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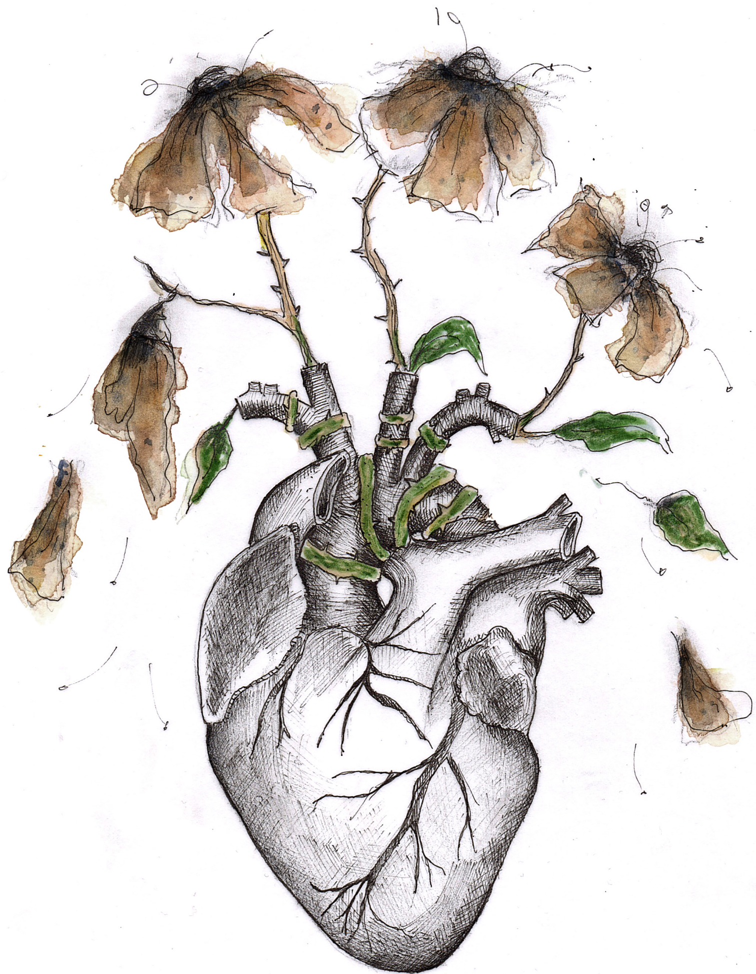
**DOCTORAL THESIS**
*Compendium of Publications*

# THORACIC AORTIC ANEURYSMS AND DISSECTIONS: GENETIC ANALYSIS OF MENDELIAN AND COMPLEX CASES



**Marina Gago Díaz**

**Programa de Doutoramento en Medicina Molecular (RD99/2011)**
**Facultade de Medicina e Odontoloxía**

SANTIAGO DE COMPOSTELA, 2016

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La Doctora **María Brión Martínez**, investigadora principal del grupo de investigación *Xenética de Enfermidades Cardiovasculares e Oftalmolóxicas* del *Instituto de Investigación Sanitaria de Santiago de Compostela*, y el Profesor Doctor **Ángel Carracedo Álvarez**, Catedrático de Medicina Legal del *Departamento de Anatomía Patolóxica e Ciencias Forenses* de la *Facultade de Medicina e Odontoloxía* de la *Universidade de Santiago de Compostela*,

CERTIFICAN:

Que la presente memoria que lleva por título *"Thoracic aortic aneurysms and dissections: genetic analysis of Mendelian and complex cases"* de la Licenciada en Veterinaria por la *Universitat Autònoma de Barcelona* y Máster en Investigación Biomédica por la *Universidade de Santiago de Compostela* **Marina Gago Díaz**, ha sido llevada a cabo bajo su dirección, considerándola en condiciones para optar al Grado de Doctor y autorizándola para su defensa ante el tribunal correspondiente.

De acuerdo con el *artigo 41* del *Regulamento de Estudos de Doutoramento*, declaran también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de **COMPENDIO DE ARTÍCULOS**, en los que la participación de la doctoranda fue decisiva para su ejecución. El resto de coautores, además, están en conocimiento de que ninguno de los trabajos aquí reunidos podrá ser presentado en ninguna otra tesis doctoral.

Y para que así conste, se expide el presente certificado en Santiago de Compostela, a 12 de diciembre de 2016.

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Fdo: Dr. María Brión Martínez

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*San Ciprián, 26 de agosto 2016*

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*A mi madre*

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# Summary (English)

The present doctoral thesis deals with the still partially unraveled genetic component of thoracic aortic aneurysms and dissections, a frequently asymptomatic but potentially lethal condition and major cause of sudden death. Believing in the relevance of the molecular diagnosis of this disease for an anticipated clinical diagnosis and prognosis, our main objective was to contribute to further elucidate the genetics behind it, from both Mendelian and complex perspectives.

The Mendelian cases we analyzed were single and familial, forensic and clinical. We looked for potentially causal genetic variants applying either candidate-gene or whole exome massive parallel sequencing approaches, respectively. When other relatives were available, either affected or unaffected, we demonstrated segregation with the disease through traditional sequencing. We were able to solve approximately 23% of the forensic single cases, and identified two strong candidate genetic variants in *TGFB2* and *PRKG1* genes in the two non-syndromic familial cases analyzed.

For the analysis of complex cases we chose a population-based approach. We selected bicuspid aortic valve patients with and without concomitant thoracic aortic dilation, and faced them against general population controls, seeking a different distribution of risk or protective allele frequencies. We were not able to identify any consistently significant association, though a promising one arose involving *HMCN2* and calcium metabolism that should be considered in future studies.

Some of the results obtained in this doctoral thesis had direct clinical consequences, supporting molecular diagnosis, reliable genotype-phenotype correlations, and risk stratification as important tools for clinical management of these patients and family members at risk, as well as the need of research to continue.

*Keywords:* bicuspid aortic valve, exome-wide association study, genetics, massive parallel sequencing, thoracic aortic aneurysms and dissections.

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# Summary (Spanish)

En la presente tesis doctoral se aborda el estudio del todavía parcialmente desconocido componente genético de aneurismas y disecciones de aorta torácica, una entidad clínica con frecuencia asintomática pero potencialmente letal y causa de muerte súbita. Su principal objetivo fue contribuir a su descubrimiento, desde una perspectiva tanto mendeliana como compleja, para alcanzar un diagnóstico y pronóstico clínicos tempranos.

Se estudiaron casos con herencia mendeliana individuales y familiares, procedentes del ámbito forense y clínico, mediante estrategias de secuenciación masiva en paralelo de genes candidatos y exoma completo, respectivamente, en búsqueda de variantes genéticas potencialmente causales. En aquellos casos en los que se tuvo acceso a otros familiares, tanto afectos como no afectos, se evaluó la segregación de las mismas con la patología mediante secuenciación tradicional. Con esta aproximación, se han resuelto aproximadamente un 23% de los casos forenses y se han identificado dos variantes genéticas candidatas en *TGFB2* y *PRKG1* en los dos casos familiares no sindrómicos analizados.

Para el análisis de los casos con herencia compleja se optó por una estrategia poblacional. Se seleccionaron pacientes afectos por válvula aórtica bicúspide y algunos también por dilatación de aorta torácica. Estos casos se enfrentaron con controles de población general, en búsqueda de diferencias en la distribución de las frecuencias de alelos de riesgo o protección. Esta estrategia de análisis no ha permitido identificar ninguna asociación significativa y consistente, excepto una prometedora en *HMCN2* relacionada con el metabolismo del calcio.

Algunos de los resultados obtenidos en esta tesis doctoral han tenido consecuencias clínicas directas, lo que demuestra la importancia del diagnóstico molecular, asociación genotipo-fenotipo y estratificación del riesgo en el manejo de estos pacientes y familiares, y han evidenciado, además, la relevancia de proseguir la investigación en este campo.

*Palabras clave:* aneurismas y disecciones de aorta torácica, estudio de asociación de exoma completo, genética, secuenciación masiva en paralelo, válvula aórtica bicúspide.

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# Summary (Galician)

Na presente tese de doutoramento abórdase a aínda parcialmente descoñecida compoñente xenética de aneurismas e diseccións da aorta torácica, unha entidade clínica con frecuencia asintomática pero potencialmente letal e causa de morte súbita. O seu principal obxectivo foi contribuír ao seu descubrimento, desde unha perspectiva tanto mendeliana como complexa, para alcanzar un diagnóstico e prognóstico clínicos temperáns.

Estudiáronse casos con herdanza mendeliana individuais e familiares, procedentes do ámbito forense e clínico, mediante estratexias de secuenciación masiva en paralelo de xenes candidatos e exoma completo, respectivamente, na busca de variantes xenéticas potencialmente causais. Naqueles casos nos que se tivo acceso a outros familiares, tanto afectos coma non afectos, avaliouse a segregación das mesmas coa patoloxía mediante secuenciación tradicional. Con esta aproximación, resolvéronse aproximadamente un 23% dos casos forenses e identificáronse dúas variantes xenéticas candidatas en *TGFB2* e *PRKG1* nos dous casos familiares non sindrómicos analizados.

Para a análise dos casos con herdanza complexa optouse por unha estratexia poboacional. Seleccionáronse pacientes afectos por válvula aórtica bicúspide e algúns deles tamén por dilatación da aorta torácica. Estes casos foron enfrontados con controis de poboación xeral, en busca de diferencias na distribución das frecuencias de alelos de risco ou protección. Esta estratexia de análise non permitiu identificar ningunha asociación significativa e consistente, agás unha prometedora en *HMCN2* relacionada co metabolismo do calcio.

Algúns dos resultados obtidos nesta tese de doutoramento tiveron consecuencias clínicas directas, o que demostra a importancia do diagnóstico molecular, asociación xenotipo-fenotipo e estratificación do risco no manexo destes pacientes e familiares. Estas evidencias resaltaron, ademais, a relevancia de proseguir a investigación neste campo.

*Palabras chave:* aneurismas e diseccións de aorta torácica, estudo de asociación de exoma completo, secuenciación masiva en paralelo, válvula aórtica bicúspide, xenética.

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# ABBREVIATIONS

AAA: Abdominal Aortic Aneurysm.

ACE: Angiotensin-Converting Enzyme.

ADPKD: Autosomal Dominant Polycystic Kidney Disease.

AOS: Aneurysm-Osteoarthritis Syndrome.

ATS: Arterial Tortuosity Syndrome.

BAV: Bicuspid Aortic Valve.

cM: centimorgan.

CNV: Copy Number Variation.

CT: Computed Tomography.

DNA: Deoxyribonucleic Acid.

ECM: Extracellular Matrix.

EDS: Ehlers-Danlos Syndrome.

EWAS: Exome-Wide Association Study.

GWAS: Genome-Wide Association Study.

ICA: Intracranial Aneurysm.

indel: insertion or deletion.

LDS: Loeys-Dietz Syndrome.

LOD: Logarithm of the Odds.

LVOT: Left Ventricular Outflow Tract.

MAF: Minor Allele Frequency.

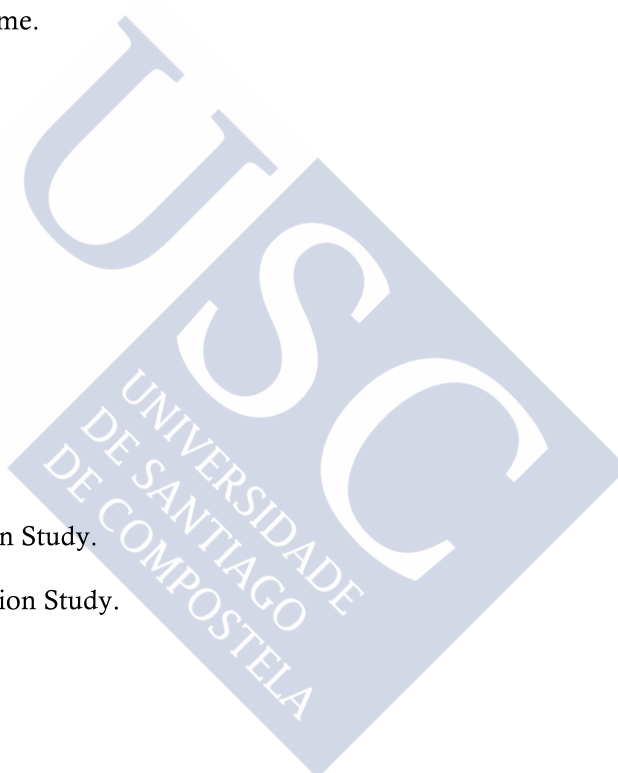
MFS: Marfan Syndrome.

miRNA: micro-Ribonucleic Acid.

MMP: Matrix Metalloproteinase.

MPS: Massive Parallel Sequencing.

MRI: Magnetic Resonance Imaging.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mRNA: messenger Ribonucleic Acid.

NSAD: Non-Syndromic Aortic Disease.

PCR: Polymerase Chain Reaction.

PDA: Patent *Ductus Arteriosus*.

p-Smad: phosphorylated Smad.

RNA: Ribonucleic Acid.

RT-qPCR: quantitative Real-Time Polymerase Chain Reaction.

SD: Sudden Death.

SMC: Smooth Muscle Cell.

SNP: Single Nucleotide Polymorphism.

STR: Short Tandem Repeat.

SV: Sinuses of Valsalva.

TAA: Thoracic Aortic Aneurysm.

TAAD: Thoracic Aortic Aneurysm and Dissection.

TAD: Thoracic Aortic Dissection.

TAV: Tricuspid Aortic Valve.

TEVAR: Endovascular Therapy.

TGF-β: Transforming Growth Factor-β.

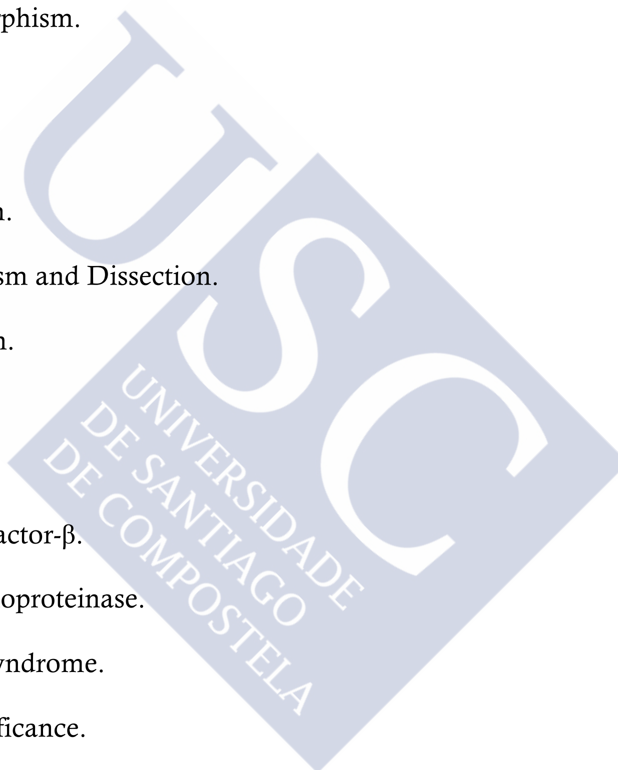
TIMP: Tissue Inhibitor of Metalloproteinase.

vEDS: vascular Ehlers Danlos Syndrome.

VUS: Variant of Unknown Significance.

WES: Whole Exome Sequencing.

WGS: Whole Genome Sequencing.



# I. INTRODUCTION

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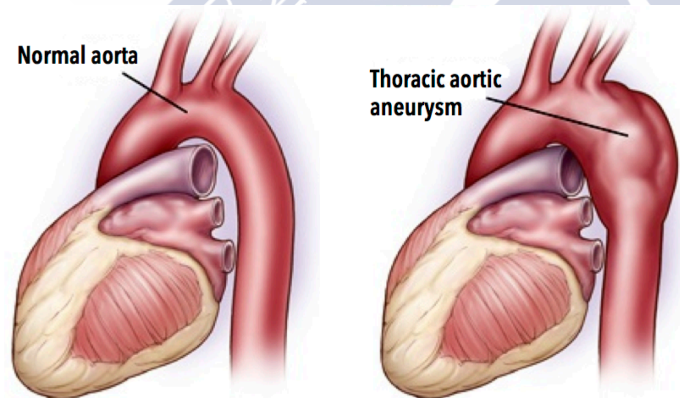
# I. INTRODUCTION

## 1. Thoracic aortic aneurysms and dissections: clinical aspects

### 1.1. Definition and epidemiology

Thoracic aortic aneurysm (TAA) is defined as the abnormal, asymptomatic, and irreversible enlargement of a weakened thoracic aortic wall over time, to at least 1.5 times the blood vessel original size, approximately (*Figure 1*)[1–3]. Although TAA is usually not inherently dangerous, it is a potentially lethal condition because as the diameter of the vessel grows, it shows a predisposition for both dissection or abrupt rupture[4,5]. These may occur even without substantial aortic enlargement[6] and they are many times the first symptom, so the morbidity and mortality rates are high[4,7]. Rapid diagnosis and decision-making are crucial to reduce an extremely poor prognosis[8].

**Figure 1. Graphic representation of a normal thoracic aorta and thoracic aortic aneurysm**

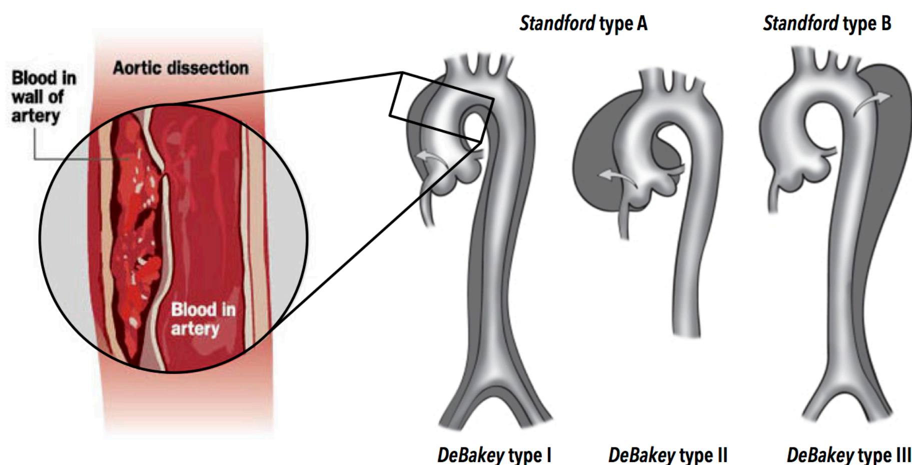



In the image on the right, the aortic dilation is located at the beginning of the descending thoracic aorta, just after the aortic arch *(adapted from the Society for Vascular Surgery, at https://vascular.org/patient-resources/vascular-conditions/thoracic-aortic-aneurysm)*[9].

Aortic dissection is defined as the acute disruption of the medial layer provoked by an acute tear in the aortic inner layer (intima), which typically permits blood to penetrate through the diseased medial layer and dissect along the plane of the aortic wall[2]. The subsequent formation of a true and a false lumen with or without communication can be followed either by an aortic rupture in the case of adventitial disruption, or by re-entering the aortic lumen through a second intimal tear[8,10]. This clinical entity is known as thoracic aortic dissection (TAD) and it is a major cause of sudden death (SD)[11], being the annual risk greater than 10%[12,13].

Two main classification systems of TAD exist: *Standford* and *DeBakey* (*Figure 2*)[14]. On the one hand, the *Standford* scheme categorizes dissection by the involvement of the ascending thoracic aorta an distinguishes between type A, which involves the ascending thoracic aorta, and type B, which does not[14–17]. On the other hand, the *DeBakey* classification categorizes dissection by the site of origin[14]. Dissections originating in the ascending thoracic aorta are subdivided into those that propagate to the aortic arch (type I), and those confined to the ascending thoracic aorta (type II), as opposed to those that originate in the descending thoracic aorta (type III), regardless of whether they propagate distally or retrograde[15].

**Figure 2. *Standford* and *DeBakey* types of aortic dissection**



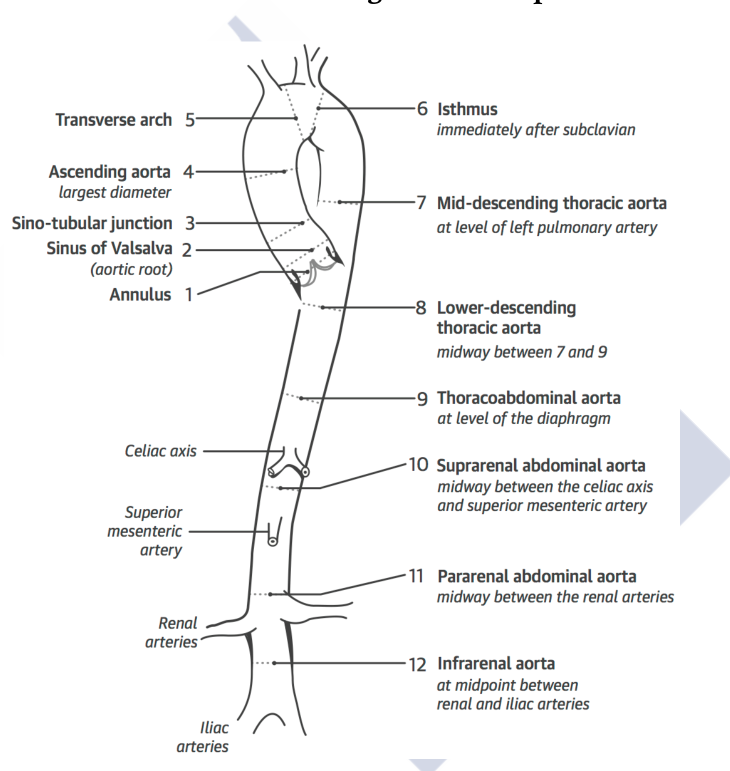

Graphic representation of an aortic dissection and the *Standford* and *DeBakey* classification systems. *DeBakey* type III can be also differentiated in subtypes III-A to III-C, depending on the thoracic or abdominal involvement according to Reul *et al.* 1975 *(adapted from Institut za Kardiovaskularne bolesti-IKVB Vojvodine at [http://www.ikvbv.ns.ac.rs/sr/za-pacijente/srce-i-bolesti-srca/547-disekcija-aorte?highlight=WyJkaXNla2NpamEiLCJhb3J0ZSIsImRpc2VrY2lqYSBhb3J0ZSJd](http://www.ikvbv.ns.ac.rs/sr/za-pacijente/srce-i-bolesti-srca/547-disekcija-aorte?highlight=WyJkaXNla2NpamEiLCJhb3J0ZSIsImRpc2VrY2lqYSBhb3J0ZSJd), and "Aortic Aneurysm. Recent Advances" (edited by Cornelia Amalinei) - chapter 12 "Numerical simulation in ulcer-like projection due to type B aortic dissection with complete thrombosis type" (Mori, F.; Ohtake, H.; Watanabe, G; and Matsuzawa, T.), 2013, DOI: 10.5772/52559 at [http://www.intechopen.com/books/aortic-aneurysm-recent-advances/numerical-simulation-in-ulcer-like-projection-due-to-type-b-aortic-dissection-with-complete-thrombos](http://www.intechopen.com/books/aortic-aneurysm-recent-advances/numerical-simulation-in-ulcer-like-projection-due-to-type-b-aortic-dissection-with-complete-thrombos))*[18–20].

Whereas the abdominal aortic aneurysm (AAA) is mainly linked to lifestyle-associated risk factors (atherosclerosis, hypertension, sex, age, and smoking)[21,22], thoracic aortic aneurysm and dissection (TAAD) can develop in association with several heterogeneous conditions with substantial phenotypic diversity[23]. They are typically classified based on the underlying pathology in sporadic (degenerative, inflammatory, autoimmune, infectious, or traumatic) or hereditary[24–26]. Although TAAD is most frequently degenerative and isolated, up to 40% of cases have been expected to be hereditary with a heterogeneous etiology and genetic background[8,26–29]. Those heritable disorders affecting the thoracic aorta can be further classified, depending on the presence or absence of clinical manifestations in other organ systems, in[30]: (i) syndromic, including well-characterized genetic syndromes such as Marfan (MFS), Loeys-Dietz (LDS), the vascular type of Ehlers-Danlos (vEDS), or aneurysm-osteoarthritis (AOS) syndromes, representing less than 5% of

all TAAD[26,31,32]; and (ii) non-syndromic, involving those familial cases without syndromic features, in which TAAD is the predominant clinical manifestation (non-syndromic aortic disease, NSAD)[26,33,34]. They are all of them characterized by genetic heterogeneity, phenotypical overlap, and a broad range of inter- and intra-familial variability[29]. While degenerative TAA is a late onset disease which tends to manifest and dissect in patients over 65 years, patients with hereditary disorders typically present between the fourth and the sixth decades[30].

Furthermore, TAAD may involve one or more aortic segments (aortic root, ascending thoracic aorta, aortic arch, or descending thoracic aorta; *Figure 3*) and has been further classified accordingly[35].

**Figure 3. Anatomic location of the aortic segments susceptible to develop aneurysm**




The *ligamentum arteriosus*, not represented in this figure, would be located between numbers 6 and 7 *(adapted from Weinsaft et al. 2016)*[36].

It is generally assumed that 60% of TAAD involve the aortic root or ascending thoracic aorta, 40% the descending thoracic aorta, 10% the aortic arch, and 10% the thoracoabdominal aorta (with some involving multiple aortic segments)[4,22,35]. Albornoz *et al.* described in 2006 520 TAA cases in which the ascending thoracic aorta was affected in 79.4% and the descending in 20.6%[37]. Both the syndromic and non-syndromic groups include many disorders in which disease predominates in the very proximal ascending thoracic aorta[5]. In fact, there are only rare exceptions in which the ascending thoracic aorta is seldom involved, such as the vEDS[5]. No matter which of the

aforementioned segments is affected[35], the most devastating complication is in any case the aortic dissection or rupture[38].

Both the etiology and location of an aneurysm may affect its rate of growth and propensity for dissection or rupture[35]. Although the type of aortic disease can vary within a family (e.g. presence or absence of dilation prior to dissection)[39], the location of the aneurysm in the proband generally influences the location of the aneurysm in the family members: probands with ascending TAA have relatives with ascending TAA, while probands with descending TAA most commonly tend to have, surprisingly, family members with AAA[33,37,40].

In fact, the descending TAA is closer to AAA and seems to have a diverse pathogenetic basis compared to the ascending TAA[37]. This latter is very highly familial, not highly correlated with atherosclerosis or hypertension, very rarely calcified, and almost never contains intramural thrombus[33,37]. Descending TAA or AAA, conversely, are strongly related to advanced age, atherosclerosis, hypertension, almost invariably calcified, and very frequently contain thrombus[33,37]. Therefore, despite affecting the same organ with a common phenotypic manifestation, TAA and AAA could be considered two different diseases[33,41].

This concept is correlated with the diverse origin of the cell lineages and the structural differences between the thoracic and abdominal lamellar units, highlighting the aortic regional heterogeneity[28]. During the early stages of embryonic development, migration and differentiation of cells from the neural crest give raise to the ascending thoracic aorta, the aortic valve, and the *ductus arteriosus*, while the proximal descending and abdominal aorta are mainly composed of mesoderm-derived cells[5,10,28]. Different smooth muscle cell (SMC) populations within a common vessel may respond in a lineage-dependent way to growth factors or other stimuli that govern developmental events and which could participate in vascular disease in later life[5]. Nevertheless, it is important to keep in mind that the TAA and AAA dichotomy is somehow artificial, not only because of the presence of thoracoabdominal aneurysms, but also because of the possibility of tandem lesions[8]. For instance, in a recent series, 27% of patients with AAA also presented a TAA, most of whom were women and seniors[8,42].

Regarding the epidemiology of this disease, TAAD is one of the major diseases affecting the aorta[39,43]. Although AAA is a much more common form of aortic aneurysm (about 80% of aortic aneurysms occur between the renal arteries and the aortic bifurcation)[35,44], TAA is still the second common site[8]. In fact, autopsy series, able to document acute aortic dissection true incidence without confounding it with myocardial infarction, have indicated that it is actually the most common lethal condition affecting the human aorta, more common than the better-appreciated

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ruptured AAA[13,45]. Although this particularly lethal nature has sparked interest in understanding TAA behavior[4,5], less is known in comparison[25].

As about 95% of TAA cases are asymptomatic[1,35] and remain undetected unless incidentally discovered[4] or if a life-threatening event occurs[10], TAA real incidence is hard to estimate[4,46]. Data published in 2009 by the *U.S. Center for Injury Control and Prevention* indicated aortic aneurysm as the 18th most common cause of death considering the whole population, and the 15th most common in individuals older than 65, accounting for 13,843 and 11,147 deaths, respectively[4,46]. Specifically regarding TAAD, and according to the data published by Milewicz *et al.* in 2008, it was considered the 15th leading cause of death in the United States[10]. Furthermore, Bickerstaff *et al.* and Ramanath *et al.* estimated in 1982 and 2009, respectively, the annual incidence of TAA to be approximately 6 to 10 cases/100,000 patients per year[4,47,48], while the incidence of aortic dissection and rupture had been estimated to approximately 3.5/100,000 patients per year[4,49,50]. Clouse *et al.* calculated in 2004 the overall incidence of TAAD to be 2.7 per 100,000/person per year[49,51]. In any case, TAA frequency appears to be increasing, although it is not yet understood if this observation could be explained by an aging population or more available and advanced imaging methods and screening[4,40].

## 1.2. Pathophysiology and risk factors

Any TAAD can result from the influence of genetics, environmental factors, or hemodynamics, which are in many cases linked, being difficult to differentiate causes from consequences:

(i) **Genetic factors:** are key for TAAD development and will be discussed in detail further on. They mainly contribute to medial degeneration[14].

(ii) **Environmental factors:** generally increase aortic wall stress, being the most frequent: (a) age; (b) hypertension; (c) male gender; (d) hyperlipidemia and atherosclerosis; (e) trauma; (f) smoking; (g) chronic cocaine abuse; (h) pregnancy; and (i) weight lifting or extreme exercise[6,25,31,35,52]. One of the most important is age, as TAAD always has an age-dependent penetrance[53]. With increasing age, and without any other predisposing factors, arterial walls typically undergo gradual stiffening, thickening, accumulation of atherosclerotic plaques, and deposition of calcium[54].

(iii) **Hemodynamics:** have either the potential to affect hereditary TAAD progression and complications, or to directly cause some forms of aortopathy, such as the post-stenotic[31,55]. In addition to intrinsic factors responsible for aortic dilation, the wall of a

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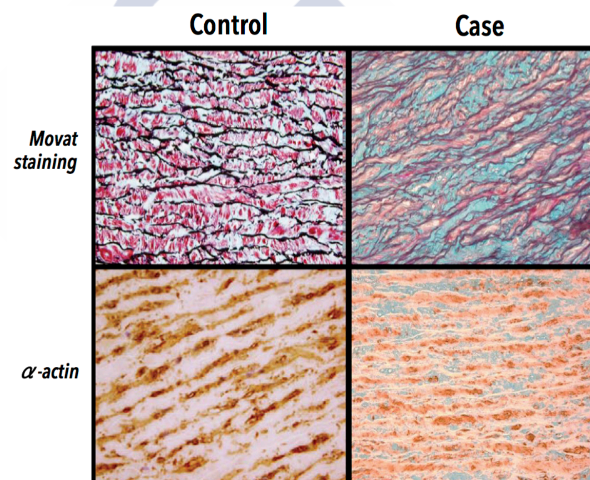
dilated aorta is submitted to increased tension and a change of flow from laminar to turbulent at the dilated site[55].

Regardless of the genetic or environmental factors behind TAAD, the pathophysiological hallmark of about 80% of cases is medial degeneration (formerly "cystic medial necrosis"; *Figure 4*)[7,52,56]. It occurs with normal aging of the aorta but can be accelerated by hypertension and brought on by genetic variation that predisposes individuals to the thoracic aortic disease[13,39,57]. In fact, it is an overlapping histopathological condition that involves a collection of different histopathological changes[52], mainly: (i) elastic fibers fragmentation and/or loss, thinning, and disorganization; (ii) SMC disorganization and nuclei loss (at least not clearly identifiable); (iii) laminar medial collapse or compaction of medial elastic fibers; (iv) medial mucoid extracellular matrix (ECM) accumulation; and (v) medial fibrosis[10,52].

**Figure 4. Healthy thoracic aorta versus medial degeneration**




Upon *Movat's pentachrome* staining (elastin stain): the case shows increased proteoglycan deposition (blue), focal mild fragmentation of elastic fibers (black), and decreased number of SMCs (red). Upon *α-actin* immunostaining: the case shows mild focal loss of SMCs. SMC = smooth muscle cell (*adapted from Guo et al. 2015*)[58].

Not all them need to be present in every TAAD case, but imply changes in the integrity and structure of the ECM within the media of the aortic wall, and lead to weakening, which in turn results in aortic dilation and aneurysm formation[30,35,52]. They can even be found in the aortic walls of affected individuals before the development of aneurysms or dissections together with altered expression of many ECM proteins (these latter not yet known to be cause or consequence)[30]. In fact, medial degeneration is not the cause of a dissection, but rather a consequential pathological finding or a side effect caused by a primary pathology[22]. Although it is clear that excessive matrix degradation is involved in TAAD development, the exact underlying mechanisms have not yet been fully unraveled[22].

The pathophysiologic processes that give rise to TAAD seem to be similar despite the diverse primary cause[30,52]. It is therefore essential to grade the corresponding medial degeneration, which may vary from very little to severe destructive alterations[52]. A more specific pathologic picture is likely to be defined in a near future allowing the recognition of histopathologic patterns typical of specific genetic aortic syndromes[8,52,59]. In fact, Halushka *et al.* have already started to do so this 2016[52]. Nevertheless, nowadays, it is not yet possible to reach a precise diagnosis based on histopathology, raising the need for alternative imaging and molecular approaches.

## 1.3. Symptoms and clinical diagnosis

Similar to other arterial diseases, TAA is commonly diagnosed either incidentally on imaging studies after a long period of subclinical development, or due to an acute presentation[8,35]. Therefore, in general, these patients feel no pain or other symptoms until a catastrophic event such as dissection or rupture occurs[6,39]. The main symptoms that happen in those acute cases and in approximately 5% of the symptomatic ones[13], are: (i) syncope; (ii) chest or back pain (abrupt, deep, aching, or throbbing); (iii) local mass effects (the trachea or mainstem bronchus lead to cough, dyspnea, inspiratory stridor, recurrent airway infection; the esophagus to dysphagia; and the recurrent laryngeal nerve to hoarseness); (iv) aortic regurgitation (diastolic murmur); (v) myocardial ischemia or infarction; (vi) congestive heart failure; (vii) large pleural effusions; and (viii) neurological symptoms (cerebral malperfusion, hypotension, distal thromboembolism, peripheral nerve compression)[8,35].

Timely recognition of TAAD patients can be life-saving as mortality rates of up to 70% have been reported[48,51], but clinical diagnosis based on symptoms cannot be considered a real diagnostic alternative. In contrast, some useful imaging procedures for early diagnosis exist (*Table 1*). These, followed by appropriate treatment, could significantly improve morbidity and mortality rates[1]:

(i) **Chest X-ray:** is generally obtained for other indications, but may detect abnormalities of the aortic contour or size as an incidental finding, prompting further imaging[8]. TAAs visible on chest X-ray films are characterized by a widening of the mediastinal silhouette, an enlargement of the aortic knob, or a tracheal deviation[35]. Nevertheless, a normal aortic silhouette is not sufficient to rule out the presence of a TAA[8].

