FII) and active factor X (FXa) immunostaining. In vitro cultures of valve interstitial cells (VICs), isolated from obtained valves were used for mechanistic experiments and PCR investigations.

Results Diabetic compared with non-diabetic individuals displayed enhanced valvular expression of NF-κB, BMP-2, FII and FXa (all $p \leq 0.001$). Moreover, the expression of NF-κB and BMP-2 positively correlated with amounts of valvular FII and FXa. Only in diabetic participants, valvular NF-κB expression was strongly associated with serum levels of HbA_{1c}, and moderately with fructosamine. Of importance, in diabetic participants, valvular expression of NF-κB correlated with aortic valve area (AVA) and maximal transvalvular pressure gradient. In vitro experiments conducted using VIC cultures revealed that glucose (11 mmol/l) upregulated expression of both NF-κB and BMP-2 ($p < 0.001$). In VIC cultures treated with glucose in combination with reactive oxygen species (ROS) inhibitor (*N*-acetyl-L-cysteine), the expression of NF-κB and BMP-2 was significantly suppressed. A comparable effect was observed for VICs cultured with glucose in combination with NF-κB inhibitor (BAY 11–7082), suggesting that high doses of glucose activate oxidative stress leading to proinflammatory actions in VICs. Analysis of mRNA expression in VICs confirmed these findings; glucose caused a 6.9-fold increase in expression of *RELA* (NF-κB p65 subunit), with the ROS and NF-κB inhibitor reducing the raised expression of *RELA* by 1.8- and 3.2-fold, respectively.

Conclusions/interpretation Type 2 diabetes enhances in loco inflammation and coagulation activation within stenotic valve leaflets. Increased valvular expression of NF-κB in diabetic individuals is associated not only with serum HbA_{1c} and fructosamine levels but also with AVA and transvalvular gradient, indicating that strict long-term glycaemic control is needed in AS patients with concomitant type 2 diabetes. This study suggests that maintaining these variables within the normal range may slow the rate of AS progression.

Keywords Aortic stenosis · Bone morphogenetic protein 2 · Coagulation factors · Diabetes mellitus · Inflammation · NF-κB · Oxidative stress

Abbreviations

AS	Aortic stenosis
AVA	Aortic valve area
BMP	Bone morphogenetic protein
CRP	C-reactive protein
F1+2	Prothrombin fragments 1+2
FII	Prothrombin

✉ Joanna Natorska
j.natorska@szpitaljp2.krakow.pl

[1] John Paul II Hospital, Kraków, Poland

[2] Jagiellonian University Medical College, Kraków, Poland

Research in context

What is already known about this subject?

- Diabetes mellitus is a risk factor for aortic stenosis
- Plasma and valvular accumulation of AGEs is associated with aortic stenosis severity
- Valvular inflammation and calcification are driven by the NF-κB pathway

What is the key question?

- Does type 2 diabetes upregulate valvular expression of NF-κB leading to enhanced inflammation, coagulation activation and valvular calcification?

What are the new findings?

- Individuals with type 2 diabetes, especially those with poorly controlled diabetes, display enhanced valvular expression of NF-κB in association with increased amounts of valvular prothrombin, active factor X and bone morphogenetic protein 2
- In individuals with type 2 diabetes, valvular expression of NF-κB correlates with valvular calcification and markers of long-term glycaemic control, HbA_{1c} and fructosamine
- Inhibition of reactive oxygen species or direct inhibition of NF-κB signalling protects valve interstitial cells exposed to high glucose concentration from calcification

How might this impact on clinical practice in the foreseeable future?

- Strict long-term glycaemic control in aortic stenosis patients with concomitant type 2 diabetes might slow down the rate of aortic stenosis progression

FVIIa-AT	Active factor VIIa–antithrombin complex
FXa	Active factor X
NAC	*N*-Acetyl-L-cysteine
PG_{max}	Maximal transvalvular pressure gradient
PG_{mean}	Mean transvalvular pressure gradient
RAGE	Receptor for AGEs
ROS	Reactive oxygen species
TF	Tissue factor
VICs	Valve interstitial cells

Introduction

Aortic stenosis (AS) is a progressive disease associated with reduction of the aortic valve orifice and leaflet mobility due to a build-up of calcium. A consequence of this defect is an impaired blood ejection from the left ventricle into the aorta. AS is the most common acquired valvular heart disease in the western adult population, with no available pharmacological treatment. The prevalence of AS in individuals >65 years of age ranges between 2% and 7% [1]. It is estimated that 4.5 million cases of AS will be present worldwide by the year 2030 [2]. Aortic valve replacement, whether surgical or percutaneous, is the only definitive treatment for AS. While both methods present excellent outcomes, surgical intervention remains the treatment of choice for the vast majority of patients [3].

The initial stage of aortic valve degeneration is endothelial damage by high shear stress [4–6]. Then, subendothelial accumulation of intracellular lipids, lipoproteins and mediators of calcification is observed, together with activation of local and systemic inflammation [7, 8].

AS shares some risk factors with atherosclerosis. Among people with AS, similar to atherosclerosis, the prevalence of diabetes is visibly higher than in the general population and appears to be increasing [9, 10]. Ljungberg et al [11] have shown in population-based cohorts in northern Sweden that the prevalence of diabetes 10 years before surgery for AS was 15.8%.

Hyperglycaemia has been proposed as one of the metabolic states enhancing aortic valve fibrosis and calcification [12–14] through a complex mechanism involving increased valvular protein glycation, of reactive oxygen species (ROS) generation, inflammation and coagulation activation [15, 16]. Although the pivotal mechanism leading to such dysregulation is not fully understood, formation of AGEs has been suggested as a factor initiating and/or escalating valvular calcification [17, 18].

Our previous study showed increased valvular expression of C-reactive protein (CRP) and its mRNA, and higher tissue factor (TF) expression in individuals with AS and concomitant type 2 diabetes compared with non-diabetic individuals [14]. Moreover, regulation of valvular inflammation is under control of NF-κB [19]. In loco activation of NF-κB leads to an upregulation of IL-6, implicated in calcification of aortic valves via bone morphogenetic protein (BMP) stimulation [20]. In addition, coagulation factors such as TF and active factor X (FXa) upregulate inflammation and fibrosis through NF-κB signalling [21, 22].

Here, we hypothesised that metabolic dysregulation seen in type 2 diabetes may lead to enhanced valvular NF-κB expression. Thus, we investigated the valvular expression of NF-κB, BMP-2 and components of the blood coagulation system in individuals with AS and concomitant type 2 diabetes.

Methods

Participants

Between August 2016 and April 2019, we recruited 50 individuals with isolated symptomatic AS and concomitant type 2 diabetes and a control group of 100 individuals with AS without diabetes of similar age and sex. All participants underwent first-time elective surgical aortic valve replacement at the Department of Cardiovascular Surgery and Transplantology at the John Paul II Hospital, Krakow, Poland [18]. Data on demographics, medical history and current treatment were collected using a standardised questionnaire. AS was diagnosed based on transthoracic echocardiography performed by an experienced cardiologist on a Toshiba APLIO 80 (Toshiba, Tokyo, Japan) ultrasound machine, and it was defined as a mean transvalvular pressure gradient (PG_{mean}) ≥40 mmHg and/or aortic valve area (AVA) <1 cm^2 [23]. Arterial hypertension was diagnosed based on a history of hypertension (BP >140/90 mmHg) or preadmission antihypertensive treatment. Type 2 diabetes was diagnosed based on fasting serum glucose ≥7.0 mmol/l on two separate occasions, HbA_{1c} ≥48 mmol/mol (6.5%), or post-load plasma glucose levels ≥11.1 mmol/l [24]. All participants had diabetes diagnosed at least 5 years before enrolment and all were receiving treatment with insulin or oral glucose-lowering agents. To exclude latent autoimmune diabetes in adults (LADA), GAD65 antibodies and C-peptide concentrations were assessed in the diabetic participants. Participants negative for GAD autoantibodies and who had C-peptide within the normal range were classified as having type 2 diabetes. Twenty-four hours prior to aortic valve replacement, all participants receiving oral glucose-lowering agents were switched to insulin. Fasting blood glucose and HbA_{1c} levels were routinely performed in all diabetic participants and in the non-diabetic participants with AS who served as a control group.

Hypercholesterolaemia was diagnosed based on medical records, cholesterol-lowering therapy, or total cholesterol ≥5.0 mmol/l. Smoking was defined as the use of one or more cigarettes per day.

The following exclusion criteria were applied: atherosclerosis requiring concomitant revascularisation; rheumatic AS; acute infection including infective endocarditis; diagnosed malignancy; chronic kidney disease; previous pericardiotomy; required concomitant valvular surgery (e.g. mitral valve repair); recent (<90 days) acute coronary syndrome or cerebrovascular episode; percutaneous coronary intervention; and pregnancy. The valvular anatomy was identified preoperatively by echocardiography and confirmed intraoperatively by a cardiac surgeon. Bicuspid valve and root/ascending aortic dilatation requiring intervention were used as an exclusion criterion. The diagnosis of atherosclerosis was based on angiographically documented coronary artery stenosis >20% diameter and such individuals were excluded from the study.

The Ethical Committee (Krakow District Medical Chamber, Poland) approved the study and all participants provided written informed consent in accordance with the Declaration of Helsinki.

Laboratory analysis

At 07:00–09:00 hour, before surgical aortic valve replacement, fasting venous blood was drawn from the antecubital veins. Citrated blood was centrifuged at 2500 *g* at 20°C for 20 min, while blood drawn into serum or EDTA tubes was centrifuged at 1600 *g* at 4°C for 10 min. All samples were stored in small aliquots at −80°C until analysis. Routine laboratory assays were used to determine lipid profile, glucose, creatinine, CRP and fibrinogen. HbA_{1c} was assessed using a turbidimetric inhibition immunoassay (TINIA) in whole-blood haemolysates (Roche Diagnostics, Mannheim, Germany). Serum fructosamine levels were measured using a colorimetric assay (Roche Diagnostics, Risch-Rotkreuz, Switzerland).

Aortic valve preparation

Valves were collected during open heart surgeries and transferred directly from the operating room to the laboratory. One valvular leaflet was used for in loco analysis and two for in vitro studies (cell cultures and mRNA expression). Valve leaflets were cryosectioned into 4.5 μm sections as previously described [14, 18, 25].

Immunofluorescence analysis

Immunostaining was performed on 50 valves obtained from diabetic individuals and on 50 randomly selected valves from individuals with AS without diabetes, according to the previously

described protocol [14, 18]. Specific primary antibodies were used against NF-κB (p65 subunit, 1:500), BMP-2 (1:200), prothrombin (FII, 1:100) and FXa (1:200) (all from Abcam, Cambridge, UK) by overnight incubation at 4°C. The corresponding secondary goat or mouse antibodies conjugated with AlexaFluor 488 (Abcam) (1:1000) were applied in the dark at 4°C for 1 h. A negative control, without primary antibody was performed for all staining. All analyses were repeated three times. The Olympus BX 43 microscope (Tokyo, Japan) equipped with dedicated software (cellSense Dimension 2.3, License Version 2, Serial Number: BRR7BPW2NQP; Münster, Germany) was used to analyse images. Positively stained areas were assessed on a continuum from the undetected level (0%) to diffused staining (100%) and were calculated by two independent observers from 30 images taken of each valve. The percentages of immunopositive areas were calculated as the extent of positive immunoreactive areas/total sample area [25]. The fluorescence intensity was computed as the ratio (%) of positively and negatively stained areas. The investigators were blinded to the sample origin. The intra-observer variability was below 6%.

ELISAs

Active factor VIIa–antithrombin complex (FVIIa-AT; Diagnostica Stago, Asnières-sur-Seine, France), TF (R&D System, Minneapolis, MN, USA) and prothrombin fragments 1+2 (F1+2; Siemens Healthcare, Marburg, Germany) were assayed quantitatively in plasma samples using commercial ELISAs in accordance with manufacturers' instructions.

Valve interstitial cells in vitro cultures

Valve interstitial cells (VICs) were isolated and cultured as previously described [26]. All experiments were performed on VICs between their third and fifth passages. To initiate calcification, VICs were cultured in a calcification medium containing β-glycerophosphate disodium hydrate salt (10 mmol/l; Sigma-Aldrich, St Louis, MO, USA), $CaCl_2$ (1.5 mmol/l; Chempur, Piekary Slaskie, Poland) and ascorbic acid (50 μg/ml; Chempur) and stimulated or not (a negative control) with TNF-α (50 ng/ml). In parallel, to investigate the influence of glucose, VICs were cultured in the calcification medium supplemented with the D-(+)-glucose (11 mmol/l; Sigma-Aldrich). BMP-2 was used as a marker of calcification and was quantified using immunofluorescence as described above. To inhibit oxidative stress generated by high concentration of glucose, the inhibitor of ROS was added (1 mmol/l *N*-acetyl-L-cysteine [NAC]; Sigma-Aldrich) to the calcification medium 1 h before glucose stimulation. Similarly, to inhibit the transcription pathway of NF-κB, an inhibitor (BAY 11-7082; Sigma-Aldrich) was added to the calcification medium at a concentration of 10^{-6} mol/l 30 min before glucose stimulation. VICs were cultured for 72 h. Each experiment was repeated three times using VICs isolated from another valve.

Relative quantification of transcripts by real-time PCR

A total of 400 ng of RNA from VICs was reverse transcribed to single-strand cDNA (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. The cDNA was amplified with TaqMan Gene Expression Assay (Hs01042014_m1 for NF-κB p65 Rel; gene symbol: *RELA*) containing both primers and probe on an ABI PRISMR 7900HT Fast Real-Time PCR System (Applied Biosystems). β-Actin (Hs99999903_m1, human ACTB Endogenous Control FAM/ MGB Probe, Non-Primer Limited; Applied Biosystems) was used as a housekeeping gene. To analyse the obtained data, the comparative threshold cycle method was applied [26].

Statistical analyses

All statistical analyses were performed using STATISTICA Version 13.3 (TIBCO Software, Palo Alto, CA, USA) software. Categorical variables were presented as numbers and percentages and were analysed by Pearson's χ^2 or two-tailed Fisher's exact test. Continuous variables were expressed as mean ± SD or median (IQR). Normality was analysed by the Shapiro–Wilk test. Differences between groups were compared using Student's *t* test for normally distributed variables or the Mann–Whitney *U* test for non-normally distributed variables. Associations between normally distributed continuous variables were calculated using Pearson's correlation coefficient, while non-parametric variables were assessed by Spearman's test. A p value of <0.05 was considered statistically significant.

Results

Baseline characteristics of participants with AS, with and without type 2 diabetes, are shown in Table 1. The median duration of diabetes was 11 (7–18) years and 36 (72%) individuals had HbA_{1c} ≥48 mmol/mol (≥6.5%).

In the whole population of diabetic participants, no associations were found between serum glucose, HbA_{1c} or fructosamine levels and echocardiographic variables. However, diabetic participants with HbA_{1c} ≥48 mmol/mol (≥6.5%) compared with HbA_{1c} <48 mmol/mol (<6.5%) were characterised by 32% higher maximal transvalvular pressure gradient (PG_{max}; 87 [64–99] vs 66 [63–80] mmHg, $p = 0.038$), 18% higher PG_{mean} (52 [43–65] vs 44 [42–51] mmHg, $p = 0.036$) and 18% lower AVA (0.7 [0.6–0.8] vs 0.85 [0.8–0.9] cm^2, $p = 0.0005$).

Table 1 Baseline characteristics of participants with AS, with or without concomitant type 2 diabetes

Variable	Diabetic participants (*n*=50)	Non-diabetic participants (*n*=100)	*p* value[a]
Age, years	70.2±6.2	67.8±5.6	0.08
Female sex, *n* (%)	31 (62)	55 (55)	0.41
BMI, kg/m^2	31.3 (28.7–34.5)	28.3 (26.6–30.9)	0.049
Risk factors, *n* (%)			
Arterial hypertension	50 (100)	90 (90)	0.05
Hypercholesterolaemia	46 (92)	84 (84)	0.21
Current smoking	8 (16)	18 (18)	0.76
Medications, *n* (%)			
β-Blockers	47 (94)	87 (87)	0.19
Aspirin	40 (80)	76 (76)	0.58
ACE inhibitors	45 (90)	85 (85)	0.40
Statins	46 (92)	76 (76)	0.025
Insulin	14 (28)	0	<0.0001
Metformin	36 (72)	0	<0.0001
Echocardiographic data			
Mean gradient, mmHg	52 (43–66)	47 (43–58)	0.047
Maximal gradient, mmHg	89.2±12.3	80±14.2	0.042
LVEF, %	60 (58–64)	59 (50–65)	0.22
AVA, cm^2	0.78 (0.60–0.82)	0.87 (0.72–0.91)	0.044
Laboratory investigation			
Fibrinogen, g/l	3.6±0.6	3.3±0.76	0.3
Creatinine, μmol/l	81 (74–100)	82 (65–95)	0.68
CRP, mg/l	1.0 (1.0–2.0)	1.8 (1.0–4.0)	0.29
Glucose, mmol/l	7.5 (5.9–9.1)	5.3 (5.0–5.6)	<0.0001
HbA_{1c}, mmol/mol	51 (45–62)	37 (34–40)	<0.0001
HbA_{1c}, %	6.8 (6.3–7.8)	5.5 (5.3–5.8)	<0.0001
Fructosamine, μmol/l	262 (241–291)	223 (220–239)	0.007
TC, mmol/l	3.8 (3.0–4.6)	4.0 (3.6–4.7)	0.11
LDL-cholesterol, mmol/l	2.3 (1.5–3.1)	2.5 (2.0–3.4)	0.12
HDL-cholesterol, mmol/l	1.2 (1.0–1.4)	1.5 (1.2–1.5)	0.12
Triacylglycerols, mmol/l	1.5 (1.2–2.0)	1.4 (1.0–1.9)	0.39

Data are presented as *n* (%), mean±SD or median (IQR)

[a] Categorical variables were analysed by the χ^2 test; the Mann–Whitney *U* or Student's *t* tests were used to compare differences between groups

ACE, angiotensin converting enzyme; DM, type 2 diabetes; LVEF, left ventricular ejection fraction; TC, total cholesterol

In loco studies

Valvular expression of NF-κB in association with valve calcification NF-κB valvular expression was observed mainly on the aortic side of the leaflets, in both diabetic and control participants (Fig. 1). However, valves from diabetic compared with control participants were characterised by a 92% higher level of NF-κB expression (38 ± 10% vs 20 ± 6%, $p = 0.001$). In non-diabetic participants, expression of NF-κB presented a diffused pattern of fluorescence, while within valves from diabetic participants the expression was more condensed (Fig. 1a). Interestingly, the highest expression of NF-κB was found in the diabetic participants with HbA_{1c} ≥48 mmol/mol (≥6.5%) (+45%) (Fig. 1b). A similar pattern of immunofluorescence was observed with regard to valvular calcification, reflected by 148% ($p < 0.001$) higher BMP-2 expression in diabetic participants compared with control participants (Fig. 1a), with the highest percentage of BMP-2-positive areas (+23%) in diabetic participants with HbA_{1c} ≥48 mmol/mol (≥6.5%) compared with those with HbA_{1c} <48 mmol/mol (<6.5%) (Fig. 1c). Moreover, positive associations between valvular NF-κB and BMP-2 were found in both diabetic and non-diabetic participants (Fig. 1d,e). Only in the diabetic group valvular NF-κB expression was weakly associated with serum glucose (Fig. 2a), strongly associated with

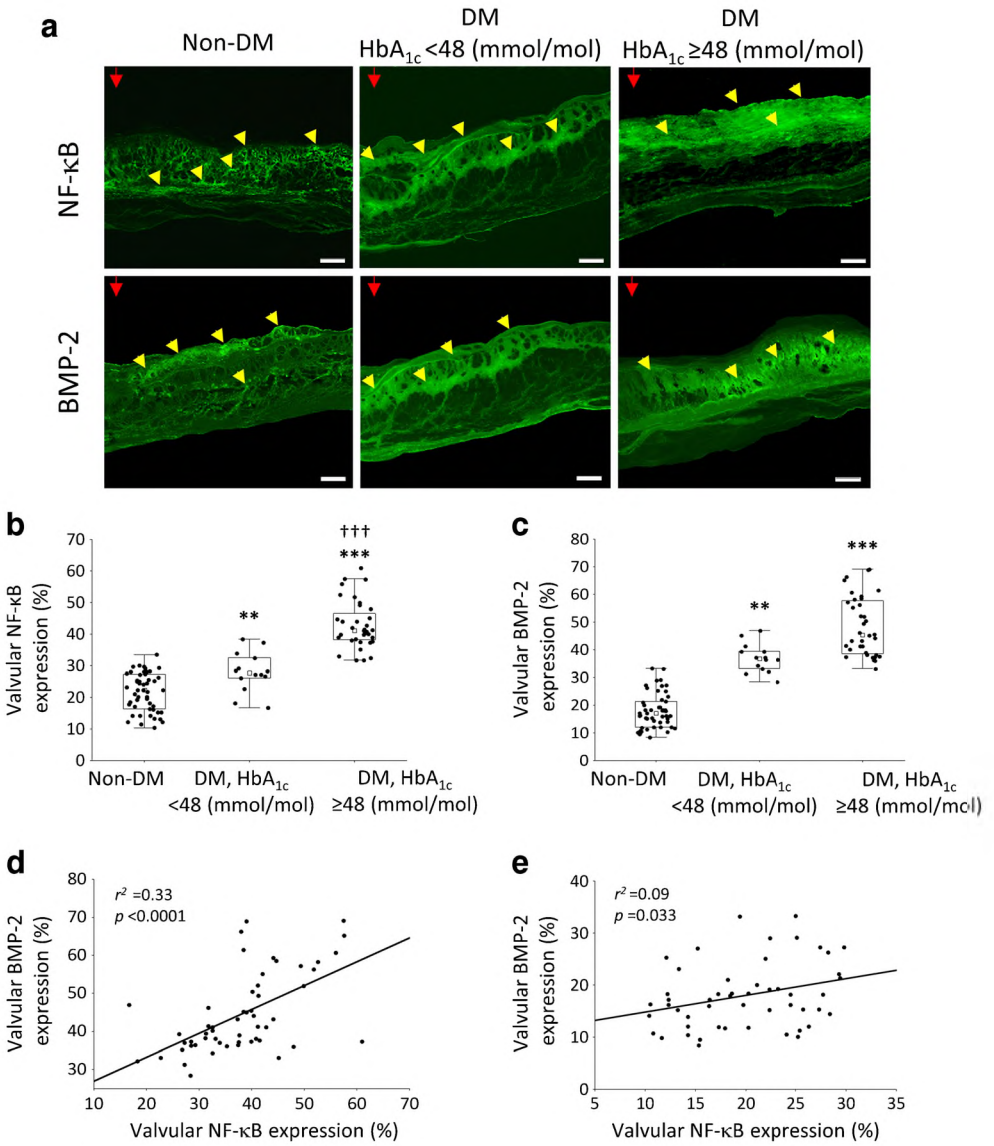


Fig. 1 The expression of NF-κB and BMP-2 within stenotic aortic valves in participants with AS and concomitant type 2 diabetes compared with participants with AS without diabetes. (**a**) Representative microphotographs of valvular NF-κB and BMP-2 expression (red arrowheads indicate aortic side of the leaflet; yellow arrowheads indicate the immunopositive area of expression). Scale bar, 200 μm. (**b**, **c**) Box plots showing valvular expression of NF-κB (**b**) and BMP-2 (**c**). Values are medians (IQR). $^{**}p<0.01$ and $^{***}p<0.001$ vs non-DM; $^{\dagger\dagger\dagger}p<0.001$ vs DM with HbA_{1c} <48 mmol/mol (<6.5%). (**d**, **e**) Associations between valvular expression of NF-κB and BMP-2 in participants with AS with (**d**) and without (**e**) concomitant diabetes. DM, AS with concomitant type 2 diabetes; Non-DM, AS without concomitant diabetes

HbA_{1c} (Fig. 2b) and moderately with fructosamine (Fig. 2c). In diabetic participants, valvular expression of BMP-2 correlated with HbA_{1c} ($r^2 = 0.65$, $p < 0.0001$) and fructosamine levels ($r^2 = 0.15$, $p = 0.006$) but not with glucose. No such associations were noted for control participants with AS but without concomitant diabetes.

Valvular expression of coagulation factors in association with NF-κB and BMP-2 In control participants with AS but without concomitant diabetes the valvular expression of FII and FXa was detected on the aortic side of the leaflets, in the endothelial and subendothelial layers, while in participants with type 2 diabetes the expression of both proteins was observed additionally in the fibrosa layer (Fig. 3a). Compared with valves from control participants, valves from diabetic patients were characterised by 113% higher expression levels of FII and 66% higher expression levels of FXa (both $p < 0.001$) (Fig. 3b). The expression of both factors was slightly higher (both $p > 0.05$) in diabetic participants with HbA_{1c} ≥48 mmol/mol (≥6.5%), compared with those with HbA_{1c} <48 mmol/mol (<6.5%). In diabetic participants, valvular NF-κB correlated positively with FII and FXa expression (Fig. 3c,d). Similar associations were observed in participants without diabetes (electronic supplementary material [ESM] Fig. 1a,b). Moreover, in diabetic participants, valvular BMP-2 was positively associated with the expression of FII and FXa (Fig. 3e,f). Both factors were co-expressed with BMP-2. In control participants, valvular FXa ($r^2 = 0.13$, $p = 0.01$) but not FII ($p = 0.38$) correlated positively with valvular BMP-2.

