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BICUSPID AORTIC VALVE-ASSOCIATED AORTOPATHY

UNRAVELING THE MOLECULAR SIGNATURE

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Stockholm 2013

To my family

ABSTRACT

Patients with bicuspid aortic valve (BAV) have an increased risk of developing ascending aortic aneurysm compared with individuals with a tricuspid aortic valve (TAV). A crucial factor involved in vascular remodeling during aneurysm development is transforming growth factor-β (TGF-β) and impaired signaling of this pathway can alter important extracellular matrix (ECM) protein such us fibronectin and collagen and thereby explaining the increased aneurysm susceptibility of BAV patients. The overall aim of this thesis was to investigate the BAV-associated aortopathy in relation to the TGF-β signaling pathway.

Alternatively spliced extra domain A (EDA) of fibronectin (FN) has an essential role in tissue repair. In paper I, the mRNA expression of FN splice forms was analyzed by Affymetrix Exon arrays in dilated and non-dilated ascending aorta of TAV (40 individuals) and BAV patients (69 individuals). EDA-FN was significantly increased only in TAV dilated aortas. Upon TGF-β treatment, vascular smooth muscle cells (vSMCs) isolated from TAV aortas were able to enhance the formation of EDA-FN whereas cells derived from BAV patients could not influence fibronectin splicing. Multivariate and univariate data analyses of mRNA expression suggested that differences in the TGF-β signaling pathway may explain the impaired EDA inclusion in BAV patients.

In paper II, multivariate techniques were applied to all exons (n = 614) of the TGF-β pathway in order to analyze alternative splicing in the TGF-β pathway. Alternative splicing mechanisms were found to be important in the development of aneurysm in both BAV and TAV patients. Furthermore, the pattern of alternative splicing is clearly different between TAV and BAV patients. Differential splicing was specific for BAV and TAV patients in 40 and 86 exons, respectively, and splicing of 61 exons were shared between the two phenotypes. This suggested that dilatation in TAV and BAV patients has different alternative splicing fingerprints in the TGF-β pathway.

In paper III, collagen homeostasis in non-dilated and dilated aortas of BAV patients was studied and compared to non-dilated and dilated aortas taken from tricuspid aortic valve patients as reference. Ascending aortas from 56 patients were used for biochemical and morphological analyses of collagen. Collagen turnover rates were similar in non-dilated and dilated aortas of BAV patients, showing that aneurysm formation in BAV is, in contrast to TAV, not associated with an increased collagen turnover. In addition, the ratio of hydroxylysyl pyridinoline (HP) to lysyl pyridinoline (LP), two distinct forms of collagen cross-linking, was lower in dilated aortas from patients with BAV, which hints at a defect in the posttranslational collagen modification associated with BAV.

In paper IV, the response to TGF-β was analyzed in primary aortic smooth muscle cells isolated from 7 BAV and 5 BAV patients and 217 genes were found differentially expressed following TGF-β1 treatment in BAV vSMCs, whereas no gene was significantly altered between treated/untreated vSMCs of TAV patients. Majority of genes were down-regulated and enriched in genes involved in angiogenesis and formation of focal adhesion. Importantly, principle component analysis based on the 217 genes demonstrated that there was a clear difference in expression of these genes in intima/media region of dilated ascending aorta of BAV and TAV patients.

In conclusion, the understanding of impairments in the TGF-β signaling pathway could be the key to unravel the molecular mechanisms underlying BAV-associated aortopathy.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Aortaklaffen är den klaff som sitter mellan hjärtats vänstra kammare och stora kroppspulsådern (aorta), och som förhindrar att blod rinner tillbaka till kammaren då hjärtat slappnar av. Vanligtvis består aortaklaffen av tre delar (kuspar), men hos ca 1-2 procent av befolkningen förekommer en missbildning där två av kusparna sammansmält och bildat en tvådelad, så kallad bikuspid aortaklaff (BAV). Patienter med BAV drabbas i högre utsträckning av både klaff- och aortakomplikationer, så som stenotisk/läckande klaff och pulsåderbråck (aneurysm) än patienter med tredelad (trikuspid) aortaklaff (TAV).

Transforming growth factor β (TGFβ) är ett protein som finns i kärlväggen och förändringar i TGFβ aktivitet har föreslagits påverka uppkomst av aneurysm. Bland annat reglerar TGFβ celltillväxt, celldöd och omsättningen av flera viktiga komponenter i kärlväggen så som t.ex. kollagen och fibronektin. En defekt reglering av TGFβ-signalering kan således leda till en förändrad sammansättning av kärlväggen med en ökad risk för utveckling av aneurysm som följd. Det övergripande syftet med den här avhandlingen var att studera TGFβ i relation till aneurysmutveckling hos patienter med BAV och TAV.

I delarbete I studerades uttrycket av EDA-fibronektin, en speciell variant av fibronektin som spelar en särskild roll i vävnadsreparation. Ett ökat uttryck av EDA-fibronektin kunde ses i vävnadsbitar från TAV-patienter med dilaterad aorta (vidgad) jämfört med TAV-patienter med icke-dilaterad aorta. Ingen skillnad i uttryck kunde dock ses mellan dilaterad och icke-dilaterad aorta från BAV-patienter. Behandling med TGFβ visades öka bildande av EDA-fibronektin i glattmuskelceller isolerade från TAV-, men inte BAV-patienter. En defekt TGFβ-signalering hos patienter med BAV skulle kunna förklara det lägre uttrycket av EDA-fibronektin i aorta hos dessa patienter, vilket i sin tur kan bidra till en försämrad vävnadsreparation.

I delarbete II kunde vi visa att det finns en tydlig skillnad i alternativ splicing av gener relaterade till TGFβ-signalering hos patienter med BAV och TAV. Alternativ splicing är en viktig regleringsmekanism som innebär att flera proteinprodukter, med potentiellt olika funktionalitet, kan bildas från samma gen. Mönstret av alternativ splicing skilde sig tydligt åt mellan de olika patientgrupperna, vilket tyder på att gener involverade i TGFβ-signalering regleras olika hos patienter med BAV respektive TAV.

I delarbete III studerades omsättning och uppbyggnad av kollagen, en strukturellt och funktionellt viktig komponent av kärlväggen som bidrar till aortans stabilitet. Hos patienter med TAV kunde en ökad omsättning av kollagen ses i dilaterad vävnad jämfört med icke-dilaterad vävnad. En sådan ökning kunde däremot inte ses hos BAV-patienter. Vidare kunde en minskad kross-linking, dvs. svagare sammansättning, av kollagenet i vävnad isolerad från dilaterad aorta ses hos patienter med BAV. En försvagad sammansättning tyder på en defekt modifiering och reglering av denna viktiga kärlväggskomponent hos patienter med BAV.

I delarbete IV studerades hur odlade glattmuskelceller, isolerade från aorta från respektive patientgrupp, svarar på TGFβ-behandling. Till skillnad från celler isolerade från TAV-patienter, kunde en stor skillnad ses i genuttryck efter TGFβ-behandling hos BAV-patienter. De flesta generna som skilde sig i uttryck var nedreglerade, och majoriteten var kopplade till nybildning av blodkärl och bildandet av stora proteinkomplex, så kallade focal adhesions som cellen använder sig av för att ankra till

kringliggande vävnad. En nedreglering av dessa gener skulle kunna bidra till en ökad aneurysmrisk genom att göra kärlväggen svagare.

Sammanfattningsvis visar resultat från den här avhandlingen på en defekt reglering av TGFβ-signalvägen hos patienter med BAV. Detta skulle kunna förklara den ökade risken för utveckling av aneurysm hos patienter med BAV.

LIST OF PUBLICATIONS

I. **Paloschi V**, Kurtovic S, Folkersen L, Gomez D, Wågsäter D, Roy J, Petrini J, Eriksson MJ, Caidahl K, Hamsten A, Liska J, Michel JB, Franco-Cereceda A, Eriksson P.
Impaired Splicing of Fibronectin Is Associated With Thoracic Aortic Aneurysm Formation in Patients With Bicuspid Aortic Valve.
Arterioscler Thromb Vasc Biol. 2011;31:691-97

II. Kurtovic S, **Paloschi V**, Folkersen L, Gottfries J, Franco-Cereceda A, Eriksson P.
Diverging alternative splicing fingerprints in the transforming growth factor-β signaling pathway identified in thoracic aortic aneurysms.
Mol Med. 2011;17:665-75

III. Wågsäter D*, **Paloschi V***, Hanemaaijer R, Hultenby K, A. Bank R, Franco-Cereceda A, Lindeman JHN, Eriksson P. *contributed equally
Impaired collagen biosynthesis and cross-linking in aorta of patients with bicuspid aortic valve.
J Am Heart Assoc. 2013;2:e000034:1-11

IV. **Paloschi V**, Gådin J, Björck H, Kjellqvist S, Maleki S, Roy J, Franco-Cereceda A, Eriksson P.
Increased aortic TGFβ signaling in patients with bicuspid aortic valve.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AAA	Abdominal Aortic Aneurysm
AoSMCs	Aortic smooth muscle cells
AS	Aortic Stenosis
ASAP	Advanced Study of Aortic Pathology
BAV	Bicuspid Aortic Valve
BMP	Bone Morphogenic Protein
ECM	Extracellular matrix
EDA	Extra domain A
EDB	Extra domain B
EDS-VIA	Ehlers-Danlos syndrome type VIA
EndMT	Endothelial Mesenchymal Transition
ER	Endoplasmic reticulum
FN	Fibronectin
GAGE	Gene-set enrichment algorithm
HP	Hydroxylysyl pyridinoline
HPLC	High-performance liquid chromatography
LH	Lysyl hydroxylase
LP	Lysyl pyridinoline
MFS	Marfan syndrome
MVA	Multivariate analysis
OPLS-DA	Orthogonal partial least squared discriminant analysis
PCA	Principal component analysis
PLOD1	Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 1
SEM	Scanning electron microscopy
SMCs	Smooth muscle cells
TAA	Thoracic Aortic Aneurysm
TAV	Tricuspid aortic Valve
TEM	Transmission electron microscopy
TGFBR1	Transforming growth factor β receptor 1
TGF-β	Transforming growth factor β
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular smooth muscle cells

1 INTRODUCTION

1.1 THORACIC AORTIC ANEURYSM (TAA)

The term "aneurysm" was probably mentioned for the first time by the Greek physician Rufus of Ephesus in the late 1st century AD, and it semantically derives from *ana* (on top of, towards the outside) and *eurunô* (widening). Galen, shortly after Rufus, uses the term *aneurysms* for arterial dilatations occurring after a trauma, describing it as "when the skin above has formed a scar, and the wound to the artery has remained unhealed". It was only later in the 16th century that, aneurysms in aorta and other arteries were recognized as a common pathology, with Jean Fernel, at Sorbonne, being the first to report on the aneurysms of internal arteries [1]. Today, aneurysms are defined as a persistent localized enlargement of vessels, exceeding 50% of the expected diameter.

Virtually every vascular entity can become aneurysmal, with the aorta being the most common location for aneurysm development in humans [2]. The most common location for aortic aneurysms is the infra-renal abdominal aorta (Abdominal Aortic Aneurysms (AAAs)), followed by thoracic aneurysms in the ascending aorta (Thoracic Aortic Aneurysms (TAAs)) (Figure 1).
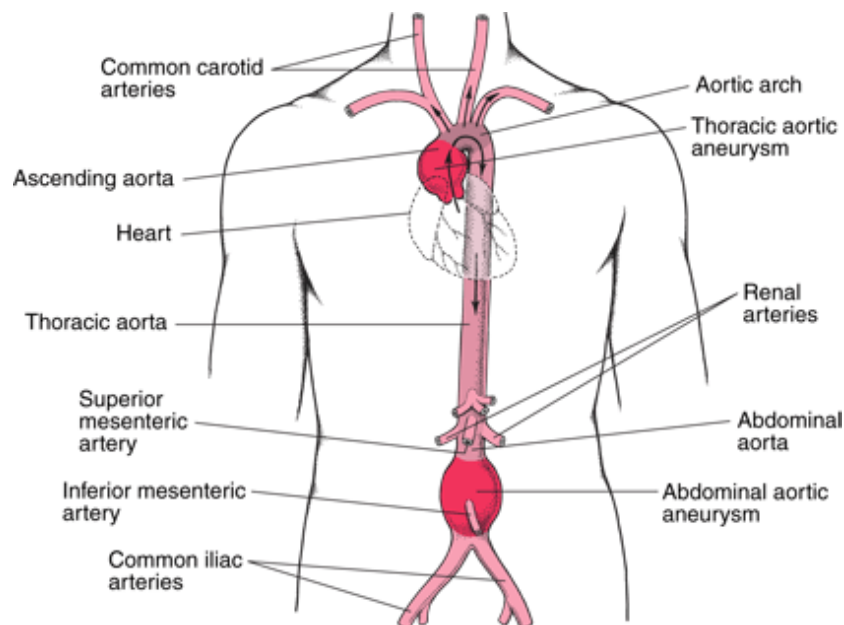

Figure 1. The two most common locations of thoracic aortic aneurysm
(from www.merckmanuals.com)

TAAs and AAAs share some pathological phenotypes, including the loss of vascular smooth muscle cells (vSMCs) and destruction of matrix elastic fibers, but, in addition to different locations, they differ in various physiological and etiological attributes and therefore are considered as two separate diseases [3].

AAAs are almost always due to atheroma associated with identifiable risk factors (i.e. male sex, hypertension and smoking) generally occurring in old age, often in the absence of history and primarily associated with atherosclerosis and inflammation [4]. In contrast, TAAs are usually not related to atheromas and often occur at a younger age with a strong genetic component involved in the etiology. At the molecular level, TAAs are defined by medial degeneration, traditionally termed *cystic medial necrosis*, a poorly understood mechanism characterized by loss of smooth muscle cells, fragmented and diminished number of elastic fibers, and increased accumulation of proteoglycans [5]. TAAs can involve the aortic root, ascending aorta, arch, descending aorta, or a combination of all these locations. The combination of aortic root dilatation and ascending aneurysm is termed "annuloaortic ectasia."

1.1.1 Thoracic Aortic Dissection

Aortic aneurysm is a costly disease in terms of both human lives and medical expenditure since it expands asymptomatically until a catastrophic event such as aortic rupture or dissection, signals the disease.

Dissection occurs when a tear in the inner wall of the aorta causes blood to flow between the layers of the wall of the aorta, forcing the layers apart. The risk factors for aortic dissection include the presence of a pre-existing dilatation, hypertension and hardening of the aortic wall due to atherosclerosis, aortic valve defects and congenital narrowing of the aorta (coarctation) [6].

Like aortic aneurysms, aortic dissections are categorized based on the location/extent of the dissection and are more frequently occurring in association with TAA (Figure 2). Type A dissection is the most common and dangerous type of aortic dissection, demanding prompt surgical treatment. It originates in the ascending aorta, just where it rises out from the heart. At this point, there is no support from nearby structures and the pressure is the highest on the aortic wall. Type B dissections involve the descending aorta and can usually be controlled by blood pressure treatments and regular monitoring.
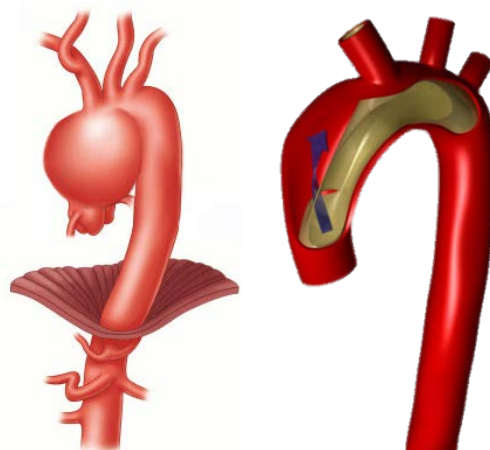

Figure 2. Ascending TAA (left) and type A dissection (right)
(from www.sphcs.org - www.radpod.org)

Individuals with certain genetic diseases, affecting the integrity of aortic wall, run higher risks of having aortic dissection than general population. These heritable disorders include Marfan syndrome (MFS), vascular Ehlers–Danlos syndrome, familial forms of TAAs or aortic dissection and will be further discussed in the next paragraph [7].

1.2 CLASSIFICATION OF TAAs

A variety of pathological conditions may give rise to TAA (inherited syndromes, congenital conditions, hypertension, atherosclerosis, infections) according to which, TAAs can be classified into different groups of idiopathic/sporadic, syndromic, familial and bicuspid aortic valve-associated.

- *Idiopathic/sporadic forms of TAAs*
  Most TAAs are classified as idiopathic/sporadic, occurring in individuals without familial transmission. The pathogenesis of those aneurysms, which is due to non-inflammatory processes such as hypertension, is often associated with medial degeneration of the elastic aortic walls. An approximately 2:1 male predilection for non-inflammatory TAA exists. Of note, atherosclerosis which provides the inflammatory component to some cases of thoracic aneurysms, accounts for only 1% of ascending TAAs while it is associated in 90% of the cases with descending TAAs [8].

  Infections are rare causes of sporadic inflammatory-type TAAs. Syphilitic aneurysms result in marked aortic root dilatation with ascending aneurysms but are extremely rare in industrialized countries, accounting for less than 1% of TAAs [9]. Other rare infectious bacterial aneurysms, termed mycotic aneurysms, [10] occur mostly in the descending aorta and are the consequence of bacteremia in patients with a history of infectious endocarditis.

- *Syndromic TAA*
  MFS is the most common inherited form of syndromic TAAs and is a multi-systemic disorder in which aneurysms occur in association with a wide range of other clinical abnormalities (eyes, skeleton, and cardiovascular system). MFS results from mutations in the fibrillin 1 gene located on chromosome 15q15-31. Since the first mutation was described in 1991 [11], more than 1,000 different (mostly missense) mutations in this gene have been identified. Recently mutations in the SMAD3 gene have also been identified in families with familial forms of TAA [12]. This TAA is termed Marfan-like syndrome for the skeletal features similarities shared with MFS.

Loeys-Dietz syndrome is an autosomal dominant connective tissue disorder that is a rare cause of TAA. It results from genetic mutations in the transforming growth factor beta receptors 1 and 2 (TGFBR1 and TGFBR2) [13].

Vascular Ehlers–Danlos syndrome (type IV) is an autosomal dominant disorder that also manifests arterial aneurysms, rupture and dissection, as well as thin, translucent skin and easy bruising. It results from mutations in the *COL3A1* gene (a type III collagen) [14] which determine extreme friability and fragility of the aortic tissue.

- *Familial non syndromic TAA*
  This group comprehends the familial TAAs and dissections in which aneurysms/dissection occur in absence of other clinical manifestations and typically follow an autosomal dominant pattern of inheritance. Six different genetic loci have been recognized in families with familial non-syndromic TAAs, but only three genes have been identified: *TGFBR2* in TAA2, *ACTA2* in TAA4 and *MYH11* in familial TAA and patent ductus arteriosus [5, 15].

