UNIVERSITÀ DEGLI STUDI DI SIENA
DIPARTIMENTO DI MEDICINA MOLECOLARE E DELLO SVILUPPO
DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE

CICLO XXIX

COORDINATORE: Prof.ssa ANTONELLA NALDINI

"Genetic bases of aortopathies: from Marfan syndrome to bicuspid aortic valve"

SETTORE SCIENTIFICO-DISCIPLINARE:

MALATTIE CARDIOVASCOLARI E DEL MUSCOLO SCHELETRICO

DOTTORANDO:

ROSINA DE CARIO

TUTOR:

PROF.SSA BETTI GIUSTI

Anno Accademico: 2015/2016

INTRODUCTION

Aortic aneurysms might represent life-threatening diseases, even though they are less common with respect to many other cardiovascular conditions. The global clinical awareness of aortic aneurysms, along with the latest methods of diagnosis, will definitely help reducing the morbidity and mortality associated with this condition. Genetic screening represents, at present, an emerging and helpful tool in supporting the clinical diagnosis of thoracic aortic aneurysms and/or dissection (TAA/D), a potential lethal condition with a rising incidence. Its clinical impact, as well as the therapeutic management, differs according to the syndromic or non-syndromic manifestations of the disease. TAA/D represents a characteristic clinical feature common to different connective tissue disorders, e.g., Marfan syndrome (MFS), Loeys-Dietz syndrome (LDS), and bicuspid aortic valve (BAV). Analysis of genes associated with these disorders helps in early detection of the disease and in differential diagnosis with important implications for the management of the individuals affected by these complex traits.

Genetic variants in transforming growth factor beta (TGF-β) receptors type 1 (TGFBR1) and type 2 (TGFBR2) genes have been associated with different hereditary connective tissue disorders sharing TAA/D. Mutations in both TGFBR1/2 genes have been described in patients with familial TAA/D and MFS, and they are consistently associated with LDS. Existing literature shows discordant data resulting from mutational screening of *TGFBR1/2* genes in MFS patients. Aim of the first part of the PhD thesis was to investigate the role of *TGFBR1/2* genetic variants in determining and/or modulating MFS cardiovascular (CV) manifestations. At this purpose, we investigated 75 unrelated patients with MFS - referred to the Center for Marfan Syndrome and Related Disorders (Careggi University Hospital, Florence) - who were subjected to *FBN1* and *TGFBR1/2* Sanger mutational screening. Forty-seven MFS patients (63%) carried a pathogenetic *FBN1* mutation. No pathogenetic mutations were detected in *TGFBR1/2* genes while common polymorphic variants where evaluated for their association with CV manifestations. When evaluated as a burden by Genetic scores, some of these variants were found out to be associated with reduced aortic Z-scores and to have a protective effect towards global aortic manifestations severity (aortic Z-score ≥2 or aortic surgery). These data reappraise the role of *TGFBR1* and

TGFBR2 as major genes in MFS patients, while suggest that *TGFBR1/2* genetic variants might play a role in modulating CV manifestation severity in MFS.

BAV is the most common congenital heart defect. Aortic dilatation is a common feature of BAV and an increased risk of aortic dissection has been reported, raising concerns for the proper timing of aortic surgery in these patients. Familial clustering has also been reported, mostly with an autosomal pattern of inheritance with reduced penetrance and variable expressivity. Actually, the heritability of BAV has been estimated to be 89%, thus suggesting the presence of a relevant genetic contribution. Furthermore, BAV has been described as an isolated trait or associated with other clinical manifestations in syndromic conditions, thus the identification of a syndromic condition in a BAV patient is clinically relevant in order to personalize indication to aortic surgery. Differences in the anatomic site of aortic wall vulnerability in MFS and BAV, were evidenced: the maximal aortic dilatation is observed at the level of the proximal thoracic ascending aorta in BAV patients, while it is mainly referred to the aortic root in MFS patients. Therefore, due to differences and similarities between BAV and MFS aortic wall pathology, the pathogenetic mechanisms underlying these conditions are still matter of debate. Seventy-nine BAV patients were enrolled during the three years of the PhD project. Fifteen out of the 79 BAV patients were analyzed by next generation sequencing (NGS) of 91 genes known or with plausibility to be associated with connective tissue disorders. Whenever possible, we performed mutational screening on the first-degree family members of the probands (3 families). Data of this thesis on a cohort of 79 consecutive BAV patients confirmed that they have a strong male predominance (~75%) and have BAV diagnosis at a median age of 42 years. This datum underlines the usefulness in this moment of the patients' clinical history for a differential diagnosis of the aortopathy eventually accompanying BAV diagnosis. The targeted NGS approach allowed the identification of rare mutations in genes previously suspected to be associated with the disease and in genes not previously associated with it; analysis on family members of 3 probands suggest that no major pathogenetic mutations in genes previously associated with BAV as well as in novel genes of the 91 included in our NGS panel are associated as major genetic determinants with this clinical phenotype in its isolated form as well as BAV/TAA. This datum, together with those obtained in our 15 BAV patients and other previously commented literature data, suggest that BAV often perceived as monogenic or oligogenic disorder might be declined as a complex multifactorial genetic trait due to the interaction of individual genetic profile, epigenetic effects, somatic mutations, and environmental factors.

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CHAPTER 1

ANATOMY AND PATHOPHYSIOLOGY OF THE HUMAN AORTA

1.1 THE NORMAL HUMAN AORTA

The aorta is the main artery in the human body, originating from the top of the left ventricle of the heart which pumps blood from the left ventricle into the aorta through the aortic valve. The aorta extends down to the abdomen, where it splits into two smaller arteries (the common iliac arteries). It transports oxygenated blood from the left ventricle of the heart to every organ, ending in the abdomen after bifurcation of the abdominal aorta in the two common iliac arteries. The size of the aorta is directly proportionate to the patient's height and weight [Devereux RB et al., 2012]. Its diameter may range from 3cm to 1.2 cm. It is typically the largest in the aortic root and smallest in the abdominal aorta. It can be divided into sections (Figure 1):

- **Aortic valve:** the aorta starts with the aortic valve just below the first branches of the aorta, the coronary arteries. It is the most important and most commonly replaced valve of the heart. The aortic valve usually has three leaflets and commissures (tricuspid) but, in some pathological conditions, such as bicuspid aortic valve (BAV, OMIM#109730), leaflets and commissures can be two in number. BAV patients have a much higher chance to develop aneurysms and dissection (splitting or separating of tissues) of the aortic root and, more frequently, of the ascending aorta.
- **Aortic root:** the aortic root is the segment above the aortic valve beginning at the aortic annulus and extending to the sinotubular junction. The left and right coronary arteries (supplying the heart with oxygenated blood) arise here from the sinuses of Valsalva. The sinotubular junction is the point in the ascending aorta where the aortic sinuses end and the aorta becomes a tubular structure.

Aortic root is typically aneurysmal in many patients with connective tissue disorder, such as Marfan syndrome (MFS, OMIM #154700).

- **Ascending aorta:** the ascending aorta (AAo) represents the segment between the sinotubular junction and the largest aortic branch vessel, beginning from the upper part of the base of the left ventricle, and passing obliquely upward, forward, and to the right, in the direction of the brachiocephalic artery. AAo describes a slight curve in its course, and it's situate, about 6 cm behind the posterior surface of the sternum. The total length is about 5 cm. AAo presents an increase in its calibre at the union with the aortic arch which is termed the *bulb* of the aorta. It is covered at its commencement by the trunk of the pulmonary artery and the right auricula and it rests posteriorly upon the left atrium and right pulmonary artery. AAo has two coronary arteries which supply the heart as its only two branches, arising near the commencement of the aorta from the aortic sinuses. It is the most anterior (toward the front of the body) portion of the aorta. The normal AAo functions as an elastic reservoir to enhance arterial flow. It stores energy during systole and dissipates it during diastole. The medial layer of the aorta consisting of elastin, collagen, and smooth muscle cells, ensures the elasticity and the tensile strength of the aortic wall. The ascending part of the aorta is more elastic than the descending part due to its greater concentration of elastic fibres.
- **Aortic arch:** the aortic arch (or arch of the aorta, or transverse aorta) begins at the level of the upper border of the second sternocostal articulation of the right side, and runs at first upward, backward, and to the left in front of the trachea; then travels backward on the left side of the trachea and finally passes downward on the left side of the body of the fourth thoracic vertebra. At this point the aortic arch continues as the descending aorta. It has typically three branches The first, and largest, branch of the arch of the aorta is the brachiocephalic trunk, originating behind the manubrium of the sternum, positioned at the right and anteriorly to the other two branches. Brachiocephalic artery supplies right arm and right portion of head and brain with oxygenated blood. Next, the left common carotid artery, carrying blood to the left head and brain, originates from the aortic arch to the left of the brachiocephalic trunk, then ascends along the left side of the trachea and through the superior mediastinum. The last branch vessel from the aortic arch is usually the left subclavian artery, supplying the left arm with blood; it comes off of the aortic

arch to the left of the left common carotid artery ascending through the superior mediastinum and along the left side of the trachea. The arch of the aorta forms two curvatures: one with its convexity upward, the other with its convexity forward and to the left. Its upper border is usually about 2.5 cm below the superior border to the manubrium sterni. Blood flows from the upper curvature to the upper regions of the body, located above the heart (namely the arms, neck, and head). Coming out of the heart, the thoracic aorta has a maximum dimension of 4 cm at the root. By the time it becomes the ascending aorta, the diameter should be <3.5-3.8 cm, and 3 cm at the arch. The diameter of the descending aorta should not exceed 2.5 cm [Mao SS et al., 2008]. There are many anomalies of the aortic arch such as the bovine arch, where branches vessels off the aortic arch are two instead of three. The operations involving the aortic arch usually require the body to be cooled down using the heart-lung machine. This (hypothermic circulatory arrest). Alternatives are complex hybrid operations that usually do not require heart-lung-machine.

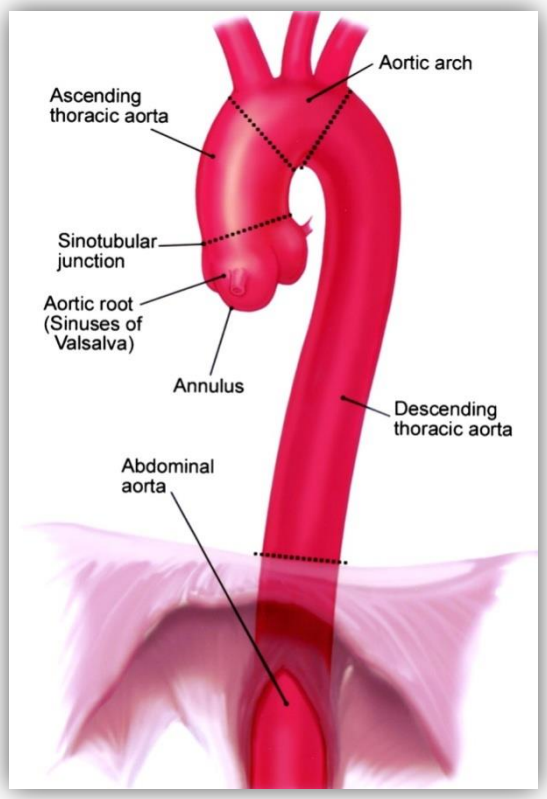


Fig 1. Anataomy of thoracic and proximal abdminal aorta (©Massachusetts General Hospital Thoracic Aortic Center)

- **Descending thoracic aorta:** the descending thoracic aorta begins at the lower border of the fourth thoracic vertebra where it is continuous with the aortic arch, and ends in front of the lower border of the twelth thoracic vertebra, at the aortic hiatus in the diaphragm where it ends at the first branch in the abdominal aorta; the celiac artery. It is contained in the posterior mediastinal cavity. The thoracic aorta has a curved shape that faces forward, and a radius of approximately 1.16 cm. It has six paired branches: bronchial arteries, mediastinal arteries, esophageal arteries, pericardial arteries, superior phrenic artery, and intercostal arteries (nine pairs). Through its various branches, it supplies blood to the esophagus, lungs, and the chest area, including the ribs and mammary glands.

The descending thoracic aorta is the most posterior (toward the back of the body) portion of the aorta. Therefore, the most common symptom from the descending thoracic aorta is back pain, and it may be confused with back pain associated with back muscles, joints and nerve pain. As for the AAo, the descending thoracic aorta can be affected by common diseases, such as atherosclerosis, aneurysms and dissections (occurring sporadically or representing one of the clinical features of an hereditary tissue disorder such as MFS, BAV, Loeys-Dietz syndrome [LDS, OMIM#609192]).

- **Abdominal aorta:** the abdominal aorta represents the largest artery in the abdominal cavity. Being a direct continuation of the descending aorta, it branches to the intestine and the kidneys and divides into left and right the two common iliac arteries. The abdominal aorta begins at the level of the diaphragm, crossing it via the aortic hiatus at the vertebral level of T12. It runs down the posterior wall of the abdomen, anterior to the vertebral column, following the curvature of the lumbar vertebrae, that is, convex anteriorly. It runs parallel to the inferior vena cava, which is located just to the right of the abdominal aorta, and becomes smaller in diameter as it gives off branches. This is thought to be due to the large size of its principal branches. At the 11th rib, the diameter is 12.2 cm long and 5.5 cm wide and this is because of the constant pressure. The abdominal aorta is clinically divided into 2 segments: 1) the paravisceral segment, off which the visceral branches arise and 2) the infrarenal segment, inferior to the renal arteries and superior to the iliac bifurcation. The branch vessels of the abdominal aorta include the celiac artery, the superior mesenteric artery, the left and right renal arteries, and the inferior mesenteric artery. Most of the major organs receive blood from branches of the abdominal aorta. The infrarenal abdominal aorta is most affected by the atherosclerotic process and is similarly the most common site of abdominal aneurysm formation. Abdominal aortic aneurysm (AAA, OMIM#100070) is more frequent than thoracic aortic aneurysm (TAA, OMIM#607086) [Larsson E et al., 2008]. Corresponding to their differing embryology, the thoracic aorta and abdominal aorta are structurally very different from one another and they also carry genetic differences underlying aneurysms formation in their specific locations, with no genetic overlap between the two types of dilatation [Kuivaniemi H et al., 2015].

1.2 MICROSCOPIC ANATOMY OF THE AORTA

The normal human adult aortic wall is composed of 3 layers, listed from the blood flow surface outward:

- **Intima:** endothelial layer on a basement membrane with minimal ground substance and connective tissue.
- **Media:** bounded by an internal elastic lamina, a fenestrated sheet of elastic fibers; layers of elastic fibers arranged concentrically with interposed smooth muscle cells; bounded by an external elastic lamina, another fenestrated sheet of elastic fibers.
- **Adventitia:** a resilient layer of collagen containing the vasa vasorum and nerves. Some of the vasa vasorum canpenetrate into the outer third of the media (Figure 2).

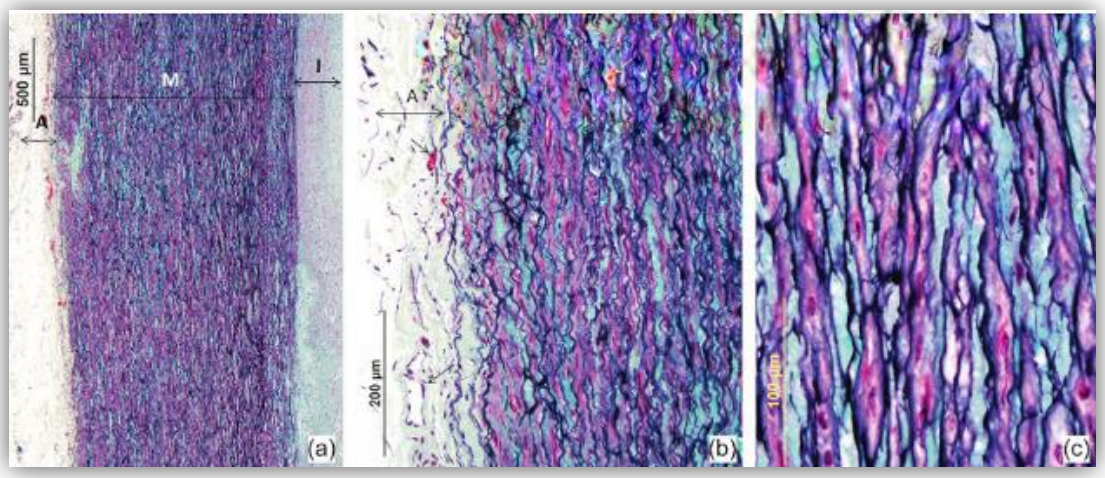


Fig 2. Aorta histology: (a) aorta has three histologic layers, the intima (I), media (M), and adventitia (A). I consists of an endothelial cell layer overlying a thin layer of connective tissue; (b) M consists of concentric fenestrated lamellae of elastic tissue, smooth muscle cells, proteoglycans (in green), collagen fibers and microfibrils of elastin in between the large elastic lamellae. A presents collagen and elastic fibers with fibroblasts and interspersed vasa vasorum (black arros); (c) the media wall; parallel elastic lamellae with smooth muscle cells, proteoglycans and collagen in between the lamellae (from Buja and Butany Cardiovascular Pathology, 4th Edition, Jan 2016)

This basic structural layout is maintained throughout all of the heart chambers but their composition and thickness vary among the different regions of the circulatory tree. Regional variations such as the stratification of the extracellular matrix (ECM) into elastin-, proteoglycan-, and collagen-rich layers confers distinct biochemical properties to the different sections and their supporting structures.

- **Valves:** valve interstitial cells are present in all valvular layers and they mediate ECM remodelling and maintenance of the valve structural integrity via the

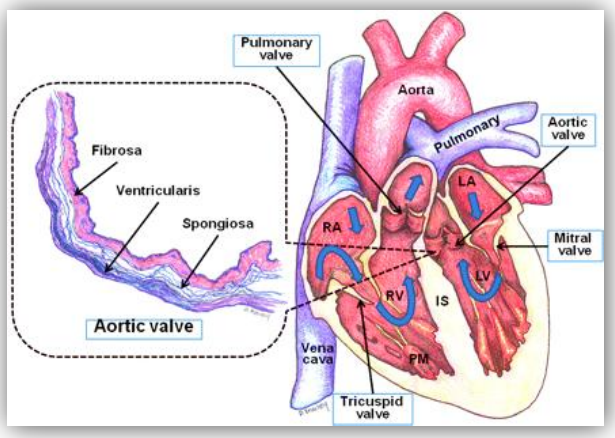


Fig 3. Systematic representation of heart chambers, valves, and valvular histology. Blue arrows indicate unidirectional blood flow through the various chambers of the heart. Histology of aortic valves, mimicking Elastic Van Giesen stain (collagen, red; elastic fibers, black). Three well-defined layers: (1) fibrosa, facing the outflow surface, consisting predominantly of dense collagen fibers; (2) spongiosa, composed of loose collagen and abundant proteoglycans (clear spaces); and (3) ventricularis, facing the inflow surface, composed of primarily collagen and elastic fibers. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; IS, interventricular septum; and PM, papillary muscles (from Elangbam CS, 2010)

synthesis of ECM components and expression of matrix-degrading enzymes and their inhibitors. In monolayer cell cultures, quiescent valve interstitial cells appear in a spindle-shaped morphology; once activated in response to valve injury or abnormal hemodynamic/mechanical stress, they exhibit a myofibroblastic phenotype with the enhanced expression of α-smooth muscle actin and other proteins of the contractile system, they also secrete cytokines such as TGF-ß in order to activate cellular repair processes including proliferation, migration and matrix remodelling. The above processes, when dysregulated, may result in clinical valvular diseases characterized by pathological fibrosis, chronic inflammation, calcification [Liu AC et al., 2007; Li C et al., 2013]. Atrio-ventricularis valves are comprised of four layers (Figure 3), the *auricularis*, the *spongiosa*, the *fibrosa* and the *ventricularis*. The first layer covers the spongiosa on the basal third of the leaflet and is mainly composed of prominent elastic fibers, collagen, and smooth muscle cells. The ventricularis layer exhibits small elastic fibers blending with the sub-endocardium of the adjacent ventricle. Both layers are extendible and allow recoil of the valve during the cardiac cycle. The central layer, the fibrosa, extending the entire length of the leaflet to the free edge, can be considered as an extension of the *annulus fibrosus* and it's composed of dense, circumferential collagen and elastic fibers constituting a connective tissue which extends into the tendinous cords and papillary muscles. This layer is characterized by a high tensile strength providing structural support to the valves. The spongiosa contains mostly proteoglycans (with hyaluronic acid, chondroitin sulphate B and AC, and heparin being the predominant glycosaminoglycans) with interspersed collagen and elastic fibers. In the mitral valve, the proximal third of the layer also contains cardiac myocytes and

capillaries extending from the left atrial myocardium. The spongiosa allows for tissue compressibility. In the tricuspid valve, valvular layers are thinner and myocytes are absent in the spongiosa. The auricularis contains a bigger amount of smooth muscle cells appearing prominent on the posterior and septal leaflets. Semilunar valves are structurally similar to the atrio-ventricular valves with few minor variations (Figure 4) [Hinton RB et al., 2011].

- **Abdominal and thoracic aorta:** the intima of the aorta consists of an endothelial cell layer overlying a basement membrane and underlying connective tissue and smooth muscle cells. The border of the intima is separated by the internal elastic lamina (IEL) which forms the boundary between the intima and the media. The smooth muscle layer of the latter has a circular arrangement along the long-axis of the aorta. Smooth muscle cells and the media are furthermore separated by multiple layers of *elastic lamellae*, representing the medial lamellar units which are defined as bands of elastin filaments with associated collagen fibers and smooth muscle cells working as one to provide elasticity.

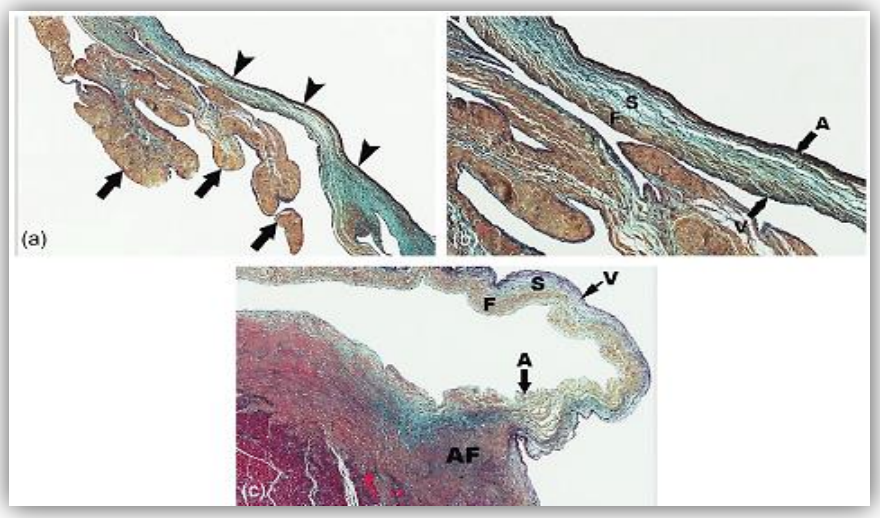


Fig 4. Cardiac valves: (a) The leaflets of the atrioventricular valves and their layers. (b) Auricularis layer (A) with prominent elastic fibers, collagen, and smooth muscle cells. The fibrosa (F) with dense collagen and some elatic fibers. The spongiosa (S) contains mostly proteoglicans with interspersed collagen and sparse elastic fibers. The ventricularis (V) with small elastic fibers. (c) The cusps of the semilunar valves and their four layers. The fibrosa is the extension of the annulus fibrosus (AF) and comprises dense collagen and small amount of elastic fibers (from Buja and Butany Cardiovascular Pathology, 4th Edition, Jan 2016)

The ascending aorta has a thinner intima, thicker media, and more medial lamellar units compared to the abdominal aorta. Compared to the abdominal aorta, the thoracic aorta has significantly higher elastin and collagen content and a lower collagen-to-elastin ratio being, as a consequence, more predisposed to compliance than the abdominal aorta. Vascular smooth muscle cells of ascending aorta originate from the neural crest, while those in the descending and abdominal aortas originate from mesoderm and endothelial cells [Gittenberger-de Groot AC et al., 1999].The early embryo develops a common set of precursor vessels that differentiate into arteries, veins and lymphatic vessels. Primitive arteries are surrounded by smooth muscle cells (SMCs) of mesodermal origin. Following extensive remodeling, the original primitive SMCs of the aortic arch and the thoracic aorta are replaced by a second wave of SMCs that migrate from the neural crest [Wiegreffe C et al., 2009]. These neural crest–derived SMCs are likely better suited to adaptively remodel the thoracic aorta to withstand the higher pulse pressure and ejection volume by laying down more elastic lamellae during development and growth. In contrast, the epigenetic programming of the SMCs in the abdominal aorta remains more similar to that of the original primitive arterial SMCs [Tromp G et al., 2010]. Moreover, the neural crest cell precursors of the thoracic aorta respond differently to various cytokines and growth factors than the mesodermal precursors of the abdominal aorta [Ruddi JM et al., 2013]. Homocysteine has been found to stimulate the proliferation and synthetic activity of neural crest vascular SMCs while those of mesodermal origin are unaffected [Dalton ML et al., 1997]. Another example is angiotensin II, a vasoactive peptide associated with vascular remodeling and atherosclerosis, which when continuously infused led to aneurysms of the suprarenal abdominal aorta in mice deficient in the gene for apolipoprotein E ($Apoe^{-/-}$) without effect on the thoracic aorta [Rateri DL et al., 2011], highlighting, in addition, the differential response of the cells comprising the thoracic aorta as compared to the abdominal aorta. A final example is transforming growth factor-beta (TGF-β), which is an established contributor to vascular development and regulator of cell growth and differentiation. When treated with TGF-β, neural crest vascular SMCs demonstrated increased DNA synthesis and collagen production, while mesodermal vascular SMCs did not respond [Gadson PF et al., 1997]. This may explain why mutations in the TGF-β receptor may lead to TAA but have

little effect on the abdominal aorta. These structural and embryological differences have important implications in aneurysm pathogenesis and incidence and affect the presentation of the two distinct pathologies (thoracic and abdominal aortic aneurysms). Blood vessels (*vasa vasorum*) reside in the adventitia, the outermost layer of the aorta, consisting of collagen fibers and fibroblasts providing support and structure to the aortic wall. Vasa vasorum are also present in the outer medial wall and they supply oxygen to the outer one-third to one-half of the thoracic aorta. The inner half is supplied from the lumen. The abdominal aorta has no medial vasa vasorum and it's thinner than the thoracic aorta, supporting less than 28 layers of vasa vasorum. The thoracic aorta consists of approximately 36 layers of elastic lamellae [Hinton RB, 2011]. It has also been suggested that there is a strong atherosclerotic background behind AAA formation (partly because AAA shares the exact same major predisposing factors as atherosclerosis), which is not the case in those with TAA and TAAD. However, many of the genes predisposing to atherosclerosis have not been shown to be implicated in AAA formation and, thus, the two are unlikely to share a common genetic background [Annambhotla S et al., 2008].

1.2.1 PATHOLOGIC PROCESSES

The aorta and its components are susceptible to age related changes which manifest themselves as a progressive decline in the elastic properties of the artic wall. Fragmentation of elastin followed by an increase in collagenous remodeling can be seen, thus resulting in an increase in collagen to elastic ratio leading to loss of aortic compliance [Schlatmann TJ et al., 1977]. The degeneration of elastin, whose integrity is essential for the maintenance of the heart pumping function, results in an increase in wall stiffness and in pulse wave velocity. With age, collagen becomes thicker, more linear in arrangement, while tropoelastin expression is 50% decreased every decade. The aorta becomes tortuous, its diameter increases with age, the largest increase occurring in the ascending aorta and the smallest in the abdominal aorta. Hypertension and atherosclerosis greatly affect the extent of age-related aortic changes, resulting in an increased abdominal aortic circumference and intimal thickness [O'Rourke MF et al., 1982; Hockson SS et al., 2010; Virmani R et al., 1991]. The main degenerative

pathologies affecting the aorta are so represented by CMN and atherosclerosis, predominantly located at the intima level the first, and at the media level the latter.

CMN: the earliest terminology of degenerative aortic histopathology was the classic description of medionecrosis by Erdheim in 1930 and, since then, different groups have used a variety of terms to describe the histopathologic changes they have observed in the ascending aortas affected by diseases and aging [Schlatmann TJ et al., 1977; Homme JL et al., 2006]. CMN is a disorder of large arteries, in particular the aorta, characterized by a medial degeneration whose individual components are represented by:

- Mucoid extracellular matrix accumulation
- Elastic fiber fragmentation and/or loss
- Elastic fiber thinning
- Elastic fiber disorganization
- Smooth muscle cell nuclei loss
- Laminar medial collapse
- Smooth muscle cell disorganization
- Medial fibrosis [Halushka MK et al., 2016].

The loss of elasticity of the aorta and its weakening can lead to the most important complications of CMN, annuloaortic ectasia with aortic insufficiency and aortic dissection.

Atherosclerosis occurs more frequently in the abdominal aorta with respect to the thoracic aorta and gives rise to cerebrovascular disease and coronary artery disease (CAD) through a slowly progressing lesion formation and luminal narrowing of arteries. Early lesion consist of an atheroma which represents an accumulation of degenerative material in the tunica intima (inner layer) of artery walls consisting of (mostly) macrophage cells or debris, containing lipids (cholesterol and fatty acids), calcium and a variable amount of fibrous connective tissue. The underlying pathology is characterized by a chronic inflammatory process of the arterial wall that occurs at predilection sites with disturbed laminar flow, such as branch points [Moore KJ et al., 2011]. Endothelial dysfunction and structural alterations including the absence of a confluent luminal elastin layer and the exposure of proteoglycans initiate the degenerative process with the subendothelial accumulation of low-density lipoprotein (LDL). Elevated levels of circulating cholesterol are transported by apolipoprotein B100 (ApoB100), which binds to negatively charged extracellular matrix proteoglycans leading to retention of LDL particles in the intima, where they are susceptible to oxidative modification by reactive

oxygen species or enzymes such as myeloperoxidase or lipoxygenases released from inflammatory cells. Oxidized lipids and LDL (oxLDL) trigger the expression of adhesion molecules and the secretion of chemokines by endothelial cells, which, together with the deposition of platelet-derived chemokines, drive intimal immune cell infiltration. Early 'fatty-streak' lesions consist of T cells and monocyte-derived macrophage-like foam cells loaded with lipids. Successive accumulation of apoptotic cells, debris and cholesterol crystals forms a necrotic core (Figure 5).

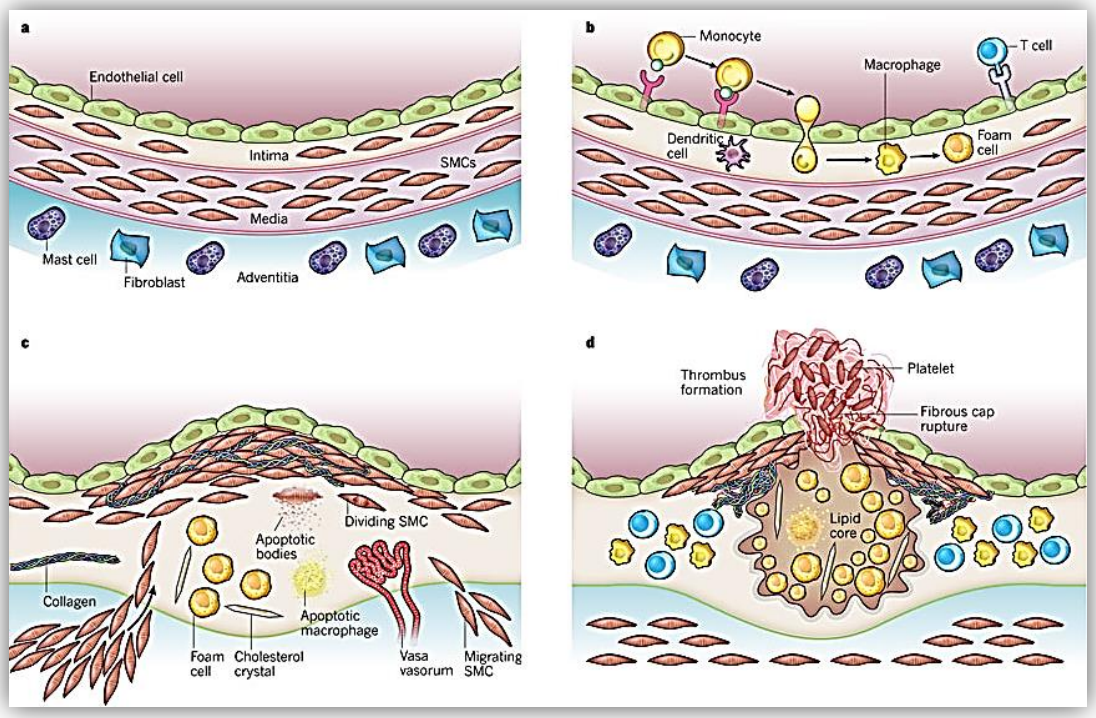


Fig 5. Stages in the development of atherosclerotic lesions: a)Elastic arteries with clearly demarcated laminae in the tunica media, where layers of elastin lie between strata of smooth muscle cells (SMCs). The adventitia, the outer layer of arteries, contains mast cells, nerve endings and microvessels. b) The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. c) Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. d, Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow (from Libby P et al., 2011)

Fibroatheromatous plaques are covered by a fibrous cap composed of collagen and smooth muscle cells (SMCs), which are replaced by macrophages in the thinning inflamed caps that are prone to rupture. The 'shoulder' regions are heavily infiltrated by T cells and mast cells, which produce enzymes and proinflammatory mediators,

contributing to adventitial inflammation of advanced plaques [Ylä-Herttuala S et al., 2013]. A series of complications affecting the plaque makes atherosclerosis symptomatic and life-threatening. A cap rich in fibrillar collagens and elastin covers the top of the atheroma and it confers considerable stability to the plaque, protecting against rupture and the devastating complication of thrombosis. Local dissection can evolve in external rupture, this occurrence being particularly common in the descending thoracic trait of the aorta. From this ulcerated plaque and from mural thrombi, emboli of different nature can dislodge inducing both thrombo- and athero-embolism. Advanced atherosclerotic lesions are characterized by a mineralization of the intima and the media layers of the aorta (intimal or atherosclerotic calcification). Histologically, intimal calcification is observed as hydroxyapatite crystals originating from secreted matrix vescicles in the vicinity of the lipid rich necrotic core and in close proximity to cellular debris and inflammatory cell infiltrate [Johnosn RC et al., 2006]. Metalloproteinases (MMPs) secreted by macrophages and smooth muscle cells (SMCs) in the shoulder regions of the plaque degrade collagens in the fibrous cap rendering the plaque vulnerable to rupture events responsible for the majority of acute coronary syndromes, which are in turn the major causes of morbidity and mortality in coronary artery disease [Galis ZS et al., 1994]. Secreted MMPs also determine an atrophy of the media which becomes thinner and weaker as a result of SMCs apoptosis as well, thus leading to fusiform aneurysms formation, a cardiovascular complication often associated with atherosclerosis. Dilatations exceeding 4.5-5.0 cm in diameter are considered at risk for rupture putting the indication for surgery [Thiene G et al., 2006].

Inflammatory disorders represent a third broad category as a predisposing factor causing aortic diseases. These occur in isolation or in the context of systemic disorders such as inflammatory vasculitides. They include infectious and non infectious causes.

Infectious aortitis: having become a rare occurrence after the advent of antibiotics, the best known infection route for this condition is via bacteremia in the setting of pre-existing damage to the endothelium due to reduced immune barrier function.

The most frequently affected patients are those with arteriosclerotic lesions of the aortic wall or arteriosclerotic aneurysms and concomitant or previous infective endocarditis, even though also patients with a healthy aortic wall can be affected by aortitis in the setting of endocarditis [Bansal RC et al., 2001]. *Treponema pallidum* represents the pathogen microrganism in classical *Syphilitic aortitis*, and it directly penetrates the

media layer of the aorta through the vasa vasorum. Cardiovascular manifestations occur during the third stage of the disease (*tertiary syphilis*or *cardiovascular syphilis*). Syphilitic aortitis is the most common manifestation and it typically involves the ascending aorta. Uncomplicated aortitis is usually asymptomatic, while symptoms include substernal dull, aching pain in about 20% of patients with aortitis and heart failure in 25%. The most common complication of untreated syphilitic aortitis is aortic regurgitation; coronary ostial stenosis occurs in 20% of patients with syphilitic aortic insufficiency. Angina, the most frequent symptom, is rarely associated with myocardial infarction. Aneurysm formation is the least common complication of aortitis, it is symptomatic in 5 to 10% of patients and it usually presents itself in a saccular shape rather than fusiform and with involvement of the ascending aorta in 50% of patients [Singh AE et al., 1999]. *Mycotic aneurysm* of the thoracic aorta is rare but can be fatal if not diagnosed early, with bacterial endocarditis which now accounts for the minority of cases reported. *Staphylococcus aureus* and *Salmonella* species are the predominant causative organisms, although many opportunistic organisms may be the cause of the condition in immunosuppressed patients and in those who use intravenous drugs. The first clinical manifestation is often the sequela of aneurysm expansion or rupture with surgery remaining the definitive treatment, albeit perioperative mortality can be as high as 63% in patients with aneurysm rupture (Figure 6) [Malouf JF et al., 2003]. *Takayasu arteritis* (TAK, OMIM#207600), is a rare chronic inflammatory arteritis primarily affecting the large vessels, in particular the aorta and its branches. Disease onset is generally seen in the second or third decade of life, with non-specific symptoms including fever, nocturnal sweating, malaise, weight loss, joint and muscle pain as well as mild anemia. As vascular lesions progress, stenosis and occlusion occur with resultant ischemia and changes in the aortic region of the abdominal section in particular. The aortic arch and its branches are affected almost equally. Clinical features include weakened or absent peripheral pulse (pulseless disease), vascular bruits, renal

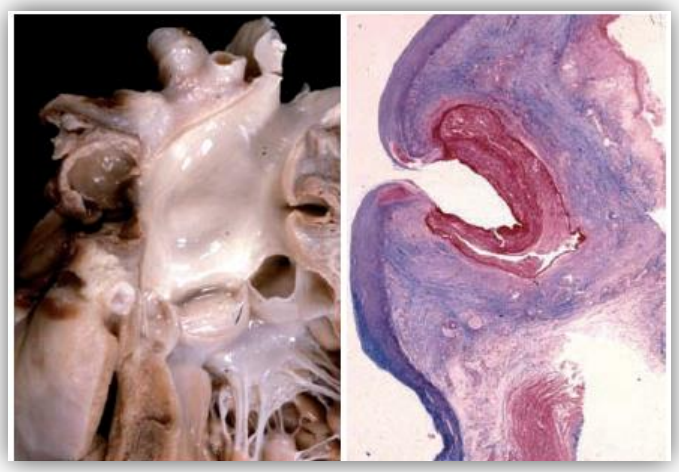


Fig 6. Rupture of the ascending aorta in mycotic aneurysm [Thiene G, 2006]

hypertension due to renal artery stenosis, retinopathy, aortic valve insufficiency due to dilation of the valve ring and subsequent dilated cardiomyopathy and myocardial ischemia due to coronary ostial stenosis. As a result of ischemic or hypertensive cerebral damage, syncope and epileptic seizures may occur [Töpel I et al., 2016].

Non infectious aortitis: *Giant cell arteritis* is is the most common form of non-infectious arteritis with the aorta itself affected by inflammatory changes in only a subgroup of patients. Stenotic and aneurysmal lesions of the supra-aortic branches represent the most common features of the disease resulting from the weakening of the aortic wall which is interested by a prominent inflammation process riddled with polinucleate giant cells [Eagleton M et al., 2012].The incidence in Europe is 15-25 cases per 100,000 inhabitants per year, considerably higher than TAK (2.6 cases per 1,000,000 inhabitants per year) [Gornik HL et al., 2008].

