DILATACIÓ AÒRTICA A LA VALVULOPATIA AÒRTICA BICÚSPIDE: APROFUNDINT EN LA FISIOPATOLOGIA A LA CERCA DE BIOMARCADORS

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Facultat de Medicina i Ciències de la Salut
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DILATACIÓ AÒRTICA A LA VALVULOPATIA AÒRTICA BICÚSPIDE: APROFUNDINT EN LA FISIOPATOLOGIA A LA CERCA DE BIOMARCADORS

Tesi doctoral presentada per:

Marta Jesús Faiges Borràs

Dirigida i tutoritzada per:

Josep Maria Alegret Colomé

Per optar al títol de:
Doctor per la Universitat Rovira i Virgili

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UNIVERSITAT ROVIRA i VIRGILI

El Dr. Josep Maria Alegret Colomé, professor titular del Departament de Medicina i Cirurgia de la Universitat Rovira i Virgili.

FA CONSTAR que:

el present treball titulat ´Dilatació aòrtica a la valvulopatia aòrtica bicúspide: aprofundint en la fisiopatologia a la cerca de biomarcadors´ que presenta **Marta Faiges Borràs** per a l'obtenció del títol de doctor s'ha dut a terme sota la seva direcció en el marc del programa de doctorat en Biomedicina al Departament de Medicina i Cirurgia de la Universitat Rovira i Virgili.

José Mª Alegret Colomé

Firmado digitalmente por José Mª Alegret Colomé
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Josep Maria Alegret Colomé

Director de la tesi doctoral

Al Pere, la Teresa i la Cinta per la seva paciència, empatia, i tolerància.

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Aquesta tesi doctoral consta d'un compendi de 3 articles publicats en revistes internacionals indexades, amb els següents factors d'impacte i quartils:

- Journal of Translational Medicine – 2017 – IF 4.197 – Q1 (28/133) categoria: Medicine, Research & Experimental
- Frontiers in Physiology – 2017 – IF 3.394 – Q1 (20/83) categoria; Physiology
- Journal of Clinical Medicine – 2023 – IF 4.964 – Q2 (55/172) categoria: Cardiac & cardiovascular Systems

Aquesta tesi ha rebut una beca de Investigación Clínica de la Sociedad Española de Cardiología l'any 2018, i una beca tipus accèssit a grup d'Inici de la Fundació Ferran l'any 2017.

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1. RESUM/ABSTRACT

1. RESUM

Paraules clau: vàlvula aòrtica bicúspide; metabolisme de les lipoproteïnes; dilatació de l'aorta ascendent; glicoproteïna; HMR; microRNA; micropartícules endotelials circulants; bioinformàtica; dany endotelial.

Antecedents i objectius: La vàlvula aòrtica bicúspide (VAB) confereix un alt risc de dilatació de l'aorta ascendent (DAA), encara que la seva progressió és molt variable. A més, els mecanismes implicats en la DAA no estan del tot clars. Entre altres fenòmens s'ha descrit que els pacients amb VAB presenten nivells elevats de micropartícules endotelials circulants (EMP) que serien manifestació de dany endotelial. Respecte als factors de risc cardiovascular clàssics, la DAA a la VAB s'ha relacionat amb la hipertensió arterial, però el paper del metabolisme lipoproteic no està del tot aclarit. Els objectius plantejats es descriuen a continuació. Des de la vessant epigenètica, hem intentat identificar microRNA (miRNA o miR) circulants associats amb la VAB i la DAA amb la intenció de proporcionar potencials biomarcadors diagnòstics i pronòstics de la malaltia. També hem volgut identificar un patró específic de miRNAs associats a EMP en el context del dany endotelial descrit a la VAB. Finalment, hem avaluat l'impacte dels perfils de lipoproteïnes i glicoproteïnes en la DAA de la VAB així com la seva progressió **Mètodes:** Els participants formen part del projecte Cardiobanc, que recull pacients amb VAB, pacients amb vàlvula aòrtica tricúspide (VAT) i DAA i controls, dels que s'obté mostres de sèrum, plasma i ADN que queden emmagatzemades al Biobanc del mateix nom de l'IISPV. En el primer estudi, vam aplicar inicialment un enfocament de *discovery* no dirigit utilitzant un microarray. Vam fer servir mostres de plasma (n = 24) d'individus sans amb VAT i pacients amb VAB sense i amb dilatació aòrtica per comparar i identificar els miRNA específics associats amb la VAB i la dilatació aòrtica. En una segona etapa, els patrons d'expressió dels candidats a miRNA es van validar mitjançant RT-qPCR en una cohort independent (n = 43). En el segon estudi, vam emprar un enfocament d'anàlisi bioinformàtica per relacionar els nivells d'EMP amb el perfil d'expressió de miRNA al plasma en individus sans i en pacients amb VAB (n = 36). A més, fent servir els miRNAs que estaven associats

significativament amb els nivells d'EMP, vam identificar miRNAs altament coexpressats o grups de miRNA reguladors de dany endotelial. Finalment, en el tercer estudi, mitjançant ressonància magnètica nuclear 1H (1H-NMR), vam analitzar i comparar els perfils de lipoproteïnes i glicoproteïnes de mostres de plasma de 152 pacients amb VAB amb aorta ascendent dilatada i no dilatada. A més, els perfils de 119 pacients van ser seguits clínicament i per ecocardiografia a llarg termini. Per a aquesta anàlisi es va calcular la velocitat de DAA (mm/any). **Resultats:** En el primer estudi, vam observar que els nivells d'expressió de miR-122, miR-130a i miR-486 circulants estan influïts significativament per la morfologia de la vàlvula aòrtica (bicúspide/tricúspide) i podrien estar implicats funcionalment en la regulació de la senyalització de factor de creixement transformador beta (TGF-β). A més, l'expressió de miR-718 es va correlacionar de forma inversa amb el diàmetre aòrtic (R = -0,63,p = 3,1 × 10−5) i va ser un predictor independent de la dilatació aòrtica (β = -0,41, p = 0,022), suggerint que els gens relacionats amb miR-718 estan implicats en la regulació de la remodelació vascular. En el segon estudi, vam identificar una xarxa de coexpressió composta per 131 miRNAs associats significativament amb els nivells d'EMP. A més, vam identificar un clúster de 19 miRNAs altament coexpressats, situats al locus 14q32 del cromosoma 14 (p = 1,9 × 10). Vam trobar que aquest clúster estava implicat en la regulació de gens amb diverses funcions, específicament implicats en el procés metabòlic del compost nitrogenat cel·lular (p = 2,34 × 10−145), el procés del sistema immunitari (p = 2,57 × 10−6), l'organització de la matriu extracel·lular (p = 8,14 × 10−5) i la via de senyalització TGF-β (p = 2,59 × 10−8). Finalment, en el tercer estudi vam identificar alguns paràmetres relacionats amb el perfil de les lipoproteïnes, com ara el colesterol remanent que inclou el colesterol de molt baixa densitat (VLDL) i el colesterol de densitat intermitja (IDL), que estaven significativament augmentats en el grup dilatat en comparació amb els del grup no dilatat. També vam observar un augment del senyal de ressonància magnètica nuclear (RMN) de la glicoproteïna A (GlycA), un marcador d'inflamació, en el grup dilatat. Després de fer una anàlisi multivariant, el colesterol remanent va romandre una variable independent relacionada amb la DAA. En el seguiment a llarg termini, els paràmetres lipoproteics proaterogènics es van relacionar amb un major creixement aòrtic. Després d'una anàlisi de regressió lineal, les partícules no HDL es van mantenir com a un predictor independent de la velocitat de DAA. **Conclusions:** Proposem que miR-122, miR-130a, miR-486 i

miR-718 són noves molècules associades a la VAB i a la dilatació aòrtica. A més, s'ha descrit un perfil d'expressió de miRNA associat a les EMP secretades en la valvulopatia aòrtica bicúspide. També hem identificat un clúster de miRNA altament coexpressat que podria intervenir en processos biològics crucials de la VAB. Finalment, els pacients amb VAB i DAA presenten un perfil més proaterogènic avaluat per 1H-RMN, especialment relacionat amb les lipoproteïnes riques en triglicèrids. Aquest perfil proaterogènic sembla contribuir a una taxa de major creixement del diàmetre de l'aorta ascendent.

1. ABSTRACT

Keywords: bicuspid aortic valve; lipoprotein metabolism; dilatation of the ascending aorta; glycoprotein; HMR; microRNA; circulating endothelial microparticles; bioinformatics; endothelial damage.

Background and objectives: The bicuspid aortic valve (VAB) confers a high risk of dilatation of the ascending aorta (DAA), although its progression appears to be highly variable. In addition, the mechanisms involved in DAA are not fully elucidated. Among other phenomena, it has been described that patients with VAB present high levels of circulating endothelial microparticles (EMP) which would be a manifestation of endothelial damage. Regarding classic cardiovascular risk factors, DAA in VAB has been linked to arterial hypertension, but the role of lipoprotein metabolism is not fully clarified. From the epigenetic side, we tried to find the circulating microRNAs (miRNAs or miRs) associated with VAB and DAA with the intention of providing potential diagnostic and prognostic biomarkers of the disease. We also hypothesized that the endothelial damage described in VAB is functionally related to the secretion of a specific pattern of EMP-associated miRNAs. We evaluated the impact of lipoprotein and glycoprotein profiles on DAA as well as its progression on VAB aortopathy. **Methods:** The participants are part of the Cardiobank project, which collects patients with VAB, patients with tricuspid aortic valve (VAT) and DAA and controls. Their samples of plasma, serum and DNA are stored in the biobank of the same name in IISPV. In the first study, we applied an untargeted discovery approach with microarray, using plasma samples (n = 24) from healthy individuals with VAT and patients with VAB without and with aortic dilatation to compare and to identify the specific miRNAs associated with VAB and aortic dilatation. In a second stage, the expression patterns of miRNA candidates were validated by RT-qPCR in an independent cohort (n = 43). In the second study, we employed a bioinformatics analysis approach to relate EMP levels to miRNA expression profile in plasma in healthy individuals and in patients with VAB (n = 36). Furthermore, using the miRNAs that were significantly associated with EMP levels, we identified highly co-expressed miRNAs or groups of miRNAs regulating endothelial damage. Finally, in the third study, using 1H nuclear magnetic resonance (1H-NMR), we analyzed and compared the lipoprotein and glycoprotein profiles of

plasma samples from 152 VAB patients with dilated and non-dilated ascending aorta. In addition, the profiles of 119 patients were followed by long-term clinically and echocardiography. DAA velocity (mm/year) was calculated for this analysis. **Results:** In the first study, miRNA microarray data and RT-qPCR analyzes revealed that the expression levels of circulating miR-122, miR-130a and miR-486 are significantly influenced by aortic valve morphology (bicuspid/tricuspid) and could be functionally involved in the regulation of transforming growth factor beta (TGF-β) signaling. In addition, miR-718 expression was inversely correlated with aortic diameter (R = -0.63,p = 3.1 × 10–5) and was an independent predictor of aortic dilatation (β = -0.41, p = 0.022), suggesting that miR-718-related genes are involved in the regulation of vascular remodeling. In the second study, we identified a co-expression network composed of 131 miRNAs significantly associated with EMP levels. Furthermore, we identified a cluster of 19 highly co-expressed miRNAs, located at locus 14q32 on chromosome 14 (p = 1.9 × 10). We found that this cluster was involved in the regulation of genes with various functions, specifically involved in the cellular nitrogen compound metabolic process (p = 2.34 × 10–145), the immune system process (p = 2, 57 × 10–6), the organization of the extracellular matrix (p = 8.14 × 10–5) and the TGF-β signaling pathway (p = 2.59 × 10–8). Finally, in the third study we identified some parameters related to the lipoprotein profile, such as the remaining cholesterol that includes very low density cholesterol (VLDL) and intermediate density cholesterol (IDL), that were significantly increased in the dilated group compared to those in the non-dilated group. We also observed an increase in the nuclear magnetic resonance (RMN) signal of glycoprotein A (GlycA), a marker of inflammation, in the dilated group. After multivariate analysis, residual cholesterol remained an independent variable related to DAA. At long-term follow-up, proatherogenic lipoprotein parameters were associated with greater aortic growth. After linear regression analysis, non-HDL particles remained an independent predictor of DAA rate. **Conclusions:** To conclude, we propose that miR-122, miR-130a, miR-486 and miR-718 are novel molecules associated with VAB and aortic dilatation. In addition, a miRNA expression profile associated with secreted EMPs has been described in bicuspid aortic valvulopathy. We also identified a highly co-expressed miRNA cluster that could be involved in crucial biological processes of VAB. Finally, patients with VAB and DAA present a more proatherogenic profile assessed by 1H-NMR, especially related to lipoproteins rich in triglycerides. This proatherogenic profile appears to contribute to a higher rate of growth in the diameter of the ascending aorta.

2. INTRODUCCIÓ

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2 a. VÀLVULA AÒRTICA BICÚSPIDE

La vàlvula aòrtica (*valva aortae*) està situada després del tracte de sortida del ventricle esquerre i abans de l'arrel aòrtica. Es tracta d'una vàlvula semilunar amb tres plecs membranosos de mida similar suspesos a la paret del vas en forma de "niu d'oreneta" que s'anomenen valves: la posterior o no coronària (*valvula semilunaris posterior*), la dreta (*valvula semilunaris dextra*) i l'esquerra (*valvula semilunaris sinistra*). Cada valva presenta dues cares: una còncava cap a la llum de l'aorta i una convexa cap a la llum del ventricle esquerre, i dos extrems: un adherit a la paret en forma de "U" i un altre lliure cap a la cavitat arterial. Al mig de la vora lliure hi trobem un petit engruiximent fibrós anomenat nòdul d'Aranci (*noduli valvularum semilunarium o nodulus Arantii*) i al seu costat una zona molt fina en forma de mitja lluna coneguda com la lúnula (*lunulae valvurarum semilunarium*). Durant la diàstole les valves es tanquen hermèticament gràcies a l'oposició de les lúnules adjacents i dels nòduls d'Aranci. A diferència de les vàlvules auriculo-ventricular, la vàlvula aòrtica no està sustentada per cap anell fibrós, sinó que es produeix una extensió de la mateixa paret arterial aòrtica en tres cavitats anomenades sinus arterials o de Valsalva. Les artèries coronàries dreta i esquerra s'originen del sinus corresponent. El punt d'unió entre cada sinus respecte al seu adjacent s'anomena comissura (figura 1).

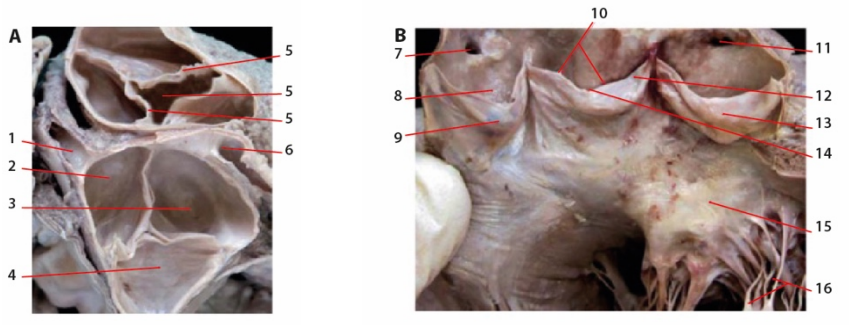

Figura 1. A) Visió superior de la vàlvula aòrtica. 1-Origen de l'artèria Coronària Esquerra. 2-Valva semilunar esquerra. 3-Valva semilunar dreta. 4-Valva semilunar posterior. 5-Vàlvula pulmonar. 6-Origen de l'artèria Coronària Dreta. **B) Visió sagital de la vàlvula aòrtica.** 7-Origen de l'artèria Coronària Dreta. 8-Sinus arterial o de Valsalva. 9-Valva semilunar dreta. 10-Lúnula de la valva semilunar. 11-Origen de l'artèria Coronària Esquerra. 12-Valva semilunar posterior. 13-Valva semilunar esquerra. 14-Nòdul d'Aranci. 15-Vàlvula mitral. 16-Cordes tendinoses. **Atlas de anatomía cardíaca. Correlación con las técnicas de imagen. 2011.**

La valvulopatia aòrtica bicúspide (VAB) és la cardiopatia congènita més freqüent, amb una prevalença entre del 0,6-2% en la població general (Hung et al., 2014; Rajamannan et al., 2004). Es tracta de la fusió de dos dels vels coronaris que s'estableix per mitjà d'un rafe o engruiximent fibrós o de la presència de només dos vels coronaris (Fedak et al., 2003; Sun B et al., 2017).

L'ecocardiografia transtoràcica (ETT) és el mètode d'elecció per al diagnòstic i seguiment de la disfunció valvular i l'afectació aòrtica dels pacients amb VAB. La sensibilitat i especificitat en la detecció de la VAB pot arribar al 92% i 96% respectivament si es disposa d'una finestra acústica adequada. Es recomana valorar la morfologia valvular en sístole per poder visualitzar el rafe. Per als casos dubtosos o per a una millor avaluació de l'aorta s'utilitza l'ecocardiograma transesofàgic (ETE), la cardio-ressonància (cardio-RM) o la tomografia axial computaritzada (TC).

Hi ha diferents classificacions de la vàlvula bicúspide segons l'aspecte que es pretén emfatitzar (Michelena et al., 2021). La classificació més clàssica és la de Sievers (Sievers et al., 2007), que distingeix els següents tipus: a) tipus 0 (VAB pura, sense rafe); b) tipus 1 (la més freqüent, amb un rafe entre els 2 vels), que se subdivideix entre fusió dels vels aòrtics esquerre i dret (L-R), dret i no coronari (R-N) o esquerre i no coronari (N-L), i c) tipus 2 (amb 2 rafes o monocúspide). Una altra classificació molt utilitzada fa referència als vels afectats, distingint-se els tipus I, II i III (Schaefer et al., 2008). El tipus I, també anomenada típica o anteroposterior es caracteritza per la fusió entre els vels dret i esquerre, amb una obertura típica en "boca de peix". És el morfotipus valvular més freqüent, afectant entre el 70-80% dels casos. El tipus II, atípica o dreta-esquerra es produeix quan el rafe es presenta entre el vel dret i el no coronari. Sol ser menys freqüent (20-30%) i ocasiona una obertura latero-lateral. Finalment, el tipus III es distingeix quan s'observa una fusió del vel esquerre i no coronari. És una variant excepcional (2-3%) (figura 2).

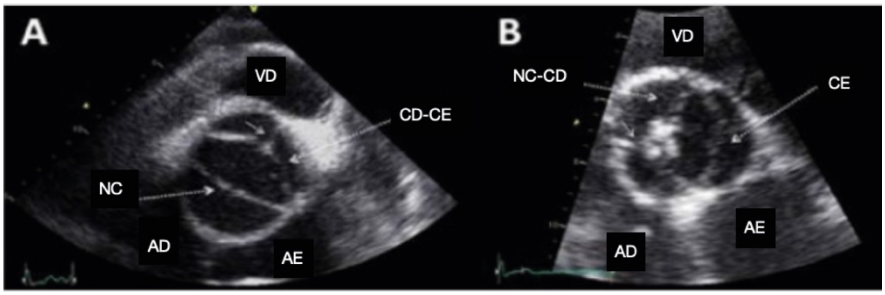

Figura 2. Morfotips de la vàlvula aòrtica bicúspide des del pla paraesternal eix curt. **A) Tipus I, típica o anteroposterior. B) Tipus II, atípica o dreta-esquerra.** Les fletxes petites senyalen el rafe. Imatges del Dr. Evangelista. VD: ventricle dret. AD: aurícula dreta. AE: aurícula esquerra. NC: vel no coronari. CD: vel coronari dret. CE: vel coronari esquerre.

Recentment, un grup internacional ha proposat una classificació basada en les característiques de la fusió de les cúspides i la geometria de les comissures. En aquesta classificació es poden distingir tres patrons de VAB relacionats amb la fusió de les cúspides i el nombre de sinus (Michelena et al., 2021). El primer patró es defineix com la VAB fusionada i es diagnostica en el 90-95% dels casos i presenta tres subtipus definits segons les cúspides involucrades. Es distingeixen tres sins i la fusió de dues de les tres cúspides (dreta-esquerra, dreta-no coronària, esquerra-no coronària). El segon tipus de VAB es coneix com el tipus VAB de dos sins. En aquest patró és possible identificar dues cúspides corresponents a sins homòlegs, no depenent de la fusió sinó de la constitució embriològica anormal. En general, les cúspides tenen la mateixa mida, no hi ha rafe i l'orifici aòrtic pot tenir dues obertures: laterolateral o anteroposterior. El tercer tipus de VAB és una VAB de fusió parcial.

Les classificacions anteriors tenen implicacions per a la pràctica clínica diària, de manera que les que tenen presents els vels i els patrons de fusió de les comissures són transcendents per valorar la dilatació aòrtica. Fins i tot, la morfologia de la vàlvula aòrtica, els canvis de flux i l'avaluació pronòstica estan ben determinats per models derivats de la selecció del patró de fusió.

La història natural de la VAB és molt variable (Liebe et al., 2006). El diagnòstic durant l'etapa infantil se sol establir per l'estudi d'un buf o clic d'ejecció aòrtica i sol cursar sense símptomes. Fora de l'estenosi aòrtica neonatal, és molt infreqüent que aparegui simptomatologia en aquesta etapa, malgrat que s'identifiqui una disfunció valvular. Si distingim per edats, els joves presenten més insuficiència mentre que

l'estenosi i la dilatació aòrtica és més freqüent a partir dels 50 anys. En termes generals, l'evolució natural de la malaltia comporta un 13-30% d'insuficiència aòrtica moderada-greu, un 30-70% d'estenosi aòrtica moderada-greu i un 2-5% d'endocarditis (Michelena et al., 2021). Pel que fa al sexe, la VAB afecta més a homes en una relació 3:1. Però a pesar que les dones solen tenir una afectació més tardana, menys insuficiència i majors comorbiditats, no existeixen diferències amb la supervivència respecte als homes.

La VAB de vegades coexisteix amb altres cardiopaties congènites com ara la coartació aòrtica, el ductus arteriós persistent, les anomalies de les artèries coronàries amb una major dominància esquerra, la comunicació interventricular o la Tetralogia de Fallot. La cardiopatia congènita associada més freqüent és la coartació aòrtica. Malgrat que la presència d'una coartació aòrtica només s'observa en el 7-10% dels pacients amb VAB, al voltant del 50% dels pacients amb coartació aòrtica tenen una VAB. Altres alteracions genètiques relacionades són la síndrome de Turner, que també comporta dilatació de l'aorta, i la síndrome de Williams.

2 b. Característiques de l'aorta ascendent en la vàlvula aòrtica bicúspide

Aorta toràcica

L'aorta es divideix segons la cavitat que ocupa en toràcica i abdominal. L'aorta toràcica se situa al mediastí superior i se subdivideix en aorta ascendent (*Pars ascendens aortae*), arc (*Arcus aortae*) i aorta descendent (*Pars*

descendens aortae). L'aorta ascendent sol fer entre 2-3,7 cm de diàmetre i 5 cm de longitud i s'hi solen distingir 3 parts: l'arrel, la unió sino-tubular i la porció tubular. A l'altura de l'arrel es localitzen l'origen de les artèries coronàries, els sinus de Valsalva i la vàlvula aòrtica. L'arc s'estén des del 2n espai intercostal al costat de l'estèrnum fins a la cara lateral esquerra del cos vertebral de T4, des d'on segueix com a aorta descendent. D'aquesta part emergeixen el tronc braquiocefàlic, l'artèria caròtida comú esquerra i l'artèria subclàvia esquerra.

L'aorta és un tipus d'artèria elàstica o de conducció. S'hi poden distingir tres capes: l'íntima, la mitjana i l'adventícia. L'íntima està formada per l'endoteli, la túnica continua en contacte amb la sang on es troba la matriu extracel·lular, i el subendoteli, amb les cèl·lules musculars llises. La matriu de l'endoteli té una triple funció: dona suport a la cèl·lula endotelial, fa de filtre de les macromolècules i modula la funció cel·lular. L'endoteli sintetitza substàncies que regulen el to i la permeabilitat vascular com ara l'òxid nítric, les prostaciclines, el tromboxà A2, les prostaglandines, l'enzim convertidor de l'angiotensina, l'endotelina-1 i els radicals lliures. Entre la capa íntima i la mitjana es troba la làmina elàstica interna formada per teixit elàstic fenestrat. La mitjana està formada per 55-60 unitats músculo-esquelètiques concèntriques d'1mm de gruix. Cada unitat conté làmines d'elastina disposades en forma d'hèlix, fenestrades i interconnectades entre si. També s'hi troben fibres de col·lagen, cèl·lules musculars llises secretadores de proelastina i glicoproteïnes, i proteoglicans. De tot això podem deduir que es tracta d'una capa metabòlicament activa. L'adventícia és una capa subdesenvolupada, formada per diversitat de cèl·lules, fibres de col·lagen, fibres

elàstiques, nervis, vasos sanguinis (*vasa vasorum*) i vasos limfàtics.

Els diàmetres aòrtics es mesuren amb l'ETT des del vorell intern a l'extern en telediàstole i s'indexen per superfície corporal i per edat. A partir del pla paraesternal eix llarg, els diàmetres que es poden mesurar són: l'anell aòrtic, els sinus de Valsalva, la unió sino-tubular, la porció tubular de l'aorta ascendent i l'arc (figura 3). Malgrat que hi ha diverses referències, en adults es solen considerar normals uns diàmetres indexats en aorta ascendent tubular < 2,0 cm/m2. En els casos de dilatació es recomana la validació dels diàmetres per mitjà de dues tècniques d'imatge a fi d'aconseguir una mesura més precisa. La cardio-RM i el TC permeten definir de forma més exacta la mida del vas a través d'una reconstrucció multiplanar.

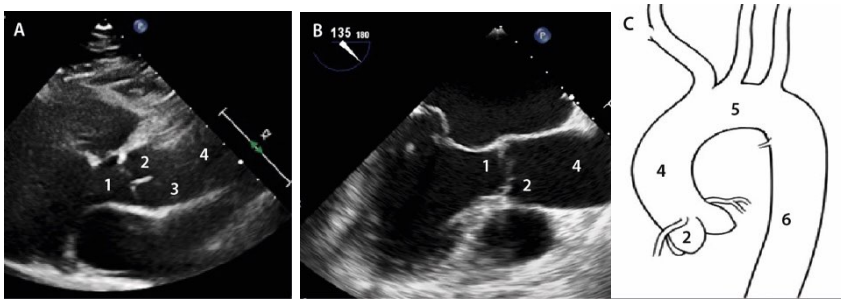

Figura 3. A) Mesura dels diàmetres aòrtics amb l'ETT en telediàstole. Pla paraesternal eix llarg. **B)** Mesura dels diàmetres aòrtics amb l'ETE. **C)** Dibuix de l'aorta toràcica. 1-Anell aòrtic. 2-Sinus de Valsalva. 3- Unió sino-tubular. 4-Porció tubular de l'aorta ascendent. 5-Arc aòrtic. 6-Aorta toràcica descendent.

Aortopatia bicúspide: histologia, classificació i història natural

La prevalença de la dilatació aòrtica significativa en individus amb VAB se situa al voltant del 50% (Verma et al., 2014), malgrat que varia en funció del criteri emprat per definir la dilatació i del rang d'edat de la població analitzada. La prevalença de la DAA tubular en pacients amb VAB en edats entre els 30 i els 60 anys està entre el 56% i el 88%, respectivament, mentre que en altres sèries en els mateixos grups d'edat es troba entre el 48% i el 57%, respectivament (Della Corte et al., 2007). Els homes semblen tenir dilatació aòrtica amb major proporció que les dones (Andrei et al., 2015).

Histopatològicament, s'observa una aortopatia degenerativa, havent-se descrit diferents alteracions histoquímiques de la paret aòrtica. Les metal·loproteïnases reguladores de la matriu (MMP) i els seus inhibidors endògens es troben en un desequilibri que afavoreix la degradació de la matriu extracel·lular. Aquest fenomen s'ha descrit en diversos estudis (Forte et al., 2017; Rabkin et al., 2014; Wu et al., 2016) amb resultats heterogenis que poden ser deguts al diferent tipus de mostres analitzades i a l'heterogeneïtat de l'aortopatia bicúspide. També s'ha descrit una disminució del contingut de fibrilina-1 en les aortes de pacients amb VAB en comparació amb les aortes de VAT (Fedak et al., 2003; Rueda-Martínez et al., 2017). Diversos estudis han objectivat una transició anormal de les cèl·lules musculars llises cap a un fenotip secretor associat a la senescència (Ailawadi et al., 2009; Grewal et al., 2014; Balint et al., 2019), així com una senyalització defectuosa de NOTCH1 que augmentaria l'apoptosi de les cèl·lules musculars llises vasculars (Harrison et al., 2019). També es

descriu una menor quantitat de fibres d'elastina, que a més són de menor gruix i més separades entre elles.

