FACULTAD DE MEDICINA
UNIVERSIDAD DE CANTABRIA

GRADO EN MEDICINA

TRABAJO FIN DE GRADO

FISIOPATOLOGÍA MOLECULAR DEL REMODELADO ECTASIANTE DE LA PARED AÓRTICA

MOLECULAR PATHOPHYSIOLOGY OF AORTIC WALL REMODELLING IN ANEURYSMAL DISEASE

Autor: Dña. Lydia Fraile Ruiz

Directores: D. Juan Francisco Nistal
Dña. Raquel García López

Santander, Junio 2016

INDEX

ABSTRACT

Arterial aneurysm is defined as a focal dilation with respect to the original artery diameter. The risk of abdominal aortic aneurysm (AAA) increases dramatically in male older than 60 years. The likelihood that an aneurysm will rupture is influenced by size, expansion rate, continued smoking and persistent hypertension. However, to date the pathogenesis of AAA still remains unclear. Ang II–dependent TGF-β activity promotes aortic aneurysm progression in experimental Marfan syndrome. Nevertheless, its role in experimental models of AAA has not been comprehensively assessed. The aim of this study was to determine the effects of BAMBI (TGF-β pseudoreceptor) on AngII induced-aneurysm model. Male ApoE-/- mice were infused with AngII administered with BAMBI neutralizing antibody and then abdominal aorta diameter was measured. Mice without antibody developed AAA including thrombus formation and rupture, leading to death. We also analysed changes of gene expression of proteins related with aorta remodelling, such as collagen I, III and fibronectin and smooth muscle cell (SMC) markers (specific isoform of α-actin, ACTA2, and myosin protein complex, MYH11). Our results provide evidence that AngII-induced AAA formation is prevented by BAMBI neutralization. Additionally, BAMBI neutralization favoured a more contractile and less rigid phenotype of abdominal aorta by increasing the expression of SMC markers and reducing the expression of collagen.

RESUMEN

Un aneurisma arterial se define como una dilatación focal respecto al diámetro original de la arteria. El riesgo de aneurisma aórtica abdominal (AAA) aumenta dramáticamente en hombres mayores de 60 años. La probabilidad de que un aneurisma se rompa está influenciado por su tamaño, ratio de expansión, no dejar de fumar y la hipertensión persistente. Sin embargo, hasta la fecha la patogénesis de la AAA permanece sin esclarecer. La actividad de TGF-β dependiente de AngII promueve la progresión de la aneurisma aórtica en estudios experimentales sobre el síndrome de Marfan. No obstante, su función en los modelos experimentales de AAA no ha sido evaluado exhaustivamente. El objetivo de este estudio ha sido determinar los efectos de BAMBI (pseudoreceptor de TGF-β) en modelos con aneurismas inducidos por AngII. Se emplearon ratones macho ApoE-/- infundidos con AngII y se les administró un anticuerpo neutralizador de BAMBI, después se midió el diámetro de la aorta abdominal. Los ratones sin anticuerpo desarrollaron AAA incluyendo la formación de trombos y ruptura, llevando a la muerte. También se analizaron los cambios en la expresión genética de proteínas relacionadas con el remodelado aórtico, como el colágeno I, III y fibronectina, y marcadores de las células musculares lisas (SMC) (isoforma específica de α-actina, ACTA2, y miosinas de cadenas pesadas, MYH11). Nuestros resultados aportan evidencia de que la neutralización de BAMBI previene la formación de AAA inducida por AngII. Además, la neutralización de BAMBI favorece un fenotipo de aorta abdominal más contráctil y menos rígido mediante el aumento de la expresión de los marcadores de SMC y reduciendo la expresión de colágeno.

Key words: Abdominal Aortic Aneurysm (AAA), TGF-β (Transforming Growth Factor - β), BAMBI (BMP and activin membrane-bound inhibitor), Vascular remodelling.

INTRODUCTION

An aneurysm is an abnormal bulge in the wall of an artery. Normally, walls of arteries are thick and muscular, allowing them to withstand a large amount of pressure. Occasionally, however, as the result of a degenerative process a weak area develops in the wall of these vessels, which allows the blood pressure to dilate the affected vascular segment, creating a ballooned area called an "aneurysm." Aortic aneurysm is defined conventionally as a permanent dilation of the aorta to greater than 1.5 times the immediately sub- or superjacent segment of normal size. Indeed, the word aneurysm originates from the Greek ανεύρυ (aneurys), which means *to dilate*.

The aorta, main arterial vessel of the body, begins just above the heart and rises straight up in the front of the chest (ascending aorta) toward the top of the mediastinum. At that level, it takes a turn, called the aortic arch, toward the back of the body. From the aortic arch, the aorta runs along the spine all the way down the chest (descending aorta), through the diaphragm, and into the abdomen. In the mid-abdomen (abdominal aorta), just opposite the belly button, the aorta splits into two branches, each of which heads off to supply blood to one of the legs. When an aneurysm is formed in the aorta it is called aortic aneurysm (AA).

1. Epidemiology

Aortic dilatation is an indolent but virulent disease that produces no or few symptoms for a long period of time but is associated with more than 80% mortality if aneurysm rupture occurs, constituting the fifteenth cause of death over all in the population of the United States and the tenth cause of death in men over 55 years. The incidence of the disease is rising in the general population as the life expectancy increases and reaches a maximum of 4% in people older than 60 years. It is estimated that 1–3% of deaths in the male population are caused by this pathology [1]. Even though the progressive dilation of the aorta does not produce major symptoms by itself, it is associated with high cardiovascular morbidity and mortality as these patients belong to a high risk subgroup. In addition, the incidence of such events seems to have risen over the past two decades [2,3]. This contrasts with trends seen in coronary heart disease and stroke, suggesting that measures introduced to reduce the impact of occlusive arterial disease have not been effective. The key points in the treatment and research related to aneurysms are an early diagnosis, an appropriate follow-up based on image techniques, strict control of cardiovascular risk factors and a timely surgery when the absolute diameter, the dilation velocity, or other qualitative characteristics of the aneurysm or the disease make surgery advisable. A ruptured aneurysm often results in fatality, as blood is lost internally into the chest or abdominal cavities. Death can occur very quickly, at times within minutes or even seconds. Currently, the only effective treatment is open surgery or endovascular conventional surgery.

The size of the aneurysm is critically important, as the risk of rupture is directly related to the diameter of the aneurysm (according to the Law of Laplace). The risk of

rupture of small aneurysms (smaller than 4.0 centimetres) is much less than the risk of rupture of large aneurysms (larger than 6.0 centimetres). However, a large proportion of dissections occur at smaller diameters, and even in patients with no visible enlargement [4].

2. Etiology

The aneurysms of the aorta can be congenital, as in Marfan syndrome, in bicuspid aortic valve, aortic coarctation or familial thoracic aortic aneurysm and dissections (TAADs); or acquired in the context of pathological processes of arteriosclerosis, systemic hypertension, isometric exercise, cocaine consumption, pregnancy or diseases of the aortic valve. Both inherited and acquired conditions share a common phenotype, the deterioration of the integrity of the wall of the aorta with subsequent expansion and aneurysm formation.