Traumatic pathology: traumatic thoracic aortic injury, although uncommon in the clinical practice of trauma medicine, is associated with a high morbidity and mortality, the majority of the cases being caused by traffic accidents. Approximately 80% of patients with thoracic aortic injury die at the scene of the trauma. In those who make it to the hospital, clinical diagnosis is difficult due to the non-specific signs and symptoms and the presence of distracting injuries. Clinical presentation may include chest or mid-scapular back pain, signs of external chest trauma or haemodynamic instability. Clinical suspicion is usually based on mechanism and severity of the injury, the haemodynamic status of the patient and/or the presence of related injuries. The diagnosis ultimately relies on appropriate imaging. Trauma to the aorta may result in an aortic laceration (presenting atypically transverse tear in the intima which may extend through the vessel wall), aortic rupture or pseudoaneurysm, aortic rupture contained by adventitia or periaortic tissue and aortic intramural haematoma. The 90% of injuries occurs at the aortic isthmus, 5% at the ascending aorta, and 5% diaphragmatic hiatus. Treatment is with an aortic stent graft or open repair and mortality is very high (>95% if untreated, ~80% die immediately and >30% if in hospital and treated) [Watanabe K et al., 2013].

Neoplastic pathology: tumors originating from the heart and great vessels have an incidence of about 0.02 % of autopsy, 70% of these being benign. Tumors of the great vessels are rare compared to the cardiac tumors. Malignant tumor that occurs in the aorta have been classified following two types, i.e., intimal or luminal type which main lesion is in the intima, and the mural type which main lesion is in the media to adventitia: in particular, the intimal type of tumor is that protruding and exposed to the

vessel lumen resulting in strong degeneration and necrosis, and it is diagnosed as intimal sarcoma [Iwabuchi K et al., 2008]. Benign tumors are more common in order of myxoma, papillary fibroelastoma, rhabdomyoma, fibroma, hemangioma, and cystic tumor of atrio-ventricular node, and malignant tumors are more common in order of angiosarcoma, unclassified sarcoma, and malignant fibrous histiocytoma.

Congenital pathology: *Supravalvular aortic stenosis* (SVAS, OMIM#185500), which may be present in a non syndromic condition or in syndromic conditions such as Williams-Beuren syndrome, is a systemic elastin (ELN) arteriopathy widly affecting the supravalvular aorta. ELN arteriopathy is genetically heterogeneous and occurs as a consequence of haploinsufficiency of the *ELN* gene on chromosome 7q11.23 resulting from either a microdeletion of the entire chromosomal region or *ELN* point mutations [Merla G et al., 2012]. ELN arteriopathy is usually characterized by a systolic murmur related to the SVAS with affected subjects becoming symptomatic the age of 20. Symptoms are similar to those related to valvular aortic stenosis (dyspnea, angina, and syncope), with aortic involvement being often responsible for the clinical outcome. Aortic stenosis results in increased resistance to blood flow that causes elevated left heart pressure and cardiac hypertrophy and may evolve to cardiac failure and death if left untreated. Variable hypoplasia and thickening can involve the ascending, transverse arch, and descending aorta and great systemic arteries containing the largest number of ELN fibers in their media are the most affected. Hypertension develops as a consequence of the lacking systemic vessel distensibility, but occasionally may be secondary to renal artery stenosis [McElhinney DB et al., 2000]. Intracranial focal and segmental stenotic artery disease can be responsible for stroke [Kalbhenn T et al., 2003]. *Coarctation of the aorta* (CoA, OMIM#120000) is the sixth most common congenital lesion accounting for 4-6% of live births with congenital heart disease [Reller MD et al., 2008]. It consists of a constricted aortic segment comprising localized medial thickening with some infolding of the media and superimposed neo intimal tissue and it represents an important cause of secondary hypertension. More commonly, the coarctation is discrete although a long segment of the aorta may be narrowed. All coarctations are juxtaductal. Abnormal arterial compliance has been implicated as a risk factor for aortic wall injury as well as advanced age is considered a risk, likely secondary to aortic wall calcification and consequently diminished aortic elasticity [Forbes TJ et al., 2007]. Dilatation of the descending aorta immediately distal to the coarctation segment (poststenotic dilatation) represents a common feature of the disease. Aortic rupture is the most serious and feared complication of endovascular

treatment for CoA, and may quickly lead to circulatory collapse and death [Rosenthal E et al., 2010]. Thoracic coarctation is often accompanied by varying degrees of hypoplasia of the isthmus of the aorta and transverse aortic arch [Rao PS et al., 2005]. This condition is often associated with CMN, both proximal and distal to the narrow segment, characterized by depletion and disarray of the medial elastic fibers which may be congenital or acquired. BAV is present in more than half of patients with a CoA overall, and approximately two thirds of those with a native or repaired CoA and an aneurysm involving the ascending or descending aorta [Oliver JM et al., 2004]. *MFS* displays an autosomal dominant inheritance, with a prevalence of 2 per 10,000. Mutations in *FBN1* gene encoding fibrillin-1 have been described in 70-90% of patients fulfilling MFS diagnosis [Loeys BL et al., 2010]. Cardiovascular manifestations include dilatation of the aorta at the level of the sinuses of Valsalva, a predisposition for aortic tear and rupture, mitral valve prolapse with or without regurgitation, tricuspid valve prolapse, and enlargement of the proximal pulmonary artery (See Chapter 3). *BAV* represents the most common congenital heart defect, as it affects 1.3% of the adult general population [Verma S et al., 2014]. Among complications associated with BAV, dilatation of the ascending aorta contributes to negatively affect the clinical outcome, thus being responsible for aortic dissection and rupture (See Chapter 4). *LDS* was described for the first time in 2005 as a novel autosomal dominant heritable connective tissue disorder (HCTD) with widespread systemic involvement [Loeys BL et al., 2005]. LDS is typically characterized by the triad of hypertelorism (widely spaced eyes), bifid uvula/cleft palate, and arterial tortuosity with aortic aneurysm and dissection. Mutations in the TGF-β receptor type I (*TGFBR1*,OMIM*190181) and type II (*TGFBR2*, OMIM*190182) genes have been linked to the disease. Recently, LDS has been subdivided in type I (if craniofacial involvement consisting of cleft palate, hypertelorism, or craniosynostosis was observed) and type II (absence of craniofacial involvement, but isolated bifid uvula). Patients with more severe craniofacial abnormalities tend to develop more severe and aggressive arterial disease. Arteriopathy, in particular aortic dilatation and dissection, represents the main cause of death (See Chapter 3). *The vascular type of EDS* (previously EDS IV; OMIM#130000) is caused by mutations in *COL3A1* (collagen type III alpha 1; OMIM*120180), the gene encoding type III collagen. Typically, dissection or rupture occurs in medium sized arteries in vEDS, although aortic involvement is sometimes observed. There is no particular predisposition at the aortic root. About half of the aneurysms/dissections occur in

thoracic or abdominal branch arteries; arteries of the head, neck and limbs are less frequently involved [Pepin M et al., 2000] (See Chapter 3).

Aortic Dissection comprises one of the more ominous acute aortic syndromes, which include the dissection variants of penetrating aortic ulcers, intramural hematomas, and symptomatic aneurysms [Vilacosta I et al., 2001]. Aortic dissection involves splitting of the aortic wall within the media, which results in the formation of an aortic false lumen that courses along with a true lumen. The hallmark of aortic dissection is an intimal tear, which allows access of pulsatile high-pressure blood into the aortic media, separating it from the outer layers. Often, the so-called intimal flap is usually an intimal-medial flap. The initiating event of dissection may be a tear in the intima. Alternatively, primary rupture of the vasa vasorum can result in an intramural hematoma, which secondarily leads to an intimal tear as blood vents from the intramural space (Figure 7). Regardless of the initiating event, the force of blood flow propagates the dissection antegrade (and, less commonly, retrograde) for a variable extent along the vessel, splitting the aortic wall, usually along the outer one third of the medial layer.

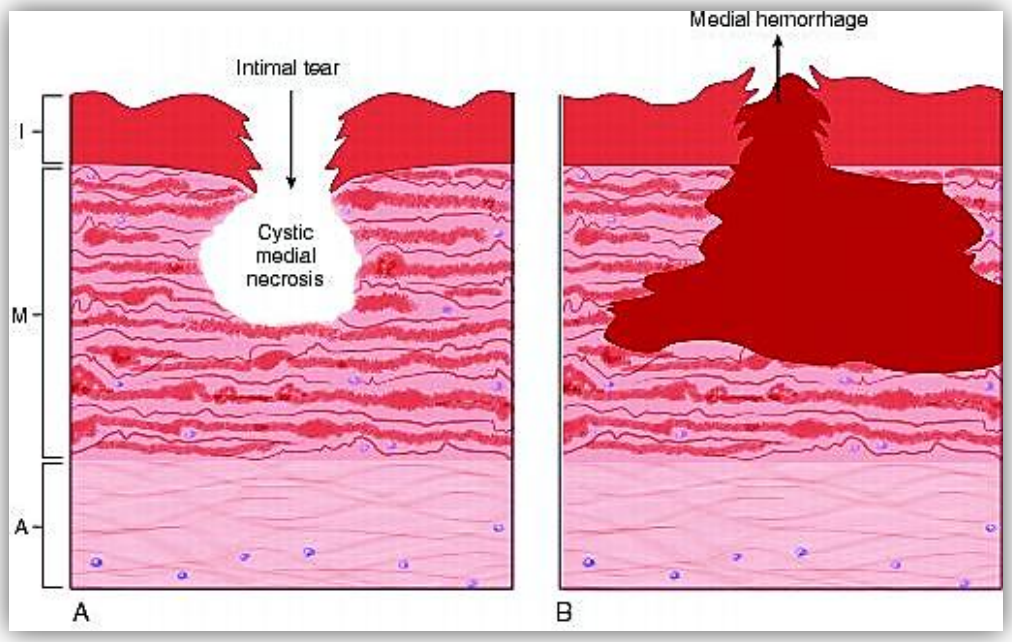


Fig. 7 Schematic representation of the initiating event of aortic dissection. (A) A tear in the intima. (B) Primary rupture of the vasa vasorum, secondarily leading to an intimal tear as blood vents from the intramural space (from http://www.clevelandclinicmeded.com/)

CHAPTER 2.

THORACIC AORTIC ANEURYSM AND DISSECTION

2.1 ANEURYSM: DEFINITION AND SITES OF FORMATION

Aneurysm derives from the Greek ανευρυσμα (aneurusma), meaning widening and can be defined as the permanent and localised dilatation of an artery, which has, at least, a 50% increased diameter when compared to the expected normal one. True aneurysm exhibits dilation of all three layers of the arterial wall (intima, media and adventitia). This condition differs from others that might resemble it, such as: - pseudoaneurysm (or false aneurysm), a pseudo-vascular space resulting from disruption of the arterial wall with extravasation of blood contained by periarterial connective tissue and not by the arterial wall layers [Razzouk A et al., 1993]; - ectasia, a dilatation of an arterial segment to a diameter at least 1.5 times that of the adjacent normal artery [Hartnell GG et al., 1985]; -arteriomegaly, a diffused increase in the dimensions of the arterial vessels, involving many vascular segments (not focal) with an increase in diameter more than 50% compared to the respective healthy vascular segments [Lawrence PF et al., 1998].

Aneurysmal degeneration can occur anywhere within the arterial circulatory tree but the most common localizations are the following:

* Aortic aneurysm: occurs in the major artery from the heart;
* Cerebral aneurysm: occurs in the brain;
* Popliteal artery aneurysm: occurs in the leg behind the knee;
* Mesenteric artery aneurysm: occurs in the intestin;
* Splenic artery aneurysm: occurs in a spleen artery.

With regard to the first type of aneurysm, it can develop anywhere along the aorta (Figure 8):

- aneurysms involving the infradiaphragmatic abdominal aorta are called **abdominal aortic aneurysms (AAA, OMIM #100070)**;
- aneurysms involving the thoracic (ascending or descending) aorta **thoracic aortic aneurysms (TAA, OMIM #611788)**;
- aneurysm located at the aortic root, are called **aneurysm of sinus of Valsalva**;

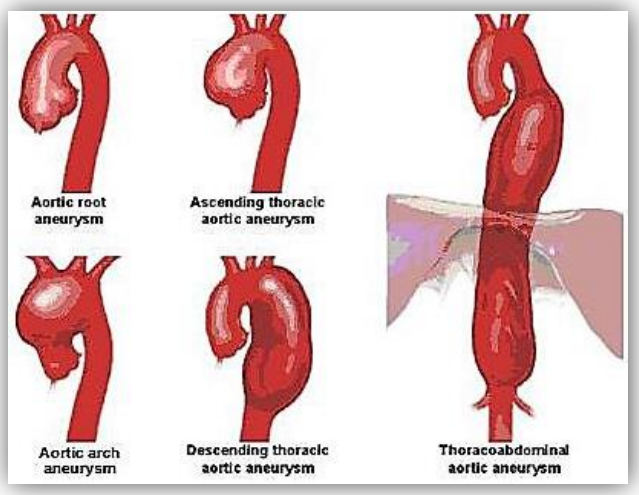


Fig 8. Different types of aortic aneurysm (from http://www.phsoregon.org/)

- aneurysm involving the thoracic and abdominal aorta are called **thoraco-abdominal aortic aneurysms**.

An aortic aneurysm can be the result of a trauma, an infection, or structural alterations in ECM proteins with a disruption and loss of elastic fibers and increased deposition of proteoglycans. All these features together define the aortic aneurysm histopathology, more accurately termed medial degeneration. Typically, the aortic media appears with areas of loss of smooth muscle cells in the aortic media, while their relative abundance in the aortic wall is not clear. It has been also reported recently the presence of inflammatory cell infiltration in this condition [Tang PC et al., 2005; He R et al., 2006]. Furthermore, both familial thoracic and abdominal aortic aneurysms demonstrate a strong genetic alterations or mutations component in their aetiology which predisposes some individuals to aortic diseases; accordingly, their detection has the potential to allow an early identification of individuals at risk [Saratzis A et al., 2014]. These genetic variants, along with biochemical alterations involved in the progression of aortic disease (which are being identified through studies of patients' aortic samples and animal models of the disease), can serve as biomarkers [Habashi JP et al., 2006; Pannu H et al., 2007] for aortic disease. According to these evidences, genetic determinants helped identifying novel aspects of vascular biology that reveal cellular and tissue events contributing to aortic aneurysm and are now understood to represent a major factor in determining aneurysmal risk for the individual patient. Genetic testing has therefore become a regular feature of clinical practice in the disease [Isselbacher EM et al., 2016].

Aortic aneurysms determine the weakening of the wall of the aorta and increase the risk of aortic rupture which leads to an internal bleeding and, if not treated immediately, to shock and death. The Aortic dissection (AoD) represents the disruption of the media layer of the aorta which may, and often does, occur without an aneurysm being present as well as an aneurysm may occur without necessarily evolving to a dissection. The term dissecting aortic aneurysm is often used incorrectly and should be

reserved only for those cases where a dissection occurs in an aneurysmal aorta [Hiratzka LF et al., 2010].

Aortic aneurysms might represent life-threatening diseases, even though they are less common that many other cardiovascular conditions. Most intact aortic aneurysms, however, do not produce symptoms aside from the abdominal pain and back pain which may develop as a consequence of their enlargement. That being said, it's also important to mention that even large aneurysms may not produce symptoms, making it extremely important for clinicians to be particularly vigilant in their evaluation of patients at risk. Aneurysms can be found on physical examination and modern medical imaging techniques [especially computed tomography (CT) and magnetic resonance imaging (MRI)], which are necessary to confirm the diagnosis and to determine the anatomic extent of the aneurysm, have now made their sizing and surveillance relatively easy. It must also be remembered that aneurysms are often first detected on an imaging study ordered for other indications, so that any suggestion of an enlarged aorta should prompt follow-up with an appropriate dedicated CT or MRI examination. The global clinical awareness of aortic aneurysms along with the latest methods of diagnosis (thus including the genetic screening in those with a family history of thoracic aortic aneurysms for example) will definitely help reducing the morbidity and mortality associated with this condition [Isselbacher EMet al., 2005].

2.2 EPIDEMIOLOGY OF THORACIC AORTIC ANEURYSM (TAA)

The true incidence of TAAs is difficult to determine, as many go undiagnosed principally because a substantial proportion of individuals with TAA die either before reaching the hospital or before the diagnosis is made; thus, these deaths are not accounted for unless an autopsy is performed. Diagnosis can also be easily missed because physicians' suspicion for TAA tends to be low, symptoms are nonspecific in many cases and are atypical or even absent in others, and diagnostic tests are often not performed when needed, or their results can be misinterpreted [Elefteriades JA et al., 2008]. Although findings from autopsy series vary widely, the prevalence of aortic aneurysms probably exceeds 3-4% in individuals older than 65 years with reported incidence 2.9 and 4.3 cases per 100,000 persons per year, increased over time [Mészáros Iet al., 2000]. In a Mayo Clinic sampling from 1980-1994, the incidence in Olmstead County, Minnesota, was 10.4 per 100,000 person years [Clouse WD et al.,

1998]. This was significantly higher than the incidence in the same population prior to 1980, possibly due to the advances in diagnostic imaging techniques.

The prevalence of TAA differs among aortic segments, with approximately the two-thirds of TAAs involving the ascending aorta (Stanford type A), around half extending distally into the descending thoracic aorta (DeBakey type I); the rest involves the ascending segment (DeBakey type II). Up to 37% of TAAs develop in the descending segment and spare the ascending aorta (Stanford type B, DeBakey type III) [Sato F et al., 2005].

Death from aneurysmal rupture is one of the 15 leading causes of death in most series. The true incidence of acute aortic dissection is difficult to determine, as many cases may go undiagnosed, but population-based studies suggest an incidence of acute aortic dissection of 3.5 per 100,000 persons; an incidence of thoracic aortic rupture of 3.5 per 100,000 persons; and an incidence of abdominal aortic rupture of 9 per 100,000 persons. Today, the mortality risk associated with TAA largely depends on the extent of the dissection, the delay between the onset of dissection and diagnosis, the availability of treatment, and the type of treatment administered (medical, surgical, or endovascular) [Le Maire SA et al., 2011]. The peak incidence of aortic dissection is in the sixth and seventh decades of life, with a mean age of 62 years among more than a thousand subjects in the International Registry of Acute Aortic Dissection (IRAD) [Nienaber CA et al., 2004].

The age-adjusted incidence of TAA among men is estimated by Clouse and colleagues at 5.2 per 100,000 per year and at 2.2 per 100,000 per year for women [Clouse WDet al., 1998] and this is equivalent to a male:female ratio of nearly 2:1. A sex-based comparison has been made on patients enrolled in IRAD regarding the demographics and history of subjects with TAA. Women turned out to be older than men at the onset of TAA (mean age 67 years for women versus 60 years for men) and they are more likely to have an history of hypertension. Men are more likely to have had cardiac surgery [Nienaber CA et al., 2004].

It is noteworthy that, despite the average age at onset of TAA is approximately 65 years, the disease tends to occur at a much younger age in patients with connective tissue disorders than in sporadic cases; Januzzi and colleagues in 2004 carried out a study of the baseline demographics and clinical history of patients with TAA, showing that those aged ≥40 years were more likely than those aged <40 years to have a history of hypertension and atherosclerosis and to have undergone previous cardiac surgery, whereas the younger patients were more likely to have MFS or a BAV [Januzzi JL et

al., 2004]. Furthermore, according to IRAD reports, hypertension, atherosclerosis, previous cardiac surgery, diabetes mellitus, and previous aortic aneurysm are more common among TAA patients who are aged ≥70 years than among those younger than 70 years [Trimarchi S et al., 2010].

2.2 PATHOGENESIS OF TAA

TAA is a multifactorial pathology resulting from the complex interaction between haemodynamics, genetic and environmental factors. TAAs often result from the generalized weakening the aortic wall deriving from CMN or medial degeneration. The typical histopathological finding of the aortic wall in patients with TAA is in fact CMN. About 80% of TAAs are secondary to medial degeneration with 15%-20% being associated with aortic dissection [Hoel AW et al., 2013]. These aneurysms seem to occur naturally with increasing age and the majority of them are associated with atherosclerosis and risk factors such as hypertension, hypercholesterolemia and smoking. As already mentioned, the balanced composition of vascular smooth muscle cells and ECM proteins, as found in the medial layer of the aorta, is critical for preserving its functional properties, particularly its mechanical compliance with pulsatile blood flow; their imbalance results therefore in excessive ECM degradation leading to progressive aortic wall deterioration and expansion up to rupture, eventually [Sinha I et al., 2006]. Key structural components of the aorta whom alteration may lead to aneurysm formation are represented by: *elastin*, which is the major protein of the ECM and provides the aorta with its elastic properties (Figure 9). Cross-linking of tropoelastin monomers by lysyl oxidase (LOX) forms elastin molecules, which in turn cross-link with microfibrils to form elastic fibers [Karnik SK et al., 2003]; *fibrillin-1 and fibrillin-2*, the first is a major structural component of microfibrils in the mature aorta and, in addition, it regulates tissue homeostasis by sequestering growth factors such as TGF-ß, influences cell signaling by binding to integrin receptors, and it can interact with cell-surface heparan sulfate proteoglycans to regulate matrix deposition [Ritty Tm et al., 2003]. Fibrillin-2 is primarily expressed in the embryonic period and is important for aortic morphogenesis [Kelleher CM et al., 2004]. Mice deficient in the *FBN1* gene ($Fbn1^{-/-}$) recapitulate the human aortic MFS phenotype, have thin and fragmented elastic fibers [Bax DV et al., 2003], and are prone to aortic aneurysm and dissection. In contrast, mice deficient in the fibrillin-2 gene ($Fbn2^{-/-}$) have normal elastic

fibers and are not reported to develop aortic aneurysms or dissection during development [Carta L et al., 2006]. In addition to its functional role, elastin is also important for vascular morphogenesis. *MAGP-1 and -2* are constitutive components of microfibrils and are capable of binding members of the TGF-ß growth factor family [Massam-Wu T et al., 2010]. However, reports indicate that inactivation of either protein's associated gene does not result in cardiovascular defects in mice [Wagenseil JE and Mecham RP, 2009]. The *fibulins* are a group of seven ECM proteins that are important for aortic wall homeostasis. Studies of aortic tissue from humans and mice with FBLN4/Fbln4 deficiency show increased TGF-ß signaling, which may result from impaired LOX-mediated TGF-ß repression [Sicot FX et al., 2008; Horiguchi M et al., 2009], while fibulin-5 is essential for lamellar unit formation [Atsawasuwan P et al., 2008], and Fbln5-deficient mice have disrupted elastic fiber assembly, as well as less compliant aortas [Maki JM et al., 2002], these findings suggesting a key role of these fibulins in elastic fiber assembly; deficiency of either one may predispose patients to aortic dissection. The *elastin microfibril interface-located proteins* (EMILIN- 1, -2, and -3) and the multimerins (multimerin 1 and 2) are glycoproteins of the ECM that have a unique N-terminus cysteine-rich EMI-domain [Colombatti A et al., 2011]. EMILIN-1 is the most well studied member of this family of proteins and is commonly found at the interface between micro-fibrils and amorphous elastin and it's also capable of binding both tropoelastin and fibulin-5; deficiency in EMILIN-1 results in abnormal elastic fiber architecture [Zanetti M et al., 2011]. However, the role of EMILIN-1 in dissection formation is not entirely clear. EMILIN-1 is known to inhibit

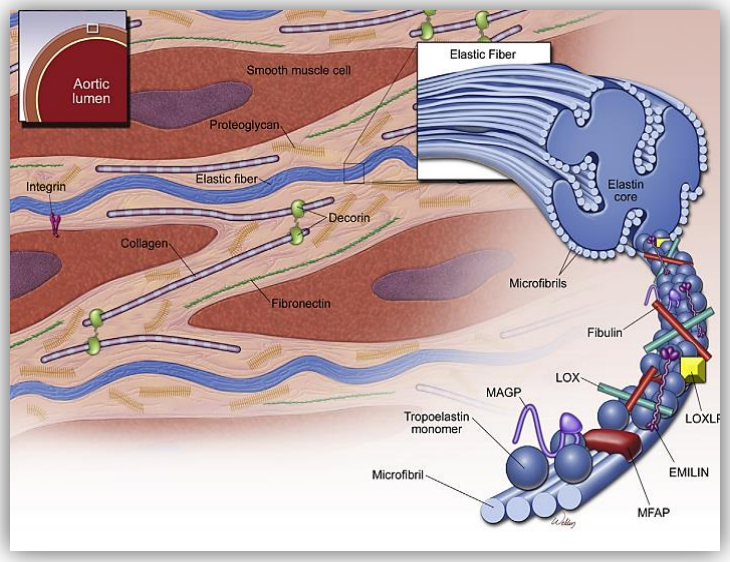


Fig 9. Key structural components of the aorta. The media of the aortic wall is composed of vascular smooth muscle cells (SMCs) and an extracellular matrix (ECM) of elastic fibers, collagen fibers, and proteoglycans. Elastic fibers are one of the major structural components of the ECM and provide extensibility to the aortic wall. Cross-linking of tropoelastin monomers by lysyl oxidase (LOX) forms elastin molecules, which in turn cross-link with microfibrils to form elastic fibers. Microfibrils provide a scaffold for tropoelastin cross-linking. Microfibrils are composed of fibrillin and several microfibrilassociated proteins (MFAPs), such as elastin microfibril interface-located protein 1 (EMILIN-1), microfibril-associated glycoproteins (MAGP-1 and -2), and fibulins. (from Wu D et al., 2013)

TGF-ß signaling by binding to its precursor, pro-TGF-ß, and inhibiting its maturation by furin convertases [Zacchigna L et al., 2006]. *Collagen* is important for sequestering cytokines and mediating cell proliferation by binding to integrins [Pozzi A et al., 1998]. Intact elastic fibers are necessary for organized collagen deposition and efficient collagen function. In thoracic aneurysm and dissection, type I and type III collagen expression is increased [Sariola H et al., 1986], as is the level of spiraled collagen [Ishii T et al., 2000]. The increased expression and disorderly deposition of collagen may correspond to a slow reparative process triggered by elastic fiber fragmentation and depletion. This fibrosis may also be due to an increase in the expression and release of sequestered growth factors [Wang X et al., 2006]. The increase in collagen could lead to increased arterial stiffness, thus enhancing the aorta's susceptibility to dissection and rupture [Sariola H et al., 1986]. Other molecules of the aorta which may be involved in aortic wall alteration and aneurysm formation are represented by *LOX family enzymes*, critical for collagen and elastin cross-linking; small, leucine-rich proteoglycans binding to key ECM molecules such as *collagen*, *tropoelastin*, *fibronectin*, and *microfibrils*; *SMCs* which show signs of apoptosis in thoracic aneurysms, appearing even stronger in areas rich in ground substance and in the vasa vasorum [He R et al., 2006]. SMC apoptosis may be mediated by the altered expression of angiotensin II type 2 receptors and may result in abnormal matrix deposition in the aortic media and adventitia [Nagashima H et a., 2005]. Using a mouse model that overexpresses diphtheria toxin in SMCs in ApoE/ mice, Clarke et al. [Clarke MC et al., 2008] showed that SMC apoptosis is associated with medial expansion, increased elastic lamina breaks, and abnormal matrix deposition, suggesting that SMC apoptosis is important for degenerative medial remodeling and may contribute to aortic aneurysm or dissection formation.

The pathogenetic mechanism underlying aneurysm formation largely involves the MMPs. TAA patients in fact have been demonstrated to carry increased levels of different members of the MMP family, such as MMP-1, MMP-2, MMP-3 and MMP-9 [Galis ZS et al., 2002]. Murine models without the MMP-9 gene (*MMP-9*, matrix metalloproteinase 9, OMIM*120361) have demonstrated attenuated TAA formation [Ikonomidis JS et al., 2005] and more recent studies showed increased MMP-9 and MMP-2 levels and low tissue inhibitor of MMPs expression in patients with thoracic aortic disease [Ikonomidis JS et al., 2006] and assessed the association between the MMP9 -8202A>G polymorphism and TAA development, although the functional role of this variant in MMP-9 expression remains to be determined [Chen L et al., 2006]. All

these evidences suggest MMPs to play a role in the destruction of the ECM in TAA, making the *MMP-9* gene an emerging target for investigation.

The pathology associated with TAA, CMN, was historically described as a non inflammatory lesion, thus the role of inflammatory cells in its the pathogenesis has only recently been investigated, leading to the evidence in according to which inflammation plays an important role in the pathogenesis of aortic aneurysms and dissections as well. T lymphocytes have been shown to be increased in TAA aortas compared to control aortas in immunohistochemical studies with monoclonal antibodies against CD3+ cells, some of them appearing flattened in the aortic media when they reside between the SMC layers [He R et al., 2006]. The lymphocytes cell shape was similar to vascular smooth muscle cells', and this may partially explain why infiltration of T lymphocytes in TAA aortas was not recognized for decades. T lymphocytes and macrophages represent the predominant inflammatory cells infiltrated in the aortas of patients with sporadic TAA by immunohistochemical staining [Guo DC et al., 2006], and their presence around vasa vasorum suggests that the infiltration into TAA aortas occurs through the adventitia layer [He R et al., 2006]. Then, RNA levels of the Th1-promoting cytokine IFN-γ and the IFN-γ inducible chemokines IP-10 and Mig are significantly increased in TAA aortas compared to control aortas, unlike mRNA levels of Th2-promoting cytokines which appear to be not detectable [Tang PC et al., 2005].

To date several heritable connective tissue disorders have been associated with mutations in *TGFBR*s (and therefore disturbances in TGF-β signaling) including LDS, familial thoracic aortic aneurysm and dissection syndrome (FTAAD, OMIM #615436 also known as familial TAA), Shprintzen-Goldberg syndrome (SGS; OMIM #182212), and hereditary hemorrhagic telangiectasia (HHT; OMIM #187300). Interestingly, each of these disorders also display unique cardiovascular manifestations, resulting in a spectrum of disorders ranging from heart valve defects to thoracic aortic aneurysms, characteristic of MFS and LDS, the prototypical disorders of aberrant enhanced TGF-β signaling [Wheeler JB et al., 2014]. TGF-β1, -2 and -3 are synthesized as 75 kDa precursor proteins composed of the mature growth factor domain at the C-terminus and an N-terminal prodomain, thelatency associated peptide (LAP) [Karina A et al. 2015]. The precursor proteins homodimerize through disulfide bond formation. The homodimers are proteolytically processed by furin-like endoproteases in the trans-Golgi network, forming the smalllatent complex (SLC) with the LAP dimer non-covalently shielding the active TGF-β dimer. Like all other members of its family, TGF-β is synthesized as the C-terminal domain of a precursor form that is cleaved before

secretion from the cell. However, the TGF-β propeptide, which is referred to as the latency associated peptide (LAP), remains noncovalently bound to TGF-β after secretion, retaining TGF-β in a latent form that cannot bind to betaglycan or the signaling receptors [Pannu H et al.,2005]. Most cell types secrete TGF-β in this biologically inert form. Although LAP may be destroyed in the process of TGF-β activation, recombinant LAP retains TGF-β masking ability, and its injection in mice can inhibit endogenous TGF-β1 action [Boileau C et al., 2007].

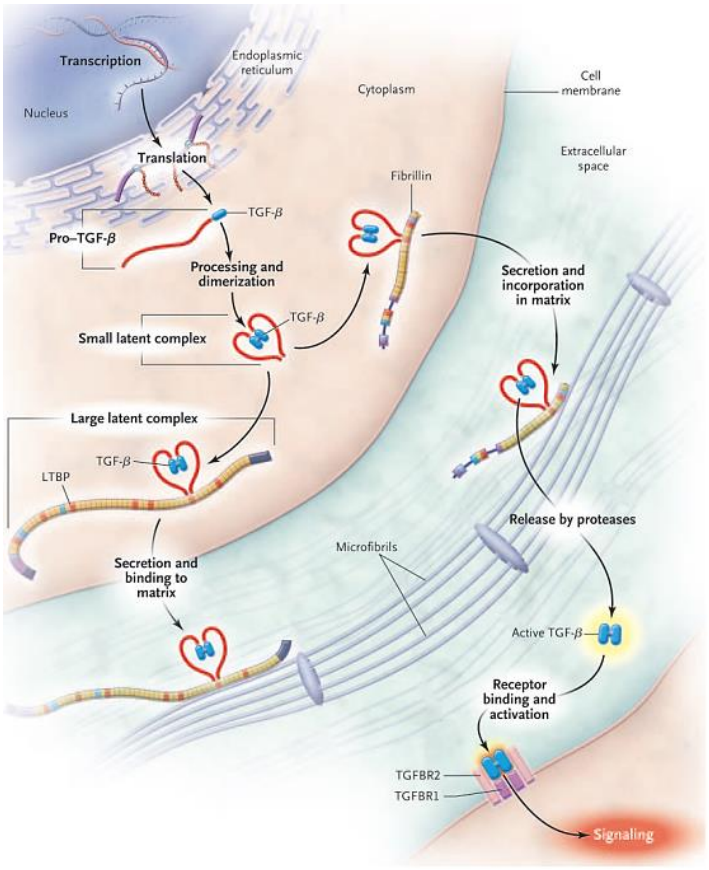


Fig 10. Small latent complexes containing TGF-β bind LTBPs intracellularly to form large latent complexes that are then secreted into the extracellular space, where they can bind the fibrillin in microfibrils. Alternatively, small latent complexes might bind fibrillin directly and become incorporated into the extracellular matrix. However latent complexes are attached to microfibrils, TGF-β becomes active through its release by proteases and then binds to receptor complexes at the cell surface (from Gelb BD et al., 2006).

The third component of the latent TGF-β complex is latent TGF-β binding protein (LTBP), which is disulfide-linked to LAP [Horbelt D et al., 2010]. LTBP is not required for the latency of the TGF-β complex but is implicated in the secretion, storage in the ECM, and eventual activation of this complex [Horbelt D et al. 2010]. LTBP comprises several forms generated from two genes and by alternative splicing: LTBP-1 in short and long forms [Ramirez F et al. 2007] and LTBP-2 [Dijke P and Arthur MH, 2007]. Structurally, LTBPs contain a core of epidermal growth factor (EGF) repeats and eight cysteine motifs organized in a fashion resembling fibrillin-1 and -2 (two microfibrillar proteins whose mutations cause MFS and congenitalcontractural arachnodactyly, CCA, OMIM#121050) respectively. Like fibrillins, LTBP undergoes crosslinking by transglutaminases, forms fibrillar structures, and associates tightly with the ECM in mesenchymal and endothelial cells [Fleischer KJ et al.,1997].

In tissue culture, LTBP associated with the ECM mediates storage of latent TGF-β and facilitates its activation [Bunton TE et al., 2001]. Latent TGF-β can be activated in vitro by acid, alkali, heat, limited proteolysis, or incubation by glycosidases [Horbelt D et al., 2010]. In tissue culture, activation of latent TGF-β may involve a combination of steps including the following: LAP proteolysis, binding to the mannose 6-phosphate/type II insulin-like growth factor receptor (Man6P/IGFR-II) via a mannose 6-phosphate group in LAP, cell-cell interactions between endothelial and vascular smooth-muscle cells, and binding to thrombospondin (Figure 10) [Boileau C et al., 2007]. TGF-ß activation may occur by integrin-independent, or integrin-related pathways. Possible mediators of integrin-independent TGF-β activation are matrix metalloproteases (MMPs) playing a role in tissue remodeling via proteolysis of the ECM [Booms P et al., 2005]. TGF-β release and activation by MMPs are well recognized phenomena. TGF-β activation is also influenced by pH, since latency associated protein (LAP) disintegrates in an acidic environment. Although it is known from in vitro experiments that in extreme pH environments (pH 1,5 or 12) TGF-β activation significantly increases, further research is needed to clarify the physiological role of this effect [Lyons RM et al., 1988].

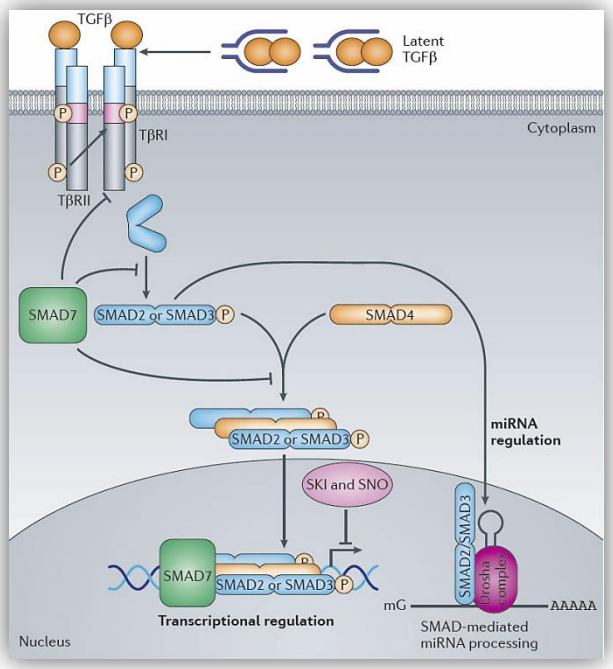


Fig 11. Schematic overview of the canonical, SMAD-dependent TGF-β signalling pathway (from Akhurst RJ and Hata A, 2012)

Another pathway of integrin-independent activation is the effect of reactive oxygen species (ROS) [Barcellos-Hoff MH and Dix TA, 1996]. Fast activation of TGF-β happens by radiation, due to alteration of LAP and the molecule interaction [Schultz-Cherry S and Murphy-Ullrich JE, 1993]. Presently, there are two accepted modalities, which explain TGF-β 1 activation by integrins containing βV. According to the first one, conformation alteration takes place in the inactive TGF-β complex induced by integrins resulting in an active form of TGF-β. αVβ6 integrin is known to be the first

TGF-β activator: when bound to the LAP arginine-glycine-aspartic acid (RGD) sequence, an alteration in conformation occurs, while the adhesion-mediated effects of intercellular influences brings about TGF-β activation via a biochemical process [Wipff PJ e Hinz B, 2008]. This process happens in case of the high rigidity of the microfibrillar system, which is not capable of withstanding the shear stress, generated by integrin bonding. In these instances, integrin bound to RGD sequences excludes TGF-β from its complex. The other modality is the mechanism of protease-dependent activation of integrin. TGF-β can beactivated by MMP-2 and MMP-9 by proteolytic degradation of the inactive (latent) TGF-β complex. αVβ6 may enhance this process as it tightens the interaction of the molecules. Integrin αVβ6 and αVβ3 are simultaneously bound to latent TGF-β complexand to MMPs as catalysts to speed up complex degradation [Yu Q and Stamenkovic I, 2000].