S'han descrit diferents classificacions de la dilatació aòrtica amb valor descriptiu o pronòstic, sense que hi hagi consens sobre quina ha de prevaldre. Fazel et al. (2008) va proposar quatre patrons de dilatació aòrtica: tipus I, on només està implicada l'arrel aòrtica; tipus II, que només afecta la porció tubular de l'aorta ascendent; tipus III, on estan dilatades l'aorta ascendent i l'arc aòrtic; i el tipus IV, que comporta una dilatació difusa de l'arrel aòrtica, l'aorta ascendent i l'arc aòrtic proximal. En canvi, Park et al. (2011) va recomanar una altra classificació que descriu la DAA basada en el potencial efecte del fenotip sobre el pronòstic. Inclou tres tipus: el tipus I afecta la DAA tubular amb preservació de la unió sinotubular, el tipus II es defineix per una ectàsia de l'aorta ascendent i arrel amb esborrament de la unió sinotubular i, el tipus III engloba una dilatació limitada a l'arrel. Della Corte et al. (2014) va suggerir una classificació més senzilla que considera dos fenotips principals: el fenotip de l'arrel aòrtica (dilatació sins aòrtics > aorta ascendent) i el fenotip ascendent (DAA > sins). Encara que més simple, aquesta classificació sembla tenir major valor pronòstic.

Respecte a la relació amb la funció valvular, en els pacients amb estenosi s'observa una major prevalença de la dilatació a l'aorta ascendent tubular (Rossi et al., 2013; Roman et al., 2017), mentre que la prevalença de la dilatació difusa de l'aorta és major en pacients amb insuficiència aòrtica (Roman et al., 2017). La prevalença de la dilatació aòrtica també varia segons el fenotip valvular. La prevalença de la dilatació aòrtica proximal és més elevada en la VAB amb fusió dreta-esquerra (Khoo et al., 2013; Li et al., 2019), mentre que la DAA i la part proximal de l'arc és més freqüent

en pacients amb fusió dreta-no coronàrica (Li et al., 2019). Globalment, la dilatació aòrtica és més freqüent en pacients amb fusió D-E que en aquells amb fusió D-NC (Khoo et al., 2013).

En pacients amb VAB, la taxa d'increment del diàmetre de l'aorta ascendent varia molt depenent de les sèries (Davies et al., 2007; Detaint et al., 2014; Etz et al., 2010; Guo et al., 2018). Aquestes diferències poden estar relacionades amb les característiques de la població, les tècniques utilitzades, així com l'heterogeneïtat de la malaltia. S'ha descrit que, com major sigui el diàmetre aòrtic basal, més ràpida és la progressió de la dilatació aòrtica (Park K. H. et al., 2017). Tanmateix, no hi ha una correlació lineal típica entre la mida aòrtica inicial i la dilatació aòrtica (Detaint et al., 2014). Della Corte et al. (2013) va analitzar retrospectivament una sèrie de 133 adults amb VAB que van ser seguits per ecocardiografia. Van trobar una taxa de creixement mitjana de 0,3 mm/any en els sinus de Valsalva i 0,6 mm/any a l'altura de l'aorta ascendent. El fenotip de l'arrel aòrtica sovint va predir una aortopatia més greu i la dilatació aòrtica va progressar més ràpidament (>0,9 mm/any) al nivell de l'aorta ascendent. No obstant això, els pacients amb VAB amb fusió D-E i un fenotip de dilatació ascendent van demostrar ser més estables i rarament van mostrar un creixement ràpid (Della Corte et al., 2013). Un altre estudi retrospectiu va trobar que la fusió D-E era un factor de risc per a una dilatació més ràpida, amb una taxa de creixement anual superior a 1,01 mm/any (Thanassoulis et al., 2008). Per altra part, Avadhani et al. (2015) va observar que els morfotips de fusió no influïen en el risc de dilatació aòrtica (0,47 mm/any D-E i 0,46 mm/any D-NC, respectivament). Una revisió sistemàtica i una metaanàlisi descriuen que la taxa de creixement de l'aorta ascendent va ser de 0,3 mm/any en

pacients amb VAT i de 0,76 mm/any en pacients amb VAB (Guo et al., 2018).

La dilatació aòrtica pot progressar a aneurisma o dissecció aòrtica. La dissecció aòrtica és la complicació més greu relacionada amb la VAB. La mortalitat hospitalària se situa al voltant del 19% (Reutersberg et al., 2019). La taxa de dissecció aòrtica en pacients amb VAB es descriu entre el 0,31 i el 0,50%. Malgrat que el risc és baix en termes absoluts, és vuit vegades més alt que el de la població general (Michelena et al., 2011; Kong et al., 2017). El coneixement de la història natural de l'aortopatia VAB és decisiu en alguns estudis observacionals retrospectius. En un estudi de cohort retrospectiu de 384 pacients sense aneurismes, després de 15 anys de seguiment, 49 pacients van desenvolupar-ne. La incidència d'aneurisma en VAB va ser 84,9 casos per cada 10.000 pacient-any, que és 86 vegades més alt risc que la població general. Després del diagnòstic d'aneurisma, el risc de dissecció aòrtica a llarg termini va ser del 7% (Michelena et al., 2011).

La coartació de l'aorta pot accelerar la progressió de la dilatació aòrtica en altres segments aòrtics, especialment a l'aorta ascendent i a nivell de la postcoartació, predisposant a l'aneurisma aòrtic, i potencialment a la dissecció aòrtica i ruptura aòrtica (Erbel et al., 2014). Malgrat que amb algun estudi discordant (Davies et al., 2007), la coartació aòrtica es descriu com un factor de risc per a la dilatació aòrtica ascendent i la dissecció aòrtica tipus A (Oliver et al., 2009; Eleid et al., 2013; Zhao et al., 2018). La severitat de la coartació també s'ha considerat com a factor afavoridor de la dilatació (Zhao et al., 2018). La perspectiva actual és que els pacients amb VAB amb antecedents de coartació aòrtica

haurien de tenir un llindar més baix per indicar la cirurgia aòrtica preventiva (Erbel et al., 2014).

De tot el dit abans es dedueix que els factors de risc relacionats amb la dilatació aòrtica són complexos i que s'han analitzat i registrat de forma diferent en els diversos estudis, on variava el diàmetre aòrtic inicial, l'edat, la tècnica d'imatge, i el temps de seguiment, dificultant tot això poder establir conclusions definitives en alguns aspectes.

Aortopatia bicúspide: fisiopatologia

Les alteracions genètiques i hemodinàmiques es postulen actualment com a causes de la formació dels aneurismes aòrtics a la VAB. Malgrat que hi ha arguments per atribuir a la predisposició genètica una part de la responsabilitat, els estudis més recents situen les alteracions hemodinàmiques com a les principals responsables de la dilatació aòrtica de la VAB. Analitzarem què se'n sap de cadascuna.

Genètica

Algunes dades clíniques recolzarien la predisposició genètica, com que es pot produir una dilatació aòrtica en pacients amb VAB sense disfunció valvular, en pacients amb VAB després d'un recanvi valvular aòrtic i en pacients amb anomalies sindròmiques lligades a mutacions. Les bases genètiques que hi estarien relacionades s'han estudiat àmpliament en els darrers anys, però només s'han identificat algunes vies. Mutacions en el gen ACTA2, que codifica per a-actina del múscul llis, poden causar aneurismes aòrtics toràcics i/o VABs (Guo et al., 2007). S'ha identificat que la

variació del gen SMAD6 s'associa amb l'aneurisma aòrtic en pacients amb VAB no sindròmic (Galvin et al., 2000; Gillis et al., 2017; Luyckx et al., 2019). Malgrat això, només algunes de les variants SMAD6 anaven lligades a aneurisma de l'aorta toràcica, mentre que la majoria de les variants presentaven fenotips clínics variables (Luyckx et al., 2019; Park et al., 2019). També s'ha identificat el NOTCH1 com un gen lligat a la susceptibilitat dels aneurismes aòrtics de la VAB, en concret variants sense sentit NOTCH1 (Harrison et al., 2019). La desregulació de la via de senyalització NOTCH1 es pot identificar a etapes precoces de la formació dels aneurismes aòrtics (Balistreri et al., 2018). Un estudi recent ha descrit que ROBO4 està implicat en la formació i el desenvolupament de l'aneurisma aòrtic a la VAB (Gould et al., 2019).

A més, GatA5, TGFBR1/2, FBN1, ADAMTSL1, ADAMTS- 4 i NOS3 també són gens candidats associats amb l'aortopatia VAB (Arrington et al., 2008; Folkersen et al., 2011; Laforest et al., 2011; Ren et al., 2017; Rocchiccioli et al., 2017; Vorkapic et al., 2017; Peterson et al., 2020). Malgrat això, aquestes mutacions només explicarien una petita part dels aneurismes relacionats amb la VAB. Com estan implicats aquests gens en la formació dels aneurismes, o si aquests gens són necessaris per a la formació de l'aneurisma, són preguntes encara sense resposta, però cada cop queda més clar que un model genètic únic no pot explicar completament l'aortopatia bicúspide.

Hemodinàmica

Moltes evidències relacionen les característiques de la dinàmica del flux transvalvular aòrtic de la VAB amb la dilatació aòrtica. Aquestes evidències s'han recollit amb diverses tècniques d'imatge, des de la clàssica ETT fins a la recentment incorporada ressonància magnètica de flux 4D. La primera evidència que relacionava el flux amb la dilatació aòrtica a la VAB es basava en la descripció de l'estenosi aòrtica com a factor de risc independent relacionat amb la dilatació/aneurisma de l'aorta ascendent. A més del paper del flux accelerat, l'excentricitat del jet i les seves característiques al llarg de l'aorta ascendent s'han relacionat amb el risc de dilatació. Malgrat que aquesta teoria no és nova, no ha sigut fins als darrers anys, amb la incorporació de l'anàlisi del flux 4D a l'aorta ascendent, quan tècnicament s'han pogut explicar en profunditat aquests mecanismes hemodinàmics (Markl et al., 2012). La ressonància magnètica de flux 4D pot mostrar de manera exhaustiva paràmetres com ara la direcció del flux, la velocitat i l'impacte sobre l'elasticitat a la paret aòrtica i la força tangencial del jet a la paret aòrtica (també anomenada força de cisallament o *wall shear stress (WSS))* (Markl et al., 2012). A més, la precisió de la ressonància magnètica de flux 4D permet mesurar la velocitat màxima de flux de la vàlvula aòrtica de forma similar a l'ecocardiografia Doppler (Rose et al., 2016). La variació del flux aòrtic a la VAB condiciona un WSS no uniforme, que es considera un dels mecanismes que condueixen a l'aortopatia, així com lesions locals de la paret aòrtica (Grewal et al., 2019). El patró de flux i la distribució local de WSS a l'aorta també estan alterats, malgrat que en diferent grau, en pacients amb VAB sense aneurisma aòrtic o fins i tot amb una funció valvular normal (Hope et al., 2010; Garcia et al., 2016). A més, el fenotip de la VAB era el factor més important

que influïa en la distribució del WSS, l'excentricitat del flux sistòlic i els fenotips de l'aortopatia (Mahadevia et al., 2014). La diversa morfologia valvular determinava canvis en la direcció, la velocitat del raig d'ejecció i els patrons WSS regionals. El perfil de velocitat i el WSS axial màxim a la VAB amb fusió D-E es distribueixen uniformement i flueixen cap a la paret aòrtica anterior i dreta (Mahadevia et al., 2014). En canvi, en la VAB D-NC s'observa un flux de sortida posterior principal dirigit a la unió sinotubular que es desplaça cap a les parets anterior o anterior dreta a l'aorta ascendent mitjana i distal (Mahadevia et al., 2014). El flux ejectiu excèntric i el WSS donaven lloc a una major dilatació aòrtica proximal en pacients amb VAB i fusió E-D mentre que en pacients amb VAB i fusió D-NC es veia una major tendència a la DAA i l'arc aòrtic proximal (Mahadevia et al., 2014; Rodríguez-Palomares et al., 2018). Atkins et al. (2014) en un estudi experimental va utilitzar la simulació de la interacció de l'estructura de fluids en un model ex vivo d'aorta porcina sotmès al flux de la VAT i la bicúspide durant 48 h en un bioreactor. La simulació va revelar l'existència d'un major WSS i més unidireccional a la convexitat de l'aorta ascendent a la VAB que a la VAT. L'expressió de MMP-2 i MMP-9 i l'activitat de MMP-2 van augmentar de forma més evident a l'aorta ascendent dels afectes de VAB (Atkins et al., 2014). Això va confirmar la sensibilitat del teixit aòrtic medial a les anomalies del WSS i va donar suport al paper de les tensions hemodinàmiques en l'aortopatia VAB (Atkins et al., 2014). Guzzardi et al. (2015) va realitzar una ressonància magnètica de flux 4D en pacients que havien patit una dissecció aòrtica i va identificar preoperatòriament la ubicació de les regions amb WSS altes i normals. Durant la cirurgia es van recollir mostres de la paret de l'aorta i es va observar una major desregulació de la matriu extracel·lular a les regions de WSS

altes, amb una major degradació de l'elastina medial i una major expressió de TGFb-1, MMP-1, MMP-2, MMP-3 i dels inhibidors tissulars específics de les MMP (TIMP) 1. La majoria dels estudis de ressonància magnètica de flux 4D actuals s'han centrat principalment en les avaluacions qualitatives i quantitatives de les característiques hemodinàmiques i de l'estrès, que poden ajudar a identificar aquells pacients en risc de desenvolupar un aneurisma de l'aorta ascendent. Els canvis en la dinàmica de flux associats a la morfologia de la vàlvula aòrtica poden precedir l'aparició de la malaltia aòrtica i poden impulsar la remodelació i la dilatació aòrtica amb el pas del temps (Piatti et al., 2017). Tanmateix, encara falten estudis clínics longitudinals a gran escala per validar aquests resultats.

2 C. Biomarcadors de dilatació aòrtica

L'evolució dels pacients amb VAB és incerta i, mentre alguns pacients desenvolupen dilatació aòrtica, altres mantenen el diàmetre aòrtic estable al llarg dels anys. Com s'ha explicat en els apartats anteriors, els mecanismes implicats en aquest fenomen no són del tot coneguts i no existeixen predictors precisos que permetin identificar al pacient que progressarà cap a una dilatació significativa. Per altra part, tampoc es disposa de biomarcadors específics de DAA identificables en sang perifèrica. Donat que la DAA és una de les principals complicacions de la VAB, i que una de les manifestacions clíniques pot ser la dissecció aòrtica, seria d'interès disposar de biomarcadors que permetessin identificar als pacients en risc de dilatació. Per altra part, també serien profitosos paràmetres per distingir els pacients amb dilatació aòrtica en un cribratge poblacional. Per

aquestes raons seria útil disposar de biomarcadors capaços d'identificar la dilatació i de predir l'evolució cap a l'augment de la mida aòrtica. Un biomarcador és aquell paràmetre utilitzat com a indicador d'un estat biològic, que es pot mesurar objectivament i pot ser avaluat com a marcador d'un procés normal, un estat patogènic o de resposta a un tractament farmacològic. Malgrat que podrien haver-hi altres tipus de biomarcadors relacionats amb la dilatació aòrtica a la VAB, com els aconseguits amb la imatge cardíaca, ens centrarem en els biomarcadors potencialment identificables en sang perifèrica, que permetrien anàlisis de cribratge poblacional. Dins d'aquests, ens focalitzarem en els que es disposa de més informació actualment i que tenen més potencial: els epigenètics i els bioquímics.

Epigenètica

L'epigenètica es refereix a canvis en l'expressió gènica que no són causades per modificacions en la seqüència gènica. Els estudis que han avaluat el paper de l'epigenètica en aquest camp s'han centrat principalment en la metilació de l'ADN i, especialment, en els miRNAs. Pan et al. (2017) va trobar per primera vegada que tant la dissecció aòrtica com la dilatació aneurismàtica aòrtica de la VAB es caracteritzaven per la pèrdua de metilació de l'ADN en llocs no CpG. Un estudi posterior va observar que la metilació de l'ADN a l'íntima-mitjana aòrtica de mostres de teixit aòrtic de pacients amb VAB es va associar amb la pèrdua de les característiques de les cèl·lules endotelials i la seva transició cap a mesenquimals (Björck et al., 2018). Però la part de l'epigenètica que sembla més prometedora en el camp dels biomarcadors és els dels miRNAs. Els miRNA són petites molècules d'ARN de 19-23 nucleòtids no codificadors que

regulen l'expressió gènica a nivell post-transcripcional com a moduladors negatius, silenciant o degradant els ARN missatgers (ARNm). D'aquesta manera participen en diversos processos biològics com ara la diferenciació cel·lular, la proliferació, l'apoptosi i el desenvolupament embrionari i tissular. Els miRNAs representen un 2-3% del genoma humà i poden regular l'expressió d'aproximadament el 60% del gens, de manera que un sol miRNA pot regular 200 gens diferents, així com un mateix ARNm pot ser regulat per múltiples miRNAs. Alguns miRNA s'han proposat com a implicats en el desenvolupament i progressió de la dilatació aòrtica del VAB. Una anàlisi de tot el miRNA en teixit d'aneurismes de l'aorta ascendent de pacients amb VAB i VAT va indicar que el miR-424-3p i el miR-3688-3p estaven significativament regulats a la baixa en pacients amb VAB en comparació amb els pacients amb VAT (Borghini et al., 2017). Wu et al. (2016) va recollir mostres de teixit aòrtic severament dilatat i no dilatat de 12 pacients amb VAB i va descriure el paper del miR-17 en la regulació de la via dels TIMP, implicada en la formació d'aneurismes aòrtics. Per altra banda, en pacients amb VAB i aorta no dilatada s'ha descrit una baixa expressió de la família miR-200 (Maleki et al., 2019). També s'ha descrit una menor expressió de miR-145 circulant en pacients VAB i dilatació de l'arrel aòrtica que s'associava amb la presència de variants rares de NOTCH1 (Girdauskas et al., 2018). Tot i que els miRNA són biomarcadors potencials molt prometedors, tot apunta que la determinació d'un sol miRNA no pot ser un predictor fiable de la malaltia aneurismàtica, i que probablement caldria un patró de miRNA per tenir una precisió acurada (Albinsson et al., 2017; Maleki et al., 2019).

Bioquímica

Respecte als paràmetres bioquímics, els que s'han estudiat fins ara estan en relació amb la fisiopatologia de la dilatació aòrtica a la VAB. Fonamentalment, s'han descrit paràmetres bioquímics circulants relacionats amb la degradació de la paret aòrtica, amb factors proinflamatoris i amb dany endotelial. Com s'ha comentat anteriorment, la desregulació de la via TIMP-MMP està implicada en la formació d'aneurismes aòrtics, havent-se descrit diferents patrons de TIMP i de MMP relacionades amb la dilatació aòrtica a la VAB.

De tota manera, actualment no es poden considerar específics ni es disposa d'estudis que avalin la seva precisió. Recentment, s'ha descrit la relació entre els nivells d'IL-6, una citoquina proinflamatòria, i els diàmetres de l'aorta ascendent en una sèrie de pacients amb anomalies genètiques de l'aorta ascendent, entre les quals es trobava la VAB. De tota manera, l'associació era molt feble (Fujita et al., 2019). Altrament, és conegut que la VAB determina un flux transvalvular aòrtic excèntric que s'havia relacionat amb la presència de disfunció endotelial (Tzemos et al., 2010). El nostre grup va postular que aquest flux excèntric podria ser responsable de dany endotelial aòrtic, i que aquest participi en l'inici de l'activació de mecanismes de degeneració de la matriu aòrtica i la posterior dilatació. Vam identificar la relació entre la dilatació aòrtica a la VAB i els nivells circulants d'EMP, que podrien estar en relació amb el dany endotelial provocat pel flux excèntric (Alegret et al., 2016). En posteriors anàlisis vam observar que els pacients amb VAB i dilatació aòrtica tenien una disminució de la capacitat antioxidant per la davallada dels nivells de α-tocoferol i del seu transportador l'apolipoproteïna A1, la principal proteïna del HDL (Martínez-

Micaelo et al., 2020). α-tocoferol és un antioxidant liposoluble transportat pel HDL amb capacitat de neutralitzar els radicals lliures endògens i té una relació inversament proporcional amb els diàmetres de l'arrel aòrtica i de l'aorta ascendent. En canvi, el seu augment provoca l'augment de la paraoxonasa 1, un enzim amb propietats antioxidants transportat per HDL. De forma paral·lela, s'ha estudiat la relació causal entre la dilatació de l'aorta abdominal i el metabolisme lipídic (Wild et al., 2012; Kubota et al., 2018). En aquest sentit, prèviament vam relacionar LDL i l'apoB com a predictors del diàmetre de l'arrel aòrtica, mentre que apoB també ho és de l'aorta ascendent (Alegret et al., 2015).

De tot l'anterior es conclou que encara no es disposa de biomarcadors precisos de dilatació aòrtica a la VAB i que l'aprofundiment en l'estudi de l'epigenètica, en concret en el paper dels miRNA, i de la lipidòmica poden aportar llum en aquest camp.

3. HIPÒTESI

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Es pot obtenir un patró de miRNA predictor de la morfologia valvular aòrtica i de la presència de DAA. Així mateix, la disfunció endotelial i el metabolisme lipídic estan implicats en la fisiopatologia de la dilatació aòrtica de la VAB i poden haver-hi biomarcadors relacionats, que ens ajudin tant en el diagnòstic com en el pronòstic de la malaltia.

4. JUSTIFICACIÓ I OBJECTIUS

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La VAB és la cardiopatia congènita més freqüent i sovint afecta adolescents i adults joves, que habitualment al llarg de la vida desenvoluparan dilatació aòrtica i molts d'ells necessitaran cirurgia de reparació aòrtica.

Actualment, els fenòmens fisiopatològics implicats en l'aortopatia bicúspide no estan del tot clars. El nostre estudi pretén contribuir a una millor comprensió dels mecanismes implicats en l'augment de la mida de l'aorta. Aquests resultats podrien tenir una translació directa a la pràctica clínica, mitjançant la identificació de predictors del risc de dilatació aòrtica a la VAB que contribuirien a optimitzar i individualitzar el seguiment, el pronòstic i el tractament dels pacients. Per tant, els resultats d'aquesta tesi podrien ser rellevants tant des del punt de vista d'aportació de coneixement com de la potencial aplicabilitat clínica.

OBJECTIU GENERAL

Aprofundir en el coneixement de la fisiopatologia de la dilatació aòrtica a la VAB, mitjançant l'estudi de l'epigenètica i del metabolisme lipoproteic, amb la finalitat d'identificar biomarcadors relacionats i factors sobre els quals actuar per alentir la seva evolució.

OBJECTIUS ESPECÍFICS

1. Identificar miRNAs específics associats a la VAB amb DAA (article 1).

2. Cercar un patró de miRNA relacionat amb el dany endotelial a la VAB (article 2).

3. Avaluar la relació de les lipoproteïnes i les glicoproteïnes sobre la DAA a la VAB per mitjà de 1H-NMR (article 3).

5. METODOLOGIA

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5 A. MOSTRA

Tots els participants d'aquest estudi formen part del projecte anomenat "Cardiobanc" vinculat al Biobanc del mateix nom de l'IISPV, que inclou pacients amb VAB, pacients amb DAA i VAT i controls. En el present estudi s'han inclòs pacients afectes de VAB sense i amb dilatació aòrtica majors de 18 anys controlats al Servei de Cardiologia de l'Hospital Sant Joan de Reus (HSJR) entre 1999 i 2020. També s'han inclòs pacients amb VAT sense i amb dilatació aòrtica controlats per un altre motiu o sense patologia.

El diagnòstic de VAB es determina analitzant la morfologia valvular aòrtica mitjançant l'ecocardiograma transtoràcic al pla paraesternal eix curt, on també es distingeix quins són els vels fusionats. També s'inclouen pacients diagnosticats de VAB amb un ecocardiograma transesofàgic o una cardioressonància magnètica (CMR). Com a mesura de l'aorta ascendent s'ha considerat el diàmetre màxim de la porció tubular de la mateixa en telediàstole i amb l'estudi ecocardiogràfic. Totes les imatges s'han realitzat o supervisat pel mateix observador (JMA).

A la inclusió del participant s'ha dut a terme una anamnesi acurada amb la recollida dels antecedents patològics, inclosos els factors de risc cardiovascular i un examen físic complet. S'ha extret una mostra de sang perifèrica per venopunció, emmagatzemant-se al Biobanc les mostres de sèrum, plasma i ADN. Els pacients inclosos han estat seguits de forma prospectiva clínica i ecocardiogràficament, recollint-se els esdeveniments

cardiovasculars apareguts i la necessitat de cirurgia cardiovascular.

En l'article 1 i 2 es van incloure pacients amb VAB i VAT mentre que a l'article 3 només pacients amb VAB. Els criteris d'inclusió i exclusió varien en els diferents articles. D'una banda, en l'article 1 i 2 s'han exclòs els pacients majors de 70 anys i els diagnosticats de malalties cardiovasculars, síndrome de Marfan, estenosi aòrtica, hipertensió, diabetis mellitus o que estaven rebent tractament farmacològic com ara estatines, IECA/ARAII i/o β-bloquejants). En canvi, en l'article 3 només s'han exclòs els Marfan i els afectes d'altres cardiopaties congènites.

També hi ha diferències amb el tipus d'estudi dels 3 articles. L'article 1 i 2 van ser estudis observacionals transversals, mentre que l'article 3 té dues parts: la primera fou un estudi observacional transversal i la segona va ser un estudi longitudinal prospectiu amb un seguiment ecocardiogràfic de com a mínim de 5 anys de durada.

5 B. Emmagatzematge i tractament de les mostres

Les mostres de sang es van recollir a primera hora del matí havent estat com a mínim 8 hores en dejú i es van processar en un termini màxim de 90 minuts després de l'extracció. Les mostres es van centrifugar a 1500 g durant 15 minuts per obtenir plasma i posteriorment aquest plasma es va tornar a centrifugar a 4000 g durant 10 minuts més per a eliminar les plaquetes. El plasma es va emmagatzemar a -80 °C al nostre banc de mostres biològiques (Biobanc IISPV - HUSJR) fins que fos necessari.

En l'article 1, per a l'extracció de l'ARN i la preparació dels microarrays de miRNA es van seguir diferents passos. L'ARN total es va extreure de 250 µL de plasma mitjançant el reactiu TRIzol segons les instruccions del fabricant (Invitrogen) i es va purificar mitjançant un minikit RNeasy (Qiagen). Per augmentar la recuperació de l'ARN, es va afegir 1 µg d'ARN portador MS2 a cada mostra de plasma. La qualitat de l'ARN total aïllat es va determinar mitjançant un bioanalitzador Agilent 2100. Els nivells d'expressió de miRNA en plasma per a 24 individus es van avaluar mitjançant microarrays de miRNA humans SurePrint G3 8 × 60 k (Agilent Technologies), que cobrien 1205 miRNAs humans (alliberament de Sanger miRBase 16). Els miRNA es van desfosforilar i es van etiquetar amb bifosfat de cianina 3-citidina, inclosa una solució d'etiquetatge (Agilent Technologies) per avaluar l'eficiència de l'etiquetatge. Les mostres es van hibridar a les matrius, inclosa una solució d'hibridació (Agilent Technologies) per controlar l'eficiència de la hibridació. Les matrius es van escanejar amb un sistema d'escàner de microarrays G2565CA amb tecnologia d'alta resolució SureScan (Agilent Technologies) mitjançant el programari Scan Control. Per al processament de dades es van utilitzar els paquets de programari Feature Extraction 11.5.11 (Agilent Technologies) i GeneSpring 12.6.1 i tots els passos es van realitzar segons el protocol del fabricant. Les dades dels microarrays es van normalitzar mitjançant el mètode robust de mitjana multiarray (RMA), implementat al paquet AgimiRNA Bioconductor, i els canvis de plec dels miRNAs circulants es van determinar mitjançant el model lineal implementat al paquet limma Bioconductor. El mètode Benjamini i Hochberg es va utilitzar per a ajustar els valors de *p* per a les proves múltiples i per a controlar la taxa de falsos positius. Les dades d'expressió de microarrays es van validar

en una cohort de pacients independent (n = 43), inclosos controls VAT sans, pacients amb VAB, pacients amb VAB dilatats i un grup addicional d'individus VAT amb dilatació aòrtica. La transcripció inversa es va fer mitjançant el kit de transcripció inversa TaqMan miRNA (Applied Biosystems) i els primers de transcripció inversa específics de miRNA proporcionats amb el TaqMan miRNA Assay (Applied Biosystems) per analitzar l'expressió de cada miRNA. La concentració final d'ARN total utilitzada va ser de 2, 5 ng/μL. La reacció es va realitzar a 16 ° C durant 30 min, 42 ° C durant 30 min i 85 ° C durant 5 min. Es va utilitzar 1, 33 μL dels ADNc obtinguts en una amplificació quantitativa posterior de qRT-PCR mitjançant la barreja mestra TaqMan Universal PCR (Applied Biosystems) i la sonda específica associada proporcionada al TaqMan miRNA Assay Kit (Applied Biosystems). Es van emprar sondes Taqman específiques per a cada gen de la següent manera: miR-122-5p (miR-122-5p; hsa-miR-122-5p), 5′-UGGAGUGUGACAAUG GUGUUUG-3′; miR-130a-3p (miR-130a-3p; hsa-miR-130a-3p), 5′-CAGUGCAAUGUUAAAAGGGC AU-3′; miR-486-5p (miR-486-5p; hsa-miR- 486-5p), 5′-UCCUGUACUGAGCUGCCCCGAG-3′ i miR-718 (miR-718; hsa-miR-718), 5′-CUUC- CGCCCCGCCGGGCGUCG -3′. Els resultats es van normalitzar a l'expressió de l'ARN nuclear petit U6 (ARNsn U6), que es va utilitzar com a control endogen. L'amplificació es va realitzar mitjançant el sistema ABI Prism 7300 SDS Real-Time PCR (Applied Biosystems, Madrid, Espanya) a 95 °C durant 10 min, seguit de 40 cicles de 95 °C durant 15 segons i 60 °C durant 1 min. El canvi de plec en el nivell de miRNA es va calcular mitjançant l'escala logarítmica 2 segons l'equació 2-ΔΔCt, on ΔCt = Ct miR-Ct U6 i ΔΔCt = mostres tractades amb ΔCt - ΔCt controls no tractats. Els gens objectiu de miR-122, miR-130a i miR-486

es van predir mitjançant DIANA-miRPath (v3; miR. gr/ tarbase), mapejats a les vies de l'Enciclopèdia de Genomes i Genomes de Kyoto (KEGG) basades en una anàlisi d'enriquiment, i prioritzat per a les vies de transducció del senyal intracel·lular que podrien ser regulades pels tres miRNAs. A causa dels gens objectiu identificats per a miR-718, per a aquest miRNA, vam combinar els gens diana predits per quatre bases de dades, és a dir, TargetScan (v7.0; targetscan.org), miRTarBase (versió 2016; miRtar-base). .mbc.nctu.edu.tw), bases de dades DIANA-microT-CDS (v3; miR.gr/microT-CDS) i miRDB (versió 2016; miRdb.org). En aquest cas, la classificació funcional dels gens diana es va dur a terme amb Gene Ontology (GO) i l'anàlisi de vies mitjançant DAVID (david.ncifcrf.gov) i WebGestalt (webgestalt.org), respectivament. La versió 3.0.1 de Cytoscape es va utilitzar per visualitzar la interacció prevista entre miRNA i ARNm. A la xarxa, les vies de transducció del senyal intracel·lular estan representades per nodes en forma d'hexàgons, els miRNAs estan representats per nodes en forma de rectangle i els gens diana estan representats per nodes en forma rodona.