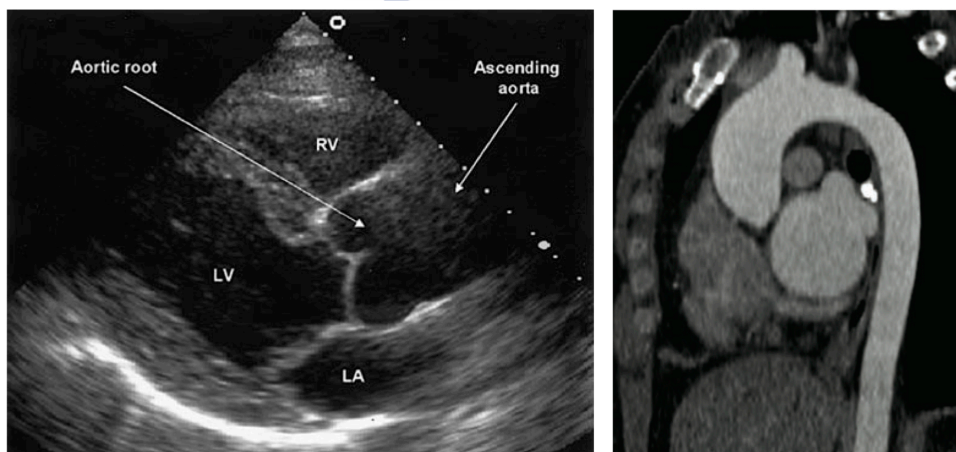
(ii) **Ultrasound:**

a. ***Transthoracic echocardiography:*** is not the technique of choice for a full assessment, but an excellent imaging modality for serial measurements of maximal aortic root and proximal diameters, evaluation of aortic regurgitation, and timing for elective surgery (*Figure 5*)[8].

b. ***Transesophageal echocardiography:*** permits improved assessment of the aorta from its root to the descending portion, but it is a semi-invasive procedure that requires sedation, strict blood pressure control, and the exclusion of any esophageal disease[8].

(iii) **Computed tomography (CT):** plays a central role in the diagnosis, risk stratification, and clinical management of all aortic diseases[8]. Non-enhanced CT, followed by CT contrast-enhanced angiography, is the generally recommended protocol, particularly when aortic dissection is suspected[8]. It is a widespread available imaging technique that requires little time for image acquisition and processing, and has the ability to obtain a complete three-dimensional dataset of the entire aorta (*Figure 5*)[8].

**Figure 5. Transthoracic echocardiography versus computed tomography visualization of the thoracic aorta**




Parasternal long-axis view of the transthoracic echocardiogram demonstrating a dilated aortic root and a less well visualized ascending thoracic aorta (left). Sagittal-reconstructed CT image of the thoracic aorta showing dilated ascending TAA (right). CT = computed tomography; TAA = thoracic aortic aneurysm *(adapted from Isselbacher 2005 and Rajiah 2013)*[35,60].

(iv) **Magnetic resonance imaging (MRI):** together with CT, it is one of the generally preferred imaging modalities to define aortic anatomy and TAA size, and evaluate evasive ascending thoracic aortas[31,35]. It reliably depicts all the features needed for clinical decision-making: maximal aortic diameter, shape, and extent of the aortic aneurysm; involvement of the aortic branches; relationship with adjacent structures; and presence of mural thrombus[8]. Nevertheless, it has some drawbacks, such as the lower accessibility, the greater difficulty to monitor unstable patients during imaging, and the lengthy acquisition times[8].

(v) **Aortography:** implies a cardiac catheterization, and has therefore been generally replaced by non-invasive techniques, but may be useful when this latter are ambiguous or incomplete[8]. It provides exact information about the shape, size, and anomalies of the

aorta, and permits the visualization of the aortic lumen, side branches, and collaterals, as well as the evaluation of the aortic valve condition and left ventricular function[8].

**Table 1. Advantages and disadvantages of the main imaging methods used for thoracic aortic disease diagnosis**

| Feature | Transthoracic echocardiography | Transesophageal echocardiography | Computed tomography | Magnetic resonance imaging | Aortography |
|---|---|---|---|---|---|
| Ease of use | +++ | ++ | +++ | ++ | + |
| Diagnostic reliability | + | +++ | +++ | +++ | ++ |
| Follow-up | ++ | + | ++ | +++ | - |
| Aortic wall visualization | + | +++ | +++ | +++ | - |
| Cost | - | - | -- | --- | --- |
| Radiation | 0 | 0 | --- | - | -- |
| Nephrotoxicity | 0 | 0 | --- | -- | --- |

The number of signs refers to the estimated potential value. “+” = positive; “-” = negative; 0 = no effect *(adapted from Erbel et al. 2014)*[8].

Many times, more than one of these proposed imaging diagnostic tools are used, and their results compared to age-appropriate normograms related to body surface area[8,61]. This is of particular interest when diameters are borderline for the decision to proceed with an intervention, and to assess enlargement rates during the follow-up[8]. The corresponding imaging study should be repeated in all patients six months after the initial diagnosis to asses evolutionary changes[31,35]. Further follow-up, that will allow to determine the rate of growth[35], is guided by the diameter, evolution, underlying diagnosis, and family history[31]. In any case, should there be a significant increase in aortic size from one study to the next, the interval between studies would need to be decreased, or an operative intervention to be scheduled[30,35].

So as TAA is a clinical entity that can appear in association to multiple diseases[4,62], once the TAA has been identified, it is also important to determine which is the underlying disorder. A complete diagnosis will ensure appropriate decisions regarding the set-up of a personalized strategy for follow-up and treatment for the patient and relatives[31,62]. Although standardized initial evaluation is absolutely necessary, it is also important to determine if vascular evaluation should be limited or not to the aorta, as well as the need for an extravascular evaluation (including skeleton, eyes, neurological and immunological systems, and digestive tract)[63]. This includes a detailed family history examining facial dysmorphology, myopia, ocular lens dislocation, premature cataracts, bifid uvula, spine disease, *pectus* deformities, cleft palate, club feet, pneumothorax, bicuspid aortic valve (BAV), intracranial aneurysm (ICA), aortic or branch vessel aneurysm, patent *ductus arteriosus* (PDA), premature vascular disease, aortic dissection, or SD[64]. For instance, if MFS was to be suspected, clinical diagnosis would rely on the revised *Ghent nosology* published by Loeys *et al.* in



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2010, which already considers all the cardiac and extracardiac symptoms that these patients can develop[65].

TAA could also be indicative of a more diffuse aortic disease so a full assessment of the aorta and aortic valve should be performed, both at baseline and during follow-up[8]. Up to 1/4 of TAA patients have concomitant AAA[35], and there is a 10% likelihood to harbor an ICA[33,66]. From the 88 TAA families evaluated by Albornoz *et al.* in 2006, a total of 197 probands and kindred had an aneurysm in some site: 66.5% had TAA, while 24.9% AAA, and 8.6% cerebral or other arterial aneurysms[37].

Finally, besides proband follow-up, systematic familial screening is also recommended in order to recognize affected family members before an aortic complication occurs, especially for those with concurrent ICAs or AAAs, a family history of SD, or persistent atypical chest pain[4,59]. As TAAD is an age-dependent clinical manifestation that may progress subclinically until later in life, lifelong follow-up is required too in all those family members at risk[31].

## 1.4. Treatment and prognosis

Considering this is not the main topic of this thesis, we have briefly summarized the main TAAD treatment options. Although lifestyle, medical, and surgical approaches exist, the effectiveness of many of them is still largely unproven.

(i) **Lifestyle alterations:** seek the reduction of cardiovascular risk factors, such as blood pressure control, smoking cessation, optimization of the lipid profile, avoidance of acute exertion (e.g. heavy weight lifting), or any other stressful activity and emotion, as they could precipitate the onset of acute TAD[4,8,31].

(ii) **Medical therapies:** are still quite limited, many times without proven clinical benefit, and frequently controversial as they often imply a lifetime decision[35,46]. Their aim is to reduce shear stress on the diseased segment of the vessel by reducing blood pressure and cardiac contractility[8].

a. ***β-blockers:*** can be either used in acute or chronical cases to reduce the heart rate and systolic blood pressure[8], or the cardiac inotropy, wall shear stress, and blood pressure stress[4,46], respectively. Although they have been used as the standard therapy in MFS and LDS patients[31,67], there is general concern about the treatment of non-MFS patients, the side effects, and the potential paradoxical adverse impact on AAA[46]. Real clinical benefit has only been demonstrated in MFS patients[46], who show slower

aortic root growth, fewer cardiovascular complications (aortic regurgitation, dissection, or surgical repair), and improved survival[10].

b. ***Angiotensin-II receptor blockers:*** may have a protective effect by inhibiting the transforming growth factor-β (TGF-β) signaling through selective blockade of the angiotensin-II receptor AT1 and its hypertensive effect[8,14]. They have experimentally resulted in the normalization of the aortic growth and improved aortic wall structure[41], and have been commonly used for the medical treatment of MFS and LDS patients[59,67]. Clinical trials involving *losartan* in MFS patients (the main representative of this pharmacological group) have been generally not conclusive and disappointing[14,41]. But a recent study performed by Franken *et al.* in 2015 demonstrated that the efficiency of *losartan* therapy depended on the type of *FBN1* mutation, significantly reducing the rate of aortic growth in haploinsufficient but not in dominant-negative MFS patients[41,68]. To date, it has not yet been demonstrated their efficacy in aortic diseases of other etiologies[8].

c. ***Angiotensin-converting enzyme (ACE) inhibitors:*** together with angiotensin II receptor blockers and β-blockers, they seem to have reduced either the progression of the aortic dilation or the occurrence of complications when used prophylactically in MFS chronic cases[8]. However, there is not yet evidence for the efficacy of these treatments in aortic disease of other etiologies[4,8,41].

d. ***Antibiotics:*** such as tetracyclines or macrolides (*doxycycline*), were proposed as a potential treatment of TAAD due to their ability to nonspecifically inhibit matrix metalloproteinases (MMPs)[4,10,46]. In fact, they had been used for the treatment of other conditions associated with increased MMP activity, such as rheumatoid arthritis[28]. Although preliminary results from animal models have been promising, they are still under development as to date no human studies have been conducted[28].

e. ***Anti-inflammatory agents:*** although the role of inflammatory and oxidative mechanisms in TAAD pathogenesis remains unclear, they have been expected to prevent or mitigate aneurysm progression through anti-cyclooxygenase inhibition, particularly in the sporadic forms of the disease[28,46].

f. ***Statins:*** have been suggested to inhibit the expansion of aneurysms by Jovin *et al.* in 2012 and Stein *et al.* in 2013[8,69,70], but their clinical usefulness is still under debate.

g. ***Immunosuppressants:*** such as *ramapycin*[46], they have been expected to be efficient especially in the treatment of the sporadic forms of the disease, the same as the anti-inflammatory agents. They are also under development.

(iii) **Surgery:** is currently the mainstay of effective treatment, especially when prophylactic[4,58], but the optimal timing remains somewhat uncertain[35]. In asymptomatic patients, it depends mainly on the size of the aneurysm, but also on the location and the underlying etiology[8,13,14]. The general recommendations for preventive surgery are five and a half centimeters for the aortic root, six for the ascending, seven for the descending, and six for the thoracoabdominal aorta[8,14,46]. The presence of a concomitant BAV, connective tissue syndrome, a positive family history of aortic dissection, systemic hypertension, aortic coarctation, or a rapidly enlarging aortic diameter (greater than three millimeters/year), often warrant earlier intervention at smaller aortic sizes[4,8,31]. Furthermore, regardless of the aortic dimensions, any symptomatic patient (5%) should be immediately referred for surgery[8,13,14]. Many different surgical procedures exist, regarding how to access the lesion, how to manage blood circulation while the surgery is taking place, the type of graft used, and even the replacement technique itself. So, like the timing, the election will depend on the underlying disease and aneurysm location.

Although TAAD is often a lethal disease if left untreated, non-dissecting patients who have been early recognized and have undergone appropriate elective surgical treatment, have a near-normal prognosis and good long-term outcomes[6,39]. Nevertheless, even when prophylactic surgery has been performed, the risk for aortic dissection may persist and surveillance is still necessary[36]. Finally, it is also important to consider that indications for scheduled surgery generally rely on available guidelines that evolve with time, so the most recent recommendations should be consulted when needed[26].

(iv) **Endovascular therapy (TEVAR):** can be either an alternative or a complement to open surgery. It has the objective of repairing dilation of the thoracic aortic wall through the implantation of a membrane-covered stent-graft across it, in order to prevent further enlargement and ultimate aortic rupture, but it must necessarily be preceded by a careful pre-procedural planning for being successful[8]. This approach avoids the pain and recovery time from a thoracotomy and the risks of aortic clamping, but its use may be limited in patients with inadequate landing zones for stent placement, excessive aortic dilation, inadequate vascular access, or severe aortic atherosclerosis[14]. Furthermore, besides the potential vascular complications at the puncture site, aortic and neurological complications, and/or endoleaks can also develop[8]. Therefore, although TEVAR is playing an increasingly important role, surgery remains necessary in many situations, as for example in patients with connective tissue disease and ascending or type A dissections, in which there has been no role for TEVAR[8,14]. Finally, the ongoing frequent

lack of information on long-term results should also be kept in mind, particularly when dealing with young patients[8].

The poor prognosis after dissection supports how critically important is to intervene before it occurs[46]. Even with the best medical and surgical election, 40% of TAAD patients do not survive acute dissections[12]. This fact emphasizes the need of timely surgical repair and complementary diagnostic tools able to anticipate the development of this life-threatening complication, as there are no clinically available methods to identify patients at risk of dissection[12].

Individualization of TAA therapy has therefore to be adapted to both the specific phenotype and genotype (as discussed below)[63]. Just as the scientific community has come to recognize that the thoracic aorta behaves differently from the abdominal, and the ascending from the descending thoracic aorta, so too has it come to appreciate that TAAs of different origins can behave quite differently[41]. The underlying mutation, as well as the underlying disease, appear to affect both risk and response to therapy[41], and should be, when possible, taken into account.

# 2. Genetic architecture of thoracic aortic disease

Once the clinical usefulness of TAAD molecular diagnosis had been demonstrated, great advances were made in this field[5,71]. Over the past years, the high genetic heterogeneity behind heritable TAAD has been strongly supported[67]. This clinical manifestation has proved to develop following a typical Mendelian pattern of inheritance (frequently autosomal dominant, but also recessive or X-linked) or in association with complex diseases, dependent on multiple genetic and environmental factors. In the near future, more genotype/phenotype data on already known and new genes will be described. Thus allowing a larger proportion of TAAD cases to be explained, with the resultant benefit, enabling medical and surgical management recommendations to be strengthened on the basis of greater evidence[67]. The following is a complete summary about the actual knowledge on the genetics behind TAAD cases, approached from the perspective of the different diseases they have been associated with, classified based on their genetic basis.

## 2.1. Mendelian diseases

They are, generally, the most accessible, as caused by a single-gene alteration affecting multiple family members following a specific pattern of inheritance[27]. Necessary and sufficient causal mutations lead to dramatic changes in protein concentration or function, while environmental factors typically play a small or non-existent role[72]. Depending on the presence of other symptoms beyond the vascular affection, Mendelian TAAD diseases can be further classified in syndromic and non-syndromic. Despite the differences among them, which will be exposed below, both groups

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include many disorders in which the disease predominates in the very proximal ascending thoracic aorta[5].

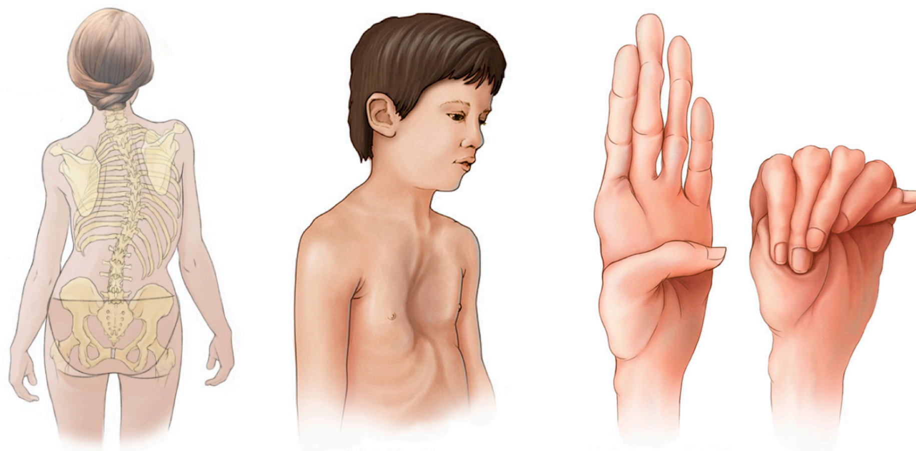
### 2.1.1. Syndromic thoracic aortic disease

Syndromic TAAD occurs in conjunction with a range of associated anomalies affecting multiple organ systems, showing prominent features of a systemic connective tissue disorder[28,41]. They tend to be caused by genes with wide expression patterns[41], and comprise the main connective tissue syndromes: MFS, LDS, and vEDS.

#### 2.1.1.1. Marfan syndrome

MFS (*OMIM* #154700[73]) is the most common familial connective tissue disorder associated with TAAD[4,74,75], affecting to one to five of every 10,000 live births[76,77]. It is a multisystemic and pleiotropic disease that produces the most significant changes in the connective fibers of the musculoskeletal, cardiovascular, and ocular systems, but may also affect the pulmonary and nervous systems, and the integumentary fibrils (*Figure 6*)[22,44]. Morbidity and mortality rates mainly depend on the development of TAAD, which frequently affects the sinuses of Valsalva (SV) causing annulo-aortic ectasia, although the entire aorta is weakened[59,74,78]. Aortic root dilation is present in 60-80% of MFS patients[13,61], which furthermore have a 40% likelihood of aortic dissection during a lifetime[33]. TAAD typically happens in early adulthood (before 40 years if left untreated[44,61]), unless they undergo prophylactic aortic repair[10]. As a consequence of these high morbidity rates, despite being a rare disease, MFS accounts for about 5% of all TAAD cases[13,61].

**Figure 6. Three of the most representative musculoskeletal manifestations of Marfan syndrome**



From left to right, scoliosis, *pectus excavatum*, and arachnocatyly *(adapted from Mayo Clinic http://www.mayoclinic.org/diseases-conditions/marfan-syndrome/symptoms-causes/dxc-20195415 and from Boston Children's Hospital at http://www.childrenshospital.org/conditions-and-treatments/conditions/scoliosis/symptoms-and-causes)*[79,80].

MFS is a monogenic disorder inherited as an autosomal dominant trait (direct father to son transmission)[2,78]. The genetic defect behind it was discovered in 1990, when Kainulainen *et al.* pinned it to chromosome 15q15-31[81]. But it was not until 1991 when Dietz *et al.* identified the causal gene within this region to be *FBN1*[82]. Mutations in the large *FBN1* gene account for approximately 70-93% of patients who meet diagnostic criteria for MFS (*Table 2*)[44,61,65]. Fibrillin-1 is a large, 2,871-amino acid glycoprotein sited in the ECM and essential for maintaining the integrity of the vessel wall and the ciliary apparatus from the ocular lens, promoting the anchorage of SMCs to the matrix of elastin and collagen[22,24,54].

To date, over 2,000 *FBN1* mutations have been identified worldwide and more than 1,000 have been associated with MFS[59,74,83], demonstrating a nearly complete penetrance in affected families[58]. They are typically heterozygous, missense, private (different in each family, but identical between family members), and widely distributed with no mutational hotspot[3,59], but nonsense and whole-gene deletions have also been routinely described[41]. These mutations often affect one of the numerous cysteine residues found in any of the 47 epidermal growth factor-like domains of the mature protein[59]. Consequently, the affected aorta exhibits markedly abnormal elastic properties that lead to progressive increases in stiffness and dilation[35].

After the revision of the *Ghent* diagnostic criteria in 2010[65], MFS has been diagnosed if aortic dilation or *ectopia lentis* were present, in addition to a *FBN1* mutation[59]. Nevertheless, it should be taken into account that, when only aortic dilation is present, the chance of finding an *FBN1* mutation is very low, and in the absence of family history, mutation screening is not warranted[59]. In contrast, the presence of *ectopia lentis* is much more specific, especially if transmitted as an autosomal dominant trait[59].

A second locus for MFS was mapped to 3p24-25, and mutations in the *TGFBR2* gene have been described in a small subset of MFS patients with a phenotype that may overlap LDS (*Table 2*)[24,84,85].

**Table 2. Marfan syndrome genetic and molecular basis**

| Gene | Locus | Protein | Percentage of affected patients | *OMIM*[73] | References |
|---|---|---|---|---|---|
| *FBN1* | 15q21.1 | Fibrillin-1 | 70-93% | *134797 | 44 |
| *TGFBR2* | 3p24.1 | tgf-βr2 | Controversial | *190182 | 10,28,84 |

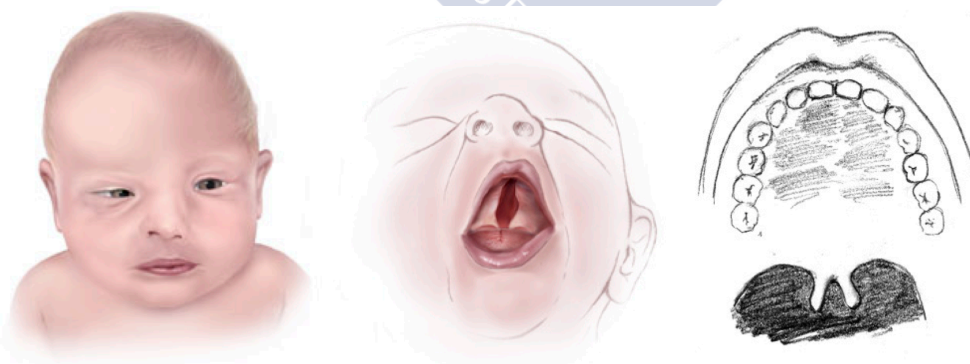
Although mostly dependent on *FBN1* mutations, the clinical spectrum of MFS is highly variable suggesting the participation of additional genetic factors capable of modulating the syndromic phenotype. Proost *et al.* performed in 2015 massive parallel sequencing (MPS) of 14 TAA candidate genes in 100 consecutive MFS patients and, besides identifying *FBN1* mutations in 96 of them, they

also detected *MYH11*, *NOTCH1*, *FLNA*, *SLC2A10*, and *COL3A1* rare genetic variants of unknown significance (VUS) in some of the *FBN1* mutation-positive patients[86]. It is still unclear whether and to what extent they contribute to the phenotype, but will undoubtedly be a matter of future discussion[86].

#### 2.1.1.2. Loeys-Dietz syndrome

LDS (*OMIM* #609192, #610168, #613795, #614816, and #615582[73]) is an exceedingly rare and aggressive aortic aneurysm syndrome first described in 2005, and with a still unknown incidence in the general population (thought to fall between MFS and vEDS)[77,87]. It is characterized by a triad of arterial tortuosity, hypertelorism, and cleft palate or bifid uvula, combined with widespread and aggressive aneurysms and dissections, as well as MFS-like cardiovascular, craoniofacial, and skeletal features (*Figure 7*)[87,88]. Since 2005, LDS cases have been subdivided into two different clinical groups: (i) the facial dysmorphogenic type or MFS-like, showing severe craniofacial features such as cleft palate, craniosynostosis, and micrognathia; and (ii) the vEDS-like type, notable for visceral rupture, easy bruising, wide/atrophic scars, joint laxity, and translucent/velvety skin[24,44,88,89]. But in contrast to the median survival MFS patients (70 if actively treated), for LDS is of 37, being the mean age for the first vascular surgical procedure about 20[28,44]. Mortality is generally due to thoracic or abdominal aortic dissection or rupture (that can occur without marked arterial dilation), or cerebral hemorrhage[44]. Still, due to the relatively limited number of patients reported to date, the full spectrum of LDS patients is likely to evolve in the upcoming years[44].

**Figure 7. Three of the most representative extracardiac manifestations of Loeys-Dietz syndrome**



From left to right, craniosynostosis, cleft palate, and bifid uvula *(adapted from the NIH U.S. National Library of Medicine at https://ghr.nlm.nih.gov/condition/loeys-dietz-syndrome and Stony Brook Medicine at https://www.stonybrookmedicine.edu/patientcare/surgery/patient-care/clinical/plastic-reconstructive-surgery/CPCF-Center)*[90,91].

LDS is inherited in an autosomal dominant manner[44], with reduced penetrance (patients carrying a pathogenic mutation may present no symptoms but remain at risk of transmitting[59]) and variable expressivity[44]. It is mainly caused by mutations in *TGFBR1* and more frequently *TGFBR2*

genes, being this latter mutated in approximately 2/3 of patients (*Table 3*)[10,44]. Mutations in both genes can cause any clinical type of LDS[88] through a paradoxical activation of the Smad pathway via its phosphorylation[24]. However, *TGFBR2* mutations have in general a more deleterious phenotype than *TGFBR1*, particularly in men[38]. Nevertheless, they can be associated with a very variable phenotype, ranging from an asymptomatic or completely normal person to a severe polymalformative syndrome with dismal prognosis[59].

**Table 3. Loeys-Dietz syndrome genetic and molecular basis**

| Gene | Locus | Protein | Percentage of affected patients | *OMIM*[73] | References |
|---|---|---|---|---|---|
| *TGFBR1* | 9q22.33 | tgf-βr1 | < 1% | *190181 | 61 |
| *TGFBR2* | 3p24.1 | tgf-βr2 | 67% | *190182 | 10 |
| *SMAD3* | 15q22.33 | Smad family member 3 | < 1% | *603109 | 61 |
| *TGFB2* | 1q41 | tgf-β2 | < 1% | *190220 | 61 |
| *TGFB3* | 14q24.3 | tgf-β3 | < 1% | *190230 | 61 |

Although it is known that most LDS mutations involve the *TGFBR2* gene, the percentage of cases caused by *TGFBR1* and *SMAD3* mutations have not yet been described. LDS = Loeys-Dietz syndrome; "-" = undetermined; "< 1%" = rare.

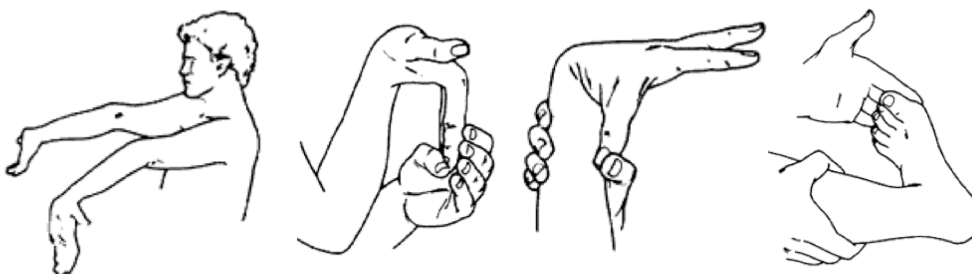
Most mutations are missense substitutions at the intracellular serine/threonine kinase domains of *TGFBR1* and *TGFBR2*, reducing receptor signaling activity in response to TGF-β binding, and suggesting loss-of-function as the relevant mechanism of pathogenesis[5,22,44]. Many of the disease-causing *TGFBR2* missense genetic variants tend to cluster within exons 4 to 7 and are much rarer than those in the *FBN1* gene, with less than 300 currently reported; *TGFBR1* mutations are even more rare[59]. This skewed mutational repertoire seems to manifest selection for receptor variants that traffic to the cell surface and bind the ligand but lack the ability to propagate signal, instead of eliciting messenger ribonucleic acid (mRNA) clearance through nonsense-mediated mRNA decay or whole allele deletions[5]. In fact, *TGFBR1* genomic deletions have been associated with Ferguson-Smith disease, a disorder of self-healing squamous epitheliomas[41].

#### 2.1.1.3. Vascular Ehlers-Danlos syndrome

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of aggressive connective tissue disorders with cutaneous, skeletal, and vascular implications[22]. They are characterized by articular hypermobility, skin hyperextensibility, tissue fragility, and significantly shortened life spans, with a 50% mortality rate by 48 years due to the spontaneous rupture of visceral organs (colon, uterus), and blood vessels (*Figure 8*)[44,88,92,93]. At least 12 characterized types of EDS exist, each of them with a different genetic basis[30,94]. From these, there are six major types: the arthrochalasia type, the classical type, the dermatosparaxis type, the hypermobility type, the kyphoscoliosis type, and the vascular

type (formerly type IV)[44]. In the classical, hypermobile, and vascular types, aortic dilation is found with a higher prevalence[30,44,77]. In fact, vEDS (*OMIM* #130050[73]) is the most life-threatening form, and affects the entire vasculature and the heart with a prevalence of 1 to 9/100,000[22,30,44,77]. It manifests mainly with arterial aneurysm, dissection and rupture, as well as thin, translucent skin, and easy bruising[28]. The vascular complications have a tendency to affect arteries of large and medium diameters, which can dissect even without prior dilation[8,22,88]. Thus, they are unpredictable and clinically difficult to manage[2,93].

**Figure 8. Some of the most representative hypermobility manifestations of Ehlers-Danlos syndrome**



From left to right, elbow, finger, and toe hypermobility characteristic of Ehlers-Danlos syndrome *(adapted from University of Washington Medicine, Orthopaedics and Sports Medicine at http://www.orthop.washington.edu/?q=patient-care/articles/arthritis/ehlers-danlos-syndrome.html)*[95].

Regarding the genetic component, vEDS is an autosomal dominant disorder caused by type III collagen alterations. As shown in *Table 4*, *COL3A1* mutations have been found in 98-99% of cases with suggestive clinical features[44]. To date, this has been the only gene associated with this specific form of EDS, with more than 70 known mutations[28].

**Table 4. Vascular Ehlers-Danlos syndrome genetic and molecular basis**

| Gene | Locus | Protein | Percentage of affected patients | *OMIM*[73] | References |
|---|---|---|---|---|---|
| *COL3A1* | 2q32.2 | Collagen 3 α-1 | 98-99% | *120180 | 44 |

*COL3A1* encodes collagen 3 α-1, the subtype of collagen most represented in the ECM of blood vessels and organ walls[24,88]. Like other collagens, it has a core triple-helical domain that depends on glycine amino acids in order to assemble[26]. Over 95% of missense mutations in *COL3A1* correspond to glycine substitutions throughout this 139-kDa protein, resulting in a kinked and structurally compromised collagen that leads to weak blood vessels and organ walls[88].

### 2.1.1.4. Others

Besides the main connective tissue syndromes, there are many other Mendelian diseases that have been associated with the development of TAAD. Although less relevant than the previous, they should be also considered when facing the genetic diagnosis of this clinical entity:

(i) **Turner syndrome (*OMIM* #313000[73]):** is a sex-chromosome aneuploidy caused by complete or partial monosomy of the chromosome X (45,XO), affecting 1 in every 2,000 to 5,000 women[8,44,51,88]. The most typical manifestations of this disease are webbed neck, short stature, and lymphedema, while the cardiovascular are generally the main cause of mortality[88]. Some of the most frequent are BAV (found in 1/3 of patients), aortic coarctation, hypertension, and TAAD[28,96,97]. This latter is present in almost 40% of Turner syndrome patients, with a 1-2% estimated risk of dissection[28,96,97]. Furthermore, up to 25% of the Turner BAV girls develop aortic dilation, as compared to 5% of those with tricuspid aortic valve (TAV)[51]. In fact, it is thought that only one in 100 Turner conceptions become live births, primarily due to severe congenital cardiovascular defects[88]. The underlying gene on the X-chromosome has not yet been identified, but some candidates exist: (i) a locus on the short arm of this chromosome, identified based on the comparison of different Turner karyotypical abnormalities[22,98]; and (ii) the *SHOX* gene haploinsufficiency, which has been associated with short stature[8,99].

(ii) **Shprintzen-Goldberg syndrome (*OMIM* #182212[73]):** is caused by sporadic *de novo* mutations in the *SKI* oncogene, which encodes a TGF-β repressor[22,41]. It shows a considerable phenotypic overlap with MFS and LDS, but also comes with mental retardation and severe skeletal muscle hypotonia[22,41]. Furthermore, TAA is in this case less penetrant and severe, possibly because of the temporal and regional expression pattern of the causal gene[22]. *SKI* plays an important role in the negative feedback loop of the TGF-β signaling pathway through Smad protein function inhibition[22,41], and mutations in this gene cluster in the N-terminal Smad2/3-binding and the *Dachshund*-homology domains[41]. It is responsible for binding Smads and other cofactors, predicting the molecular mechanism to be the loss of function of the SKI protein[41].

(iii) **Autosomal dominant polycystic kidney disease (ADPKD; *OMIM* #173900, and #613095[73]):** is a relatively common disease, with an estimated prevalence of 1 in 1,000, but without TAAD major involvement. It is characterized by progressive cyst development and bilaterally enlarged polycystic kidneys, and caused by mutations in *PKD1* or *PKD2* (85 and 15% of cases, respectively), encoding polycystin-1 and polycystin-2[44]. Polycystin-2 forms a nonselective cation channel calcium ion permeable, whereas polycystin-1 activates and stabilizes that channel[44]. Both are detected in vascular SMCs and endothelial cells of all major vessels, including the aorta and intracranial arteries[44]. *PKD*1 and *PKD2* mutations may disrupt calcium homeostasis and

consequently affect transcription of the genes involved in blood vessel structural integrity, or cause an increase in both vascular SMCs proliferation and apoptosis[44].

(iv) **Arterial tortuosity syndrome (ATS; *OMIM* #208050[73]):** is a very rare autosomal recessive disease characterized by arterial tortuosity, elongation, stenosis, and aneurysm formation in the major arteries[8,28,33]. Patients generally display altered facial features (elongated face, blepharophimosis and down-slanting palpebral fissures, beaked nose, highly arched palate, and micrognathia), and various signs of a more generalized connective tissue disorder of the skin (soft and hyperextensible skin) and skeleton (arachnodactyly, chest deformity, joint laxity, and contractures), overlapping those found in MFS[8]. ATS has been associated with the *SLC2A10* gene, which encodes the facilitative glucose transporter GLUT10[8].

(v) **Aneurysm-osteoarthritis syndrome (AOS; *OMIM* #613795[73]):** is a new and rare autosomal dominant condition that combines early-onset joint abnormalities (osteoarthritis and osteochondritis dissecans), and aortic aneurysms and dissections all over the arterial tree[8,33]. Mild craniofacial, skin, and skeletal features may also be found, overlapping with MFS and LDS[8]. AOS has been associated with mutations in the *SMAD3* gene, which encodes an intracellular effector of TGF-β signaling[8].

### 2.1.2. Non-syndromic aortic disease

The existence of familial but non-syndromic TAAD was recognized in the 1980s[28,100]. Although TAAD cases in the absence of overt connective tissue syndromic features may be sporadic[35], up to 20% of these individuals have affected first-degree family members, indicating that some familial predisposition may exist (19.3% by Coady *et al.* 1999 and 21.5% by Albornoz *et al.* 2006)[25,37,101,102]. These cases are generally termed NSAD or familial TAAD[58], and although they are characterized by rapidly growing ascending thoracic aneurysms, the descending thoracic aorta can also be affected[25,88]. Elefteriades *et al.* reported in 2013 that the 21% was sustained, no matter the number of families analyzed[33]. But the true rate of inheritance could be even higher than reported, as many family members may harbor aneurysms without being aware of their presence[13].

NSAD is a monogenic condition that mainly segregates as an autosomal dominant trait (76.9% of Albornoz *et al.* NSAD cases) with decreased penetrance (primarily in women) and variable expression[39,71]. But other modes of inheritance have been reported, such as X-linked dominant and recessive[25,103], and even in some probands with healthy parents, *de novo* mutations can occur[26]. The disease presentation is also variable, not only regarding the age of onset, location of the aneurysm, or degree of aortic dilation prior to dissection, but also in the presence of additional congenital



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defects (BAV, PDA) and vascular diseases elsewhere (ICAs, coronary artery disease, and occlusive cerebrovascular disease)[10,104].