There are two distinct ways of intracellular signaling, through which elevated TGF-β level can exert its physiological effects: the so-called canonical and non-canonical signal transduction (Figures 11 and 12) [Gomez D et al., 2011]. The former pathway is mediated by the Smad proteins. After TGF-ß release from ECM, the type-II receptor then activates the type-I receptor via transphosphorylation [Wrana JL et al., 1994]. The kinase domain of the activated type-I receptor propagates the intracellular signal through the phosphorylation of specific receptor-regulated Smad proteins (R-Smads; Smad 1, 2, 3, 5, and 8), which are the second messengers of the canonical TGF-β signaling pathway. For example, activation of the type-I receptor TGFBR1, results in the phosphorylation of Smad 2 or 3; while activation of the type-I receptor ALK-1 results in the phosphorylation of Smad 1, 5 or 8. The choice of Smad is likely tissue-specific and context dependent. The phosphorylated R-Smad then interacts with a common Smad or “co-Smad” (Smad4), which induces translocation of the complex to the nucleus. The nuclear Smad complex along with multiple co-regulatory factors form a transcription regulating complex capable of activating or repressing TGF-β associated genes [Feng XH and Derynck R, 2005]. Activation of the TGF-β system stimulates a number of diverse cellular processes, such as cell growth, proliferation and apoptosis and therefore requires strict regulation at multiple levels. An example of this regulation, is the negative feedback of inhibitory Smads (I-Smads; Smad6 and 7) induced by TGF-β stimulation [Park SH et al., 2005]. Smad6 exerts its effects by binding directly to type-I receptors and blunting R-Smad phosphorylation [Imamura T et al., 1997]. Smad6 also inhibits signaling by competing with Smad4 for receptor Smad binding sites, reducing nuclear translocation [Hata A et al., 1998]. Smad7 inhibits TGF-β signaling

by targeting TGFBR1 and 2 for ubiquitination and subsequent degradation, through the recruitment of Smurfs 1 and 2 (Smad ubiquitination regulatory factor 1 and 2) [Wick SJ et al., 2006]. Additionally, many regulatory proteins influence the bioavailability of TGF-β, such as the structurally related scavenging proteoglycans decorin and biglycan, which bind and reduce its availability for signaling [Droguett R et al., 2006].

Recent studies have expanded upon the hypothesis that TGF-β signaling can occur independently of Smad mediators, through alternative pathways. Several alternative signaling pathways exist: (1) type-1 receptors signaling in the absence of Smads [Yu L et al., 2002; Itoh S et al., 2003; Wilkes MC et al., 2003]; (2) type-2 receptors signaling without type-1 receptors [Ozdamar B et al., 2005]; (3) R-Smad signaling to parallel pathways [Inamichi Y et al., 2005]; and (4) activation of R-Smads independent of TGFBRs [Runyan CE et al., 2004]. However, downstream intra-cellular mediators of these alternative pathways are not as well understood as the Smad proteins. Studies of noncanonical signaling in FBN1 deficient mice have proved helpful in this regard. Carta et al. demonstrated in vivo that p38 mitogen-activated protein kinase (p38 MAPK) mediated phosphorylation of Smad2/3, which was attenuated with p38 MAPK inhibitors suggesting independence from TGFBR1 [Carta L et al., 2009]. Further studies performed by independent groups elucidated the TGF-β dependent activation of p38 MAPK independently of Smad proteins. Tumor necrosis factor receptor- associated factor 6 (TRAF6), an E3 ubiquitin ligase, was demonstrated to associate with TGFBR1 in a TGF-β dependent manner [Yamashita M et al., 2008]. This newly formed complex recruits and activates TGF-β associated kinase 1 (TAK1), which activates p38 MAPK via phosphorylation [Sorrentino A et al., 2008]. In addition to p38 MAPK, TGF-β can activate many other signal pathways not directly involving Smads: extracellular-signalregulated kinase 1 and 2 (ERK1/2) [Lee MK et al., 2007], c-Jun N-terminal kinase (JNK) [Yamashita M et al., 2008], and phosphoinositide 3-kinase-Akt (PI3K-Akt) [Wilkes MC et al., 2005].

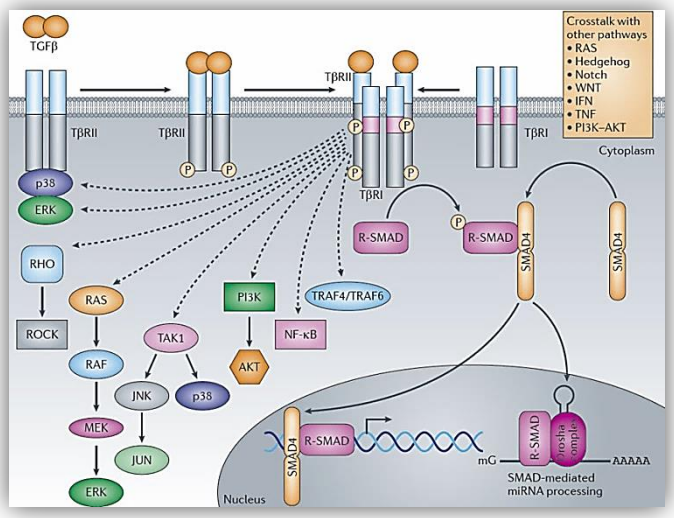


Fig 12. Schematic overview of the non canonical TGFβ signalling pathway (from Akhurst RJ and Hata A,, 2012)

As already mentioned, TGF-β signaling is known to contribute to a number of disparate and opposing physiologic processes including angiogenesis, proliferation, differentiation, apoptosis, and wound healing, and is an established modulator of ECM structure and composition. Importantly, the canonical and noncanonical pathways appear to exert differential effects on the connective tissues within the ECM. Stimulation via the canonical pathway induces profibrotic effects including increased ECM protein deposition (collagen and elastin) [Ignotz RA and Massague J, 1986], decreased expression MMPs [Yan C and Boyd DD, 2007], and increased proteolytic inhibition (tissue inhibitors of MMPs, TIMPs) [Kwak HJ et al., 2006]. Alternatively, stimulation via the noncanonical pathway degrades matrix proteins through increased proteolysis via MMPs (2, 9, and 13) [Kim ES et al., 2004] and increased MMP activation via plasminogen activators [Laiho M et al., 1986]. The balance between ECM deposition and degradation is tightly regulated, and the mechanism of the loss of balance in TAAs is not clearly understood. For example, recent evidence uncovered paradoxical effects in the TGF-β signaling pathway, specifically identifying mutations in *TGFBR*s that resulted in elevated TGF-β signaling [Loeys BL et al., 2005]. Several mechanisms, however, have been proposed. First, that heterozygous mutation of *TGFBR*s may enhance TGF-β signaling in functional TGFBR complexes by facilitating ligand interaction much like the role of type-III TGFBRs (endoglin and betaglycan). This was hypothesized after authors of a study using transgenic mice with a fibroblast-specific heterozygous *TGFBR2* mutation noticed increased pulmonary and dermal collagen deposition rather than a dominant negative phenotype which was expected to reduce deposition [Denton CP et al., 2003; Wong SH et al., 2000]. Second, alternate pathways of receptor recycling could also explain the enhanced signaling seen. Internalization of TGFBRs is mediated by either clathrin- or caveolin- dependent endocytosis [Di Guglielmo GM et al., 2003]. A plasma membrane protein, Smad anchor for receptor activation (SARA) binds TGFBR2 and mediates interactions with Smad2 and the clathrin endocytosis pathway leading to receptor recycling [Di Guglielmo GM et al., 2003]. A mutation that enhanced interaction of TGFBR2 with SARA could increase signaling and favor recycling over degradation. Alternatively, the Smurfs (Smad ubiquitination regulatory factors) mediate TGF-β receptor interactions with the caveolin pathway [Nakao A et al., 1997]. The interaction of the TGFBR complex with Smad7 recruits Smurf1 and 2, activating the caveolin pathway and leading to proteasomal degradation of the receptors. In this case, a TGFBR mutation that decreased interaction with either Smad7 or the Smurf proteins could decrease receptor

degradation and prolong signaling. Mutated TGFBRs may enhance signaling because their ability to form functional signaling platforms is unaffected by mutation. These signaling platforms are sites where multiple signaling intermediates aggregate. This was demonstrated when Smad3 was found to be phosphorylated by PI3K at residues not within TGFBR1's target site after TGF-β stimulation and increased collagen expression [Runyan CE et al., 2004]. This suggests that those mutated *TGFBR*s with intact TGF-β binding but deficient kinase domains may still induce TGF-β dependent signaling through alternate pathways. Thus, enhanced TGF-β signaling may drive vascular ECM destruction through non-Smad signaling pathways alone, as a result of imbalanced homeostasis having direct implications for the formation and progression of TAA and dissection. Lending support to the pivotal role of TGF-β in connective tissues and the cardiovascular system, heritable mutations in downstream and upstream mediators of TGF-β signaling also display symptoms overlapping with TGFBR mutation syndromes (See Chapter 2.3).

Albeit progressive dilatation, aortic aneurysms usually remain asymptomatic until dissection or rupture occurs. Despite the availability of highly effective prophylactic measures, there are still difficulties in identifying the at-risk subjects, which hamper the implementation of such surgical interventions. Indeed, there are no high-yield risk factors that can be used for screening the general population. The understanding of the genetic basis underlying the development of aortic aneurysms appears therefore critical in order to better screen the population, which can, as a consequence, lead to early intervention, and better clinical outcomes [Pomianowsky P et al., 2013].

Although this condition may occur sporadically, it displays familial clustering in >20% of the cases, in particular, family history confers a significant increased (six- to 20-fold) relative risk of TAAD which is not conveyed by cardiopulmonary comorbidity [Olsson C et al., 2013]. A positive family history is known to represent a risk factor for the development of TAAD and can be considered in the evaluation and identification of patients at increased risk. Evidences exist indicating that between 15% and 30% of patients with an aortic aneurysm and/or dissection have a positive family history of TAAD risk [Elefteriades JA and Pomianowsky P, 2013]. When TAAs appear to be familial, clinicians tend to describe them as being either syndromic (associated with abnormalities of other organ systems) or nons yndromic (with manifestations restricted to the cardiovascular system). Conditions that are usually considered non syndromic

include familial thoracic aneurysms and dissections (also known as familial TAA), as well as TAA associated with bicuspid aortic valve (BAV).

2.3 SYNDROMIC TAAs

Syndromic aortic aneurysms occur in patients in association with other connective tissue disorders such as MFS, LDS, AOS (AOS, OMIM#613795), arterial tortuosity syndrome (ATS, OMIM#208050), EDS and conditions associated with disorders of TGF-ß signalling. All of these disorders are multisystem diseases. External physical features associated with connective tissue disorders (CTDs) have included findings in the tegumental, musculoskeletal, ocular, craniofacial, and cardiovascular systems. It should be noticed however that the presence of these external CTD features alone does not indicate cardiovascular risk despite the fact that some familial aortic conditions are correctly classified as CTDs. Although associations with CTD and aortic disease were once exclusive, improved phenotyping has now described extracardiac phenotypes in some non-CTD aortic conditions, signs of which may be less familiar to cardiologists who encounter aortic disease patients. Iinvestigation in such families has led to the discovery of variants in genes specific to the cardiovascular system, such as those whose functions are specific to the SMCs of the aortic media [Isselbacher EM et al., 2016]. The diagnosis of many of the syndromic aneurysms can be therefore supported by both the recognition of their characteristic dysmorphic features and gene testing (Table 1) [Elefteriades JA and Pomianowsky P, 2013].

Table 1. Non-syndromic and main syndromic disorders associated with thoracic aortic aneurysm (from Giusti B et al., 2016)

Classification	Frequency	Gene Name	Gene Symbol	Inher.
Non-Syndromic				
Aortic aneurysm, familial thoracic 1 (AAT1)	Rare	-	*Chr.11q23.3-q24*	AD
Aortic aneurysm, familial thoracic 1 (AAT2)	Rare	-	*Chr.5q13-q14*	AD
Aortic aneurysm, familial thoracic 3 (AAT3)	3% of TAA	Transforming growth factor-beta receptor, type II	*TGFBR2*	AD
Aortic aneurysm, familial thoracic 4 (AAT4)	1-2% of TAA	Myosin, heavy chain 11, smooth muscle	*MYH11*	AD
Aortic aneurysm, familial thoracic 5 (AAT5)	2% of TAA	Transforming growth factor-beta receptor, type I	*TGFBR1*	AD
Aortic aneurysm, familial thoracic 6 (AAT6)	10-15% of TAA	Actin, alpha-2, smooth muscle, aorta	*ACTA2*	AD
Aortic aneurysm, familial thoracic 7 (AAT7)	1% of TAA	Myosin light chain kinase	*MYLK*	AD
Aortic aneurysm, familial thoracic 7 (AAT8)	Rare	Protein kinase, cGMP-dependent, regulatory, type I	*PRKG1*	AD
Syndromic				
Marfan Syndrome	1:5,000-10,000	Fibrillin 1	*FBN1*	AD
Loeys-Dietz Syndrome 1	Rare	Transforming growth factor-beta receptor, type I	*TGFBR1*	AD
Loeys-Dietz Syndrome 2	Rare	Transforming growth factor-beta receptor, type II	*TGFBR2*	AD
Loeys-Dietz Syndrome 3 or Aneurysm osteoarthritis syndrome	Rare	Mothers against decapentaplegic homolog 3	*SMAD3*	AD
Loeys-Dietz Syndrome 4	Rare	Transforming growth factor-beta 2	*TGFB2*	AD
Vascular Ehlers-Danlos Syndrome	1:100,000	Collagen, type III, alpha-1	*COL3A1*	AD
Arterial tortuosity syndrome	Rare	Solute carrier family 2 (facilitated glucose transporter), member 10	*SLC2A10*	AR

MFS_ is the best known aortic aneurysm syndrome and is caused by heterozygous mutations in the *FBN1* gene, which result in both a decrease in the amount of elastin in the aortic wall and a loss of elastin's normally highly organized structure. As a consequence, the aorta exhibits markedly abnormal elastic properties that lead to progressive increases in stiffness and dilatation [Jeremy RW et al., 1994]. In addition to their structural role, fibrillins perform a regulatory role by influencing cell-signaling events; this regulatory action is dominated by the interaction between fibrillins and the TGF-βs that control multiple aspects of cellular behaviour, including differentiation, motility, and proliferation. Multiple members of the TGF-β superfamily bind fibrillin; therefore, fibrillin deficiency indirectly influences ligand availability (See Chapter 3). MFS is the most common and in many ways the archetype of the familial connective tissue disorders associated with TAA. Patients with MFS show a high predisposition for aortic aneurysm centered anatomically at the sinuses of Valsalva, which often extends

into the proximal portion of the tubular ascending thoracic aorta to create a pear-shaped aortic dilatation that is sometimes referred to as annuloaortic ectasia [Isselbacher EM et al., 2016].The diagnosis of MFS has multiple implications for a patient, given the more aggressive pattern of the disease in comparison with other potential etiologies. Davies et al., found that the mean rate of growth for all TAA was 0.1 cm/y, which was greater for dissected aneurysms versus non dissected ones, and was greater for those with MFS versus those without [Davies RR et al., 2002]. Those evidences have led to implications regard the need for specific medical treatment, the counselling for follow-up, pregnancy and family planning, and advice for aortic surgery, which are indicated at lower threshold of aortic size in MFS than in non-MFS patients [Giusti B et al., 2016]. In MFS patients aortic surgery is recommended when aortic size is ≥50 mm, or ≥45 mm when other risk factors are present. These risk factors include family history of aortic dissection, rapid increase in aortic size, severe aortic and/or mitral regurgitation, and desire of pregnancy in women [Vahanian A et al., 2012].

LDS_a subset of patients previously thought to have MFS do not have identifiable mutations in *FBN1*. Most of these patients are now classified as having LDS, an autosomal dominant disorder of connective tissue with multisystem involvement. Cardiovascular features of LDS include TAD, widespread arterial tortuosity and aneurysms, and persistent ductus arteriosus. LDS patients have Marfanoid features with hypertelorism, cleft palate/bifid uvula, craniosynostosis, club feet, easy bruising, and dystrophic scars [Loeys BL et al., 2005]. In contrast to MFS, LDS patients can have more variable phenotypes than MFS patients. Vascular disease in LDS is not confined to the aorta, but can affect the entire arterial tree. Dissections occur at younger ages and at smaller diameters than in MFS, with *TGFBR2* mutations having a more deleterious phenotype than *TGFBR1* mutations, particularly in men [Loeys BL et al., 2006; Tran-Fadulu V et al., 2009]. Further evidence for causality of the TGF-ß pathway in TAD was provided recently by the discovery of mutations in *SMAD2* (Mothers against decapentaplegic homolog 2, OMIM*601366), *SMAD3* (Mothers against decapentaplegic homolog 3, OMIM*603109), *SMAD4* (Mothers against decapentaplegic homolog 4, OMIM*600993), *TGFB2* (transforming growth factor beta 2, OMIM*190220), *TGFB3* (transforming growth factor beta 3, OMIM*190230), and *SKI* (V-SKI avian sarcoma viral oncogene homolog, OMIM*164780) [Micha D et al., 2015; van de Laar IM et al.,2011; Andrabi S et al., 2011; Lindsay ME et al., 2012; Bertoli-Avella AM et al., 2015; Schepers D et al., 2015]. The classical TGF-β signaling

pathway involving the Smad-mediated cascade has been characterized by signals that not only induce ECM deposition but also matrix degradation, thus implicating the pathway as being critical to the structure and composition of ECM. Enhanced TGF-β signaling with downstream canonical pSmad2 upregulation has been described as the predominant mechanism in both MFS and LDS aneurysm formation [Loeys BL et al., 2005]. Rapidly progressive aortic aneurysmal disease is a distinct feature of LDS, requiring close monitoring. Individuals with LDS 1/2 with severe craniofacial features are at particularly high risk, known to have ruptures at early ages and at smaller dimensions than those with other aneurysm syndromes (unlike the increased risk of aortic dissection at or above the 5.0 cm aortic root dimension in MFS, dissections have occurred in individuals with LDS 1, 2, or 3 at aortic dimensions of 3.9-4.0 cm1.3 and has been reported in LDS 4 at a dimension <5.0cm) [MacCarrick G et al., 2014]. The decision to undergo aortic surgery is typically based on the absolute dimension of the aorta, rate of progression, valve function, severity of non cardiac features, family history, and genetic testing, which is recommended at diagnosis to review the multisystem manifestations of LDS, to help develop an imaging surveillance plan, and to coordinate interdisciplinary care. Additionally, a genetics professional can assist in reviewing the family history to determine whether echocardiogram screening or familial genetic testing is warranted, especially in the families of LDS patients with mild external features [MacCarrick G et al., 2014].

AOS_is a newly described autosomal dominant syndrome with variable expression which presents with aortic aneurysms in the setting of atypical osteoarthritis and dysmorphic features. The frequency of AOS is 2% among TAA patients and is caused by mutations in the *SMAD3* gene on chromosome 15 (15q22.33), encoding a protein critical for cellular signaling downstream of the TGF-β receptors [Regalado ES et al., 2011]. Mutations in *SMAD3* result in aortic aneurysms, dissections, arterial tortuosity, early onset osteoarthritis, and cutaneous anomalies. In one series, aortic aneurysms were present in 71% of patients with *SMAD3* mutations, mainly at the level of the sinus of Valsalva but also affecting the abdominal aorta and/or other arteries such as the splenic, common iliac, mesenteric, renal, vertebral, and pulmonary arteries. The mean age of death, due to aortic dissection, has been reported at 54±15 years and occurred at mildly increased aortic diameters (4.0-6.3 cm) [van de Laar IM et al., 2012]. Arterial tortuosity was diagnosed in 48% of patients. Velvety skin, striae, and umbilical/inguinal hernias are common and joint abnormalities; osteochondritis dissecans (OCD), meniscal

abnormalities, intervertebral disc degeneration, and early onset osteoarthritis have been reported in all cases of AOS [van de Laar et al., 2011]. AOS can be complicated by dissections at relatively small diameters, so that early elective aneurysm repair seems to be appropriate to avoid vascular catastrophes. In addition, although rate of aneurismal growth is not entirely elucidated yet, it has become clear that growth can be fast and unpredictable. The current general consensus in atherosclerotic aneurysmal disease is that vascular treatment is indicated in asymptomatic visceral artery aneurysms >2.0 cm and iliac artery aneurysms >3.0 cm 20-25 [Santilli SE et al., 2000; Van Petersen A et al., 2011]. However, due to the sometimes rapid aneurysmal growth and occurrence of dissections in only mildly dilated arteries, a more aggressive treatment strategy seems to be necessary in AOS patients, which should be offered genetic testing and counseling for *SMAD3* gene mutations when AOS is suspected. In addition, at least a transthoracic echocardiogram should be performed to look for an aortic root aneurysm. If a mutation is identified, additional counseling of family members is strongly recommended [van der Linde D et al., 2013].

ATS_is an autosomal recessive disorder caused by mutations in the *SLC2A10* (solute carrier family 2 member 10; OMIM#606145) gene on chromosome 20 (20q13.1), which encodes the facilitative glucose transporter GLUT10 (18-20). GLUT10 is localized to the gene promoter region of decorin, a natural inhibitor of TGF-β. Mutations in the *SLC2A10* gene are thought to result in down-regulation of decorin and thereby up-regulation of TGF-β signaling. Mutations causing this syndrome are the loss-of-function category. The clinical spectrum of ATS includes: arachnodactyly, joint laxity or contractions, hypertelorism, cleft palate, bifid uvula, micrognathia, down-slanting palpebral fissures, and arterial tortuosity with aneurysm formation [Coucke PJ et al., 2006].The cardiovascular system is the major source of morbidity and mortality. Cardiovascular manifestations include congenital widespread tortuosity of the large and middle-sized arteries. There is increased risk at any age for aneurysm formation and dissection both at the aortic root and throughout the arterial tree. In some cases, arterial dissections and stroke, focal stenosis of the aorta and large stenotic stretches, stenosis of the main and peripheral pulmonary arteries (which may lead to pulmonary hypertension), large-vein dilation, valvular regurgitation and mitral valve prolapse can be seen [Callewaert BL et al., 2008].The more striking lesion is the degree of arterial tortuosity and lengthening associated with aneurysms and stenosis confined to arterial vessels, which is much more severe than in other more CTDs (MFS and EDS). In such

cases ascending aortic dilation usually is clinically evident with a time-dependent progression, often leading to aortic valve incompetence. Due to the rarity of the disease, there are no standardized treatment protocols or guidelines for affected individuals, even if surgical intervention for arterial and pulmonary abnormalities has been successful in specific cases reported in the medical literature, such as valve-sparing aortic root replacement [Bottio T et al., 2007].

vEDS_ this autosomal recessive condition is caused by defects in the enzymatic activity of lysyl hydroxylase, encoded by the *PLOD1* (procollagen-lysine1,2-oxoglutarate 5-dioxygenase 1; OMIM#153454) gene. Aortic dilation/dissection and rupture of medium sized arteries have been observed [Wenstrup RJ et al., 1989]. Patients with the so-called "cardiac valvular subtype of EDS" , which associates severe cardiac valvular problems and features of the classic type of EDS (atrophic scars, skin hyperelasticiy and joint hypermobility), were found to have a complete deficiency of the proα2-chain of type I collagen (*COL1A2*, collagen, type 1, alpha-2, OMIM#120160). Most recently, patients with arginine to cysteine substitutions in the proα1-chain of type I collagen (*COL1A1*, collagen, type 1, alpha-1, OMIM*120150) displayed classic EDS but evolved to a vascular EDS-like phenotype later in life, with increased risk for spontaneous arterial rupture, most prominently affecting the femoral and iliac arteries [Schwarze U et al., 2004]. Vascular aneurysms/dissections or gastrointestinal perforation constitutes the presenting signs in a majority of adults with vEDS. The average age for the first major arterial or gastrointestinal complication is 23 years. Among patients with vEDS ascertained because of a medical complication associated with the disorder, in 25% the complication was present by age 20 and in 80% by age 40 [Oderich GS et al., 2005]. The median age of death in patients with vEDS is 48 years. The diagnosis of vEDS is made on clinical grounds (using major and minor diagnostic criteria) and is confirmed by genetic or protein-based testing [Pepin M et al., 2000]. Surgical intervention is notoriously dangerous in EDS IV due to excess vessel fragility [Elefteriades JA and Pomianowsky P, 2013].

2.4 NON-SYNDROMIC TAAs

In non syndromic TAAs, abnormalities are limited to the cardiovascular system. Conditions that are usually considered non syndromic include FTAAD, where more than one person in the family is affected, sporadic TAA, where only a single person in the family is known to have an aneurysm, well as TAA associated with BAV.

FTAAD_it predominantly displays an autosomal dominant pattern of inheritance, with varying degrees of penetrance and variable expressivity, although other forms of inheritance, including recessive patterns, have been reported [Elefteriades JA and Pomianowsky P, 2013]. In comparison to sporadic TAAD patients, familial TAAD individuals tend to be younger at presentation, suggesting a more aggressive clinical phenotype [Olsson C et al., 2013]. Beyond a positive family history of TAAD, criteria for diagnosing FTAAD include:

a. detection of dilatation at any trait of the thoracic aorta involving either the sinuses of Valsalva, the ascending aorta, or both and
b. the exclusion of MFS, LDS, Ehlers-Danlos syndrome vascular type, and other syndromic causes of TAAD.

FTAAD typically presents earlier in life with respect to sporadic aneurysms, has a higher annual growth rate, and does not demonstrate association with traditional risk factors for aortic disease such as dyslipidemia or coronary artery disease [Albornoz G et al., 2006].

FTAADs are in addition genetically heterogeneous. The main theme that emerges in the non syndromic TAA inventory converges on components of the contractile apparatus of the VSMCs. Mutations in five genes (*MYH11, TGFBR1, TGFBR2, MYLK*, and *ACTA2*) have this far been identified and all have autosomal dominant inheritance. Together these account for only 23% of familial non syndromic cases. Copy number variants (CNVs) in *MFAP4* (microfibrillar associated protein 4, OMIM#600596) in cases of aortic valve disease, with high expression of *MFAP4* in the developing valves and ascending aorta [Hitz MP et al., 2012]. In line with these observations, a study of CNVs in familial and sporadic TAAD found 47 enriched CNVs in TAAD compared with normal population control subjects [Prakash SK et al., 2010]. Gene ontology, expression profiling, and network analysis showed that genes within TAAD CNVs regulate smooth muscle cell adhesion or contractility and interact with the smooth muscle-specific isoforms of α-actin and b-myosin, which are known to cause familial TAAD. Two

additional chromosomal regions have been implicated (5q13-14 and 11q23.3-24) but no genes have as yet been identified. Novel genes have been difficult to map by linkage analysis, probably because of incomplete penetrance and/or locus heterogeneity [Guo D et al., 2001; Vaughan CJ et al., 2001].

MYH11 mutations account for 2% of non syndromic TAADs, and have been often with patent ductus arteriosus (PDA, OMIM#607411) [Zhu L et al., 2006].The myosin superfamily is composed of a large class of motor molecules which interact with actin filaments and result in force generation through ATP hydrolysis. Heterozygous *MYH11* mutations result in TAA and are thought to act via a dominant negative mechanism with a reduced penetrance; *MYH11*mutations identified in FTAAD families are predominantly splice-site mutations resulting in in-frame deletions and missense mutations. It has been observed, however, that *MYH11* mutations do not always cosegregate with disease within affected families, which suggests the presence of additional modifiers of the phenotype. Again of function mechanism might be at play for *MYH11* in microduplications of chromosome 16p13.1, which were found in adult-onset TAAD, but that did not co-segregate with disease in familial TAAD. Also, patients with 16p13.1 duplications were more likely to carry a second rare copy number variant (CNV), which lead to the possibility that *MYH11* mutations require modifiers, or are themselves modifiers, in TAAD pathogenesis [Harakalova M, et al., 2013 Kuang SQ et al., 2011].

Mutations in another component of the VSMC contractile apparatus, the *MYLK* gene (encoding myosin light chain kinase, OMIM*600922), have been found in a minority of FTAAD patients (accounting for ~1% of subjects). Mutations in this gene follow an autosomal dominant pattern of inheritance, and are thought to cause disease as a consequence of haploinsufficiency. Acute aortic dissection with little to no enlargement of the aorta has been described in *MYLK* mutation carriers. *MYLK* mutations disrupt kinase binding to calmodulin and reduce kinase activity in vitro, and mice with VSMC-specific knockdown of Mylk develop medial degeneration of the aorta [Wang L et al., 2010].

Mutations in the *ACTA2* gene, (actin, alpha-2, smooth muscle, aorta, OMIM*102620) which encodes the VSMC-specific actin isoform required for VSMC contraction, are the most prevalent mutations resulting in TAD and account for 10%-15% of all FTAAD mutations [Guo DC et al., 2007]. Interestingly, although *ACTA2* mutations are thought to act through a dominant negative mechanism, they are not fully penetrant, and only half of mutation carriers have aortic disease. Indeed, livedo

reticularis is the most penetrant manifestation in mutation carriers. A specific genotype-phenotype correlation exists for the p.Arg179His mutation, which causes a severe cerebrovascular phenotype, stroke, coronary artery disease, and variable presentations of pulmonary hypertension, bladder, and gastrointestinal problems [Munot P et al., 2012; Guo DC et al., 2009]. *ACTA2* mutations entail a high risk of aortic dissection, with most aneurysms <5.0 cm before dissection [Regalado ES et al., 2015]. For this reason, early surgical intervention should be considered even when minimal changes in aortic diameter are recognized.

BAV/TAA_with a population incidence of 1%, BAV is the most common form of congenital heart disease. In ~40% to 50% of those with BAV, there is an associated dilatation of the ascending thoracic aorta (or aortic root). Therefore, BAV-associated TAA (BAV/TAA) is likely the most common type of aneurysm affecting humans [Isselbacher EM et al., 2016]. Because the aortic valve and ascending aorta receive important cellular contributions from the neural crest during cardiac morphogenesis, a common developmental basis for BAV with TAAD has been postulated [Elefteriades JA and Pomianowsky P, 2013]. Numerous mouse models of BAV have been developed, including mice targeted for *NKX2.5* (nk2 homeobox 5, OMIM*600584), *GATA5* (Gata-binding protein 5, OMIM*611496), *NOS3* (nitric oxide synthase 3, OMIM*163729), *MATR3* (matrin 3, OMIM*164015), *ADAMTS5* (A disintegrin and metalloproteinase with thrombospondin motifs 5, OMIM*605007), *BRG1*(SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4, OMIM*603254), *HOXA1* (Homeobox A1, OMIM*142955) [Laforest B et al., 2011; Quintero-Rivera F et al., 2015; Akerberg BN et al., 2015; Makki N and Capecchi MR, 2012; Lee TC et al., 2000; Biben C et al., 2000]. However, aneurysmal disease has not been reported in these models, and penetrance of BAV is reduced even in the homozygous knockout animals, while elastic fibre degradation, a trend toward increased matrix metalloproteinase 2expression, and increased smooth muscle cell apoptosis (reminiscent featuresof TAAD) have been observed in double heterozygous $Notch1^{+/-}$ and $Nos3^{-/-}$ mice [Koenig SN et al., 2015]. These and other evidences ensured that a large number of candidate genes were considered for mutational screening in BAV with and without aortopathy [Bonachea EM et al., 2014]. These studies led to the identification of some rare variants in a minority of sporadic and familial cases, but it appears that only mutations in the transcriptional regulator *NOTCH1*(Notch homolog 1, translocation-associated, OMIM*190198) would be consistently associated with non

syndromic BAV with and without TAAD in humans, accounting for 4% of cases [Garg V et al., 2005; McKellar SH et al., 2007]. The prevalence of mutations in genes causing syndromic TAAD, such as *FBN1*, *TGFBR1*, and *TGFBR2*, in isolated BAV with TAAD is probably extremely low, which confirms the stringent genotype-phenotype correlation of these genes with their respective syndromic disorders [Pepe G et al., 2014; Girdauskas E et al., 2011; Arrington CB et al., 2008]. The understanding of BAV-associated TAAD is therefore limited by the lack of mechanistic insight into BAV pathogenesis. Several scenarios have been suggested to explain the difficulty to tease apart genetic factors in BAV/TAAD [Andelfinger G et al., 2016]: (1) genetic heterogeneity and reduced penetrance: to date some factors have been associated with BAV/TAAD but they explain only a minority of cases. In addition to that, linkage studies have not been replicated, which is conceivable with high genetic heterogeneity of strong genetic factors. If many different genes can lead to BAV/TAAD, many factors could be private, and/or difficult to find with current sample sizes. (2) Complex trait: statistical genetic analysis suggests that BAV is oligogenic. It is conceivable that a multitude of variants with low minor allele frequencies (eg, <5%) interact, in variable composition, in BAV pathogenesis, thus making segregation patterns and causal contributions very difficult to define, requiring, as a consequence, very large-scale studies. (3) Variants in noncoding sequence: advanced technologies, such as the whole-exome sequencing, have allowed rapid screening of the entire coding sequence of the human genome, which account for the vast majority of Mendelian phenotypes [Chong JX et al., 2015]. However, BAV/TAAD does not behave as a strictly Mendelian trait, and it is possible that whole-exome sequencing has overlooked causal or contributory variants in regulatory sequences. This inability to identify simple mendelian loci with whole-exome approaches suggests that many pedigrees may be attributable to polygenic influence rather than simply incomplete penetrance. (4) Interaction with epigenetic factors: abnormal flow patterns in the ascending aorta occurs even in the absence of a hemodynamically significant gradient, being a direct consequence of the altered leaflet morphology; this could imply valve-related hemodynamics as a contributing factor in the development of aortopathy [Guzzardi DG et al., 2015] and several genes related to epithelial to mesenchymal transformation, such as *KLF2* (KRUPPEL-LIKE FACTOR 2, OMIM*602016), *ET1* (endothelin 1, OMIM*131240), and *NOS3*, have been demonstrated to be shear stress responsive [Groenendijk BC, et al., 2004].

The discovery of numerous genes causative of an inherited predisposition for TAAD has made the adaptation of clinical management according to the underlying gene mutation progressively more important. The genotype-phenotype relationships that have emerged over the past years, in fact have flown into treatment guidelines endorsed by medical societies (Table 2) [Hiratzka LF et al., 2010]. Immediate consequences of gene discovery in TAAD are the gene-specific guidance for surgical intervention and the genetic diagnosis in family members, which have a direct bearing on the management of syndromic and nonsyndromic TAAD, but less so for the more prevalent forms of BAV with TAAD [Andelfinger G et al., 2016].

Table 2. Size thresholds for surgical intervention and screening modalities and for patients and family members with genetic aortopathy

Aortopathy	***Gene(s) involved***	***Size thresholds for surgical intervention***	***Part of aorta affected***	***Screening of family members***	***Imaging modality***
Marfan syndrome (MFS)	*FBN1*	Ao root*: 5.0 cm Asc aorta*: 5.0 cm Arch: 5.5-6.0 cm Desc: 5.5-6.0 cm	Asc: ++ Arch: + Desc: +	Clinical: Ghent criteria Genetic: in some cases Imaging: yes	Echo and CT vs MRI
Loeys-Dietz Syndrome (LDS)	*TGFBR1* *TGFBR2* *SMAD3* *TGFB2* *TGFB3*	Ao root: 4.0-5.0 cm Asc aorta: 4.2-5.0 cm Arch: 5.5-6.0 cm Desc: 5.5-6.0 cm	Asc aorta: ++ Arch: + Desc: +	Clinical: yes Genetic: yes Imaging: yes	Echo andCT vs MRI
Aneurysm-osteoarthritis syndrome (AOS)	*SMAD3*		Asc aorta: ++ Desc: +	Clinical: yes Genetic: yes Imaging: yes	Echo and CT vs MRI
Familial thoracic aortic aneurysm (FTAAD)	*TGFB2* *TGFBR1* *TGFBR2* *MYH11* *MYLK* *ACTA2*	Ao root: 4.5-5.0 cm Asc aorta: 4.5-5.0 cm Arch: 5.5-6.0 cm Desc: 5.5-6.0 cm	Asc aorta: ++ Arch: + Desc: +	Clinical: yes Genetic: yes Imaging: yes	Echo and CT vs MRI
Ehlers-Danlos type IV (vEDS)	*COL3A1*		Asc aorta: + Desc: +	Clinical: yes Genetic: yes Imaging: yes	Echo and CT vs MRI
Bicuspid aortic valve (BAV)	Likely multiple genes	Ao root: 5.0-5.5 cm Asc aorta: 5.0-5.5 cm Arch: 5.5 cm Desc: 6.5 cm	Asc aorta: ++ Arch: + Desc: -	Clinical: yes Genetic: no Imaging: yes	Echo and CT vs MRI

Summary of guidelines for genetic stratification of patient management in TAD.
++, most common site involvement; þ, next common site of involvement; Ao, aortic; Asc, ascending; CT, computed tomography; Desc, descending thoracic
aorta; Echo, echocardiogram; FBN1, fibrillin-1; MRI, magnetic resonance imaging; TGFBR, transforming growth factor b receptor.
* Lower intervention thresholds apply for women who anticipate pregnancy (from Andelfinger G et al., 2016).

Large studies have assessed that extrapolation of size thresholds for intervention for the BAV/TAAD is not possible, because a generous approach to aortic resection deduced from experience in MFS would expose most BAV patients to incremental surgical risk without clear evidence of long-term benefit [Itagaki S et al., 2015]. As already mentioned, MFS patients are susceptible to thoracic aortic aneurysms with a greater incidence of aortic dissection but the low risk of aortic complications in subjects with an aortic size <5.0 cm has led to the recommendation, for the aortic root and ascending aorta, of a 5.0 cm size threshold to be appropriate. A family history of premature aortic dissection, however, warrants consideration of surgical intervention at smaller diameters [Jondeau G et al., 2012]. LDS patients carrying a mutation in *TGFBR1*, *TGFBR2*, or *SMAD3* will benefit from surgery when the ascending aorta is between 4.0 and 4.2 cm [Hiratzka LF et al., 2010]. However, there's a paucity of data on the risk of aortic complications in these syndromes because of their overall low prevalence. FTAAD patients carrying causative mutations in *MYH11*, *ACTA2*, or *MYLK* should receive surgery when the diameter of the ascending aorta is between 4.5 cm and 5.0 cm; aortic dissections have been described at minimal dilations of the ascending aorta in this group [Wang L et al., 2010]. Genetic testing should be oriented on clinical findings as the confirmation (or exclusion) of a genetic predisposition toward TAAD, can be helpful in tailoring management. The development and standardization of highthroughput sequencing technologies (HTS) or next generation sequencing (NGS) also for diagnostic purpose are determining a lowering of cost and time of analysis, with successful identification of disease causing mutations in 4%-27% of cases [Giusti B et al., 2016; Koboldt DC et al., 2013; Campens L et al., 2015; Wooderchak-Donahue W et al., 2015]. All these findings taken together highlight both the challenges and opportunities that face us in determining optimal treatment strategies for TAAs according to the evidence that not all aneurysms, even when they involve the same aortic segment, behave similarly. The underlying cause and even the underlying genetic mutation appear to affect both risk and response to therapy. Therefore, efforts to better define individual patients' specific genetic defects may inform future research and define the groups in which specific therapies may be most efficacious [Isselbacher EM et al., 2016].

CHAPTER 3.

MARFAN SYNDROME

3.1 BACKGROUND HISTORY

MFS represents a spectrum of disorders caused by a heritable genetic defect of connective tissue that has an autosomal dominant mode of transmission. The defect itself has been isolated to the fibrillin-1 gene (*FBN1*; OMIM#134797) on chromosome 15, which codes for the connective tissue protein fibrillin. Abnormalities in this protein cause a myriad of distinct clinical problems, of which the musculoskeletal, cardiac, and ocular system problems predominate. MFS was first described more than 100 years ago by a Parisian professor of pediatrics, Antoine-Bernard Marfan, who reported the association of long slender digits and other skeletal abnormalities in a 5-year-old girl.
Thereafter many different body districts were proved to be involved and the cardinal manifestations are described in the clinical picture related with the syndrome. In 1955, Victor McKusick described a range of pleiotropic and phenotypic variability in MFS, including cardiovascular aspects, ectopia lentis, skeletal pectus excavatum and kyphoscoliosis. He also established the disease to be hereditary with an autosomal dominant mode of transmission, including it inside the connective tissue disorders nosological cathegory [McKusick VA et al., 1955]. Today, the disorder that bears his name has benefited from decades of clinical and molecular investigation. To ensure accurate communication between health care providers, researchers and patients, the Berlin nosology predefined clinical criteria for MFS in 1986 [Beighton Pet al., 1986]. In 1995 *FBN1* gene mutations were identified to be associated with the onset of the disease [Dietz HCet al., 1995] and, as a consequence, previuos criteria were recognised to possibly lead to an overdiagnosis of patients with a positive family history of MFS. In fact, these patients were sometimes only affected by non-specific connective tissue findings themselves, without carrying the mutation present in more typically affected family members.