1.2.1 Bicuspid aortic valve-associated TAA

An important number of non-inflammatory aneurysms are associated with congenital condition due to the presence of a bicuspid aortic valve (BAV), a topic that will be further discussed in the next paragraph. BAV can be highly heritable [16] as well as a sporadic event with low penetrance and its association with TAA is poorly understood. Therefore, BAV-associated aneurysm requires a special classification among the TAAs.

The age of disease manifestation is largely affected by different TAA etiologies and three different clinical populations can be identified within the group of patients with TAA, as illustrated in figure 3 [17, 18]. Patients with inherited disorders (also called monogenic TAAs) have an early disease onset at childhood or adolescence and require surgery very early in their life. Patients with idiopathic aneurysms (also referred as to a degenerative pathology) are the oldest at the time of undergoing surgery and patients with BAV are somewhere in between the monogenic and the idiopathic TAAs.
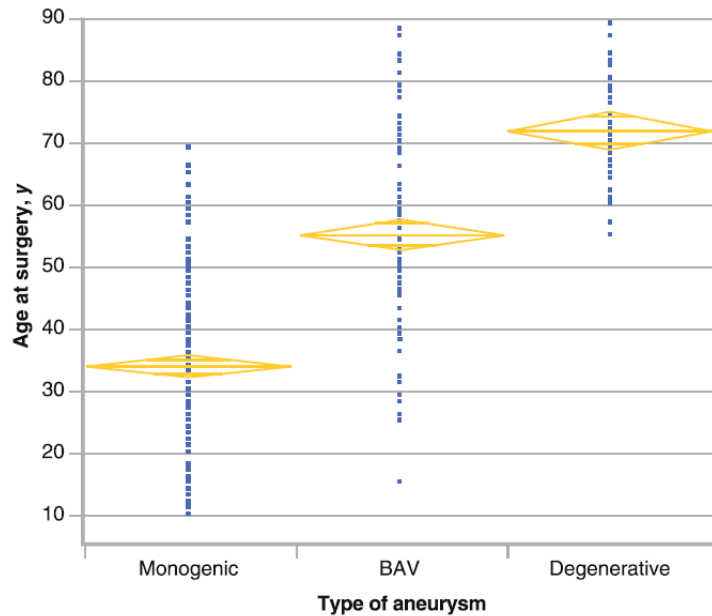
Figure 3. Age at surgery of different types of TAAs patients. Permission obtained from [18].

1.3 BICUSPID AORTIC VALVE (BAV) AND TAA

A bicuspid aortic valve is an aortic valve that only has two leaflets (cusps), instead of three (Figure 4). BAV has been recognized as a common congenital abnormality for centuries and was firstly described by Leonardo da Vinci [19]. BAV results from abnormal aortic cusp formation during valvulogenesis, where adjacent cusps fuse to form a single aberrant cusp, larger than its counterpart yet smaller than 2 normal cusps combined.
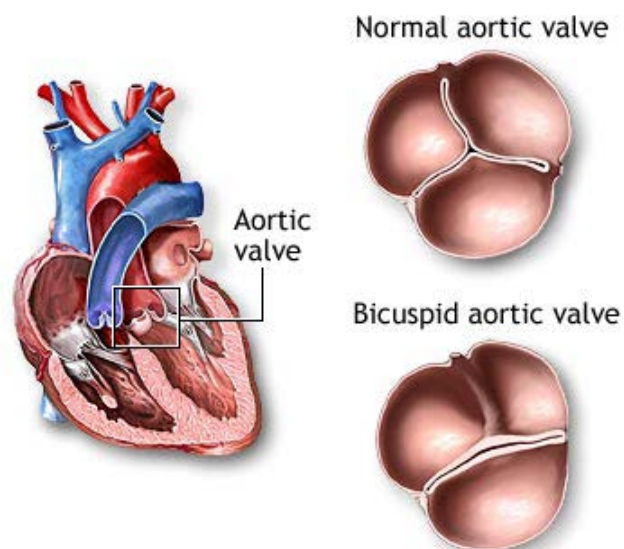

Figure 4. Schematic representation of normal TAV and BAV
(from www.scripps.org)

BAV is often referred to as a syndrome since in addition to the valve abnormalities can be accompanied by various left heart lesions such as hypoplastic left heart, arch hypoplasia and aortic coarctation [20]. In adulthood, complications are common: the presence of a BAV represents a high likelihood of requiring interventions on the aortic valve and/or aorta which makes the burden of diseases associated with BAV more significant than any other congenital cardiac defect [21].

Aortic stenosis (AS) is the most frequent complication of BAV, consisting of calcium deposits on the leaflets with consequent hardening and stiffness of the valve, a condition that in many cases requires aortic valve replacement (Figure 5 b).

Aortic regurgitation/insufficiency can also occur in the presence of a BAV, caused by cusp prolapse, fibrotic retraction, or dilation of the sinotubular junction (Figure 5 c).

Figure 5. Normal TAV (a). Diseased stenotic BAV (b) and diseased regurgitant BAV (c)
(from www.heart-valve-surgery.com - www.quizlet.com - www.docstoc.com)

As mentioned above, there is a significant clinical association between TAA and BAV. BAV is the most common cardiovascular malformation which affects approximately 1-2% of the general population with a male predominance of approximately 2:1 [22-25]. TAAs affect about 50% of patients with BAVs and are associated with increased risk of dissection and rupture [23, 26, 27].

The commonly observed phenotypes of aneurysm associated with BAV disease are shown in figure 6. The most common type is a supracoronary enlargement with preservation of the sinotubular junction (type 1). The next most common demonstrates ectasia of the ascending aorta and root with loss of the sinotubular junction (type 2). The least common is similar in appearance to the aneurysm typical of MFS and is confined to the root itself (type 3) [28].
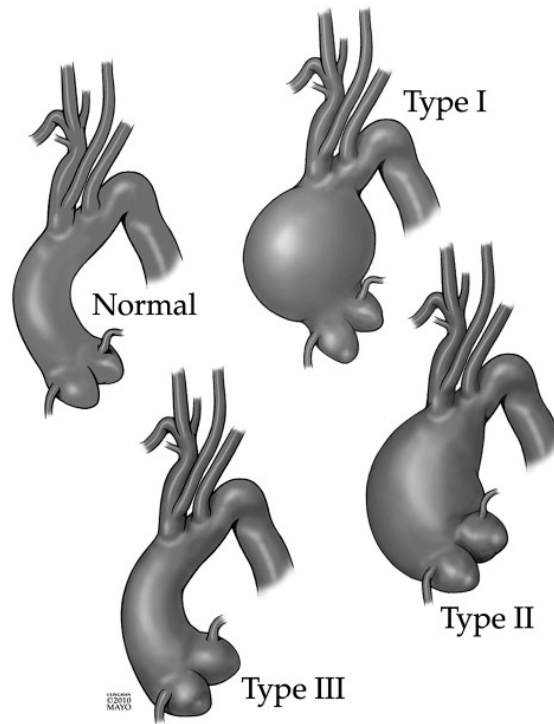
Figure 6: Types of ascending aortic aneurysms. Permission obtained from [28].

The autosomal dominant inheritance of BAV (Online Mendelian Inheritance in Man #109730, www.omim.org) in some families is well-documented; however, the inheritance of BAV with TAA is less clear. Two major hypotheses describing the increased prevalence of ascending aortic dilatation, rupture, and dissection in patients with BAV have been proposed [29].

The first is that a genetic or developmental abnormality present in patients with BAV decreases aortic wall strength and predisposes it to complications [30]. Family-based studies suggest that BAV-TAA syndrome follows an autosomal dominant pattern of inheritance in their families [31]. Moreover, the strongest genetic link found in human so far seems to be mutations in the NOTCH1 gene in a subset of patients with combined BAV-TAA [32]. However, more recently, with the development of more advanced measurement techniques, the contribution of hemodynamic factors to the development of aneurysm in BAV has also gained further ground. The second theory relies on the higher blood velocity and eccentric flow jets caused by a BAV which can lead to increased shear stress on the ascending aortic wall and thereby increasing the risk of ascending aortic dilatation, dissection, and rupture [33].

1.4 HEART VALVE FORMATION: A SOURCE FOR UNDERSTANDING THE PATHOGENESIS OF BAV CONGENITAL MALFORMATION

Heart valves formation is a coordinated process in which growth factor signaling and extracellular matrix proteins meticulously orchestrate the migration of specific primordial cells that will constitute the heart valves, an event occurring simultaneously to the heart chambers formation (Figure 7). The developing heart tube contains an outer layer of myocardium and an inner lining of endothelial cells separated by an ECM referred to as the cardiac jelly. The formation of the cardiac jelly, a gelatinous substance present between the endothelium and myocardium of the embryonic heart, starts in the middle of the third week after conception in humans and is thought to be stimulated by bone morphogenetic protein-4 (BMP4) [34].

During heart valve formation, a subset of endothelial cells overlying the future valve site are specified to delaminate, differentiate, and migrate into the cardiac jelly, a process referred to as endothelial-mesenchymal transition or transdifferentiation (EndMT). This is followed by a process of localized swellings of both cardiac jelly and mesenchymal cells which will eventually result into the formation of cardiac cushions, the primordial structures of the valves (Figure 8). Cardiac cushions undergo extensive remodeling from bulbous swellings to eventual thinly tapered heart valves [35].
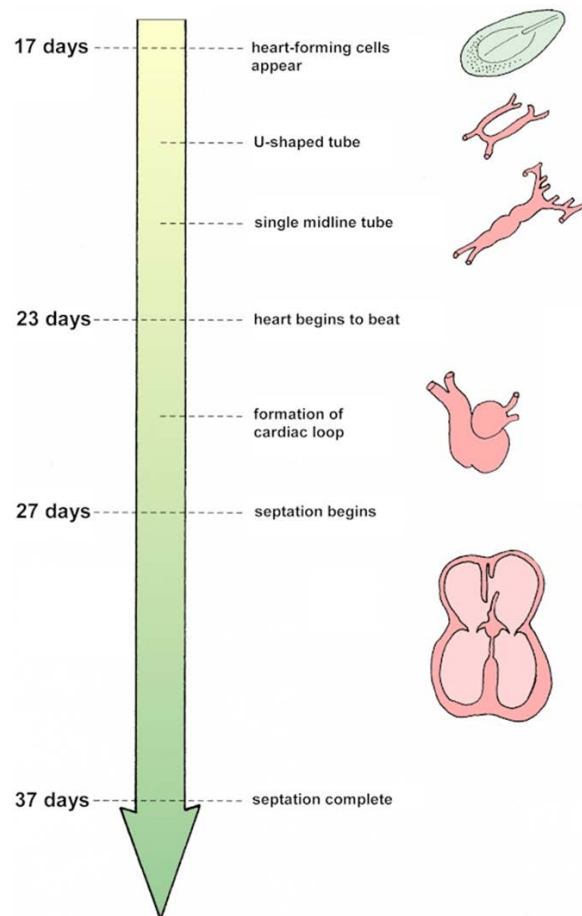

Figure 7. Development of the embryonic heart from 17 days after conception to 37 days (from www.bionalogy.com)
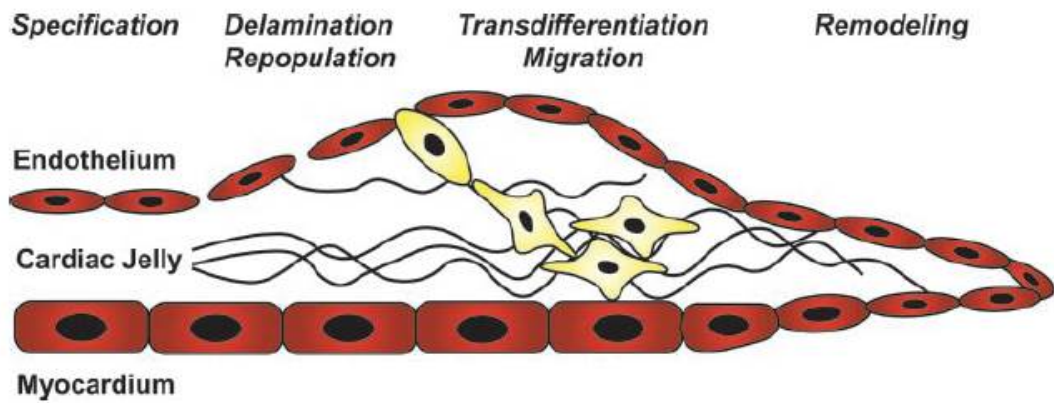
Figure 8. Valvulogenesis. Permission obtained from [35].

The human embryonic heart starts to contract at 65 beats per minute at the beginning of the fourth week which a few days later turn into a unidirectional flow [36]. Although cardiac contraction and EndMT start at the same time, the contribution of haemodynamics and growth factors to valvulogenesis at this stage remains unknown and shear forces might be relevant for fine-tuning morphogenesis [37].

Both the formation of cardiac jelly and EndMT process require the constant molecular regulation of different signaling pathways. The coordinated interaction of Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor beta (TGF-β), BMP, NOTCH, hyaluronan and Wnt/β-catenin pathways results in a proper mesenchymal invasion into the cardiac jelly [38]. The signaling pathway responsible for the initiation of EndMT is the BMP/ TGF-β pathway [35, 39].

Endothelial cells receive the myocardial signal and are activated to secrete TGF-β as a latent complex. Activated endothelial TGF-β triggers the initial phenotypic changes of EndMT in an autocrine fashion. Myocardial BMP acts synergistically with endothelial TGF-β to enhance the TGF-β induced mesenchyme formation.

Heart valve development is characterized by increasing complexity and organization of the extracellular matrix (ECM). Before EndMT, the ECM of endocardial cushions is rich in hyaluronan. After EndMT, the mesenchymal cells in the cushions, express a network of collagens and metalloproteinases to promote cell migration [40]. During late gestation and soon after birth, the valve leaflets become stratified into highly organized collagen, proteoglycan, and elastin-rich compartments. Given the important role of ECM in the formation and maturation of the valves, it is not surprising that abnormal expression and distribution of ECM proteins in the valves could give rise to developmental abnormalities such as BAV condition.

The pathogenesis of congenital BAV malformations is still unknown. Some investigators have suggested that environmental factors such as impaired blood flow during valvulogenesis may be responsible for the incorrect fusion of cusps [19]. Others have argued that BAV can have a genetic cause, supported by high association of BAV with congenital abnormalities of the aorta (coarctation of the aorta and patent ductus arteriosis) [41]. Interestingly, bicuspid aortic valves are also found in combination with

other genetic disorders including Turner's syndrome. Approximately 35% of patients with Turner's syndrome and up to 80% of patients with coarctation of the aorta have an associated BAV although the significance of these associations is unclear [42].

1.5   TGF-Β AND TAA FORMATION

TGF-β family members have critical and specific roles during embryogenesis, such as the heart valve formation, and in maintaining the homeostasis state of adult tissues. Perturbations in their signaling pathways have been linked to a diverse set of developmental disorders and diseases, including cancer, fibrosis, autoimmune and cardiovascular diseases [43].

TGF-β superfamily includes TGF-βs, BMPs and the activins/inhibins. All TGF-β family ligands are generated as dimeric precursor proteins and are subsequently cleaved by proteases before secretion. The cellular signal is elicited by binding of the ligand to a complex of type II and type I serine/threonine kinase transmembrane receptors. Each member of the TGF-β superfamily binds to a characteristic combination of type I and type II receptors.

In case of TGF-β1, the most extensively studied member of the superfamily, a 25 kDa homodimeric peptide is formed and, upon interaction with the receptor complex, initiates an intracellular signaling termed *canonical pathway*. This involves SMAD2 and SMAD3, which heterodimerize with the common-mediator SMAD4. The complex is then translocated to the nucleus, where it binds directly or in complex with other transcription factors to DNA and regulates the expression of target genes. Inhibitory SMADs (SMAD6 and SMAD7) bind to the receptors, and prevent the phosphorylation and signaling activity of pathway-dependent SMADs [44].

A *non-canonical* intracellular signaling cascade through p38 MAP kinase can also be activated by TGF-β1 [45] (Figure 9).
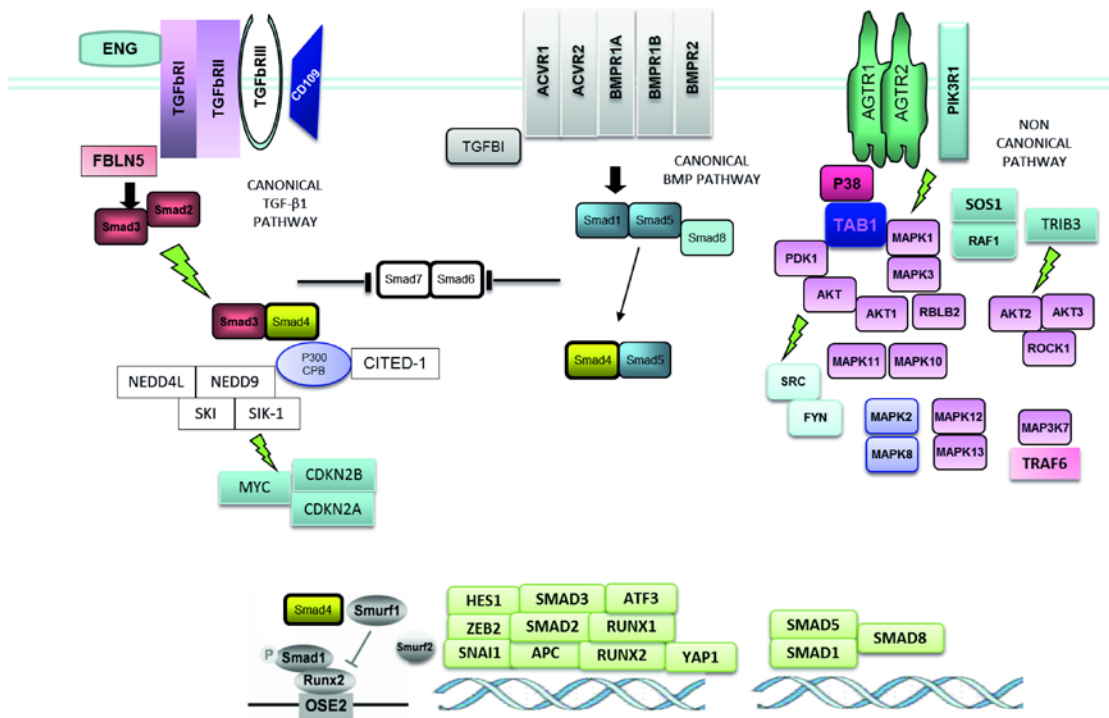
Figure 9. TGF-β signaling pathway *canonical* (left) and *non-canonical* (right)

TGF-β pathway is important in matrix regulation in health and disease, and an increased TGF-β activity is a key factor in the development of various forms of TAAs.