Congenital aortic aneurysms are divided into two broad categories: syndromic (associated with abnormalities of other organ systems) and non-syndromic (with manifestations restricted to the aorta) [5]. Syndromic aortic aneurysms occur in patients with: Marfan syndrome (MFS), Loeys-Dietz syndrome (LDS), aneurysm osteoarthritis syndrome (AOS), arterial tortuosity syndrome (ATS), Ehlers-Danlos Syndrome type IV (EDS) [5,6] and TGFβ mutations. Many of the syndromic aneurysms can be diagnosed by their characteristic dysmorphic features and by gene testing (*Table 1*) [7].

Table 1 Associations with syndromic and non-syndromic aneurysms

Classification	Chromosome	Gene	Protein	Location	Frequency	Inheritance
Syndromic						
Marfan	15q21.1	*FBN1*	Fibrillin 1	ECM	1:5,000-10,000	AD
Loeys-Dietz	3p24-25	*TGFBR2,*	TGFβ-R2	Cell surface	Rare	AD
	9q33-34	*TGFBR1*	TGFβ-R1			
Ehlers-Danlos	2q24.3-31	*COL3A1*	Type III collagen	ECM	1:10,000-25,000	AD
ATS	20q13.1	*SLC2A10*	GLUT10	Intracellular	Rare	AR
AOS	15q22.2-24.3	*Smad3*	SMAD3	Intracellular	Rare	AD
TGFB2	1q41	*TGFB2*	TGFβ2	Intracellular	Rare	AD
Non-Syndromic						
TAAD2	3p24-25	*TGFBR2*	TGFβ-R2	Cell surface	~3 % of TAA	AD
TAAD4	10q23-24	*ACTA2*	Smooth muscle actin	Intracellular	10-15% of TAA	AD
TAAD5	9q33-34	*TGFBR1*	TGFβ-R1	Cell surface	~2 % of TAA	AD
TAAD-PDA	16p12-13	*MYH11*	β-MHC	Intracellular	1-2% of TAA	AD
	3q21.1	*MYLK*	MLCK	Intracellular	~1% of TAA	

AD, autosomal dominant; AR, autosomal recessive; ATS, arterial tortuosity syndrome; AOS, aneurysm osteoarthritis syndrome; TAA, thoracic aortic aneurysm

Aortic aneurysms may occur both in the thoracic region (TAA) and abdominal region (AAA). Although AAAs have similarities to TAAs, they differ mainly in the location of the disease and the embryonic origin of vascular smooth muscle cells (VSMC) (Table 2) [8]. Furthermore, in contrast to TAA syndromes, AAA is usually not caused by a single gene defect, but multiple genetic and environmental factors are thought to participate in its development.

Table 2

Similarity and difference between AAAs and TAAs

Features	AAAs	TAAs
Loss of VSMCs	Yes	Yes
Increased MMPs	Yes	Yes
Increased ECM degradation	Yes	Yes
Age is a risk factor	Yes	Yes
Locations	Abdominal aorta	Ascending aorta, arch and descending thoracic aorta
Origins of VSMCs	VSMCs of abdominal aorta originate from somites	The embryonic origin of medial VSMCs in the ascending aorta and arch arise from neural crest, and VSMCs in descending thoracic aorta from somites.
Increased inflammatory cell infiltration	Yes	Minimal

3. Thoracic Aortic Aneurysms

Thoracic aortic aneurysms and aortic dissections affect the ascending aorta and are inherited in an autosomal dominant manner in up to 20% of patients [9,10]. A majority of patients with a family history of TAAD do not have a known syndrome associated with aneurysm formation, such as MFS or Loeys–Dietz syndrome [9,11].

Marfan syndrome is an autosomal dominant heritable disorder of connective tissue that affects the cardiovascular [12], skeletal, ocular, pulmonary, and nervous systems [12] and is usually caused by mutations in the *FBN1* gene, which encodes fibrillin-1 [13]. MFS is traditionally considered to result from the structural weakness of connective tissue. However, recent investigations on molecular mechanisms indicate that increased transforming growth factor-*β* (TGF-*β*) activity plays a crucial role in the pathogenesis of MFS and related disorders, such as LDS, which is caused by mutation in TGF-*β* signalling-related genes [12].

In 2004–2005, mutations in genes encoding TGF-*β* receptors 1 and 2 (*TGFBR1* and *TGFBR2*, respectively) were identified in a subset of patients with MFS2 [11], which is currently referred to as Loeys–Dietz syndrome. Although LDS patients present several characteristics of MFS, LDS is characterized by rapidly progressive aortic/arterial tortuosity and aneurysmal disease that is known to result in ruptures at an early age and at smaller dimensions, and by widely spaced eyes (hypertelorism) [14] and bifid uvula or cleft palate. Until recently, mutations in TGFB2 [15] and *SMAD3* [16], which encode main members of the TGF-*β*/SMAD signal transduction pathway, were also reportedly associated with diseases that resemble MFS. Fibrillin-1 also regulates TGF-*β* bioavailability, and thus traditional definitions for the pathogenesis of MFS and related diseases have been dramatically revised. In particular, the dysregulation of TGF-*β* is considered the main contributor to the major extra-ocular features of MFS [17].

In patients with MFS, cystic medial necrosis (CMN) is present in the medial layer of the aortic wall, and is characterized by fragmentation and disorganization of elastic fibres, fibrosis with collagen production, accumulation of amorphous matrix components, and loss of cell nuclei [18]. In addition, inflammatory T lymphocytes and macrophages reportedly infiltrate aortic media and adventitia, and those numbers were negatively correlated with patient ages at referral for prophylactic surgical repair, suggesting that inflammation might affect disease progression [19]. Normally, inflammatory proteolytic enzymes such as elastase and/or certain physiological stimuli lead to microfibril degradation, which contributes to local TGF-*β* activation [20]. In contrast, mutated fibrillin-1 in MFS leads to failed sequestration of TGF-*β*, and the ensuing overactivity of TGF-*β* signalling cascades is considered crucial in the pathogenesis of MFS as seen in Figure 1 [12].

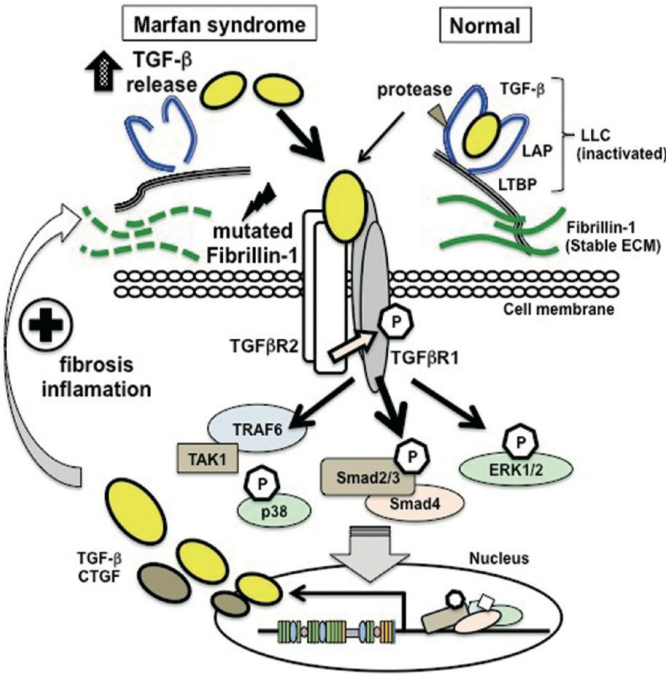

Figure 1. Dysregulation of TGF-*β* bioavailability in MFS caused by mutations in the FBN1 gene. Latent TGF-*β* is activated by physical and enzymatic stimuli under normal conditions (right side). Mutated fibrillin-1 in MFS leads to failed ECM sequestration of TGF-*β* and subsequent activation of TGF-*β* signalling cascades (left side), which play critical roles in the pathogenesis of MFS. CTGF indicates connective tissue growth factor; ECM, extracellular matrix; LLC, large latent complexes; LAP, latency-associated peptide; and LTBP, latent TGF-*β* binding protein.