Therefore, in 1996 the Ghent nosology was reported as new diagnostic criteria to ensure stringent guidelines for the diagnosis of MFS in unequivocally affected individuals and to separate other connective tissue disorders from MFS [De Paepe A et al., 1996]. The Ghent criteria made use of a classification for the different clinical features, which were divided into *major criteria* with high diagnostic specificity and *minor criteria* in which an organ system is involved. The presence of a major criterion in two different systems and a minor criterion in a third system were required to make the diagnosis of MFS. The prevalence of a *FBN1* mutation in patients fulfilling the criteria for MFS currently is 91-95%. Nevertheless, the sensitivity of the Ghent nosology was relatively low as most features of MFS are age dependent, which often postpones the diagnosis of MFS in children with a positive family history. Another concern regarding the sensitivity of the Ghent nosology in diagnosing MFS were the highly variable and diverse features, which are present as well in the general population and in other connective tissue disorders [Aalberts JJ et al., 2010].

These further revisions of the Ghent criteria have ultimately resulted in the revised Ghent nosology of 2010, in which the presence of aortic root dilatation in a personal and/or family history was required for the diagnosis. In absence of aortic root dilatation, identification of a pathogenic *FBN1* mutation is required in order to establish the diagnosis. Presence of aortic root dilatation according to Z-score and ectopia lentis is now sufficient for establishing the diagnosis of MFS in individual patients. All other clinical features contribute to a "systemic score", which guides diagnosis when ectopia lentis or aortic disease is absent. Less specific manifestations of MFS have been removed or made less influential in the diagnostic evaluation. The major criterion "dural ectasia" from the old Ghent criteria is converted into the systemic score in the revised criteria while a more prominent role is provided for molecular genetic screening of the *FBN1* gene and other relevant genes [Loeys BL et al., 2010].

3.2 EPIDEMIOLOGY

A number of studies have assessed the prevalence of MFS. The incidence of classic MFS was considered about 2-3 per 10,000 individuals, although this estimate depends on complete recognition of all affected and genetically predisposed individuals. Various factors could have contributed to an underestimate of disease prevalence. First, the phenotype becomes more apparent with increasing age in most families. Second, many of the outward manifestations are common in the general population and many physicians miss the diagnostic significance of these findings. Third, while the disorder

segregates as a dominant trait in families, about 25% of cases are sporadic due to de-novo mutations; a family history of MFS is not always present as an obvious risk factor. Fourth, although it is known that mutations in the *FBN1* gene, which encodes the matrix protein fibrillin-1, are the predominant (if not the only) cause of classic MFS, there is no rapid molecular diagnostic test [Judge DP and Dietz HC, 2005].

The earliest study comes from Northern Ireland and it reports 1.5 affected persons per 100,000 of the population. A second study that screened 29,067 children from 18 provinces and cities of the People's Republic of China from 1951 to 1987 reported five children with MFS, giving a prevalence of 17.2 per 100,000 of the population. Taken together, all studies provide lower prevalence rates than do the widely cited estimate of one per 5,000 individuals. Interestingly, these studies document a wide range from 1.5-17.2 per 100,000 [von Kodolitsch Y et al., 2015]. Nevertheless, MFS diagnosis can be arduous considering all age-dependent features; it is also observed a phenotyping variability among patients.

The disease occurs worldwide, with no predilection for either sex or ethnicity [Tsang A et al., 2013]. Tall stature with dolichostenomelia (long-bone overgrowth) leads to increased incidence in certain athletes, including basketball and volleyball players, sometimes prompting a recommendation for screening by echocardiography [Kinoshita N et al., 2000].

MFS is a multisystem disorder with manifestations typically involving the cardiovascular, skeletal, and ocular systems. Besides these, other districts can be involved such as the integumentary system, nervous and the pulmonary system. A report in the early 1970s on life expectancy and cause of death in MFS describes that life expectancy for affected individuals was about two-thirds that of unaffected individuals, with life-table mortality curves deviating in infancy between controls and affected individuals [Lamont Murdoch Jet al., 1972]. Death cause was cardiovascular (aortic dissection, congestive heart failure, or cardiac valve disease) in over 90% of cases. Medical, surgical and basic research advances over the last two decades have had a major positive impact on the clinical management of MFS patients.

Life expectancy has increased significantly, more discriminating diagnostic criteria have been developed, a number of new clinical entities have been recognized, and exciting opportunities for drug-based therapy have emerged [Cook JR, 2014]. Indeed, advances in medical and surgical therapies have resulted in a life-expectancy in classic MFS approaching that of the general population [Pyeritz RE et al., 2012].

3.3 PATHOGENESIS

Despite advances in our understanding of the genetics of MFS and other related disorders, the pathogenic and molecular mechanisms leading to the development of the phenotype are complex and currently the focus of much research. Early pathogenetic models for MFS focused on the structural weakness of the tissues imposed by microfibrillar deficiency. Fibrillins constitute the major backbone of multifunctional microfibrils in elastic and nonelastic extracellular matrices. siRNA knockdown experiments demonstrated that the assembly of fibrillin-1 is strictly dependent on the presence of extracellular fibronectin fibrils. The properties of these microfibrils are often compromised in MFS. The fibrillinopathies arise from mutations in fibrillins and are frequently associated with aberrant microfibril assembly [Hubmacher, 2008].

The observed genotype-phenotype correlations may indicate different underlying pathophysiologic mechanisms, including genetic (dominant negative vs. haploinsufficiency) and functional (structural function of fibrillin-1 vs. mediator of TGF-ß signaling) mechanisms. The MFS phenotype is developmentally acquired and manifests the dynamic interplay between primary tissue predisposition, physiologic stress, time, and both productive and deleterious compensatory events. Modifying factors are likely to be important for the phenotypic expression of this disease.

3.3.1 Dominant-negative mechanism and haploinsufficiency

Since the discovery that *FBN1* mutations cause MFS, the pathogenesis of the disorder has been the subject of intense investigation and controversy. *FBN1* encodes fibrillin-1, a 350 kDa glycoprotein, highly conserved among different species, first identified by Sakai and colleagues to be the principal component of the extracellular matrix microfibrils and which is present in all tissues with phenotypic manifestations of the disorder [Sakay LY et al., 1986]. Each fibrillin monomer contains a large number of epidermal growth factor-like motifs, most capable of binding calcium ions, and a few motifs resembling the binding protein for transforming growth factor beta. In vitro polymerization of fibrillin monomers produces 'beads on a string' structures that look on electron microscopy much like microfibrils purified from the extracellular matrices of a variety of tissues (see Chapter 3.6.2).

In patients with MFS, the medial layer of the aorta shows extensive abnormalities, including the fragmentation, disorganization and loss of the elastic lamina and its replacement by a basophilic material made up of glucosaminoglycans

[Schlatmann TJ et al., 1977]. Areas with CMN develop. Although sometimes thought incorrectly to be pathognomonic of MFS, this medial degeneration is nonspecific and can be seen in all types of thoracic aortic aneurysms.

FBN1 gene contains 65 exons spanning 235 kb of genomic DNA. Most mutations occur within the 47 tandemly repeated epidermal growth factor-like domains, many disrupting one of the six predictably spaced cysteine residues that interact via disulfide linkage to determine domain folding or residues that affect calcium binding to fibrillin-1 (see Chapter 3.6.3). Mutations in *FBN1* were believed to cause structural weakness of the aortic wall, explaining the progressive dilation of the aortic root and the histological changes described as a consequence of the enhanced cleavage and proteolytic degradation [Reinhardt DP et al., 1997].

Several observations have suggested that a traditional dominant-negative mechanism or haploinsufficiency may play a role. According to the dominant-negative model of pathogenesis, the product of the mutant allele interferes with the function of the wild-type gene product. Large, multiprotein aggregates may be especially sensitive to defects in monomeric components. A dominant-negative mechanism has been inferred based on the dominant inheritance: - fibrillin-1 monomers aggregate to form complex structures called microfibrils, providing an opportunity for interference; - the dramatic paucity of matrix-incorporated fibrillin-1 observed in heterozygous patient samples (far less than 50% that would be predicted from simple loss of contribution of one allele); - some patients with mutations associated with low production of mutant protein have a mild, subdiagnostic variant of the disorder [Dietz HCet al., 1993; Nijbroek G et al., 1995]. However, there are reports of patients with classic severe disease who also presented very low levels of mutant transcripts, in a way refuting this evidence of an association between abundance of mutant transcript and severity of disease [Halliday D et al., 1999].

Transgenic experiments with mutant fibrillin-1 support the haploinsufficiency hypothesis (half-normal production of normal protein), rather than the production of mutant protein, as mechanism for MFS pathogenesis suggesting its critical role in reaching the threshold loss of fibrillin-1 function needed for clinical expression of the disorder. Transgenic overexpression of mutant fibrillin-1, in the context of two normal *FBN1* alleles, was shown to be insufficient to cause the vascular changes of MFS seen in mice heterozygous for a comparable missense mutation C1039G and the transgenic addition of a wild-type allele to C1039G heterozygous mice rescued the aortic phenotype [Judge DP et al., 2004]. Another recent study demonstrated that identical

mutations expressed in the same domain context, result in alternative trafficking fates of mutant proteins, which has highlighted the further complexity in the molecular pathology of MFS. A cooperative dependence of protein folding of cb-EGF12 on cb-EGF13 accounted for the ER retention associated with defective calcium binding to cbEGF13. Such complex folding behavior may be the underlying molecular mechanism of MFS. The mutant proteins retained as a consequence of this misfolding have an intracellular dominant effect or cause MFS via haploinsufficiency [Whiteman Pet al., 2007]. These evidences suggest that normal complement of fibrillin-1 might be needed to initiate productive microfibrillar assembly, making the haploinsufficiency-imposed reduction in microfibrillar abundance to be critical to the context within which a dominant negative effect can achieve clinical significance, even if it may or may not be sufficient in isolation to lead to classic MFS [Judge DP et al., 2005].

Certain manifestations of the disease (such as lens luxation, dural ectasia, or joint hyperlaxity) are easily explained as a consequence of connective-tissue weakness. However, this model cannot explain other phenotypic features of MFS, such as the disproportionate growth of the long bones or myxoid changes in the mitral valve.

3.3.2 Transforming growth factor beta (TGF-ß) signaling

As a growing body of evidence supports the view that elevated TGF-β levels may play a central role in the development of the manifestations specific to MFS; all factors that can modulate active TGF-β levels should be studied in order to understand the diversity and variable expressivity observed in the phenotypes of the patients with the syndrome. One such factor is the activation of the latent TGF-β complex. Polymorphisms in gene-encoding molecules that participate in the activation process may be responsible for the variable expressivity. Among the known activating pathways proteases, integrins, pH alteration and free oxygen radicals are all capable triggers for TGF-β activation [Lyons RM et al., 1988; Barcellos-Hoff MH and Dix TA, 1996; Schultz-Cherry S and Murphy-Ullrich JE, 1993; Wipff PJ and Hinz B, 2008].

From the perspective of MFS, the mechanosensor function of TGF-β seems to be important. Elasticity of the microfibrillar system affects the extent of TGF-β release from the latent complex. When connective tissue is inelastic, the elevated active TGF-β level, by increasing the expression of certain proteases including MMPs, induces the remodelling of the ECM [Booms P et al., 2005]. This negative feedback cycle in the mechano-signalling process eventually allows the fine tuning of the connective tissue elasticity [Booms P et al., 2005]. The aforementioned activating modalities, by altering

the tissue concentration of the active form of TGF-β, may affect the dynamic equilibrium maintained by the mechano-transduction feedback and thus modify the severity of the manifestations in MFS [Wipff PJ e Hinz B, 2008]. Therefore, better understanding of this function together with the clarification of the possible role of gene polymorphisms, may help to explain the important phenotypical variations in Marfan patients. This may offer a potential research opportunity for the development of drugs to modify the course of MFS by diminishing TGF-β tissue levels. ERKl/2 protein takes part in the regulation of Smad2-Smad4 complex, and may represent an important therapeutic target. TGF-β activation induces the phosphorylation cascade in Jun N terminal associated Kinase (JNK) p38/MAPK pathways as well. JNK activates c-JUN and c-FOS transcriptional factors in the cell nucleus [Holm TM et al., 2011]. Based on mice model experiments, JNK inhibition might be a therapeutic target, since blocking this pathway may reduce MMPs and mRNS, which play a role in Marfan pathology [Gomes LR etal., 2012]. Besides, inhibiting the signalling pathway of p38/MAPK also reduces MMPs level, while blocking ERK 1/2 pathway enhanced the level of membrane associated MMP inhibitors. Because of conflicting data on the canonical and non-canonical pathways, it is not clear which is more important in the development of the manifestations of MFS. In MFS, Smad2 protein plays an exclusive role by forming a complex with Smad4, CBP and P300 Smad2 regulates transcription andinduces MMP production. Gomez et al. concluded in 2011 [Gomez D et al., 2011] that the signalling pathway of TGF-β/Smad2 plays a crucial role in the development of thoracic aortic aneurysms of different etiology. Increased expression of Smad2 may cause histological aortic wall changes.

High concentration of active TGF-β predominates in the aforementioned mechanisms and leads to the different features of MFS. Thus, more collagen reduces the compliance of the aortic wall, which loses structural integrity and becomes dilated and aneurysmal. High interstitial concentration of TGF-β is also responsible for reduced muscle mass, characteristic to Marfanoid habitus as TGF-β hinders mitosis and differentiation of satellite cells which are important in the development of muscle fibres. Moreover, a high TGF-β level inhibits the fusion of satellite cells and the downstream signalling pathways of endogenous bone morphogenetic protein 2 (BMP-2) inhuman stem cells, which is indispensable for bone and cartilage development [PereiraL et al. 1993]. Because of this interaction between BMP-2 and TGF-β, the high availability of the latter molecule may be responsible for the tall stature in Marfan. While an increased level of elastase enhances elastin degradation, MMPs seem to be responsible for the

disintegration of elastic fibres [Bunton TE et al., 2001]. Therefore overproduction of these proteases reduces connective tissue elasticity, and leads to weakness of the aortic wall.

3.3.3 Matrix metalloproteinases (MMPs)

MMPs are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodeling and degradation of the ECM, including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. MMPs are usually minimally expressed in normal physiological conditions and thus homeostasis is maintained. However, MMPs are regulated by hormones, growth factors, and cytokines, and are involved in ovarian functions. Endogenous MMP inhibitors (MMPIs) and tissue inhibitors of MMPs (TIMPs) strictly control these enzymes. Over-expression of MMPs results in an imbalance between the activity of MMPs and TIMPs that can lead to a variety of pathological disorders [Raspollini MR et al., 2005].

A number of studies have shown the evidence of a correlation between the expression of these proteins and the development and progression of some cardiovascular manifestations in MFS in patients. TAA is the largest and potentially fatal cardiovascular complication in MFS which can lead to aortic rupture, dissection and death. The development and expansion of aneurysm is the result of a multifactorial process influenced by both cellular and extracellular mechanisms [Barbour JR et al., 2007]. The major histopathological feature of this complication is represented by the degradation of the elastic fibers of the media [Rodriguez-Pla A et al., 2005]. Recent studies on mouse models of MFS showed that the progression of TAA is in many cases associated with upregulation of matrix metalloproteinases MMP-2 and MMP-9 and the consequent series of molecular changes including extensive degeneration of the elastic fibers, endothelial dysfunction and reduction of smooth muscle cells contractility. Therapeutic treatments MMP-2 and MMP-9 proteins inhibitors, could represent a potentially effective strategy in decelerating progression of some of the histopathological features associated with TAA also in patients with MFS [Chung AW et al., 2007].

The correlation between MMPs regulation and TAA formation in MFS individuals, is related to some features identified in murine models of MFS in which increased mRNA levels together with an enhanced protein expression of MMP-2 and MMP-9 were observed: an alteration between the same proteins and their specific endogenous inhibitors (TIMP-1/MMP-9 and MMP-2/TIMP-2), loss of integrity of the

elastic fibers associated with a reduction in resistance of the vessel wall. These are therefore possible molecular mechanisms underlying the development and expansion of TAA in MFS individuals and may contribute to the degradation of mechanical properties and structural integrity of the aortic wall, one of the most important cardiovascular complications seen in MFS patients [Ikonomidis JS et al., 2006; Chung AW et al., 2007; Monaco M et al., 2007].

3.4 CLINICAL MANIFESTATIONS

MFS is characterized by a wide range of clinical manifestations. Most patients who have MFS are usually diagnosed incidentally when they present for a routine physical examination for various reasons, such as a pre-employment, physical or screening examination prior to participation in sports. Typically, patients with MFS present with tall stature, ectopia lentis, aortic root dilatation, and a positive family history. MFS primarily involves the skeletal, ocular, and cardiovascular systems. Common cardiovascular manifestations, most of which are substantial contributors to mortality, include annuloaortic ectasia with or without aortic valve insufficiency, aortic dissection, aortic aneurysm, pulmonary artery dilatation, and mitral valve prolapse. Scoliosis, pectus excavatum and carinatum, arachnodactyly, and acetabular protrusion are common musculoskeletal manifestations. Dural ectasia is a characteristic central nervous system manifestation. In some MFS patients, there is also pulmonary and ocular involvement. Presentation of the disease varies greatly, even among family members. Some persons with MFS experience only mild clinical manifestations, whereas others have severe problems. Early identification and treatment of these conditions contribute to an improved quality of life and a life expectancy close to the average for the general population.

3.4.1 Cardiovascular (CV) manifestations

CV manifestations are the most serious complications, determining the prognosis and survival in MFS as they represent the major cause of morbidity and mortality in the disease. Dysregulated TGF-β represent the pathogenic contribution to CV abnormalities in MFS and a few clinically related diseases, such as LDS, SGS, AOS and TAA. MFS displays a number of abnormalities of the thoracic and abdominal aorta, ranging from abnormal aortic stiffness [Dormand H et al., 2013] to aortic aneurysm and dissection. Tissue histologic specimens from the aorta of MFS patients show elastic lamellae fragmentation, disorganization of the aortic architecture with excessive collagen and mucopolysaccharides accumulation, and a relative decrease of vascular smooth muscle cells. The earliest recognition of the tissue abnormalities underlying aortic dilatation in MFS is degeneration of the medial layer, with fragmentation, disarray, andloss of elastic lamina and replacement by basophilic-staining proteoglyan. The process is associated with signs of ongoing inflammation and MMPs activation (Figure 13) [Wu D et al., 2013].

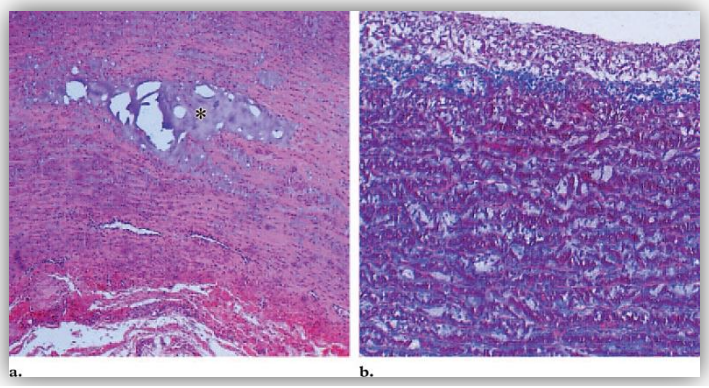


Fig 13. Histologic specimens from the aorta in a 24-year-old man show evidence of cystic medial necrosis. **(a)** Photomicrograph (original magnification, X40; hematoxylin-eosin stain) shows deposition of mucopolysaccharide (*). **(b)** Photomicrograph (original magnification, X100; Masson trichrome stain) demonstrates disruption of the elastic lamina (from Ha HI et al., 2007)

TAAs can occur early in life and can be also detected in the fetus by echocardiography (hereinafter abbreviated as echo). Acute TAD represents the leading cause of premature death in untreated individuals with MFS and it follows a period of progressive dilatation of the ascending aorta [Robinson PN et al., 2006]. The periodic magnetic resonance imaging (MRI) or computed tomography (CT) scan, as appropriate, is necessary since the echo does not allow the visualization of the entire thoracic aorta. However, the rate of progression and risk of dissection are unpredictable on a single-patient basis [Pepe G et al., 2016]. In case of long-term follow up of patients, for example, it can be advisable to have Cardiovascular magnetic resonance (CMR) which is not limited by acoustic windows and is free from ionising radiation. The entire aorta can be imaged and complications including aneurysm formation, dissection, and previous surgery are well visualised [Dormand H, Mohiaddin RH, 2013]. The clinical standardized follow-up of patients with MFS includes limitation in sports,

administration of beta-receptor blockers [Milleron O et al., 2015], and an echo test (every 1 year or 2 years), with subsequent prophylactic aortic root surgery in patients with an aortic diameter >5 cm. However, in patients with a family history of aortic dissection, a rapid increase of the aortic diameter (>0.3 cm/y on repeated measurements using the same imaging technique, at the same aorta level, with side-by-side comparison and confirmation by another technique), or presence of severe aortic or mitral regurgitation (or if a pregnancy is planned), aortic surgery should be performed when the diameter is >4.5 cm [Baumgartner H et al., 2010].

The risk of aortic dissection, which is the most serious manifestation of MFS, increases as the aorta enlarges and it develops more often in young patients with MFS than it does in the general population. Surgical replacement of the aortic root with a composite graft does not end the disease process [Aldrich HR et al., 1992]. The key histopathologic feature of TAD is medial degeneration, a process characterized by smooth muscle cell depletion and ECM degradation [Wu D et al., 2013]. TAD may occur as a result of TAA; nonetheless, aortic diameter is an unreliable predictor of TAA rupture or dissection because a significant proportion of TAD happens at diameters well below the suggested threshold for prophylactic aortic surgery. Thus, future prospective studies should include a search for potential predictors of TAD [Pepe G et al., 2016].

Almost all of MFS patients, by the age of 60 years, will have developed aortic root dilatation at varying degrees and three quarters of them would have undergone aortic root replacement on the basis of increased aortic diameter to critical levels and/ or symptomatic aortic valve insufficiency (or Stanford type “A” dissection), according to a study conducted by Détaint D. and colleagues in 2010 [Détaint D et al., 2010]. Thus, an optimal timing of these operations is highly required to avoid those complications undermining life expectancy and quality of life of MFS patients, although unreasonable early cardiac surgical interventions (with smaller aortic diameters than that stated by the European Society of Cardiology and the American College of Cardiology) in young adulthood or childhood may lead to re-operation [Pepe G et al., 2016].

It has to be noticed the lack of good indicators or biomarkers of TAADs since TAD may also develop in patients with the aorta having normal or smaller diameters than those reported in the current surgical criteria [Song HK et al., 2012]. Increased total serum levels of TGF-β1 have been investigated as a potential marker for TAAD in MFS and found out to be correlated with larger aortic root diameters, faster aortic root growth and earlier aortic root surgery when TGF-β1 level is >140 pg/mL in a cohort of 99 MFS patients carrying a *FBN1* mutation; after a follow-up of 38 months TGF-β has

been used as a therapeutic biomarker for effectiveness of losartan on the aortic root dilatation rate (AoDR) in patients with MFS and its plasma level was found out to be decreased in association with an increase of AoDR [Franken R et al., 2013], suggesting that the initiator of aorta dilatation is increased AngII, and not elevated TGF-β. TGF-β now seems to be a marker of aortic damage instead of being considered the initial cause of aortic dilatation [Franken R et al., 2015].

Molecular genetics may help in the selection of pharmaceutical therapy management of MFS; a study conducted by Franken R and colleagues in 2015 reported that losartan reduces AoDR only in patients carrying an *FBN1* mutation resulting in a haploinsufficiency, while patients carrying dominant negative *FBN1* mutations (mainly missense mutations) do not gain any advantage from this drug [Franken R et al., 2015], which can be possibly explained by the fact that a dominant negative *FBN1* mutation (qualitative defect) may alter functions or foldings of the protein, affecting interactions with fibrillin 1 and/or other proteins, leading to a disorganized ECM. That could lead to alterations in the strength of the fibrillin matrix, affecting the release of binding proteins, such as TGF-β. Noteworthy, plasma levels of TGF-β turned out to be increased in MFS and related to TAADs. Losartan is only effective on patients with haploinsufficiency possibly for the aortic wall, being thinner because of the decreased fibrillin matrix, may suffer from hypertension, which, as a consequence, alone or with aortic stretch, may directly damage the aorta by activating angiotensin II (AngII) receptor type 1 (AT1R), which, in turn, responds to the damage by producing TGF-β (Figure 14). Local AngII has been associated with aneurysm formation. Patients with MFS with haploinsufficiency have a more beneficial effect from losartan due to the greater content of AngII in the aortic wall [Franken R et al., 2015]. Losartan, by blocking AT1R, decreases TGF-β production and blood pressure, as well as increases pro-inflammatory responses, myofibroblast differentiation, and production of reactive oxygen species (all AngII-mediated detrimental processes in the aortic wall) [Franken R et al., 2013]

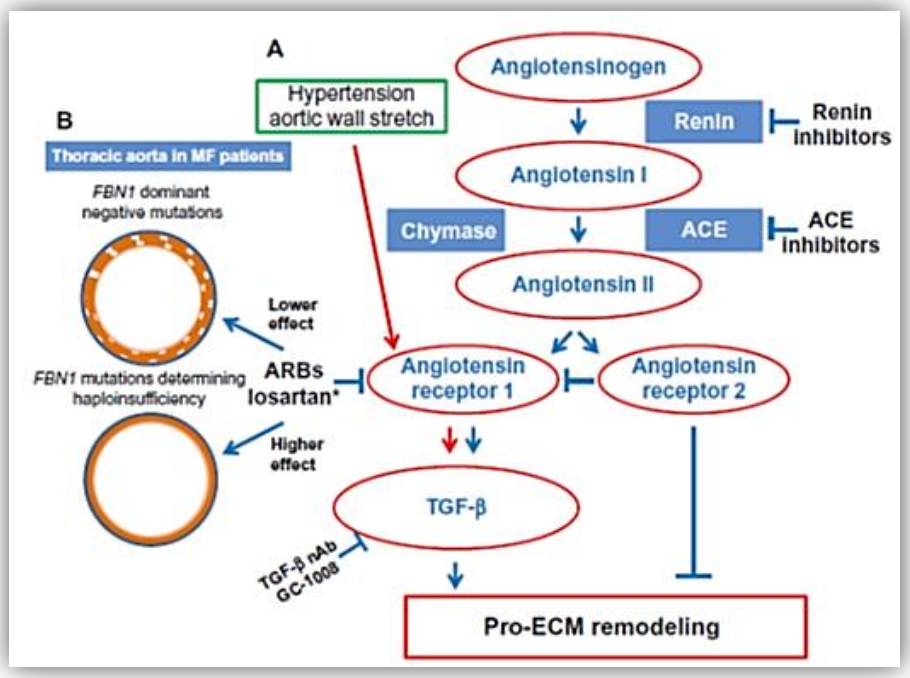


Fig 14. Effects of drug therapies on thoracic aortic wall of MF patients.(A) Biological targets and therapies upstream of TGF-β in Marfan syndrome. Renin converts angiotensinogen to angiotensin I, which is further converted to angiotensin IIby ACE, as well as tissue enzymes including chymase, thus bypassing the inhibition of ACEinhibitors. Angiotensin IIthen binds to either type 1 or type 2 angiotensin receptors. (B) Schematic representation of thoracic aortic wall in patients with Marfan syndrome with *FBN1* dominant negative mutations or haploinsufficiency, as well as the possible explanation of the better effect of losartan in patients with haploinsufficiency. Hypertension and wall stretching represent trigger factors for upregulation of type 1 angiotensin receptor, which in turn increases TGF-β production. *Administration of Losartan decreases TGF-β production and increases proinflammatory response, myofibroblast differentiation, and reactive oxygen species.
Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; ECM, extracellular matrix; *FBN1*, the gene encoding fibrillin 1; MF, Marfan (patients); TGF-β, transforming growth factor-β; TGF-β nAb GC-1008, human pan-TGF-β neutralizing antibody (from Pepe G et al., 2016)

Other CV manifestations include mitral valve elongation and myxomatous thickening usually leading to mitral valve prolapse (MVP, OMIM#157700), which is the most common valvular abnormality in MFS, with a highly variable reported prevalence of 28%-75% in comparison with an overall prevalence of 2.4% in the general population [Pyeritz RE et al., 1983]. MVP in children can be complicated by severe mitral regurgitation and myocardial dysfunction, resulting in heart failure [Morse RP et al., 1990]. Cardiac disease has been well documented in neonatal MFS patients, who typically present with a combination of valvular insufficiency, atrial and ventricular septal defects, and congestive heart failure [Fujiseki Y et al., 1985]. LV dysfunction, including both systolic and diastolic function, in adult MFS patients has been considered a secondary phenomenon due to valvular insufficiency and LV volume overload. Increased arterial stiffness may contribute to cardiac dysfunction by altering hemodynamic load on the LV. Although long considered a secondary event, a number

of studies have identified primarly heart disease in the absence of MVP or TAA, thereby suggesting an intrinsic problem with myocardial function in MFS (Figure 15) [Alpendurada F et al., 2010; Cook JR et al., 2014].

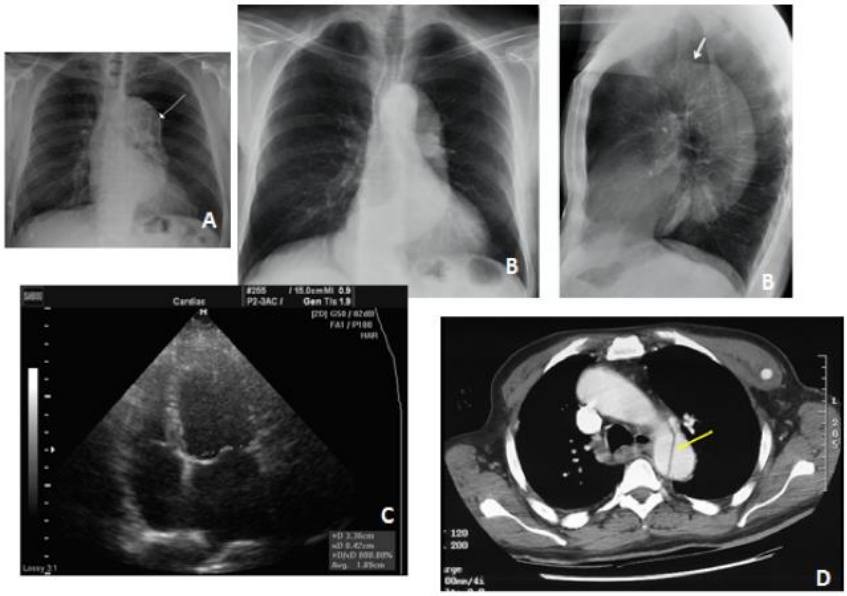


Fig 15. Clinical signs of the vascular system in Marfan syndrome. (A) Thoracic aortic aneurysms, (B) frontal and sideward view of a descending aorta aneurysm, (C) mitral valve prolapse, (D) thoracic aorta dissection.

3.4.2 Musculoskeletal manifestations

Among the most common skeletal manifestations are tall stature (>95th percentile by age/race/sex) dolichostenomelia or long limbs, arachnodactyly or long fingers, hyperextensible joints, scoliosis, kyphosis and "straight back", pectus deformities of the anterior chest, congenital contractures (particularly of the fingers or elbows), and generalized osteopenia. Long bones are thin, with subcutaneous fat and muscle atrophy exaggerating the elongated morphology of the arms and legs. Hypotonicity, hypermobility, and subluxations create the potential for deformity at virtually any joint [Dietz HC, 2001].

Patients may have premature arthritis. Bone growth may be asymmetric. Arms pan may equal or exceed height. The ratio of the length of the upper body segment (measured from the crown to the top of the symphysis) to the length of the lower segment (measured from symphysis to floor) is often two standard deviations below normal. Such exaggerated limb length or dolichostenomelia, however, is non-specific and non-diagnostic, although suggestive. Techniques for describing and quantifying body proportions are imprecise, overlapping both the normal spectrum and other causes for growth disturbances. Scoliosis exaggerates alterations in both the upper body segment: lower segment and arm span: height ratios.

Skull anomalies, often asymtomatic and therefore rarely radiographed, include dolichocephaly, long face with retrognathia, frontal sinus enlargment and high-arched or gothic palate. Spinal anomalies are common and often clinically significant. Scoliosis or kypho-scoliosis, Schmorl's nodes, straight back, or flattened thoracic kyphosis, and spondylolisthesis may be seen.

Scoliosis, seen in over half of all patients, is often a double major or right thoracic curve. Unlike idiopathic scoliosis, it is seen in as many males as females; is diagnosed in younger patients, and is more progressive, severe, and rigid, often requiring surgery. Scoliosis and straight back may contribute to cardio-pulmonary compromise. Lost thoracic kyphosis is associated with reduced total lung and forced vital capacity and with decreased residual volume. Dural ectasia produces posterior vertebral body scalloping. 63% of patients have a widened lumbo-sacral canal, often with thinned pedicles and laminae, or neural foraminal erosions. This may be associated with neurologic signs, pelvic meningoceles, or arachnoid cysts. The MFS patient's potential to pool or dilute intrathecal drugs should be recognized. It is hypothesized that ectasia reflects the action of gravity on weak connective tissue; the presence or severity in this pleiotropic syndrome. The chest is classically elongated and hyperinflated. Deformity and asymmetry are produced by longitudinal overgrowth of ribs, and by pectus excavatum and carinatum deformities, which may appear in isolation, in combination, or sequentially. Rib asymmetry may modify pectus findings. Pectus excavatum may combine with straight back to narrow the chest's antero-posterior diameter. Cardiopulmonary compromise may indicate the need for pectus repair.

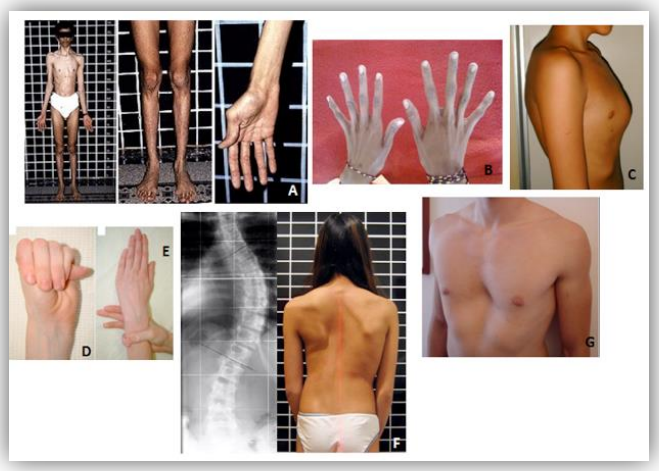


Fig 16. Musculoskeletal manifestations in Marfan syndrome. (A) Classic habitus in which is visible the tallstature and dolichostenomelia; (B) arachnodactyly; (C)(D)(E)(F) scoliosis; (G) pectus excavatum

Arachnodactyly, although not pathognomonic, is one of the best known features of MFS and can be striking. No simple diagnostic criteria are available. The clinical thumb and wrist signs may overlap the normal spectrum. The metacarpal index is more quantitative but time consuming; values greater than 8.8 for males and 9.4 for females

are abnormal. Flexion deformities and clinodactyly are common. Advances bone age is reflected in and documented by the hand radiograph. The weight-bearing lower extremities may show limb-lenght discrepancies or dislocations at the hip, knee, or patella.

Acetabular protrusion and slipped capital femoral epiphysis have been described, although they are not unique to this population. Tibial subluxation, patella alta, or genu recurvatum reflects ligamentous laxity at the knee. The foot echoes the arachnodactyly more commonly documented in the hand and may show pronation, pes planus, calcaneo planovalgus, club foot or vertical talus, or less clinically significant deformities such as calcaneal spur, hallux valgus, hammer toe, or disproportionately long first- digit (Figure 16) [Magid D et al., 1990; Avivi E et al., 2008].

3.4.3 Ocular manifestations

Most people with MFS are myopic and have astigmatism and dislocated lenses. However, most of them are asymptomatic. Either eye pain or visual loss are rare. The main ocular symptoms of MFS are the following :

- Blurred vision and monocular diplopia caused by progressive lens subluxation and resulting severe astigmatism.
- Headache, dizziness, and blurred vision, caused by complete anterior lens dislocation, and may result in damage to the corneal endothelium and secondary glaucoma.
- Eye pain and visual loss are caused by posterior lens luxation to the vitreous that may induce phacolytic uveitis and retinal detachment.

Signs include refractive instability and disturbances caused by lens movements, iridodonesis, phacodonesis, and recessed angle. The zonules and the lens equator may be seen in the papillary aperture. The cornea may be edematous, the intraocular pressure high, and in case of phacolytic uveitis cells and flare may be seen in the anterior or posterior chamber. Retinal detachment may be found [Nemet AY et al., 2006]. MFS is often associated with lenticular abnormalities, the most frequent of which are ectopia lentis and microspherophakia [Meire FM et al., Wallace RN et al., 1991].

Ectopia lentis (i.e., lens subluxation), is usually bilateral, symmetric, and stable from early childhood. These patients may complain of decreased or fluctuating vision and monocular diplopia. Findings include iridodonesis (tremulous iris) and irregular astigmatism. Lens dislocation is an important feature of several familial syndromes that also include skeletal dysplasias. MFS is the most prevalent of these. Bilateral lens dislocation occurs in 60-87% of patients. Fibrillin abnormalities may alter the lens development and its mechanism, and take part in the pathogenesis of ectopia lentis or other lenticular abnormalities in MFS [Traboulsi EI et al., 2000]. Histologically, in the normal human eyes, the zonules and superficial capsule, where zonules attached to the lens, are rich in fibrillin. Staining of fibrillin within the capsule gradually decreased toward the center of the lens, and the anterior and posterior central capsule is entire free of fibrillin [Wheatley HM et al., 1995]. In capsules that were dissected from lenses extracted intra-capsularly from MFS patients, ABC immunoperoxidase staining with monoclonal anti-fibrillin antibody showed fine disorganized fibrillin-positive fragments dispersed in the anterior capsule. There was only light staining for fibrillin encircling the equator, which serves as insertion platform for most zonular fibers. Their zonules were few and often attenuated and broken, and electron microscopy showed the zonule fibrils to be finer and lacking the normal parallel alignment with haphazardly arranged. As explained above, abnormal ciliary processes and zonules contribute to lens dislocation in MFS. For the same reason, coloboma of the lens may occasionally be found. Anterior subluxation may occur, but less commonly than in homocystinuria and Weill-Marchesani syndrome. Dislocation of the lens was found to be positively correlated with increased axial length, which is one of the minor criteria of the old Ghent diagnostic criteria [De Paepe A et al., 1996].