The first indication of TGF-β1 being responsible for aneurysm pathogenesis came from the research field of monogenic forms of TAAs thanks to the discovery of the molecular mechanisms underlying MFS [46]. Fibrillin1, together with other extracellular matrix proteins, contributes to the sequestering of TGF-β1 by maintaining it in a bound and inactive state. Mutant versions of fibrillin1 are incapable of doing so and result in the abundantly accessible TGF-β1 to its receptors. Target genes of TGF-β1 such as metalloproteinase (MMP2 and MMP9) [47] contribute to matrix proteolysis which is the hallmark of TAA. Hence, the increased TGF-β1 activity explains the histological abnormalities observed in aortas of patients with MFS. This includes elastin fragmentation, vascular smooth muscle cells apoptosis as well as other associated-phenotypes, such as bone overgrowth and mitral valve anomalies [48]. Treatment of mutant mice with systemic TGF-β1 neutralizing antibodies reversed some of Marfan phenotypes [49].

As already mentioned, Loeys–Dietz syndrome is another syndromic form of TAA caused by mutations in *TGFBR1* and *TGFBR2,* resulting in aortic dissection and dilatation at early ages. Most mutations are missense mutations that commonly affect the intracellular kinase domain of the receptor, reducing receptor signaling activity in response to TGF-β1. Paradoxically, cells obtained from the aortic wall of patients with Loeys–Dietz syndrome had an increased TGF-β1 activity, highlighting the central role of increased and dysregulated TGF-β1 signaling in aneurysm formation [13]. The

hypothesis proposed to explain this paradox assumes that an alternative TGF-β1 signaling pathway via direct stimulation of TGF-βR2 without TGF-βR1 could be activated. Moreover, the angiotensin II receptor, can initiate a rapid SMAD3-phosphorylation in a TGF-β independent fashion [50, 51]. Cross talks among different signaling can account for SMADs phosphorylation despite TGF-β receptor insufficiency.

1.6 EXTRACELLULAR MATRIX COMPONENTS IN THE AORTIC WALL: THE LOSS OF HOMEOSTASIS IN TAA PATIENTS

Apart from serving as conduit for the blood originating from the left ventricle, the thoracic aorta performs several sophisticated functions. In fact, the ascending aorta allows the propagation of the pulse wave through the vasculature and ultimately influences the efficiency and distribution of blood flow.

The propagation of the pulse wave depends on the structure of the thoracic aorta itself, which combines intrinsic contractile properties with the elastic recoiling capacity. The capacity to withstand the highest blood pressure in the vasculature resides in the structure of the thoracic aorta. To each ventricle ejection at systole, the thoracic aorta responds with a dilatation followed by a return to its initial diameter during diastole.

All arterial walls have the same basic triple layer composition—intima, media and adventitia. These layers are separated from each other by two layers of thick elastic fibers, the internal and external elastic laminae. Depending on their location in the vascular tree, these layers vary considerably in thickness, composition and biological properties.

The basic structural and functional unit in the aortic wall is the lamellar unit [52]. Each lamellar unit is composed of a vascular smooth muscle cell lying between two layers of elastin fibers and surrounded by extracellular matrix proteins containing microfibrils and proteoglycans. Lamellar units are intercalated by collagen bundles (Figure 10).
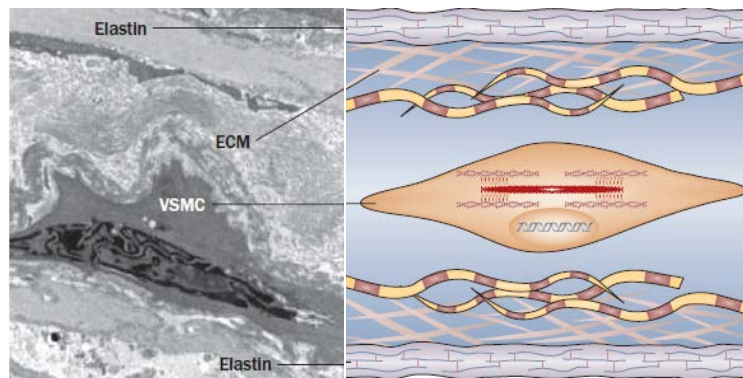
Figure 10. The lamellar unit
Electron micrograph of a single lamellar unit in the medial layer of the aortic wall
Permission obtained from [5].

The interaction and cross-talk between the vSMCs and extracellular matrix components in the aortic wall warrants the various functions of the aorta.

During TAA formation the aortic wall homeostasis can be lost due to:
- dysregulation of one or more of the important players (cells, collagen, elastin, microfibrills).
- dysregulation of important regulatory pathways controlling the aortic wall normal function (tissue repair).

1.6.1 Vascular SMCs

The medial layer of the aorta is populated mainly by vSMCs, which are circumferentially aligned and interspaced by thin layers of elastic fibers. VSMCs are not terminally differentiated [53]: they possess both contractile and secretory properties and depending on the needs (homeostasis state) can alternate quiescent, contractile or proliferative states.

The latter corresponds to the synthetic phenotype that comes into play when a repair of ECM components is required. The vSMCs synthetic properties depend on biochemical signals such as TGF-β1 stimulus. Moreover, vSMCs can transform mechanical stimuli into biological responses (also known as mechano-transduction) [54], leading to intracellular responses and extracellular changes (synthesis, alignment and repair of particular ECM components).

VSMCs loss is a common hallmark of aneurysmal aortic wall independent of the types of TAAs. The importance of integrity of vSMC layer in aorta is manifested by the fact that two SMC specific gene mutations have been linked to the formation of familial non syndromic TAAs: *ACTA2* and *MYH11* encoding for a smooth muscle cell specific actin and a smooth muscle myosin heavy chain family, respectively. *ACTA2* mutations interfere with actin filament assembly and are predicted to decrease SMCs contraction [55]. *MYH11* mutations are associated with marked aortic stiffness and

examination of pathological aortas in tissues bearing this mutation showed large areas of medial degeneration with very low vSMCs content [56].

Interestingly, the embryological origin of SMCs along different region of the aorta is different, highlighting the differences between aortic pathologies in the thorax and abdomen. During the early stages of embryonic development, migration and differentiation of cells from the neural crest give rise to the ascending aorta, the aortic valve and the ductus arteriosus. The abdominal aorta, on the other hand, is mainly composed of cells derived from mesoderm (Figure 11). Differences in cell lineage result into different cellular responses to TGF-β. TGF-β1 stimulation of neural crest-derived SMCs resulted in a significant increase in cell proliferation, activation collagen production whereas the stimulation of mesoderm-derived SMCs did not have the same effect [57]. The different embryonic origin combined with the structural differences between the thoracic and abdominal lamellar units, accounts for the regional heterogeneity within the aorta and the unique properties of the thoracic aorta as described earlier.

Figure 11. Developmental origin of cells populating the ascending aorta. aAo: ascending aorta, dAo: descending aorta, INN: innominate artery, LCA: left carotid artery, LSCA: left subclavian artery, PT: pulmonary trunk, RCA: right carotid artery, RSCA: right subclavian artery. Permission obtained from [58].

1.6.2 Collagen

Type I and type III collagen are the most abundant collagen fibers in the aortic wall [59]. They are present in the adventitia and media layer and provide tensile and mechanical strength to the aorta. Collagen also participates in the activation of

intracellular pathways by interacting with trans-membrane receptors on vSMCs as well as with other ECM proteins. Collagen fibers also act as reservoirs for soluble enzymes and cytokines in the ECM, thereby regulating their function (Figure 12).

Mutations affecting the collagen fibers, such as the ones found in *COL3A1* observed in type IV Ehler-Danlos syndrome, can influence both the structure and the function of the aortic wall.
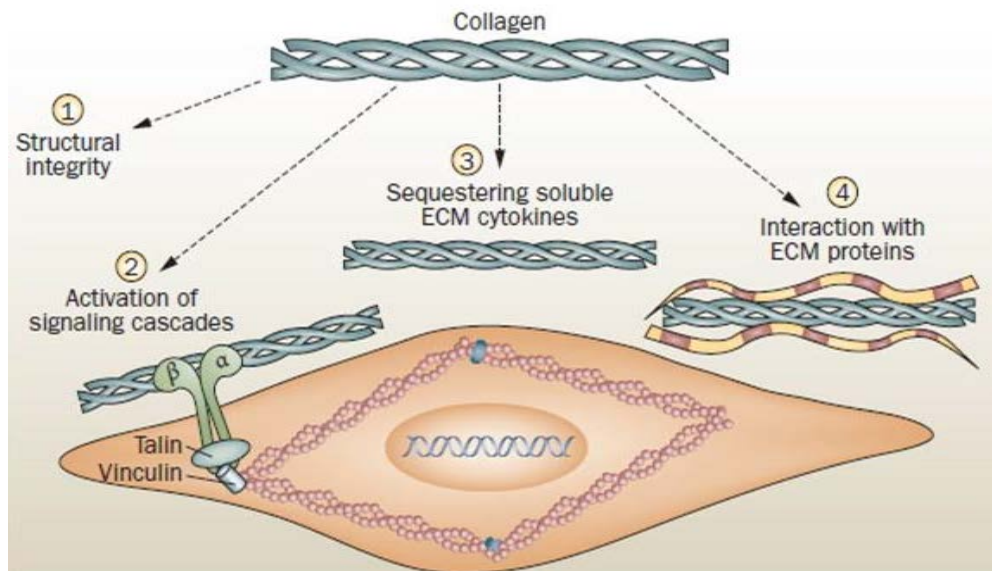

Figure 12. Roles of collagen fibers in the ECM.
Permission obtained from [5].

Collagen has the vital role in maintaining the shape of aorta. Impairments of the collagen homeostasis, either affecting the quality or the quantity of the fibers, underlie the actual dilation and the ultimate mechanical failure of the vessel wall [60].

Collagen biosynthesis - α1 and α2 peptide chain synthesis is carried out on ribosomes located on the endoplasmic reticulum (ER) membrane. The nascent pre-pro-α-chains protrude after translation into the lumen of the rough ER with the help of signal recognition particles and receptors. After removal of the signal peptide, the procollagen α-chains undergo multiple post-translational modifications, including hydroxylation of specific proline and lysine residues, and glycosylation of hydroxylysine. The hydroxylation of lysine residues into hydroxylysines is catalyzed by different lysyl hydroxylases (LH or PLOD).

After undergoing these post-translational modifications the pro- α-chains associate into a triple α-helix through disulfide bounds (formed by two alpha-1 chains and one alpha-2 chain). The α-triplehelix or procollagen is packaged within the Golgi compartment into secretory vesicles and transported outside the cell where is converted to collagen.

The formation of intermolecular and/or intra-molecular cross-links is the final step in the biosynthesis of collagen, essential for physical and mechanical properties of

collagen fibrils as well as for its stabilization. Stabilization of collagen fibers is achieved by the formation of covalent cross-links between neighboring collagen molecules. The major type, hydroxylysyl pyridinoline (HP), is made of three hydroxylysines, and a less abundant form, lysyl pyridinoline (LP) consists of two hydroxylysines and one lysine (Figure 13).

Hydroxylation of lysine residues is a fundamental step in the maturation of collagen, determining the chemical nature of intermolecular cross-links [61]. Depending on the lysine position in the triple helix, specific lysyl hydroxylases will perform the reaction. PLOD2 acts on lysine present in the telopeptide of the triple helix and the conversion into hydroxylysine is fundamental for the formation of cross-linking in general (via generation of pyridinolines) [62]. On the contrary, PLOD1 acts on lysine residues located within the triple helix [63] thereby influencing the amount of hydroxylysine available which ultimately affects the relative amount of HP and LP.
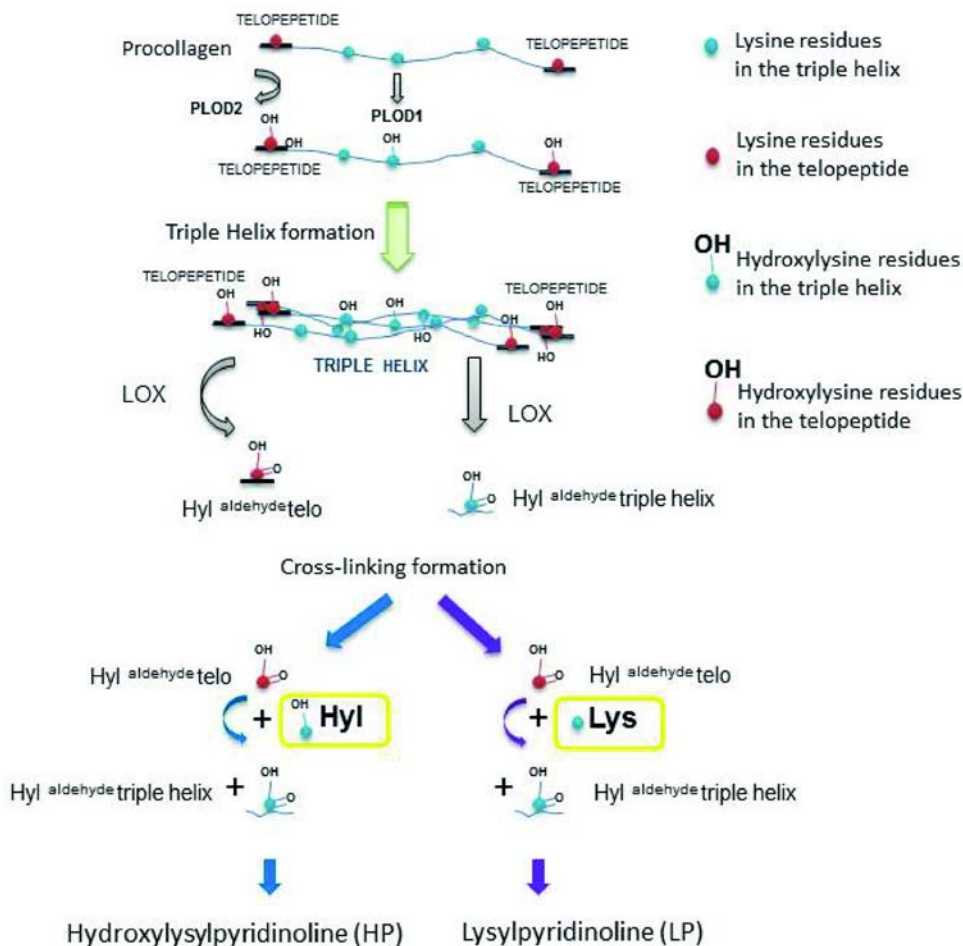

Figure 13. Collagen cross-linking/fibril formation

Mutations in *PLOD1* have been shown to be the cause of the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VI-A) [63, 64], a syndrome associated with severe aortopathy including aneurysm and dissection. *Plod1*$^{-/-}$ mice have a decreased aortic HP/LP ratio and an increased risk of aortic rupture compared to the wild-type mice [65].

1.6.3 Elastin

Elastin is the most abundant ECM protein in the aortic wall, constituting up to 50% of the dry weight of the thoracic aorta. In addition to the structural role, elastin has an active role in regulation of vSMCs in the media layer of the aortic wall through direct interaction with integrin receptors. Elastin modulates cytoskeletal actin organization and limit cell proliferation and migration [66]. In aneurysmal thoracic aortas elastin is fragmented but, in contrast to other ECM proteins, disruption of elastin does not result in aneurysm rupture. Instead, elastin abnormalities result in an uncontrolled proliferative state of vSMCs, which highlights the regulatory role of this protein on vSMCs homeostastis [67] (Figure 14).
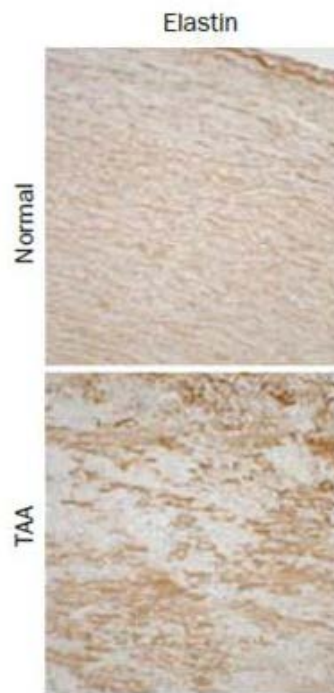

Figure 14. Elastin fragmentation in TAA.
Permission obtained from [5].

1.6.4 Fibronectin

Fibronectin (FN) is a ubiquitous ECM glycoprotein that is assembled into a fibrillar matrix in all tissues and throughout all stages of life. Its assembly is a cell-mediated process [68] and is essential for life [69]. Cells mediate FN matrix assembly through integrin binding to the RGD (Arg-Gly-Asp) cell-binding domain. The primary receptor for FN matrix assembly is α5β1 integrin.

FN is a multi-modular protein and has domains for interacting with other ECM proteins, cell surface receptors, glycosaminoglycans, and other FN molecules. This combination of domains allows FN to bind simultaneously to cells and to molecules within the surrounding matrix (Figure 15).
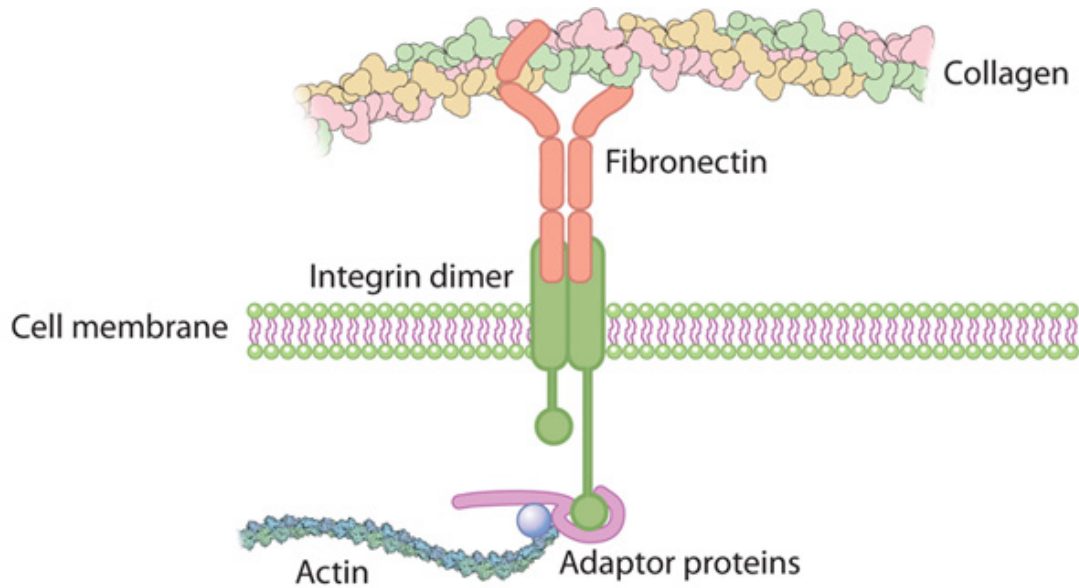
Figure 15. Communication cell-ECM through fibronectin
Permission obtained from Nature Education.