En l'article 2, els nivells circulants d'EMP es van determinar en les mateixes cohorts utilitzades per a les anàlisis de validació de microarrays i RT-qPCR que es van fenotipar prèviament (Alegret et al., 2016), però per a aquest estudi es van seleccionar individus per maximitzar el poder de l'anàlisi de miRNA. Els nivells d'EMP es van caracteritzar a partir de la presència d'antígens de superfície específics de l'endoteli, la composició dels quals depèn de l'origen cel·lular de les micropartícules i del procés de generació (Jiménez et al., 2003). En el nostre estudi anterior (Alegret et al., 2016), vam determinar que la presència de CD31 és un marcador que pot discriminar les micropartícules alliberades de les

cèl·lules endotelials sotmeses a danys per causes hemodinàmiques secundàries al flux aòrtic anòmal. La concentració d'EMP es va determinar en un citòmetre de flux EPICS-XL (Beckman Coulter) a una velocitat baixa i en un temps de parada de 30 segons. Es va utilitzar el kit estàndard de mida de partícules nano fluorescents (Spherotech) per a l'estandardització d'instruments i es van afegir fluorosferes de comptatge de flux (Beckman Coulter) com a calibrador intern per a calcular les quantitats de micropartícules. Les EMP de plasma es van marcar incubant 50 µl de plasma pobre en plaquetes amb l'anticòs corresponent, anti-CD31-PE (Beckman Coulter), anti-CD42b-FITC (Beckman Coulter) o anti-CD45-PE (Beckman Coulter), a temperatura ambient a la foscor durant 20 minuts (Ci et al., 2013). A continuació, es van afegir 500 µl de PBS i es van determinar els nivells d'EMP seguint el procés descrit anteriorment (Jiménez et al., 2003; Sutherland et al., 2010; Ci et al., 2013). Les EMP es van definir com a partícules > 0, 1 i < 1 µm de mida, i el seu origen endotelial es va identificar en funció de la seva afinitat amb antígens específics de la superfície cel·lular, és a dir, CD31 i CD42b. Per a avaluar l'abast de la possible contaminació amb micropartícules derivades de leucòcits, es van determinar els nivells circulants de micropartícules CD31+CD45+ en totes les mostres. Vam trobar que <4,5% de les micropartícules CD31+ coexpressaven CD45+; aquest resultat és coherent amb els informes anteriors d'altres autors (Amabile et al., 2005; Pirro et al., 2006). Els nivells d'EMP van ser mesurats per tècnics de forma cega, sense conèixer l'estat clínic dels pacients. L'ARN total es va extreure de 250 µl de plasma mitjançant el reactiu TRIzol segons les instruccions del fabricant (Invitrogen) i es va purificar mitjançant un minikit RNeasy (Qiagen). Per augmentar la recuperació de l'ARN, es va afegir 1 µg d'ARN portador MS2

a cada mostra de plasma. La qualitat de l'ARN aïllat total es va determinar mitjançant el Bioanalyzer Agilent 2100. Els nivells d'expressió de miRNA plasmàtics es van avaluar mitjançant microarrays de miRNA humans Sure Print G3 de 8 × 60 k (Agilent Technologies) que cobrien 1.205 miRNAs humans (Sanger miRBase release 16). Els miRNA es van desfosforilar i es van etiquetar amb bifosfat de cianina 3-citidina inclosa una solució d'etiquetatge de punta (Agilent Technologies) per avaluar l'eficiència de l'etiquetatge. Les mostres es van hibridar a les matrius amb la inclusió d'una solució de punta d'hibridació (Agilent Technologies) per controlar l'eficiència de la hibridació. Les matrius es van escanejar amb un sistema d'escàner de microarrays G2565CA amb tecnologia d'alta resolució SureScan (Agilent Technologies) mitjançant el programari Scan Control. S'han utilitzat paquets de programari per al tractament de dades. Les dades dels microarrays (dipositats a Gene Expression Omnibus amb el número d'adhesió de la sèrie GEO GSE101616) es van normalitzar mitjançant el mètode robust de mitjana multiarray (RMA) (Irizarry et al., 2003) implementat al paquet AgimiR Bioconductor. Els miRNAs circulants es van determinar mitjançant el model lineal implementat al paquet limma Bioconductor (Ritchie et al., 2015). Els mètodes Benjamini i Hochberg es van fer servir per a ajustar els valors *p* per a proves múltiples i per a controlar la taxa de falsos positius (Benjamini et al., 1995). La xarxa de miRNA es va construir a partir dels perfils d'expressió d'aquells miRNAs associats significativament amb EMP (Spearman p <0, 05). La xarxa de coexpressió es va inferir mitjançant models gràfics gaussians (GGM) implementats al paquet R GeneNet (Schäfer et al., 2005). Breument, es va estimar una matriu de correlació parcial calculant la correlació parcial entre els perfils d'expressió de cada parell de miRNA. Es va fer servir

una probabilitat de vora posterior bayesiana> 0,95 (corresponent a una taxa local de falsos positius <5%) per determinar la importància de les correlacions parcials per parelles resultants. A la xarxa de coexpressió resultant, que es va visualitzar mitjançant el programari Cytoscape (Cline et al., 2007), els nodes representen el conjunt de miRNAs que estaven significativament correlacionats amb els nivells d'EMP, i les vores uneixen els parells de miRNAs l'expressió dels quals era no condicionalment independent, definida com la correlació parcial per parelles un cop eliminats els efectes comuns dels altres miRNAs del subconjunt (Opgen-Rhein et al., 2007). Els assajos de miRNA TaqMan (Applied Biosystems) es van usar per quantificar l'expressió dels miRNA seleccionats. Breument, la transcripció inversa es va realitzar mitjançant el kit de transcripció inversa TaqMan miRNA (Applied Biosystems) i els oligonucleòtids específics de miRNA proporcionats amb el TaqMan miRNA Assay (Applied Biosystems). La concentració final d'ARN total utilitzat va ser de 2, 5 ng/µL. La reacció es va realitzar a 16 ○ C durant 30 min, 42 ○ C durant 30 min i 85 ○ C durant 5 min. Hem utilitzat 1, 33 µL dels ADNc obtinguts en una posterior amplificació quantitativa qRT-PCR mitjançant la barreja mestra TaqMan Universal PCR (Applied Biosystems, Madrid, Espanya) i la sonda específica associada proporcionada al TaqMa(n)R miRNA Assay Kit (Applied Biosystems). Es van utilitzar sondes TaqMan específiques per a cada gen: let-7d (hsa-let-7d-5p), let-7g(hsa-let-7g-5p),miR-122(hsa-miR-122-5p),miR-130a(hsa-miR-130a-3p), miR-337 (hsa-miR-337-5p), miR-409 (hsa-miR-409-3p), miR-486 (hsa-miR-486-5p), miR-494 (hsa-miR-494) i miR-718 (hsa-miR-718). Els resultats es van normalitzar a l'expressió de l'ARN nuclear petit U6 (ARNsn U6), que es va utilitzar com a control endogen. L'amplificació es va realitzar en un

termociclador 7900HT (Applied Biosystems) a 95 ○ C durant 10 minuts, seguit de 40 cicles de 95 ○ C durant 15 segons i 60 ○ C durant 1 minut. El canvi de plec en el nivell de miRNA es va calcular mitjançant l'escala log 2 d'acord amb l'equació 2 - Ct, on Ct = Ct miR-Ct U6 i Ct = mostres tractades amb Ct - controls no tractats. Les reaccions de control negatiu, sense ARN, tenien valors de cicle de quantificació indetectables (Cq). La visualització de loci genètics de miRNAs coexpressats a través dels cromosomes es va realitzar mitjançant PhenoGram (Wolfe et al., 2013). Per determinar la importància de la sobrerepresentació de les regions cromosòmiques en el conjunt de miRNA co-expressat generat, vam aplicar una prova hipergeomètrica. Els llocs objectiu de miRNA putatius per als candidats a miRNA es van identificar mitjançant una anàlisi bioinformàtica a partir de bases de dades de predicció d'objectius de miRNA, incloses DIANA-microT-CDS (Paraskevopoulou et al., 2013), DIANA-TarBase v7.0 (Vlachos et al., 2015) i TargetScan v6.2 (Agarwal et al., 2015). L'anàlisi d'enriquiment funcional dels gens diana de miRNA es va portar a cap mitjançant miRNAPath v3.0 (Vlachos et al., 2015). La visualització i l'anàlisi topològica de la xarxa es va fer mitjançant el programari R i Cytoscape.

En l'article 3 el perfil de lipoproteïnes es va mesurar en mostres de sèrum (250 µL) mitjançant la prova Liposcale® basada en 1H-NMR, una prova de ressonància magnètica nuclear de nova generació de Biosfer Teslab (Reus, Espanya). Es van calcular les concentracions de les quatre classes principals de lipoproteïnes: VLDL, IDL, LDL i lipoproteïnes d'alta densitat (HDL). També es van determinar els nombres de partícules de nou subclasses: nombres de partícules grans, mitjanes i petites de les quatre lipoproteïnes. Breument, les concentracions de partícules i

els coeficients de difusió es van obtenir a partir de les amplituds mesurades i l'atenuació dels seus senyals de RMN del grup metil de lípids espectroscòpicament diferents mitjançant el pols d'espectroscòpia 1H-NMR ordenat per difusió 2D. El senyal de metil estava equipat a la superfície amb nou funcions lorentzianes associades a cada subclasse de lipoproteïnes: subclasses grans, mitjanes i petites de cadascuna de les classes principals de lipoproteïnes. L'àrea de cada funció Lorentziana es va relacionar amb la concentració de lípids de cada subclasse de lipoproteïnes, i la mida es va calcular a partir del seu coeficient de difusió. Les diferents subclasses de lipoproteïnes corresponien als següents rangs de mida de diàmetre: VLDL gran, de 68,5 a 95,9 nm; VLDL mitjà, de 47 a 68,5 nm; VLDL petit, de 32,5 a 47 nm; LDL gran, de 24 a 32,5 nm; LDL mitjà, de 20,5 a 24 nm; LDL petit, de 17,5 a 20,5 nm; HDL gran, de 10,5 a 13,5 nm; HDL mitjà, de 8,5 a 10,5 nm; i HDL petit, de 7,5 a 8,5 nm. Les partícules no HDL es defineixen com la suma de totes les partícules de lipoproteïnes menys el colesterol HDL. RemCholesterol es defineix pel colesterol total menys el colesterol HDL i el colesterol LDL. El perfil de la glicoproteïna es va determinar mitjançant l'anàlisi de la regió espectral específica de 1H-RMN on aquests enllaços proteïna-sucre ressonen (2, 15-1, 90 ppm). Per a cada funció, vam determinar l'àrea total (proporcional a la concentració), l'alçada, la posició i l'amplada de banda. L'àrea de la GlycA va proporcionar la concentració de grups acetils d'enllaç proteic N-acetilglucosamina i N-acetilgalactosamina, i l'àrea de la glicoproteïna B va proporcionar la concentració d'àcid N-acetilneuramínic. L'àrea de la glicoproteïna F sorgeix de la concentració en els grups acetils de N-acetilglucosamina, N-acetilgalactosamina i àcid N-acetilneuramínic no unit a proteïnes (fracció lliure). L'alçada es va calcular com la

diferència entre la línia de base i el màxim dels pics de RMN corresponents, i el valor d'amplada corresponia a l'amplada del pic a mitja alçada.

5 C. ANÀLISI ESTADÍSTICA

En l'article 1, les mitjanes de dos grups es van comparar mitjançant la prova t de Student. Per comparar les freqüències de les variables categòriques, es va utilitzar una prova de Chi quadrat o una prova exacta de Fisher, quan correspongués. Els efectes de la morfologia de la vàlvula i la dilatació aòrtica es van avaluar mitjançant l'anàlisi de la variància (ANOVA). La prova de Tukey es va usar per a les comparacions per parelles. Es va utilitzar una correlació de Pearson per identificar les relacions lineals entre els nivells d'expressió de miRNA i el diàmetre de l'aorta ascendent. Es van fer servir models de regressió lineal cap enrere per identificar els predictors independents de l'expressió dels miRNAs circulants. Els valors de p <0,05 es van considerar significatius. Les anàlisis estadístiques es van realitzar mitjançant el programari SPSS versió 21.0 (IBM, Chicago, IL, EUA).

En l'article 2, a causa de la distribució esbiaixada a la dreta dels valors, els nivells plasmàtics d'EMP van experimentar una transformació logarítmica natural i es van expressar com a recomptes transformats en log per µl (log EMPs / µl). Els nivells d'EMP es presenten com a mitjana ± desviació estàndard. Proves de chi quadrat o proves exactes de Fisher quan escau, s'han utilitzat per comparar les freqüències de les variables categòriques. Els efectes de la morfologia de la vàlvula i la dilatació de l'arrel aòrtica es van avaluar mitjançant ANOVA. La prova de Tukey es va usar per

a les comparacions per parelles. Una p <0,05 es va considerar significativa. L'anàlisi estadística es va realitzar mitjançant el programari SPSS, versió 21.0 (IBM, Chicago, IL, EUA).

En l'article 3, es van fer estudis estadístics per comparar la distribució de les diferents variables en una anàlisi transversal (al principi: no DAA vs. DAA), i en una anàlisi longitudinal en funció de la velocitat de DAA (DAA Velocity). Les variables categòriques s'expressen en percentatges i les diferències significatives es van identificar mitjançant la prova de chi quadrat o la prova exacta de Fisher, segons correspongués. Les variables quantitatives, representades com a mitjana (desviació estàndard (DE)), es van analitzar mitjançant la prova t de Student. Es va utilitzar la correlació de Pearson o Spearman, segons la distribució, per identificar les relacions lineals entre el diàmetre de l'aorta ascendent i els paràmetres inclosos. En l'anàlisi longitudinal dels perfils de glicoproteïnes i lipoproteïnes, a més, es va construir una regressió logística amb un model de pas endavant per a analitzar les variables independents de les lipoproteïnes i les glicoproteïnes relacionades amb la DAA. Es van considerar valors significatius de p <0,05. Totes les anàlisis es van realitzar amb SPSS v25 (IBM Corp., Armonk, NY, EUA) i els gràfics es van dissenyar amb Prism v9 (GraphPad Software, La Jolla, CA, EUA).

5 D. ASPECTES ÈTICS

Aquest treball s'ha dut a terme d'acord amb els principis de la Declaració d'Hèlsinki. El projecte Cardiobanc va ser aprovat pel Comitè d'Ètica en Investigació Clínica de l'Hospital Universitari Sant Joan de Reus l'any 1999. Una posterior actualització va ser aprovada pel Comitè d'Ètica en Investigació en Medicaments de l'Institut d'Investigació Sanitària Pere Virgili (CEIMm IISPV) (03-06-19 / 6proj4 i 114/2020). També va ser aprovat pel CEIMm IISPV l'inici del Biobanc de mostres sanguínies (Cardiobanc) al Servei de Cardiologia de l'Hospital Verge de la Cinta. Els participants en el projecte van ser informats de la seva naturalesa i van signar un consentiment informat acceptant la seva participació.

6. RESULTATS

6. RESULTATS

ARTICLE 1: SPECIFIC CIRCULATING MIRNA SIGNATURE OF BICUSPID AORTIC VALVE DISEASE

Hem investigat quins miRNA són específics de la VAB i que, per tant, podrien utilitzar-se com a biomarcadors diagnòstics i pronòstics de la malaltia. Aquest estudi ha tingut dues fases: una primera de *discovery* no dirigida utilitzant un microarray i una segona de validació fent servir RT-qPCR. A la primera fase, a través d'un microarray hem analitzat les mostres de plasma d'individus sans amb VAT, pacients amb VAB sense dilatació i pacients amb VAB i DAA per comparar i identificar els miRNA específics associats amb la VAB i la dilatació aòrtica. En una segona etapa, els patrons d'expressió dels miRNA candidats es van validar mitjançant RT-qPCR en una cohort independent amb quatre grups: VAB amb i sense dilatació i VAT amb i sense dilatació. Les dades de microarrays de miRNA i les anàlisis RT-qPCR van revelar que el fenotipus valvular VAB s'associa a una disminució dels nivells de miR122 i miR486 i a un augment de miR130a. Aquests miRNA tenen com a diana 32 gens implicats en la via del TGF-β1. Per tant, miR122, miR130a i miR486 s'han relacionat amb la morfologia valvular regulant l'expressió gènica via el TGF-β1. D'altra banda, s'ha observat una correlació inversa entre miR-718 i el diàmetre de l'aorta ascendent. A partir d'aquesta cohort de validació, hem construït múltiples models lineals logístics utilitzant els perfils d'expressió miR-718 com a variable predictiva i el diàmetre de l'aorta com a variable de resultat. S'han tingut en compte l'edat i la hipertensió com a possibles factors de confusió. Finalment, hem identificat 4 gens diana de miR-718

relacionats amb el remodelat dels vasos sanguinis i 3 gens relacionats amb la via d'adhesió focal. Per tant, miR-718 s'ha identificat com a biomarcador de dilatació aòrtica via l'expressió de gens implicats en el remodelat dels vasos sanguinis.

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Specific circulating microRNA signature of bicuspid aortic valve disease

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Abstract

Background: We aimed to determine the circulating miRNA expression profile associated with BAV and aortic dilation to provide diagnostic and prognostic biomarkers for BAV and/or aortic dilation.

Methods and results: We applied a miRNome-wide microarray approach using plasma samples (n = 24) from healthy tricuspid aortic valve individuals, BAV patients and BAV patients with aortic dilation to compare and identify the specific miRNAs associated with BAV and aortic dilation. In a second stage, the expression patterns of the miRNA candidates were validated by RT-qPCR in an independent cohort (n = 43). The miRNA microarray data and RT-qPCR analyses revealed that the expression levels of circulating miR-122, miR-130a and miR-486 are significantly influenced by the morphology of the aortic valve (bicuspid/tricuspid) and could be functionally involved in the regulation of TGF-β_1 signalling. Furthermore, the expression pattern of miR-718 in the plasma was strongly influenced by dilation of the ascending aorta. miR-718 expression was inversely correlated with the aortic diameter ($R = -0.63$, $p = 3.1 \times 10^{-5}$) and was an independent predictor of aortic dilation ($\beta = -0.41$, $p = 0.022$). The genes targeted by miR-718 are involved in the regulation of vascular remodelling.

Conclusions: We propose that miR-122, miR-130a, miR-486 and miR-718 are new molecular features associated with BAV and aortic dilation principally by the activation of TGF-β_1 pathway and vascular remodelling mediated by VEGF signalling pathways.

Keywords: microRNA, Bicuspid aortic valve, Aortic dilation

Background

Bicuspid aortic valve (BAV) represents the most common congenital cardiac malformation and is commonly associated with the development of aortic valve dysfunction and the progressive dilation of the ascending aorta. In patients with BAV, the dilation of the aorta is associated with aortic regurgitation [1] and the risk of aortic dissection and thus often requires prophylactic aortic surgery [2–6]. The causes of aorta dilation have been debated for several years, and changes in the flow characteristics within the ascending aorta have been suggested as the main cause [7, 8]. However, the mechanisms involved have not been fully elucidated.

MicroRNAs (miRNAs) are endogenously expressed 19–23nt-long noncoding RNAs (ncRNAs) that silence gene expression at the post-transcriptional level, largely via imperfect base-pairing interactions that occur preferentially within the 3′ untranslated regions (UTRs) of target mRNAs. Due to the ability of miRNAs to target hundreds of mRNAs, they are considered as potent post-transcriptional regulators, and in fact, it is suggested that a significant portion of the human genome is regulated by miRNAs. Aside from their intracellular function, miRNAs can be exported or released by cells into the circulating blood in highly stable forms [9]. miRNA signatures have been proposed as potential biomarkers with the potential to improve disease diagnosis and prognosis in clinical practice and have been identified as useful biomarkers for a wide range of cardiovascular diseases, including cardiovascular calcification [10], vulnerable coronary artery disease [11], heart failure [12], myocardial infarction [13], abdominal aortic aneurysm [14] and

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coronary artery disease [15]. Despite the large number of miRNAs identified thus far, the biological roles of most miRNAs and the molecular mechanisms underlying their function remain largely unknown.

In this study, we applied a miRNome-wide microarray approach using the plasma of healthy tricuspid aortic valve (TAV) individuals and BAV patients [with or without aortic dilation (BAV_{dil})] to compare and identify specific miRNAs associated with BAV and the dilation of the ascending aorta. In a second stage, the expression levels of the miRNA candidates were validated in an independent cohort, providing new insights into the complex mechanisms that underlie BAV and aortic dilation.

Methods

Study population

The participants included in this study belong to a cohort of prospective patients with BAV that were followed-up in our facilities. Upon enrolment, the participants were prospectively entered into a specific database, underwent a blood draw and provided informed written consent. The samples were stored until needed in our biological sample bank (Biobanc IISPV-HUSJR). BAV diagnosis was made when two aortic leaflets were clearly visualized, with or without a raphe, either on the parasternal short-axis view of a transthoracic echocardiogram [16], on a transesophageal echocardiogram [16] or on a cardiac magnetic resonance [17]. Explorations were performed or supervised by the same observer (JMA). This study was approved by the Institutional Review Board (the Clinical Ethics Committee) of our institution.

The study design was composed of two evaluations to determine the possible miRNAs associated with BAV and aortic dilation. First, we studied the association of BAV with the modulation of the circulating miRNOme profile by determining the miRNA expression profile in the plasma of patients diagnosed with BAV ($BAV_{non\text{-}dil}$; n = 9) or BAV and aortic dilation (BAV_{dil}; n = 9) using microarrays, and then, we compared these with the expression profiles of healthy TAV control subjects ($TAV_{non\text{-}dil}$; n = 6). For this stage and to improve the study power, we selected three groups composed of patients with characteristics that were extremely homogeneous (Table 1) to exclude possible confounding factors, such as age, sex or BMI. In addition, patients with aortic stenosis, hypertension, diabetes mellitus or pharmacologic treatment (including statins, ACE/ARII and/or β-blockers) were excluded. An aortic diameter lower than 19 mm/m^2 or higher than 21 mm/m^2 was used as the criterion to differentiate between the non-dilated and dilated patients, respectively.

In the second evaluation, we validated the miRNA candidates by RT-qPCR in a new cohort of individuals comprising TAV (n = 19) and BAV individuals (n = 24) (Table 2). The BAV group comprised $BAV_{non\text{-}dil}$ (n = 12) and BAV_{dil} (n = 12) patients. The TAV group comprised healthy controls $TAV_{non\text{-}dil}$ (n = 12), and we also included a new group of patients with TAV and aortic dilation (TAV_{dil}; n = 7) in this evaluation to determine the specific effects associated with valve morphology or aortic dilation per se. Patients with Marfan syndrome were excluded.

Blood sampling

Blood samples were collected in overnight fasting conditions in the early morning and were processed within 90 min after collection. The samples were centrifuged at

Table 1 Clinical and echocardiographic characteristics of the patients included in the microarray

	Control	BAV	BAVdil	p
Age (years)	41 ± 4	33 ± 3	41 ± 4	0.177
Sex (male/female)	(6/0)	(6/0)	(6/0)	1.000
Body weight (kg)	74.2 ± 2.2	75.0 ± 4.2	79.0 ± 5.7	0.743
Hypertension	0 (0%)	0 (0%)	0 (0%)	1.000
Hypercholesterolemia	0 (0%)	0 (0%)	2 (22.2%)	0.162
Smoker	1 (20.0%)	2 (22.2%)	3 (33.3%)	0.814
Aortic stenosis (mean gradient ≥20 mm Hg)	0 (0%)	0 (0%)	0 (0%)	1.00
Aortic regurgitation (≥II)	0 (0%)	3 (33.3%)	6 (66.7%)	0.031*
Aortic valve gradient (mean, mm Hg)	3.75 ± 0.3	4.6 ± 0.8	7.22 ± 1.6	0.190
Left ventricle diastolic diameter (mm)	51.67 ± 1.5	50.75 ± 1.0	55.11 ± 1.3	0.050
Left ventricle systolic diameter (mm)	32.33 ± 0.9	31.88 ± 2.7	33.78 ± 1.4	0.484
Left ventricular ejection fraction (%)	74.40 ± 3.2	71.25 ± 1.2	71.33 ± 2.6	0.641
Ascending aorta diameter (mm/m^2)	15.05 ± 0.4	15.74 ± 0.7	20.71 ± 1.1	0.001**

BAV bicuspid aortic valve patients, *BAVdil* patients with bicuspid aortic valve and aortic dilation

* Significant values ($p < 0.05$)

** Significant values ($p < 0.01$)

Table 2 Clinical and echocardiographic characteristics of the independent cohort used in the validation study

	TAV	TAVdil	BAV	BAVdil	p
Age (years)	39 ± 3	65 ± 3	33 ± 3	42 ± 3	<0.001**
Sex (male/female)	(8/4)	(5/2)	(11/1)	(6/6)	0.171
Body weight (kg)	70.1 ± 3.7	76.8 ± 3.4	73.25 ± 4.4	66.6 ± 4.2	0.374
Hypertension	0 (0%)	5 (71.4%)	1 (8.3%)	3 (25%)	0.002**
Hypercholesterolemia	1 (8.3%)	1 (14.3%)	0 (0%)	0 (0%)	0.391
Smoker	0 (0%)	0 (0%)	1 (8.3%)	3 (25%)	0.105
Aortic stenosis (mean gradient ≥20 mm Hg)	0 (0%)	0 (0%)	1 (8.3%)	2 (16.7%)	0.507
Aortic regurgitation (≥II)	0 (0%)	4 (57.1%)	7 (46.7.3%)	4 (33.3%)	0.012*
Aortic valve gradient (mean, mm Hg)	3.4 ± 0.2	3.0 ± 0.1	6.7 ± 1.0	16.5 ± 5.8	0.117
Left ventricle diastolic diameter (mm)	50.1 ± 1.2	53.0 ± 2.2	54.8 ± 2.0	52.3 ± 0.9	0.182
Left ventricle systolic diameter (mm)	29.9 ± 0.7	35.8 ± 2.9	34.7 ± 0.7	32.2 ± 0.7	0.048*
Left ventricular ejection fraction (%)	77.3 ± 1.6	61.0 ± 11.7	69.1 ± 2.6	70.7 ± 1.2	0.063
Ascending aorta diameter (mm/m^2)	15.9 ± 0.6	23.2 ± 0.9	17.6 ± 0.8	25.1 ± 1.2	<0.001**

BAV bicuspid aortic valve patients, *BAVdil* patients with bicuspid aortic valve and aortic dilation

* Significant values ($p < 0.05$)

** Significant values ($p < 0.01$)

1500*g* for 15 min to obtain plasma. The plasma samples were stored at −80 °C in our biological sample bank until needed (Biobanc IISPV-HUSJR).