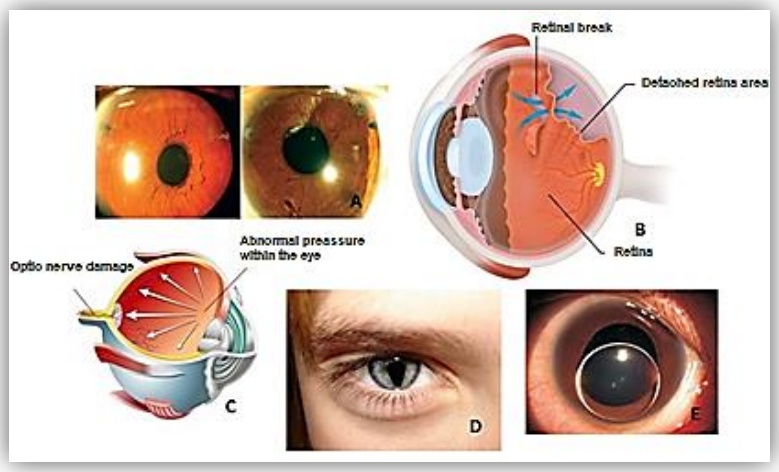


Fig 17. (A) Microspherophakia ; (B) Retinal detachment; (C) glaucoma; (D) coloboma; (E) ectopia lentis

Microspherophakia is a familial and typical bilateral condition in which the crystalline lens is small and relatively spherical with increased antero-posterior

thickness. The cortical fibers are approximately 20% of their normal thickness, which suggests a typical rather than arrested fibergenesis [Dayal MB et al., 1960]. A lens coloboma represents incomplete lens formation due to failure of the fetal fissure to close completely. It is often accompanied by other ocular colobomas of the eyelid, iris, lens, choroid, or optic disk. In MFS it may represent secondary changes of filamentary degeneration of zonular fibers [Schlote T et al., 1997]. Flattened corneas in MFS patients have been reported for many years, and were included in the Ghent criteria, as a minor sign. Corneas should be measured with keratometry. The reason for the flattened cornea is not known. It may be secondary to the increased dimensions of the globe in MFS patients, the scleral thinning and the associated flattened curvature of the cornea, or the pathology is primarily corneal due to fibrillin gene mutations and associated corneal misdevelopment. *FBN1* is a possible part of anchoring function in the cornea, as the microfibrils may act as a flexible mechanical anchor at epithelial- mesenchymal basement membrane interface.

The second most common ocular manifestation in MFS is myopia, which is found in 34-44% of patients with MFS, as compared to 4.8% in the general population in one study. Maumenee and coworkers (1981) reported that only 16.3% had myopia of 7.00D or more, and about 50% had myopia of 3.00 D or more. The mean average axial length is 24.9 mm in patients with MFS, being greater (25.96 mm) in those with ectopia lentis than those without (23.39 mm). [Maumenee H, 1978].

There is significantly elevated prevalence of strabismus in patients with MFS, ranging from 19% to 39%. Abnormal afferent visual inputs to cortical centers caused by ectopia lentis, craniofacial abnormalities, and genetic factors may all contribute to the higher prevalence of strabismus in this disease [Izquierdo NJ et al., 1994]. A case of retinal artery occlusion in a patient with MFS was described, assumed to be secondary to a possible thromboembolism from MVP (Figure 17) [Butt Z et al., 1992].

3.4.4 Central nervous system manifestations

Dural ectasia (DE) occurs in 63 to 90% of MFS patients according to three important studies: Pyeritz RE et al., 1988; Villeirs GM et al., 1999; Fattori R et al.,1999. Along with aortic root dilatation/dissection and ectopia lentis, DE has been one of the major criteria for clinical diagnosis of MFS [De Paepe et al., 2006], although recently it has been included among the heterogeneous group of systemic features that has to reach a score of 7 or more to be considered a major criterion [Loeys B et al.,2010].

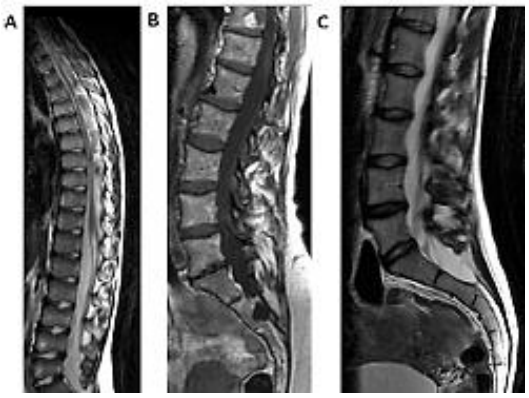


Fig 18. MRI images of dural ectasia. (a) Duralectasia grade 1, with a mean of dural sacdimension values between 14 and 18 mm; (b)dural ectasia grade 2, with a mean of dural sacdimension values greater than 18 mm and up to20 mm; (c) dural ectasia grade 3, with valuesmean upper 20 mm, with anterior sacralmeningocele.

DE usually occurs in the most caudal portion of the lumbosacral spinal column, at the point of greatest cerebrospinal fluid pressure in the upright patient. The consequences of DE include bony erosion or anterior meningoceles. DE is rare in the normal population and it is a sensitive and specific marker that can assist in confirmation of a diagnosis of MFS in young patients. It is usually associated with increased cerebrospinal fluid pressure, trauma or spinal surgery. In MFS lumbosacral dura mater affected by a defect in *FBN1*, due to highest pressure of cerebrospinal fluid for gravity action in the upright posture, results expanded (Figure 18).

3.5 CLINICAL DIAGNOSIS

The diagnosis of MFS relies on defined clinical criteria (Ghent nosology), outlined by international expert opinion to facilitate accurate recognition of this genetic aneurysm syndrome and to improve patient management and counselling. These Ghent criteria, comprising a set of major and minor manifestations in different body systems, have proven to work well since, with improving molecular techniques, confirmation of the diagnosis is possible in over 95% of patients. However, concerns with the nosology are that some of the diagnostic criteria have not been sufficiently validated, are not applicable in children or necessitate expensive and specialised investigations. These Ghent criteria were more stringent than the Berlin criteria, mitigating over-diagnosis of MFS and providing better guidelines to differentiate MFS from related, ‘overlapping’ conditions such as the MASS phenotype (myopia, mitral valve prolapse, borderline and non-progressive aortic root dilatation, skeletal findings and striae) and MVP syndrome (MVPS) [De Paepe A et al., 1996]. The Ghent nosology employs a set of ‘major’ and ‘minor’ manifestations in numerous tissues including the skeletal, ocular, cardiovascular, and pulmonary systems and the dura, skin and integument. Major manifestations include ectopia lentis, aortic root dilatation/dissection, DE or a

combination of ≥4 out of eight major skeletal features. The diagnosis of MFS in an index patient requires major involvement of at least two organ systems with minor involvement of a third organ system. In the presence of a pathogenetic *FBN1* mutation or a first degree relative who was unequivocally diagnosed based upon Ghent nosology, the presence of one major and one minor manifestation in different organ systems is sufficient to make the diagnosis.

The Ghent nosology clearly attempted to accommodate the fact that some people with ectopia lentis, skeletal findings and even *FBN1* mutation have less cardiovascular risk than seen in classic MFS, by allowing the diagnosis of familial ectopia lentis in the absence of a second major Marfan manifestation. However, inadequate data were available to evaluate the critical issue of whether cardiovascular risk could be predicted by the presence of non-cardiac features, such as DE or major versus minor skeletal involvement. At the other extreme, it is not justified to diagnose MFS in patients with typical lens dislocation and aortic root enlargement simply because they lack minor skeletal or skin findings. To address some of these issues, an international panel of experts in the diagnosis and management of MFS was convened in Brussels, Belgium by the National Marfan Foundation (USA) and charged with considering modifications to the Ghent criteria. Other factors under consideration included the specialised nature, availability and cost of diagnostic tests for selected manifestations (DE), the need to define certain diagnostic categories better (eg, familial ectopia lentis, MASS phenotype and MVPS), to define features that should trigger alternative diagnoses and a desire to complement diagnostic criteria with follow-up, and management guidelines for various patient groups including children who do not yet fulfill the diagnostic criteria but may do so in the future. This proposal for a revised nosology (Table 3) was based on critical review of clinical characteristics in large published patient cohorts, [Loeys B et al., 2004; Faivre L et al., 2008; Faivre L et al., 2009] and expert opinions of the panel members with extensive experience in applying the current criteria, the differential diagnosis of MFS, and the strengths and limitations of molecular genetic testing. Several guiding principles were followed: maximal use of evidence based decision making; attention to practical (patient centric) implications; a focus on features and criteria that distinguish MFS from other disorders; and definition of purposeful thresholds for diagnosis. As a result, five major changes in the diagnostic guidelines are proposed. More weight is given to two cardinal features of MFS, aortic root aneurysm/dissection and ectopia lentis. In the absence of findings that are not expected

in MFS, the combination of ectopia lentis and aortic root enlargement/ dissection should be sufficient to make the diagnosis:

1. a more prominent role is assigned to molecular genetic testing of *FBN1* and other relevant genes (eg, *TGFBR1* and *TGFBR2*), thus not making, in the practice, *FBN1* testing a formal requirement (which represents a financial burden in some countries, and does not yet have 100% sensitivity and specificity), but allows its appropriate use when available;
2. some of the less specific manifestations of MFS were either removed or made less influential for the diagnosis, avoiding the use of obligate thresholds that lacking clear validation or general availability;

Table 3: -Ao, aortic diameter at the sinuses of Valsalva above indicated Z-score or aortic root dissection; EL,ectopia lentis; ELS, ectopia lentis syndrome, without discriminating features of Sphrintzen–Goldberg syndrome (SGS), Loeys–Dietz syndrome (LDS), and the vascular form of Ehlers–Danlos syndrome (vEDS)

In the absence of family history:
(1)Ao (Z ≥2) AND EL=MFS
(2) Ao (Z ≥2) AND FBN1=MFS
*(3) Ao (Z ≥2) AND Syst (≥7pts)=MFS**
(4) EL AND FBN1 with known Ao=MFSEL with or without Syst AND with an FBN1 not known with Ao or no FBN1=ELS
Ao (Z < 2) AND Syst (≥5 with at least one skeletal feature) without EL=MASS MVP AND Ao (Z <2) AND Syst (<5) without EL=MVPS
In the presence of family history:
(5) EL AND FH of MFS (as defined above)=MFS
(6) Syst (≥7 pts) AND FH of MFS (as defined above)=MFS
*(7) Ao (Z≥2 above 20 years old, ≥3 below 20 years) +FH of MFS (as defined above)=MFS**

3. the new criteria formalise that, when a patient has sufficient findings to satisfy the criteria for MFS but also shows unexpected findings, additional diagnostic considerations and testing are required, particularly if these signs segregate with disease in the family or if they are suggestive of a specific alternative diagnosis.

Particular attention is placed on Sphrintzen–Goldberg syndrome (SGS), LDS, and the vascular form of Ehlers–Danlos syndrome (vEDS), which shows overlap, at some extent, with MFS;

4. this current nosology should help to allay concerns regarding delayed or ambiguous diagnoses by providing context specific recommendations for patient counselling and follow-up.

In the revised nosology, new diagnostic criteria have been defined for a sporadic patient and for an index patient with a positive family history. In the absence of a conclusive family history of MFS, the diagnosis can be established in four distinct scenarios:

1. the presence of aortic root dilatation (Z-score ≥2) or dissection and ectopia lentis allows the unequivocal diagnosis of MFS, irrespective of the presence or absence of systemic features except when they are indicative of SGS, LDS or vEDS;
2. the presence of aortic root dilatation (Z-score≥2) or dissection and the identification of a pathogenetic *FBN1* mutation (Table 3) is sufficient to establish the diagnosis even in the absence of ectopia lentis. An overview of criteria that enhance confidence in the pathogenetic potential for MFS of particular *FBN1* mutations is provided in Table 4. These include missense mutations that substitute or create cysteine residues, alter one of the conserved residues for calcium binding in epidermal growth factor-like (EGF) domains, create a premature termination codon (nonsense mutations), delete or insert coding sequence, or disrupt the consensus sequence for pre-mRNA splicing;
3. evidence for pathogenicity of other types of missense mutations would include its absence in at least 400 ethnically matched control chromosomes and cosegregation with disease in the family, or de novo occurrence in a sporadic case (with confirmation of paternity). Definitive evidence of linkage to a predisposing *FBN1* haplotype can substitute for an *FBN1* mutation for diagnostic purposes, but this linkage analysis requires at least six informative meioses in the patient's family to confirm the MFS associated *FBN1* allele. The absence of a mutation in the *FBN1* gene despite complete screening is possible in MFS;
4. in the presence of ectopia lentis but absence of aortic root dilatation/dissection, the identification of an *FBN1* mutation previously associated with aortic disease is required before making the diagnosis of MFS. If the *FBN1* mutation is not

unequivocally associated with cardiovascular disease in either a related or unrelated proband, the patient should be classified as 'ectopia lentis syndrome'.

Table 4 . Diagnostic criteria for the evaluation of causative *FBN1* mutation [Loeys BL et al., 2010]

Mutation previously shown to segregate in Marfan family
De novo (with proven paternity and absence of disease in parents) mutation (one of the five following categories): (1) Nonsense mutation (2) Inframe and out of frame deletion/insertion (3) Splice site mutations affecting canonical splice sequence or shown to alter splicing on mRNA/cDNA level (4) Missense affecting/creating cysteine residues (5) Missense affecting conserved residues of the EGF consensus sequence ((D/N)X(D/N)(E/Q)Xm(D/N)Xn(Y/F) with m and n representing variable number of residues; D aspartic acid, N asparagine, E glutamic acid, Q glutamine, Y tyrosine, F phenylalanine)
Other missense mutations: segregation in family if possible + absence in 400ethnically matched control chromosomes, if no family history absence in 400ethnically matched control chromosomes
Linkage of haplotype for n≥6 meioses to the FBN1 locus

In an individual with a positive family history of MFS (where a family member has been independently diagnosed using the above criteria), the diagnosis can be established in the presence of ectopia lentis, or a systemic score ≥7 points or aortic root dilatation with Z-score≥2 in adults (≥20 years old) or Z-score≥3 in individuals <20 years old. Special consideration should be given to young individuals (<20 years old). In sporadic cases, these children may not fit in one of the four proposed scenarios. If insufficient systemic features (<7) and/or borderline aortic root measurements (Z-score <3) are present (without *FBN1* mutation), the use of the term 'non-specific connective tissue disorder' has been proposed until follow-up echocardiographic evaluation shows aortic root dilation (Z-score≥3). If an *FBN1* mutation is identified in sporadic or familial cases but aortic root measurements are still below Z-score=3, the use of the term 'potential MFS' has been proposed until the aorta reaches threshold. Neonatal MFS is not

considered as a separate category, but rather represents the severe end of the MFS spectrum [Loeys BL et al., 2010].

3.5.1 Organ system specific considerations

*Cardiovascular criteria*_A key diagnostic criterion in the new nosology is aortic root aneurysm or dissection. Aortic root measurements should be done parallel to the plane of the aortic valve and perpendicular to the axis of blood flow. The largest correctly measured root diameter obtained from at least three transthoracic images should be corrected for age and body size and interpreted as a Z-score. There are no special criteria for diagnosing MVP in MFS and standard practices should be applied. Pulmonary artery (PA) dilation is often seen in MFS, but it is not specific to the diagnosis, and complications of pulmonary artery disease occur rarely. MFS patients can develop aortic enlargement or dissection at segments distant from the aortic root, particularly at the proximal descending thoracic aorta and in the abdomen, and the frequency of this finding appears to be increasing with the prolonged survival due to improved management of disease at the aortic root. Descending aortic aneurysm or dissection in the absence of aortic root enlargement can also occur in MFS but this is rare and has a low specificity for MFS, so that this finding is not included in the diagnostic criteria.

*Ocular criteria*_The most prominent ocular features of MFS are myopia and EL. The diagnosis of EL is based on slit-lamp examination after maximal dilatation of the pupil. Dislocation of the lens in MFS is most typically upward and temporal, but deviation in any direction may occur. If lens subluxation is deemed equivocal or minimal, manifesting only as a scalloped or ruffled lens margin at extremes of gaze, the eye exam should be repeated later before a definitive diagnosis of EL can be made. Increased globe length and corneal flattening are seen in MFS, but they have unclear specificity and are not routinely measured by ophthalmologists. Given that myopia is very common in MFS, is routinely monitored, and tends to show early onset, high severity and rapid progression.

*Systemic criteria*_Three points are assigned to the combination of wrist and thumb signs. The thumb sign is positive when the entire distal phalanx of the adducted thumb extends beyond the ulnar border of the palm with or without the assistance of the patient or examiner to achieve maxim. The wrist sign is positive when the tip of the thumb

covers the entire fingernail of the fifth finger when wrapped around the contralateral wrist. If either of the two signs is absent, only one point is assigned.

Two points were assigned to each of five other specific systemic manifestations including anterior chest deformity, hindfoot deformity, spontaneous pneumothorax, DE and acetabular protrusion. Pectus carinatum is believed to be more specific for MFS than pectus excavatum and is assigned two points. Subjective qualifiers in the original Ghent criteria such as 'requiring surgery' have been eliminated, but the examiner should be confident that a positive finding extends beyond normal variation of chest contour in the general population before assigning one point. DE is a sensitive but not a specific sign of MFS and, as such, is no longer considered on equal footing with lens dislocation or aortic root enlargement. An additional technical exam for detection of acetabular protrusion can be helpful but is not mandatory. On an x-ray anterior-posterior pelvis angle, the medial protrusion of the acetabulum at least 3 mm beyond the ilio-ischial (Kohler) line is diagnostic. One point is assigned to eight other manifestations, one cardiovascular (MVP), one ocular (myopia, ≥3 diopters) and six features from other organ systems. These are considered less specific features for MFS and can be observed in other connective tissue disorders or as normal variation in the general population. The combined presence of reduced upper segment to lower segment (US/LS) ratio (for white adults <0.85; <0.78 in black adults; no data have been assessed in Asians) and increased arm span to height ratio (for adults >1.05) in the absence of significant scoliosis contributes one point to the systemic score. Scoliosis can be diagnosed either clinically if, upon bending forward, a vertical difference of least 1.5 cm between the ribs of the left and right hemithorax is observed or if a Cobb's angle (angle between a line drawn along the superior end plate of the superior end vertebra and a second line drawn along the inferior end plate of the inferior end vertebra of the scoliosis measured on anterior-posterior view of the spine) of at least 20° is seen on radiographs. In the absence of scoliosis, one point can be contributed by the presence of an exaggerated thoracolumbar kyphosis. Elbow extension is considered reduced if the angle between the upper and lower arm measures 170° or less upon full extension.

One point can be assigned based upon facial characteristics if the patient shows at least three of the five typical facial characteristics including dolichocephaly, downward slanting palpebral fissures, enophthalmos, retrognathia and malar hypoplasia. Striae atrophicae are considered significant as a diagnostic feature if they are not associated with pronounced weight changes (or pregnancy) and if they have an uncommon location such as the mid back, lumbar region, the upper arm, axillary region

or thigh. The following criteria were removed from the current nosology because of lack of perceived specificity: joint hypermobility, highly arched palate, and recurrent or incisional herniae (Table 5).

Table 5: Manifestations and signs included in systemic score and systemic score calculation: maximum score = 20 points; score ≥ 7: systemic involvement [Loeys BL et al., 2010]

Clinical features	***Score***
Wrist *and* thumb signs	**3** (wrist or thumb sign: **1**)
Pectus carinatum deformity	**2** (pectus excavatum or chest asymmetry: **1**)
Hindfoot deformity	**2** (plain pes planus: **1**)
Pneumothorax	**2**
Dural ectasia	**2**
Protrusio acetabuli	**2**
Reduced US/LS *and* increased arm/height *and* no severe scoliosis	**1**
Scoliosis or thoracolumbar kyphosis Kyphosis	**1**
Reduced elbow extention	**1**
Facial features (3/5)	**1** (dolichocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, and retrognathia)
Skin striae	**1**
Myopia, ≥3 diopters	**1**
Mitral valve prolapse (all types)	**1**

3.5.2 Differential diagnosis and correlated fibrillinopathies

There are several connective tissue diseases sharing some of the features and clinical manifestations with MFS which have to be considered in the differential diagnosis (Table 6). Some of them are generically classified as fibrillinopathies for they are hereditary and associated with mutations in the fibrillins genes. The marked overlap of their phenotypes and the progressive nature of many of their manifestations make differential diagnosis a challenge, and periodic re-evaluation is often necessary [Cañadas V et al., 2010]. Some of the most complex diseases showing notable analogy to MFS include the MASS phenotype and LDS. The acronym 'MASS' refers to a subgroup of patients with present enough signs to consider that a systemic connective tissue disorder may be present, but that couldn't be diagnosed of any of the known syndromes. MASS (OMIM#157700) phenotype is characterized by the following

manifestations: myopia, mitral valve prolapse, mild aortic dilation, skin and skeletal abnormalities [De Paepe A et al., 1996]. Although it involves multiple systems, the cutaneous and skeletal manifestations are nonspecific and aortic dilation is always mild and non progressive. Diagnosis always requires at least two systems to be affected. The MASS phenotype has been associated with mutations in the *FBN1* gene; MASS represents, therefore, a fibrillinopathy.

*LDS*_Unlike MFS, in which arteriopathy seems to be confined to the ascending aorta, tortuosity and dilatation of abdominal aorta, pelvic vessels, and intracranial vessels can develop in patients with LDS. Aortic dilatation and dissection occur at an earlier age and at smaller aortic diameters than in patients affected of MFS. Finally, it is important to point out that aortic aneurysms and dissection can occur in multiple members of an affected family that has no other syndromic manifestations. As with *FBN1* mutations, the phenotype associated with *TGFBR1/2* mutations can be variable, even within families, and can be associated with skeletal features of MFS leading to overlapping phenotypes in the old Ghent nosology. In order to avoid persistent ambiguity even under the proposed criteria, molecular testing should be strongly considered because it influences the clinical management. It has been proposed that patients with *TGFBR1/2* mutations who lack outward discriminating features of LDS should be designated LDS2, highlighting the potential for more aggressive vascular disease than seen in MFS (OMIM#190181 and 190182) (See Chapter 1) [Loeys BL et al., 2010].

*BAV*_ A subset of individuals with BAV present with ascending aortic aneurysm; however, such patients usually lack ocular or other systemic findings that strongly contribute to MFS diagnosis. Skeletal findings such as pectus deformity and scoliosis can be observed in these families. BAV and aortic aneurysm can occur together in some family members but independently in others, indicating that they can be variably penetrant consequences of a common underlying genetic defect. Unlike MFS, this condition commonly shows maximal or exclusive dilatation in the ascending aorta above the sinotubular junction. Mutations in the *NOTCH1* (Notch homolog 1, translocation-associated, OMIM#190198) and *KCNJ2* (potassium channel inwardly rectifying sub family J member 2, OMIM#600681) genes have been identified , but these account for only a small fraction of BAV patients, who may have prominent valve calcification or associated forms of congenital heart disease. Linkage analysis

reveals genetic heterogeneity with putative loci on chromosomes 18q, 5q and 13q (See Chapter 4) [Martin LJ et al., 2007].

*FTAAD*_The acronym refers to a clinically and genetically heterogeneous group of disorders where thoracic aortic disease predominates. The age of onset and rate of progression of aortic dilatation is highly variable and conditions that include variable or subtle systemic manifestations of a connective tissue disorder have been included in this designation. Mutations have been identified in *FBN1*, *TGFBR1/2*, *MYH11* (myosin heavy chain 11; OMIM#160745), and *ACTA2* (actin alpha 2 aorta OMIM#102620), the latter two encoding components in the smooth muscle cell contractile apparatus (See Chapter 2).

*vEDS*_The main feature is represented by vascular and tissue fragility. Other cardinal features distinguishing vEDS from MFS include translucent skin, easy bruising, dystrophic scarring and a tendency for intestinal or uterine rupture. Three other rare types of EDS have been associated with vascular problems. The kyphoscoliotic type (previously type VI EDS) is characterised by kyphoscoliosis, joint laxity, and muscle hypotonia (See Chapter 2).

*ATS*_is a rare autosomal recessive connective tissue disorder, characterised by severe tortuosity, stenosis, and aneurysms of the aorta and medium sized arteries [Wessels MW et al., 2004]. Skeletal and skin involvement is common. The underlying genetic defect is homozygosity or compound heterozygosity for loss-of-function mutations in *SLC2A10*, the gene encoding the facilitative glucose transporter GLUT10 [Coucke PJ et al., 2006]. The condition is lethal in infancy in a subset of patients, but some survive into adulthood and seem to do well [Callewaert BL et al., 2008] (See Chapter 2).

*ELS*_this condition presents an autosomal dominant pattern of inheritance and associates ectopia lentis as well as variable skeletal manifestations that are reminiscent of the MFS. Heterozygous pathogenic variants in *FBN1* are often causative of the disease. It remains unclear whether some individuals in affected families are destined to show later onset of progressive aortic enlargement. A regimen of intermittent cardiovascular imaging should be maintained. This phenotypic designation is appropriate if the aortic root Z-score is less than 2.0 and the patient does not have an *FBN1* pathogenic variant previously associated with aortic enlargement irrespective of

the systemic score. This diagnosis can only be established for individuals age 20 years or older [Loeys BL et al., 2010]. Autosomal recessive inheritance of isolated ectopia lentis (ectopia lentis et pupillae OMIM#225200) can be caused by pathogenic variants in *ADAMTSL4* (ADAMTS-like 4; OMIM#610113) and is not associated with other manifestations of MFS [Ahram D et al., 2009].

*Weill-Marchesani syndrome*_(WMS; OMIM#277600) The lens dislocation is typically associated with microspherophakia (small, rounded and thickened crystalline lens) and a shallow anterior eye chamber. WMS patients are short with brachydactyly and joint stiffness. Both autosomal dominant and recessive forms of WMS have been described and are caused by *FBN1* mutations or mutations in the *ADAMTS10* (ADAMTS-like 10; OMIM#608990) gene, respectively [Faivre L et al., 2003; Dagoneau N et al., 2004].

*Homocystinuria*_(OMIM#236270) is often easily differentiated from MFS by the presence of mental retardation and thrombosis, and can be excluded by urine amino acid analysis in the absence of pyridoxine supplementation. In homocystinuria, the lens usually dislocates downward due to complete loss of support by ciliary zonules [Judge DP and Dietz HC, 2005].

*Stickler syndrome*_(OMIM#108300) Typical ocular signs include vitreal degeneration, retinal detachment, myopia and open angle glaucoma. Early cataracts are common, but lens subluxation is not. Other potential discriminating features from MFS include cleft palate, hearing loss and epiphysial changes of the bones [Loeys BL et al., 2010].

*Shprintzen–Goldberg syndrome*_(SGS; OMIM #182212) is a rare craniosynostosis syndrome characterised by some of the systemic features found in MFS (pectus abnormalities, scoliosis, arachnodactyly), craniofacial dysmorphism (exophthalmos, hypertelorism, downslanting palpebral fissures, maxillary and mandibular hypoplasia, high arched palate and low set ears) and developmental delay. So far, only two SGS patients have shown an *FBN1* mutation. Another patient reported as SGS was felt to have LDS based on arterial tortuosity and the presence of a bifid uvula [Sood S et al., 1996; Kosaki K et al., 2006]. Other important distinguishing features between SGS and either LDS or MFS are the high incidence of cognitive impairment and the low frequency of vascular disease in the former.

*CCA*_is an autosomal dominant disorder characterised by a Marfan-like body habitus and arachnodactyly. Most affected individuals have ‘crumpled’ ears that present as a folded upper helix, and contractures of major joints (knees and ankles) at birth. The proximal interphalangeal joints of the fingers and toes have flexion contractures (camptodactyly). Kyphosis/scoliosis is present in about half of affected individuals. Mild enlargement of the sinuses of Valsalva has been reported, but there is no evidence that the aortic dilatation progresses to dissection or rupture. CCA is caused by mutations in *FBN2* (OMIM#612570), the gene encoding the ECM protein fibrillin-2 [Godfrey M, 2001].

Table 6. Marfan syndrome differential diagnosis [Yuan SM and Jingl H, 2010]

Differential disorder	*Phenotype expression*	*Sharing presentation*	*Differential presentation*	*Differential test*	*Etiology*
Familial thoracic aortic aneurysm and dissection syndrome (FTAAD)	Aortic root and aneurysm and dissection	Aneurysm of the thoracic aorta	No eye or musculoskeletal findings	No differentiating tests	Breakdown of the extracellular matrix proteins elastin and collagene by proteases such as collagenase,elastae, various metalloproteinases and plasmin
Bicuspid aortic valve (BAV)	Maximum dilatation often occurs further up in the ascneding aorta, beyond the sinotubular junction	Occasionally occurs with Marfan syndrome	No eye or musculoskeletal findingsa	Echo,thorax computed tomography or MRI can reveal signs of bicuspid aortic valve	Inadequate production of fibrillin-1
Ehlers-Danlos Syndrome (EDS)	An inherited heterogeneous group of connective tissue desorders,characterized by abnormal collagene synthesis, affecting skin, ligaments, joints,blood vesselsand other organs. Marked joint, hypermobility, papyraceous scars and mitral valve prolapse	Aortic aneurysm and dissection at any age	Type IV variety most commonly affects the aorta, characterized by thin skin and bleeding disorders with increased bruising	Skin biopsy for abnormal collagen and DNA testing for gene mutation	EDS IV (mutations in *COL3A1*); EDS VI (homozygous and compuond heterozygous mutations in the lysyl hydroxylase gene); EDS VIIA and VIIB (*COL1A1*mutation) EDS VIIC (deficiency of pro collagene-N-proteinase); EDS IX (decreased lysys oxidase activity)
Homocysti-nuria	Marfanoid habitus, ectopia lentis, mental ritardation and osteoporosis	Ectopia lentis	Deficit of cistathionine-β-synthase; tall stature, long-bone overgrowth and ectopia lentis but typically without aortic enlargement or dissection	Raised concentration of plasma homocysteine	Mutations in the beta receptor genes (*CBS*,*MTHFR*, *MTR* e *MTRR)*
MASS phenotype	Mitral valve prolapse, mild aortic dilatation, striae atrophicae and skeletal involvement	Mitral valve prolapse, aortic root dilatation and skin and skeletal conditions	Long limbs, deformity of the thoracic cage, striae atrophicae, mitral valve prolapse and mild and nonprogressive dilation of the aortic root	Careful followup is needed to distinguish tha MASS phenotype from emerging marfan syndrome, especially in children	*FBN1* mutation

Table 6. Marfan syndrome differential diagnosis [Yuan SM, Jingl H, 2010]

Differential disorder	***Phenotype expression***	***Sharing presentation***	***Differential presentation***	***Differential test***	***Etiology***
Congenital contractural arachnoda-ctyly (CCA)	Multiple flexion contractures, arachnodactyly, severe kyphoscoliosis, abnormal pinnae and muscular hypoplasia	Skeletal features with Marfan syndrome such as marfanoid habitus, arachnodactyly, camptodactyly and kyphoscoliosis	Multiple joint contractures and crumpled ears in the absence of significant aortic root dilation are characteristic and rarly found in Marfan syndrome	Crumpled appearance of ear helix and congenital contractures, typically without ocular and CV complications	*FBN1* mutation
Isolated ectopia lentis	A rare syndrome characterized by dislocation of eye tenses, which often occurs at birth	Ectopia maybe associated with mild skeletal findings	Mental retardation, impaired joint mobility and stiff joints, but without cardiovascular abnormalities	No	*FBN1* mutation
Stickler syndrome	A relatively common genetic disorder characterized by very flexible joints, typical facial characteristics, hearing loss and associated eye problems	Tall stature, mitral valve prolapse and retinal detachment	Retrognathia, midfacial hypoplasia, without aortic involvement	No	*COL2A1* mutation
Loeys-Dietz Syndrome (LDS)	Genetic disorder that effects blood vessels in the body, especially the aorta. It was first described in the medical literature in January 2005	Aortic dilation, aneurism and dissection, and mitral valve prolapse	No associated lens dislocation. Aortic dissection occurs at much smaller diameter. Bifid uvula or cleft palate, arterial tortuosity and hypertelorism	Not currently available	Mutations in the genes encoding transforming growth factor beta receptor 1 (*TGFBR1*) or 2 (*TGFBR2*)
Shprintzen-Goldberg Syndrome (SGS)	Craniosynostosis (involving the coronal, sagittal or lambdoid surure), distinctive craniofacial features, skeletal abnormalities, neurological abnormalities, mild- to moderate mental retardation and brain abnormalities	Mitral valve prolapse, mitral regurgitation and aortic regurgitation	Aortic root dilatation is most likely not found	Not currently available	Uncertain, possibly *FBN1* mutations

3.6 MOLECULAR GENETICS OF MARFAN SYNDROME

MFS is a systemic disorder of connective tissue with autosomal dominant inheritance. Approximately 75% of individuals with MFS have an affected parent while 25% carries a *de novo* mutation. The identification of a *FBN1* gene mutation, encoding fibrillin-1, a protein component of microfibrils, accounts for more than 90% of MFS. The penetrance of the fibrillin mutation is high and the phenotypic expression (degree of affectation) is variable. The first *FBN1* mutation was found in MFS patient in 1991, after that and it was confirmed that mutations in the fibrillin gene locus on chromosome 15 were responsible for MFS. Since then, 1847 *FBN1* mutations have been identified in affected patients [www.umd.be/*FBN1*, last update 28/6/2014; Collod-Beroud G et al., 2003]. Several lines of evidence have also shown that many *FBN1* mutant alleles cause MFS phenotypes through a dominant-negative effect [Faivre L et al., 2007] though there is a considerable number of patients with mutations resulting in haplo-insufficiency owing to gene deletion, splicing mutations or nonsense mutations causing nonsense-mediated mRNA decay. Therefore, decreased protein synthesis as well as the dominant-negative effect induced by the mutant protein is thought to be a pathogenic mechanism for MFS [Judge D et al., 2004].

3.6.1 *FBN1* gene structure

The sequence of Fibrillin-1 c-DNA (complementary DNA) is nowadays completely known as well as the nucleotide sequences of the junction of each exon-intron. The protein is encoded by 65 exons of *FBN1* gene, which is located on human chromosome 15q21. The size of the gene had been estimated at 110 kb. There are sequences of 134 base pairs at the 5’ and 916 of the 3' end that are transcribed but not translated. The gene covers more than 237,000 base pairs and the longer transcript consists of 11695 pairs of bases. The larger dimensions intron is placed between exon 6 and exon 7, starting with the numbering from the exon containing the ATG start codon, and has a 58474 base pairs length. With few exceptions, each exon encodes a single domain protein: 50 of the 57 cysteine-rich domains are encoded by individual exons. The exception are mainly the D region where exons 9/10, 16/17, 37/38, 41/42 and 50/51 encode each for a domain 8-cys while exons 20-21 encodes for the hybrid #2 domain and exon 63 encodes only for two domains cbEGF-like. The amino acid residues of transition between each region are encoded by exons 2, 10, 11 and 64. All the exons encoding the regions B, C and D of the fibrillin-1 begin and end respectively in 5 'and 3'. Can be noticed the splice site in

the 5' intron 62 where a GC is found instead of a GT dinucleotide, mandatory in the donor joint [Biery NJ et al., 1999].

3.6.2 Fibrillin-1 protein: domain structure and supramolecular assembly

*FBN1*gene encodes fibrillin-1, an ECM protein that is defective in MFS. Fibrillin-1 is a 350-kDa ECM protein, the principal constituent of 10-nm microfibrils [Sakai et al., 1986]. ECM microfibrils have a wide array of functions including the maintenance of elastic fibers and anchoring epithelial cells to the interstitial matrix. Fibrillin-1 has a highly modular organisation (which is typical of many extracellular proteins), with the striking repetition of two disulphide-rich modules, the epidermal growth factor-like (EGF) domain and the transforming growth factor β binding protein-like (TB or eightcysteine) domain. The protein is made up of 47 EGF domains the, 43 of which are of the Ca2+ binding type ("cbEGF" domains). Each of these is characterised by six cysteine residues which normally disulphide bond in a 1-3, 2-4, 5-6 arrangement and a calcium binding consensus D/N-x-D/N-E/Q-xm-D/N*-xn-Y/F (where *m* and *n*are variable and indicates possible post-translational β-hydroxylation). The calcium binding site has a pentagonal bipyramidal geometry with six out of seven ligands provided by intra-domain oxygen atoms including both side chain oxygen atoms and backbone carbonyl groups.

Two of the cbEGF domain, part of the calcium binding consensus do not ligand directly to calcium, as they are involved in stabilising the structure of the calcium binding site itself. The identity of the seventh ligand is unknown and may be a water molecule or an inter-domain or intermolecular protein ligand. Fibrillin-1 cbEGF domains occur as multiple copies and, in most cases, each set of cbEGF repeats is separated from the next by a TB domain. The region around domain hyb2 is of particular interest because it contains all three of these interface types and is also at the beginning of the "neonatal" region of fibrillin affected by mutations resulting in the neonatal MFS phenotype.

Based on domain structure and interface data, a model of the fibrillin-1 fragment spanning the region from cbEGF7-cbEGF13 was produced. The results suggest a linear organization of the region, with as light twist in the region of hyb2 and TB3 in which these two domains appear inverted with respect to each other. Several experiments have shown that the removal of calcium from the tandem repeated cbEGF domains determines a loss of the extended conformation of the protein and the rigidity of each cbEGF-like interdomain. Furthermore, the presence of EDTA reduces the distances

between regions interbead, appearing disorganized, and therefore a reduction of the periodicity.

Morphological changes were observed on microfibrils, resulting markedly curved and losing their linearity. The ion also exerts an important role in conferring the protein a suitable conformation for its recognition by other components of the ECM [El-Hallous Eet al., 2007]. Calcium is finally required for heterotypic and homotypic interaction between the fibrillin-1 and fibrillin-2 [Marson A et al., 2005].

*8cys/TB domains*_in fibrillins they are composed of 69-78 residues, eight of which are cysteines. The four disulfide bonds paired in a 1-3, 2-6, 4-7, 5-8 arrangement. The C-terminus of the domain was found to be flexible which may be of functional significance. These globular domains often appear to break up linear regions within fibrillin-1 molecules. Linker regions between 8cys/TB modules and adjacent domains in fibrillin-1 consist of 6-8 residues at the N-terminal side and 11-19 residues on the C-terminal side of the8cys/TB domain [Lee SS et al., 2004]. If these linker regions are flexible, the kinks and bends observed in fibrillin-1 molecules may be required for proper alignment of molecules within the assembled microfibril. Some evidence suggests that these domains mediate specific protein-protein interactions. 8cys/TB4 in both fibrillin-1 and 2 contains an αvβ3 integrin-binding RGD sequence. Homology modeling localized this sequence to the tip of a hairpin loop thus suggesting it would be accessible fo rintegrin binding. A homologous domain from LTBP-1 has been shown to covalently associate with TGF-β1-LAP via disulfide exchange during secretion, presumably via one of the surface located disulfide bonds. An insertion of two amino acid residues FP in the LTBP-1 sequence is adjacent to Cys 1062, which is predicted to form a solvent exposed disulfide. This hydrophobic dipeptide, together with additional surface residues, may contribute to specific interactions with TGF-β1-LAP which facilitate disulfide exchange [Lack J et al., 2003].

*Hybrid domains*_two of them are present in fibrillins, and in LTBPs, which have a single hybrid domain. These modules appear to combine features of an 8cys/TB module and an EGF module, however their evolutionary derivation is unknown. Little is known about the structure and function of these domains. They contain eight conserved cysteine residues in an unknown pattern of disulfides. The hybrid domains are embedded between the other major types of domains, and are located within the N-terminal portions of the fibrillins or the LTBPs. The first hybrid domain in both

fibrillin-1 and fibrillin-2 contains an additional cysteine residue that is not present in the second hybrid domains of the fibrillins or in the hybrid domains of the LTBPs. Therefore, at least one of the nine cysteine residues in the first hybrid domain must be unpaired. A cysteine residue at position 204 in fibrillin-1 was identified as a free thiol by sequential alkylation and subsequent sequencing of a small recombinant subdomain expressed in mammalian cells. It was also shown that this cysteine is located at or near the surface of the domain and is thus available for intermolecular disulfide bonding [Reinhardt DP et al., 1996].

