INTEGRACIÓN MULTIÓMICA EN EL ESTUDIO DE LAS COMPLICACIONES ASOCIADAS A LA VALVULOPATÍA AÓRTICA BICÚSPIDE

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Integración multiómica en el estudio de las complicaciones asociadas a la valvulopatía aórtica bicúspide

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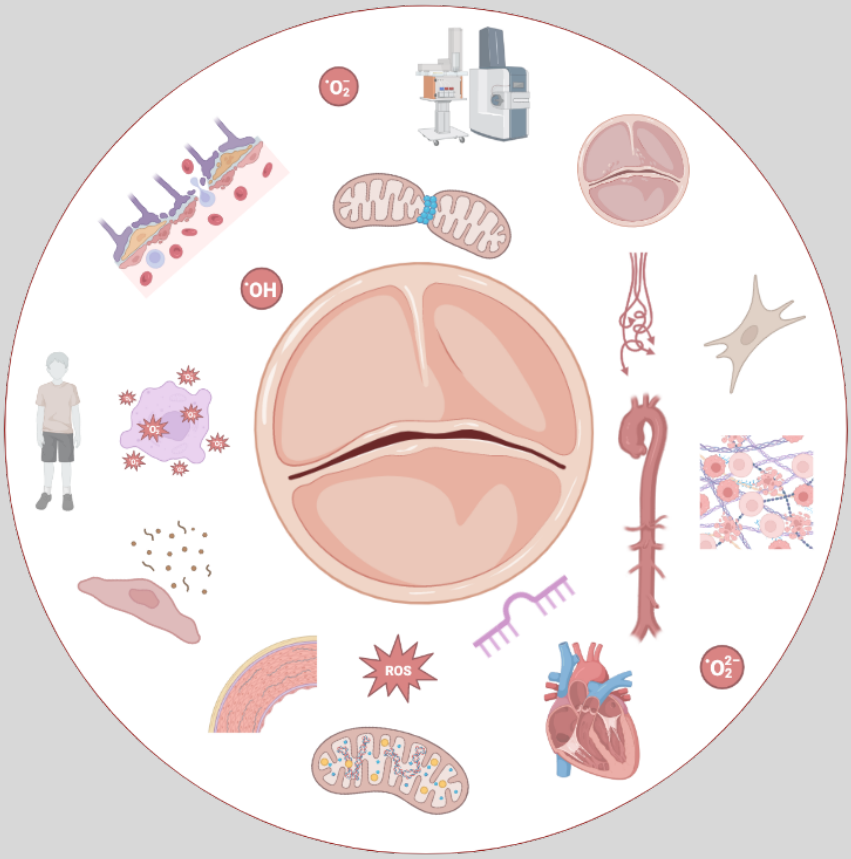

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FAIG CONSTAR que aquest treball, titulat "Integración multiómica en el estudio de las complicaciones asociadas a la valvulopatía aórtica bicúspide", que presenta Borja Antequera González per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Medicina i Cirurgia d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "Integración multiómica en el estudio de las complicaciones asociadas a la valvulopatía aórtica bicúspide", que presenta Borja Antequera González para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Medicina y Cirugía de esta universidad.

I STATE that the present study, entitled "Integración multiómica en el estudio de las complicaciones asociadas a la valvulopatía aórtica bicúspide", presented by Borja Antequera González. for the award of the degree of Doctor, has been carried out under my supervision at the Department of Medicine and Surgery of this university.

En Reus, a 26 de Mayo de 2023

El/s director/s de la tesi doctoral
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Neus Martínez Micaelo

Agradecimientos:

Hace casi 4 años en Cádiz, trabajando en mi antiguo doctorado mientras intentaba conseguir alguna beca predoctoral, de repente recibí un mail indicando me habían aceptado para trabajar en la Universitat Rovira i Virgili en… ¿Reus?. No conocía la ciudad, ni a nadie, pero me gustaba el tema y decidí aceptar esa oportunidad única para empezar de nuevo. Comenzó bien, la verdad. Me gustaba la ciudad, mis compañeros de trabajo y, en general, mi vida allí. Todo iba viento en popa. Incluso estábamos a punto de empezar todos los experimentos cuando de repente: la pandemia comenzó. ¿Quién imaginaba lo que cambiarían nuestras vidas durante años o incluso para siempre en muchos aspectos? Es bien sabido que hacer una tesis doctoral no es nada sencillo, pero tener que hacerla en mitad de una pandemia en un sitio desconocido sin gente a la que conozcas, lo hizo aún más duro. Tras muchos altibajos, puede que más bajos que altos, estoy hoy aquí, en 2023, entregando mi tesis doctoral. Borja, ¿creías nunca que llegaría este momento? Pues ha llegado. Mentiría si dijera que no he pensado en rendirme, pero gracias a vosotros, no lo he hecho:

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Este es nuestro trabajo.

Índice

Abreviaturas

Abreviatura	Nombre completo
1H-NMR	Resonancia magnética nuclear 1H
4D-MRI	Imágenes de resonancia magnética en 4D
ACTA2	Alfa actina 2
ADMA	Dimetilarginina asimétrica
ApoA1	Apolipoproteína A1
CAVD	Calcificación de la válvula aórtica
CKm	Creatin-quinasa mitocondrial
COX7C	Subunidad del citocromo c oxidasa 7c
CRP	Proteína C reactiva
DA	Dilatación aórtica
DRP1	Proteina relacionada con dinámica 1
E-slectina	Molécula de adhesión leucocito-célula endotelial
EA	Estenosis de la válvula aórtica
ECs	Células endoteliales
EMPs	Micropartículas endoteliales
endoMT	Transición endotelial-mesenquimal
eNOS	Óxido nítrico sintasa endotelial
ETT	Ecocardiografía transtorácica
FCGR2a	Receptor gamma Fc 2a
FIS1	Proteina de fisión 1
IGF-1	Factor de crecimiento insulina tipo 1
KLF2	Factor kruppel-like 2
LC/MS	Cromatografía líquida/espectrometría de masas
MEC	Matriz extracelular
MFF	Factor de fisión mitocondrial
MFN1	Mitofusina 1
MFN2	Mitofusina 2

miRNA	MicroARN
MMPs	Metaloproteasas de la matriz extracelular
MRI	Imagen por resonancia magnética
mRNA	ARN mensajero
MTND5P10	Pseudogen mitocondrial 10
N-cadherin	Cadherina neural
NF-kB	Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
NO	Óxido nítrico
NOX	NADPH oxidasas
Nrf2	Factor nuclear eritroide 2
OPA1	Atrofia óptica de tipo 1
RMN	Resonancia magnética nuclear
RNAseq	Secuenciación de ARN
RNU12	ARN pequeño nuclear 12
ROS	Especies reactivas de oxígeno
RT-qPCR	Reacción en cadena de la polimerasa con transcripción inversa
SCARNA12	ARN específico pequeño de cuerpo Cajal 12
TAVR	Reemplazo de válvula aórtica transcatéter
TGFß1	Factor de crecimiento transformante ß1
VA	Válvula aórtica
VAB	Válvula aórtica bicúspide
VAT	Válvula aórtica tricúspide
VCAM-1	Molécula de adhesión de células vasculares 1
VE-cadherin	Cadherina vascular-endotelial
vECs	Células valvulares endoteliales
VECs	Células vasculares endoteliales
VEGFR2	Receptor 2 del factor vascular endotelial de crecimiento
vICs	Células valvulares intersticiales
VSMCs	Células vasculares de la musculatura lisa
α-SMA	Actina de músculo liso alfa

Introducción

Introducción

1. Valvulopatía aórtica bicúspide

1.1 Anatomía y función de la válvula aórtica

La válvula aórtica (VA) es la válvula cardíaca que conecta el ventrículo izquierdo con la aorta ascendente[1]. La VA se encuentra en la raíz aórtica y su principal función es permitir el flujo de sangre durante la apertura de la válvula (sístole), y evitar la regurgitación de la sangre al ventrículo izquierdo al cerrarse (diástole)[2].

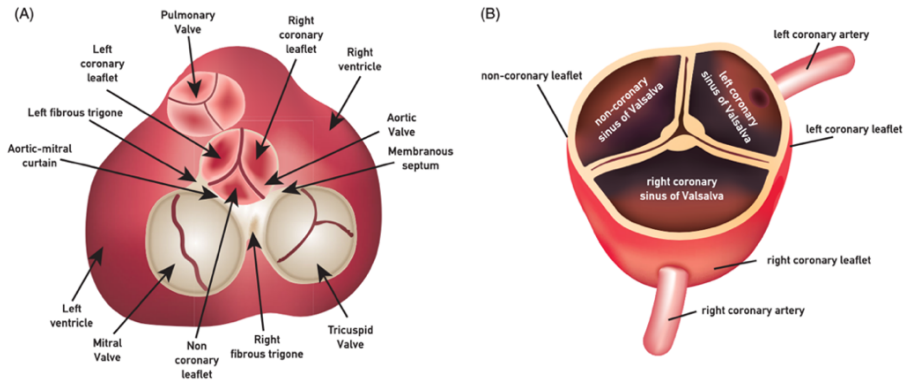

Figura 1. Visión general de la VA. Visualización de las distintas partes que conforman la VA, así como su localización en el corazón. Fuente: Katsi et. al, 2021[3].

La VA es una válvula semilunar compuesta por 3 cúspides o valvas, 3 senos de Valsalva y 3 triángulos fibrosos intervalvas (figura 1)[4]. Al no estar sustentada por un anillo fibroso, su dinámica de apertura y cierre se produce por una extensión de la propia pared aórtica en 3 bolsillos denominados senos de Valsalva, que permiten el espacio necesario para la apertura y cierre de las valvas durante el ciclo cardíaco[5]. En la mayoría de los casos, en dos de estos senos se encuentra el orificio de las arterias coronarias derecha e izquierda, que le dan nombre tanto a los senos de Valsalva como a las valvas que componen la VA. Las valvas son las estructuras más importantes de la VA, ya que modulan su correcta apertura y cierre[2]. Cada una tiene dos caras: una cóncava hacia la luz de la aorta y otra convexa hacia el ventrículo izquierdo. Las valvas se denominan según su posición y origen de las arterias coronarias en: derecha e izquierda (ambas coronarias) y valva no conoraria o posterior.

La VA está compuesta por 3 capas denominadas fibrosa, esponjosa y ventricular (figura 2)[1,3]. Además, una capa de células endoteliales valvulares (vECs) recubre cada extremo. La capa fibrosa se puede definir como una zona densa, rica en colágeno, además de las células valvulares intersticiales (vICs) y fibroblastos. La capa esponjosa es la capa central y está compuesta por tejido conectivo laxo rico en glucosaminoglicanos y proteoglicanos[6]. Finalmente, encontramos la capa ventricular, que es la zona más flexible, ya que se encuentra formada por abundante elastina, además de hallar células musculares de tejido liso. Por lo tanto, la VA está compuesta por 3 capas diferenciadas por su distinta composición celular y de la matriz extracelular (MEC)[6]. Cabe destacar que la interacción de los distintos tipos celulares, así como su MEC, proporcionan un papel clave en el correcto funcionamiento y durabilidad de la válvula. Las vICs se presentan por lo general con fenotipo de fibroblasto y suelen ser quiescentes[7]. Además, también se encuentran otros tipos celulares como células vasculares de músculo liso y miofibroblastos[8].

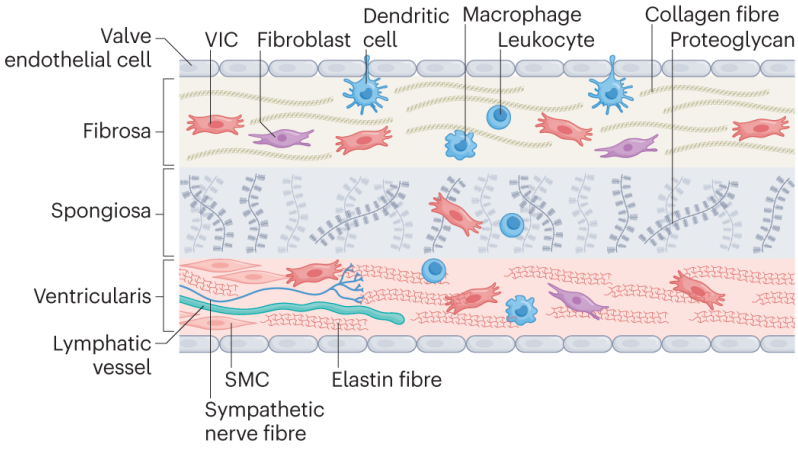

Figura 2. Histología de la VA. Representación gráfica de las 3 capas que conforman la VA junto con el endotelio. VIC: célula valvular intersticial; SMC: célula muscular lisa. Figura adaptada de Moncla et. al, 2023[9].

El conjunto de células que componen la VA y la MEC forman una perfecta red que juega en sintonía para mantener un correcto funcionamiento valvular[10,11]. Se conoce que diversos factores como alteraciones en el metabolismo lipoproteico, estrés oxidativo o cambios hemodinámicos son capaces de afectar al funcionamiento de las células que conforman la válvula, incluyendo las vECs[12]. La interacción entre vECs y vICs es esencial para mantener la estructura y función de la propia válvula, por lo que desajustes en su funcionamiento podrían tener un papel demoledor en la integridad de ésta[3].

1.2 ¿Qué es la válvula aórtica bicúspide?

La válvula aórtica bicúspide (VAB) se considera la malformación cardíaca congénita con más prevalencia, afectando a entre un 1 y 2% de la población general, siendo más frecuente en hombres[13,14]. La morfología típica de la VA se define por la composición de 3 valvas: derecha, izquierda y no coronaria. La fusión de dos de estas valvas durante la embriogénesis forma una VA con una morfología bivalva, dando lugar a la VAB. La fusión entre 2 valvas más frecuentes se produce entre las coronarias izquierda y derecha, aunque todos los tipos de fusiones han sido observados en mayor o menor proporción[15,16]. Esta unión de las valvas puede producir una comisura de unión (fusionada con rafe) o no mostrarse (pura) (figura 3). Aunque existen diversas clasificaciones de la morfología en la VAB, la de Sievers es la más clásica y distingue tres tipos: el tipo 0 (sin rafe, conocida como VAB pura), el tipo 1 (en la que dos de las valvas se fusionan formando un rafe) y la tipo 2 (dos fusiones formando una válvula unicúspide)[15]. Diversos estudios avalan que las diferentes morfología posibles en la VAB están asociadas con distintos grados de severidad de la propia valvulopatía, así como la predisposición a sus complicaciones asociadas como son la estenosis de la válvula aórtica (EA) o la dilatación de la aorta (DA) y, en general, a una degeneración más rápida de la propia válvula[17].

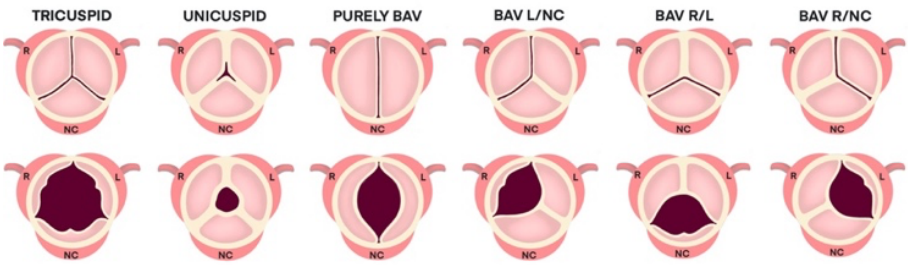

Figura 3. Clasificación de la morfología de la VA. Representación de los distintos tipos de morfologías observados en la VA. Figura adaptada de Dutta et. al, 2021[18].

1.3 Diagnóstico y complicaciones asociadas

El diagnóstico de la VAB se suele realizar mediante ecocardiografía transtorácica (ETT)[19]. En ella, su diagnóstico se establece cuando se visualizan en sístole únicamente dos valvas, con o sin rafe, en la vista de eje corto paraesternal de una ETT. Si no se puede

establecer el diagnóstico por dificultad de visualización de las valvas, técnicas más precisas como la imagen por resonancia magnética (MRI) o la tomografía axial computarizada (TAC), se pueden utilizan para confirmar esta valvulopatía.

La VAB confiere un riesgo de disfunción valvular y de DA[20,21]. La disfunción valvular puede manifestarse como una insuficiencia o EA. La insuficiencia aórtica es la disfunción valvular más prevalente en jóvenes, mientras que la predominancia de EA se incrementa con el envejecimiento. La DA es muy prevalente en la VAB y también progresa con el envejecimiento[20,21]. Su progresión incrementa el riesgo de disección aórtica, entidad con una elevada mortalidad. Los pacientes con VAB son sometidos a controles cardiológicos en los que se analiza el funcionamiento valvular así como la morfología de la aorta, entre otros parámetros, mediante ETT, TAC o MRI[19].

Cuando se identifican diámetros de la aorta ascendente que confieren un riesgo significativo de disección de la aorta o cuando la disfunción valvular es grave y con repercusión clínica, se procede al tratamiento quirúrgico. Actualmente no existe un tratamiento farmacológico que evite la progresión de las complicaciones asociadas como la EA y DA. Por ello, resulta necesario profundizar en el estudio sobre la VAB, así como sus complicaciones asociadas. Es necesario indagar sobre la fisiopatología de esta valvulopatía y descubrir nuevos biomarcadores fiables que nos permitan distinguir a los pacientes de distinto riesgo para poder optimizar así su tratamiento y seguimiento a largo plazo. Destacar también que la identificación de nuevos biomarcadores nos abriría las puertas hacia dianas terapéuticas novedosas que pudieran ayudar a ralentizar la degeneración valvular, así como reducir las aortopatías asociadas a la VAB.

1.4 Degeneración valvular asociada: la estenosis aórtica

La EA es una de las complicaciones más comunes en los individuos diagnosticados con VAB y se podría definir como el estrechamiento del orificio de la VA debido al incremento de rigidez y, a veces, calcificación de las valvas que la conforman[22]. En la válvula aórtica tricúspide (VAT), los factores de riesgo cardiovasculares tradicionales como fumar, el síndrome metabólico, hipertensión o alteraciones en el metabolismo lipoproteico, se conoce que afectan al desarrollo y progresión de la EA. Estas alteraciones, junto a otros factores como el estrés oxidativo, promueven la

activación de miofibroblastos, que secretan citoquinas proinflamatorias, metaloproteasas y factores osteogénicos, promoviendo a su vez la calcificación (figura 4)[23–26]. Todo ello provoca cambios tanto a nivel celular como extracelular, volviendo a la VA cada vez menos flexible y más rígida, perdiendo su funcionalidad y promoviendo el desarrollo de la EA[27]. El estado más avanzado y calcificado de la EA se conoce como la calcificación de la válvula aórtica (CAVD)[28]. En la VAB, se debate que los mecanismos pueden no ser idénticos y la morfología anormal de la válvula puede contribuir a una degeneración más rápida[29]. Los cambios hemodinámicos que se producen en la VAB, así como el gran estrés mecánico al que las células está sometidas debido a las alteraciones en su dinámica de apertura, parecen también influir en el desarrollo de la EA[30,31]. Estas células serían capaces de percibir este estrés mecánico y transformarlo en respuestas bioquímicas, alterando tanto el endotelio como su interacción con el resto de células de la VA y la MEC.[32–34]

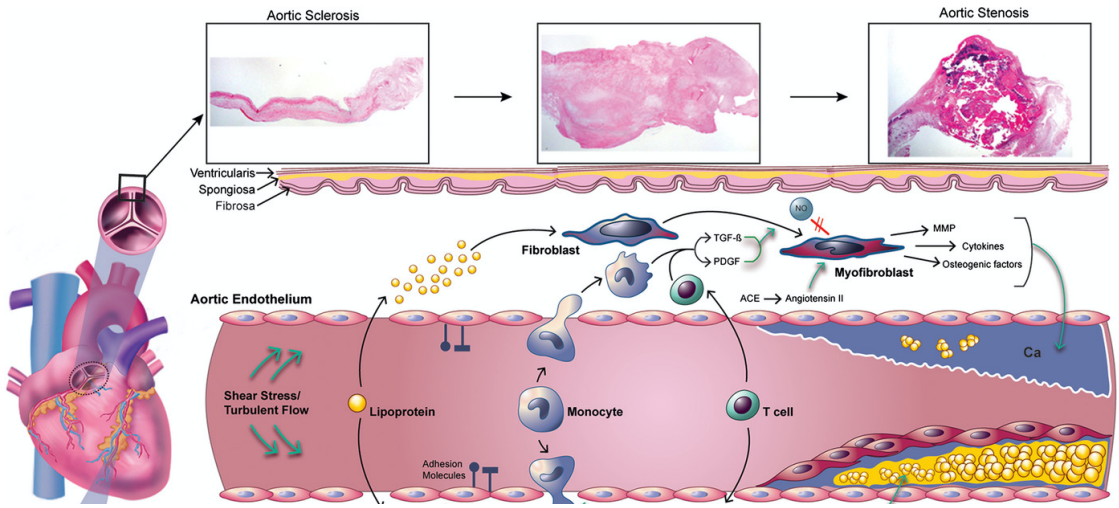

Figura 4. Fisiopatología de la EA. En esta representación gráfica se observan los diferentes factores de riesgo que influyen en el desarrollo de la EA, así como su progresión desde la esclerosis hasta una estenosis más severa. MMP: metaloproteasas de la matriz extracelular; PDGF: Factor de crecimiento derivado de plaquetas; TGF-ß: factor de crecimiento transformante ß. Adaptación de Milin et al, 2014[27].

El diagnóstico de la EA en la VAB suele establecerse mediante ecocardiografía, cuantificando el área valvular aórtica, la velocidad del flujo y el gradiente medio transaórtico[19]. En función de estos parámetros, la EA se clasifica como de grado leve, moderado o severo. Cuando la EA evoluciona hacia la significancia clínica, la única

opción viable hoy en día es proceder a reemplazarla mediante intervencionismo percutáneo o quirúrgico[35].

1.5 La dilatación aórtica y su asociación con la válvula aórtica bicúspide

1.5.1 Anatomía de la aorta torácica

La aorta es la arteria de mayor calibre y lleva la sangre oxigenada proveniente del ventrículo izquierdo, a través de la VA, hasta la zona abdominal. La aorta torácica es la parte de la aorta que se sitúa en el mediastino superior y se compone de 3 partes: la aorta ascendente, el arco aórtico y la aorta descendente[36]. La aorta ascendente es el primer segmento de la aorta y en ella distinguimos así mismo 3 partes: la raíz aórtica, la unión sinotubular y la porción tubular o comúnmente denominada, aorta ascendente[36].

La aorta es una arteria elástica que está formada fundamentalmente por 3 capas. Desde dentro hacia fuera se denominan íntima, media y adventicia, y cada una tiene aspectos diferenciales (figura 5)[37,38]. La capa íntima está formada por el propio endotelio y tejido conjuntivo subintimal, donde se encuentran las células vasculares musculares lisas (VSMCs), además de la MEC. El endotelio está formado por células endoteliales vasculares (VECs) que interaccionan con las VSMCs, así como la MEC, siendo esta red de interacciones vital para el correcto funcionamiento de la arteria. Además, entre la capa íntima y media encontramos una lámina elástica fenestrada. A continuación, nos encontramos con la capa media, formada mayoritariamente por VSMCs que secretan pro-elastina, glicoproteínas y proteoglicanos; así como fibroblastos y tejido conjuntivo, siendo ésta la más elástica. La capa media está formada por unidades músculo-esqueléticas que contienen elastina dispuestas en forma de hélice e interconectadas entre sí. En último lugar se encuentra la capa adventicia que es rica en colágeno, aportando rigidez, además de diversos tipos celulares. Esta capa se encuentra muy irrigada por vasos sanguíneos, vasos linfáticos y nervios.

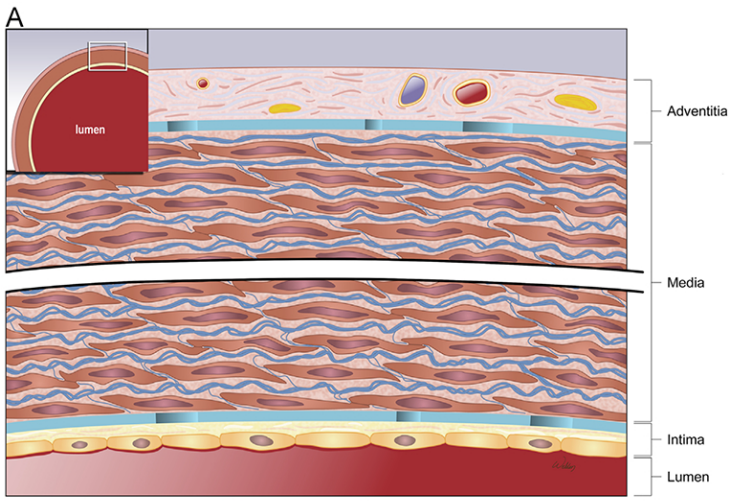

Figura 5. Estructura de la pared aórtica. En esta representación gráfica se observan las diferentes capas que conforman la pared aórtica, así como sus componentes celular y extracelulares. Adaptación de Shen et al., 2017[39].

1.5.2 Prevalencia y clasificación de la dilatación aórtica

Los pacientes con VAB presentan una elevada prevalencia de DA[40]. Ésta varía según las series, pero alrededor del 50% de los pacientes con VAB tienen una DA significativa. La DA en pacientes con VAB afecta fundamentalmente a la raíz aórtica y/o a la aorta ascendente[41], y puede progresar con el envejecimiento[42]. Aunque la DA asociada a la VAB suele ocurrir en adultos, diversos estudios han sugerido que su desarrollo parece comenzar a edades tempranas[40,43,44]. Por ello, el seguimiento de estos pacientes desde niños es transcendente para prevenir cualquier tipo de complicación inesperada asociada a la VAB.

El diagnóstico de la DA se basa en la medida del diámetro aórtico mediante ETT, TAC o MRI, tomándose el diámetro máximo al final de la diástole. Se han descrito diversos criterios de DA[19], algunos casos en medida absoluta y en otros ajustados por parámetros como la superficie corporal[36]. Se suele utilizar como criterio de DA un diámetro de la aorta ascendente ≥40 mm en medidas absolutas o ≥21 mm/m2 cuando se indexa por la superficie corporal[19]. La indexación es especialmente importante en pacientes pediátricos, donde se suelen utilizar fórmulas de z-score en los que se tiene en cuenta la edad y variables antropométricas como el peso y la talla[45].

La dilatación de un fragmento de la aorta por encima del 50% del diámetro medio se conoce como aneurisma y puede ocurrir en cualquier parte de sus segmentos: raíz aórtica, aorta ascendente, etc (figura 6)[46,47]. Cuando esta aneurisma continúa su desarrollo y no es tratada a tiempo, puede llegar a provocar una rotura de la capa interna de la aorta o disección aórtica, entidad con una muy elevada mortalidad. Por lo tanto, un correcto diagnóstico y seguimiento de estos pacientes es fundamental para prevenir este tipo de lesiones en la aorta y ser tratadas lo antes posible.

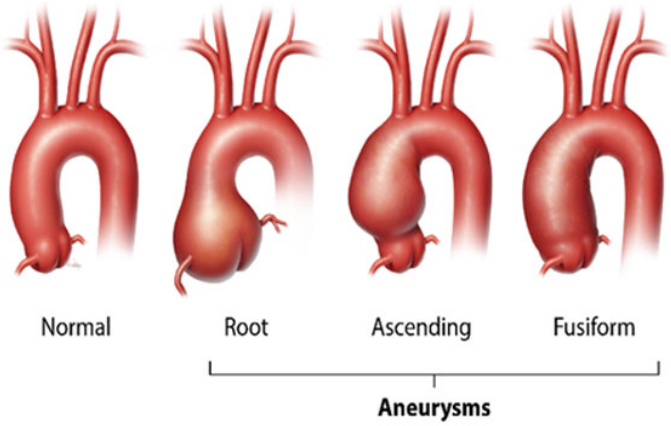

Figura 6. Dilatación y aneurisma. Representación de la progresión de DA a distintos tipos de aneurismas en la aorta torácica. Adaptación de Pinard et al., 2019[48].

1.5.3 Fisiopatología de la dilatación aórtica

En los últimos años se han descubierto numerosos mecanismos implicados en la fisiopatología de la DA en la VAB, y sin duda, sus componentes celulares y extracelulares juegan un papel muy importante (figura 7)[49]. En pacientes con VAT, los factores de riesgo de desarrollo de DA incluyen el envejecimiento, los procesos inflamatorios, cambios en el flujo hemodinámico, estrés oxidativo, etc. Las VSMCs y VECs, así como la MEC, juegan en equipo para mantener una arteria firme que pueda soportar el flujo de sangre pero a la vez elástica para permitir los procesos de vasodilatación[50]. En la VAB, parece que la fisiopatología de la DA puede diferir con respecto a lo que ocurre en VAT. Numerosas mutaciones en genes relacionados con la aortopatía se han identificado en la VAB como en el caso del receptor 1/2 de TGFß (TGFBR1/2), NOTCH1 o alfa actina 2 (ACTA2), destacando la posible predisposición genética en la VAB[51–53]. Además, se han observado características histopatológicas específicas de la DA en la VAB. Diversos estudios han visto alterada la expresión de varios tipos de MMPs y sus inhibidores en la

VAB, como MMP2 y MMP9 [23,54,55]. Cambios de expresión en las MMPs pueden provocar la degradación y desorganización de la MEC, afectando a la estructura y función de la aorta. Otras alteraciones en la MEC también han sido observadas, como por ejemplo la reducción del contenido de fibrilina 1, colágeno o elastina, en las arterias de pacientes con VAB en comparación con individuos con VAT.[55,56]. Por otro lado, perturbaciones en el fenotipo de las VSMCs también han sido relacionadas con el desarrollo de VAB y DA debido a desajustes en la vía de señalización NOTCH1, entre otras, clave en la diferenciación y funcionamiento de estas células[57]. Alteraciones en esta vía también han sido relacionadas con la transición endotelial-mesenquimal (endoMT) en la VAB, la cual parece verse perturbada en esta patología[58]. Esta balanza podría verse desequilibrada a favor de la transición a un fenotipo mesenquimal, deteriorando por lo tanto la función principal del endotelio como barrera semipermeable[58–60]. En numerosos estudios, el progreso de la DA se ha visto afectado notablemente por el incorrecto funcionamiento del endotelio en la VAB[53,61]. Finalmente, cambios epigenéticos en la aortopatía asociada a la VAB también han sido identificados al compararla con la DA que se desarrolla en VAT[62,63].

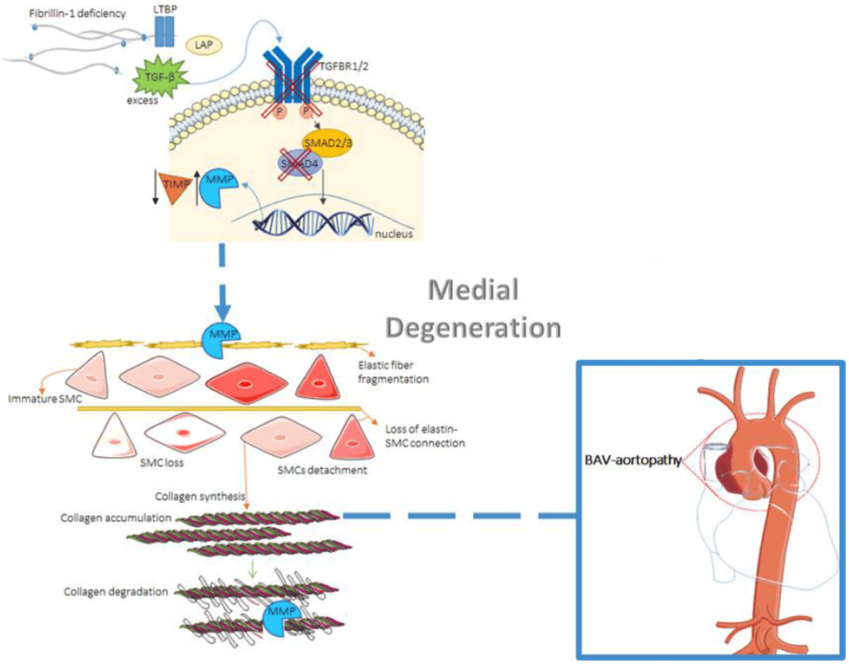

Figura 7. Fisiopatología de la DA y su progreso hacia una aneurisma en la VAB. Degradación de la pared aórtica debido, entre otros factores, a cambios en TGFß, MMPs, elastina, fenotipo de las VSMCs, etc. LAP: péptido asociado a latencia; MMP: metaloproteasas de la matriz extracelular; SMC: células musculares lisas; TIMP: inhibidor de las metaloproteasas. Adaptación de Sophocleous et al., 2018[64]

1.5.4 Alteraciones hemodinámicas y genéticas

Existe controversia sobre si las causas de la DA son genéticas o hemodinámicas, o lo que parece más plausible, la conjugación de ambas. Algunos datos clínicos apoyan la predisposición genética, como puede ser la presencia de DA en pacientes con VAB sin disfunción valvular o en pacientes con anomalías sindrómicas ligadas a mutaciones[65]. Como se ha comentado anteriormente, el descubrimiento de numerosas mutaciones asociadas a la VAB y la DA apoyan la teoría de la predisposición genética. Mutaciones en diversos componentes de la vía de señalización del factor de crecimiento transformante ß1 (TGFß1) o NOTCH-1 han sido halladas en individuos con VAB y DA[53,66]. Por otro lado, variantes en ROBO4 y SMAD6 también han sido relacionadas con el desarrollo y formación de aneurismas en la VAB[67,68]. Otros candidatos que han sido asociados con la aortopatía presente en la VAB, además de los mencionados anteriormente, son ACTA2, ADAMTSL1, NOS3, GATA5, etc[49,51,69]. Sin embargo, las pacientes con mutaciones identificadas asociadas a la aortopatía son una minoría y, por lo tanto, resulta complicado atribuir sus complicaciones solo a una predisposición genética.