Plasma total TGF-*β*1 levels were elevated in patients with MFS, and were lowered by treatment with the angiotensin II receptor blocker (ARB) losartan [22]. However, relationships between genotypes, changes in signals, and phenotypes remain incompletely characterized. In addition, more than 3,000 mutations have been identified in the *FBN1* gene, and these are mostly unique in families [23].

Additionally, familial TAAD is genetically heterogeneous and four loci have been mapped for non-syndromic familial TAAD to date: TAAD1, FAA1, TAAD2 and a locus at 16p [11]. The gene at the TAAD2 locus on 3p24 – 25 has been identified as TGFβR2, implicating the TGF-β pathway in aortic disease [14]. More recently, the defective gene at the 16p locus has been identified as SMC-specific myosin heavy chain (MYH11) through the detection of mutations in two families with TAAD, suggesting that a viable contractile machinery in SMCs is also essential to prevent aortic disease [24].

4. Abdominal Aortic Aneurysms (AAA)

Abdominal aortic aneurysm (AAA) indicates an irreversible dilatation of the terminal aortic segment. Normally, the diameter of the abdominal aorta is between 1,8 cm and 2 cm whereas in AAA it is more than 3 cm.

This pathology is responsible for the death of thousands of patients in developed countries each year. Its prevalence reaches 1.5% in the total population, reaching 5% in men over 65 years and even 12% in men between 75 and 84 years. With the possible introduction of more advanced screening techniques for AAA, smaller AAA will be diagnosed. Approximately 80% of aortic aneurysms occur between the renal arteries and the aortic bifurcation [25]. Surgery is currently considered the only appropriate therapy when AAA diameter exceeds 55 mm [26]. Decision-making in regard to elective repair of AAA requires careful assessment of factors that influence rupture risk, operative mortality, and life expectancy. Individualized consideration of these factors in each patient is essential, and the role of patient preference is of increasing importance. It is not possible or appropriate to recommend a single threshold diameter for intervention which can be generalized to all patients [27]. Based upon the best available current evidence, 55 mm is the best threshold for repair in an “average” patient. However, subsets of younger, good-risk patients or aneurysms at higher rupture risk may be identified in whom repair at smaller sizes is justified. Conversely, delay in repair until larger diameter may be best for older, higher-risk patients, especially if endovascular repair is not possible [27]. However, no pharmacology treatments have been proven effective in limiting AAA progression or reducing risk of rupture [28]. Insufficient understanding of the mechanisms underlying its pathogenesis limits the prevention and treatment of this disease. Therefore, it is an important health problem, especially in our population. It is estimated that 1–3% of deaths in the male population is caused by this pathology [1].

4.1. Risk factors

Male sex, age, arterial hypertension, diabetes, Caucasian race, dyslipidemia and cigarettes are cardiovascular risk factors that play an important role in the development of AAA [29,30,31]. Furthermore, genetic alterations connected to collagen synthesis and familiarity are also risk factors specific to AAA [32]. AAA is therefore considered a multifactorial disorder in which both environmental and genetic factors contribute to its development [25]. Although the involvement of these factors is recognized, the *primum movens* has not yet been identified. The pathogenesis of AAA is complex and needs further investigation. Different theories have been proposed, but none have been accepted as fully established or accepted [33, 34]. It would seem that the interaction of several factors may determine changes in the vessel thus causing dilatation.

In addition to size, the risk of AAA rupture, and death, depends upon the rate at which the aneurysm is expanding. The evidence suggests that aneurysms expand at an average rate of 0.3 to 0.4 centimetres per year [27]. There can be significant

variability in the rate of expansion, both from one patient to another and for a given patient from year to year. Many patients have long periods with little change in aneurysm size. Larger aneurysms tend to expand faster than smaller aneurysms. Aneurysms that expand rapidly (for example, more than 0.5 cm over six months) are at high risk of rupture. Growth tends to be more rapid in smokers, and less rapid in patients with peripheral artery disease or diabetes mellitus [35].

4.2. Clinical presentation

The majority of AAAs are asymptomatic and are most often detected as an incidental finding on ultrasonography (USG), abdominal computed tomography (CT) or magnetic resonance imaging performed for other purposes. Most AAAs are silent until they rupture, although some are identified during evaluation for abdominal symptoms. Patients with ruptured AAAs classically present with shooting abdominal or back pain and a pulsatile abdominal mass. Aneurysm rupture typically causes severe hypotension. Aneurysms producing symptoms, especially pain and tenderness on palpation, are at increased risk for rupture [25].

AAAs can also present with complications due to thrombosis, embolization or, rarely, as clinically overt disseminated intravascular coagulation causing haemorrhagic and thrombotic complications [36,37].

4.3. Diagnosis

The diagnosis of an AAA should ideally be made before the development of clinical symptoms to prevent rupture. Approximately 30% of asymptomatic AAAs are discovered as a pulsatile abdominal mass on routine physical examination [25]. Physical examination may reveal a pulsatile, expansile mass at or above the umbilicus. The vascular examination should include abdominal auscultation because the presence of a bruit may indicate aortic or visceral arterial atherosclerotic disease, or rarely an aortocaval fistula (machinery murmur)[38].

Large aneurysms in thin people are easy to detect. The accuracy of the clinical examination is tremendously reduced by obese body habitus and small aneurysm size [38]. However, the physical examination has considerably variable inter-observer sensitivity for detection of AAAs. The sensitivity of physical examination for the identification of an AAA ranges from 22% to 96%, and even an experienced physician may miss palpating an AAA in the presence of obesity or abdominal distension [39].

An asymptomatic AAA is often discovered incidentally because of the performance of abdominal USG, CT or magnetic resonance imaging for other purposes. An AAA may also be found with plain x-rays showing some calcification in the wall of the aneurysm. However, they are not reliable because some aneurysms do not have sufficient calcification to be detected[25].

Abdominal USG is considered the screening modality of choice for AAAs because of

its high sensitivity of 95% to 100% and a specificity of nearly 100%, as well as its safety and relatively low cost [40]. USG has excellent test characteristics for diagnosing and following an AAA. Thrombus or echodense calcifications in or adjacent to the aortic wall may also be seen and both are quite common. Disadvantages of abdominal USG are that it is operator dependent and, in 1% to 2% of cases, overlying bowel gas or obesity hinders proper imaging of the abdominal aorta [41].

CT scanning evaluates the abdomen in more detail in patients with a specific abdominal complaint. It also assesses the shape of the aneurysm with more comprehensive anatomical details of the mesenteric and iliac arteries, and also provides better imaging of suprarenal aneurysms [42]. CT angiography is also essential in tailoring stent grafts in cases for which endovascular treatment is indicated. Disadvantages of CT scanning compared with USG include increased cost, requirement for contrast, exposure to radiation with repeated scans and limitation of accuracy in localizing the aneurysm neck in some cases compared with contrast angiography [42]. Magnetic resonance angiography is probably more accurate than CT, but is more expensive and not universally available [43].