*The N- and C-terminal domains*_they display two prominent features: the presence of an even number of cysteine residues, four in the N-terminal and two in the C-terminal domains; the presence of a basic sequence of the pattern BXBB (B=basicamino acid residue, K or R) in each domain. The 4-cysteine domain in the N-terminalof fibrillins is homologous to similar 4-cysteine domains in the N-terminal extended forms of the LTBPs. The C-terminal domains of the fibrillins are homologous to the C-terminal domains of all four members of the fibulin family, and thus a new type of extracellular module of approximately 120 amino acid residues in length has been proposed. This type of homology is not shared by the LTBPs. The cysteine residues in the N- and C-terminal domains are candidate residues for intermolecular disulphide bonding. However, recently it was demonstrated that all four cysteine residues within the N-terminal domain are involved in intra-molecular disulfide bonds in a recombinant polypeptide of fibrillin-1, whereas no information is yet available about the form of the two cysteine residues within the C-terminal domain [Reinhardt DP et al., 1996]. The cleavage site for processing at the C-terminal end has been directly identified by sequencing of the C-terminal propeptide that was released from a larger recombinant polypeptide during or after secretion into the culture medium. This propeptide started at position S2732 directly C-terminal to the R2728KRR sequence (Figure 19).

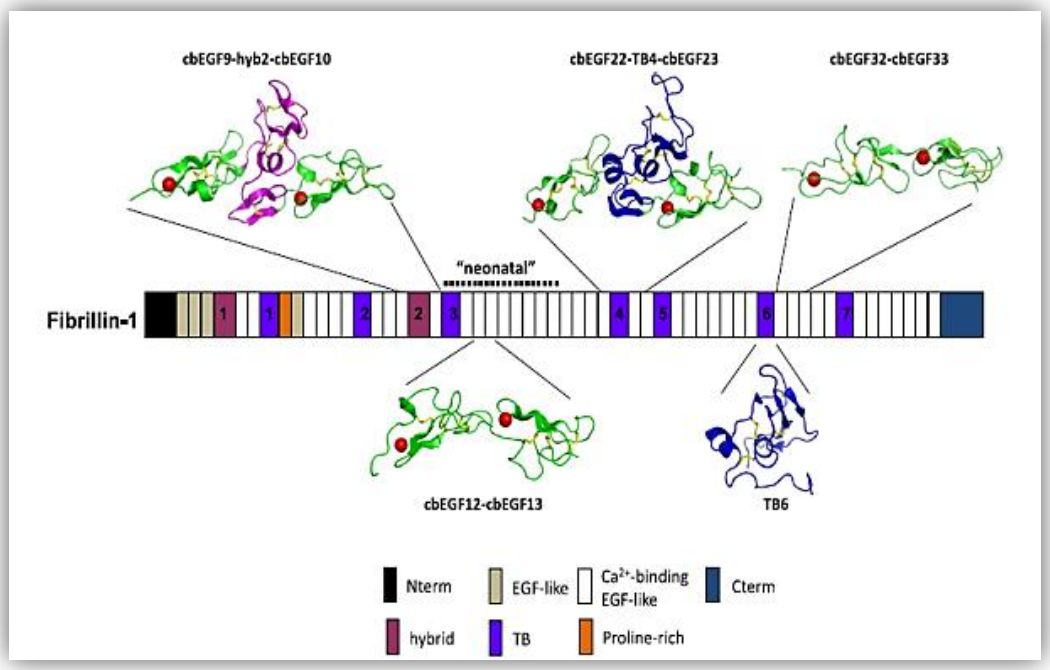


Fig 19. Primary structure of fibrillin-1 (from Jensen SA et al., 2012)

Fibrillin-1 is synthesized from a precursor, the profibrillin-1, which contains 2871amino acids and can be divided into a region that constitutes the signal peptide for the extracellular secretion and among this 5 distinct structural domains named A, B, C, D and E. The first exon contains the ATG translation start site and encodes a signal peptide followed by a consensus sequence for the processing of the propeptide. In vitro studies have suggested that the protein receaves at N-terminal, followed by a series of twelve basic amino acids within domain A. Domain B, which corresponds to the region covered by exons 2 to 10, begins with 5 regions rich in cysteine and contains three regions not EGF-like calcium binding, a structural motif containing 9 cystein eresidues (domain hybrid motif # 1), a domain TB and two cbEGF-like regions. The C domain, which corresponds to exon 10, is constituted by 58 amino acids and is characterized by a high content of proline residues (42% of the total) that could allow this region to fold into a three dimensional space serving as a molecular hinge. The domain D is larger in size and includes 2240 amino acids grouped into 49 repeating patterns rich in cysteine over a EGF-like non-calcium-binding region, 41-like motifs cbEGF of which 12 arranged in the center of the region consecutively to one another 6reasons why TB and a "hybrid motif # 2" (exon 11-63). The domain and the C-terminal, consisting of 148 amino acids, covers exons 64-65 and is characterized by the presence of two consecutive cysteine residues within a sequence that is highly conserved both in the fibrillin-1 and fibrillin-2 in, which could confer to this particular region a functional

significance. In addition to this, this domain contains a stretch ofbasic amino acids that constitute a site of proteolytic cleavage extracellularly by the enzyme PACE (Paired basic Amino acid Cleaving Enzyme) [Handford PA,2000]

Fibrillin-1 contains one Arg-Gly-Asp (RGD) site. Common to all three mammalian fibrillins is a RGD site present in the TB4 domain at the tip of an extensible loop. This site mediates the interactions with αvβ3, α5β1 and αvβ6 integrins. Human fibrillin-2 and fibrillin-3 contain a second RGD site in TB3 and cbEGF18, respectively. It is not known whether these RGD sites represent functional integrin-binding epitopes.

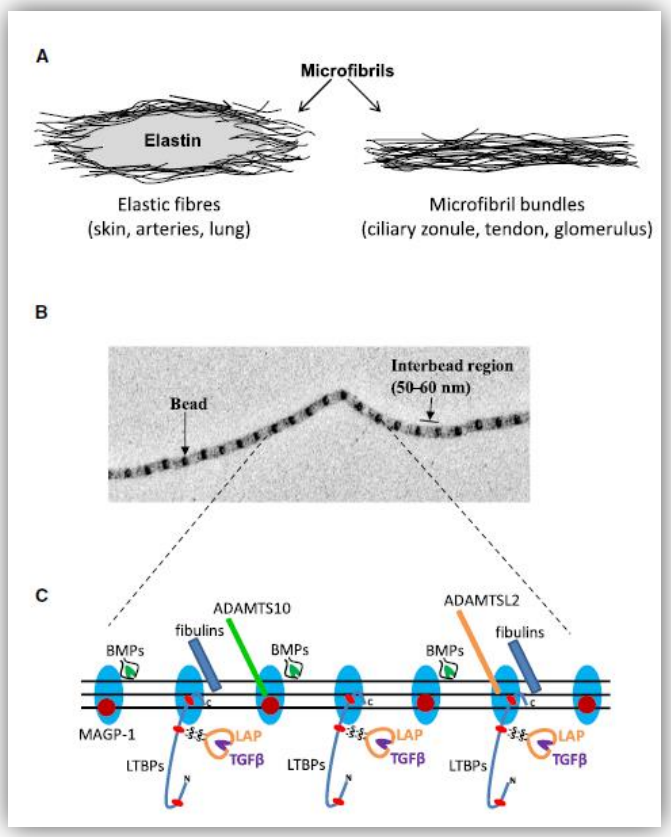


Fig 20. Microfibril Structure and Interactions (A) Fibrillin microfibrils are associated with elastin in tissues such as the skin, arteries and lung. (B) When viewed by rotary-shadowing electron microscopy, isolated microfibrils appear as 10 to 12 nm diameter beaded filaments with an average periodicity of 50–60 nm. (C) Microfibrils are known to be binding sites for several ECM proteins such as fibulins, microfibril-associated glycoproteins [MAGPs (red circles)], and members of the ADAMTS family, and function as sequestration sites for growth factors such as TGFb and BMPs in connective tissues. Inactive TGFb, bound to its LAP, binds to microfibrils through the LTBPs. The interaction involves disulphide bond formation between the LAP and the second TB domain (red ovals on LTBP) of LTBPs 1, 3, and 4 (from Jensen SA et al., 2012)

Another differential feature between the three fibrillins is the number and position of predicted N-linked glycosylation sites which are 15 in fibrillin-1. It has been shown for fibrillin-1 that only the processed form becomes incorporated into the ECM, suggesting that profibrillin-1 processing within its terminal domains plays a regulatory role for its assembly into microfibrils. Both the N- and the C-terminal domains are proteolytically processed at these sites. It has been suggested the following new model for fibrillin-1 assembly. The C terminus of fibrillin-1 multimerizes through a disulphide bond-mediated mechanism into bead-like structures. This multimerization enhances the avidity of the N-to-C self-interaction site contained within cbEGF41–43 for binding to the N-terminal region of fibrillin-1. When the avidity of a nascent bead has sufficiently increased, the peripheral N-terminal sites begin to bind to the C-terminal sites in appropriate adjacent beads. Such a universal mechanism is compatible with all parallel

head-to-tail models of fibrillin-1 organization in microfibrils, independent of whether the molecules were staggered or unstaggered or whether the interbead arms were spanning one, two, or even three bead-to-bead distances. Parallel staggered or unstaggered fibrillin-1 organization requires unidirectionality of the peripheral arms. This feature could be conferred to a nascent bead by increasing avidities of the next appropriate downstream bead causing the N termini to bind. Alternatively, microfibril-associated proteins such as MAGP-1 could potentially act to confer unidirectionality to the fibrillin molecules (Figure 19) [Hubmacher D et al., 2008].

Fibrillin microfibrils have essential roles in elastic fiber formation and elastic tissue homeostasis. A role for fibrillin microfibrils in tissue elasticity has been implied by their ability to increase periodicity from 56 to 150 nm. The length of a single fibrillin molecule has been reported to be between 143 nm, 148 nm, and 160 nm, and has been suggested to contain specific sites of variable flexibility. It is still poorly understood how fibrillin monomers are assembled and arranged within microfibrils. Several models were proposed to explain the molecular organization of fibrillin monomers. A‘folding/pleating model’ proposes a head-to-toe parallel alignment of fibrillin monomers where molecules are highly folded and form characteristic beads (Figure 21) [Wang MC et al., 2009]. A combined study presented showed a detailed explanation of the ‘folded model’ where it was proposed that a complex molecular folding of 160-nmfibrillin molecules allows reversible extension. This packing arrangement could explain the appearance of different periodicities observed in extended fibrils and is believed to be the basis of the elasticity of fibrillin-rich microfibrils. The molecular folded model was also supported by a detailed analysis of theoretical axial mass distribution, where profiles calculated for the folded alignment fitted the observed microfibril mass maps. Another model suggested a staggered arrangement of individual molecules. In this arrangement, it is possible that unfolded fibrillin monomers span over two or three interbead regions. [Kuo CL et al., 2007]. Lee et al. proposed a mechanism in which the interdomain linker regions between TB and cbEGF domains act like a

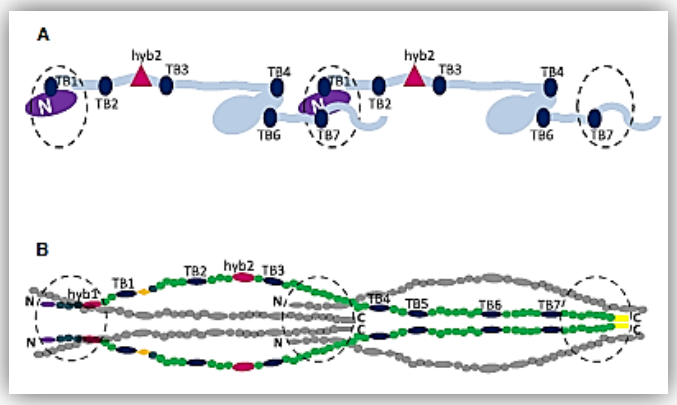


Fig 21. (A) The “pleated” model for fibrillin 1 protein structure involves folding back of the monomer, especially in the region around domains TB4 and TB5. (B) The extended model based on observations made by electron microscopy allows the identification of collagenase cleavage sites (from Jensen SA, 2012)

molecular spring that unfolds in response to application of a stretching force [Lee SS et al., 2004]. The high calcium affinities of most fibrillin TB, cbEGF, and hyb-cbEGF domain interfaces suggest an obvious recoil mechanism.

Under physiological conditions, these domain interfaces would be in a fully calcium bond form. Under extension, unfolding of the domain interface would occur, resulting in a reduction of calcium affinity and loss of the bound calcium ion. A return to the relaxed state would then be driven by the calcium-dependent stabilization and refolding of domain interfaces [Jensen SA et al., 2009].

The scaffold of the vibrant extracellular space is made primarily of fibronectin, collagenous and elastic fibers, and microfibrils, which provide tissues with resilience and elasticity and also serve as docking platforms for soluble factors involved in intercellular communication. Latent transforming growth factor beta (TGF-β) binding proteins (LTBPs), components of microfibrils, represent a family of matrix proteins that exert important and complex functions in the extracellular space. Recent studies in genetically targeted mice have however revealed that fibrillin-1 and fibrillin-2mutations perturb signaling events mediated by TGF-β superfamily members. As such, these studies have established a new biological paradigm whereby fibrillin-rich microfibrils are structural networks that specify the local concentration and timely release of signaling molecules during morphogenesis and tissue remodeling [Jensen SA et al., 2009].

3.6.3 The Universal Mutation Database (UMD)

Primary mutations in the *FBN1* gene have been associated with a wide range of phenotypes that show considerable variation in the timing of onset, tissue distribution, and severity. The spectrum of overlapping disorders presenting with *FBN1* mutations define the molecular group of ‘‘type 1 fibrillinopathies’’ [Collod-Beroud G and Boileau C, 2002; Robinson PN et al., 2002]. In 1994, it has been devised a generic software package called UMD (Universal Mutation Database). This software includes an optimized structure to assist and secure data entry and to allow the input of various clinical data.

In an effort to standardize the information regarding *FBN1* mutations, UMD software has been optimized in 1995 to create a computerized database; the database currently contains information about the published mutations of the *FBN1* gene. Some information has been exclusively reported in meetings, other information has been contributed by the co-authors of published papers [Collod-Béroud G et al., 1997 and

1998]. The mutation records of the database provide point mutations, large and small deletions, insertions, and splice mutations in the *FBN1* gene. The database cannot accommodate complex mutations (Figure 22).

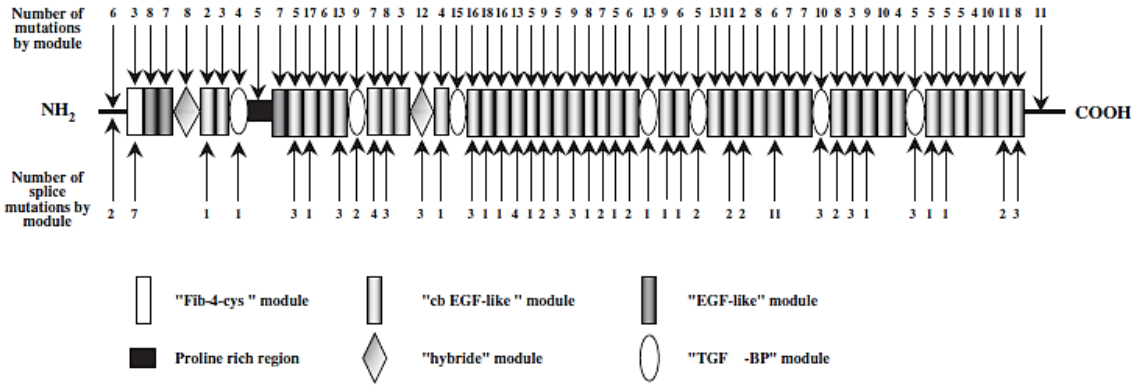


Fig 22. Distribution of *FBN1*mutations per module. The number of splice mutations (bottom) and other mutations (top) are listed (from Collod-Béroud G et al., 2003).

As in previous editions, mutation names are numbered with respect to the *FBN1* gene cDNA sequence obtained from GenBank (accession number L13923; complete coding sequence of HUM-FIBRILLIN Homo sapiens fibrillin mRNA). Intron-exon boundaries are as defined by Pereira et al. [1993] and module organization is from SWISS-PROT (accession number P35555). The database presents the molecular and clinical data on the causative mutations of MFS and type 1 fibrillinopathies in a standardized, easily accessible, and summarized form. For each mutation, information is provided at several levels: at the gene level (exon and codon number, wild-type and mutant codon, mutational event, mutation name); at the protein level (wild-type and mutant amino acid, affected domain); and at the clinical level (absence or presence of skeletal, ocular, cardiovascular, central nervous system, and other various manifestations). With the increased amount of data, the percentage of recurrence has become more evident and now represents 12% (56 recurrent mutations representing 156 events). When information about transmission type (available for 398 mutations) is examined, there are a surprising number of de novo mutations as compared to transmitted mutations (188 de novo versus 210 familial). One can ask if this extreme representation can really be explained by a selection bias: each family accounting for one. De novo mutations may represent more than the usually suspected 25%. Exonic mutations (492 mutations) are present in all exons, however they are significantly underrepresented in exon 45 (cbEGF-like#27) and exon 57 (8 cystein#7) and overrepresented in exon 13 (encoding

cbEGF-like#4), exon 26 (cbEGF-like#12), exon27 (cbEGF-like#13), exon 28 (cbEGF-like#14), and exon 43 (cbEGF-like#25). Overrepresentation in exons 26 to 28 can be explained by the fact that almost allt he mutations identified in neonatal cases of MFS1 are located within this area (exons 24-32). Furthermore, mutations in this region are more likely to be associated with a severe clinical phenotype [Tiecke F et al., 2001], which probably led to a bias in patien tselection for mutation detection.

*Deletions*_when they involve contiguous EGF-like domains, their effects vary depending on their location within the fibrillin-1 molecule. Deletion of the three contiguous cbEGF-like domains encoded by exons 44-46 resulted in a severe phenotype with onset in infancy and a rapidly progressing clinical course. Inframe deletion of exons 42-43 was characterized in a patient presenting with bilateral ectopia lentis and Marfanoid skeletal features [Liu W et al., 2001], and finally, deletion of the cbEGF-like domains encoded by exons 60-62 in the C-terminal domain of fibrillin-1 resulted in a much less severe phenotype characterized by a moderate Marfanoid habitus [Kainulainen K et al.,1992].

*Small insertion/deletion mutations*_they account for 12.9% of the total mutations create a PTC. These act as dominant negatives, but display a highly variable clinical phenotype, ranging from severe to mild. The severity of the phenotype is directly related to the quantitative expression of the mutant allele and to the percentage of truncated proteins incorporated in the microfibrils. In the 55 small deletions reported, 18 single base pair deletions can be the result of a mechanism of slipped mispairing, four small deletions (5791_5793delGTT, 8525_8529delTTAAC, 755_762 del, and 3603_3668del) are flanked by direct repeats, and three mutations are deletions of a repeated sequence (635_636delCA, 3355_3358delAGAG, and 4920_4923 delTGAA). For the 30 other deletions, the mechanisms must be determined by searching for the presence of palindromic sequences, inverted repeats, or symmetric elements that facilitate the formation of secondary-structure intermediates. Twenty-seven insertions have been reported so far. Six are insertions within runs of identical bases and can be explained by slippage mispairings at the replication fork. Eleven single base pair insertions correspond to the duplication of an existing base. Seven insertions are small duplications of existing sequences. The mechanisms for the remaining three insertions must be determined by searching [Dietz HC, 2001].

*Splice mutations*_the pre-mRNA splicing machinery recognizes exons and joins them together to form mRNAs with intact translational reading frames. Splicing requires canonical sequences at the intron/exon boundary. Three categories of mutations can be identified. The first one corresponds to mutations in canonical sequences and represents, for the *FBN1* gene, 60 of the 73 splice mutations that cause abnormal splicing patterns using the nearest and strongest consensus splice site. The second category (10 mutations) corresponds to mutations not located in canonical sequences. Over the last few years, several studies have indicated that distinct sequence elements distant from the splice sites are also needed for normal splicing. These sequence elements can affect splice-site recognition during constitutive splicing and can also play important roles in directing alternative splicing [Cooper TA and Mattox W, 1997]. They can be auxiliary splicing elements (ASEs), which are required for cell-specific modulation of alternative splicing within introns that flank alternative exons, or exonic splicing enhancers (ESEs) within both coding and noncoding exons, which direct specific recognition of splice sites during constitutive and alternative splicing. Two exonic mutations, a nonsense mutation 6339T>G, and a silent exonic mutation 6354C>T, have been reported as inducing in-frame skipping of the entire exon 51, and demonstrate the existence of an ESE sequence in exon 51 [Dietz HC and Kendzior RJ Jr, 1994; Caputi M et al., 2002]. Eight other mutations could belong to this category, with mutations up to 53 bp away from the canonical sequence. For most patient cDNA analysis is not available and abnormal splicing has not been demonstrated, so causality is still uncertain. Finally, the third category of mutations is provided by single base pair changes that introduce novel splice sites that substitute for the wild-type sites. A single recurrent mutation that may create a potential donor splice site has been reported, but an abnormal splicing pattern has not been demonstrated (3294C>T). This alteration was not observed during screening of 504 chromosomes for reference a. In the majority of splice-site mutations, exon-skipping results in an in-frame mRNA product. This results in a mutant fibrillin-1 missing an integral domain. Mutant alleles produce abnormal monomers that interfere considerably with the assembly of fibrillin molecules in the microfibrilliar network. In a small number of patients (nine cases), the skipping of an exon causes a frameshift, a premature termination codon (PTC), and reduced mutant RNA levels because of nonsense-mediated decay of the mutant transcript [Frischmeyer PA and Dietz HC, 1999]. Furthermore, MFS patients have been reported in whom the donor splice site mutation results in the incorporation of intronic sequence (IVS46+1G>A,

IVS27+1G>A) into the transcript or in the use of a cryptic splice site inducing partial exon deletion (IVS18+2T>C, IVS37+5G>T) [Collod-Béroud G et al., 2003].

*Nonsense and missense mutations*_they represent respectively 10.9% and 60.3% of mutations. Among missense mutations, more than three-quarters are located in the calcium-binding modules. These mutations can either introduce or substitute cysteine residues potentially implicated in disulfide bonding. Pulse-chase studies on fibrillin-1 secretion from MFS patient fibroblasts have shown that these mutations often result in a delay in secretion or intracellular retention of profibrillin-1. Since three disulphide bonds are required to maintain the native cbEGF-like module fold, suppression or addition of cysteine residues would result in cbEGF-like module misfolding, which impairs trafficking. Most of the remaining mutations of these modules affect residues of the calcium consensus sequence and result in reduced calcium affinity, which may in turn destabilize the interface between two consecutive cbEGF-like modules. Calcium binding would rigidify the interdomain region between two cbEGF-like modules and allow multiple tandem cbEGF-like modules to take on a rigid, rod-like conformation. An increased protease susceptibility due to reduced calcium affinity is a mechanism also reported for missense mutations. This pathological mechanism emphasizes the importance of calcium binding for the structural integrity of fibrillin-1[Vollbrandt T et al., 2004].

Mutations which do not belong to one of these subclasses may likely be involved in protein-protein interactions. Other modules are carriers of one-quarter of missense mutations and their pathological mechanisms have yet to be clearly demonstrated. The global molecular analysis of the *FBN1* mutations reveals two classes of mutations. The first, which represents more than one-third of the mutations (38.6%), corresponds to mutations predicted to result in shortened fibrillin-1 molecules; 61 nonsense mutations, 71 splicing errors, 23 insertions and duplications, 51 deletions, and 10 inframe deletions are in this class. They act as dominant negative but display a highly variable clinical phenotype, the severity of which is directly related to the quantitative expression of the mutant allele and to the percentage of truncated or shortened proteins incorporated in the microfibrils [Nijbroek G et al., 1995; Karttunen L et al., 1998].

The second class represents less than two-thirds (60.3%) of the mutations and corresponds to missense mutations, most among them are located in cbEGF-like modules (78%). They can be sub-classified into: 1) mutations creating or substituting cysteine residues potentially implicated in disulfide bonding and consequently in the

correct folding of the monomer; and 2) amino acids implicated in calcium binding and subsequently in interdomain linkage, rigidification of monomer, and in protease susceptibility [seewww.hgvs.org/mutamen for updated recommendations].

To assist in diagnosis, an independent database for *FBN1* polymorphisms was created to report all the polymorphisms published or contributed. The aim is to make available a complete set of *FBN1* gene variations (mutations + polymorphisms) to the community, which may be used to quickly identify a causative mutation, thus saving time and money on testing for a polymorphism in a control population. In a first step along with the molecular data already published, we report information regarding: population ethnicity in which the polymorphism was first described; other populations tested, if done; the number of tested chromosomes; and the frequency of each allele. Another step will be to report patients in which these polymorphisms have been found. In the future, this information would be of particular interest and assistance in determining if an *FBN1* genotype could be associated with greater severity or moderate phenotypes in coordination with other *FBN1* mutations, ECM proteins, or enzyme genotypes. These data should provide a tool for beginning to understand the great phenotypic variability associated with a mutation in different probands or in the same family [Benke K et al., 2015].

3.6.4 Somatic and germline mosaicism in *FBN1*

Mosaicism in germ cells has been recognized, over the past few years, as an important and relatively frequent mechanism at the origin of genetic disorders. There are two possibilities for the existence of such a mosaicism: one is that the mutation occurs in a germ cell that continues to divide. The other possibility is that the mutation occurs very early in a somatic cell before the separation to germinal cells and is therefore present both in somatic and germinal cells. Depending on various factors, such as the gene involved and/or the degree of mosaicism, the carrier of a somatic and germline mosaicism may be asymptomatic or may present with various symptoms of the disease. The prevalence of MFS has been estimated at 1/5,000, and 25% of patients represent sporadic cases and this high mutation rate should be associated with cases of germline mosaicism. Therefore, it was surprising that, until recently [Montgomery RA etal. 1998; Rantamäki T, et al. 1999], no instance of somatic or germline mosaicism had been reported in MFS. Furthermore, since *FBN1* mutations are also associated with phenotypes overlapping MFS, mosaicism could also be identified in these subtypes. Collod-Béroud et al. in 1999, identified a germline mosaicism at the Centre Hospitalier

in Amiens. The diagnostic criteria used were those reported by Beighton et al. (1988). The parents and his brother, were unaffected. The patient, a 16-year-old boy, presented dilation of the ascending aorta, MVP with regurgitation, highly arched palate, arachnodactyly (positive wrist and thumb signs), tall stature (199 cm, 4 SD, 76 kg), and scoliosis. His 9-year-old brother displayed dilation of the ascending aorta (32 mm at the sinuses of Valsalva, 6 SD above the mean when standardized to age and body surface area), arachnodactyly (positive wrist and thumb signs), dolichostenomelia (arm span-to-height ratio 1.05), tall stature (144 cm, 3 SD, 31 kg), highly arched palate, and joint hypermobility. No other typical anomaly of MFS (including ectopia lentis) was found in either subject. Both parents were examined thoroughly, and the diagnosis of MFS was excluded for both. Blood samples were collected from the four family member and the finding of the alteration in the father's white blood cells and the recurrence of the disease in his children implied somatic and germline mosaicism in the father. Careful reassessment of clinical examination of the father (performed systematically before the identification of the mosaicism) revealed no skeletal or ocular sign but minor findings: discreet dilation of the ascending aorta and minimal aortic regurgitation. Their observation shows that somatic and germline mosaicism are associated with MFS-like features and should be looked for in parents of sporadic cases presenting with these MFS-like features. In fact, if mild or isolated features of the disease are found in one of the parents, genetic counseling should take into account the possible presence of the disease in another child. Somatic mosaicism could also explain the mild and incomplete features often seen in patients referred to MFS clinics for diagnosis. Again, caution is warranted in the follow-up of these patients and in evaluation of the risk of transmission. In 1999 another study has been performed by Rantamäki, reporting a de novo *FBN1* mutation that was identified in two sisters with MFS born to clinically unaffected parents [Rantamäki T et al.,1999]. The paternity and maternity were unequivocally confirmed by genotyping. Although one of the parents had to be an obligatory carrier for the mutation, and there was no detection of the mutation in the leukocyte DNA of either parent. To identify which parent was a mosaic for the mutation several tissues were analyzed from both parents. Although the mutation could not be identified in a sperm sample from the father or in samples of multiple tissue from the mother, concluding that the mother was the likely mosaic parent and that the mutation must have occurred during the early development of her germline cells. It is difficult to estimate how many of the sporadic patients with MFS actually represent offspring of a mosaic parent, especially if only leukocyte DNA has been analyzed by non quantitative

conventional methods with limited detection sensitivity. The degree of mosaicism may not be apparent in the leukocytes but would become evident if other tissues studied. The mutation was identified by restriction-site analysis of PCR products in the hair bulbs and buccal cells of the proband's father but was not detectable from the father's leukocyte DNA. Although not shown earlier, mosaicism could be a more common phenomenon in MFS than has been realized so far. Somatic mosaicism may even be one modifying factor responsible for the phenotypic variability among unrelated patients with MFS. Most importantly, the present demonstration of germ-line mosaicism in MFS has implications for genetic counseling. The recurrence risk in the families with "sporadic" patients should, therefore, be estimated to be higher than the new mutation rate alone. Whenever a single child is identified with an *FBN1* mutation and more children are desired, the parents should be tested, and tissues other than leukocytes should be analyzed. Furthermore, prenatal diagnosis should be offered [Le Gloan L et al., 2016].

3.6.5 Other genes encoding for the fibrillins family

Three closely related fibrillins have been described. Fibrillin-2 and the recently discovered fibrillin-3 have a domain organisation identical to that of fibrillin-1 and an overall level of amino acid identity of between 61% and 69%. Several lines of evidence suggest that the fibrillins have both overlapping and unique functions. All three fibrillins are structural components of microfibrils; however, fibrillin-2 and fibrillin-3 are preferentially expressed in embryonic developmental stages, whereas fibrillin-1 is expressed from the gastrula to throughout adult life [Robinson PN et al., 2006]. The human genome contains three fibrillins: *FBN1* and *FBN2* (OMIM*612570), both well characterized, and *FBN3* (OMIM*608529), reported only as a cDNA sequence. Like *FBN2*, the highest expression levels of *FBN3* were found in fetal tissues, with only low levels in postnatal tissues. Immunolocalization demonstrated fibrillin-3 in extracellular microfibrils abundant in developing skeletal elements, skin, lung, kidney, and skeletal muscle. Unlike the other two fibrillins, *FBN3* expression is high in brain [Corson GM et al., 2004]. The human *FBN1* and *FBN2* genes have a similar structure, with several long introns taking up the first half of the gene while the second half shows a high density of exons. The human *FBN1* gene covers more than 237,000 bps and the transcript is a total of 11,695 bps. The larges tintron is 58,474 bps. The *FBN2* gene covers over 250,000 bps in humans and the longest transcript is 11,132 bps. The largest intron is 54,341 bps,

between coding exons 5 and 6. This structure of an exon-poor 5′ region of the gene followed by an exon-dense 3′ region is observed in the majority of mammals with annotated *FBN1*and *FBN2* genes. The gene structure is different from *FBN1* and *FBN2*. The *FBN3* gene is about 82,000 bps with the exons (63 total) and introns relatively evenly dispersed throughout [Davis MR and Summers KM, 2012]. *FBN2* mutations have been associated with congenital CCA. There are at least 40 recorded *FBN2* mutations resulting in CCA. These *FBN2* mutations reside primarily between exon 24 and exon 33, a cbEGF rich region of fibrillin-2, while only three are recorded near the N terminus, in exons 8, 9 and 17. The majority present as missense and deletion mutations, thought to impair calcium binding and protein folding through impeding disulfide bond formation. Interestingly, the *FBN2* mutations are found in the region corresponding to those mutations commonly observed in *FBN1* in the severe neonatal MFS. *FBN2* mutations leading to a premature termination codon are significantly underrepresented (only one nonsense mutation is reported) contrasting with an overrepresentation of in-frame mutations (splice-site mutations are likely all in-frame and the unique duplication results in in-frame insertion). This particular distribution of mutation types is similar in the homologous *FBN1* region (exons 24-32). Nevertheless, contrary to *FBN1*, where mutations in this region are mainly missense (56.9% for 12.5% of splice-site mutations), the mutations currently reported in the *FBN2* gene are evenly distributed between missense (50%) and splice-site mutations (46.9%) [Callewaert BL et al., 2008; Frédéric MY et al., 2009]. Interestingly, the *FBN2* mutations are found in the region corresponding to those mutations commonly observed in *FBN1* in the severe neonatal MFS. This region in the two proteins, though displaying 71.2% homology (Mega Align, Clustal W alignment; not shown), is likely to perform distinct roles in ECM development, based on the varying phenotype of neonatal MFS and CCA and different expression patterns. CCA rarely shows the severe cardiovascular effects seen in MFS patients. However some *FBN2* mutations result in early lethality due to cardiovascular defects. One case showed atrial and ventricular septal defects, aortic arch abnormalities and gastrointestinal defects ultimately resulting in premature death [Davis RM and Summers KM, 2012]. There is no specific condition known to be caused by *FBN3* mutations, although genetic variation at the *FBN3* locus was recently linked to polycystic ovary syndrome (PCOS). Polymorphism of microsatellite D19S884, located in intron 55 of *FBN3*, was associated with an increase in susceptibility to PCOS [Urbanek M et al., 2007]. Another study failed to replicate the

PCOS association. The expression of *FBN3* early in development and in oocytes could be linked to a role in ovarian development. Further examination of the impact of *FBN3*mutations on phenotype is hampered by the lack of a functional *Fbn3* gene in rodents, so that models will have to be created in other animals [Corson GM et al., 2004].

3.6.6 LTBP proteins

The LTBPs are large secreted glycoproteins structurally related to fibrillins. The first described LTBP (LTBP1) was identified in platelet releasate as a component of a high molecular weight complex, which also included a dimer of TGF-β and a dimer of its propeptide [Miyazono K et al., 1988]. Since the characterization of LTBP1, three other LTBPs were identified (LTBP2-4). LTBP3 and LTBP4 also interact with latent TGF-β, whereas LTBP2 does not [Saharinen J and Keski-Oja J, 2000].The four LTBP proteins have a modular and highly repetitive structure, primarily composed of two distinct cysteine-rich motifs: one, characterized by six cysteines, is similar to the epidermal growth factor (EGF) module, and the other defined by eight intramolecularly bound cysteines (8-cys or TB domain), represents the family's signature domain [Todorovic V et al., 2005]. TBPs can also possess an LTBP-exclusive 4-cys domain near the N-terminus. Each LTBP includes four 8-cys domains interspersed with numerous EGF-like motifs (15-20), many of which contain consensus Ca^{2+}-binding sequences. The first 8-cys domain is known as the hybrid domain, since it shares similarities both with 8-cys and EGF-like motifs (Figure 23). Between the second 8-

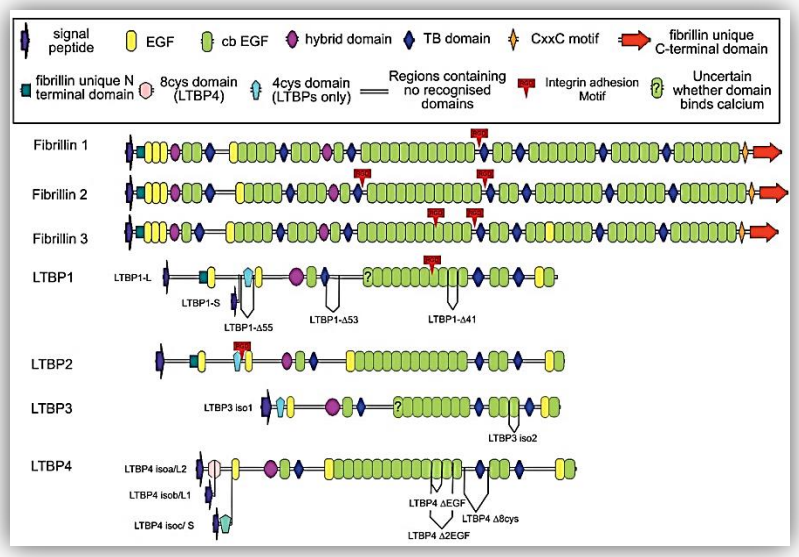


Fig 23. The domain organization of the human TB domain proteins All known human TB proteins are shown, with domain symbols summarized in the box at the top of the Figure. Note the high conservation of fibrillin domain organization compared with LTBPs. Some of the alternatively spliced forms of the LTBPs demonstrated to occur in humans are also illustrated (from Robertson I et al., 2015)

cysteine domain and the stretch of repeating EGF-like motifs, there is an unstructured proline rich region called the *hinge domain*, which shows the highest degree of sequence diversity amongst the four LTBPs [Annes JP et al., 2003]. Another hinge region is localized between the fourth 8-cys domain and the downstream EGF-like motif of LTBP-1. The hinge sequences are sensitive to proteolytic cleavage [Taipale J et al., 1995; Unsold C et al., 2001; Dallas SL et al., 2002; Ge Gand Greenspan DS, 2006], which plays an important role in regulation of LTBP function. In addition to proteolytic processing, alternative splicing and usage of different promoters contribute to the structural variability of LTBPs. LTBP1 and LTBP4 exist as two major isoforms-long and short, transcribed from independent promoters [Koski C et al., 1999; Kantola AK et al., 2010].Transcriptional regulation of *LTBP2* and *LTBP3* genes might be similar, considering that Northern blot analysis of *LTBP2* and *LTBP3* mRNA in different tissues shows the presence of two major transcripts [Moren A et al., 1994; Shipley JM et al., 2000; Dabovic B et al., 2002]. Different variants of each LTBP show distinct expression patterns and diverse affinities toward their binding partners [Koli K et al., 2001]. The interactions of LTBPs with their intra- and extra-cellular ligands determine their localization, function, and action. LTBP1 and 3 bind all three TGF-β LAP isoforms with high affinity, whereas LTBP4 shows a weak binding capacity only for TGF-β1 LAP. LAP binds to the third 8-cys domain (8-cys-3) in LTBP [Gleizes PE et al., 1996; Saharinen J etal., 1996]. Close inspection of all 8-cys domains in fibrillins and LTBPs demonstrated that the 8-cys-3 motifs of LTBP1, 3, and 4 possess a two amino-acid insertion between the sixth and the seventh cysteine [Saharinen J and Keski-Oja J, 2000]. LTBPs function as chaperones by binding LAP within the endoplasmic