La otra teoría que ha ido cogiendo gran impulso en los últimos años ha sido la hemodinámica gracias al desarrollo de nuevas técnicas de imagen como las imágenes de resonancia magnética en 4 dimensiones (4D-MRI)[30,70–72]. Esta técnica es capaz de medir con precisión la velocidad del flujo y su dirección, entre otros parámetros, permitiendo la observación de características diferenciales de la pared aórtica en función de las características del flujo, así como la zona de impacto[73]. La morfología anómala de la VA en la VAB determina cambios en flujo, promoviendo un flujo turbulento con incremento de la fuerza de cizalladura (figura 8)[74]. Es reconocido que los cambios hemodinámicos están asociados con disfunción endotelial y el desequilibrio de su homeostasis[32,60]. En la VAB, los cambios hemodinámicos han sido asociados a la DA en numerosas ocasiones[75]. Además, diferentes tipos de flujos hemodinámicos y zonas de impacto han sido a su vez relacionados con distintos tipos de aneurismas presentes en la VAB[76]. En definitiva, se hipotetiza que ambas podrían tener un papel clave en el desarrollo de complicaciones asociadas a la VAB, y que la importancia de cada una variaría entre pacientes.

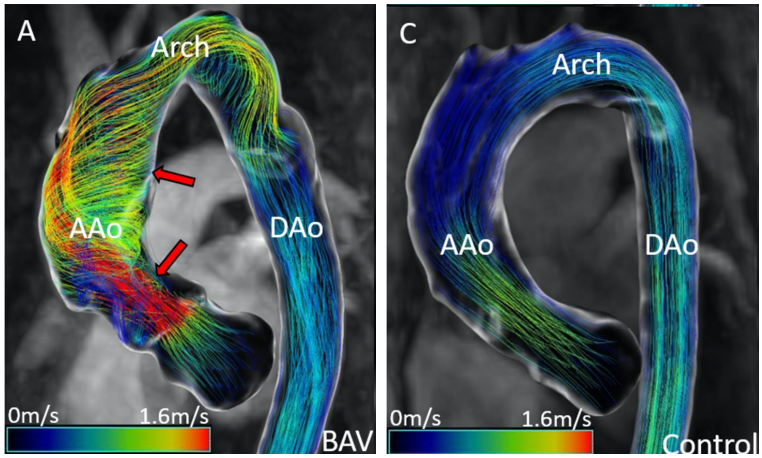

Figura 8. Visualización por 4D-MRI del flujo hemodinámico en la aorta torácica en distintas morfologías valvulares aórticas. Los cambios hemodinámicos producidos por la VAB influyen en el progreso de la DA, promoviendo un flujo turbulento, a diferencia de VAT control en la que el flujo es laminar. AAo: aorta ascendente; Arch: arco; DAo: aorta descendente; BAV: válvula aórtica bicúspide. Adaptación de Gordon et al., 2020[77].

1.6 Válvula aórtica bicúspide en pacientes pediátricos

Al tratarse de una enfermedad congénita, la VAB se encuentra presente desde su nacimiento. En la infancia la VAB suele diagnosticarse por el estudio de un soplo o clic de eyección aórtica. Los niños con VAB muestran diámetros aórticos mayores que los niños con VAT[40,43,44]. En la infancia y en la adolescencia ya se puede observar una mayor prevalencia de DA, aunque no suelen ser dilataciones severas[22]. Por otro lado, la EA también es un proceso que suele desarrollarse a edades más tardías, a excepción de la EA neonatal. Finalmente, los jóvenes con VAB presentan una mayor predisposición a la insuficiencia que en el caso de individuos de igual edad con VAT. Excepto en el caso de la EA neonatal grave o la VAB asociada a coartación aórtica, que pueden requerir intervención urgente en el lactante, durante la infancia no suelen observarse complicaciones graves, aunque la detección precoz de esta valvulopatía, así como su correcto seguimiento, es trascendental[78]. Por lo tanto, la correcta detección de la VAB en niños, así como su seguimiento periódico, es de gran importancia para evitar los riesgos asociados a esta valvulopatía[79]. Así mismo, es importante conocer sus características y pronóstico de cara a los consejos sobre la actividad deportiva que los pacientes puedan realizar, aspecto de gran importancia para muchos niños y jóvenes.

2. Endotelio, estrés oxidativo y mitocondrias como desencadenantes en las enfermedades cardiovasculares

2.1 Endotelio

2.1.1 Definición y funciones

Las ECs que componen el endotelio tapizan todo el sistema vascular en monocapa, desde arterias hasta pequeños capilares. Aunque el endotelio era considerado hasta hace no demasiados años un tejido básico con funciones rudimentarias como la permeabilidad al agua, hoy en día se sabe que este juega un papel fundamental en numerosos procesos, siendo fundamental en la homeostasis vascular (figura 9)[57,80–83]. Además de ser una barrera semipermeable entre el interior del tejido vascular y la sangre, regulando nutrientes, el endotelio participa en el mantenimiento del tono vascular, interaccionando con las células de alrededor e influyendo en su fenotipo y diferenciación[84,85]. El endotelio también se encarga de modular la permeabilidad a macrófagos, el balance redox, la inflamación, el metabolismo, etc. Sin embargo, su correcto funcionamiento puede verse alterados por diversos factores.

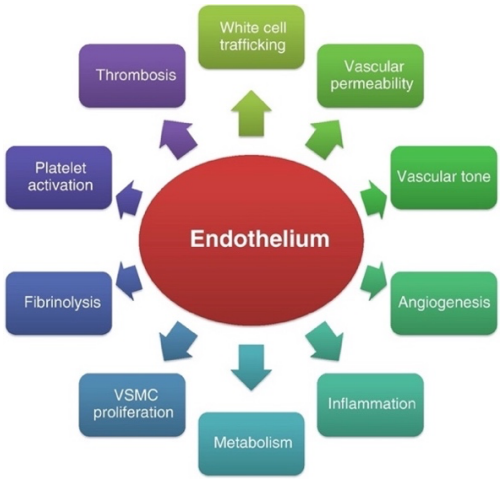

Figura 9. Importancia del endotelio. Esquema con alguna de las funciones más importantes del endotelio. Fuente: Sena et al., 2013[85].

2.1.2 Disfunción endotelial

La disfunción endotelial se puede definir como el incorrecto funcionamiento de las ECs que forman el endotelio[86]. Esta alteración es multifactorial y diversos procesos

pueden participar en ella: hipertensión, envejecimiento, inflamación, hipercolesterolemia, cambios hemodinámicos, estrés oxidativo, etc[87]. Estos procesos provocan una serie de alteraciones fenotípicas en las ECs, que imposibilita su correcto funcionamiento. Una de las alteraciones más frecuentes que lleva a la disfunción endotelial es la insuficiente producción de óxido nítrico (NO)[88,89]. El tono vascular se mantiene gracias a los vasodilatadores y vasoconstrictores, siendo el NO secretado por las ECs uno de los vasodilatadores más importantes. Además, éste también tiene propiedades antioxidantes y antiinflamatorias. El NO producido por las ECs se difunde hacia el tejido vascular muscular liso provocando su relajación (figura 10)[89]. El NO se produce en estas células gracias a la óxido nítrico sintasa endotelial (eNOS), siendo esta enzima la mayor productora de NO en la red vascular. La alterada producción y déficit de NO conlleva un estado proliferativo, proinflamatorio y trombótico de las ECs[90]. Una de las vías de reducción de la biodisponibilidad de NO es su interacción con las especies reactivas de oxígeno (ROS), produciendo ONOO-. Esto provoca alteraciones en la célula entre las que se incluye la nitración de proteínas y la modulación de la función mitocondrial, provocando en última instancia su apoptosis[91].

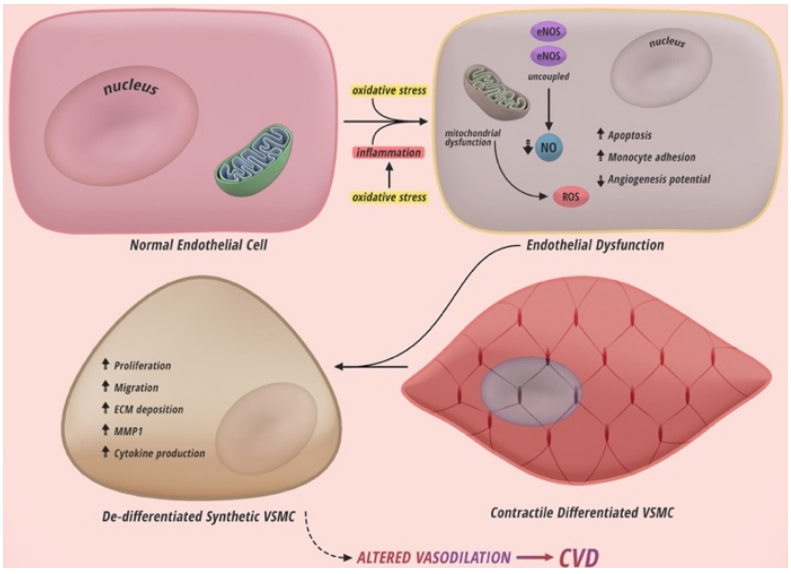

Figura 10. Disfunción endotelial e interacción con las VSMCs. El estrés oxidativo, junto con la inflamación, son capaces de promover el daño endotelial por la disminución de producción de NO e incremento de ROS por las mitocondrias. Esto provoca cambios en estas células, así como en el fenotipo de las VSMCs y la MEC. CVD: enfermedad cardiovascular; ECM: matriz extracelular; eNOS: óxido nítrico sintasa endotelial; MMP1: metraloproteasa de la matriz extracelular 1; NO: óxido nítrico; ROS: especies reactivas de oxígeno; VSMC: célula muscular lisa vascular. Fuente: Shaito et al., 2022[92].

2.1.3 Efecto del estrés mecánico en el endotelio

Las alteraciones del flujo hemodinámico provocan en las ECs un complejo y continuo estrés mecánico, promoviendo cambios en su fenotipo por mecanotransducción[60,93]. La mecanotransducción endotelial es un proceso de conversión de los cambios mecánicos en respuestas bioquímicas. Las ECs contienen numerosas moléculas capaces de responder a cambios de estrés mecánico al que está sometidas. Entre ellos se puede incluir el receptor 2 del factor vascular endotelial de crecimiento (VEGFR2), proteínas de unión (ocludinas, cadherina vascular-endotelial (VE-Cadherin)), glucocálix, integrinas, etc. Además del mecanosensor, también es necesario un ejecutador de la respuesta bioquímica, denominado mecanoadaptador[93,94].

Los cambios hemodinámicos pueden modificar la producción de las ROS y el balance redox en las ECs, en gran parte gracias a las distintas isoformas de las NAPDH oxidasas (NOX)[95]. En condiciones normales, las ROS se producen en las ECs en equilibrio gracias a la acción de antioxidantes como el factor nuclear eritroide 2 (Nrf2) y el factor *kruppel-like 2* (KLF2). En condiciones fisiológicas, el incremento de ROS suele ir acompañado con un incremento de antioxidantes para mantener el equilibrio. Sin embargo, el estrés mecánico al que puede estar sometido de forma crónica el endotelio puede inducir el incremento del estrés oxidativo, inflamación y promover la disfunción endotelial[96]. Finalmente, un alto estrés oxidativo continuado puede llegar a promover la autofagia.

La inflamación, que tiene un papel fundamental en el progreso de la aterosclerosis o la disfunción endotelial, también está relacionada con el estrés cortante al que las ECs están sometidas. El flujo laminar reduce la expresión de moléculas de adhesión como la molécula de adhesión de células vasculares 1 (VCAM-1) y la molécula de adhesión leucocito-célula endotelial (E-selectina), limitando la unión de leucocitos[97–99]. Adicionalmente, la respuesta de las ECs a citoquinas inflamatorias se ha visto que está condicionada por el flujo hemodinámico al que están expuestas[99,100]. En las ECs, una de los mecanismos proinflamatorios de mayor importancia, capaz de responder a los cambios mecánicos, es el factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-kB)[101].

Además de los cambios que ocurren en las ECs, éstas interaccionan con al resto de células, como las VSMCs, en función del flujo o estrés mecánico al que estén sometidas[102]. Flujos alterados, no laminares, inducen la secreción de moléculas como TGFß1 o el factor de crecimiento insulina tipo 1 (IGF-1), que inducen la proliferación de las VSMCs, así como el remodelado vascular. En cambio, el NO que se produce en flujos no alterados, laminares, inhibe el remodelado de la MEC, reduce la expresión de MMPs y promueve un fenotipo contráctil de las VSMCs (figura 11)[102]. Finalmente, también se ha observado que las ECs liberan diferentes microRNAs (miRNAs) en función del flujo hemodinámico y estrés mecánico al que están sometidas[103,104].

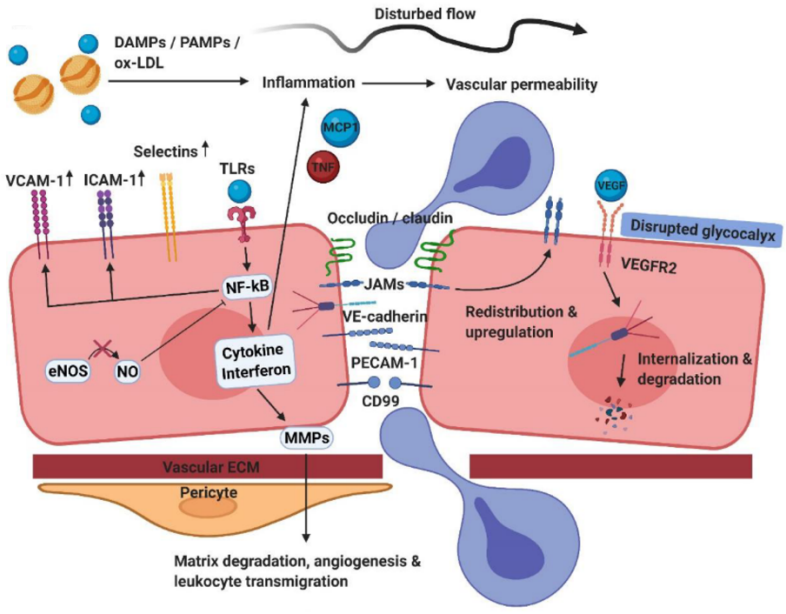

Figura 11. El endotelio bajo condiciones patológicas. Las alteraciones en el flujo promueven la disfunción endotelial al reducir la producción de NO, activar la vía NF-kß y promover la remodelación de la MEC. DAMPs: patrones moleculares asociados a daño; ECM: matriz extracelular; eNOS: óxido nítrico sintasa; ICAM-1: molécula de adhesión intercelular 1; JAMs: moléculas de adhesión tipo *junction*; MCP1: Proteína quimiotáctica de monocitos 1; Nf-kB: factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas; NO: óxido nítrico; oxLDL: lipoproteina de baja densidad oxidada; PAMPs: patrones moleculares asociados a patógenos; PECAM-1: molécula de adhesión de células endoteliales-plaquetas; TLR: receptor tipo toll; VCAM-1: proteína de adhesión de células vasculares 1; VE-cadherin: cadherina vascular-endotelial; VEGF: factor de crecimiento vascular endotelial; VEGFR2: receptor 2 del factor de crecimiento vascular endotelial. Adaptación de Sluiter et al., 2021[105].

2.1.4 Evidencias de la alteración de la función endotelial en la valvula aórtica bicúspide

Aunque numerosos estudios avalan la presencia de disfunción endotelial en la VAB, su papel es aún discutido[106]. Se han encontrado expresiones diferenciales en los niveles de eNOS en tejido valvular aórtico en función de la morfología valvular, e incluso esta expresión era variable según la zona de impacto del flujo hemodinámico. La vía de señalización NOTCH es una de las vías que parece estar afectada en la VAB. El NO procedente de las ECs juega un papel fundamental en esta vía de señalización al incrementar la expresión de los genes diana en las VICs, promoviendo su correcto funcionamiento y protegiéndolas de la calcificación[107]. Otros marcadores típicos de disfunción endotelial como la dimetilarginina asimétrica (ADMA) o biomarcadores más novedosos como micropartículas endoteliales (EMPs) han sido correlacionados con la VAB y la DA asociada a ella[108,109]. Además, a nivel celular, también se ha comprobado que las ECs que circulan en plasma como células endoteliales progenitoras o células endoteliales formadoras de colonias, tienen una menor capacidad migratoria *in vitro*, así como de formación de colonias[110,111]. Esto podría traducirse *in vivo* a un peor funcionamiento del endotelio y su función de barrera semipermeable. Finalmente, los estudios más recientes destacan desajustes en el proceso de endoMT[58]. Este proceso es esencial en el desarrollo del corazón y la curación de heridas, entre otros. Durante la endoMT, las ECs pierden sus propiedades de adhesión y modifican su citoesqueleto para adquirir un fenotipo mesenquimal[112,113]. Los marcadores endoteliales son sustituidos por marcadores de tipo mesenquimal como cadherina neural (N-cadherin) o actina de músculo liso alfa (α-SMA). Este proceso está controlado por distintas vías de señalización como la de TGFß, NOTCH o WNT-ß-catenina [114]. Además, se ha descubierto que la regulación de este proceso también puede verse afectada por las alteraciones hemodinámicas[115]. Por último, diversas investigaciones que habían puesto sus esfuerzos en estudios epigenéticos han sido capaces de relacionar patrones de miRNAs con disfunción endotelial en la VAB y sus complicaciones asociadas, como la DA y la EA[62,63,116].

2.2 Mitocondrias

2.2.1 Estructura y metabolismo

Las mitocondrias son unos de los organelos más cruciales en las células, pues en ellas tienen lugar procesos tan importantes como la producción de ATP[117]. Además, las mitocondrias son las principales fuentes de las ROS, influyendo sobre el estrés oxidativo, inflamación, homeostasis del calcio y pudiendo incluso regular la muerte celular[118,119]. Estos organelos están rodeados por dos membranas, una interna y otra externa, quedando separadas por un espacio intermembranoso[120]. La membrana interna forma numerosos pliegues hacia el interior (matriz) de la mitocondria, siendo estas dos zonas las más activas e importantes. La matriz contiene tanto las enzimas responsables del metabolismo oxidativo como el ADN mitocondrial. El piruvato procedente de la glicólisis que proviene del citosol se transporta a la mitocondria para completar su oxidación para conseguir el producto final deseado, el ATP (figura 12)[121]. El piruvato se oxida a acetil CoA, entrando en el ciclo de Krebs; de forma similar a lo que ocurre con la oxidación de los ácidos grasos. Este conjunto de procesos provoca grandes cantidades de NADH y FADH2 reducidos que servirán para producir ATP durante la fosforilación oxidativa que ocurre en la membrana interna en la cadena de transporte de electrones[122].

La localización de las mitocondrias también varía entre tipos celulares. Por ejemplo, en las VECs se encuentran ancladas al citoesqueleto para responder a los estímulos del estrés cortante del flujo de sangre, mientras que en las VSMCs se encuentran inmóviles cerca del retículo sarcoplasmático para la regulación de la homeostasis del calcio[123,124].

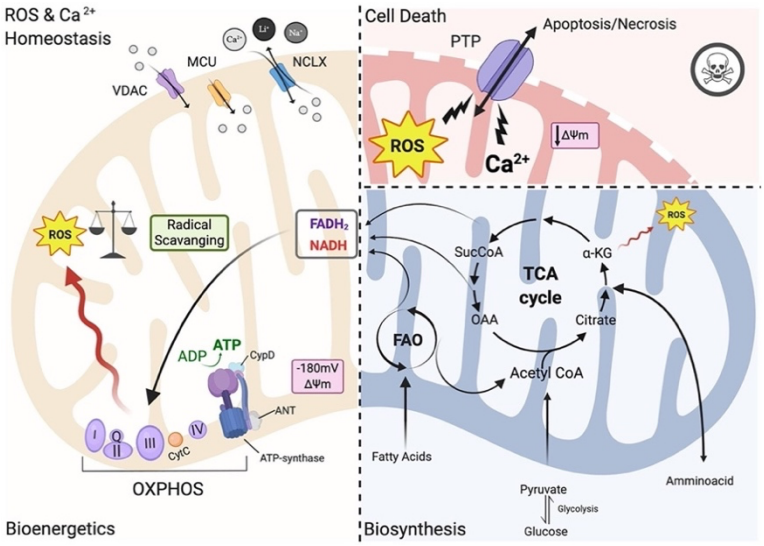

Figura 12. Funciones de la mitocondria y ROS. En la parte de abajo, a la derecha, observamos su función de biosíntesis en la que el piruvato procedente de la glicólisis entra en la mitocondria, se transforma en Acetil CoA e inicia el ciclo de Krebs. A la izquierda se puede observar la síntesis de ATP durante la forforilación oxidativa, así como la homeostasis del calcio y regulación de las ROS. Finalmente, a la derecha superior observamos el proceso de apoptosis celular controlado por la mitocondria. Acetil CoA: acetil coenzima A; ANT: ADP/ATP translocasa; ATP: adenosín trifosfato; CypD: ciclofilina D; cytC: citocromo C; FAO: oxidación de ácidos grasos; MCU: complejo uniportador de calcio mitocondrial; NCLX: intercambiador de Na+/Ca2+ mitocondrial; OAA: oxalacetato; OXPHOS: fosforilación oxidativa; PTPC: complejo de poro de transición de permeabilidad; ROS: especies reactivas de oxígeno; TCA: ciclo de Krebs; VDAC: proteínas de canal selectivas de aniones dependientes de voltaje; α-KG: alpha cetoglutarato deshidrogenasa; Δψm: potencial de la membrana mitocondrial. Adaptación de Ramaccini et al., 2018[125].

2.2.2 Dinámica de mitocondrias

El número de mitocondrias y su tamaño no es estático, sino que en condiciones normales se encuentran en un equilibrio de fusión y fisión denominado dinámica de mitocondrias (figura 13). Estos procesos están controlados por las proteínas de fusión y fisión. La fusión está controlada en mamíferos por las mitofusinas 1 y 2 (MFN1 y MFN2) y la proteína atrofia óptica de tipo 1 (OPA1)[126]. Tanto MFN1 y MFN2 son GTPasas que se encuentran en la membrana externa, mientras que OPA es una proteína transmembrana que se encuentra en la membrana interna. Por otro lado, la fisión se lleva a cabo por otras GTPasas conocidas como la proteína relacionada con dinámica 1 (DRP1), la proteína de fisión 1 (FIS1), el factor de fisión mitocondrial (MFF) y las proteínas de dinámica mitocondrial de 49 y 51 kDa[127].

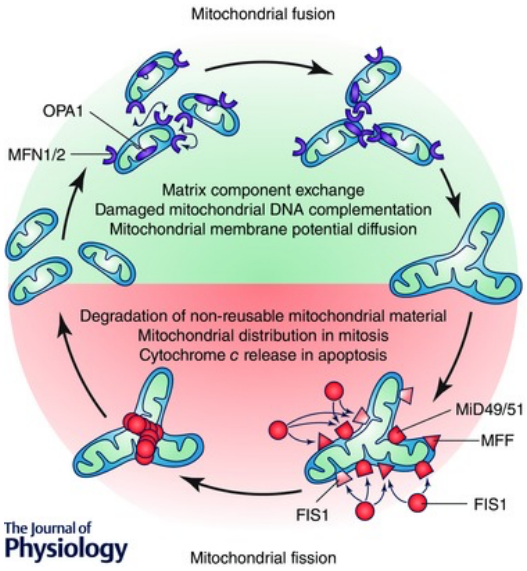

Figura 13. Dinámica de las mitocondrias: fusión y fisión. FIS1: proteína de fisión mitocondrial 1; MiD49/51: proteínas dinámicas mitocondriales de 49 y 51 kDa ; MFF: factor de fisión mitocondrial ; MFN1/2: mutofusina 1/2; OPA1: proteína de atrofia óptica 1. Adaptación de Vásquez-Trincado et al., 2016[117].

El desajuste de este equilibrio puede llevar a una fusión excesiva, generando mitocondrias de gran tamaño, megamitocondrias, que suelen ser disfuncionales y eliminadas por mitofagia por la propia célula[128]. Por el contrario, una fisión excesiva conduce a la fragmentación de mitocondrias. La fragmentación se produce gracias a DRP1, que forma un complejo proapoptótico y promueve la apertura del poro de transición de permeabilidad en la mitocondria. Esto provoca a su vez la salida del citocromo c, fragmentación de la propia mitocondria e inducción de la apoptosis celular[129]. Se ha comprobado que existen alteraciones en el equilibrio de dinámica de las mitocondrias que provocan un incorrecto funcionamiento de estos organelos en diversas patologías[117,130].

2.2.3 Disfunción mitocondrial vascular: biomarcador y diana terapéutica

La disfunción mitocondrial puede estar causada por diversos factores, pero de forma general se podría definir como el incorrecto funcionamiento de las mitocondrias. Al tener funciones tan importantes como ser consideradas las principales productoras de ATP, regulación de la homeostasis del calcio y generación de las ROS, su disfunción influye sobre numerosos procesos y vías de señalización[119]. El incremento de producción

de ROS (H2O2, O2-, ONOO-, radicales hidroxilo, etc) que tiene lugar, en gran parte, en las mitocondrias de las ECs y/o la disminución de la capacidad antioxidante puede conllevar a la disfunción endotelial al reducirse la biodisponibilidad de NO[131–133]. Esto puede ocurrir por la reacción de O2- con NO para producir ONOO-, lo cual incrementa aún más el estrés oxidativo (figura 14)[134,135]. Adicionalmente, las ROS como O2- también son capaces de oxidar el BH4, que es un cofactor esencial para eNOS, lo cual lo reduce a BH2, provocando el desacoplamiento de eNOS, que produce menos NO y más O2-[136]. La producción excesiva de ROS también promueve la inflamación, que contribuye a la disfunción endotelial y endurecimiento de las arterias[137–139]. Esto ocurre debido a la previamente indicada menor biodisponibilidad de NO, que afecta directamente a la interacción de las ECs con las VSMCs (figura 14). A su vez, esto influye sobre la función y fenotipo de éstas últimas, estimulando la degradación de elastina y deposición de colágeno en la MEC[140].

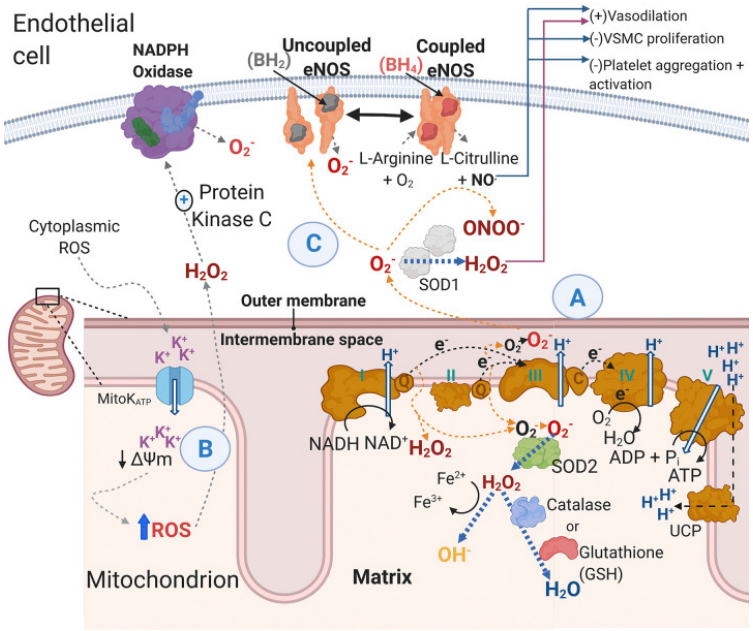

Figura 14. Consecuencias de la generación de ROS en ECs. La disfunción mitocondrial en ECs provoca un incremento de ROS y la alteración de la interacción con otras células como las VSMCs. H2O2: peróxido de hidrógen; MitoK+ATP: canales de potasio dependientes de ATP; ONOO−: peroxynitrato; NO: óxido nítrico; O−2, superóxido; ROS: especies reactivas de oxígeno; SOD: superóxido dismutasa; eNOS: óxido nítrico sintasa endotelial; VSMC: células musculares lisas vasculares; UCP: proteína de desacoplamiento-; Δψm: potencial de la membrana mitocondrial. Adaptación de Kirkman et al., 2021[141].

El incremento de las ROS fomenta la descompensación del potencial de membrana en las mitocondrias, que lleva consigo la activación de numerosas vías de señalización que promueven, a su vez, la expresión de genes como respuesta al estrés y finalmente apoptosis[142]. Estudios recientes han observado que cambios en el ambiente como un incremento oxidativo o alteraciones hemodinámicas a las que las células están sometidas, están asociados a desajustes en el metabolismo de las mitocondrias, así como a las dinámicas de fusión/fisión[143–145]. A su vez, estos desajustes de equilibrio en las dinámicas de las mitocondrias están relacionados con condiciones patológicas y senescencia celular[131]. En ECs, cuando el número de mitocondrias dañadas es demasiado alto, la mitofagia se detiene para dar paso a la apoptosis celular[146]. En los últimos años, el estudio de la disfunción mitocondrial se ha asociado y puesto en el punto de mira en numerosas patologías (tabla 1). Entre ellas destacan la enfermedad de Parkinson, diferentes tipos de cáncer y enfermedades musculares o cardiovasculares[147]. El estudio del incorrecto funcionamiento mitocondrial en estas patologías ha permitido su uso como biomarcador, además de ayudar a profundizar en el conocimiento de su desarrollo y fisiopatología.

Tabla 1. Patologías en las que se ha hallado disfunción mitocondrial. Fuente: Diaz-Vegas et al., 2020. [147]

Patología	Tipo celular	Disfunción mitocondrial	Cambios a nivel celular	Ref.
Obesidad	Adipocito	Fragmentación mitocondrial Sobreproducción de ROS	Incremento en proliferación y diferenciación	148,149
Resistencia a la insulina	Célula muscular esquelética	Fragmentación mitocondrial Desajustes en Ca2+ Sobreproducción de ROS Disrupción de la MAM	Desajustes en la señalización de la insulina, reducción de consumo de glucosa y translocación de GLUT4	150–155
	Adipocito	Sobreproducción de ROS	Deterioro del tráfico de GLUT4 y la captación de glucosa	153,154,156
Diabetes Mellitus tipo 2	Células ß	Desajuste en la respiración mitocondrial Síntesis de ATP dañada Mitofagia alterada Desequilibrio dinámica de mitocondrias	Reducción de la secreción de insulina	151,157–159
Enfermedades cardiovasculares	Célula muscular lisa	Daño en el ADN mitocondrial	Aumento de proliferación	160
	Cardiomiocito	Fragmentación mitocondrial Disrupción de la MAM Desajustes en Ca2+ Sobreproducción de ROS Bajo consumo de oxígeno	Hipertrofia, deterioro de la contracción, crisis energética, sobrecarga de calcio y muerte celular	155,161–164

ROS: especies reactivas de oxígeno; MAM: membranas asociadas a las mitocondrias; GLUT4: transportador de glucosa tipo 4.

En concreto, en el campo de las enfermedades cardiovasculares, la disfunción mitocondrial está siendo utilizada incluso como diana terapéutica y diferentes enfoques se encuentran hoy en día en estudio para intentar revertirla (tabla 2)[141,165,166]. El tratamiento con antioxidantes en los casos en donde existe un estrés oxidativo incrementado debido a esta disfunción mitocondrial ha conseguido una mejora en la función vascular en ciertos ensayos[167–171].

Tabla 2. La disfunción mitocondrial como diana terapéutica. Fuente: Wu et al., 2022. [172]

Estrategia	Agente	Función	Patología	Fase/modelo animal	Ref.
Reguladores y antioxidantes mitocondriales OXPHOS	CoQ10	OXPHOS y Antioxidante	Insuficiencia Cardíaca	Ensayo clínico fase IV	173
	MitoQ	OXPHOS y Antioxidante	Insuficiencia Cardíaca	Modelo de ratón de sobrecarga de presión	174
	Elamipretida	Antioxidante, interactúa con la cardiolipina	Insuficiencia Cardíaca	Modelo canino IC inducida por embolización	175
			Insuficiencia Cardíaca	Ensayo clínico fase II	176
			Infarto de Miocardio	Ensayo clínico fase II	177
	Resveratrol	Antioxidante	Insuficiencia Cardíaca	Ensayo clínico fase I	178
	Melatonina	Antioxidante	Insuficiencia Cardíaca	Modelo de rata de IC inducida por isoproterenol	179,180
			Infarto de Miocardio	Ensayo clínico fase II	181
Reguladores de control de calidad mitocondrial	CSA	Inhibidor de mPTP	Infarto de Miocardio	Ensayo clínico fase III	182

CoQ10: coenzima Q 10; CSA: ciclosporina A; IC: insuficiencia cardíaca; OXPHOS: fosforilación oxidativa.