This clinical heterogeneity predicts a corresponding genetic heterogeneity[10]. Mutations in several genes, including *FBN1*, *TGFBR1*, *TGFBR2*, *TGFB2*, *SMAD3*, *MYH11*, *ACTA2*, *MYLK*, and *PRKG1* have been reported to cause NSAD in approximately 25% of families (*Table 5*)[58]. Of this, approximately 20% was explained by *ACTA2* (10-15%), *TGFBR2* (3%), *TGFBR1* (1-2%), and *MYH11* (1-2%)[24,33,40,43,51]. Mutations in each of these NSAD genes have further particularities, as for example the relatively low penetrance of *ACTA2* mutations (50%) and their high risk of aortic dissection[105], or the incomplete segregation between thoracic aortic disease and *MYH11* mutations[106], suggesting that additional modifying genes could be contributing to the phenotype, at least in certain families[38,51].

**Table 5. Non-syndromic aortic disease genetic and molecular basis**

| Gene | Locus | Protein | Percentage of affected patients | *OMIM*[73] | References |
|---|---|---|---|---|---|
| *FBN1* | 15q21.1 | Fibrillin-1 | - | *134797 | [27] |
| *ELN* | 7q11.23 | Elastin | - | *130160 | [51] |
| *LOX* | 5q23.1 | Lysyl oxidase | - | *153455 | [23,107] |
| *MFAP5* | 12p13.31 | Microfibrillar-associated protein 5 | - | *601103 | [11] |
| *TGFBR1* | 9q22.33 | tgf-βr1 | 1-2% | *190181 | [61] |
| *TGFBR2* | 3p24.1 | tgf-βr2 | 3% | *190182 | [61] |
| *SMAD3* | 15q22.33 | Smad family member 3 | 2% | *603109 | [31] |
| *TGFB2* | 1q41 | tgf-β2 | Controversial | *190220 | [108,109] |
| *TGFB3* | 14q24.3 | tgf-β3 | - | *190230 | [51] |
| *ACTA2* | 10q23.31 | α-smooth muscle actin | 10-15% | *102620 | [24,31,61] |
| *MYH11* | 16p13.11 | Smooth muscle myosin heavy chain 11 | 1-2% | *160745 | [24,61] |
| *MYLK* | 3q21.1 | Myosin light chain kinase | 1% | *600922 | [61] |
| *PRKG1* | 10q11.2-q21.1 | Protein kinase cGMP-dependent type 1 | < 1% | *176894 | [61,110] |
| *MAT2A* | 2p11.2 | Methionine adenosyltransferase 2α | - | *601468 | [58] |
| *FOXE3* | 1p33 | Forkhead box 3 | - | *601094 | [41,111] |

cGMP = cyclic guanosine monophosphate; "< 1%" = rare; "-" = undetermined.

Nevertheless, many NSAD families do not depend on these known loci, which suggests that many more are yet to be discovered[10,88].

## 2.2. Complex diseases

Besides the rare single-gene disorders causing TAAD, other common diseases have been associated with the development of the same clinical manifestation that do not obey simple Mendelian genetics, but respond to a complex inheritance[72]. In these cases, the individual risk

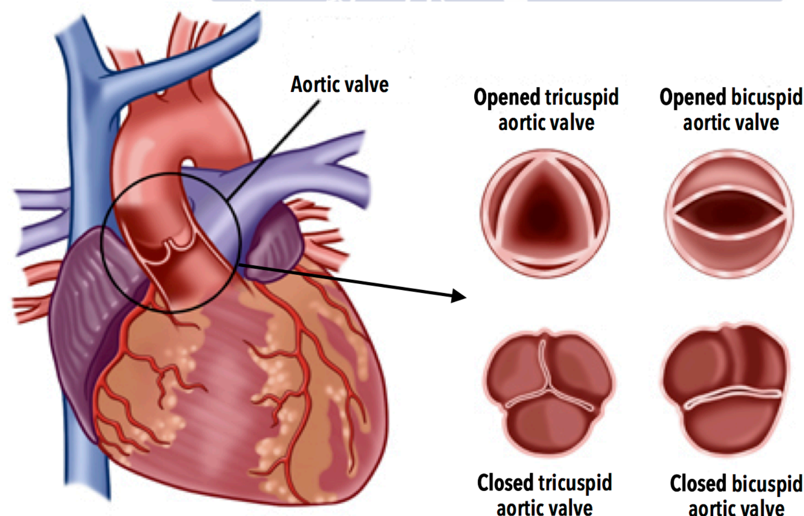
depends on the combination of multiple environmental and heritable factors, mainly polymorphisms with a minor allele frequency (MAF) greater than 1%, rather than rare mutations[72,112]. The effect of each common variant on the phenotype tends to be far more modest and, in fact, each polymorphism alone is neither necessary nor sufficient to cause disease[72].

BAV, coarctation of the aorta, and the tetralogy of Fallot are some of the complex and congenital diseases that have been associated with TAAD. They have been proposed to share genetic risk factors with it, and therefore the thoracic aortic disease would not only develop as a consequence of the hemodynamic alterations they cause. In some cases, as shown below, this last hypothesis is still under debate.

### 2.2.1. Bicuspid aortic valve

BAV (*OMIM* #109730[73]) is the most common congenital cardiovascular malformation in humans, with a prevalence of 0.5-2% in the general population[113–115] and a male:female ratio ranging from 2:1 to 4:1[116–119]. This embryological defect results from the abnormal formation of the three aortic valve leaflets during valvulogenesis; adjacent cusps fuse into two or, in a small percentage of cases, one single cusp (unicuspid aortic valve)[55,113,120]. The two leaflets are usually of unequal size and sometimes, an apparent raphe or false commissure is present in the larger one (*Figure 9*)[115,120].

**Figure 9. Illustration of an opened and closed normal tricuspid and bicuspid aortic valve**

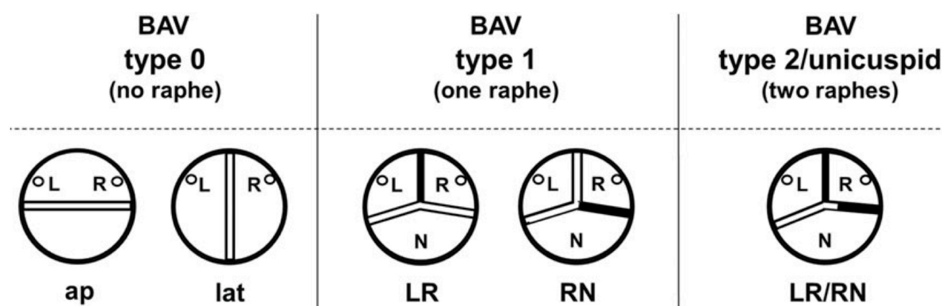



The abnormal formation of the three leaflets of the aortic valve during valvulogenesis leads to the fusion of adjacent cusps into two instead of three. The third reminiscent cusp is represented in the image as a raphe (*adapted from the Cleveland Clinic Heart and Vascular Institute – Valley Health System at* http://valleyheartandvascular.com/Thoracic-Aneurysm-Program/Bicuspid-Aortic-Valve-(BAV).aspx)[121].

Several BAV subtypes have been recognized (*Figure 10*)[122]. The classification system most widely used has been the one described by Sievers and Schmidtke in 2007, which was based on three

characteristics: (i) the number of raphes; (ii) the functional status of the valve; and (iii) the spatial position of cusps or raphes [51,123]. Regarding the latter, the most frequent pattern is the fusion of the right and left coronary cusps, representing 70% of BAV cases, while the right and non-coronary, and the left and non-coronary fusion patterns correspond to 10-20% and 5-10%, respectively[8,51,124].

**Figure 10. The most frequent bicuspid aortic valve types based on the *Sievers* classification system**




As it can be seen in the figure, this classification system depends on the number and spatial orientation of raphes or pathologically developed commissures. ap = anterior-posterior; BAV = bicuspid aortic valve; lat = lateral; L = left coronary sinus; N = non-coronary sinus; R = right coronary sinus *(adapted from Sievers et al. 2016)*[122].

Although in most cases BAV is asymptomatic and incidentally diagnosed as a benign lesion early in life[22,115,125], it has been also associated with serious long-term health risks, mainly cardiovascular complications, that occur in over 1/3 of patients and result in considerable morbidity and mortality[113,116,125]. These potentially life-threatening complications include TAAD, progressive aortic valve regurgitation or stenosis, infective endocarditis, interrupted aortic arch, cervicocephalic arterial dissections, ductus diverticulum aneurysm[44,114,115], and other forms of left-sided heart obstruction (aortic coarctation, mitral stenosis, and hypoplastic left heart syndrome)[41]. Some of them, such as aortic stenosis and infective endocarditis, have an age-related incidence[120]: (i) in childhood and early adult life, critical aortic stenosis and infective endocarditis are the commonest problems; (ii) from early adulthood to early middle-age, aortic regurgitation (especially secondary to infective endocarditis) and aortic dissection are the major causes of morbidity; and (iii) thereafter, aortic stenosis is increasingly common until the 70s[120]. Most importantly, they could be prevented by appropriate intervention such as antibiotic prophylaxis if diagnosed in time, being early detection crucial to the clinical management of these patients[113,116].

Abbott first described an association between thoracic aortic disease and BAV in 1928 in a case series of coarctation of the aorta[38,126]. Although it has been generally considered that up to 50% of BAV cases develop TAAD affecting the tubular ascending thoracic aorta[41,124,127,128], Tadros *et al.* estimated, in 2009, the lifetime prevalence of ascending TAA in BAV patients to be even 70-80%[12,129]. It could likely be considered the most common type of aneurysm affecting humans[41]. The incidence of aortic dissection during a BAV patient lifetime has been estimated to be 5-6%,



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compared to the 40% of MFS patients[36,38,120,130]. But being the BAV prevalence much higher (0.5-2 versus 0.01-0.02%[120]), it causes many more cases of aortic dissection (by orders of magnitude) than the better appreciated connective tissue syndrome[33,120].

The repeated association of these two entities in the literature, their common embryologic origin (neural crests), the development of aortic dilation even in the absence of significant aortic valve dysfunction, and the non-protection of BAV patients from subsequent aneurysm formation by aortic valve replacement, suggested that hemodynamics alone was not the only driving force, and that BAV and TAAD could be manifestations of a single-gene defect[38,44,116,131,132]. TAAD might therefore be either genetic, with common genetic pathways for aortic dilation and BAV, or consecutive to altered aortic flow patterns in BAV, or a combination of both[8]. To date, all hypotheses are still present, and will be further discussed in *section IV*.

Although it was historically considered a sporadic malformation, heritability studies reported high familial clustering of BAV[125]. Its inheritance pattern has been mainly considered either autosomal dominant with incomplete penetrance and variable expressivity, or complex and polygenic[125,133–135]. While Emanuel *et al.* found in 1978 a minimum familial incidence of 14.6% for this disease[136], Cripe *et al.* estimated in 2004 BAV heritability to be 0.89 ± 0.06 (*p-value* < 0.0000001)[113]. Furthermore, Huntington *et al.* estimated in 1997 the prevalence in first-degree relatives to be 9% (in some cases with different combinations of BAV, TAA, and aortic coarctation)[59,116,125], while a more recent study found it to be 10.1%[125,137]. But, despite the high prevalence and overwhelming genetic evidence for heritability, BAV molecular genetics remain largely unknown, due at least partially to the heterogeneity within the bicuspid spectrum[33,38,113]; different BAV subtypes could depend on distinct underlying genetic factors[8,44,138].

To date, no single gene has been definitely associated with the development of the valvular malformation, either alone or in combination with TAAD[44,58]. Linkage analysis studies have identified some critical regions at 5q, 13q, and 18q[44,139], and even a set of candidate genes and proteins have been proposed based on BAV mouse models (*ACTA2*, *TGFBR*2, *FBN1*, *NO*S*3*, *UFD1L*, *GATA5*, *MATR3*, *NKX2.5*, etc.)[38,140–144]. Furthermore, *NOTCH1*[145], *GATA5*[146,147], and *STR1*[24,148] have been somehow associated with dominantly inherited BAV in a small number of families that represented a small proportion of all patients with BAV/TAA[51,114,145]. To date, *NOTCH1* has been the only proven candidate gene to be consistently associated with BAV with and without thoracic aortic disease and calcification in humans, contributing to 4% of sporadic, and also familial cases[38,41,125,145,149–151].

*NOTCH1* gene encodes a signaling and transcriptional regulator transmembrane receptor with 2,556 amino acids involved in cellular differentiation, cell fate, lateral inhibition, and multiple cell

aspects of cardiac embryogenesis and vascular development (including aortic and pulmonary valve development, and maintenance of the aorta and other great vessels)[114,145]. But the rate of *NOTCH1* mutations in familial forms of BAV and their association with different clinical complications remains incomplete[114].

Besides the two first *NOTCH1* germline mutations identified in two BAV families by Garg *et al.* in 2005[114,145], two other novel mutations were found by Mohamed *et al.* in patients with sporadic BAV in 2006. Furthermore, McKellar *et al.* demonstrated in 2007 the presence of two other novel *NOTCH1* mutations among patients with BAV and sporadic TAAs, providing evidence that they may also confer susceptibility to aortic aneurysm formation[114,149]. Finally, McBride *et al.* demonstrated in 2008 that mutations in this gene were also found in cases of left ventricular outflow tract (LVOT) malformations, including aortic valve stenosis, aortic coarctation, hypoplastic left heart syndrome, and a high rate of BAV, which was proposed as the *forme fruste* of the more serious LVOT malformations[114,152].

### 2.2.2. Coarctation of the aorta

This complex disease of the vasculature (*OMIM* 120000[73]) is defined as the narrowing of the thoracic aorta distal to the left subclavian artery, and affects 5-10% of all cases of congenital heart disease[8,52]. It is typically located near the *ductus arteriosus* insertion, but can also develop ectopically affecting the thoracic ascending, descending, or abdominal aorta[8]. It is considered complex, as it can be influenced by environmental factors such as maternal diabetes or organic solvent exposure[153].

It appears apparently sporadic in the majority of cases[153] and, when isolated, it is generally identified and repaired in infancy, and rarely associated with TAD[8,52,154,155]. Nevertheless, Francis *et al.* considered it in 2016 not in isolation, but as one manifestation of a spectrum of left-sided, primarily obstructive cardiac defects, ranging from relatively minor or aortic valve abnormalities to severe hypoplastic left heart syndrome[153]. Lewin *et al.* found in 2004 that up to 42% of first-degree relatives of patients with coarctation of the aorta displayed some form of left-sided cardiac congenital abnormality[153,156]. In fact, a strong association between this disease and BAV exists; 50-80% of aortic coarctation patients will also have a congenital BAV, being the risk of developing TAAD much higher than in the population with only BAV[52]. It is still unknown if this increased risk for TAAD depends on a common genetic risk factor or on the hypertension caused by the narrowing of the vessel[14]. These endeavors are made much more challenging by the huge variability in presentation, the co-existence of multiple congenital lesions in individual patients, and the difficulty of defining large families with often-divergent phenotypes for segregation analysis.

### 2.2.3. Tetralogy of Fallot

This congenital cardiac malformation (*OMIM* #187500[73]) consists of an interventricular communication (also known as ventricular septal defect), obstruction of the right ventricular outflow tract, override of the ventricular septum by the aortic arch, and right ventricular hypertrophy[77]. The prevalence of this combination of lesions has been estimated in 1 to 5/10,000, which accounts for approximately 7-10% of all congenital cardiac malformations[77].

Thoracic aortic involvement in tetralogy of Fallot patients generally appears after having undergone repair, developing insidious aortic root dilation later in life[52]. Before assuming a common genetic origin for tetralogy of Fallot and TAAD, it should be further evaluated if the thoracic aortic disease could be only a consequence of the cardiac surgery, and not dependent on the specific congenital defect behind it. Although mainly defined as multigenic or multifactorial, an autosomal dominant pattern of inheritance has also been described for some cases[77]. Among the genetic factors behind the development of this disease, recent experience points to microdeletions of chromosome 22 instead of the previously described trisomies 13, 18, and 21[77].

## 2.3. Genotype-phenotype correlation

Taking advantage from the variable expressivity that characterizes most TAAD cases, a very powerful tool for the personalized management of these patients would be to assign clinical phenotypes to the different genes and genotypes[86]. Not all mutated genes carry the same degree of vascular risk, and not all patients with a gene mutation may develop aortopathy leading to clinical events[64].

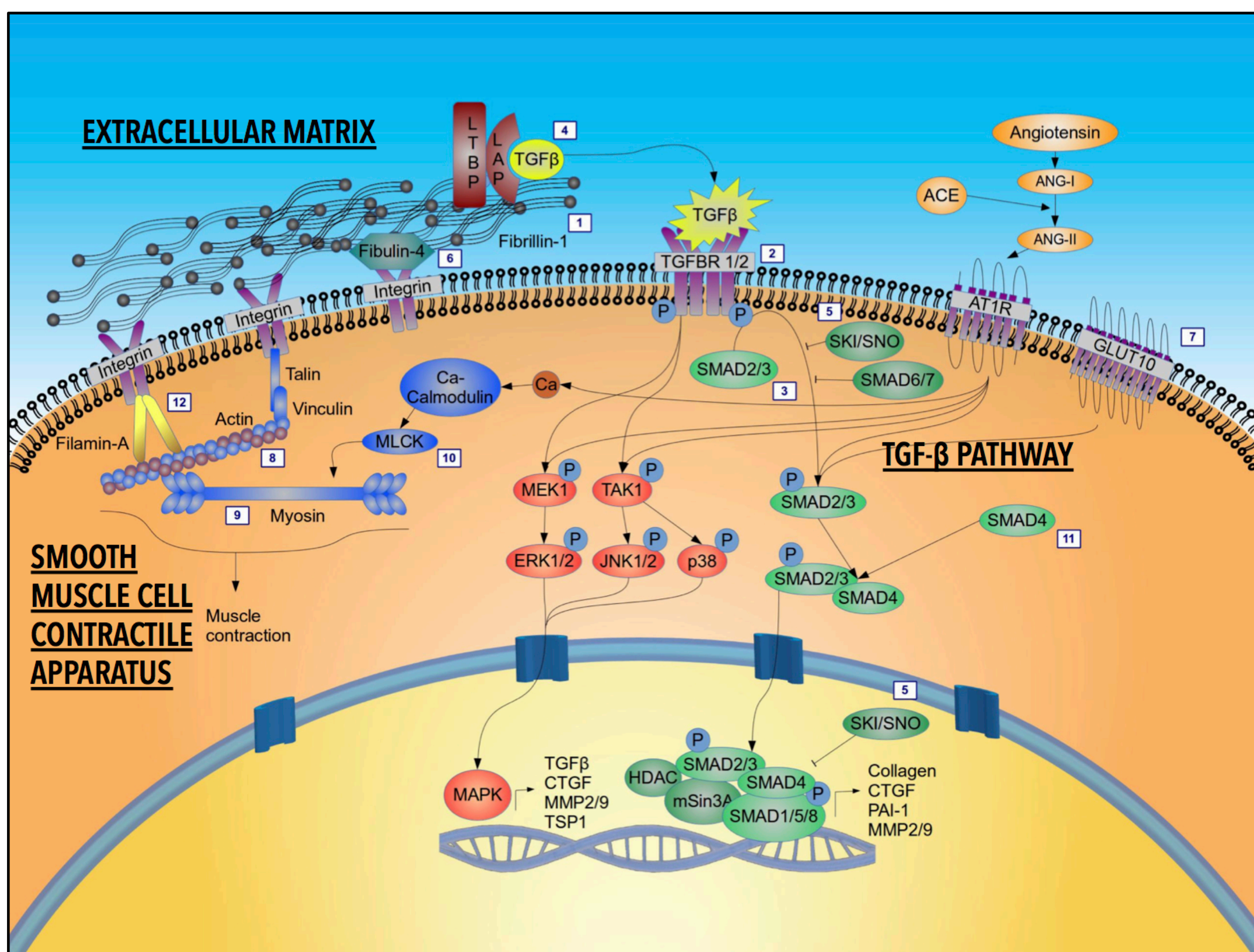
One of the initial steps the scientific community performed was to classify the already known causal genes based on the signaling pathway they belong to. Therefore, after the genetic basis of several TAAD cases had been established, three main pathomechanisms emerged (*Figure 11*)[38]:

(i) **Impairment of ECM synthesis and decoupling of SMC from the ECM:** leading to aortic wall weakness[38].

(ii) **Perturbation of the TGF-β signaling pathway:** while inactivation of this signaling pathway has been shown to contribute to tumorigenesis[43], up-regulation has been defined as a major hallmark of TGF-β related vasculopathies[11,38]. Although originally only the canonical (Smad-dependent) TGF-β pathway was evaluated in aneurysmal disease, more recently, it has become clear that the non-canonical (Smad-independent) is also associated with aneurysm formation[22].

(iii) **Disruption of the vascular SMC contractile apparatus:** analogously to hypertrophic and dilated cardiomyopathy pathogenesis, SMC contractile function has been shown to play a key role in maintaining the structural integrity of the thoracic aorta and preventing the development of aortic aneurysms[10,38].

**Figure 11. Summary of the main thoracic aortic aneurysm and dissection pathways**




They involve the extracellular matrix, smooth muscle cell contractile apparatus, and TGF-β signaling. Green represents the canonical (Smad-dependent) and red the non-canonical (Smad-independent) TGF-β pathway. Numbers indicate the corresponding syndrome caused by mutations in the protein: 1 = Marfan syndrome; 2 = Loeys-Dietz syndrome types 1 and 2; 3 = Loeys-Dietz syndrome type 3; 4 = Loeys-Dietz syndrome type 4; 5 = Shprintzen-Goldberg syndrome; 6 = cutis laxa type 1B; 7 = arterial tortuosity syndrome; 8-10 = non-syndromic aortic disease; 11 = juvenile polyposis and hemorrhagic telangiectasia syndromes; 12 = Ehlers-Danlos syndrome with periventricular heterotopia. ACE = angiotensin-converting enzyme; ANG = angiotensin; ERK = extracellular signal–regulated kinase; HDAC = histone deacetylase; JNK = Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MEK = mitogen-activated protein kinase/extracellular signal-regulated kinase; MLCK = myosin light chain kinase; MMP = matrix metalloproteinase; PAI = plasminogen activator inhibitor; TAK = transforming growth factor-β-activated kinase; TGF = transforming growth factor; TGFBR = transforming growth factor-β receptor; and TSP = thrombospondin *(adapted from Gillis et al. 2013)*[22].

The definition of the genotype-phenotype correlations based on this classification facilitates TAAD diagnosis, as shown in *Table 6*. All the genes included in this table have been associated with TAA, but the extent of other vascular and extra-vascular features (skeletal, ophthalmologic, neurological, or immunological) vary, enabling a more accurate diagnosis[63].

**Table 6. Thoracic aortic aneurysm and dissection genes classified based on the underlying pathogenesis**

| Gene | Chr[73] | Protein | Disease | Differential diagnosis based on clinical manifestations | References |
|---|---|---|---|---|---|
| **Extracellular matrix** | | | | | |
| *FBN1* | 15q21.1 | Fibrillin-1 | MFS | TAAD (root, annuloaortic ectasia, infrequent descending), pulmonary artery dilation, AAA (infrequent), aortic regurgitation, arterial tortuosity, MV prolapse and regurgitation, ventricular dysfunction, *ectopia lentis*, arachnodactyly, *pectus* deformities, scoliosis, flat feet, increased arm span, tall stature, hypermobile joints, abdominal wall laxity, highly arched palate, dental crowding, dolichocephalia, dural ectasia, skin *striae distensae*, inguinal hernias, pneumothorax, lung emphysema | 10,11,14,22,24,27–31,38,41,44,51,52,61,63,64,67,74,75,78,82,83,88,104,157–159 |
| | | | NSAD | TAAD, BAV, mild skeletal MFS features | 8,10,11,22,27,51,63,74,75,86,104,160,161 |
| | | | MASS phenotype (MV, aorta, skin, and skeletal features) | Aortic enlargement, MV prolapse, skin and skeletal findings | 29,75 |
| | | | Shprintzen-Goldberg syndrome | TAA (ascending), craniosynostosis, chest wall deformities, arachnodactyly, cleft palate (Marfanoid features) | 2,63,73,74,162 |
| | | | Weill-Marchesani syndrome | TAAD (ascending), MV insufficiency, AV stenosis, short stature, brachydactyly, joint stiffness and contractures, *ectopia lentis* | 2,22,29,38,63,73,83,163,164 |
| *FBN2* | 5q23.3 | Fibrillin-2 | Congenital contractural arachnodactyly or Beals syndrome | TAA (root, rare), MV prolapse, BAV, atrial septal defect, arachnodactyly, dolichostenomelia, *pectus* deformities, kyphoscoliosis, congenital joint contractures, crumpled ears | 2,22,57,75,158,161,165–167 |
| *COL1A1* | 17q21.33 | Collagen 1 α-1 | Osteogenesis imperfecta, type 1 | TAA (rare), MV disease, mild joint hypermobility, hearing loss, short stature, thin and velvety skin, fractures | 5,10,22,74,161,168 |
| | | | Classical EDS, type 1 | TAA (root), loose-jointedness, fragile and bruisable skin, cigarette-paper skin scars | 73,158 |
| | | | Arthrochalasic EDS, type 7A | TAA, bilateral congenital hip dislocation, joint hyperlaxity, recurrent partial dislocations, hyperextensible skin, tissue fragility, atrophic scars, muscular hypotonia | 77,158 |
| *COL1A2* | 7q21.3 | Collagen 1 α-2 | Osteogenesis imperfecta | TAA (rare), MV disease, mild joint hypermobility, hearing loss, short stature, thin and velvety skin, fractures | 5,10,74,161,168–171 |
| | | | Valvular EDS, type 7B | TAAD (root, borderline), MV and severe AV insufficiency and regurgitation, joint laxity and hypermobility, skin hyperextensibility | 5,22,65,74,166,172,173 |
| *COL3A1* | 2q32.2 | Collagen 3 α-1 | Vascular EDS, type 4 | TAD (infrequent TAA), AAA, vascular fragility and varicose veins, severe valvular insufficiency, diffuse vascular disease, MV prolapse, thin and translucent skin, easy bruising, dystrophic scars, thin pinched nose, thin lips, prominent ears, hollow cheeks, ICA, intestinal and uterine ruptures, small joint hypermobility | 2,10,14,24,28,34,38,41,44,52,61,63–65,67,74,75,78,88,158,159,166,174,175 |

| Gene | Chr[73] | Protein | Disease | Differential diagnosis based on clinical manifestations | References |
|---|---|---|---|---|---|
| *COL4A3* | 2q36.3 | Collagen 4 α-3 | Alport syndrome | TAA, AAA, hypertension, deafness, myopia, renal failure | 28,176,177 |
| *COL4A5* | Xq22.3 | Collagen 4 α-5 | Alport syndrome | TAAD (ascending, rare), AAA, hypertension, deafness, myopia, renal failure | 5,22,41,64,74,158,178,179 |
| *COL5A1* | 9q34.3 | Collagen 5 α-1 | Classical EDS, types 1 and 2 | TAAD, hyperextensible and doughy and velvety skin, joint laxity, atrophic scars, orthopedic complications, bowel rupture | 75,158,166,180,181 |
| *COL5A2* | 2q32.3 | Collagen 5 α-2 | Classical EDS, types 1 and 2 | TAAD, hyperextensible and doughy and velvety skin, joint laxity, atrophic scars, orthopedic complications, bowel rupture | 75,158,166,180,181 |
| *EFEMP2* | 11q13.1 | Fibulin-4 | Autosomal recessive cutis laxa, type 1B | TAA (ascending), arterial tortuosity, aortic stenosis, arachnodactyly, hypertelorism, highly arched palate, joint hypermobility | 5,22,28,38,41,63,64,74,86, 158,166,182 |
| *ELN* | 7q11.23 | Elastin | Autosomal dominant cutis laxa, type 1 | TAAD (ascending, rare), BAV, MV and AV regurgitation, loose redundant skin, premature aged appearance, emphysema | 5,22,41,74,158,166,183, 184 |
| | | | NSAD | Isolated TAAD | 51 |
| *PLOD1* | 1p36.22 | Lysyl hydroxylase 1 | Kyphoscoliotic EDS, type 6 | TAAD (rare), arterial rupture, kyphoscoliosis, joint laxity, muscle hypotonia, ocular alterations | 2,5,22,41,65,74,158,185, 186 |
| *LOX* | 5q23.1 | Lysyl oxidase | NSAD | TAAD (root, ascending), BAV, *pectus* deformities, *striae* | 23,41,107 |
| *MFAP5* | 12p13.31 | Microfibrillar-associated protein 5 | NSAD | TAAD (root), paroxysmal atrial fibrillation, MV prolapse, *pectus* deformities, arachnodactyly, highly arched palate | 11,38,41,51,67 |
| *BGN* | Xq28 | Biglycan | Syndromic TAAD | TAAD (early onset), hypertelorism, *pectus* deformities, joint hypermobility, contractures, mild skeletal dysplasia | 187 |
| **TGF-β pathway** | | | | | |
| *TGFBR1* | 9q22.33 | tgf-βr1 | LDS, type 1 | TAAD (root, especially men, aggressive), other arterial aneurysms and dissections (cerebral and abdominal, especially women), arterial tortuosity (highly penetrant), MV prolapse, PDA, bifid uvula, cleft palate, hypertelorism, craniosynostosis, club feet, scoliosis, *pectus* deformities, thin and velvety skin, dystrophic scarring, easy bruising, mental retardation, Marfanoid features | 5,11,14,22,24,28,31,38,41, 44,51,52,61,63–65,67,71, 74,75,87–89,104,109, 157–159,166,188 |
| | | | NSAD | TAAD (aggressive) | 7,8,10,11,28,31,33,38,41, 51,61,64,75,78,86,104, 158,166,189 |
| *TGFBR2* | 3p24.1 | tgf-βr2 | LDS, type 2 | TAAD (root, descending, aggressive), dilation and dissection of medium sized arteries (abdominal aorta, cerebral, carotid, pulmonary, popliteal, splanchnic, renal), highly penetrant arterial tortuosity, MV prolapse, PDA, bifid uvula, cleft palate, hypertelorism, craniosynostosis, club feet, scoliosis, *pectus* deformities, thin and velvety skin, dystrophic scarring, easy bruising, mental retardation, Marfanoid features | 5,11,14,22,24,28,31,38,41, 43,44,51,52,61,63–65,67, 71,74,75,87–89,104, 109,157–159,166 |

| Gene | Chr[73] | Protein | Disease | Differential diagnosis based on clinical manifestations | References |
|---|---|---|---|---|---|
| | | | MFS, type 2 | PDA, cardiac septal defects, cervical spine instability | 2,10,11,24,28,29,51,57,63,71,84–86,158 |
| | | | NSAD | TAAD (ascending, highly penetrant, aggressive), other arterial aneurysms (cerebral, carotid, and popliteal arteries, descending aorta), PDA, BAV, *pectus* deformities, joint hypermobility | 2,7,8,10,11,14,22,27,28,31,38,41,43,44,51,61,63,64,74,75,78,86,89,103,104,158,159,166,189 |
| *TGFB2* | 1q41 | tgf-β2 | LDS, type 4 | TAA (root), other arterial aneurysms and dissections, arterial tortuosity, MV prolapse, PDA, hypertelorism, cleft palate, bifid uvula, club feet, soft and translucent skin, mild Marfanoid features | 22,31,33,38,41,51,52,61,64,67,74,108,158,166 |
| | | | NSAD | TAAD, MV prolapse | 8,11,31,38,44,51,63,64,109,158,166 |
| *TGFB3* | 14q24.3 | tgf-β3 | LDS, type 5 | TAAD, AAA, branch vessel disease (rare) | 38,41,51,61,64,67 |
| | | | NSAD | TAAD | 51 |
| *SMAD2* | 18q21.1 | Smad family member 2 | - | Aortic and peripheral arterial aneurysms and dissections | 38,41,190 |
| *SMAD3* | 15q22.33 | Smad family member 3 | AOS or LDS, type 3 | TAA (root, descending), diffuse arterial aneurysms (cerebral, pulmonary, mesenteric, celiac, hepatic, splenic, internal mammary, and iliac arteries), arterial tortuosity, hypoplastic left heart syndrome, PDA, atrial fibrillation, early-onset osteoarthritis, flat and club feet, scoliosis, *pectus* deformities, soft skin, hypertelorism, bifid uvula, dental malocclusion, dolichostenomelia, arachnodactyly, scoliosis, joint hypermobility, acetabular protrusion, peripheral neuropathy, autoimmune features, recurrent hernias, osteoarthritis, intervertebral disc degeneration, osteochondritis dissecans | 11,22,31,38,41,51,52,61,63,64,67,74,75,88,109,157,158,166,191 |
| | | | NSAD | TAAD, AAA, ICA, bilateral iliac aneurysms | 11,22,24,31,51,75,78,104,157,166 |
| *SMAD4* | 18q21.2 | Smad family member 4 | JPS /HHTS | TAA, MV prolapse, other arteriovenous malformations, intestinal polyposis, telangiectasia | 22,38,41,63,74,158,192 |
| *SLC2A10* | 20q13.12 | Glucose transporter 10 | Arterial tortuosity syndrome | TAA, vascular tortuosity, elongation and stenosis, micrognathia, dolicocephaly, down-slanting palpebral fissures, beaked nose, highly arched palate, malar hypoplasia, hypertelorism, archnodactyly, joint laxity | 5,22,28,41,52,61,64,65,74,75,86,158,166,193 |
| *SKI* | 1p36.33-p36.32 | v-SKI sarcoma oncogene homolog | Shprintzen-Goldberg syndrome | TAA (root), MV prolapse, craniosynostosis, chest wall deformities, aranchnodactly, cleft palate, camptodactyly, scoliosis, joint hypermobility, severe skeletal muscle hypotonia, mild-to-moderate intellectual disability | 22,31,38,41,52,64,73,74,86,158,162,194 |
| *ENG* | 9q34.11 | Endoglin | Hereditary hemorrhagic telangiectasia, type 1 | TAA, medium-sized arterial aneurysms, other arteriovenous malformations, telangiectasia, nose bleeding | 5,22,195 |
| *ACVRL1* | 12q13.13 | Activin receptor-like kinase I | HHTS, type 2 | TAA, medium-sized arterial aneurysms, other arteriovenous malformations, telangiectasia, nose bleeding | 5,22,196 |

| Gene | Chr[73] | Protein | Disease | Differential diagnosis based on clinical manifestations | References |
|---|---|---|---|---|---|
| **Smooth muscle cell contractile apparatus** | | | | | |
| *ACTA2* | 10q23.31 | α-smooth muscle actin | NSAD | TAAD (ascending, rarely descending, aggressive), ICA, aortic coarctation, arterial tortuosity, BAV, premature coronary artery disease and myocardial infarction, PDA, *livedo reticularis*, iris *flocculi*, ischemic stroke, features of Moyamoya disease, congenital mydriasis | 5,7,8,10,11,14,22,24,28, 31,33,38,41,44,51,52,59, 61,63,64,67,74,75,78,86, 88,102,157–159,166,189, 197–199 |
| | | | Multisystemic SMC dysfunction syndrome (single *ACTA2* mutation) | TAAD (early onset), other arteries aneurysm, coronary artery disease, PDA, pulmonary hypertension, congenital mydriasis, intestinal malrotation, bladder hypotonia, periventricular white matter hyperintensities, stroke | 22,38,41,51,67,200 |
| *MYH11* | 16p13.11 | Smooth muscle myosin heavy chain 11 | NSAD-PDA | TAAD (ascending, rarely descending), PDA, arterial tortuosity, BAV (rare) | 5,7,8,10,11,14,22,24,27, 28,31,38,41,44,51,52,59, 61,63,64,67,74,75,86,88, 103,157–159,166,189,201, 202 |
| *FLNA* | Xq28 | Filamin A | EDS-like periventricular nodular heterotopia | TAAD (root), MV prolapse with or without insufficiency, arterial tortuosity, BAV, aortic coarctation, coagulopathy, joint hypermobility, skin hyperextensibility, miscarriages of male fetuses, brain malformations, epilepsy | 10,22,28,38,41,63,64,67, 74,86,153,158,166,203 |
| *MYLK* | 3q21.1 | Myosin light chain kinase | NSAD | TAAD (ascending, aggressive), PDA, hypertension, gastrointestinal abnormalities | 8,11,22,31,33,38,41,51,61, 63,64,67,74,75,86,158, 166,189,204,205 |
| *PRKG1* | 10q11.2-q21.1 | Protein kinase cGMP-dependent type 1 | NSAD | TAAD (early onset), other arterial aneurysms and dissections, arterial tortuosity | 8,11,31,38,41,51,61,63,64, 67,74,86,110,158,166 |
| *MAT2A* | 2p11.2 | Methionine adenosyltransferase 2α | NSAD | TAAD, BAV | 38,41,51,58,67,206 |
| *FOXE3* | 1p33 | Forkhead box 3 | NSAD | TAAD | 41,111,206 |
| **Miscellanea** | | | | | |
| *PTPN11* | 12q24.13 | Protein-tyrosine phosphatase 2C | Noonan, type 1 and LEOPARD syndromes | TAA (ascending, rare), hypertrophic cardiomyopathy, coronary artery aneurysms, atrial septal defect, PDA, BAV, aortic coarctation, short stature, chest abnormalities, hypertelorism, cleft palate, broad neck, developmental delay, hearing loss, lentigines | 2,5,10,22,28,73,74,153, 161,207–209 |
| *PKD1* | 16p13.3 | Polycistin-1 | ADPKD | TAAD (root, ascending, rare), saccular ICA, coronary arteries and atrial septal aneurysms, cervicocephalic arteries dissection, MV prolapse, bilaterally enlarged polycystic kidneys and progressive cyst development, renal failure, dolichoectasias, abdominal wall hernias | 2,10,22,28,34,44,52,74, 210 |
| *PKD2* | 4q22.1 | Polycistin-2 | ADPKD | TAAD (root, ascending, rare), ICA, coronary arteries aneurysms, atrial septal aneurysm, cervicocephalic arteries dissection, MV prolapse, bilaterally enlarged polycystic kidneys and progressive cyst development, renal failure, dolichoectasias, abdominal wall hernias | 2,10,22,28,34,44,74,211 |