4.4. Pathophysiology of AAA formation

Pathological progressive dilatation of the aorta is mainly characterized by an imbalance between the phenomena of apoptosis, proliferation and migration of smooth muscle cells (SMC) and between synthesis and degradation of ECM. This disruption of vessel wall homeostasis is related in large part to localized transmural inflammation. To date, it is still not clear which is the determining cause of the first inflammatory activation and the initial recruitment of immune cells in the aortic wall [44].

Degradation of the aortic wall structural elements and loss of VSMCs within the medial layer is associated with progressive aortic dilatation and eventual rupture [45]. The release of cytokines, inflammatory mediators, growth factors, matrix metalloproteinases (MMPs), other proteases etc. bring about a chronic inflammatory state. The infiltrating cells identified in AAA biopsies are dominated by monocytes, macrophages and lymphocytes, which produce proinflammatory mediators that result in the irreversibility of the process [46].

The medial layer of the aorta confers elasticity and strength to the aortic wall and is composed of alternating layers of SMCs and elastic fibres. The SMC elastin-contractile unit is a structural unit that links the elastin fibres to the SMCs and is characterized by the following: (1) layers of elastin fibres that are surrounded by microfibrils; (2) microfibrils that bind to the integrin receptors in focal adhesions on the cell surface of the SMCs; and (3) SMC contractile filaments that are linked to the focal adhesions on the inner side of the membrane. The genes that are altered to cause AAA encode proteins involved in the structure or function of the SMC elastin-contractile unit. Included in this gene list are the genes encoding proteins that are structural components of elastin fibres and microfibrils, FBN1, microfibrillar associated protein 5 (MFAP5), elastin (ELN), and fibulin-4 (FBLN4) [47].

Also included are genes that encode structural proteins in the SMC contractile unit, including ACTA2 and MYH11, along with MYLK and PRKG1, which encode kinases that control SMC contraction. The major function of VSMCs is to contract in response to the stretch resulting from pulsatile blood flow, a process that is dependent on the cyclic interaction between thin filaments, composed of the SMC-specific isoform of α-actin (SM α-actin, encoded by *ACTA2*), and thick filaments, composed of SMC-specific β-myosin. The importance of SM α-actin for SMC function is supported by several lines of evidence [47,48].

Fibronectin, collagen, elastin and proteoglycans are the main components of the extracellular matrix (ECM) of the arterial vessel wall. Fibronectin is known to interact with cell surface and with collagen and proteoglycans [49], and it is believed to provide a linkage between the cells and ECM. Fibronectin is localized in the region of the endothelial basement membrane and in the subendothelium of the normal arterial intima [49]. The alteration of the connective tissue present in the aortic wall plays an important role in the development of aneurysms given that elastic and collagen fibres are the main determinants of the mechanical properties of the aorta. Elastin is stabilized by a series of cross-links between molecules and can be degraded by specific proteases that have elastase properties. The elastic fibres associated with SMCs tend to be more abundant in the tunica media of the aortic wall [50]. Collagen, on the other hand, is an important component of the tunica media and also of the tunica adventitia. In particular two types of collagen, type I and type III, oppose the tensive force of blood pressure and give support in maintaining the structural integrity of the arterial wall [49].

One of the most important histological features of aneurysmal tissue is the fragmentation of elastic fibres and the decreased concentrations of elastin during aneurysm growth until its eventual rupture [50,51]. The loss of elastic fibres can lead to an initial step toward the formation of aneurysm [50]. The degradation of fibres seems to be caused by inflammatory components, which lead to the production of cytokines. This in turn attracts other immune cells which arrive at this site via the bloodstream [52]. The alterations of elastin and collagen are the consequence of the release of proteases by arterial wall cells, such as SMCs of the tunica media, fibroblasts of the adventitia and cells constituting inflammatory infiltrates.

5. Transforming Growth Factor (TGF)-β

Transforming growth factor beta (TGF-β) is a family of cytokines that forms a serine/threonine kinase complex and binds to TGF-β receptors, which are composed of type 1 and type 2 receptor subunits. After the binding of TGF-β, the type 2 receptor kinase phosphorylates and activates the type 1 receptor kinase that activates a signalling cascade [53] that is depicted in Figure 2. This leads to the activation of different downstream substrates and regulatory proteins, inducing transcription of different target genes that participate in differentiation, chemotaxis, proliferation, and activation of many immune cells [54].