RNA extraction and miRNA microarray preparation

Total RNA was extracted from 250 µL of plasma using the TRIzol reagent according to the manufacturer's instructions (Invitrogen) and purified using an RNeasy minikit (Qiagen). To increase RNA recovery, 1 µg of MS2 carrier RNA was added to each plasma sample. The quality of the total RNA isolated was determined using an Agilent 2100 Bioanalyser.

The plasma miRNA expression levels for 24 individuals were assessed using SurePrint G3 human 8 × 60 k miRNA microarrays (Agilent Technologies), covering 1205 human miRNAs (Sanger miRBase release 16). The miRNAs were dephosphorylated and labelled with cyanine 3-cytidine biphosphate, including a labelling spike-in solution (Agilent Technologies) to assess the labelling efficiency. The samples were hybridized on the arrays, including a hybridization spike-in solution (Agilent Technologies) to monitor the hybridization efficiency. The arrays were scanned with a G2565CA Microarray Scanner System with SureScan High Resolution Technology (Agilent Technologies) using Scan Control software. The Feature Extraction 11.5.11 (Agilent Technologies) and GeneSpring 12.6.1 software packages were used for data processing, and all steps were performed according to the manufacturer's protocol.

Microarray analysis of the miRNA expression data

The data from microarrays were normalized using the robust multi-array average (RMA) method [18], implemented in the AgiMicroRna Bioconductor package, and the fold changes of the circulating miRNAs were determined using the linear model implemented in the limma Bioconductor package [19]. The Benjamini and Hochberg method was used to adjust the p values for multiple testing and to control the false discovery rate [20].

MicroRNA quantification by real-time qRT-PCR

The microarray expression data were validated in an independent patient cohort ($n = 43$), including healthy TAV controls, BAV patients, BAVdil patients, and an additional group of TAV individuals with aortic dilation. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the miRNA-specific reverse-transcription primers provided with the TaqMan MicroRNA Assay (Applied Biosystems) to analyse the expression of each miRNA. The final total RNA concentration used was 2.5 ng/µL. The reaction was performed at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. We used 1.33 µL of the obtained cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems) and the associated specific probe provided in the TaqMan MicroRNA Assay Kit (Applied Biosystems). Specific Taqman probes were used for each gene as follows: microRNA-122-5p (miR-122-5p; hsa-mir-122-5p), 5′-UGGAGUGUGACAAUGGUGUUUG-3′; microRNA-130a-3p (miR-130a-3p; hsa-mir-130a-3p), 5′-CAGUGCAAUGUUAAAAGGGCAU-3′; microRNA-486-5p (miR-486-5p; hsa-mir-486-5p), 5′-UCCUGUACUGAGCUGCCCCGAG-3′

and microRNA-718 (miR-718; hsa-mir-718), 5′-CUUCCGCCCCGCCGGGCGUCG-3′. The results were normalized to the expression of the U6 small nuclear RNA (U6 snRNA), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale according to the equation $2-\Delta\Delta Ct$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples − ΔCt untreated controls.

Functional and pathway enrichment analysis of the miRNA target genes

The target genes of miR-122, miR-130a and miR-486 were predicted using DIANA-miRPath (v3; microrna.gr/tarbase) [21], mapped on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways based on an enrichment analysis, and prioritized for the intracellular signal transduction pathways that could be regulated by the three-miRNAs.

Because of the limited target genes identified for miR-718, for this miRNA, we combined the targets genes predicted by four databases, namely, the TargetScan (v7.0; targetscan.org) [22], miRTarBase (2016 version; mirtarbase.mbc.nctu.edu.tw) [23], DIANA-microT-CDS (v3; microrna.gr/microT-CDS) [21] and miRDB (2016 version; mirdb.org) [24] databases. In this case, the functional classification of the target genes was carried out with Gene Ontology (GO) and pathway analysis using DAVID (david.ncifcrf.gov) [25] and WebGestalt (webgestalt.org) [26], respectively.

Cytoscape version 3.0.1 [27] was used to visualize the predicted miRNA-mRNA interaction. In the network, the intracellular signal transduction pathways are represented by hexagons-shaped nodes, the miRNAs are represented by rectangle-shaped nodes and the target genes are represented by round-shaped nodes.

Statistical analysis

The means of two groups were compared using Student's t test. A Chi squared test or a Fisher's exact test, when appropriate, was used to compare the frequencies of the categorical variables. The effects of the valve morphology and aortic dilation were assessed using analysis of variance (ANOVA). Tukey's test was used for the pairwise comparisons. A Pearson's correlation was used to identify linear relationships between the miRNA expression levels and the diameter of the ascending aorta. Backward linear regression models were used to identify the independent predictors of the expression of the circulating miRNAs. p values <0.05 were considered significant. The statistical analyses were performed using SPSS software version 21.0 (IBM, Chicago, IL, USA).

Results

Identification of the circulating miRNome profiles

Global miRNA expression profiling was performed on the plasma samples of the TAV controls and the $BAV_{non\text{-}dil}$ and BAV_{dil} patients to identify the miRNA candidates, and the expression of these miRNAs was altered in BAV because of the valve morphology and/or because of the aortic dilation. The clinical characteristics of the patients involved in this stage are provided in Table 1. After processing the microarray data corresponding to the miRNA expression profiles, 260 miRNAs were identified as expressed in at least 10% of the samples. We performed a paired comparison between the groups to identify miRNA candidates for not only BAV but also aortic dilation. In this manner, we first analysed the effect of the valve morphology (comparing patients with $TAV_{non\text{-}dil}$ and $BAV_{non\text{-}dil}$) on the miRNome, and as shown in the volcano plot (Fig. 1a), the expression levels of miR-122 and miR-486 differed significantly from the remainder, indicating that the expression levels of these two miRNAs in plasma might be associated with the morphology of the aortic valve.

In a second analysis, we compared the two most divergent groups ($TAV_{non\text{-}dil}$ and BAV_{dil}) to integrate the complexity associated with the valve morphology and the dilation of the ascending aorta. This analysis indicated that the expression of two other miRNAs, miR-130a and miR-718 (Fig. 1b), was significantly different between the plasma of the $TAV_{non\text{-}dil}$ and BAV_{dil} patients.

RT-qPCR validation of the miRNA candidates in an independent cohort

The miRNA candidates selected using the microarray approach were validated by RT-qPCR in a new set (n = 43), which comprised $TAV_{non\text{-}dil}$ healthy controls and $BAV_{non\text{-}dil}$ and BAV_{dil} patients, and we also included a new group of patients with TAV and aortic dilation (TAV_{dil}) to determine the specific pattern associated with the valve morphology or aortic dilation *per se*.

The expression levels of miR-122, miR-130a and miR-486 are associated with the morphology of the aortic valve

We compared the plasma expression of the miRNA candidates, miR-122, miR-130a, miR-486 and miR-718, which depended on the morphology of the valve and was independent of the presence or absence of dilation in the ascending aorta (Fig. 2a).

This validation study corroborated that the expression of three of the miRNA candidates, miR-122, miR-130a, and miR-486, differed significantly between the

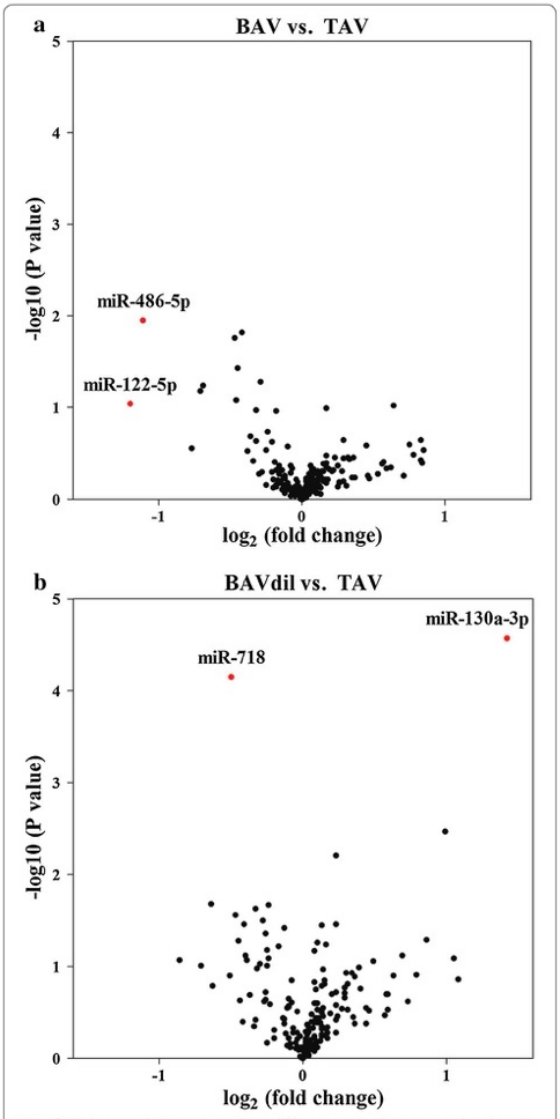

Fig. 1 Volcano plot representing differences in the expression levels of the circulating miRNAs depending on **a** the bicuspid or tricuspid morphology of the aortic valve and **b** the morphology of the valve together with the dilation of the aorta. The log-fold change is plotted against the −log10 (p value)

plasma of TAV individuals and that of BAV patients (BAV vs. TAV), and therefore, their expression levels in the circulation were influenced by the morphology of the valve. The bicuspid valve morphology was associated with decreased circulating levels of miR-122 and miR-486 instead of the increased levels reported for miR-130a.

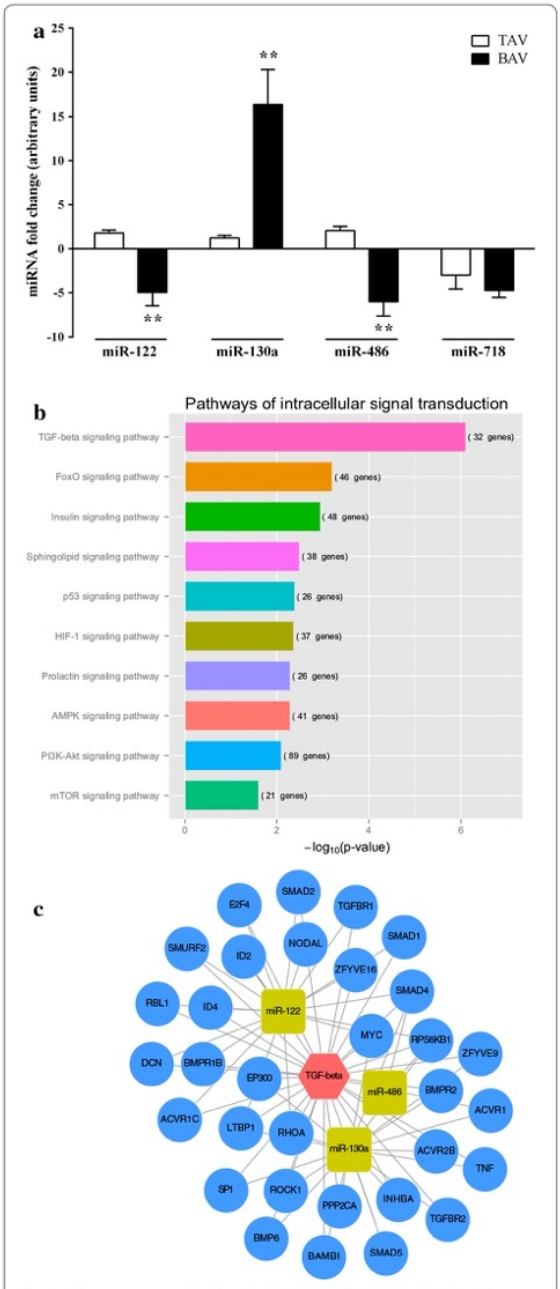

Fig. 2 The expression levels of miR-122, miR-130a and miR-486 are influenced by the morphology of the aortic valve. **a** The validation of the miRNA candidates for aortic morphology was performed by RT-qPCR. *Two asterisks* Significant values ($p < 0.001$; unpaired t test). **b** The pathways of intracellular signal transduction were significantly enriched by the predicted target genes of miR-122, miR-130a and miR-486. **c** Interactive miRNA-gene network regarding the TGF-beta signalling pathway. *TAV* tricuspid aortic valve, *BAV* bicuspid aortic valve, *TGF* transforming growth factor

We performed a functional categorization of the miRNA target genes to gain further insight into the biological role of miR-122, miR-130a and miR-486 in BAV (Fig. 2b). The targets of each of the selected miRNAs were predicted, mapped on KEGG pathways based on an enrichment analysis, and used to prioritize the intracellular signal transduction pathways that could be regulated by the three miRNAs. Using this approach, we identified the transforming growth factor-beta (TGF-β_1) signalling pathway as the most significantly enriched pathway ($p = 7.73 \times 10^{-7}$), with a total of 32 genes targeted by the four selected miRNAs (Fig. 2c).

Effect of aortic dilation on the miR-130a and miR-718 expression levels in plasma

Next, we validated whether the modulation of the miRNA expression profile of miR-130a and miR-718 in the plasma was related to the dilation of the aorta. We first analysed the effect of the aortic dilation on miR-130a and miR-718, comparing the expression levels between the dilated and non-dilated patients independent of the aortic valve morphology (n = 43; Fig. 3A). The up-regulation of miR-130a expression in the plasma of the BAV patients was only influenced by the morphology of the aortic valve, and the dilation of the aorta had no modulatory effect. In contrast, the plasma expression of miR-718 was significantly affected by the dilation of the ascending aorta, and this dilation-promoted effect was masking the differential expression consequence of the valve morphology not observed in the comparisons used in the previous validation.

We explored these morphology- and aortic-dilation-mediated effects by comparing the expression of miR-130a (Fig. 3B) and miR-718 (Fig. 3C) between the four sub-groups depending on the valve morphology and the dilation of the ascending aorta ($TAV_{non\text{-}dil}$, TAV_{dil}, $BAV_{non\text{-}dil}$ and BAV_{dil}). This analysis corroborated that up-regulated miR-130a expression was associated only with the bicuspid morphology of the aortic valve, and no effect of the aortic dilation was observed. Furthermore, the down-regulated expression of miR-718 observed in BAV patients was significantly more prevalent in the presence of aortic dilation compared with the expression in the non-aortic dilated patients and was independent of the tricuspid or bicuspid aortic valve morphology.

The expression of miR-718 in plasma is a predictor of aortic dilation

The role of miR-718 as a biomarker of aortic dilation was supported by the inverse correlation between miR-718 plasma expression and the diameter of the ascending aorta ($R = -0.629$, $p = 3.1 \times 10^{-5}$; Fig. 4a) in all of the patients in the validation cohort; this correlation was independent of valve morphology (TAV and BAV) or the presence or absence of aortic dilation (dilated and non-dilated). Furthermore, using this validation cohort, we built a multiple logistic and linear models using the miR-718 expression profiles as the predictive variables and the dilation of the aorta (represented as a categorical variable or as the diameter of the aorta) as the outcome variable in addition to age and hypertension as possible confounding factors (Table 3). This analysis confirmed that the expression of miR-718 in the plasma is an independent predictor of the dilation of the aorta.

To provide a better understanding of the possible functional relevance of miR-718 down-regulation, we characterized the putative biological functions of this miRNA based on the identification of the miR-718 target genes. The predicted miRNA-gene interaction network (Fig. 4b) was composed of miR-718 and the 167 target genes. We also performed a functional enrichment analysis to predict the potential biological function of the miR-718 target genes. DAVID was used to determine the GOs associated with biological processes that might be overrepresented within the miR-718 target genes. Four of the target genes were significantly enriched for the GO biological process "blood vessel remodelling" ($p = 1.9 \times 10^{-2}$). Using WebGestalt, we performed a functional analysis based on the pathways that might be associated with the miR-718 targets; 3 of the miR-718 target genes were significantly enriched for the "focal adhesion pathway" ($p = 9.0 \times 10^{-4}$).

Discussion

This study found that the miRNA expression pattern in plasma is influenced by the morphology of the aortic valve and the dilation of the ascending aorta. Using comprehensive miRNA expression profiling and further RT-qPCR validation, we identified a circulating miRNA signature, including miR-122, miR-130a and miR-486, whose regulation is dependent on the tricuspid or bicuspid morphology of the aortic valve. Furthermore, the plasma expression of miR-718 may be a biomarker of aortic dilation, that is, an increased ascending aorta diameter is associated with the down-regulation of miR-718. We explored the biological implications of the differential expression of these miRNAs based on target gene prediction and the putative biological functions and processes regulated by the target genes. In this manner, the differential modulation of the bicuspid valve morphology-associated miRNAs might affect the TGF-β_1 signalling pathway, and the dysregulation of miR-718 might be associated with the progression of aortic dilation by modulating the focal adhesion and blood vessel remodelling processes.

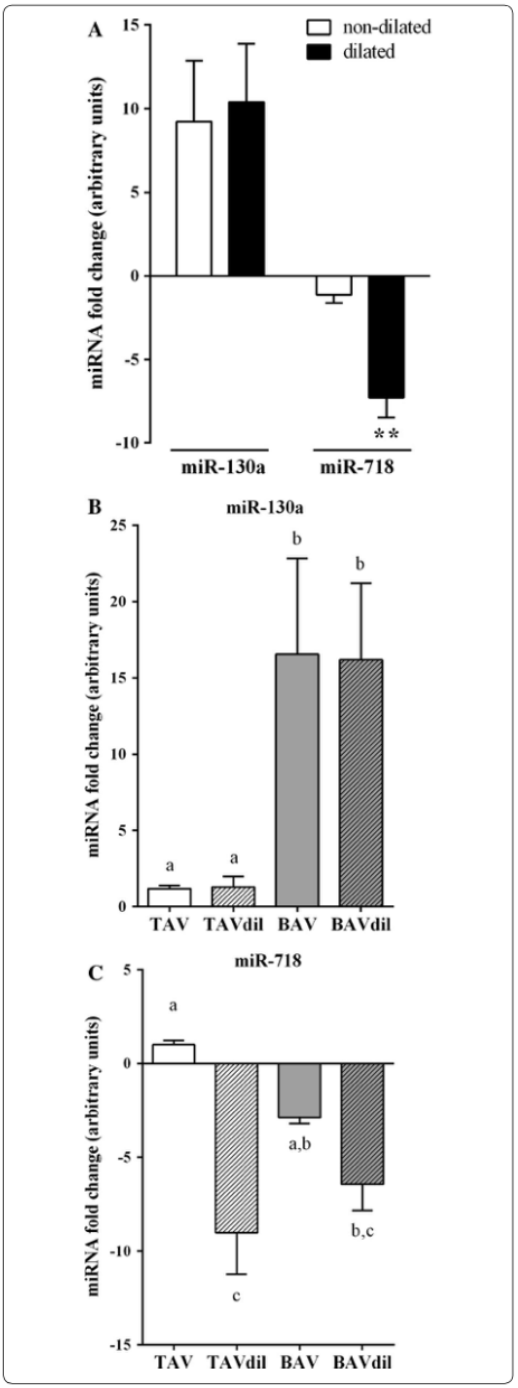

Fig. 3 The expression of circulating miR-718 is affected by the dilation of the ascending aorta. **A** RT-qPCR validation of the effect of the ascending aortic dilation on the expression levels of miR-130a and miR-718 in the plasma. The specific effects of the valve morphology and aortic dilation were analysed for miR-130a (**B**) and miR-718 (**C**). *Two asterisks* Significant values ($p < 0.001$; unpaired t test). The *bars* with different letters are significantly different (one-way ANOVA, Tukey test, $p < 0.05$)

The molecular features and mechanisms that underlie BAV and the dilation of the aorta are poorly understood, and to our knowledge, the miRNA expression profile and putative gene regulation in BAV disease and aortic dilation identified in this study by screening in plasma of BAV patients has not been previously reported. Previous studies have analysed the expression of miRNAs in aortic tissue segments of patients with BAV and animal models. Nigam et al. [28] determined that miR-26a, miR-30b and miR-195 were decreased in the aortic valve leaflets of patients requiring aortic valve replacement due to aortic stenosis compared to those requiring replacement due to aortic insufficiency, and both pathologies are associated with BAV. Using porcine valvular interstitial cells and human valve leaflets, Yanagawa et al. [29] determined that the down-regulation of miR-141 in stenotic bicuspid leaflets regulates BMP-2-mediated valve calcification. Wu et al. [30] performed a paired comparison of the miRNA expression between severely dilated and normal-appearing, less-dilated aortic samples, associating the differential regulation of the miR-17 gene cluster with the predisposition to dilation through the dysregulation of the matrix metalloproteinases-tissue inhibitors of matrix metalloproteinases (TIMP-MMP) pathway. However, although the determination of the miRNA expression profile in tissue samples provides valuable information regarding the pathophysiological mechanisms underlying diseases, this expression signature often cannot be extrapolated to the bloodstream. Thus, unlike these tissue-specific miRNAs, the determination of a circulating miRNA expression profile allows for the integration of the complexity associated with multiple tissues, as is in the case of complex diseases, in addition to serving as biomarkers of these diseases.

In clinical practice, the diagnosis of BAV and ascending aorta dilation is currently performed exclusively based on imaging techniques (i.e., echocardiography, TAC, and magnetic resonance) and the subsequent interpretation by an expert. Moreover, both BAV and the dilation of the aorta are normally presented as asymptomatic clinical conditions, and the molecular causes underlying both of these pathologies are unknown. Therefore, determining plasma molecular biomarkers of BAV and the progression of aortic dilation would be extremely useful in

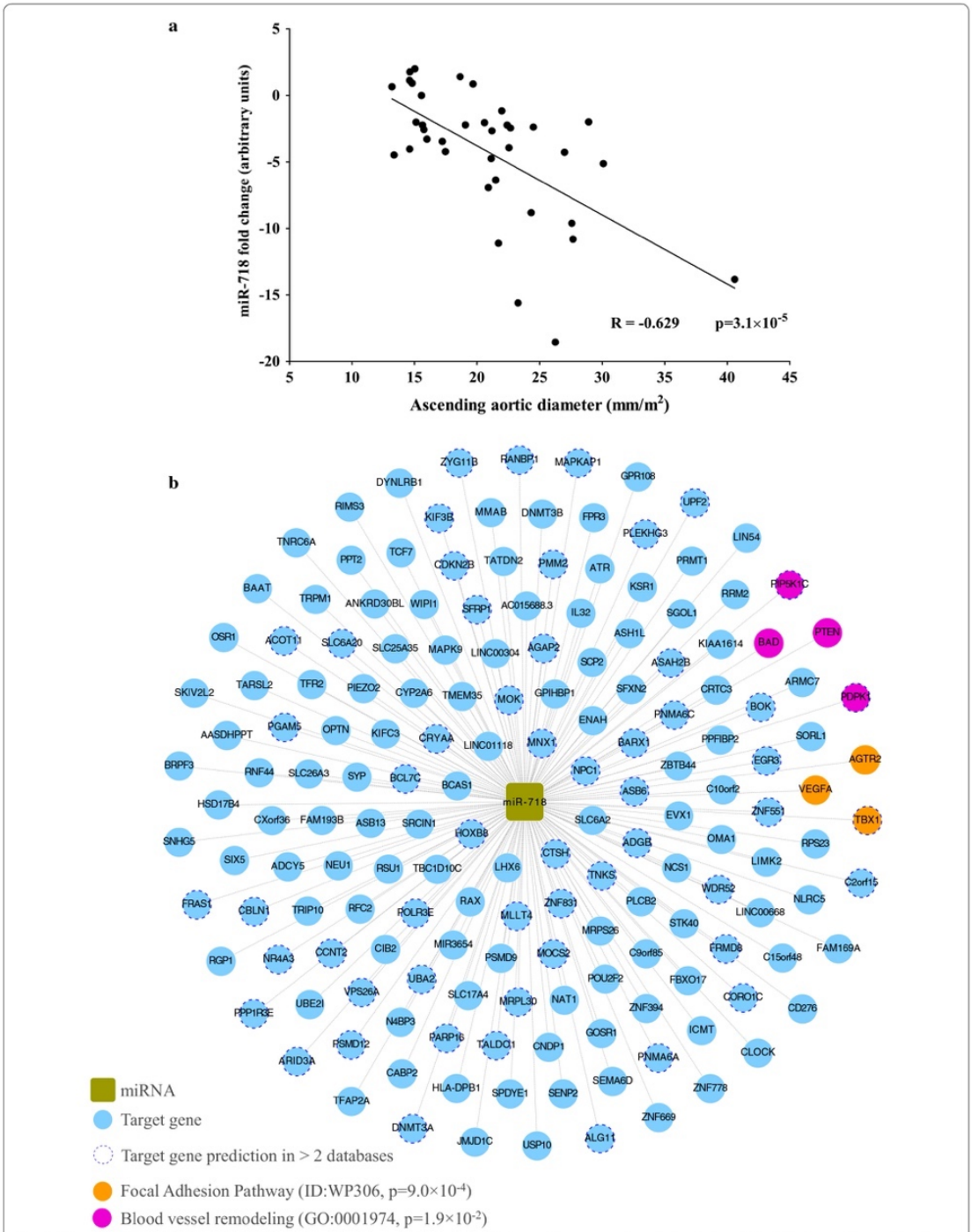

Fig. 4 Functional implications of miR-718 expression in ascending aortic dilation. **a** The expression of circulating miR-718 is inversely correlated with the diameter of the ascending aorta. **b** miR-718 target gene network resulting from the functional enrichment analysis

Table 3 Logistic and linear regression analyses of the miR-718 expression levels as a predictor of aortic dilation

	Aortic dilation		Indexed aortic diameter (×mm/m^2)	
	β	p	β	p
Age (years)	0.317	0.118	0.234	0.177
Hypertension	0.050	0.801	0.301	0.078
miR-718 expression	−0.407	0.022*	−0.541	0.002*

The analysis was performed in the independent validation cohort, including TAV, TAVdil, BAV and BAVdil (n = 43)

* Significant values ($p < 0.05$)

expanding the basic knowledge of the aortic dysfunction, as well as for the identification of new therapeutic targets that might significantly improve the diagnosis, prognosis and treatment of BAV and aortic dilation. Moreover, the determination of biomarkers in the plasma has an added value because in the plasma, the miRNA levels are reproducible and consistent among individuals, and the miRNAs appear to be protected from endogenous ribonuclease-induced degradation [31]. In addition, in contrast to other tissues, the plasma is more easily accessible by non-invasive means, which might improve the feasibility of these molecules as useful biomarkers in routine clinical practice.

The bicuspid morphology of the aortic valve is associated with an abnormal blood flow and shear stress at the ascending aorta that leads to damage and apoptosis of endothelial cells at the cellular level, involving the activation of multiple biological processes, such as endothelial dysfunction, inflammation, oxidative stress, angiogenesis and extracellular matrix remodelling [32–34]. Considering this BAV-associated environment, we hypothesized that circulating miRNAs might be involved in the paracrine communication between cells and contribute to the regulation of disease progression, and thus, the circulating levels of miRNAs might reflect the hemodynamic alteration of the blood flow resulting from the bicuspid morphology of the aortic valve.

In addition to their role as biomarkers for various diseases, miRNAs can be considered potential dynamic master regulators of signalling pathways due to their capacity to specifically control the expression of key components of signal transduction at multiple levels. Therefore, based on a functional analysis, our data supported the crucial role of the dysregulation of genes involved in the TGF-β_1 signalling pathway in BAV disease [35]. In addition, we propose that miR-122, miR-130a and miR-486 might serve as molecular effectors in the BAV associated dysregulation of TGF-β_1 receptor. TGF-β_1 is a crucial factor mediating tissue fibrosis and extracellular matrix (ECM) remodelling in ascending aorta. TGF-β_1 is a pleiotropic cytokine secreted and stored in the ECM as a biologically inactive form that is complexed with latent TGF-β_1 binding protein (LTBP-1) in the ECM. Activated TGF-β_1 binds to a heterodimeric receptor complex comprising two subunits (TGFβR1 and TGFβR2) whose genes are targeted by miR-122 and miR-130a, respectively, activating Smad proteins, which mediates the intracellular signalling of TGF-β_1 [36]. The phosphorylation of SMAD2 (the gene targeted by miR-122) and SMAD3 results in the formation of heterooligomeric complexes with SMAD4 (the gene targeted by miR-122, miR-130a and miR-486). TGF-β_1 signalling has been reported to play divergent roles in aortic dilation depending on its location (i.e. abdominal or thoracic aortic aneurysms) or aortic valve morphology [37]. As limitation of this study, the role of these miRNAs in the regulation TGF-β1 signalling pathway was determined based on computational predictions and it was not experimentally confirmed.