FN is encoded by a ~8-kb mRNA yielding FN subunits that range in size from 230–270 kDa depending on alternative splicing. Two intra-molecular disulfide bonds form within each type I and type II module to stabilize the folded structure. Type III modules are seven-stranded β-barrel structures that lack disulfide bonds (Figure 16).
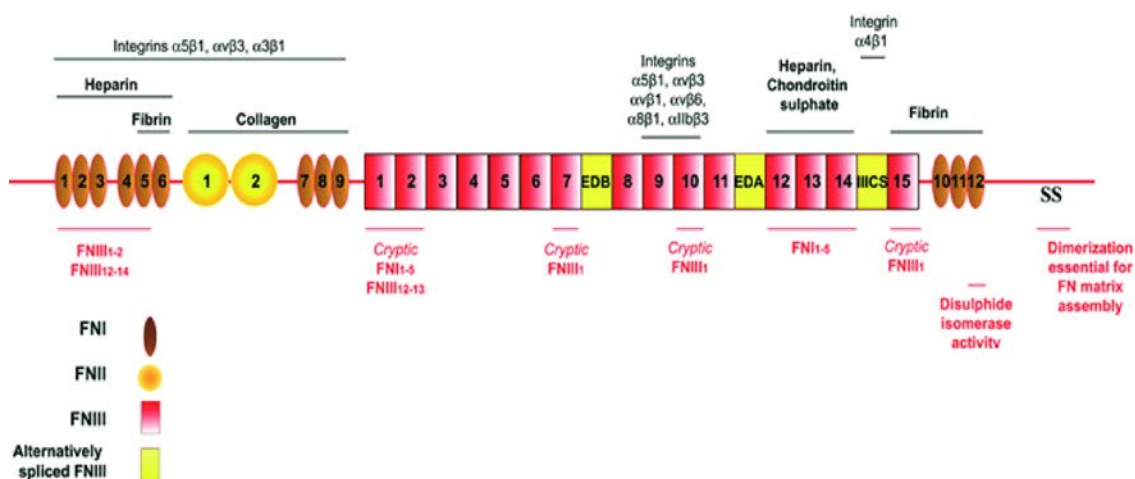

Figure 16. Fibronectin modules. Permission obtained from [70]

The exact composition of FN depends on the tissue type and/or cellular conditions. Alternative splicing occurs by exon skipping at EIIIA/EDA (Extra Domain A) and EIIIB/EDB (Extra Domain B) and by exon subdivision at region IIICS, producing more than 20 different isoforms in humans.

FN exists in two major forms, termed plasma FN and cellular FN. Cellular FN which is made by several cell types including endothelial cells, fibroblasts, smooth muscle cells, macrophages and chondrocytes, is deposited in the ECM as highly insoluble FN filaments. Plasma FN is produced by hepatocytes accounting for 1% serum protein. Cellular forms of FN differ from plasma FN for the inclusion of EDA and EDB domains [71]. *In vivo* studies with experimental animal embryos have shown that cellular forms of FN are expressed in development [72, 73].

Cellular FNs are the only isoforms present during embryogenesis and exerts important role in developmental processes such as directing the migration of cardiac precursors anteriorly towards the midline to form a single heart tube. The heart tube undergoes right-ward looping to change from anterior/posterior polarity to left/right polarity. Proper positioning and function of the valves is critical for chamber formation and proper blood flow [74]. Once development is complete, the amount of insoluble cellular FNs decrease in a wide range of tissues [75] but it is up-regulated again during the pathogenesis of many disorders, including cancer and fibrosis [76-78]. The "embryonic" splicing pattern is temporally re-established in adult life also in physiological tissue repair and angiogenesis [73, 79].

1.6.5 Tissue repair

The tissue repair process is characterized by the appearance of activated myofibroblasts, a transient population of cells that have a phenotype in between fibroblasts and smooth muscle cells [80]. Myofibroblasts can derive from the loss of contractile phenotype (or acquisition of "synthetic phenotype") of a smooth muscle cell, differentiation of a progenitor resident cell in the stromal tissue, recruitment of mesenchymal precursors, or epithelial to mesenchymal transdifferentiation of epithelial cells. Myofibroblasts, by virtue of acquiring smooth muscle type actin-myosin (α-SMA) complex (Figure 17), can contract the edges of the wound and speed up the wound repair, an event that is regulated by TGF-β1 [81].
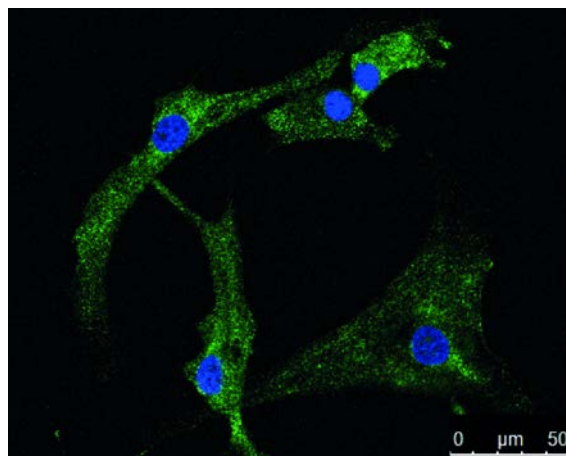

Figure 17. Myofibroblasts positive for α-SMA

Meanwhile, the production of ECM is heavily activated and the FN containing EDA exon is one of the most important activated proteins in this process. The promotion of EDA inclusion is influenced by TGF-β1 [82, 83] (Figure 18).

TGF-β1, EDA-FN and myofibroblasts activation can all be contextualized in the thoracic aorta undergoing dilatation. Weakening of the aortic wall (depending on different TAA etiologies) results in ECM degradation. As a physiological response, tissue repair is triggered, driven by TGF-β that exerts its action by activating myofibroblasts, ultimately targeting fibronectin and its splicing variants to the damaged vascular tissue.
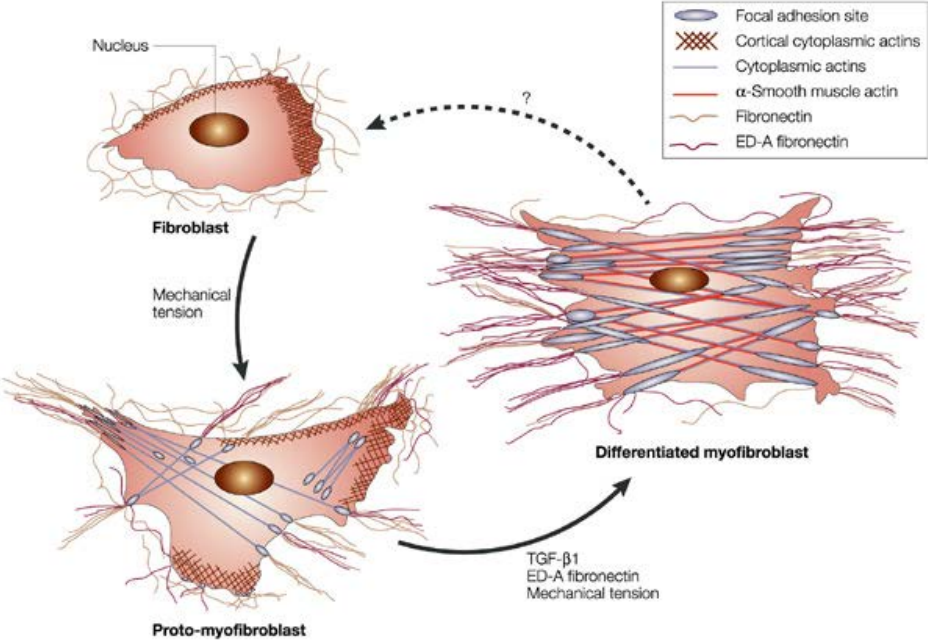

Figure 18. TGF-β1 via increase of EDA-FN promotes the differentiation into mature myofibroblast. Permission obtained from [80].

2 AIMS OF THE STUDY

The overall aim of this thesis was to characterize the molecular events underlying the differences in aneurysm development in BAV compared to TAV.

Specific aims:

- As the alternative spliced EDA-fibronectin is a crucial player in wound healing response, the aim of Paper I was to investigate the expression of EDA-fibronectin with aneurysm development in BAV and TAV aortas.

- Since the production of the EDA-fibronectin depends on TGFβ signaling, the aim of Paper II was to investigate if alternative splicing events occurring in TGFβ pathway could discriminate aneurysm development in BAV and TAV.

- As TGFβ was pointed as a discriminator of BAV and TAV aneurysm and it is known to be dysregulated in monogenic form of TAA, the aim of paper IV was to investigate whether there are inherent differences between BAV and TAV cell response to TGFβ treatment.

- Given the important role of collagen in providing strength to the aortic wall, the aim of paper III was to evaluate collagen synthesis and cross-linking in patients with BAV using normal TAV patients as a reference.

3 COMMENTS ON METHODS

This chapter refers to material and methods used during the work underlying this thesis. For further details, see the Materials and Methods section of Paper I-IV, respectively.

3.1 PATIENTS

All the paper presented in this thesis are based on the ASAP study (Advanced Study of Aortic Pathology) [84].

ASAP is a prospective study enrolling a total of 600 consecutive patients undergoing elective surgery due to aortic valve disease and/or ascending aortic aneurysm at the Cardiothoracic Surgery Unit at Karolinska University Hospital. Exclusion criteria included indications for other concomitant valve surgery, presence of significant coronary artery disease and/or previous cardiac surgery. This study was approved by the Ethics Committee at the Karolinska Institutet and patients were included after informed, written and signed consent.

The ascending aorta was considered to be non-dilated at a diameter of 4.0 cm or less and dilated at a diameter of 4.5 cm or more [85]. Subjects with aortic dimensions of 4.1 to 4.4 cm were not included in the studies. Aortic biopsies were taken from the anterior (convex) part of the aorta i.e. the site of aortotomy a few cm above the aortic valve.

All patients were operated on through a midline sternotomy using cardio-pulmonary bypass and cardiac arrest. For isolated aortic valve replacement a biological or a mechanical valve prosthesis was used. Patients with only dilatation of the ascending aorta received a single tubular graft prosthesis while patients with combined valve and ascending aortic pathology were operated on using a valve prosthesis combined with a supracoronary graft or alternatively a mechanical or a biological composite graft.

3.2 RNA AND PROTEIN ANALYSES

The intima-medial layer of the aortic specimens was isolated by adventicectomy [86]. RNA and proteins were extracted from the intima-media fraction for expression analysis purposes. The RNA samples were hybridized and scanned at the Karolinska Institute microarray core facility.

Gene arrays are powerful tools that allow to measure expression of thousands of genes from a single sample. We chose to use the Affymetrix GeneChip® Human Exon 1.0 ST array, a special type of array that enables two complementary levels of analysis:

gene expression and alternative splicing (Figure 19). In fact, it is designed to cover each exon of the human genome with approximately four probes per exon and roughly 40 probes per gene. The "gene-level" expression analysis implies that the multiple probes on different exons are summarized into an expression value of all transcripts from the same gene.
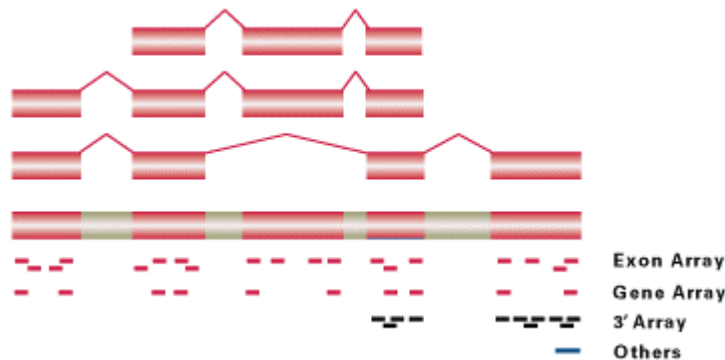

Figure 19. Exon array design
(from www.bcm.edu)

The "exon-level" analysis whole-genome scale was especially relevant in paper II, where we investigated the splicing of 26 selected genes belonging to the TGF-β pathway. The expression level of each of the 614 exons (all the exons accounting for the 26 genes) was normalized with respect to whole transcript (gene) expression (splice index).

$$\text{Splice Index} = \log_2 (\text{exon intensity/transcript expression level})$$

In parallel to RNA expression profiling studies, the aortic media biopsies were also used for validation at the protein level. Tissues were freshly dissected, homogenized to obtain protein lysates and western blot analysis and immunohistochemistry were performed.

3.3 STATISTICAL ANALYSES

When dealing with microarray data it is important to correct for random events that falsely appear significant. In a typical microarray experiment sometime, more than 10,000 separate hypotheses are tested. If a standard p-value cut-off of 0.05 is used, 500 genes would be falsely believed "significant" by chance. Therefore the importance of setting a multiple testing correction is pivotal. In all papers paired and unpaired Student's T-tests were used and the significant p-values for each comparison were adjusted by applying the Benjamini and Hochberg False Discovery Rate method.

In paper I the gene set enrichment analysis was performed comparing TAV and BAV patients. This algorithm tests if a particular group of genes show more differential

expression than it could be expected from a randomly selected group of genes of the same size [87].

In order to understand the attributes of the 217 genes found in paper IV a gene ontology analysis was done. This allowed us to gain a broad overview of our gene set and find out which gene ontology categories are 'enriched' for our 217 genes.

In paper I, II and IV the TGF-β signaling pathway was investigated by the usage of a multivariate analysis (MVA) approach, a statistical technique used to analyze data that arise from more than one variable. The screening of several patients with regard to gene/exon level expression generates multidimensional data. Each patient will thus have a unique expression profile that is a summary of all the genes/exons present in the data set, an expression fingerprint for that particular patient. Patients that show similar profiles to each other will group together in the MVA plot.

For example in paper II and IV each patient could be positioned in a multidimensional space by the expression of genes involved in the TGF-β signaling pathway. With MVA it is possible to consider all the variables simultaneously and observe the existing inter-dependency among them. Two tools are used in MVA: Principal component analysis (PCA) and orthogonal partial least squared projection to latent structure discriminant analysis (OPLS-DA). PCA is a mathematical algorithm that can reduce the dimensionality of the data while keeping most of the variation in the data set.

In our study, for each patient we had more than 10,000 dimensional gene expression profiles which were projected onto the principal components. Along PC1 the variation in the data is maximal. The second principal component (pc2), perpendicular to pc1, accounts for the second largest variation in the data and results into a two-dimensional plot (Figure 20 a). By adding a third principal component we further increase the capability of the PCA model to describe the variation unexplained by the two previous components and eventually better detect interdependency among variables that can result in clustering of the sample (Figure 20 b).
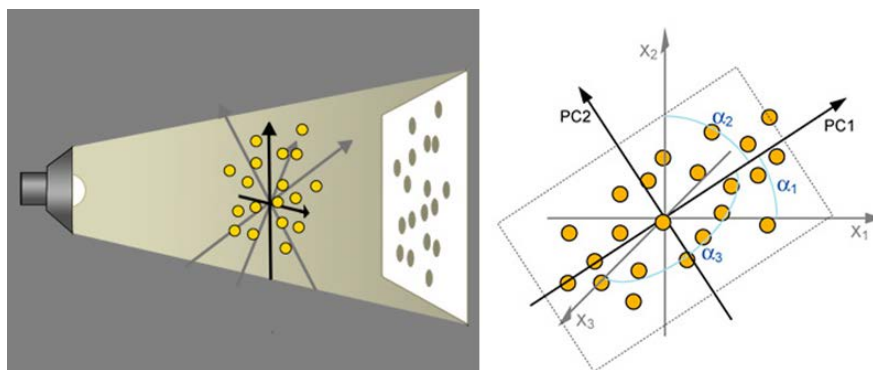

Figure 20 a. Projection of multiple variables into pc1 and pc2
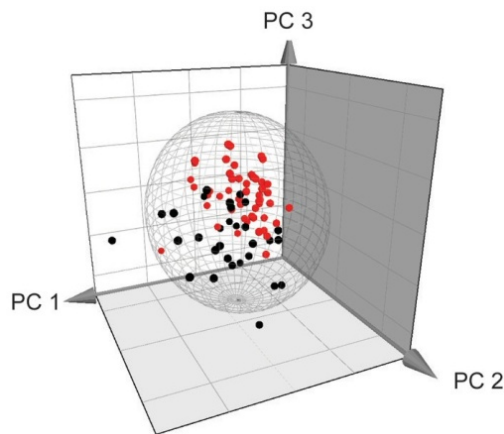(from www.umetrics.com)

Figure 20 b. 3D PCA, each dot represents a patient and its position in the 3D space depends on the gene expression profile measured by the exon array platform.

PCA is useful to get an overview of a dataset and was chosen to visualize graphically the distribution and clustering of the ASAP patients with BAV or TAV, based on the expression profile of selected genes. Additionally, with OPLS-DA it is possible to analyze together the quantitative relationship between a data matrix **X**, in our case expression levels of different 614 exons belonging to TGF-β pathway, and a vector (or matrix), **Y**, containing qualitative values (class belonging to BAV or TAV phenotype). The main idea of OPLS-DA is to separate the systematic variation in **X** into two parts, one that is linearly related to **Y** and one that is unrelated (orthogonal) to **Y**. In this way it is possible to remove systematic variation from **X** that is not correlated to the response set **Y**, thereby giving the opportunity to study only the variation that is correlated to the classification of interest. It is also termed as *supervised* analysis since it implies the assignment to a certain group, in our case being for example a BAV or a TAV based on medical records.

3.4 CELL ISOLATION

Human aortic SMCs were isolated from TAV and BAV aortic biopsies. Depending on the size of the biopsy, AoSMCs were either obtained by enzymatic digestion or by explant outgrowth technique [88].

The enzymatic method was used in case of dilated aorta biopsies (Figure 21): the cells preparation consisted of an immediate dissection to separate medial and adventitial layers followed by enzymatic digestion. Medial samples were incubated in a collagenase 0.3% solution for 2 hours at 37°C under shaking, to obtain SMC cultures.
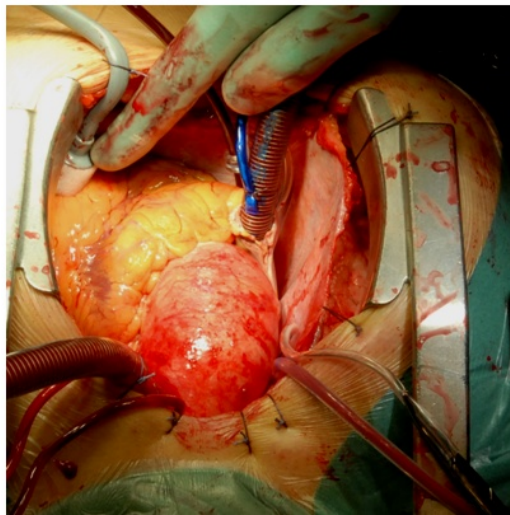
Figure 21. Dilatation of the ascending aorta

In the case of non-dilated aortas, small biopsies were available and the explant technique was the method of preference (Figure 22). The media was firstly separated from adventitia layer and then kept in culture wells with specific medium for approximately 10 days, time necessary in order to detect SMCs outgrowth from the tissue.
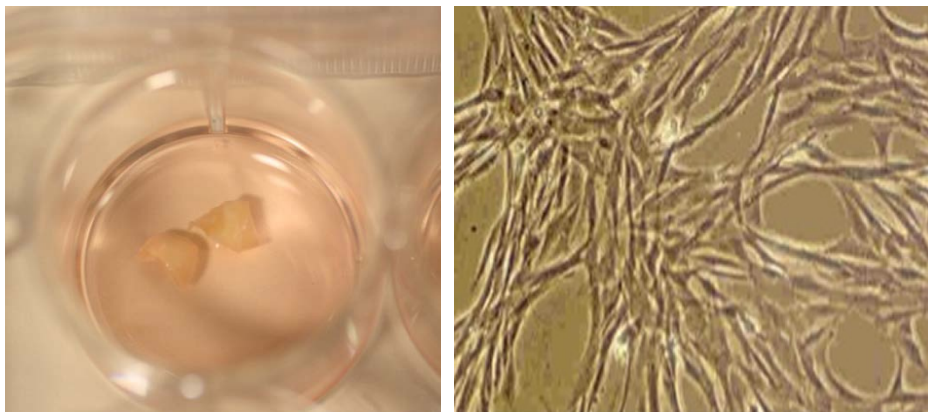

Figure 22. Explant outgrowth

In paper IV valves biopsies were also used for the isolation of myofibroblasts. The enzymatic method was preferred in this case. The valves could be stenotic (with different amount of calcium deposits) or regurgitant (very thin leaflets) (Figure 23). To facilitate the digestion process of the stenotic valves the hard and calcified deposits were removed.