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| Gene | Chr[73] | Protein | Disease | Differential diagnosis based on clinical manifestations | References |
|---|---|---|---|---|---|
| *TSC2* | 16p13.3 | Tuberin | Tuberous sclerosis | TAA (thoracoabdominal, descending), renal cysts, seizures, mental retardation | 22,74,212–214 |
| *JAG1* | 20p12.2 | Jagged1 | Alagille syndrome | TAA, ICA, aortic coarctation, pulmonary branch stenosis, hypertelorism, embryotoxon, myopia, cholestasis, renal involvement, butterfly vertebrae | 5,10,22,74,153,209,215, 216 |
| *MED12* | Xq13.1 | Mediator complex subunit 12 | Lujan-Fryns syndrome | TAA (extremely rare), Marfanoid features, intellectual disability | 5 |
| *ADAMTS10* | 19p13.2 | ADAM metallopeptidase-thrombospondin type 1 motif 10 | Weill-Marchesani syndrome | TAAD | 166 |
| *NOTCH1* | 9q34.3 | Notch1 | BAV-TAAD syndrome | TAAD (ascending, rare), early calcified BAV, aortic coarctation | 5,22,28,38,41,51,67,86, 125,145,153,158,166 |
| *GATA5* | 20q13.33 | Transcription factor GATA-5 | BAV-TAAD syndrome (controversial) | TAAD, BAV, aortic coarctation | 153,158 |
| *NKX2.5* | 5q35.1 | Homeobox protein Nkx-2.5 | Tetralogy of Fallot (controversial) | TAAD (root), ventricular septal defect, obstruction of the right ventricular outflow tract, override of the ventricular septum by the aortic root, right ventricular hypertrophy, aortic coarctation | 77,153,209 |
| *FOXC1* | 6p25.3 | Forkhead box C1 | Aortic coarctation (controversial) | TAAD (infrequent dissection if isolated), aortic coarctation, BAV | 153,217 |
| *LRP1* | 12q13.3 | Low-density lipoprotein receptor-related protein 1 | Sporadic TAD | TAD | 206 |
| *ULK4* | 3p22.1 | Unc-51-like kinase 4 | Sporadic TAD | TAD | 206 |
| *45, XO* | - | - | Turner syndrome | TAAD (ascending), BAV, aortic coarctation, other arterial aneurysms (brachial and carotid arteries), hypertension, short stature, unusual facial appearance, neck webbing, widely spaced nipples, lymphedema, gonadal dysgenesis | 2,10,14,28,35,52,74,88, 153,161,209,218 |

In the "Differential diagnosis based on clinical manifestations" column, cardiovascular features appear first. The greater number of references, the stronger the genotype-phenotype association is. A low number of references could also indicate a recent description. AAA = abdominal aortic aneurysm; ADPKD = autosomal dominant polycystic kidney disease; AOS = aneurysm-osteoarthritis syndrome; AV = aortic valve; BAV = bicuspid aortic valve; cGMP = cyclic guanosine monophosphate; Chr = cytogenetic location; EDS = Ehlers-Danlos syndrome; ICA = intracranial aneurysm; JPS/HHTS = Juvenile polyposis syndrome/hereditary hemorrhagic telangiectasia syndrome; LDS = Loeys-Dietz syndrome; MFS = Marfan syndrome; MV = mitral valve; NSAD = non-syndromic aortic disease; PDA = patent *ductus arteriosus*; SMC = smooth muscle cell; TAA = thoracic aortic aneurysm; TAAD = thoracic aortic aneurysm and dissection; TAD = thoracic aortic dissection.

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In spite of the huge amount of information included in *Table 6* and although for some of the genes (*FBN1*, *TGFBR1,* and *TGFBR2*) a wide spectrum of phenotypes has already been described, the full range of phenotypes associated with most of them has not been fully studied up to now[86]. The following are some examples of those more detailed and desired genotype-phenotype correlations:

(i) ***FBN1* and MFS:** the *FBN1* cysteine residue in position 1408 has been associated up to four times with MFS in patients with specific clinical manifestations[26,219–221]. Poninska *et al.* found in 2016 this mutational hotspot to cause the disease in monozygotic female twins suffering from *ectopia lentis*, which required prophylactic aortic surgery at early ages (around 30). In contrast, other *FBN1* mutations affecting multiple MFS patients have shown remarkable clinical variability that impedes the establishment of an accurate genotype-phenotype correlation, such as the p.I2585T first detected by Liu *et al.* in 1997[222] or p.Y2639C by Mátyás *et al.* in 2002 (NM_000138)[223].

(ii) ***TGFBR2* and NSAD:** Pannu *et al.* first associated in 2005 the presence of *TGFBR2* mutations with the development of NSAD in four unrelated families from the 80 they sequenced[43]. Strikingly, all four families had *TGFBR2* mutations in the arginine 460 of the intracellular kinase domain of the receptor, suggesting the existence of another mutational hotspot[10,28,103]. Law *et al.* further supported this hypothesis in 2006, with the description of an independent large NSAD family with a *TGFBR2* mutation that also disrupted arginine 460[10,224]. Mutations in this specific amino acid are thought to disrupt F-helix D-helix communications within the three-dimensional structure of the receptor, thus reducing signal transduction, but without affecting the extracellular TGF-β binding domain, preserving its ability to bind TGF-β[28].

(iii) ***SKI* and Shprintzen-Golderg syndrome:** most of the so far discovered genetic variants causing this syndrome have been located to exon 1, between amino acid residues 21 and 117, and clustered between the regions encoding R-Smad binding domain and the start of Dachshund-homology domain. Poninska *et al.* also identified in 2016 the disease-causing genetic variant p.T20K affecting residue 20 (NM_003036.3), which also belongs to the R-Smad binding domain, further supporting the implication of this region in the Shprintzen-Goldberg syndrome pathogenesis[26,225]. The clinical pattern they found was highly complex, with a proband affected by TAA and dilation of the pulmonary artery, mitral ring, and left ventricle, multiple craniofacial and skeletal findings, joint hypermobility, intellectual disability, myopia, astigmatism, and hypertrophy of the third tonsil[26].

(iv) ***ACTA2* and NSAD:** in *ACTA2*-associated vasculopathy distinct allele-specific differences determining the disease severity have been described[41]. Guo *et al.* associated in 2009 the *ACTA2* R258C and R258H amino acid changes with a high risk for cerebrovascular disease, including both vascular occlusive disease and fusiform aneurysms[198]. Luyckx and Loeys proposed in 2015 that mutations affecting arginine 258 seemed to predispose to TAAD and premature stroke, whereas mutations at arginine 118 and 149 were more frequently associated with premature coronary artery disease[51]. Finally, once again Poninska *et al.* detected in 2016 a novel p.N117S amino acid change at the adjacent residue 117 to be associated with TAAD and myocardial infarction (NM_001613.2)[26].

(v) ***ACTA2* and multisystemic SMC dysfunction syndrome:** another *ACTA2* specific genotype-phenotype correlation exists for the p.Arg179His mutation. Milewicz *et al.* identified in 2010 *de novo ACTA2* genetic variants at arginine 179 leading to a generalized smooth cell dysfunction. Patients presented with coronary artery disease, congenital mydriasis, pulmonary hypertension, intestinal malrotation, hypotonic bladder, stroke, and periventricular white matter hyperintensities[38,51,200].

(vi) ***PRKG1* and NSAD:** the specific gain-of-function mutation p.Arg177Gln in exon 3 has been identified by Guo *et al.* in 2013 in a handful of families, which developed type A and B dissections at young age, arterial tortuosity, and hypertension (NM_001098512.2)[51,110].

As seen in *Table 6*, a precise definition of the phenotype is always necessary for the discovery of the associated or causal genotype[112]. The role of the clinician in detecting the phenotype has been crucial to this pursuit, and will continue to be even more so as we further refine and specify subphenotypes[112]. Clinical manifestations not previously recognized as part of a heritable disorder may contribute to the identification of novel TAAD genes[34]. For instance, in families with TAAD associated with either PDA or *livedo reticularis*, Zhu *et al.* and Guo *et al.*, respectively, used the associated clinical features as phenotypic markers in those family members in which the aortic disease was not penetrant; this approach was determinant for mapping the defective gene[197,201].

Hereditary TAAD has therefore been shown as a genetically and phenotypically heterogeneous clinical manifestation with important clinical overlap between the different underlying diseases[11,31], making patient classification based exclusively on their phenotype often difficult. Therefore, as more experience is gained, this type of genotype-phenotype correlations will be further common and particularly useful for establishing an accurate differential clinical and molecular diagnosis of TAAD patients[63].

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# 3. State-of-the-art in genetic approaches to thoracic aortic disease

When seeking to identify pathogenic sequence variants, the technological and analytical approaches should be selected based on the most likely genetic architecture of the disease of interest[226]. For instance, rare, high-penetrant, protein-coding genetic variants causing rare diseases could be cost-effectively captured by whole exome sequencing (WES), while the genotyping of linkage panels remains useful for identifying haplotypes segregating with large Mendelian (especially dominant) diseases in families[226]. Optimal approaches to discovering rare pathogenic genetic variants in common diseases remain unclear: WES, deep and low-coverage whole genome sequencing (WGS), and high-throughput genotyping arrays with enhanced coverage of protein-coding variants have been applied in research settings[226]. Nevertheless, many times, decisions depend on historical evidence, ease of recruiting study individuals, and the decreasing costs of a specific technology[227].

The following are some examples of the genetic approaches that, to date, have been used to try to unravel the genetic component behind TAAD. We have sorted them chronologically, and also referred to the genetic architecture they have been effective with, willing to give context to the upcoming studies that conform this doctoral thesis.

## 3.1. Linkage analysis

It has been the conventional approach to large families, and very successful for mapping genes underlying monogenic Mendelian diseases[112,228]. Genetic variants that cause these disorders generally impart large effect sizes, are sufficient to cause disease, and often rare[112,228]. Negative selection reduces the frequency of those genetic variants that cause severe phenotypes, characterized by early-onset morbidity and mortality[112,228]. In contrast, for most common diseases, linkage analysis has achieved only a limited success[229], due to, mainly: (i) low heritability of most complex traits; (ii) imprecise definition of phenotypes (late-onset diseases); (iii) low statistical power to detect common markers with modest effects that explain only a small fraction of the overall heritability; (iv) participation of non-coding regulatory variants; or (v) phenocopies[72,228,229].

Linkage analysis can be either a candidate-gene or genome-wide approach[228]. It relies entirely on the tendency for shorter haplotypes (10-20 centimorgan, cM) to be passed on to the next generation intact, without recombination events at meiosis[227,228]. Markers that flank the disease-causing gene are less likely to be separated by a recombination event than are markers far away from it[72,228]. Therefore, they are more likely to be transmitted together from parent to offspring and segregate with the disease in families[72,228]. Those polymorphic markers (historically microsatellites or short

tandem repeats, STRs, but more recently also single nucleotide polymorphisms, SNPs) inherited more commonly than by chance by the affected family members, will indicate they are at least in close physical proximity to the deoxyribonucleic acid (DNA) region containing the responsible gene[112,227].

As a result of the many rare Mendelian diseases that have been associated with the development of TAAD, and the relatively low number of affected patients needed to perform this type of study, linkage analysis has been the approach chosen by the vast majority of TAAD investigators to date[7]. Hereunder, we detail some examples that have led to important discoveries in the field.

Guo *et al.* chose in 2001 a genome-wide linkage approach based on randomly selected polymorphic markers spaced approximately 10 cM, to find the defective gene behind multiple cases of NSAD in two different families[57]. After excluding linkage to *FBN1*, they found three chromosomal loci with multiple markers demonstrating evidence of linkage to the phenotype (a 38-cM region of chromosome 3q, a 52-cM region of chromosome 5q, and a 26-cM region of chromosome 16q), and repeated linkage analysis using further markers in these regions for fine genetic mapping[57]. The analysis of 15 extra Caucasian and Japanese families in which TAAD segregated in an autosomal dominant manner with decreased penetrance, confirmed the linkage of nine of them to a specific haplotype located in the previously identified chromosomal region 5q13-14 (*TAAD1* locus), with a maximum logarithm of the odds (LOD) score of 4.74 (D5S2029)[51,57].

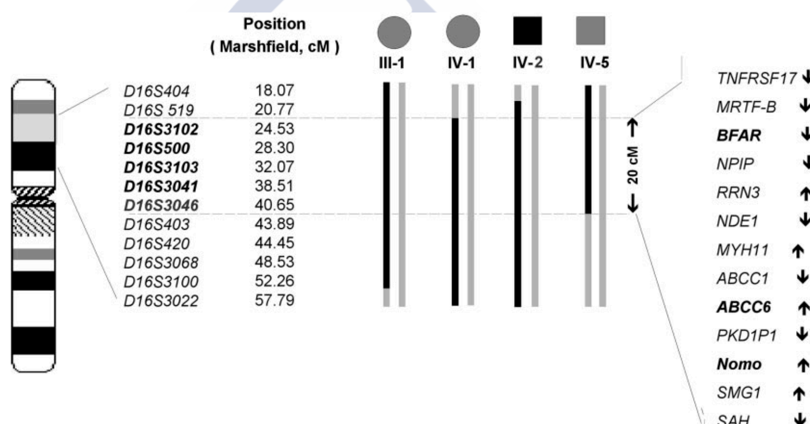
Vaughan *et al.* also performed in 2001 a genome-wide linkage analysis based on polymorphic STRs seeking the genetic factor behind one northern European familial case of thoracic and abdominal aortic affection, after *FBN1*, *FBN2*, *COL3A1, TGFBR2,* and the *TAAD1* locus at 5q had been excluded[161]. They analyzed highly polymorphic STRs dispersed throughout the genome and identified a 2.3 cM locus on chromosome 11q23-24 with a maximum multipoint LOD score of 4.4 in one of the families (*FAA1* locus)[161].

Hasham *et al.* carried out in 2003 a genome-wide scan to map the defective gene causing NSAD in a large four-generation Swiss-German family with multiple members affected by TAAD, but without the ocular or skeletal features of MFS[218]. Using 380 autosomal polymorphic sequences spaced at 10-cM intervals, and after the exclusion of the *FBN1*, *TAAD1*, and *FAA1* loci, they identified a 25 cM chromosomal region on 3p24-25 with a maximum multipoint LOD score of 4.28 (D3S2336; *TAAD2* locus)[218]. They further confirmed linkage through the analysis of 26 additional markers and DNA from 51 family members[218].

Khau Van Kien *et al.* linked in 2005 the chromosomal region 16p12.2-p13.13 to the NSAD and PDA phenotypes present in a large French family from which 40 first-degree non-consanguineous

relatives from 3 generations, and 9 unrelated spouses were available for genome scan analysis (*Figure 12*)[53]. The whole-genome linkage scan involved 382 well-defined highly polymorphic STRs distributed with an average spacing of 10 cM[53]. They first considered only seven NSAD affected family members and detected LOD scores greater or equal to 1.5 for several adjacent markers on chromosome 16[53]. When they also considered the 5 individuals with PDA as affected, they observed stronger values in this region, with a maximum LOD score of 3.56 for marker D16S3075[53]. The multipoint analysis they performed showed another peak LOD score of 4.14 near marker D16S3103[53]. They subsequently enrolled 29 extra family members for haplotype status analysis and 16 markers within the 16p12-p13 region for refined mapping[53]. They identified a single locus within a critical interval of 20 cM, responsible for both TAAD and PDA, and with aortic stiffness as an early hallmark of the disease, occurring even in asymptomatic carriers[53].

**Figure 12. Linkage results obtained by Khau Van Kien *et al.* in 2005**




Aside from the identification of the associated region, they further examined it, as well as the potential candidate genes contained in it *(adapted from Khau Van Kien et al. 2005)*[53].

Again Guo *et al.* identified in 2007 a large family with autosomal dominant inheritance of TAAD with decreased penetrance, associated with pronounced and persistent *livedo reticularis* in arms and legs[197]. To map the causal gene, they carried out a genome-wide linkage analysis involving 50,000 different SNPs on seven family members[197]. After the identification of two linkage peaks at 10q23-24 and 17p11-13 with maximum multipoint LOD scores of 1.80 and 1.81, respectively, they typed 31 STRs on 10q and 17p in 27 different family members and considered the TAAD and *livedo reticularis* phenotypes combined[197]. Based on this approach, they excluded the 17p locus and found a maximum LOD score of 4.40 on chromosome 10q23-24[197]. They further delineated the critical genetic region through positional cloning using recombinants, limiting it to a 17.2 Mb interval between the STRs D10S1765 and D10S1264[197].

As a starting point for the posterior genome-wide association study (GWAS) carried out by LeMaire *et al.* in 2011, Keramati *et al.* performed in 2010 a genome-wide linkage analysis that

involved more than 10,000 SNPs. They analyzed 11 affected, two unaffected, and two unknown living relatives from a large three-generation Iranian family affected by early-onset NSAD[27]. They specified the linkage analysis inheritance model as autosomal dominant with 90% penetrance, 1% phenocopy rate, and a disease allele frequency of 0.001[27]. Based on this specification, they mapped the causal gene to a single interval with a significant LOD score of 3.6 that peaked at the *FBN1* gene locus[27]. They then reconstructed the 15q haplotype revealing recombination immediately prior to rs668842 and after rs1072994, refining the linked interval to less than 500 kilobases[27].

Finally, Boileau *et al.* performed in 2012 a genome-wide linkage analysis of two large unrelated families (US and French) affected by autosomal dominant TAAD with decreased penetrance, along with ICAs and subarachnoid hemorrhages[109]. This procedure allowed them to identify a candidate locus at chromosome 1q41, which was shared between the two families with multipoint LOD scores greater than 2.0 and 1.4, respectively[109].

## 3.2. Expression profiling

Chronologically, the second genetic approach to thoracic aortic disease that emerged had to do not with sequence or structural, but regulatory variation, involving changes in the expression levels of specific candidate genes. This has been another useful technique for the identification of candidate genes, but, as it is not directly linked to this doctoral thesis' course, the following are just some examples of the advances made in this field, from different perspectives:

(i) Koullias *et al.* evaluated in 2004 the expression pattern of the proteolytic enzymes MMP-1, MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2, by aortic tissue microarray immunostaining of 47 TAAD patients and seven controls free of any vascular disease[230]. MMP-1, MMP-9, and TIMP-2 expression was significantly increased in cases compared to controls[13,230]. These results gave support to the hypothesis that MMPs act as a prime agents of deleterious change in the affected aortic wall, and that increased proteolysis plays an important pathophysiological role in the development and progression of the aortic disease[13,230]. Wilton *et al.* further supported this hypothesis in 2008. They quantified MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 gene expression in 82 samples of ascending aorta and aortic valve by real-time reverse transcription polymerase chain reaction (PCR)[231]. Although they did not find differences among the expression patterns in BAV versus TAV, patients with larger aortic diameters had increased MMP-2/TIMP-1, reinforcing that modifying MMP expression may have a role in aneurysm development[231].

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(ii) Wang *et al.* postulated in 2007 that gene expression patterns in peripheral blood cells may correlate with TAA disease status[232]. With the objective of identifying a distinct gene expression signature able to define individuals at risk for TAA, they compared the expression patterns of 30,000 ribonucleic acids (RNA) from 58 TAA patients and 36 controls[13,232]. They were able to establish a 41-biomarker array that could discriminate quite well between patients with and without asymptomatic aneurysm from a single blood test with an accuracy greater than 80%[13,232]. From those 41 biomarkers, those corresponding to already identified genes were: *PCDHA5*, *SBSN*, *SLC6A18*, *OR5B21*, *LOC388751*, *ENPP4*, *IL18R1*, *RASSF3*, *C10orf99*, *SYNGAP1*, *RGS3*, *ELSPBP1*, *SLC25A24*, *VPS72*, *IMPDH2*, *HADHSC*, *SNRPC*, *PHB*, *VKORC1*, *HMOX2*, *RUVBL1*, *AKR1B1*, *RPN1*, *EDF1*, *CDK4*, *APOA1BP*, *HYPK*, *ATP5G1*, *SSU72*, *MED6*, *MIF*, *WDR58*, *ATAD3B*, *NOSIP*, and *NUDT5*[232]. They also provided insights into the mechanisms of development of aortic aneurysms and highlighted potential targets for therapeutic intervention, regarding mainly interleukin signaling, T-cell activation, and apoptosis[232].

(iii) Li *et al.* evaluated in 2015 if dysregulated gene expression of ACE and ACE2, which regulate the renin-angiotensin system, could contribute to TAAD formation[233]. They assessed ACE and ACE2 gene expression by quantitative real-time PCR (RT-qPCR) in aortic tissues from 12 patients with acute TAD, 16 with TAA, and other 16 with coronary heart disease[233]. They found a significantly lower level of ACE and ACE2 gene expression in dissection samples when compared to aneurysm and coronary heart disease groups, which were furthermore strongly correlated[233]. They therefore concluded that the down-regulation of their mRNA expression levels may play an important role in aneurysm progression and subsequent dissection[233].

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## 3.3. Candidate-gene sequencing

Taking advantage from the former linkage analysis results or from established hypothesis about the pathogenesis of the disease of interest, another of the common approaches to TAAD has been traditional or MPS of candidate genes. In general, the entire candidate gene or genes are sequenced in patients and controls, searching for a single or a set of genetic variants enriched or depleted in the disease cases[228]. The studies using this approach have been multiple and have frequently involved familial cases affected by monogenic Mendelian diseases developing with TAAD. We have gathered here some of the most relevant for the field, representing both traditional sequencing and MPS approaches, and trying to show their *pros* and *cons*.

### 3.3.1. Traditional sequencing

This basic sequencing approach continues to be used with multiple finalities: (i) to sequence previously described candidate genes looking for already known or new causal mutations (mutational screening); (ii) to further explore linkage analysis regions willing to identify the causal mutation; (iii) to perform segregation studies in families; and, in the clinical diagnosis setting, (iv) to validate MPS results.

Regarding TAAD, traditional sequencing was firstly used to further explore previously described linkage regions. Due to the limitations inherent to any candidate-gene approach, it was many times unsuccessful, but still, led to important conclusions that have been very useful to date. This was the case of Guo *et al.* in 2001[57]. They at some point realized that the cardiovascular complications of the NSAD families they had previously linked to 5q13-14 (*section I, subsection 3.1*) were similar to those observed in MFS patients[57]. As the condition was furthermore inherited in an autosomal dominant manner, they hypothesized that the defective gene could be a connective tissue protein[57]. They sequenced three candidate genes present in the critical interval that encoded for the connective tissue proteins versican, thrombospondin 4, and cartilage-linked protein, but were unable to reveal any variation that accounted for or segregated with the disease phenotype[57].

Willing to identify the causal gene at the 3p24-25 loci, Pannu *et al.* performed in 2005 bidirectional traditional sequencing of all eight coding exons of *TGFBR2* (*TAAD2* locus) in 80 unrelated NSAD cases with type A dissections[43]. They found heterozygous germline *TGFBR2* genetic variants (NM_003242) in four of those unrelated families, which were also affected by ascending and descending aneurysms of other arteries (carotid, cerebral, pulmonary)[43]. Strikingly, all four mutations resulted in an amino acid substitution at arginine 460 in the serine/threonine kinase intracellular domain of the receptor[43]. This results supported the specific genotype-phenotype correlation described in *section I, subsection 2.3*, as well as the existence of a mutational hotspot for NSAD that interferes with the receptor ability to transduce signals.

Zhu *et al.* further explored in 2006 the two familial cases with TAAD and PDA linked to 16p12.2-p13.13 by Khau Van Kien *et al.* in 2005 (*section I, subsection 3.1*)[53,201]. Using bidirectional traditional sequencing, they discovered *MYH11* as the causal gene[53,201]. Besides sequencing several candidate genes contained in that 20-cM interval (*NOMO1*, *ABCC6*, and *BFAR*), which showed no mutation, they sequenced all *MYH11* exons in two affected individuals of each family and two controls[201]. For the first family, they identified a substitution at the splice-donor site of intron 32 (IVS32+1G>T) that resulted in an in-frame deletion of 71 amino acids, and a missense genetic variant in exon 37 (G5361>A, R1758Q), in all subjects carrying the disease haplotype

(NM_002474)[201]. In the second family, they detected another 24-amino acid deletion within exon 28.[201]. Both deletions affected the coiled-coiled region of *MYH11* and were not found in 340 normal chromosomes[201,234]. Finally, they confirmed their segregation with the disease in other available relatives, once again by bidirectional traditional sequencing[201].

Guo *et al.* performed in 2007 bidirectional traditional sequencing to further delineate their 17.2 Megabases candidate region at chromosome 10q23-24 (*section I, subsection 3.1*)[197]. From the 12 candidate genes present in this region, they identified a missense genetic variant in *ACTA2* that resulted in the amino acid substitution R149C at a strongly conserved position (NM_001613). It segregated with the affected haplotype and was absent in 384 controls[197]. Finally, they sequenced the *ACTA2* gene in 97 unrelated TAAD families and identified 14 additional families with *ACTA2* candidate genetic variants that segregated with TAAD and were absent in 192 controls[197].

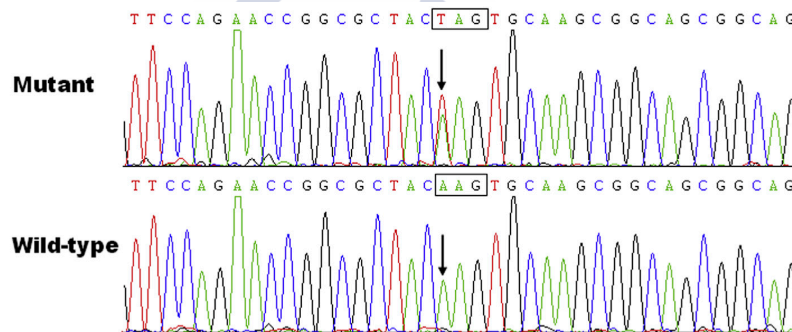
Another common use of this approach has been the direct candidate-gene sequencing itself, also known as mutational screening. Pannu *et al.* applied it in 2007, for instance, to further define the *MYH11* mutational spectrum. They performed bidirectional traditional sequencing of probands from three TAAD-PDA families along with probands from 93 unrelated families with TAAD but without PDA[234]. They identified three *MYH11* candidate genetic variants in just two of the three TAAD-PDA families. The three of them were missense, but two were located at the coiled-coiled domain (L1264P, 3791T>C and R1275L, 3824G>T), while the third at the adenosine triphosphatase head region (R712Q, 2153C>T) (NM_002474, NM_022844)[234]. All of them segregated with the TAAD and/or PDA phenotype, and were not present in 360 control chromosomes[234]. No disease causing *MYH11* variants were identified in the other 94 families analyzed[234].

With the same objective of determining the mutational spectrum of *SMAD3* in individuals with thoracic aortic disease, Regalado *et al.* sequenced in 2011 181 probands with familial TAAD, and identified three *SMAD3* mutations in four families: c.836G>A (p.R279K; detected twice), c.715G>A (p.E239K), and c.235C>T (p.A112V; NM_005902.3). They all resulted in a combined LOD score of 5.21 and were absent in 2,300 control exomes[104]. They therefore concluded that *SMAD3* mutations were responsible for approximately 2% of familial TAAD cases, and that they led to an inherited predisposition for TAAD, ICAs, and AAA[104].

Foffa *et al.* evaluated in 2013 the potential contribution of *NOTCH1*, *GATA5*, *TGFBR1*, and *TGFBR2* germline mutations in 11 unrelated Italian patients with familial BAV, by directly sequencing all their coding exons[114]. They found two novel *NOTCH1* mutations (p.P284L and p.Y1619X; NM_017617.3) in two unrelated families that segregated with the disease and were not found in 200 unrelated chromosomes from ethnically matched controls[114]. In contrast, they did not identify any pathogenetic mutation in *GATA5*, *TGFBR1*, and *TGFBR2* genes[114].

Again focused on BAV instead of TAAD, Qu *et al.* sequenced in 2014 the coding exons and flanking introns of the *NKX2.5* gene in 142 unrelated patients, all the available relatives, and 200 unrelated controls (*Figure 13*)[124]. They chose *NKX2.5* as a candidate gene for BAV pathogenesis because it had been considered key for the normal cardiovascular development and valvulogenesis in vertebrate species, as well as a synergistic transactivational partner of *GATA5*[124,147,235]. Furthermore, targeted deletion of *Nkx2.5* resulted in partially penetrant BAV in mice, similarly to what had been observed in Gata5-null mice[124,143,236]. They were able to identify the single novel loss-of-function heterozygous *NKX2.5* nonsense mutation c.574A>T (p.K192X) in one of the families they sequenced (NT_023133)[124,237]. This mutation segregated with the disease in an autosomal dominant manner with complete penetrance[124], and it was absent in 400 control chromosomes and the *NCBI dbSNP*[238], *Human Gene Mutation Database*[239], and *1000 Genomes Project* databases[124,240].

**Figure 13. Traditional sequencing electropherograms of affected and unaffected patients by Qu *et al.***




Above, a mutant carrying the *NKX2.5* mutation and below, the corresponding control. The arrow points to the heterozygous nucleotides A/T in the proband, and the homozygous nucleotides A/A in the control subject *(adapted from Qu et al. 2014)*[124].

### 3.3.2. Candidate-gene massive parallel sequencing

In the molecular diagnosis of genetically heterogeneous TAAD, simultaneous testing of multiple genes is often indicated[31,86]. While traditional sequencing has not always been cost- and time-effective[31,86], candidate-gene MPS has proved to be a very useful approach for this purpose[228]. This latter offers several advantages over traditional molecular techniques, such as the rapid, cost-effective detection, and differentiation of some of the TAAD disorders with phenotypic overlap[75]. Many TAAD candidate genes have been analyzed through MPS to date. The following are some of the most recent examples:

(i) Campens *et al.* defined in 2013 a set of 17 TAAD candidate genes including: *ACTA2*, *ADAMTS10*, *COL3A1*, *ELN*, *FBLN4*, *FBN1*, *FBN2*, *FLNA*, *MYH11*, *MYLK*, *NOTCH1*, *SKI*, *SLC2A10*, *SMAD3*, *TGFB2*, *TGFBR1*, and *TGFBR2*[31].

(ii) Proost *et al.* analyzed in 2015 14 TAAD candidate genes: *ACTA2*, *COL3A1*, *EFEMP2*, *FBN1*, *FLNA*, *MYH11*, *MYLK*, *NOTCH1*, *SKI*, *SLC2A10*, *SMAD3*, *TGFB2*, *TGFBR1,* and *TGFBR2*, in 55 individuals, and reported 15 causative genetic variants and six VUS, achieving 27% clinical sensitivity[26,86].

(iii) Wooderchak *et al.* sequenced in 2015 the 10 TAA candidate genes *ACTA2*, *COL3A1*, *FBN1*, *FBN2*, *MYH11*, *MYLK*, *SLC2A10*, *SMAD3*, *TGFBR1*, and *TGFBR2,* and further analyzed copy number variations (CNV) through a comparative genomic hybridization array. They analyzed a series of 175 individuals with features of aortopathies[75]. From these, 18 tested positive for a pathogenic genetic variant (10.3%; eight novel) and 32 were carriers of VUS (18.3%)[75]. Such a high negative rate (71.4%) led them to incorporate seven extra TAAD candidate genes: *CBS*, *COL5A1*, *COL5A2*, *PLOD1*, *SKI*, *SMAD4*, and *TGFB2*[75].

(iv) Poninska *et al.* evaluated in 2016 the diagnostic yield of candidate-gene MPS in 51 TAAD unrelated patients and 64 relatives, seeking novel genetic variants and strong genotype-phenotype correlations. With this purpose, they sequenced the 10 TAAD candidate genes *ACTA2*, *COL3A1*, *FBN1*, *MYH11*, *MYLK*, *SKI*, *SMAD3*, *TGFB2*, *TGFBR1*, and *TGFBR2* in 22 patients, and the whole exome of another 29[26]. They found 22 rare genetic variants, six which had been previously reported as pathogenic in TAAD patients (27.3%), and 16 novel (73.7%)[26]. They assumed three of the novel genetic variants to be causative, nine likely causative, four VUS, and two benign, and obtained a diagnostic yield of 35.3%, which could nonetheless be slanted due to the severe disease suffered by the selected patients[26].

It is always important to take into account that the candidate-gene lists are constantly evolving with the discovery of new TAAD genes[67], and many more will probably be extensively described in a near future.

## 3.4. Association studies (candidate-gene, exome-wide, genome-wide)

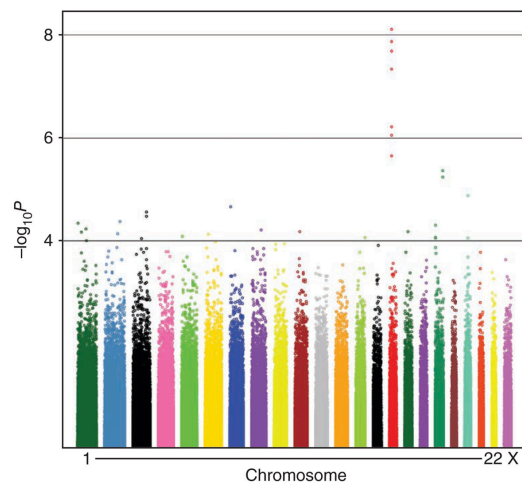
Although both previous approaches to single-gene disorders have been very useful to the scientific community, they have not offered sufficient resolution to identify neither the causal genes behind small families or sporadic cases, nor the susceptibility alleles to common and complex phenotypes[54,112]. Population-based association studies were proposed to fill the second knowledge gap and identify common variation, with comparatively modest effects, and unlikely to be under strong negative selection, which could be contributing to the risk of developing complex phenotypes.