2.3 Estrés oxidativo y antioxidantes

La producción excesiva de ROS provoca el conocido estrés oxidativo. Las ROS están constituidas por radicales de oxígeno libre (superóxidos, radicales hidroxilo…) y no radicales (H2O2, O3…)[183]. Estas moléculas aparecen de forma natural en el organismo siendo esenciales en diferentes funciones celulares como vías de señalización, metabolismo, defensa ante microorganismos, apoptosis, etc[184]. El problema comienza cuando se produce una desregulación de los niveles de las ROS y la balanza queda desequilibrada a favor de éstas. Las ROS provienen en su mayoría de las mitocondrias debido a la acción de las NOX[185]. Sin embargo, el organismo tiene sistemas antioxidantes, tanto enzimáticos como no enzimáticos, que previenen y controlan este estrés oxidativo, como la dismutasa superóxido y el tocoferol[186]. El tocoferol es una vitamina (vitamina E) liposoluble que actúa como antioxidante, además de ser un nutriente muy importante en ciertos tejidos (figura 15)[187].

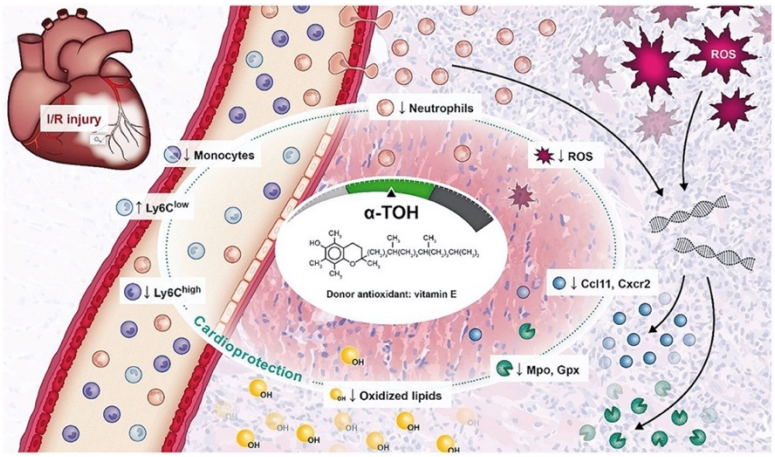

Figura 15. Papel del alfa-tocoferol como antioxidante. α-TOH: alfa tocoferol; Ccl11: eotaxina 1; Cxcr2: receptor de quimiocina CXC 2; Gpx: glutatión peroxidasa; Lys6c:antígeno 6 linfocito ; Mpo: mieloperoxidasa; ROS: especies reactivas de oxígeno; I/R: lesión por isquemia/reperfusión. Adaptación de Wallert et al., 2019. [188]

Alteraciones en este equilibrio provocan una descompensación de ROS vs. antioxidantes, promoviendo el estrés oxidativo. Este puede acarrear graves consecuencias, ya que las ROS son capaces de dañar proteínas, lípidos e incluso ADN, provocando en última instancia necrosis y apoptosis celular[189]. El incremento del estrés oxidativo, junto con la inflamación, es una de las principales causas de disfunción

endotelial, claves ambos en el desarrollo y fisiopatología de numerosas enfermedades, incluidas las cardiovasculares[90].

3. Ómicas y estudios no dirigidos en enfermedades cardiovasculares

3.1 Transcriptómica y metabolómica

El nacimiento de las ómicas en los últimos años ha sido de gran ayuda para poder analizar de forma simultánea multitud de parámetros que pueden estar alterados en una situación, desbancando en la mayoría de las ocasiones a las tradicionales técnicas dirigidas. Además, el conjunto de ellas, conocido como un estudio multiómico, nos permite tener una imagen más completa de lo que está ocurriendo en una patología y comprender los mecanismos de forma más profunda por los que ésta se desarrolla.

La transcriptómica es la ciencia que estudia los transcritos de RNA en un organismo. Además de los RNAs mensajeros (mARN) que acabarán formando las proteínas, estudia otro tipo de ARN que no codifican proteínas, llamados RNAs no codificantes. Aunque hasta hace unos años se pensaba que estos RNAs no codificantes no tenían ninguna función, este tipo de RNAs tienen un papel fundamental en la regulación de la traducción de los mARN[190]. Un ejemplo de ellos son los microRNA (miRNAs), ampliamente estudiados hoy en día en diversas patologías y utilizados como biomarcadores o dianas terapéuticas[191]. La transcriptómica, junto con el resto de ómicas, es una ciencia muy útil que nos puede ayudar a conocer los procesos de la fisiopatología de diversas enfermedades debido a la amplitud de observación de todo el transcriptoma. Para ello se realiza la secuenciación de RNA (RNAseq), en la que diferentes tipos muestras (plasma, tejido…) pueden ser analizadas para obtener la secuencia de millones de moléculas de RNA[192]. Posteriormente éstas son alineadas con un genoma de referencia y se cuantifican para finalmente analizar posibles diferencias de expresión. Este estudio no dirigido es muy útil cuando no se tiene una molécula de RNA o un grupo concreto y pequeño de interés. Sin embargo, si ya se conocen las moléculas a cuantificar, su expresión se suele analizar individualmente mediante la reacción en cadena de la polimerasa con transcripción inversa (RT-qPCR), ya que es una técnica dirigida mucho más económica y rápida[193].

De igual manera, la metabolómica es otra ómica que estudia el estado metabolómico de un individuo en un momento concreto. Esta ciencia cuantifica los metabolitos de un individuo y puede ayudar a comprender de forma más completa el desarrollo y fisiopatología de diferentes enfermedades. Al igual que ocurre con la transcriptómica, un estudio metabólico puede ser no dirigido, lo cual implica que no se interfiere en la selección de los metabolitos que se van a cuantificar, o dirigido, en el que se conocen y seleccionan las moléculas de interés[194,195]. Entre los métodos más utilizados en la metabolómica destaca la cromatografía líquida/espectrometría de masas (LC/MS) y la resonancia magnética nuclear 1H (1H-NMR), ayudándonos esta última a identificar diferentes perfiles metabólicos, como pueden ser el perfil lipídico o el de metabolitos de bajo peso molecular.

3.2 Epigenética: definición y microRNAs

La epigenética es la ciencia que estudia la heredabilidad y los cambios estables que se producen en los genes sin que existan alteraciones en la propia secuencia de ADN. Éstos comprenden desde modificaciones de las histonas y metilación del ADN, que son capaces de alterar el estado de empaquetamiento del cromosoma, hasta RNAs no codificantes[196]. Entre los RNA no codificantes más destacados y estudiados en la actualidad destacan los miRNAs, que son un tipo de RNAs no codificantes de tamaño pequeño. Los miRNAs son moléculas de RNA endógenas de cadena simple de 18 a 24 nucleótidos. La biogénesis de los miRNAs comprende un compendio de procesos hasta formar los miRNAs maduros que comienza en el núcleo, donde se transcribe el pri-miRNA con el cap 5’ y la cola de poly-A 3’, que es posteriormente eliminada por Drosha para generar el pre-miRNA y ser transportado fuera del núcleo por la Exportina 5 (figura 16). En el citoplasma, el complejo Dicer es el encargado de completar la maduración de estas moléculas hasta los miRNAs maduros. Estos miRNAs maduros pueden ser exportados mediante exosomas, microvesículas o directamente secretados fuera de la célula al unirse a proteínas de unión al RNA para ejercer su función reguladora.

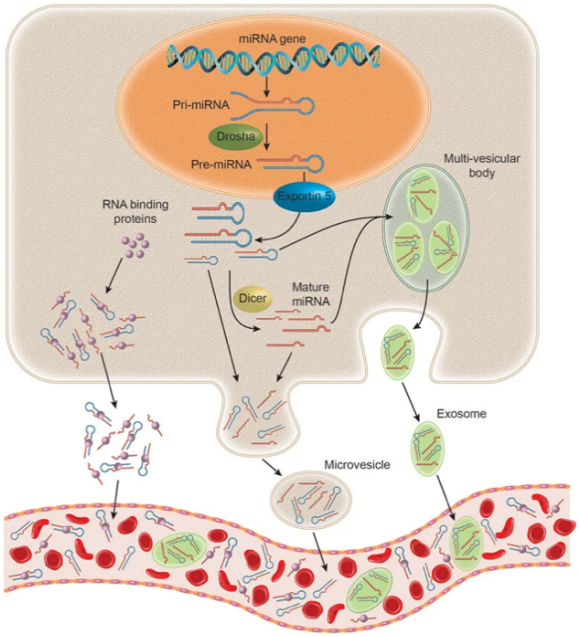

Figura 16. Biogénesis de los miRNAs. Proceso de formación de los miRNAs desde su transcripción hasta su maduración y posterior exportación. Adaptación de Barwari et al., 2016. [197]

3.2.1 microRNAs como biomarcadores y dianas terapéuticas

Los miRNAs, debido a su gran función reguladora, tienen una gran influencia en la mayoría de procesos biológicos y su expresión puede ver alterada en diversas patologías (figura 17). Los miRNAs actúan regulando de forma negativa la traducción de proteínas, uniéndose de forma complementaria a una zona del ARN mensajero (ARNm)[198]. Esto, sumado a su gran estabilidad y facilidad de obtención en plasma, los ha puesto en el punto de mira para la identificación de nuevos biomarcadores que pudieran ser de utilidad en el diagnóstico o pronóstico de distintas enfermedades[198]. Además, en la actualidad, existen numerosos estudios sobre la utilización de ellos como diana terapéutica, modificando su expresión mediante su silenciamiento o sobreexpresión[198].

Entre las patologías en las que se han estudiado los patrones de miRNAs se encuentra la VAB[62,63,116,199]. La expresión diferencial de diversos miRNAs ha sido relacionada con la presencia de la VAB, así como de sus complicaciones asociadas. En concreto, nuestro grupo relacionó anteriormente un patrón característico de miRNAs en la VAB con DA de la aorta ascendente en plasma de adultos[62,63]. En este caso, el miR-

718 mostró gran correlación con el tamaño de la aorta ascendente indexada. Otros estudios también han mostrado sus esfuerzos por obtener biomarcadores en forma de miRNAs para la VAB con DA o EA en muestras de plasma. Sin embargo, ninguno de estos está siendo utilizado como marcador establecido para diagnosticar o predecir la evolución de esta valvulopatía.

El desarrollo de biomarcadores fiables que nos permitan un correcto diagnóstico y pronóstico de los pacientes con VAB es de vital importancia para optimizar su seguimiento y poder estratificar a los pacientes según su riesgo. Además, tal y como ocurre en otras patologías, el patrón de miRNAs podría ayudar a comprender de forma más profunda la fisiopatología de la DA y la EA asociadas a la VAB. Estos miRNAs podrían incluso utilizarse en un futuro como dianas terapéuticas para ralentizar su progresión.

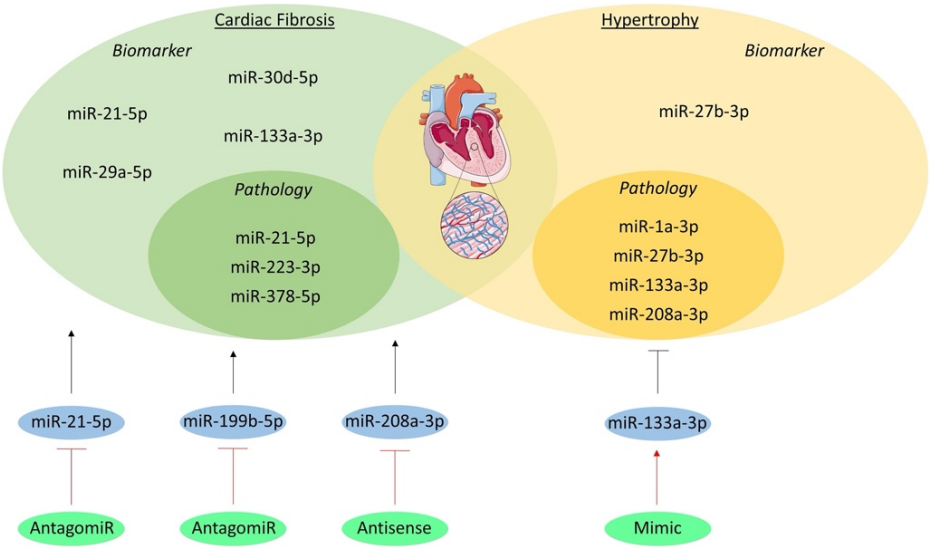

Figura 17. miRNAs que participan en el remodelado cardíaco. Algunos de ellos se utilizan como biomarcadores (en verde o amarillo), mientras que otros están siendo utilizados como dianas terapéuticas (en azul). Fuente: Peters et al., 2020. [200]

4. Justificación del estudio

El seguimiento de la progresión de la DA y de la EA en la VAB se realiza fundamentalmente mediante ETT y otras técnicas de imagen hasta la necesidad de una intervención quirúrgica. Actualmente no existen biomarcadores definitivos que nos

ayuden a predecir la evolución del proceso de degeneración de la válvula, EA, así como la progresión de la DA, más allá de las técnicas de diagnóstico por imagen. Sin embargo, estas técnicas tienen una gran variabilidad y sus determinaciones pueden variar de forma considerable en función del especialista que las realice. Además, estas pruebas diagnósticas nos informan del estado del paciente en un momento concreto, pero no nos ayudan a predecir su evolución. Por ello, los marcadores actuales establecidos resultan insuficientes para este tipo de pacientes, y la búsqueda de nuevos biomarcadores que nos ayuden a estratificar a los pacientes por riesgo de evolución es fundamental. Asimismo, la identificación de nuevos biomarcadores ayudaría a una comprensión más completa de su fisiopatología, necesaria para descifrar qué mecanismos difieren entre la DA y EA que ocurre en la VAB y VAT. Finalmente, y como perspectivas futuras, el conocimiento de los mecanismos implicados en su fisiopatología, así como los biomarcadores identificados, podrían contribuir en el descubrimiento de nuevas dianas terapéuticas para promover la ralentización de las complicaciones asociadas a la VAB, complementarias al riguroso seguimiento por ETT.

Hipótesis y objetivos

Hipótesis y objetivos:

Teniendo en cuenta que:

- En pacientes con VAB hay una mayor incidencia de DA y EA a edades más tempranas.
- Los mecanismos que acontecen en la fisiopatología de la DA y EA en la VAB parecen diferir de los que ocurren en VAT.
- La morfología anómala de la VAB provoca cambios en el flujo sanguíneo, promoviendo un régimen más turbulento en la VA y aorta, así como alteraciones en la dinámica de apertura de la propia válvula.
- Los cambios hemodinámicos están relacionados con la homeostasis vascular, así como disfunción endotelial y desajustes en la función mitocondrial.
- Existe una predisposición de disfunción endotelial y mayor estrés oxidativo en la VAB.
- El patrón transcriptómico y perfil metabolómico varía en presencia de patologías, incluida la VAB, y, en concreto, de sus complicaciones asociadas.

Postulamos que:

Existen diferencias en la fisiopatología de la DA y la EA asociada a la VAB con respecto a los individuos con VAT, y su patrón diferencial podría ser útil como biomarcador de diagnóstico y pronóstico para ayudar a estratificar los pacientes según su riesgo.

Nuestro objetivo principal es:

Profundizar en el conocimiento de los factores o mecanismos que intervienen en la degeneración más rápida de la VA (EA) y la DA asociada a la VAB mediante la integración de distintas ómicas.

Estudio 1.

Objetivo principal: Estudiar la existencia de un patrón diferencial metabolómico en plasma en la VAB y su DA asociada en comparación con pacientes con VAT con ausencia o presencia de DA.

Objetivos secundarios:

- Determinar diferentes perfiles metabolómicos característicos para los distintos tipos de pacientes en función de su morfología valvular y DA.
- Valorar el perfil metabolómico asociado a la morfología valvular o DA y estudiar la implicación funcional de los metabolitos diferencialmente expresados.
- Crear un modelo óptimo que nos permita distinguir su morfología valvular y presencia de DA gracias a los metabolitos analizados.

Estudio 2.

Objetivo principal: Evaluar un patrón de miRNAs previamente relacionado con la VAB y DA en adultos en una cohorte de pacientes pediátricos.

Objetivos secundarios:

- Estudiar si el patrón de miRNAs relacionado con la VAB y DA puede trasladarse directamente a una cohorte de pacientes pediátricos.
- Analizar las vías de señalización que puedan verse afectadas por los cambios de expresión de los miRNAs alterados.
- Profundizar en la fisiopatología de la DA en niños con VAB.

Estudio 3.

Objetivo principal: Identificar la presencia de un patrón diferencial transcriptómico y metabolómico en tejido valvular aórtico con EA en función de la morfología valvular (VAT vs. VAB).

Objetivos secundarios:

- Establecer un patrón transcriptómico diferencial mediante RNAseq para VAT y VAB, ambas con EA, y estudiar las implicaciones funcionales de éste.
- Establecer un perfil metabolómico diferencial mediante 1H-NMR para VAT y VAB, ambas con EA, y estudiar las implicaciones funcionales de éste.
- Crear un perfil multiómico mediante la integración de los datos clínicos, transcriptómicos y metabolómicos específico de la EA que se desarrolla en la VAB.
- Analizar las implicaciones funcionales que pueden llevar a estas alteraciones de expresión de RNAs y metabolitos para diferenciar la fisiopatología de la EA que acontece en VAB de la que ocurre en VAT.

Resultados

ESTUDIO 1

Plasma Metabolomic Profiling Associates Bicuspid Aortic Valve Disease and Ascending Aortic Dilation with a Decrease in Antioxidant Capacity
Neus Martínez-Micaelo, Carme Ligero, Borja Antequera-González, Alexandra Junza, Oscar Yanes and Josep M. Alegret
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doi: 10.3390/jcm9072215
Estado: Publicada el 13/07/2020
Posición autoría: Tercer coautor
Área: Medicina General e Interna
Cuartil: Q1
IF: 4,24 (2020)

ESTUDIO 2

miR-130a expression is related to aortic dilation in bicuspid aortic valve children
Borja Antequera González†, Rosa Collell Hernández†, Neus Martínez Micaelo, Cristina Marimon Blanch, Bàrbara Carbonell, Joaquín Escribano, Josep M. Alegret
Pediatric Research (en revisión desde el 12/04/2023)
Revista científica: Pediatric Research
Estado: en revisión
Posición autoría: Primer co-autor
Área: Pediatría
Cuartil: Q1
IF: 3,953 (2021)

ESTUDIO 3

Unraveling the role of mitochondrial dysfunction in the pathophysiology of aortic stenosis in bicuspid aortic valve disease: integrating RNA-seq and metabolomic profiling
Autores: **Borja Antequera González**, Neus Martínez Micaelo, Carlos Sureda-Barbosa, Laura Galian-Gay, MS Siliato Robles, Artur Evangelista, Josep M. Alegret
Revista científica: Journal of Molecular and Cellular Cardiology
Posición autoría: Primer autor
Estado: En revisión
Área: Sistemas Cardíaco y Cardiovascular
Cuartil: Q2
IF: 5,763 (2021)

Journal of
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MDPI

Article

Plasma Metabolomic Profiling Associates Bicuspid Aortic Valve Disease and Ascending Aortic Dilation with a Decrease in Antioxidant Capacity †

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Abstract: Background: The bicuspid aortic valve (BAV) is the most common cardiac congenital disease and is associated with an increased risk of developing ascending aorta dilation; which can have fatal consequences. Currently; no established risk biomarkers exist to facilitate the diagnosis and prognosis of BAV. Methods: Using an untargeted metabolomic approach; we identified the levels of metabolites in plasma samples and compared them depending on the bicuspid or tricuspid morphology of the aortic valve. Including those patients with ascending aortic dilation and/or aortic stenosis (n = 212), we analyzed the role possibly played by alpha-Tocopherol in BAV disease; considering its association with the pathophysiological characteristics of BAV and biomarkers related to inflammation, oxidative stress and endothelial damage, as well as characteristics related to alpha-Tocopherol functionality and metabolism. Results: We found that BAV patients; especially those with ascending aortic dilation; presented lower antioxidant capacity; as determined by decreased plasma levels of alpha-Tocopherol; paraoxonase 1 and high-density lipoprotein (HDL), as well as increased levels of C-reactive protein (CRP; a biomarker of inflammation) and endothelial microparticles (EMPs; an endothelial damage biomarker). By applying random forest analyses; we evaluated the significant screening capacity of alpha-Tocopherol; CRP and EMPs to classify patients depending on the morphology of the aortic valve. Discussion: Our findings support the role of decreased antioxidant capacity; increased inflammation and endothelial damage in the pathogenesis of BAV and the progression of aortic dilation. Moreover; determining the plasma levels of alpha-Tocopherol; CRP and EMPs could improve BAV diagnosis in large populations.

Keywords: bicuspid aortic valve; ascending aortic dilation; metabolomics; alpha-Tocopherol; antioxidant; inflammation

1. Introduction

Bicuspid aortic valve (BAV) is the most common cardiac congenital malformation, affecting 1–2% of the population and occurring when two of the three leaflets (or cups) of the aortic valve did not

separate during fetal development (or less frequently, when congenitally only two leaflets are presents), leading to a bicuspid configuration, rather than the normal three (tricuspid aortic valve; TAV) [1].

As such, BAV patients present a high prevalence of aortic dilation (greater than 50%) which, if untreated, can lead to fatal consequences, such as aortic dissection and/or rupture [2,3]. Bicuspid morphology of the aortic valves causes turbulent blood flow and abnormal hemodynamical patterns in the proximal ascending aorta [4], generating shear stress that impacts the endothelial cells that line the inner surface and leading to dysfunctional aortic endothelium [5,6].

In clinical practice, early detection of BAV is difficult because most of the patients are asymptomatic for a long time and echocardiography is the technique most frequently used to achieve a conclusive diagnosis. Currently, no effective strategies exist to prevent the progression of BAV disease and its related disorders; therefore, the development of new and efficient approaches require a more detailed understanding of the molecular mechanisms for BAV pathogenesis [7]. Echocardiographic measurement of the ascending aortic diameter is the most current method used to monitor aortopathy in BAV patients. Therefore, development of new screening methods and identification of non-invasive biomarkers that could be examined in a high-throughput format could facilitate and improve the prompt diagnosis of BAV.

Untargeted metabolomic profiling is considered a powerful analytic tool for the identification of disease-related biomarkers and pathways that can be used to develop new diagnostic and treatment methods [8]. Considering our previous publications that describe an association between BAV and the circulating levels of biologically active molecules, including microparticles and microRNAs [9–11], in this study, we hypothesized that the complex etiology of the BAV valvulopathy and aortopathy could be associated with a differential circulating profile of metabolites, which could supply an improvement in the diagnostic methods and could also elucidate the molecular mechanisms and functional implications underlying BAV.

2. Methods

2.1. Study Participants

In this study, we included patients belonging to a cohort of TAV controls and BAV patients prospectively included and followed-up in our facilities. The participants were informed and provided written consent. BAV was diagnosed when two aortic leaflets were visualized, with or without a raphe, in the parasternal short-axis view of a transthoracic or transesophageal echocardiogram [12] or on a cardiac magnetic resonance image [13]. Explorations were performed or supervised by the same observer (JM.A.). 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 de Sant Joan* and the *Institut d'Investigació Sanitària Pere Virgili*.

The design of this study included two evaluations of four independent cohorts of age 18–78 years, including TAV individuals (TAV), BAV patients with no aortic dilation (BAV), patients with BAV and ascending aorta dilation at the time of diagnosis (BAVdil) and patients with TAV and ascending aorta dilation (TAVdil) (n = 212). The patient characteristics are described in Table 1. First, we determined the influence of the aortic valve morphology on the plasma metabolome by comparing the circulating metabolite profile between TAV and BAV patients. For this purpose and to exclude the effects of possible confounding factors, we included only those TAV (n = 33) and BAV patients (n = 62) with no aortic dilation (i.e., patients with a diameter of the ascending aorta less than 37 mm) and with no aortic stenosis (defined as patients with a mean transaortic pressure gradient less than 20 mmHg). However, in the second evaluation and to gain insight into the molecular mechanisms underlying the complexity of BAV pathology and the predictive value of the identified metabolites, we included patients with both morphologies (tricuspid or bicuspid) and aortic dilation (TAVdil; n = 35 and BAVdil; n = 82), as well as patients with aortic stenosis independent of the morphology of the aortic valve.

Table 1. Clinical and echocardiographic characteristics of patients included in this study.

	TAV	BAV	TAVdil	BAVdil	*p*-Value
n	33	62	35	82	
Age (years)	47 ± 2	41 ± 2	63 ± 2	52 ± 2	3.9×10^{-11} **, b,c,d
Sex (male/female)	(25/8)	(45/17)	(22/13)	(58/24)	0.670
Body weight (kg)	71.32 ± 2.4	71.00 ± 2.0	80.50 ± 11.5	76.52 ± 1.8	0.183
Severe aortic stenosis (mean gradient ≥ 40 mm Hg)	0 (0%)	3 (5%)	0 (0%)	19 (23%)	2.1×10^{-5} **
Aortic valve gradient (mean, mm Hg)	3.57 ± 1.0	12.04 ± 1.8	2.85 ± 0.8	24.75 ± 2.6	5.5×10^{-12} **, b,d
Left ventricle diastolic diameter (mm)	50.22 ± 0.9	52.20 ± 0.8	53.48 ± 1.0	53.79 ± 1.0	0.100
Left ventricle systolic diameter (mm)	30.35 ± 1.2	32.56 ± 0.8	30.01 ± 0.9	33.36 ± 0.9	0.175
Left ventricular ejection fraction (%)	71.92 ± 1.9	70.72 ± 1.2	70.86 ± 1.8	69.18 ± 1.1	0.568
Aortic root diameter (mm)	33.06 ± 0.8	33.93 ± 0.8	41.94 ± 1.2	39.32 ± 0.6	2.4×10^{-13} **, b,c
Ascending aorta diameter (mm)	30.81 ± 0.6	32.56 ± 0.4	44.88 ± 0.7	44.36 ± 0.6	5.5×10^{-47} **, b,c

TAV tricuspid aortic valve; *BAV* bicuspid aortic valve patients; *BAVdil* patients with bicuspid aortic valve and aortic dilation * Significant values (Overall $p < 0.01$); a, $p < 0.05$ TAV vs. BAV; b, $p < 0.05$ BAV vs. BAVdil; c, $p < 0.05$ TAV vs. TAVdil; d, $p < 0.05$ BAVdil vs. TAVdil. ** p-value < 0.01.

Patients diagnosed with Marfan syndrome or diabetes mellitus, or those receiving pharmacologic treatment (including statins, ACE/ARBs and/or β-blockers), were excluded.

2.2. Blood Sampling and Analyses

Blood samples were collected under overnight fasting conditions and were 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 obtain platelet-poor plasma. The plasma was stored at −80 °C in our biological samples bank (Biobanc IISPV–HUSJR) until needed.

Total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, direct high-density lipoprotein (HDL) cholesterol, high-sensitivity C-reactive protein (CRP), lipoprotein (a) (Lpa), apolipoprotein B100 and apolipoprotein A1 were measured using standardized enzymatic and immunoturbidimetric assays (Spinreact and Horiba ABX Montpellier) adapted to a Cobas Mira Plus autoanalyzer (Roche Diagnostics).

The levels of circulating endothelial microparticles (EMPs) were determined as previously described [9]. Briefly, EMPs were measured using flow cytometry (EPICS-XL; Beckman Coulter) at a low rate and 30 s stop time. The Nano Fluorescent Particle Size Standard Kit (Spherotech) and Flow Count fluorospheres (Beckman Coulter) were used in instrument standardization and as an internal calibrator for microparticle amount calculation, respectively.

Plasma 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, CD31 and CD42b. Possible contamination with leukocyte-derived microparticles was discarded by measuring <4.5% of microparticles co-expressing $CD31^+CD45^+$. EMPs were measured by trained technicians who were blind to the clinical status of the patients as well as to the results.

2.3. Metabolomics Profiling

A biphasic extraction with methanol in 0.1% formic acid and dichloromethane was used to extract metabolites. Methanol in 0.1% formic acid was added to plasma samples followed by vortexing and 3 volumes of dichloromethane and 1 volume of water were added. After a 30-min incubation at 4 °C, samples were centrifuged (15,000 rpm, 15 min at 4 °C) and the organic phase was collected for drying under nitrogen. Pellets were resuspended in methanol:toluene (9:1) for LC/MS analysis.

Untargeted liquid chromatography/mass spectrometry (LC/MS) analyses were performed using an ultrahigh-performance liquid chromatography (UHPLC) system (1290 Agilent) coupled to a 6550 ESI-QTOF MS (Agilent Technologies) instrument operating in positive (ESI+) electrospray ionization mode. Metabolites were separated by reverse-phase chromatography with an Acquity UPLC C18-RP instrument (ACQUITY UPLC BEH C18 1.7 μM, Waters). Mobile phase A was acetonitrile/water (60:40) (10 mM ammonium formate) and mobile phase B was isopropanol/acetonitrile (90:10) (10 mM

ammonium formate). Solvent modifiers were used to enhance ionization and to improve the LC resolution in positive ionization modes. The separation was conducted under the following gradient—0 in 15% of B; 0–2 min 30% of B; 2–2.5 min 48% of B; 2.5–11 min 82% of B; 11–11.5 min 99% of B; 11.5–12 min 99% of B; 12–12.1 min 15% of B; and 12.1–15 min 15% of B. The ESI conditions were applied as follows—gas temperature = 150 °C; drying gas = 13 L min^{-1}; nebulizer = 35 psig; fragmentor = 150 V; and skimmer = 65 V. The instrument was set to work over an m/z range of 50–1200 with an acquisition rate of 3 spectra/sec. For compound identification, tandem mass Spectrometry (MS/MS) analyses were performed in targeted mode with a default iso-width (width at half-maximum of the quadrupole mass bandpass used during MS/MS precursor isolation) of 4 m/z. The collision energy was fixed at 20 and 40 V.

LC/MS data were processed using XCMS [14] software (version 1.34.0) to detect and align mzRT features. XCMS analysis of these data produced a matrix containing the retention time, m/z value and integrated peak area of each feature for each sample. We constrained the initial number of features via the following criteria. Only features above an intensity threshold of 5.000 were retained for further statistical analysis. Quality control samples (QCs) consisting of pooled samples from each condition were injected at the beginning and periodically throughout the worklist. The performance of the LC/MS platform for each mzRT feature detected in the samples was assessed by calculating the relative standard deviation of these features on pooled samples (CVQC) [15]. Statistical analyses were performed using the specmine package [16] in R Bioconductor.

2.4. Random Forest Model Construction and Validation

A model was constructed to estimate the capacity to predict or classify patients based on the morphology of the aortic valve. Therefore, the selection of the variables that make the largest contributions to classification was conducted using association based on Spearman's correlation and linear regression analysis and random forest variable importance measures. The analyses were performed using the randomForest package [17] in R (Bioconductor). We build a model to discriminate patients depending on the morphology of their aortic valve. This model was evaluated including patients with no aortic dilation (BAV and TAV, $n = 95$) or including the four previously described groups (TAV, TAVdil, BAV and BAVdil, $n = 212$). Patients were randomly divided into two subsets—63% of the individuals were used to train the model and 37% of the individuals were used to test the developed models. The training data set was evaluated with a 10-fold cross-validation fold procedure. The statistical parameters for assessing the predictive performance of the random forest classifier were based on the accuracy, sensitivity and specificity.

2.5. Statistical Analysis

Data are expressed as the mean ± standard error mean (SEM). Continuous variables were compared between groups using the Wilcoxon test. Correlations were evaluated using Spearman's Rho. Statistical analyses were performed using SPSS software (IBM SPSS Statistics, United States, version 22.0) and R (Bioconductor). *P*-values < 0.05 were considered to be significant.

3. Results

3.1. Circulating Profile of Metabolites Depends on the Morphology of the Aortic Valve

To analyze whether BAV valvulopathy could be related to a differential profile of circulating metabolites, we compared the circulating metabolome of patients with bicuspid or tricuspid morphology of the aortic valve using an untargeted metabolomics approach. In this analysis, we selected patients with homogeneous characteristics, no aortic dilation and with no aortic stenosis to exclude the effects of possible confounding factors.

Using this high-throughput approach, we detected 2289 distinct metabolite features in plasma samples. As an exploratory analysis, using unsupervised principal component analysis (PCA), we firstly

corroborated the absence of outliers, trends, patterns or clusters in the data prior to statistical analysis. Moreover, using a supervised partial-least-squares discrimination analysis (PLS-DA), we determined that by measuring the metabolomic plasma profile, patients can be classified depending on the bicuspid or tricuspid morphology of their aortic valves (Figure 1). Thus, from the 2289 metabolite features measured, 2 unique metabolites were significantly different between TAV and BAV, namely, alpha-Tocopherol (fdr = 2.17×10^{-3}) and choline (fdr = 8.44×10^{-3}) and both metabolites presented lower abundance in the plasma of BAV patients than in that of TAV patients.