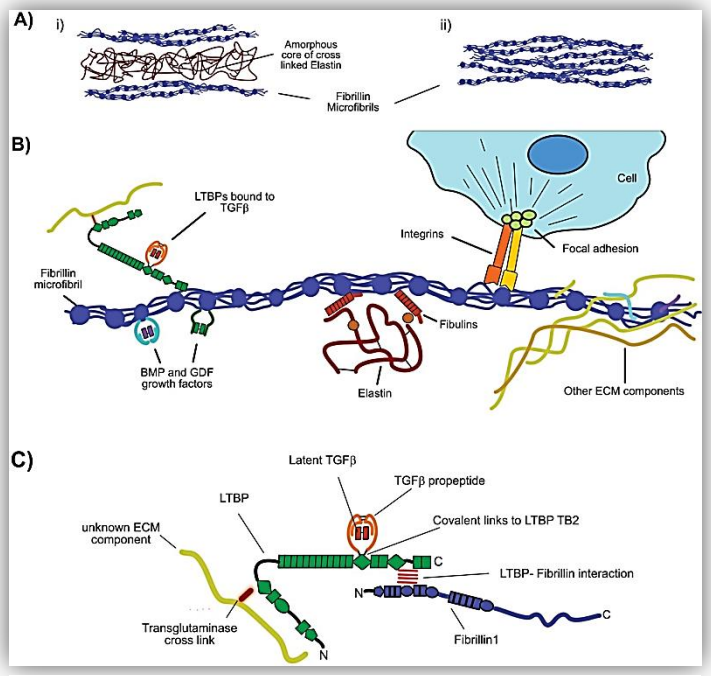


Fig 24. The roles for TB domain proteins in the ECM (A) Fibrillin and elastin association (i) in blood vessel wall, lung and skin,and independent fibrillin (ii)in the suspensory ligaments of the eye, peridontal ligament and mesangium of the kidney glomerulus. (B) Some of the key cell matrix interactions mediated by fibrillin microfibrils: (C) The current model of LTBP organization in the ECM for maintenance of latent TGFβ (from Robertson I et al., 2015)

reticulum. In the absence of LTBP, the reactive cysteine (cys33) within LAP bonds with a free cysteine of the mature cytokine, causing misfolding and slow secretion of the SLC [Rifkin DB, 2005]. Interestingly, LTBP3 cannot be secreted from cells if not complexed to SLC [Chen Y et al., 2002]. Secreted LLC is either soluble or sequestered within the ECM through LTBP interactions with multiple extracellular proteins. Latent TGF-β is then stored in the extracellular space until required, and when the need arises it is liberated from its propeptide, in a process commonly referred to as "latent TGF-β activation". Several different mechanisms of TGF-β activation have been described, involving diverse activators such as proteases, the integrins αvβ6, αvβ3, αvβ5, and αvβ8, thrombospondin-1, F-spondin, neuropilin-1, reactive oxygen species, and low orhigh pH [Chandramouli A et al., 2011]. The molecular targets of these divergent latent TGF-β activators can be LTBPs and/or LAPs. LTBPs orchestrate TGF-β action at multiple levels: (1) They ensure correct folding and efficient secretion, (2) they direct temporal and spatial deposition in the extracellular space through their own secretion dynamics and interaction with ECM proteins, and (3) they regulate activation. Incorporation of LTBPs into the ECM is crucial for regulation of latent TGF-β storage and activation. However, significant amounts of LTBP1 and LTBP4 are secreted free of SLC, much like LTBP2, which cannot interact with SLC, suggesting that LTBPs perform roles unrelated to TGF-β. LTBPs interact with many extracellular proteins, but interactions with fibronectin and fibrillins seem to be crucial for proper localization and function. The initial studies of LTBP1 deposition into the ECM identified three potential ECM-interacting domains, two localized at the N-terminus and one localized at the C-terminus [Unsold C et al., 2001]. N-terminal fragments were readily incorporated into the matrices and association of the binding domains with the ECM was enhanced by soluble, cell-derived factors. Similar results were obtained with binding peptides from LTBP2, LTBP3, and LTBP4. The N-terminal ECM-binding sequences of LTBP1, 2, and 4 associate with fibronectin and this interaction is promoted by transglutaminase [Rifkin DB et al., 2005]. The purified N-terminal fragment of LTBP3 co-localized with fibronectin in pre-formed lung fibroblast ECM, and so did the endogenous LTBP3 at the time of its initial incorporation into mature matrices [Koli K et al., 2005], suggesting an association of LTBP3 and fibronectin. However, no biochemical studies were performed thus far to investigate this possibility. LTBPs and fibrillins are highly homologous proteins, but are easily distinguished since fibrillins are much larger (~350 kDa) with almost double the number of EGF-like and 8-cys repeats compared to LTBPs (Figure 24). Fibrillins are major structural

components of connective tissue microfibrils and elastic fibers [Olivieri J etal., 2010]. Microfibril extracts from tissues do not contain LTBPs, indicating that LTBPs are not disulfide bonded to microfibrils [Isogai Z et al., 2003]. However, all LTBPs, with the exception of LTBP3, bind to fibrillin-1, the major component of microfibrils through their C-terminus [Isogai Z et al., 2003]. LTBP3 is unique because it cannot be secreted if not bound to latent TGF-β. Because the binding capacity of LTBP3 to fibrillin-1 was assessed only with a C-terminal peptide homologous to thefibrillin-1-binding LTBP1 and LTBP4 domains, which lack the TGF-β LAP-binding sequence (8-cys-3), this C-terminal peptide may have assumed a conformation different from the one of LTBP3-LLC. It would be advisable to use the full length LTBP3-LLC for testing its binding abilities toward fibrillins. LTBP1 and LTBP4 can also bind fibrillin-2, whereas LTBP2 cannot [Isogai Z et al., 2003]. Competitive binding studies showed that the binding site on fibrillin-1 for LTBP2 is the same or in close proximity to that for LTBP1, suggesting that LTBP2 might be a negative regulator of LTBP1 interaction with microfibrils. LTBP4 may also compete with its family members for binding to fibrillin-1, since its binding site lies within the same fibrillin-1 peptide as that of LTBP1 and LTBP2. Competitive binding of LTBPs to microfibrils may represent an additional level of control of microfibril composition and function. Some LTBPs (LTBP1, 2, and 4) associate with heparin and heparan sulfate, in a manner that might affect cell adhesion, migration, and micro- and elastic fibers sembly [Chen Y et al., 2007; Kantola AK et al., 2008; Parsi MK et al., 2010]. Elastogenesis and structural organization of elastic fibers also depend on fibulin-4 and -5. LTBP2 interacts with fibulin-5 and plays an important role in the targeting of fibulin-5 to microfibrils to form elastic fibers [Hirai M et al., 2007]. LTBP1 binds to fibulin-4 [Massam-Wu T et al., 2010], whose role in elastogenesis is to tether lysyl-oxidase (LOX) to tropoelastin to facilitate cross-linking [Horiguchi M et al., 2009], whereas LTBP4 interacts with fibulin-5. Fibulin-2, -4, and -5 can bind to fibrillin-1 and their binding sites are within the domain responsible for interaction with LTBPs [Ono RN et al.,2009]. Therefore, LTBPs and fibulins may compete for interaction with fibrillin-1. However, Kielty's group showed that fibulin-4 can simultaneously bind fibrillin-1 and LTBP1, suggesting that fibulin-4 might function as a mediator of LTBP1 association with microfibrils [Massam-Wu T et al., 2010]. Recently, a few diverse factors have been shown to bind to LTBPs. For example, LTBP1 interacts with ADAMTSL2, a secreted glycoprotein of unknown function [Le Goff C et al., 2008]. *ADAMTSL2* mutations in humans cause geleophysic dysplasia, an autosomal recessive disorder characterized by thick skin, short stature, fingers and toes,

and cardiac valve malfunction that can lead to premature death. The functional significance of LTBP1 interaction with ADAMTSL2 is unclear, but skin fibroblasts from affected individuals secret enhanced levels of active TGF-β, suggesting that ADAMTSL2 might regulate TGF-β activity, possibly in an LTBP1-dependent fashion. LTBP2 and LTBP4 have been implicated in the formation of elastic fibers. Elastogenesis requires coaecervation of tropoelastin and is modulated by fibulin-4 and5, cell-surface glycosaminoglycans, and cross-linking enzymes LOX and LOX-like (LOXL). Fibulin-5 and elastin deposition in cell culture is dependent on fibrillin-1 [Hirai M et al., 2007]. However, in the absence of LTBP2, fibulin-5 targets tropoelastin mainly to fibrillin-2. Therefore, LTBP2 might function as a molecular switch that regulates the differential usage of microfibrils during assembly of elastic matrices. Genetic disorders arising as a result of mutations in LTBP genes in mice and humans underline the importance of the LTBP family. Deficiency of the long form of Ltbp1 (Ltbp1L) in mice causes serious disruption of great vessels and cardiac valve development, resulting in perinatal death [Todorovic V et al., 2007; Todorovic V et al., 2011]. However, mice with deletion of the first exon of the *LTBP1* gene common for both the long and the short form display only mild cranio-facial abnormalities and live a normal lifespan [Drews Fet al., 2008]. Null mutations in *LTBP2* have been associated with ocular malformations, such as development of a small and dislocated lens and glaucom [Robertson I et al.,2010]. Homozygous mutation within the seventh EGF-like domain in *LTBP3* caused a dramatic decrease in *LTBP3* expression and was manifested as oligodontia (agenesis of ≥ six teeth), short stature and scoliosis [Noor A et al., 2009]. *Ltbp3* null mice are also shorter than their littermates and have multiple skeletal and craniofacial abnormalities, including kyphosis, extension of the mandible beyond the maxilla, and cranial doming.

As the animals age, their long bones and vertebrae become osteoarthritic and osteopetrotic and they develop pulmonary emphysema [Dabovic B et al., 2002; Colarossi C et al., 2005]. Autosomal recessive mutations in *LTBP4* cause Urban-Rifkin-Davis syndrome (URDS), which is characterized by multi-organ developmental defects, such as aberrant lung, gastrointestinal, genitourinary, musculoskeletal, and dermal organogenesis [Urban Z et al., 2009]. However, it is possible that LTBP4 plays exclusively a structural role and that increased TGF-β activity is actually a consequence of abnormal elastic fibers and perturbed ECM, which, in turn, leads to a promiscuous activation of latent TGF-β, similarly to what is seen in human and mouse mutants for fibrillin-1 [Olivieri J et al., 2010].

3.6.7 *TGFBR1* and *TGFBR2* genes

Propagation of TGF-βs cytokines signalling into the cell is mediated by a family of type 1 and type 2 receptors including the type 1 and type 2 TGFβ receptor. TGFβ binds first to type 2 receptors, allowing subsequent incorporation of type 1 receptors into a ligand-receptor complex involving a TGFβ dimer and four receptor molecules. The signal is then propagated into the cell by means of phosphorylation of the Smad proteins [Massague J et al., 2000]. *TGFBR1* gene is located on chromosome 9 in 9q22 position and is composed of 9 exons while *TGFBR2* is located on chromosome 3 in 3q22 position and is composed of 7 exons [Loeys BL et al., 2005].

Linkage to chromosome 3p24.2–p25 was demonstrated for a large family with a Marfan-like phenotype for whom linkage to *FBN1* and *FBN2* had previously been excluded. This disorder has been termed MFS type 2 and shares some of the cardiovascular and skeletal features of classic MFS [Boileau C et al., 1993]. More recently, a new aortic aneurysm syndrome with hypertelorism, bifid uvula or cleft palate, and generalised arterial tortuosity with ascending aortic aneurysm together with other findings such as craniosynostosis, mental retardation, and congenital heart disease was described; LDS, was shown to be associated with mutations in the genes for either *TGFBR1* or *TGFBR2* resulting in perturbations of TGFβ signalling [Loeys BL et al., 2005]. *TGFBR1* and *TGFBR2* mutations have been also shown in milder forms of LDS, as well as autosomal dominant forms of thoracic aortic aneurysm and aortic dissection (TAA/TAAD) associated with no syndromic features and decreased penetrance (Pannu H et al., 2005; Matyas G et al., 2006; Attias D et al., 2009; Isselbacher et al., 2016). Given the high number of data suggesting a significative association between genetic variants in TGF-ß receptors and different syndromes sharing TAA and aortic dissection as overlapping clinical features, it seems reasonable to hypothesize that the dysregulated TGF-ß signalling pathway may be involved in aneurysm formation in general [Baas AF et al., 2010]. LDS patients have in fact been described as showing not only TAA but also aneurysms at the abdominal aorta and head and neck [Loeys BL, 2006], while subjects carrying *TGFBR2* mutations had aneurysm at different locations such as descending aorta, carotid, subclavian or popliteal arteries [Hasham SN, 2003].

SGS is characterized by craniosynostosis and other craniofacial features, marfanoid skeletal abnormalities, and developmental. Although a question was raised on the involvement of *FBN1*abnormality in SGS, at least two *FBN1* mutations were identified [Kosaki K. et al.,2006; Sood et al., 1996]. Furthermore, the initial study of five SGS patients failed to reveal mutations in either *TGFBR1* or *TGFBR2*, but Kosaki

et al. reported in 2006 a *TGFBR2* mutation in a SGS patient with craniofacial and skeletal abnormalities, mild developmental delay, and cardiovascular features, including aortic regurgitation, annuloaortic ectasia and sigmoid configuration of the brachiocephalic, left common carotid and left subclavian arteries. Robinson et al., however, in 2006 suggested that this patient might be more appropriately diagnosed as having LDS, due to the presence of bifid uvula and arterial manifestations.

The various mutations, previously located in exons 4 and 9 [Stheneur C et al., 2008] as well as gene disruption by chromosomal structural abnormality, suggest that loss of function mutations of *TGFBR2* are responsible for a wide spectrum of connective tissue disorders, but no simple genotype-phenotype correlation has been observed. It is intriguing that domain-specific germline mutations of *TGFB1* cause Camurati-Engelmann disease (CED, OMIM#131300) [Kinoshita A et al., 2000] associated with marfanoid habitus, despite the absence of connective tissue fragility. *TGFB1* mutations in CED were shown to cause increased TGFβ signaling [Janssens K et al., 2003]. These findings suggest that abnormal TGFβ signaling could be responsible for the skeletal features of MFS. This hypothesis is corroborated by earlier studies describing the roles of TGFβ signaling in skeletal development (Alvarez J and Serra R, 2004; Serra R et al.,1999). The vascular consequences associated with *TGFBR2* and *TGFBR1* mutations include aortic dissections in young age and aneurysms at sites distant from the aortic root.

A number of studies carried out mutational *TGFBR1* and *TGFBR2* screening in classical MFS patients satisfying the Ghent criteria [Mizuguchi T et al., 2004; Singh KK et al., 2006; Matyas G et al., 2006; Disabella I et al., 2005; Ki CS et al., 2005; Loeys BL et al., 2005]; some led to the identification of pathogenetic mutations [Mizuguchi T et al., 2004; Singh KK et al., 2006; Matyas G et al., 2006; Disabella I et al., 2005], while others failed in detecting any [Ki CS et al., 2005; Loeys BL et al., 2005; Baetens et al., 2011]. Due to the evidences indicating the pivotal role of TGF-ß pathway in remodeling of aortic wall [Azhar M et al., 2003] and of pathogenetic mutations of TGF-b signaling genes in disorders in which TAA or AAA represent the major phenotype of one of the clinical manifestations, the role as susceptibility factors for aneurysmal disease of polymorphisms in *TGFBR1/2* genes (e.g. TGFBR1*6A, TGFBR2rs764522 and rs1036095) was also investigated with discordant results [Lucarini L et al., 2007; Golledge J et al., 2009; Baas AF et al., 2010; Biros E et al., 2011].The role of *TGFBR1* and *TGFBR2* genetic variants in classical MFS remains therefore unclear.

CHAPTER 4.

BICUSPID AORTIC VALVE

4.1 HISTORY

Bicuspid aortic valve (BAV) disease is the most common congenital cardiac defect, affecting 1.3% of the adult general population (Figure 25) [Verma S et al., 2014]. The precise aetiology of the BAV has not been clearly defined, and it is a precursor of complications occurring a decade earlier in life than in a tricuspid aortic valve (TAV). It can cause aortic valve disease (stenosis and/or regurgitation), infective endocarditis and thrombus formation and is associated with ascending aortic aneurysm (AA) and dissection [Michelena HI et al., 2011]. The majority of BAV occurs sporadically; however, familial BAV has been reported with prevalence in first-degree relatives of affected individuals being as high as 9%, suggesting an autosomal dominance pattern with reduced penetrance [Huntington K et al., 1997].

The earliest description of the BAV has been attributed to Leonardo da Vinci who over 400 years ago sketched the bicuspid variant of the aortic valve [Braverman AC et al., 2005] and it was in 1844 that Paget first brought to attention the propensity of the BAV to develop disease; in 1858 Peacock noted the tendency of these valves to develop obstructive lesions initially, with subsequent incompetence [Roberts W et al., 1970]. In 1886 Osler emphasized the clinical significance of the BAV by describing 18 cases of BAV with a predilection of these valves to the development of infective endocarditis [Braverman AC et al., 2005]. Dates back to the 1950s the finding of some investigators who observed that the propensity to develop isolated calcific aortic stenosis occurring in

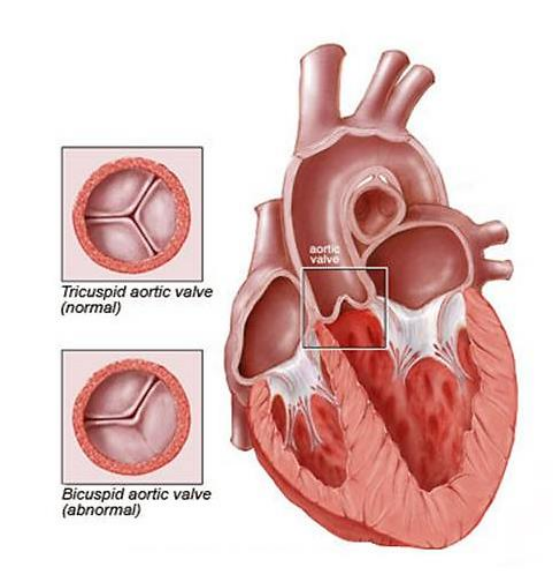


Fig 25. Tricuspid (normal) and bicuspid (abnormal) aortic valve (from *www.lucinafoundation.org*)

the setting of BAV was the result of an intrinsic property of the BAV rather than the consequence of rheumatic disease [Campbell M and Dauntze R, 1953; Smith D et al., 1955; Bacon A et al., 1959].

Then, it was the autopsy studies of Wauchope which established that BAV is the commonest congenital anomaly of the heart [Wauchope G, 1928]. However, the association of BAV with diseases of the aorta was first commented by Abbott in 1927 with the description of an association between congenital BAV with aortic dissection [Acierno LJ, 1994]. Subsequently, between 1970 and 1990, anatomic pathology studies corroborated the association aortic dissection until year 1984 when the clinicopathologic significance of the aortic valve was emphasized by the work of Larson and Edwards who noted that BAV was associated with at least a nine-fold greater risk of aortic dissection [Edwards WD et al., 1978; Larson EW and Edwards WD, 1984]. In more contemporary times, with the advent of imaging, clinical studies indicate that aortic valve dysfunction and dilatation of the ascending aorta are the most common complications associated with BAV [Michelena HI et al., 2011], such that the BAV condition is recognized as a valvulo-aortopathy [Michelena HI et al., 2014], particularly in adults. Dilatation of the ascending aorta is one risk factor associated with increased risk of catastrophic aortic outcomes such as dissection and rupture [Coady MA et al., 1999], which are the most feared complications in BAV disease. All the recent developments have helped to clarify the incidence of BAV in the general population and advancements in human genetics have begun to elucidate the etiology of aortic disease associated with BAV.

4.2 EPIDEMIOLOGY

BAV is the most common congenital cardiac malformation with an estimated prevalence between 0.5% and 2% [Verma S et al., 2014]. Because the bicuspid valve may be entirely silent during infancy, childhood, and adolescence, these incidence figures may be underestimated and are not generally included in the overall incidence of congenital heart disease. Incidence does not appear to be affected by geography. A recent report suggests much lower than expected prevalence in African-Americans [Khan W et al., 2008]. The male-to-female ratio is 2:1 or greater. Sex is not a predictive variable in the natural history of bicuspid aortic valve. A recent prospective echocardiographic study in newborn infants showed a prevalence of bicuspid aortic

valve in 7.1 per 1,000 male newborns versus 1.9 per 1,000 female newborns [Tutar E et al., 2005].

Bicuspid aortic valve may be identified in patients of any age, from birth through the 11th decade of life. It may be only an incidental finding at autopsy. Bicuspid aortic valve may remain silent and be discovered as an incidental finding on echocardiographic examination of the heart.

Critical aortic stenosis and infective endocarditis may be considered relatively early sources of morbidity for patients with bicuspid aortic valve. Critical aortic stenosis may occur in infancy and may be associated with a bicuspid valve. Occasionally, bicuspid aortic valve is diagnosed after a patient has developed infective endocarditis with systemic embolization. Stenosis of a bicuspid aortic valve is more likely to develop in persons older than 20 years and is caused by progressive sclerosis and calcification. High levels of serum cholesterol have been associated with more rapidly progressive sclerosis of the congenitally bicuspid aortic valve [Chui MC et al., 2001]. Children who develop early progressive, pathologic changes in the bicuspid aortic valve are more likely to develop valve regurgitation than stenosis [Spaziani G et al., 2014].

4.3 MORPHOLOGY AND PATHOLOGIC FEATURES OF BAV

The anatomic features of BAV are characterized by smooth cusps margins and the fusion of two cusps of unequal size. These cusps often include a central raphe (or false commissure, the hypoplastic commissure between two partially fused cusps) which are usually in the centre of the larger cusp (Figure 26). The most common variant of BAV is the result of the fusion between the left and right coronary cusps (L-R BAV), also known as the latero-lateral cusps position which is seen in 70-86% of the BAV cases, while the fusion of the right and noncoronary cusps (R-N BAV; antero-posterior cusps position) is observed in 12-28% and the left and noncoronary cusps (L-N BAV) in 0.5-3% of the cases [Russo CF et al., 2008]. This BAV classification in 3 main phenotypes is based on

Fig 26. On the left, bicuspid aortic valve with raphe, on the right bicuspid aortic avlve without fusion between the two cusps (from Gatto L et al., 2013)

the presence and the position of raphes and it describes with sufficient detail the morphology of the bicuspid valve, giving a complete description of the phenotype itself and which it can be easily reproducible and immediately understandable. Sievers and Schmidtke in 2007 [Sievers HH and Schmidtke C, 2007] proposed a different system of classification based on 3 characteristics: the number of raphes, the spatial position of the cusps or raphes, and the functional status of the valve (Figure 27). The complexity of this arrangement however has not been successful in the clinical setting also given the difficulty of its use in patients with inadequate ultrasound windows [Longobardo L et al., 2016]. Multidetector computed tomography recently provided an integrated classification of bicuspid aortic valve phenotypes and aortopathy with interesting clues about the relationship between bicuspid aortic valve phenotypes and aortic dilatation patterns (Figure 28) [Kang JW et al., 2013]. Indeed, there are numerous systems proposed (Table 7), and no general consensus on the most manageable description in clinical practice but the classification considering raphe position and cusp size is simple, complete, and reproducible, and could better define anatomy visualized in the majority of echocardiography laboratories [Longobardo L et al., 2016].

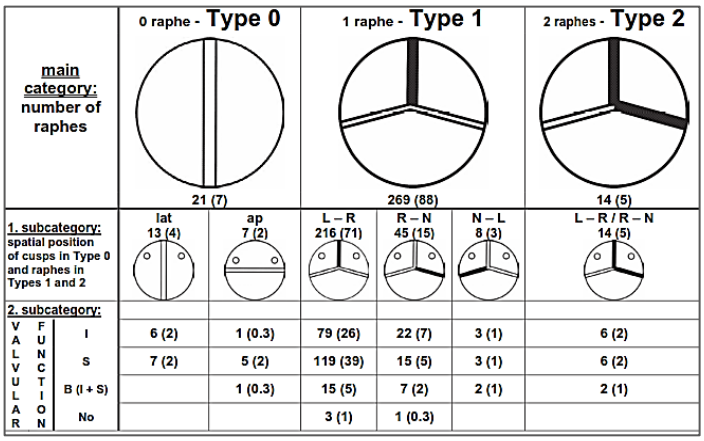


Fig 27. Bicuspid aortic valve morphology classification according to Sievers and Schmiedtke, 2007

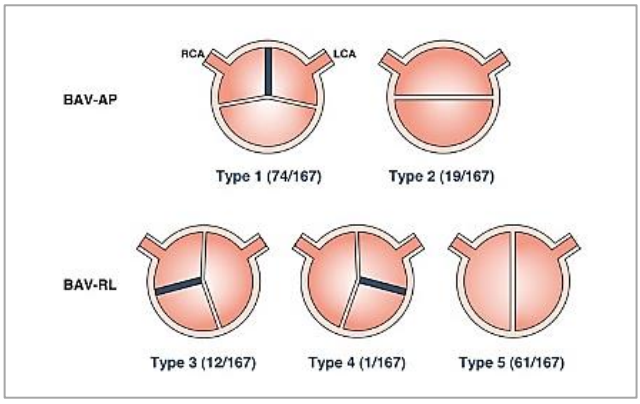


Fig 28. Bicuspid aortic valve morphology classification according to Kang, 2013

BAV is characterised by a wide heterogeneity of morphology which was initially considered natural variation rather than a suspected issue [Longobardo L et al., 2016]. This heterogeneity was depicted as "a continuous spectrum" of morphologies by Sievers and Schmidtke [Sievers HH, Schmidtke C, 2007]. The 2 main BAV phenotypes appear to have different disease entities. In children, right-noncoronary bicuspid aortic valve seems to be associated with valve stenosis and regurgitation, whereas right-left bicuspid aortic valve is associated more with aortic coarctation [Ciotti GR et al., 2006].

In adults, instead, the phenotype more often associated with aortic stenosis appear to be the right-left BAV, which also showed an increased risk of rapid aortic dilatation [Thanassoulis G et al., 2008], being therefore considered a determinant for a more aggressive surgical treatment [Sievers HH et al., 2014]. Fernández and colleagues in 2009 [Fernandez B et al., 2009] showed the different embryological pathogenesis of right-noncoronary and right-left bicuspid aortic valves in $NOS3^{-/-}$ mice and Syrian hamsters, this evidence possibly explaining the strange heterogeneity of prognosis described above. If confirmed in humans, these findings would support the theory that BAV is not a single valve alteration characterized by a wide heterogeneity, but a heterogeneous cluster of diseases, caused by specific genetic mutations and associated with different complications, outcomes, and prognoses [Longobardo L et al., 2016].

In the normally functioning tricuspid aortic valve, the aortic cusps are relatively similar in size, opening into their respected sinuses during systole, and coapting equally during diastole to equalize pressure dynamics across the aortic root, while, in BAV, aortic cusps often do not fully open [Robicsek F et al., 2004] and coaptation is often eccentric [Conti CA et al., 2010]. Together, these abnormalities can produce an elliptical orifice area and flow turbulence, perhaps predisposing to early valve degeneration and calcification leading to clinically significant aortic stenosis, up to a decade earlier than individuals with tricuspid aortic valves [Davies RR et al., 2007]. Echocardiographic studies have shown that in BAV patients, cuspal sclerosis typically begins in the second decade of life while calcification is prominent in most middle-aged patients [Beppu S et al., 1993]. This early degeneration may be related to more aggressive inflammatory changes of the aortic valve, characterized by increased macrophage infiltration and neovascularization [Moreno PR et al., 2011].

Aortic insufficiency in BAV disease is often mild to moderate in severity and concurrent with aortic stenosis; its development can be attributed to several different BAV characteristics. Firstly, as a result of differences in leaflet dimensions, 15-20% of all BAVs have incomplete closure [Lewin MB and Otto CM, 2005]. Redundancy in the fused leaflet also predisposes BAV to cuspal prolapse leading to the onset of aortic insufficiency [Sadee AS et al., 1992]. Furthermore, dilatation of the aortic root and sinotubular junction are common traits of BAV disease. This dilatation is often progressive and can lead to deterioration of valvular function. Studies have shown a 36% decrease in coaptation height and a 41% decrease in contact pressure [Conti CA et al., 2010] between leaflets in BAV, both of which are likely to be further exacerbated by dilatation of the aortic root and sinotubular junction. Isolated severe insufficiency is

relatively uncommon in the setting of BAV and when present, is often related to infective endocarditis [Roman MJ et al., 1987].

BAV is commonly associated with other congenital cardiovascular defects. The most robust association occurs with coarctation of the aorta, where up to 3/4 of individuals with aortic coarctation also have coexistent BAV [Roos-Hesselink B et al., 2003]. In this specific BAV population, morphological fusion of the left-coronary and right-coronary cusps appear to be a preponderant [Ciotti GR et al., 2006]. BAVs are also more commonly linked to other left-sided obstructive lesions including interrupted aortic arch [Schreiber C et al., 1997], Shone's complex [Bolling SF et al., 1990], and hypoplastic left heart syndrome [Hinton RB et al., 2007]. Other congenital lesions associated with BAV include PDA, ventricular septal defect, and atrial septal defects. Several studies have also, noted variations in the coronary anatomy in patients with BAV with an increased prevalence of left dominant coronary circulation and shorter left main coronary arteries [Higgins CN and Wexler L, 1975; Lerer PK and Edwards WD, 1981].

Table 7 Main Classifications of Bicuspid Aortic Valve Morphology (from Longobardo L et al., 2016)

Authors	*Classification System*	*Classification Determinants*	*Main Findings*
Roberts, 1970	Right-left Anteroposterior	Raphe Cusp position	Aortic stenosis was the most common valve dysfunction. Aortic regurgitation caused death or surgical intervention at a young age. Coronary arteries usually arise in front of the cusp with the raphe.
Brandenburg et al., 1983	Type A: posterior and anterior commissures and raphe located at 4-5, 9-10, and 1-2 o'clock, respectively Type B: posterior and anterior commissures and raphe located at 6, 1-2, and 9-10 o'clock, respectively Type C: commissures at 3 and 9 o'clock, with no raphe	Commissures Raphe position, (on a clock face)	Very high sensitivity, specificity, and diagnostic accuracy of 2-dimensional echocardiography in bicuspid aortic valve (BAV)
Sabet et al., 1999	Right-left Right-noncoronary Left-noncoronary With or without raphe	Raphe Raphe position	Aortic stenosis was the most common valve dysfunction. Raphe most common between right and left cusps. Absence of raphe occurred in 36% of cases.
Sievers and Schmidtke, 2007	Type 0: no raphe Type 1: one raphe Type 2: 2 raphes First subcategory: anteroposterior (AP), lateral (LAT), left-right (L-R), right-noncoronary (R-N), noncoronary-left (N-L) Second subcategory: predominant insufficiency (I), predominant stenosis (S), balanced insufficiency and stenosis (B) and normal function (No)	Main category: number of raphes First subcategory: spatial arrangement of the free edge of the cusps Second subcategory: functional status of the valve	The most common BAV phenotype was Type 1, L-R, S. "Pure" BAV with no raphe (Type 0) was quite rare. The least common phenotype was Type 2.
Kang et al., 2013	Type 1: AP BAV with raphe Type 2: AP BAV without raphe Type 3: R-N BAV with raphe Type 4: L-N BAV with raphe Type 5: R-N BAV or L-N BAV without raphe	Cusp and raphe presence and orientation	Aortic stenosis was predominant in patients with R-L BAV, aortic regurgitation in AP BAV. A normal aorta was the most common phenotype in AP BAV. Type 3 aortopathy was the most common phenotype in R-L BAV patients.

4.4 BAV-ASSOCIATED AORTOPATHY: AORTIC DILATATION AND DISSECTION

4.4.1 Pathophisiology

"Aortopathy" in BAV disease refers to progressive dilatation of the aorta which may include the root, the tubular ascending aorta, aortic arch and the descending thoracic aorta. Once again heterogeneity seems to be the rule as any, a combination of, or all segments can be involved (Figure 29). It is now well known that BAV patients have an increased risk of aortic dilatation and dissection; the prevalence of aortic dilatation increases with age, and surgical intervention to prevent dissection or rupture occurs more frequently at a younger age in patients with BAV than it does in patients with normal tricuspid aortic valve [Michelena HI et al., 2011]. The most common segment involved in BAV patients is the tubular ascending aorta (60-70% of BAV dilated aortas) [Michelena HI et al., 2014]. Growing rates for the tubular ascending segment in BAV adults have recently been reported to range from 0.4 to 0.6 mm/year [Detaint T et al., 2013], and these rates are generally independent of the valvular phenotype (i.e., right-left or right-noncusp fusion). In addition, absolute echocardiographic baseline aorta diameters do not

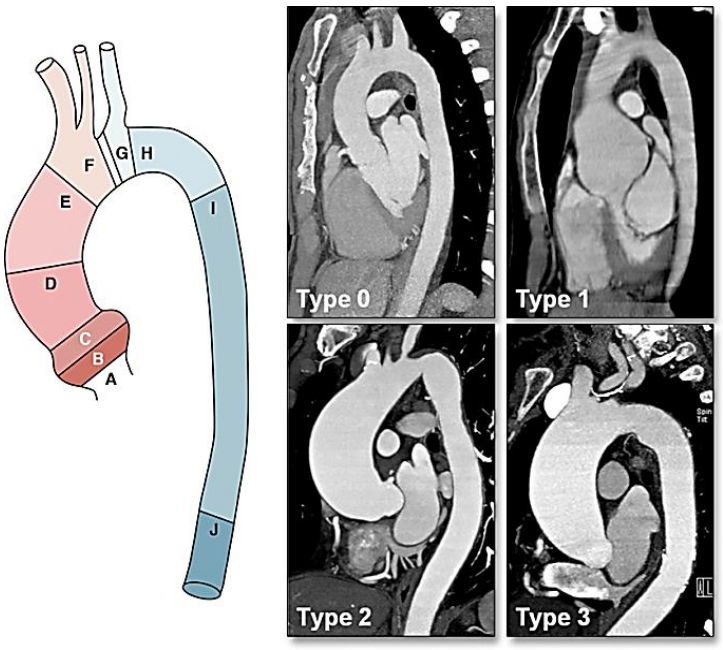


Fig 29. The 10 Levels Used in Aortic Dimension Measurements With Representative MDCT Images of Bicuspid Aortopathy Phenotypes
Type 0 is a normal aorta; type 1 is characterized by dilated aortic root. If the aortic enlargement involves the tubular portion of the ascending aorta, it is classified as type 2, whereas in type 3, there is diffuse involvement of the entire ascending aorta and the transverse aortic arch. A indicates the aortic annulus; B, sinuses of Valsalva; C, sinotubular junction; D, tubular portion of the ascending aorta; E, proximal to the innominate artery (or common trunk in case of a bovine arch); F, distal to the innominate artery (or common trunk); G, proximal to the left subclavian artery; H, distal to the left subclavian artery; I, proximal descending aorta, and J, distal descending thoracic aorta at the level of the diaphragmatic hiatus. MDCT = multidetector computed tomography (from Kang JW et al., 2013).

proportionally predict the rate of dilatation [Della Corte A et al., 2013], thus, systematic interval imaging follow-up is required in all BAV patients, regardless of baseline diameter [Michelena HI et al., 2015].

The pathogenesis of aortic aneurysm in BAV still remains to be fully elucidated. Many authors support the *abnormal hemodynamic theory*, whereby the abnormal hemodynamic stress on the aortic wall induced by eccentric turbulent flow through the bicuspid valve leads to subsequent aortopathy. The presence of increased systolic velocities has been demonstrated by echocardiography at the anterolateral ascending aortic wall. The degree and direction of flow jet may be crucial for determining the risk of segmental aneurysm formation. The evidence, obtained by cardiac magnetic resonance imaging of the ascending aorta of bicuspid aortic valve patients, of an abnormal blood flow that causes an asymmetrical, elevated wall shear stress [Hope MD et al., 2010 and 2011] and the demonstration that the various aortic valve morphologies determine different patterns of abnormal blood flow, could provide a valid explanation for aortic aneurysms and the relationship between aortic valve phenotype and aortic dilatation patterns proposed by many authors [Schaefer BM et al., 2008; Yuan SM et al., 2010]. According to the different direction of the flow jet, distributed along the right-posterior aortic wall in right-noncoronary bicuspid aortic valve and more toward the right-anterior wall in right-left bicuspid aortic valve, patients with right-left bicuspid aortic valve presented more often an involvement of the tubular portion of the ascending aorta, whereas patients with right-noncoronary bicuspid aortic valve showed a much higher prevalence of aortic root dilatation or a diffuse involvement of both the entire ascending aorta and the transverse aortic arch [Mahadevia R et al., 2014]. The evidence of a “regionality” of the aortic alterations in bicuspid aortic valve patients due to the asymmetrical wall shear stress reflects the histopathological findings of more severe changes in the convexity (right anterolateral wall) than in the concavity of the dilated ascending aorta [Cotrufo M et al., 2005]. Wall alterations in the ascending aortas of bicuspid aortic valve patients include cystic medial necrosis, elastic fiber fragmentation, loss of smooth muscle cells, and changes in their orientation in the medium layer of the aortic wall. These findings, common in several diseases that cause ascending aortic aneurysms, show a worse severity in bicuspid aortic valve patients compared with patients with trileaflet valves and aneurysms (Figure 30) [de Sa M et al., 1999]. VSMCs play a key role in the physiological and pathological remodeling of the aortic media as they are responsible for producing ECM proteins. High rates of VSMCs apoptosis and medial degeneration are present even in nondilated ascending aortas [Isselbacher EM et

al., 2005], suggesting an intrinsic underlying abnormality in patients with BAV. The convexity of the aorta is especially prone to high rates of VSMC apoptosis as well as medial degeneration which may explain the higher incidence of aortic dilatation in this region [Conti CA et al., 2010]. Nataatmadja and coworkers speculate that the loss of VSMCs is the primary cause of aortic wall weakness in patients with MFS and that the histological changes seen in BAV could be part of a continuum of aortopathy with aneurysms in tricuspid aortic valves and MFS patients representing the extremes [Nataatmadja M et al., 2003]. The histological changes in BAV are similar, though less severe than those found in patients with MFS [Bonderman T et al., 1999]; however, they appear to be more severe and occur at an early age than in patients with tricuspid valve [Schmid FX et al., 2004]. In the aortic media, MMPs activity is regulated by the presence of their inhibitors TIMPs and a growing body of evidence support the hypothesis of an increased MMP:TIMP ratio acting in aneurysm formation in individuals with BAV disease [Losenno KT et al., 2012]. In a study of surgically excised aortic valves, Wilton and colleagues [Wilton E et al., 2008] were unable to find differences in the level of expression of MMPs and TIMPs; however, they did find that there was a significant difference in the ratio of MMP-2 to TIMP-1 in patients with BAV compared to those with a TAV. Similarly, Lemaire and coworkers found a significant increase in MMP-2/TIMP-2 ratio in BAV aneurysms compared to control aortas. A recent and very interesting investigation by the group of Ikonomidis et al. [Ikonomidis JS et al., 2012] discovered that each BAV morphology has a unique pattern of MMP and TIMP activity. Their investigation revealed that individuals with L-R morphology have an elevated MMP/TIMP score ratio, suggesting that extracellular matrix degradation in these patients may be more aggressive.