Associations of valvular factors with echocardiographic variables In participants with AS and concomitant type 2 diabetes, valvular NF-κB expression correlated with AVA and PG_{max} (Fig. 4a,b). In the control group of participants, we found the

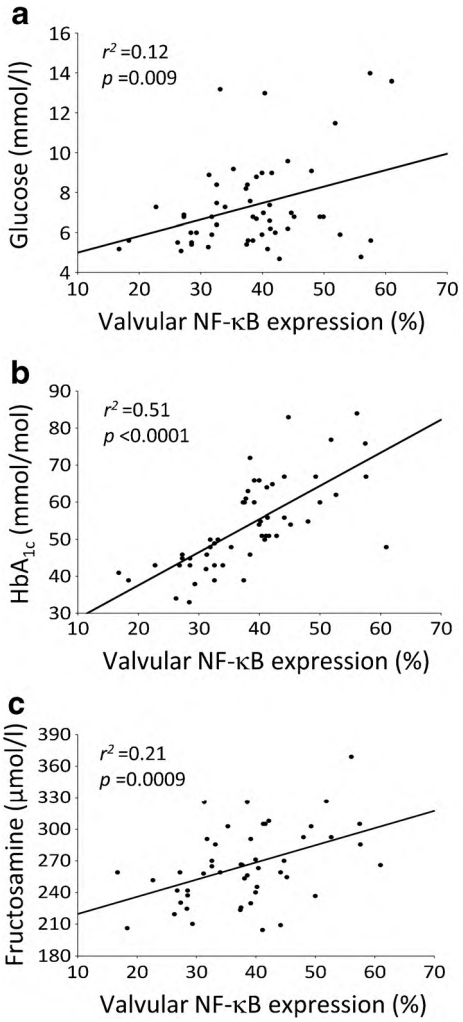


Fig. 2 Associations between valvular NF-κB expression and serum markers of glycaemic control in participants with AS and concomitant type 2 diabetes. Scatterplots represent the correlation between valvular NF-κB expression and serum levels of glucose (**a**), valvular NF-κB expression and serum concentrations of HbA_{1c} (**b**), and valvular NF-κB expression and serum levels of fructosamine (**c**)

inverse association solely between valvular NF-κB and AVA (ESM Fig. 1c). In diabetic participants, we also observed that valvular BMP-2 expression was associated with AVA and PG_{max} (Fig. 4c,d), while in control participants, BMP-2 expression correlated solely with AVA (ESM Fig. 1d). In diabetic participants both valvular FII and FXa were associated with AVA (Fig. 4e,g) and PG_{max} (Fig. 4f,h). Even when participants were matched based on PG_{max} (median [IQR] 87 [75–95] for diabetic participants vs 90 [83–94] mmHg for control participants, $p = 0.36$), those with type 2 diabetes ($n = 17$) vs without diabetes ($n = 19$) had higher valvular expression levels of NF-κB (+77%, $p < 0.0001$), BMP-2 (+118%, $p < 0.0001$), FII (+107%, $p < 0.0001$) and FXa (+65%, $p < 0.0001$).

Plasma markers of coagulation Participants in the type 2 diabetes group compared with the control group had 59% higher plasma concentrations of FVIIa-AT (median [IQR] 89 [79–112] vs 56 [48–71] pmol/l, $p < 0.0001$) but not TF (median [IQR] 1.38 [1.26–1.53] vs 1.29 [1.17–1.44] pmol/l, $p = 0.07$) or F1+2 (median [IQR] 196 [146–238] vs 182 [172–192] pmol/l, $p = 0.42$). However, diabetic participants with HbA_{1c} <48 mmol/mol (<6.5%), compared with those with HbA_{1c} ≥48 mmol/mol (≥6.5%), had slightly lower plasma TF and FVIIa-AT concentrations (Fig. 5a). No difference for F1+2 was observed (median [IQR] 188 [97–255] vs 190 [148–217] pmol/l, $p = 0.66$).

Only in the diabetic participants we found a positive association between plasma FVIIa-AT and serum fructosamine levels (Fig. 5b), while plasma TF correlated positively with both HbA_{1c} (Fig. 5c) and fructosamine (Fig. 5d). No associations between F1+2 and HbA_{1c} or fructosamine levels were found. Similarly, no correlations of plasma TF, FVIIa-AT or F1+2 with echocardiographic variables in participants with or without type 2 diabetes were noted (data not shown).

In vitro studies

Expression of NF-κB and BMP-2 in VICs cultures VICs activated with TNF-α showed upregulated expression of NF-κB (+75 ± 10%, $p < 0.001$) accompanied by higher expression of BMP-2 (+80 ± 12%, $p < 0.001$) when compared with unstimulated cells (Fig. 6a). A comparable effect was observed after incubation of VICs at high glucose concentration (+56 ± 10% for NF-κB and +52 ± 9% for BMP-2, both $p < 0.001$). The expression of NF-κB was downregulated in VICs incubated with glucose plus ROS inhibitor (−29 ± 7%, $p < 0.01$) or NF-κB inhibitor (−31 ± 7%, $p < 0.01$) (Fig. 6a). A similar effect was observed for BMP-2 expression after treatment of VICs with glucose plus ROS inhibitor (−31 ± 8%, $p < 0.01$) or NF-κB inhibitor (−33 ± 8%, $p < 0.01$) (Fig. 6a).

Relative expression of NF-κB mRNA in VICs Stimulation of VICs by TNF-α resulted in a 7.8-fold increase in *RELA* expression compared with non-stimulated VICs cultures (Fig. 6b). VICs treated with glucose showed a 6.9-fold increase in *RELA* expression compared with no treatment, while pre-incubation of VICs with glucose in combination with ROS or NF-κB inhibitors suppressed the *RELA* expression by 1.8-fold and 3.2-fold compared with VICs treated with glucose alone (Fig. 6b).

Discussion

This study is the first to demonstrate that individuals with severe isolated AS and concomitant type 2 diabetes, compared with no concomitant diabetes, exhibit enhanced valvular expression of NF-κB in association with increased expression of valvular FII, FXa and BMP-2. In diabetic

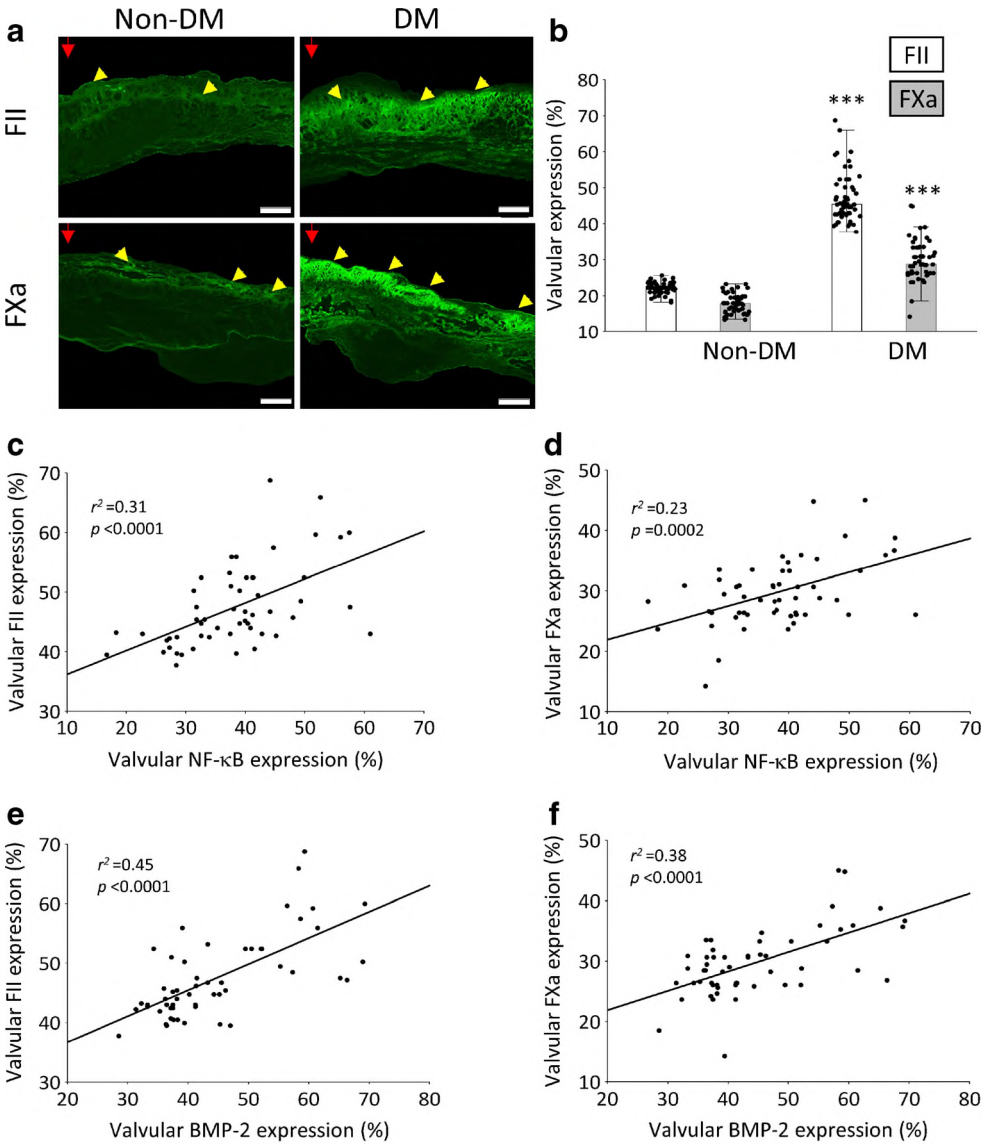


Fig. 3 The expression of valvular FII and FXa within stenotic aortic valves in participants with AS and concomitant type 2 diabetes compared with participants with AS without diabetes. (**a**) Representative microphotographs of valvular FII and FXa expression (red arrowheads indicate aortic side of the leaflet; yellow arrowheads indicate the immunopositive area of expression). Scale bar, 200 μm. (**b**) Bar graph showing valvular expression levels of FII and FXa. Values are medians (IQR).***$p<0.001$ vs non-DM. (**c**–**f**) The scatterplots show correlations between valvular NF-κB and FII (**c**), NF-κB and FXa (**d**), BMP-2 and FII (**e**), and BMP-2 and FXa (**f**) in participants with AS and concomitant type 2 diabetes. DM, AS with concomitant type 2 diabetes; Non-DM, AS without concomitant diabetes

individuals, valvular expression of NF-κB correlated with PG_{max}, AVA and biomarkers of long-term glycaemic control, namely HbA_{1c} and fructosamine. Poorly controlled type 2 diabetes was associated with the highest in loco expression of inflammatory and calcification markers, as well as higher concentrations of plasma coagulation factors, such as TF and FVIIa-AT. Moreover, in vitro experiments conducted on VICs isolated from stenotic aortic valves confirmed that high concentrations of glucose generate inflammation through NF-κB-mediated signalling, leading to subsequent cellular calcification. We also showed that inhibition of either ROS or NF-κB prevents VICs calcification. These data are in line with our previous report showing that AS patients with poorly controlled type 2 diabetes are characterised by higher transvalvular pressure gradients and higher valvular accumulation of AGEs associated with AS severity and serum levels of HbA_{1c} and fructosamine [18].

Previous reports on the association between diabetes and the incidence of AS progression are inconsistent [27–31]. Aronow et al [27] and Kamalesh et al [28] showed a positive association between diabetes and AS progression in individuals with mild and moderate AS, respectively, but no such data are available for severe AS. Katz et al [29] found that both diabetes and the metabolic syndrome were independently associated with an increased prevalence of valvular calcification. Finally, an increased risk of AS development in individuals with type 2 diabetes was shown by Larsson et al [30] in a cohort study comprised of more than 70,000 participants. Testuz et al [31] found no association between AS progression (in individuals with at least mild asymptomatic AS) and

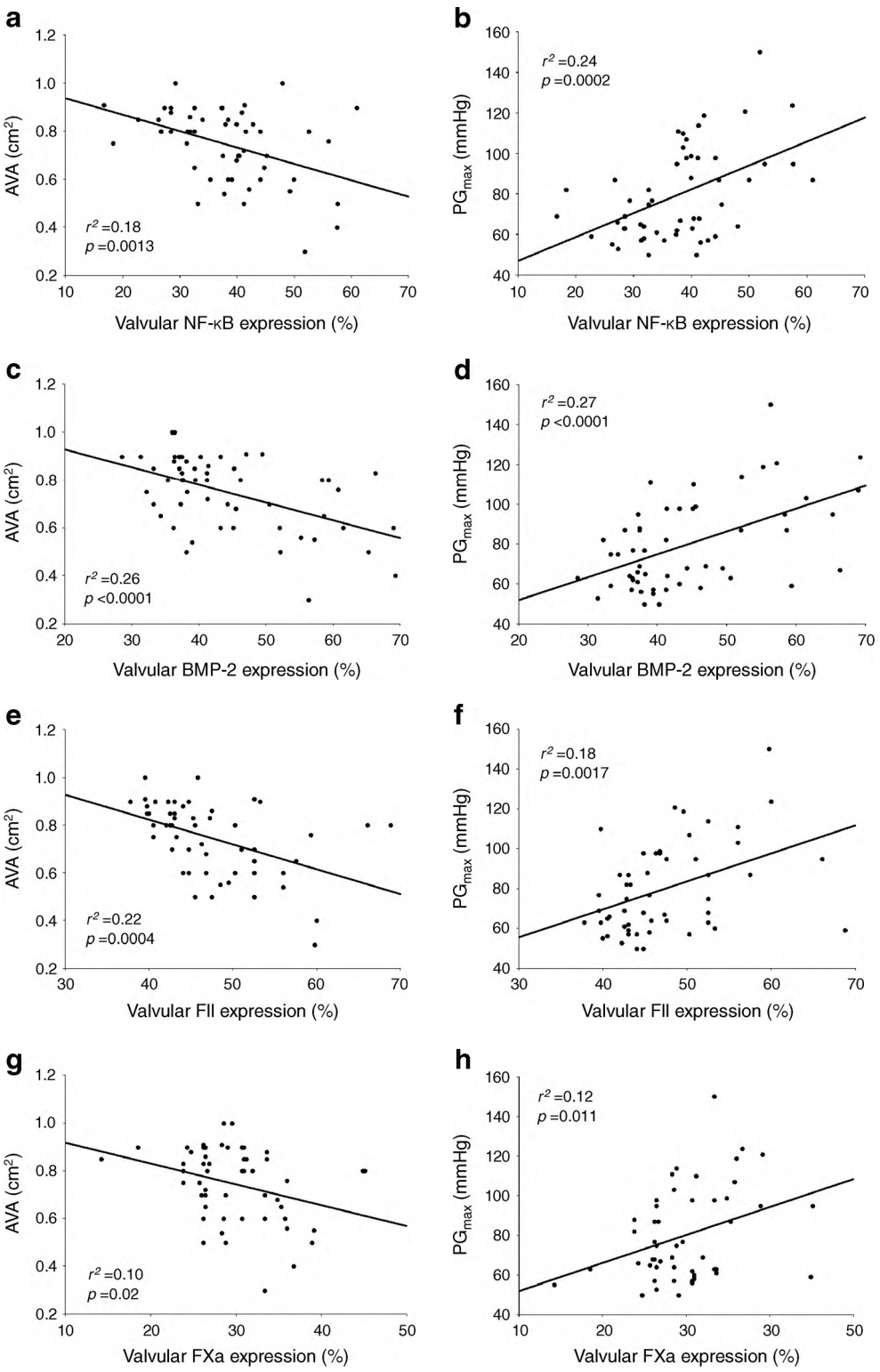


Fig. 4 Associations between valvular expression of inflammatory, calcification and coagulation factors and disease severity in participants with AS and concomitant type 2 diabetes. The scatterplots show correlations between valvular NF-κB and AVA (**a**), valvular NF-κB and PG_{max} (**b**), valvular BMP-2 and AVA (**c**), valvular BMP-2 and PG_{max} (**d**), valvular FII and AVA (**e**), valvular FII and PG_{max} (**f**), valvular FXa and AVA (**g**), and valvular FXa and PG_{max} (**h**)

diabetes or the metabolic syndrome. However, in their study, only short-term glucose control (reflected by fasting glucose levels) was assessed. Arguably, as demonstrated by our previous research [18], long-term glycaemic control may be of key importance. The present data confirmed that only HbA_{1c} and fructosamine were associated with valvular inflammation and calcification, while glucose levels showed only a very weak association. Importantly, the highest in loco expression of both NF-κB and BMP-2 was seen in individuals with poorly

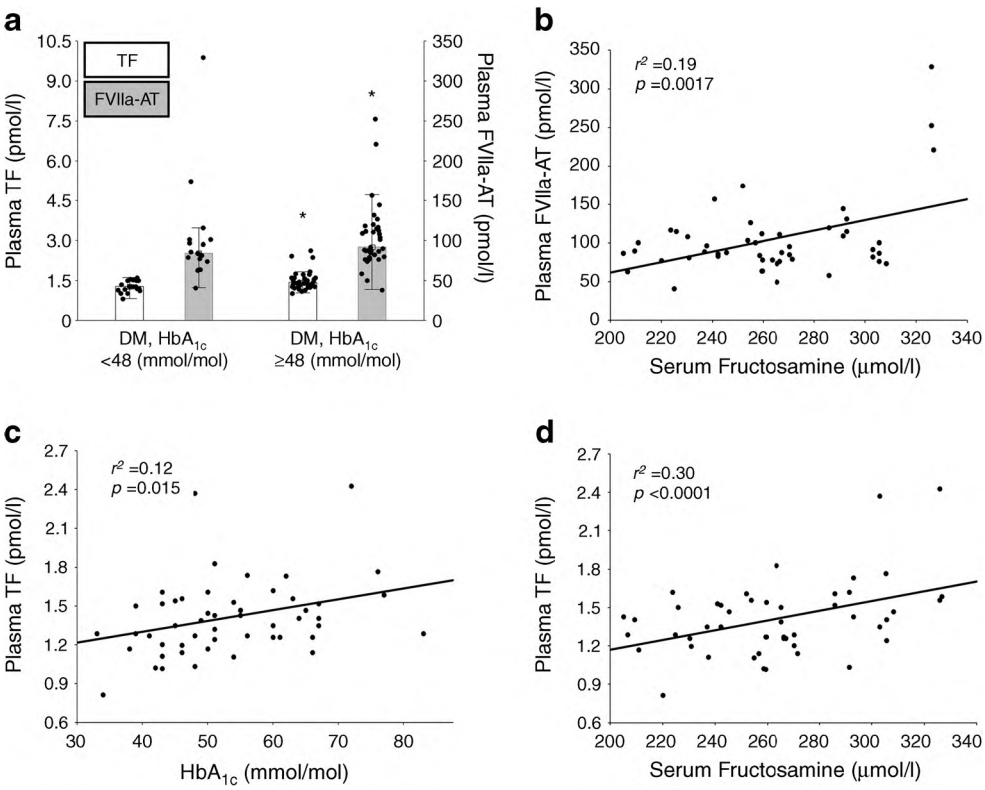


Fig. 5 Plasma levels of TF and FVIIa-AT in participants with AS and concomitant type 2 diabetes. (**a**) Bar graphs showing plasma levels of TF and FVIIa-AT in diabetic participants with HbA_{1c} <48 mmol/mol (<6.5%) and HbA_{1c} ≥48 mmol/mol (≥6.5%). Values are medians (IQR). *p<0.05 vs DM with HbA_{1c} <48 (mmol/mol). (**b**–**d**) The scatterplots show correlations between serum levels of fructosamine and plasma concentrations of FVIIa-AT (**b**), serum concentrations of HbA_{1c} and plasma levels of TF (**c**) and serum levels of fructosamine and plasma levels of TF (**d**). DM, type 2 diabetes

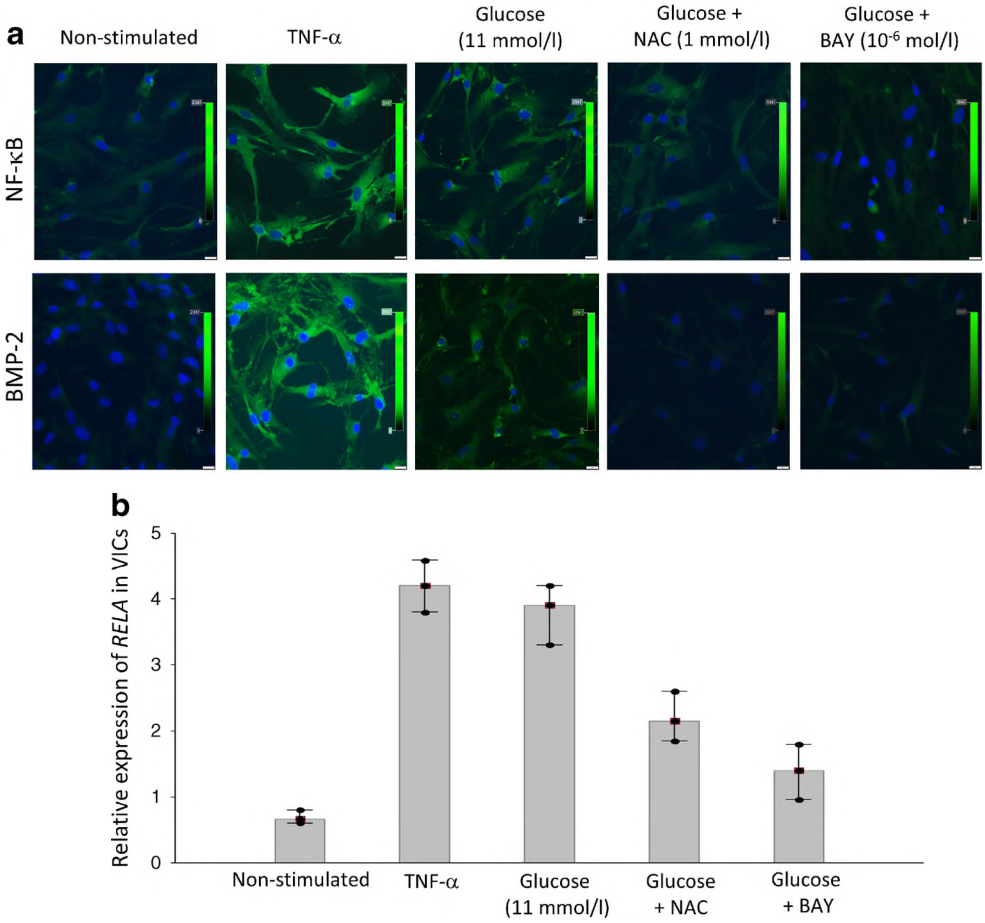


Fig. 6 The influence of glucose (11 mmol/l) and specific inhibitors of ROS (NAC) and transcription pathway NF-κB (BAY 11-7082 [BAY]) on the expression of NF-κB and BMP-2 in VICs isolated from aortic stenotic valves obtained during surgery. (**a**) Representative microphotographs of immunostaining in VIC cultures. Scale bar, 20 μm. (**b**) Relative expression of *RELA* in VIC cultures after stimulation. Data are presented as mean±SD

controlled diabetes. This data supports the hypothesis that maintaining long-term glycaemic variables within normal values in individuals with type 2 diabetes who have mild-to-moderate AS may slow the rate of AS progression. However, further studies are warranted to elucidate this issue.

Taken together, we propose the following mechanism underlying the influence of type 2 diabetes on AS progression: hyperglycaemia leads to enhanced accumulation of AGEs/receptor for AGEs (RAGE) and, as a consequence, enhanced production of ROS within valves [18]. Further, ROS escalate valvular inflammation via aggravated macrophage activation and NF-κB pathway expression with upregulated expression of BMP-2-4, osteopontin, osteocalcin, Smad1/5/8, and Runt-related transcription factor 2 (Runx-2), resulting in increased calcium deposition [32]. The findings by Vadana et al [32] are in line with our hypothesis. They showed that high glucose concentration (25 mmol/l) resulted in remodelling of VICs, defined as increased production of matrix metalloproteinases and extracellular matrix proteins, and increased expression of proinflammatory cytokines [32, 33], cell adhesion molecules and integrins [33]. Since inhibition of the NF-κB pathway not only decreased NF-κB expression at the protein and mRNA level but also decreased BMP-2 expression, the present study extended observations by Vadana et al [32] and showed that glucose-driven VIC activation is mediated via the NF-κB pathway and might be responsible for faster valve calcification and dysfunction.

While these findings bear the limitations inherent to flow [3] and observer-dependent echocardiographic measurements, one can speculate that they reflect a more pronounced expression of NF-κB in individuals with a heavier calcific burden on the aortic valve. Optimally, our findings should be verified by a flow-independent method of calcification assessment, like computed tomography (CT)-based calcium scoring [34].

Coagulation

We are the first to show that individuals with type 2 diabetes and AS have significantly higher valvular expression levels of FII and FXa. Moreover, poorly controlled diabetes was associated with the highest plasma TF and FVIIa-AT concentrations.

It has been shown that increased accumulation of AGEs/RAGEs is able to increase TF expression [35], platelet aggregation [36, 37] and fibrin stabilisation, and reduce the sensitivity of fibrin to degradation by plasmin [37, 38]. The current data suggests that poorly controlled diabetes is associated with a systemic prothrombotic state that can influence AS severity. However, we did not find enhanced thrombin generation in the participants with type 2 diabetes. As the associations between type 2 diabetes and its complications are rather longitudinal, one might hypothesise that prolonged exposure to hyperglycaemia predisposes to a more extensive calcific burden. Apparently, a diabetic individual may have a more calcified valve compared with a non-diabetic individual at the time of symptom presentation and surgical intervention. It remains to be established how diabetes biologically affects AS at the earlier stages of the disease. This is technically more difficult, as the surgical removal of the diseased valve is warranted at the very late stage of disease progression in isolated AS.

Study limitations

This study has several limitations. Any significant atherosclerosis was used as an exclusion criterion, although the role of atherosclerosis cannot be completely omitted. First, the number of participants in the subgroups with well and poorly controlled diabetes was small. However, this is a unique cohort of a relatively high number of individuals with poorly controlled type 2 diabetes concomitant to AS. Second, we did not assess all haemostasis-related proteins, such as von Willebrand factor, which was shown by Ljungberg et al [39] to be implicated in AS development and thus may influence valvular inflammation. Valvular expression of particular factors was determined semi-quantitatively and therefore these estimations may be less precise. However, microscopic analyses were performed by two independent experienced investigators. Moreover, the presented analysis cannot determine whether type 2 diabetes enhances the expression of the investigated proteins in valvular cell populations other than VICs, as this was beyond the scope of this study. Third, modification of VIC culture conditions, such as glucose concentration or different incubation times, might be considered in order to investigate the longitudinal action of glucose on VICs. It would also be of interest to conduct in vitro studies using co-culture of VICs and macrophages in order to examine the crosstalk between these two cell populations co-existing within stenotic aortic valves. In our opinion, the effect of glucose in co-culture could be even more intense. Finally, this study was performed in individuals with isolated severe AS and our results cannot be extrapolated to individuals with mild or moderate AS. Moreover, AS severity was measured as transvalvular gradients and AVA but not as a peak velocity, which is currently recommended for assessing AS severity [23].