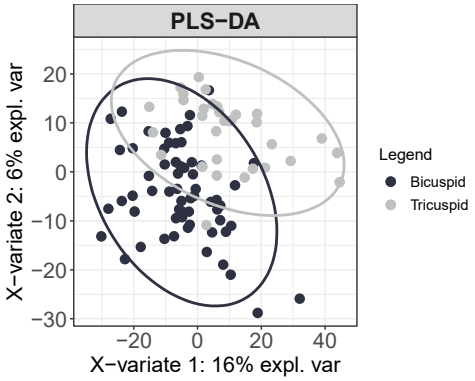

Figure 1. Plasma metabolome differs depending on the morphology of the aortic valve. Multivariate supervised partial-least-squares discrimination analysis PLS-DA of the 2289 total metabolite features detected in plasma classifies between tricuspid aortic valve (TAV) and bicuspid aortic valve (BAV), patients (95% confidence interval ellipses are shown for the TAV and BAV groups). (n = 95).

3.2. *Functional Implication of the Identified Metabolites*

Dilation of the ascending aorta and aortic stenosis are the two most common comorbidities associated with BAV; therefore, to improve the translational character and the clinical applicability of the obtained results, we analyzed whether the circulating levels of alpha-Tocopherol and choline remained significantly different between TAV and BAV patients when we consider those patients with an ascending aortic dilation (TAVdil and BAVdil) and also include those patients with aortic stenosis in the corresponding groups (Figure 2).

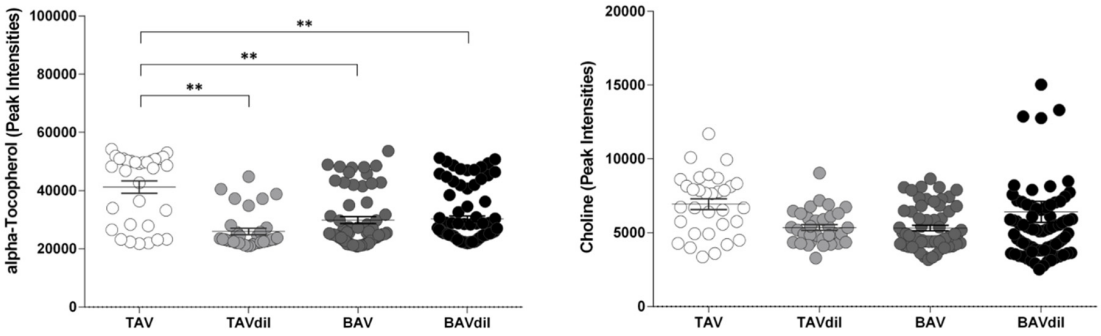

Figure 2. Peak intensities measured for the alpha-Tocopherol and choline levels in TAV and BAV patients. n = 202. ** p-value < 0.01.

We observed that although the levels of alpha-Tocopherol were higher in TAV individuals than in patients with BAV, BAVdil and TAVdil, no significant changes were observed in the circulating levels of choline. We performed a 3-way ANOVA including the valve morphology, ascending aortic dilation and aortic stenosis as independent variables and alpha-Tocopherol or choline as dependent variables and we found that only the interaction between the valve morphology (BAV vs TAV) and

dilation resulted significative (p-value = 0.042) for the alpha-Tocopherol levels and no main effects nor interactions resulted significative for the choline levels.

To analyze the relationship between alpha-Tocopherol plasma levels and BAV disease and whether it might play a role in the progression of the disease, we analyzed the association of the circulating levels of alpha-Tocopherol with the pathophysiological characteristics of BAV disease, including echocardiographic and other confounding clinical variables. Using multivariate linear regression analysis (Table 2), we observed that the diameter of the ascending aorta was the only variable independently and negatively associated with the plasma levels of alpha-Tocopherol.

Table 2. Multivariate linear analysis of variables related to the plasma levels of alpha-Tocopherol.

	Coefficient	Std-Coefficient	p-Value
	alpha-Tocopherol		
Age (years)	−47.99 (−157.20 to 61.31)	−0.75	0.388
Aortic root (mm)	120.60 (−207.20 to 448.39)	0.75	0.469
Ascending aorta (mm)	−370.60 (−643.81 to −97.39)	−2.68	8.13×10^{-3} **
Mean transaortic pressure gradient (mm Hg)	25.91 (−58.78 to 110.61)	0.046	0.547
Left ventricle diastolic diameter (mm)	29.72 (−316.57 to 376.01)	0.021	0.169
Left ventricle systolic diameter (mm)	133.85 (−418.85 to 686.54)	0.071	0.633
Left ventricular ejection fraction (%)	165.13 (−76.42 to 406.68)	0.157	0.179

** p-value < 0.01.

Using this approach to elucidate the biological implication and the functionality of alpha-Tocopherol in BAV disease, we considered both the cellular and molecular mechanisms that have been associated with BAV, including inflammation, oxidative stress and endothelial damage [6]. Furthermore, we also considered characteristics related to alpha-Tocopherol function and metabolism, including its role as the most important lipid-soluble antioxidant carried by lipoproteins in plasma. Therefore, we first analyzed the effect of aortic valve morphology and ascending aortic dilation on the plasma levels of parameters associated with lipid and lipoproteins, inflammation, oxidative stress and endothelial damage.

We determined that the bicuspid morphology of the aortic valve and dilation of the ascending aorta were related to higher levels of endothelial microparticles (EMPs) and C-reactive protein (CRP), which are biomarkers related to endothelial damage and inflammation, respectively but these patients also had lower levels of apolipoprotein A1 (ApoA1), the major HDL protein (Table 3).

Table 3. Effect of aortic valve morphology and ascending aortic dilation on the plasma levels of parameters associated with lipid metabolism, inflammation, oxidative stress and endothelial damage.

	TAV	BAV	TAVdil	BAVdil	p-Value
Total cholesterol (mmol/L)	5.61 ± 0.3 (5.04–6.18)	4.77 ± 0.2 (4.43–5.01)	5.20 ± 0.3 (4.60–5.79)	5.07 ± 0.1 (4.79–5.33)	0.171
Triglycerides (mmol/L)	1.36 ± 0.2 (0.96–1.76)	0.97 ± 0.7 (0.82–1.11)	1.48 ± 0.2 (1.13–1.84)	1.33 ± 0.1 (1.06–1.60)	0.139
LDL (mmol/L)	3.45 ± 0.2 (3.00–3.90)	2.87 ± 0.1 (2.58–3.15)	3.26 ± 0.2 (2.80–3.72)	2.97 ± 0.1 (2.74–3.20)	0.172
HDL (mmol/L)	1.55 ± 0.1 (1.32–1.77)	1.47 ± 0.5 (1.37–1.57)	1.26 ± 0.1 (1.14–1.39)	1.49 ± 0.5 (1.39–1.59)	0.049 *
ApoA1 (mg/dL)	160.67 ± 7.3 (144.67–176.66)	133.47 ± 4.4 (124.60–142.35)	139.53 ± 4.5 (130.02–149.03)	140.61 ± 3.7 (133.22–148.00)	0.007 ** a, e
ApoB100 (mg/dL)	118.50 ± 6.9 (103.27–133.73)	88.67 ± 4.1 (80.38–96.95)	112.88 ± 6.2 (99.65–126.12)	99.22 ± 3.7 (91.79–106.64)	0.005 ** a, c
C-reactive protein (mg/dL)	1.06 ± 0.3 (0.38–1.72)	1.71 ± 0.3 (1.14–2.29)	1.84 ± 0.5 (0.86–2.82)	2.42 ± 0.4 (1.70–3.13)	0.046 * e
Paraoxonase 1 (ng/mL)	85.23 ± 6.5 (70.94–99.94)	94.61 ± 8.8 (76.67–112.55)	78.12 ± 8.0 (61.25–94.99)	87.12 ± 7.1 (72.86–101.37)	0.647
Endothelial microparticles (log part/μL)	2.49 ± 0.4 (1.55–3.43)	3.68 ± 0.3 (3.05–4.27)	3.24 ± 0.3 (2.55–3.92)	3.78 ± 0.2 (3.35–4.20)	0.020 * e
Albumin (g/dL)	4.64 ± 0.3 (4.55–4.73)	4.53 ± 0.1 (4.53–4.65)	4.39 ± 0.1 (4.27–4.52)	4.52 ± 0.1 (4.46–4.58)	0.010 * c

* Significant values (Overall $p < 0.05$), ** Significant values (Overall $p < 0.01$), a, $p < 0.05$ TAV vs. BAV; b, $p < 0.05$ BAV vs. BAVdil; c, $p < 0.05$ TAV vs. TAVdil; d, $p < 0.05$ BAVdil vs. TAVdil; e, $p < 0.05$ TAV vs. BAVdil.

Using an analysis of covariance (ANCOVA) we found that the levels of alpha-Tocopherol remained significantly different between groups (p-value < 0.01) while considering the effect of ApoA1 (p-value = 0.423) or HDL (p-value = 0.773).

Furthermore, using Spearman's correlation coefficient, we found that plasma levels of alpha-Tocopherol were negatively associated with the diameter of the ascending aorta, the aortic root and the age. The plasma levels of alpha-Tocopherol were also associated with circulating concentrations of ApoA1 and albumin, the two carriers of alpha-Tocopherol in plasma and paraoxonase 1, an enzyme with antioxidant properties transported in HDL (Table 4).

Table 4. Linear relationship between the plasma levels of the identified metabolites and the parameters associated with lipid metabolism, inflammation, oxidative stress and endothelial damage.

	Alpha-Tocopherol	
	R	***p*-Value**
Aortic root (mm)	−0.172	0.012 *
Ascending aorta (mm)	−0.271	7.20×10^{-5} **
Mean transaortic pressure gradient (mm Hg)	−0.083	0.230
Age (y)	−0.179	0.009 **
Lipid Metabolism		
Total cholesterol (mmol/L)	0.052	0.514
Triglycerides (mmol/L)	0.023	0.770
ApoA1 (mg/dL)	0.209	0.008 **
ApoB100 (mg/dL)	0.082	0.305
Inflammation		
C-reactive protein (mg/dL)	0.042	0.607
Oxidative stress		
Paraoxonase 1	0.195	0.030 *
Endothelial damage		
Endothelial microparticles (log part/µL)	0.097	0.263
Liver damage		
Albumin (g/dL)	0.248	0.002 **

* p-value < 0.05; ** p-value < 0.01.

3.3. Prediction of the Morphology of the Aortic Valve or the Dilation of the Ascending Aorta in BAV Patients

The results obtained in the previous analyses enable us to consider the role of plasma biomarkers based on its functional implication in aortic valve dysfunction and also as predictive circulating signatures of aortic valve morphology. Thus, we evaluated the predictive capacity of the differential metabolomic signature in plasma together with the variables that make the largest contributions to classification. To this end, we used the random forest algorithm to construct a model able to discriminate patients depending on the morphology of the aortic valve (Table 5 and Figure 3). The model was constructed using two approaches, one including all patients independent of the morphology of the aortic valve and the dilation of the ascending aorta and the other excluding those patients with ascending aortic dilation.

Table 5. Evaluation of multivariable biomarker models including alpha-Tocopherol, endothelial microparticles (EMPs) and C-reactive protein (CRP) for the prediction of aortic valve morphology.

***p*-Value**	**OOB Error**	**Accuracy (95% CI)**	**Sensitivity**	**Specificity**	**AUC**
		TAV + TAVdil + BAV + BAVdil			
3.9×10^{-3} **	21.7%	0.89 (0.77–0.96)	0.91	0.86	0.92
		TAV + BAV			
7.6×10^{-3} **	17.5%	0.95 (0.75–0.99)	0.93	1.00	0.99

** p-value < 0.01.

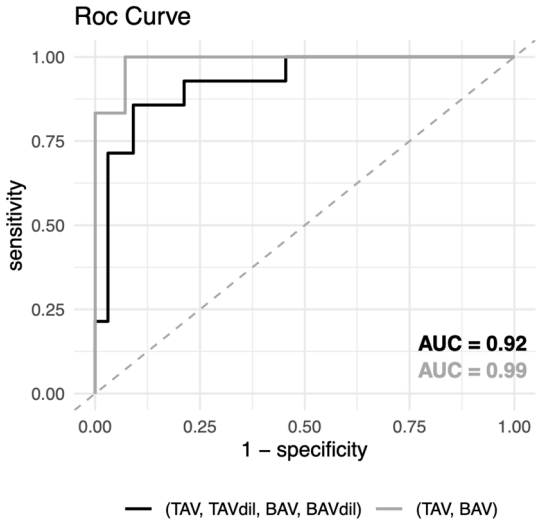

Figure 3. ROC curves of cross-validated random forest classification of test data.

We determined that the alpha-Tocopherol, EMPs and CRP circulating levels were variables able to discriminate patients based on their valve morphologies. Thus, the model constructed using these variables presented an accuracy of 0.89, a test sensitivity of 0.91, a specificity of 0.86 and a *p*-value (accuracy > no information rate) = $3.9 \times 10^{-}$ when we do not exclude patients depending on aortic diameter. However, in the case in which only TAV and BAV patients with no ascending aortic dilation were included in the model construction, the test sensitivity and specificity values were 0.95 and 1.00, respectively and the *p*-value (accuracy > no information rate) = 7.6×10^{-3}.

4. Discussion

Using an untargeted metabolomics approach, we have identified significant associations between the plasma levels of alpha-Tocopherol and the morphology of the aortic valve and diameter of the ascending aorta. Moreover, we have determined that BAV patients, especially those with ascending aortic dilation, are characterized by increased levels of circulating biomarkers of inflammation, such as CRP, endothelial damage by measuring EMPs and decreased antioxidant capacity due to the lower circulating levels of alpha-Tocopherol, as well as its main carrier in plasma, the HDL lipoprotein, measured as ApoA1. Finally, we have evaluated how the circulating levels of alpha-Tocopherol, together with CRP and EMPs, are significant predictors of the aortic valve morphology, thereby identifying alpha-Tocopherol as the most important classification variable.

The pathogenesis of ascending aortic dilation in BAV is not completely understood. Two main non-mutually exclusive theories exist associating BAV aortopathy with genetic or hemodynamic causes. Focusing on hemodynamic causes, the bicuspid morphology of the aortic valve causes an abnormal biochemical environment, a consequence mainly of the non-physiological hemodynamic impact on the ascending aorta. This morphology-associated helical flow alteration that propagates flow eccentricity leads to increased wall shear stress on the endothelium and progressive dilation of the ascending aorta [18,19]. Although BAV disease is asymptomatic initially, the lack of an early diagnosis can lead to identifying the case in advanced stages or after an event. Therefore, in clinical practice, the existing approaches to achieving a conclusive diagnosis and to monitoring aortopathy in BAV patients are based on images obtained by transthoracic echocardiography [20]. A clear need exists for novel diagnostic and molecular tools to improve BAV screening, disease stratification and monitoring response to therapeutic interventions [21].

Untargeted metabolomics is a powerful tool that can supply a high-resolution snapshot of the complete set of metabolites, leading to the identification of specific metabolic signatures that can determine any perturbation of single or multiple metabolites and the related biochemical pathways [22],

thereby offering new insights into the molecular mechanisms underlying complex diseases. Therefore, despite our untargeted metabolomics approach that identified the alpha-Tocopherol and the choline, only alpha-Tocopherol levels remained significantly different when we included patients with dilated ascending aorta and aortic stenosis, the two common complications related to BAV progression, in the analysis. Alpha-Tocopherol, as the primary form of the vitamin E, is a fat-soluble antioxidant with the capacity to neutralize endogenous free radicals and is carried in the blood primarily through HDL lipoproteins [23] but would also be bound to albumin [24]. Supporting this observation, we found a significant positive correlation between plasma levels of alpha-Tocopherol and ApoA1, albumin and paraoxonase 1, an enzyme transported by HDL with antioxidant and anti-atherogenic properties [25]. Interestingly, in addition to its antioxidant capacity, alpha-Tocopherol also acts as a regulator of the expression of genes involved in lipid metabolism and inflammation [26]. The plasma levels of alpha-Tocopherol have not been previously related to BAV pathology but nevertheless, a previous study Wang et al. [27] identified choline as one of the different metabolites in the serum of BAV patients before aortic valve replacement surgery compared with healthy individuals. However, the patients included in that study presented severe conditions (aortic valve dysfunction and/or aortic dilatation) leading to surgery indication; thus, those conditions together with BAV could affect the circulating metabolome profile. Choline is an essential nutrient for human health, being the liver the central organ responsible for its metabolization. Choline is involved in several physiological functions, phosphorylated, choline acts as the substrate for the synthesis of phosphatidylcholine, a key component of eukaryotic cellular membranes when its oxidized, choline participates as a methyl-donor in the methylation processes and in the case to be acetylated, choline generates acetylcholine, an important neurotransmitter [28,29]. Further, choline also participates in the solubilization of bile salts and is required for the generation of VLDL lipoproteins [30]. Choline has cardioprotective effects by downregulating inflammation, restoring endothelium structure and by inhibiting the generation of reactive oxygen species [28,31].

The results presented in this study showed that circulating levels of alpha-Tocopherol were higher in TAV individuals than in patients with BAV but also in those patients with dilated ascending aorta, that is, including TAVdil and BAVdil patients. Therefore, we speculated that the decrease in the measured levels of alpha-Tocopherol could occur because of cellular and molecular events related to pathogenesis of BAV and vascular wall remodeling associated with dilation of the ascending aorta, rather than as a consequence of the bicuspid morphology of the aortic valve itself. BAV patients normally present increased, even not clinically significant, diameters of the ascending aorta than healthy TAV ones (32.56 ± 0.4 and 30.81 ± 0.6 $p = 0.003$, respectively; Table 1) and according to reported in [32]. However, histological differences exist in the dilated ascending aorta between BAV and TAV patients and several studies have determined that the inherent defects of the aortas in BAV patients lead to altered wall mechanical properties, which might contribute to the progressive dilation of the ascending aorta [33,34].

Using the alpha-Tocopherol levels together with the variables associated with the bicuspid morphology of the aortic valve, that is, the levels of EMPs and CRP, we constructed a model that significantly classified individuals depending on the morphology of the aortic valve. Interestingly, alpha-Tocopherol was identified as the most important variable. Therefore, measuring the levels of alpha-Tocopherol together with the parameters associated with inflammation and endothelial damage might be useful as a more feasible alternative for BAV screening and could be complementary to echocardiogram examination in large populations, as well as being useful in risk stratification of patients susceptible to developing ascending aortic dilation.

However, considering that the alpha-Tocopherol is functionally related to lipid and lipoprotein metabolism, we must consider whether the endothelial damage and pro-inflammatory state observed in BAV patients, especially in BAV patients with ascending aorta dilation, could be a consequence not only of hemodynamic forces and shear stress due to the bicuspid morphology of the aortic valve but also of an alteration to lipid and lipoprotein metabolism. In support of this hypothesis, selected authors have suggested that several molecular mechanisms that promote atherosclerosis are also present in

BAV and that these alterations could underlie the progression of aortic stenosis and dilation [35]. The results reported in this study supported the hypothesis that BAV and dilation of the ascending aorta are associated with increased endothelial damage and inflammation. However, although the results presented in this study support the hypothesis that the lipid and lipoprotein metabolism might play an important role in BAV disease, we cannot establish causality.

Accordingly, additional studies are necessary to further evaluate whether different lipoproteins profiles in plasma affect the endothelium in BAV patients depending on susceptibility to developing ascending aorta dilation. Additionally, further research is warranted to determine whether alpha-Tocopherol administration might influence the progression of BAV pathology.

Limitations: We excluded from the analysis patients with diabetes mellitus and those receiving certain pharmacological treatments such as statins, ACE inhibitors/ARBs and beta-blockers, to minimize the possibility that the excluded variables could influence the identification of related metabolites with the BAV. Subsequent studies carried out in a large population including the excluded variables should validate the generalization of our results.

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Abbreviations

Tricuspid aortic valve	TAV
Tricuspid aortic valve with ascending aortic dilation	TAVdil
Bicuspid aortic valve	BAV
Bicuspid aortic valve with ascending aortic dilation	BAVdil
endothelial microparticles	EMPs
C-reactive protein	CRP
apolipoprotein A1	ApoA1
apolipoprotein B100	ApoB100

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Pediatric RESEARCH

miR-130a expression is related to aortic dilation in bicuspid aortic valve children

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miR-130a expression is related to aortic dilation in bicuspid aortic valve children

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Impact:

- miR-130a expression in plasma is related to aortic dilation in bicuspid aortic valve (BAV) children.
- To our knowledge, this is the first study that analyses miRNA patterns in bicuspid aortic valve children with aortic dilation.
- miR-130a expression in plasma could be a biomarker in order to help differentiate low-to high-risk BAV children, which is vitally important for advanced care planning.

Abstract:

Purpose: Bicuspid aortic valve disease (BAV) is present in 0.5-2% of the population and can promote aortic dilation, eventually leading to fatal consequences. Although some biomarkers have been proposed in adults, no studies have tested these candidates in children. We aimed to evaluate 4 miRNAs previously described to be related to BAV disease and aortic dilation in adults in a pediatric cohort. Methods: Eighty participants ≤ 17 years old (4-17; mean 12) were included. From the BAV group, 40% had a dilated aorta (z score>2). RT–qPCR were performed in plasma samples to quantify miR-122, miR-130a, miR-486, and miR-718 using the delta-delta Ct method. Functional and enrichment analyses of miR-130a were also performed. Results: miR-130a expression in plasma was found to be significantly lower in BAV patients with a dilated aorta verses nondilated patients (p= 0.008) and healthy TAV controls (p= 0.004). Furthermore, miR-130a expression in plasma was inversely correlated with ascending aorta (r=0.318, p=0.004) and aortic root z scores (r=0.322; p=0.004). Enrichment analysis showed that miR-130a target genes are related to the TGFß signalling pathway. Conclusions: miR-130a expression in plasma is decreased in aortic dilated BAV children compared to nondilated BAV children , helping differentiate low- to high-risk patients.

Introduction:

Bicuspid aortic valve (BAV) disease is present in 0.5-2% of the general population and is defined by the abnormal morphology of the valve, composed of two cusps instead of three, usually by the fusion of two of them[1–3]. This morphological anomaly can lead to valve dysfunction (aortic insufficiency, aortic stenosis) and is related to a high prevalence of ascending aorta dilation[4,5]. Specifically, aortic dilation is present in more than 50% of the BAV population, and its progression can lead to fatal consequences, including aortic aneurysm and dissection[6,7]. Although the mechanisms that underlie aortic dilation are not clear, haemodynamic alterations and genetic abnormalities in BAV disease have been proposed for the defective structure of the aorta[8,9]. Additionally, some studies have suggested that oxidative stress and consequently endothelial dysfunction are present in BAV disease[10–12].

BAV disease in children is usually asymptomatic, although it has been estimated that 1 of 50 adolescents have clinically significant valve disease[13,14]. Aortic dilation has been previously described by many clinicians to have correlations with children, indicating that the progression of aortic dilation may develop at younger ages[15–17]. Although the risk of aneurysm progression in pediatric patients is scarce, identifying children with BAV disease and aortic dilation is vitally important for advanced care planning.

Currently, there are no definite established biomarkers for BAV disease and aortic dilation that help differentiate high- to low-risk patients. While some studies have linked aortic dilation to different microRNA (miRNA) patterns in BAV adults, none of them have studied its possible potential in children[18–22]. The use of miRNAs for biomarker purposes has been widely extended in the last few years, and miRNAs are currently used in many cardiovascular diseases[23]. miRNAs are RNA molecules of 18-24 nucleotides that are capable of modifying gene expression at the posttranscriptional level by binding to messenger RNA (mRNA)[24]. miRNAs are highly expressed in blood, as well as every cell, and their expression pattern can be specific for different pathologies, making them perfect candidates for biomarker purposes[25]. Our group has previously presented a miRNA pattern present in BAV patients with aortic dilation in an adult cohort. Hence, in this study, we aimed to evaluate the potential of these 4 miRNA candidates (miR-122, miR-130a, miR-486, and miR-718) as biomarkers of aortic valve morphology as well as aortic dilation in the pediatric population.

Material & methods:

Study population:

This study included a cohort of 80 patients aged ≤ 17 years old (4-17) who were prospectively included and followed-up in our facilities. Half of them, 40, corresponded to consecutive children diagnosed with BAV disease. The other half of them corresponded to a group of 40 healthy TAV controls adjusted by age and sex recruited during follow-ups in sports medicine. The diagnosis of BAV disease was made when two aortic leaflets were clearly visualized, with or without a raphe, on the parasternal short-axis view of a transthoracic echocardiogram. A dilated aortic root or ascending aorta was diagnosed when the aortic diameter was measured in diastole using the leading edge technique, with a z score>2 for the Paris reference source[26]. Patients with an aortic root or ascending aorta z score >2 were classified as dilated (BAV_DIL), whereas the other patients were considered nondilated (BAV_nonDIL). All explorations were performed or supervised by the same observer. Upon enrollment, participants were prospectively entered into a specific database, underwent a blood draw and provided informed written consent. Samples were stored until needed in our biological sample bank (Biobanc IISPV-HUSJR).

Blood sampling:

Blood samples were collected in overnight fasting conditions and were processed within 90 mins after collection. The samples were centrifuged at 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:

RNA was extracted and purified from 200 µL of plasma using the miRNeasy serum/plasma advanced kit following the manufacturer's instructions (#217204, Qiagen). To increase RNA recovery, 1 µg of MS2 carrier RNA was added to each plasma sample (#10165948001, Roche).

miRNA quantification by RT–qPCR:

For quantification of miR-122, miR-130a, and miR-486, reverse transcription was performed using the extracted RNA with the cDNA Synthesis Kit TaqMan™ Advanced (Applied Biosystems, #A28007). TaqMan™ Fast Advanced Master Mix kit (Applied Biosystems, #4444963) with TaqMan™ Advanced probes were used for quantification of miR-122 (477855_mir), miR-130a (477851_mir), and miR-486 (478128_mir). The results were normalized to miR-16 (477860_mir) and relatively quantified using the delta delta Ct method. For mi-R718 quantification, the miRCURY LNA RT Kit (Qiagen, #339340) was used for reverse transcription, and the miRCURY LNA SYBR Green PCR Kit (Qiagen, #339346) and hsa-miR-718 miRCURY LNA miRNA PCR Assay (Qiaegn, #YP00205702) were used for qPCR. The results were also normalized to miR-16 (Qiagen, #YP02119603).

Functional and pathway enrichment analysis of miR-130a

Target genes of miR-130a were predicted using the latest version available of miRwalk (v. Jan22)[27]. Visualization of the strongest validated gene targets was performed with miRTargetLink v.2.0 [28]. Pathway analysis (Reactome 2022 database) and disease association study (DisGeNET database) of the predicted target genes were performed with Enrichr [29,30]. The representation of these results was visualized using Prism v9 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis:

Statistical studies were performed to compare the distribution of the different variables between different groups. 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 analysed using Student's t test. Pearson's or Spearman correlation, depending on the distribution, was used to identify the linear relationships between variables. One-way ANOVA was performed when comparing more than 2 groups, and Tukey's test was used for individual comparisons. Furthermore, a logistic regression with a forward stepwise model was constructed, and parameters with the strongest correlations to the dependent variable were included. Linear

forwards regression was also 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. AUC analysis was also performed to evaluate the quality of the model and prediction of aortic dilation. 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).

Results:

Clinical and echocardiographic characteristics of the cohort:

The complete cohort (n: 80) was divided into three groups: TAV (n: 40) vs. BAV without aortic dilation (BAV_nonDIL, n: 24) vs. BAV with aortic dilation (BAV_DIL, n: 16). Clinical and echocardiographic characteristic comparisons can be found in Table 1. We observed greater aortic diameters and aortic valve gradients, as well as a higher prevalence of aortic insufficiency, in both BAV groups when compared to the healthy TAV control group. These parameters were also higher in the BAV_DIL group than in the BAV_nonDIL group. Age, sex, and body surface area (BSA) were not significantly different between the groups.

miRNA pattern is related to aortic dilation in BAV children

After quantifying miR-122, miR-130a, miR-486, and miR-718 expression in plasma, the complete miRNA pattern was compared (Table 2 and Figure 1). We identified miR-130a as the only miRNA differentially expressed between the three groups (p=0.008). Individual comparisons also showed that miR-130a dCt was significantly increased when comparing TAV vs. BAV_DIL (p=0.004) and BAV_nonDIL vs. BAV_DIL (p=0.013), but no significant changes were found when comparing TAV vs. BAV_noDIL (p=0.326) between the three groups. The rest of the miRNAs measured (miR-122, miR-486, and miR-718) did not show any significant changes between groups in the child cohort.

Furthermore, an area under the curve (AUC) graph was generated with baseline clinical parameters (sex, age, and BSA) to analyse the power prediction of a set of parameters (Figure 2). Different curves were prepared by adding valve morphology and subsequently miR-130a dCt. The results showed that adding

valve morphology increased the AUC from 0.63 to 0.75 (p 0.002), but it reached 0.90 when adding miR-130a dCt ($p<0.001$) (Table 3).

Subsequently, we evaluated the relationship between miR-130a and aortic diameter parameters. We performed a correlation study between aortic root and ascending aorta z scores and the miRNA pattern (Table 4). In this case, miR-130a dCt was the only miRNA related to both z scores in the complete cohort (TAV + BAV) and in BAV children (Figure 3).

A multivariate linear analysis was also performed for both aortic root and ascending aorta z scores to determine miR-130a dCt independency. The initial model included miR-130a dCt, sex, age and BSA. In both cases, only miR-130a dCt persisted as an independent predictor of the z score in children (Table 5).

Functional and pathway enrichment analysis of miR-130a:

To provide a better understanding of the relevance of miR-130a downregulation in aortic dilated BAV children, we characterized the putative biological functions of this miRNA based on the identification of the miR-130a target genes. The predicted miRNA-gene interaction network was composed of miR-130a (hsa-miR-130a-3p) and 250 target genes, but only the 32 strongest related genes were included in Figure 4 for better visualization.

A functional enrichment analysis was also performed to predict the associated signalling pathways and related diseases of the miR-130a target genes. Although numerous signalling pathways were predicted to be related to miR-130a expression, many of them are linked to the TGFß signalling pathway, oxidative stress, and extracellular vesicle metabolism. The top 10 miR-130a target gene-associated pathways are represented in Figure 5. In addition to cell signalling processes, an association between miR-130a target genes and related diseases was analysed and is represented in Figure 6. Cardiovascular diseases were the diseases most related to the miR-130a target genes.

DISCUSSION

In this study, we analysed the miRNA pattern in plasma in children with BAV disease and aortic dilation to confirm the previously found alterations that occur in adult patients. Although miRNA

expression seemed to behave differently at younger ages, we found miR-130a to be highly related to aortic dilation in children, specifically in BAV children. miR-130a expression in plasma was significantly lower in dilated patients, and both ascending aorta and aortic root z scores were strongly correlated with miR-130a dCt.

BAV disease can significantly increase the possibility of diverse associated aortopathies, such as aortic dilation[4,31]. Aortic dilation is present in more than 50% of BAV patients, and it is known to progress faster in these patients. Furthermore, its latest stages can be fatal, concluding in aortic aneurysm and dissection[4,31]. Aortic dilation in BAV children can easily go unnoticed since it does not show clear symptoms until later stages. Therefore, it seems of special importance to have biomarkers that can help identify high-risk patients to increase the plan for a better follow-up strategy. In the last few years, miRNAs have been widely studied in different pathologies, mainly in adults, resulting in important discoveries of independent biomarkers for cardiovascular diseases, among others[23,32]. Furthermore, miRNA differential expression has also helped elucidate the pathophysiology and development of diverse cardiovascular diseases[32]. Unfortunately, in children, the number of studies is very small, and to date, we have not been able to find any studies regarding miRNA expression in a cohort of BAV children, which also adds value to our results.

Although it is not yet fully understood, the mechanisms that underlie aortic dilation include numerous and diverse genetic abnormalities, as well as haemodynamic alterations generated by the anomalous valve morphology[8,9,33]. These changes in blood flow can increase wall shear stress in the aorta and promote extracellular matrix dysregulation and elastic fibre degeneration, which may induce aortic dilation[34–36]. Furthermore, these blood flow alterations can also promote medial degeneration by metalloprotease pathways[37,38]. These factors could contribute to faulty aortic media in BAV patients. On the other hand, some studies suggest that endothelial dysfunction and oxidative stress could also play an important role in BAV disease, as well as in aortic dilation. Endothelial dysfunction has widely been discussed in BAV disease[12]. While some studies have focused on plasma biomarkers such as asymmetric dimethylarginine (ADMA)[39], others have focused their research on *in vitro* studies, showing impaired functionality of circulating endothelial cells in BAV disease[40]. Recent studies have also suggested that endothelial-to-mesenchymal transition, induced by TGFß, may be altered in BAV disease[41,42]. Our group

has previously discussed poor antioxidant capacity in aortic dilated adult BAV patients, as well as higher expression of endothelial microparticles in plasma, a biomarker for endothelial dysfunction[18,43]. A specific miRNA pattern has also been proposed in BAV disease and aortic dilation in adults, which was also linked to TGFß signalling and endothelial damage[18,19].