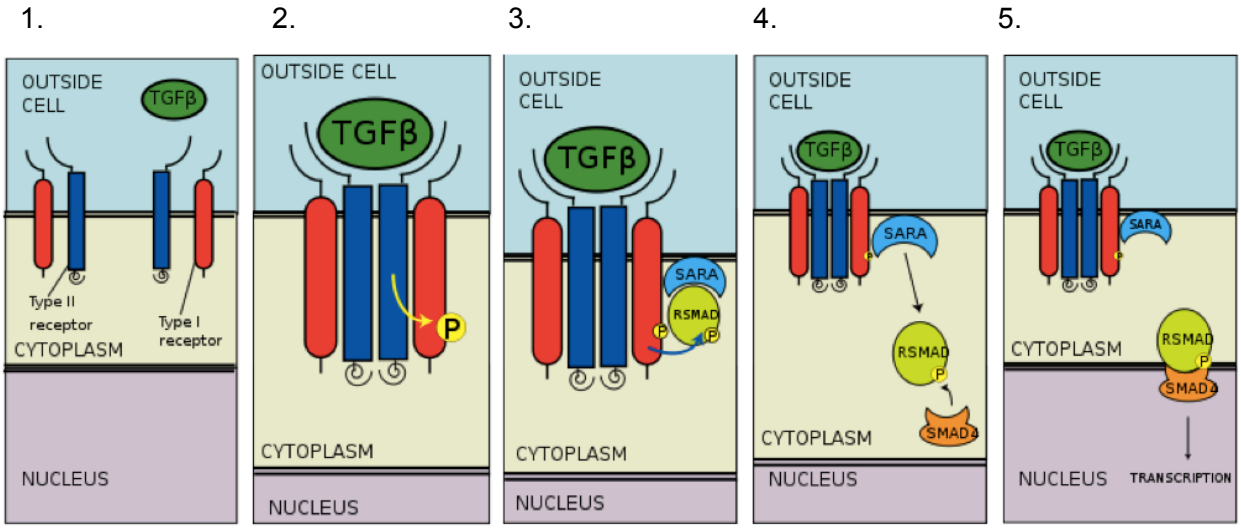

Figure 2. The **TGFB signaling pathway** is involved in many cellular processes in both the adult organism and the developing embryo including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions. In spite of the wide range of cellular processes that the TGFβ signaling pathway regulates, the process is relatively simple. **1. Ligand binding.** Signaling begins with the binding of a TGF beta superfamily ligand to a TGF beta type II receptor. The type II receptor is a serine/threonine receptor kinase, which catalyzes the phosphorylation of the Type I receptor. Each class of ligand binds to a specific type II receptor [54]. In mammals there are seven known type I receptors and five type II receptors [54]. When the receptor-ligand binding occurs via local action, this is classified as paracrine signalling. **2. Receptor recruitment and phosphorylation.** The TGF beta ligand binds to a type II receptor dimer, which recruits a type I receptor dimer forming a hetero-tetrameric complex with the ligand. These receptors are serine/threonine kinase receptors. The binding of a TGF beta family ligand causes the rotation of the receptors so that their cytoplasmic kinase domains are arranged in a catalytically favorable orientation. The Type II receptor phosphorylates serine residues of the Type I receptor, which activates the protein. **3. SMAD phosphorylation.** There are five receptor regulated SMADs: SMAD1, SMAD2, SMAD3, SMAD5, and SMAD9. There are essentially two intracellular pathways involving these R-SMADs. The binding of the R-SMAD to the type I receptor is mediated by a zinc double finger FYVE domain containing protein. Two such proteins that mediate the TGF beta pathway include SARA (The SMAD anchor for receptor activation) and HGS (Hepatocyte growth factor-regulated tyrosine kinase substrate). SARA recruits an R-SMAD and permits the binding of the R-SMAD to the Type I receptor [55]. The Type I receptor phosphorylates the serine residue of the R-SMAD. Phosphorylation induces a conformational change in the MH2 domain of the R-SMAD and its subsequent dissociation from the receptor complex and SARA [55]. 4. **CoSMAD binding.** The phosphorylated RSMAD has a high affinity for a coSMAD (e.g. SMAD4) and forms a complex with one. The phosphate group does not act as a docking site for coSMAD, rather the phosphorylation opens up an amino acid stretch allowing interaction. **5. Transcription.** The phosphorylated R-SMAD/coSMAD complex enters the nucleus where it binds transcription promoters/cofactors and causes the transcription of DNA. TGF betas cause the transcription of mRNAs involved in apoptosis, ECM neogenesis and immunosuppression. It is also involved in G1 arrest in the cell cycle.

TGF-β is secreted by many cell types, including macrophages, in a latent form in which it is complexed with two other polypeptides, latent TGF-beta binding protein (LTBP) and latency-associated peptide (LAP). Serum proteinases such as plasmin catalyze the release of active TGF-β from the complex. This often occurs on the surface of macrophages where the latent TGF-β complex is bound to CD36 via its ligand, thrombospondin-1 (TSP-1). Inflammatory stimuli that activate macrophages enhance the release of active TGF-β by promoting the activation of plasmin. Macrophages can also endocytose IgG-bound latent TGF-β complexes that are secreted by plasma cells and then release active TGF-β into the extracellular fluid [53]. Among its key functions is regulation of inflammatory processes, particularly in the gut [56]. TGF-β also plays a crucial role in stem cell differentiation as well as T-cell regulation and differentiation [57]. As such, it is a highly researched cytokine in the fields of cancer, auto-immune diseases, and infectious disease.

The TGF-β superfamily includes endogenous growth inhibiting proteins; an increase in expression of TGF-β often correlates with the malignancy of many cancers and a defect in the cellular growth inhibition response to TGF-β. Its immunosuppressive functions then come to dominate, contributing to oncogenesis [55,58]. The disregulation of its immunosuppressive functions is also implicated in the pathogenesis of autoimmune diseases, although their effect is mediated by the environment of other cytokines present [56].

TGF-β has also a range of biological actions relevant to AAA. It is an important regulator of vascular remodelling, and has effects on both ECM synthesis and degradation [59]. TGF-β signalling has been implicated in many aspects of cellular performance with respect to aneurysm initiation and progression. Under defined circumstances, TGF-β signalling regulates innate immune responses [56], vascular inflammation, SMC growth or apoptosis [50], cellular differentiation or activation [60], and MMP-dependent proteolysis [61], and all of these activities are implicated in the pathogenesis of aneurysms. Despite decades of intensive study in TGF- β pathway, the net effects of this signalling in aneurysm pathogenesis and, perhaps more importantly, the application of its antagonism into therapeutic strategies, have engendered confusion and controversy.

Given the role of TGF-β in the regulation of matrix production and remodeling [62], the initial studies suggested a protective role for this cytokine in limiting disease progression [63]. However, such idea has been challenged by the discovery that endogenous vascular TGF-β activity is increased in patients with Marfan and Loeys-Dietz syndromes, and contributes to vessel dilatation and aneurysm formation [64]. More recent studies reported increased TGF-β expression, retention, and/or signaling in syndromic and nonsyndromic aneurysms of the ascending aorta [65] and suggested a pathogenic role for TGF-β activity in driving thoracic aneurysm formation [66].

On the other hand, recent studies suggest that TGF-β activity plays a different role depending on the type of aneurysm [8,67]. This implies that the pathogenic character of TGF-β in Marfan syndrome cannot be extrapolated to other types of aneurysm.

There is evidence that TGF-β in animal models can protect against the formation, progression and rupture of AAA decreasing inflammatory cell infiltration, degradation of the ECM and apoptosis phenomena in SMCs. Therefore, the signalling pathway of this cytokine could provide new therapeutic targets in AAA [67]. However, this theory is prefixed with the results observed in patients with Marfan and Loeys-Dietz syndrome, where in the endogenous activity of TGF-β vascular is increased, contributing to vascular dilation and aneurysm formation, mainly in the thoracic aorta [22,64].

Thus, in a mouse model of induced AAA by Angiotensin II, the activity of TGF-β opposes the development of AAA in Angiotensin II treated mice, whereas treatment with TGF- β neutralizing antibody aggravates it. TGF-β probably acts protectively by different mechanisms, including inhibition of inflammatory processes, cell infiltration, reduction in degradation of ECM, limiting apoptosis of VSMCs and promoting the formation of new ECM [8,67].

Different studies indicate the association of a chronic inflammatory process with loss of SMCs of the media and a destructive remodelling of the ECM as an important histopathological phenomenon underlying the formation of AAA. Mechanistic studies in animal models of disease indicate the critical role of MMPs in the degradation of collagen and elastin with consequent degradation of the middle layer [68]. Because of the role of TGF-β in regulating production and remodelling of ECM [62], recent studies consider that this cytokine might play a protective role in the AAA, limiting disease progression [63]. Other studies suggest that TGF-β1 is protective in AAA disease, as for atherosclerotic plaques. Using endovascular gene therapy, Dai et al. increased the local expression of TGFβ1 in the wall of an aneurysm induced in the rat model, and demonstrated that increased TGFβ1 expression appeared to arrest aneurysm expansion [63].

6. BMP and activin membrane-bound inhibitor (BAMBI)

The cellular response elicited by TGF-βs is tightly controlled by multiple mechanisms at each step in their signalling pathway [53]. BAMBI (BMP and activin membrane-bound inhibitor) is a 260-aa transmembrane protein that is very highly conserved in vertebrates from humans to zebrafish [69]. BAMBI has been assigned to the transforming growth factor-β (TGFβ) superfamily due to the high homology of its extracellular domain to the TGFβ type I receptors (TGFβRI). Similar to TGFβRI, BAMBI can dimerize with itself, as well as associate stably with all type I (except ALK2) and type II TGFβ superfamily receptors, in a ligand-independent manner [69]. It has, therefore, been postulated that BAMBI can inhibit, or modulate, TGFβ/bone morphogenetic protein (BMP)/activin-mediated signalling by preventing the homo- and heterodimerization of type I/II receptors that is required for transducing TGFβ/BMP/activin-dependent signals [69,70]. Interestingly, and in contrast to TGFβRI, BAMBI is devoid of an intracellular kinase domain. Indeed, the aminoacid sequence of its intracellular domain does not resemble any known domains, making it difficult to predict the function or mode of action of this domain.