We can speculate that the opposite regulation of mir-122 (decreased expression levels) and miR-130a (increased expression levels) that we observed could be associated with the imbalance of TGFβR1 and TGFβR2 subunits. This speculation is supported by previous findings where the postnatal disruption of the *Tgfbr2* gene expression was associated with decreased canonical TGF-β_1 signalling and accelerated aneurysm growth in a murine model of Marfan syndrome [38]. Moreover, Forte et al. [39], reported that ascending aorta aneurysms in BAV patients was associated with an imbalance between the TGFβR1 and TGFβR2 subunits, although, in that case the authors associated this imbalance by an intrinsic defect of TGFβR1 expression. This imbalance between both subunits of the TGF-β_1 receptor could be indicating a redirection towards the activation of the non-canonical TGF-β_1 signalling [37], which is related to the induction of the ECM degradation and MMP activity, both biological processes associated with BAV and aortic dilation.

In addition to the biological and cardiology-relevant implication of each miRNA individually, miR-122 accounts for 70% of the total miRNAs in the adult liver [40] and is considered a central player in liver biology and in the regulation of cholesterol and fatty acid metabolism [41]. Interestingly, Beaumont et al. [42] demonstrated that the down-regulation of miR-122 was associated with the up-regulation of TGF-β_1 expression, increasing the severity of myocardial fibrosis in patients with aortic stenosis, which is the most frequent valvular complication in BAV patients [43]. miR-130a has important roles in the cardiovascular system due to its capacity to promote the proliferation of endothelial cells and vascular smooth muscular cells. In fact, miR-130a has been described as a regulator

of the angiogenic phenotype of vascular endothelial cells through its ability to modulate the expression of the homebox genes GAX and HOXA5 [44]. Furthermore, the expression of miR-130a in the aorta is correlated with vascular remodelling in spontaneously hypertensive rats (SHRs) [45]. Regarding miR-486, Holliday et al. [46] demonstrated that the expression of miR-486 in human aortic valvular endothelial cells was shear dependent, and Navon et al. [47] suggested that a reduction of miR-486 supports tissue remodelling characterized by increased proliferation. Therefore, our findings regarding the miRNA signature associated with the morphology of the aortic valve support the notion that miR-122, miR-130a and miR-486 play a key role in BAV disease and add new knowledge regarding the molecular effectors of the TGF-β_1 pathway dysregulation associated with BAV.

We propose miR-718 as a new non-invasive biomarker of dilation of the ascending aorta. We demonstrated its potential as molecular feature associated with an aortic dilation in patients with a dilated ascending aorta that was independent of the tricuspid or bicuspid morphology of the aortic valve. We found only 4 references [48–51] in PubMed when searching in the literature for information on the biological function of miR-718; to date, no one has referred to the biological function of miR-718 expression in the cardiovascular field, including its function in relation to aortic valve morphology or the dilation of the aorta.

Using a bioinformatics approach, we predicted the putative target genes of miR-718 and the biological function and processes that it might regulate. The miR-718 target genes were significantly enriched for the "blood vessel remodelling" process and "focal adhesion pathway". Ascending aorta dilation is defined as a degenerative vascular disorder due to the destructive remodelling of the aortic wall and the degradation of the ECM proteins, leading to the recruitment and infiltration of immune system cells mediated by the secretion of adhesion molecules. Interestingly, vascular endothelial growth factor A (VEGFA) is one of the miR-718 target genes that was significantly enriched for the "focal adhesion pathway". VEGFA acts on endothelial cells by increasing vascular permeability and angiogenesis, inducing vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis [52]. These biological implications of the miR-718 target genes might explain the relation between miR-718 and ascending aorta dilation.

In BAV, as in other disease-associated complications, the emergence of the ascending aorta dilation cannot be considered as a discrete alteration in time; instead, it is a continuous process associated with the pathophysiological implications of BAV, and such a complex process involves multiple cell signalling pathways that might be integrated to reflect the complexity of the disease. In this manner, our results are consistent with previous findings that postulated the crosstalk between TGF-β_1 and VEGF together with the Notch signalling pathways, to act coordinately in space and time in the regulation of vascular morphogenesis [53]. With this consideration, our circulating miRNA signature, including miR-122, miR-130a, miR-486 and miR-718 expression, integrates the complexity associated with not only the bicuspid morphology of the aortic valve but also the progressive dilation of the ascending aorta. Furthermore, whether these miRNAs are directly involved in the aortic wall pathogenesis of BAV disease or if they are a consequence of the increased aortic shear stress generated by the anomalous aortic flow caused by BAV remains unknown.

Conclusions

We proposed miR-122, miR-130a, miR-486 and miR-718 as new molecular features associated with BAV and aortic dilation by the regulation of TGF-β_1 and vascular remodelling mediated by the VEGF signalling pathway.

Abbreviations

TAV: tricuspid aortic valve; TAVnondil: tricuspid aortic valve with non-dilated aorta; BAV: bicuspid aortic valve; BAVnondil: bicuspid aortic valve with non-dilated aorta; miRNAs: microRNAs; LV: left ventricular; WSS: wall systolic stress; MMP: matrix metalloproteinases; TIMP: inhibitors of matrix metalloproteinases; ECM: extracellular matrix; TGF-β_1: transforming growth factor-beta; VEGFA: vascular endothelial growth factor A.

Authors' contributions

NMM performed the experiments, analyzed the data, and wrote the manuscript; RBD performed the experiments; IB performed the experiments and analyzed the data; MF contributed in sample collection and writing the manuscript; JMA conceived and designed the study, analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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All relevant data are within the paper and its supporting information files.

Ethics approval and consent to participate

Study approved by the local ethical commitee (Hospital Universitari de Sant Joan, Reus); each patient signed a written informed consent.

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ARTICLE 2: miRNAs CLUSTERED WITHIN THE 14q32 LOCUS ARE ASSOCIATED WITH ENDOTHELIAL DAMAGE AND MICROPARTICLE SECRETION IN BICUSPID AORTIC VALVE DISEASE

Aquest estudi es va dissenyar en dues avaluacions en què es van determinar els nivells d'EMP i el perfil d'expressió de miRNA en dues cohorts independents d'individus amb VAT i pacients amb VAB amb o sense dilatació aòrtica (n = 60). En la primera avaluació, es va utilitzar plasma dels pacients diagnosticats de VAB, amb o sense dilatació aòrtica, i subjectes sans control amb VAT (n = 24) per determinar els nivells d'EMP i el perfil de miRNA mitjançant microarrays. Per millorar la potència de l'estudi, en aquesta etapa vam seleccionar grups formats per pacients amb característiques extremadament homogènies per excloure possibles factors de confusió, com ara l'edat, el sexe o l'índex de massa corporal. Per a estudiar quins miRNA podrien estar relacionats amb les EMP es van incloure pacients amb VAB en els que s'esperaven nivells elevats d'EMP, i controls VAT sans en els quals se sospitaven nivells baixos d'EMP. A la segona avaluació, els miRNA associats a EMP es van validar mitjançant RT-qPCR en una nova cohort (n = 36) de controls sans VAT i pacients amb VAB amb o sense dilatació aòrtica. Els pacients amb VAB van presentar un increment proporcional dels nivells d'EMP en funció del diàmetre aòrtic, que desapareixia després de la cirurgia reparadora de l'aorta ascendent. D'altra banda, vam identificar 175 miRNA relacionats amb l'expressió d'EMP. Per mitjà d'una anàlisi doble, bioinformàtica i amb PCR, vam determinar 7 miRNAs localitzats al locus 14q32 del cromosoma 14 que estan relacionats amb l'elevació d'EMP. Aquest locus és un clúster de miRNA altament coexpressat per les EMP a la VAB.

Aquest clúster també es coneix com la regió genòmica DLK1-MEG3. Els gens codificats en aquest locus intervenen en el metabolisme del nitrogen, el sistema immunitari, l'organització de la matriu extracel·lular i la via del TGF-β i, per tant, estan vinculats en el procés de disfunció endotelial. Concretament, 19 dels 131 miRNAs inferits a la xarxa de coexpressió, es localitzen dins d'aquesta regió cromosòmica. Entre tots els miRNAs, el miR-494 és el que més s'associa a malaltia cardiovascular. Per tant, els miRNA associats a EMP situats dins del locus 14q32 modulen l'expressió d'una sèrie de gens vinculats a la disfunció endotelial i, per tant, a la seva progressiva dilatació.

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MicroRNAs Clustered within the 14q32 Locus Are Associated with Endothelial Damage and Microparticle Secretion in Bicuspid Aortic Valve Disease

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Martínez-Micaelo N, Beltrán-Debón R, Aragonés G, Faiges M and Alegret JM (2017) MicroRNAs Clustered within the 14q32 Locus Are Associated with Endothelial Damage and Microparticle Secretion in Bicuspid Aortic Valve Disease. Front. Physiol. 8:648. doi: 10.3389/fphys.2017.00648

Background: We previously described that PECAM$^+$ circulating endothelial microparticles (EMPs) are elevated in bicuspid aortic valve (BAV) disease as a manifestation of endothelial damage. In this study, we hypothesized that this endothelial damage, is functionally related to the secretion of a specific pattern of EMP-associated miRNAs.

Methods: We used a bioinformatics approach to correlate the PECAM$^+$ EMP levels with the miRNA expression profile in plasma in healthy individuals and BAV patients (n = 36). In addition, using the miRNAs that were significantly associated with PECAM$^+$ EMP levels, we inferred a miRNA co-expression network using a Gaussian graphical modeling approach to identify highly co-expressed miRNAs or miRNA clusters whose expression could functionally regulate endothelial damage.

Results: We identified a co-expression network composed of 131 miRNAs whose circulating expression was significantly associated with PECAM$^+$ EMP levels. Using a topological analysis, we found that miR-494 was the most important hub within the co-expression network. Furthermore, through positional gene enrichment analysis, we identified a cluster of 19 highly co-expressed miRNAs, including miR-494, that was located in the 14q32 locus on chromosome 14 ($p = 1.9 \times 10^{-7}$). We evaluated the putative biological role of this miRNA cluster by determining the biological significance of the genes targeted by the cluster using functional enrichment analysis. We found that this cluster was involved in the regulation of genes with various functions, specifically the "cellular nitrogen compound metabolic process" ($p = 2.34 \times 10^{-145}$), "immune system process" ($p = 2.57 \times 10^{-6}$), and "extracellular matrix organization" ($p = 8.14 \times 10^{-5}$) gene ontology terms and the "TGF-β signaling pathway" KEGG term ($p = 2.59 \times 10^{-8}$).

Conclusions: Using an integrative bioinformatics approach, we identified the circulating miRNA expression profile associated with secreted PECAM$^+$ EMPs in BAV disease. Additionally, we identified a highly co-expressed miRNA cluster that could mediate crucial

biological processes in BAV disease, including the nitrogen signaling pathway, cellular activation, and the transforming growth factor beta signaling pathway. In conclusion, EMP-associated and co-expressed miRNAs could act as molecular effectors of the intercellular communication carried out by EMPs in response to endothelial damage.

Keywords: microRNA, bicuspid aortic valve, aortic dilation, circulating endothelial microparticles, bioinformatics, endothelial damage, co-expression network

INTRODUCTION

Bicuspid aortic valve (BAV), the most common cardiac congenital malformation (occurs in 1–2% of the population), is associated with valve dysfunction and is a risk factor for aortopathy (Tzemos et al., 2008; Alegret et al., 2013). The progressive dilation of the aorta, if untreated, can lead to fatal consequences such as aortic dissection and/or rupture. The mechanisms that underlie aortic dilatation have been a matter of debate for years (Padang et al., 2013); the proposed causes include anomalous flow in the ascending aorta generated by the anomalous dynamics of BAV (Kim et al., 2012; Bissell et al., 2013) and genetic causes responsible for the anomalous structure of the aortic media (Biner et al., 2009; Pepe et al., 2014).

In a previous study, we reported that circulating endothelial microparticles (EMPs) are elevated in BAV patients and related to aortic dilation (Alegret et al., 2016). Circulating EMPs are a type of extracellular microvesicle (100–1,000 nm) that bud directly from the plasma membranes of endothelial cells upon activation, injury, or apoptosis and that are involved in intercellular communication. Specifically, we identified $PECAM^+$ EMPs, which are a kind of EMP that express CD31 and are released in endothelial damage, as those related to BAV disease.

Micro-ribonucleic acids (miRNAs) are endogenously expressed, 19- to 23-nt-long noncoding RNAs that regulate gene expression at the post-transcriptional level, mostly via imperfect base-pairing interactions that occur preferentially within the 3′ untranslated regions (UTRs) of target mRNAs. MiRNA genes are distributed across diverse genomic locations, and although some miRNAs are isolated, ∼50% are found in clusters transcribed as polycistronic miRNA transcripts (Mourelatos et al., 2002). MiRNAs are considered potent post-transcriptional regulators because each miRNA has multiple to several 100 target genes; therefore, inhibition of a single miRNA can lead to the activation of multifactorial physiological processes. In addition to functioning intracellularly, miRNAs can be exported or released by cells into the circulating blood in very stable forms. Microparticles are reportedly the major carriers of miRNAs in the blood (Diehl et al., 2012). MiRNA signatures have been proposed as potentials with the potential to improve disease diagnosis and prognosis in clinical practice and have been identified as useful biomarkers for a wide range of cardiovascular diseases. For example, in a previous study, we proposed miR-122, miR-130a, miR-486, and miR-718 as molecular features associated with BAV and aortic dilation (Martínez-Micaelo et al., 2017).

Currently, there are no effective strategies to prevent the progression of BAV disease, including the aortic dilation, and the development of new strategies requires more detailed understanding of the molecular mechanisms associated with BAV and progressive dilation of the aorta. Therefore, in this study, we hypothesized that changes in blood flow in the ascending aorta caused by the bicuspid morphology of the aortic valve would induce endothelial damage, resulting in the secretion of a specific signature of EMP-associated miRNAs. We used a bioinformatics approach to integrate the $PECAM^+$ EMP levels with the miRNA expression profile in plasma of healthy individuals and BAV patients. In addition, from the miRNAs that were significantly associated with $PECAM^+$ EMP levels, we inferred a miRNA co-expression network using a Gaussian graphical modeling approach.

METHODS

Study Population

The patients included in this study belonged to a cohort of BAV patients who were prospectively included and followed-up in our facilities. Upon enrolment, the participants were informed and prospectively entered into a specific database, underwent a blood draw and provided informed written consent. The samples were stored in our biological samples bank (Biobanc IISPV—HUSJR) until they were needed. A BAV diagnosis was made when two aortic leaflets were clearly visualized, with or without a raphe, in the parasternal short-axis view of a transthoracic echocardiogram (Alegret et al., 2005), on a transesophageal echocardiogram (Alegret et al., 2005), or on a cardiac magnetic resonance image (Gleeson et al., 2008). Explorations were performed or supervised by the same observer (JMA). Our database and biobank also included a group of healthy tricuspid aortic valve (TAV) controls. This study was approved by the Institutional Review Board (the Clinical Ethics Committee) of our institution.

This study was designed in two evaluations in which EMP levels and the miRNA expression profile were determined in two independent cohorts of TAV individuals and BAV patients with or without aortic dilation ($n = 60$). In the first evaluation, plasma from the patients diagnosed with BAV, with or without aortic dilation, and healthy TAV control subjects ($n = 24$) was used to determine the levels of EMPs and the miRNA profile using microarrays. To improve the study's power, for this stage, we selected groups composed of patients with characteristics that were extremely homogeneous (Supplementary Table 1) to exclude possible confounding factors, such as age, sex, or BMI. We included subjects in whom high levels of circulating $PECAM^+$ EMPs were expected, the BAV patients, and subjects in whom low levels of $PECAM^+$ EMPs were expected, the healthy TAV controls, to study miRNAs whose expression levels could be related to $PECAM^+$ EMPs. Patients diagnosed with cardiovascular diseases, Marfan syndrome, aortic stenosis, hypertension, or diabetes mellitus or who were receiving

pharmacologic treatment (including statins, ACE/ARII, and/or β-blockers) were excluded. In the second evaluation, the EMP-associated miRNA candidates were validated by RT-qPCR in a new cohort ($n = 36$) of TAV healthy controls and BAV patients with or without aortic dilation (Supplementary Table 2).

Blood Sampling

Blood samples were collected under overnight fasting conditions and were processed within 90 min after collection. The samples were centrifuged at 1,500 g for 15 min to obtain plasma, which was further centrifuged at 4,000 g for 10 min to obtain platelet-poor plasma. The plasmas were stored at −80°C in our biological samples bank (Biobanc IISPV—HUSJR) until they were needed.

Determination of Circulating Levels of EMPs

The circulating $PECAM^+$ EMP levels were determined in the same cohorts used for the microarray and RT-qPCR validation analyses that were previously phenotyped (Alegret et al., 2016), but for this study, individuals were selected to maximize the power of the miRNA analysis. The levels of EMPs were characterized based on the presence of endothelial-specific surface antigens, the composition of which depends on the cellular origin of the microparticles and the generating process (Jimenez et al., 2003). In our previous study (Alegret et al., 2016), we determined that the presence of CD31 (PECAM) is a marker that can discriminate between microparticles released from endothelial cells subjected to endothelial damage produced by haemodynamic causes due to the anomalous aortic flow associated with BAV and microparticles released by other triggering stimuli, including cell activation or apoptosis, in TAV and BAV patients. The concentration of circulating $PECAM^+$ EMPs was determined on an EPICS-XL (Beckman Coulter) flow cytometer at a low rate setting and a 30 s stop time. The Nano Fluorescent Particle Size Standard Kit (Spherotech) was used for instrument standardization, and Flow-Count fluorospheres (Beckman Coulter) were added as an internal calibrator to calculate microparticle amounts.

Plasma EMPs were labeled by incubating 50 μl of platelet-poor plasma with the corresponding antibody, anti-CD31-PE (Beckman Coulter), anti-CD42b-FITC (Beckman Coulter), or anti-CD45-PE (Beckman Coulter), at room temperature in the dark for 20 min as previously described (Ci et al., 2013). Then, 500 μl of PBS was subsequently added, and the EMP levels were determined as previously described (Jimenez et al., 2003; Sutherland et al., 2010; Ci et al., 2013). EMPs were defined as particles >0.1 and <1 μm in size, and their endothelial origin was identified based on their affinity to specific cell surface antigens, namely, CD31 and CD42b. To evaluate the extent of possible contamination with leukocyte-derived microparticles, the circulating levels of $CD31^+CD45^+$ microparticles were determined in all samples. We found that <4.5% of $CD31^+$ microparticles co-expressed $CD45^+$; this result is consistent with previous reports by other authors (Amabile et al., 2005; Pirro et al., 2006). EMP levels were measured by trained technicians who were blind to the clinical status of the patients as well as to the results.

RNA Isolation and Preparation of miRNA Microarrays

Total RNA was extracted from 250 μl of plasma using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and purified using an RNeasy minikit (Qiagen). To increase RNA recovery, 1 μg of MS2 carrier RNA was added to each plasma sample. The quality of total isolated RNA was determined using the Agilent 2100 Bioanalyzer.

The plasma miRNA expression levels were assessed using Sure Print G3 human 8 × 60 k miRNA microarrays (Agilent Technologies) covering 1,205 human miRNAs (Sanger miRBase release 16). The miRNAs were dephosphorylated and labeled with cyanine 3-cytidine biphosphate including a labeling spike-in solution (Agilent Technologies) to assess labeling efficiency. The samples were hybridized on the arrays with the inclusion of a hybridization spike-in solution (Agilent Technologies) to monitor hybridization efficiency. The arrays were scanned with a G2565CA Microarray Scanner System with SureScan High-Resolution Technology (Agilent Technologies) using Scan Control software. The Feature Extraction 11.5.11 (Agilent Technologies) and GeneSpring 12.6.1. software packages were used for data processing.

Analysis of miRNA Microarray Expression Data and Integrative Analysis and Gene Co-expression Network

The data from microarrays (deposited at Gene Expression Omnibus under GEO Series accession number GSE101616) were normalized using the robust multi-array average (RMA) method (Irizarry et al., 2003) implemented in the AgiMicroRna Bioconductor package, and the fold changes in circulating miRNAs were determined using the linear model implemented in the limma Bioconductor package (Ritchie et al., 2015). The Benjamini and Hochberg methods were used to adjust p-values for multiple testing and to control the false discovery rate (Benjamini and Hochberg, 1995). The miRNA co-expression network was constructed from the expression profiles of those miRNAs whose expression was significantly associated with $PECAM^+$ EMPs (Spearman $p < 0.05$). The co-expression network was inferred using graphical Gaussian models (GGMs) implemented in the R package GeneNet (Schäfer and Strimmer, 2005). Briefly, a partial-correlation matrix was estimated by computing the partial correlation between the expression profiles of each miRNA pair. Bayesian posterior edge probability >0.95 (corresponding to a local false discovery rate of <5%) was used to determine the significance of the resulting pairwise partial correlations. In the resulting co-expression network, which was visualized using Cytoscape software (Cline et al., 2007), the nodes represent the set of miRNAs that were significantly correlated with $PECAM^+$ EMP levels, and the edges link the pairs of miRNAs whose expression was not conditionally independent, defined as the pairwise partial correlation once the common effects of the other miRNAs in the subset were removed (Opgen-Rhein and Strimmer, 2007).

MiRNA Quantification by Real-Time qRT-PCR

TaqMan microRNA assays (Applied Biosystems) were used to quantify the expression of selected miRNAs. Briefly, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the miRNA-specific oligonucleotides provided with the TaqMan MicroRNA Assay (Applied Biosystems). The final concentration of total RNA used was 2.5 ng/μL. The reaction was performed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. We used 1.33 μL of the obtained cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Specific TaqMan probes were used for each gene: let-7d (hsa-let-7d-5p), let-7g (hsa-let-7g-5p), miR-122 (hsa-mir-122-5p), miR-130a (hsa-mir-130a-3p), miR-337 (hsa-mir-337-5p), miR-409 (hsa-mir-409-3p), miR-486 (hsa-mir-486-5p), miR-494 (hsa-mir-494), and miR-718 (hsa-mir-718). The results were normalized to the expression of the U6 small nuclear RNA (U6 snRNA), which was used as an endogenous control. Amplification was performed in a 7900HT thermocycler (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale according to the equation $2^{-\Delta\Delta Ct}$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct$ = ΔCt treated samples-ΔCt untreated controls. Negative control reactions, with no RNA, had undetectable quantification cycle values (C_q).

Genomic Region Overrepresentation Analysis

The genetic loci visualization of co-expressed miRNAs across chromosomes was performed using PhenoGram (Wolfe et al., 2013). To determine the significance of the overrepresentation of chromosome regions in the generated co-expressed miRNA set, we applied a hypergeometric test.

Target Prediction and Functional Pathway Analysis

Putative miRNA target sites for the miRNA candidates were identified by bioinformatics analysis using miRNA target prediction databases, including DIANA-microT-CDS (Paraskevopoulou et al., 2013), DIANA-TarBase v7.0 (Vlachos et al., 2015a), and TargetScan v6.2 (Agarwal et al., 2015). Functional enrichment analysis of miRNA target genes was conducted using miRPath v3.0 (Vlachos et al., 2015b). Visualization and topological analysis of the network was done using R and Cytoscape software.

Statistical Analysis

Because of the right-skewed distribution of the values, the $PECAM^+$ EMP plasma levels underwent a natural logarithmic transformation and were expressed as log-transformed counts per μl (log $PECAM^+$ EMPs/μl). EMP levels are presented as the mean ± SEM. Chi-squared tests, or Fisher exact tests when appropriate, were used to compare the frequencies of the categorical variables. The effects of valve morphology and aortic root dilation were assessed using ANOVA. Tukey's test was utilized for pairwise comparisons. $p < 0.05$ were considered significant. The statistical analysis was performed using SPSS software, version 21.0 (IBM, Chicago, IL, USA).

RESULTS

Identification of Circulating miRNA Sets Associated with $PECAM^+$ EMP Levels

To explore the circulating $PECAM^+$ EMP-associated miRNAs, we first corroborated our previously reported finding that BAV patients have increased levels of $PECAM^+$ EMPs in plasma compared with TAV healthy controls (Supplementary Figure 1A). Furthermore, the results of this analysis also supported the role of $PECAM^+$ EMP circulating levels as a biomarker of aortic dilation in BAV disease, as an increased diameter of the aortic root or ascending aorta was associated with higher levels of $PECAM^+$ EMPs (Supplementary Figure 1B).

We used a microarray-based screening approach to determine the miRNA expression pattern that could be related to endothelial damage. The expression of 1,205 miRNAs were evaluated in plasma samples from BAV patients and TAV healthy controls, and after the miRNA microarray expression data were processed, 277 miRNAs were identified as expressed in at least 5% of the samples. We prioritized the potential miRNA candidates based on the linear relationship between $PECAM^+$ EMP circulating levels and the miRNA expression (Supplementary Figure 1C), and based on this analysis, we found that the expression of 175 miRNAs was significantly associated with the circulating levels of $PECAM^+$ EMPs ($p < 0.05$).

Construction of a miRNA Co-expression Network Based on the EMP-Associated miRNAs

Once we identified the 175 miRNAs whose expression patterns in plasma significantly correlated with the levels of $PECAM^+$ EMPs, we took into consideration that about half of all described miRNAs are co-expressed in clusters of miRNAs. For this reason, we used a Gaussian graphical model approach to map the simultaneous expression of miRNA pairs into a co-expression network.

The resulting miRNA co-expression network is composed of a single connected component formed by 131 miRNAs (FDR<5%) linked by 391 edges (**Figure 1A**). We first evaluated the biological implications of these co-expressed miRNAs in terms of intercellular communication in BAV-induced endothelial damage (e.g., whether they are expressed in endothelial cells and whether their expression levels have been previously associated with cardiovascular diseases; **Figure 1B**). More interestingly, we also focused on miRNAs that were described in previous studies as related to BAV or whose expression might be sensitive to blood flow. We found in the literature that 98 of the 131 co-expressed miRNAs were previously reported as expressed in

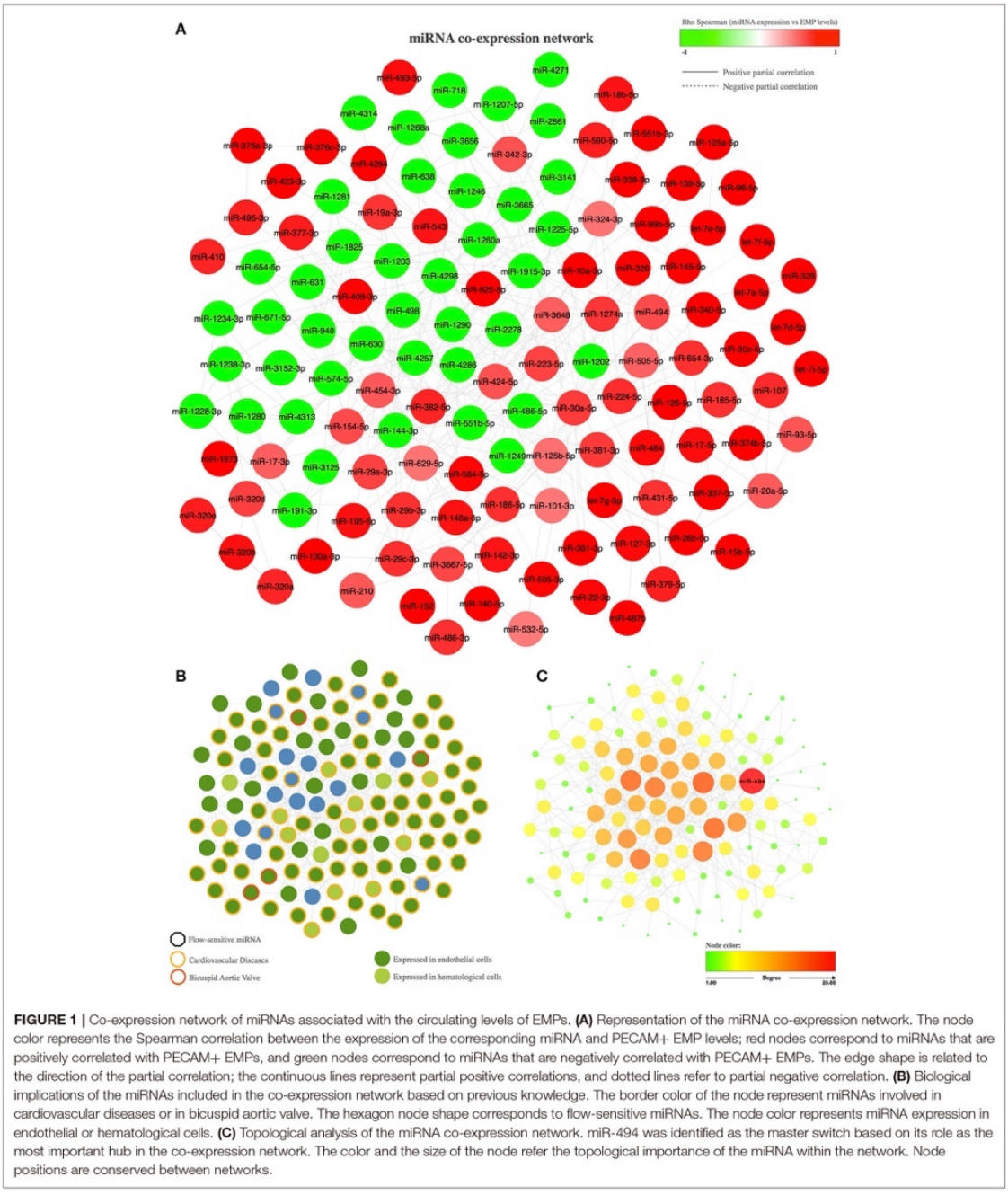

FIGURE 1 | Co-expression network of miRNAs associated with the circulating levels of EMPs. **(A)** Representation of the miRNA co-expression network. The node color represents the Spearman correlation between the expression of the corresponding miRNA and PECAM+ EMP levels; red nodes correspond to miRNAs that are positively correlated with PECAM+ EMPs, and green nodes correspond to miRNAs that are negatively correlated with PECAM+ EMPs. The edge shape is related to the direction of the partial correlation; the continuous lines represent partial positive correlations, and dotted lines refer to partial negative correlation. **(B)** Biological implications of the miRNAs included in the co-expression network based on previous knowledge. The border color of the node represent miRNAs involved in cardiovascular diseases or in bicuspid aortic valve. The hexagon node shape corresponds to flow-sensitive miRNAs. The node color represents miRNA expression in endothelial or hematological cells. **(C)** Topological analysis of the miRNA co-expression network. miR-494 was identified as the master switch based on its role as the most important hub in the co-expression network. The color and the size of the node refer the topological importance of the miRNA within the network. Node positions are conserved between networks.

endothelial cells. Moreover, 88 miRNAs included in the co-expression network had been related to cardiovascular disease. More specifically, modulation of the expression of 4 of them had been associated with BAV, and 9 miRNAs had been described as blood flow sensitive. We also carried out a topological analysis of the network and identified miR-494 as the most important

hub within the co-expression network, that is, the miRNA with the largest degree; the highest betweenness, stress, and closeness scores; and the highest radiality coefficient (**Figure 1C**).