We previously performed a discovery microarray to identify a differential miRNA pattern corresponding to BAV morphology and aortic dilation. In that study, four miRNAs were identified: miR-122, miR-130a, miR-486, and miR-718[18,19]. Therefore, we aimed to test whether this miRNA pattern was similarly expressed in BAV children with or without aortic dilation. Our results showed that even miRNA patterns in BAV children seem to be differ from those in BAV adults. Nevertheless, miR-130a emerged as an interesting biomarker candidate for aortic dilation in children, specifically in BAV children. miR-130a was inferiorly expressed in plasma in children with aortic dilation compared to nondilated children. Furthermore, miR-130a dCT showed strong correlations to aortic root and ascending aorta z score, both in the complete cohort, but above all, in the BAV children group.

Although the role of miR-130a needs further research, the enrichment analysis has helped us link miR-130a, aortic dilation and different processes, such as TGFß signalling, oxidative stress, or even extracellular vesicle transport. Our previous work has already discussed poor antioxidant capacity, endothelial damage, and TGFß imbalance in aortic dilation in BAV adults due to alterations in miRNA patterns [12,19,43,44]. Bibliography research has complemented these findings, since many studies in adults have already associated differential miR-130a expression with different cardiovascular diseases. It has been shown to have a cardioprotective role and can even help predict cardiovascular risk in some studies[45,46]. Furthermore, its decreased expression has been associated with endothelial cell senescence [47]. Other studies have also discussed its role in the regulation of the vascular pattern during embryogenesis [48] and have linked miR-130a to the attenuation of cardiac fibrosis through TGF-ß/SMAD signalling [49]. Finally, TGFß signalling has also been associated with endothelial-to-mesenchymal transition (endoMT), which has also been highlighted to be altered in the ascending aortas of BAV patients[41,42]. Combined, this data has helped us hypothesize that miR-130a expression may be related to alterations in diverse signalling processes, such as the TGFß signalling pathway, which could lead to endothelial dysfunction and endoMT alterations [12,44].

Hence, the endothelium homeostasis imbalance may alter the endothelial barrier in the aorta, leading to faster aortic dilation in these BAV children. Nevertheless, future studies focused on analysing functional alterations caused by miR-130a should be assessed to confirm this hypothesis.

Conclusions:

We propose miR-130a as a solid biomarker candidate for aortic dilation in a pediatric population with BAV. Enrichment analysis also showed that miR-130a expression is related to TGFß signalling alterations, which could lead to endothelial dysfunction, a mechanism involved in the pathogenesis of aortic dilation.

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.

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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.

Author's Contributions: JM. A and R. CH conceptualized the experiments. B. AG wrote the original draft of the main manuscript, as well as figure preparation. JM. A and R. CH reviewed and edited the main manuscript for the final version. B. AG performed all the experiments, analysed the results and prepared the figures. R. CH was in charge of funding acquisition. N. MM: Helped in data curation and data analysis. J. supervised the methodology and results. C. MB, R. CH, B. C and JM. performed and reviewed the echocardiography. All authors reviewed the manuscript.

Conflicts of Interest: JM. A has a patent in miR-718 expression in aortic dilation in bicuspid aortic valve adults.

Figure Legend:

Figure 1. miRNA expression comparison between TAV, BAV_nonDIL and BAV_DIL patients. TAV: tricuspid aortic valve children; BAV_nonDIL: bicuspid aortic valve children without a dilated aorta; BAV_DIL: bicuspid aortic valve children with a dilated aorta. Data are presented as the mean ± SEM. Nonsignificant p values are not included in this figure.

Figure 2. Aortic dilation prediction with AUC graph in children.

Figure 3. Representation of the association between miR-130a dCt and aorta z scores in children**.** AA: Ascending aorta; AR: Aortic root.

Figure 4. Visualization of the strongest related miR-130a target gene predictions.

Figure 5. Related pathways to miR-130a gene targets.

Figure 6. Associated diseases to miR-130a gene targets.

Table 1. Clinical and echocardiographic characteristics:

				p. value			
	TAV	BAV_nonDIL	BAV_DIL	Overall	TAV vs. BAV_nonDIL	TAV vs. BAV_DIL	BAV_nonDIL vs. BAV_DIL
Age (years)	11.7 (3.0)	11.3 (3.9)	11.4 (3.8)	0.932	0.719	0.802	0.958
Sex (female)	13 (32.5%)	5.0 (20.8%)	6 (37.5%)	0.372	0.307	0.538	0.164
Weight (kg)	46.9 (16.4)	47.9 (22.0)	41.8 (15.8)	0.530	0.857	0.278	0.335
Height (cm)	153.4 (18.4)	149.8 (23.3)	147.3 (18.7)	0.546	0.494	0.264	0.725
BSA (m^2)	1.4 (0.3)	1.4 (0.4)	1.3 (0.3)	0.581	0.871	0.261	0.465
LVEDD (mm)	43.1 (4.9)	42.9 (5.5)	43.9 (6.0)	0.826	0.864	0.608	0.583
LVESD (mm)	28.3 (3.5)	27.4 (3.9)	28.5 (4.9)	0.610	0.388	0.799	0.406
EF (%)	63.8 (5.2)	65.6 (6.4)	64.0 (10.8)	0.593	0.221	0.937	0.563
Aortic root (mm)	22.75 (2.7)	24.5 (4.2)	26.7 (4.0)	**0.001**	0.086	**0.001**	0.113
Aortic root z score	-2.30 (0.8)	-0.8 (0.6)	1.9 (1.0)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Ascending aorta (mm)	20.8 (2.1)	23.9 (3.9)	30.1 (5.3)	**<0.001**	**0.001**	**<0.001**	**<0.001**
Ascending aorta z score	-0.2 (0.8)	1.2 (0.5)	3.6 (0.9)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
AVG (mean; mmHg)	5.9 (0.6)	7.9 (4.4)	15.1 (9.8)	**<0.001**	**0.044**	**0.001**	**0.008**
AI (≥1)	0 (0.0%)	9 (37.5%)	12 (75%)	**<0.001**	**0.001**	**<0.001**	**0.013**

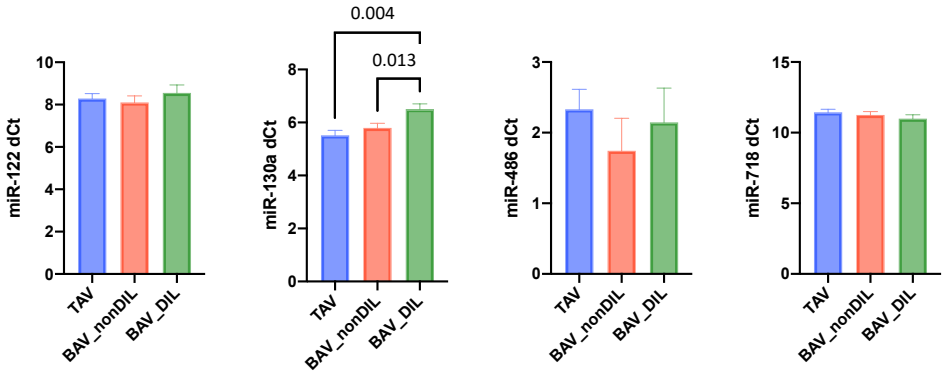
TAV: tricuspid aortic valve children; BAV_nonDIL: bicuspid aortic valve children without a dilated aorta; BAV_DIL: bicuspid aortic valve children with a dilated aorta; BSA: body surface area; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; EF: ejection fraction; AVG: aortic valve gradient AI: aortic insufficiency

Table 2. ANOVA between three groups:

				p. value			
	TAV	BAV_non DIL	BAV_DIL	Overall	TAV vs. BAV_nonDIL p. value	TAV vs. BAV_DIL p. value	BAV_non DIL vs. BAV_DIL p. value
miR-122 dCt	8.3 (1.5)	8.1 (1.5)	8.6 (1.5)	0.646	0.634	0.543	0.646
miR-130a dCt	5.5 (1.2)	5.8 (0.8)	6.5 (0.8)	**0.008**	0.326	**0.004**	**0.013**
miR-486 dCt	2.3 (1.7)	1.7 (2.3)	2.1 (2.0)	0.576	0.298	0.802	0.576
miR-718 dCt	11.4 (11.4)	11.3 (1.1)	11.0 (1.2)	0.478	0.589	0.249	0.478

TAV: tricuspid aortic valve children; BAV_nonDIL: bicuspid aortic valve children without a dilated aorta; BAV_DIL: bicuspid aortic valve children with a dilated aorta.

Figure 1. miRNA expression comparison between TAV, BAV_nonDIL and BAV_DIL patients



TAV: tricuspid aortic valve children; BAV_nonDIL: bicuspid aortic valve children without a dilated aorta; BAV_DIL: bicuspid aortic valve children with a dilated aorta. Data are presented as the mean ± SEM. Nonsignificant p values are not included in this figure.

Figure 2. Aortic dilation prediction with AUC graph in children:

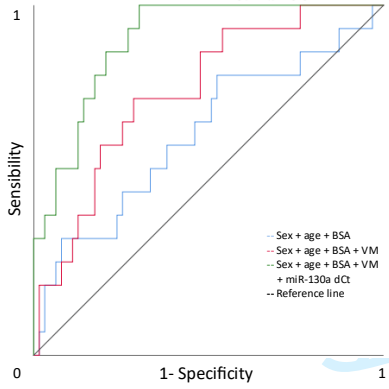


Table 3. Aortic dilation prediction models with AUC analysis:

	AUC	Error Dev.	p value	95% CI
Age + Sex + BSA	0.63	0.09	0.014	0.47-0.79
Age + Sex + BSA + VM	0.75	0.07	0.002	0.63-0.88
Age + Sex + BSA + VM + miR-130a dCt	0.90	0.04	<0.001	0.83-0.97

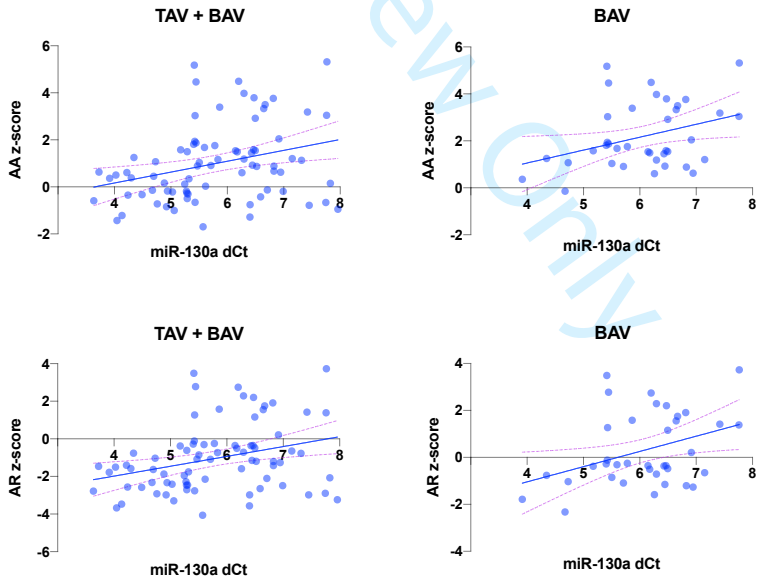
BSA: Body surface area; VM: Valve morphology

Table 4. Correlation analysis between microRNAs and aortic z scores

		AA z score	p value	AR z score	p value
TAV + BAV	**miR-122 dCt**	0.048	0.673	0.057	0.619
	miR-130a dCt	**0.318**	**0.004**	**0.322**	**0.004**
	miR-486 dCt	-0.054	0.633	-0.035	0.758
	miR-718 dCt	-0.103	0.361	-0.108	0.339
BAV	**miR-122 dCt**	0.136	0.402	0.137	0.404
	miR-130a dCt	**0.345**	**0.034**	**0.368**	**0.025**
	miR-486 dCt	0.017	0.914	0.019	0.905
	miR-718 dCt	-0.12	0.453	-0.122	0.455

AA: Ascending aorta; AR: Aortic root

Figure 3. Representation of the association between miR-130a dCt and aorta z scores in children



AA: Ascending aorta; AR: Aortic root

Table 5. Multivariate linear regression of parameters related to ascending aorta and aortic root z score

			B	Error Dev	ß	t	p. value
TAV+ BAV	Ascending Aorta	Constant	-1.693	0.968		-1.749	0.084
		miR-130[a] (by dCt)	0.464	0.164	0.308	2.822	**0.006**
	Aortic Root	Constant	-4.076	1.076		-3.790	**<0.001**
		miR-130a (by dCt)	0.524	0.182	0.315	2.870	**0.005**
BAV	Ascending Aorta	Constant	-1.141	1.528		-0.746	0.460
		miR-130a (by dCt)	0.549	0.249	0.345	2.207	**0.034**
	Aortic Root	Constant	-3.633	1.706		-2.130	**0.040**
		miR-130a (by dCt)	0.648	0.277	0.368	2.339	**0.025**

Figure 4. Visualization of the strongest related miR-130a target gene predictions:

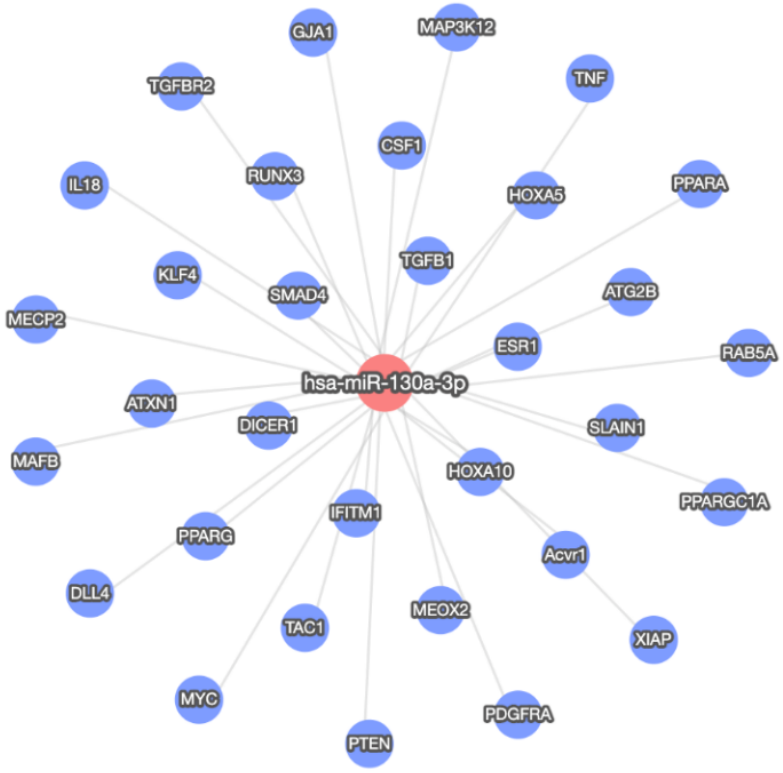


Figure 5. Related pathways to miR-130a gene targets:

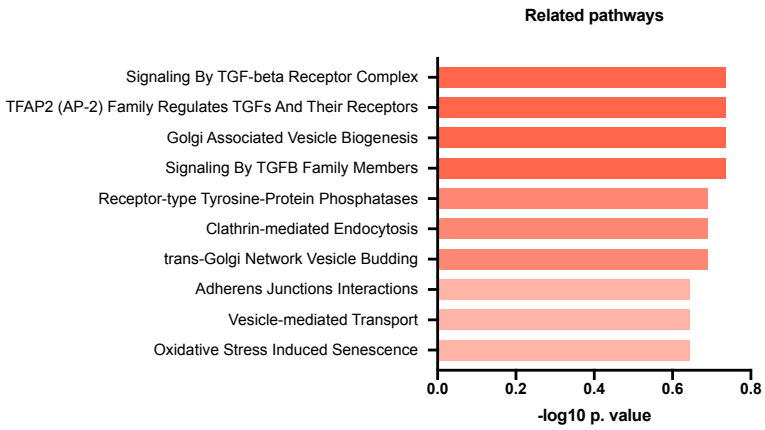
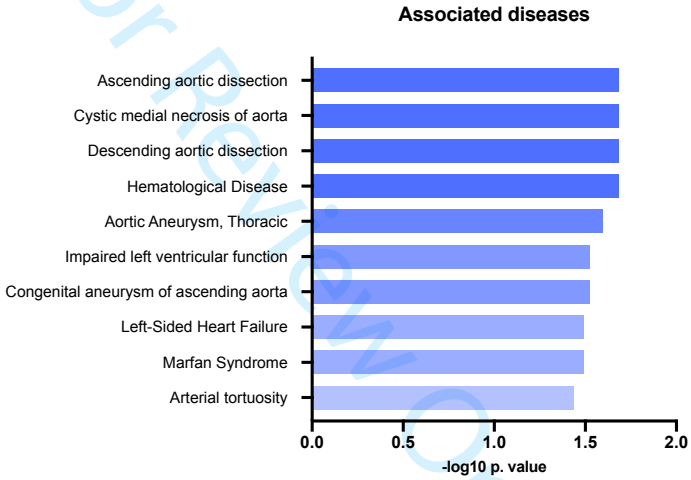


Figure 6. Associated diseases to miR-130a gene targets:



Journal of Molecular and Cellular Cardiology

Unraveling the role of mitochondrial dysfunction in the pathophysiology of aortic stenosis in bicuspid aortic valve disease: integrating RNA-seq and metabolomic profiling

--Manuscript Draft--

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Abstract:	Purpose: Bicuspid aortic valve (BAV) disease is associated with faster aortic valve degeneration and a high prevalence of aortic stenosis (AS). In this study, we aimed to identify differences in the pathophysiology of AS between BAV and tricuspid aortic valve (TAV) patients in a multiomics study integrating metabolomics and transcriptomics, as well as clinical data. Methods: Eighteen patients underwent aortic valve replacement due to severe aortic stenosis: 8 of them had a TAV, while 10 of them had a BAV. RNA sequencing (RNA-seq) and proton nuclear magnetic resonance spectroscopy (1H-NMR) were performed in these tissue samples to obtain the RNA profile and lipid and low-molecular-weight metabolites. These results, combined with clinical data, were posteriorly compared, and a multiomic profile specific for AS in BAV disease was obtained. Results: H-NMR results showed that BAV patients with AS had different metabolic profiles than TAV patients. RNA-seq also showed differential RNA expression between the groups. Functional analysis helped connect this RNA pattern to mitochondrial dysfunction. Integration of RNA-seq, 1H-NMR and clinical data helped create a multiomic profile that suggested that mitochondrial dysfunction and oxidative stress are key players in the pathophysiology of AS in BAV disease. Conclusions: The pathophysiology of AS in BAV disease differs from patients with a TAV and has a specific RNA and metabolic profile. This profile is associated with mitochondrial dysfunction and increased oxidative stress.
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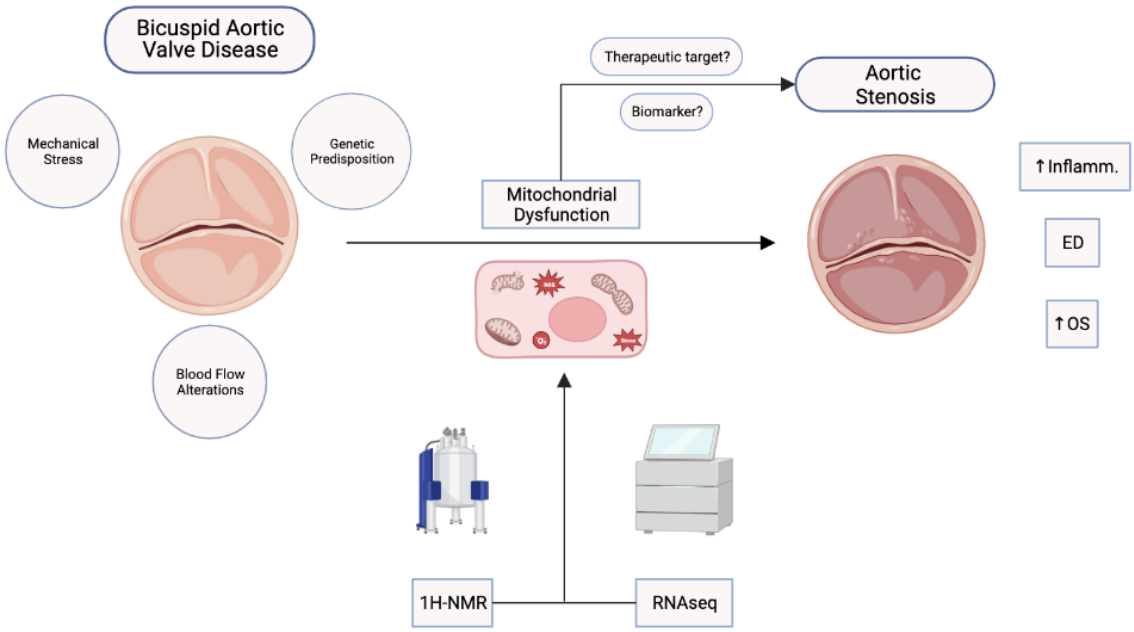

Graphical abstract. ED: endothelial damage; Inflamm.: inflammation; OS: oxidative stress; RNAseq: RNA sequencing; 1H-NMR: 1H nuclear magnetic resonance

Unraveling the role of mitochondrial dysfunction in the pathophysiology of aortic stenosis in bicuspid aortic valve disease: integrating RNA-seq and metabolomic profiling

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Abstract:

Purpose: Bicuspid aortic valve (BAV) disease is associated with faster aortic valve degeneration and a high prevalence of aortic stenosis (AS). In this study, we aimed to identify differences in the pathophysiology of AS between BAV and tricuspid aortic valve (TAV) patients in a multiomics study integrating metabolomics and transcriptomics, as well as clinical data. **Methods:** Eighteen patients underwent aortic valve replacement due to severe aortic stenosis: 8 of them had a TAV, while 10 of them had a BAV. RNA sequencing (RNA-seq) and proton nuclear magnetic resonance spectroscopy (1H-NMR) were performed in these tissue samples to obtain the RNA profile and lipid and low-molecular-weight metabolites. These results, combined with clinical data, were posteriorly compared, and a multiomic profile specific for AS in BAV disease was obtained. **Results:** H-NMR results showed that BAV patients with AS had different metabolic profiles than TAV patients. RNA-seq also showed differential RNA expression between the groups. Functional analysis helped connect this RNA pattern to mitochondrial dysfunction. Integration of RNA-seq, 1H-NMR and clinical data helped create a multiomic profile that suggested that mitochondrial dysfunction and oxidative stress are key players in the pathophysiology of AS in BAV disease. **Conclusions:** The pathophysiology of AS in BAV disease differs from patients with a TAV and has a specific RNA and metabolic profile. This profile is associated with mitochondrial dysfunction and increased oxidative stress.

Keywords:

Bicuspid aortic valve; Aortic dilation; Aortic stenosis; Mitochondrial dysfunction; Oxidative stress; Endothelial damage

Introduction:

Aortic stenosis (AS) is defined as the narrowing of the orifice of the aortic valve due to the stiffness, and sometimes calcification, of the aortic valve leaflets that promote a greater pressure gradient across the valve[1]. AS one of the most common form of heart disease, and its prevalence has increased to 10% of the population in their eight decade[1]. Currently, there is no effective pharmacological treatment, and surgical processes such as transcatheter aortic valve replacement (TAVR) or aortic valve replacement are usually necessary[2]. Of the latter cases, at least 25% of patients 80 years of age or older referred for aortic valve replacement have a bicuspid aortic valve (BAV)[3]. BAV disease is the most common type of congenital heart disease, affecting up to 2% of the general population [4–6]. BAV disease is defined by a two-cusp morphology instead of the normal three-cusp morphology of the aortic valve, which promotes an anomalous transvalvular flow[7–9]. This pathology is associated with quicker degeneration of the valve, with AS developing at younger ages than in individuals with a tricuspid aortic valve (TAV).

In individuals with a tricuspid aortic valve, known AS development risk factors include traditional cardiovascular factors such as smoking, metabolic syndrome, high low-density lipoprotein (LDL) cholesterol and hypertension, among others[10,11]. In BAV disease, the hemodynamic alterations and increased mechanical stress that the anomalous aortic valve is already subjected are believed to also contribute to AS[10]. Despite the fact that there are few studies comparing AS pathophysiology in terms of aortic valve morphologies, some studies have suggested that they may not be equal[12,13]. Although not yet fully understood, endothelial dysfunction is thought to play a key role in AS, due to mechanical stress, leaflet thickening, fibrosis and

calcification in its latest stages[1]. Alterations in lipoprotein metabolism and oxidative stress are also believed to affect the endothelium[14–17]. All these factors seem to affect the hemostasis and permeability of the endothelium, promoting activation of valvular interstitial cells (vICs) (myofibroblasts), which secrete proinflammatory cytokines, matrix metalloproteases and osteogenic factors[14–17]. This directly affects the function of the aortic valve, which loses elasticity and becomes more rigid[18]. The interplay between vECs and vICs, as well as the extracellular matrix (ECM), plays an important role in maintaining proper optimal valve function, and any alterations in any of its components can put it at risk[19].

Untargeted studies in the omics field have helped elucidate the pathophysiology of numerous diseases and have become powerful tools for the identification of new biomarkers[20,21]. Furthermore, untargeted data results can help open pathways to identify the mechanisms that underlie these pathologies and discover new therapeutic targets. In this study, aortic valves from patients with severe aortic stenosis (TAV and BAV) who underwent aortic valve replacement were analyzed. 1H-NMR for metabolomics and RNA-seq for transcriptomics were performed in tissue samples from the aortic valves. The aim of this study was to further understand the differences between the two valves in the pathophysiology of AS and the mechanisms involved in the faster degeneration of the BAV.

Methods:

Patients:

We included 18 patients (8 TAV, 10 BAV) who underwent aortic valve replacement due to severe AS. Patient selection was adjusted for sex, and we tried to select younger patients with TAV due to the known younger age of AS patients with BAV. Patients in which surgery was indicated due to ascending aorta aneurysm or ischemic heart disease were excluded. We also excluded patients with a grade of aortic regurgitation ≥III or chronic kidney disease (eGFR < 45 ml/min).

Aortic tissue extraction:

In this study, 18 patients with an average age of 71 (57-83) years old were included. Aortic valve samples from patients who underwent aortic valve replacement were obtained, snap frozen in liquid nitrogen and stored at -80 °C at the Vall d'Hebron Biobanc. Samples were classified as TAV or BAV samples, both with severe AS.

Tissue homogenization:

Aortic valve tissues were transferred to a mortar under dry ice. Liquid nitrogen was quickly added to prevent denaturalization. Each sample separately was ground into powder and stored in 1.5 mL tubes. Homogenates were stored at -80 °C until use.

Protein extraction:

Aliquots of the samples with the same weight were prepared and kept cold with dry ice and liquid nitrogen to prevent denaturalization. Next, lysis buffer (#IS007 n4, Cloud Clone) was added and supplemented with 1.5 mM PMFS. Each sample was vortexed and stored on ice for 1 hour and then centrifuged at 14,000 rpm to remove debris.

The supernatant (protein phase) was transferred to a new tube and stored at -80 °C until further use.

RNA-seq:

RNA was extracted using the PureLinkTM RNA Mini Kit (ref: 12183018A, Invitrogen) and eluted with 30 µl of RNAse free water. RNA was used for the creation of libraries. RNA was concentrated using speedvac, and then libraries were created with a TruSeq small RNA Library kit (Illumina) following the manufacturer's instructions. DNA libraries were obtained and quantified by microfluidic electrophoresis on the Agilent TapeStation and the Agilent DNA High Sensitivity ScreenTape kit. Then, an equimolar pool was created at a concentration of 450 pM and later amplified by synthesis by adding labeled nucleotides with NextSeq2000 equipment from the Illumina platform. The generated libraries were read maintaining the direction of the transcripts and using 36 cycles. The resulting reads were separated per sample and converted to fastq format. Each sample was aligned to the human GRCh38 reference genome using HiSAT2, and the resulting alignments were used to assemble the transcripts with StringTie, also generating matrix counts of transcripts and their respective genes. Features present in less than 25% of the samples or with a coefficient of variation <10% were removed. Differential expression analysis was performed using DESeq2.

Gene enrichment analysis:

The complete list of significantly altered RNAs was input in Enrichr-KG, an online platform that provides summaries of numerous databases and visualization of collective functions of gene lists[22]. Data results were manually curated to visualize them graphically.

Lipid profile and low-molecular-weight metabolite quantification by 1H-NMR:

For metabolomic analysis by nuclear magnetic resonance spectroscopy (1H-NMR), frozen homogenate aortic valve samples were sent on dry ice to Biosfer Teslab®[23–25]. The 1H-NMR spectra were recorded on a BrukerAvance III 600 spectrometer, with the following certifications and validations: PCT/EP2014/075873, ISO 9001:2015; IVD-CE, ISO 13485,2015; ISO 13485:2016; License of the Spanish Agency for Medicines and Health Products number 6855-PS, 2016. Quality assurance assessments are carried out annually by the company SGS, certified ES19/86886, 2019, and by the company TÜV Rheinland, certified 0.04.15155, 2015.

A target set of 21 low-molecular-weight metabolites (LMWMs), including acetate, alanine, glucose, valine, isoleucine, leucine, gluconate, myo-inositol, lactate, glutamate, pyruvate, glutamine, 3-hydroxybutyrate, glycerol, glycine, formate, mannitol, choline, o-phosphocholine, creatine and histidine, were identified and quantified in the 1D Carr–Purcell–Meiboom–Gill (CPMG) spectra using a Dolphin adaptation. Each metabolite was identified by checking all its resonances across the spectra and then quantified using line shape fitting methods on one of its signals.

Lipophilic extracts were also obtained using the BUME method with slight modifications[26]. BUME was optimized for batch extractions with diisopropyl ether (DIPE) replacing heptane as the organic solvent since the 1H-NMR fingerprint of heptane heavily overlaps the fatty acid signals. This procedure was performed with a BRAVO liquid handling robot that has the capacity to extract 96 samples at a time. The upper lipophilic phase was completely dried in a Speedvac until evaporation of organic solvents

and frozen at -80 °C until NMR analysis. Lipid extracts were reconstituted in a CDCl3:CD3OD:D2O (16:7:1, v/v/v) solution containing 1.18 mM tetramethylsilane (TMS) as a chemical shift reference and transferred to 5 mm NMR glass chemistry tubes. 1H-NMR spectra were measured at 600.20 MHz. A 90° pulse with water presaturation sequence (ZGPR) was used.

Quantification of lipid signals in 1H-NMR spectra was carried out with LipSpin[27], an in-house MATLAB-based software. Resonance assignments were made on the basis of literature values[28]. The 13 lipid species obtained by this NMR approach included cholesterol (free and esterified), triglycerides, unsaturated fatty acids (omega-6+omega-7 and omega-9), saturated fatty acids, polyunsaturated fatty acids, glycerophospholipids, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, linoleic acid and arachidonic+eicosapentaenoic acid.

Statistical Analysis and Multiomics integrative analysis:

Data were analyzed using descriptive statistics. Quantitative variables are expressed as the median and 25-75 interquartile range and were compared using the nonparametric Wilcoxon rank-sum test. Qualitative or dichotomous variables are represented using percentages, and the chi-square test ($\chi 2$) and Fisher's exact test were utilized to compare proportions and examine relationships.

In this study, we examined the predictive capacity of individual metabolites for aortic valve morphology using a separate model that was adjusted for age. Odds ratios were scaled to a 1-standard deviation (SD) increment in log-transformed metabolite concentration to facilitate comparisons across metabolites, and logistic regression was

used to assess their associations with aortic valve morphology. The ggforestplot R package was employed for this analysis.

To identify key variables and patterns that distinguish between tricuspid and bicuspid valve morphologies, we performed an integrative analysis using sPLS-DA in the mixOmics R package. Clinical data, low-molecular-weight metabolites, lipids, and differentially expressed RNAs were integrated in this analysis. Relevant signatures that were highly correlated and associated with aortic valve morphology were visualized using Cytoscape version 3.9.1. These variables were subsequently included in a linear fitting model to predict the valve morphology. To evaluate the accuracy of the selected variables in discriminating between valve morphologies, we computed the area under the curve (AUC) and the 95% confidence interval of a receiver operating characteristics (ROC) curve. Patients were randomly assigned to training (50%) and test (50%) sets, and we performed 10-fold cross-validation with 100 replicates on the training data during the model construction process. The model was then tested on the hold-out data. All statistical analyses were conducted using R version 4.1.1.

Ethics

Written informed consent was obtained from all patients who participated in this study. This study was approved by the Research Ethics Committee (CEIm) of Hospital Universitari Vall d'Hebron.