Cells, either AoSMCs or myofibroblasts were held in culture and treated between passages 3 to 6 with TGF-β1 for 6 hours in serum free medium to avoid any interaction of TGF-β1 with the medium growth factors.
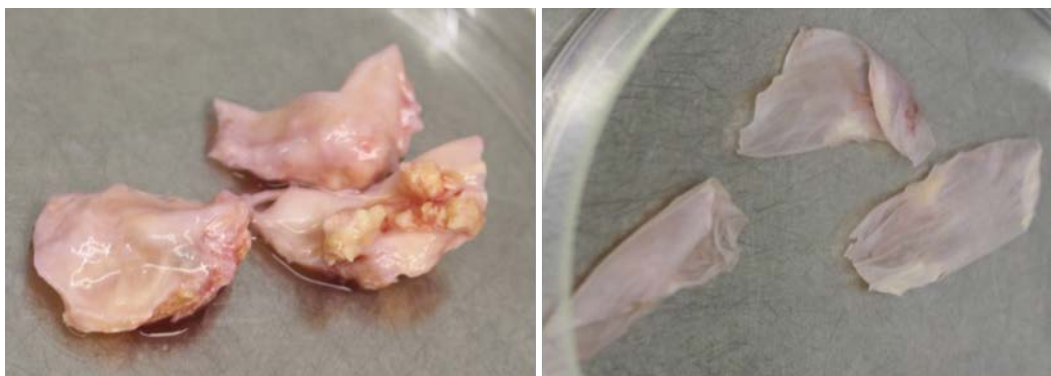
Figure 23. Appearance of removed stenotic (left) and regurgitant valve (right)

3.5   COLLAGEN ANALYSES

In paper III the amount of collagen and its cross-linking forms was measured by High-performance liquid chromatography (HPLC) a technique used to separate a mixture of compounds. The separation of the sample mixture occurs through the interaction with the column particles and spectrophotometric techniques are used to measure the concentration after the elution. After deparaffinization in xylene of 10-µm slices of paraffin-embedded tissue, the samples were hydrolyzed in 1 mL 6 M HCl in 5-mL Teflon sealed glass tubes and then dried and re-dissolved in 1 mL of water containing 10 µmol/L of pyridoxine (internal standard for the cross-links HP and LP) and 2.4 mmol/L of homoarginine (internal standard for amino acids). Derivatization of the amino acids with 9-fluorenylmethyl chloroformate and reversed-phase HPLC of amino acids and crosslinks was performed on a reversed-phase column (150mm×4.6 mm) [89, 90]. The content of collagen, pentosidine (marker for collagen half-life), proline, hydroxyproline, hydroxylysyl, HP and LP (mature collagen cross-linking forms) were measured. Collagen cross-links with fluorescent properties are derived from two different pathways: those initiated by enzymes such as lysyl hydroxylase and lysyl oxidase (HP and LP) and those derived from the non-enzymatic glycation (pentosidine). The crosslinks were optimally detected by their native fluorescence by switching wavelengths of the detector during the assay. The quantities of the cross-link HP were expressed as the number of residues per collagen molecule, assuming 300 hydroxyproline residues per triple helix. This procedure is well established, because hydroxyproline is a collagen-specific amino acid and because the prolyl hydroxylation level in collagen is stable [91]. Amino acids, including the collagen-specific hydroxylysine, hydroxyproline and the glycosylated hydroxylysines were eluted with a multistep gradient system and were separated in less than 30 min, followed by reaction with ninhydrin and fluorimetric detection [92].

Picrosirius red staining was used for the assessment of collagen fibers in aortas: thick mature, tightly packed, and better-aligned collagen fibers were orange, and thin immature fibers were green. To further investigate the collagen structure in the aortas, we performed both scanning electron microscopy (SEM) that provides high-

resolution, three-dimensional images of the extracellular matrix morphology and transmission electron microscopy (TEM) for a closer observation of the collagen filaments size. For SEM, aortic biopsy samples were fixed, rinsed in distilled water and placed in ethanol followed by pure acetone. After drying, specimens were mounted on an aluminum stub and coated with carbon before the analysis at SEM. For TEM, aortic tissues were fixed by immersion in 2% glutaraldehyde and 1% paraformaldehyde and post-fixed in 2% osmium tetroxide. Semi-thin sections were prior stained with toluidine blue and used for light microscopic analysis in order to select areas of the media layer for closer analysis. From those areas ultra-thin sections (40 to 50 nm) were cut, contrasted with uranyl acetate followed by lead citrate, and examined in a transmission electron microscope.

4 RESULTS AND DISCUSSION

This chapter highlights the main findings of each paper. For a more detailed description of the results, see the Result section in Paper I-IV, respectively.

4.1 IMPAIRED SPLICING OF FIBRONECTIN IS ASSOCIATED WITH TAA FORMATION IN PATIENT WITH BAV

Loss of smooth muscle cells and degeneration of extracellular matrix are the main outcomes of an aneurysm developing in the ascending aorta. Fibronectin plays a crucial role in the maintenance of the extracellular matrix homeostasis [69]. Alternatively spliced *extra domain* A of fibronectin appears to be important for cell migration and proliferation, mechanisms that are central for normal functions such as tissue repair and maintenance of tissue integrity and therefore may be involved in aneurysm formation and progression.

Since TAA is a common complication in patients with BAV, in paper I, we analyzed the expression of EDA-fibronectin in dilated and non-dilated ascending aorta of TAV and BAV patients. Microarray data from a total of 109 patients revealed that EDA-FN expression was significantly higher in dilated aorta of TAV compared to BAV patients, a result that was confirmed also at the protein level (Figure 24-25).
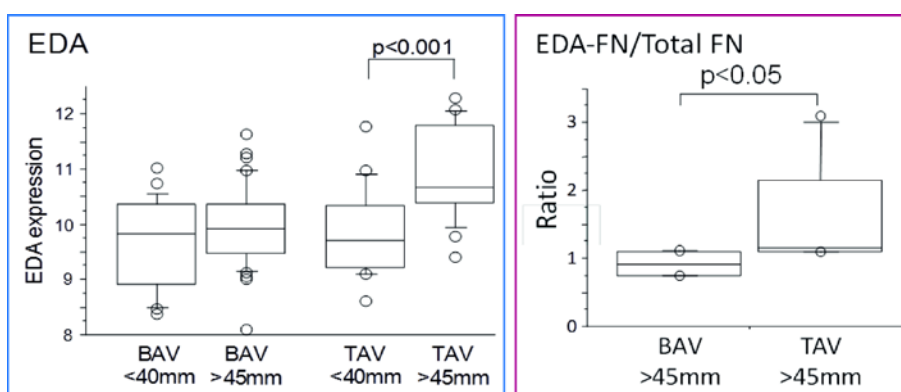

Figure 24. EDA exon expression is higher in dilated TAV compared to non-dilated but remains unchanged in BAV. Real-time PCR was used to measure the ratio between FN containing EDA and total FN forms and highlighted the significantly higher presence of EDA-FN isoform in TAV dilated compared to BAV.
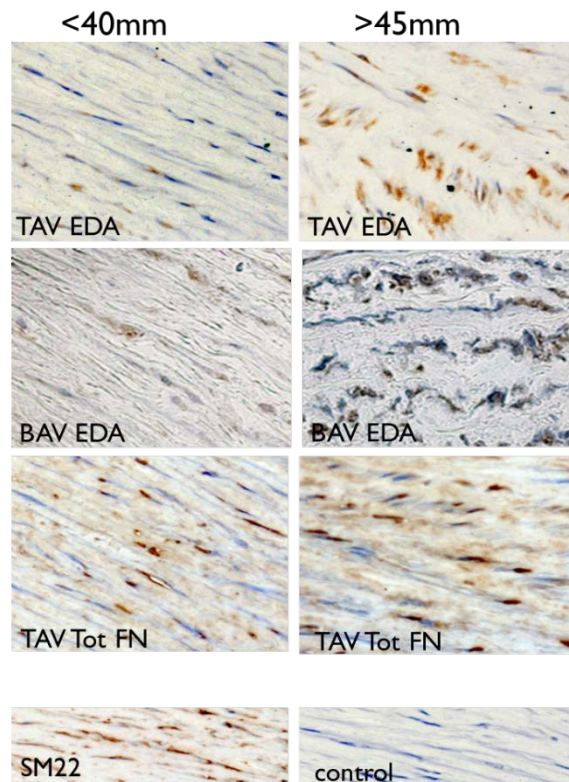
Figure 25. Staining of EDA containing FN in non-dilated (<40mm) and dilated (>45mm) aortic sections. The staining of EDA containing FN was much more pronounced in dilated aorta from TAV patients compared with dilated aorta from BAV patients. Total FN staining showed no different expression in TAV dilated compared to non-dilated.

EDA-FN is normally not expressed in adulthood, unless as a response to some triggering factors, for instance to tissue damage [73]. As expected, we found that the major isoform in the aortic tissue was the FN without EDA. However, EDA-FN isoform could also be detected, exclusively in TAV patients. In particular we observed a higher presence of EDA-FN in association with the dilatation in the aorta (Figure 26).
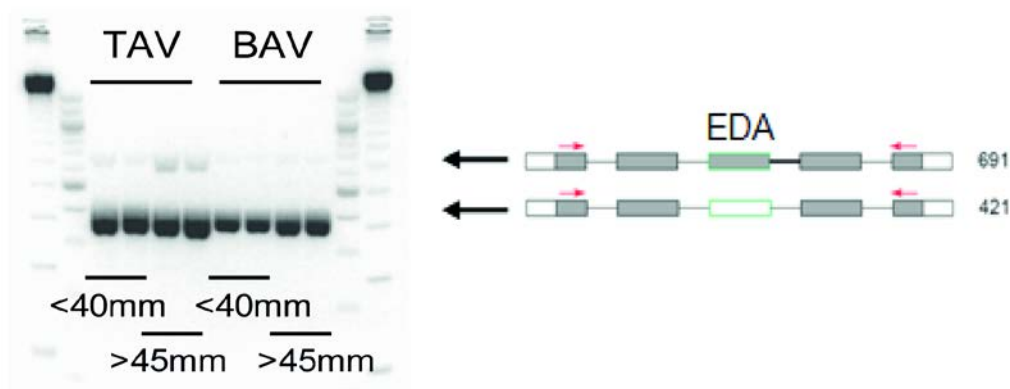

Figure 26. RT-PCR was performed with primers spanning EDA exon. The main isoform (lower band) does not include EDA exon. EDA-FN (upper band) is only present in TAV aortas, with higher intensity in dilatation.

Inclusion of EDA exon is promoted by TGF-β treatment [82, 83]. Defects in the TGF-β signaling pathway has attracted attention in the field of TAA as the cause of the aortic wall degeneration that leads to aneurysm. Therefore we investigated the effect of TGF-β signaling in vSMCs isolated from BAV and TAV aortas as a possible cause of the different FN splicing observed in the tissue. We found that only TAV cells respond to TGF-β1 treatment by stimulating the inclusion of EDA exon (Figure 27).

Figure 27. TGF-β1 stimulation influence positively the inclusion of EDA only in vSMCs isolated from TAV aorta. The main isoform in culture is the EDA-FN (upper band). (Real-Time PCR on the left and RT-PCR analysis on the right)

A possible explanation is that TGF-β treatment can trigger different cascades in BAV and TAV cells (Figure 28) resulting in a deficiency of BAV cells to perform "exon inclusion" splicing mechanism. Using the gene-set enrichment (GAGE) algorithm we examined whether the expression of TGF-β pathway related genes on average differed more than what was expected for the same number of randomly selected genes between BAV and TAV patients. This analysis revealed that TGF-β genes on average showed more significant differences between BAV and TAV than genes in general. The significant differences were detected only in dilated aortas (GAGE probability values = 0.0248).
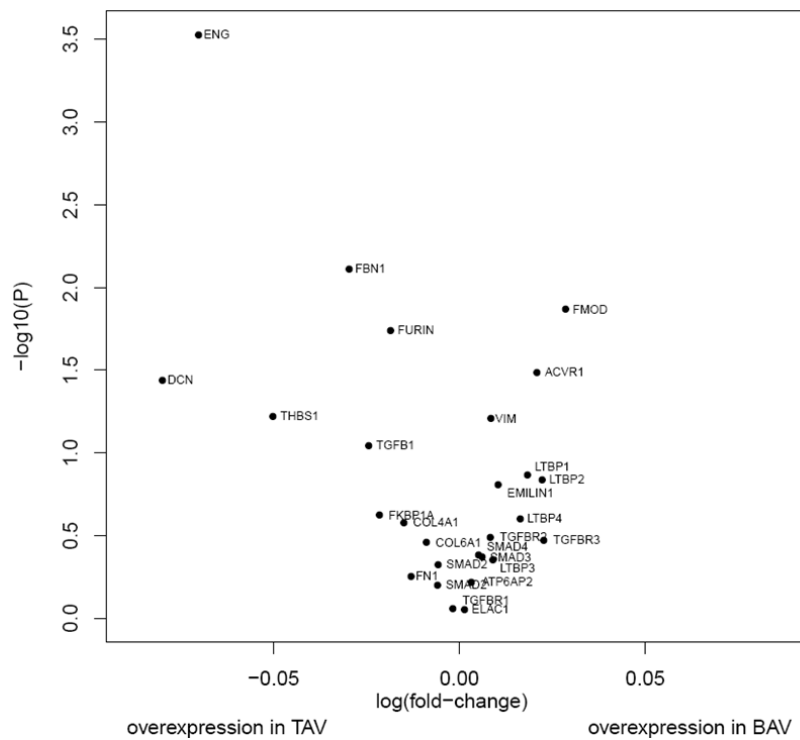
Figure 28. Volcano plot of the TGF-β pathway in BAV and TAV patients. Fold change between the 2 groups is shown on the *x*-axis, and the probability value for a *t* test of differences between samples is shown on the *y*-axis.

To conclude, in paper I we demonstrated that BAV patients have impaired mechanism for the inclusion of EDA in response to injury such as aneurysm formation, which is a possible explanation for their increased TAA susceptibility. The defect in FN splicing signifies impairments of the TGF-β signaling pathway.

4.2 DIVERGING ALTERNATIVE SPLICING FINGERPRINTS IN TGFB SIGNALING PATHWAY IDENTIFIED IN TAA

As a follow up of paper I, we pursued with TGF-β signaling pathway and investigated the alternative splicing phenomenon within the main players of the TGF-β cascade. Differential splicing is a common phenomenon that can influence the function of proteins and thus being an important process in human diseases. In paper II we have investigated the occurrence of differential splicing in the TGF-β pathway associated with TAA in patients with BAV and TAV. Exon array data from 81 patients, 51 dilated (15 TAV, 36 BAV) and 30 non-dilated (14 TAV, 16 BAV) were utilized in this study. All known exons of TGF-β pathway related genes were subjected to alternative splicing analysis.

For this purpose a comprehensive gene-list consisted of all the important players in TGF-β pathway, including fibronectin, was created. This list contains 26 genes which

correspond to 614 exons. Importantly, the expression of each exon was normalized to the expression of the entire transcript by calculating the splice index. In order to identify differentially spliced TGF-β exons and their potential contribution to dilated aorta in patients with TAV and BAV, multivariate data techniques such as PCA and OPLS-DA were adopted together with FDR corrected two-sided Student's T-test.

By applying PCA based on the splice index of the "614 TGF-β exons", we showed that it was possible to discriminate the dilated and non-dilated aorta in BAV and TAV patients (Figure 29).
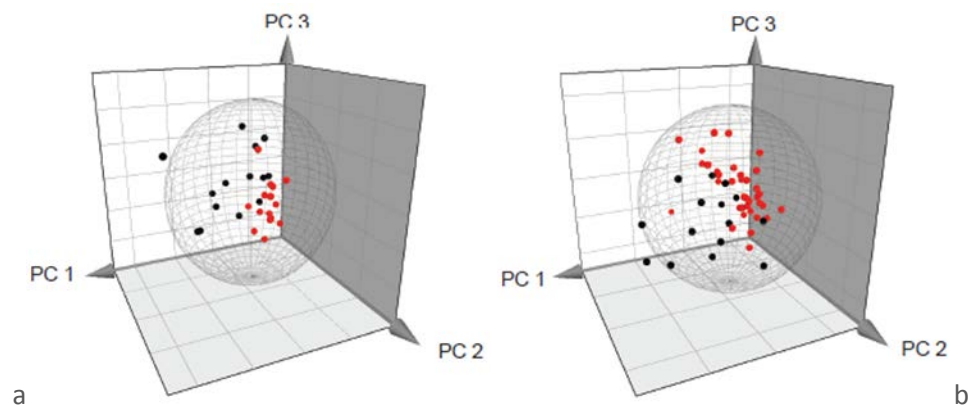

Figure 29. Three-dimensional scores plot showing PC1-PC3 plane of non-dilated (black) and dilated (red) thoracic aorta samples with TAV (a) and BAV (b) patients separately.

The first conclusion drawn from this analysis was that alternative splicing is an important mechanism in aneurysm development, both in BAV and TAV patients. To find out which exons were responsible for separation and therefore were important for the dilatation process, we used FDR-corrected *t* tests between dilated and non-dilated aortas. When this method was applied to TAV patients, 147 exons showed significant differences in splicing, with a cutoff *P* value of 0.023. The corresponding analysis for BAV patients resulted in 101 significant exons with a cutoff *P* value of 0.013. The results were applied to a Venn diagram which showed that among the significant exons 86 were TAV and 40 were BAV differentially spliced, respectively (Figure 30).