Conclusions

The current study showed that type 2 diabetes enhances valvular expression of NF-κB and activation of coagulation within aortic stenotic valves and in circulating blood. Enhanced NF-κB expression was associated with AVA and PG_{max}. The level of valvular NF-κB expression was associated with HbA_{1c} and fructosamine levels, strongly supporting the concept that strict long-term glycaemic control is needed in AS patients with concomitant type 2 diabetes. Whether

maintaining these variables within the normal range might slow the rate of AS progression at earlier stages in the setting of diabetes remains to be established.

Supplementary Information The online version contains peer-reviewed but unedited supplementary material available at https://doi.org/10.1007/s00125-021-05545-w.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding This work was supported by the grant from the Polish National Science Centre (UMO-2015/19/B/NZ5/00647 to JN).

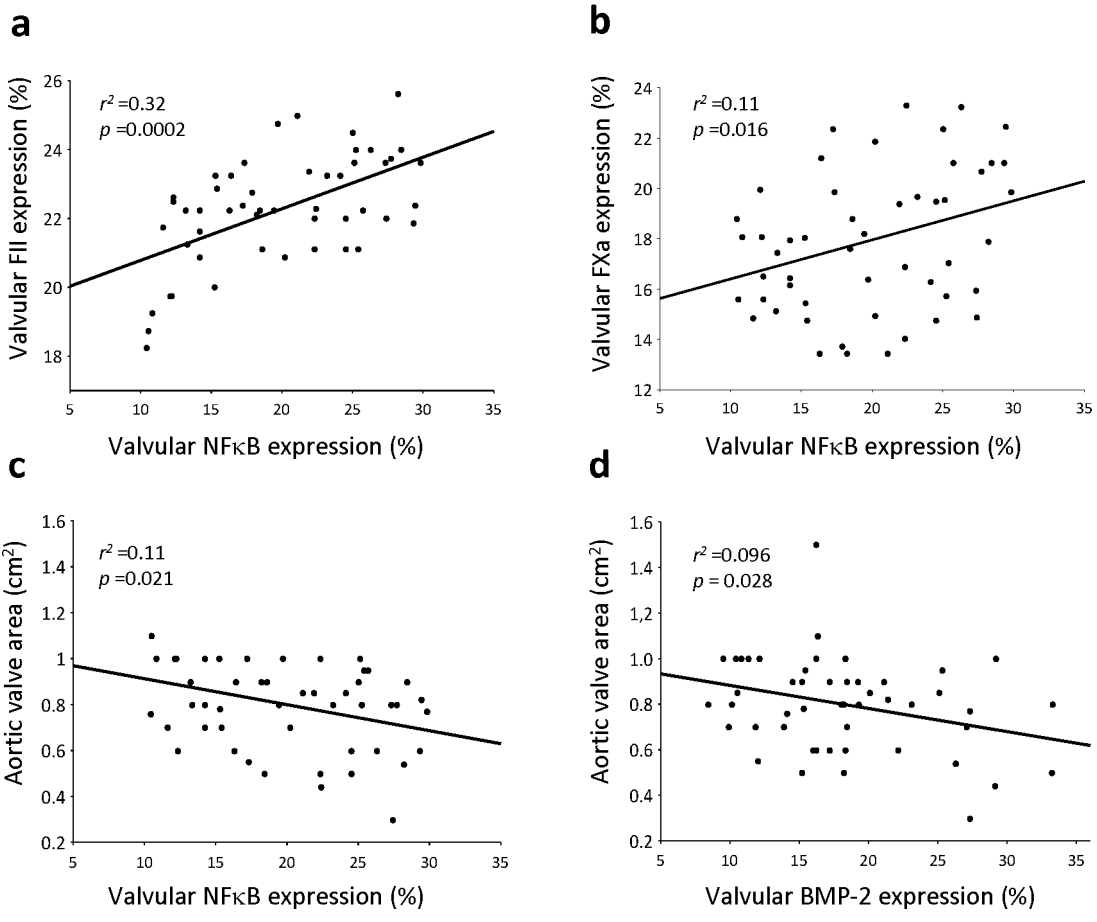
Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement MK acquired and analysed data, and drafted and revised the manuscript. PM recruited participants, acquired and analysed data, and drafted the manuscript. MZ contributed to the experimental design and interpretation of data and drafted the manuscript. AU contributed to the experimental design, analysed the data, and revised the manuscript. JN designed the experiments, acquired and analysed data, and drafted and revised the manuscript. All of the authors approved the final version to be published. JN is responsible for the integrity of the work as whole.

References

1. Lindroos M, Kupari M, Heikkilä J, Tilvis R (1993) Prevalence of aortic valve abnormalities in the elderly: an echocardiographic study of a random population sample. J Am Coll Cardiol 21(5): 1220–1225. https://doi.org/10.1016/0735-1097(93)90249-Z
2. Deeb GM, Reardon MJ, Chetcuti S et al (2016) 3-Year Outcomes in High-Risk Patients Who Underwent Surgical or Transcatheter Aortic Valve Replacement. J Am Coll Cardiol 67:2565–2574. https://doi.org/10.1016/j.jacc.2016.03.506
3. Baumgartner H, Hung J, Bermejo J et al (2017) Recommendations on the Echocardiographic Assessment of Aortic Valve Stenosis: A Focused Update from the European Association of Cardiovascular Imaging and the American Society of Echocardiography. J Am Soc Echocardiogr 30(4):372–392. https://doi.org/10.1093/ehjci/jew335
4. Natorska J, Undas A (2015) Blood coagulation and fibrinolysis in aortic valve stenosis: links with inflammation and calcification. Thromb Haemost 114:217–227. https://doi.org/10.1160/TH14-10-0861
5. Miller JD, Weiss RM, Heistad DD (2011) Calcific Aortic Valve Stenosis: Methods, Models, and Mechanisms. Circ Res 108:1392–1412. https://doi.org/10.1161/CIRCRESAHA.110.234138
6. Pawade TA, Newby DE, Dweck MR (2015) Calcification in aortic stenosis: the skeleton key. J Am Coll Cardiol 66:561–577. https://doi.org/10.1016/j.jacc.2015.05.066
7. Yetkin E, Waltenberger J (2009) Molecular and cellular mechanisms of aortic stenosis. Int J Cardiol 135(1):4–13. https://doi.org/10.1016/j.ijcard.2009.03.108
8. Mohty D, Pibarot P, Després JP et al (2008) Association between plasma LDL particle size, valvular accumulation of oxidized LDL, and inflammation in patients with aortic stenosis. Arterioscler Thromb Vasc Biol 28:187–193. https://doi.org/10.1161/ATVBAHA.107.154989
9. Movahed MR, Hashemzadeh M, Jamal MM (2007) Significant increase in the prevalence of non-rheumatic aortic valve disease in patients with type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes 115(2):105–107. https://doi.org/10.1055/s-2007-949656
10. Culler SD, Cohen DJ, Brown PP et al (2018) Trends in Aortic Valve Replacement Procedures Between 2009 and 2015: Has Transcatheter Aortic Valve Replacement Made a Difference? Ann Thorac Surg 105(4):1137–1143. https://doi.org/10.1016/j.athoracsur.2017.10.057
11. Ljungberg J, Johansson B, Engström KG et al (2017) Traditional Cardiovascular Risk Factors and Their Relation to Future Surgery for Valvular Heart Disease or Ascending Aortic Disease: A Case-Referent Study. J Am Heart Assoc 6(5):e005133. https://doi.org/10.1161/JAHA.116.005133
12. Banovic M, Athithan L, McCann GP (2019) Aortic stenosis and diabetes mellitus: an ominous combination. Diab Vasc Dis Res 16(4):310–323. https://doi.org/10.1177/1479164118820657
13. Drolet MC, Roussel E, Deshaies Y, Couet J, Arsenault M (2006) A high fat/high carbohydrate diet induces aortic valve disease in C57BL/6J mice. J Am Coll Cardiol 47(4):850–855. https://doi.org/10.1016/j.jacc.2005.09.049
14. Natorska J, Wypasek E, Grudzień G et al (2012) Does diabetes accelerate the progression of aortic stenosis through enhanced inflammatory response within aortic valves? Inflammation 35(3): 834–840. https://doi.org/10.1007/s10753-011-9384-7
15. Khan MS, Tabrez S, Rabbani N, Shah A (2015) Oxidative Stress Mediated Cytotoxicity of Glycated Albumin: Comparative Analysis of Glycation by Glucose Metabolites. J Fluoresc 25(6): 1721–1726. https://doi.org/10.1007/s10895-015-1658-2
16. Yan SF, Ramasamy R, Schmidt AM (2009) The receptor for advanced glycation endproducts (RAGE) and cardiovascular disease. Expert Rev Mol Med 11:e9. https://doi.org/10.1017/S146239940900101X
17. Saku K, Tahara N, Takaseya T et al (2020) Pathological Role of Receptor for Advanced Glycation End Products in Calcified Aortic Valve Stenosis. J Am Heart Assoc 9(13):e015261. https://doi.org/10.1161/JAHA.119.015261
18. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J (2020) Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol 19(1):92. https://doi.org/10.1186/s12933-020-01068-7
19. Gee T, Farrar E, Wang Y et al (2020) NFκB (Nuclear factor κ-Light-Chain Enhancer of Activated B Cells) Activity Regulates Cell-Type-Specific and Context-Specific Susceptibility to

Calcification in the Aortic Valve. Arterioscler Thromb Vasc Biol 40(3):638–655. https://doi.org/10.1161/ATVBAHA.119.313248
20. El Husseini D, Boulanger MC, Mahmut A et al (2014) P2Y2 receptor represses IL-6 expression by valve interstitial cells through Akt: implication for calcific aortic valve disease. J Mol Cell Cardiol 72: 146–156. https://doi.org/10.1016/j.yjmcc.2014.02.014
21. Borensztajn K, Peppelenbosch MP, Spek CA (2008) Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. Trends Mol Med 14(10):429–440. https://doi.org/10.1016/j.molmed.2008.08.001
22. Rothmeier AS, Ruf W (2012) Protease-activated receptor 2 signaling in inflammation. Semin Immunopathol 34(1):133–149. https://doi.org/10.1007/s00281-011-0289-1
23. Otto CM, Nishimura RA, Bonow RO et al (2021) 2020 ACC/AHA Guideline for the Management of Patients With Valvular Heart Disease: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. Circulation 143(5):e72–e227. https://doi.org/10.1161/CIR.0000000000000923
24. American Diabetes Association (2014) Diagnosis and classification of diabetes mellitus. Diabetes Care 37(Suppl 1):S81–S90. https://doi.org/10.2337/dc14-S081
25. Natorska J, Marek G, Hlawaty M, Sadowski J, Tracz W, Undas A (2011) Fibrin presence within aortic valves in patients with aortic stenosis: association with in vivo thrombin generation and fibrin clot properties. Thromb Haemost 105(2):254–260. https://doi.org/10.1160/TH10-09-0612
26. Wypasek E, Natorska J, Mazur P et al (2020) Effects of rivaroxaban and dabigatran on local expression of coagulation and inflammatory factors within human aortic stenotic valves. Vasc Pharmacol 130: 106679. https://doi.org/10.1016/j.vph.2020.106679
27. Aronow WS, Ahn C, Kronzon I, Goldman ME (2001) Association of coronary risk factors and use of statins with progression of mild valvular aortic stenosis in older persons. Am J Cardiol 88(6):693–695. https://doi.org/10.1016/S0002-9149(01)01821-5
28. Kamalesh M, Ng C, El Masry H, Eckert G, Sawada S (2009) Does diabetes accelerate progression of calcific aortic stenosis? Eur J Echocardiogr 10(6):723–725. https://doi.org/10.1093/ejechocard/jep048
29. Katz R, Wong ND, Kronmal R et al (2006) Features of the metabolic syndrome and diabetes mellitus as predictors of aortic valve calcification in the Multi-Ethnic Study of Atherosclerosis. Circulation 113(17):2113–2119. https://doi.org/10.1161/CIRCULATIONAHA.105.598086
30. Larsson SC, Wallin A, Håkansson N, Stackelberg O, Bäck M, Wolk A (2018) Type 1 and type 2 diabetes mellitus and incidence of seven cardiovascular diseases. Int J Cardiol 262:66–70. https://doi.org/10.1016/j.ijcard.2018.03.099
31. Testuz A, Nguyen V, Mathieu T et al (2017) Influence of metabolic syndrome and diabetes on progression of calcific aortic valve stenosis. Int J Cardiol 244:248–253. https://doi.org/10.1016/j.ijcard.2017.06.104
32. Vadana M, Cecoltan S, Ciortan L et al (2020) Molecular mechanisms involved in high glucose-induced valve calcification in a 3D valve model with human valvular cells. J Cell Mol Med 24(11): 6350–6361. https://doi.org/10.1111/jcmm.15277
33. Ciortan L, Macarie RD, Cecoltan S et al (2020) Chronic High Glucose Concentration Induces Inflammatory and Remodeling Changes in Valvular Endothelial Cells and Valvular Interstitial Cells in a Gelatin Methacrylate 3D Model of the Human Aortic Valve. Polymers (Basel) 12(12):E2786. https://doi.org/10.3390/polym12122786
34. Pawade T, Clavel MA, Tribouilloy C et al (2018) Computed Tomography Aortic Valve Calcium Scoring in Patients With Aortic Stenosis. Circ Cardiovasc Imaging 11(3):e007146. https://doi.org/10.1161/CIRCIMAGING.117.007146
35. Sugimoto K, Ohkawara H, Nakamura Y, Takuwa Y, Ishibashi T, Takeishi Y (2014) Receptor for advanced glycation end products - membrane type1 matrix metalloproteinase axis regulates tissue factor expression via RhoA and Rac1 activation in high-mobility group box-1 stimulated endothelial cells. PLoS One 9(12):e114429. https://doi.org/10.1371/journal.pone.0114429
36. Ahrens I, Chen YC, Topcic D et al (2015) HMGB1 binds to activated platelets via the receptor for advanced glycation end products and is present in platelet rich human coronary artery thrombi. Thromb Haemost 114(5):994–1003. https://doi.org/10.1160/TH14-12-1073
37. Yamagishi S, Nakamura N, Suematsu M, Kaseda K, Matsui T (2015) Advanced Glycation End Products: A Molecular Target for Vascular Complications in Diabetes. Mol Med 21(Suppl 1): S32–S40. https://doi.org/10.2119/molmed.2015.00067
38. Singh R, Barden A, Mori T, Beilin L (2001) Advanced glycation end-products: a review. Diabetologia 44(2):129–146. https://doi.org/10.1007/s001250051591
39. Ljungberg J, Janiec M, Bergdahl IA, Holmgren A, Hultdin J, Johansson B, Näslund U, Siegbahn A, Fall T, Söderberg S (2018) Proteomic Biomarkers for Incident Aortic Stenosis Requiring Valvular Replacement. Circulation 138(6):590–599. https://doi.org/10.1161/CIRCULATIONAHA.117.030414



ESM Figure 1. Associations between valvular expression of NFκB and (a) prothrombin (FII), (b) active FX (FXa), and (c) AS severity measured as aortic valve area as well as association between expression of (d) valvular BMP-2 and AS severity in participants with AS but without diabetes.

RESEARCH LETTER

Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis

Magdalena Kopytek[1,2], Michał Ząbczyk[1,2], Piotr Mazur[3,4], Jakub Siudut[2], Joanna Natorska[1,2]

1 Department of Thromboembolic Disorders, Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland
2 Krakow Centre for Medical Research and Technologies, John Paul II Hospital, Kraków, Poland
3 Department of Cardiovascular Surgery, Mayo Clinic, Rochester, Minnesota, United States
4 Department of Cardiovascular Surgery and Transplantology, Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland

Introduction Aortic stenosis (AS) is the most prevalent cause of acquired valvular heart disease in the aging population with no available pharmacotherapy to reduce or inhibit the disease progression. Within the stenotic aortic valves, a prominent accumulation of lipoproteins has been observed, being an essential component of AS development.[1] Lipoprotein(a) (Lp[a]) is a major carrier of phospholipids and their oxidized forms (OxPLs), which shows both proatherogenic and prothrombotic properties.[2] Data indicated that OxPLs are co-expressed with Lp(a) within the stenotic leaflets and promote valvular calcification.[1,3] A genome-wide association study[4] revealed that *LPA* rs10455872 polymorphism was significantly associated with a 2-fold increased risk of aortic leaflet calcification. Kamstrup et al[5] demonstrated that OxPL levels were associated with increased risk of AS (odds ratio [OR] of 2.0 (1.3–3.1) for 91st to 95th percentile levels). Recently, Siudut et al[6] reported that in AS Lp(a) and oxidized low-density lipoprotein contribute to hypofibrinolysis reflected by prolonged clot lysis time (CLT), and hypofibrinolysis has been shown to be associated with AS severity.[7] It is tempting to speculate that OxPL associated with Lp(a) contribute to hypofibrinolysis and thus AS severity.

The aim of this study was to evaluate whether in patients with severe AS elevated serum concentrations of OxPL are associated with increased Lp(a) level, impaired fibrinolysis, and AS severity.

Patients and methods Between October 2018 and November 2020, we recruited 70 patients with symptomatic severe AS. Fifty patients had Lp(a) concentration equal to or above 50 mg/dl and 20 had a Lp(a) level below 50 mg/dl, with a threshold for Lp(a) being equal to or above 50 mg/dl according to a recent European Atherosclerosis Society consensus statement.[8] All AS patients underwent first-time elective surgical aortic valve replacement at the Department of Cardiovascular Surgery and Transplantology at the John Paul II Hospital, Kraków, Poland. Data on medical history, current treatment, and demographics were collected using a standardized questionnaire. Severe AS was defined as mean transvalvular pressure gradient (PG_{mean}) equal to or above 40 mm Hg, peak transvalvular velocity (V_{max}) equal to or above 4.0 m/s, and aortic valve area (AVA) equal to or below 1 cm^2 on transthoracic echocardiography. Arterial hypertension and hypercholesterolemia were diagnosed as previously described.[6,9]

The exclusion criteria for AS patients included atherosclerotic vascular disease requiring revascularization, acute infection including infective endocarditis, rheumatic AS, diabetes mellitus, advanced chronic kidney disease, need for concomitant valvular surgery (eg, mitral valve repair), percutaneous coronary intervention, recent (<3 months) acute coronary syndrome or cerebrovascular episode, diagnosed malignancy, and pregnancy. The valvular anatomy was confirmed intraoperatively by a cardiac surgeon, and patients with bicuspid valve and root/ascending aortic dilatation requiring intervention were excluded from the study. The diagnosis of atherosclerosis was based on angiographically documented coronary artery stenosis greater than 20% of the diameter and such patients were excluded from the study to avoid any influence of nonobstructive atherosclerosis.[10]

The ethics committee approved the study (8/KBL/OIL/2019 and 53/KBL/OIL/2022) and

Correspondence to:
Joanna Natorska, PhD,
Department of Thromboembolic Disorders, Institute of Cardiology, Jagiellonian University Medical College, ul. Prądnicka 80, 31-202 Kraków, Poland,
phone: +48 12 614 21 08,
email: j.natorska@szpitaljp2.krakow.pl
Received: September 29, 2022.
Revision accepted: November 14, 2022.
Published online: November 17, 2022.
Pol Arch Intern Med. 2022; 132 (11): 16372
doi:10.20452/pamw.16372

all participants provided their written informed consent in accordance with the Declaration of Helsinki.

Fasting venous blood was drawn between 7:00 and 9:00 AM before the aortic valve replacement. Routine laboratory assays were used to assess glucose, creatinine, lipid profile, C-reactive protein and fibrinogen. Lp(a) was evaluated using immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Plasminogen and α_2-antiplasmin activity in plasma samples were measured by chromogenic assays (Siemens Healthcare, Marburg, Germany).

Aortic valves were collected during open heart surgery, embedded in Cryomatrix (Thermo Scientific, Kalamazoo, Michigan, United States), and sectioned into 4.5 µm slices with a Leica CM1520 cryostat. Immunostaining was performed on 30 valves from the patients with serum Lp(a) level equal to or above 50 mg/dl and 20 valves from the patients with Lp(a) level below 50 mg/dl, as described previously.[11] The primary antibody was used against OxPL (E06; Avanti Polar Lipids, Alabaster, Alabama, United States). The secondary goat antibody conjugated with AlexaFluor 488 (Abcam, Cambridge, United Kingdom; 1:1000) was applied in the dark. Olympus BX43 microscope (Tokyo, Japan) was used to visualize and analyze the images. The percentage of immunopositive areas was calculated as previously,[11] and 15 serial step sections were analyzed per each valve by 2 independent observers.

The levels of human tissue plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1) antigen, and thrombin activatable fibrinolysis inhibitor (TAFI) (all Hyphen Biomed, Neuville-sur-Oise, France), along with OxPL (Cell Biolabs, San Diego, California, United States) concentrations were assayed quantitatively in plasma or serum samples[12] using the enzyme-linked immunosorbent assay in accordance with the manufacturers' instructions.

CLT in plasma samples was measured as described previously.[13] Briefly, citrated plasma was mixed with thrombin (0.5 U/ml, Merck, Darmstadt, Germany), exogenous tPA (18 ng/ml, Boehringer Ingelheim, Germany), phospholipids (15 µM, Rossix, Mölndal, Sweden), and calcium ions. CLT was assessed turbidimetrically. The interassay coefficient of variation was below 6%.

Statistical analysis All statistics were performed using the STATISTICA software (Version 13.3, TIBCO Software, Palo Alto, California, United States), and models were performed using R 4.1.1 package (The R Foundation for Statistical Computing, Vienna, Austria). Categorical variables were presented as numbers and percentages, while continuous variables were expressed as mean and SD or median and interquartile range (IQR). Categorical variables were analyzed by the Pearson χ^2 test or the 2-tailed Fisher exact test. Normality was analyzed by the Shapiro–Wilk test. Differences between the groups were compared using the *t* test or the Mann–Whitney test, as appropriate. Associations between the variables were calculated using squared Pearson (*r*) or Spearman (*R*) correlation coefficients, as appropriate. The univariable linear regression models were performed to identify associations between CLT and laboratory, echocardiographic, and demographic variables. The variables that were associated with the prolonged CLT with a significance level below 0.2 in the univariable models or were clinically important were selected, and the multivariable linear model was fitted using stepwise regression with minimization of the Akaike information criterion, and adjusted for body mass index (BMI) with CLT as a dependent variable. The final model was validated using bootstrap resampling and examination of the residuals. Variance inflation factors were used to assess the multicollinearity. A *P* value below 0.05 was considered significant.

Results The AS patients with Lp(a) level equal to or above 50 mg/dl did not differ from individuals with Lp(a) level below 50 mg/dl with regard to demographic and risk factors, used medications, or laboratory parameters (Supplementary material, *Table 1*). The AS patients with Lp(a) level equal to or above 50 mg/dl had by 10% higher V_{max} ($P < 0.001$), 13% higher PG_{mean} ($P = 0.04$), 15% higher PG_{max} ($P < 0.001$), and 11% lower AVA ($P = 0.003$) than the patients with Lp(a) level below 50 mg/dl (Supplementary material, *Table 1*).

Valvular expression of OxPL was detected within all studied stenotic valves (**FIGURE 1A** and **1B**), at the aortic side of the leaflets, and presented a condensed pattern of fluorescence. However, the patients with serum Lp(a) level equal to or above 50 mg/dl had enhanced valvular OxPL expression (21.4% [3.0] vs 16.6% [2.1] immunopositive area, $P < 0.001$) and by 35% higher serum OxPL concentration ($P = 0.03$) than those with Lp(a) level below 50 mg/dl. Valvular amounts of OxPL were associated with serum OxPL concentration ($R = 0.55$, $P = 0.002$) and with Lp(a) concentration ($R = 0.84$, $P < 0.001$). Moreover, the patients with Lp(a) level equal to or above 50 mg/dl were characterized by 11% longer CLT ($P < 0.001$), 38% higher plasma PAI-1 ($P = 0.003$), and 12% higher TAFI levels ($P = 0.007$) than those with Lp(a) level below 50 mg/dl (Supplementary material, *Table 1*). We did not observe any differences in plasma levels of tPA or plasminogen and α_2-antiplasmin activity between the investigated groups (Supplementary material, *Table 1*). Interestingly, solely in the AS patients with Lp(a) level equal to or above 50 mg/dl, serum OxPL concentrations correlated with CLT, plasma levels of PAI-1, and TAFI (**FIGURE 1C–1E**), but not with tPA, plasminogen, or α_2-antiplasmin activity. Serum OxPL concentrations were also strongly associated with V_{max} ($R = 0.7$, $P < 0.001$), PG_{mean} ($R = 0.62$, $P < 0.001$), and weakly with AVA ($R = -0.32$, $P = 0.02$). As expected, serum Lp(a) levels positively correlated with CLT, PAI-1 concentration, and AS severity reflected by V_{max} and PG_{mean} (data not shown).