Results:

Clinical characteristics of the cohort:

Clinical and echocardiographic characteristics are shown in Table 1. The results showed that BAV patients were younger than TAV patients (Table 1). Furthermore, although it did not reach statistical significance, there was a trend toward a higher mean transaortic valvular gradient (mTAVG) and ascending aorta diameter, as well as a higher prevalence of hypertension and diabetes mellitus, in the BAV group than in the TAV group.

Table 1. Clinical characteristics of the cohort.

	TAV (n=8)	BAV (n=10)	p value
Sex			1.000
Female	2 (25.0%)	3 (30.0%)	
Age (y)	77.5 [76.8;78.5]	65.5 [61.2;73.2]	**0.008**
Weight (kg)	78.0 [66.2;106]	75.5 [66.5;82.8]	0.699
Height (cm)	168 [162;172]	168 [156;176]	0.948
BMI (kg/m2)	27.5 [26.4;36.1]	26.9 [25.5;28.1]	0.439
Hypertension	7 (87.5%)	4 (40.0%)	0.066
Diabetes mellitus	6 (75.0%)	2 (20.0%)	0.054
Dyslipidemia	6 (75.0%)	3 (30.0%)	0.153
Smoking			0.588
Ex	2 (25.0%)	1 (10.0%)	
No	6 (75.0%)	7 (70.0%)	
Yes	0 (0.00%)	2 (20.0%)	
Statin	5 (62.5%)	3 (30.0%)	0.342
Renal insufficiency	1 (12.5%)	1 (10.0%)	1.000
Ischemic cardiopathy	5 (62.5%)	2 (20.0%)	0.145
mTAVG (mmHg)	45.0 [42.5;50.0]	55.5 [49.0;62.8]	0.062
AR (≤II)	1 (12.5%)	4 (40.0%)	0.348
LVDD (mm)	48.0 [45.0;52.0]	46.0 [40.8;48.0]	0.447
LVSD (mm)	33.0 [29.5;37.5]	30.0 [24.0;35.0]	0.306
LVEF (%)	60.0 [46.8;64.6]	60.5 [56.2;69.5]	0.475
Ascending aorta (mm)	36.5 [33.5;38.8]	41.5 [37.8;43.0]	0.068
Aortic root (mm)	36.0 [31.0;37.0]	36.5 [34.0;42.8]	0.349

BMI: body mass index; mTAVG: mean transaortic valvular gradient; AR: aortic regurgitation; LVDD: left ventricular diastolic diameter; LVSD: left ventricular systolic diameter; LVEF: left ventricular ejection fraction

RNA pattern depends on aortic valve morphology in AS:

RNA-seq of 18 aortic valve samples from patients with AS and different aortic valve morphologies (BAV and TAV) was performed. After statistical analysis, the expression of 75 RNA molecules was found to be significantly different between the two groups (p value <0.05). The complete data can be found in Supplementary Table 1. After performing multiple comparisons and applying an adjustment method, five of the 75 molecules remained statistically significant with an adjusted p value<0.05. These five RNAs are described and represented in Figure 1.

A)

	Log2 FC	p. value	p. adj
COX7C	21.87	<0.001	<0.001
MTND5P10	-2.57	<0.001	<0.001
FCGR2A	14.62	<0.001	<0.001
SCARNA2	-1.05	<0.001	0.010
RNU12	5.34	<0.001	0.040

B)

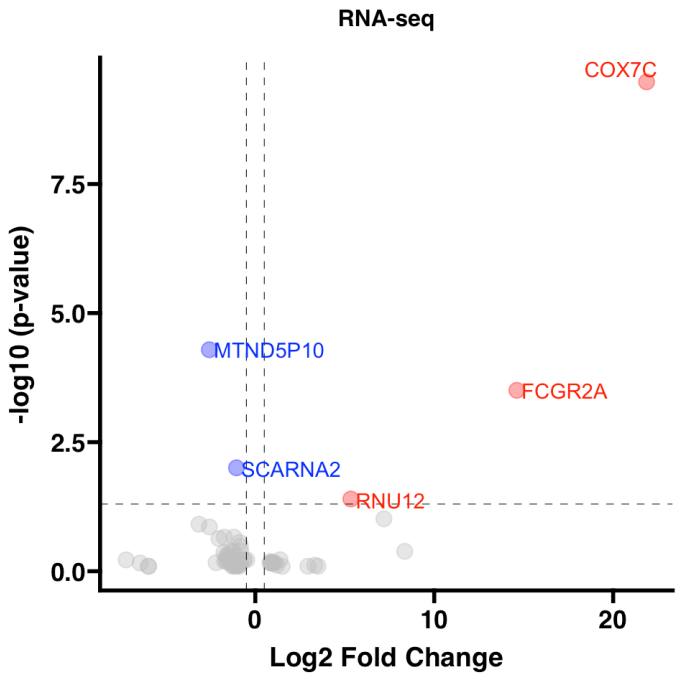


Figure 1. Five most significant RNAs in the TAV vs. BAV RNA-seq comparison. Data are presented in a Table (A) and can be easily visualized in the volcano plot (B), where the overexpressed RNAs are represented as red dots, and the downregulated RNAs are represented as blue dots.

For discovery purposes, the original 75 differentially expressed RNAs before multiple comparisons were used. Functional analysis of the differentially expressed RNAs was performed. The results showed that these RNAs were predominantly related to mitochondrial function and dynamics. Furthermore, the pathology databases also connected this RNA pattern to different cardiovascular diseases and processes (Figure 2).

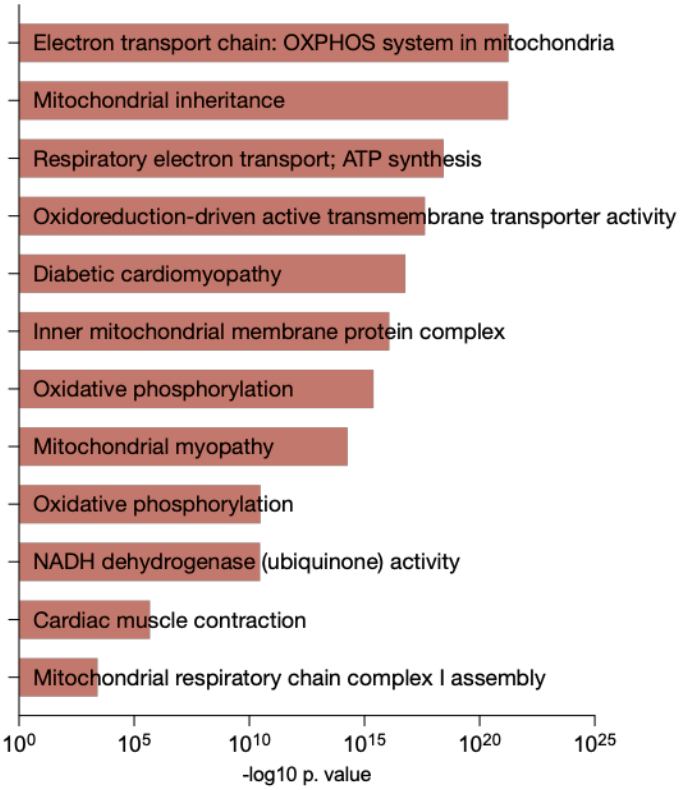


Figure 2. Visualization of RNA enrichment analysis with different databases. OXPHOS: oxidative phosphorylation; ATP: adenosine triphosphate; NADH: nicotinamide adenine dinucleotide (reduced).

<u>The metabolic profile seems to be specified by the aortic valve morphology:</u>

The 1H-NMR analysis showed that some metabolites were differentially expressed in AS when compared by aortic valve morphology. Gluconate and choline levels were decreased in aortic valve samples from BAV individuals, while alanine was increased in stenotic TAV patients (Table 2). However, no differences in lipid metabolites between the groups were found (Table 3).

Table 2. Low-molecular-weight metabolite analysis by 1H-NMR:

Metabolites	TAV (n=8)	BAV (n=10)	p value
Acetate (µmol/gr)	0.79 [0.18;1.43]	0.50 [0.21;0.67]	0.183
Alanine (µmol/gr)	0.08 [0.06;0.13]	0.15 [0.11;0.19]	**0.046**
Glucose (µmol/gr)	1.05 [0.83;1.83]	1.48 [0.74;1.73]	0.753
Valine (µmol/gr)	0.07 [0.07;0.08]	0.07 [0.06;0.08]	0.549
Isoleucine (µmol/gr)	0.03 [0.03;0.04]	0.04 [0.03;0.06]	0.201
Leucine (µmol/gr)	0.06 [0.04;0.07]	0.05 [0.04;0.07]	0.655
Gluconate (µmol/gr)	0.65 [0.45;1.19]	0.27 [0.09;0.44]	**0.045**
Myo-Inositol (µmol/gr)	0.38 [0.36;0.42]	0.23 [0.19;0.31]	0.188
Lactate (µmol/gr)	2.93 [2.08;3.76]	2.96 [2.29;4.17]	0.929
Glutamate (µmol/gr)	0.38 [0.30;0.45]	0.25 [0.13;0.31]	0.126
Pyruvate (µmol/gr)	0.02 [0.01;0.02]	0.02 [0.01;0.02]	0.462
Glutamine (µmol/gr)	0.28 [0.19;0.29]	0.23 [0.18;0.31]	0.655
3-Hydroxybutyrate (µmol/gr)	0.04 [0.03;0.18]	0.08 [0.07;0.10]	0.380
Glycerol (µmol/gr)	0.04 [0.03;0.07]	0.04 [0.02;0.05]	0.286
Glycine (µmol/gr)	0.11 [0.06;0.14]	0.14 [0.09;0.17]	0.657
Formate (µmol/gr)	0.16 [0.14;0.19]	0.22 [0.18;0.27]	0.050
Mannitol (µmol/gr)	0.92 [0.56;1.43]	0.34 [0.26;0.75]	0.167
Choline (µmol/gr)	0.08 [0.06;0.09]	0.05 [0.04;0.06]	**0.041**
O-Phosphocholine (µmol/gr)	0.16 [0.09;0.16]	0.10 [0.08;0.12]	0.149
Creatine (µmol/gr)	0.04 [0.04;0.05]	0.04 [0.03;0.07]	0.886
Histidine (µmol/gr)	0.08 [0.08;0.08]	6.47 [4.02;8.91]	0.221

Table 3. Lipid profile quantification by 1H-NMR:

Lipids	**TAV (n=8)**	**BAV (n=10)**	**p value**
Esterified cholesterol (µmol/gr)	61.3 [53.5;73.5]	76.1 [68.6;93.0]	0.214
Free cholesterol (µmol/gr)	43.5 [36.9;47.8]	51.0 [35.4;56.7]	0.477
Triglycerides (µmol/gr)	12.0 [9.29;14.2]	15.3 [9.77;19.4]	0.594
Glycerophospholipids (µmol/gr)	10.8 [10.3;11.1]	11.5 [10.8;12.0]	0.286
Phosphatidylcholine (µmol/gr)	8.23 [7.48;9.45]	9.57 [7.83;10.3]	0.248
Sphingomyelin (µmol/gr)	13.7 [11.6;15.1]	17.7 [12.2;22.2]	0.214
Lysophosphatidylcholine (µmol/gr)	1.76 [1.60;1.92]	2.12 [1.49;2.20]	0.328
Polyunsaturated fatty acids (µmol/gr)	129 [115;158]	128 [110;143]	0.790
Linoleic acid (µmol/gr)	53.6 [47.9;56.9]	59.1 [48.9;68.7]	0.286
Saturated fatty acids (µmol/gr)	77.2 [71.6;81.2]	81.1 [67.8;91.8]	0.424
Omega-6 and fatty acids (µmol/gr)	16.9 [13.6;18.2]	19.6 [14.8;21.6]	0.374
Omega-9 fatty acids (µmol/gr)	49.4 [46.3;72.8]	69.9 [57.7;84.5]	0.248
ARA + EPA (µmol/gr)	16.1 [15.2;22.1]	16.3 [15.4;19.6]	0.790

ARA: arachidonic acid; EPA: eicosapentaenoic acid

Age differences between the groups were eliminated by adjusting metabolites by age. The association of each metabolite with age-adjusted valve morphology was also determined by logistic regression and is represented in Figure 3. This method helped us solidify our previous results, where alanine was found to be upregulated in BAV disease with AS, whereas gluconate, myo-inositol, glutamate and choline were found to be downregulated in TAV disease with AS.

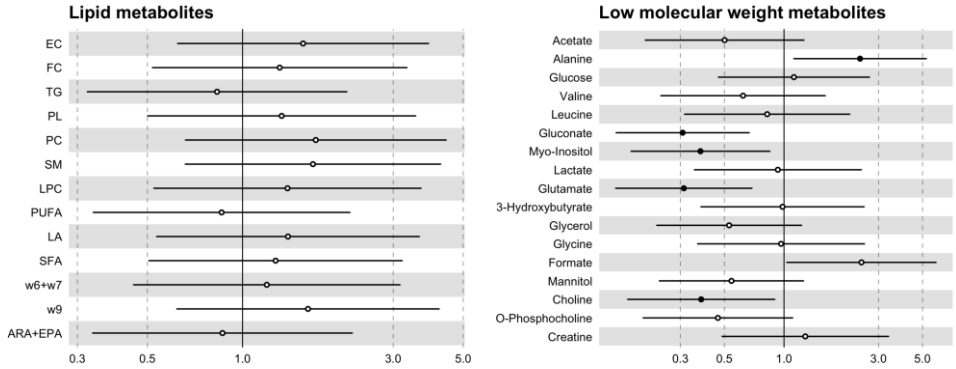
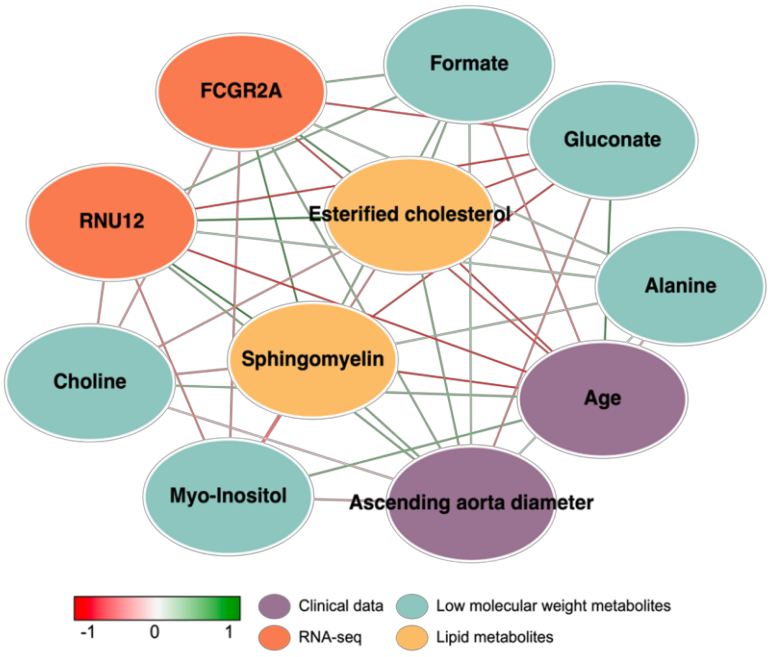


Figure 3. Association between lipid profile and low-molecular-weight metabolites with age-adjusted valve morphology. Odds ratio for aortic valve morphology (95% CI) per 1-SD increment in metabolite concentration. The black circle indicates a p value < 0.05. EC: esterified cholesterol; FC: free cholesterol; TG: triglycerides; PL: phospholipids; PC: phosphocholine; SM: sphingomyelins; LPC: lysophosphatidylcholine; PUFA: polyunsaturated fatty acids; LA: linoleic acid; SFA: saturated fatty acids; w6+w7: omega 6 and 7; w9: omega 9; ARA+EPA: arachnoid acid+eicosapentanoic acid

Integrative analysis of clinical, 1H-NMR and RNA-seq data

The aim of data integration was to find a pattern of variables highly associated with each other that could classify patients according to their valvular morphology. The data used for integration were as follows: clinical data, low-molecular-weight metabolites, lipids and differentially expressed RNAs determined by RNA-seq. Through integration, the selected variables formed the network shown in Figure 4A. PLS-DA represented in Figure 4B shows the potential of the selected variables to distinguish between patients based on their aortic valve morphology.

A)



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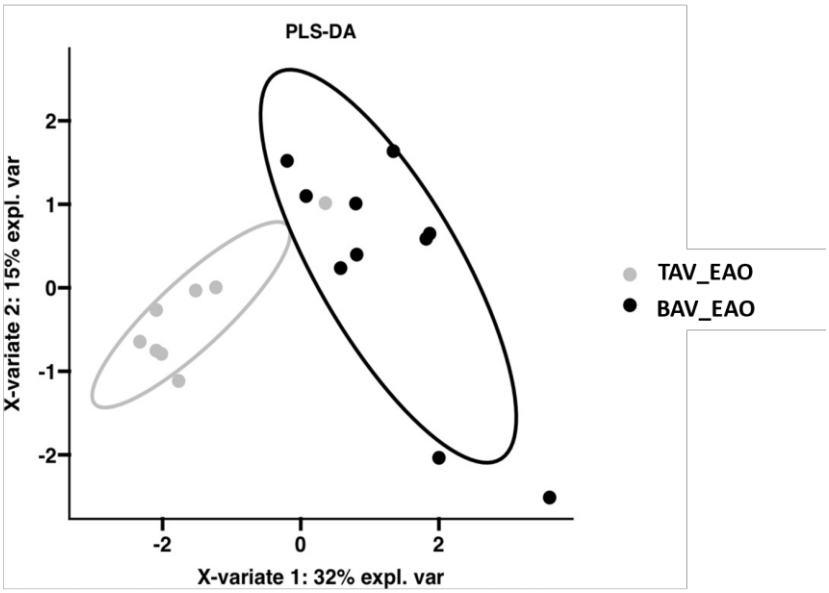

Figure 4. Visualization of data integration. A) Network with parameters symbolized with nodes classified by color and associations with each other represented with red or green lines. B) PLS-DA with X variables explaining 32% and 15% of the cases divided by aortic valve morphology.

The previously selected variables were included in a multivariate analysis to validate the proposed integrational model. The model was developed using 50% of the patients as training data, and the performance of the model was assessed using the remaining 50% of samples as a test set. Figure 5A illustrates the contribution of each variable in the classification of patients based on their valve morphology. The ROC curve depicted an area under the curve (AUC) of 0.9 (95% CI = 0.67-1), indicating high predictive accuracy. The model demonstrated an overall accuracy of 0.89, a sensitivity of 1, a specificity of 0.75, a p value of 0.041, and an out-of-bag (u) error value of 0.28, as shown in Figure 5B.

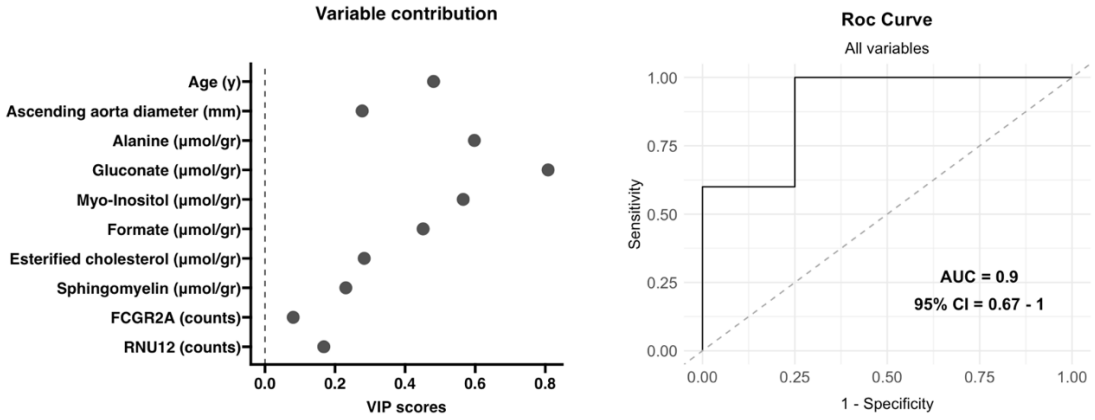

Figure 5. Evaluation of the multiomics integration model. This model included the variables shown on the left with their contributions (VIP scores) and an ROC curve that classified aortic valve morphology on the right.

Discussion:

In the present study, we found a specific metabolic profile for AS patients with BAV when compared to TAV patients by 1H-NMR in aortic valve tissues. Metabolic data showed that gluconate, myo-inositol, glutamate and choline were downregulated in BAV individuals compared to TAV individuals. On the other hand, alanine was upregulated. The results of RNA-seq in aortic valve tissue also showed a different RNA pattern when comparing both groups, which was bibliographically related to mitochondrial dysfunction and associated with numerous cardiovascular diseases. Furthermore, integration of the clinical, 1H-NMR and RNA-seq data demonstrated that the pathophysiology of AS may be specified by the aortic valve morphology and that the mechanisms that underlie this pathology are not the same between BAV and TAV patients.

Abnormal aortic valve morphology is known to cause hemodynamic alterations. Although few studies have focused on vECs in BAV disease, some have noted the activation of these vECs by abnormal shear stress patterns[29–32]. Paracrine signaling, calcification and catabolic enzyme secretion have also been associated with activity of vECs. Similar to what happens in the aortic media, these changes in hemodynamics could be capable of altering correct vEC functioning[33–35]. Altered blood flow can exert different effects in endothelial cells, including inflammation, increased oxidative stress, and mitochondrial function, due to mechanotransduction[36,37]. Furthermore, abnormal valve dynamics, such as those in BAV disease, could also influence this process by the increased mechanical stress during opening the leaflets are submitted to[38]. Endothelial cells can sense and transduce the increased shear stress into different intracellular biochemical signals[39]. This could play a key role in AS progression when combined with the highly discussed predisposition of endothelial dysfunction present in BAV

disease[40]. The interplay between vECs and vICs, as well as the ECM, is essential to maintaining correct vascular function and homeostasis[41,42]. Furthermore, some studies have found that shear stress can even play a role in mitochondrial homeostasis in endothelial cells by controlling ATP generation and mitochondrial membrane potential[43,44]. Additionally, low shear stress prevents oxidative stress by enhancing the expression of mitochondrial antioxidant enzymes as well as mitochondrial impairment produced by inflammatory molecules such as TNFα[43]. Although mitochondrial dysfunction has been discovered in numerous cardiovascular diseases, no definitive answers have been found in BAV disease[45]. Furthermore, mitochondrial dysfunction has recently been the focus for new therapeutic targets in numerous diseases[45,46].

Mitochondrial dysfunction is present in many diseases, and recently, numerous studies have identified its key role in many cardiovascular diseases. Mitochondria are crucial organelles that not only function in ATP production but are also the primary source of reactive oxygen species (ROS), triggering oxidative stress, and can regulate cell death and survival[47,48]. Mitochondria are not static organelles; they are constantly subjected to an equilibrium of fusion and fission termed mitochondrial dynamics[49,50]. Both processes have an equilibrated interplay in physiological conditions that support cell and mitochondrial homeostasis. Nevertheless, in pathological conditions, these processes have been observed to be imbalanced[50]. Mitochondrial damage has also been related to alterations in metabolism[51]. In our study, gluconate, myo-inositol, glutamate and choline were observed to be downregulated in BAV individuals with AS compared to TAV individuals with AS. The expression alterations of these molecules have been previously related, to a greater or a lesser extent, to mitochondrial damage or dysfunction[52–55]. Specifically, myo-inositol has recently been found to serve as an

inhibitor of mitochondrial fission[53]. On the other hand, alanine was found to be upregulated in BAV patients compared to TAV patients. High levels of alanine have been related to a reduction in ATP generation in the mitochondria, enhancing oxidative stress[56]. Therefore, it seems that the metabolic profile present in BAV disease may be related to altered mitochondrial dynamics and increased oxidative stress.

RNA-seq also showed a differential RNA profile between the BAV and TAV groups with severe AS. The 5 most significant RNAs were presented, and some of them had already been linked with alterations in mitochondrial homeostasis. COX7C, the most significantly altered RNA, corresponds to a subunit of cytochrome c oxidase, the terminal component of the mitochondrial respiratory chain, which drives oxidative phosphorylation[57]. This nuclear-encoded subunit is hypothesized to function in the regulation and assembly of the complex[58]. The second most differentially expressed RNA, MTND5P10, is a mitochondrial pseudogene whose function remains unknown. Nevertheless, aberrant expression in mitochondrial pseudogenes has already been observed in other pathologies, such as Alzheimer's disease[59]. FCGR2a plays a key role in immune processes and is known to mediate changes in the plasma membrane potential of mitochondria and inflammation[60]. The other two most significant RNAs, SCARNA2 and RNU12, were less abundant but also bibliographically related to mitochondria: SCARNA2 is believed to play a role in the miR-342-3p-EGFR/BCL2 pathway[61], which controls mitochondrial apoptosis, and RNU12 also seems to be associated with altered mitochondrial electron transport in other pathologies[62].

Omics has already been used in the study of the pathophysiology of AS and its progression to calcific aortic valve disease (CAVD), mainly in TAV patients[20]. Some

studies have observed a different proteomic architecture in the ECM of valve cells in CAVD compared to healthy control valves[63]. Furthermore, the metabolomic profile was also found to be AS progression dependent, where lysophosphatidic acid was positively associated with the AS grade[64]. A review with a multiomic focus also highlighted the different patterns found in omic sciences for AS[20]. Nevertheless, few comparative assays have compared AS development according to aortic valve morphology. A transcriptomic analysis showed similar mechanisms in TAV and BAV, including increased inflammation, downregulation of NOTCH-1 signaling and ECM alterations.[12] Nevertheless, dysregulation of fetal gene programs was present, demonstrating that although similar, AS development in BAV disease differs from that in TAV[12]. In our study, a larger sample size for transcriptomics, combined with metabolomics, helped us discover a multiomic-specific profile for AS in BAV disease. This helped us connect AS in BAV disease to mitochondrial dysfunction.

Abnormal bicuspid aortic valve dynamics, including opening restrictions and blood flow alterations, promote high shear stress and turbulent eccentric flow, which could stimulate endothelial dysfunction in the aortic valve leaflets[7–9,40]. We hypothesize that these dynamic alterations, combined with the predisposition of endothelial dysfunction inherent to BAV disease, could alter vECs, including correct mitochondrial function, partly through mechanotransduction pathways. These alterations could be part of the explanation for the increased oxidative stress and endothelial dysfunction observed in BAV disease[40,65,66].

Limitations:

The main limitation of our study was the sample size for aortic valve tissues. As there were 18 samples, all of them were used for transcriptomics and metabolomics, providing a rigorous omic analysis but leaving no additional samples to validate the results obtained. Age differences between both groups were also present. Therefore, the metabolic profile was adjusted by age, and the complete integration of clinical, metabolic and transcriptomic data was performed. These findings only strengthened our previous results. Furthermore, no subsequent studies analyzing mitochondrial dysfunction or dynamics *in vivo* or *in vitro* were performed, as our initial study was only for discovery, not validation. Mitochondrial dysfunction should be specifically studied in BAV disease with AS in larger cohorts, and its utility as a new therapeutic target should also be assessed.

Conclusions:

Aortic valve tissue samples from patients with BAV disease have different metabolic and RNA profiles than those from TAV individuals. This indicates that the pathophysiology of AS differs between BAV individuals and TAV individuals. In BAV disease, AS was related to increased oxidative stress and mitochondrial dysfunction, which could be directly related to the endothelial dysfunction and hemodynamic alterations present in this disease. New therapeutic targets focusing on rescuing mitochondrial and endothelial function should be assessed to evaluate their utility in slowing aortic valve degeneration in BAV individuals.

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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.

Conflicts of Interest: authors declare no conflict of interest.

Author's Contributions: JM. A and N. MM conceptualized the experiments. B. AG wrote the original draft of the main manuscript. N. MM performed the statistical analysis. B. AG processed tissue samples. B. AG helped in data curation and data analysis. JM. A and N. MM reviewed and edited the main manuscript for the final version. B. AG performed all the experiments, analyzed the results and prepared the figures. JM. A was in charge of funding acquisition. L. G-G performed the echocardiography and recruited the participants. A. E supervised the process. MS. SR, NE. PC and C. S-B performed the aortic valve replacement. All authors reviewed and approved the manuscript.

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Resumen

Resumen

Justificación

La VAB es la malformación congénita más frecuente en humanos, existiendo en un 1-2% de la población. Pese a ello, hoy en día existen todavía numerosas incógnitas sobre su origen multicausal, así como sobre la fisiopatología de sus complicaciones asociadas como son la DA y la EA. La DA es una de las complicaciones asociadas más frecuentes en la VAB. Aunque la DA también ocurre en pacientes con VAT, su prevalencia es muy superior en pacientes con VAB. La progresión de la DA puede llegar a ocasionar una aneurisma, y finalmente una disección aórtica, siendo potencialmente mortal. La DA es asintomática y puede comenzar a edades tan tempranas como en pacientes pediátricos y adolescentes. Por ello se practican controles periódicos con técnicas de diagnóstico por imagen para evaluar la progresión de los diámetros aórticos. Actualmente no existen tratamientos farmacológicos óptimos que ayuden a ralentizar este proceso acelerado en la VAB ni biomarcadores alternativos consolidados para una detección precoz, así como pronóstico. Por lo tanto, parece necesaria la búsqueda de nuevos biomarcadores de DA en la VAB que nos permitan estratificar a los pacientes según su riesgo. Además, esto nos ayudaría a entender mejor la fisiopatología de la DA en la VAB, así como su mayor prevalencia en este tipo de pacientes. Por otro lado, existe una degeneración más rápida de la VA en pacientes con VAB, lo que promueve la aparición de la EA. Este estrechamiento de la válvula puede manifestarse desde el propio nacimiento (estenosis neonatal) o lo que es más habitual, ir desarrollándose a lo largo de la vida del paciente (estenosis adquirida). Aunque la EA puede ocurrir también en pacientes con VAT, el desarrollo de la misma parece diferir con respecto a los pacientes con la VAB.

La alteración del flujo y/o predisposición genética de la propia válvula son las principales teorías causantes de las complicaciones asociadas a la VAB. Aunque cada vez se conoce más sobre la fisiopatología de la DA y la EA, los mecanismos que provocan su desarrollo y vías implicadas aún no están completamente dilucidados. Dadas las graves consecuencias que pueden acarrear la progresión de la DA y la EA acelerada en la VAB, su seguimiento rutinario es fundamental.

Por lo tanto, el propósito de este estudio multiómico fue, gracias a la integración de la metabolómica y transcriptómica, profundizar en los mecanismos que ocurren en la VAB, así como en sus complicaciones asociadas, la DA y la EA. Dada la complejidad de esta valvulopatía, la identificación de nuevos marcadores de diagnóstico y pronóstico, que suplementen a las técnicas de diagnóstico por imagen, es también necesaria. Adicionalmente, esto supondría conocer mejor el desarrollo y la fisiopatología de las complicaciones asociadas a la VAB. Finalmente, la identificación de nuevos biomarcadores podría abrir la puerta a dianas terapéuticas alternativas que ayuden a frenar el desarrollo de estas patologías asociadas a la VAB.

Metodología

Para la realización de estos 3 estudios se ha utilizado una cohorte de pacientes de dos centros diferentes, así como de tipos de muestra diferente: plasma y tejido valvular aórtico. Para los estudios 1 y 2 se usaron muestras de plasma procedentes del biobanco Cardiobanc del IISPV (Reus). Estas incluían pacientes adultos para el primer estudio, de distinta morfología valvular y presencia de posibles complicaciones asociadas a la VAB. En el caso del segundo estudio, los pacientes eran menores de edad y se incluyeron también niños con VAB y DA o sin ella, además de controles sanos con VAT. Finalmente, para el estudio 3 se utilizaron muestras de tejido valvular aórtico de pacientes adultos con VAB o VAT sometidos a recambio valvular por EA severa. En este caso, las muestras procedían del Hospital Universitari Vall d'Hebron. Todos los participantes, o tutores legales en el caso de los menores de edad, fueron informados y dieron su consentimiento por escrito. Los estudios se realizaron de acuerdo con los principios de la Declaración de Helsinki. Los dos primeros estudios fueron aprobado por el Comité Ético de Investigación Clínica del Hospital Universitari de Sant Joan y una posterior revisión por el Comité de Ética de Investigación con Medicamentos (CEIm) del Institut d'Investigació Sanitària Pere Virgili. En el caso del tercer estudio, éste fue aprobado por el CEIm del Hospital Universitari Vall d'Hebron.

Estudio 1. En una cohorte de pacientes adultos con VAB o VAT, con presencia o ausencia de DA, se utilizó la técnica no dirigida LS/MC para analizar el metaboloma y encontrar expresiones diferenciales de metabolitos en plasma. Tras ello se estudió la función del alfa-tocoferol, que fue el metabolito hallado que se expresaba

diferencialmente entre grupos. La posible relación de la DA, el estrés oxidativo, la inflamación y la disfunción endotelial que se dan en esta patología fue, a su vez, asociada con la funcionalidad y metabolismo del alfa-tocoferol.