To further validate the results obtained in the microarray analysis and the EMP-miRNA correlations, we determined the expression of 7 of miRNAs included in the miRNA co-expression network (let-7d, let-7g, miR-130a, miR-337, miR-409, miR-494, and miR-718; **Figure 2**) by RT-qPCR in a new cohort of TAV individuals and BAV patients ($n = 36$). In this way, by correlating the RT-qPCR-determined expression data for each of the selected miRNAs with the circulating levels of PECAM$^+$ EMPs, we validated and reaffirmed the sensitivity and power of our bioinformatics approach for the identification and selection of PECAM$^+$ EMP-associated miRNA candidates.

Genomic Region Enrichment Analysis for the miRNA Co-expression Network

Because co-expressed miRNA pairs tend to reside in close genomic proximity, we performed a positional gene enrichment analysis to identify chromosome regions that were overrepresented among the PECAM$^+$ EMPs-associated and co-expressed miRNAs.

We constructed chromosome ideograms to map the genomic locus of each of the miRNAs included in the co-expression network (**Figure 3A**). We found that 21 of the 131 PECAM$^+$ EMP-associated co-expressed miRNAs were located on chromosome 14 and that 19 of them were located in the same chromosome region, the 14q32 locus. We confirmed the statistical significance of this 14q32 genomic region overrepresentation using a hypergeometric test ($p = 1.90 \times 10^{-7}$). This genomic location consists of the miRNA clusters A and B and is also known as the *Dlk1-Dio3* miRNA cluster. Specifically, we identified 4 of the 8 miRNAs located within miRNA cluster A (miR-127-3p, miR-337-3p, miR-431-5p, and miR-493-5p) and 15 of the 42 miRNAs located within miRNA cluster B (miR-154-5p, miR-376a-3p, miR-376c-3p, miR-377-3p, miR-379-5p, miR-381-3p, miR-382-5p, miR-409-3p, miR-410, miR-487b, miR-494, miR-495-3p, miR-543, miR-654-3p, and miR-654-5p; **Figures 3B–C**).

Functional Analysis of the 14q32 miRNA Cluster

To gain deeper insight into the biological significance of these findings, we determined the biological significance of the regulation of the 14q32 miRNA cluster based on the functional implications of the genes targeted by the selected miRNAs. We carried out a functional enrichment analysis of the set of genes putatively targeted by the 19 EMP-related miRNAs located in the 14q32 cluster. Interestingly, this functional analysis revealed that the genes targeted by the 14q32 co-expressed miRNAs were categorized with the "cellular nitrogen compound metabolic process" ($p = 2.34 \times 10^{-145}$), "immune system process" ($p = 2.57 \times 10^{-6}$), and "extracellular matrix organization" ($p = 8.14 \times 10^{-5}$) GO terms as well as the "TGF-β signaling pathway" ($p = 2.59 \times 10^{-8}$) KEGG term, among others (**Figure 4**).

DISCUSSION

Using a bioinformatics approach, our study reported that the endothelial damage observed in BAV disease results in the differential regulation of a post-transcriptional regulatory miRNA network associated with the increased release of endothelial-derived microparticles. By determining the circulating miRNAs associated with PECAM$^+$ EMP levels, we inferred a miRNA co-expression network. Furthermore, based on the results of genomic enrichment analysis, we focused on a cluster of highly co-expressed miRNAs located at the 14q32 locus on chromosome 14 that could be acting as molecular effectors in BAV-related pathophysiological processes, including endothelial damage. We showed that the miRNAs contained within the 14q32 miRNA cluster could mediate crucial biological processes in BAV disease, such as nitric oxide biosynthesis, immune activation, reorganization of the extracellular matrix and TGF-β signaling.

Bicuspid morphology of the aortic valve is associated with haemodynamic abnormalities that result in increased wall shear stress on the endothelial layer, which contributes to dilation of the ascending aorta (Braverman et al., 2005). At the cellular level, this disturbed blood flow is associated with rearrangement of the extracellular matrix and with endothelial damage and dysfunction (Davignon and Ganz, 2004). Furthermore, in our previous studies, we demonstrated that BAV and dilation of the aorta are associated with endothelial-mediated release of microparticles (Alegret et al., 2016). Thereby, when designing this study, we hypothesized that the characteristics of blood flow through the ascending aorta in patients with BAV disease are related to aortic endothelial damage and play key a role in EMP generation.

It is important to consider that on the one hand, circulating miRNAs take very stable forms, mainly packaged in transport particles; circulating microparticles have been reported as the major carriers of miRNAs in the blood (Diehl et al., 2012). On the other hand, although most published studies have focused mainly on differences in the expression of single miRNAs instead of studying miRNA co-expression networks, miRNAs are significantly enriched in clusters in discrete genomic regions (Lau, 2001; Kim and Nam, 2006), and miRNAs in the same cluster might be transcribed in a polycistronic manner (Baskerville, 2005; Wang et al., 2016) and likely regulate functionally related genes (Kim et al., 2009; Wang et al., 2011). We considered the determination and analysis of the miRNA co-expression network resulting from the integration of PECAM$^+$ EMP levels with the expression of circulating miRNAs as an advantageous strategy to unravel the molecular mechanisms underlying the pathophysiological processes in BAV disease for the following reasons: changes in haemodynamic forces in the vascular system might alter the expression of miRNAs in endothelial cells (Marin et al., 2013); BAV promotes endothelial dysfunction, which results in the release of PECAM$^+$ EMPs in plasma; microparticles are the main carriers of miRNAs in plasma; and miRNA-coding genes are enriched in discrete genomic regions.

Using a bioinformatics approach, we identified 175 miRNAs that were significantly associated with PECAM$^+$ EMP levels.

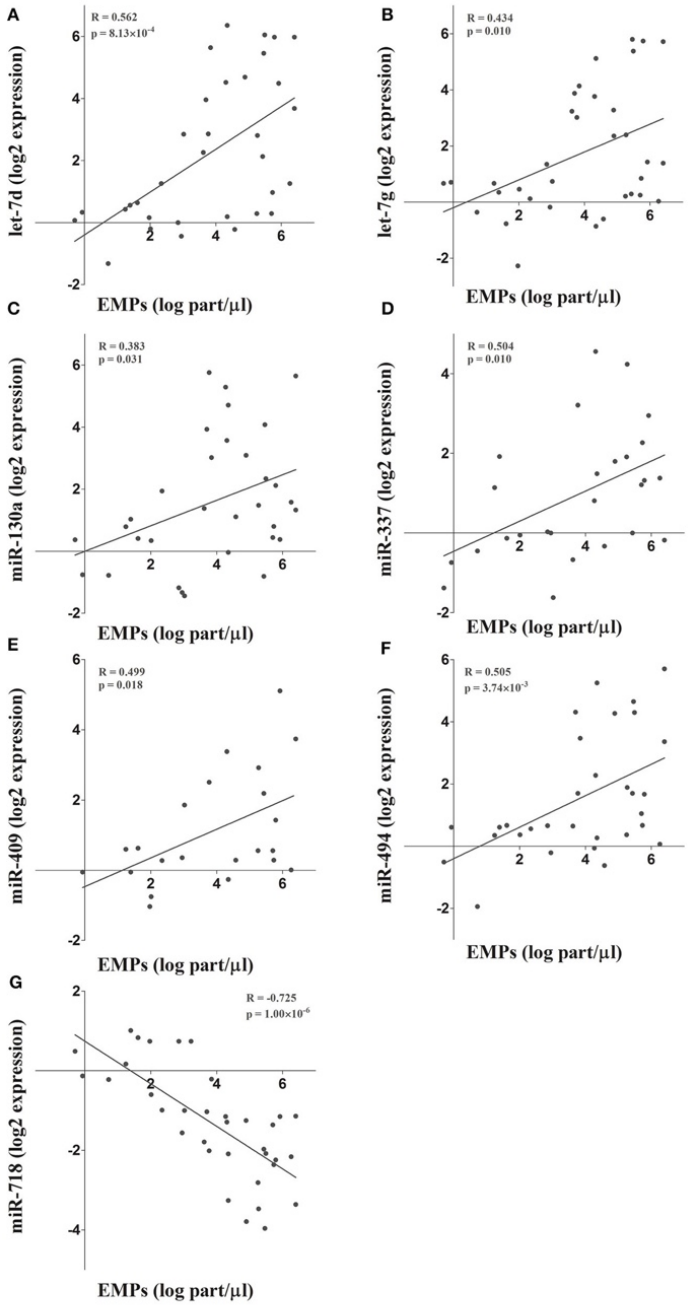

FIGURE 2 | (A-G) The microarray results and the integrative analysis were further validated by determining the expression of 7 miRNAs included in the co-expression network. The expression of these miRNAs was determined by RT-qPCR and correlated with circulating PECAM+ EMP levels.

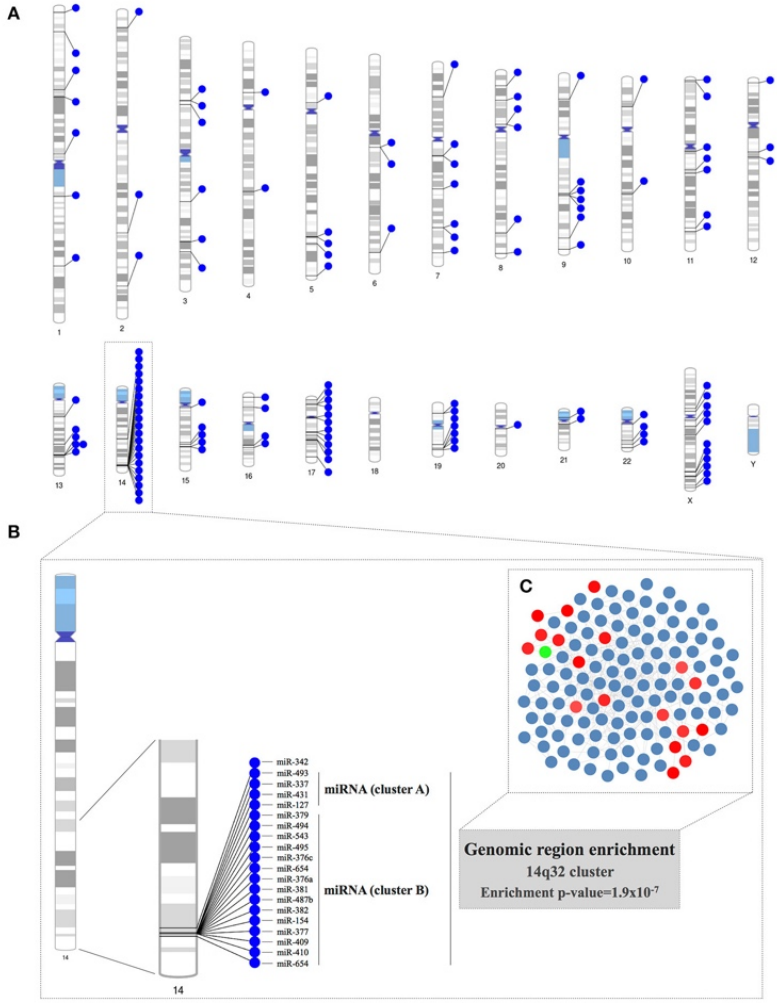

FIGURE 3 | Genomic region enrichment analysis of the miRNA co-expression network. **(A)** The locus of each of the miRNAs included in the co-expression network was mapped in the chromosome ideograms to visualize the genomic loci of co-expressed miRNAs. **(B)** Detailed genomic localization of the co-expressed miRNAs located on chromosome 14. The genomic region overrepresentation was corroborated by the results of a hypergeometric test ($p = 1.9 \times 10^{-7}$). **(C)** The miRNAs located in the 14q32 locus are highlighted within the miRNA co-expression network.

Of these miRNAs, 131 exhibited significant pairwise partial correlations and were inferred into a co-expression network to analyse miRNA co-expression patterns. Co-expression network analysis is considered a powerful method to extract strong regulatory associations that could be responsible for modulating transcriptional networks underlying biological processes. Accordingly, we first analyzed previous knowledge regarding the biological implications of the inferred network and found that 75% of the co-expressed miRNAs had been previously described as miRNAs expressed in endothelial cells and that 67% of them had been associated with cardiovascular diseases. We also analyzed the topology of the co-expression network and found that miR-494 was the most important hub of the network because it was the most highly connected miRNA. miR-494 was also the most influential miRNA within the network based on centrality parameters, which showed that miR-494 was most often found on the shortest path between two other miRNAs. These results indicate that miR-494 might have a large regulatory impact on the biological functions that could be modulated by the co-expression network.

We further analyzed the miRNA co-expression network in terms of enrichment for specific genomic locations, and we

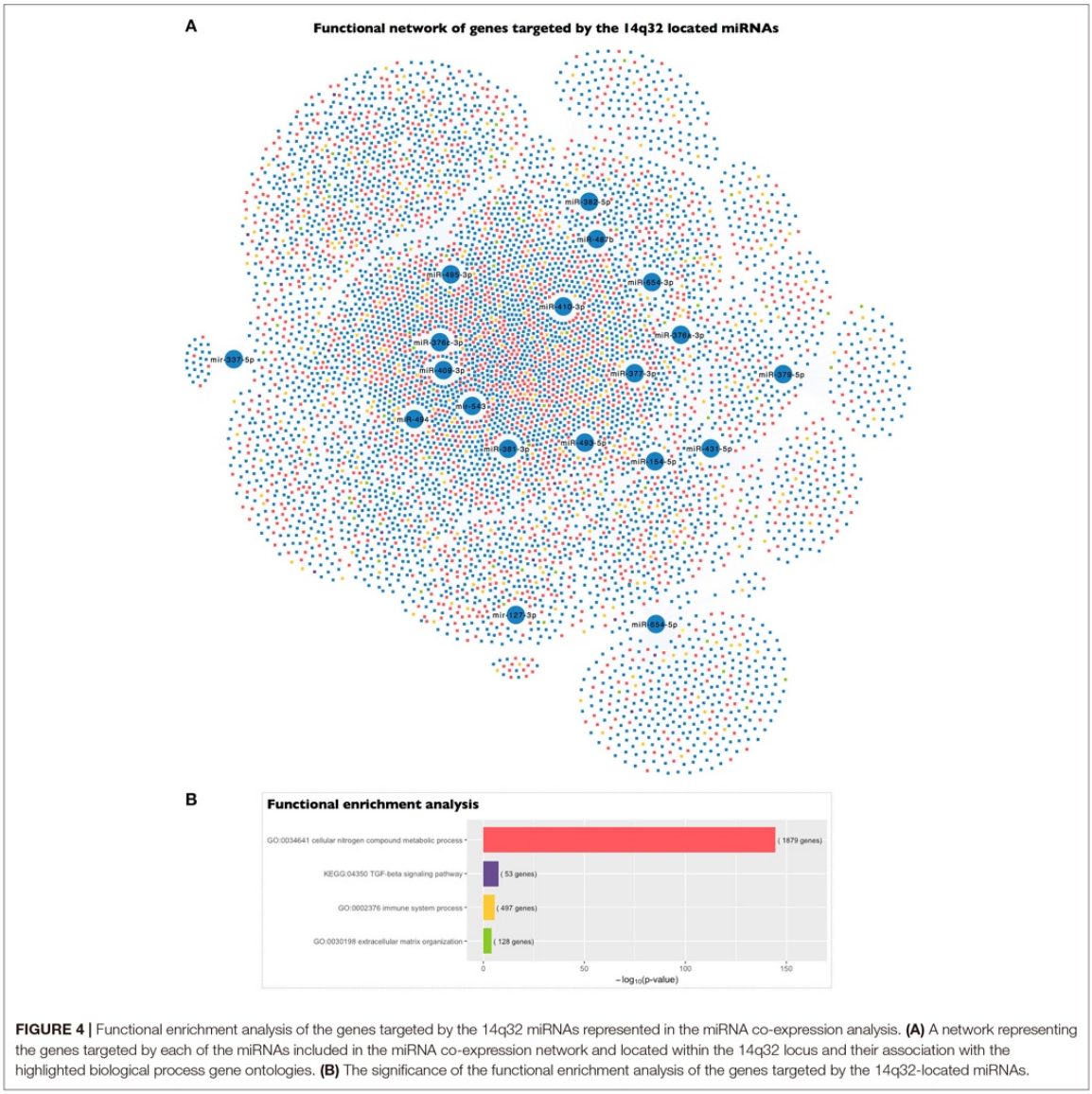

FIGURE 4 | Functional enrichment analysis of the genes targeted by the 14q32 miRNAs represented in the miRNA co-expression analysis. **(A)** A network representing the genes targeted by each of the miRNAs included in the miRNA co-expression network and located within the 14q32 locus and their association with the highlighted biological process gene ontologies. **(B)** The significance of the functional enrichment analysis of the genes targeted by the 14q32-located miRNAs.

found that the 14q32 locus on chromosome 14 was significantly overrepresented as a highly co-expressed miRNA cluster for the PECAM^{+} EMP-associated and co-expressed miRNAs in BAV. This genomic region contains the largest miRNA cluster found in the human genome (Benetatos et al., 2013). Specifically, we found that 19 of the 131 miRNAs inferred in the co-expression network, including miR-494, are located within this chromosome region. This 14q32 miRNA cluster is also known as the imprinted *DLK1-MEG3* genomic region. This region contains the protein-coding genes *DLK1*, *RTL1*, and *DIO3*, which are expressed from the paternally inherited chromosome. The region also contains multiple long and short non-protein coding RNAs, including *MEG3*, *MEG8*, the miRNA cluster and small nucleolar RNA (snoRNA) genes, which are expressed from the maternally inherited chromosome. The imprinting of a genomic region refers to biased expression of the genes contained in either the paternally or maternally inherited chromosome instead of the more common biallelic expression. Although, miRNAs located within the 14q32 region have been proposed as candidates for various diseases, including cancer, psychiatric illness, alcoholism, and non-alcoholic fatty liver disease, to date, there is no published data relating the 14q32 miRNA cluster with

cardiovascular diseases (Benetatos et al., 2013; Okamoto et al., 2016).

Based on the functional enrichment of the genes targeted by the PECAM$^+$ EMP-associated and co-expressed miRNAs located within the 14q32 locus, we could determine the biological significance of the regulation of this miRNA cluster. We found that the genes targeted by the 14q32 co-expressed miRNAs are strongly involved in the regulation of genes in categories such as "cellular nitrogen compound metabolic process," "immune system process," "extracellular matrix organization," and "TGF-β signaling pathway." The "cellular nitrogen compound metabolic process" gene ontology term includes the "nitric oxide biosynthetic pathway" subcategory. Nitric oxide (NO) is a pivotal endothelium-derived substance that plays a crucial role in the homeostasis of the cardiovascular system by modulating endothelium-dependent vasodilation; in fact, impairment of NO production or activity has been proposed as a major mechanism of endothelial dysfunction (Davignon and Ganz, 2004). Moreover, modulation of the expression and activity of endothelial nitric oxide synthase (eNOS), the specific enzyme that produces NO in endothelial cells, by fluid shear stress and the implication of this modulation in the development of BAV are well established (Ranjan et al., 1995; Aicher et al., 2007; Vion et al., 2013). Thus, in the context of BAV, the valvulopathy and the associated abnormal haemodynamics are related to the altered distribution of aortic wall shear stress, which promotes regional disruption of the eNOS pathway in a manner that is primarily mediated by the differential expression and activity of eNOS. Endothelial cells release EMPs in response to cellular stress and cell activation (Dignat-George and Boulanger, 2011). Consistent with this fact and the role of PECAM$^+$ EMP-related and co-expressed miRNAs in the regulation of endothelial dysfunction, we found that among the genes targeted by the 14q32-located miRNAs, those with functional implications related to modulation of immune system processes and to extracellular matrix organization were significantly enriched. In fact, interactions between inflammatory activation and endothelial dysfunction have been previously described in patients with BAV (Ali et al., 2014). In addition, we also found that the 14q32-located miRNAs present in our co-expression network targeted genes that were related to the TGF-β signaling pathway. The TGF-β family is composed of several cytokines with diverse functions, including the regulation of tissue repair and fibrosis, extracellular matrix remodeling, and inflammation as well as cell proliferation and migration (Bobik, 2006). Previous studies have implicated the impairment of TGF-β signaling in BAV pathology and the development of BAV-associated aortopathy (Forte et al., 2016). Additionally, functional interactions between TGF-β and NO have been demonstrated in endothelial cells (Saura et al., 2005). All these data suggest that the 14q32 miRNA cluster may play a pivotal role in the induction of BAV-associated endothelial damage.

Furthermore, in addition to studying the post-transcriptional implications of the regulatory functions of the 14q32 miRNA cluster, we thought it would be interesting to understand the regulatory mechanisms that could affect the activation or repression of the expression of this miRNA cluster. The imprinted status of the maternally expressed RNAs of the *DLK1-MEG3* locus, including the 14q32 miRNA cluster, is regulated by epigenetic mechanisms, specifically by the methylation of two differentially methylated regions (DMRs) located upstream of the transcription activation site of *MEG3* (Murphy et al., 2003; Kameswaran et al., 2014). The hypermethylation of either of these two DMRs has been associated with decreased expression of the maternal transcript (Kagami et al., 2010). In various models, such as in human type 2 diabetic islets, repression of the 14q32 miRNA cluster is strongly correlated with hypermethylation of the *MEG3*-DMR, and modifications at this region increase susceptibility to disease (Kameswaran et al., 2014). Interestingly, Boon et al. (2016) proposed that aging induces the expression of *MEG3* and that *MEG3*-mediated changes in the epigenetic regulation of gene expression contributes to aging-related endothelial dysfunction. Moreover, Gordon et al. (2010) suggested that *MEG3* may regulate the expression of *VEGF*; they showed that loss of *MEG3* leads to up-regulation of the expression of genes in the VEGF and Notch signaling pathways in mouse brains. Furthermore, DNA methylation mechanisms are responsive to disturbed flow (Dunn et al., 2014); in fact, endothelial gene expression can be regulated by flow-dependent epigenetic mechanisms (Jiang et al., 2015). Therefore, the disturbed flow associated with BAV may promote not only the secretion of EMPs but also the *MEG3*-mediated epigenetic regulation of the 14q32 miRNA cluster and thus may play a key role in the regulation of endothelial damage in BAV disease. On the other hand, DLK1 is the best-studied non-canonical Notch ligand and acts as an inhibitor of Notch signaling *in vitro* (Baladrón et al., 2005). In this manner, regulation of the expression of genes located in the 14q32 locus could also modulate the crosstalk between TGF-β and the VEGF and Notch signaling pathways, which have been previously described to act co-ordinately in space and time in the regulation of vascular morphogenesis (Holderfield and Hughes, 2008).

LIMITATIONS

We studied the miRNAs associated with the secretion of PECAM$^+$ EMPs in the context of BAV-induced endothelial damage, but we did not determine if this pattern of expression of PECAM$^+$ EMPs-associated miRNAs is also deregulated when the endothelial damage is caused by other conditions.

In summary, we propose that the changes in hemodynamic forces in the vascular system that are caused by the bicuspid morphology of the aortic valve might result in the expression of a specific pattern of PECAM$^+$ EMP-associated miRNAs that regulates a potent post-transcriptional network that might be involved in the regulation of endothelial damage. We reported that the highly co-expressed and PECAM$^+$ EMP-associated miRNAs clustered within the 14q32 locus might modulate a functional regulatory miRNA co-expression network that targets functionally related genes, which could be the molecular effectors linking the impairment of various signaling pathways (VEGFA, TGF-β, NOTCH) involved in the pathophysiology of BAV disease. Thus, we propose that the 14q32 miRNAs may act as master switches for endothelial damage and that inhibition of

either individual or a combination of 14q32 miRNAs might offer a new therapeutic approach for BAV disease.

AUTHOR CONTRIBUTIONS

NM: Performed the experiments, analyzed the data, and wrote the manuscript; RB and GA: Performed the experiments; MF: Contributed in sample collection and writing the manuscript; JA: Conceived and designed the study, analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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ARTICLE 3: GLYCOPROTEIN AND LIPOPROTEIN PROFILES ASSESSED BY 1H-NMR AND ITS RELATION TO ASCENDING AORTIC DILATATION IN BICUSPID AORTIC VALVE DISEASE

En aquest estudi es va avaluar la relació entre el perfil de lipoproteïnes i glicoproteïnes analitzades mitjançant 1H-NMR en sang perifèrica amb la DAA en pacients amb VAB, així com amb la seva progressió. Es van analitzar mostres de 152 pacients amb VAB amb aorta ascendent dilatada i no dilatada. A més, 119 d'aquests pacients van ser seguits prospectivament clínica i ecocardiogràficament a llarg termini. Per a aquesta anàlisi es va calcular la velocitat de DAA (mm/any). Es va trobar que diversos paràmetres relacionats amb el perfil de lipoproteïnes, com ara el colesterol remanent que inclou el colesterol VLDL i el colesterol IDL, estaven significativament augmentats en el grup amb DAA en comparació amb els del grup no dilatats. També es va observar que el senyal de 1H-NMR de la GlycA augmentava en el grup dilatat. A més, després de fer una anàlisi multivariant, el colesterol remanent va romandre com a variable independent relacionada amb la dilatació aòrtica. Durant el seguiment a llarg termini, a l'anàlisi univariant els paràmetres lipoproteics proaterogènics es van relacionar amb la velocitat de progressió de la DAA. I finalment, després d'una anàlisi de regressió lineal, les partícules no HDL es van mantenir com a predictores independents de la velocitat de progressió de la DAA.

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Glycoprotein and Lipoprotein Profiles Assessed by 1H-NMR and Its Relation to Ascending Aortic Dilatation in Bicuspid Aortic Valve Disease

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Abstract: Introduction: The bicuspid aortic valve (BAV) confers a high risk of ascending aorta dilatation (AAoD), although its progression seems highly variable. Furthermore, the implication of lipoprotein metabolism and inflammation in the mechanisms that underlie AAoD is not fully established. The aim of this study consisted of evaluating the impact of the lipoprotein and glycoprotein profiles in AAOD as well as its progression in BAV aortopathy. Methods: Using 1H-nuclear magnetic resonance (1H-NMR), we analyzed and compared the lipoprotein and glycoprotein profiles of plasma samples from 152 BAV patients with dilated and nondilated ascending aorta. Additionally, these profiles were also compared for 119 of these patients who were prospectively followed-up clinically and by echocardiography in the long-term (5 years). Ascending aorta dilation velocity (mm/year) was calculated for this analysis. Results: Several parameters related to the lipoprotein profile including remnant cholesterol, small LDL and IDL-cholesterol were found to be significantly increased in the dilated group compared to those in the nondilated group. The glycoprotein A-nuclear magnetic resonance (NMR) signal, a novel inflammation biomarker, was also observed to be increased in the dilated group. After performing multivariate analysis, remnant cholesterol remained an independent variable related to AAoD. In the long-term follow-up, proatherogenic lipoprotein parameters were related to ascending aorta dilatation velocity ascending. After a lineal regression analysis, non-HDL particles remained as an independent predictor of ascending aorta dilation velocity. Conclusions: Patients with BAV and AAoD presented a more pro-atherogenic profile assessed by 1H-NMR, especially related to triglyceride-rich lipoproteins. This pro-atherogenic profile seems to contribute to the higher growth rate of ascending aorta diameter.