The basic principle behind any association study entails the comparison of allele frequency distributions between cases and controls[54,228], testing for dependence among a genotype and the disease phenotype; if a genotype was truly related to a disease, it would be found more or less frequently in cases than controls[72]. Akin to linkage analysis, a disease-causing genetic variant that arose many generations ago in close proximity to a DNA marker will be strongly co-inherited, leading to an association at the population level due to shared common ancestry[72].

Therefore, within association studies, one can opt to genotype: (i) putative causal variants, typically functional SNPs in biologically relevant genes (direct association, constrained by the current knowledge); (ii) polymorphic markers expected to be in linkage disequilibrium across the population with the true disease-causing genetic variant within the same gene or even, potentially, nearby genes (indirect association); or (iii) combine the former strategies[72]. Nevertheless, in general, genome- or exome-wide association studies emerged to avoid hypothesis-based approaches[228]. They seek to identify the genetic factors that contribute to common and complex genetic disorders using large samples of unrelated individuals, and genotyping hundreds of thousands of genetic variants, frequently SNPs[54,72]. Because in this latter case no assumptions are made about the genomic location of the causal genetic variants[228], they are the generally preferred approaches.

According to the *GWAS Catalog* register[241], the only GWAS that has been published to date involving TAA patients was conducted by LeMaire *et al.* in 2011[3]. In a three-stage GWAS, they evaluated the genetic basis of sporadic TAAD (*Figure 14*)[3]. They first compared 765 sporadic TAAD cases with 874 controls, and identified five SNPs at the 15q21.1 locus (rs10519177, rs4774517, rs755251, rs1036477, and rs2118181) associated with the trait of interest at genome-wide significance (*p-value* $\leq 5 \cdot 10^{-8}$), and with an odds ratio of 1.6-1.8[3].

**Figure 14. *Manhattan* plot representing LeMaire *et al.* stage 1 genome-wide association results**




The *x*-axis represents the chromosomic location, whereas the *y*-axis the significance level as the $-\log_{10}$ of the *p-value (adapted from LeMaire et al. 2011)*[3].

They validated their findings in two independent sporadic TAAD cohorts that comprised: (i) 385 sporadic TAAD cases, 192 of whom also had BAV, and 159 controls; and (ii) 163 sporadic TAAD cases, 157 of whom had BAV, and 476 controls[3]. They were able to replicate all five association signals in both replication stages, whether or not BAV was present[3]. They proposed those common genetic variants at the 15q21.1 locus to be probably acting through *FBN1*, suggesting a common pathogenesis of MFS and sporadic TAAD[3].

Although not directly involving TAAD patients, Thanassoulis *et al.* performed in 2013 a GWAS of aortic and mitral valve calcification including 6,942 individuals from the general population[54,242]. They identified a genetic variant in the *LPA* gene encoding lipoprotein(a) to be associated with an increased risk of aortic sclerosis and stenosis, as well as lipoprotein(a) levels[54,242]. This finding was subsequently confirmed by Arsenault *et al.* and Kamstrup *et al.* in 2014[243,244], reinforcing the hypothesis that the process of valvular age-related degeneration shares key pathophysiologic features with the observed in the atherosclerotic vessels[54]. In a second analysis of the same cohorts, they showed that a polygenic score comprising alleles associated with increased LDL-cholesterol also conferred increased risk of aortic valve calcium deposit and stenosis, further supporting a role of blood lipids beyond lipoprotein(a) in the development of aortic valve calcification[245].

Guo *et al.* further evaluated in 2016 the genetics behind sporadic TAD through an exome-wide association study (EWAS) involving 753 sporadic TAD cases and 2,259 controls, matched for age, gender, and hypertension[206]. They identified SNPs in *FBN1*, *LRP1*, and *ULK4* to be significantly associated with sporadic TAD, and replicated these results in two independent cohorts (129 and 123 sporadic TAD cases, and 181 and 13,646 ethnicity-matched controls, respectively)[206]. Genomic CNV analysis independently confirmed the association of *ULK4* with sporadic TAD, and further supported the contribution of *ULK4* genetic variants to an increased risk for thoracic aortic disease through a different mechanism than hypertension[206].

Given that aneurysms are known to co-occur, Van 't Hof *et al.* performed in 2016 a mega-analysis of *1000 Genomes Project*-imputed GWAS data from ICAs, AAAs, and TAAs, willing to identify shared genetic risk factors[240,246]. The four series they gathered involved: 1,516 ICA cases and 4,305 controls, 818 AAA cases and 3,004 controls, and LeMaire *et al.* 760 TAA cases and 2,212 controls[3,240,246]. Four of the known risk loci (9p21, 18q11, 15q21, and 2q33, previously described as risk loci for ICA and AAA, ICA, TAA, and ICA, respectively) showed consistent effect direction in all four cohorts[246]. But when they calculated polygenic scores based on ICA, AAA, and TAA-associated SNPs, and tested these scores for association with the other aneurysm cohorts, they found no polygenic effects[246]. They also estimated the heritability of each type of aneurysm, obtaining values of 0.15 for AAA, 0.31-0.34 for ICA, and 0.40 for TAA, and performed a genetic correlation

analysis that did not show significant correlation between any pair of aneurysm types[246]. Finally, they evaluated the effect of 14 bonafide risk SNPs from previously published ICA, AAA, and TAA GWAS on the other aneurysm types in extended aneurysm cohorts involving 6,548 ICA cases and 16,843 controls, and 4,391 AAA cases and 37,904 controls. They found nominally significant associations between the ICA risk locus 18q11 and AAA (rs1166154, *RBBP8*), and the TAA risk locus 15q21 and AAA (rs2118181, *FBN1*), both of which were previously unknown to be associated with AAA, supporting the existence of a shared genetic background of ICA, AAA, and TAA[246].

Finally, this same case-control strategy has been applied to the identification of risk or protective CNVs. Based on LeMaire *et al.* 2011 genotypes, Kuang *et al.* investigated also in 2011 the association of recurrent CNVs with TAAD[3,189]. With this approach, they identified 16p13.1 duplications, involving nine genes including *MYH11*, in eight out of 765 sporadic TAAD cases, which at the same time were present in four of 4,569 controls matched for ancestry[189]. They went on to replicate the findings in another independent cohort of 467 patients[189]. This same 16p13.1 region had already been implicated in a variety of neuropsychiatric disorders such as autism, schizophrenia, epilepsies, and attention-deficit hyperactivity disorder[189]. They concluded that recurrent CNVs may predispose to disorders involving more than one organ system, an observation critical to the understanding of their role in human disease and that may apply to other recurrent CNVs involving multiple genes[189].

Prakash *et al.* also applied in 2010 and 2016 genome-wide genotyping approaches to the identification of CNVs associated with a higher risk for TAAD[12,247]. The 2010 study involved 418 sporadic TAAD cases, 5,088 *dbGaP* controls, and a replication cohort of 387 unrelated individuals, and led to the identification of 47 CNV regions that were enriched or unique to TAAD patients compared to population controls[247,248]. Most of the genes within these CNVs participated in the regulation of SMC adhesion or contractility, known to cause familial TAAD when affected[247]. In fact, they for example demonstrated that 16p13.1 duplications, involving *MYH11*, increased the risk of TAAD over 10-fold[12,247]. The 2016 study involved 108 early-onset TAAD patients who were enrolled in the *National Registry of Genetically Triggered Thoracic Aortic Aneurysms and Cardiovascular Conditions (GenTAC)*[249], looking for CNVs that could contribute to the development of early-onset TAAD among young patients with BAV, aortic coarctation, and other LVOT defects[12]. They compared these results to 7,013 *dbGaP* controls without any history of vascular disease, 805 sporadic TAAD patients with late-onset aortic disease, and 192 probands from families with at least two affected relatives[12,248]. They again identified 47 recurrent CNVs containing 105 protein-coding genes in all the different subgroups of affected patients, which were furthermore absent or extremely rare in controls[12]. The largest and most prevalent of the recurrent CNVs were at Xq28 (two duplications and two deletions) and 17q25.1 (three duplications)[12]. Finally, they also observed that the enriched

genes were mainly related to aortic valve defects or arterial aneurysms (*DOCK8*, *ARSB*), LVOT and aortic development (*LIMS3*, *DTX2*, *PTCH1*, *HOXA3*), the maintenance the integrity of vascular SMC interactions with the ECM (*LAMB1*, *BGN*, *FBN1*, *CDH13*), and the regulation of ECM proteolysis (*CTSL2*, *cystatins*).

## 3.5. Whole exome and genome massive parallel sequencing

These new sequencing technologies arose to fill the knowledge gap left by candidate-gene sequencing and population-based association studies regarding single-gene disorders occurring in sporadic cases or small families. Briefly, these high-throughput technologies are based on parallel sequencing of millions of DNA fragments simultaneously[112]. Multiple reads of the same DNA fragment are aligned with a reference sequence and compared to identify those positions that differ between them, and that can therefore be classified and annotated as genetic variants[112].

Two of these MPS strategies exist, the first centered on the protein-coding regions of the genome, known as exome, and the second one involving the whole genome and therefore generating huge amounts of data. In any case, a robust study design is necessary for being able to prioritize the large number of genetic variants that randomly segregate with the phenotype[112].

### 3.5.1. Whole exome sequencing

Although the exome constitutes only approximately 1.5% of the human genome[250] (30 Megabases of genomic DNA, 180,000 protein-coding exons, 21,000-23,000 genes, and about 13,000 nonsynonymous and 7,000 potentially functional variants[67,112]), this is still the most commonly used MPS approach, as approximately 85% of mutations causing Mendelian diseases have been found in coding regions or canonical splice sites[251–253]. Andelfinger *et al.* stated in 2016 that these mutations account for the vast majority of Mendelian phenotypes (> 4,000), whereas only a vanishing minority is caused by mutations in non-coding sequences (approximately 20)[38]. Compared with the candidate-gene MPS approach, this huge number of candidate genetic variants increases the chance of finding the true causal, as well as VUS that pose another challenge to researchers[67,112]. Regarding specifically thoracic aortic disease, this approach has been widely used for the molecular diagnosis of familial TAAD, the following being some examples.

Regalado *et al.* performed in 2011 WES of 2 distant relatives affected by TAAD, willing to identify the causative mutation behind a large family with dominant inheritance of TAAD, AAA, and ICAs[104]. They focused on the analysis of novel, heterozygous, nonsynonymous, and insertion or deletion (indel) genetic variants present in both affected family members, and absent in the *NCBI dbSNP*[238] and *1000 Genomes Project* databases[240], as well as in 21 in-house control exomes[104]. With this

approach, they identified a novel frameshift genetic variant introducing a premature stop codon in the *SMAD3* gene (c.652delA:p.N218fs; NM_005902.3)[104]. It segregated with the vascular disease with a LOD score of 2.52 and was absent in approximately 2,300 control exomes[104]. They afterwards performed a mutational screening of *SMAD3* mutations in TAAD patients, as described in *section I, subsection 3.3.1*.

Although less frequently, WES has also been used to further evaluate linkage analysis results. For instance, this is what Boileau *et al.* did in 2012 with the candidate TAAD locus they identified at chromosome 1q41 (*section I, subsection 3.1*)[109]. From the two affected families, they selected two affected and distant relatives, and three affected and one unaffected individual, respectively[109]. They prioritized the sequencing results for novel, heterozygous, rare, and nonsynonymous, nonsense, or indel genetic variants, shared between the affected relatives[109]. They identified rare *TGFB2* genetic variants in both families[109]: c.1021_1025del-TACAA (p.Tyr341Cysfs*25) in exon 6, with a two-point linkage LOD score of 3.3 and which was absent in 3,795 control exomes; and p.Cys229* in exon 4, with complete penetrance, a LOD score of 4.4, and which again was absent in over 10,000 chromosomes from the *NHLBI Exome Sequencing Project* database[254], respectively (NM_003238.3)[109]. Both genetic variants segregated with the disease with a combined LOD score of 7.7[109].

Guo *et al.* performed in 2013 WES of two distantly related affected individuals and identified the potentially causal *PRKG1* genetic variant c.530G>A (p.R177Q; NM_001098512.2), which segregated with the autosomal dominant inheritance of the predisposition to thoracic aortic disease[110]. It furthermore demonstrated complete penetrance for TAAD with early-onset aortic dissections in the majority of family members, as young as 17 years of age[110].

Again Guo *et al.* applied in 2015 a similar strategy to two affected family members belonging to a large NSAD familial case[58]. They initially identified 25 missense genetic variants that were shared between the two affected relatives with a MAF under 0.05% in the *NHLBI Exome Sequencing Project*[254] and *1000 Genomes Project* databases[58,240]. From these, they prioritize a *MAT2A* sequence variant that fell under a linkage peak and disrupted the coding sequence of the gene by introducing the nucleotide change c.1031A>C (p.Glu344Ala; NM_005911.5)[58]. To confirm that *MAT2A* genetic variants predisposed individuals to NSAD, they further analyzed exome and traditional sequencing data from 78 NSAD probands and 447 additional probands in whom no genetic variants responsible for the disease had been identified, respectively[58]. With this latter approach, they identified the extra candidate *MAT2A* mutation c.1067G>A (p.Arg356His)[58].

Kuang *et al.* performed in 2016 WES of two distantly related patients from a large family with at least eight cases of acute aortic dissection inherited in an autosomal dominant manner[111]. They prioritized the sequencing data towards shared heterozygous genetic variants with MAF less than

0.05% in the *NHLBI Exome Sequencing Project* database[111,254]. They identified the rare genetic variant c.457G>C in *FOXE3* (p.Asp153His; NM_012186.2), which was the only one that segregated with the disease (LOD score of 3.0)[111]. It disrupted an evolutionarily conserved amino acid belonging to the DNA-binding domain, and was furthermore present just once in 13,000 chromosomes from the *NHLBI Exome Sequencing Project* database[111,254].

A different way of prioritizing the huge amount of results produced by this technology is to first analyze in detail a set of preferred candidate genes, and should no mutations be present in any of them, extend the analysis to the detection of new causal genes. This was the approach implemented by Ziganshin *et al.* in 2015. They sequenced the whole exome of 102 TAAD patients, but underwent a more focused analysis of 21 already known candidate genes: *ACTA2*, *ADAMTS10*, *COL1A1*, *COL1A2*, *COL3A1*, *COL5A1*, *COL5A2*, *ELN*, *FBLN4*, *FLNA*, *FBN1*, *FBN2*, *MYH11*, *MYLK*, *NOTCH1*, *PRKG1*, *SLC2A10*, *SMAD3*, *TGFB2*, *TGFBR1*, and *TGFBR2*[166]. Up to 72.5% of the patients they analyzed had no functionally important genetic variants, 3.9% had a deleterious mutation in *FBN1*, *COL5A1*, *MYLK*, and *FLNA,* and, finally, 21.6% had previously unknown but suspicious VUS with potential implications for clinical management[166]. Taking advantage of the WES approach, they also identified a stop mutation in the *MIB1* gene (Notch pathway), and another mutation in the *TGFB3* gene, known to be associated with LDS but to date not with isolated TAAD[166]. They finally reported four patients with medically actionable incidental findings not related with the aortic disease, which included mutations in: *KCNE2* (drug-induced cardiac arrhythmias), *PTPN11* (LEOPARD syndrome), *PTCH2*, and *SDHAF*[166]. Another example of this alternative prioritization protocol can be found in *section I, subsection 3.3.2.*

Despite the WES high efficiency here shown, some of the main shortcomings of this MPS approach are: (i) incomplete capture; (ii) inadequate coverage of all exons; (iii) incorrect mapping of the reads[112]; and (iv) the still lack of knowledge about the true distribution of disease-associated genetic variants between the coding and non-coding sequences[227]. For all this reasons, in some cases the preferred approach has already been the one described below.

### 3.5.2. Whole genome sequencing

This is the most comprehensive approach towards the understanding of a complex disease[228], as it enables the identification of all DNA sequence variants (common and rare) and hence, the opportunity to not only discover the causal variants, but also modifiers that influence the phenotypic expression of the disease[112]. Nevertheless, the huge amount of data it generates is nowadays often unapproachable and the results are challenging to interpret[228]. Regarding TAAD, this field has just started, but will be absolutely central in future research.

Lee *et al.* performed in 2016 WGS of two affected first-cousins that did not have any potentially causal genetic variant in the set of known TAAD genes, willing to identify new causal genes[23]. The requirements they pre-established for any prioritized genetic variant were: (i) to be shared in a heterozygous state between the two affected relatives; (ii) to have a MAF in all populations from the *NHLBI Exome Sequencing Project* and *Exome Aggregation Consortium* databases of 0.01% or less[254,255]; (iii) to not be present in an internal database of individuals sequenced for other rare, nonvascular, Mendelian disorders; and (iv) to exert a functional impact on the gene product as missense, nonsense, frameshift, or splice site genetic variants. After applying this prioritization protocol, they identified a missense genetic variant in the *LOX* gene (c.893T>G:p.Met298Arg), which segregated with the disease in the family[23].

Despite the usefulness of these high-throughput MPS approaches, which have allowed the scientific community to shorten the turnaround time, increase mutation uptake, and reduce the overall cost of molecular testing[86], it is important to remember that nowadays, they are not always justified[31]. For instance, in a patient affected by TAA in combination with lens luxation, genetic diagnosis could be restricted to the analysis of *FBN1*[31]. Furthermore, traditional sequencing keeps being the reference technology for clinical molecular diagnosis, and so all DNA sequence variation identified by MPS platforms should be confirmed and validated either by repeating independent MPS reactions, traditional sequencing, or at least by genotyping. This current recommendation will probably evolve in a near future, once MPS quality standards are established.

## 3.6. Epigenetics

Besides all the aforementioned approaches defined to detect sequence changes, the existence of other types of regulatory genetic variation should be further taken into account. Briefly, the term "epigenetics" refers to heritable but reversible DNA or histone modifications, and micro-ribonucleic acid (miRNA) mediation affecting gene expression, without modifying the DNA sequence[55]. While the primary sequence of the human genome is largely preserved in all human cell types, the epigenomic landscape of each cell can vary considerably, contributing to distinct gene expression programs and biological functions[256], and providing another important source of genetic variation. Furthermore, it is fundamental during development and throughout life (regulating the differentiation and functionality of all cell types, and allowing cells to react quickly to environmental changes through variation in chromatin accessibility), and it also plays a central role in several diseases progression[55]. Despite recent technological advances, research regarding TAAD epigenetics has been sparse[55]. The large majority of data concerning the role of epigenetics in aortopathy was

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obtained in AAAs[55]. Nevertheless, there is increasing evidence for epigenetic alterations associated with TAAs that involve miRNA, DNA-methylation profile, or histone modifications[55].

For instance, willing to unravel the relation between the TAA etiological diversity and the common TGF-β signaling cell phenotype, Gomez *et al.* analyzed in 2011 48 TAA (14 MFS, 15 BAV, and 19 degenerative) and 10 control individuals, from which they obtained both aortic tissue extracts, SMCs, and fibroblasts[257]. After performing siRNA transfections to verify the TGF-β/Smad2 specificity, real-time PCR, and immunoblotting, they found that all TAA types shared a complex dysregulation of the Smad2 signaling, characterized by an SMC-specific Smad2 overexpression in the tunica media[55,257]. The cell specificity and heritability of this overexpression suggested the implication of the epigenetic control of Smad2 expression[257]. To prove it, they demonstrated by chromatin immunoprecipitation the association between Smad2 overexpression and the increase in H3K9/14 acetylation and H3K4 methylation, affecting the vicinity of a transcription start site on the Smad2 promoter[257]. That cell-specific epigenetic activation of Smad2 in TAAs of different etiologies suggested that the genetic background was not the direct cause of Smad2 alterations, but could trigger long-term environmental modifications, leading to epigenetic reprogramming[55]. Two years later, Gomez *et al.* performed additional experiments on TAA samples and aneurysmal SMCs, and demonstrated that the histone acetylases p300 and P300/CREB-binding-protein associated protein also played a major role in Smad2 promoter activation, suggesting that other loci could be dysregulated in addition to the Smad2 promoter[55,258].

Regarding miRNAs analysis, Jones *et al.* chose in 2011 differential RT-qPCR to analyze single, specific miRNAs[259]. They revealed a decrease in *miR-1*, *miR-21*, *miR-29a*, *miR-133a*, and *miR-486* in TAAs, and a significant relationship between specific miRNAs expression levels (*miR-1*, *miR-21*, *miR-29a*, and *miR-133a*) and aortic diameter[55,259].

With the objective of identifying miRNAs that might contribute to aortic dissection, Liao *et al.* performed in 2011 a miRNA profile comparison between 6 TAD patients and 6 age-matched controls[260]. Besides miRNA microarray, they also performed reverse transcription qPCR to verify the expression pattern of a subset of differentially expressed miRNAs in a greater sample (12 cases and 8 controls)[260]. They found significant differences in the overexpression of 18 and subexpression of 56 miRNAs in TAD patients[260]. Further target gene-related pathway analysis suggested the involvement of focal adhesion and MAPK signaling pathways in TAD pathogenesis[260].

Patuzzo *et al.* performed in 2012 a larger miRNA microarray analysis pooling ascending thoracic aortas obtained from sporadic TAA-TAV patients, and compared them with matched control samples[55,261]. The overall array data they obtained suggested a significant repression of the focal adhesion pathway and a potential *miR-29b*-mediated down-regulation of ECM proteins in

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TAA patients[55,261]. Among the 99 dysregulated miRNAs, only 16 were up- or down-regulated in both male and female patients, suggesting that vascular gene expression could be sex-related[55,261].

Finally and briefly, Shah *et al.* performed in 2015 the analysis of DNA-methylation signatures in TAV and BAV patients with TAAs, and reported a different methylation profile in TAV and BAV patients submitted to surgery[55,262]. Some of the selected genes (*PTPN2*, *RIPK1*) were differentially expressed, whereas others, such as *ACTA2*, *TBX5*, and *PRDM16*, showed a different methylation profile, but not a different expression level[55,262].

Therefore, epigenetic alterations seem to not only play a key role in TAA pathogenesis, being potential therapeutic or preventive targets, but also to be possible biomarkers of the disease[55]. As stated later on, this is one of the main pending issues for future TAAD research.

## 3.7. Functional modeling

Besides gene and genetic variant identification, the fuller picture requires knowledge of the gene product function, as well as the place of DNA, RNA, and proteins in the living environment of an integrated organism[227]. With the term functional modeling we are not only referring to *in vivo* experiments, but also some more affordable *in vitro* experiments, that can sometimes be even more appropriate. In any case, it is essential to consider their relevance to human disease and their ability to mimic human environment to reliably interpret them. The following are just some examples of the main functional approaches that have been applied to heritable TAAD cases:

(i) **Depiction of protein functional domains:** Pannu *et al.* developed in 2005 a homology model of the *TGFBR2* cytoplasmatic domain that behaved as a mutational hotspot, with the objective of identifying the structural basis of the contribution of R460 genetic variants to the development of NSAD (*section I, subsections 2.3 and 3.3.1*)[43]. They used the crystal structure of *TGFBR1* kinase domain as a template, and demonstrated a key role for the R460 residue in maintaining the structural integrity of the *TGFBR2* catalytic loop for effective signaling[43].

(ii) **Functional assays involving the encoded protein:** Barbier *et al.* performed in 2014 immunoblot analysis of dermal fibroblasts to demonstrate that pure or at least partial haploinsufficiency was the pathomechanism behind the *MFAP5* genetic variants they identified through WES in two NSAD families[11]. In those cases in which c.472C>T was present in heterozygosis (p.Arg158*; NM_003480.3), they detected no extracellular signal for the truncated protein, suggesting an endoplasmic-reticulum-associated degradation pathway leading to pure haploinsuffiency[11]. On the other hand, when the heterozygous c.62G>T genetic variant was present (p.Trp21Leu; NM_003480.3), they detected the resulting protein

with the expected molecular weight, but apparently lower intracellular levels[11]. They further investigated a possible effect of *MFAP-5* loss-of-function mutations on the TGF-β signaling pathway by the assessment of TGF-β1 and nuclear phosphorylated Smad (p-Smad) 2/3, and found TGF-β signaling to be greatly enhanced[11].

(iii) **Cellular models:** Barbier *et al.* further confirmed their previous results by site-directed mutagenesis and transfection assays, and also performed histological evaluation of the aortic tissue collected from one of the probands at the time of surgery, revealing disorganization of the media with loss of SMCs and proteoglycan accumulation[11]. Loss-of-function *MFAP5* might affect the structural function of the vessel by directly altering the ECM organization and/or deposition of elastic fibers, leading to fibers disruption and aortic dilation[11].

(iv) **Mouse models:** trying to give support to the association among *FBN1*, TGF-β and MFS, Habashi *et al.* generated in 2006 targeted mutant mouse model of MFS, heterozygous for the cysteine substitution C1039G. They found that fibrillin-1 deficient mice had increased TGF-β when compared with wild-type mice[10,263], suggesting that a reduction in fibrillin-1-containing microfibrils increased the bioavailability of active TGF-β in tissues[10,263]. They were furthermore able to prevent the aortic dilation and some of the non-cardiovascular manifestations of MFS (impaired alveolar septation) through TGF-β antagonism[10,263].

As an example of an alternative use of mouse models, Garg *et al*. performed in 2005 *in situ* hybridization at multiple stages during cardiogenesis, with the objective of determining the correlation between *Notch1* expression and cardiac valves development[145]. At mouse embryonic day 11.5, *Notch1* mRNA transcripts were abundant in the outflow tract mesenchyme (which gives rise to the valve) and endocardium[145]. By embryonic day 13.5, when septation of the common arterial trunk occurred, *Notch1* was expressed at high levels in the endothelial layer, mesenchyme, and aortic valve leaflets[145]. These variable patterns of *Notch1* expression over embryogenesis time confirmed the role for Notch signaling in the morphological development of the aortic valve[145].

Finally, regarding BAV instead of TAAD, Sans-Coma *et al.* described a spontaneous animal model of BAV which will be undoubtedly valuable in future studies: the *Mesocricetus auratus* stripe of *Syrian* hamsters[264–267]. This mouse model has a relatively high (near 40%) incidence of BAV in the general population and concomitant structural alterations of the ascending aortic wall, but it has not been associated with other congenital cardiac malformations[264,268].

(v) **Zebrafish models:** Guo *et al.* performed in 2015 different functional experiments willing to give support of causality to the *MAT2A* genetic variation present in certain NSAD familial cases[58]. Among them, they used zebrafish to determine whether the human wild-type or

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mutant *MAT2A* were able to rescue the *mat2aa*-MO-knockout-induced embryonic defects[58]. They demonstrated that human wild-type *MAT2A* mRNA was significantly more efficient in rescuing the zebrafish abnormal cardiovascular development than the *MAT2A* mRNA altered to express either the rare p.Glu344Ala or p.Arg356Trp they identified in NSAD familial cases through WES (NM_005911.5; *section I, subsection 3.5.1*)[58].

Kuang *et al.* also used in 2016 a zebrafish model to support the causality of the *FOXE3* gene in thoracic aortic disease (*section I, subsection 3.5.1*)[111]. As the gene of interest had no known role in the aorta, they explored the effect of a *foxe3* morpholino oligomer-induced knock-down in aortic development in zebrafish embryos[111]. They found the aortic arch to be disrupted in 70% of *foxe3* morphants, without alterations in the cardiac outflow tract[111].

(vi) **CRISPR-Cas 9:** taking advantage from the latest functional modeling developments, Lee *et al.* demonstrated in 2016 the causality of the *LOX* genetic variant they identified through WGS in a family with autosomal dominant TAAD (*section I, subsection 3.5.2*)[23]. For this matter, they used the clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats-associated protein-9 nuclease (CRISPR-Cas9) genome engineering tool[23]. They introduced the human genetic variant in the homologous position of a mouse genome to study the *in vivo* effects, creating mice that were either heterozygous or homozygous for the human allele[23]. They discovered that the heterozygous mutant mice displayed abnormal aortas with a disorganized assembly of elastic lamellae in the aortic wall, whereas the homozygous died from perineonatal ascending TAA and spontaneous hemorrhage[23]. They therefore concluded that the *LOX* missense genetic variant was associated with human aortic disease, likely through insufficient cross-linking of elastin and collagen, two of the major structural components that comprise the aortic wall[23]. Mäki *et al.* and Hornstra *et al.* had previously supported the functional significance of *LOX* in the maintenance of the normal arterial wall integrity in 2002 and 2003, respectively[269,270].

# 4. Clinical and forensic implementation of the molecular diagnosis of thoracic aortic disease

## 4.1. On to the clinical setting

As a consequence of all the previously described findings regarding the genetic component of TAAD, the desired precision medicine is nowadays an emerging reality in the clinical management of this potentially life-threatening disease.

Compared to other cardiovascular malformations, in which genetics is just useful for familial screening, in heritable TAAD it is about confirming the clinical diagnosis, performing proper surveillance and the corresponding familial screening, determining the best timing for surgery, and establishing an accurate prognosis based on the risk stratification and specific underlying mutation[38,64,75,158]. An extra potential benefit that could also be implemented in the near future would be the prenatal or preimplantation genetic diagnosis[86], for the identification of fetuses or embryos at high risk of developing TAAD[209].

Given all these advantages, cardiologists and vascular surgeons should consider offering genetic testing as part of the routine care of any individual affected by TAAD, should any of the following factors be present: (i) medical history or physical examination findings (or both) consistent with a syndromic form of TAAD; (ii) family history of TAAD, SD, or syndromic features; (iii) early age of onset of TAAD (<65 years), especially in the absence of other cardiovascular risk factors, such as hypertension, smoking, or hyperlipidemia; (iv) involvement of the aortic root and ascending thoracic aorta, aortic arch, or descending thoracic aorta; or (v) medial degeneration[67].

In any case, genetic counseling is absolutely required, independently of the purpose of counseling itself (e.g. early diagnosis or future prenatal diagnosis)[61,67]. It should be always performed both before and after mutation screening analysis[61,125]. During pretest counseling, the patients should be informed of: (i) the specific test carried out and its limitations; (ii) the risk of a negative result arising from the fact that all genes implicated in the given phenotype have not been identified; (iii) how difficult is to determine the pathogenic potential of some genetic variants; (iv) the potential impact on cardiac and extracardiac management; and (v) if a genetic familial disorder is identified, his/her responsibility to inform the family[67,209]. In the specific case of pregnant women, potential risks that should be discussed include progressive aortic enlargement or dissection, and complications such as aortic stenosis and/or regurgitation, heart failure, or delivery complications[125]. After genetic testing, counseling should be performed to review the results and discuss the implications with the patient and family[209].

In summary, the clinical landscape is changing in two important ways because of advances in genetics, that should continue to improve sensitivity[33,75]. Firstly, we are getting closer to molecular definition of clinical risk stratification and prognosis[31,33,67], and to achieve pre-symptomatic diagnosis of this silent killer disease[33,161]. Secondly, as the scientific community learns more about the particular clinical characteristics of patients affected by specific mutations, we are closing in on a personalized, gene-tailored strategic management of TAAD patients, based on the particular operating characteristics, and in which new therapeutics would be targeted by molecular mechanisms[33,41,51,161]. Although aortic size currently remains as the main criteria for prophylactic

surgical intervention in TAA patients, it has already been proved that patients with specific genetic variants require an earlier intervention at smaller aortic sizes[166]. Even certain genetic variants exist that have been exclusively associated with aortic dissection without aneurysm, so that aortic size loses its predictive ability[166,198]. All these evidences have already led to a recommendation by the *American College of Cardiology Foundation* and the *American Heart Association*, reflected in the corresponding guidelines for clinical management of thoracic aortic disease patients[8,38,50,67,166].

## 4.2. On to forensic medicine

Apart from the evident main connective tissue syndromes, the asymptomatic nature of many developing TAAD[22] leads to the aortic dissection being frequently the first manifestation of the disease, causing SD without a previous diagnosis of thoracic aortic affection.

Depending on the underlying cause of death, SD can be divided into cardiac and non-cardiac, being the non-cardiac causes mainly pulmonary embolism, and metabolic, neurological, or infectious conditions[271–273]. In elderly people, cardiac SD is mainly caused by coronary artery disease, whereas in young people (1-39 years of age), a complete *post-mortem* examination fails to reveal a cause of death in up to 30% of all cases[271,274,275]. These are generally designated as autopsy-negative unexplained SD cases[271]. It is estimated that just 30% of this latter carry sequence variants in known cardiac genes causing lethal cardiac arrhythmia in a morphological normal heart[271,276,277]. Nevertheless, several non-categorized SD cases, like those disguised as heart attacks, have been expected to result from TAAD life-threatening complications[22].

In these latter, *post-mortem* genetic testing also represents an interesting tool to confirm the cause of death, elucidate potential disease-causing mechanisms, and further anticipate the development of the same condition in relatives carrying the causal mutation. Although recent findings suggest that the risk for aortic dissection is determined by the specific underlying gene defect[22], to date, the molecular diagnosis of this disease has been sparsely used in this field.

Many times, as recently happened to Neubauer *et al.* in 2016, TAAD genes have been identified by chance in cases expected to be caused by the main channelopathies or cardiomyopathies[271]. They sequenced the whole exome of five supposedly unexplained SD cases and identified a potentially causal mutation in *TGFB2* in one of them[271]. This example further supports the need to consider the main TAAD genes in the molecular autopsy routine approaches to cardiac SD cases.

# II. MOTIVATION & OBJECTIVES

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# II. MOTIVATION & OBJECTIVES

The thoracic aorta is the second most common site for aneurysms, a potentially devastating condition and major cause of SD due to the increased risk for aortic dissection or rupture[4,5,11]. Although mortality rates up to 70% have been reported for TAAD[48,51], compared to AAA, much less is known about it[25].

This clinical entity can develop as the result of a wide range of diseases[52], which can at the same time be classified in either sporadic or genetic. Up to 40% of TAAD cases are expected to be hereditary with a heterogeneous genetic background[26], and 80% of those remain unexplained at the molecular level[11]. As TAA is generally asymptomatic and undiagnosed until a catastrophic event occurs[35,43], unraveling the genetic component behind it could help anticipate and prevent fatal consequences and personalize clinical management, supporting appropriate surgical decision-making, as well as determining risk stratification and prognosis[206].

Among hereditary diseases causing TAAD, some are clearly Mendelian, but others complex. From those Mendelian, the main connective tissue syndromes are more easily diagnosed based on the clinical appearance, as symptoms are not only present in the thoracic aorta but also in many more visible organ systems, such as the musculoskeletal. In contrast, NSAD cases tend to be incidentally diagnosed. Although up to 20% of NSAD patients have a positive family history[25,37,101], the currently known NSAD genes explain a small percentage of all cases with a genetic origin, so many remain unexplained at the molecular level[11].

On the other hand, one of the complex diseases which has been frequently associated with TAAD is BAV, the most common congenital cardiovascular malformation in humans, with a prevalence of 0.5-2% in the general population[8,44,113–115]. In the complex diseases, genetic variants confer risk but cannot cause disease if alone, so the genetic approach to identify them is completely different compared to those Mendelian, and generally relies on population-based cohorts. Despite the relative ease to gather BAV patients, little is known about the genetics behind the development of this valvular malformation and associated conditions. Once again, BAV genetics could help to classify patients based on the risk for aortic dissection or rupture, the most relevant clinical condition associated with this disease.