Estudio 2. En este caso, se pretendió validar en una cohorte pediátrica un patrón de miRNAs previamente identificados en adultos por su relación con la VAB y DA. En este estudio se incluyeron muestras de plasma de pacientes menores a 18 años (media de 12, rango 4-17) con VAB y VAT. Del grupo de VAB, un 40% se definió como dilatados al tener un z-score>2. Tras aislar el ARN en plasma por kits comerciales, se utilizó la técnica RT-qPCR para cuantificar el miR-122, miR-130a, miR-486 y miR-718 y posteriormente el método delta-delta Ct para calcular los valores de expresión relativa. Finalmente se realizaron análisis funcionales y de enriquecimiento con los genes diana del miR-130a.

Estudio 3. En este caso se utilizaron muestras de tejido valvular aórtico de pacientes que habían sido sometidos a recambio valvular aórtico por una EA grave. De ellos, 8 tenían VAT mientras que los 10 restantes tenían VAB. Tras el procesamiento y homogeneización del tejido, se procedió a estudiar el transcriptoma con RNAseq y metaboloma mediante 1H-NMR. Estos resultados se combinaron con los datos clínicos de los pacientes para estudiar las diferentes fisiopatologías de la EA en función de la morfología valvular.

Resultados

Estudio 1. Tras el análisis metabolómico no dirigido en plasma, se encontró que los pacientes con VAB, y en especial con DA, tenían una menor capacidad antioxidante. Esto se determinó por los niveles bajos en alfa-tocoferol, paraoxonasa 1 y HDL. Además, se relacionó a su vez con inflamación y disfunción endotelial al verse incrementados los niveles de proteína C reactiva (CRP) y los niveles de EMPs. Tras realizar un análisis con *random forest*, se confirmó la gran capacidad del alfa-tocoferol junto a CRP y EMPs como modelo de diagnóstico de la VAB.

Estudio 2. En este caso, tras analizar la expresión de los 4 miRNAs mencionados anteriormente en plasma de una cohorte pediátrica, se observó una disminución de la expresión de miR-130a en pacientes con VAB y DA en comparación con los pacientes VAB sin dilatación y también con controles con VAT sanos. Además, la expresión de miR-130a se pudo correlacionar inversamente con los z-scores de la aorta ascendente y la raíz aórtica. Finalmente, se realizó una curva ROC con distintos parámetros que sirvió para demostrar la gran capacidad del miR-130a en plasma como biomarcador de diagnóstico de DA en pacientes pediátricos con VAB.

Estudio 3. Los resultados de 1H-RMN mostraron un perfil metabolómico diferente entre los pacientes con VAB y los que tenían VAT, ambos con EA. La RNAseq también mostró un patrón de RNAs diferente entre ambos grupos. El análisis funcional de estos RNAs nos permitió asociarlo en VAB con disfunción mitocondrial y diferentes enfermedades cardiovasculares. Además, el perfil metabolómico coincidía bibliográficamente con esta disfunción mitocondrial. La integración de los datos ayudó a crear un perfil multiómico específico de la VAB y EA, y que supone que la fisiopatología de la EA varía según la morfología valvular.

Conclusiones generales:

- Parece existir una menor capacidad antioxidante, mayor inflamación y daño endotelial en la VAB, así como en el desarrollo de la DA, caracterizado por un incremento de CRP y EMPs y disminución del alfa-tocoferol.
- El modelo de predicción de la morfología valvular compuesto por alfa-tocoferol, CRP y EMPs podría ser de utilidad en su diagnóstico.
- La expresión de miR-130a en plasma era inferior en los pacientes pediátricos con VAB y DA en comparación con los VAB no dilatados y controles.
- El miR-130a se postula como biomarcador de DA en pacientes pediátricos con VAB.
- Los pacientes con VAB tienen un perfil metabolómico y transcriptómico diferente con respecto a los que tienen VAT, ambos con EA, indicando diferencias en su fisiopatología.

- Estos cambios se asociaron con disfunción mitocondrial y estrés oxidativo, hipotéticamente debido a los cambios hemodinámicos y de estrés mecánico presentes en la VAB.
- La disfunción mitocondrial observada en tejido podría ser un mecanismo presente en la VAB relacionado con la disfunción endotelial, inflamación e incremento del estrés oxidativo observado a nivel sistémico.
- Se debería valorar la restauración de la función mitocondrial como diana terapéutica en estos pacientes para frenar la progresión de esta patología y sus complicaciones asociadas.

Discusión

Discusión

Los resultados obtenidos en el presente trabajo ponen de manifiesto la utilidad de las ómicas (metabolómica y transcriptómica) y su integración en el estudio de las complicaciones asociadas a la VAB, en concreto la DA y la EA. Gracias a la utilización de LC/MS para estudiar el metaboloma en plasma, se consiguieron asociar los niveles de alfa-tocoferol con las distintas morfologías valvulares y el tamaño de la aorta ascendente. Los pacientes con VAB tenían unos niveles bajos de alfa-tocoferol en comparación con los individuos con VAT, lo cual se relacionó posteriormente con una peor capacidad antioxidante. Esto, además, fue complementado por el descenso observado en los niveles de HDL, que fue medida como ApoA1. Por otro lado, se observó que los pacientes con VAB mostraban en plasma un perfil más inflamatorio y de disfunción endotelial, caracterizado por un incremento de CRP y EMPs. Finalmente, se consiguió crear un modelo predictor de la VAB con CRP, EMPs y alfa-tocoferol, siendo esta última la variable de mayor contribución. En el siguiente apartado del estudio se analizó la capacidad predictora de DA en población pediátrica con VAB de un perfil de miRNAs plasmáticos que previamente se había asociado a la VAB y DA en una población adulta por nuestro grupo en un estudio transcriptómico. Se observó una disminución significativa de los niveles de miR-130a en plasma en pacientes pediátricos con VAB y DA en comparación con VAB o VAT sin DA. Además, la expresión de miR-130a se correlacionó inversamente con los z-scores de la aorta ascendente y raíz aórtica. Finalmente, también se creó un modelo con gran valor diagnóstico de DA pacientes pediátricos con VAB añadiendo la expresión de miR-130a a los parámetros básicos de edad, sexo, peso, talla y morfología valvular. Continuamos con el último apartado del trabajo, en el que se analizaron muestras de tejido valvular aórtico de pacientes con EA severa sobre VAB o VAT. En este caso, la transcriptómica nos permitió hallar un patrón diferencial de RNAs específico de la VAB que difería de la VAT. Este perfil fue a su vez relacionado, por estudios funcionales, con la disfunción mitocondrial. Los estudios adicionales de metabolómica confirmaron estos resultados al existir también diferencias en diversos metabolitos entre ambos grupos. Esta alteración podría estar causada, en parte, por los cambios hemodinámicos inherentes a la VAB, así como el estrés mecánico al que están sometidas las células valvulares debido a su dinámica de apertura anómala. Gracias al conjunto de resultados, se relacionaron las alteraciones hemodinámicas con la

disfunción mitocondrial, que podría ser uno de los mecanismos implicados en la disfunción endotelial y peor capacidad antioxidante observada anteriormente en la VAB.

Las ómicas han conseguido superponerse a las tradicionales técnicas dirigidas dada su inmensa capacidad de detección de distintos parámetros (metabolitos, RNAs…) de forma simultánea. Estas técnicas no dirigidas están siendo usadas gracias a su gran utilidad para comprender mejor los mecanismos que intervienen en numerosas patologías, como las enfermedades cardiovasculares[195,201,202]. Además, la profundización sobre el conocimiento de su fisiopatología ha supuesto el descubrimiento de nuevos biomarcadores e, incluso, dianas terapéuticas[197]. La integración de diversas ómicas, los estudios multiómicos, consiguen una visión aún más amplia gracias a su enfoque multidisciplinar y constituyen el método más óptimo para descifrar las fisiopatologías más complejas o aún no dilucidadas, como ocurre en la VAB[203,204]. La VAB conlleva riesgo de DA y EA[22], entidades que pueden progresar durante el seguimiento. En cualquiera de los casos, parece ser que la fisiopatología de ambas complicaciones varía con respecto a lo que ocurre en pacientes con VAT. Por ello, en este trabajo se propuso realizar un estudio multiómico, mediante la integración de la metabolómica y transcriptómica, para indagar en los mecanismos que acaecen tanto en la VAB como en sus complicaciones asociadas, la DA y la EA.

En primer lugar, se utilizó la metabolómica en el estudio de expresiones diferenciales de metabolitos en la VAB y su DA asociada. En nuestro caso, aunque se identificó tanto el alfa-tocoferol como la colina como biomarcadores de la morfología valvular, solo se mantuvo significativo el alfa-tocoferol al añadir pacientes con VAB y sus complicaciones asociadas, DA y EA. Cabe destacar que ciertos pacientes tenían valores de diámetro de la aorta o EA muy extremos, propuestos para cirugía, y podrían tener un metaboloma tan alterado que puede explicar la pérdida de significancia en el caso de la colina. Además, el grupo con VAB, aún sin DA significativa, tenía de forma inherente un diámetro mayor de la aorta ascendente en comparación con los individuos con VAT (32.56 ± 0.4 vs. 30.81 ± 0.6; p=0.003). Esto nos hizo hipotetizar que la disminución del alfa-tocoferol en la VAB estaba asociada a la patogénesis de ésta junto al remodelado vascular que ocurre en la progresión de la DA. El alfa-tocoferol es la forma primaria de la vitamina E, que tiene una gran capacidad antioxidante para neutralizar radicales libres endógenos[187]. Esta vitamina es transportada en sangre en su mayoría por

HDL, aunque también va unida a la albúmina[205,206]. Encontramos una relación entre los niveles de alfa-tocoferol, apoA1, albúmina y paraoxonasa 1, enzima transportada por HDL con capacidad antioxidante y antiaterogénica[207]. Además, el alfa-tocoferol actúa como regulador del metabolismo lipoproteico e inflamación. En el caso de la colina, que ha sido identificada previamente en la VAB por encontrarse diferencialmente expresada, se trata de un nutriente esencial que es metabolizado en el hígado mayoritariamente[208]. Tiene funciones cardioprotectoras al regular la inflamación, la estructura del endotelio e inhibir la formación de ROS[209–212]. Gracias a la información obtenida de los metabolitos diferencialmente expresados por LC/MS, relacionamos estos hallazgos con otros parámetros indicadores de disfunción endotelial e inflamación, EMPs y CRP. Con estas dos variables y el alfa-tocoferol se pudo crear un modelo que diferenciaba con gran confianza a los pacientes según su morfología valvular.

Con relación a la DA asociada a la VAB, un estudio transcriptómico previo de nuestro grupo señaló la existencia de un patrón de miRNAs plasmáticos relacionado con la dilatación de la aorta ascendente y la morfología valvular en adultos[62,63]. En este estudio, se identificaron 4 miRNAs (miR-122, miR-130a, miR-486 y miR-718) diferencialmente expresados entre grupos, siendo el miR-718 el que mayor correlación tenía con el diámetro de la aorta ascendente. Tras análisis funcionales, se comprobó cómo este patrón de miRNAs estaba relacionado con el remodelado vascular y el funcionamiento de ECs. Sin embargo, estos miRNAs solo habían sido estudiados en una población adulta. Como se ha comentado anteriormente, la DA es una aortopatía muy común entre los pacientes con VAB y puede aparecer en edades pediátricas. En edad pediátrica, su detección precoz es trascendente para optimizar el seguimiento y orientar sobre la actividad deportiva. La DA es asintomática, por lo que su diagnóstico se basa en el estudio dirigido por técnicas de imagen. Por todo ello, resultaba necesario intentar validar este conjunto de miRNAs en pacientes pediátricos y comprobar si podían ser de utilidad en una población pediátrica. Para ello se analizaron estos 4 miRNAs mediante RT-qPCR en una cohorte pediátrica que incluía pacientes con VAB con presencia o ausencia de DA y en controles sanos con VAT. En este estudio se observó que la expresión de miR-130a se encontraba significativamente disminuida en plasma en los niños con VAB y DA en comparación con VAB sin DA o controles sanos con VAT. Además, la expresión de miR-130a se correlacionaba inversamente con el z-score de la

aorta, tanto ascendente como raíz aórtica, en la cohorte pediátrica. Tras realizar un análisis de enriquecimiento y comprobar cuáles eran los genes diana de miR-130a, se comprobó que éste estaba ligado al estrés oxidativo y a la vía de señalización de TGFß1[213]. El miR-130a tiene un gran papel cardioprotector y su baja expresión ha sido relacionada con la senescencia endotelial[214]. Además, ha sido utilizado incluso como biomarcador para predecir el riesgo cardiovascular en distintos estudios[213,215]. La vía de señalización a la que más parece afectar la expresión de miR-130a, TGFß1, está a su vez asociada con la endoMT, proceso que se debate estar alterado en la VAB y pone en riesgo la funcionalidad correcta del endotelio[58,216]. Por último, la expresión de miR-130a también ha sido asociada con la atenuación de la fibrosis cardíaca por la vía de señalización TGFß/SMAD[217].

El conjunto de resultados epigenéticos y metabolómicos nos ayudan a entender mejor la fisiopatología de la DA en la VAB y refuerzan los resultados previos de nuestro grupo en los que se describen la disfunción endotelial e incremento del estrés oxidativo e inflamación en la VAB y la DA[62,63,108]. En pacientes con VAB, la DA se desarrolla desde etapas muy tempranas y hemos comprobado que puede identificarse gracias a los cambios epigenéticos, como ocurre con la expresión de miR-130a en plasma de pacientes pediátricos. En adultos, además de las alteraciones epigenéticas observadas en trabajos previos, hemos observado diferencias a nivel metabolómico entre los distintos tipos de morfología valvular, siendo más marcadas en presencia de DA. Todo este conjunto de resultados nos permite hipotetizar que la VAB con DA parece llevar asociada disfunción endotelial, una menor capacidad antioxidante y mayor inflamación inherente a esta malformación valvular.

La presencia de DA suele estar muy relacionada con la EA en la VAB. En general, el desarrollo de EA en la VAB promueve un incremento de presión y velocidad de flujo en la aorta que se hipotetiza que puede acelerar el desarrollo de la DA. Al ser la EA otra de las complicaciones de la VAB ligada a un progresivo proceso degenerativo y con un espectro de evolución muy variable, también dirigimos nuestro estudio a su fisiopatología y las posibles diferencias en su desarrollo con respecto a VAT. Para ello, utilizamos la metabolómica (1H-NMR) y la transcriptómica (RNA-seq) en tejido valvular aórtico de pacientes sometidos a recambio valvular por EA, tanto de pacientes con VAB y pacientes

con VAT. Gracias al análisis integrativo pudimos obtener un perfil multiómico específico de la VAB con EA al comparar los pacientes con VAT y EA. En este aspecto, nuestros resultados sugirieron que la fisiopatología de la EA en la VAB difiere de la que ocurre en VAT, y que esta está relacionada con una posible disfunción mitocondrial. En la RNA-seq sobre tejido valvular aórtico con EA se obtuvieron 75 RNAs diferencialmente expresados entre pacientes con VAB y VAT. Tras el análisis funcional de los genes asociados, se pudo comprobar que el patrón de RNAs estaba relacionado en su mayoría con disfunción mitocondrial, así como alteraciones en sus dinámicas. Además, gracias a las bases de datos de enfermedades, también se pudo asociar este perfil con diversas enfermedades cardiovasculares. Tras estudiar de forma individual los 5 RNAs diferencialmente expresados con mayor significancia, pudimos confirmar los anteriores resultados sobre una posible implicación de la disfunción mitocondrial. En primer lugar se encuentra COX7C, que se trata de la subunidad del citocromo c oxidasa 7c, clave en la cadena respiratoria y fosforilación oxidativa[218,219]. En segundo lugar encontramos el pseudogen mitocondrial 10, (MTND5P10) sin función conocida. Sin embargo, asociaciones entre pseudogenes mitocondriales y diversas patologías ya han sido observadas, como ocurre en el Alzheimer[220]. El tercero que observamos fue el receptor gamma Fc 2a (FCGR2a), que tiene un papel muy importante en los procesos inmunes, inflamatorios y en el potencial de membrana de la mitocondria[221]. Finalmente encontramos el ARN específico pequeño de cuerpo Cajal 12 (SCARNA12) que se ha visto implicado en la apoptosis mitocondrial y el ARN pequeño nuclear 12 (RNU12), que se ha asociado con alteraciones en la cadena de transporte de electrones en otras patologías[222,223].

Tras analizar el perfil metabólico en tejido valvular aórtico con EA, se consiguió también relacionar la expresión diferencial de los metabolitos con alteraciones en la homeostasis y función de las mitocondrias. En concreto, estudios previos señalan el papel del mioinositol en la inhibición de la fisión de la mitocondria, cuya expresión estaba incrementada en los pacientes con VAB[224]. Por otro lado, altos niveles de alanina, como observamos en la VAB, están relacionados con una reducción de la generación de ATP en la mitocondria e incremento del estrés oxidativo[225]. Además, en este estudio se observó una disminución de la colina en los pacientes con VAB en comparación con los que tenían VAT, lo cual había sido observado en el análisis metabolómico anterior en plasma.

Finalmente, la integración de los datos clínicos, transcriptómica y metabolómica nos ayudó a perfilar un patrón multiómico distintivo de la EA en la VAB en comparación con lo que ocurre en VAT, confirmando ciertas diferencias en su fisiopatología.

Tanto el patrón de RNAs como el perfil metabólico expresados en tejido valvular aórtico específico de la VAB con EA fueron relacionados mediante ensayos funcionales y bibliografía con disfunción mitocondrial y alteraciones del estrés oxidativo. La disfunción mitocondrial se encuentra presente en numerosas patologías, entre ellas las enfermedades cardiovasculares[141]. Las mitocondrias son organelos esenciales puesto que además de ocuparse de la producción de ATP, son la principal fuente de ROS, y regulan el estrés oxidativo y la muerte celular[226,227]. Estos organelos no son estáticos y están sometidos a un equilibrio constante de fusión y fisión mediante diversas proteínas que controlan estas dinámicas[130]. Se ha comprobado que el propio estrés cortante al que están sometidas las ECs puede influir en la generación de ATP y el potencial de membrana de las mitocondrias[144]. Cambios en las características del flujo pueden provocar alteraciones en las enzimas antioxidantes de las mitocondrias, así como un incremento de la inflamación[228]. De forma similar a lo que ocurre en la pared aórtica, las ECs de la válvula también son sensibles a las alteraciones hemodinámicas presentes de forma inherente en la VAB[229]. Las ECs poseen numerosos mecanorreceptores que las hacen sensibles a los cambios mecánicos, pudiendo influir la mecanotransducción[230]en procesos como la inflamación, el estrés oxidativo o la función mitocondrial[231,232]. El funcionamiento de estas células podría verse modificado por este mecanismo, influyendo en la señalización paracrina, calcificación, secreción de enzimas, etc[23–25]. El incorrecto funcionamiento de las vECs influye sobre su interacción con el resto de células que conforman la válvula, las vICs[233]. Estas células se activan promoviendo la inflamación y calcificación, así como el remodelado de la MEC, haciendo la VA menos funcional y más rígida de forma progresiva, promoviendo la EA[27].

Aunque existen diversos estudios ómicos en tejido valvular aórtico sobre la EA y su progresión a CAVD, la mayoría se han realizado en pacientes con VAT[204]. Estudios proteómicos han demostrado que existe una estructura diferente de la MEC en la CAVD cuando se compara con válvulas control sanas, mientras que un estudio metabolómico ha propuesto el ácido lisofosfatídico como biomarcador del grado de EA, ambos en pacientes

con VAT[201,202]. Un estudio de transcriptómica compara la VAT y la VAB con EA, y sugiere que aunque existen mecanismos similares en su fisiopatología, ciertos genes se encontraban desregulados en la VAB[29]. En nuestro estudio, el incremento del número de muestras y su combinación con la metabolómica nos ha permitido encontrar un perfil multiómico específico de la EA en la VAB asociado a la disfunción mitocondrial. Hipotetizamos que el flujo hemodinámico alterado por la propia morfología anómala de la válvula, sumado al incremento de estrés mecánico por la limitación de apertura, podría promover la disfunción mitocondrial. A su vez, esto provocaría el incorrecto funcionamiento de las vECs, en parte mediante esta disfunción mitocondrial mediada por mecanismos de mecanotransducción. Este complejo de alteraciones podría explicar el incremento de estrés oxidativo y disfunción endotelial observada en la VAB anteriormente (figura 18).

La combinación de los 3 estudios parece llevarnos a un punto común: la fisiopatología de la DA y la EA varía en función de la morfología valvular. En los dos primeros estudios sobre la VAB y la DA pudimos comprobar qué ocurría a nivel sistémico mediante metabolómica en plasma y epigenética: un perfil inflamatorio, incremento del estrés oxidativo y disfunción endotelial. En el estudio de metabolómica y transcriptómica en tejido valvular aórtico con EA, pudimos identificar uno de los posibles mecanismos que podrían estar asociados con este perfil oxidante, inflamatorio y de daño endotelial: la disfunción mitocondrial. Este último estudio nos permitió obtener una nueva pieza clave en el puzle de esta heterogénea y compleja patología. Del conjunto de los resultados de estos estudios se pueden obtener biomarcadores que pueden servir como diagnóstico de VAB y DA, como el conjunto de alfa-tocoferol, EMPs y CRP en plasma. Además, se ha validado la gran eficacia de miR-130a como biomarcador de DA en niños con VAB. Finalmente, la comprensión más profunda de los mecanismos de desarrollo de estas patologías asociadas supone la aparición de nuevas posibles vías terapéuticas que ayuden en la ralentización de la progresión de estas patologías asociadas, como es el caso de la disfunción mitocondrial. En este caso, se debería valorar el papel que tendrían las terapias indicadas para mejorar la función mitocondrial y, por ende, la endotelial, con el propósito de reducir el estrés oxidativo observado en los pacientes con VAB.

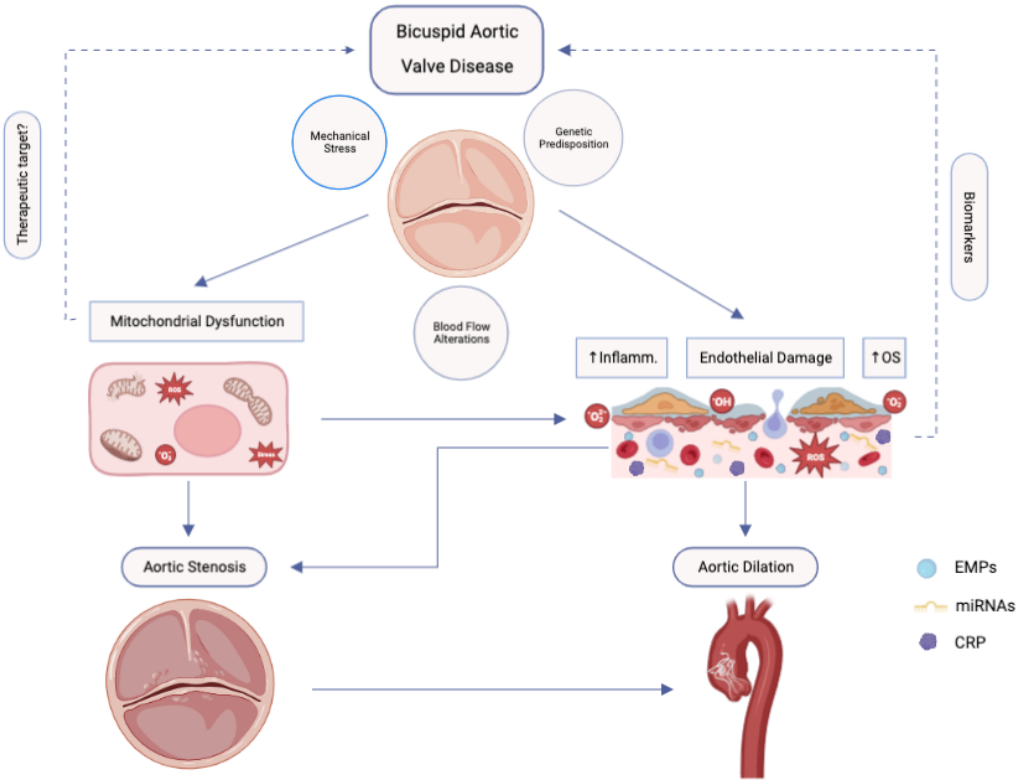

Figura 18. Esquema resumen del papel y asociación del estrés oxidativo, inflamación y disfunción endotelial existente en la VAB y sus complicaciones asociadas, DA y EA, así como uno de los posibles mecanismos implicados en ellas: la disfunción mitocondrial. CRP: proteína C reactiva; EMPs: micropartículas endoteliales; Inflamm.: inflamación; miRNAS: microRNAs; OS: Estrés oxidativo; ROS: especies reactivas de oxígeno.

Limitaciones

En el caso de los 3 estudios existen limitaciones a considerar, siendo de interés general el tamaño muestral; que podría verse incrementado para consolidar los resultados obtenidos. En concreto, en el primer estudio, se excluyeron pacientes con diabetes mellitus y con tratamientos farmacológicos de estatinas o betabloqueantes, lo que implicaría la necesidad de validar estos resultados en una población más amplia y heterogénea. En el segundo estudio, se utilizó un patrón de miRNAs procedentes de un estudio transcriptómico previo en adultos, y solo pudo identificarse uno de ellos, el miR-130a, como biomarcador de DA en niños con VAB. En este caso, se debería incrementar el tamaño muestral de los grupos para conseguir solidificar los resultados obtenidos. En el caso del último estudio, la utilización de tejido valvular aórtico dificulta un gran tamaño muestral. Cabe destacar que el total de las muestras de tejido valvular aórtico se utilizaron para los ensayos metabolómicos y transcriptómicos, lo que por un lado provee de un análisis ómico más riguroso, pero no deja lugar a la validación posterior de estos

resultados en una cohorte independiente. Por lo tanto, estos resultados deberían validarse; y este estudio supone únicamente la hipotetización de posibles mecanismos asociados a la EA en la VAB, basándonos en los resultados del perfil multiómico obtenido.

Perspectivas de futuro

El conjunto de estos estudios aporta nuevos datos sobre cómo interpretar qué estaba ocurriendo a nivel sistémico en los pacientes con VAB y sus complicaciones asociadas, así como proponer uno de los posibles mecanismos asociados a ello. Sin embargo, no se han llegado a identificar las posibles vías de señalización concretas que podrían estar implicadas en estas alteraciones mencionadas anteriormente. Además de ello, se debería comprobar si la disfunción mitocondrial puede ser observada a nivel sistémico y analizar así su utilidad como biomarcador en plasma.

Tras confirmarse estos hallazgos, el siguiente paso sería el estudio de la funcionalidad mitocondrial *in vitro,* por ejemplo en células endoteliales circulantes obtenidas fácilmente a partir de muestras de sangre de pacientes con VAB o en las propias vECs aisladas de la VA tras un recambio valvular. Este tipo de estudios nos ayudarían a confirmar las alteraciones mitocondriales (producción de ATP y ROS, dinámicas…) y el propio daño endotelial (biomarcadores de daño endotelial, ensayos funcionales) que podría acontecer en la VAB. Estas células también podrían ser sometidas *in vitro* a estímulos mecánicos y flujos diferentes para comprobar nuestra hipótesis de las alteraciones hemodinámicas como posible causa. Estos ensayos ayudarían a determinar también la posibilidad de utilización de la disfunción mitocondrial como diana terapéutica en las complicaciones asociadas a la VAB.

Por otra parte, en estos cultivos celulares, se podría analizar la función de los miRNAs identificados mediante su silenciamiento o sobreexpresión con inhibidores o mímicos, respectivamente. Además de ello, se debería valorar la posibilidad de un estudio transcriptómico realizado directamente en una cohorte pediátrica para rescatar biomarcadores de utilidad que en muestras de adultos no se obtuvieron. Finalmente, sería muy interesante identificar un patrón de miRNAs específico de la EA asociada a la VAB, que pudiera servir de diagnóstico y pronóstico para pacientes con esta degeneración valvular.

Conclusiones

Conclusiones:

- Existe una asociación entre el alfa-tocoferol y la morfología valvular, siendo los niveles de este menores en la VAB en comparación con individuos con VAT en plasma, indicando una menor capacidad antioxidante.

- Los niveles de EMPs y CRP en plasma, indicadores de disfunción endotelial e inflamación, se encontraron incrementados en los pacientes con VAB en comparación con los individuos con VAT.

- El modelo de predicción de la morfología valvular compuesto por alfa-tocoferol, EMPs y CRP podría ser de utilidad en el diagnóstico de la VAB.

- El patrón completo de miRNAs relacionado con la VAB y DA en adultos no puede trasladarse directamente a pacientes pediátricos.

- La expresión del miR-130a está relacionada con el z-score de la aorta, raíz y ascendente, en pacientes pediátricos y su expresión es menor en niños con VAB y DA en comparación con niños con VAB sin DA y controles sanos con VAT.

- El miR-130a está relacionado con la disfunción endotelial así como el estrés oxidativo y tiene un papel cardioprotector.

- La adición de miR-130 como biomarcador a otros parámetros básicos como edad, sexo, peso, talla y morfología valvular mejora significativamente la predicción de DA en niños con VAB.

- Tanto el perfil metabolómico como el patrón de RNAs observados en tejido valvular aórtico con EA está determinado por la morfología valvular.
- La integración de ambos permite crear un modelo multiómico específico de la EA presente en la VAB, indicando su distinta fisiopatología en comparación con la EA desarrollada en pacientes con VAT.

- El perfil multiómico obtenido permite el reconocimiento de la disfunción mitocondrial como un posible mecanismo que promueve el incremento del estrés oxidativo y el daño endotelial en la VAB.

- Las alteraciones hemodinámicas y mecánicas de apertura en la VAB podrían fomentar un mayor estrés en las células de la válvula, que promoverían la disfunción mitocondrial, y este, a su vez, un mayor estrés oxidativo y daño endotelial.

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Publicaciones vinculadas a la tesis

Publicaciones vinculadas a la tesis:

Publicaciones utilizadas para la elaboración de la tesis:

1. Artículo científico: Plasma Metabolomic Profiling Associates Bicuspid Aortic Valve Disease and Ascending Aortic Dilation with a Decrease in Antioxidant Capacity
   Autores: Neus Martínez-Micaelo, Carme Ligero, **Borja Antequera González**, Alexandra Junza, Oscar Yanes and Josep M. Alegret
   Revista científica: Journal of Clinical Medicine (MDPI)
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   Área: Medicina General e Interna
   Cuartil: Q1
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2. Artículo científico: miR-130a expression is related to aortic dilation in bicuspid aortic valve children
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Anexo 1

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REVIEW
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Bicuspid Aortic Valve and Endothelial Dysfunction: Current Evidence and Potential Therapeutic Targets

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Bicuspid aortic valve (BAV), the most frequent congenital heart malformation, is characterized by the presence of a two-leaflet aortic valve instead of a three-leaflet one. BAV disease progression is associated with valvular dysfunction (in the form of stenosis or regurgitation) and aortopathy, which can lead to aneurysm and aortic dissection. This morphological abnormality modifies valve dynamics and promotes eccentric blood flow, which gives rise to alterations of the flow pattern and wall shear stress (WSS) of the ascending aorta. Recently, evidence of endothelial dysfunction (ED) in BAV disease has emerged. Different studies have addressed a reduced endothelial functionality by analyzing various molecular biomarkers and cellular parameters in BAV patients. Some authors have found impaired functionality of circulating endothelial progenitors in these patients, associating it with valvular dysfunction and aortic dilation. Others focused on systemic endothelial function by measuring artery flow-mediated dilation (FMD), showing a reduced FMD in BAV individuals. Novel biomarkers like increased endothelial microparticles (EMP), which are related to ED, have also been discovered in BAV patients. Finally, latest studies indicate that in BAV, endothelial-to-mesenchymal transition (EndoMT) may also be de-regulated, which could be caused by genetic, hemodynamic alterations, or both. Different hypothesis about the pathology of ED in BAV are nowadays being debated. Some authors blamed this impaired functionality just on genetic abnormalities, which could lead to a pathological aorta. Nevertheless, thanks to the development of new and high-resolution imaging techniques like 4D flow MRI, hemodynamics has gained great attention. Based on latest studies, alterations in blood flow seem to cause proper modification of the endothelial cells (ECs) function and morphology. It also seems to be associated with aortic dilation and decreased vasodilators expression, like nitric oxide (NO). Although nowadays ED in BAV has been reported by many, it is not clear which its main cause may be. Comprehending the pathways that promote ED and its relevance in BAV could help further understand and maybe prevent the serious consequences of this disease. This review will discuss the ED present in BAV, focusing on the latest evidence, biomarkers for ED and potential therapeutic targets (**Figure 1**).