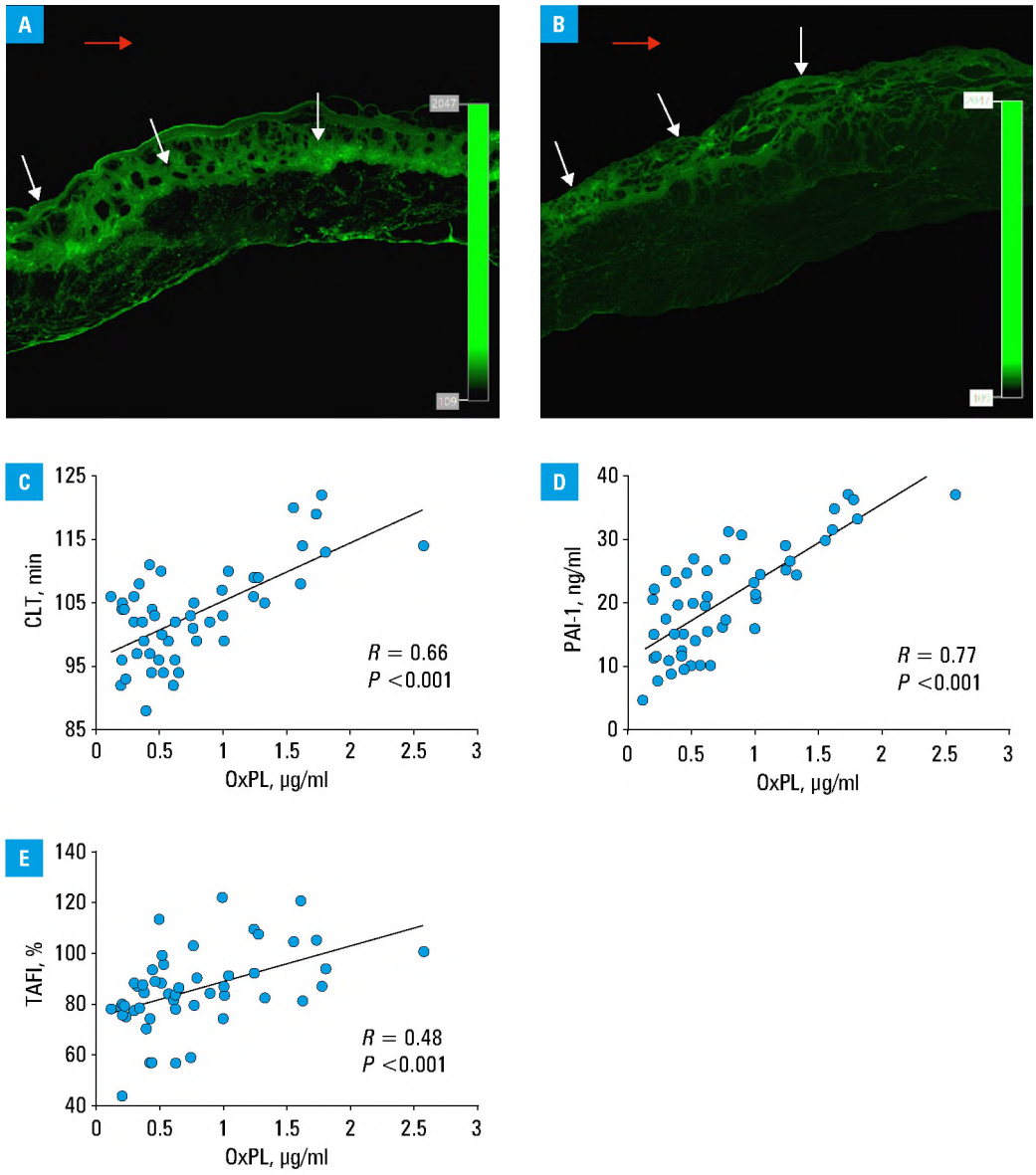


FIGURE 1 Valvular expression of OxPLs and associations between plasma OxPLs and markers of fibrinolysis. Representative microphotographs show valvular expression of OxPLs (green) within stenotic leaflets from patients with serum Lp(a) level equal to or above 50 mg/dl (**A**) and below 50 mg/dl (**B**). The red arrow indicates the aortic side of the leaflet, and the white arrows indicate the immunopositive areas of expression. Original magnification × 4. The scatterplots show associations between serum OxPL concentrations and (**C**) CLT, (**D**) plasma PAI-1 levels, and (**E**) plasma TAFI levels in AS patients with Lp(a) level equal to or above 50 mg/dl (n = 50).
Abbreviations: AS, aortic stenosis; CLT, clot lysis time; Lp(a), lipoprotein(a); OxPLs, oxidized phospholipids; PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor

The univariable linear regression analysis showed that OxPL, Lp(a), PAI-1, TAFI, disease severity, and age, but not plasminogen activity were associated with CLT in the AS patients with Lp(a) level equal to or above 50 mg/dl (Supplementary material, *Table 2*). The multiple linear regression analysis adjusted for BMI showed that higher OxPL levels predicted prolonged CLT in severe AS patients with Lp(a) level equal to or above 50 mg/dl (Supplementary material, *Table 2*).

Discussion This study is the first to show that in patients with severe AS and Lp(a) level equal to or above 50 mg/dl OxPLs are associated with impaired fibrinolysis and a have stronger impact on CLT than Lp(a). We also observed enhanced valvular OxPL expression in the patients with increased serum Lp(a) concentrations.

Lp(a) is the major plasma pool of OxPL and, as shown by Leibundgut et al,[14] the second plasma pool of OxPLs is circulating with plasminogen, which in physiological conditions facilitates fibrinolysis by conversion of plasminogen to plasmin. However, at increased Lp(a) levels, OxPLs impair fibrinolysis by inhibiting tPA-mediated plasminogen activation and inhibition of plasminogen binding to fibrin.[14]

The present study extended the observations of Siudut et al,[6] and showed a stronger association of OxPL with hypofibrinolysis in AS patients

than with Lp(a). In addition, given that in AS patients hypofibrinolysis is linked to the disease severity,[6,7] our observation that OxPL level correlates with AS severity seems justified.

Moreover, we showed that valvular OxPL expression was related to both serum OxPL and Lp(a) concentrations, which may suggest that OxPL-lowering therapies could be of importance in retardation of AS progression.

The study limitations should be acknowledged. The number of enrolled patients was limited, especially those with Lp(a) level below 50 mg/dl. However, the study was adequately powered and it represents typical patients with symptomatic severe AS in clinical practice. Moreover, the levels of OxPL and markers of fibrinolysis were measured once at enrolment, thus they may not reveal some associations. Our results cannot be directly extrapolated to individuals with mild or moderate AS.

In conclusion, our study showed that in patients with severe AS increased OxPL levels were associated with prolonged CLT and the disease severity. Larger studies are needed to confirm the observed associations.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

ARTICLE INFORMATION

ACKNOWLEDGEMENTS None.

FUNDING This work was supported by the Polish National Science Centre (UMO-2018/29/B/NZ5/02629 to JN and UMO-2021/41/N/NZ5/03323 to MK).

CONFLICT OF INTEREST None declared.

HOW TO CITE Kopytek M, Ząbczyk M, Mazur P, et al. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022; 132: 16372. doi:10.20452/pamw.16372

REFERENCES

1 Yu B, Hafiane A, Thanassoulis G, et al. Lipoprotein(a) induces human aortic valve interstitial cell calcification. JACC Basic Transl Sci. 2017; 2: 358-371.

2 Anuurad E, Boffa MB, Koschinsky ML, Berglund L. Lipoprotein(a): a unique risk factor for cardiovascular disease. Clin Lab Med. 2006; 26: 751-772.

3 Yeang C, Wilkinson MJ, Tsimikas S. Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. Curr Opin Cardiol. 2016; 31: 440-450.

4 Thanassoulis G, Campbell CY, Owens DS, et al; CHARGE Extracoronary Calcium Working Group. Genetic associations with valvular calcification and aortic stenosis. N Engl J Med. 2013; 368: 503-512.

5 Kamstrup PR, Hung MY, Witztum JL, et al. Oxidized phospholipids and risk of calcific aortic valve disease: the Copenhagen general population study. Arterioscler Thromb Vasc Biol. 2017; 37: 1570-1578.

6 Siudut J, Natorska J, Wypasek E, et al. Apolipoproteins and lipoprotein(a) as factors modulating fibrin clot properties in patients with severe aortic stenosis. Atherosclerosis. 2022; 344: 49-56.

7 Natorska J, Wypasek E, Grudzień G, et al. Impaired fibrinolysis is associated with the severity of aortic stenosis in humans. J Thromb Haemost. 2013; 11: 733-740.

8 Kronenberg F, Mora S, Stroes ESG, et al. Lipoprotein(a) in atherosclerotic cardiovascular disease and aortic stenosis: a European Atherosclerosis Society consensus statement. Eur Heart J. 2022; 43: 3925-3946.

9 Natorska J. Diabetes mellitus as a risk factor for aortic stenosis: from new mechanisms to clinical implications. Kardiol Pol. 2021; 79: 1060-1067.

10 Maddox TM, Stanislawski MA, Grunwald GK, et al. Nonobstructive coronary artery disease and risk of myocardial infarction. JAMA. 2014; 312: 1754-1763.

11 Kopytek M, Mazur P, Ząbczyk M, et al. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021; 64: 2562-2574.

12 Larsen JB, Hvas AM. Fibrin clot properties in coronary artery disease: new determinants and prognostic markers. Pol Arch Intern Med. 2021; 131: 16113.

13 Pieters M, Philippou H, Undas A, et al; Subcommittee on Factor XIII and Fibrinogen, and the Subcommittee on Fibrinolysis. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH. J Thromb Haemost. 2018; 16: 1007-1012.

14 Leibundgut G, Arai K, Orsoni A, et al. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. J Am Coll Cardiol. 2012; 59: 1426-1437.

Supplementary material

Kopytek M, Ząbczyk M, Mazur P, et al. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022; 132: 16372. doi:10.20452/pamw.16372

Table S1. Baseline characteristics of patients with severe aortic stenosis (AS) stratified according to serum Lp(a) levels

Variable	AS with Lp(a) ≥50 mg/dL (n=50)	AS with Lp(a) <50 mg/dL (n=20)	P value
Age, years	66 [60-70]	68 [60-70]	0.83
Male, n (%)	34 (68)	9 (45)	0.11
BMI, kg m^{-2}	28.7 (4.7)	28.1 (4.5)	0.60
Risk factors, n (%)			
Arterial hypertension	47 (94)	16 (80)	0.10
Hypercholesterolemia	46 (92)	18 (90)	0.99
Current smoking	9 (18)	4 (20)	0.99
Medications, n (%)			
Beta-blockers	33 (66)	17 (85)	0.15
Acetylsalicylic acid	32 (64)	14 (70)	0.78
ACE inhibitors	31 (62)	12 (60)	0.99
Statins	37 (74)	16 (80)	0.76
Echocardiographic parameters			
V_{max}, m/s	4.6 [4.4-4.8]	4.2 [4.0-4.5]	<0.001
Mean gradient, mmHg	52 [47-62]	46 [43-56]	0.044

Maximal gradient, mmHg	83 [79-91]	72 [64-81]	<0.001
AVA, cm^2	0.8 [0.7-0.8]	0.9 [0.8-0.9]	0.003
Laboratory investigations			
Fibrinogen, g/L	3.4 (0.8)	3.3 (0.7)	0.61
Creatinine, µmol/L	76 [70-89]	78 [71-105]	0.32
CRP, mg/L	2.0 [1.0-3.8]	1.9 [1.0-4.2]	0.95
Glucose, mmol/L	5.3 [5.0-5.6]	5.4 [5.2-5.6]	0.27
Total cholesterol mmol/L	4.1 [3.5-4.8]	3.8 [3.4-4.5]	0.20
LDL-cholesterol, mmol/L	2.6 [2.1-3.3]	2.3 [1.8-2.7]	0.19
HDL-cholesterol, mmol/L	1.3 [1.0-1.5]	1.3 [1.2-1.6]	0.19
Triglycerides, mmol/L	1.3 [0.9-1.8]	1.2 [0.9-1.4]	0.44
OxPL, µg/mL	0.62 [0.38-1.0]	0.46 [0.27-0.63]	0.03
Lp(a), mg/dL	81.9 [67.8-121.1]	6.7 [2.9-11]	<0.001
Plasma markers of fibrinolysis			
PAI-1, ng/mL	20.6 (8.5)	14.9 (4.8)	0.003
tPA, ng/mL	5.7 (2.0)	5.9 (2.4)	0.75
α_2-antiplasmin, %	96.2 (7.5)	94.3 (5.8)	0.31
Plasminogen, %	101.6 (13.2)	99.0 (16.5)	0.48
TAFI, %	85.7 (15.8)	76.7 (10.9)	0.007
CLT, min	103 [97-108]	93 [90-96]	<0.001

Data presented as numbers (percentages), mean (standard deviation) or medians [interquartile range]. P-values of <0.05 were considered statistically significant.

Abbreviations: ACE inhibitors, angiotensin converting enzyme inhibitors; AS, aortic stenosis; AVA, aortic valve area; BMI, body mass index; CLT, clot lysis time; CRP, C-reactive protein; Lp(a), lipoprotein (a); OxPL, oxidized phospholipids; PAI-1, plasminogen activator

inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator; V_{max}, peak transvalvular velocity.

Table S2. Factors associated with clot lysis time (CLT) in patients with severe aortic stenosis and Lp(a) ≥50 mg/dL

	Univariable		
Variable	Estimate	95% CI	P value
OxPL, µg/mL	9.17	6.12;12.22	<0.001
Lp(a), mg/dL	0.14	0.09;0.19	<0.001
PAI-1, ng/mL	0.47	0.26;0.69	<0.001
TAFI, %	0.15	0.02;0.28	0.03
tPA, ng/mL	0.77	-0.29;1.83	0.15
Plasminogen, %	-0.13	-0.29;0.03	0.12
α_2-antiplasmin, %	0.07	-0.22;0.36	0.63
V_{max}, m/s	10.9	5.00;16.95	<0.001
Mean gradient, mmHg	0.31	0.09;0.52	<0.001
Max gradient, mmHg	0.29	0.14;0.45	<0.001
AVA, cm^2	-12.4	-30.2;5.49	0.17
Age, years	-0.29	-0.54;-0.04	0.02
BMI, kg m^{-2}	0.29	-0.17;0.75	0.21
Fibrinogen, g/L	2.29	-0.39;4.97	0.09
Creatinine, µmol/L	-0.04	-0.18;0.11	0.59

CRP, mg/L	0.11	-0.49;0.71	0.72
Glucose, mmol/L	0.18	-3.86;4.22	0.93
Total cholesterol mmol/L	1.6	-0.46;3.66	0.13
LDL-cholesterol, mmol/L	1.83	-0.35;4.00	0.10
HDL-cholesterol, mmol/L	0.46	-1.73;2.66	0.67
Triglycerides, mmol/L	-0.47	-3.57;2.63	0.76
	Multivariable		
OxPL, µg/mL	7.87	4.71;11.03	<0.001
Plasminogen, %	-0.11	-0.23;0.01	0.08
Age, years	-0.19	-0.39;0.02	0.07
Fibrinogen, g/L	1.31	-0.78;3.40	0.21

CI, confidence interval. Abbreviations explained in Table S1. Multivariable model adjusted for BMI.

cells

MDPI

Communication

PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis

Magdalena Kopytek [1,2], Michał Ząbczyk [1,2], Piotr Mazur [3,4], Anetta Undas [1,2] and Joanna Natorska [1,2,*]

[1] Thromboembolic Disorders Department, Institute of Cardiology, Jagiellonian University Medical College, 80 Pradnicka St., 31-202 Krakow, Poland; m.kopytek@szpitaljp2.krakow.pl (M.K.); michalzabczyk@op.pl (M.Z.); anetta.undas@uj.edu.pl (A.U.)
[2] Krakow Centre for Medical Research and Technologies, John Paul II Hospital, 80 Pradnicka St., 31-202 Krakow, Poland
[3] Department of Cardiovascular Surgery, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, USA
[4] Department of Cardiovascular Surgery and Transplantology, Institute of Cardiology, Jagiellonian University Medical College, 80 Pradnicka St., 31-202 Krakow, Poland
* Correspondence: j.natorska@szpitaljp2.krakow.pl; Tel.: +48-12-614-21-08; Fax: +48-12-614-21-20

Abstract: Aortic stenosis (AS) is associated with hypofibrinolysis, but its mechanism is poorly understood. We investigated whether LDL cholesterol affects plasminogen activator inhibitor 1 (PAI-1) expression, which may contribute to hypofibrinolysis in AS. Stenotic valves were obtained from 75 severe AS patients during valve replacement to assess lipids accumulation, together with PAI-1 and nuclear factor-κB (NF-κB) expression. Five control valves from autopsy healthy individuals served as controls. The expression of PAI-1 in valve interstitial cells (VICs) after LDL stimulation was assessed at protein and mRNA levels. PAI-1 activity inhibitor (TM5275) and NF-κB inhibitor (BAY 11-7082) were used to suppress PAI-1 activity or NF-κB pathway. Clot lysis time (CLT) was performed to assess fibrinolytic capacity in VICs cultures. Solely AS valves showed PAI-1 expression, the amount of which was correlated with lipid accumulation and AS severity and co-expressed with NF-κB. In vitro VICs showed abundant PAI-1 expression. LDL stimulation increased PAI-1 levels in VICs supernatants and prolonged CLT. PAI-1 activity inhibition shortened CLT, while NF-κB inhibition decreased PAI-1 and SERPINE1 expression in VICs, its level in supernatants and shortened CLT. In severe AS, valvular PAI-1 overexpression driven by lipids accumulation contributes to hypofibrinolysis and AS severity.

Keywords: aortic stenosis; fibrinolysis; LDL; nuclear factor kappa B; plasminogen activator inhibitor 1; valve interstitial cells

Citation: Kopytek, M.; Ząbczyk, M.; Mazur, P.; Undas, A.; Natorska, J. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. *Cells* **2023**, *12*, 1402. https://doi.org/10.3390/cells12101402

Academic Editor: Ezequiel Álvarez

Received: 23 March 2023
Revised: 11 May 2023
Accepted: 15 May 2023
Published: 16 May 2023

1. Introduction

Aortic stenosis (AS) is the most common acquired valvular heart disease in the elderly population from industrialized countries with no available pharmacological treatment to slow down or inhibit the disease progression [1]. The pathomechanism of aortic valve calcification is a complex and tightly regulated process, associated with activation of multiple molecular pathways. It involves accumulation of lipoproteins, chronic inflammation and activation of the coagulation system [2,3]. These actions lead to stimulation of valvular interstitial cells (VICs), playing a pivotal role in valvular calcification [4]. AS patients are also characterized by hypofibrinolysis, expressed as prolonged clot lysis time (CLT). CLT provides information about overall plasma fibrinolytic capacity, reflecting the simultaneous effect of procoagulant and profibrinolytic factors [5]. Impaired fibrinolysis was shown to be positively associated with AS severity and the thickness of fibrin deposited within stenotic valves [6,7]. However, the role of the fibrinolytic system in the development and progression of AS is not yet understood. The expression of major fibrinolytic protein—plasminogen, and its inhibitor—plasminogen activator inhibitor type 1 (PAI-1),

controlling the activity of both tissue (tPA) and urokinase plasminogen activators (uPA), has been demonstrated within human stenotic aortic valves, as well as in vitro in valvular myofibroblasts cultures [8]. PAI-1 is of key importance in various chronic and acute pathophysiological processes [9], and the expression of *SERPINE1* gene encoding *PAI-1* is controlled by nuclear factor- κB (NF-κB) transcription pathway, which can be induced through pro-fibrotic signaling cascades [10]. PAI-1 secretion is strongly influenced by pro-inflammatory cytokines [11], abundantly present within stenotic aortic valves [12]. Our recent study demonstrated NF-κB expression within stenotic aortic valves as well as in VICs on both protein and mRNA levels [13]. Recently, Siudut et al. [14] showed in severe AS patients that serum lipids and apolipoproteins predicted hypofibrinolysis measured as CLT. However, it is still not clear whether impaired fibrinolysis contributes to faster AS progression or that more severe AS leads to hypofibrinolysis. Considering that LDL mediates inflammation within stenotic valves, we decided to investigate this particular lipoprotein in the context of hypofibrinolysis.

The aim of this study was to evaluate whether LDL is responsible for PAI-1 overexpression as the main factor driving hypofibrinolysis in AS.

2. Materials and Methods

2.1. Patients

We enrolled 75 patients with symptomatic severe AS between February 2019 and January 2021. All AS patients underwent first-time elective surgical aortic valve replacement at the Department of Cardiovascular Surgery and Transplantology at the John Paul II Hospital, Krakow, Poland. Data on medical history, current treatment and demographics were collected using a standardized questionnaire. Severe AS was defined as aortic valve area (AVA) < 1 cm^2 and/or mean transvalvular pressure gradient (PG_{mean}) ≥ 40 mmHg [15] on transthoracic echocardiography. All studies were evaluated by an experienced cardiologist. Arterial hypertension was diagnosed based on a history of hypertension (blood pressure > 140/90 mmHg) or preadmission antihypertensive treatment. Hypercholesterolemia was diagnosed based on total cholesterol level ≥5.0 mM, medical records or cholesterol-lowering therapy.

The exclusion criteria for AS patients were: atherosclerotic vascular disease requiring revascularization, acute infection including infective endocarditis, rheumatic AS, diabetes mellitus, advanced chronic kidney disease, need for concomitant valvular surgery (e.g., mitral valve repair), percutaneous coronary intervention, recent (<3 months) acute coronary syndrome or cerebrovascular episode, diagnosed malignancy, and pregnancy. The valvular anatomy was confirmed intraoperatively, and patients with bicuspid valve and root/ascending aortic dilatation requiring intervention were excluded from the study. The diagnosis of atherosclerosis was based on angiographically documented coronary artery stenosis >20% diameter and such patients were excluded from the study.

2.2. Laboratory Analysis

Fasting venous blood was drawn from the antecubital vein between 7:00 and 9:00 AM in AS patients (before aortic valve replacement). Citrated blood (9:1 of 0.106 M sodium citrate) was centrifuged at 2500× *g* for 20 min at 20 °C, while blood drawn into EDTA or serum tubes was centrifuged at 1600× *g* for 10 min at 4 °C and stored at −80 °C until analysis. Routine laboratory assays were used to determine glucose, creatinine, lipid profile, C-reactive protein (CRP), and fibrinogen.

2.3. Aortic Valves Preparation

Aortic valves were collected during open heart surgery. One valvular leaflet was used for in loco analysis and one for in vitro cell cultures. The third leaflet was secured for future analysis. To decalcify the incised aortic valves, they were incubated in 15% EDTA (Sigma -Aldrich, St. Louis, MO, USA) at 4 °C for 10 days. Decalcification was confirmed by calcium determination in 6 M HCl. After treatment, valves were rinsed with phosphate buffered saline (PBS), embedded in Shandon Cryomatrix frozen embedding medium (Thermo Fisher

Scientific, Kalamazoo, MI, USA) for tissue cryopreservation and cryosectioned vertically into 4.5 µm slices using a Leica CM 1520 cryostat. Transverse sections were taken from the mid and commissural areas of the leaflet and stored at −20 °C until immunostaining. The control valves (n = 5) were obtained at autopsy from apparently healthy individuals of similar age without cardiac disorders.

2.4. Histochemical and Immunofluorescence Staining

Valvular staining was performed on randomly selected 50 AS and 5 control valves. Lipid detection was performed using Sudan black dye followed by counterstaining with nuclear fast red solution. Immunofluorescence was conducted according to the previously described protocol [16] using primary antibodies against PAI-1 (1:500; Abcam, Cambridge, UK), tPA (1:100; Novus Biologicals, Centennial, CO, USA), α_2-antiplasmin (1:250; Santa Cruz Biotechnology, Dallas, TX, USA), plasminogen (1:500; GeneTex, Irvine, CA, USA), fibrin degradation products (D-dimer, 1:100; Bioss Antibodies, Woburn, MA, USA) and NF-κB (p65, 1:500, Abcam) and the corresponding secondary donkey or goat antibodies conjugated with AlexaFluor 488 or 594 (Abcam) (1:1000). Double-label immunofluorescence analysis was performed for PAI-1 and NF-κB. A negative IgG isotype control was performed routinely. The percentage of immunopositive areas and the fluorescence intensity (FI) were calculated as described previously [16]. The data were analyzed by two independent investigators blinded to the sample origin. The intra- and inter-observer variability was below 7%.

2.5. Valve Interstitial Cells In Vitro Cultures

VICs were isolated from stenotic aortic valves as previously described [17]. Experiments were performed on VICs between their third and fifth passages. When the cells reached 90–95% confluence, they were subcultured in 6-well plates in concentration of 1×10^5 for immunofluorescence staining and 2×10^5 for mRNA analysis in 2 mL of cell culture medium per well. VICs cultured in a standard medium (DMEM: low glucose medium, without L-glutamine and with sodium pyruvate; Biowest, Nuaillé, France) served as a negative control. To initiate the process of calcification, VICs were cultured in a calcification medium as previously described by us [18]. To induce inflammation, VICs were cultured in the calcification medium supplemented with TNF-α (50 ng/mL; Santa Cruz Biotechnology) [18] or LDL (300 µg/mL, reflecting hyperlipidemia; Sigma-Aldrich) [19].

The VICs expression of fibrinolytic proteins was assessed using immunofluorescence, as described above.

Mechanistic experiments were performed to suppress PAI-1 activity or to inhibit NF-κB transcription pathway. PAI-1 inhibitor—TM5275 (MedChemExpress LLC, Monmouth Junction, NJ, USA), which converts PAI-1 to its inactive form—was added 30 min before LDL (final concentration, 100 µM) [20]. To inhibit NF-κB, BAY 11-7082 (Sigma-Aldrich, final concentration, 10^{-6} M) was added 30 min before TNF-α or LDL stimulation [13,21].

All VICs were cultured for 7 days. The number of immunopositive cells was quantified per 100 consecutive cells per slide and 3 slides per each condition. Each experiment was repeated three times using VICs isolated from randomly selected stenotic valves.

VICs supernatants were collected after each experiment and stored at −20 °C until analysis. PAI-1 concentration in supernatants was assayed quantitatively using commercial ELISA kit (Hyphen Biomed, Neuville-sur-Oise, France) in accordance with manufacturer's instructions.