For all these reasons, and believing in the relevance of the genetic component of TAAD, the main objective of this doctoral thesis was to contribute to its elucidation, from both Mendelian and complex perspectives. To achieve this main objective, we proposed the following secondary objectives:

1. To apply a combination of traditional and MPS approaches, based on either candidate genes or WES, to the analysis of different Mendelian TAAD cases, and prove the utility of this molecular diagnostic tool in both the clinical and forensic fields.
    a. To exhaustively gather the most phenotypic details from every available TAAD Mendelian case, willing to further characterize the genotype-phenotype correlations that we believe are key for a personalized clinical management.
    b. To define a new candidate-gene MPS strategy involving the main known TAAD genes, and prove it useful for the molecular autopsy of TAD-SD cases.
    c. To define a robust WES approach involving both affected and unaffected relatives for the analysis of the two NSAD familial cases and identification, based on a pre-established prioritization protocol, of potentially causal genetic variants.
    d. To perform segregation analysis through traditional sequencing to support candidate genetic variants causality, and identify family members carrying them and are therefore at risk of developing the disease.
2. To identify risk or protective alleles for BAV, the main complex disease associated with TAAD, from a population-based perspective.
    a. To collect as much phenotypic information as possible from all BAV cases and create a detailed dataset including epidemiology, risk factors, clinical manifestations, family history, and echocardiography data, among others.
    b. To perform an EWAS facing BAV cases versus general population controls, and analyze the genotypes distribution using both single variant and gene-based association tests.
    c. To repeat the former association analysis after the reclassification BAV cases based on the presence or absence of concomitant thoracic aortic dilation.
    d. If any significant association signal arose from any of the previous association analysis, to replicate it in an independent population-based cohort.

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e. To impute association results for a possible future participation in worldwide consortiums, willing to increase sample size and statistical power for the detection of the mildest association signals.

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# III. MATERIALS AND METHODS, RESULTS & DISCUSSION

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# III. MATERIALS AND METHODS, RESULTS & DISCUSSION

## 1. Whole exome sequencing for the identification of a new mutation in *TGFB2* involved in a familial case of non-syndromic aortic disease

*Marina Gago-Díaz, Alejandro Blanco-Verea, Gisela Teixidó-Turà, Irene Valenzuela, Miguel Del Campo, Mar Borregan, Beatriz Sobrino, Jorge Amigo, David García-Dorado, Artur Evangelista, Ángel Carracedo, María Brión*

**Clin Chim Acta. 437:88-92, July 2014, doi: 10.1016/j.cca.2014.07.016**

https://www.ncbi.nlm.nih.gov/pubmed/25046559

## 2. *PRKG1* and genetic diagnosis of early-onset thoracic aortic disease

*Marina Gago-Díaz, Alejandro Blanco-Verea, Gisela Teixidó, Francesca Huguet, Marta Gut, Steven Laurie, Ivo Gut, Ángel Carracedo, Artur Evangelista, María Brion*

**Eur J Clin Invest. 46(9):787-794, August 2016, doi: 10.1111/eci.12662**

https://www.ncbi.nlm.nih.gov/pubmed/27442293

## 3. The genetic component of bicuspid aortic valve and aortic dilation. An exome-wide association study

*Marina Gago-Díaz*, María Brion*, Pastora Gallego, Francisco Calvo, Juan Robledo-Carmona, Daniel Saura, Violeta Sánchez, Javier Bermejo, Teresa Sevilla, Christopher Newton-Cheh, Ángel Carracedo, J. Daniel Muehlschlegel, David García-Dorado, Simon C. Body, Artur Evangelista*

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**J Mol Cell Cardiol. 102:3-9, November 2016, doi: 10.1016/j.yjmcc.2016.11.012**

https://www.ncbi.nlm.nih.gov/pubmed/27894865

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# 4. *Post-mortem* genetic testing should be recommended in sudden cardiac death cases due to thoracic aortic dissection

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**To be submitted**

*ABSTRACT:* acute thoracic aortic dissection and rupture, the main life-threating complications of the corresponding aneurysm, are an important cause of sudden cardiac death. Molecular diagnosis has already been proved useful for an early diagnosis of the 40% of heritable thoracic aortic aneurysms and dissections, which is essential for proper clinical management and patient's survival. Nevertheless, the corresponding forensic field remains largely unexplored. The main goal of this study has been to explore and validate a new candidate-gene massive parallel sequencing assay involving 22 different genes as a diagnostic tool for acute thoracic aortic dissection autopsy cases. Massive parallel sequencing of 22 thoracic aortic disease candidate genes was performed in 17 cases of thoracic aortic dissection using *AmpliSeq* and *Ion Proton* platforms. All genetic variants were filtered by location, type, and frequency at the *Exome Aggregation Consortium* and an internal database, and further classified based on the *ACMG recommendations* published in 2015. The presence or absence of the selected genetic variants was confirmed through traditional sequencing. From the total of 10 potentially pathogenic genetic variants identified in seven out of the 17 initial samples, their classification according to the *ACMG recommendations*, proved two of them as pathogenic, two as likely pathogenic, one as possibly benign, and the remaining five as variants of uncertain significance. Therefore, the molecular autopsy yield represented a 23% of the total number of samples analyzed, approximately. This massive parallel sequencing candidate-gene approach, involving 22 already known thoracic aortic disease genes, has proved useful for the molecular autopsy of aortic dissection sudden cardiac death cases, and should therefore be progressively incorporated in the forensic field. Among its main advantages, it would help to anticipate the clinical diagnosis and establish an accurate risk stratification of any family member at risk of developing the disease.

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**_Post-mortem_ genetic testing should be recommended in sudden cardiac death cases due to thoracic aortic dissection**

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**ABSTRACT**

**Background:** acute thoracic aortic dissection and rupture, the main life-threating complications of the corresponding aneurysm, are an important cause of sudden cardiac death. Molecular diagnosis has already been proved useful for an early diagnosis of the 40% of heritable thoracic aortic aneurysms and dissections, which is essential for proper clinical management and patient's survival. Nevertheless, the corresponding forensic field remains largely unexplored. The main goal of this study has been to explore and validate a new candidate-gene massive parallel sequencing assay involving 22 different genes as a diagnostic tool for acute thoracic aortic dissection autopsy cases.

**Materials and Methods:** massive parallel sequencing of 22 thoracic aortic disease candidate genes was performed in 17 cases of thoracic aortic dissection using *AmpliSeq* and *Ion Proton* platforms. All genetic variants were filtered by location, type, and frequency at the *Exome Aggregation Consortium* and an internal database, and further classified based on the *ACMG recommendations* published in 2015. The presence or absence of the selected genetic variants was confirmed through traditional sequencing.

**Results:** from the total of 10 potentially pathogenic genetic variants identified in seven out of the 17 initial samples, their classification according to the *ACMG recommendations*, proved two of them as pathogenic, two as likely pathogenic, one as possibly benign, and the remaining five as variants of uncertain significance. Therefore, the molecular autopsy yield represented a 23% of the total number of samples analyzed, approximately.

**Conclusions:** this massive parallel sequencing candidate-gene approach, involving 22 already known thoracic aortic disease genes, has proved useful for the molecular autopsy of aortic dissection sudden cardiac death cases, and should therefore be progressively incorporated in the forensic field. Among its main advantages, it would help to anticipate the clinical diagnosis and establish an accurate risk stratification of any family member at risk of developing the disease.

## INTRODUCTION

Besides the main structural and arrhythmogenic cardiomyopathies, acute thoracic aortic dissection (TAD) or rupture is another important cause of sudden cardiac death (SCD), understood as the natural unexpected decease with cardiac origin of an apparently healthy individual[1,2]. With an incidence of approximately 3.5/100,000 patients per year[3], the annual risk of this specific type of SCD has been estimated to be greater than 10%[4,5].

Although TAD may sometimes develop abruptly, it is generally the result of a growing thoracic aortic aneurysm (TAA). The progressive dilation and weakening of the aortic wall could at some point lead to an acute tear in the intima[6]. This, typically permits blood to penetrate through the diseased medial layer and dissect along the plane of the aortic wall, forming a true and false lumens with or without communication[6–8]. In a worst case scenario, this lesion would be followed by adventitial disruption and lethal aortic rupture[7].

Approximately, 95% of TAAs are asymptomatic until a life-threatening complication as such occurs as the first manifestation[3,9,10], and even acute aortic dissections can be misdiagnosed and difficult to recognize[11]. To further challenge their clinical diagnosis, thoracic aortic aneurysms and dissections (TAADs) have been associated with a wide variety of underlying diseases, typically classified in sporadic (degenerative, inflammatory, autoimmune, infectious, or traumatic, without familial inheritance) or hereditary[12–14]. In contrast to abdominal aortic aneurysms, which are mainly attributed to atherosclerosis, up to 40% of TAADs have been expected to be hereditary with a genetic background as heterogeneous as their broad phenotypic spectrum[14–16]. Those heritable disorders affecting the thoracic aorta can be further classified as syndromic or non-syndromic, depending on the presence or absence of clinical manifestations in other organ systems. Among the 5% corresponding to the syndromic forms of the disease, the most relevant cases involve the main connective tissue syndromes: Marfan, Loeys-Dietz, vascular Ehlers-Danlos, and Aneurysm-Osteoarthritis[17,18]. On the other hand, in those non-syndromic, TAAD is the predominant clinical manifestation[14].

No matter the underlying disease, several common non-genetic risk factors for acute TAD exist, such as hypertension, atherosclerosis, male gender, and advanced age[11]. Furthermore, the pathophysiological hallmark of about 80% of TAADs has been described as medial degeneration (formerly “cystic medial necrosis”)[19,20]. It occurs with

normal aging of the aorta but can be accelerated by hypertension and brought on by genetic variation that predisposes individuals to the thoracic aortic disease[5,21,22]. The last consensus statement from the *Society for Cardiovascular Pathology* and the *Association for European Cardiovascular Pathology* published by Halushka *et al.* in 2016 described it as a unique, but overlapping histopathological condition, involving a collection of different histopathological changes[20]. Those key for the definition of this condition were: (i) elastic fibers fragmentation and/or loss, thinning, and disorganization; (ii) smooth muscle cell disorganization and nuclei loss (at least not clearly identifiable); (iii) laminar medial collapse or compaction of medial elastic fibers; (iv) medial mucoid extracellular matrix accumulation; and (v) medial fibrosis[8,20].

Although a more specific clinical and pathological picture is likely to be defined in the near future, allowing the recognition of each disease particularities, alternative molecular approaches have already been demonstrated useful in the clinical setting, specially for those patients with non-specific clinical symptoms or a rapid deterioration[7,11,20,23]. The specific underlying genotype could help confirm the diagnosis, perform proper surveillance, determine the best time for surgery, establish an anticipated risk stratification and prognosis, and perform cascade screening for other family members at risk, with the aim of reducing morbidity and mortality[16,24–27]. In fact, the *American College of Cardiology Foundation* and the *American Heart Association* have already recommended that the underlying genetic variation should dictate the timing of aortic repair, the current standard treatment[16,28]. But, despite the positive clinical consequences that the molecular diagnosis has among family members and the high mortality rates obtained when TAAD is left undiagnosed or untreated[11], to date, the corresponding forensic field remains largely unexplored.

Willing to demonstrate the potential benefits of incorporating the molecular diagnosis in the forensic field, the main goal of this study was to explore and validate candidate-gene massive parallel sequencing (MPS) as a diagnostic tool for acute TAD autopsy cases. In comparison with traditional sequencing of single genes, MPS has proved to be cost- and time-effective for the molecular diagnosis of genetically heterogeneous diseases, in which simultaneous testing of multiple genes is often indicated[17,29–31]. For this matter, a new MPS candidate-gene assay was designed, involving 22 genes already known to be associated with the development of thoracic aortic disease.

## MATERIALS AND METHODS

### Study samples

Blood or tissue samples (myocardium and spleen) were collected from 17 TAD cases from *Unidad de Valoración del Riesgo de Muerte Súbita Familiar* (*Hospital Universitario La Fe* and *Institutos de Medicina Legal de la Comunidad Valenciana*), 15 of which were obtained from autopsies. The *Comité Ético de Investigación Clínica de Galicia* was the institution in charge of approving the corresponding informed consent, signed by the legal representative. Clinical details of the 17 studied cases are shown in *Table 1*.

Genomic DNA from the blood samples was extracted using the *phenol-chlorophorm* method, following manufacturer's instructions. After extraction, the double strand DNA was quantified with *Qubit™ fluorometer*, and DNA purity and integrity were evaluated based on *Nanodrop* 260/230 and 260/280 ratios and agarose gels 0.8% (*Invitrogen, Thermo Fisher Scientific Inc, Waltham, MA, USA*), respectively. Those still doubtful cases were further analyzed with the *2200 Tape Station* (*Agilent Technologies, Santa Clara, CA, USA*).

### Candidate-gene massive parallel sequencing approach

An *AmpliSeq Custom* assay was designed using the *AmpliSeq Designer v4.2* tool (https://ampliseq.com/). The target region, sized 145.52 Kb, comprised the coding exons (padding 5 bp) of a total of 22 genes previously associated with TAAD, detailed in *Table 2*.

Library preparation was performed using the *Ion AmpliSeq Library kit 2.0* (*Thermo Fisher Scientific Inc, Waltham, MA, USA*) according to the manufacturing protocol (*Publication Part Number MAN0006735, Revision B.0*). Briefly, 10 nanograms of DNA were used for multiplex PCR amplification, followed by digestion of the PCR primers and ligation of a specific barcode-sequence adaptor to each sample for identification. All the reactions were performed at half volume of the original fixed in the protocol. Libraries were quantified using the *Ion Library Quantification Kit* (*Thermo Fisher Scientific Inc, Waltham, MA, USA*) and an equimolar pool of 20 samples were prepared at a final concentration of 40 pM. Template preparation was performed on the *Ion Chef* and sequencing on the *Ion Proton* using *the Ion PI Hi-QTM Chef* and *Sequencing Chemistry*, respectively.

**Data analysis: genetic variant identification and annotation, prioritization protocol, and *in silico* predictions of causality**

The *Torrent Suite Software v5.0.2* was used for the alignment of each sample to the *hg19* human reference genome with the *Torrent Mapping Alignment Program (TMAP) v5.0.7,* while genetic variant calling was performed with the *Torrent Variant Caller* (*TVC) v5.0.7*, and genetic variant annotation with *Annovar*[32].

A pre-established prioritization protocol was applied to the TAD cases, which consisted in six different steps. First of all, seeking genetic variants expected to disrupt protein function, those located in exonic or splicing flanking regions were selected, and the referred as synonymous excluded. A frequency filter towards rare genetic variants with minor allele frequency in the non-Finnish Europeans from the *Exome Aggregation Consortium* database either unknown or below 1% was then applied[33]. Next, again focusing on rare variants, those reported more than four times in an internal database of around 100 exomes with a highly variable background were excluded. Finally, the genetic variants present in two or less samples from the same MPS run and those that seemed real during the visualization of the raw sequencing results using the *Integrative Genomics Viewer* were prioritized for validation[34,35].

Available information of each of the prioritized genetic variants was revised in the literature. Their frequency in two extensive reference databases was consulted, the *Exome Variant Server* from the *NHLBI GO Exome Sequencing Project* and the *Exome Aggregation Consortium*[33,36,37]. Prediction of pathogenicity was assessed based on the conservation score and the following four pathogenic prediction tools: *Polyphen-2*[38], *Mutation Taster*[39], *SIFT*[40], and *CADD*[41]. Considering all the available information, candidate genetic variants were further classified according to the *ACMG recommendations* published in 2015[42].

**Traditional sequencing for massive parallel sequencing results confirmation**

Traditional sequencing by capillary electrophoresis was chosen as the alternative technology to confirm MPS results. Those exons and the corresponding exon-intron boundaries harboring any of the prioritized genetic variants were sequenced.

## RESULTS

### Technical performance: coverage and variant calling

The coverage of the submitted region in the assay design was 97.26%, which was achieved based on 789 amplicons distributed in two pools of 401 and 388 primer pairs. The percentage of each gene sequence covered with this assay and the register of the non-covered base pairs are represented in *Table 3*.

As the majority of the samples were forensic cases with an increased probability of DNA degradation, before starting any prioritization protocol, we ensured the corresponding coverage exceeded the minimum read depth of 30x, the recommended standard in most publications. Only one sample, *TAD_13*, did not reach the required threshold, probably as a consequence of a low-quality DNA, and was therefore excluded from the subsequent analysis. The average percentage of gene sequence with a depth of coverage greater than 30x in the remaining 15 samples is also shown in *Table 3*.

### Prioritized genetic variants

*Table 4* contains a summary of the results obtained after each step of the prioritization protocol, shown as medians calculated after the exclusion of *TAD_13*. Briefly, from a median of 32.5 candidate genetic variants, 32 were located in exons or flanking splicing regions, 14 were not synonymous, and just 2.5 had a frequency below 1% and could be therefore considered not polymorphisms, but rare genetic variants. From those, a median of one was present less than four times in the internal exome database and also in a maximum of two samples from the same MPS run. After the evaluation of this latter with the *Integrative Genomic Viewer*[34,35], *Table 5* shows each of the genetic variants that overcame the whole prioritization protocol, met *ACMG recommendations*[42], and were further confirmed by traditional sequencing.

### Molecular autopsy yield

Unexpectedly, we were able to confirm the presence of a total of 10 potentially pathogenic mutations in seven out of the 17 initial samples, representing approximately a 41% of the total samples analyzed. The classification of these genetic variants according to *ACMG recommendations*[42] (*Table 5*), proved two of them as pathogenic and two other as likely pathogenic. Of the six remaining variants, one was classified as possibly benign and the other five as variants of uncertain significance (VUS), partly due to lack of information. Therefore, the molecular autopsy yield achieved with this

approach has been of four out of 17 cases in which an established molecular diagnosis was reached, which represents a 23%, approximately.

**DISCUSSION**

In the present study, we have transferred to the forensic field a molecular diagnostic tool based on MPS of candidate genes, an approach that Proost *et al*., among others, had already shown in 2016 to be clinically useful[29]. In this case, 22 different genes were analyzed in a total of 17 TAD SCD cases, resolving about 23% of all of them. Despite the general complications associated with forensic samples, this latter yield was closed to the obtained by Prost *et al*. in 2016. In fact, only *TAD_13* had low-quality DNA, demonstrating the usefulness of the PCR-based targeted enrichment methods for MPS[31]. In addition, the only 10 nanograms of starting DNA is another important advantage, especially when compared with the higher amounts needed for the hybridization-based target enrichment of *post-mortem* samples.

Although the TAAD outcomes are often fatal[11], the diagnosis of aortic dissection remains clinically and histopathologically challenging. It is difficult to accurately distinguish it from conditions such as acute coronary artery syndrome, pericarditis, pulmonary thromboembolism, or cholecystitis/pancreatitis[11]. Even during a macroscopic physical examination, a false lumen completely occupied by thrombus could disguise the aortic wall tear[11]. All these existing confounding factors support the need for alternative diagnostic approaches to be widely implemented.

In the clinical setting, *European Society of Cardiology* current recommendations already suggest to include the molecular diagnosis of TAAD as part of routine clinical care for any affected patient and relatives[7]. Hiratzka *et al*. had already conditioned TAAD clinical management based on the genetic background in 2010[28]. No matter the underlying disease causing TAAD, it is essential to accurately recognize which patients would benefit the most from a genetic diagnosis[16]. Bowdin *et al*. proposed in 2016 different situations in which genetic testing should be recommended: (i) a medical history and/or examination consistent with a syndromic form of the disease; (ii) a family history of TAAD, SCD, or family members with syndromic features; (iii) an early-age development (under 65 years), especially in the absence of cardiovascular risk factors such as hypertension, smoking, or hyperlipidemia; (iv) the involvement of the aortic root and ascending aorta, aortic arch, or descending aorta; or (v) a histopathological examination compatible with medial degeneration[16].

The potential clinical consequences of a molecular TAAD diagnosis based on strong genotype-phenotype correlations, such as a personalized surgical timing or anticipated prognosis, are more obvious. We defend this also concerns other family members at risk, because early diagnosis of the latent stage of the heritable disease would allow preventive management[2]. They could undergo cascade screening to anticipate the development of this potentially fatal condition, no matter if the proband was originally a clinical or forensic case.

In fact, nowadays, the molecular autopsy has already been implemented for the diagnosis of other cardiovascular diseases causing SCD, such as the hyperthrophic or dilated cardiomyopathies. The candidate-gene MPS strategy is particularly useful for those mendelian cases expected to be monogenic, caused by necessary and sufficient high impact mutations. By way of example, Meder *et al*. defined a candidate-gene MPS approach for the hypertrophic and dilated cardiomyopathies in 2011, Hertz *et al*. did likewise with the main channelopathies (long and short QT and Brugada syndromes) in 2014, and finally, Pua *et al*. embraced all the main inherited cardiac conditions in the assay they published in 2016[43–45]. Based on the relatively large number of genes that had already been associated with TAAD, we developed a new candidate-gene MPS approach involving the 22 most frequently associated, shown in *Table 2*. In contrast with the previously mentioned cardiovascular diseases, that have been extensively explored, this has been one of the first candidate-gene MPS approaches to TAAD, despite also being a potentially lethal condition. Once again, candidate-gene MPS has been proved a useful approach for the molecular diagnosis and molecular autopsy of genetically heterogenic conditions, allowing simultaneous testing of an increasing number of TAAD candidate genes. We believe that many more will be discovered in the coming years, resulting in a constant update of any MPS design[16].

However, any MPS approach entails challenges regarding genetic variants interpretation. When a large numbers of genes are tested at the same time, there is a higher chance of finding VUS[16]. They therefore need to be accompanied by a corresponding prioritization protocol, adapted to the disease characteristics. The one here described is just an example. It is essential to remember that every prioritization step implies a certain bias, and we could be missing important genetic variants as well as detecting others of unknown significance. For this reason, MPS results should be re-evaluated as the scientific community progresses in the field. In the case of a deceased individual, if there were to be sufficient banked DNA, an updated design could be

offered in the same way as for living individuals[16]. The interpretation of MPS results is even more difficult when dealing with a frequently asymptomatic disease with an incomplete penetrance and variable expression, which means that not all carriers of the causal mutation develop the clinical manifestations and that a wide range of clinical manifestations exists.

Once a putative causal mutation has been identified, the next step would be to support causality through *in silico* predictions of pathogenicity and conservation, cascade screening, and functional studies. In this case, we were able to perform the first, and would offer the segregation analysis to every willing family member. This latter provides one of the strongest evidences of causation, but it is not always feasible.

Based on all the aforementioned advantages in both the clinical and forensic fields, we propose TAAD molecular autopsy to be offered at clinical centers and not only restricted to research laboratories. In the forensic field, the identification of a potentially causal genetic variant often leads to appropriate genetic counseling in relatives, that could benefit the most from this diagnostic and prognostic tool.

**CONCLUSIONS**

The MPS candidate-gene approach here proposed, involving 22 already known TAAD genes, has proved useful for the molecular autopsy of acute TAD SCD cases, with a diagnostic yield of about 23%. Considering the survival rate is directly related to a prompt diagnosis and individualized treatment, this alternative diagnostic tool should be progressively incorporated in the forensic field and appropriately correlated with clinical and histopathological findings. It would help to anticipate prognosis and establish accurate risk stratification of any family member at risk of developing the same disease.

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## TABLES

| Sample | Age | Thoracic aorta phenotype | Other manifestations and family history |
|---|---|---|---|
| ***TAD_1*** | 48 | DeBakey type I TAD | Hepatic cavernous hemangioma, hypertension, dyslipidemia, ictus, intracranial aneurysm |
| ***TAD_2*** | 33 | DeBakey type II TAD | - |
| ***TAD_3*** | 27 | DeBakey type II TAD, aortic root dilation | BAV, left ventricle hypertrophy. Father abdominal and TAA (root, ascending) and ischemic cardiomyopathy |
| ***TAD_4*** | 34 | TAD | - |
| ***TAD_5*** | 40 | Two intervened TAD (alive) | Two brothers with TAD, one surgically treated and another deceased |
| ***TAD_6*** | - | DeBakey type I TAD and re-TAD (alive) | Father SD at 70, aunt SD at <30, uncle angiectasias, cousin fatal TAD at 50, cousin-son TAD at 26, cousin-son TAA |
| ***TAD_7*** | 51 | DeBakey type I TAD | Pulmonary hypertension, steatohepatitis, colloid goiter |
| ***TAD_8*** | 44 | TAD | - |
| ***TAD_9*** | 35 | DeBakey type II TAD, aortic root dilation | Moderate coronary arteriosclerosis, myocardial fibrosis |
| ***TAD_10*** | 48 | DeBakey type II TAD | Hypertrophic cardiomyopathy |
| ***TAD_11*** | 58 | DeBakey type I TAD, annuloaortic ectasia | Left ventricle hypertrophy |
| ***TAD_12*** | 49 | DeBakey type II TAD | Left ventricle hypertrophy, severe coronary arteriosclerosis |
| ***TAD_13*** | 46 | DeBakey type I TAD, aortic root dilation | Moderate coronary arteriosclerosis |
| ***TAD_14*** | 49 | DeBakey type II TAD | Left ventricle hypertrophy |
| ***TAD_15*** | 34 | DeBakey type I TAD, aortic root dilation | BAV |
| ***TAD_16*** | 43 | DeBakey type I TAD | BAV, left ventricle hypertrophy |
| ***TAD_17*** | 46 | Standford type A TAD (root, right TSA, left iliac artery) | - |

**Table 1. Clinical details of the 17 enrolled TAD patients.** BAV = Bicuspid Aortic Valve, SD = Sudden Death, TAA = Thoracic aortic Aneurysm, TAD = Thoracic Aortic Dissection, TSA = Supra-Aortic Trunk.

| Metabolic pathway | Gene | Locus (OMIM) | Differential diagnosis | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MFS | LDS | EDS | NSAD | Osteogenesis imperfecta | Cutis laxa | JP/HHTS | ATS | SGS | BAV-TAA syndrome | Noonan/LEOPARD syndromes |
| ***Extracellular matrix*** | *FBN1* | 15q21.1 | x | | | x | | | | | x | | |
| | *COL1A1* | 17q21.33 | | | x | | x | | | | | | |
| | *COL1A2* | 7q21.3 | | | x | | x | | | | | | |
| | *COL3A1* | 2q32.2 | | | x | | | | | | | | |
| | *EFEMP2* | 11q13.1 | | | | | | x | | | | | |
| | *ELN* | 7q11.23 | | | | x | | x | | | | | |
| | *PLOD1* | 1p36.22 | | | x | | | | | | | | |
| ***TGF-β signaling*** | *TGFBR1* | 9q22.33 | | x | | x | | | | | | | |
| | *TGFBR2* | 3p24.1 | x | x | | x | | | | | | | |
| | *TGFB2* | 1q41 | | x | | x | | | | | | | |
| | *TGFB3* | 14q24.3 | | x | | x | | | | | | | |
| | *SMAD3* | 15q22.33 | | x | | x | | | | | | | |
| | *SMAD4* | 18q21.2 | | | | | | | x | | | | |
| | *SLC2A10* | 20q13.12 | | | | | | | | x | | | |
| | *SKI* | 1p36.33-p36.32 | | | | | | | | | x | | |
| ***Smooth muscle cell contractile apparatus*** | *ACTA2* | 10q23.31 | | | | x | | | | | | | |
| | *MYH11* | 16p13.11 | | | | x | | | | | | | |
| | *FLNA* | Xq28 | | | x | | | | | | | | |
| | *MYLK* | 3q21.1 | | | | x | | | | | | | |
| | *PRKG1* | 10q11.2-q21.1 | | | | x | | | | | | | |
| ***Miscellanea*** | *NOTCH1* | 9q34.3 | | | | | | | | | | x | |
| | *PTPN11* | 12q24.13 | | | | | | | | | | | x |

**Table 2. Description of the 22 candidate genes considered for MPS and the main diseases affecting the thoracic aorta they have been associated with.** *COL1A1* and *COL1A2* cause the classical form of EDS, while *COL3A1* has been associated with the vascular and *PLOD1* with the kyphoscoliotic forms. Alike, *TGFBR1* participates in the development of LDS type 1, *TGFBR2* in type 2, *TGFB2* in type 4, *TGFB3* in type 5, and *SMAD3* in type 3, also known as Aneurysm-Osteoarthritis syndrome. *TGFBR2* association with MFS type 2 has been controversial. Most MYH11 mutations have been associated with NSAD and concomitant patent ductus arteriosus. ATS = Arterial Tortuosity Syndrome; BAV = Bicuspid Aortic Valve; JP/HHT = Juvenile Polyposis/Hereditary Hemorrhagic Telangiectasia Syndrome; LDS = Loeys-Dietz Syndrome; MFS = Marfan Syndrome; NSAD = Non-syndromic Aortic Disease; SGS = Shprintzen-Goldberg Syndrome; vEDS = vascular Ehlers-Danlos Syndrome.

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| **Gen** | **Target (bp)** | **Assay design** | | **Mean % of the gene covered > 30x** |
|---|---|---|---|---|
| | | **Missed (bp)** | **Covered (%)** | |
| *ACTA2* | 1,214 | 0 | 100 | 83.90 |
| *COL1A1* | 4,905 | 291 | 94.07 | 95.31 |
| *COL1A2* | 4,621 | 0 | 100 | 99.64 |
| *COL3A1* | 4,911 | 13 | 99.74 | 96.85 |
| *EFEMP2* | 1,432 | 51 | 96.44 | 98.83 |
| *ELN* | 2,734 | 0 | 100 | 97.34 |
| *FBN1* | 9,266 | 0 | 100 | 100 |
| *FLNA* | 8,414 | 498 | 94.08 | 95.66 |
| *MYH11* | 6,391 | 119 | 98.14 | 97.67 |
| *MYLK* | 6,055 | 99 | 98.36 | 95.49 |
| *NOTCH1* | 8,088 | 658 | 91.78 | 89.62 |
| *PLOD1* | 2,374 | 0 | 100 | 94.80 |
| *PRKG1* | 2,517 | 0 | 100 | 90.04 |
| *PTPN11* | 1,936 | 24 | 98.76 | 98.44 |
| *SKI* | 2,257 | 77 | 96.59 | 92.12 |
| *SLC2A10* | 1,676 | 38 | 97.73 | 98.75 |
| *SMAD3* | 1,452 | 43 | 97.04 | 86.78 |
| *SMAD4* | 1,769 | 0 | 100 | 99.90 |
| *TGFB2* | 1,409 | 100 | 92.90 | 93.28 |
| *TGFB3* | 1,309 | 26 | 98.01 | 87.41 |
| *TGFBR1* | 1,602 | 107 | 93.32 | 92.73 |
| *TGFBR2* | 1,859 | 0 | 100 | 99.89 |

**Table 3. Assay design expected versus observed coverage per gene.** bp = base pairs.

| **Prioritization step** | **Median of the remaining genetic variants** |
|---|---|
| ***Total number*** | 32.5 |
| ***Exonic and located in splicing flanking regions*** | 32 |
| ***Nonsynonymous, frameshift or stop codon*** | 14 |
| ***Minor allele frequency ≤ 1%*** | 2.5 |
| ***Presence in internal database ≤ 4 times*** | 1 |
| ***Presence in ≤ 2 samples from the same MPS run*** | 1 |

**Table 4. Description of the prioritization protocol applied to the candidate gene massive parallel sequencing results.** MPS = Massive Parallel Sequencing.

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| Sample | Gen | Ref seq | Nucelotide change | AA change | Zigosity | *ACMG* classification | References |
|---|---|---|---|---|---|---|---|
| *TAD_1* | *ELN* | NM_000501 | c.767C>T | p.(Ala256Val) | het | VUS | |
| | *SMAD3* | NM_005902.3 | c.1140G>A | p.(Trp380*) | het | Pahogenic | |
| *TAD_2* | *PLOD1* | NM_000302.3 | c.1495C>T | p.(Arg499Trp) | het | VUS | |
| | *FBN1* | NM_000138.4 | c.2243G>A | p.(Cys748Tyr) | het | Pahogenic | 46 |
| *TAD_4* | *FBN1* | NM_000138.4 | c.165-4T>A | | het | VUS | |
| *TAD_6* | *FBN1* | NM_000138.4 | c.7412C>G | p.(Pro2471Arg) | het | Likely pathogenic | 47 |
| *TAD_10* | *COL1A2* | NM_000089.3 | c.304C>T | p.(Pro102Ser) | het | Likely benign | |
| *TAD_16* | *MYLK* | NM_053025.3 | c.454C>T | p.(Arg152Cys) | het | VUS | |
| *TAD_17* | *MYH11* | NM_002474.2 | c.3766_3768del | p.(Lys1256del) | het | Likely pathogenic | 48,49 |
| | | | c.3611C>T | p.(Ala1204Val) | het | VUS | |

**Table 5. Summary of the candidate-gene massive parallel sequencing results.** ACMG = *American College of Medical Genetics*; het = heterozygosis, VUS = Variant of Unknown Significance.

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| **Sample** | ***TAD_1*** | | ***TAD_2*** | | ***TAD_4*** | ***TAD_6*** | ***TAD_10*** | ***TAD_16*** | ***TAD_17*** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gen** | *ELN* | *SMAD3* | *PLOD1* | *FBN1* | *FBN1* | *FBN1* | *COL1A2* | *MYLK* | *MYH11* | *MYH11* |
| ***RefSeq*** | NM_000501 | NM_005902.3 | NM_000302.3 | NM_000138.4 | NM_000138.4 | NM_000138.4 | NM_000089.3 | NM_053025.3 | NM_002474.2 | NM_002474.2 |
| **Nucleotide change** | c.767C>T | c.1140G>A | c.1495C>T | c.2243G>A | c.165-4T>A | c.7412C>G | c.304C>T | c.454C>T | c.3766_3768del | c.3611C>T |
| **Aminoacid change** | p.(Ala256Val) | p.(Trp380*) | p.(Arg499Trp) | p.(Cys748Tyr) | - | p.(Pro2471Arg) | p.(Pro102Ser) | p.(Arg152Cys) | p.(Lys1256del) | p.(Ala1204Val) |
| **Zigosity** | Het | Het | Het | Het | Het | Het | Het | Het | Het | Het |
| **Type of variant** | Missense | Stopgain | Missense | Missense | Splicing | Missense | Missense | Missense | Inframe deletion | Missense |
| ***dbSNP*** | - | - | rs149124387 | - | rs761159501 | rs193922233 | rs189557655 | rs559933360 | rs730880147 | rs772621139 |
| ***ESP European American*** | - | - | 1/8600 | - | - | - | 3/8600 | - | - | - |
| ***ExAC European non-Finnish*** | 1/66694 | - | 21/65764 | - | 1/65818 | 1/66724 | 90/66708 | 3/65334 | 5/66730 | - |
| ***Human Splicing Finder*** | - | - | - | - | 0.1% | - | - | - | - | - |
| ***Polyphen-2*** | Benign | - | Probably damaging | Probably damaging | - | Probably damaging | Benign | Probably damaging | - | Benign |
| ***Mutation Taster*** | Polymorphism | - | Disease causing | Disease causing | - | Disease causing | Disease causing | Disease causing | - | Disease causing |
| ***SIFT*** | Deleterious | - | Deleterious | Deleterious | - | Tolerated | Tolerated | Deleterious | - | Tolerated |
| ***CADD*** | 16.22 | - | 24.5 | 32 | - | 27 | 23.1 | 25.9 | - | 7.282 |
| ***PhyloP*** | Weakly conserved | - | Weakly conserved | Highly conserved | - | Highly conserved | Moderately conserved | Weakly conserved | - | Weakly conserved |
| **Aminoacid conservation** | Moderately conserved | - | Moderately conserved | Highly conserved | - | Highly conserved | Highly conserved | Weakly conserved | - | Moderately conserved |
| ***ClinVar*** | - | - | - | - | - | RCV000181602.2; RCV000029777.1 | - | - | RCV000157334.3; RCV000254668.1 | RCV000182469.1 |
| ***ClinVar* Pathogenicity** | - | - | - | - | - | Uncertain significance | - | - | Uncertain significance | Likely benign |
| ***HGMD*** | - | - | - | CM021593 | - | CM157985 | - | - | CD132994 | - |
| ***HGMD* disease** | - | - | - | MFS | - | AAA | - | - | TAAD | - |
| ***HGMD* variant class** | - | - | - | DM | - | DM? | - | - | DM | - |
| ***ACMG* classification** | VUS | Pathogenic | VUS | Pathogenic | VUS | Likely pathogenic | Likely benign | VUS | Likely pathogenic | VUS |
| **References** | - | - | - | [46] | - | [47] | - | - | [48,49] | - |

**Table S1. Extended summary of the candidate-gene massive parallel sequencing results.** ESP = *Exome Sequencing Project*; HGMD = *Human Gene Mutation Database*; ACMG = *American College of Medical Genetics;* MFS = Marfan syndrome; AAA = Abdominal Aortic Aneurysm; TAAD = Thoracic Aortic Aneurysm and Dissection; DM = Denotes a Mutation reported to be disease-causing; DM? = Denotes a Mutation reported as likely disease-causing, but with questionable pathogenicity; VUS = Variant of Uncertain Significance.