Several studies have suggested that BAMBI is involved in pathogenesis of human diseases. Human BAMBI, initially named nma, is down-regulated in metastatic melanoma cell lines [71] and in a subset of high grade bladder cancer [72]. Its elevated expression was suggested to attenuate the TGF-β-mediated growth arrest in colorectal and hepatocellular carcinomas [58] and to induce cell growth and invasion of human gastric carcinoma cells [70]. A recent study has also suggested that BAMBI is involved in Toll-like receptor 4- and lipopolysaccharide-mediated hepatic fibrosis [73].

Little is known about the physiological functions regulated by BAMBI, however, it has been observed that an imbalance of TGF-β signalling, secondary to overexpression of BAMBI, plays an important pathophysiological role in the development of processes of inflammation and fibrosis [74]. Along this lines, the group of Dr. Nistal has previously reported that BAMBI plays a modulatory role of profibrotic TGF-β dependent signalling in the myocardium under pressure overload in both murine models and humans [75].

The group of Dr Nistal is currently focused in the study of BAMBI and its role in the development of AAA in an AngII induced mouse model. The aim of this project is to demonstrate that BAMBI plays a modulator role in AngII-induced aneurysms in the abdominal aortic wall.

We analyse the action of BAMBI neutralizing antibody in ApoE -/- mouse model of AAA induced by AngII. These strain of mice used in the study contain a disruption of the endogenous murine apolipoprotein E (ApoE) gene. ApoE, a glycoprotein, is a structural component of very low density lipoprotein (vLDL) synthesized by the liver and intestinally synthesized chylomicrons. ApoE mediates high affinity binding of chylomicrons and vLDL particles to the LDL receptor, allowing for specific uptake of these particles by the liver, preventing the accumulation of cholesterol rich particles in the plasma.

The development of mouse models of atherosclerosis has been the most critical advancement in the elucidation of factors affecting lipid metabolism, atherogenesis and vascular wall impairment. Among available models, the apolipoprotein E–deficient (ApoE−/−) mouse is particularly popular because of its propensity to develop spontaneously atherosclerotic and vascular lesions on a standard chow diet.

In this line of investigation, it is postulated that the inactivation of the pseudoreceptor BAMBI confers protection against the development and progression of AAA. This would lead to find potential therapeutic targets with novel character in the abdominal aneurysm.

METHODS

1. Equipment available

- Animal-housing unit, Faculty of Medicine, University of Cantabria
- Experimental operating room for small animals: microsurgical equipment, stereo-microscope Zeiss Stemi 2000 CS, cold light source KL 1500 LCD Zeiss, thermal blanket Cibertec RTC1 for mice.
- Inhalation of anesthetic (isoflurane) system to mice and rats.
- Laboratory for echocardiogram performance: ultrasonic microscopy and RMV scanheads (VisualSonics Vevo 770) detectors of cardiac function (ECG) and respiratory systems and micro injection.
- Molecular Biology Laboratory: ultra fast centrifuges, conventional PCR and real-time electrophoresis tanks for DNA / RNA, -80ºC freezer.

2. Experimental design and methods

2.1. Studies in mouse models with AAA

2.1.1. Animals: Adult (16–20 weeks old) male ApoE-deficient (ApoE−/−) in a C57BL/6 genetic background [75]. Mice were housed in a room kept at 22 °C with 12:12 h light/dark cycle and provided with standard food and water ad libitum. The study was approved by the University of Cantabria Institutional Laboratory Animal Care and Use Committee (approval ID 2008/05) and conducted in accordance with the “European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (European Communities Council Directive 86/606/EEC). All efforts were made to minimize animal suffering.

2.1.2. Experimental model of AAA:

- Chronic administration of angiotensin II. 0’1 mL of Angiotensin II was infused via subcutaneous osmotic pumps at 1.2ug/kg/min for a maximum of 14 days.
- Treatment with BAMBI neutralizing antibody. Anti-BAMBI antibody was kindly provided by Drs Ramon and Jesus Merino. Briefly, the antibody was produced by growing hybridoma cells within the peritoneal cavity of a mouse. When injected into a mouse, the hybridoma cells multiply and produce fluid (ascites) in its abdomen. This fluid contains a high concentration of antibody which was harvested and purified afterwards. 40 mg/kg/week of purified anti-BAMBI antibody was injected intraperitoneally in ApoE-/- mice under chronic administration of angiotensin II.

2.1.3. Time monitoring of abdominal aortic dilatation and the development of aneurysm: a reference pattern (time and severity of aortic dilatation) in animal models AAA was established. Abdominal aortic diameter was measured weekly (between 1 and 2 weeks) using echocardiographic images (VisualSonics Vevo-770), a non-invasive technique to observe the aneurysm evolution over time in the same animal.

2.1.4. Sampling: The animals were sacrificed under deep anaesthesia after 2 weeks, when presenting the highest levels of aortic dilatation. Aorta suprarenal section was dissected and frozen down at -80 C to perform molecular biology studies.

2.2. Echocardiographic measurements

Transabdominal echocardiography was performed with ultrasound equipment [Agilent Sonos 5500 (Philips/Hewlett Packard) using 15-MHz linear and 12-MHz sectorial scanheads and Vevo-770 (VisualSonics, Toronto, ON, Canada) using a high-resolution transducer centered at 30 MHz]. Two dimensionally guided, short axis, B-mode recordings of the AA were recorded by an operator blinded to the study groups.

Preparation for imaging. After sedation, the animal was shaved to increase probe contact. The ultrasound device was placed on the anterior abdominal side for ultrasound examination of the abdominal aorta (both suprarenal and infrarenal segments). Imaging depth was set at 2–3 cm when applying zoom; frame rate was approximately 50 frames/s [67]. Mice were placed on the measuring table to a 0 degree tilt with abundant gel in the abdomen.

The transducer was moved until the aorta was strictly parallel to the surface of the device, in order to place this vessel strictly perpendicular to the B-Mode measurement. PW Doppler mode was activated to help localize the abdominal aorta among vessels and structures that can be found in this area (Figure 3).

Images of the abdominal aorta were captured by transversal cut in B-mode and M-mode. In addition, images were captured in a longitudinal section. The B-mode line was placed to cross the renal aorta 1–3 mm down the renal arteries for measurement of aortic diameter (Figure 4). Targeted M-mode–derived measurements of aortic diameters were obtained, i.e., intima-to-intima systolic diameter (Figure 5). In the case of aortic aneurysm, targeted B-mode–derived measurements of the aorta were made at the level of the highest dilatation of the aneurysm.