2.6. CLT in VICs Supernatants

Modified CLT was performed based on Pieters method [22] using a mixture of human PAI-1-deficient plasma (25 µL; Innovative Research, Novi, MI, USA) and 5 µL of supernatants containing VICs-released PAI-1. Supernatants were diluted 1:5 due to the fact that undiluted supernatants from VICs treated with pro-inflammatory factors prolonged CLT > 300 min, which is considered as the upper limit of detection. To remove cell debris from VICs, supernatant samples were centrifuged at 1000× g for 5 min. Briefly, 30 µL

mixture of human PAI-1 deficient plasma and VICs supernatants was mixed with 15 μM phospholipid vesicles (Rossix, Mölndal, Sweden), 15 mM $CaCl_2$, 20 ng/mL tPA (Boehringer Ingelheim, Ingelheim am Rhein, Germany) and 0.5 U/mL human thrombin (Merck, Darmstadt, Germany). The turbidity was measured at 405 nm, at 37 °C. CLT was defined as the time from the midpoint of the clear-to-maximum-turbid transition to the midpoint of the maximum-turbid-to-clear transition. The experiment was repeated three times using VICs supernatants from other cell cultures. All samples were tested in triplets. Intra-assay and inter-assay coefficients of variation were <8%.

2.7. Relative Quantification of Transcripts by Real-Time PCR

A total of 400 ng of VICs RNA was reverse transcribed to single strand cDNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. The cDNA was amplified with TaqMan Gene Expression Assays (Hs00167155_m1 for PAI-1, Gene Symbol: *SERPINE1*) containing both primers and probe on an ABI PRISMR 7900HT Fast Real-Time PCR System (Applied Biosystems). Beta-actin (Hs99999903_m1, human ACTB Endogenous Control FAM/MGB Probe, Non-Primer Limited; Applied Biosystems) was used as a housekeeping gene. The comparative threshold cycle method ($R = 2^{-\Delta\Delta Ct}$) was applied to analyze the obtained data.

2.8. Statistical Analyses

All statistics were performed using the STATISTICA software (Version 13.3, TIBCO Software, Palo Alto, CA, USA). Categorical variables were presented as numbers and percentages, while continuous variables were expressed as mean ± standard deviation (SD) or median and interquartile range [IQR]. Categorical variables were analyzed by Pearson's χ^2 or two-tailed Fisher's exact test. Normality was analyzed by the Shapiro–Wilk test. Differences between the groups were compared using the Student's *t*-test or Mann–Whitney U test, as appropriate. Analysis of variance (ANOVA) was used to compare continuous variables between multiple groups. Post-hoc comparisons were performed with the Tukey–Kramer HSD test. Associations between variables were calculated using Pearson's or Spearman's correlation coefficients, as appropriate. p-value < 0.05 was considered statistically significant.

3. Results

3.1. Patients' Characteristics

Baseline characteristic of AS patients is shown in Table 1. Most of the AS patients were treated with angiotensin converting enzyme inhibitors, beta-blockers and acetylsalicylic acid. Due to concomitant atrial fibrillation, 12 (16%) AS patients were taking non-vitamin K antagonist oral anticoagulants (NOACs), with an average duration of treatment less than 2 years (19.8 ± 11.8 months) (Table 1). Of note, AS patients not receiving statin treatment (n = 18) compared to those treated with statins were characterized by increased concentrations of LDL cholesterol (3.2 [2.7–4.2] mmol/L vs. 2.3 [1.9–2.9] mmol/L, $p = 0.0013$).

Table 1. Baseline characteristics of patients with aortic stenosis (AS).

Variable	AS Patients (n = 75)
Age, years	66 [60–71]
Male, n (%)	46 (61.3)
BMI, kg m^{-2}	28 [25.7–30.6]
Risk factors, n (%)	
Arterial hypertension	67 (89.3)
Hypercholesterolemia	64 (85.3)
Current smoking	13 (17.3)
Medications, n (%)	
Beta-blockers	54 (72)

Table 1. *Cont.*

Variable	AS Patients (n = 75)
Acetylsalicylic acid	51 (68)
ACE inhibitors	48 (64)
Statins	57 (76)
Rivaroxaban	5 (6.7)
Apixaban	3 (4)
Dabigatran	4 (5.3)
Echocardiographic parameters	
Mean gradient, mmHg	50 [44–58]
Maximal gradient, mmHg	82 [74–94]
AVA, cm^2	0.8 [0.7–0.9]
LVEF, %	60 [55–65]
Laboratory investigations	
Fibrinogen, g/L	3.4 ± 0.74
Creatinine, µmol/L	76 [70–92]
CRP, mg/L	2.0 [1.0–4.0]
Glucose, mmol/L	5.4 [5.0–5.6]
Total cholesterol mmol/L	4.1 [3.5–4.8]
LDL cholesterol, mmol/L	2.5 [2.0–3.3]
HDL cholesterol, mmol/L	1.6 [1.3–1.7]
Triglycerides, mmol/L	1.1 [0.9–1.7]

Data presented as numbers (percentages), mean ± standard deviation or medians [interquartile range]. Abbreviations: ACE inhibitors, angiotensin converting enzyme inhibitors; BMI, body mass index; CRP, C-reactive protein; LVEF, left ventricular ejection fraction.

3.2. In Loco Studies

Massive intracellular lipid accumulation was observed within stenotic aortic valves (18.3 ± 2% of immunopositive area), but not in control leaflets (Figure 1A,B). Valvular expression of all studied fibrinolytic proteins and their inhibitors, along with D-dimer, was detected within stenotic aortic leaflets, mainly on the aortic side of the leaflets, but not in control valves (Figures 1 and 2). The expression of PAI-1 (24.6 ± 4.1%) was observed in the fibrosa and spongiosa layers and presented a condensed pattern of fluorescence (Figure 1D). NF-κB expression was not detected within control valves (Figure 1E), while double staining revealed 84% co-expression of valvular PAI-1 and NF-κB within stenotic valves (Figure 1F). Valvular PAI-1 expression positively correlated with lipids accumulation and AS severity measured as PGmean (Figure 3A,B). The expression of plasminogen (16.6 ± 3.9%), α_2-antiplasmin (12.2 ± 4.1%) and tPA (8.4 ± 3.6%) was observed in the subendothelial and fibrosa layers (Figure 2). Valvular expression of D-dimer was observed in the fibrosa and partially in spongiosa layers (Figure 2). However, the pattern of fluorescence was diffused; therefore, the FI was determined instead of the positive areas percentage. Almost 200% higher FI was observed for D-dimer-positive areas (1136 ± 223 vs. 391 ± 129 FI; $p < 0.0001$).

No differences between patients treated with NOACs or NOAC naïve were found in valvular expression of the investigated proteins (for PAI-1: 22.9% vs. 24.7%, for plasminogen: 15.6% vs. 16.8%, for α_2-antiplasmin: 11.5% vs. 12.4%, for tPA: 8.7% vs. 8.1%; all $p > 0.05$, respectively).

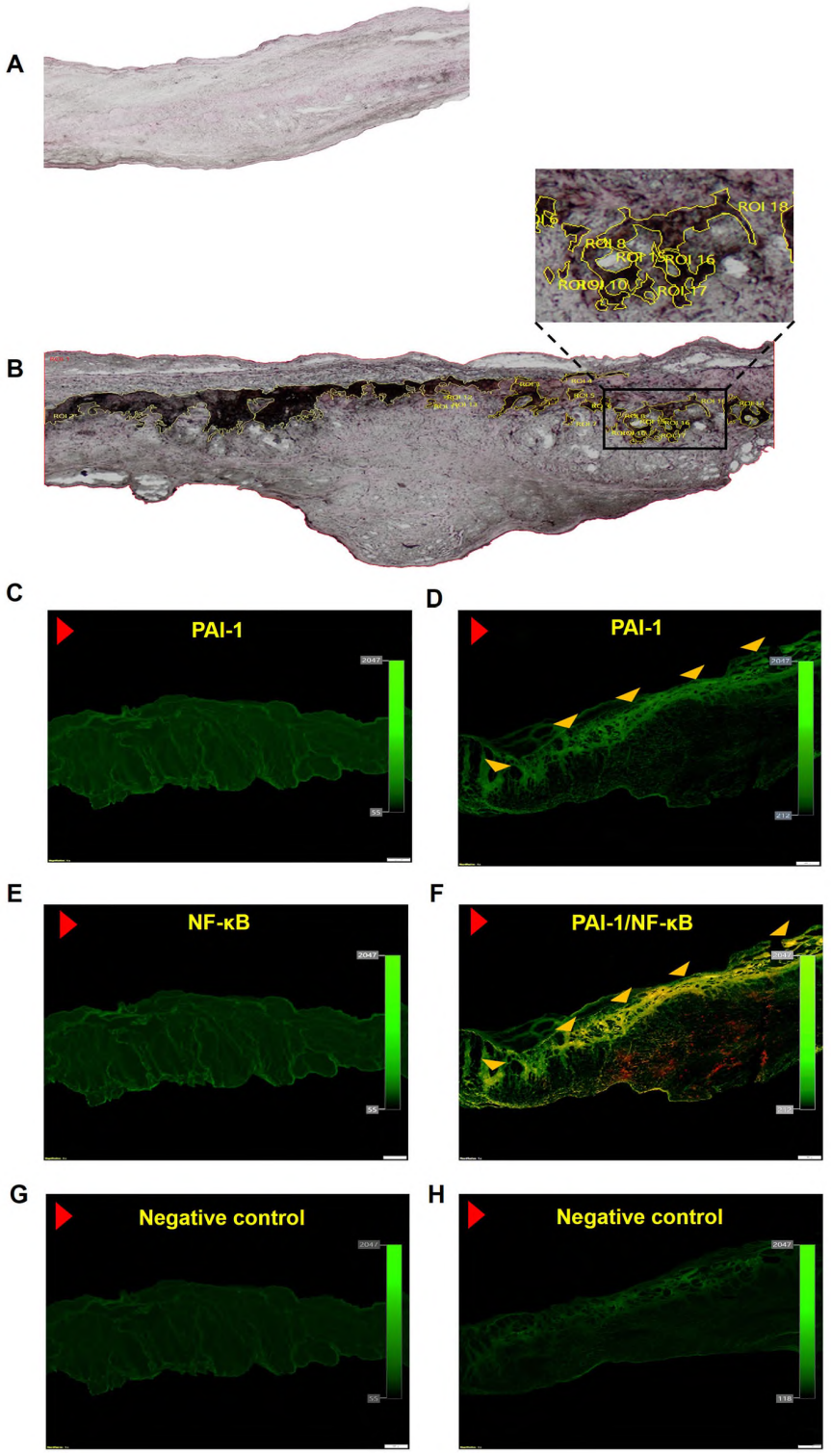


Figure 1. Valvular lipids accumulation together with plasminogen activator inhibitor 1 (PAI-1) and nuclear factor-κB (NF-κB) expression. Representative microphotographs of lipids accumulation in (**A**) control valve and (**B**) stenotic valve, original magnification 4×. Regions of interest (ROI) are marked in yellow. Valvular expression of (**C**) PAI-1 in control leaflets, (**D**) PAI-1 in stenotic leaflets. (**E**) NF-κB in control leaflets, and (**F**) colocalization (yellow) of PAI-1 (green) and NF-κB (red). IgG isotype control for healthy (**G**) and stenotic (**H**) valves. Red arrowhead indicates aortic side of the leaflet; yellow arrowheads indicate the immunopositive areas. Scale bar 200 µm, original magnification 4×.

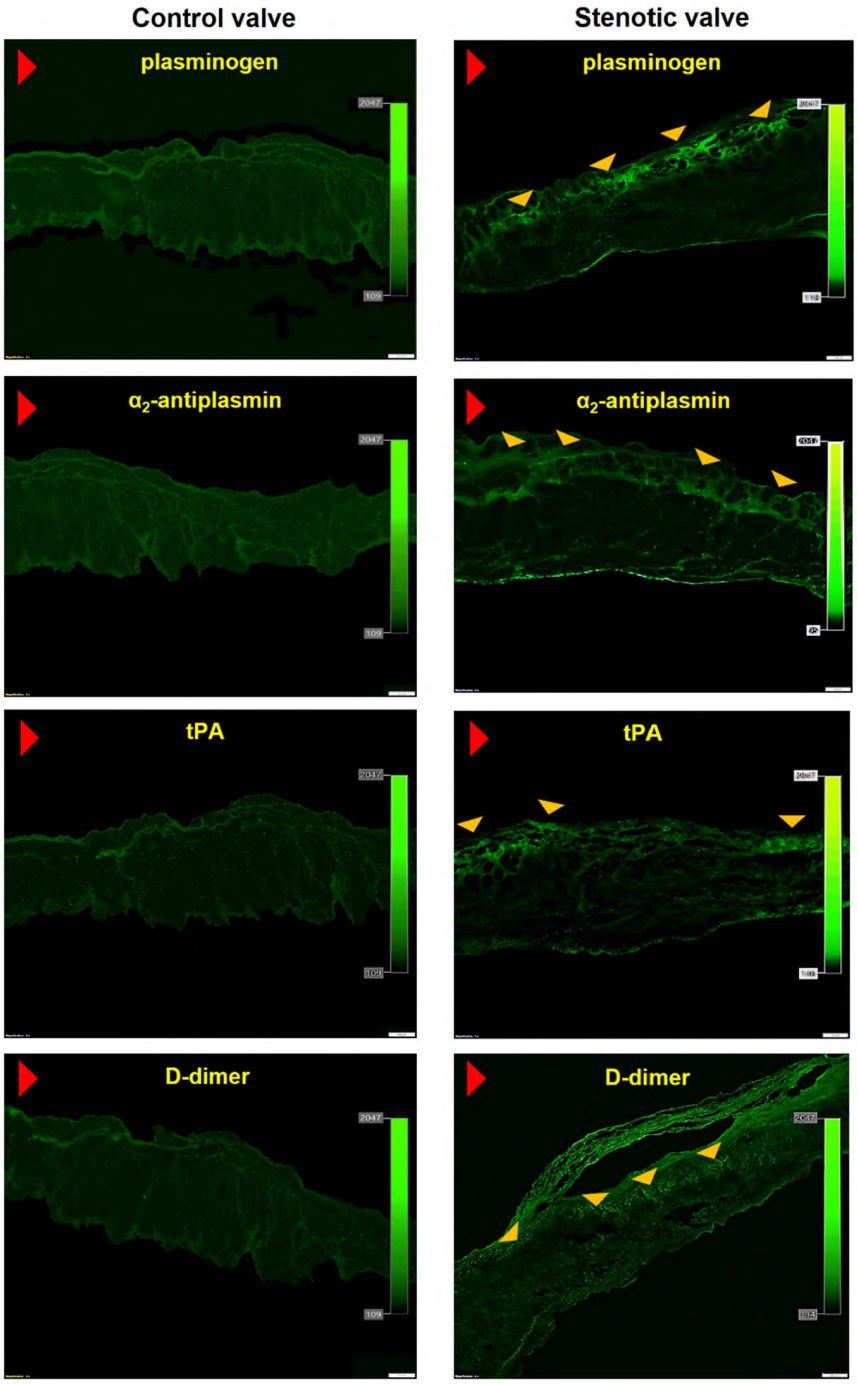


Figure 2. Valvular expression of fibrinolytic proteins and inhibitors. Representative microphotographs showing valvular expression of plasminogen, α_2-antiplasmin, tissue plasminogen activator (tPA), and D-dimer in control (left panel) and stenotic aortic leaflets (right panel). Red arrowhead indicates aortic side of the leaflet; yellow arrowheads indicate the immunopositive areas. Scale bar 200 µm, original magnification 4×.

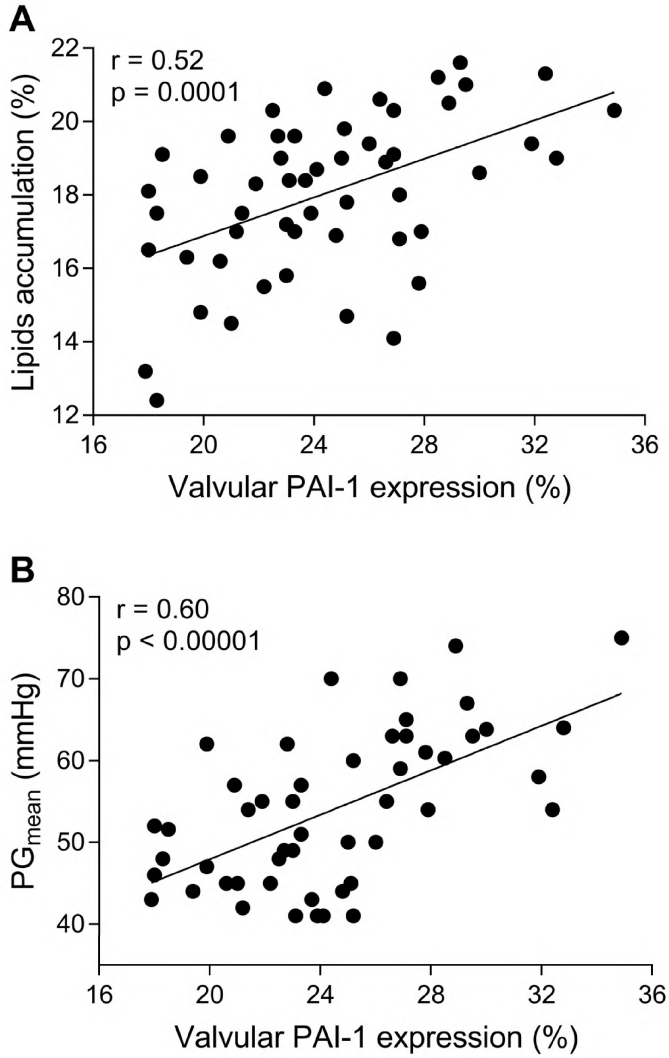


Figure 3. Valvular PAI-1 correlated with lipids accumulation and AS severity. The scatterplots showing associations between (**A**) valvular PAI-1 expression and lipids accumulation, (**B**) valvular PAI-1 expression and mean transvalvular pressure gradient (PG_{mean}). Associations between continuous variables were calculated using Pearson's or Spearman's correlation coefficients. N = 50, *p*-values of <0.05 were considered statistically significant.

3.3. *In Vitro Studies*

Independently of culture condition, VICs showed constant expression of PAI-1 (100% of cells) (Figure 4A). VICs cultured in control or calcification medium did not express tPA or α_2-antiplasmin, while plasminogen was poorly expressed in VICs cultured in the calcification medium (12 ± 2% of cells) (Figure 4B).

After TNF-α or LDL stimulation, 18 ± 3% or 21 ± 3% of VICs, respectively, expressed plasminogen, and 15 ± 2% or 17 ± 3% of VICs, respectively, expressed α_2-antiplasmin but a trace expression of tPA (<10% of VICs) was observed (Figure 4B). After the use of TM5275 inhibitor, which was supposed to reduce the ability of PAI-1–tPA complex formation, the expression of tPA in VICs cultures was still at a very low level (Figure 5A).

LDL stimulation increased PAI-1 levels in VICs supernatants by 32% (p = 0.0005) compared to VICs cultured in the calcification medium (Figure 5B). Similarly, TNF-α stimulation increased PAI-1 concentrations in supernatants by 25% (p = 0.011) compared to VICs cultured in the calcification medium.

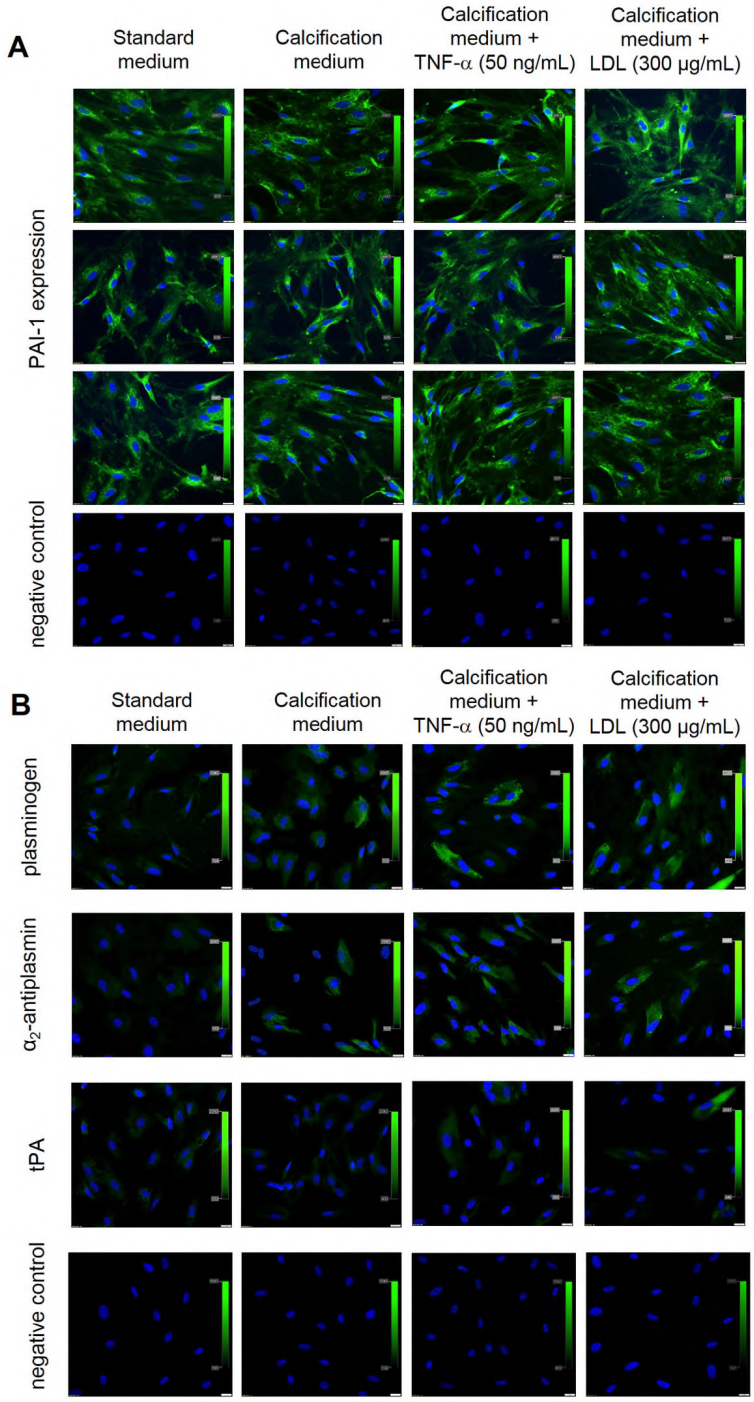


Figure 4. Expression of fibrinolytic proteins in valve interstitial cells (VICs) according to different culture conditions. Representative microphotographs of VICs cultured in different conditions tested for (**A**) PAI-1 expression and IgG isotype control, (**B**) plasminogen, α_2-antiplasmin or tPA expression, and IgG isotype control. Cell nuclei are stained blue (DAPI), protein expression is green. Scale bar 20 µm, original magnification 40×. The experiment was repeated three times using VICs isolated from different valves.

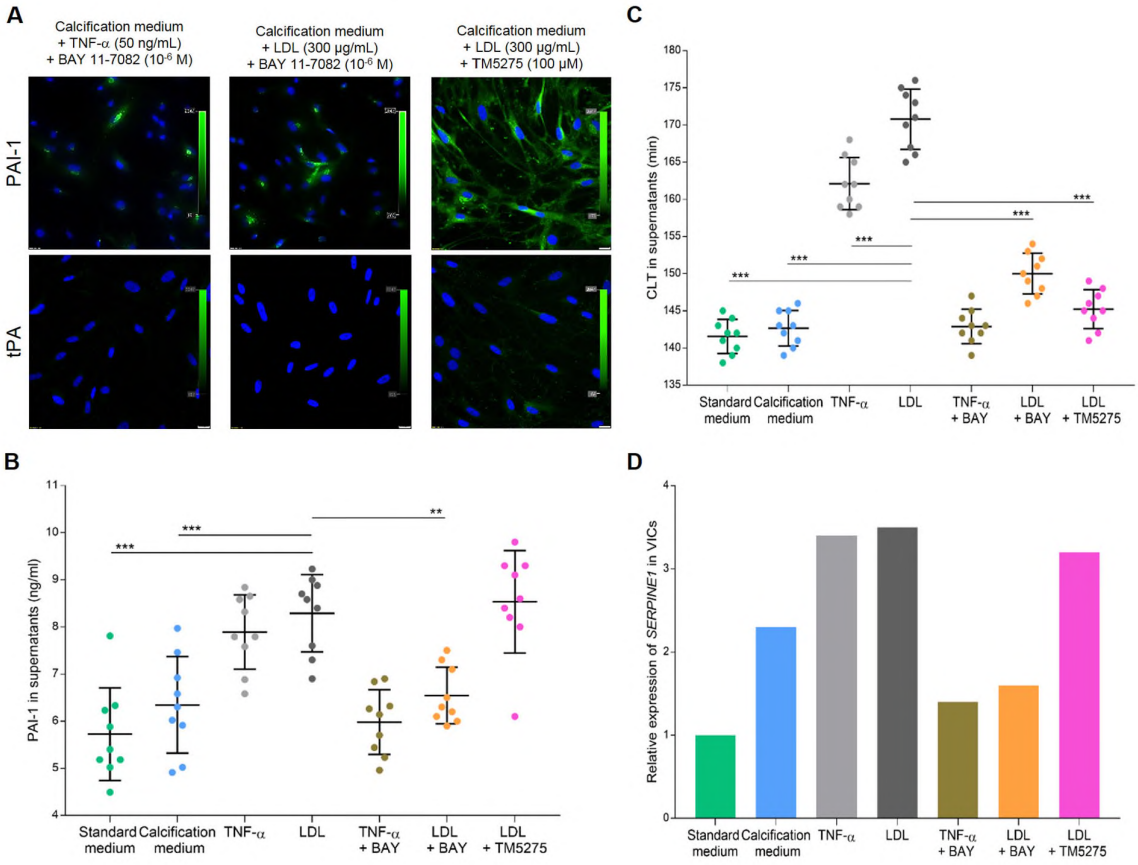


Figure 5. Effect of BAY 11-7082 and TM5275 on PAI-1 inhibition in VICs cultures. (**A**) Representative microphotographs of PAI-1 and tPA immunostaining in VICs stimulated with TNF−α or LDL in combination with NF−κB inhibitor (BAY 11-7082) or PAI-1 inhibitor (TM5275). Cell nuclei are stained blue (DAPI), PAI−1 is green. Scale bar 20 µm, original magnification 40×. The dot plots show (**B**) PAI−1antigen levels and (**C**) clot lysis time (CLT) in VICs supernatants from different culture conditions. Data presented as mean ± SD. ** $p < 0.01$ and *** $p < 0.001$ compared to VICs cultured in the calcification medium supplemented with LDL. Statistical analysis was performed by ANOVA and post-hoc tests. (**D**) Box plot shows relative gene expression of *SERPINE1* in VICs cultured in different conditions. Real-time PCR data are presented as mRNA expression fold change. All experiments were repeated three times using cells, supernatants or lysates from different VICs cultures; all samples were tested in triplets.