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# IV. GENERAL DISCUSSION

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# IV. GENERAL DISCUSSION

## 1. Thoracic aortic aneurysms and dissections generalities

TAAD, estimated to be the 15$^{th}$ leading cause of death in the United States[10], is a frequently asymptomatic clinical entity that many times remains silent until a devastating event occurs. It can appear in association with multiple diseases with a widely different origin, some of them dependent on genetic factors. At least in this latter, it would be worthy to develop new diagnostic tools for routine checkups able to anticipate the disease development, and therefore reduce associated morbidity and mortality rates. But the knowledge about the genetic component of TAAD is still limited and, despite the many discoveries made in the recent years, many other clues are still pending. The scientific community needs to solve them all to make the application of TAAD genetic diagnosis in daily clinical practice real.

One of the most basic issues that should be clarified in the near future is, in our opinion, the nomenclature guidelines. Probably as a consequence of the rapid progression of TAAD research over the past decade, some incongruences regarding TAAD classification arose, hindering in many cases the appropriate interpretation of research results. Along this doctoral thesis, we have classified TAAD as sporadic or hereditary based on the underlying pathology, and understood the term "sporadic" as isolated and non-genetic. We therefore expected sporadic TAAD to be degenerative, inflammatory, autoimmune, infectious, or traumatic, and not to show any familial aggregation or transmission. On the other hand, we defined those hereditary TAAD as genetic, affecting multiple family members, and further classified them on syndromic or non-syndromic, depending on the presence or absence of manifestations in other organ systems. This same classification system was adopted, for instance, by El-Hamamsy *et al.* and Forte *et al.* in 2009 and 2016, respectively, who classified TAAs in syndromic, familial non-syndromic, or sporadic[28].

But LeMaire *et al.* and Balisteri findings in 2011 and 2015, respectively, suggested that some genetic involvement might also exist behind sporadic TAAD cases (*section I, subsection 3.4*)[3,24,278]. Probably based on the fact that sporadic cases might also depend on genetic risk factors (understood as a complex and not Mendelian contribution), Ziganshin *et al.* and Andelfinger *et al.* established a different classification system in 2015 and 2016, respectively, including the sporadic form of the disease in the non-syndromic category[38,166].

We are aware this evolution in nomenclature responds to new discoveries in the field, but at the same time support the necessity to reach an agreement regarding a reference nomenclature that should be used by every professional working on this topic, no matter the specialty, facilitating multidisciplinary communication amongst teams. We propose to use the term sporadic to refer to those non-familial complex cases that could depend or not on genetic factors, and define a different term for the non-genetic TAAD cases.

Aside from the nomenclature, another issue as basic as the disease pathogenesis is still pending, thus further slowing down the identification of new candidate genes. It is not even clear why defects in structural or regulatory matrix elements, signaling molecules, or contractile proteins culminate in aneurysms affecting the thoracic and sometimes abdominal aorta, rather than a diffusely fragile and dilated arterial tree[5]. If hemodynamic stress were the answer, it remains unclear why lesions occur on both the high- and low-pressure (the root of the pulmonary artery) sides of the circulation[5]. Another source of confusion affecting the disease pathogenesis has been the widespread use of the common term "medial degeneration", which has indicated disparate histopathologies in different studies[52]. As a result, it has not often been possible to distinguish subtle histopathologic variation among diseases[52]. Luckily, Halushka *et al.* have already begun to clarify this latter issue in 2016, looking for a more detailed histopathological description dependent on the underlying disease[52].

Finally, another important pending task is the improvement of the genotype-phenotype correlations. It is not just about the thoracic aortic disease itself, but the characterization of all multisystemic features associated with genetic variants in each specific gene. To date, there is still confusion as the genetic component of TAAD has not been completely unraveled. Even similar genetic defects have been shown to lead to drastically different phenotypes, suggesting the participation of regulatory elements (*Table 6*)[38]. As stated before, besides the anticipation of the clinical consequences and diagnosis of other family members at risk, the identification of the causal mutation behind a heritable TAAD case could be used as a prognostic factor[26]. It could help to assess the *a priori* probability of finding dissection in a given patient, and be therefore very useful in reaching a personalized clinical management.

## 2. Mendelian thoracic aortic diseases. Candidate genes versus whole exome sequencing

Despite being rare, many different Mendelian diseases exist that can develop with TAAD, and so the analysis of the genetics behind them has had a key role in this field. In those Mendelian cases, thoracic aortic disease can appear isolated or concomitantly with other clinical manifestations in multiple organ systems, being categorized as non-syndromic and syndromic, respectively. While the

syndromic cases can be, in general, accurately defined based on the clinical features, those non-syndromic correspond to a by-exclusion category that, in our opinion, will be further delineated in the upcoming years, maybe leading to the definition of new syndromes[88].

As immersed in the MPS era, and given the relative cost-effectiveness of MPS approaches compared to single-gene sequencing[125], we chose them as the preferred technology, although traditional sequencing is still the reference approach for MPS results confirmation and cascade screening[158]. The MPS technology can be used for either sequencing multiple candidate genes or the whole exome or genome. Despite the common technology, the approaches are different and the selection of either of them will depend on the specific needs. In this doctoral thesis, we have demonstrated the utility of both of them, and have supported in all cases the importance of evaluating their appropriateness in advance.

On the one hand, we performed the molecular autopsy of 17 TAD cases through a candidate-gene MPS strategy that involved 22 already known TAAD genes (*section III, subsection 4)*. Despite the inherent limitation of any candidate-gene approach, they have been many times demonstrated useful, at least for: (i) performing the whole differential diagnosis of TAAD cases with a well-defined phenotype; and (ii) identifying the more ambiguous cases that could be perfect candidates for a wider approach looking for new causal genes. In *section III, subsection 4*, we were able to prove the time and cost-efficiency of this approach, which allowed the identification of a candidate genetic variant in approximately 23% of cases. When applied to SD forensic cases, the molecular diagnosis is especially beneficial for other family members at risk, in which the development of such a devastating complication could be anticipated based on their mutational status[279]. Nevertheless, as the large majority (80-90%) of TAAD patients have a poorly understood genetic predisposition[26,41], a candidate-gene MPS approach is not always the best choice.

Another very common approach towards the genetic component of Mendelian diseases is the analysis of large families with multiple affected relatives. We chose WES to analyze two different NSAD familial cases in which the main non-syndromic genes had already been excluded (*section III, subsections 1* and *2*). WES is a targeted approach too, but much wider, providing an unbiased assessment of the genetic variants present in all the protein-coding regions of the genome (known as exome). Since approximately 85% of causative mutations for Mendelian diseases have been found in the coding regions or canonical splice sites, WES can be considered an efficient approach to identify them[251].

Despite the evident usefulness of this last approach, which has already been widely applied for the analysis of TAAD families, as exposed in *section I, subsection 3.5.1*, it also has some limitations that should always be taken into account:

(i) **The low percentage of the whole genome that is actually sequenced:** the exome involves approximately 1.5% of the whole genome, and causative mutations could be located a distance from coding regions[112,250,251].

(ii) **The type of genetic variants detected:** as it does not cover the whole CNVs spectrum or epigenetic modifications affecting gene expression[251]. In fact, CNVs have been widely considered relevant for inherited thoracic aortic disease. Kuang *et al.* determined in 2011 whether recurrent CNVs contribute to TAAD pathogenesis through the screening of 765 individuals with adult-onset sporadic TAAD (*section I, subsection 3.4*)[189]. They identified recurrent 16p13.1 duplications in 1% of sporadic TAAD cases compared with 0.09% of controls[189]. Hitz *et al.* found in 2012 CNVs to contribute to at least 10% of their left-sided congenital heart disease cases (including BAV and coarctation of the aorta)[280]. Finally, Arslan-Kirchner *et al.* recommended in 2016 that in patients with a highly suggestive phenotype in whom initial MPS or traditional sequencing proved negative, a supplementary deletion/duplication diagnostic test should be performed for genes with a known proportion of large genomic deletions/duplications[158].

(iii) **The coverage:** approximately 2-3% of exomes are not efficiently captured by the regular exome capture arrays for WES, especially concerning regions with high GC content[251].

As the cost of sequencing declines, it is expected that deep WGS will soon become the technology of choice, with no limitations regarding the location or type of causal mutations, or genetic architectures[226].

Despite the targeted nature of either the candidate-gene or WES MPS approaches, the number of potentially pathogenic genetic variants identified is frequently unmanageable. For this reason, it is necessary to pre-determine a prioritization protocol towards rare genetic variants expected to disrupt protein function, and so to be causal. Although Milewicz *et al.* reported in 2014 that WES identifies approximately 15,000-20,000 single nucleotide and short indel genetic variants in each individual[251], the total number we obtained after WES of multiple affected and unaffected family members was significantly greater. As expected, the main complication derived from the selection of the appropriate straightforward prioritization strategy, which had to be able to identify the putative disease-causing genetic variants from this large starting number[251]. False positive rates would vary depending on the stringency of the prioritization criteria, as well as the WES data quality[251].

In our case, the prioritization protocol was partially common to both the analysis of the individual forensic cases and the NSAD families, although in the first case we analyzed single and in the second multiple individuals, affected and unaffected, belonging to the same family. Therefore,

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regarding the two NSAD familial cases, we first performed a strongly reductive prioritization step that consisted in the comparison between either different affected or both affected and unaffected individuals, looking for the common or uncommon genetic variants, respectively. The more distantly related these individuals were, the more efficient this prioritization step would be[251]. Thereafter, the common prioritization steps we applied were the following: (i) selection of genetic variants located in exonic or splicing flanking regions; (ii) exclusion of those synonymous or unknown; (iii) exclusion of those categorized as polymorphisms with a frequency greater than 1% in reference databases such as the *NHLBI Exome Sequencing Project*[254]; (iv) selection of the genetic variants with a frequency of the mutated allele greater than 20% (to make sure they were real and not artifacts); and (v) selection of those novel or rare, based on their frequency on an internal database with more than 100 exomes obtained from patients with a highly variable background. More details about each specific prioritization protocol can be found in *section III*.

In any case, when analyzing the outcomes of a prioritization protocol as such, it is essential to remember that every prioritization step implies a certain error, and that we therefore could be missing important genetic variants. For this reason, especially when unresolved, we recommend to re-evaluate MPS results as the scientific community progresses in the field. The interpretation of MPS results is even more difficult when dealing with a frequently asymptomatic disease with an incomplete penetrance, which means that not all carriers of the causal mutation develop the clinical manifestations. This was the case of the first NSAD family, in which this handicap was compensated by the analysis of an increased number of affected and unaffected relatives.

Despite the previously exposed challenges, we were able to identify the strong candidate genetic variant c.C1042T:p.R348C in the *TGFB2* gene (NM_001135599.2), one of the main ligands of the TGF-β pathway. This candidate genetic variant was present in three affected and absent in six unaffected family members, confirming its segregation with the disease. But, at the same time, it was found in two supposedly unaffected relatives, indicating either a possible later development in young individuals or an incomplete penetrance in those seniors. Furthermore, it was not present in an affected individual in which the TAA was an expected consequence of a long duration hypertensive cardiomyopathy (*section III, subsection I*). In our opinion, the incomplete penetrance present in many Mendelian diseases supports the existence of currently unknown modifying factors that also need to be unraveled to establish accurate genotype-phenotype correlations. With that same objective, the extra-cardiovascular features present in this supposedly non-syndromic familial case should be further re-evaluated. The fact that they were partially common to those described by Lindsay *et al.* and Boileau *et al.* in 2012[108,109] suggested the existence of a new *TGFB2*-syndrome that would need a detailed description of a greater number of affected cases to be accurately defined.

The MPS results we obtained from the second NSAD familial case were easier to interpret, as they involved six instead of two whole exomes. We sequenced the whole exome of four affected and two unaffected individuals, and extended the segregation analysis to a further eight relatives, both affected and unaffected. Through the application of the previously described prioritization protocol, we identified the c.530G>A:p.Arg177Gln candidate genetic variant in *PRKG1* (NM_001098512), an aortic SMC function gene. Based on the results published by Guo *et al.* in 2013[110], we were expecting the penetrance of this genetic variant to be complete. It was present in seven affected and absent in five unaffected family members. In contrast, we detected it in a 33-year-old woman that was not affected at the time of diagnosis, and did not find it in a 50-year-old man recently diagnosed as slightly affected. The variable age of onset of the disorder also makes it difficult to exclude an individual as unaffected even with normal imaging studies. While the frequency of the clinical checkups of the woman increased considerably as she was young enough to develop the disease, the male was considered affected by non-genetic factors, and so at low risk of developing an aggressive thoracic aortic disease (*section III, subsection 2*). In summary, WES could be considered a useful approach for the identification of potentially causal genetic variants in large familial cases with multiple affected and unaffected relatives, enabling the definition of new causal genes.

Whether candidate-gene or WES approaches are chosen, when identifying new causal genes and mutations, it is essential to give strong genetic support for causation through cascade screening, as well as *in silico* and functional studies[226]. We were able to perform the *in silico* predictions of causality and conservation for all the candidate genetic variants identified in the individual forensic cases, while in the two NSAD familial cases we also conducted the corresponding segregation analysis. Although the first have been widely used, it is important to keep in mind that they only give estimated predictions based on *in silico* assumptions, and sometimes even discordant results. Therefore, they can support a hypothesis, though the final decision should not rely exclusively on them.

Besides the segregation study, it is also important to describe multiple familial cases linked to the same causal gene (*section III, subsections 1 and 2*), which is not always easy when dealing with rare diseases. Once the new causal gene has been postulated as a strong candidate, the mechanism underlying causality should be always proven by functional studies. Many, but unfortunately not all genetic variants that have been causally associated with rare and common genetic disorders represent robust and correct conclusions[226]. False assignments of pathogenicity can have severe consequences for patients, resulting in incorrect prognostic, therapeutic, or reproductive advice, and even for the research enterprise, resulting in misallocation of resources for basic and therapeutic research[226].

Finally, although well defined, the penetrance and clinical picture of TAAD syndromes are highly heterogenic. The severity of the disease can vary from one family to another, but also between different members of a given family. It even happens in MFS cases, the most studied TAAD syndrome, despite carrying the same mutation[59]. Besides discovering new aortopathy genes, etiologic genetic variants located in non-coding regions (deep intronic or regulatory) should also be considered, as they could also be playing a role in the disease[75]. A great deal of novel research is now aimed at understanding the modifying factors (genetic as well as environmental) that explain the huge MFS variability[59]. The genetic component of any TAAD disease will not be completely unraveled until potential regulatory genetic variants are linked to the baseline mutations, leading to the description of clinically relevant prognostic factors.

## 2.1. *TGFB2* and TGF-β signaling pathway: paradoxical up-regulation

The continuous implication of TGF-β signaling genes in the pathogenesis of TAAD, no matter the underlying disease (either syndromic or non-syndromic[7,28,59]), suggested the dysregulation of this pathway as essential in aortic aneurysm development[22,52]. TGF-β molecules are regulatory cytokines highly expressed in the cardiovascular system (e.g. the arterial wall of vascular SMCs), and kept in latent state in the ECM[14,22,38]. They participate in both the embryonic development and adult tissue homeostasis[14,22,38].

The common mechanism behind those diverse mutations in different components of the TGF-β pathway remains largely unknown, and has engendered some controversy in the field[22]. Briefly, although most TGF-β genetic variants have been predicted loss-of-function, aortic tissue from affected individuals has repeatedly shown increased amounts of nuclear p-Smad2 in aortic SMCs[22,109,191,281]. This paradoxical up-regulation of TGF-β signaling has been shown to lead to an increased MMP activity and ECM breakdown[14].

The disease in which TGF-β pathomechanism has most widely been evaluated is MFS, caused by *FBN1* mutations. Fibrillin-1 molecules assemble into microfibrils, which have an important structural function, both in the aortic wall and the ciliary apparatus supporting the ocular lens[22]. This knowledge has for a long time supported the "weak tissue model", which hypothesized that *FBN1* mutations caused a purely structural deficiency leading to aneurysm and *ectopia lentis*[22]. However, it has never provided a sufficient explanation for the musculoskeletal symptoms, challenging the idea of a pure structural role for fibrillin-1[22]. It did not take long for the alternative TGF-β hypothesis to appear, capable of justifying the aortic and ocular, as well as the musculoskeletal symptoms, such as bone overgrowth and mitral valve anomalies[28]. Fibrillin-1 deficiency alters matrix sequestration of the TGF-β latent complex, leading to the uncontrolled release of TGF-β and as a consequence, the

activation of the TGF-β pathway[22]. The use of anti-TGF-β antibodies gave further support to this hypothesis. Neptune *et al.* evaluated in 2003 the developmental impairment of the pulmonary alveolar septation in a *Fbn1*-deficient mouse model, and were able to fully rescue the lung phenotype by perinatal administration of a polyclonal TGF-β neutralizing antibody, which prevented the TGF-β signaling increase[22,38,282]. Habashi *et al.* demonstrated in 2006 that anti-TGF-β antibodies were also effective in preventing aortic dilation in the mouse model of MFS (*section I, subsection 3.7*)[263].

Independently of the underlying disease, several possible explanations for this pathomechanism have been proposed, although all of them will need to be validated with further experimental evidence[22]:

(i) **Impaired auto-regulation of TGF-β signaling[5,38,51]:** has been considered the most likely mechanism[22], leading to an altered balance between the canonical and non-canonical TGF-β signaling[51]. The non-canonical pathway aims to compensate for the loss of the canonical, resulting in overcompensation. The activation of the non-canonical, Smad-independent pathway, has been furthermore proved to induce ECM degradation through increased MMP activity[55].

(ii) **Altered TGF-β receptors trafficking on the cell surface[5,51]:** limiting their presence on the cell surface and their interaction with the corresponding TGF-β ligands.

(iii) **Shift in the use of different TGF-β ligands (TGF-β1/2/3)[51]:** preliminary evidence has suggested that *TGFB2*-deficient patients and mice have an increased expression of *TGFB1*, causing a shift from *TGFB2*- to *TGFB1*-driven signaling[22,108].

The results we obtained during the analysis of the first NSAD familial case by WES (*section III, subsection 1*) were the perfect example of this general controversy, as the *TGFB2* candidate genetic variant was also expected to be loss-of-function. Nevertheless, we did not have the opportunity to perform any functional study to evaluate p-Smad2 levels and further confirm that hypothesis.

Another pending issue is the ambiguous clinical phenotype of altered-*TGFB2* carriers. Although they were initially considered non-syndromic, many of them had demonstrated clinical features of both MFS and LDS (TAAD, mitral valve disease, club feet, bifid uvula, and skeletal features)[22]. A detailed clinical description of a greater number of families is needed to further determine if *TGFB2* genetic variants cause a unique phenotype or, in contrast, a subtype of any of the already known syndromes they overlap with. LDS was recently defined in 2005, and a lot of research has yet to be carried out. These pending tasks will be facilitated by the already developed mouse model haploinsufficient for *Tgfb2*, which was shown by Lindsay *et al.* in 2012 to perfectly recapitulate the human phenotype with TAA involving both the aortic annulus and root[22,108,283]. This mouse model

has also revealed a signature of increased canonical (p-Smad2) and non-canonical signaling pathways by Western blot and immunohistochemical analysis of aortic wall tissues[22,108,283].

Once TGF-β signaling had been established as central in TAAD pathogenesis, it was automatically suggested as a therapeutic target. But, it is important to consider that this pathway could play divergent roles in AAAs and TAAs[55,284]. Active TGF-β in thoracic aortas has been associated with increased signaling and aneurysms, whereas in AAAs it has been shown to stabilize the already formed aneurysm, to decrease MMPs levels and inflammation, and to increase tissue inhibitors of MMPs[55,284]. Furthermore, in spite of the promising evidence surrounding TGF-β signaling, attention should not be withdrawn from other potentially causal alterations. As Jondeau *et al.* suggested in 2014, further research in this field is needed to definitely determine if the TGF-β upraise within the SMCs may actually be responsible for TAAD or a compensatory mechanism, being just a marker of thoracic aortic disease[63]. Despite the known interactions between the three pathways linked to TAAD development, to date, it has not been possible to definitely link all of them to TGF-β disruption. For instance, although collagen production has been proved TGF-β dependent, there is no direct evidence that *COL3A1* mutations affect TGF-β pathway regulation in a direct way[22].

Finally, besides sequence variation, it should also be considered that, on the basis of its pleiotropic nature, systemic interference with the epigenetic regulation of the TGF-β pathway could also imply severe adverse events. On this topic, Gomez *et al.* demonstrated in 2011[257] that increased p-Smad2 was associated with an epigenetic alteration of the Smad2 pathway in SMC derived from TAA of all origin (genetic or not), which could be interpreted as an adaptive phenomenon (*section I, subsection 3.6*)[59].

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## 2.2. *PRKG1* and smooth muscle cell contractile apparatus. Implications

The pathological mechanism behind the alteration of this second pathway is easier to understand, as any dilation can be understood as a deficit of contraction. For this reason, when the first SMC-contractile apparatus genetic variant was identified, mutations in other components of the same metabolic pathway were automatically expected. It has been proposed that the alteration of the transmission of the tensile forces resulting from these specific mutations may be responsible for SMCs modification, leading to its disappearance through apoptosis after the release of MMPs, and worsening the alteration of the ECM structure[59].

Actin and myosin are the two main contractile proteins within SMCs, and polymerize to form the thin and thick filaments, respectively[59,110]. Any genetic perturbation that decreases actin-myosin interaction tends to cause TAAD, either through the altered function of any of the two major

proteins in the contractile complex (*ACTA2* and *MYH11* genes), or the kinases controlling phosphorylation of the regulatory light chain within vascular SMCs (*MYLK* and *PRKG*1 genes)[41,251]. Nevertheless, although the scientific community has already shown these four genes as strong candidates, mutations in additional genes encoding SMC contractile proteins, such as *CALM1*, *CALD1*, *CNN1*, and *RLC*, have not yet been linked to NSAD[251].

In the second NSAD familial case resolved by WES (*section III, subsection 2*), we identified a recurrent genetic variant in *PRKG1*, a gene strongly expressed in all smooth muscle types[285]. That exact same *PRKG1* genetic variant (rs397515330) had been previously described by Guo *et al.* in 2013 to constitutively active the protein kinase cyclic guanosine monophosphate-dependent type 1 (*section I, subsection 3.5.1*)[41,110]. The increased activity of this protein resulted in decreased phosphorylation of myosin light chain, inhibition of the actin-myosin interaction, decreased contraction of vascular SMCs, and thoracic aortic dilation[41,110].

Intriguingly, TGF-β up-regulation has also been observed in the aortic walls of TAA patients with *ACTA2* and *MYH11* mutations[7,22,281]. This might not be completely surprising because the vascular SMC contractile apparatus is linked to the cell surface integrins through the intermediate filaments, which at the same time are known major regulators of TGF-β activity[22]. This potential link between TGF-β up-regulation and vascular SMC contractility alterations further supports the central role of TGF-β signaling in TAAD pathogenesis[22].

## 3. Bicuspid aortic valve as a complex embryologic defect

Besides the typical Mendelian TAAD cases, in this doctoral thesis we have also explored the genetic component behind BAV, the most common cardiovascular malformation in humans which has been repeatedly associated with an increased risk of aortic dilation in patients younger than those in which TAAD had an idiopathic origin[125,286–288]. Probably as a consequence of the frequently asymptomatic nature of BAV, no matter how devastating the consequences of the concomitant aortic dissection could be, the pathogenesis of this disease remains largely unclear[113].

Historically, the development of TAAD in patients with BAV was considered a purely hemodynamic consequence of the altered pattern of the blood flow through an abnormal valve, causing a mechanical stress against the aortic wall and contributing to the "post-stenotic dilation"[51]. Although very logical, over the years, some data suggested otherwise, and multiple defenders of the common embryological and genetic origin revoked this hypothesis; the following are just some examples: (i) Pachulski *et al.* found in 1991 that the aortic root diameter was significantly larger among patients with BAV compared to controls, even in the absence of hemodynamically significant aortic stenosis[115,289]; (ii) Hungtington *et al.*, while assessing the frequency of familial



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clustering of congenital BAV in 30 different families in 1997, identified four individuals from 11 affected families with ascending thoracic aortic dilation but without BAV[116]; (iii) Nistri *et al*. found in 1999 52% of young patients with normally functioning BAV suffering from aortic dilation[127]; (iv) Gleason supported in 2005 that BAV-TAAD patients were not protected from subsequent aneurysm formation by aortic valve replacement[30]; (v) Michelena *et al.* found in 2008 that amongst 212 young patients with normally functioning or minimally dysfunctional BAV, 30% developed TAA over a mean follow-up period of 10 years[125,290]; (vi) also in 2008, Della Corte *et al.* demonstrated that at a histological level, the rate of matrix disruption and SMC apoptosis at the aortic wall was increased in BAV patients[291]; and (vii) Biner *et al.* reasserted in 2009 that BAV and TAAD could occur independently in different individuals from BAV families[28,125,292].

All these examples of the co-existence of TAA and normally functioning BAV supported the theory that developmental mechanisms were more significant than hemodynamic disturbances, which happened to contribute but might be not necessary to cause aortic aneurysm in BAV patients[51,125]. As a result of the increasing evidence, Prakash *et al.* defended in 2016 that BAV and TAAD shared a common developmental origin and were caused by defects in the same genes or interacting genes (*section I, subsection 3.4*)[12]. This potentially common genetic background led the scientific community to strive to unravel it, and not just to consider BAV as any other risk factor. Nevertheless, to date, no definite consensus exists whether the dilated thoracic aorta is genetically determined or a result of the year-long, valve-related, hemodynamic stress[122]. In our opinion, according to the statement made by Sievers *et al.* in 2016, both genetic and hemodynamic factors could be playing an important role[122].

Despite the relatively high frequency of this valvular malformation and relative ease to gather affected patients, almost nothing is known about the genetics behind its development. There is not even an agreement on whether it should be understood as a single or multiple diseases, or as a Mendelian or complex trait. Traditionally, a polygenic model defined by the interaction of multiple genes and environmental factors was hypothesized to account for different forms of congenital heart disease, but it is now understood that their genetic component extends beyond a single unified paradigm[209]. Several scenarios (or a combination of them) have been proposed to explain why it has been so extraordinarily difficult to identify the genetic factors causing BAV: (i) genetic heterogeneity and reduced penetrance; (ii) complex inheritance; and (iii) genetic variants in the non-coding sequence and epigenetic factors[38].

As very little is known about the development of BAV and BAV-associated TAAD at the molecular level, the complex disease hypothesis has been the most embraced[22,51,122]. Although both manifestations have been associated with an autosomal dominant pattern of inheritance with

incomplete penetrance and variable clinical expression[39,125,293], the inability to identify simple Mendelian loci even with whole-exome approaches suggested that many pedigrees may be attributable to a polygenic influence, rather than simply incomplete penetrance[41]. But the possibility of an overestimated heritability should also be considered. In fact, Robledo-Carmona *et al.*, through the analysis of 100 consecutive BAV families in 2013, found a low BAV recurrence rate in first-degree relatives (4.6%), and a morphologic concordance in family members of 68.6%, which seemed to be occurring by chance[134]. At the same time, it is difficult to understand an embryological defect as a conjunction of several genetic and environmental factors acting coordinately over time. Could BAV be understood as multiple instead of a single disease? Could some cases be dependent on genetic factors, while others on both genetic and environmental factors, as it happens with TAAD[125]? Should therefore the scientific community consider the possibility of either a Mendelian or complex inheritance patterns in BAV cases?

In vertebrates, the heart is the first functional organ that forms during the development of embryos[124]. Cardiac valve morphogenesis, which occurs early in fetal development, is a complex morphogenetic process that requires the temporal and spatial cooperation of cardiac cell commitment, differentiation, proliferation, and migration[124]. Either environmental or genetic factors may interrupt this biological process, leading to abnormal valvulogenesis and the formation of a BAV[124]. Multiple signaling pathways have been involved in this process, including, but not limited to, members of the TGF-β superfamily, VEGF, Notch, Wnt/β-catenin, Tbx20, and Gata4[125,294,295]. The identification of *NOTCH1* mutations causing BAV emerged from the analysis by Garg *et al.* in 2005 of a large family of European-American descent spanning five generations with 11 cases of congenital heart disease, nine of which had aortic valve disease (*section I, subsection 3.7*)[145]. This could only have been possible if the disease of interest responded to a somehow Mendelian inheritance pattern.

Besides the multiple comorbidities surrounding BAV, it has also been proposed that it could be understood not as a single disease, but a combination of currently unknown different pathologies[59]. For instance, Schaefer *et al.* described in 2008 the relationship between thoracic aortic dilation rates and the BAV cusp fusion pattern[296], with greater rates of SV and ascending thoracic aortic dilation associated with the right-left coronay sinus phenotype[55,297]. As defended by Calloway *et al.* in 2011, different BAV morphologies may represent different developmental abnormalities[298], justifying the current negative results with the confusion derived from having mixed patients that actually suffered from different diseases. The concordance rate of BAV subtype between siblings has been described to be 76%[51,298]. Although being just an example, we strongly believe greater efforts should be performed during sample collection to accurately phenotype each and every affected patient. The

first step should be the definition of those crucial phenotypic characteristics based on which patients would need to be further sub-classified.

In conclusion, high heterogeneity of BAV and BAV-associated aortic disease reflects the complexity of the underlying genetic component, as well as the potential participation of other gene expression regulatory mechanisms (epigenetics), and hemodynamics[55]. Although unraveling the complex genetic architecture of BAV-TAA will absolutely require joint efforts, large-scale collections, and deep-sequencing approaches[41], it is in any case worth pursuing, especially when thinking about the potential to predict TAAD-risk based on the underlying genetic risk factors.

## 4. Exome-wide association approach to bicuspid aortic valve. Comparison with genome-wide association study. Imputation

The identification of genetic risk factors that predispose to the development of complex diseases is a common pending goal for genetic medicine. Considering BAV a complex trait strongly associated with the development of TAAD, we have chosen an association study as the best approach to at least partially unravel the genetic component behind it. Any complex disease depends on the participation of environmental factors as well as multiple risk alleles, many of them common but also rare, and the identification of any of them would be very valuable for the establishment of the genetic basis of these conditions.

The main difference between GWAS and EWAS is in relation with the frequency and distribution of the set of genotyped genetic variants and the linkage blocks they belong to. Typical GWAS involve millions of polymorphic markers homogeneously distributed across the whole genome and have the resolution to detect common, but generally not rare, associations[112]. On the other hand, EWAS involve a lower number of genetic variants distributed only across the exome, that represents the 1.5% of the genome with protein-coding goals[112,250]. Genetic variants located in protein-coding regions are expected to be relevant for human disease and, in general, have a lower frequency as a result of their direct effect on health and the subsequent negative selection. Many more rare than common variants exist[112], but many are at the same time bounded to specific populations, which hinders their selection when creating association arrays wanted to be meaningful for the whole population.

As valid as a common GWAS, we opted for an EWAS that involved 319,000 protein-coding genetic variants, 80% of which were categorized as rare with a MAF below 1%, and analyzed with it 565 unrelated BAV cases and 484 general population controls (*section III, subsection 3*). The rarer a genetic variant is, the more difficult it is to find frequency differences between cases and controls, and therefore larger sample sizes are needed to detect significant associations. In the specific case of



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BAV, an almost completely genetically unraveled disease, there is a further complication that consists in the absence of previous association results. Furthermore, BAV multiple comorbidities, such as TAAD, should also be taken into account, as they have the higher clinical impact on BAV patients. For this reason, from a clinical point of view and willing to maximize the genotype data[228], we repeated the association analysis considering some of these extra clinical features and combined association results for replication. A better understanding of the relationship between the valvulopathy and the aortopathy is necessary to reduce the potentially life-threatening complications associated with the presence of BAV[122].

Besides the genetic and phenotypic heterogeneity of available cohorts, population frequency of the disease, and available sample sizes, in this case the predicted distributions of allele frequencies and effect sizes for pathogenic variant were uncertain, making predictions about efficacy imprecise[226]. So although we conducted the corresponding formal power calculations that supported the usefulness of our approach, we were not able to identify any significant association result with *p-value* under $5 \cdot 10^{-8}$, corresponding to the conservative *Bonferroni* threshold proposed by Risch and Merikangas in 1996 (equivalent to a *p-value* of 0.05 after a *Bonferroni* correction for one million independent tests)[228,229]. But the *Bonferroni* correction is generally punitively conservative, requiring inappropriately low *p-values* (and therefore inappropriately large sample sizes)[228,229,299]. With a high density of markers, significant linkage desequilibrium between many markers, and redundancy between single markers and the multi-marker haplotypes that might also be tested for association, the assumption of independence among tests is strongly violated[228,229]. Furthermore, in this case, the significance threshold should be lower, as the number of association tests performed was significantly smaller than one million (being the high number of the genotyped markers that resulted monomorphic in Spanish population a great disadvantage).

For all these reasons, we decided to replicate seven of the most significant association signals that reached a *p-value* $< 1 \cdot 10^{-4}$ in an independent dataset, although strictly non-significant (rs8001733, rs42663, rs17382301, rs4889554, rs13294886, rs10492585, and rs3740526). Unfortunately, any of the association signals selected for replication was consistently significant across cohorts, possibly as a result of low power, spuriousness, or population singularities. Nevertheless, willing to avoid similar efforts and, conversely, seeking large collaborative ones, we believe in the relevance of publishing the different attempts made by the scientific community on the genetics of complex diseases, even being negative. Regardless of the chosen approach and the number of polymorphic markers genotyped, any association study should rely on accurate and comprehensive genotyping, evaluation of a large number of carefully phenotyped patients, and of course replication of significant associations[72,228].