Keywords: bicuspid aortic valve; BAV; lipoprotein metabolism; remnant cholesterol; ascending aortic dilatation; glycoprotein; HMR

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1. Introduction

Bicuspid aortic valve (BAV) disease is the most common congenital cardiac malformation, with a prevalence between 0.6 and 2% in the general population [1–4]. In this condition, two cusps are identified in the aortic valve instead of three, usually because two of them are fused by a raphe [5]. This alteration produces a high risk of valvular

dysfunction such as stenosis or regurgitation and aortic dilatation [6,7]. In fact, ascending aorta dilatation (AAoD) might affect up to 50–77% of BAV patients [8,9]. A significant AAoD confers a higher risk of aortic dissection or rupture.

The mechanisms that underlie AAoD are not yet definitively clear. The proposed causes include anomalous flow in the ascending aorta generated by the odd dynamics of BAVs and genetic abnormalities responsible for the defective structure of the aortic media, to which cardiovascular risk factors such as hypertension may contribute [1,8,10–13]. Additionally, some studies have suggested that oxidative stress and consequently, endothelial dysfunction, may be present in BAV disease [14–16]. In our previous work, we showed a decreased antioxidant plasma metabolic profile in BAV individuals with AAoD [17].

Furthermore, it is not fully clear whether lipoprotein metabolism could play a key role in AAoD progression. While lipoprotein metabolism seems to be directly related to abdominal aortic dilatation, its role in AAoD, and specifically in BAV disease, remains unknown [18,19]. Although an association between low-density lipoprotein (LDL) and apolipoprotein B (ApoB) and aortic dilatation in BAV patients has previously been highlighted by our group, the research in this area remains insufficient [20]. Hence, an extensive analysis that comprises different lipoprotein subclasses seems necessary to obtain new insights into lipoprotein metabolism and its relation to AAoD in BAV disease.

The aim of this study was to analyze the complete lipoprotein and glycoprotein profiles using ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) in a BAV population with a long-term prospective follow-up, assessing its relationship with the rate of AAoD. This information would provide important physio pathological information that could potentially help to predict a faster rate of AAoD; therefore, possible complications could be prevented such as aortic aneurysm and dissection by improving treatment and medical follow-up.

2. Materials and Methods

2.1. Study Participants

This study included those patients recruited in our BAV database who were older than 18 years, followed-up for 5 years, and with a plasma sample stored in our biological sample bank at the baseline. Patients with Marfan syndrome were excluded. The participants were prospectively entered into the database and underwent an echocardiogram and blood sample collection upon enrollment. Once patients were included, a clinical and echocardiographic follow-up was performed at least once a year by the same cardiologist in a monographic consultation. Written informed consent was obtained from all patients who participated in this study. BAV disease was diagnosed when two aortic leaflets were clearly visualized, with or without raphe, on the parasternal short-axis view of a transthoracic echocardiogram. If it was inconclusive, transesophageal echocardiogram or cardiac magnetic resonance imaging was performed according to the clinical criteria to obtain a definitive diagnosis. The valve morphotype (i.e., the pattern of cusp fusion) was categorized as typical (fusion between right and left coronary cusps) or atypical (other morphotypes, mainly fusion between right and noncoronary cusps). The maximum diameter at the end-diastole was selected as the ascending aorta measure. All echocardiographic studies were performed or reviewed upon inclusion by the same observer. An exhaustive medical history including cardiovascular risk factors, a complete physical examination, and anthropometry data were recorded.

At the baseline, the patients were divided into two groups: dilated (ascending aorta diameter $\geq$40 mm) [21] and nondilated (ascending aorta diameter <40 mm) groups. The baseline measurement of the ascending aorta was compared with that recorded in the 5-year follow-up control (range $\pm$1 year). The ascending aorta dilation velocity during follow-up was calculated considering the difference in the ascending aorta diameter between the first and last echocardiograms in the follow-up and dividing it by the follow-up duration (mm/year) for the longitudinal analysis. Severe aortic regurgitation was defined according to an integrated approach considering qualitative, semi-quantitative, and quantitative

parameters [22]. Severe aortic stenosis was defined when the mean aortic gradient was ≥40 mmHg or when the aortic valve area was ≤1 cm^2 [22]. Patients with an ascending aorta diameter ≥50 mm were excluded because they could be considered for surgery. For patients who underwent aortic valve or ascending aorta replacement, the last follow-up before surgery was selected. We included 152 patients who met the established criteria at baseline, who constituted the transversal analysis group. From them, 33 had an ascending aorta diameter ≥50 mm or underwent cardiac surgery before the 5-year follow-up. Therefore, 119 patients constituted the longitudinal analysis group.

This study was conducted according to the principles of the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee (03-06-19/6proj4 and 114/2020) of our institutions, the Hospital Universitari Sant Joan and the Institut d'Investigació Sanitària Pere Virgili.

2.2. Plasma Preparation

Blood samples were collected overnight under fasting conditions and processed within 90 min of collection. The samples were centrifuged at 1500× *g* for 15 min to obtain plasma, which was further centrifuged at 4000× *g* for 10 min to remove platelets. The plasma was stored at −80 °C in our biological sample bank (Biobanc IISPV—HUSJR) until needed.

2.3. ^{1}H-NMR Lipid and Glycoprotein Profile Evaluation

The lipoprotein profile was measured in serum samples (250 µL) using the ^{1}H-NMR-based Liposcale® test, a new generation nuclear magnetic resonance test by Biosfer Teslab (Reus, Spain). The lipid concentrations (i.e., triglycerides and cholesterol) of the four main classes of lipoproteins (very low-density lipoprotein (VLDL); intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL)), and the particle numbers of nine subclasses (large, medium, and small particle numbers of each of the following: VLDL, LDL, and HDL) were determined as previously reported [23,24]. Briefly, the particle concentrations and diffusion coefficients were obtained from the measured amplitudes and attenuation of their spectroscopically distinct lipid methyl group NMR signals using the 2D diffusion-ordered ^{1}H-NMR spectroscopy pulse. The methyl signal was surface fitted with nine Lorentzian functions associated with each lipoprotein subclass: large, medium, and small subclasses of each of the main lipoprotein classes. The area of each Lorentzian function was related to the lipid concentration of each lipoprotein subclass, and the size was calculated from their diffusion coefficient. The different lipoprotein subclasses corresponded to the following diameter size ranges: large VLDL, 68.5 to 95.9 nm; medium VLDL, 47 to 68.5 nm; small VLDL, 32.5 to 47 nm; large LDL, 24 to 32.5 nm; medium LDL, 20.5 to 24 nm; small LDL, 17.5 to 20.5 nm; large HDL, 10.5 to 13.5 nm; medium HDL, 8.5 to 10.5 nm; and small HDL, 7.5 to 8.5 nm. Non-HDL particles are defined as the sum of all lipoprotein particles minus HDL. RemCholesterol is defined by the total cholesterol minus HDL-cholesterol and LDL-cholesterol.

The glycoprotein profile was determined by analyzing the specific ^{1}H-NMR spectral region where these protein–sugar bonds resonate (2.15–1.90 ppm) by deconvoluting the spectra by using three Lorentzian functions, as previously reported [24]. For each function, we determined the total area (proportional to concentration), height, position, and bandwidth. The area of glycoprotein A (GlycA) provided the concentration of acetyl groups of protein-bond N-acetylglucosamine and N-acetylgalactosamine, and the area of glycoprotein B (GlycB) provided the concentration of N-acetylneuraminic acid. The glycoprotein F (GlycF) area arises from the concentration in the acetyl groups of N-acetylglucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid unbound to proteins (free fraction). H/W ratios, which reflect the aggregation state of the sugar-protein bonds, were also reported for GlycA and GlycB [25,26]. Height was calculated as the difference from the baseline to maximum of the corresponding NMR peaks, and the width value corresponded to the peak width at half height.

2.4. Statistical Analysis

Statistical studies were performed to compare the distribution of the different variables in a transversal analysis (at baseline: non AAoD vs. AAoD), and in a longitudinal analysis depending on the ascending aorta dilation velocity (AAoD Velocity). Categorical variables are expressed as percentages, and significant differences were identified using the chi-square test or Fisher's exact test, as appropriate. The quantitative variables, represented as the mean (standard deviation (SD), were analyzed using the Student's *t*-test. Pearson's or Spearman correlation, depending on the distribution, was used to identify the linear relationships between the ascending aorta diameter and the parameters included in the glycoprotein and lipoprotein profiles. Furthermore, a logistic regression with a forward stepwise model was constructed to analyze the independent lipoprotein and glycoprotein variables related to AAoD. Parameters with the strongest correlations to the dependent variable but lower correlation between them were included. In the longitudinal analysis, a linear forward regression was performed. In both multivariate analyses, a forward stepwise method was performed, which helped reduce the number of significant parameters that were selected in the final model. p values < 0.05 were considered significant. All analyses were performed using SPSS v25 (IBM Corp., Armonk, NY, USA), and graphs were designed using Prism v9 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Evaluation of the Relation of Lipoprotein and Glycoprotein Profiles to AAoD in BAV Disease: Transversal Study

3.1.1. Baseline Clinical Characteristics

The study included 152 patients with BAV, predominantly men (72.4%), with a mean age of 47.6 (17.2). The mean ascending aorta diameter at the baseline was 39.1 (7.4) mm, and 69 patients (45.4%) had an AAoD. Severe aortic valve dysfunction was present in 47 patients (30.9%): aortic regurgitation (17.1%), aortic stenosis (12.5%), or both (1.3%).

Baseline characteristics revealed that patients with AAoD were significantly older than patients from the nondilated group (41.7 (14.6) vs. 54.8 (17.3) years; $p < 0.001$). Patients with AAoD had a higher body mass index (BMI) and a higher prevalence of type 2 diabetes and hypertension. Regarding the echocardiographic parameters, a higher aortic valve gradient (mean) ($p = 0.002$) was observed in the dilated group. All data are represented in Table 1.

Table 1. Baseline clinical and echocardiographic characteristics of the BAV patients in the transversal analysis, depending on the presence of a dilated ascending aorta.

	NonDIL (n: 83)	DIL (n: 69)	
	Mean (SD) or n (%)	**Mean (SD) or n (%)**	***p* Value**
Sex (male %)	61 (73.5%)	54 (78.3%)	0.430 ***
Age (years)	41.7 (14.6)	54.8 (17.3)	<0.001 ***
Body mass index (kg/m^2)	24.60 (4.01)	27.53 (4.23)	<0.001 ***
Smoking (%)	34 (41.0%)	26 (37.7%)	0.792
NYHA Scale (≥II)	1 (1.2%)	2 (2.9%)	0.887
Hypertension	19 (22.9%)	36 (52.2%)	0.001 **
Type 2 diabetes	3 (3.6%)	10 (14.5%)	0.024*
Peripheral artery disease	2 (2.4%)	3 (4.3%)	0.517
Stroke	1 (1.2%)	2 (2.9%)	0.465
Coronary artery disease	1 (1.2%)	3 (4.3%)	0.231
Aortic regurgitation (≥2)	34 (41.0%)	30 (43.5%)	0.986
LVEDD (mm)	51.6 (6.1)	52 (5.8)	0.692
LVESD (mm)	31.2 (7.2)	33.2 (6.3)	0.078
Aortic root (mm)	33.9 (5.1)	39.9 (5.7)	<0.001 ***

Table 1. *Cont.*

	NonDIL (n: 83)	DIL (n: 69)	
	Mean (SD) or n (%)	**Mean (SD) or n (%)**	***p* Value**
Ascending aorta (mm)	33.7 (4.1)	45.4 (4.6)	<0.001 ***
AV gradient (mean) (mmHg)	13.1 (13.5)	22 (19)	0.001 **
Statins	9 (10.8%)	10 (14.5%)	0.501
BAV morphology (typical)	47 (56.6%)	45 (65.2%)	0.243

BAV: bicuspid aortic valve; NonDIL: nondilated ascending aorta; DIL: dilated ascending aorta; NYHA: New York Heart Association; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; AV Gradient: aortic valve gradient; Typical: right and left coronary leaflet fusion. * Significant values ($p < 0.05$), ** Significant values ($p < 0.01$), *** Significant values ($p < 0.001$).

3.1.2. Lipoprotein and Glycoprotein Profiles Assessed by ^{1}H-NMR: Nondilated Vs. Dilated

The relationship between the lipoprotein and glycoprotein profiles and AAoD evaluated by ^{1}H-NMR was assessed. Several variables related to cholesterol and triglycerides, most of them triglyceride-rich lipoproteins as well as glycoproteins, showed a linear relationship with the ascending aorta diameter (Supplementary Table S1). The most representative correlations between these parameters and the ascending aorta diameter are shown in Figure 1. The complete correlation values can be seen in the Supplementary Materials (Table S1).

Our results showed significantly higher concentrations in the dilated group in several parameters regarding cholesterol metabolism including VLDL-C, IDL-C, LDL-C, total cholesterol, and remnant cholesterol (Table 2). Furthermore, our results revealed higher concentrations of several parameters related to triglyceride metabolism in the AAoD group, reflected in numerous parameters, most of them triglyceride-rich lipoproteins and total triglycerides. Patients included in the AAoD group also presented a higher number of VLDL and LDL particles and, specifically, higher concentrations of small VLDL and LDL particles.

Finally, diverse ratios regarding the different lipoprotein compositions were studied. In our study, we found that the IDL-TG/IDL-C ratio was significantly higher in the nondilated group than in the dilated group of BAV patients, whereas the HDL-TG/HDL-C ratio was significantly lower.

The glycoprotein profile was also assessed by ^{1}H-NMR and then compared between the two groups (Table 3). Our results showed a significantly higher glycoprotein A NMR signal in the AAoD group. Nevertheless, the height–weight ratio did not show significant differences between the groups. Furthermore, no significant differences were observed in the other measured parameters including glycoprotein F and glycoprotein B or the height/weight ratio.

Logistic regression was used to analyze the relationship between the ascending aorta dilation and diverse parameters, which were differentially expressed between both groups including age, hypertension, type 2 diabetes, remnant cholesterol, Glyc-A signal, LDL-P, BMI, and AV gradient (mean) (Table 4). The model obtained was composed of two independent parameters, remnant cholesterol and age, where both represent a risk for AAoD in BAV patients.

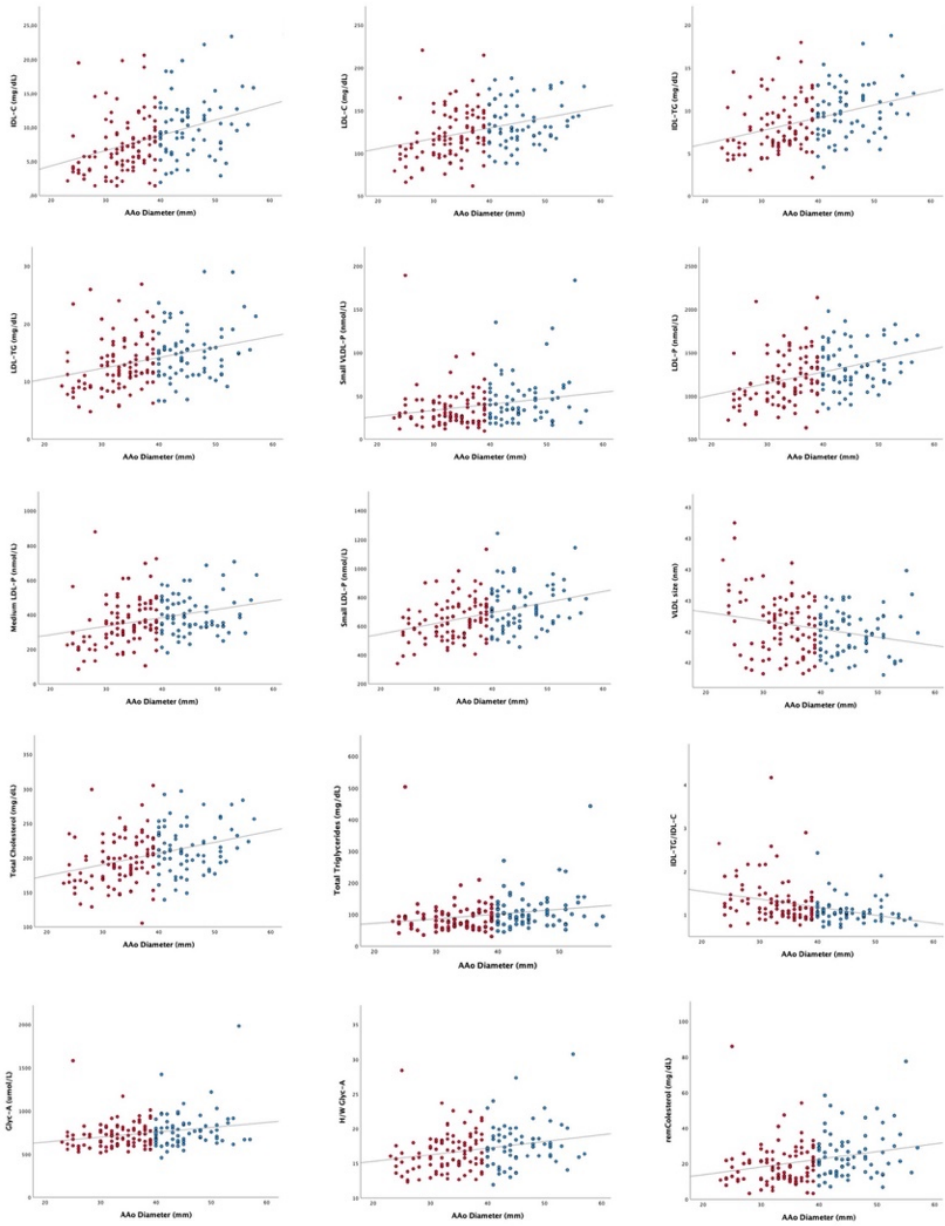

Figure 1. Association between the lipoprotein and glycoprotein profiles and the ascending aorta diameter in the bicuspid aortic valve patients. Red dots correspond to BAV patients without AAD while blue dots correspond to BAV patients with AAD. Complete correlations and *p* values can be found in Supplementary Table S1. AAo: ascending aorta; remCholesterol: remnant cholesterol; TG: triglycerides; C: cholesterol; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein; *p*: particles; GlycA: glycoprotein A signal; H/W GlycA: height/weight ratio for glycoprotein A signal.

Table 2. Comparison of the lipoprotein profile assessed by HNMR-1 in the transversal analysis, depending on the presence of a dilated ascending aorta.

	NonDIL (n:83)	DIL (n:69)	
	Mean (SD)	**Mean (SD)**	***p* Value**
VLDL-C (mg/dL)	12.08 (8.96)	15.48 (10.39)	0.032 *
IDL-C (mg/dL)	7.76 (4.20)	10.16 (4.35)	0.001 **
LDL-C (mg/dL)	125.17 (30.14)	135.8 (24.69)	0.02 *
HDL-C (mg/dL)	55.08 (10.35)	54.37 (9.06)	0.657
VLDL-TG (mg/dL)	59.48 (50.63)	76.72 (55.01)	0.046 *
IDL-TG (mg/dL)	8.44 (3.02)	10.07 (2.90)	>0.001 ***
LDL-TG (mg/dL)	13.55 (4.48)	15.12 (4.52)	0.033 *
HDL-TG (mg/dL)	9.61 (3.70)	10.84 (3.86)	0.046 *
VLDL-P (nmol/L)	41.33 (30.35)	53.36 (35.37)	0.026 *
Large VLDL-P (nmol/L)	1.07 (0.75)	1.28 (0.74)	0.074
Medium VLDL-P (nmol/L)	5.08 (5.97)	6.36 (5.66)	0.180
Small VLDL-P (nmol/L)	35.18 (24.25)	45.71 (29.45)	0.017 *
LDL-P (nmol/L)	1223.88 (291.67)	1348.36 (253.78)	0.006 **
Large LDL-P (nmol/L)	198.54 (37.91)	208 (33.88)	0.110
Medium LDL-P (nmol/L)	370.51 (139.25)	406.07 (111.42)	0.089
Small LDL-P (nmol/L)	654.83 (142.33)	734.29 (154.03)	0.001 **
HDL-P (µmol/L)	26.26 (5.01)	26.59 (3.87)	0.649
Large HDL-P (µmol/L)	0.26 (0.04)	0.27 (0.04)	0.140
Medium HDL-P (µmol/L)	9.44 (1.74)	9.35 (1.78)	0.754
Small HDL-P (µmol/L)	16.55 (3.85)	16.97 (2.78)	0.455
VLDL-Z (nm)	42.3 (0.24)	42.24 (0.17)	0.080
LDL-Z (nm)	21.13 (0.24)	21.07 (0.25)	0.110
HDL-Z (nm)	8.28 (0.08)	8.26 (0.06)	0.173
TOTAL-C (mg/dL)	200.1 (35.32)	215.81 (33.76)	0.006 **
TOTAL-TGs (mg/dL)	91.08 (55.95)	112.75 (60.14)	0.023 *
VLDL-TG/VLDL-C	5.38 (1.84)	5.22 (1.15)	0.526
IDL-TG/IDL-C	1.23 (0.40)	1.08 (0.27)	0.006 **
LDL-TG/LDL-C	0.11 (0.03)	0.11 (0.02)	0.671
HDL-TG/HDL-C	0.18 (0.07)	0.20 (0.07)	0.024 *
remCholesterol (mg/dL)	19.84 (11.91)	25.64 (13.15)	0.005 **

NonDIL: nondilated ascending aorta; DIL: dilated ascending aorta; remCholesterol: remnant cholesterol; TG: triglycerides; C: cholesterol; HDL: high density lipoprotein; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein; P: particles. * Significant values ($p < 0.05$), ** Significant values ($p < 0.01$), *** Significant values ($p < 0.001$).

Table 3. Comparison of the glycoprotein profile assessed by ^{1}H-NMR in the transversal analysis, depending on the presence of a dilated ascending aorta.

	NonDIL (n:83)	DIL (n:69)	
	Mean (SD)	**Mean (SD)**	***p* Value**
GlycB (µmol/L)	364.99 (52.77)	371.58 (55.55)	0.455
GlycF (µmol/L)	236.35 (78.75)	257.74 (91.11)	0.123
GlycA (µmol/L)	723.67 (154.61)	789.09 (214.47)	0.031 *
H/W GlycB	4.59 (0.67)	4.67 (0.70)	0.470
H/W GlycA	16.87 (2.77)	17.74 (3.08)	0.068

Non-DIL: nondilated ascending aorta; DIL: dilated ascending aorta; H/W: height/weight. * Significant values ($p < 0.05$).

Table 4. Multivariate analysis of the variables related to ascending aorta dilation in the transversal analysis.

		95% CI of OR		
	OR	**Inf.**	**Sup.**	***p* Value**
Age (year)	1.041	1.017	1.065	0.001 **
Remnant cholesterol (by mg/dL)	1.042	1.007	1.079	0.019 *

* Significant values ($p < 0.05$), ** Significant values ($p < 0.01$).

3.2. Effect of the Lipoprotein and Glycoprotein Profiles in the Progression of Ascending Aorta Diameter in BAV Disease: Longitudinal Analysis

3.2.1. Ascending Aorta Diameter Progression in BAV Patients: A Long-Term Follow-Up

In order to analyze the lipoprotein and glycoprotein profile impact in the ascending aorta diameter progression in BAV disease, 119 patients from the whole group with a mean age of 48.4 (15.6) years who were prospectively followed-up for 5 years (mean 4.7 (0.7)) were included in a longitudinal analysis. The mean and median aortic dilatation rate were 0.54 (0.93) mm/year and 0.20 mm/year, respectively. Baseline ascending aorta measurement was found to be negatively correlated to AAoD velocity (Figure 2). The complete analysis of the baseline clinical and echocardiographical characteristics can be found in Table 5.

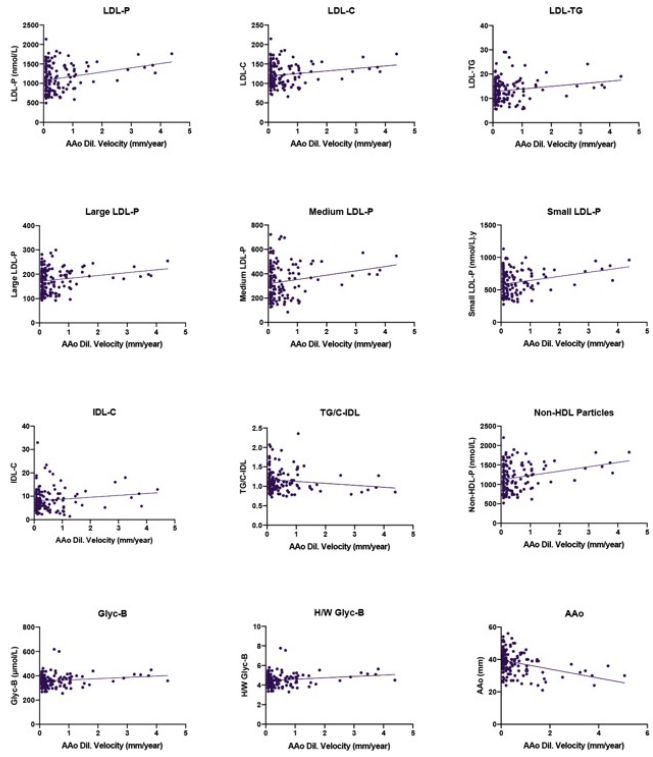

Figure 2. Association between the lipoprotein and glycoprotein profiles and the ascending aorta diameter in the bicuspid aortic valve patients. AAo: ascending aorta (mm) at baseline; AAo Dil. Velocity: ascending aorta dilation velocity; ascending aorta; TG: triglycerides; C: cholesterol; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; P: particles; GlycA: glycoprotein A signal; H/W GlycA: height/weight ratio for glycoprotein A signal.

Table 5. Baseline clinical and echocardiographic characteristics of the BAV patients in the longitudinal analysis, depending on the ascending aorta dilation velocity.

	Ascending Aorta Dilation Velocity (mm/Year)	
	Pearson/Spearman Correlation	***p* Value**
Sex (male %)	0.044	0.622
Age (years)	0.062	0.491
Body mass index (kg/m^2)	0.008	0.933
Smoking (%)	0.024	0.804
NYHA scale (≥II)	−0.004	0.974
Hypertension	−0.036	0.699
Type 2 diabetes	−0.155	0.166
Peripheral artery disease	0.103	0.367
Stroke	0.103	0.368
Coronary artery disease	−0.077	0.504
Aortic regurgitation (≥II)	0.071	0.537
LVEDD (mm)	0.207	0.060
LVESD (mm)	0.121	0.290
Aortic root (mm)	−0.146	0.106
Ascending aorta (mm)	−0.369 **	<0.001
AV gradient (mean) (mmHg)	−0.027	0.765
Statins	−0.057	0.538
BAV morphology (typical)	−0.025	0.835

BAV: bicuspid aortic valve; NonDIL: nondilated ascending aorta; DIL: dilated ascending aorta; NYHA: New York Heart Association; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; AV Gradient: aortic valve gradient; Typical: right and left coronary leaflet fusion. ** Significant values ($p < 0.01$)

3.2.2. Evaluation of the Lipoprotein and Glycoprotein Profiles' Influence in AAoD Progression in BAV Aortopathy: A Longitudinal Study

The complete lipoprotein and glycoprotein profiles were also evaluated in relation to the ascending aorta dilation velocity (mm/year) (AAoD Velocity). Several parameters were found to be positively correlated to AAoD Velocity in the lipoprotein profile including IDL-C, LDL-C, LDL-TG, non-HDL-P, and LDL-P as well as its three different sizes (Figure 2). The only parameter negatively correlated to AAoD. Velocity was TG-C/IDL ratio. Complete data can be found in Table 6.

When analyzing the glycoprotein profile, the Glyc-B signal as well as H/W Glyc-B were found to be positively correlated to AAoD. Velocity (Figure 2). The rest of the parameters measured did not show any significant correlation to AAoD Velocity and can be observed in Table 7.

Furthermore, a linear regression was performed in order to identify possible predictors of the AAoD Velocity out of several parameter which were positively or negatively correlated: Ascending aorta (at baseline), LDL-TG, H/W Glyc-B, small LDL-P, IDL-TG, IDL-C and Non-HDL particles and the final model is represented in Table 8 In this analysis, AAo and Non-HDL-P remained as independent parameters in order to determine AAoD Velocity.