Mechanistic experiments revealed that PAI-1 expression in VICs stimulated with LDL was strongly suppressed by the NF-κB inhibitor (about 80% of cells; Figure 5A) and its concentration in VICs supernatants decreased by 22% ($p = 0.0025$; Figure 5B) compared to VICs cultured with LDL alone. The TM5275 PAI-1 inhibitor did not downregulate PAI-1 expression in VICs cultures (Figure 5A) or the level of PAI-1 antigen in VICs supernatants ($p = 0.99$; Figure 5B) compared to VICs cultured with LDL alone.

LDL stimulation prolonged CLT by 20% ($p < 0.001$) compared to calcification medium (Figure 5C). Regardless of LDL stimulation, NF-κB inhibition as well as PAI-1 activity inhibition shortened CLT by 12% and 15%, respectively, compared to VICs stimulated with LDL alone (both $p < 0.001$; Figure 5C).

Relative gene expression analysis in VICs cultures showed that stimulation with calcification medium resulted in a 1.3-fold increase in *SERPINE1* expression compared to standard medium (Figure 5D). Pro-inflammatory treatment of VICs resulted in about 2.5-fold increase in *SERPINE1* expression compared to standard medium, while NF-κB inhibition suppressed the *SERPINE1* expression by about 2-fold compared to VICs stimulated with TNF-α or LDL alone (Figure 5D). The TM5275 inhibitor treatment did not

downregulate *SERPINE1* expression compared to VICs treated with LDL alone, and it was 2.2-fold higher than in VICs cultured in the standard medium (Figure 5D).

4. Discussion

Our study showed for the first time that, in severe AS patients, PAI-1 overexpression is driven by LDL activation of VICs. We also showed constant expression of PAI-1 in VICs, regardless of pro-inflammatory stimulation, while the expression of profibrinolytic proteins, such as plasminogen and tPA, was observed only after VICs activation. Moreover, for the first time we showed valvular α_2-antiplasmin expression.

Valvular fibrin accumulation accounting for about 40% of the total valve area suggested impaired fibrinolysis in AS [23]. The current study reported the presence of D-dimer, a fibrin degradation product, within all valvular layers, demonstrating that fibrinolysis occurs in loco and that VICs PAI-1 overexpression contributed to hypofibrinolysis. The abundant amounts of PAI-1 with a condensed pattern of expression were found within AS valves, suggesting that this protein is synthesized de novo. Of note, a similar PAI-1 expression pattern has been reported in lung cancer [24]. We also showed for the first time expression of α_2-antiplasmin, both within stenotic valves and in in vitro VICs cultures. The valvular expression of α_2-antiplasmin was weak and localized subendothelially. Similarly, the α_2-antiplasmin expression in VICs was very weak, even after the pro-inflammatory stimulation with TNF-α or LDL. It remains to be established, using quantitative methods such as RT-PCR or proteomics, whether VICs have the ability to synthesize α_2-antiplasmin. It may be of importance to better understand VICs contribution to hypofibrinolysis in AS.

We confirmed that VICs stimulated with LDL showed enhanced *SERPINE1* expression and PAI-1 synthesis, resulting in fibrinolysis inhibition. NF-κB inhibitor suppressed PAI-1 expression on both protein and mRNA levels in VICs along with shortened CLT, regardless of pro-inflammatory stimulation with TNF-α or LDL. On the other hand, PAI-1 activity inhibition with TM5275 did not affect *SERPINE1* expression and PAI-1 antigen level but as expected promoted fibrinolysis. Therefore, we suspect that LDL may contribute to the imbalance between coagulation and fibrinolysis within stenotic aortic valves via inflammatory stimulation of VICs, resulting in PAI-1 overexpression. Valvular fibrin deposition, at least in part driven by limited ability to fibrin dissolution, contributes to valve dysfunction, hemodynamic disturbances, and increased shear stress, which activates NF-κB-related expression of pro-inflammatory genes [25]. Increased shear stress can also stimulate endothelial cells to release PAI-1 [26], contributing to systemic hypofibrinolysis. Alexopoulos et al. [27] observed that PAI-1 was strongly implicated in the pathogenesis of atherosclerosis, which shares similar mechanisms with AS. Moreover, in a murine model of atherosclerosis PAI-1 inhibitors, PAI-039 and MDI-2268, inhibited atherosclerosis development [28]. Our study also suggests that NF-κB pathway could also be a potential target for PAI-1 inhibition. NF-κB pathway inhibitors are now extensively tested in clinical studies for therapeutic intervention [29].

We also showed low in loco expression of plasminogen and tPA localized subendothelially and the trace expression of both proteins in VICs cultures, suggesting that they are released by valvular endothelial cells, rather than synthesized by VICs. Kochtebane et al. [8] identified small amounts of plasminogen, uPA, tPA, and PAI-1 within all the three tissue layers of human stenotic aortic valves and in myofibroblast cultures, however, with a large variability between valves. uPA was the only protein with enzymatic activity in myofibroblasts lysates [8]. The myofibroblasts expression of free tPA was not confirmed by the Western blot, and tPA was shown exclusively as a complex with PAI-1. We observed negligible amounts of free tPA in VICs cultures, even after the use of TM5275 inhibitor, which reduces the ability to form PAI-1–tPA complexes, reveal free tPA, and convert active PAI-1 to its inactive form [20]. Our data suggest that tPA synthesis by VICs is very limited and its release is associated with valvular endothelium or delivered to stenotic aortic valves with the bloodstream. In our opinion, the disproportion between expression of PAI-1 and profibrinolytic factors contributes to hypofibrinolysis in AS.

This study has several limitations. First, the group size was limited, and type II errors cannot be excluded. However, the study represents typical patients with symptomatic severe AS in clinical practice. Second, the valvular proteins expression was determined semi-quantitatively and estimations may be less precise. However, microscopic analysis was performed by two independent experienced investigators. Third, we did not assess the expression of uPA. We assessed hypofibrinolysis based on plasma CLT, which is the tPA-dependent model. tPA activation requires binding to fibrin, while uPA is fibrin independent and activates plasminogen in solution. Lastly, this study was performed in individuals with severe AS and tricuspid valves; thus, our results cannot be directly extrapolated to individuals with mild, moderate or bicuspid AS. Further studies on larger cohorts are needed to eliminate an influence of phenotypic aspects of the valve and aortic root characteristics.

5. Conclusions

Our data demonstrate that, in severe AS patients, PAI-1 is abundantly released by VICs, probably due to chronic valvular inflammation caused by LDL. PAI-1 overexpression leads to disturbed balance between coagulation and fibrinolysis, which may contribute to valvular dysfunction and AS progression.

Author Contributions: M.K. designed the experiments, performed experiments, acquired and analyzed data, drafted and revised the manuscript. M.Z. contributed to the experimental design and interpretation of data and drafted the manuscript. P.M. recruited participants, acquired and analyzed data. A.U. analyzed the data and critically revised the manuscript. J.N. designed the experiments, acquired and analyzed data, drafted and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Polish National Science Centre (UMO-2018/29/B/NZ5/02629 to J.N.) and the reserve of the Dean of the Faculty of Medicine, Jagiellonian University Medical College (N41/DBS/000003).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethical Committee (Krakow Medical District Chamber, Poland, approval number: 8/KBL/OIL/2019).

Informed Consent Statement: All participants provided written informed consent for inclusion before they participated in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ongoing experiments.

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

References

1. Lindroos, M.; Kupari, M.; Heikkilä, J.; Tilvis, R. Prevalence of aortic valve abnormalities in the elderly: An echocardiographic study of a random population sample. *J. Am. Coll. Cardiol.* **1993**, *21*, 1220–1225. [CrossRef] [PubMed]
2. Pawade, T.A.; Newby, D.E.; Dweck, M.R. Calcification in Aortic Stenosis: The Skeleton Key. *J. Am. Coll. Cardiol.* **2015**, *66*, 561–577. [CrossRef] [PubMed]
3. Natorska, J.; Undas, A. Blood coagulation and fibrinolysis in aortic valve stenosis: Links with inflammation and calcification. *Thromb. Haemost.* **2015**, *114*, 217–227. [CrossRef] [PubMed]
4. Liu, A.C.; Joag, V.R.; Gotlieb, A.I. The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. *Am. J. Pathol.* **2007**, *171*, 1407–1418. [CrossRef] [PubMed]
5. Stubblefield, W.B.; Alves, N.J.; Rondina, M.T.; Kline, J.A. Variable Resistance to Plasminogen Activator Initiated Fibrinolysis for Intermediate-Risk Pulmonary Embolism. *PLoS ONE* **2016**, *11*, e0148747. [CrossRef]
6. Natorska, J.; Wypasek, E.; Grudzień, G.; Sadowski, J.; Undas, A. Impaired fibrinolysis is associated with the severity of aortic stenosis in humans. *J. Thromb. Haemost.* **2013**, *11*, 733–740. [CrossRef] [PubMed]
7. Siudut, J.; Natorska, J.; Wypasek, E.; Wiewiórka, Ł.; Ostrowska-Kaim, E.; Wiśniowska-Śmiałek, S.; Plens, K.; Legutko, J.; Undas, A. Impaired Fibrinolysis in Patients with Isolated Aortic Stenosis is Associated with Enhanced Oxidative Stress. *J. Clin. Med.* **2020**, *9*, 2002. [CrossRef]
8. Kochtebane, N.; Choqueux, C.; Passefort, S.; Nataf, P.; Messika-Zeitoun, D.; Bartagi, A.; Michel, J.-B.; Anglés-Cano, E.; Jacob, M.-P. Plasmin induces apoptosis of aortic valvular myofibroblasts. *J. Pathol.* **2010**, *221*, 37–48. [CrossRef]

9. Jung, R.G.; Motazedian, P.; Ramirez, F.D.; Simard, T.; Di Santo, P.; Visintini, S.; Faraz, M.A.; Labinaz, A.; Jung, Y.; Hibbert, B. Association between plasminogen activator inhibitor-1 and cardiovascular events: A systematic review and meta-analysis. *Thromb. J.* **2018**, *16*, 12. [CrossRef] [PubMed]
10. Rahman, F.A.; Krause, M.P. PAI-1, the Plasminogen System, and Skeletal Muscle. *Int. J. Mol. Sci.* **2020**, *21*, 7066. [CrossRef]
11. Cesari, M.; Pahor, M.; Incalzi, R.A. Plasminogen activator inhibitor-1 (PAI-1): A key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc. Ther.* **2010**, *28*, e72–e91. [CrossRef] [PubMed]
12. Kaden, J.J.; Dempfle, C.E.; Grobholz, R.; Fischer, C.S.; Vocke, D.C.; Kılıç, R.; Sarıkoç, A.; Piñol, R.; Hagl, S.; Lang, S.; et al. Inflammatory regulation of extracellular matrix remodeling in calcific aortic valve stenosis. *Cardiovasc. Pathol.* **2005**, *14*, 80–87. [CrossRef] [PubMed]
13. Kopytek, M.; Mazur, P.; Ząbczyk, M.; Undas, A.; Natorska, J. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. *Diabetologia* **2021**, *64*, 2562–2574. [CrossRef] [PubMed]
14. Siudut, J.; Natorska, J.; Wypasek, E.; Wiewiórka, Ł.; Ostrowska-Kaim, E.; Wiśniowska-Śmiałek, S.; Plens, K.; Musialek, P.; Legutko, J.; Undas, A. Apolipoproteins and lipoprotein(a) as factors modulating fibrin clot properties in patients with severe aortic stenosis. *Atherosclerosis* **2022**, *344*, 49–56. [CrossRef] [PubMed]
15. Vahanian, A.; Beyersdorf, F.; Praz, F.; Milojevic, M.; Baldus, S.; Bauersachs, J.; Capodanno, D.; Conradi, L.; De Bonis, M.; De Paulis, R.; et al. ESC/EACTS Scientific Document Group. 2021 ESC/EACTS Guidelines for the management of valvular heart disease. *Eur. Heart J.* **2022**, *43*, 561–632. [CrossRef]
16. Kopytek, M.; Ząbczyk, M.; Mazur, P.; Undas, A.; Natorska, J. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. *Cardiovasc. Diabetol.* **2020**, *19*, 92. [CrossRef] [PubMed]
17. Meng, X.; Ao, L.; Song, Y.; Babu, A.; Yang, X.; Wang, M.; Weyant, M.J.; Dinarello, C.A.; Cleveland, J.C., Jr.; Fullerton, D.A. Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: Potential roles in aortic valve inflammation and stenosis. *Am. J. Physiol. Cell Physiol.* **2008**, *294*, C29–C35. [CrossRef]
18. Wypasek, E.; Natorska, J.; Mazur, P.; Kopytek, M.; Gawęda, B.; Kapusta, P.; Madeja, J.; Iwaniec, T.; Kapelak, B.; Undas, A. Effects of rivaroxaban and dabigatran on local expression of coagulation and inflammatory factors within human aortic stenotic valves. *Vascul. Pharmacol.* **2020**, *130*, 106679. [CrossRef] [PubMed]
19. Gnanaguru, G.; Choi, A.R.; Amarnani, D.; D'Amore, P.A. Oxidized Lipoprotein Uptake Through the CD36 Receptor Activates the NLRP3 Inflammasome in Human Retinal Pigment Epithelial Cells. *Investig. Ophthalmol. Vis. Sci.* **2016**, *57*, 4704–4712. [CrossRef]
20. Yasui, H.; Suzuki, Y.; Sano, H.; Suda, T.; Chida, K.; Dan, T.; Miyata, T.; Urano, T. TM5275 prolongs secreted tissue plasminogen activator retention and enhances fibrinolysis on vascular endothelial cells. *Thromb. Res.* **2013**, *132*, 100–105. [CrossRef] [PubMed]
21. Matilla, L.; Roncal, C.; Ibarrola, J.; Arrieta, V.; García-Peña, A.; Fernández-Celis, A.; Navarro, A.; Álvarez, V.; Gainza, A.; Orbe, J.; et al. A Role for MMP-10 (Matrix Metalloproteinase-10) in Calcific Aortic Valve Stenosis. *Arterioscler. Thromb. Vasc. Biol.* **2020**, *40*, 1370–1382. [CrossRef] [PubMed]
22. Pieters, M.; Philippou, H.; Undas, A.; De Lange, Z.; Rijken, D.C.; Mutch, N.J. Subcommittee on Factor XIII and Fibrinogen, and the Subcommittee on Fibrinolysis. An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: Communication from the SSC of the ISTH. *J. Thromb. Haemost.* **2018**, *16*, 1007–1012. [CrossRef] [PubMed]
23. Natorska, J.; Marek, G.; Hlawaty, M.; Sadowski, J.; Tracz, W.; Undas, A. Fibrin presence within aortic valves in patients with aortic stenosis: Association with in vivo thrombin generation and fibrin clot properties. *Thromb. Haemost.* **2011**, *105*, 254–260. [CrossRef] [PubMed]
24. Robert, C.; Bolon, I.; Gazzeri, S.; Veyrenc, S.; Brambilla, C.; Brambilla, E. Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor progression. *Clin. Cancer Res.* **1999**, *5*, 2094–2102. [PubMed]
25. D'Auria, F.; Centurione, L.; Centurione, M.A.; Angelini, A.; Di Pietro, R. Tumor Necrosis Factor Related Apoptosis Inducing Ligand (Trail) in endothelial response to biomechanical and biochemical stresses in arteries. *J. Cell. Biochem.* **2015**, *116*, 2427–2434. [CrossRef] [PubMed]
26. Eren, M.; Painter, C.A.; Gleaves, L.A.; Schoenhard, J.A.; Atkinson, J.B.; Brown, N.J.; Vaughan, D.E. Tissue- and agonist-specific regulation of human and murine plasminogen activator inhibitor-1 promoters in transgenic mice. *J. Thromb. Haemost.* **2003**, *1*, 2389–2396. [CrossRef] [PubMed]
27. Alexopoulos, N.; Katritsis, D.; Raggi, P. Visceral adipose tissue as a source of inflammation and promoter of atherosclerosis. *Atherosclerosis* **2014**, *233*, 104–112. [CrossRef]
28. Khoukaz, H.B.; Ji, Y.; Braet, D.J.; Vadali, M.; Abdelhamid, A.A.; Emal, C.D.; Lawrence, D.; Fay, W.P. Drug Targeting of Plasminogen Activator Inhibitor-1 Inhibits Metabolic Dysfunction and Atherosclerosis in a Murine Model of Metabolic Syndrome. *Arterioscler. Thromb. Vasc. Biol.* **2020**, *40*, 1479–1490. [CrossRef]
29. Ramadass, V.; Vaiyapuri, T.; Tergaonkar, V. Small Molecule NF-κB Pathway Inhibitors in Clinic. *Int. J. Mol. Sci.* **2020**, *21*, 5164. [CrossRef]

Received: 28 December 2020 | Revised: 19 February 2021 | Accepted: 22 February 2021

DOI: 10.1111/eci.13527

REVIEW ARTICLE

WILEY

Aortic valvular stenosis: Novel therapeutic strategies

Joanna Natorska[1,2] | **Magdalena Kopytek**[1,2] | **Anetta Undas**[1,2]

[1]John Paul II Hospital, Kraków, Poland

[2]Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland

Correspondence
Anetta Undas, Institute of Cardiology, Jagiellonian University School of Medicine, 80 Pradnicka St, 31-202 Kraków, Poland.
Email: mmundas@cyf-kr.edu.pl

Funding information
This work was supported by the grant from the Polish National Science Centre (UMO-2015/19/B/NZ5/00647 to J.N)

Abstract

Background: Aortic stenosis (AS) prevalence is estimated to reach 4.5 million cases worldwide by the year 2030. AS is a progressive disease without a pharmacological treatment. In the current review, we aimed to investigate novel therapeutic approaches for non-surgical AS treatment, at least in patients with mild-to-moderate AS.

Materials and Methods: The most recent and relevant papers concerned with novel molecular pathways that have potential as therapeutic targets in AS were selected from searches of PubMed and Web of Science up to February 2021.

Results: Growing evidence indicates that therapies using proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, simvastatin/ezetimibe combination, cholesteryl ester transfer protein inhibitors or antisense oligonucleotides targeting apolipoprotein(a) reduce the risk of AS progression. It has been shown that enhanced valvular lipid oxidation may drive AS development by leading to the activation of valvular interstitial cells (VICs), the most abundant valvular cells having a major contribution to valve calcification. Since VICs are able to release pro-inflammatory cytokines, clotting factors and proteins involved in calcification, strategies targeting these cell activations seem promising as therapeutic interventions. Recently, non-vitamin K antagonist oral anticoagulants (NOACs) have been shown to inhibit activation of VICs.

Conclusion: Several novel molecular pathways of AS development have been identified over the past few years. Therapies using PCSK9 inhibitors, simvastatin/ezetimibe combination, lipoprotein(a)-lowering therapy are highly promising candidates as therapeutics in the prevention of mild AS progression, while preclinical studies show that NOACs may inhibit valvular inflammation and coagulation activation and slower the rate of AS progression.

KEYWORDS

aortic stenosis, lipid-targeting therapy, non-vitamin K antagonist oral anticoagulants

Key messages

- Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors may prevent or slow the progression of aortic stenosis (AS)
- Simvastatin in combination with ezetimibe reduced the rate of aortic valve replacement in patients with mild AS and high low-density cholesterol levels

- Cholesteryl ester transfer protein inhibitors or antisense oligonucleotides targeting apolipoprotein(a) can reduce the risk of AS progression
- Non-vitamin K antagonist oral anticoagulants (NOACs) have been shown to inhibit valvular interstitial cells activation, which may attenuate valvular inflammation and slower the rate of AS progression

1 | INTRODUCTION

Aortic stenosis (AS) is associated with a progressive reduction in the aortic valve orifice and leaflet mobility which leads to an impaired ability of blood ejection from the left ventricle into the aorta. The three main causes of AS include calcification of the valve, rheumatic valve disease and the presence of bicuspid aortic valve. In this review, we focus on the first type of AS, which is the most common acquired valvular heart disease in the adult population of both Europe and North America. The prevalence of AS in patients >65 years of age ranges between 2% and 7%.[1] It is estimated that approximately 4.5 million patients with AS will be diagnosed worldwide by the year 2030.[2] To date, there is no pharmacological treatment to prevent or at least retard AS progression. The ASTRONOMER (Aortic Stenosis Progression Observation: Measuring Effects of Rosuvastatin) trial demonstrated that lipid-lowering therapy with rosuvastatin does not reduce the rate of AS progression.[3] Similarly, the results of other randomized clinical trials such as SEAS (The Simvastatin and Ezetimibe in Aortic Stenosis) and SALTIRE (Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression) have confirmed that neither simvastatin/ezetimibe nor atorvastatin is able to inhibit AS progression.[4,5] Thus, transcatheter aortic valve replacement and surgical aortic valve replacement are the only options for AS treatment, but predicted 30-day surgical mortality for both procedures ranges from 4% to 8%.[6,7] The occurrence of AS has been found to be associated with cardiovascular risk factors such as age,[8] male gender[9] hypercholesterolaemia,[10] diabetes mellitus,[10] arterial hypertension[10] or cigarette smoking,[9] yet the precise mechanisms triggering AS are not fully understood. AS has been considered as an atherosclerosis-like process.[11] However, valvular interstitial cells (VICs) present within aortic valves play a pivotal role in specific pathologic alterations and are responsible for differences between the pathobiology of AS and atherosclerosis.[12] In the current review, we aimed to investigate novel therapeutic approaches for non-surgical AS treatment.

2 | METHODS

The most relevant research on potential novel therapeutic approaches in AS was reviewed. We included papers regarding the inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) and other lipid-targeting therapies, such as simvastatin/ezetimibe combination, cholesteryl ester transfer protein (CETP) inhibitors or antisense oligonucleotides targeting apolipoprotein(a) and recent findings regarding non-vitamin K antagonist oral anticoagulants (NOACs). Taking into account the similarities between atherosclerosis and AS, we decided to include articles on strategies focusing on inhibiting the calcification process in atherosclerosis. We also provide information about novel molecular pathways leading to valvular inflammation and calcification in AS, including VIC activation, oxidative stress generation, involvement of lipids, coagulation proteins, matrix metalloproteinases (MMPs) and accumulation of advanced glycation end products (AGEs). All studies were examined in detail. Basic research and clinical articles were selected from PubMed and Web of Science from January 2010 to February 2021, supported by seminal papers from previous years.

3 | RESULTS

We present the recent literature regarding potential therapeutic strategies to inhibit AS progression, in those with mild-to-moderate severity. Main clinical findings are summarized in Figure 1.

3.1 | Aortic valve calcification

Normal aortic valve has three layers: ventricularis, spongiosa and fibrosa. The ventricularis is composed mainly of elastin-rich fibres aligned in a circular pattern on the ventricular side of the leaflet. The spongiosa is a layer of loose connective tissue at the base of the valve, containing fibroblasts, mesenchymal cells and a mucopolysaccharide-rich matrix. The fibrosa consists of fibroblasts and collagen fibres arranged in a radial direction on the aortic side of the leaflet. These layers cooperate to provide the strength and flexibility of the aortic leaflets in response to years of repetitive movement.[13,14] The predominant cells found in all three layers are VICs.[15] VICs are of mesenchymal origin and maintain the structural integrity of the valve.[15]

Histopathologic studies of AS indicate the involvement of cell-dependent mechanisms that regulate calcium load on the valve leaflets,[16] involving macrophages,[17] lymphocytes,[17] mast cells,[18] neutrophils,[19] cardiac chondrocytes[20] and VICs,[15] which, in a vicious cycle, differentiate into

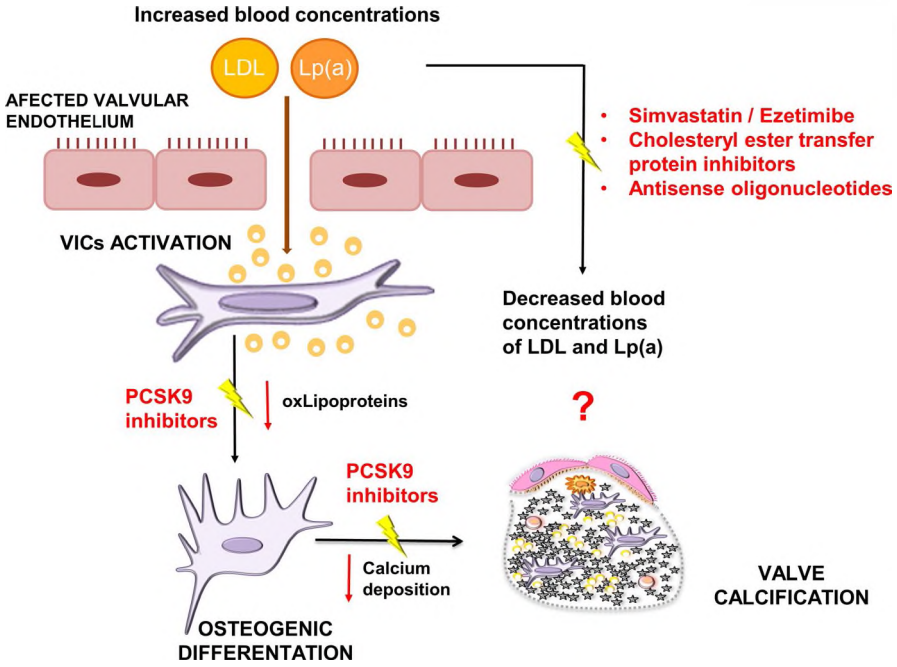


FIGURE 1 Potential lipid-lowering strategies to inhibit the progression of aortic stenosis. In response to endothelial damage, lipoproteins and phospholipids infiltrate aortic valve leaflets. Oxidation of lipoproteins activates valvular interstitial cells (VICs) and promotes valvular mineralization and calcification. Therapy with proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors lowers the level of lipids in the circulating blood, inhibits infiltration of oxidized lipids and lipoproteins (oxLipoproteins) into valve leaflets and thus attenuates valvular calcification. Other lipid-targeting therapies, such as simvastatin/ezetimibe combination which reduces serum LDL concentrations as well as lipoprotein (a) [Lp(a)]-lowering strategies such as cholesteryl ester transfer protein inhibitors (CETP) or antisense oligonucleotides targeting apolipoprotein(a), may slower the rate of aortic stenosis progression

myofibroblasts that cause fibrosis by expressing α-actin and osteoblast-like cells that cause calcification by expressing alkaline phosphatase, osteopontin and bone morphogenetic protein-2 and bone morphogenetic protein-4 (BMP-2, -4).[21]

In response to basement membrane disruption, accumulation of lipoproteins, oxidative stress and infiltration of the inflammatory cells,[22,23] the evolving VIC environment triggers the intracellular signalling cascades and activation of transcriptional pathways. It has been shown that regulation of valvular inflammation is controlled by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), perceived as a master regulator of the inflammatory response.[24,25] NF-κB is activated by tumour necrosis factor-α (TNF-α) and/or transforming growth factor-β[26,27] and leads to an upstream production of interleukin-6 (IL-6), which has been implicated in calcification of aortic valve leaflets via BMPs, especially BMP-2 stimulation.[28] BMPs stimulate aortic leaflet calcification by activating Smad1/5/8 and Wnt/β-catenin signalling pathways. Smad1/5/8 activation leads to up-regulation of master osteoblast transcription factor Runt-related transcription factor 2, which in turn increases the expression of proteins directly associated with calcification.[27] Moreover, it has been observed that upon pro-inflammatory stimulation VICs express increased amounts of IL-6, IL-32, IL-34, MMPs 1-3, osteopontin and osteocalcin.[29] Based on these findings, VICs may have a major contribution to valvular calcification and studies regarding potential therapeutic options affecting the pathways of VIC activation are highly warranted.