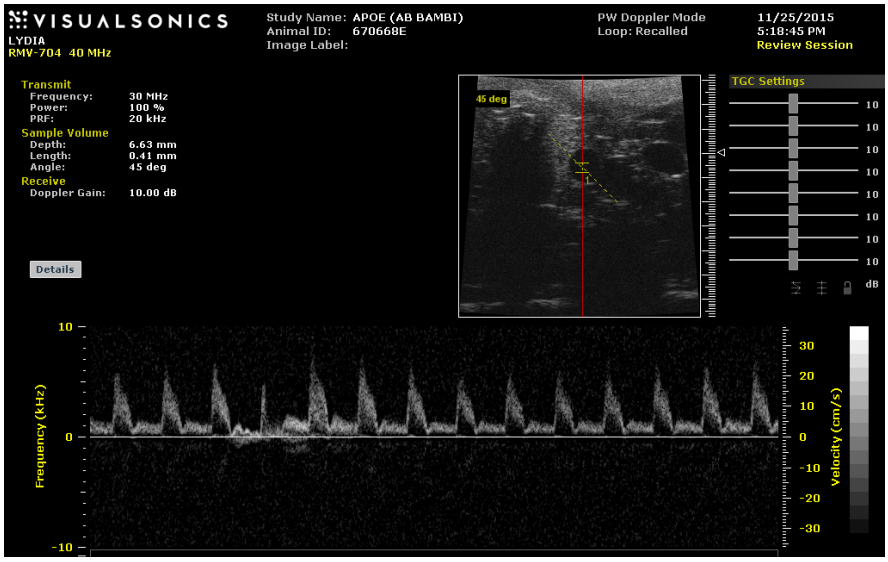

Figure 3. Ecocardiographic measurement of abdominal aorta in one of the subjects of the study (mouse 670668E). PW Doppler mode was used for testing of the correct vessel, whose flow was measured in cm/s.

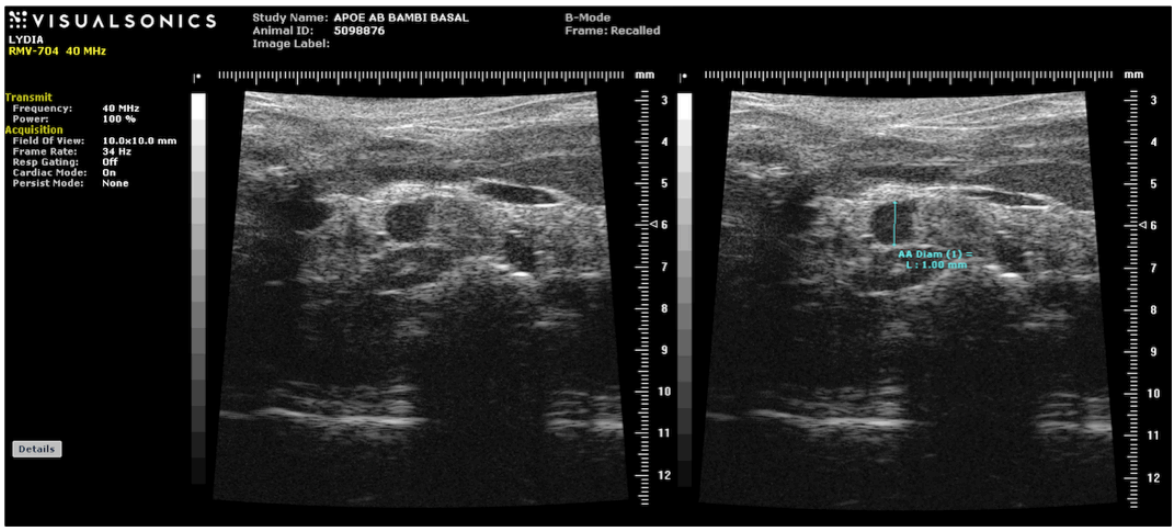

Figure 4. Ecocardiographic measurement of abdominal aorta in one of the subjects of the study (mouse 5098876). B-Mode was used for measuring the diameter of AA (after testing that it was the correct vessel). In this subject, basal measurement (before implantation of Angiotensin II pump) was 1.00 mm.

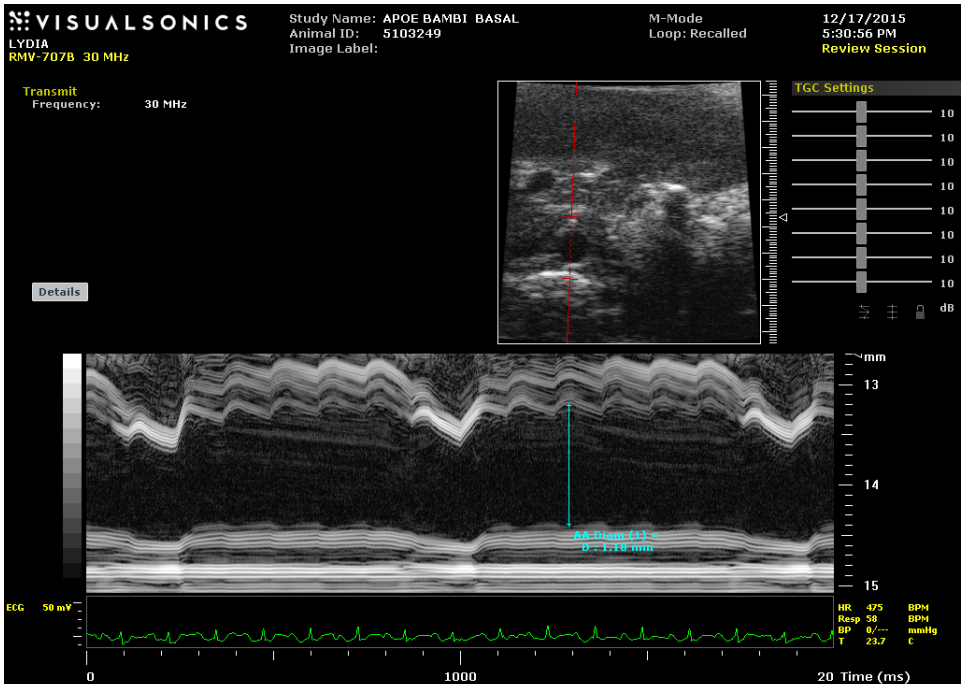

Figure 5. Ecocardiographic measurement of abdominal aorta in one of the subjects of the study (mouse 5103249). M-Mode was used for measuring the diameter of AA (after testing that it was the correct vessel), intima-to-intima systolic diameter. In this subject, basal measurement (before implantation of Angiotensin II pump) was 1.18 mm.

2.3. Trizol RNA isolation from mouse's aorta

Total RNA was obtained from mouse suprarenal aorta by Trizol extraction method (Invitrogen). Samples were mechanically disrupted in presence of Trizol. Briefly, Trizol extraction method consists in the extraction of RNA maintaining the integrity while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transferring of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

2.4. Reverse transcription (RT) into cDNA from RNA

RNA was reverse transcribed using *RevertAid H Minus First Strand cDNA synthesis kit (*Thermo Scientific). This kit is a complete system used for efficient synthesis of first strand complementary DNA (cDNA) from mRNA or total RNA templates (Figure 6). The kit uses RevertAid H Minus M-MuLV Reverse Transcriptase, which has a point mutation that completely eliminates RNase H activity. Therefore, degradation of RNA does not occur during first strand cDNA synthesis.

2.4.1. Components of the kit: RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL), RiboLock RNase Inhibitor (20 U/µL) 5X Reaction Buffer 250 mM, 10mM dNTP Mix, Random Hexamer Primer 100 uM, Water (nuclease-free).

2.4.2. For cDNA synthesis the reaction was incubated 10 min at 25 ºC followed by 60 min at 42 ºC. The reaction was ended by heating at 70 ºC for 10 min.

2.5. quantitative PCR (qPCR)

Real-time PCR, also known as quantitative PCR (qPCR), is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference. qPCR measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of nucleic acid. In traditional PCR, which is based on end-point detection, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid.