Apart from discovering that alternative splicing pattern in the TGFβ pathway is different between dilated and non-dilated aortas, this statistical analysis showed that diverging alternative splicing fingerprints are associated with dilatation in TAV and BAV patients.
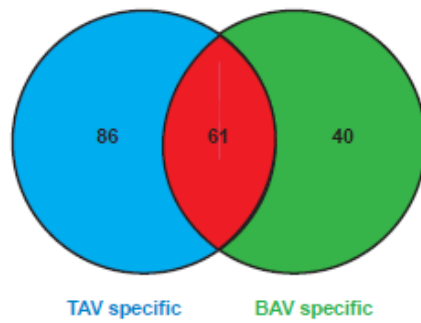
Figure 30. Venn diagram based on FDR corrected t-test:
TAV specific (blue), BAV specific (green), and common (red) exons.

Furthermore, OPLS discriminant analysis was used to extract the contribution to the model (difference between dilated and non-dilated) of the most significant exons, information that is demonstrated by the loading plot (for BAV and TAV), as shown below (Figure 31).
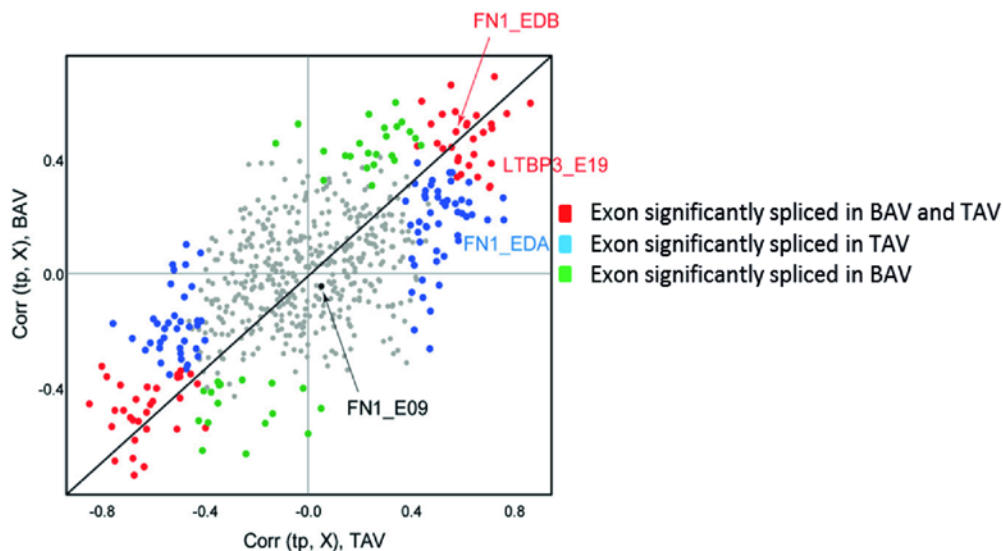
Figure 31. Loadings for TAV model versus BAV model. Significant exons (FDR corrected t-test) in TAV only (blue), BAV only (green) and shared (red) are far from the origo. Non-significant exons are marked in grey.

In accordance with paper I findings, EDA exon of FN, appeared as one of the highest exon responsible for the separation between non-dilated and dilated TAV patients and was validated with RT-PCR on RNA extracted from aortic biopsies (Figure 32-33).
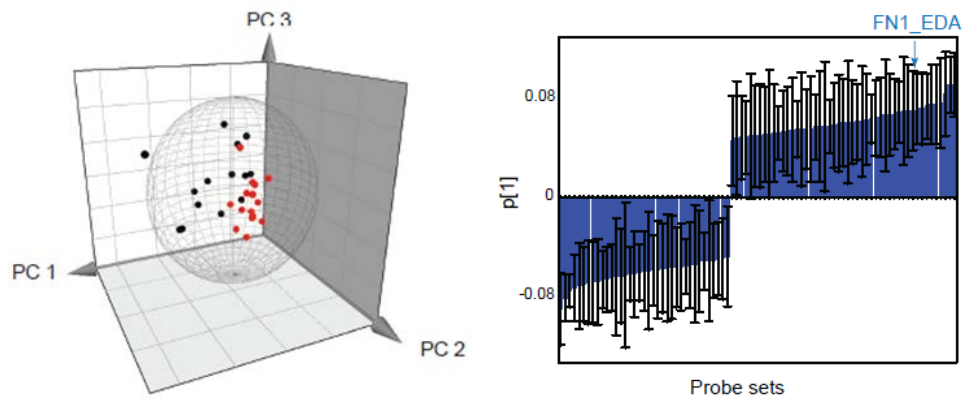
Figure 32. PCA of non-dilated (black) and dilated (red) TAV (left). Bar plot for the loadings of each of the significant exons indicating their contribution to the first PC (Pc1): EDA exon is highlighted.
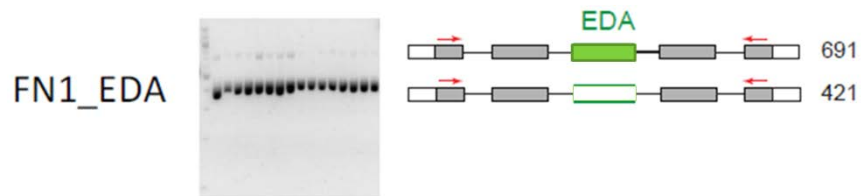
Figure 33. RT-PCR validation of alternative splicing of FN1_EDA.

Conclusions of paper II imply that the dilatation in the two different valve types proceeds via different alternative splicing mechanisms in the TGF-β pathway. The splicing of EDA exon of FN which was observed only in TAV dilated aortas (exon-inclusion) can therefore depend on specific TGF-β pathway splicing pattern (Figure 34).
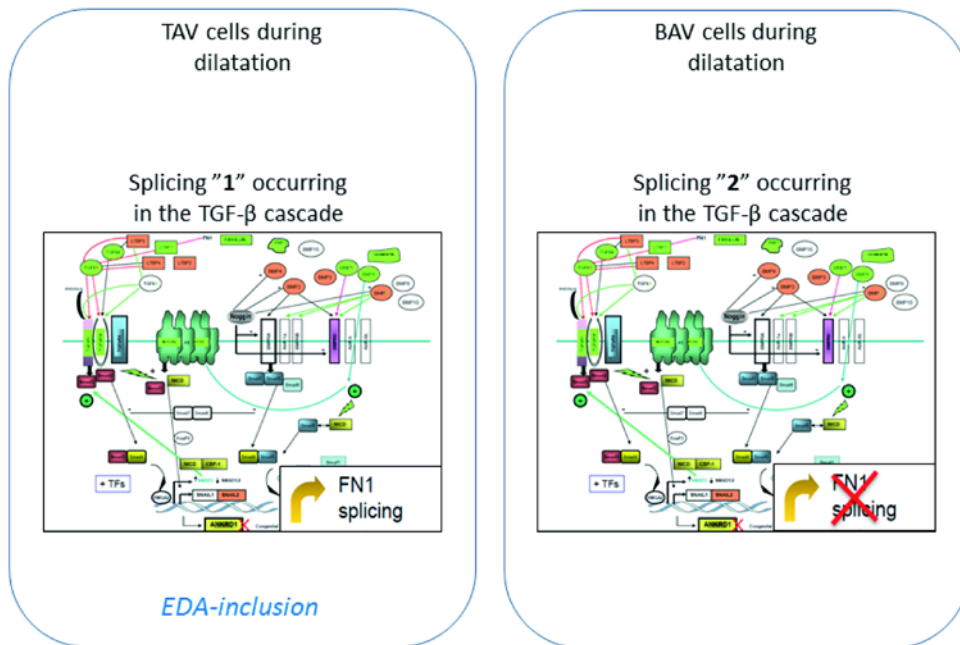
Figure 34. Simplification of the two alternative splicing fingerprints of BAV and TAV resulting in EDA exon inclusion.

4.3 IMPAIRED COLLAGEN BIOSYNTHESIS AND CROSS-LINKING IN AORTA OF PATIENTS WITH BAV

In paper III, we investigated the ECM constituting the aortic wall in BAV and TAV aortic specimens with a particular focus on collagen, the crucial player endowing aorta with strength and resistance.

Masson trichrome staining and high-performance liquid chromatography (HPLC)-based measurements showed that there was a significant increase of collagen in the dilated aortas of TAV (Figure 35). This was interpreted as a response of the TAV aorta to aneurysm formation in an attempt to compensate for ECM degeneration, hallmark of dilatation process.
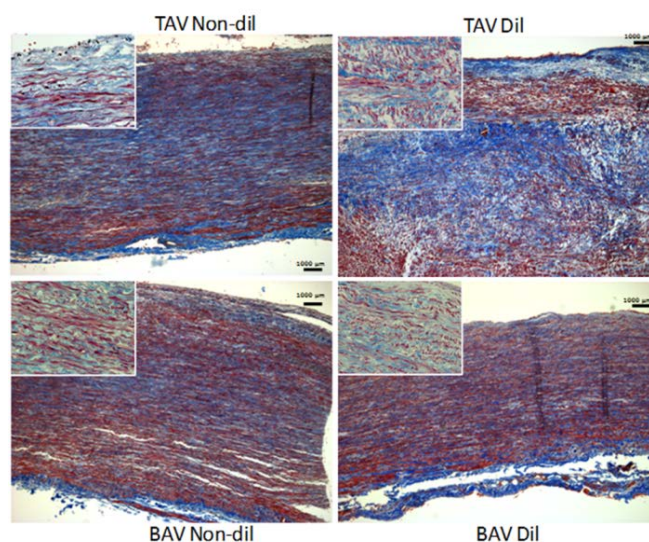
Figure 35. Masson trichrome staining of dilated and non-dilated aortas from patients with BAV and TAV

Moreover, further analysis demonstrated that this collagen is probably newly synthesized, as suggested by the small diameter of the collagen fibers measured by electron microscopy (Figure 36).
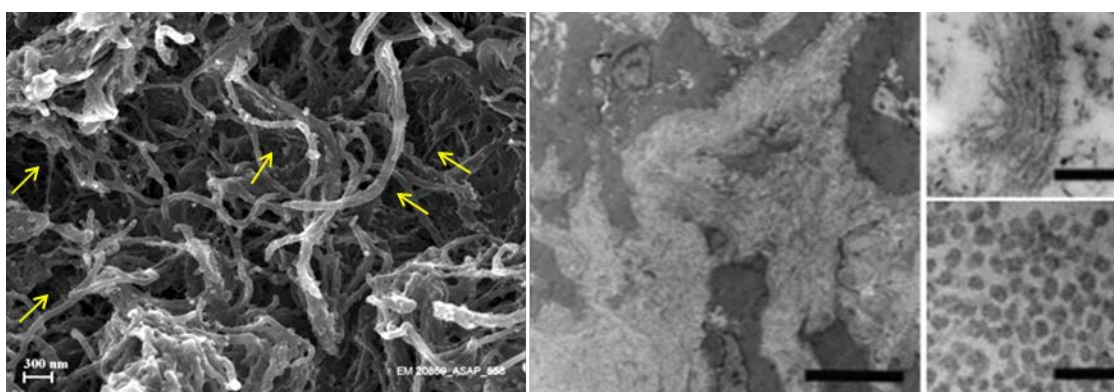
Figure 36. Thin collagen fibers in dilated aorta of TAV patients:
scansion (left) and transmission (right) electron microscopy.

Interestingly, comparisons between non-dilated and dilated aortas of BAV patients showed no substantial difference in the amount of collagen. In addition, when analyzing the structure of non-dilated aortas, we found that in BAV patients compared to TAV, the collagen fibers are less mature as a consequence of a higher collagen turnover. The extent of collagen turnover was assessed by HPLC-based measurement of pentosidine, a marker of non-enzymatic collagen glycation [93]. In fact, under normoglycemic conditions, collagen glycation, a process that contributes to the maturation of the fibers, is largely determined by the half-life of collagen: if the collagen turnover is high (i.e. continuous proteolysis and new production), the chances to undergo glycation modifications are little and this will be reflected by a low relative level of pentosidine (Figure 37). The fact that BAV non-dilated aortas have less mature fibers was also observed by Picosirius red staining (Figure 37) that allows

distinguishing thick, mature and tightly packed collagen fibers (staining in orange-red at the polarized light) from thin and immature fibers (staining in green).
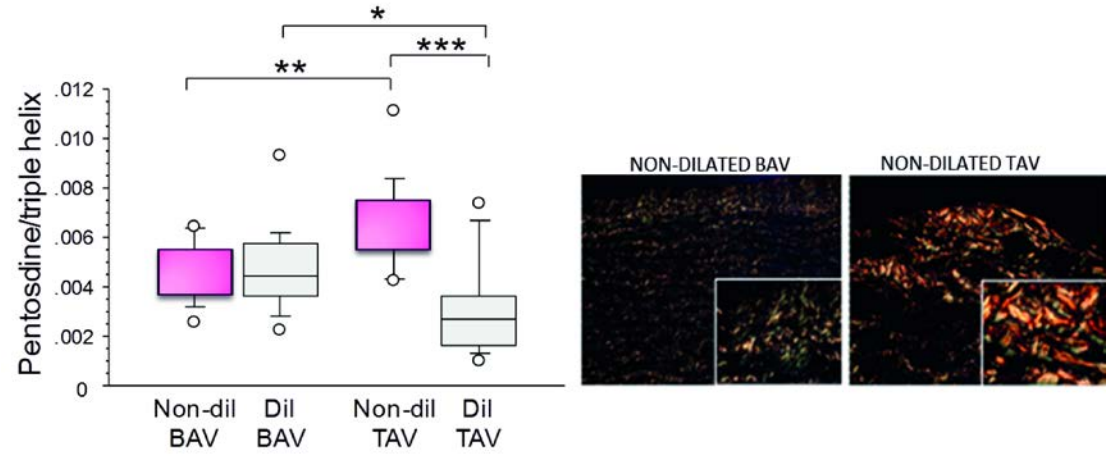
Figure 37: Pentosidine measurement (left, colored pink) and Picosirius red staining (right) of BAV and TAV non-dilated aortas.

In this study we also further defined the types of mature collagen fibers. During collagen maturation, apart from early glycation events, a long series of post translational enzymatic modifications are necessary in order to obtain mature fibers. Cross-link reactions occur between the different collagen chains and eventually terminate with the formation of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) [61].

The ratio of HP/LP was found lower in dilated aortas from patients with BAV compared to TAV, indicating that BAV is associated with a defect in post-translational collagen modification. We could explain this by a deficiency of PLOD1 enzyme (the lysyl hydoxylase responsible for HP formation), found in BAV aortas (Figure 38).
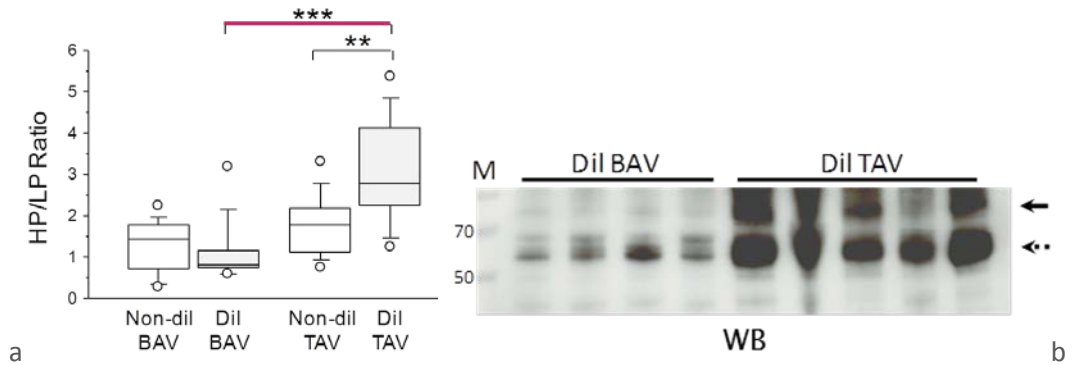
Figure 38. a) HP/LP ratio measurement. b) Western Blotting with antibody recognizing PLOD1.

The HP/LP ratio is believed to be 8:1 in the human aorta [94], while it decreases in the bone due to higher concentration of LP (LP/HP is 0.3:1) [95]. The higher content of LP in BAV compared to TAV, could explain the increased aortic stiffness previously reported in children with bicuspid aortic valves [96]. This will contribute to a reduced elasticity and may explain the increased aortic aneurysm formation in BAV patients.

From our results we could evince that during the dilatation process in BAV aortas, the collagen is not efficiently produced, both due to the lower synthesis/turnover as well as to impaired post-translational modification.

4.4 INCREASED AORTIC TGFB SIGNALING IN PATIENTS WITH BAV

In paper I and II we demonstrated that impairments in TGF-β signaling are associated to aneurysm development in patients with BAV. In project IV we further pursued the attempt to characterize a BAV-TGFβ signaling and therefore focused on the response of AoSMCs isolated from TAV and BAV aortas to TGF-β1.

We found that BAV AoSMCs responded to TGF-β1 treatment by provoking a change in the expression of 217 genes. On the contrary, TGF-β1 treatment of TAV cells did not affect any significant change in gene expression. The changes resulted in both up and down-regulation (Figure 39).
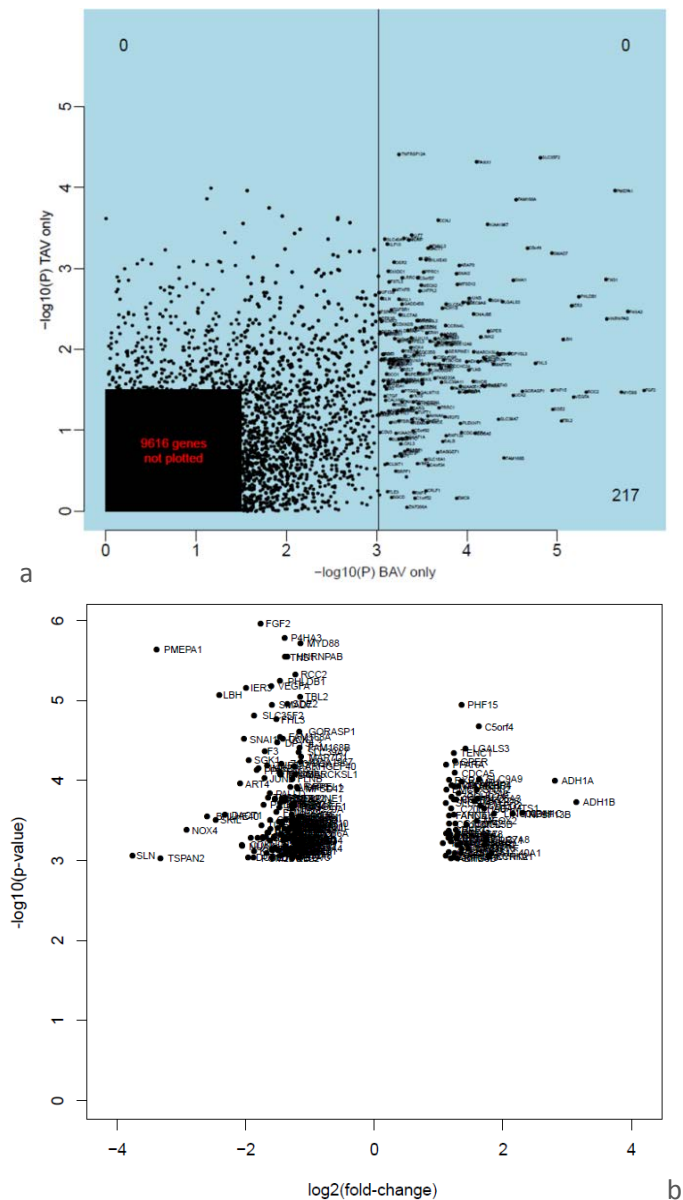
Figure 39. a) 2-dimentional plot showing differentially expressed genes in BAV and TAV from paired t-test comparison (from un-treated to treated). The line outlines cut off after FDR correction. b) Volcano plots for the 217 differentially expressed genes in AoSMCs derived from BAV patients after TGF-β1 treatment. Dots to the left represent significantly down-regulated genes after TGF-β1 treatment and dots to the right represent significantly up-regulated genes.