3.2 | Lipid involvement in AS pathomechanism

Within stenotic aortic valves, an accumulation of lipoproteins has been observed. These include low-density lipoprotein (LDL), oxidatively modified LDL (oxLDL), oxidatively modified phospholipids (oxPLs) and lipoprotein (a) [Lp(a)]—the major phospholipid carrier.[22,23,30,31] High expression of valvular oxLDL was associated with enhanced influx of macrophages, T cells and leukocytes as well as with increased TNF-α expression.[23,32,33] Valvular macrophages in an uncontrolled way capture the oxLDLs and esterify them, which leads to the formation of foam cells and enhancement of valvular inflammation.[17,34] oxPLs have been shown to promote valvular mineralization and calcification via up-regulation of reactive oxygen species and inflammatory cytokines release by macrophages.[30] It has been also demonstrated that valvular expression of oxPLs co-localized with Lp(a).[31] Notably, valvular amounts of Lp(a) were significantly higher in stenotic valves compared with healthy donor valves.[31] In VICs in vitro cultures, Lp(a) stimulation resulted in enhanced calcification reflected by increased

TABLE 1 Summary of the main pharmacological targets of AS and their effects in clinical trials

Author, year of publication	Study type	Mean age, sample size, country	Treatment	Condition	Main findings
Stein et al (2012)[46]	Randomized clinical trial	18-65 years, n = 133, United States, Canada, the Netherlands, Russian Federation, South Africa	PCSK9 inhibitor: REGN727	Healthy volunteers and patients with familial or non-familial hypercholesterolemia	REGN727 reduced LDL cholesterol levels in healthy volunteers and in patients with familial or non-familial hypercholesterolaemia.
Bergmark et al (2020)[52]	Randomized clinical trial (The FOURIER trial)	63 ± 9 years (evolocumab), 62 ± 9 (placebo) n = 27 564, 49 countries	PCSK9 inhibitor: evolocumab	Patients with stable atherosclerotic cardiovascular disease taking statins	Higher Lp(a) concentrations, but not Lp(a)-corrected LDL cholesterol levels were associated with a higher risk of AS events. Long-term therapy with evolocumab may reduce AS progression rate.
Hovingh et al (2015)[56]	Randomized clinical trial (The TULIP trial)	18-75 years n = 364, the Netherlands and Denmark	CEPT inhibitor: TA-8995, in combination with atorvastatin or rosuvastatin	Patients with mild dyslipidaemia	TA-8995 reduced the concentrations of LDL cholesterol and increased the levels of HDL cholesterol.
Nicholls et al (2016)[55]	Randomized clinical trial	58.3 ± 12 years, n = 398, United States and Europe	CEPT inhibitor: evacetrapib	Mildly hypercholesterolaemic patients	Evacetrapib reduced the concentrations of Lp(a), LDL particle and small LDL particle.
Bowman et al (2017)[54]	Randomized clinical trial (The REVEAL trial)	67 ± 8 years n = 30 449, United Kingdom	CEPT inhibitor: anacetrapib	Patients with atherosclerotic vascular disease taking atorvastatin	Long-term therapy with anacetrapib resulted in a lower incidence of major coronary events and reduced Lp(a) and LDL cholesterol levels.
Tsimikas et al (2015)[57]	Randomized clinical trial	18-65 years n = 206, United Kingdom	Antisense oligonucleotides: ISIS-APO(a)$_{Rx}$	Healthy volunteers with BMI < 32 kg m^{-2} and Lp(a) ≥25 nmol/L	ISIS-APO(a)$_{Rx}$ reduced Lp(a) concentrations in a dose-dependent manner.
Viney et al (2016)[58]	Randomized clinical trial	18-75 years n = 64 for IONIS-APO(a)$_{Rx}$, n = 58 for IONIS-APO(a)-LRx, Canada, the Netherlands, Germany, Denmark, United Kingdom	Antisense oligonucleotides: IONIS-APO(a)$_{Rx}$ and IONIS-APO(a)-LRx	Healthy volunteers with elevated Lp(a)	Both IONIS-APO(a)$_{Rx}$ and IONIS-APO(a)-LRx reduced Lp(a) concentrations.
Greve et al (2019)[60]	Randomized clinical trial (Secondary analysis of the SEAS trail)	45-85 years, n = 1687, 7 European countries	Simvastatin/ezetimibe combination	Asymptomatic patients with mild-to-moderate AS	Simvastatin/ezetimibe combination reduced the rate of aortic valve replacement in patients with mild AS and LDL cholesterol levels > 4 mmol/L.

alkaline phosphatase (an enzyme involved in calcification) activity and increased formation of calcification nodules.[31] Within the valve, lipoprotein-associated phospholipase A2 uses oxPLs and generates lysophosphatidylcholine which has an impact on in vitro mineralization.[35-37] Furthermore, some biological roles of lysophosphatidylcholine might be mediated by autotaxin, derived from oxPLs.[38,39] It has been previously demonstrated by Bouchareb et al[40] that autotaxin-lysophosphatidic acids are associated with the pathogenesis of AS and may play a pivotal role in mediating inflammation and fibro-calcific remodelling of stenotic valves through NF-κB. Torzewski et al[39] suggested that the autotaxin-Lp(a)-oxPL axis might be a key determinant of valve calcification.

3.2.1 | Lipid-targeting therapy in AS

A genome-wide association study revealed that a single nucleotide polymorphism (rs10455872) at the locus of the Lp(a) gene was associated with increased risk of valvular calcification (odds ratio per allele, 2.05).[41] On the other hand, secondary analysis of the ASTRONOMER trial demonstrated that elevated Lp(a) and oxPLs predicted a worse outcome in patients with mild-to-moderate AS (n = 220).[42] Therefore, novel therapeutic strategies based on two fully humanized monoclonal antibodies (alirocumab and evolocumab) that bind free plasma PCSK9 and promote degradation of this enzyme have been proposed.[43-46] PCSK9 is predominantly produced in the liver and its role is to bind the LDL receptor, resulting in higher blood LDL cholesterol levels.[47,48] Wang et al[49] have demonstrated that plasma PCSK9 levels are associated with the presence of AS, but not with the severity of the disease. While valvular expression of PCSK9 is evident in both calcified and non-calcified valve leaflets, calcified leaflets are characterized by significantly higher PCSK9 levels.[50] Moreover, stimulation of VICs with an osteogenic medium resulted in PCSK9 overexpression and the inhibition of PCSK9 was associated with reduced calcium deposition.[50] Langsted et al[51] showed in humans that the PCSK9 R46L loss-of-function mutation was associated with lower LDL and Lp(a) levels, as well as with reduced risk of AS. Importantly, an analysis of the FOURIER randomized clinical trial (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk) revealed that long-term treatment with evolocumab reduced the incidence of AS (hazard rate [HR] 0.48; 95% confidence interval [CI]: 0.25-0.93) compared with placebo arm.[52] Notably, Lankin et al[53] demonstrated that PCSK9 inhibitor emocoucumab reduces blood concentrations of LDL and oxLDLs in patients with coronary artery disease and atherosclerosis. Randomized clinical trials investigating different CETP inhibitors showed that in patients with atherosclerotic vascular disease, anacetrapib reduced the level of Lp(a) by 25% and LDL by 41%, and increased the level of HDL by 104% compared with placebo.[54] Similar effects were observed for evacetrapib (alone or in combination with statins) and for the novel CETP inhibitor—TA-8995 (as monotherapy or co-administered with a statin).[55,56] Other potential lipid-lowering therapies with antisense oligonucleotides targeting apolipoprotein(a) (IONIS-APO(a)Rx, ISIS-APO(a)Rx, and IONIS-APO(a)-LRx), which effectively decrease plasma Lp(a) concentrations[57,58] are also currently under investigation.[59] Antisense oligonucleotides as a class of RNA therapeutic drugs are designed to reduce the synthesis of apo(a) and therefore the concentration of Lp(a) in circulating blood.[57] However, their potential role in AS treatment is not clear yet and clinical studies are needed. Interestingly, a secondary analysis from the SEAS trial showed that simvastatin/ezetimibe combination reduced the need for aortic valve replacement in patients with mild AS and high LDL cholesterol levels (>4 mmol/L) (HR 0.4, 95% CI 0.2-0.9), but not in subjects with moderate AS.[60]

Taken together, lipid-lowering therapies show promise in inhibiting valvular calcification, at least in patients with mild-to-moderate AS (Figure 1). However, large clinical trials showing that PCSK9 inhibitors are beneficial in AS patients have not been performed to date. An ongoing phase 2 trial (NCT03051360) is currently testing the hypothesis that a PCSK9 inhibitor will reduce aortic valve macro- or microcalcification in patients with mild-to-moderate AS. A summary of the main clinical trials regarding pharmacological AS treatment is presented in Table 1.

3.3 | Preclinical models of AS therapy

3.3.1 | Involvement of blood coagulation in AS progression

The expression of several coagulation factors has been documented within stenotic aortic valves.[29,61-64] The valvular co-expression of tissue factor and fibrin has been shown, suggesting that blood coagulation is activated in loco. Moreover, in AS patients with a maximal transvalvular pressure gradient ≥75 mm Hg, positive associations between the amount of valvular fibrin and both maximal and mean transvalvular gradients have been demonstrated.[62] Breyne et al[63] suggested an involvement of valvular tissue factor and thrombin generation in the calcification of stenotic aortic valves. Our group has shown that factor VII, factor X, active factor X and their protease-activated receptors (PAR1 and PAR2) are present within aortic stenotic valves.[29] Notably, the proteolytic activation of PARs by coagulation proteases such as thrombin or active factor X initiates the activation of cellular signalling pathways that influence various valvular and cell-specific issues

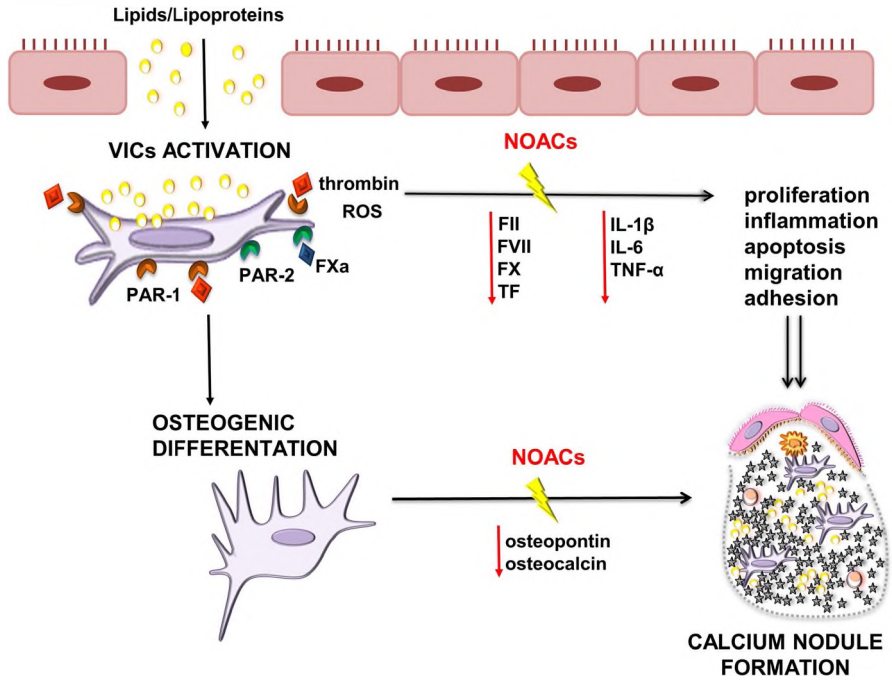


FIGURE 2 Non-vitamin K antagonist oral anticoagulants (NOACs) as potential drug candidates for AS treatment. Lipid accumulation leads to valvular inflammation. Upon inflammation and up-regulation of reactive oxygen species (ROS) generation, valvular interstitial cells (VICs) are able to express protease-activated receptors (PARs) and coagulation proteins, such as tissue factor (TF), prothrombin (FII), factor (F) VII, FX and release pro-inflammatory cytokines. Proteolytic activation of PARs either by thrombin or activated FX results in downstream signalling that influences multiple valvular transcription-regulated and cell-specific processes. As a consequence of chronic valvular inflammation VICs differentiate into fibroblasts with an osteoblastic phenotype, releasing osteopontin and osteocalcin, which are responsible for valvular calcification. Non-vitamin K antagonist oral anticoagulants (NOACs) might inhibit valvular inflammation and coagulation, preventing VIC activation and osteogenic differentiation

including proliferation, migration, adhesion, apoptosis, inflammation and coagulation.[65,66] It might be hypothesized that inhibition of active factor X could attenuate not only thrombin generation but also the activation of PARs.[66] Of major importance, it has been shown that upon a pro-inflammatory stimulation, VICs are able to express tissue factor, thrombin, factor VII, active factor X, PAR1 and PAR2 on both protein and mRNA levels. These observations suggest that VICs can synthesize coagulation proteins under stimulation.[29]

3.3.2 | Coagulation-inhibiting therapy

NOACs, including dabigatran (thrombin inhibitor), rivaroxaban or apixaban (active factor X inhibitors), are known for their anti-inflammatory properties, in addition to their anticoagulant effects. In apolipoprotein-E-deficient mice, dabigatran and rivaroxaban (at concentrations encountered in vivo) attenuate atherosclerotic plaque progression by reduction in lipid deposition and macrophage accumulation.[67-70] Wypasek et al[29] have shown that in cultures of human VICs, therapeutic concentrations of rivaroxaban (1 or 10 ng/mL) inhibited the expression of coagulation factors, while dabigatran (25 ng/mL) inhibited thrombin and PAR1 expression. Moreover, pre-incubation of VIC cultures with rivaroxaban decreased expression of pro-inflammatory and calcification factors.[29] The double-blind COMPASS trial showed a reduced rate of the primary composite outcome, including cardiovascular death, stroke or myocardial infarction among 27 395 patients with documented atherosclerosis taking low dose of rivaroxaban (2.5 mg twice daily) plus aspirin (100 mg once daily) as a secondary prevention.[71] Taking into consideration the similarities in risk factors and crucial pathomechanisms between atherosclerosis and AS, it might be assumed that rivaroxaban is able to attenuate valvular coagulation activation and inflammation, at least in patients with concomitant heart disease.

Additionally, it has been shown in humans that valvular calcification can be also affected by anticoagulant therapy with vitamin K antagonists (VKAs). However, long-term treatment with VKAs has been associated with increased risk of cardiovascular calcification.[72] VKAs, besides blocking gamma-carboxylation of coagulation factors, prevent the activation of several proteins not related to coagulation, among them matrix γ-carboxyglutamate protein, a potent inhibitor of calcification, that requires vitamin K–dependent post-translational modification.[73]

Taken together the results of in vitro and clinical investigations, it might be hypothesized that long-term anticoagulation with NOACs could serve as a therapeutic option,[74] at least in AS patients with mild-to-moderate AS and indications for anticoagulant therapy (Figure 2). In vitro and clinical studies testing this hypothesis are currently ongoing.[29,75]

3.3.3 | An impact of valvular cell populations on AS progression

MMPs are zinc-dependent endopeptidases, which have been implicated in valvular calcification by cleaving extracellular matrix components as well as non-matrix proteins.[76] Data from in vitro studies have demonstrated that inhibition of NF-κB prevents up-regulation of MMP1, MMP3 and MMP9 production from fibroblasts and vascular smooth muscle cells.[77] The valvular expression of several MMPs has been shown within aortic stenotic valves.[78,79] An in vitro study by Matilla et al[79] revealed that MMP-10 in VIC cultures increased expression of inflammatory (IL-1β), fibrotic (α-smooth muscle actin, vimentin, collagen) and osteogenic (BMP-4, BMP-9, osteopontin, Sox9 and Smad1/5/8) markers. The addition of a physiological MMP inhibitor (the tissue inhibitor of metalloproteinases type 1) to cultured human VICs prevented the release of pro-inflammatory and osteogenic factors. Moreover, monoclonal antibodies against MMP-10 inhibited the osteogenesis, resulting in much lower rate of VIC calcification.[79] Thus, it might be hypothesized that MMPs are potential therapeutic targets for delaying the progression of aortic valve calcification in AS. Clinical studies of the efficacy of MMP-10 antibodies therefore might realize new therapeutic possibilities for patients with mild-to-moderate AS.

Of note, the presence of neutrophil extracellular traps (NETs) specific biomarkers within stenotic valves has been demonstrated.[19] Since it has been shown in vitro that oxLDL-treated macrophages induced NET formation by enhanced reactive oxygen species production,[80] and NETs are involved in the activation of blood coagulation via local accumulation of tissue factor-bearing NETs,[81] it might be suggested that NETs in AS intensify valvular inflammation and coagulation. Moreover, histones are cross-linked within fibrin network[82] and they might impede lysis of valvular fibrin deposits. Importantly, the amounts of valvular citrullinated histones H3 were associated with AS severity. These data might indicate a novel link between valvular NETosis and AS progression.[19] It would be of interest whether the DNAse I-triggered clearance of NETs is able to attenuate VIC activation and slower their calcification rates. As far as we know no such experiments have been performed to date.

3.4 | Advanced glycation end products (AGEs) enhance valvular inflammation

It has been observed that diabetes mellitus concomitant with AS is associated with increased valvular inflammation, measured by valvular C-reactive protein expression, which was positively associated with tissue factor expression on a transcription level.[83] In the natural history of diabetes mellitus, AGEs accumulate in plasma and tissues.[84] AGEs are generated through non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids and, through binding to a cell surface receptor for advanced glycation end products (RAGE), are capable of modulating multiple cellular processes.[85-87] Binding of AGEs to RAGE results in increased oxidative stress, NF-κB–controlled gene expression[88-90] and, importantly, osteoblastic differentiation of VICs.[91] In 2014, Hofmann et al[92] demonstrated in a RAGE knock-out mouse that both AGEs and RAGE are involved in aortic leaflet calcification and subsequent AS. This observation has been supported by the finding that in AS patients with concomitant diabetes mellitus, hyperglycaemia is accompanied by 6.6- and 12-fold increase in valvular and plasma AGEs, respectively. These changes were correlated with AS severity, measured by aortic valve area.[93] Higher levels of RAGE in AS patients with concomitant diabetes were observed in plasma (1.3-fold higher) and within aortic stenotic valves (1.8-fold higher) compared with non-diabetic AS patients. However, only plasma RAGE levels correlated with aortic valve area and maximal transvalvular pressure gradient.[93] Additionally, in AS patients with concomitant diabetes mellitus, those with glycated haemoglobin A1c > 7% had a 1.2-fold higher valvular expression of AGEs, which correlated positively with mean transvalvular pressure gradient.[93] Interestingly, in patients with well-controlled type 2 diabetes, the influence of hyperglycaemia on AS severity was negligible.[93]

The current evidence may be highly significant for patients with AS and concomitant diabetes and suggests that in this group, long-term glucose dynamic monitoring by measuring glycated haemoglobin or fructosamine levels is needed. It is worthwhile suggesting that in diabetic AS patients, besides glycaemic control, targeting an inhibition of the AGEs/RAGE axis or its interaction with oxidative stress might be a novel approach in preventing AS progression.[94]

The summary of the main basic research studies regarding potential treatment in AS patients is presented in Table 2.

4 | CONCLUSIONS AND FUTURE PERSPECTIVES

The pathogenesis of AS is multifactorial, and despite well-known demographic and cardiovascular risk factors, several novel molecular mechanisms contributing to AS progression

TABLE 2 Summary of the main pharmacological targets of AS and their effects in basic research

Author, year of publication	Study type	Material source	Main findings
El Husseini et al (2014)[28]	In loco/In vitro	Aortic valves obtained from AS patients and valvular interstitial cells isolated from aortic valves	P2Y purinoceptor 2, which stimulates NF-kB pathway activity, is overexpressed in interstitial valvular cells, enhancing their calcification. Inhibition of valvular inflammation may be a therapeutic target in AS.
Bouchareb et al (2015)[40]	In loco/In vitro	Aortic valves obtained from AS patients and valvular interstitial cells isolated from aortic valves	Autotaxin secreted by valvular interstitial cells is transported by Lp(a) and promotes valvular inflammation and calcification, thus could represent a novel therapeutic target in AS.
Yu et al (2017)[31]	In loco/In vitro	Aortic valves obtained from AS patients and valvular interstitial cells isolated from aortic valves	Lp(a) abundant in calcified aortic valves enhanced valvular calcification, suggesting that it can serve as a therapeutic approach.
Torzewski et al (2017)[39]	In loco	Aortic valves obtained from AS patients	Increased levels of Lp(a)-associated molecules, present in plasma and aortic valve leaflets in AS patients are the key determinants of AS due to initiation of valvular inflammation, fibrosis and calcification.
Perrot et al (2020)[50]	In loco/In vitro	Aortic valves obtained from AS patients and valvular interstitial cells isolated from stenotic aortic valves	Aortic stenosis was less prevalent in carriers of the PCSK9R46L variant. PCSK9 is produced and secreted by aortic valves; thus, PCSK9 inhibition might lower calcification in aortic valve cells.
Matilla et al (2020)[79]	In loco/In vitro	Aortic valves obtained from AS patients and valvular interstitial cells isolated from stenotic aortic valves	Matrix metalloproteinases-10 is overexpressed in stenotic aortic valves. Matrix metalloproteinases could be a therapeutic target for delaying AS progression.
Wypasek et al (2020)[29]	In loco/In vitro	Valvular interstitial cells obtained from stenotic aortic valves	NOACs at therapeutic concentrations inhibit valvular inflammation and coagulation activation, which may lead to retardation of AS progression.
Kopytek et al (2020)[93]	In loco	Aortic valves obtained from AS patients with concomitant type 2 diabetes	Type 2 diabetes concomitant to AS increased valvular inflammation and oxidative stress, which were associated with AS severity. Poorly controlled diabetes may lead to increased AS progression.

have been identified. Therapies using PCSK9 inhibitors, simvastatin/ezetimibe combination, CETP inhibitors or antisense oligonucleotides targeting apo(a) have been shown to reduce blood lipid concentrations and thus have a potential to slow the rate of AS progression in patients with moderate AS or even prevent disease progression in patients with mild AS. Moreover, it has been shown that enhanced oxidation of valvular lipids leads to activation of VICs, which have a major contribution to valvular calcification and AS progression. Thus, strategies pointing to the inhibition of VIC activation are promising. Additionally, therapy with NOACs might have multitargeted beneficial effects and merits further investigation.

CONFLICT OF INTEREST

None declared.