Table 6. Comparison of the lipoprotein profile at baseline assessed by ^{1}H-NMR in the longitudinal analysis depending on the ascending aorta dilation velocity.

	Ascending Aorta Dilation Velocity (mm/Year)	
	Pearson/Spearman Correlation	***p* Value**
TOTAL-C (mg/dL)	0.166	0.071
TOTAL-TG (mg/dL)	0.095	0.302
TG/C-VLDL	0.035	0.705
TG/C-IDL	−0.182 *	0.047
TG/C-LDL	0.107	0.248
TG/C-HDL	0.031	0.740
Small VLDL %	0.156	0.090
Small LDL %	0.022	0.812
Small HDL %	0.142	0.123
VLDL-C (mg/dL)	0.088	0.341
IDL-C (mg/dL)	0.184 *	0.045
LDL-C (mg/dL)	0.199 *	0.030
HDL-C (mg/dL)	−0.131	0.155
VLDL-TG (mg/dL)	0.077	0.403
IDL-TG (mg/dL)	0.165	0.073
LDL-TG (mg/dL)	0.216 *	0.018
HDL-TG (mg/dL)	−0.026	0.776
VLDL-P (nmol/L)	0.092	0.321
Large VLDL-P (nmol/L)	0.072	0.436
Medium VLDL-P (nmol/L)	0.038	0.685
Small VLDL-P (nmol/L)	0.101	0.274
LDL-P (nmol/L)	0.269 **	0.003
Large LDL-P (nmol/L)	0.216 *	0.018
Medium LDL-P (nmol/L)	0.226 *	0.014
Small LDL-P (nmol/L)	0.283 **	0.002
HDL-P (µmol/L)	−0.091	0.327
Large HDL-P (µmol/L)	−0.053	0.565
Medium HDL-P (µmol/L)	−0.161	0.081
Small HDL-P (µmol/L)	−0.027	0.770
VLDL-Z (nm)	−0.176	0.055
LDL-Z (nm)	−0.101	0.272
HDL-Z (nm)	−0.134	0.145
Non-HDL-P (nmol/L)	0.270 **	0.003
remCholesterol (mg/dL)	0.080	0.351

remCholesterol: remnant cholesterol; TG: triglycerides; C: cholesterol; HDL: high density lipoprotein; IDL: intermediate density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein; P: particles. * Significant values ($p < 0.05$), ** Significant values ($p < 0.01$).

Table 7. Comparison of the glycoprotein profile at baseline assessed by ^{1}H-NMR in the longitudinal analysis depending on the ascending aorta dilation velocity.

	Ascending Aorta Dilation Velocity (mm/Year)	
	Pearson/Spearman Correlation	***p* Value**
Glyc-B (µmol/L)	0.182 *	0.048
Glyc-F (µmol/L)	0.091	0.326
Glyc-A (µmol/L)	0.135	0.144
H/W Glyc-B	0.189 *	0.039
H/W Glyc-A	0.146	0.113

H/W: Height/Weight. * Significant values ($p < 0.05$).

Table 8. Linear regression of the variables related to the velocity of ascending aorta dilation in the longitudinal analysis.

		95% C.I		
	ß	**Inf.**	**Sup.**	***p* Value**
AAo (by mm)	−0.356	−0.073	−0.027	<0.001 ***
Non-HDL-P (by nmol/L)	0.285	<0.001	0.001	0.001 **

AAo: ascending aorta at baseline (mm); Non-HDL-P: non-HDL particles (nmol/L). ** Significant values ($p < 0.01$), *** Significant values ($p < 0.001$).

4. Discussion

In this study, we analyzed the lipid and glycoprotein profiles via ^{1}H-NMR in a cohort of BAV patients. We observed that patients with AAoD presented a proatherogenic and proinflammatory profile compared to that in nondilated patients, specified by higher levels of triglyceride-rich lipoproteins and glycA, a biomarker directly related to inflammation [24,27]. Remnant cholesterol, which includes VLDL-C and IDL-C, has emerged as an independent variable related to AAoD in BAV disease. Furthermore, this proatherogenic lipoprotein profile also seemed to contribute to a higher dilation rate of the ascending aorta in the long-term follow-up. In this case, non-HDL particles, a parameter which includes LDL-C, VLDL-C, IDL-C, and Lp(a) cholesterol, have also emerged as an independent variable related to a faster AAoD during follow-up.

Current knowledge about the pathophysiological mechanisms involved in the dilatation of the ascending aorta observed in BAV disease places the effect of the abnormal flow generated by the irregular opening of the valve as the main cause of the dilation [28]. Unlike the anatomically typical tricuspid aortic valve, which gives rise to central flow, the anomalous opening of the BAV promotes eccentric flow, which determines high asymmetrical shear stresses, which could be decisive in dilation genesis [29]. In addition to these factors, aging contributes to the aortic wall degeneration, and as a consequence, to aortic dilation. Furthermore, there could also be genetic abnormalities in the structure of the aortic wall, which contribute to its predisposition to dilation [30]. In addition to these factors, the added effect that may determine a proatherosclerotic lipoprotein and proinflammatory glycoprotein profile remains unknown.

While wide evidence from epidemiological and experimental studies supports the involvement of dyslipidemia and inflammation in the physiopathology of abdominal aorta dilation, its role in AAoD, specifically in BAV aortopathy, is still uncertain. Our group had previously described an association between LDL/apolipoprotein B and AAoD in BAV disease in a smaller cohort [20]. Nevertheless, in the present study, we deepened the analysis of the relationship between the lipoprotein and glycoprotein profiles and BAV aortopathy-associated AAoD as well as its progression using ^{1}H-NMR, a rigorous technique that provides the complete number and size of the different lipoproteins. Our results showed that BAV patients with AAoD had higher levels of lipoproteins with a high content of triglycerides (cholesterol particles rich in triglycerides), conferring a pro-atherogenic and pro-inflammatory profile that would contribute to the progression of AAoD [31]. Different observational and Mendelian randomization studies have described the atherogenicity of TG- and TG-rich lipoproteins [32]. Proposed mechanisms associated with major adverse cardiovascular events and remnant cholesterol include local inflammation as well as atherosclerotic plaque formation [33]. Most of the TG rich particles including small VLDL and IDL and LDL particles easily penetrate into the arterial wall and are then taken up by macrophages, leading to foam cell formation [34]. These mechanisms could be involved in arterial wall damage that would contribute to AAoD. Furthermore, the PESA study revealed that serum TG levels were associated with vascular inflammation as well as subclinical atherosclerosis, irrespective of LDL-C levels [35]. Regarding the abdominal aorta, in addition to LDL, a significant association has been described between abdominal aorta aneurysm and triglycerides, suggesting that this proinflammatory effect of TG could also be involved in the development of aortic dilation [36].

The finding of the relation of non-HDL particles with a faster ascending aorta dilation velocity in the longitudinal study is consistent with that observed in the transversal analysis. Non-HDL particles have been highlighted by many to be an excellent cardiovascular risk biomarker, instead of LDL-C [37–39]. The number of particles, rather the amount of the cholesterol, has repeatedly been demonstrated to be a better CVD marker when these two parameters are discordant [40]. In addition to LDL, non-HDL includes the TG-rich lipoproteins that constitute the remnant cholesterol. Therefore, our results in the transversal and in the longitudinal study suggest the potential role of the pro-atherogenic lipoproteins contributing to the ascending aorta dilation in BAV patients, highlighting the effect of the TG-rich lipoproteins. This led us to hypothesize that the co-occurrence of hemodynamic alterations and lipidomic factors in BAV patients could simultaneously affect the function of the ascending aorta endothelium, enhancing vascular permeability and therefore accelerating and aggravating the pathological processes that may be caused by each factor individually.

To understand the possible role of lipoprotein metabolism in the progression of ADD in BAV patients, it is important to consider that although ADD-BAV patients presented higher levels of total cholesterol, LDL-C, and triglycerides than nondilated BAV patients, those values remained within the normal thresholds of the current clinical practice guidelines. This gives rise to the belief that a greater representation of patients with high LDL or TG-rich lipoproteins could have led to a stronger relationship between these parameters and ascending aorta dilation.

The use of echocardiography for measuring aortic diameters instead of computed tomography or cardiac magnetic resonance, techniques with lower variability, is a limitation of our study. However, it must be taken into account that in clinical practice, computed tomography or cardiac magnetic resonance is often not performed in BAV patients without aortic dilatation. Nevertheless, echocardiographic studies have been carried out prospectively with a predetermined methodology and by the same observer, in order to reduce the variability of the measurements. On the other hand, we identified the ascending aorta diameter at the baseline as a variable inversely related to the velocity of ascending aorta diameter progression. Therefore, patients with lower ascending aorta diameters had a higher velocity of ascending aorta diameter progression than patients with higher ascending aorta diameters. The number of patients included in our study was limited and it focused on the glycoprotein and lipoprotein profiles. Therefore, in a larger sample, other variables could be added as variables related to the rate of progression of aortic dilatation. Finally, the study included only BAV patients, so our results cannot be generalized to patients with a tricuspid aortic valve.

Our results suggest the potential benefit of an early implementation of prevention measures aimed at improving the lipidemic profile of BAV patients [20], focused on TG-rich lipoproteins, in order to contribute to slow down the dilation of the ascending aorta. Further studies in the long-term should assess the real impact of these actions.

5. Conclusions

We observed that patients with BAV-related AAoD presented a proatherogenic and proinflammatory profile when compared to nondilated patients, specified by higher levels of triglyceride-rich lipoproteins. This proatherogenic lipoprotein profile also seemed to contribute to a higher dilation rate of the ascending aorta in long-term follow-up.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12010332/s1, Table S1: Correlations between ascending aorta diameter and glycoprotein and lipoprotein profiles.

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Institutional Review Board Statement: This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board and Ethics Committee (03-06-19/6proj4 and 114/2020) of our institutions, the Hospital Universitari Sant Joan and the Institut d'Investigació Sanitària Pere Virgili.

Informed Consent Statement: Informed consent was obtained from all subjects involved 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 ethical reasons.

Conflicts of Interest: N.A. is a stock owner of Biosfer Teslab, and has a patent to commercialize the lipoprotein and glycoprotein profiles described in the present manuscript.

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7. DISCUSSIÓ

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Aquesta tesi inclou un compendi de tres articles en els quals, mitjançant l'estudi de l'epigenètica i del metabolisme lipoproteic, aprofundim en el coneixement de la fisiopatologia de la DAA a la VAB i descrivim diversos factors relacionats, alguns dels quals podrien potencialment ser utilitzats com a biomarcadors.

Malgrat que la prevalença de DAA a la VAB és molt elevada, entre el 25 i el 35% dels pacients amb VAB no desenvoluparan DAA (Verma et al., 2014; Della Corte et al., 2014). Al mateix temps, la velocitat de progressió de la DAA és molt variable. Manquen biomarcadors relacionats amb la VAB i la DAA, que podrien ser molt útils en el cribatge i en la valoració de l'evolució dels diàmetres aòrtics a la VAB. Nosaltres ens hem centrat en l'epigenètica (Nappi et al., 2022). L'epigenètica és l'estudi dels factors que activen o inactiven els gens sense canviar la seqüència de l'ADN. S'han identificat diversos mecanismes epigenètics que participen en els canvis fenotípics, entre els quals destaquem els miRNAs (He et al., 2004). Els miRNA van ser descrits per primera vegada l'any 1993 per Lee et al. a l'Universitat de Harvard. Els miRNA són ARN endògens monocatenaris i no codificants de 18-22 nucleòtids, que regulen l'expressió dels ARNm (Soler-Botija et al., 2019). Els miRNA s'uneixen a l'ARN complementari silenciant aquestes seqüències i per tant, escurçant aquest ARNm i modificant la proteïna codificada. Els miRNA tenen potencial per ser utilitzats com a biomarcadors, com a dianes terapèutiques o per aprofundir en la fisiopatologia de les malalties. En aquest estudi hem identificat un conjunt de miRNA circulants, en concret miR-122, miR-130a i miR-486, amb una regulació lligada al morfotip valvular sigui VAB o VAT. A més, vam veure que la

dilatació aòrtica està associada a una disminució de l'expressió de miR-718, sent un potencial biomarcador d'aortopatia. Aquest ha sigut el primer estudi que relaciona un patró específic de miRNA detectat en sang amb la VAB. Estudis anteriors, havien analitzat l'expressió de miRNA en segments de teixit aòrtic de pacients amb VAB i en models animals. És el cas de Nigam et al. (2010) que va observar una disminució dels nivells de miR-26a, miR-30b i miR-195 en els vels dels pacients amb estenosi aòrtica severa. Yanagawa et al. (2012) també va determinar una reducció de miR-141 en els vels de VAB amb estenosi significativa. No obstant, l'anàlisi de miRNA a través de la biòpsia pot tenir interès en l'estudi de la fisiopatologia però no pot ser utilitzat com a biomarcador. En canvi, l'anàlisi de miRNA en sang perifèrica sí que té aquesta capacitat. Els nivells plasmàtics de miRNA són reproduïbles i consistents entre individus perquè els miRNA semblen estar protegits de la degradació induïda per les ribonucleases endògenes. A més, els valors es poden reproduir i extrapolar a qualsevol laboratori i seria factible el seu ús en la pràctica clínica habitual com a biomarcador de progressió de dilatació en la VAB. Altrament, l'augment del diàmetre de l'aorta ascendent s'associa a disminució dels nivells de miR-718. Els processos biològics implicats es relacionarien amb la modulació de l'adhesió focal i la remodelació dels vasos sanguinis (Leng et al., 2014). Aquesta DAA és deguda a un trastorn vascular degeneratiu secundari a la transformació de la paret aòrtica i a la degradació de les proteïnes de la matriu, que provoca el reclutament i la infiltració de les cèl·lules del sistema immunitari a través de la secreció de molècules d'adhesió (Xue et al., 2014). Curiosament, el factor de creixement endotelial vascular A (VEGFA) és un dels gens diana del miR-718 per la "via d'adhesió focal". VEGFA actua sobre les cèl·lules endotelials augmentant la permeabilitat vascular i l'angiogènesi, induint la vasculogènesi i el creixement de les cèl·lules endotelials, promovent la migració cel·lular i inhibint l'apoptosi (Leng et al.,

2014). Aquestes implicacions biològiques dels gens diana miR-718 podrien explicar la relació entre miR-718 i la DAA.

Per altra banda, en estudis anteriors del nostre grup vam observar que la DAA en la VAB s'associa a l'alliberament d'EMP (Alegret et al., 2016). Les EMP són les principals partícules que contenen miRNA a la sang (Diehl et al., 2012). En aquest sentit, vam identificar 175 miRNAs que estaven significativament associats amb els nivells d'EMP. El 75% d'aquests miRNAs s'havien descrit prèviament com a miRNAs expressats en cèl·lules endotelials i el 67% d'ells havien estat associats a malalties cardiovasculars. Vam identificar 19 miRNA associats i coexpressats per les EMP que estan situats al locus 14q32 del cromosoma 14 i que constitueixen el cúmul de miRNA més gran del genoma humà (Benetatos et al., 2013). Altres miRNA en el mateix locus s'han descrit en diferents patologies com ara el càncer, les malalties hepàtiques i els trastorns psiquiàtrics, essent el primer cop que es defineix com a un locus relacionat amb les malalties cardiovasculars. Les EMP poden actuar com a nexes intercel·lulars en resposta al dany endotelial a través de la biosíntesi de l'òxid nítric, l'activació del sistema immune, la reorganització de la matriu extracel·lular i la senyalització del TGF-β (Holderfield et al., 2008; Bobik et al., 2006; Saura et al., 2005). En aquest sentit, el TGF-β1 és una citocina pleiotròpica secretada i emmagatzemada a la matriu extracel·lular que participa en els processos de fibrosi i remodelació tissular. El TGF-β1 activat s'uneix a un receptor amb dues subunitats: el TGFβR1 i el TGFβR2. En la VAB es produeix una disminució dels nivells miR-122 i un augment de miR-130a, que comporta un desequilibri de les subunitats TGFβR1 i TGFβR2 respectivament, i indueix a la degradació de la matriu extracel·lular i a l'activació de les MMP. A més, nivells baixos de miR-122 s'associen a majors nivells de TGF-β1 i més fibrosi miocàrdica. Per exemple, un model murí

de síndrome de Marfan amb el bloqueig de l'expressió del gen de TGFβR2 es va associar amb l'aparició d'aneurismes (Neptune et al., 2003; Emrich et al., 2019; Braverman et al., 2015). Diversos estudis han investigat la relació de miRNA, MMP i TIMP en l'aortopatia bicúspide, observant diferents nivells de MMP-2, MMP-9, TIMP-1 i TIMP-9 (LeMaire et al., 2005). Wu et al. (2016) va analitzar mostres de teixit de l'aorta sense i amb dilatació i va trobar una associació entre els gens miR-17 i la predisposició a la dilatació, deguda a la desregulació del teixit de les MMP. En aquest estudi miR-17 i MMP-2 estaven augmentats mentre que TIMP-1, TIMP-2 i TIMP-3 havien disminuït, plantejant així la hipòtesi d'una DAA influenciada per la VAB. L'any 2020, Naito et al. va estudiar els teixits d'aneurismes d'aorta toràcica en pacients amb VAB, i va descobrir una relació positiva entre miR-133a i TIMP-1 i 2, i una correlació inversa entre miR-143a i MMP-2, aportant noves evidències sobre la relació entre miRNA i MMP/TIMP en la dilatació aòrtica dels pacients amb VAB. En relació amb miR-130, estudis recents han demostrat experimentalment que el contingut en miR-130 de les vesícules extracel·lulars procedents de fibroblastes es relaciona amb la seva capacitat de reparació tissular (Gangadaran et al., 2022). A més, l'expressió de miR-130a inhibeix l'expressió GAX i HOXA5, actuant com a un regulador del fenotip angiogènic de les cèl·lules endotelials vasculars (Chen et al., 2008; Mozzini et al., 2019). Per una altra banda, s'ha observat que la disminució de miR-486 contribueix a una disminució de l'apoptosi, una alteració en la migració cel·lular i un major creixement i proliferació tissular (Holliday et al., 2011; Navon et al., 2009). Pisano et al. (2021) va objectivar nivells més alts de miR486 en sang de pacients amb més degeneració de la capa mitjana de la paret aòrtica, suggerint que podria ser un bon biomarcador del risc de dissecció aòrtica. Per tant, l'estudi dels miRNA està aportant coneixement en el camp de la fisiopatologia de la DAA. Amb

els resultats obtinguts postulem que el flux aòrtic anòmal generat per la VAB provocaria un dany endotelial amb l'alliberament d'EMP. Diversos estudis han relacionat els morfotips de la VAB amb l'alteració del flux sanguini a l'aorta ascendent (Markl et al., 2012; Grewal et al., 2019). A diferència de la VAT, on predomina un flux transvalvular aòrtic preferentment central, a la VAB se sol observar un flux excèntric amb diferents patrons de flux a l'aorta ascendent que en bona part depèn del morfotip de VAB. Aquest jet excèntric resultant provoca un augment de l'estrès de la paret i contribueix a la seva progressiva dilatació. Els canvis hemodinàmics descrits alteren la histologia de l'aorta contribuint al desenvolupament de la DAA. Dins d'aquest procés, el dany endotelial seria un dels primers fenòmens observats, traduint el contingut en miRNA per efecte del flux anòmal. S'ha suggerit que l'alteració del flux sanguini a causa de la VAB pot provocar una desregulació dels mecanismes epigenètics que condueixi a una expressió gènica anòmala i contribueixi a l'aparició les complicacions associades a la VAB, complementant la teoria hemodinàmica de la seva patogènesi (Junco-Vicente et al., 2021).

Altrament, la dislipèmia és un factor de risc cardiovascular clàssic. El seu mecanisme d'acció a la dilatació aòrtica es basa en un augment dels processos inflamatoris que produirien un dany endotelial que afavoriria el procés d'ateroesclerosi. Però el seu paper en l'aortopatia bicúspide no està aclarit. En el nostre estudi vam poder observar que les lipoproteïnes riques en triglicèrids s'associen a un major diàmetre de l'aorta ascendent en la VAB. En aquest sentit, els pacients amb VAB i dilatació aòrtica tenen un perfil lipídic més proaterogènic. El nostre grup havia descrit prèviament una associació entre l'LDL/apolipoproteïna B i la dilatació aòrtica en la malaltia de VAB en una cohort més petita (Alegret et al., 2015). I, tot i que

l'àmplia evidència d'estudis epidemiològics i experimentals recolza la implicació de la dislipèmia i la inflamació en la fisiopatologia de la dilatació de l'aorta abdominal (Harrison et al., 2018), el seu paper en l'aorta toràcica, i en concret en l'aortopatia VAB, encara és incert. Respecte al paper dels factors de risc a la DAA de la VAB, López et al. (2021) recentment ha descrit la relació entre un creixement aòrtic ràpid i la HTA en pacients amb VAB. Per tant, factors de risc clàssics com la dislipèmia i la HTA potencialment estarien relacionats amb l'aortopatia en la VAB. Amb aquestes premisses, en el present treball hem analitzat el perfil de lípids i glicoproteïnes mitjançant 1H-RMN dels pacients amb VAB, tècnica que proporciona el nombre complet i la mida de les diferents lipoproteïnes, podent fer d'aquesta forma una anàlisi profunda i global sobre la implicació del metabolisme lipoproteic en la DAA de la VAB. Els nostres resultats suggereixen que les lipoproteïnes proaterogèniques contribueixen a la DAA en pacients amb VAB (Castañer et al., 2020), destacant l'efecte de les lipoproteïnes riques en triglicèrids i la glicoproteïna A, un paràmetre directament relacionat amb la inflamació (Fuertes-Martín et al., 2018; Fuertes-Martín et al., 2020). Diferents estudis han descrit l'aterogenicitat de les lipoproteïnes riques en triglicèrids a través de la inflamació local i la formació de la placa ateroescleròtica (Jorgensen et al., 2013). Aquestes partícules riques en triglicèrids també s'anomenen colesterol remanent i són les VLDL i IDL. El colesterol remanent penetra fàcilment a la paret arterial i després és absorbit pels macròfags, donant lloc a la formació de cèl·lules escumoses (Miller et al., 2010). Aquests mecanismes podrien estar implicats en el dany de la paret arterial que contribuiria a la DAA. Així, el colesterol remanent, es postula com una variable independent relacionada amb la dilatació aòrtica en la malaltia de la VAB. I, en aquest cas, les partícules no HDL, un paràmetre que inclou el colesterol LDL, VLDL, IDL i la

lipoproteïna A, també han sorgit com una variable independent relacionada amb un creixement aòrtic més ràpid durant el seguiment. En aquest sentit, tant l'anàlisi transversal com el longitudinal van relacionar aquestes partícules no HDL amb una major velocitat de DAA. Diferents autors han destacat que les partícules no HDL són un millor biomarcador de risc cardiovascular que el LDL (Johannesen et al., 2021; Aggarwal et al., 2021). Per altra banda, la dinàmica del flux de la VAB ocasionaria el dany endotelial descrit anteriorment que augmentaria la permeabilitat del vas i que es relacionaria amb una major predisposició a la proaterogeneitat. En aquest sentit, l'estudi PESA va revelar que els nivells sèrics de triglicèrids estaven associats amb la inflamació vascular i l'ateroesclerosi subclínica, independentment dels nivells de LDL (Raposeiras-Roubin et al., 2021).

Aquest treball té diverses limitacions. En els tres estudis els diàmetres aòrtics es van mesurar utilitzant l'ecocardiografia, que és una tècnica amb una major variabilitat si es compara amb el TC o la cardio-RM. Tanmateix, per minimitzar aquest error, es va definir una metodologia predeterminada i es va avaluar pel mateix observador (JMA). A més, en l'estudi de les EMP, la relació amb el TGF-β1 no es va confirmar experimentalment. D'altra banda, en l'estudi sobre la relació del perfil lipídic amb la DAA, només es van analitzar glicoproteïnes i lipoproteïnes, sense tenir en compte variables relacionades amb altres vies metabòliques potencialment relacionades amb la taxa de progressió de la dilatació aòrtica. A més, en l'estudi 3 només es van incloure pacients amb VAB, de manera que els nostres resultats no es poden generalitzar a pacients amb DAA i VAT. I finalment, malgrat que els pacients VAB amb dilatació van presentar nivells més alts de colesterol total, LDL i triglicèrids que els no dilatats, els valors mitjans es van

mantenir dins de valors que es consideren normals a les guies de pràctica clínica actuals. Pensem que una major mida mostral podria haver demostrat una relació més forta entre aquests paràmetres i la DAA.

Els nostres resultats obren la porta a futurs estudis que els complementin i avancin en el coneixement de la DAA a la VAB. Per una part, l'anàlisi dels miRNA en els pacients amb VAB entenem que té potencial interès en el cribratge i l'estratificació de risc. Caldrà validar els nostres resultats en altres poblacions amb un nombre ampli de participants per a poder concretar el seu potencial ús com a biomarcador. Per una altra part, el dany endotelial i el metabolisme lipídic semblen estar implicats en la fisiopatologia de la dilatació aòrtica de la VAB. Plantegem que la coexistència d'alteracions hemodinàmiques i factors lipídics en pacients amb VAB podria afectar sinèrgicament a la funció de l'endoteli de l'aorta ascendent, augmentant la permeabilitat vascular i, per tant, accelerant i agreujant els processos patològics de degeneració de la paret aòrtica que poden ser provocats per cada factor individualment. Estudis posteriors que complementin les tècniques d'imatge, com ara la ressonància 4D-flow per valorar el flux aòrtic, i marcadors de dany endotelial poden donar llum a aquesta hipòtesi. Dels nostres resultats es desprèn el potencial benefici de la modificació del perfil lipídic cap a un patró menys pro-aterogènic com a mesura per alentir la DAA a la VAB. Caldrien estudis d'intervenció prospectius que validin aquesta hipòtesi. Cal tenir present la dificultat de realitzar un estudi d'aquestes característiques, donada la necessitat d'un seguiment a molt llarg termini amb una població molt àmplia i fet amb tècniques d'imatge precises com el TC. Altrament, caldria definir la intervenció a dur a terme. A l'assaig clínic BICATOR el tractament amb estatines durant 3 anys sembla no modificar significativament la progressió de la DAA en la

VAB (Evangelista et al; Congreso SEC 21; article en revisió). Actualment, el descobriment de miRNAs implicats en el metabolisme dels lípids i les lipoproteïnes ha posat el focus en el seu potencial ús en el camp de la investigació de l'ateroesclerosi i les malalties cardiometabòliques (Fernández-Tussy et al., 2021). I podria ser un camp d'investigació futur per a nous treballs que unificarien la dislipèmia i l'epigenòmica.

8. CONCLUSIONS

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CONCLUSIÓ GENERAL

Diversos factors epigenètics i del metabolisme lipoproteic estan relacionats amb la fisiopatologia de la DAA a la VAB.

CONCLUSIONS ESPECÍFIQUES

1-miR-122, miR-130a, miR-486 estan relacionats amb el morfotip de VAB mentre miR-718 s'associa a la DAA (article 1).

2-Existeix un patró de miRNA associat a EMP que està relacionat amb el dany endotelial a la VAB en un clúster de gens situats al locus 14q32 del cromosoma 14 (article 2).

3-Les lipoproteïnes riques en triglicèrids s'associen a un major creixement del diàmetre de l'aorta ascendent en la VAB (article 3).

9. BIBLIOGRAFIA

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10. LLISTAT D'ABREVIATURES

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ARNm:	ARN missatgers
Cardio-RM:	Cardio-ressonància
DAA:	Dilatació de l'aorta ascendent
EMP:	Micropartícules endotelials circulants
ETE:	Ecocardiograma transesofàgic
ETT:	Ecocardiograma transtoràcic
GlycA:	Glicoproteïna A
HDL:	*High density lipoprotein* o lipoproteïnes d'alta densitat
IDL:	*Intermediate low-density lipoprotein* o lipoproteïnes de densitat intermitja
LDL:	*Low density lipoprotein* o lipoproteïnes de baixa densitat
L-R:	Fusió esquerra i dreta
miRNA o miR:	MicroRNA
MMP:	Metaloproteïnasses reguladores de la matriu
N-L:	Fusió esquerra i no coronari
1H-NMR:	Ressonància magnètica nuclear bidimensional
RMN:	Ressonància magnètica nuclear
R-N:	Fusió dreta i no coronària
TC:	Tomografia axial computaritzada
TGF-β:	Factor de creixement transformador beta
TIMP:	Inhibidors tissulars específics de les MMP
VAB:	Vàlvula aòrtica bicúspide
VAT:	Vàlvula aòrtica tricúspide
VLDL:	*Very low density lipoprotein* o lipoproteïnes de molt baixa densitat
VEGFA:	Factor de creixement endotelial vascular A
WSS:	*Wall shear stress* o forces de cisallament

DILATACIÓ AÒRTICA A LA VALVULOPATIA AÒRTICA BICÚSPIDE: APROFUNDINT EN LA FISIOPATOLOGIA A LA CERCA DE BIOMARCADORS

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