Keywords: bicuspid aortic valve, endothelial dysfunction, hemodynamics, endothelial cells, biomarker, CVD, biomarkers, therapeutic target

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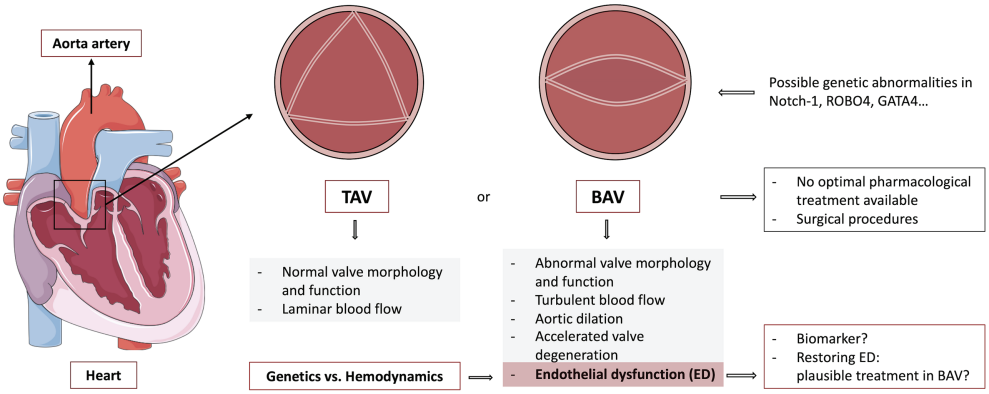

FIGURE 1 | Graphical abstract of the review: a heart is represented at the left, signalizing the aorta, as well as the zone where the aortic valve is found. Different morphologies of this valve are also shown, pointing out the generalities of a healthy tricuspid aortic valve (TAV) and bicuspid aortic valve (BAV) disease. Additionally, endothelial dysfunction (ED) and its importance in BAV disease are also discussed. TAV, Tricuspid aortic valve; BAV, Bicuspid aortic valve; Notch-1, Neurogenic locus Notch homolog protein 1; ROBO4, Roundabout guidance receptor 4; GATA4, GATA binding protein 4. The figure was created by using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

INTRODUCTION

BAV Disease

Bicuspid aortic valve (BAV) is the most frequent cardiac congenital malformation, affecting 1–2% of population, with a higher prevalence in men (3:1; Tzemos et al., 2008; Alegret et al., 2013). It is characterized by the presence of the BAV (two leaflets) instead of a tricuspid one (three leaflets), and it is usually associated with valve dysfunction and aortopathy. BAV is a complex disease that is caused by the abnormal separation of the primordial semilunar valve during embryogenesis, resulting in different phenotypes of the aortic valve (Martin et al., 2015). The most popular classification of BAV is based on the presence/absence of a raphe (type), followed by two supplementary characteristics: the spatial position of the raphe and cusps, as well as the functional status of the valve (Sievers and Schmidtke, 2007). Depending on these phenotypic characteristics of the valve, different outcomes and progression of this disease have been found (Kang et al., 2013).

As said before, BAV is a complex disease, which cannot be reduced to just a disorder of valvulogenesis (Siu and Silversides, 2010). The abnormal morphology modifies valve dynamics and promotes eccentric blood flow, which gives rise to alterations of the flow pattern and wall shear stress (WSS) of the ascending aorta. BAV disease is related to ascending aorta dilation (AD), that becomes very frequent and it appears at a much younger age than patients with a normal tricuspid aortic valve (TAV), which can lead to an increased risk of aneurysm formation and aortic dissection (Michelena et al., 2008, 2011; Tzemos et al., 2008, 2010; Alegret et al., 2013). Further, aortic valve dysfunction (stenosis, regurgitation, or both) is frequently observed. Valve dysfunction may be caused by the congenital malformation itself and by the predisposition to accelerated degeneration. In consequence, surgical procedures such as aortic valve replacement and ascending aorta repair in the presence of ascending aorta dilatation may be required throughout the life of these patients (**Figure 1**).

ENDOTHELIAL DYSFUNCTION IN BAV DISEASE: EVIDENCE AND BIOMARKERS

Endothelium and ED

Recently, evidence of endothelial dysfunction (ED) present in BAV has surfaced (Vaturi et al., 2011; Ali et al., 2014; Alegret et al., 2016; Gavriliuk et al., 2016; Balistreri et al., 2018; van de Pol et al., 2019). ED stands for the abnormal functioning of the endothelial cells (ECs) that cover the inner surface of blood vessels, the endothelium. Although it was considered a rudimentary tissue with basic functions like permeability to water and electrolytes not many years ago (Wilson and Lerman, 2001), numerous functions in which endothelium plays a pivotal role have been identified in the last few years (Kern et al., 1983; Darland and D'Amore, 2001; High et al., 2008; Baeten and Lilly, 2017). It acts as a semi-permeate barrier between blood and the inner portion of the vascular tissue maintaining the homeostasis and regulating the exchange of nutrients, fluids, and macrophages. Endothelium also participates in maintaining vascular tone and it can alter vascular smooth muscle cells (VSMCs) phenotype and differentiation (Kern et al., 1983; Darland and D'Amore, 2001; Ignarro et al., 2001; High et al., 2008; Baeten and Lilly, 2017). Vascular tone is maintained by many vasoconstrictors and dilatators, where nitric oxide (NO) remains as one of the most important vasodilators (Griffith et al., 1984). It is secreted by ECs and it is critical for many physiologic and pathologic processes (Dimmeler and Zeiher, 1999; Garg, 2016). NO is produced by the endothelial nitric oxide synthase (eNOS), present in the cell membrane caveolae (Hecker et al., 1994). When activated, NO diffuses to vascular smooth muscle, causing relaxation by activating guanylate cyclase, which increases cyclic guanosin monophosphate (cGMP),

leading to the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) by protein kinase G (PKG) (Arnold et al., 1977; Halbrügge et al., 1990; Elmarakby et al., 2007). VASP facilitates vasodilation by promoting actin elongation and protecting these filaments against capping.

ED in BAV Disease: Latest Findings

Numerous studies have found associations between eNOS expression, BAV morphology, and aortic dilation (Aicher et al., 2007; Kotlarczyk et al., 2016; Odelin et al., 2019). While some of them indicate a decreased eNOS expression in human BAV tissue in comparison to TAV (Aicher et al., 2007), others focused on concrete aortic regions (Kotlarczyk et al., 2016). One group found out that BAV had higher eNOS expression (both gene and protein) in the R aortic region, which has the greater curvature of the ascending aorta, as well as an increased pSer1177-eNOS (activated eNOS; Kotlarczyk et al., 2016). Nevertheless, they did not find any changes in pSer239VASP, an established biomarker of NO bioavailabity related to endothelial integrity and NO-stimulated soluble guanylate cyclase/cGMP-dependent kinase I (sGC/cGK-I) pathway (Oelze et al., 2000). They hypothesized a possible uncoupled production of NO in favor to produce O_2, increasing the oxidative stress. Superoxide radicals can react with NO, forming peroxynitrite, a potent oxidant, which causes general ED, as well reducing the bioavailability of NO (Knepler et al., 2001; Peluffo et al., 2009).

Notch signaling pathway is believed to be involved in vascular remodeling and repairing in the cardiovascular system (Kopan and Ilagan, 2009; Balistreri et al., 2018). Endothelial-derived NO increases the expression of Notch signaling target genes in valvular interstitial cells (VICs), protecting these cells and the proper valve from calcification. Neurogenic locus Notch homolog protein 1 (Notch-1) messenger RNA (mRNA) is expressed during valve formation as well as in adult murine aortic valves, leading up to believe that it could participate in the processes of valvulogenesis and the progression of valve disease (Krebs et al., 2000; Garg et al., 2005). Notch-1 represses runt-related transcription factor 2 (Runx2), a regulator of osteoblast cell fate, and it also seems to increase SRY-Box transcription factor 9 (Sox9) expression, which is able to prevent calcification (Garg et al., 2005). It also regulates bone morphogenetic protein 2 (Bmp2), which also seems to take part in calcification prevention (Nigam and Srivastava, 2009). When ED is present, NO expression can be deregulated and calcium deposition may be promoted, leading up to calcific aortic valve disease (CAVD). This calcification could promote valve malfunctioning in form of stenosis and/or regurgitation. Thus, ED seems participate in the first steps in the development of the CAVD (Kostyunin et al., 2019).

Latest researches have focused on the endothelial to mesenchymal transition (EndoMT) and its deregulation in BAV. While EndoMT is essential for pivotal processes, like cardiac development or wound healing, its abnormal activation can lead to different pathologies (Kalluri and Weinberg, 2009). This endothelial transition process starts by losing cell adhesion properties, polarity, and junctions by endocytosis (Le Bras et al., 2012).Later, a restructuration of their cytoskeleton is done in order to become motile and acquire a mesenchymal state. These cells lose typical endothelial markers, like vascular endothelial cadherin (VE-cadherin) or von Willebrand factor (vWF), in order to gain mesenchymal markers, like neural cadherin (N-cadherin) or alpha smooth muscle actin (α-SMA). This process is regulated by several signaling pathways, like transforming growth factor ß (TGF-ß), wingless-related integration site-ß-catenin (WNT-ß-catenin), or Notch (Lamouille et al., 2014). Therefore, alterations in these pathways can deregulate EndoMT. This transition can also be regulated by different micro RNAs (miRNAs) like miR-200 family, which seems to repress the process (Hill et al., 2013). Recent studies have shown deregulation of the EndoMT process in ECs in altered hemodynamics situations too (Muylaert et al., 2016). Others suggest that in BAV, the state of transition of EndoMT is more advanced than in TAV, leading up to a higher EndoMT process, losing essential endothelial characteristics to maintain homeostasis and successful wound healing (Maleki et al., 2019). Furthermore, different studies indicate that EndoMT seems to be more aggressive in dilated BAV. An extensive review of the possible alteration of EndoMT in thoracic aortic aneurysm can be found here (Maleki et al., 2019).

Biomarkers for ED in BAV Disease

Although nowadays numerous biomarkers for ED have been discovered in BAV (**Table 1**), there is a lack of a definitive one. For example, while some studies pointed out that asymmetric dimethylarginine (ADMA) has been found to be increased in BAV patients in comparison to TAV patients (Drapisz et al., 2013), others did not find significant differences based just on the valve morphology (Ali et al., 2014). Recent publications have also revealed different miRNAs patterns related to ED in BAV (Martínez-Micaelo et al., 2017; Poggio et al., 2019). In this regard, our group discovered circulating endothelial microparticles (EMPs) – which are related to ED present in BAV patients plasma in higher concentrations than TAV, being also associated with aortic dilatation (Alegret et al., 2016). Recent studies have shown impaired functionality or decreased number of endothelial progenitor cells (EPCs) in BAV (Vaturi et al., 2011; van de Pol et al., 2019). Specifically, one group studieds functionality of peripheral blood endothelial colony-forming cells (pbECFCs) isolated from BAV patients compared to healthy ones as possible a biomarker (van de Pol et al., 2019). They found a decreased migratory capacity in the pbECFCs isolated from BAV patients vs. pbECFCs from healthy controls. Their study also indicated that colony-forming ability typical of these highly-proliferative cells was significantly reduced in pbECFCs isolated from BAV patients with aortic dilatation. Additionally, the systemic effects of ED on regulation of vascular tone has also been evaluated by measuring the brachial artery flow-mediated dilation (FMD; Vogel, 2001) in BAV patients, and these studies have also concluded that BAV patients have a lower FMD when compared to healthy ones (Tzemos et al., 2010; Ali et al., 2014; Wang et al., 2018).

Therefore, it is hard to deny that ED is probably directly related to BAV and its worse prognosis, especially with aortic

TABLE 1 | Summary of the ED biomarkers discovered in BAV disease.

	Biomarker	Findings in BAV	References
Plasma biomarkers	ADMA	Higher levels of ADMA-inhibitor of eNOS have been found in some BAV patients	Drapisz et al. (2013); Ali et al. (2014)
	EMPs	Higher concentrations of EMPs has been associated with BAV and, specifically, aortic dilation	Alegret et al. (2016)
	miRNAs	Diverse miRNAs patterns have been identified to be related to ED in BAV	Martínez-Micaelo et al. (2017); Poggio et al. (2019)
ECs assays	EPCs	Impaired functionality and/or decreased number of circulating EPCs in BAV	Vaturi et al. (2011)
	ECFCs	Impaired functionality and/or decreased number of circulating ECFCs in BAV	van de Pol et al. (2019)
Non-invasive	FMD	Lower levels of FMD are related to BAV and its aortopathies	Tzemos et al. (2010); Ali et al. (2014); Wang et al. (2018)

BAV, bicuspid aortic valve; eNOS, endothelial nitric oxide synthase; ECs, endothelial cells, ADMA, asymmetric dimethylarginine; EMPs, endothelial microparticles; miRNAs, micro RNAs; EPCs, endothelial progenitor cells; ECFCs, endothelial colony forming cells; FMD, flow-mediated dilation.

dilatation and aortic stenosis (Vaturi et al., 2011; Ali et al., 2014; Alegret et al., 2016; Gavriliuk et al., 2016; Balistreri et al., 2018; van de Pol et al., 2019). Nevertheless, the pathways that could promote ED and its relevance in BAV are not fully known. Additionally, it is not yet fully elucidated if this malfunctioning is due to a genetical predisposition of these ECs to dysfunction, or it is caused by the proper alteration of hemodynamics that modifies ECs phenotype and function. Thus, further ED research in BAV should be assessed in order to concrete the specific molecular pathways that take place in this complex disease.

GENETICS AND ED IN BAV DISEASE

While BAV malformation may be caused by genetic abnormalities that occur sporadically, with familiar clustering in some cases (Gale et al., 1977; Brown et al., 2003), the main cause of associated ED and aortopathies in BAV, as well as the pathways involved, are still a topic of discussion.

Genetic Abnormalities in BAV Disease Related to ED

While there is much controversy between data related to BAV associated aortopathies and their first-degree relatives (FDRs), one group found significant differences in ED biomarkers in FDRs of BAV compared to FDRs of TAV individuals (Li et al., 2020). Further studies with a larger sample size and definitive ED biomarkers should be performed in order to make definitive conclusions. Accordingly, one group performed a genome-wide association study (GWAS) comparing BAV vs. control patients (Fulmer et al., 2019). They found out that a variety of single nucleotide polymorphisms (SNPs) in Exocyst-Cilia Machinery system could be the origin of the BAV phenotype. This recent study also indicates that removal of exocyst complex component 5 (Exoc5), specific of the endocardium, was enough to cause BAV malformation and promote calcified aortic valve stenosis in adulthood. This fact could support the genetic hypothesis of BAV phenotype and the presence of deregulation of essential processes in ECs, possibly endorsing ED.

Numerous studies have found relation between BAV disease, associated aorthopaties, and mutation in components of Notch pathway (Garg et al., 2005; High et al., 2008; Wang et al., 2015). Furthermore, Notch expression has been found to be deregulated in adults ECs of some BAV patients (Balistreri et al., 2018). Other genetic mutations are known to cause a variety of BAV phenotypes, as well as their associated aortopathies (Park et al., 2003; Pu et al., 2004; Odelin et al., 2019). One example of genetical abnormality, which can result in BAV is roundabout guidance receptor 4 (ROBO4), a specific vascular receptor that prevents ECs to migrate (Park et al., 2003). This study indicated that ROBO4 expression is significantly decreased in ECs of some BAV patients. Mutations of ROBO4 seem to promote deregulation of EndoMT, making endothelium lose its typical barrier function, as it reduces tight junction protein 1 (TJP1) and VE-cadherin expression, decreasing adherent and tight junctions in ECs (Maleki et al., 2019). This group found that in homozygotic null ROBO4 mice, BAV disease, as well as valve stenosis, was occasionally present. Genetical abnormalities in GATA binding protein 4 (GATA4) gen, which is a well-known regulator or cardiac morphogenesis (Pu et al., 2004), have also been found recently in BAV patients. Furthermore, some studies indicated that this alteration significantly impaired the EndoMT process, a critical step in heart valve development (Yang et al., 2017). Additionally, they found that these differences in expression were not significant when comparing BAV patients with or without AD. These findings suggest that this genetic abnormality could maybe not be the only cause of related aortopathies and ED in these BAV patients (Yang et al., 2017). Similar to GATA4, endothelial GATA binding protein 5 (GATA5) null-mice also results in a BAV morphology (Laforest et al., 2011). Another example of genetic mutation that promotes BAV malformation involves early growth response 2 (Krox20), which has also been found to be mutated in some BAV patients (Odelin et al., 2019). Krox20 binds to the promoter of nitric oxide synthase 3 (Nos3), promoting the expression of Nos3. These mutations could decrease Nos3 expression and therefore NO, deregulating vasodilation and Notch signaling pathway, both critical in maintaining vascular health (Odelin et al., 2019). Although different genes can be discussed, all these mutations are related to an incorrect endothelial function, and their deregulation can be related to ED (**Figure 2**).

In brief, numerous genetical abnormalities are known to cause BAV malformation and related aortopathies. BAV is believed to have an autosomal dominant inheritance pattern with incomplete penetrance and variable expressivity (Huntington et al., 1997; Braverman et al., 2005). Some of these mutations also seem to provoke ED by altering their proliferation-apoptosis equilibrium, deregulating EndoMT process, reducing their ability to act as a barrier, modifying their phenotype, etc (Maleki et al., 2019). ED is nowadays thought to be directly related to progression of BAV disease, as we have discussed before. Nevertheless, there has not been found yet a common genetic abnormality between all BAV patients. Diverse genetic mutations have been found in different BAV patients, bearing out the complexity and heterogeneity of this disease. Moreover, patients with different outcomes or not found genetic alterations that cause BAV are also present. Therefore, it seems like even though genetical predisposition can easily play an important role, it may not be the only one. Finally, the consensus of a definitive and trustworthy ED biomarker should be assessed in order to validate these findings.

HEMODYNAMICS IN BAV DISEASE: IS IT THE KEY PLAYER?

Until quite recently, the most accepted theory about aortic dilation was based on the existence of a genetical predisposition which resulted in BAV disease and associated aortopathies. Nevertheless, the heterogeneity of this disease makes hard to believe that only genetics could play a role in BAV. Furthermore, the development of new advances in imaging techniques has opened a topic on discussion about how alterations in blood flow could affect the endothelium and the vascular system. Specifically, in BAV, abnormal valve opening usually leads to an eccentric and turbulent blood flow in ascending aorta.

Blood Flow and ECs

Endothelium homeostasis is mostly maintained by the laminar blood flow that ECs are exposed. In laminar flow areas, ECs produce beneficial factors for their proper survival, quiescence, and barrier function. In these areas, coagulation, proliferation of VSMCs and leucocyte extravasation is suppressed (Reinhart-King et al., 2008). Nevertheless, in turbulent blood flow areas where shear stress is altered, their phenotype is modified, increasing the permeability of the endothelium, deregulating proliferation-apoptosis equilibrium and enhancing adhesion properties for monocytes (Zhou et al., 2014). Normally, ECs phenotype varies largely in the endothelium depending on the place these cells are covering due to different blood flow pattern, showing heterogeneity across vascular beds (Aird William, 2007). These cells have a variety of mechanical receptors, which can perceive alterations in blood flow hemodynamics and produce different biological responses. One of the most studied mechanotransducers in ECs is a complex composed by platelet endothelial cell adhesion molecule-1 (PECAM-1), VE-cadherin, vascular endothelial growth factor receptor (VEGFR) 2 and 3 (Osawa et al., 1997; Tzima et al., 2005; Conway et al., 2013). Blood flow increases tension of PECAM-1, triggering the association of this protein with vimentin cytoskeleton (Conway et al., 2013). This tension promotes the activation of a proto-oncogene tyrosine-protein (Src) kinase, leading up to the transactivation of VEGFR and the activation of phosphatidylnositol 3-Kinase (PI3k) and eNOS, which in turn products NO in order to induce vasodilation (Fleming et al., 2005; Tzima et al., 2005). Integrins are also activated by PI3K, which are involved in cell alignment in laminar flow. When turbulent flow is present, tissue remodeling is active and inflammatory pathways are activated (Buschmann et al., 2003). This process seems to be controlled by blood flow, suppressing it when there is no blood flow alteration, and activating it when there are changes in hemodynamics. A complete review about shear stress involved in vascular remodeling can be found here (Baeyens et al., 2016).

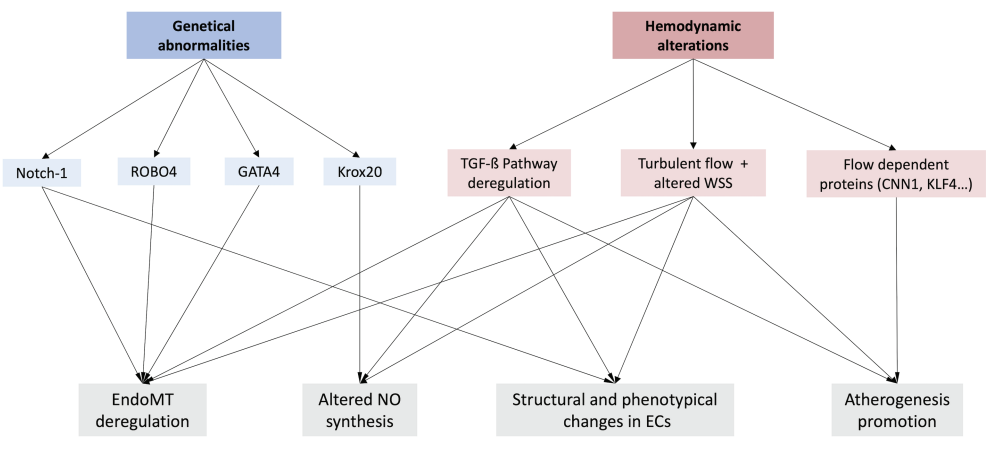

FIGURE 2 | Representation of the two main hypothesis about a possible ED in BAV disease (genetical abnormalities and/or hemodynamic alterations), as well as some of the most relevant phenomenon these variations may provoke. Notch-1, Neurogenic locus Notch homolog protein 1; ROBO4, Roundabout guidance receptor 4; GATA4, GATA binding protein 4; Krox20, Early growth response 2; EndoMT, Endothelial-to-mesenchymal transition; NO, Nitric oxide; TFG-ß, Transforming growth factor ß; WSS, Wall shear stress; CNN1, Calponin-1; KFL4, Krüppel-like Factor 4.

ECs can translate biomechanical forces into specific responses. When endothelium is intact, it regulates VSMCs proliferation by endothelial heparan sulfate proteoglycans (HSPG), controlled by mechanosensitive pathways like transforming growth factor beta (TGF-β; Ettenson et al., 2000). Nevertheless, when hemodynamics and shear stress alterations are present, as in BAV, deregulations in these pathways provoke proper VSMCs growth deregulation (Baker et al., 2008). TGF-β induction by shear stress seems to be related to the activation of krüppel like factor 2 (KLF2) signaling cascade, which is essential for maintaining the correct endothelial functioning (Baker et al., 2008; Walshe et al., 2013). Among others, KLF2 induces eNOS activity and production, which could be translated to an increased NO production, essential to maintain vascular tone and the endothelium (Fontijn et al., 2015). As said before, TGF-β also plays a key role in the EndoMT process (Ten Dijke et al., 2012). Therefore, its deregulation by alteration in hemodynamics could induce proper endothelial phenotypic changes, modifying the endothelium and homeostasis. This pathway deregulation seems to contribute to induce BAV related aortopathies like aortic aneurysm (Angelov et al., 2017; Rocchiccioli et al., 2017).

Hemodynamics Alterations in BAV Disease

Recently, numerous studies have reported that changes in flow characteristics and WSS are present in BAV disease, and these alterations directly affect the endothelium (Meierhofer et al., 2013; Baeyens et al., 2016; Chistiakov et al., 2017). Thanks to development of new and high resolution imaging techniques like time-resolved three-dimensional phase contrast MRI (Kozerke et al., 2001; Frydrychowicz et al., 2007) also known as 4D flow MRI, hemodynamics have gained attention in the BAV field. This technique allows quality and quantitative flow measurement, like flow velocity, flow direction, wall shear stress, complex flow patterns, etc (Sträter et al., 2018). The progress of development of new imaging techniques in the cardiovascular field has also helped acquire more fast and accurate data in order to obtain prognosis or diagnosis of several diseases. Specially, in BAV, it has helped further understand the hemodynamics alterations produced by morphological abnormalities of the aortic valve. Recently, it has been described that hemodynamics depends directly on BAV phenotype (Rodríguez-Palomares et al., 2018). Several studies have also shown differences between the arterial wall of BAV and TAV patients, mostly at the intimal area (Meierhofer et al., 2013). It has also been indicated that regions with altered WSS are more likely to extracellular matrix dysregulation and elastic fiber degeneration, possibly inducing aortic dilation (Bissell et al., 2013; Meierhofer et al., 2013; Guzzardi et al., 2015). Others have shown that blood flow alterations are capable of promoting medial degeneration by metalloprotease pathways (Atkins and Sucosky, 2014; Andreassi and Della Corte, 2016). Furthermore, when comparing the areas of the artery with the highest impact of blood flood (jet) and more laminar flow in the artery between these BAV patients and TAV patients, noticeable differences were found (Grewal et al., 2019). Thickening of the intimal in the jet area was observed when compared to another area with less turbulent flow. This study also linked this thickening with an increased cobblestone morphology of ECs, indicating that alterations in hemodynamics can affect ECs morphology. Nevertheless, this study did not profound on the possible EndoMT ongoing. Others did focus on EndoMT in BAV and found that hemodynamics can deregulate this process that is believed to cause loose of elasticity in arterial walls and therefore, aortic dilation (Mahler et al., 2014; Moonen et al., 2015).

Flow-Dependent Proteins

In BAV, the expression of some flow-dependent proteins has been found to be altered. A study indicated the influence of hemodynamics alterations in the expression of calponin 1 (CNN1), which is induced in turbulent flow areas in ECs (Hsu et al., 2019). CCN1 binds to its receptor, activating nuclear factor kappa B (NF-kB), which promotes ED and atherogenesis. It is also been observed that laminar blood flow and high shear stress makes ECs more resistant to oxidation by promoting autophagy. However, this effect can be attenuated by the presence of turbulent blood flow and altered shear stress (Liu et al., 2015). Another example of flow-dependent protein would be krüppel-like factor 4 (KLF4), which levels are also known to be decreased in ECs at the jet impacts areas due to turbulent flow. This protein contributes to regulate inflammation and it has atheroprotective characteristics (Jiang et al., 2014). Hence, any of these alterations could deregulate endothelium homeostasis, promoting ED, and in consequence, an increased risk of aortic dilation. Additionally, our group found increased levels of EMPs in BAV patients with aortic dilation compared to healthy subjects (Alegret et al., 2016). These microparticles are related to endothelial damage and their expression is inversely correlated with WSS (Vion et al., 2013). Therefore, changes in hemodynamics which promote an eccentric flow in BAV could alter EMPs levels as a sign of ED (**Figure 2**).

Although lots of evidence from hemodynamics importance has emerged lately in the BAV field, some studies pointed out that it could be not the only factor affecting aorthopaties and ED in BAV patients. While some genetic alterations in many key genes that participate in pivotal pathways in ECs are found in some BAV individuals, the genetical pathology of most of them remains unknown. Lately, researchers have focused on hemodynamics alterations as the main cause of associated pathologies in BAV. Although using a small cohort of patients, one group made a point in favor of this hypothesis, indicating that aortic dilatation progression in BAV patients who underwent valve replacement became similar to TAV patients (Regeer et al., 2016). This could mean hemodynamics alterations could play a key role in development of BAV associated aortopathies. As explained before, most of the recent evidence shows that alteration of blood flow is the fundamental key to ED, kicking off the process of degeneration of the aortic media. However, some experimental studies in BAV patients without significant alterations in blood flow yet severe aortopathies that have focused on genetic predisposition, suggesting that it may also play a complementary role.

ED AS A POTENTIAL BIOMARKER AND THERAPEUTIC TARGET IN BAV DISEASE

Based on the latest evidence, ED might be related to BAV and its associated aortopathies (Vaturi et al., 2011; Ali et al., 2014; Alegret et al., 2016; Gavriliuk et al., 2016; Balistreri et al., 2018; van de Pol et al., 2019). While the mechanisms and pathways involved in this process are not fully elucidated, ED seems to be linked to the progression of BAV disease, leading up to aortopathy and valvular dysfunction. Therefore, it seems reasonable that ED could be used as an additional general prognosis biomarker of valvular dysfunction and related aortopathies in BAV. Many methods that are currently used for measuring ED could be useful: FMD, plasma biomarkers, functionality assays of cultured ECs "*in vitro*," miRNA patterns, EMPs, etc. In any case, we strongly believe that ED should be further studied in BAV in order to concrete its importance as well as its related signaling pathways.

Potential Therapeutic Targets for ED in BAV Disease

If ED is related to BAV aortopathies and its worse prognosis, it could be hypothesized that restoring a correct endothelial function could at least help slow down BAV aortopathy progression. Thus, a variety of potential treatments could be considered in order to handle ED in BAV disease.

Nowadays, stem cell therapy and gene therapy are a reality in many disease treatments, and they are showing promising results, as well as, health safety. Many trials have been performed in some diseases that involve ED, like peripheral artery disease (PAD; Frangogiannis, 2018; Paschalaki and Randi, 2018). In this field, we should talk about endothelial progenitor cells (EPCs). EPCs can be classified in early endothelial progenitor cells (eEPCs) and late endothelial progenitor cells, or most commonly called endothelial colony forming cells (ECFCs; Fadini et al., 2012). The first subgroup (eEPCs) seems to act preferably in a paracrine way, secreting numerous angiogenic factors, like VEGF (Yang et al., 2015). On the other hand, ECFCs are highly proliferative and can promote angiogenesis *in vitro* and *in vivo* and are involved in wound healing (Fadini et al., 2012). A recent study has found that ECFCs in BAV have less clonogenic potential in BAV with dilated aorta (van de Pol et al., 2019). ECFCs cell therapy could help restore correct endothelial function and maybe attenuate disease progression (Banno and Yoder, 2018). ECFCs can be obtained from peripheral blood (Ingram et al., 2004) or white adipose tissue (Lin et al., 2013), which makes easy to obtain them. Allogenic cell therapy could be easily performed or even autologous cell therapy. ECFCs can even be cultured and pre-activated "*in vitro*" before injection in order to increase their yet great potential (Rosova et al., 2008; Mena et al., 2014, 2018). Nowadays, combined cell therapy is also used and it seems to have great results (Banno and Yoder, 2018). Additionally, if severe genetic abnormalities are present in patients ECs, gene editing could be performed before injecting them again or using allogenic therapy should be considered (High and Roncarolo, 2019). Nevertheless, until date, no cell therapy has been performed in BAV patients that we have found. The possible benefit of this therapy would be in line with which was the main cause of ED. It also must be said that if associated ED in BAV is mainly due to an alteration in hemodynamics, cell therapy may not be the optimal treatment, as the blood flow would remain altered, and therefore, it could continue promoting ED.

Pharmacological treatment may also be analyzed as a way of rescuing correct endothelial functioning in BAV disease. Numerous drugs classically used as standard treatment in cardiovascular diseases might slightly influence endothelial function: angiotensin-converting enzyme 1 (ACE1) inhibitors, statins, beta blockers, antioxidants, etc.; yet there is a lack of definitive clinical evidence (Su, 2015). Specifically, diverse studies have shown that pitavastatin seems to significantly improve FMD (Katsiki et al., 2018). Other studies suggest that Rac family small GTPase 1 (Rac1) could ameliorate human endothelial function in veins, as well as reducing oxidative stress, in a NO-dependent manner (Carrizzo et al., 2017); becoming an interesting target for improving ED in BAV disease.

Finally, a silencing therapy for specific miRNAs related to ED in BAV with antisense oligonucleotides could also be an interesting approach (Lima et al., 2018). As we explained before, different miRNAs patterns related to ED have been observed in BAV. Thus, silencing these specific ED-related miRNAs may help restoring endothelial function, as well as slowing BAV disease progression (Martínez-Micaelo et al., 2017; Poggio et al., 2019).

Nevertheless, if the theory that ED is mainly related to alterations in hemodynamics in the aortic flux due to BAV abnormal morphology was considered, it would be questionable to believe the possibility of a completely successful treatment while the altered blood flow remains intact. While the only definitive treatment to redirect blood flow comprise replacing or repairing the BAV nowadays, this option is not always recommended as it involves inherent intervention risks, as well as potential long-term complications of the repaired or new valve (Tillquist and Maddox, 2011; Nishimura et al., 2014; Baumgartner et al., 2017).

DISCUSSION

In summary, evidence of ED in BAV has surfaced in the latest years, showing a direct relation with BAV related aortopathies and worse prognosis of BAV. Further research should be performed in order to understand the pathways involved in this BAV related ED. New advances in imaging techniques showed an important effect of alterations in hemodynamics in ED, although a genetical predisposition should also be considered. In both cases, endothelium seems to be altered and ED might be promoted. As discussed before, specific biomarkers for ED could be powerful biomarkers for studying BAV prognosis, as well as its related aortopathies. Additionally, we hypothesize that restoring correct endothelial functioning could maybe help slow down BAV disease progression, mainly in patients where the blood flow is not extremely altered.

Further studies focused on improving endothelial functioning in BAV patients should be performed in order to assess its utility and importance in this complex and heterogeneous disease.

AUTHOR CONTRIBUTIONS

BA-G as the first author, wrote the major part of this paper, as well as searching for bibliography and unifying latest reports about any kind of endothelial dysfunction in bicuspid aortic valve. NM-M and JA helped in the writing process, supervised the manuscript and approved it. All authors contributed to the article and approved the submitted version.

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