ORCID

Anetta Undas https://orcid.org/0000-0003-3716-1724

REFERENCES

1. Osnabrugge RLJ, Mylotte D, Head SJ, et al. Aortic stenosis in the elderly: disease prevalence and number of candidates for transcatheter aortic valve replacement: a meta-analysis and modeling study. *J Am Coll Cardiol*. 2013;62:1002-1012.
2. Yutzey KE, Demer LL, Body SC, et al. Calcific aortic valve disease: a consensus summary from the Alliance of Investigators on Calcific Aortic Valve Disease. *Arterioscler Thromb Vasc Biol*. 2014;34(11):2387-2393.
3. Chan KL, Teo K, Dumesnil JG, Ni A, Tam J, ASTRONOMER Investigators. Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. *Circulation*. 2010;121(2):306-314.
4. Rossebo AB, Pedersen TR, Boman K, et al. Intensive lipid lowering with simvastatin and ezetimibe in aortic stenosis. *N Engl J Med*. 2008;359(13):1343-1356.
5. Cowell SJ, Newby DE, Prescott RJ, et al. Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression (SALTIRE) Investigators. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. *N Engl J Med*. 2005;352(23):2389-2397.
6. Makkar RR, Thourani VH, Mack MJ, et al.; PARTNER 2 Investigators. Five-year outcomes of transcatheter or surgical aortic-valve replacement. *N Engl J Med*. 2020;382(9):799-809.
7. Piroli F, Franchin L, Bruno F, De Filippo O, D'Ascenzo F, Conrotto F. New advances in the prevention of transcatheter aortic valve implantation failure: current and future perspectives. *Kardiol Pol*. 2020;78(9):842-849.
8. Lindroos M, Kupari M, Valvanne J, Strandberg T, Heikkilä J, Tilvis R. Factors associated with calcific aortic valve degeneration in the elderly. *EurHeart J*. 1994;15:865-870.
9. Stewart BF, Siscovick D, Lind BK, et al. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. *J Am Coll Cardiol*. 1997;29(3):630-634.
10. Aronow WS, Schwartz KS, Koenigsberg M. Correlation of serum lipids, calcium, and phosphorus, diabetes mellitus and history of systemic hyper-tension with presence or absence of calcified or thickened aortic cusps or root in elderly patients. *Am J Cardiol*. 1987;59:998-999.
11. Mohler ER 3rd. Are atherosclerotic processes involved in aortic-valve calcification? *Lancet*. 2000;356(9229):524-525.
12. Akerström F, Barderas MG, Rodríguez-Padial L. Aortic stenosis: a general overview of clinical, pathophysiological and therapeutic aspects. *Expert Rev Cardiovasc Ther*. 2013;11(2):239-250.
13. Freeman RV, Otto CM. Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. *Circulation*. 2005;111(24):3316-3326.
14. Warren BA, Yong JL. Calcification of the aortic valve: its progression and grading. *Pathology*. 1997;29(4):360-368.
15. Mulholland DL, Gotlieb AI. Cell biology of valvular interstitial cells. *Can J Cardiol*. 1996;12(3):231-236.
16. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. *Circ Res*. 2011;108(11):1392-1412.
17. Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. *Circulation*. 1994;90(2):844-853.
18. Wypasek E, Natorska J, Grudzień G, Filip G, Sadowski J, Undas A. Mast cells in human stenotic aortic valves are associated with the severity of stenosis. *Inflammation*. 2013;36(2):449-456.
19. Kopytek M, Kolasa-Trela R, Ząbczyk M, Undas A, Natorska J. NETosis is associated with the severity of aortic stenosis: links with inflammation. *Int J Cardiol*. 2019;286:121-126.
20. Mohler ER 3rd, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS. Bone formation and inflammation in cardiac valves. *Circulation*. 2001;103(11):1522-1528.
21. Liu AC, Joag VR, Gotlieb AI. The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. *Am J Pathol*. 2007;171(5):1407-1418.
22. Yetkin E, Waltenberger J. Molecular and cellular mechanisms of aortic stenosis. *Int J Cardiol*. 2009;135(1):4-13.
23. Mohty D, Pibarot P, Després J-P, et al. Association between plasma LDL particle size, valvular accumulation of oxidized LDL, and inflammation in patients with aortic stenosis. *Arterioscler Thromb Vasc Biol*. 2008;28(1):187-193.
24. Meng X, Ao L, Song Y, et al. Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis. *Am J Physiol Cell Physiol*. 2008;294(1):C29-C35.
25. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc Res*. 2010;86(2):211-218.
26. Jian B, Narula N, Li QY, Mohler ER 3rd, Levy RJ. Progression of aortic valve stenosis: TGF-beta1 is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis. *Ann Thorac Surg*. 2003;75(2):457-465; discussion 465–6.
27. Yu Z, Seya K, Daitoku K, Motomura S, Fukuda I, Furukawa K. Tumor necrosis factor-α accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway. *J Pharmacol Exp Ther*. 2011;337(1):16-23.
28. El Husseini D, Boulanger M-C, Mahmut A, et al. P2Y2 receptor represses IL-6 expression by valve interstitial cells through Akt: implication for calcific aortic valve disease. *J Mol Cell Cardiol*. 2014;72:146-156.
29. Wypasek E, Natorska J, Mazur P, et al. Effects of rivaroxaban and dabigatran on local expression of coagulation and inflammatory factors within human aortic stenotic valves. *Vascul Pharmacol*. 2020;130:106679.
30. Yeang C, Wilkinson MJ, Tsimikas S. Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. *Curr Opin Cardiol*. 2016;31(4):440-450.
31. Yu B, Hafiane A, Thanassoulis G, et al. Lipoprotein(a) induces human aortic valve interstitial cell calcification. *JACC Basic Transl Sci*. 2017;2(4):358-371.
32. Galeone A, Paparella D, Colucci S, Grano M, Brunetti G. The role of TNF-α and TNF superfamily members in the pathogenesis of calcific aortic valvular disease. *Sci World J*. 2013;2013:875363.
33. Olsson M, Thyberg J, Nilsson J. Presence of oxidized low density lipoprotein in nonrheumatic stenotic aortic valves. *Arterioscler Thromb Vasc Biol*. 1999;19(5):1218-1222.
34. Natorska J, Undas A. Blood coagulation and fibrinolysis in aortic valve stenosis: links with inflammation and calcification. *Thromb. Haemost*. 2015;114(2):217-227.

35. Lehti S, Käkelä R, Hörkkö S, et al. Modified lipoprotein-derived lipid particles accumulate in human stenotic aortic valves. *PLoS One*. 2013;8(6):e65810.
36. Wiltz DC, Han RI, Wilson RL, Kumar A, Morrisett JD, Grande-Allen KJ. Differential aortic and mitral valve interstitial cell mineralization and the induction of mineralization by lysophosphatidylcholine in vitro. *Cardiovasc Eng Technol*. 2014;5(4):371-383.
37. Mahmut A, Boulanger M-C, El Husseini D, et al. Elevated expression of lipoprotein-associated phospholipase A2 in calcific aortic valve disease: implications for valve mineralization. *J Am Coll Cardiol*. 2014;63(5):460-469.
38. Nakanaga K, Hama K, Aoki J. Autotaxin – an LPA producing enzyme with diverse functions. *J Biochem*. 2010;148(1):13-24.
39. Torzewski M, Ravandi A, Yeang C, et al. Lipoprotein(a) associated molecules are prominent components in plasma and valve leaflets in calcific aortic valve stenosis. *JACC Basic Transl Sci*. 2017;2(3):229-240.
40. Bouchareb R, Mahmut A, Nsaibia MJ, et al. Autotaxin derived from lipoprotein(a) and valve interstitial cells promotes inflammation and mineralization of the aortic valve. *Circulation*. 2015;132(8):677-690.
41. Thanassoulis G, Campbell CY, Owens DS, et al. CHARGE Extracoronary Calcium Working Group. Genetic associations with valvular calcification and aortic stenosis. *N Engl J Med*. 2013;368(6):503-512.
42. Capoulade R, Yeang C, Chan KL, Pibarot P, Tsimikas S. Association of mild to moderate aortic valve stenosis progression with higher lipoprotein(a) and oxidized phospholipid levels: secondary analysis of a randomized clinical trial. *JAMA Cardiol*. 2018;3(12):1212-1217.
43. Stein EA, Swergold GD. Potential of proprotein convertase subtilisin/kexin type 9 based therapeutics. *Curr Atheroscler Rep*. 2013;15(3):310.
44. Manniello M, Pisano M. Alirocumab (praluent): first in the new class of PCSK9 inhibitors. *P T*. 2016;41(1):28-53.
45. Chan JCY, Piper DE, Cao Q, et al. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. *Proc Natl Acad Sci USA*. 2009;106(24):9820-9825.
46. Stein EA, Mellis S, Yancopoulos GD, et al. Effect of a monoclonal antibody to PCSK9 on LDL cholesterol. *N Engl J Med*. 2012;366:1108-1118.
47. Lagace TA, Curtis DE, Garuti R, et al. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. *J Clin Invest*. 2006;116:2995-3005.
48. De Luca L, Corsini A, Uguccioni M, Colivicchi F. Statins plus ezetimibe in the era of proprotein convertase subtilisin/ kexin type 9 inhibitors. *Kardiol Pol*. 2020;78(9):850-860.
49. Wang WG, He YF, Chen YL, et al. Proprotein convertase subtilisin/kexin type 9 levels and aortic valve calcification: a prospective, cross sectional study. *J Int Med Res*. 2016;44(4):865-874.
50. Perrot N, Valerio V, Moschetta D, et al. Genetic and in vitro inhibition of PCSK9 and calcific aortic valve stenosis. *JACC Basic Transl Sci*. 2020;5(7):649-661.
51. Langsted A, Nordestgaard BG, Benn M, Tybjærg-Hansen A, Kamstrup PR. PCSK9 R46L loss-of-function mutation reduces lipoprotein(a), LDL cholesterol, and risk of aortic valve stenosis. *J Clin Endocrinol Metab*. 2016;101(9):3281-3287.
52. Bergmark BA, O'Donoghue ML, Murphy SA, et al. An exploratory analysis of proprotein convertase subtilisin/kexin type 9 inhibition and aortic stenosis in the FOURIER trial. *JAMA Cardiol*. 2020;5(6):709-713.
53. Lankin VZ, Tikhaze AK, Viigimaa M, Chazova IE. PCSK9 inhibitor causes a decrease in the level of oxidatively modified low-density lipoproteins in patients with coronary artery diseases. *Ter Arkh*. 2018;90(9):27-30.
54. HPS3/TIMI55–REVEAL Collaborative Groups, Bowman L, Hopewell JC, Chen F, et al. Effects of anacetrapib in patients with atherosclerotic vascular disease. *N Engl J Med*. 2017;377(13):1217-1227.
55. Nicholls SJ, Ruotolo G, Brewer HB, et al. Evacetrapib alone or in combination with statins lowers lipoprotein(a) and total and small LDL particle concentrations in mildly hypercholesterolemic patients. *J Clin Lipidol*. 2016;10(3):519-527.e4.
56. Hovingh GK, Kastelein JJP, van Deventer SJH, et al. Cholesterol ester transfer protein inhibition by TA-8995 in patients with mild dyslipidaemia (TULIP): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet*. 2015;386(9992):452-460.
57. Tsimikas S, Viney NJ, Hughes SG, et al. Antisense therapy targeting apolipoprotein(a): a randomised, double-blind, placebo-controlled phase 1 study. *Lancet*. 2015;386(10002):1472-1483.
58. Viney NJ, van Capelleveen JC, Geary RS, et al. Antisense oligonucleotides targeting apolipoprotein(a) in people with raised lipoprotein(a): two randomised, double-blind, placebo-controlled, dose-ranging trials. *Lancet*. 2016;388(10057):2239-2253.
59. Gencer B, Mach F. Potential of lipoprotein(a)-lowering strategies in treating coronary artery disease. *Drugs*. 2020;80(3):229-239.
60. Greve AM, Bang CN, Boman K, et al. Relation of lipid-lowering therapy to need for aortic valve replacement in patients with asymptomatic mild to moderate aortic stenosis. *Am J Cardiol*. 2019;124(11):1736-1740.
61. Natorska J, Marek G, Hlawaty M, et al. Evidence for tissue factor expression in aortic valves in patients with aortic stenosis. *Pol Arch Med Wewn*. 2009;119(10):636-643.
62. Natorska J, Marek G, Hlawaty M, Sadowski J, Tracz W, Undas A. Fibrin presence within aortic valves in patients with aortic stenosis: association with in vivo thrombin generation and fibrin clot properties. *Thromb Haemost*. 2011;105(2):254-260.
63. Breyne J, Juthier F, Corseaux D, et al. Atherosclerotic-like process in aortic stenosis: activation of the tissue factor-thrombin pathway and potential role through osteopontin alteration. *Atherosclerosis*. 2010;213(2):369-376.
64. Kapusta P, Wypasek E, Natorska J, et al. Factor XIII expression within aortic valves and its plasma activity in patients with aortic stenosis: association with severity of disease. *Thromb Haemost*. 2012;108(6):1172-1179.
65. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med*. 2011;364(18):1746-1760.
66. Spronk HM, de Jong AM, Crijns HJ, Schotten U, Van Gelder IC, Ten Cate H. Pleiotropic effects of factor Xa and thrombin: what to expect from novel anticoagulants. *Cardiovasc Res*. 2014;101(3):344-351.
67. Hara T, Fukuda D, Tanaka K, et al. Rivaroxaban, a novel oral anticoagulant, attenuates atherosclerotic plaque progression and destabilization in ApoE-deficient mice. *Atherosclerosis*. 2015;242(2):639-646.
68. Kadoglou NPE, Moustardas P, Katsimpoulas M, et al. The beneficial effects of a direct thrombin inhibitor, dabigatran etexilate, on the development and stability of atherosclerotic lesions in

apolipoprotein E-deficient mice: dabigatran etexilate and atherosclerosis. *Cardiovasc Drugs Ther*. 2012;26(5):367-374.
69. Lee IO, Kratz MT, Schirmer SH, Baumhäkel M, Böhm M. The effects of direct thrombin inhibition with dabigatran on plaque formation and endothelial function in apolipoprotein E-deficient mice. *J Pharmacol Exp Ther*. 2012;343(2):253-257.
70. Zhou Q, Bea F, Preusch M, et al. Evaluation of plaque stability of advanced atherosclerotic lesions in apo E-deficient mice after treatment with the oral factor Xa inhibitor rivaroxaban. *Mediators Inflamm*. 2011;2011:432080.
71. Connolly SJ, Eikelboom JW, Bosch J, et al. COMPASS investigators. Rivaroxaban with or without aspirin in patients with stable coronary artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet*. 2018;391(10117):205-218.
72. Weijs B, Blaauw Y, Rennenberg R, et al. Patients using vitamin K antagonists show increased levels of coronary calcification: an observational study in low-risk atrial fibrillation patients. *Eur Heart J*. 2011;32(20):2555-2562.
73. Schurgers LJ, Uitto J, Reutelingsperger CP. Vitamin K-dependent carboxylation of matrix Gla-protein: a crucial switch to control ectopic mineralization. *Trends Mol Med*. 2013;19(4):217-226.
74. Desperak P, Hudzik B, Gąsior M. Assessment of patients with coronary artery disease who may benefit from the use of rivaroxaban in the real world: implementation of the COMPASS trial criteria in the TERCET registry population. *Pol Arch Intern Med*. 2019;129(7–8):460-468.
75. Di Lullo L, Tripepi G, Ronco C, et al. Cardiac valve calcification and use of anticoagulants: preliminary observation of a potentially modifiable risk factor. *Int J Cardiol*. 2019;278:243-249.
76. Kaden JJ, Dempfle C-E, Grobholz R, et al. Interleukin-1 beta promotes matrix metalloproteinase expression and cell proliferation in calcific aortic valve stenosis. *Atherosclerosis*. 2003;170(2):205-211.
77. Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-kappa B reduces matrix metalloproteinase-1,-3 and-9 production by vascular smooth muscle cells. *Cardiovasc Res*. 2001;50:556-565.
78. Perrotta I, Russo E, Camastra C, et al. New evidence for a critical role of elastin in calcification of native heart valves: immunohistochemical and ultrastructural study with literature review. *Histopathology*. 2011;59(3):504-513.
79. Matilla L, Roncal C, Ibarrola J, et al. A role for MMP-10 (matrix metalloproteinase-10) in calcific aortic valve stenosis. *Arterioscler Thromb Vasc Biol*. 2020;40(5):1370-1382.
80. Zhang Y-G, Song Y, Guo X-L, et al. Exosomes derived from oxLDL-stimulated macrophages induce neutrophil extracellular traps to drive atherosclerosis. *Cell Cycle*. 2019;18(20):2674-2684.
81. Rao AN, Kazzaz NM, Knight JS. Do neutrophil extracellular traps contribute to the heightened risk of thrombosis in inflammatory diseases? *World J Cardiol*. 2015;7(12):829-842.
82. Locke M, Longstaff C. Extracellular Histones Inhibit Fibrinolysis through Noncovalent and Covalent Interactions with Fibrin. *Thromb Haemost*. 2020 Nov 1. https://doi.org/10.1055/s-0040-1718760. Epub ahead of print. PMID: 33131044.
83. Natorska J, Wypasek E, Grudzień G, et al. Does diabetes accelerate the progression of aortic stenosis through enhanced inflammatory response within aortic valves? *Inflammation*. 2012;35(3):834-840.
84. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med*. 1988;318(20):1315-1321.
85. Francis-Sedlak ME, Moya ML, Huang JJ, et al. Collagen glycation alters neovascularization in vitro and in vivo. *Microvasc Res*. 2010;80(1):3-9.
86. Wautier MP, Tessier FJ, Wautier JL. Advanced glycation end products: a risk factor for human health. *Ann Pharm Fr*. 2014;72(6):400-408.
87. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab*. 2001;280(5):E685-E694.
88. Verma N, Manna SK. Advanced glycation end products (AGE) potently induce autophagy through activation of RAF protein kinase and nuclear factor κB (NF-κB). *J Biol Chem*. 2016;291(3):1481-1491.
89. Chen YJ, Chan DC, Chiang CK, et al. Advanced glycation end-products induced VEGF production and inflammatory responses in human synoviocytes via RAGE-NF-κB pathway activation. *J Orthop Res*. 2016;34(5):791-800.
90. Koulis C, Watson AMD, Gray SP, Jandeleit-Dahm KA. Linking RAGE and Nox in diabetic micro- and macrovascular complications. *Diabetes Metab*. 2015;41(4):272-281.
91. Li F, Cai Z, Chen F, et al. Pioglitazone attenuates progression of aortic valve calcification via down-regulating receptor for advanced glycation end products. *Basic Res Cardiol*. 2012;107(6):306.
92. Hofmann B, Yakobus Y, Indrasari M, et al. RAGE influences the development of aortic valve stenosis in mice on a high fat diet. *Exp Gerontol*. 2014;59:13-20.
93. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. *Cardiovasc Diabetol*. 2020;19(1):92.
94. Yang P, Feng J, Peng Q, Liu X, Fan Z. Advanced glycation end products: potential mechanism and therapeutic target in cardiovascular complications under diabetes. *Oxid Med Cell Longev*. 2019;2019:9570616.

How to cite this article: Natorska J, Kopytek M, Undas A. Aortic valvular stenosis: Novel therapeutic strategies. *Eur J Clin Invest*. 2021;00:e13527. https://doi.org/10.1111/eci.13527

Kraków, dnia 01.06.2023

dr hab. n. med. Joanna Natorska
Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol. 2020 Jun 17;19(1):92. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- konceptualizacji badań,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu, poprawie i prowadzeniu nadzoru nad manuskryptem.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Joanna Natorska
.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu

Kraków, dnia 01.06.2023

dr hab. n. med. Joanna Natorska
Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021 Nov;64(11):2562-2574. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- konceptualizacji badań,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu, poprawie i prowadzeniu nadzoru nad manuskryptem.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Joanna Natorska
.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu

Kraków, dnia 01.06.2023

dr hab. n. med. Joanna Natorska
Zakład Chorób Zatorowo-Zakrzepowych, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowskie Centrum Badań i Technologii Medycznych, Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022 Nov 25;132(11):16372. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- konceptualizacji badań,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu, poprawie i prowadzeniu nadzoru nad manuskryptem.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Joanna Natorska
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań,

Kraków, dnia 01.06.2023

dr hab. n. med. Joanna Natorska
Zakład Chorób Zatorowo-Zakrzepowych, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowskie Centrum Badań i Technologii Medycznych, Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023: 12(10):1402. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- konceptualizacji badań,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu, poprawie i prowadzeniu nadzoru nad manuskryptem.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Joanna Natorska
.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań,

Kraków, dnia 01.06.2023

dr hab. n. med. Joanna Natorska
Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Aortic valvular stenosis : Novel therapeutic strategies. Eur J Clin Invest. 2021 Jul;51(7):e13527. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- opracowaniu koncepcji,
- interpretacji danych,
- sporządzeniu, poprawie i prowadzeniu nadzoru nad manuskryptem.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Joanna Natorska
.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Prof. dr hab. n. med. Anetta Undas
Zakład Chorób Zatorowo-Zakrzepowych
Instytut Kardiologii
Uniwersytet Jagielloński Collegium Medicum
Krakowskie Centrum Badań i Technologii Medycznych
Krakowski Szpital Specjalistyczny im. Jana Pawła II

Kraków, dnia 02.06.2023

OŚWIADCZENIE

Jako współautor pracy*:. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol. 2020 Jun 17;19(1):92. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J.

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- interpretacji danych,
- naniesieniu poprawek do manuskryptu i akceptacji końcowej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu

Prof. dr hab. n. med. Anetta Undas
Zakład Chorób Zatorowo-Zakrzepowych
Instytut Kardiologii
Uniwersytet Jagielloński Collegium Medicum
Krakowskie Centrum Badań i Technologii Medycznych
Krakowski Szpital Specjalistyczny im. Jana Pawła II

Kraków, dnia 02.06.2023

OŚWIADCZENIE

Jako współautor pracy*: Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021 Nov; 64(11):2562-2574. Kopytek M, Mazur P, Ząbczyk M, Undas A, Natorska J. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- interpretacji danych,
- naniesieniu poprawek do manuskryptu i akceptacji końcowej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Prof. dr hab. n. med. Anetta Undas
Zakład Chorób Zatorowo-Zakrzepowych
Instytut Kardiologii
Uniwersytet Jagielloński Collegium Medicum
Krakowskie Centrum Badań i Technologii Medycznych
Krakowski Szpital Specjalistyczny im. Jana Pawła II

Kraków, dnia 02.06.2023

OŚWIADCZENIE

Jako współautor pracy*: PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023; 12(10):1402. Kopytek M, Ząbczyk M, Mazur P, Undas A, Natorska J. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- interpretacji danych,
- naniesieniu poprawek do manuskryptu i akceptacji końcowej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Prof. dr hab. n. med. Anetta Undas
Zakład Chorób Zatorowo-Zakrzepowych
Instytut Kardiologii
Uniwersytet Jagielloński Collegium Medicum
Krakowskie Centrum Badań i Technologii Medycznych
Krakowski Szpital Specjalistyczny im. Jana Pawła II

Kraków, dnia 02.06.2023

OŚWIADCZENIE

Jako współautor pracy*: Aortic valvular stenosis : Novel therapeutic strategies. Eur J Clin Invest. 2021 Jul;51(7):e13527. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- przygotowaniu części manuskryptu,
- naniesieniu poprawek do manuskryptu i akceptacji końcowej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr hab. n. med. Michał Ząbczyk
Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol. 2020 Jun 17;19(1):92. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr hab. n. med. Michał Ząbczyk
Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021 Nov;64(11):2562-2574. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr hab. n. med. Michał Ząbczyk
Zakład Chorób Zatorowo-Zakrzepowych, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowskie Centrum Badań i Technologii Medycznych, Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022 Nov 25;132(11):16372. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy

Kraków, dnia 01.06.2023

dr hab. n. med. Michał Ząbczyk
Zakład Chorób Zatorowo-Zakrzepowych, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowskie Centrum Badań i Technologii Medycznych, Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023; 12(10):1402. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- konceptualizacji badań,
- analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów (lub pomiarów (podział wykonanych [illegible]) opracowaniu

Kraków, dnia 01.06.2023

dr n. med. Piotr Mazur
Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Accumulation of advanced glycation end products (AGEs) is associated with the severity of aortic stenosis in patients with concomitant type 2 diabetes. Cardiovasc Diabetol. 2020 Jun 17;19(1):92. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- rekrutacji pacjentów,
- analizie i interpretacji danych,
- naniesieniu poprawek do manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr n. med. Piotr Mazur
Uniwersytet Jagielloński Collegium Medicum

OŚWIADCZENIE

Jako współautor pracy*:. Diabetes concomitant to aortic stenosis is associated with increased expression of NF-κB and more pronounced valve calcification. Diabetologia. 2021 Nov;64(11):2562-2574. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- rekrutacji pacjentów,
- analizie i interpretacji danych,
- naniesieniu poprawek do manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr n. med. Piotr Mazur
Klinika Chirurgii Serca, Naczyń i Transplantologii, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Department of Cardiovascular Surgery, Mayo Clinic, USA

OŚWIADCZENIE

Jako współautor pracy*:. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022 Nov 25;132(11):16372. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- rekrutacji pacjentów,
- analizie i interpretacji danych,
- naniesieniu poprawek do manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr n. med. Piotr Mazur
Klinika Chirurgii Serca, Naczyń i Transplantologii, Instytut Kardiologii, Uniwersytet Jagielloński Collegium Medicum,
Department of Cardiovascular Surgery, Mayo Clinic, USA

OŚWIADCZENIE

Jako współautor pracy*:. PAI-1 Overexpression in Valvular Interstitial Cells Contributes to Hypofibrinolysis in Aortic Stenosis. Cells. 2023; 12(10):1402. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- rekrutacji pacjentów,
- analizie i interpretacji danych,
- naniesieniu poprawek do manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.

Kraków, dnia 01.06.2023

dr n. med. Jakub Siudut
Krakowskie Centrum Badań i Technologii Medycznych, Krakowski Szpital Specjalistyczny im. Jana Pawła II

OŚWIADCZENIE

Jako współautor pracy*:. Oxidized phospholipids associated with lipoprotein(a) contribute to hypofibrinolysis in severe aortic stenosis. Pol Arch Intern Med. 2022 Nov 25;132(11):16372. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji polegał na**:

- pozyskaniu i analizie części danych.

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez mgr Magdalenę Kopytek jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Magdaleny Kopytek polegający na**:

- opracowywaniu koncepcji,
- wykonywaniu części eksperymentalnej,
- pozyskaniu, analizie i interpretacji danych,
- sporządzeniu i poprawie manuskryptu.

.....
(podpis współautora)

*należy podać tytuł, nazwę czasopisma, wolumen, rok, strony

**np. opracowywaniu pomysłu badań, stworzeniu hipotezy badawczej, opracowaniu koncepcji badań, wykonywaniu określonych eksperymentów i/lub pomiarów (najlepiej wskazać których), opracowaniu i interpretacji wyników tej pracy, przygotowaniu manuskryptu pracy.