Gene ontology analysis specifically applied to the down-regulated genes revealed enrichment in pathways related to: positive regulation of angiogenesis, cell-cell and cell substrate adhesion, focal adhesions, cell migration and cytoskeleton, and cell proliferation. These pathways are all involved in wound healing repair processes [97], and the fact that they are down-regulated in response to TGF-β1 in BAV cells can be interpreted as defective signaling of this pathway in the repair process.

To further investigate the relevance of the *in vitro* AoSMC culture system for the *in vivo* aorta of the patients, we analyzed the differential expression of the 217 genes identified in the cells, in the ASAP database. Principal component analysis showed

that, aneurysmal aortas from BAV and TAV patients could be separated in two clusters by considering the expression levels of the 217 genes (Figure 40). Thus, the finding in our *in vitro* system (217 targeted genes) could be validated *in vivo* and further strengthened the hypothesis that an impaired TGF-β1 response may explain the higher propensity of BAV to aneurysm development compared to TAV patients.
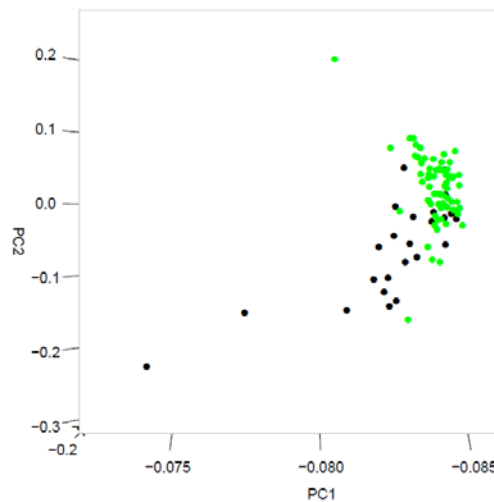
Figure 40. PCA based on the expression of 217 TGFβ-regulated genes in the intima/media layer of dilated ascending aorta. Green dots represent patients with BAV and black dots represent patients with TAV.

The TGF-β1-BAV effect on the 217 genes most likely depends on the intracellular cascade. We therefore analyzed the differential expression of intracellular TGF-β1 cascade in BAV and TAV aortas (Figure 41). Interestingly, already before dilatation, TGBR1 expression was significantly higher in BAV aortas comparing to TAV (Figure 42). Moreover, the expression of SMAD7, an inhibitory SMAD protein [98], and TGFBR3, a potential negative regulator of TGF-β signaling were also lower in non-dilated BAV compared to TAV. These comparisons collectively suggested that the TGF-β signaling is higher in BAV as compared to TAV patients.
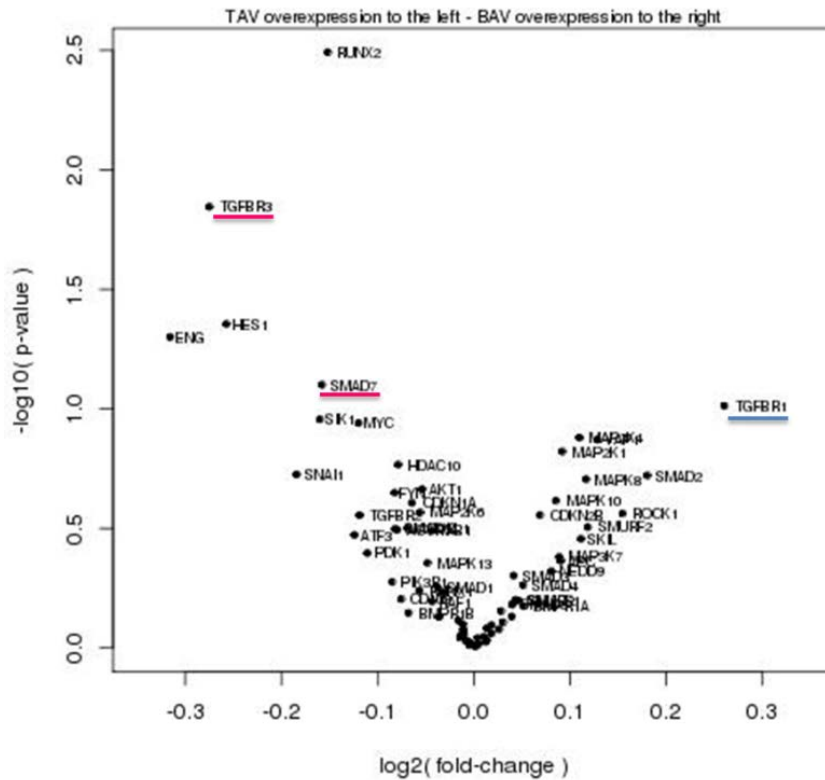
Figure 41. Volcano plots for the expression of 75 genes involved in the intracellular TGF-β signaling pathway in intima/media layer of non-dilated ascending aorta.
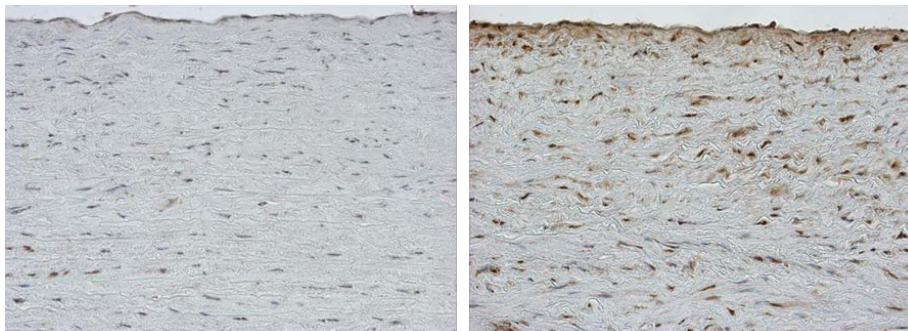
Figure 42. Immunohistochemistry staining of TGBR1 in non-dilated aortas from BAV (right) and TAV (left)

To test whether the BAV-specific response to TGF-β is genetically determined and could be observed in other cell types we analyzed the differential expression of the 217 genes induced by TGF-β1 in fibroblasts isolated from the aortic valves of BAV and TAV patients and we found no specific effect in BAV fibroblasts, an indication that the differential response of BAV patients to TGF-β is aorta-specific (Figure 43). This was an important finding that led us to speculate that local epigenetic alteration in cells isolated from aorta may play a role in enhanced susceptibility of BAV to aneurysm.
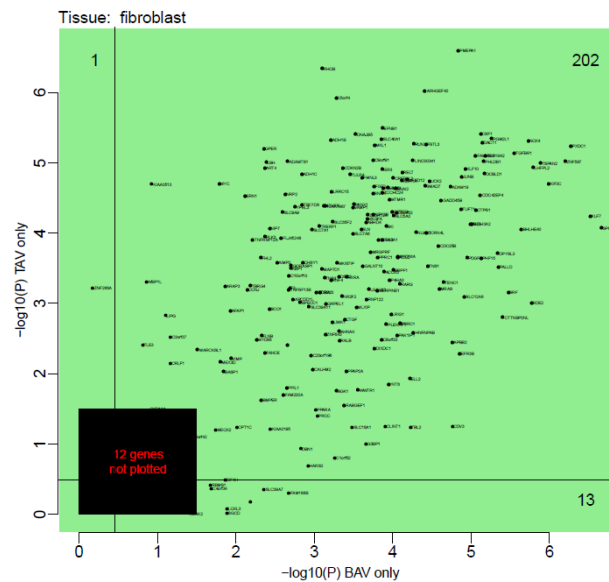
Figure 43. Two-dimentional plot showing the expression of 217 genes in fibroblasts: almost all of the genes (202) are significantly changed from control to treatment in both BAV and TAV valve cells.

In conclusion, BAV and TAV AoSMCs differed in TGF-β1 response, with cells isolated from BAV being more responsive than TAV. The 217 BAV-TGFβ target genes identified in cells were important in discriminating BAV and TAV aneurysmal aortas. Also, the expression of genes belonging to intracellular TGF-β cascade, such as TGFBR1, differed in non-dilated aorta of BAV patients (Figure 44). These results provide further support for the hypothesis that defective TGF-β signaling may underlie the higher fragility of aorta in BAV.
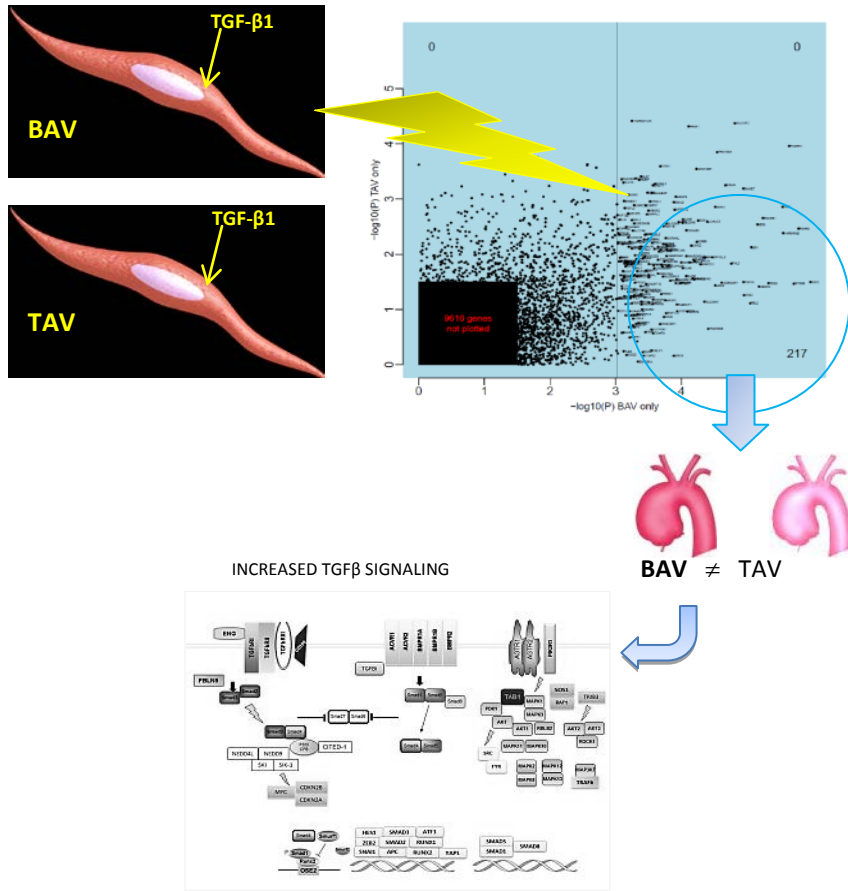
Figure 44. Translational model: form *in vitro* to *in vivo*

5   GENERAL DISCUSSION AND CONCLUSIONS

Individuals carrying BAV have higher risk than general population to develop a TAA. Studies presented in this thesis highlight some possible reasons for the increased susceptibility of BAV to form aneurysm.

During aneurysm formation the homeostasis status of the aortic wall is slowly lost: SMCs goes through apoptosis and ECM becomes extensively degraded, resulting in the loss of aortic structure and eventual enlargement into a pathological aneurysm. Tissue repair mechanisms start up by some specialized cells being recruited to the damaged area to repair the damage caused by the dilatation process. These cells are the myofibroblasts, contractile cells deriving from the resident and/or circulating progenitors cells [99, 100]. A possible source of the myofibroblasts, in the case of aortic wall, may be either fibroblasts located in the adventitia layer or the smooth muscle cells in the media layer (Figure 45).
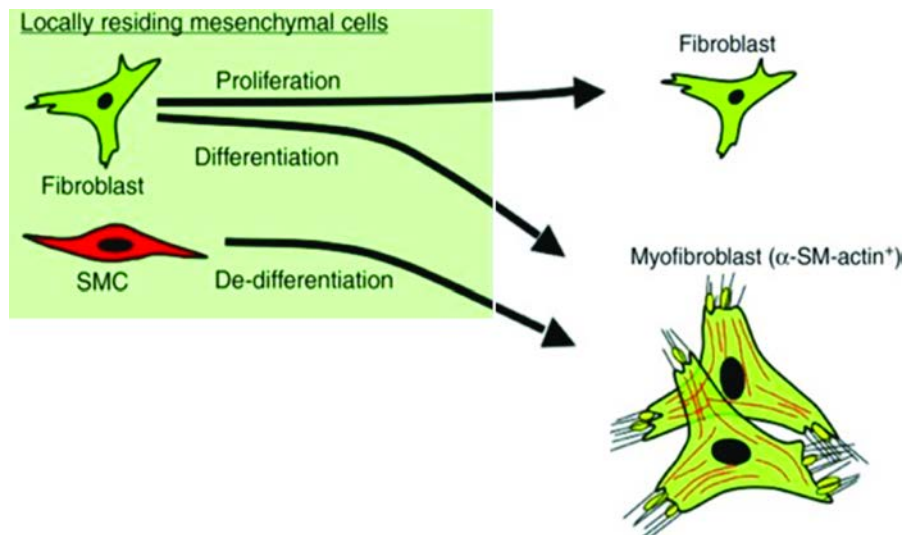
Figure 45. Origin of myofibroblasts in the aortic wall. Permission obtained from [101]

VSMCs have in their innate genetic program the capacity to shift from contractile to synthetic phenotype [102]. The latter can possibly lead to the proto-myofibroblast stage: these cells have secretory properties, high proliferation rate and have lost the capacity to express smooth muscle α-actin [103]. Proto-myofybroblasts and synthetic vSMCs are very similar cells and are not easily distinguishable. However it is the proto-myofibroblast that has the capability to develop into myofobroblast (the actual repairing cell) and this event is driven by TGFβ-1. Therefore, in principle, the task of repairing the damage in the media may be performed by a specific subtype of vSMCs, i.e. the subtype that after shifting to synthetic phenotype can become proto-myofibroblast first and eventually myofibroblasts where smooth muscle α-actin is re-expressed (Figure 46).
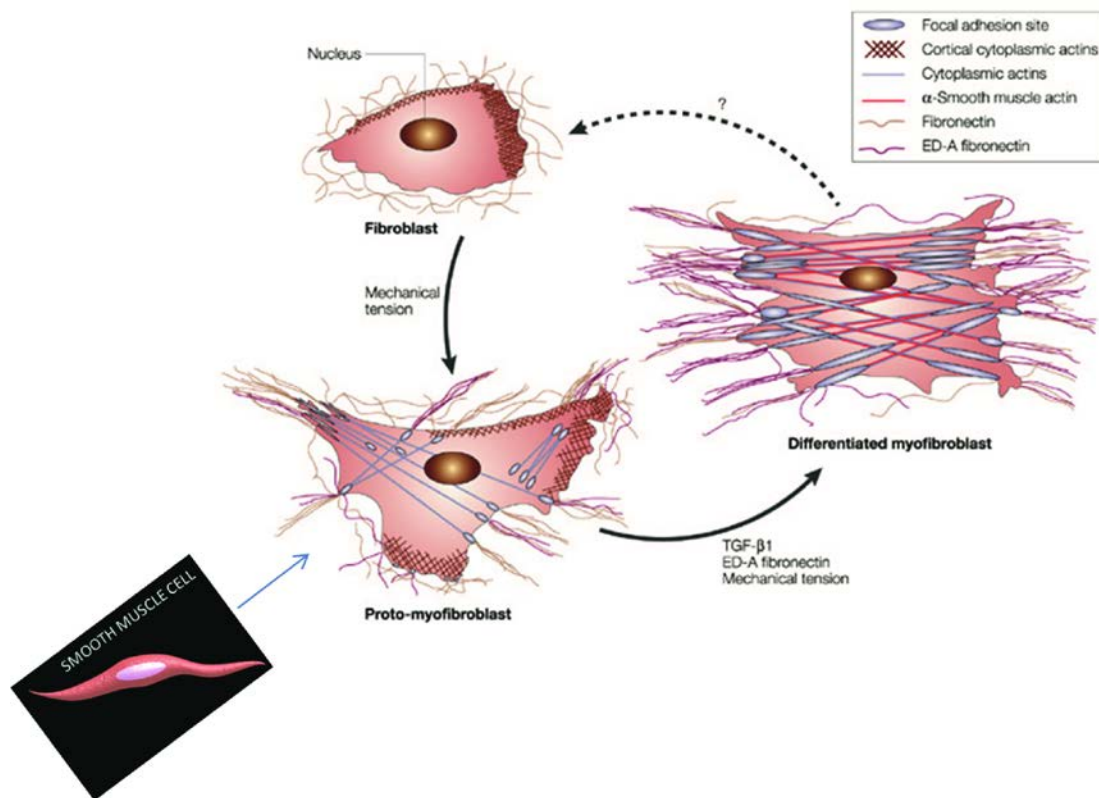
Figure 46. Myofibroblast activation from SMC.

The expression of *ACTA2*, the gene encoding for smooth muscle α-actin, is known to be dependent on TGF-β1 via RhoA signaling pathway [104]. *ACTA2* expression is increased in different types of TAAs, suggesting an activation of myofibroblasts which means that within the dilated aortic wall there is a certain attempt to compensate for ECM degradation. TGF-β, by activating myofibroblasts in the context of wound healing, also promotes the expression of EDA-fibronectin, an alternatively spliced form of fibronectin, with higher capacity of mediating cell-matrix-cell interactions [105]. In the aorta of normal tricuspid aortic valve undergoing dilatation, we have found signs of tissue repair process activation: vSMCs switch to myofribroblasts and become capable of activating the production of EDA-fibronectin. On the contrary, the aortas of BAV individuals were less efficient in producing EDA-fibronectin and this may well be explained by the differences in regulation of TGF-β cascade between BAV and TAV patients.