One of them, in fact the most significant, rs13294886 in *HMCN2*, was especially promising as it is related, together with *NOTCH1*, with calcium metabolism. It still has to be clarified the relation between BAV, valvular calcification, and *NOTCH1*[28]. This latter also participates in valvular and vascular morphogenesis, guiding neural crest cell migration and epithelial-mesenchymal transformation[28]. Could the association between BAV and *NOTCH1* be spurious hiding the real association between *NOTCH1* and calcific aortic stenosis? Although the hemodynamic alterations induced by BAV may contribute to calcification, several of the TAV family members analyzed by Garg *et al.* in 2005 also developed valvular calcification (*section I, subsection 3.7*)[145]. Furthermore, they demonstrated *Notch1* normally represses *Runx2* activation, and so the expression of osteoponin, osteocalcin, and other osteoblast-specific genes[145]. But the complete mechanism has not yet been unraveled. Further studies involving the *NOTCH1* signaling pathway in the adult calcific process may identify preventive and pharmacological approaches to slow this age-related disease[145]. It is for this reason that we disagree with the recommendation that Foffa *et al.* made in 2013 about the genetic screening for *NOTCH1* mutations (*section I, subsection 3.3.1*). They stated *NOTCH1* should be considered for BAV diagnosis and family screening, as part of the standard clinical management of patients with a family history of BAV[114]. In our opinion, it is too soon to perform genetic diagnosis of BAV cases, as the genetics of this common valve disease are still largely unknown.

Taking advantage from the multiplex approach we chose to perform the replication of the original association results, we also tried to replicate five of the *FBN1* association signals described by LeMaire *et al.* in 2011 as being associated with sporadic TAAD and BAV (rs10519177, rs4774517, rs755251, rs1036477, and rs2118181; *section I, subsection 3.4*)[3]. Although the results we obtained were negative, we could not assert the exact concordance of the evaluated phenotypes and thus we considered them not definitive. In our opinion, *FBN1* is key in TAAD, but not in BAV development. In any case, prior evidence of association of a specific genetic variant with the disease should be continuously re-evaluated with newly available information[226].

This need of a continuous re-evaluation of association signals and the general requirement of hundreds to thousands of patients and controls to identify genetic variation associated with complex traits, leads investigators to pool different patient cohorts to increase sample size[226]. All the negative results obtained from BAV GWAS and EWAS in the past years (many of them not published) suggested that this should be the next step. Although such consortium-based approaches are desirable, investigators should always be mindful of systematic differences among cohorts stemming from technical biases, population stratification, and genetic and phenotypic heterogeneity[226].

To do so, it is necessary to previously match the association results obtained from each cohort through imputation of the respectively unknown positions, based on the comparison of the

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corresponding linkage disequilibrium blocks with increasingly large reference panels. At this stage, EWAS entails a significant disadvantage compared to GWAS. Rare variants belong to linkage blocks different to those that contain the common ones and, as expected, they are much more difficult to be inferred as they are much less represented in the population.

Despite this potential limitation, we were able to impute a relatively high number of high quality genetic variants that will form part of an international effort pursuing BAV genetics, which will be developed in the near future. But, in our opinion and as stated before, it should not only be about the valvular malformation itself, but also the concomitant aortic dilation, being this latter the real potentially life-threatening condition.

## 5. Current limitations of the molecular diagnosis of thoracic aortic disease

Molecular diagnosis of thoracic aortic disease is a very promising field that currently lacks completeness, and therefore has not been fully implemented neither in the routine clinical care, nor in the forensic field. The scenario is not as simple as first thought. TAAD is a clinical entity that can appear in association with multiple diseases, which can, at the same time, depend or not on genetic factors. Regarding those with a genetic origin, their pattern of inheritance can be either Mendelian or complex, and the Mendelian cases, syndromic or non-syndromic. Finally, besides the typical sequence genetic variation, other mutations located in non-coding regions or those regulatory affecting gene accessibility and expression, could also play a role and should be further considered[75]. In fact, the existence of families with incomplete segregation and variable expressivity support the participation of other so far unknown genetic factors contributing to the observed phenotypes, as for instance modifying genes that may exaggerate or suppress the development of additional symptoms[22,26].

To date, not even the *a priori* simplest cases have been completely understood, and the search for novel causal genes depends on the possibility that each gene may contribute to the disease in only a small proportion of families[251]. The relatively high number of NSAD cases in which the genetic component is still unknown[58] supports the existence of many unraveled TAAD genes that should be identified to offer an accurate molecular diagnosis. But the identification of the causal mutations depends on having chosen the correct assay based on the expected inheritance pattern of the disease, which should be clearly defined from the very beginning. What has already been demonstrated is that it is not always easy to do so.

Life expectancy of TAAD patients is mainly determined by the risk of aortic dissection[22], so all efforts should be made in order to enable an early diagnosis and anticipate this fatal condition. To

date, the main predictor of aortic dissection is still the aortic diameter (adjusted by age, weight, and sex)[22], but recent findings suggest that it is also determined by the specific underlying genetic defect. Although the definition of the risk of dissection and the best timing for surgery associated with the different TAAD causal genes has already started, a lot of work has yet to be done.

The truth is that clinical management strategies for the medical and surgical treatment of TAAD patients are becoming increasingly gene-tailored[22]. As the results of genetic and genomic testing are increasingly being used in medical decision-making, the potential harm due to genetic variants misinterpretation is substantial[226,279]. Boycott *et al.* proved in 2013 that as many as 30% of all disease-causing genetic variants cited in the literature may have been misinterpreted[279,300]. The interpretation of findings has always been and will always be a cornerstone of genetic interrogation and precision medicine[279]. Specially when the genetic component of a disease is still partially unraveled, it is critical that healthcare providers be made aware of the varying levels of certainty in the evidence for implicating a genetic variant in the disease, both through the consistent use of genetic variant classification terminologies and the description of the supporting evidence or lack thereof[226].

Molecular diagnosis is especially important in those TAAD cases in which clinical features are not sufficient to reach a final diagnosis, for prenatal diagnosis in couples at risk, or in families in which mortality associated with TAAD along generations has been present[61]. Nowadays, it does not play a key role for the determination of the best timing for surgery in those patients in whom diagnosis has been fully accomplished clinically[61]. For example, a MFS patient with an aortic diameter greater than 50 millimeters at the level of the SV and a positive family history of aortic dissection should be automatically referred to aortic surgery, no matter the molecular diagnosis[61]. But, in any case, the identification of a pathogenetic mutation may help to anticipate the prognosis, as well as perform prevention and careful follow-up in relatives at risk[26,61]. Identifying these at risk family members and facilitating family screening is paramount to reducing the long-term health risks associated with inherited TAAD if not early identified and managed appropriately[125].

This objective could also be reached in the forensic field, in which TAAD has been hardly explored to date. In this specific case, it is not only about the molecular autopsy, but more importantly the potential identification of the same causal genetic variant in other family members at risk. As shown in *section III, subsection 4*, the molecular autopsy yield was relatively high, considering we only sequenced 22 candidate genes. We believe that many more will be incorporated in the upcoming years, and that the corresponding candidate-gene design will be regularly updated reflecting the discovery of new TAAD genes. The identification of putative causal genetic variants would allow mutational screening in relatives at risk, which could be performed using traditional sequencing.

# 6. Future research

Despite the great improvement made in TAAD genetics, as stated before, a lot of work has yet to be done in this promising field, in which molecular diagnosis has a direct impact on patient clinical management, prognosis, and life expectancy. We hereunder refer to the main pending issues that, in our opinion, should be addressed in the near future:

(i) **Further phenotypic definition:** because most TAAD gene discoveries have been made in the recent years, it is reasonable to anticipate that the full range of phenotypic diversity caused by genetic variation in these genes will evolve over the upcoming years[38].

(ii) **Identification of new causal genes and genetic variants**: one of the main topics which has already been explored, but in our opinion should continue, is the identification of new TAAD causal genes and genetic variants through a combination of traditional sequencing and high-throughput MPS technologies. These latter could especially contribute to detailed catalogues of genetic variation in both patients and the general population, which could help to establish better genotype-phenotype correlations, and therefore guide clinical management and prognosis. However, for these technologies to have the greatest medical impact, the scientific community should be able to reliably separate genuine disease-causing mutations from the broader background of genetic variants present in all human genomes, which are rare and potentially functional, but not actually pathogenic for the disease or phenotype under investigation[226]. The excessive false-positive reports of causality impede the translation of genomic research findings into the clinical diagnostic setting, and hinder the biological understanding of the disease[226]. Therefore, it is not just about the identification of additional causal genes, but previously associated genetic variants and recurring VUS should be continuously re-evaluated and re-classified if necessary, as new information becomes available[38,226].

(iii) **Powerful association studies, involving either SNPs or CNVs, looking for both common and rare genetic variants conferring susceptibility to complex TAAD:** when seeking the genetic component behind a complex disease, the preferred approach is generally an association study instead of sequencing. No matter whether involving sporadic TAAD or BAV patients, it seems worthwhile to expand future efforts in aortic research more aggressively into large-scale population-based studies[41]. To accomplish this purpose, imputation of directly genotyped markers to increasingly large reference panels will be needed. Being this more accurate if working with GWAS and not EWAS results, as haplotypes defined by common variation are more widely known. Besides involving typical

sequence variation, these association studies should also be performed for CNVs, willing to further justify their relevance for the development of TAAD, which has to date been strongly considered but, in fact, sparcely evaluated.

(iv) **Epistasis (gene-gene interactions) and epigenetics:** besides sequence variation, they are also thought to play an important role in complex traits and will require more attention[7,228]. Given the key role of miRNAs, histone modifications, and DNA methylation in the regulation of gene expression, a more in-depth comprehension of the epigenetic alterations associated with dilative thoracic aortopathies could be of great help in clarifying their pathogenesis and, possibly, identifying preventive and therapeutic strategies[55].

(v) **Functional studies:** whenever possible, functional studies should be performed to support causality or, alternatively, define new biomarkers of the disease. In our opinion, over time, the major challenge in aortic aneurysmal research will likely shift from gene identification to the assessment of gene product function in large vessel homeostasis[74]. Great effort and investment should be assigned to this second step, which comes right after candidate gene identification. Besides the already used animal models, the ability to de-differentiate and reprogram human cells (embryonic and induced pluripotent stem cells) into virtually any cell type holds great promise in generating relevant human cells, tissues, and organs[54,74], and specifically cell-based models of aortic aneurysm[74]. Nevertheless, the *NIH Roadmap Epigenomics Consortium* published some confusing results in 2015 regarding the diverse epigenetic fingerprint of original and induced pluripotent stem cell cardiomyocytes, supporting the need for further investigation in this field[256].

(vi) **Genetic component of sporadic TAAD:** some of the genes which have already been involved in monogenic TAAD, even CNVs, have been redundantly associated with sporadic forms of the same disease, which were previously considered non-dependent on genetic factors[41,189]. In our opinion, they should be understood as complex, so genetic variants would confer susceptibility to TAAD but would not be sufficient to cause the disease if alone. El-Hamamsy *et al.* suggested in 2009 that efforts regarding the pathogenetic mechanism behind this specific type of TAAD should focus on inflammatory and oxidative pathways[28].

(vii) **Development of new therapies:** new insights in the pathogenetic mechanisms behind inherited TAAD cases could also result in the development of new treatment options different to surgery, some of which have already been investigated in large clinical trials[22]. With every new TAAD gene identified, the hope for an effective therapy increases[22].

(viii) **Continuous update of the treatment guidelines:** considered the mainstay for sharing knowledge between basic scientists and clinical practitioners, in many of the recent

discovered TAAD genes, such as *TGFB2*, there is still little experience, and specific treatment guidelines will need more discussion[22]. Nevertheless, we believe that they will be continuously updated in the upcoming years, intending to improve life quality and expectancy of any TAAD patient.

# V. CONCLUSIONS

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# V. CONCLUSIONS

1. Both Mendelian and complex genetic factors influence TAAD development and are important for the determination of the individual risk to develop this life-threatening condition.

2. The 22 candidate-gene MPS molecular autopsy is a useful approach for the identification of the genetics behind Mendelian TAAD individual cases, with a success rate of approximately 23%. An accurate molecular diagnosis of aortic dissection SD cases favors an anticipated clinical diagnosis and prognosis, as well as cascade screening.

3. WES is a robust approach for the identification of the genetic factors causing NSAD in large familial cases, especially in those in which the main candidate genes have previously been excluded.

4. Given the huge amount of data generated with any MPS approach, it is essential to predefine the corresponding prioritization protocol, in which sequencing multiple affected and unaffected family members considerably reduces the number of candidate genetic variants. Nevertheless, the bias implied in any prioritization protocol should always be taken into account, as well as any candidate genetic variant further evaluated through segregation analysis and functional studies.

5. The definition of accurate genotype-phenotype correlations is essential for an early diagnosis and prognosis of TAAD patients. An exhaustive phenotypic description is an essential tool for guiding further genetic investigation.

6. The identification of a new *TGFB2* genetic variant (c.C1042T:p.R348C) in a large NSAD familial case with multiple affected relatives supports this gene as a strong candidate for TAAD diagnosis, TGF-β pathway relevance, and the still unraveled and paradoxical up-regulation hypothesis.

7. The identification of the c.530G>A:p.Arg177Gln *PRKG1* genetic variant in a new NSAD familial case with an early onset of the disease supports the inclusion of this gene in the TAAD molecular diagnosis, especially when aggressive, and the relevance of this specific genotype-phenotype correlation.

8. The presence of multiple extra-cardiovascular features in the two NSAD families analyzed suggests the potential future definition of new syndromes, which will need more exhaustively-phenotyped affected cases to be further delineated.

9. The molecular diagnosis of TAAD cases is a useful diagnostic tool to confirm clinical diagnosis, anticipate prognosis, and adapt clinical management of either the proband or other family members at risk based on the underlying genetic variant.

10. BAV was confirmed as a complex congenital malformation with a highly variable presentation, involving the clinically relevant thoracic aortic complications. Phenotypic variability should be strongly considered when performing genetic analysis of BAV patients, since at least part of the genetic complexity of this disease depends on the phenotypic variation and patient misclassification.

11. The EWAS here performed was not sufficiently powered to detect any consistently significant association signal, neither by considering just the BAV phenotype nor after BAV patients re-classification based on the presence or absence of concomitant thoracic aortic dilation. Nevertheless, a promising one arose involving *HMCN2* and calcium metabolism that should be considered in future studies.

12. The lack of replication of the five *FBN1* association signals that LeMaire *et al.* linked in 2011 to sporadic thoracic aortic disease questioned their association with the BAV phenotype.

13. Despite previous skepticism, EWAS results were reliably imputed against the *Phase 3 1000 Genomes Project* reference panel, reaching a competitive number of high-quality inferred genotypes that will be used for future collaborative efforts, willing to increase sample size and statistical power.

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# EXTENDED SUMMARY (SPANISH)

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# EXTENDED SUMMARY (SPANISH)

Los aneurismas y disecciones de aorta torácica son una entidad clínica con frecuencia asintomática pero potencialmente letal y causa de muerte súbita debido al incremento en el riesgo de disecciones o rupturas aórticas[4,5,11]. A pesar de su alta tasa de mortalidad, cercana al 70%, a día de hoy, todavía son, en muchos aspectos, desconocidos[25,48,51].

Uno de los múltiples factores que dificultan su estudio es el hecho de que pueden manifestarse como resultado de un amplio abanico de enfermedades[52], que a grandes rasgos se clasifican en genéticas o esporádicas. Se estima que hasta un 40% tienen un origen genético, altamente heterogéneo, y en el 80% de los casos desconocido[11,26,67]. Conocer los factores genéticos implicados en su desarrollo podría llegar a ser especialmente útil para la prevención de sus consecuencias fatales y anticipación de su pronóstico, la personalización del manejo clínico (la elección del mejor momento para someter al paciente a cirugía) y la estratificación del riesgo al que está expuesto cada paciente[206].

Algunas de las patologías de origen genético que cursan con este tipo concreto de aneurismas son claramente mendelianas, mientras que otras dependen de un componente genético más complejo. De las mendelianas, los principales síndromes del tejido conectivo son las más fácilmente diagnosticables, ya que la sintomatología no solo se manifiesta en la aorta torácica, sino también en otros sistemas más aparentes, como el musculoesquelético. Por el contrario, el diagnóstico de los casos mendelianos no sindrómicos suele ser accidental. A pesar de que hasta un 20% de los pacientes con aneurismas y disecciones de aorta torácica no sindrómicos cuentan con historia familiar[25,37,101], el conjunto de genes que se ha asociado hasta la fecha con el desarrollo de esta patología tan solo explica un pequeño porcentaje de todos ellos. Muchos siguen pendientes de un diagnóstico molecular certero[11].

Por otro lado, una de las patologías genéticamente complejas que se ha asociado en repetidas ocasiones con el desarrollo de este tipo de aneurismas es la válvula aórtica bicúspide, la malformación cardiovascular congénita más frecuente en humanos con una prevalencia del 0.5-2% en población general[8,44,113–115]. En cualquier patología con herencia compleja, las variantes genéticas implicadas confieren un determinado riesgo, pero de forma individual no son suficientes para causar la patología. Por este motivo, su abordaje es completamente diferente al empleado en casos

mendelianos, y generalmente poblacional. A pesar del relativo fácil acceso a pacientes con válvula aórtica bicúspide, el componente genético que está detrás esta malformación valvular y otras manifestaciones asociadas sigue siendo un gran desconocido. De la misma manera que en el caso anterior, resultaría muy útil para la estratificación de los pacientes en base al riesgo de disección de aorta torácica, la manifestación clínicamente más relevante y que compromete la vida del paciente.

Por todo ello, el principal objetivo de esta tesis doctoral fue contribuir a elucidar el componente genético de los aneurismas y disecciones de aorta torácica, tanto desde un punto de vista mendeliano como complejo. Para alcanzarlo, los objetivos secundarios propuestos fueron los siguientes:

1. Desarrollar una herramienta de diagnóstico molecular basada en la combinación de secuenciación tradicional y masiva en paralelo, tanto de genes candidatos como de exoma completo. Probar su utilidad tanto en el ámbito forense como en el clínico mediante el análisis de diferentes casos de aneurismas y disecciones de aorta torácica con un patrón de herencia mendeliano.
    a. Describir de forma exhaustiva cada una de las características fenotípicas de todos los casos mendelianos de aneurismas y disecciones de aorta torácica disponibles, con el objetivo de mejorar las correlaciones genotipo-fenotipo, que consideramos fundamentales para alcanzar la meta de un manejo clínico personalizado.
    b. Definir una nueva estrategia de secuenciación masiva en paralelo de genes candidatos previamente asociados con casos de aneurismas y disecciones de aorta torácica. Probar su utilidad para la autopsia molecular de casos de muerte súbita por disección de aorta torácica.
    c. Diseñar una segunda aproximación a estos casos basada en la secuenciación del exoma completo de miembros de una misma familia, tanto afectos como no afectos, para el análisis de dos casos familiares de aneurismas y disecciones de aorta torácica no sindrómicos y la identificación de variantes genéticas potencialmente causales.
    d. Llevar a cabo un análisis de la segregación de las variantes genéticas candidatas con el fenotipo mediante secuenciación tradicional, para respaldar la causalidad de las mismas e identificar otros familiares portadores y en riesgo de desarrollar la patología.
2. Identificar alelos de riesgo o protección frente al desarrollo de válvula aórtica bicúspide, la patología compleja más frecuentemente asociada con el desarrollo de aneurismas y disecciones de aorta torácica , utilizando en este caso una aproximación poblacional.



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a. Generar una base de datos con las características fenotípicas de cada uno de los casos con válvula aórtica bicúspide, que incluya, entre otras cosas, información epidemiológica, factores de riesgo, manifestaciones clínicas, historia familiar y mediciones ecocardiográficas.

b. Llevar a cabo un estudio de asociación de exoma completo que enfrente los casos de válvula aórtica bicúspide con controles de población general. Analizar la distribución de sus genotipos mediante análisis de asociación por variante y gen.

c. Reclasificar los casos de válvula aórtica bicúspide en base a la presencia o ausencia de dilatación concomitante de aorta torácica y repetir los mismos análisis de asociación anteriores.

d. En el caso de que se detectase alguna señal de asociación significativa, replicarla en una cohorte poblacional independiente.

e. Imputar los resultados de asociación para una posible participación futura en consorcios internacionales, en busca de incrementar el tamaño muestral y por lo tanto el poder estadístico para la detección de señales de asociación con efecto intermedio.

Las aproximaciones genéticas empleadas para alcanzar cada uno de los objetivos anteriores fueron diversas y dependientes del patrón de herencia de cada caso, bien mendeliano o complejo. Mientras que en aquellos casos mendelianos una sola variante genética causal puede ser suficiente para provocar el desarrollo de la patología, los complejos dependen de la interacción de múltiples factores genéticos a lo largo del tiempo con el ambiente. En los *artículos 1* y *2*, se abordó el estudio del componente genético de dos casos familiares no sindrómicos de aneurismas y disecciones de aorta torácica mediante secuenciación masiva en paralelo de exoma completo y secuenciación tradicional para el posterior estudio de segregación (*SureSelectXT Human All Exon version 4 kit* y *5500 SOLiD System*, *BigDye Terminator chemistry version 3.1* y *ABI Genetic Analyser 3730*). En el *artículo 1*, se seleccionaron dos familiares afectos con el parentesco más lejano posible, buscando reducir al máximo las variantes genéticas compartidas únicamente por su proximidad filogenética, y no por el fenotipo común. El protocolo de priorización de las mismas, por lo tanto, contó con un primer paso de selección de las variantes comunes a los dos individuos afectos. A continuación, se priorizaron aquellas exónicas o de *splicing*; no sinónimas, capaces de provocar un cambio en la pauta de lectura o de parada; con una frecuencia por debajo del 1% en la base de datos de referencia *Exome Sequencing Project*[254]; con al menos un 20% del alelo mutado; presentes cuatro veces o menos en una base de datos interna con información de al menos 100 exomas con antecedentes patológicos diversos; que demostraron ser reales al ser visualizadas en el *Integrative Genomics Viewer*[301,302]; y

localizadas en genes de rutas metabólicas involucradas en el desarrollo de la patología, como TGF-β o el aparato contráctil del músculo liso aórtico. Asumiendo un patrón de herencia autosómico dominante con penetrancia incompleta y expresión variable, este extenso protocolo de priorización dio lugar a la identificación de una variante genética candidata en *TGFB2*: c.C1042T:p.R348C (NM_001135599.2). Este gen candidato ya había sido previamente asociado con casos sindrómicos de aneurismas y disecciones de aorta torácica por Boileau *et al.* y Lindsay *et al.* en 2012[108,109]. El análisis del exón portador de dicha variante candidata en otros 11 familiares, tanto afectos como no afectos, demostró su segregación con el fenotipo, y diferentes predictores de patogenicidad *in silico* también la respaldaron como variante genética causal.

En el *artículo 2*, se secuenció el exoma de cuatro familiares afectos y dos no afectos de una segunda familia con aneurismas y disecciones de aorta torácica no sindrómicos y de inicio temprano, esta vez con la plataforma *Illumina HiSeq 2000*. El primer paso del protocolo de priorización, por lo tanto, supuso la selección de las variantes genéticas comunes a los cuatro afectos y además, no presentes en los dos familiares no afectos. Como era de esperar, fue más efectivo, reduciendo considerablemente el número de variantes genéticas candidatas. La aplicación del protocolo de priorización restante, muy similar al descrito para el artículo anterior, dio lugar a la identificación en este caso de una variante genética candidata en *PRKG1* (c.530G>A:p.Arg177Gln; NM_001098512, rs397515330), un gen involucrado en la regulación de la contracción del músculo liso aórtico. Una vez más, los predictores *in silico* y el estudio de segregación en otros ocho familiares afectos y no afectos respaldaron a la variante candidata como causal. A mayores, Guo *et al.* ya la habían asociado en 2013 con el desarrollo de esta patología y respaldado su causalidad mediante estudios funcionales que demostraron que el mecanismo molecular de base era la ganancia de función derivada de una mayor actividad de la proteína codificada por *PRKG1*[110].

En el *artículo 3*, el planteamiento fue completamente diferente al de los dos anteriores, ya que en este caso se abordó el estudio del componente genético de una patología con herencia compleja, la válvula aórtica bicúspide, muy vinculada al desarrollo concomitante de aneurismas y disecciones de aorta torácica. Con el objetivo de identificar factores de riesgo o protección frente al desarrollo de esta patología, se optó por una estrategia poblacional en la que se enfrentaron 570 casos de válvula aórtica bicúspide (con o sin dilatación concomitante de la aorta torácica) con 484 controles de población general, para determinar las diferencias en la distribución de las frecuencias de los aproximadamente 319,000 genotipos contenidos en el ensayo *Axiom Exome* de *Affymetrix*[303]. Con el objetivo de cubrir un espectro determinado de variación genética, las variantes genéticas de un solo nucleótido e inserciones y deleciones contenidas en este ensayo son fundamentalmente exónicas y en su gran mayoría raras.

El análisis de asociación para cada variante individual se llevó a cabo mediante regresión logística con *PLINK*[304] y la librería de *R GenABEL*[305], mientras que en el análisis de asociación por gen la herramienta de análisis fue la librería *SkatMeta* de *R*. Estos mismos análisis de asociación se llevaron a cabo por triplicado, considerando en primer lugar el total de casos con válvula aórtica bicúspide, que a continuación fueron subclasificados según la presencia o no de dilatación concomitante de la aorta torácica. En todo momento, las covariables que se consideraron en el modelo de regresión logística fueron la edad, el sexo y los 10 primeros *eigenvectors* obtenidos a partir del análisis de subestructuración poblacional llevado a cabo con el programa *GCTA*[306]. A mayores, con el objetivo de sacarle el mayor partido a los resultados de asociación, también se llevó a cabo la imputación de los mismos en base a los 82 millones de variantes genéticas contenidas en el panel de referencia *1000 Genomes Project Phase 3* con las herramientas de análisis *SHAPEIT v2*, *IMPUTE2 v2.3.2* y *SNPTEST v2.5*[307–310].

A pesar de que ninguno de los resultados obtenidos alcanzó el umbral de significación estimado en base a las recomendaciones de *Bonferroni*, se seleccionaron siete señales de asociación con *p-valor* $< 1 \cdot 10^{-4}$ (rs8001733, rs42663, rs17382301, rs4889554, rs13294886, rs10492585 y rs3740526) para replicar en una cohorte independiente de 895 casos de válvula aórtica bicúspide y 1.483 controles. La más significativa de todas ellas fue rs13294886, perteneciente al gen *HMCN2*, involucrado de la misma manera que *NOTCH1* en el metabolismo del calcio. También se consideraron otros cinco marcadores que habían sido descritos por Le Maire *et al.* en 2011 como asociados con aneurismas y disecciones de aorta torácica esporádicos (rs10519177, rs4774517, rs755251, rs1036477 y rs2118181)[3]. La réplica de estos 12 resultados se llevó a cabo con la plataforma de genotipado *IPLEX Gold MassARRAY* y el meta-análisis con *GWAMA v2.1*[311], pero sin tampoco dar lugar a ningún resultado significativo.

Finalmente, el enfoque del *artículo 4* fue fundamentalmente forense, y no clínico. Se seleccionaron 17 casos forenses de muerte súbita por disección de aorta torácica y se diseñó para su análisis una estrategia de secuenciación masiva en paralelo de 22 genes candidatos (*FBN1*, *COL1A1*, *COL1A2*, *COL3A1*, *EFEMP2*, *ELN*, *PLOD1*, *TGFBR1*, *TGFBR2*, *TGFB2*, *TGFB3*, *SMAD3*, *SMAD4*, *SLC2A10*, *SKI*, *ACTA2*, *MYH11*, *FLNA*, *MYLK*, *PRKG1*, *NOTCH1* y *PTPN11)* que se enriquecieron por PCR (*Ion AmpliSeq Library kit 2.0*) y se secuenciaron con la plataforma *Ion Proton*. En este caso, se priorizaron aquellas variantes genéticas exónicas o de *splicing*; no sinónimas, capaces de provocar un cambio en la pauta de lectura o de parada; con una frecuencia por debajo del 1% en la base de datos de referencia de europeos no finlandeses del *Exome Aggregation Consortium*[255]; presentes cuatro veces o menos en una base de datos interna con información de al menos 100 exomas con antecedentes patológicos diversos; presentes como máximo en dos de las muestras analizadas en

una misma tanda del secuenciador; y, por último, que demostraron ser reales al ser visualizadas en el *Integrative Genomics Viewer*[301,302]. Con esta aproximación, se identificaron variantes genéticas potencialmente causales en siete de los 17 casos analizados. No obstante, tan solo cuatro cumplieron con las recomendaciones publicadas por el *American College of Medical Genetics and Genomics* en 2015[312], suponiendo un rendimiento del procedimiento diagnóstico de aproximadamente un 23%. A pesar de las múltiples ventajas clínicas que la comunidad científica ya ha reconocido en relación con el diagnóstico molecular de esta patología, su aplicación en el ámbito forense ha sido muy escasa hasta la fecha. En estos casos, los principales beneficiarios serían aquellos familiares en riesgo de ser portadores de la misma variante genética, y que podrían anticiparse al desarrollo de disecciones o rupturas de aorta torácica y por lo tanto de muerte súbita cardíaca.

En base a las diferentes aproximaciones utilizadas para el estudio de casos tanto mendelianos como complejos de aneurismas y disecciones de aorta torácica y a los resultados obtenidos con cada una de ellas, las conclusiones de esta tesis doctoral fueron las siguientes:

1. Tanto los factores genéticos mendelianos como complejos están involucrados en el desarrollo de aneurismas y disecciones de aorta torácica y son importantes para la definición del riesgo individual a padecer esta manifestación clínica potencialmente letal.

2. La autopsia molecular por secuenciación masiva en paralelo de 22 genes candidatos es una herramienta diagnóstica útil para la identificación del componente genético de casos mendelianos de aneurismas y disecciones de aorta torácica, con una tasa de éxito de aproximadamente un 23%. Un diagnóstico molecular preciso de casos de muerte súbita por disección de aorta torácica favorece un diagnóstico y pronóstico clínicos anticipados, así como el estudio de segregación en familiares.

3. La secuenciación de exoma completo es una aproximación robusta para la identificación de los factores genéticos detrás de casos familiares de enfermedad aórtica no sindrómica, especialmente aquellos no vinculados a los principales genes candidatos.

4. Dada la gran cantidad de datos que cualquier aproximación de secuenciación masiva en paralelo genera, es fundamental predefinir el protocolo de priorización correspondiente, en el que la secuenciación de múltiples familiares tanto afectos como no afectos resulta especialmente efectivo para reducir el número de variantes genéticas candidatas. De todas maneras, toda priorización conlleva un sesgo implícito que refuerza la necesidad de someter cualquier variante genética candidata al correspondiente estudio de segregación en otros familiares, así como a estudios funcionales.

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5. La definición de correlaciones genotipo-fenotipo detalladas facilita el establecimiento de un diagnóstico y pronóstico anticipados de aquellos pacientes con aneurismas y disecciones de aorta torácica. Una descripción fenotípica exhaustiva es fundamental para cualquier investigación sobre el componente genético asociado.

6. La identificación de una nueva variante genética en *TGFB2* (c.C1042T:p.R348C) en uno de los casos familiares de enfermedad aórtica no sindrómica lo respalda como gen candidato para el diagnóstico de aneurismas y disecciones de aorta torácica, así como la relevancia de la ruta metabólica de TGF-β y de la hipótesis de su sobre-expresión en esta patología.

7. La identificación de la variante genética c.530G>A:p.Arg177Gln en *PRKG1* en un nuevo caso familiar de enfermedad aórtica no sindrómica de aparición a edades tempranas respalda su consideración en el diagnóstico molecular de casos de aneurismas y disecciones de aorta torácica agresivos, así como la relevancia de esta correlación fenotipo-genotipo concreta.

8. La presencia de múltiples manifestaciones extra-cardiovasculares en los dos casos familiares de enfermedad aórtica no sindrómica indica la posible existencia de nuevos síndromes por definir en un futuro, que necesitarán de la descripción exhaustiva del fenotipo de más casos para tomar forma.

9. El diagnóstico molecular de los casos de aneurismas y disecciones de aorta torácica es una herramienta diagnóstica útil para confirmar el diagnóstico clínico de la patología, anticipar su pronóstico, y adaptar el manejo clínico de los casos índices y otros familiares potencialmente portadores de la misma variante genética.

10. La válvula aórtica bicúspide es una malformación valvular congénita compleja que se manifiesta de forma altamente variable, y se asocia con complicaciones de aorta torácica clínicamente relevantes. Esta variabilidad fenotípica debe ser muy tenida en cuenta durante el análisis genético de pacientes afectos por válvula aórtica bicúspide, ya que, al menos en parte, la complejidad genética de la patología depende de la variabilidad fenotípica y de la incorrecta clasificación de los pacientes.

11. El estudio de asociación de exoma completo llevado a cabo no contó con el suficiente poder estadístico como para detectar ninguna señal de asociación significativa, ni en base únicamente a la presencia o ausencia de válvula aórtica bicúspide, ni tras la re-clasificación de los pacientes en base a la presencia o ausencia de dilatación concomitante de la aorta torácica. De todas maneras, sí se identificó una prometedora

en el gen *HMCN2*, relacionado con el metabolismo del calcio, a tener en cuenta en proyectos futuros.

12. El fallo en la réplica de las cinco señales de asociación en *FBN1* vinculadas en 2011 por LeMaire *et al.* al desarrollo de aneurismas de aorta torácica esporádicos cuestiona su asociación con el fenotipo de válvula aórtica bicúspide.

13. A pesar del escepticismo previo, los resultados del estudio de asociación de exoma completo fueron imputados con éxito en base el panel de referencia *Phase 3 1000 Genomes Project*, alcanzando un número competitivo de posiciones inferidas con alta calidad que serán utilizadas en proyectos colaborativos futuros en búsqueda de un mayor tamaño muestral y poder estadístico.

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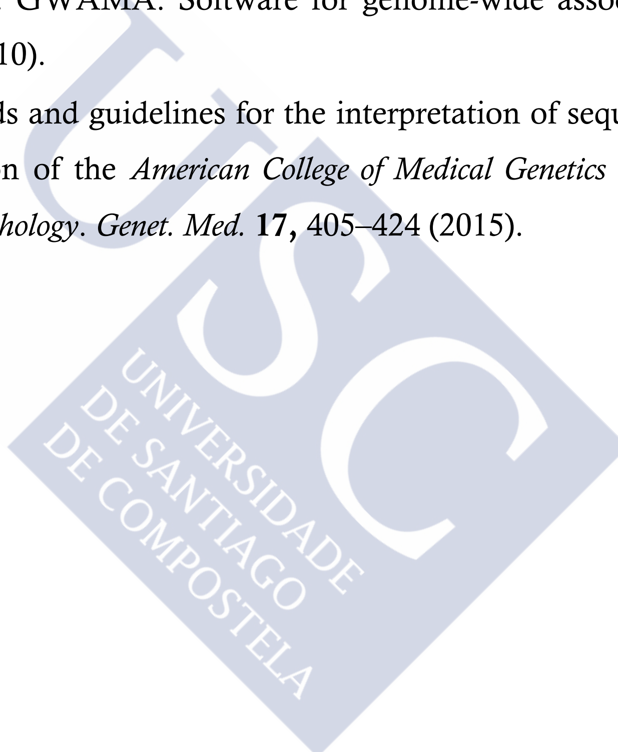

*This doctoral thesis deals with the still partially unraveled genetic component of thoracic aortic aneurysms and dissections, a frequently asymptomatic but potentially lethal condition and major cause of sudden death, from both Mendelian and complex perspectives.*
*Using candidate-gene and whole exome massive parallel sequencing approaches, we were able to solve 23% of the individual forensic cases and two familial non-syndromic cases, in which we identified TGFB2 and PRKG1 potentially causal genetic variants. In contrast, our population-based approach to complex bicuspid aortic valve cases did not identify any consistently significant association. Nevertheless, the direct clinical consequences that some of these results had, supported molecular diagnosis, reliable genotype-phenotype correlations, and risk stratification as important tools for clinical management of these patients and family members at risk, as well as the need of research to continue.*

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