Every real-time qPCR contains a fluorescent reporter molecule a TaqMan® probe, which was used in this study, to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

Real-time PCR systems were improved by the introduction of fluorogenic-labeled probes that use the 5′ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. In this study, TaqMan probes were used for performing the qPCR. These are dual labeled, hydrolysis probes that increase the specificity of real-time PCR assays. TaqMan probes contain:

- A reporter dye (for example, FAMTM dye) linked to the 5′ end of the probe
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe
- MGB moiety attached to the NFQ

TaqMan MGB probes also contain a minor groove binder (MGB) at the 3´ end of the probe. MGBs increase the melting temperature (Tm) without increasing probe length; allowing for the design of shorter probes [76]. The explanation of how these probes work is seen in Figure 7.

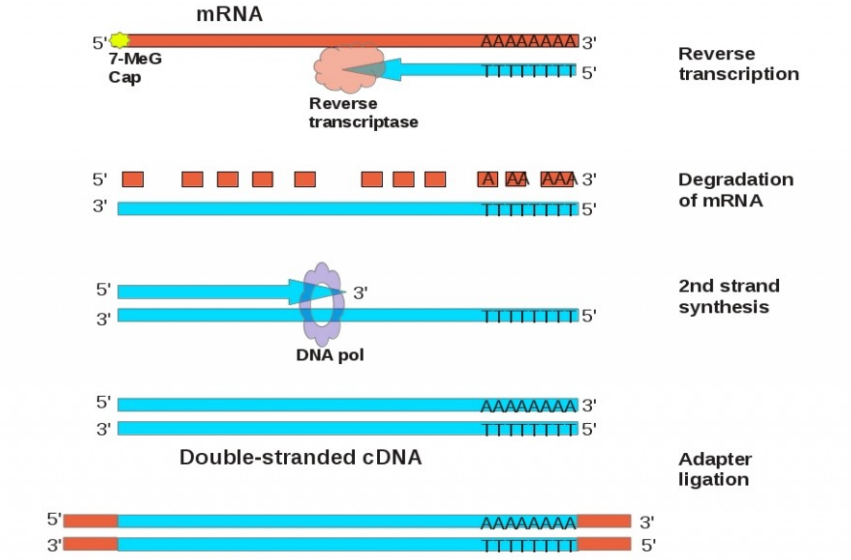

Figure 6. A cDNA is synthesized from mRNA using reverse transcriptase and olig-dT primers. The reverse transcriptase will also degrade the mRNA from the cDNA:mRNA hybrid molecule. DNA polymerase uses the mRNA fragments or random primers to synthesize the 2nd strand of the cDNA molecule. Original illustration by J. Choi

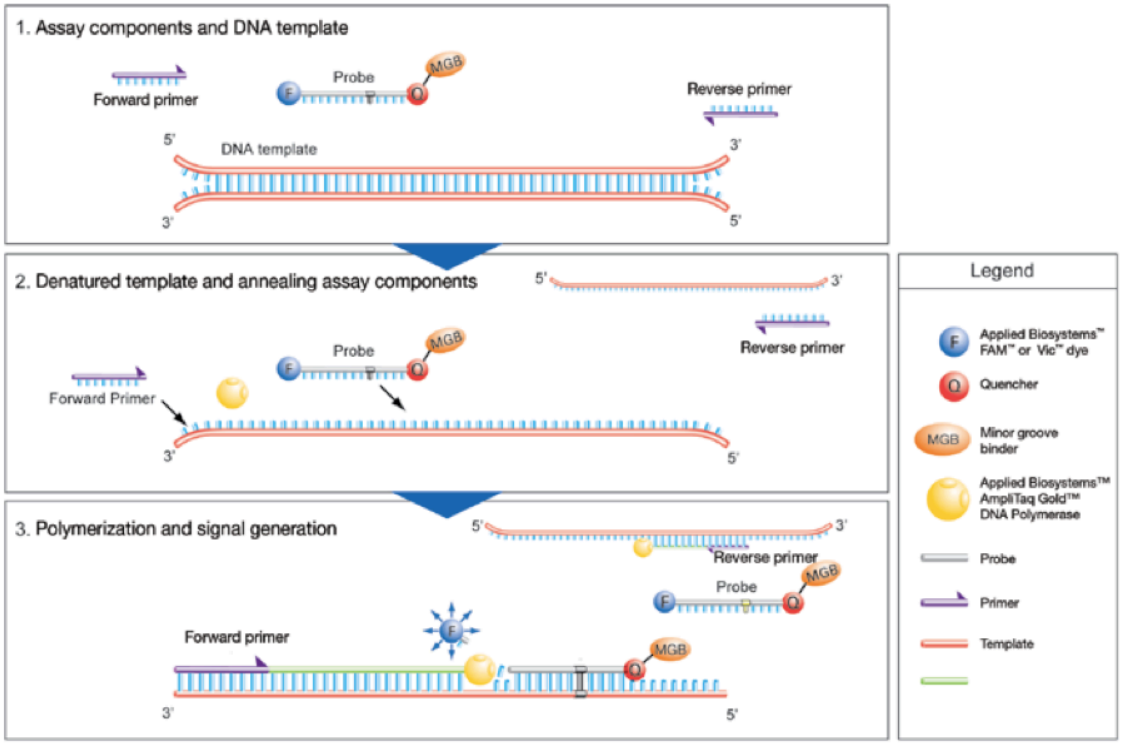

Figure 7. Explanation of steps taken during gene expression assays. **1.** At the start of the real-time PCR reaction, the temperature is raised to denature the double-stranded cDNA. During this step, the signal from the fluorescent dye on the 5´ end of the probe is quenched by the MGB–nonfluorescent quencher on the 3´ end of the probe. **2.** In the next step, the

reaction temperature is lowered to allow the primers and probe to anneal to their specific target sequences. **3.** Taq polymerase synthesizes a complementary DNA strand using the unlabeled primers and template. When the polymerase reaches the TaqMan probe, its endogenous 5´ nuclease activity cleaves the probe, separating the dye from the quencher. With each cycle of PCR, more dye molecules are released, resulting in an increase in fluorescence intensity proportional to the amount of amplicon synthesized.

In this study the cDNA products from aorta mice were amplified by qPCR in a MX-3000P Stratagene thermocycler. The specific TaqMan assays (Thermo SCIENTIFIC) used were, TGF-β1, TGF-β2, TGF-β3, muscle myosin heavy chain 11 (MYH11), smooth muscle alpha (α)-2 actin (Acta2), collagen I (Col I), collagen III α1 (Col III), fibronectin-1 (FN), α-smooth muscle actin (α-SMA) and 18S. For each assay tested, 0,5 ul of cDNA was amplified with TaqMan® Universal PCR Master Mix in a 10 µL reaction volume. Samples were amplified for 1 cycle (50 ºC for 2 min, 95 ºC for 10 min) followed by 40 cycles using universal cycling conditions (95ºC for 15 s, 60ºC for 1 min). The expression levels of the studied genes were normalized to the housekeeping gene, ribosomal 18S. Duplicate transcript levels were determined in a minimum of three independent experiments.

2.6. Statistics

All the assays in mice were performed in a minimum of five individuals per group (n = 5 to 7). Values are reported as means ± S.E.M. The GraphPad Prism 5.01 packages were used. For experiments including only one comparison, P values refer to unpaired 2-tailed Student's t test. Survival curves were compared with log-rank (Mantel-Cox) test. $P \leq 0.05$ was considered statistically significant.