Histological examinations of aortas from MFS and BAV patients have shown area of cystic medial necrosis without a change in inflammatory signals, compared to the control aortas [106]. In BAV aortas apoptosis may initiate the wound healing response, but this process cannot be properly completed due to the defective function of the relevant pathways. In support to this hypothesis, our group showed that inflammation related genes were up regulated in aneurysm development in TAV, but not in BAV patients [107]. This observation could be interpreted as aneurysm development in TAV is associated with inflammation. As inflammation plays a role in activating tissue repair mechanisms, the down regulation of inflammatory genes in

BAV aneurysmal aortas could be the outcome of a reduced wound healing mechanism in BAV patients. Irrespective of the underlying mechanisms, the conclusions drawn from these studies highlight the fact that BAV-TAA and TAV-TAA are two different diseases. Importantly, in paper III we showed that the aneurysmal aorta of TAV patients is characterized by a remarkable increase of collagen production, which indicates that a fibrotic event is going on to compensate for the dilatation process and strengthen the aorta. The absence of detectable fibrosis in BAV aortas indicated that the reparative mechanisms are not activated in BAV. Myofibroblasts are the cells responsible for inducing fibrosis [100] (Figure 47). Interestingly, *CEBPB,* a gene recently reported to be involved in pulmonary fibrosis [108] was down regulated in BAV aortas compared to TAV [107, 109].

As myofibroblasts (derived from resident vSMCs) are activated by TGF-β to induce fibrosis and collagen and EDA-fibronectin are targets of TGF-β signaling pathway, the reason for the absence of aneurysm-associated fibrosis in BAV could depend on impairments in the TGF-β cascade in BAV aortas.
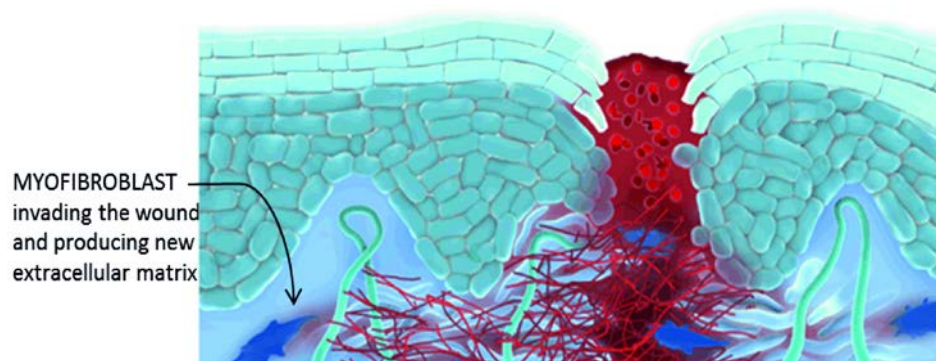
Figure 47. Fibrosis process in the context of wound healing repair.
(from www.invitrogen.com)

TGF-β is still the pathway that attracts the highest interest in the field of TAA. The causative link between defective TGF-β signaling and monogenic forms of TAAs (Marfan syndrome, Loyes-Dietz disease) has been well established. Therefore, the investigation of the role of this pathway in non syndromic TAA patients has been given a high priority in this thesis. Paper II was devoted to study the differences in the splicing pattern of genes composing the TGF-β signaling cascade between BAV and TAV patients. When we analyzed the exons constituting the genes belonging to TGF-β pathway we found that BAV and TAV had a different sets of alternatively spliced exons, which can be interpreted into two different signaling pathways associated with the dilatation, one with a specific set of exons for BAV and one with different set of exons for TAV. Moreover, we found that cells directly isolated from BAV responded massively when treated with TGF-β which would categorize BAV-aneurysms somewhere closer to the monogenic form of TAAs. However the absence of this response in fibroblasts isolated from BAV aortic valves implies that TGF-β response is

aorta specific and may be due to the local epigenetic alteration of vSMCs in BAV. Specifically, the exclusive hyper-activation of BAV vSMCs cells exposed to TGF-β resulted in down-regulation of genes involved in angiogenesis and focal adhesion formation, which is in line with the model proposed by Maleki et al (AORTA, in press), where the balance and fine-tuning of TGF-β, between the pro and anti angiogenic processes may be disturbed in BAV. As angiogenesis is part of the wound healing response, the findings in paper IV well fit with this model. However there are still several undiscovered issues regarding the initiation of dilatation process in both BAV and TAV. Genetic predisposition can be a contributing factor to aneurysm in both BAV and TAV. In TAV senescence and inflammatory processes may enhance aneurysm formation. In BAV an epigenetic factor such as disturbed flow caused by abnormal valve geometry may facilitate the process of aortic wall weakening and destruction. Our observation that the specific response to TGF-β1 is particular for the cells isolated from aorta and not the valves gives support to the importance of an epigenetic factor such as the flow in individuals carrying a BAV. Chronic exposure to the disturbed flow may cause epigenetic changes with the outcome of an imbalanced and deregulated TGF-β1 response in BAV cells.

In summary, differences in the TGF-β signaling can explain the increased aneurysm susceptibility of BAV. In our view, in patients with TAV, where a proper tissue repair response is active, the TGF-β pathway is specifically regulated and directed towards EDA-fibronectin and collagen production during aneurysm formation. In contrast, patients with BAV have an over activated TGF-β signaling both before and during aneurysm formation. The impaired TGF-β signaling in BAV patients may compromise the aortic wall homeostasis thus leading to increased risk for these patients to develop aneurysm (Figure 48).

BAV aorta

INCREASED
ANEURYSM
SUSCEPTIBILITY

↓Tissue repair

↓Collagen, EDA-FN

SMOOTH MUSCLE CELL

+ TGFβ-1

BAV-TGFβ signaling

↓Angiogenesis

↓Anchoring junction
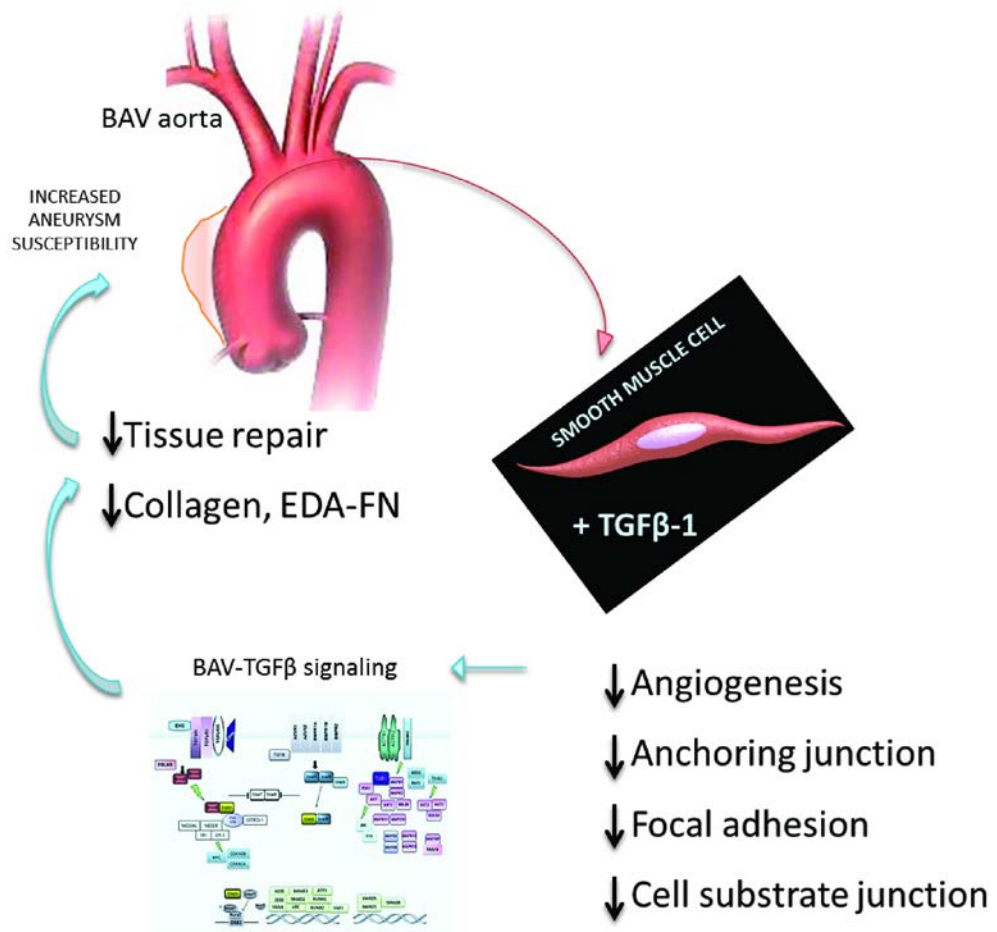
↓Focal adhesion

↓Cell substrate junction

Figure 48. BAV aorta is an aneurysm prone vessel.

6   ACKNOWLEDGEMENTS

I have met very many remarkable people along this PhD journey that have contributed to this work in one way or another. I would like to express my sincere gratitude to:

Per Eriksson, my supervisor and the scientist I would like to become one day.
You have shown me the beauty and greatness of research. From you, I learned the most important thing: to be passionate about my projects and to enjoy the challenges. Also, you are much more than just a successful and brilliant scientist, I can't put in words how proud I feel for being your student!

My co-supervisor *Joy Roy*. Even when not there, you actually WERE there, with all the *live* mail responses and the unplanned meeting which constantly turned out into fruitful and brainstorming discussions. Thanks for sharing your knowledge with me and for always finding the time to help out!

My co-supervisor *Dick Wågsäter*, thanks for believing in me when I first came to Sweden as an Erasmus student!

Anders Franco-Cereceda, I always think of you as an extra supervisor. I could learn so much from watching you in the operation theatre and beyond! You and Per, the perfect duet, managed to build up one of the most international competitive human study on thoracic aortic aneurysm, an excellent example of translational medicine. I feel much honored you have decided to print my thesis for the ASAP patients.

I am very thankful to all the *600 ASAP patients* that apart from making this study possible and contributing to expand the aneurysm research field worldwide have made me feel my work was worthy every single moment of those years.

Veronica, literally a trailblazer in this dissertation process. I know I will be extremely thankful to you the 31st of May and I know you will be amazing. I see the relationship with you and Anders as a perfect example of clinic-lab interaction.

Anders Hamsten, the Karolinska Institutet President. I feel so proud of myself just for writing it. I am very glad for you and for KI. It was a privilege for me to be a member of your Unit. Thanks for explaining genetics the way you do. It is amazing how with one of your sentence after attending a complicated lecture everything became crystal clear!

The Doktorand room with its present and past members! We really share life in there and for some sort of stars alignment there is perfect match of personalities. It also contains very true friends and not only brilliant colleagues.

Shohreh you have got the fight! Your indignity towards any sort of injustice reminds me of my mom. I loved the fact that you keep challenging me with vivid discussions ranging from politics to science. Also thank you for reading every sentence of this thesis, your presence in the group is commendable. *Hanna*: you know me so well. Our travelling history put our friendship in another level. Thanks for being my "thesis Routard", taking me to Mexico and alert me with deadlines and planning.

Joelle, my Sista, we will have so many adventures together, I know. Life is not perfect but we must try to reach perfection in what we are doing, I love that, among many other qualities of you! *Joanna*, my giuggiola, I love talking to you, it always makes me feeling in peace, especially now, during your pregnancy, you have extra powers. *Hovsep,* all these years you have been at my side not only desk-wise. I could never lie to you. Thanks for the fun, the football conversations, the hanging in Milano. You are simply the best! *Anna,* you were the Boss of the room. I miss your impulsivity and your generous personality at work, but we didn't lose each other. I still feel as an Aminoff's family member myself! *Sergey,* thanks for our special Russian-Italian connection, for being protective like a big brother. I love the way you read life and people and as you know I am really fond of you. *Katia,* thanks for being such a caring and sensitive friend. And, of course, for loving the art museums as much as I do, another example of Russian-Italian connection! *Loiusa*, the stars alignment has put you close to Jesper in the Doktorand room, which I think is really funny, since I see some sort of communication affinity there, perhaps Linus-based. Thanks for being so funny and brilliant, and for translating some of Jesper's exercise into biological terms in our Statistic Club! *Jesper*, I really enjoyed the intense scripting and certainly you succeeded in making me loving R. You have a genuine hippy approach to life which is a super good quality! *Sanela,* thanks for so patiently sharing with me your multivariate way to see things: it is certainly good to be multidimensional in life! I learned a lot from your sense of organization and professionalism. *Peter,* the smartest "kid" I ever met and also my neighbor, thanks for the support in Humlebeack. *Tianling*, for the nice time spent together, and for opening my very conservative food mind to the Chinese cuisine! *Cius,* the warmest person on earth, I am grateful to be a friend of yours. *Petra*, your laugh is as loud as Olivera's, I miss that! Thanks for the sportive and fun times

Of course the room could not contain all the other important people I would like to thank from Eriksson-Hamsten group.

Laura, my Kandinsky, thanks for the NO-boundary level of friendship! You are for me what dulche de leche is for you! Thanks for introducing me to Lu, and thanks to *Lucilla* for the family-like Argentinian dinners. Indeed Argentinians and Italians are the two halves of the same apple! *Tess,* thanks for starting with me and being there all the

way: we discovered Stockholm together and without your smile and presence Stockholm is less familiar. *Barbro*, I am the repository of some of your amazing tips. It has been a privileged to learn from you. *Fariba*, so amazingly helpful and sweet, *Olivera* you are my example of wonder woman as well as my Western guru, thanks for all the help! *Karin L,* thanks for all the help with the experiments and also for your positivity and generosity. *Ewa* and *Rachel,* thanks for being so challenging during the Wednesdays meeting, and meanwhile never made people uncomfortable. *Ferdinand,* for being direct and for the funny chats we have when you come to look for Hovsep. Our meetings actually always end up in smiling! *Angela*, I admire your devotion to work which doesn't prevent you from finding time to help everyone and importantly discuss football! *Karin H*, the few Swedish sentences I have learnt are the ones you have patiently taught me. *Mick,* we have clicked immediately at the first proteins extraction: thanks for having my level of jokes and the smart comments at our meetings! *Emina,* my perfect lab-mate, thanks for your diligence and sociability. *Paula,* for always giving me a warm smile and for making *Sigge* smiling so much!

And of course, the third floor Hamsten people. *Ami,* thanks for being so open minded and classy at the same time and for the spontaneous chats from which I could always find very intriguing travel tips. *Lasse,* thanks for all the history we have. Among everything I want to thank you for the bus trip Banff-Vancouver! *Maria Sabater,* thanks for your joviality and for all the big national Swedish events we have celebrated together, and for your half of the apple, *Pau*, who sent me to the Blue Bar in Formentera! *Rona,* thanks for being the party Queen, and for occupying the Procardis server to slow down Jesper and give me some breaks. *Alex,* you are such an example to me, always performing the coolest wet-lab experiments! *Maggie*, thanks for your colorful and strong personality! *Kalle,* thanks for always talking to me in Italian and for loving Milano so much. *Bengt* I could not forget you: we presented a signaling network project that never took off! *Maria M,* thanks for your professionalism and knowledge and the brilliant questions in the Wednesday meetings.

Lars, finally someone into miRNA! Your research is refreshing and something tells me I should thank you in advance for the grilling questions! Also very many appreciations for sharing with me some of the Italian best habits, such as the Spritz love! *Maria G,* thanks for the life conversations and for being so careful with matching earrings, certainly a common passion! *Sarah Jayne,* I am still doing the same Haga round we used to run together, thanks for making me addicted as well. Some addictions need to be shared I guess! And thanks for remembering me from San Francisco. *Sanne,* my new gym buddy, thanks for the friendly Dutch touch you add to the group! *Hong,* for the contribution in the journal clubs and the nice chats every time we meet.

Philip, you are not really in our group, but you are in the third floor! You made me love or rather dislike less things like ice and snow! Also thanks for giving me a very nice picture of Gothenburg people! *Ingrid,* my roomie, you taught English to me and even to my father! Thanks for the connection we have built. *Dima,* for being a supportive friend, you are a natural gentleman. *Nevin,* thanks for all the fun. You should feel

responsible for my English-American vocabulary! You didn't manage with my accent thou! *Alison*, my Canadian soul mate. Thanks for being my alter ego, for the crazy and improvised meeting in Chicago. It feels we will always meet somewhere in the globe. I really hope you'll touch the Swedish ground and surprise me in June! *Daphne,* Erasmus companion and friend. Thanks for showing up totally unexpected in Schipol, no one ever did that for me! *Asta,* thanks for opening to me your very big heart and for keeping in contact since the Erasmus times.

Eventually I have found friends also outside Karolinska.
Stefan, the one and the only Swedish guy for me! Thanks for being my friend for all these years and for showing me how "insane" the Swedes can be! *Tatiana,* for the friendship, for making me the biggest kiwi cake I have ever seen for my birthday and bringing it inside the club! *Anna,* I love your romantic way to see life. It is contagious! I am so proud of you for exporting Swedish culture to Italy and keeping high the honor of both Italy and Sweden in Trentino, where you are without any doubts the coolest mom!
To my Italian friends in Stockholm. In the beginning of my Swedish experience I thought I wanted to avoid the Italians abroad but that didn't last long, you can't escape from them! *Matteo*, my motivator! Thanks for your cheerful personality, for being my buddy and for the daily friendships contact. I need that! And for being such a sport geek, you make me feel normal! The inhabitants of Valentin Sabbats gata: *Davide,* indeed I did a good job on flygbussarna. I am thankful I have you here, you are a support and you care. Also thanks to you I could meet *Disisto,* and *Arianna*. Disi everything you say to me makes me laugh, thanks for that, maybe one day we will start to take each other seriously. Ari, I am glad you moved up here. I really admire your determination plus you are so charmingly cool and sweet.
Isabella, thanks for all the tennis matches, especially the ones we play very early during weekends, because playing tennis in the morning is the best! Thanks for the time we spent on the field and outside! *Stefano,* thanks for all your massive parties and the boat trips, you showed me Stockholm in the best way imaginable! *Sara,* I am honored you put me on your wall in Barcelona! Thanks for knowing what is important in life and for always make things happening. And for giving me the custody of *Enrico,* after you left! Thanks Enri for your very contagious positive attitude! *Ivan,* for believing in Grillo and for getting so excited about the Fibronectina! And of course, Pino's café, the most authentic Italian place in Stockholm, thanks to *Pino* and *Herman* for letting me start all the mornings with the good foot and a good laugh!

To my friends and family in Italy.
Roberta, I never felt disoriented when coming back to Italy thanks to you. Thanks for always calling me, the minute I was landing in Bergamo. For treating me like one of your sisters. In our consolidated friendship there is one very important aspect I want to thank you for and that is sharing one big love with me: the Sea! Our never ending

swims, very far from the shore are quality life time moments to me! *Lucia,* my little chemist and scientist. Thanks for your true friendship and for being a dream colleague during the University's thesis. I learned to be more patient in the laboratory life and I hope you will not give up research because your passion and diligence are so much of example to me! *Agnese*, the ironwoman, the willpower and the way you pursue your goals have always impressed me. Thanks for all the innumerable adventures.

My Parents for teaching me what "un-conditional love" means. My mom's everyday Skype calls and my dad's 24hs visits really made me feel a "daughter". It would be enough for me to have inherited 10% of my mom's intelligence and spirit and 10% of my dad's sense of devotion and curiosity to be perfectly ok in this world. My Bro, no one can touch him! Thanks for being precisely the way you are. It's good to be two! Thanks to my numerous relatives for organizing all the loud and noisy forced celebrations, which I indeed enjoy very much. Especially thanks to my aunt *Rinella* and my cousin *Daniela* for their huge and generous heart, they are more similar than they think.

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