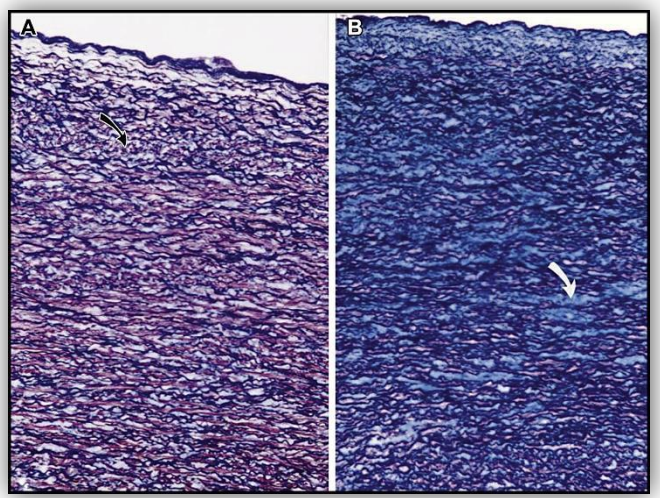


Fig 30. Comparison between ascending aorta alterations in tricuspid aortic valve and bicuspid aortic valve patients. (A) Transverse section of the ascending aorta from a 54-year-old patient with a normal tricuspid aortic valve. Circumferential arrangement of smooth muscle cells and elastic tissue plates. Small foci of accumulation of collagen (pale yellow white areas) and changes in the smooth muscle cell orientation (arrow). (B) Transverse section of the ascending aorta from a 33-year-old patient with bicuspid aortic valve disease. Significant medial destruction, accumulation of mucoid material (arrow; pool of mucopolysaccharide), marked fragmentation of elastic tissue, and large areas of loss of normal medial muscle (from: de Sa M et al, 1999)

The risk of developing TAAD is increased in patients with BAV (up to 75% of cases) with respect to those with three-leaflet aortic valves [Tadros TM et al., 2009]. Mild ascending aortic dilatation presents in middle to older age. On the other hand, aortic root dilatation is less common in BAV and it has been correlated with younger age of onset and male sex [Della Corte A et al., 2007]. A study conducted by Michelena HI and coworkers in 2008 [Michelena Hi et al., 2008] among 212 young patients with normally functioning or minimally dysfunctional BAV, reported that 30% of them developed TAA over a mean follow-up period of 10 years. TAA can therefore coexist with a normally functioning BAV supporting the theory in according to which developmental mechanisms should be more significant than hemodynamic disturbances, even if the precise mechanisms have not been established yet [Freeze SL et al., 2016]. Aortic root dilation is relatively prevalent (32%) in first-degree relatives of BAV patients according to Biner and coworkers [Biner S et al., 2009], who documented that aortic root dilation was also present in relatives with threeleaflet aortic valve morphology, these observations being consistent with the theory that BAV and TAA are both manifestations of a common developmental abnormality of the aorta. However, despite the "proposed" controversy regarding aortic dilatation representing an inherited/intrinsic predisposition versus being the result of abnormal aorta fluid hemodynamics, there is enough evidence to consider both as culprits in the origin and perpetuation of aortic dilatation in BAV [Michelena HI et al., 2015]. Aortic dilatation is an intrinsic tendency, given that non-BAV first-degree relatives of BAV patients with dilated aortas may exhibit aneurismal aortic disease [Loscalzo ML et al., 2007]. In addition, a lower incidence of aneurysm formation was observed in tricuspid valve patients who carried the same degree of aortic stenosis of BAV patients [Keane MG et al., 2000]. Moreover, although the aortic dilatation rate slows after aortic valve replacement (AVR) [Kim YJ et al., 2012], AVR does not halt aortic dilatation progression in BAV [Yasuda H et al., 2003], suggesting an intrinsic process. Besides, recent studies show that fluid hemodynamics and aortic wall stress in the BAV aorta are abnormal even in the absence of echocardiographic overt valvular dysfunction [Bissell MM et al., 2013], representing proof of concept that a "normally-functioning" BAV is intrinsically dysfunctional [Robicsek F et al., 2004], and also suggesting that hemodynamic-induced stress may contribute to aortic dilatation [Bissell MM et al., 2013]. Blood flow imaging with 3-dimensional time-resolved, phasecontrast magnetic resonance allows descriptive flow characterization and quantification of aortic wall shear stress [Hope MD et al., 2011]. Eccentric systolic jets resulting in abnormal right-

handed helical ascending aorta flow have been identified in BAV patients, even in normally functioning BAVs with normal aortic diameters [Meierhofer C et al., 2013]. Moreover, increased jet eccentricity was associated with greater aortic growth in BAV patients in a small retrospective cohort [Hope MD et al., 2012], suggesting that quantification of the hemodynamic contribution in BAV aortopathy could become a novel "imaging biomarker" for dissection risk-stratification [Hope MD et al., 2012]. Flow abnormalities appear to be dependent on BAV phenotype [Meierhofer C et al., 2013; Mahadevia R et al., 2014], such that right-left cusp fusion appears related more to proximal flow derangement with root dilatation and right-noncusp fusion related to more distally directed flow derangement with tubular ascending and arch dilatation, but the clinical implication of these observations is not yet fully defined. Although important transcriptional, protein and histopathologic alterations have been detected in aortas of BAV patients [Ikonomidis JS et al., 2006; Lehoux S and Tedgui A, 2003; Nataatmadja M et al., 2003], it remains to be elucidated whether these biochemical imbalances occur spontaneously or are the result of altered cellular mechanics and gene expression in response to shear stress in the BAV aorta, or both.

TAA in the presence of BAV poses a risk for aortic dissection but its incidence is relatively low if compared to the high incidence of progressive dilatation [Michelena Hi et al., 2015]. The largest contemporary community-based study of 416 BAV patients (age 35 ± 21 years) with the longest follow-up to date (mean 16±7 years) showed a risk of aneurysm of 26% at 25 years after the initial echocardiographic BAV diagnosis, incidence of approximately 85 cases per 10,000 patient-years, representing greater than 80 times the risk of aneurysm formation of the general population. This high aneurysm incidence translated into a risk of elective aorta repair surgery of 23% at 25 years, and an incidence of aortic dissection of approximately 3 cases per 10,000 patient-years (0.03%) which was low but still approximately 8 times higher than expected for the general population [Michelena HI et al., 2011]. In 1286 BAV patients followed after isolated AVR, the frequency of aortic dissection at 15 years was 1% [McKellar SH et al., 2010], highlighting the presence of a continued dissection risk after AVR in unrepaired aortas. This overall relatively low dissection incidence in BAV is only partially explained by prophylactic aortic repair, as official specific diameter cut offs for BAV aorta intervention were not published until 2006 [Bonow RO et al., 2006] and continue to be controversial in medical and surgical circles [Della Corte A et al., 2014], with some groups advocating aggressive aorta repair and others advocating a conservative approach. Michelena HI speculates that the following observations may

also account for the low dissection incidence: 1) compared to trileaflet aortic valve patients with ascending aortic dissection, BAV patients with dissection have a higher maximal aortic dimension before dissection and at the time of dissection [Eleid MF et al., 2013], thus, it is possible that dissections occur at larger diameters in BAV patients; 2) despite an equally fast progression of dilatation of the tubular ascending aorta in BAV compared to the root in Marfan syndrome patients, a high proportion of BAV patients do not progress at all [Detaint D et al., 2014], and [Edwards WD et al., 1978] when comparing trileaflet aortic valve patients to BAV patients with aortic aneurysms (mean baseline diameter 4.6 mm), a similar incidence of aortic catastrophes was observed [Davies RR et a., 2007] (although more BAV patients underwent elective repair), suggesting that the clinical outcome of BAV aortopathy may behave similar to that of the general population with aneurysms and less as a connective tissue disease [Michelena HI et al., 2015].

4.4.2 Genetic aspects of BAV

Although most BAV cases are sporadic, early studies identified familial clustering of BAV and its occurrence in monozygotic twins, supporting an underlying genetic abnormality [Della Corte A et al., 2016]. Subsequently, familial studies have established that the genetic effect (heritability) on BAV formation is as high as 89%, with ~9% prevalence in first-degree relatives [Huntington K et al., 1997]. Based on this evidence, it is advisable for first degree family members of BAV patients to receive an echocardiographic screening to detect BAV and potential valvular and extravalvular complications [Hiratzka LF et al., 2010].

An autosomal dominant inheritance pattern with decreased penetrance and variable expressivity is the most likely model, but the male predominance as well as association of BAV with Turner syndrome (45X chromosomal pattern) also support an X-linked etiology [Michelena Hi et al., 2014]. BAV also occurs as a component of other genetic syndromes, such as DiGeorge syndrome (DGS, OMIM#188400), LDS syndrome and Shone's complex [John AS et al., 2009; Loeys BL et al., 2005; Escarcega RO et al., 2014]. BAV is also found in association with other congenital heart disorders involving the left ventricular outflow tract (LVOT), such as hypoplastic left heart syndrome, coarctation of the aorta, and some ventricular septal defects, patent ductus arteriosus and atrial septal defects [Prakash SK et al., 2014]. The transcriptional regulator *NOTCH1* gene (chr 9q34.3), encoding a single-pass transmembrane receptor

and functioning in a highly conserved pathway, has been associated to the development and acceleration of calcium deposition in nonsyndromic BAV in humans [Mohamed SA et al., 2006]. The Notch signaling pathway plays a critical role in cell fate determination and differentiation during organogenesis and it regulates osteogenesis. However, a consistent contribution of *NOTCH1* gene variants to the development of BAV is yet to be clearly defined. Genome wide marker-based linkage analyses demonstrated a linkage of BAV to loci on chromosomes 18q, 5q and 13q in families with autosomal dominant inheritance of the disease. These regions are likely to contain genes whose mutations result in BAV and/or associated cardiovascular manifestations, suggesting their potential role in valvulogenesis and cardiac development. Recently, it has been reported the essential role of *GATA5* (GATA-binding protein 5) in aortic valve morphogenesis and endocardial cell differentiation [Laforest B et al., 2011]. However, the identification of rare *GATA5* variants has also been reported in control patients as well, suggesting that further investigation is required to define whether they can contribute to disease [Royer-Bertrand B and Rivolta C, 2015]. BAV has also been associated with a reduced *UFD1L* (ubiquitin fusion degradation 1-like; OMIM*601754) gene expression. The *UFD1L* gene encodes a component of a multienzyme complex involved in the degradation of ubiquitin fusion proteins during embryogenesis and its downregulation, probably due to an abnormal behavior of neural crest cells, may lead to reduced degradation activities. These results highlight the important role of the *UFD1L* gene in the development of ectoderm-derived structures, including neural crest cells in aortic leaflets formation, supporting the hypothesis of a genetic background in pathogenesis of BAV [Mohamed SA.et al., 2005]. Other genes have been associated with BAV. Wooten and colleagues demonstrated an association between BAV and a locus containing *AXIN1* and *PDIA2* genes in a cohort of 68 BAV probands and 830 control subjects [Wooten EC et al., 2010]. *AXIN1* (AXis INhibitor 1, OMIM*603816) is a critical member of the Wnt pathway and acts as a crucial regulator of both heart valve formation and cardiac neural crest development [Niessen K et al., 2008]. It also influences TGF-β signaling [Wang J et al., 2006]. The potential role of *PDIA2* (Protein Disulfide Isomerase family A, member 2, OMIM* 608012) in heart valve formation is unknown. Further genotyping is required to determine the relative contribution of *AXIN1* and *PDIA2* variants to BAV. About 12% of BAV/TAA patients carry mutations in *ACTA2,* a distinct subgroup characterized by livedo reticularis on lower limbs, and iris flocculi [Guo DC et al., 2007]. Regarding *TGFBR1* and *TGFBR2* genes analyses, only one incompletely clinically depicted BAV/TAA case reports a *TGFBR2* mutation

[Girdauskas E et al., 2011] while others demonstrated absence of *TGFBR1* and *TGFBR2* mutations in BAVs with aortic dilation [Arrington CB et al., 2008]. Animal studies have also been performed to understand the genetic bases of BAV disease. In mice, in particular, it has been demonstrated the association between BAV and the homozygous deletion of the endothelial nitric oxide synthase gene (*Nos3*); moreover, the haploinsufficiency in the cardiac homeobox gene *Nk2-5*, is linked to a higher incidence of BAV [Biben C et al., 2000].

Recently, a 4-fold increase in the prevalence of BAV in a large cohort of unrelated MFS patients (4.7%) with respect to the general population screened by echocardiography including primary school students in Italy (0.5%) was demonstrated [Basso C et al., 2004]. Indeed, out of a cohort of 257 unrelated MFS patients, BAV was unequivocally identified in 12 (4.7%). Three patients accepted to perform mutation screening analysis. Previously described mutations were detected in 2 out of 3 patients [Attanasio M et al., 2008], they involved conserved cysteine residues and are thought to be causative because they alter fibrillin-1 structure. These findings are consistent with data showing decreased *FBN1* mRNA or protein content in a subgroup of BAV patients, which suggest that *FBN1* may be one of the genes associated with BAV. Interestingly, the 12 MFS/BAV patients were males, a more common finding in BAV than in MFS cohorts. Moreover, the association between the two disorders seems to cause a more severe involvement of the aorta with a higher percentage of TAA requiring surgical intervention. MFS/BAV patients did not present further congenital heart diseases, aortic valve calcification or livedo reticularis and all of them patients satisfied the clinical diagnostic criteriafor MFS also according to the newly revised Ghent criteria. It is of interest that 2 out of our 3 MFS/BAV patients undergoing mutation analysis carried a *FBN1* mutation, adding further support to the diagnosis of MFS. Although a reduced *FBN1* content has been reported in the aortic media of BAV patients, it has been stated that in multiple clinical disorders associated with *FBN1* alterations there is no propensity for congenital aortic valve malformations [De Paepe et al., 1996]. These data challenged this consideration, prompting the need for the mutation screening analysis of BAV patients not fulfilling the clinical criteria for MFS. Moreover, from the practical point of view, the paper of Nistri et al. underscored the need to optimize the visualization of aortic valve morphology in MFS patients and the importance of comprehensive clinical evaluation of BAV patients to detect clinical features suggestive for inherited connective disorders [Nistri S et al., 2012].

In 2014, our group performed a study aimed to screen for *FBN1* mutations ten patients with BAV and thoracic aortic dilation not fulfilling the clinical criteria for MFS [Pepe G et al., 2014]. *FBN1* mutations were identified in two patients and this was the first study reporting pathogenetic *FBN1* mutations in patients with BAV and aortic dilatation/aneurysm in whom MFS and other more severe type 1 fibrillinopathies were clinically excluded according to the updated Ghent criteria. The detection of *FBN1* point mutations in patients with BAV/TAA without MFS, adds to the striking clinical heterogeneity of both BAV and type I fibrillinopathies showing that aortic dilatation/aneurysm may develop in a subgroup of BAV patients as a manifestation of an inherited connective tissue disorder [Pepe G et al., 2014].

Low frequency variants in *SMAD6*, which is highly expressed in the cardiac valves and outflow tract of the embryonic heart, have also been implicated as potential contributors to human BAV [Tan HL et al., 2012].

Additional genes (*eNOS* and *ACVR1*, Activin A Receptor type 1, OMIM*102576) have also been implicated as potential contributors to BAV from animal models, but their relevance to the genetics of human BAV remains to be defined [Lee TC et al., 2000; Thomas PS et al., 2012]. Finally, multiple other common genetic variants [e.g. angiotensin-converting enzyme (ACE) and matrix metalloproteinase (MMP) polymorphisms)] may act as modifier genes in the pathogenesis of BAV aortopathy, contributing to the variability of clinical phenotypes [Foffa I et al., 2012; Martìn M et al., 2013].

4.5 USE OF A TARGETED, COMBINATORIAL NGS APPROACH FOR THE STUDY OF BAV

BAV is associated with a number of long-term health risks if not identified early and managed appropriately. Pre-test genetic counseling is critical for all patients undergoing genetic testing. It has particular importance for individuals with BAV or TAAD who have low to moderate Ghent or Beighton scores for syndromic connective tissue disorders. Discussion with the patient should include the possibility of significant changes to medical management should genetic testing confirm a syndromic diagnosis. Of particular concern are the recommendations for surgical intervention at smaller

aortic diameters in certain syndromic conditions as well as additional management or surveillance recommendations [Freeze SL et al., 2016]. Given the lack of clear recommendations as to when cardiac screening should begin or at what intervals should be repeated for BAV and other LVOT defects, close communication between genetics and cardiology providers will be crucial to developing an individualized care plan for each affected individual and at-risk family members based on family history of specific left-sided lesions [McBride KL and Garg V, 2011].

Currently, the recent advances in next generation sequencing (NGS) technologies provide an unprecedented opportunity to reach a more comprehensive dissection of BAV heritability and associated aortopathy [Della Corte A et al., 2016]. In fact, given the relative cost-effectiveness of multi-gene panels and low yield of single gene sequencing, a larger TAAD panel could be considered over single-gene sequencing for patients with BAV and aortic dilation. Figure outlines an algorithm for considering molecular and/or cytogenetic testing for BAV cases [Freeze SL et al., 2016].

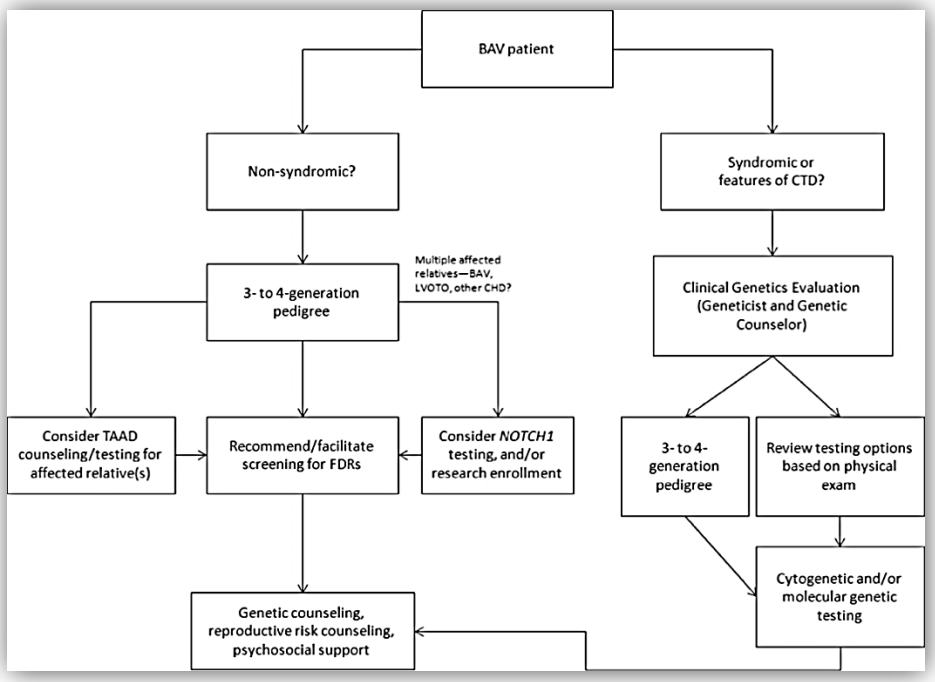


Fig 31. Clinical algorithm for patients with BAV. Acronyms used in this table: CTD = connective tissue disorder(s); TAAD = thoracic aortic aneurysms/dissections; FDRs = first-degree relatives (from Freeze SL et al., 2016)

Targeted NGS of a carefully selected part of the genome (a specific set of genes relevant to a disease phenotype) produces a more manageable data set compared with broader approaches, making analysis easier and faster [Wooderchak-Donahue WL et al., 2012]. A NGS design utilizing targeted sequencing of pooled BAV patient samples

identified 33 putative disease-causing variants, including variants in 26 genes not previously associated with human BAV, and merits further testing in replication cohorts [Bonachea EM et al., 2014]. Recently, targeted sequencing of the coding regions of nine genes previously associated with BAV (*NOTCH1*, *AXIN1*, *EGFR*, *ENG*, *GATA5*, *NKX2-5*, *NOS3*, *PDIA2* and *TGFBR2*) was performed in 48 patients with BAV [Dargis N et al., 2016]. Nine novel and 19 potentially pathogenic variants were identified bythis targeted NGS, but they were not associated with BAV in a case-control population. Sex-specific genetic variants associated with BAV were observed, further supporting the notion that the genetic basis of BAV development is sex dependent [Dargis N et al., 2016]. Based on genome-wide single nucleotide polymorphism array, a very recent study that identified 47 recurrent copy number variations (CNVs) in patients with early onset thoracic aortic disease, particularly in patients with BAV, and absent or extremely rare in controls [Prakash S and Kuang SQ, 2016]. These findings suggest that rare CNVs may disrupt the expression of cardiac or vascular developmental genes in these regions, further highlighting the genetic heterogeneity of BAV and the multiple disease mechanisms leading to aortopathy [Prakash S and Kuang SQ, 2016]. Recent data revealed that some epigenetic alterations, such as changes in DNA methylation and histone modification or regulation through microRNA (miRNA), are present in the DNA of developing heart in both experimental [Lewandowski SL et al., 2015] and human congenital heart disease [Lewandowski SL et al., 2015]. These epigenetic aberrations may contribute to the cause and/or pathogenesis of the malformation through deregulation of the expression of genes that are relevant for heart development [Vecoli C et al., 2014]. In addition, the epigenetic control may be sensitive to the biomechanical cues associated with BAV-related disturbed flow [Chen LJ et al., 2013], playing an important role in regulating gene functions in BAV [Gomez D et al., 2011].

Collectively, available evidence supports the notion that the clinical heterogeneity of BAV involves complex interactions between primary genetic defects, other genetic factors (gene modifiers), epigenetic factors (DNA methylation or histone modifications, miRNA), and hemodynamic abnormalities in aortic mechanics and valve morphology, setting the need of more integrated studies on the connections between genetic factors and pathological flow patterns in order further understand the clinical heterogeneity seen in BAV aortopathy as well as to develop methods for individualization of care [Della Corte et al., 2016].

CHAPTER 5

MATERIAL AND METHODS

5.1 GENOMIC ISOLATION FROM BLOOD SAMPLES

The genomic isolation has been made from blood sampling, collecting the sample through venipuncture, drawing the blood into a test tube containing an anticoagulant (EDTA) to stop it from clotting, using the GeneCatcher technology. GeneCatcher™ technology has been designed and optimized for scalable and automated genomic DNA isolation from blood samples using the Tecan® Freedom EVO® liquid handling platform (Tecan Group Ltd, Switzerland) (Figure 32); it requires no centrifugation or filtration steps and uses reagents that will not clog lines or cause vapor pressure buildup. Furthermore, a magnetic rack (separator) allows processing of several samples simultaneously during the magnetic bead-separation steps. In order to obtain the best results with the GeneCatcher™ gDNA 0.3-1 mL Blood Kit, the 24-well Magnetic Separator is recommended. These magnetic separators are compatible with the volumes used in the kit protocols and provide effective magnetic strength from neodymium magnets, which are aligned with plate wells or tubes, respectively. How GeneCatcher™ technology works (Figure 33):

Fig 32. Tecan Freedom EVO platform

The novel three-step clean-up process of GeneCatcher™ technology are DNA capture, DNA purification and DNA elution.

GeneCatcher™ gDNA 0.3-1 ml Blood Kit:

- STEP 1:
- Follow the steps below to purify up to 100 µg of genomic DNA from 0.3–1 mL of human blood.

- Add 60 µL of GeneCatcherTM Magnetic Beads to each well of a 24-well deep-well plate.

- Add 2.5 mL of Lysis Buffer (L13) to each well and agitate the plate gently to mix.

- Add 1 mL of blood to each well and agitate gently to mix.

- Incubate at room temperature for 5 minutes with occasional mixing.

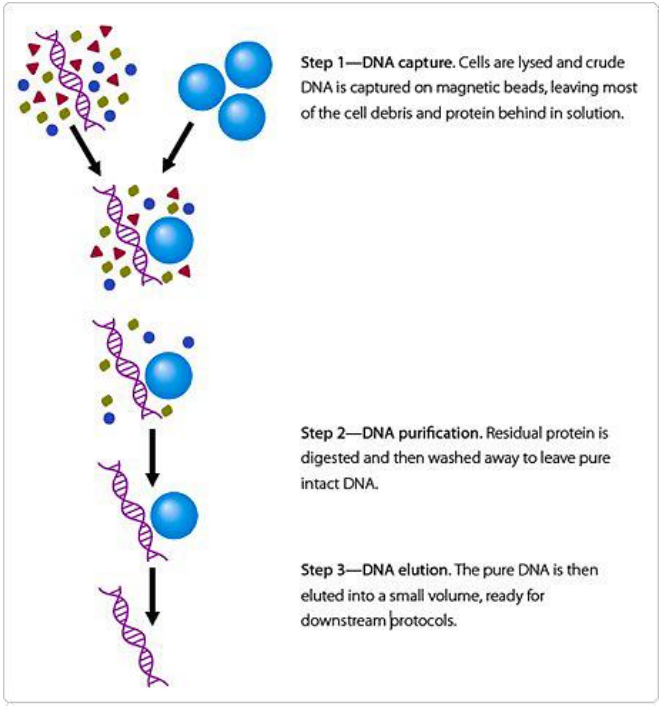


Fig 33. Gene Catcher™ technology workflow

- Place the plate on the 24-well Magentic Separator for 3 minutes.

- Remove and discard the supernatant, then remove the plate from the magnet.

- Add 2.5 mL of Lysis Buffer (L13) to each well, and agitate the plate for 10–20 seconds to mix.

- Place the plate on the magnetic separator for 1 minute.

- Remove and discard the supernatant, then remove the plate from the magnet.

- Add 0.5 mL of Protease Buffer and 10 µL of Protease to each well.

- Gently swirl the plate until the pellets are fully dispersed.

- Incubate at 65°C for 10 minutes.

- Allow the plate to cool to room temperature, and agitate gently to ensure that the pellets are dispersed.

- STEP 2:

- Set water bath at 65°C.

- Vortex the GeneCatcherTM Magnetic Beads to resuspend.

- Add 0.5 mL of Protease Buffer and 10 µL of Protease to each well.

- Gently swirl the plate until the pellets are fully dispersed.

- Incubate at 65°C for 10 minutes.

- Allow the plate to cool to room temperature, and agitate gently to ensure that the pellets are dispersed.

- Add 0.5 mL of 100% isopropyl alcohol (IPA) to each well, and agitate until a visible aggregate has formed.

- Place the plate on the magnetic separator for 30 seconds.

- Remove and discard the supernatant, then remove the plate from the magnet.

- Add 1 mL of 50% (v/v) aqueous IPA, and agitate the plate for 15 seconds.

- Place the plate on the magnetic separator for 30 seconds, then remove the supernatant and discard.

- Gently add 150 µL of Wash Buffer (W11) to the side of each plate well, and incubate for 30 seconds.

- Remove and discard the supernatant, then remove the plate from the magnet.

- STEP 3:

-Add 250 µL of Elution Buffer (E5) to each well, and agitate the plate gently until each pellet has been dislodged from the well wall.

- Incubate at 65°C for 30 minutes.

- Allow the plate to cool to room temperature, and agitate gently to ensure that the pellets are fully dispersed.

- Place the plate on the magnetic separator until the supernatant is totally clear and colorless.

- Remove the supernatant containing the purified DNA to a clean the plate.

5.2 QUANTITATION AND QUALITY ASSESSMENT OF THE GENOMIC DNA

Nucleic acid samples can be readily checked for concentration and quality using the NanoDrop 1000 Spectrophotometer. Absorbance measurements made on any spectrophotometer, including the Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the NanoDrop™ 8000 Spectrophotometer (Figures 34 and 35), will include the absorbance of all molecules in the sample that absorb at the wave-length of interest permitting us to mesure the concentration of the genomic DNA. Since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, they will contribute to the total absorbance of the sample. Therefore to ensure accurate results nucleic acid samples will require purification prior to measurement. The NanoDrop 1000 Spectrophotometer will accurately measure DNA samples up to 3700 ng/ul without dilution. To do this, the instrument automatically detects the high concentration and utilizes the 0.2 mm pathlength to calculate the absorbance. To obtain a quality assesment it's necessary to analyse the following data: 260/280: ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is 5-2 appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. 260/230: ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

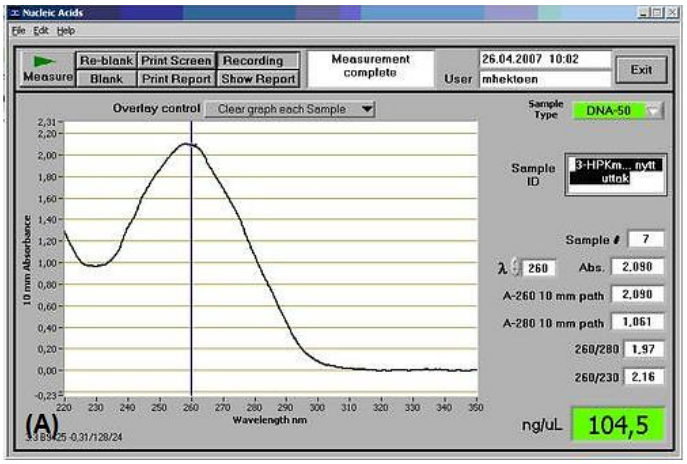


Fig 34. Quality assessment of DNA

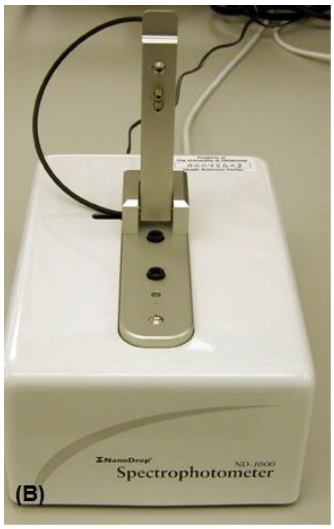


Fig 35. Nanodrop

5.3 NGS: NEXT GENERATION SEQUENCING

NGS platforms share a common technological feature massively parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. This design is a paradigm shift from that of Sanger sequencing, which is based on the electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions or, in one format, by iterative cycles of oligonucleotide ligation. As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide-sequence output in a single instrument run, depending on the platform.

5.3.1 Roche 454 platform

In 2007 Roche Applied Science acquired 454 Life Sciences and introduced the 454 instrument as the first commercially available NGS platform (Figure 36). This method permitted to obtain sequencing from different DNA molecules: genomic DNA, DNA derived from PCR, cDNA and BAC. For sequencing, a library of template DNA is prepared by fragmentation via nebulization. Fragments several hundred base pairs in length are end-repaired and ligated to adapter oligonucleotides. The library is then diluted to single-molecule concentration, denatured, and and hybridized to individual beads containing sequences complementary to adapter oligonucleotides. The beads are compartmentalized into water-in-oil microvescicles, where clonal expansion of single DNA molecules bound to the beads occurs during emulsion PCR. After amplification, the emulsion is disrupted, and the beads containing clonally amplified template DNA are enriched. The beads are again separated by limiting dilution, deposited into individual picotiter-plate wells, and combined with sequencing enzymes. Loaded into the GS FLX, the picotiter plate functions as a flow cell wherein iterative pyrosequencing is performed by successive flow addition of the 4 dNTPs. A nucleotide-incorporation event in a well

Fig 36. 454-GS FLX+ System

containing clonally amplified template produces pyrophosphate release and picotiter-plate well-localized luminescence, which is transmitted through the fiber-optic plate and recorded on a charge-coupled device camera (the pyrophosphate release represents a point of innovation). With the flow of each dNTP reagent, wells are imaged, analyzed for their signal-to-noise ratio, filtered according to quality criteria, and subsequently algorithmically translated into a linear sequence output. With the newest chemistry, termed "Titanium," a single GS FLX run generates approximately 1,106 sequence reads, with read lengths of ≥400 bases yielding up to 500 million base pairs (Mb) of sequence. A recognized strength of the 454 technology is the longer read length, which facilitates de novo assembly of genomes. The patients in our study were submitted to high throughput genotyping using a targeted sequencing strategy. Using a targeted capture approach, we designed a panel of 91 genes regarded as relevant in BAV, syndromic and non-syndromic aortopathy or involved in vessels or valves growth and remodeling processes by reviewing literature *(COL11A1, MTHFR, PLOD1, ADAMTSL4, MFAP2, PTGS2, SKI, TGFB2, CAPN2, AGT, MTR, TGFBR3, FGF8, RET, ACTA2, B3GAT3, LTBP3, EFEMP2/fbln4, LRP5, CCND1, LRP6, COL2A1, LRP1, DCN, LTBP2, TGFB3, FBLN5, ADAMTS17, CHST14, FBN1, SMAD3, MYH11, ABCC6, MAPK3, PDIA2, AXIN1, MMP2, CRYBA1, COL1A1, ACE, KCNJ2, EMILIN2, SMAD2, SMAD4, LTBP4, TGFB1, ADAMTS10, MMADHC, ACVR1, COL3A1, COL5A2, FN1, COL6A3, EMILIN1, LTBP1, JAG1, EMILIN3, MMP9, SLC2A10, GATA5, CBS, COL6A1, COL6A2, UFD1L, MAPK1, VHL, ZPLD1, MYLK, AGTR1, PDCD10, TGFBR2, FBN2, NKX2-5, B4GALT7, ADAMTS2, AGGF1, MTRR, NOS3, HOXA1, CCM2, KRIT1, COL1A2, TGFBR1, PTGS1, ENG, COL5A1, NOTCH1, GNAQ, AGTR2, FLNA, ELN).* Genomic DNA libraries were prepared according to Roche sample preparation protocol (Rapid Library Preparation Method Manual, Roche NImblegen Inc., Madison,WI) (Table 8). The customized capture array was designed to capture all coding exons, and flanking intron sequences of the 91 genes, according to

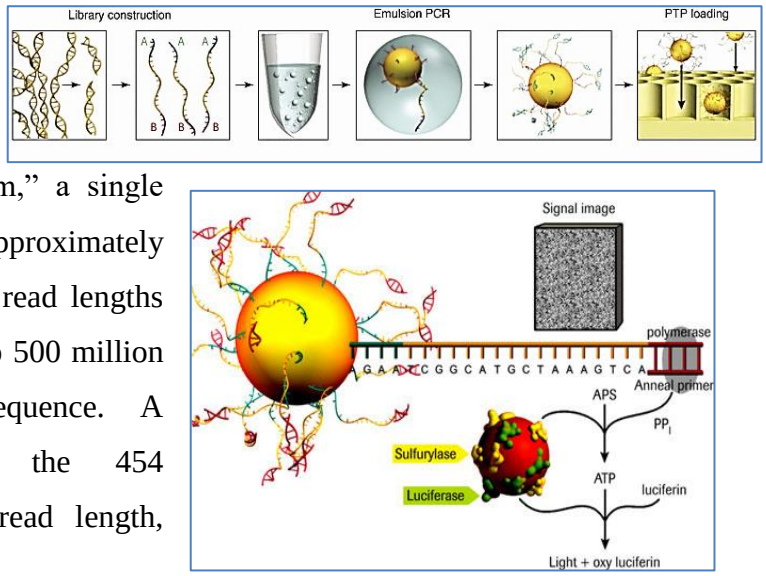


Fig 37. 454-GS FLX+ Systemworkflow

the manufacturer's protocol (NimbleGen Arrays User's Guide: 454 Optimized Sequence Capture). Hybridization and washing procedures were performed according to Roche standard protocol. Sequencing run was then performed with the 454 Roche instrument. Sequencing runs have a mean coverage depth 45x.The standard flow files (SFF) were analyzed with the GS Mapper software, version 2.6 (Roche Diagnostic, Basel, Switzerland).The gene variants calls were also evaluated by using an in-house pipeline developed by our bioinformaticians. Raw reads data were aligned to the human reference genome (hg19) with BWA version 0.7.12. Alignment and coverage statistics were generated with GATK version 3.3, Depth of Coverage version 3.0, SAMTOOLS version 1.2, and custom R scripts. Detection of SNVs and insertions/deletions (indels) was performed with the GATK UnifiedGenotyper. Detected variants were annotated with Variant Effect Predictor version. NGS experiments had a median coverage ~98% and a 45X depth of coverage.

Table 8. Targeted capture gene list

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
COL11A1	NM_001854	chr1:103342023-103574052	7191	Homo sapiens collagen, type XI, alpha 1 (COL11A1), transcript variant A, mRNA
MTHFR	NM_005957	chr1:11845787-11866160	7150	Homo sapiens methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR), mRNA
PLOD1	NM_000302	chr1:11994724-12035599	3047	Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1), mRNA
ADAMTSL4	NM_019032	chr1:150521898-150533412	4209	Homo sapiens ADAMTS-like 4 (ADAMTSL4), transcript variant 1, mRNA
MFAP2	NM_002403	chr1:17300999-17307173	1124	Homo sapiens microfibrillar-associated protein 2 (MFAP2), transcript variant 2, mRNA
PTGS2	NM_000963	chr1:186640944-186649559	4507	Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
SKI	NM_003036	chr1:2160134-2241652	5707	Homo sapiens v-ski sarcoma viral oncogene homolog (avian) (SKI), mRNA
TGFB2	NM_001135599	chr1:218518676-218617961	5966	Homo sapiens transforming growth factor, beta 2 (TGFB2), transcript variant 1, mRNA
CAPN2	NM_001748	chr1:223900119-223963720	3492	Homo sapiens calpain 2, (m/II) large subunit (CAPN2), transcript variant 1, mRNA
AGT	NM_000029	chr1:230838272-230850336	2587	Homo sapiens angiotensinogen (serpin peptidase inhibitor, clade A, member 8) (AGT), mRNA

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
MTR	NM_000254	chr1:236958581-237067281	10558	Homo sapiens 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), mRNA
TGFBR3	NM_003243	chr1:92145900-92351836	6475	Homo sapiens transforming growth factor, beta receptor III (TGFBR3), transcript variant 1, mRNA
FGF8	NM_033163	chr10:103529887-103535759	1036	Homo sapiens fibroblast growth factor 8 (androgen-induced) (FGF8), transcript variant F, mRNA
RET	NM_020975	chr10:43572517-43625797	5629	Homo sapiens ret proto-oncogene (RET), transcript variant 2, mRNA.
ACTA2	NM_001141945	chr10:90694980-90708687	1798	Homo sapiens actin, alpha 2, smooth muscle, aorta
B3GAT3	NM_012200	chr11:62382768-62389647	2073	beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I) (B3GAT3), mRNA
LTBP3	NM_001130144	chr11:65306030-65325699	4721	Homo sapiens latent transforming growth factor beta binding protein 3 (LTBP3), transcript variant 1, mRNA
EFEMP2/fbln4	NM_016938	chr11:65633912-65640405	2096	Homo sapiens EGF containing fibulin-like extracellular matrix protein 2 (EFEMP2), transcript variant 1
LRP5	NM_002335	chr11:68080108-68216743	5161	Homo sapiens low density lipoprotein receptor-related protein 5 (LRP5), mRNA
CCND1	NM_053056	chr11:69455873-69469242	4034	Homo sapiens cyclin D1 (CCND1), mRNA
LRP6	NM_002336	chr12:12268961-12419811	10088	Homo sapiens low density lipoprotein receptor-related protein 6 (LRP6), mRNA
COL2A1	NM_001844	chr12:48366748-48398285	5087	Homo sapiens collagen, type II, alpha 1 (COL2A1), transcript variant 1, mRNA
LRP1	NM_002332	chr12:57522282-57607125	14905	Homo sapiens low density lipoprotein receptor-related protein 1 (LRP1), mRNA
DCN	NM_001920	chr12:91539835-91572329	2305	Homo sapiens decorin
LTBP2	NM_000428	chr14:74964886-75079034	8568	Homo sapiens latent transforming growth factor beta binding protein 2 (LTBP2), mRNA
TGFB3	NM_003239	chr14:76424442-76448092	3183	Homo sapiens transforming growth factor, beta 3 (TGFB3), mRNA.
FBLN5	NM_006329	chr14:92335755-92414046	2637	Homo sapiens fibulin 5 (FBLN5), mRNA
ADAMTS17	NM_139057	chr15:100511643-100882183	6331	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 17 (ADAMTS17), mRNA

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
CHST14	NM_130468	chr15:40763160-40765357	2313	Homo sapiens carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 14
FBN1	NM_000138	chr15:48700503-48937985	11695	Homo sapiens fibrillin 1 (FBN1), mRNA
SMAD3	NM_005902	chr15:67358195-67487533	6256	Homo sapiens SMAD family member 3 (SMAD3), transcript variant 1, mRNA
MYH11	NM_001040113	chr16:15802668-15932109	6942	Homo sapiens myosin, heavy polypeptide 11
ABCC6	NM_001171	chr16:16243422-16317328	5125	Homo sapiens ATP-binding cassette, sub-family C (CFTR/MRP), member 6 (ABCC6), transcript variant 1, mRNA
MAPK3	NM_002746	chr16:30125426-30134630	1902	Homo sapiens mitogen-activated protein kinase 3 (MAPK3), transcript variant 1, mRNA.
PDIA2	NM_006849	chr16:332615-337209	1726	Homo sapiens protein disulfide isomerase family A, member 2 (PDIA2), mRNA
AXIN1	NM_003502	chr16:337440-402676	3675	Homo sapiens axin 1 (AXIN1), transcript variant 1, mRNA
MMP2	NM_004530	chr16:55513081-55540586	3549	Homo sapiens matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase) (MMP
CRYBA1	NM_005208	chr17:27573875-27581502	806	Homo sapiens crystallin, beta A1 (CRYBA1), mRNA
COL1A1	NM_000088	chr17:48261457-48279000	5927	Homo sapiens collagen, type I, alpha 1 (COL1A1), mRNA
ACE	NM_000789	chr17:61554422-61575741	4969	Homo sapiens angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (ACE), transcript variant 1, mRNA
KCNJ2	NM_000891	chr17:68165676-68176183	5397	Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA
EMILIN2	NM_032048	chr18:2847028-2914090	4009	Homo sapiens elastin microfibril interfacer 2 (EMILIN2), mRNA
SMAD2	NM_001003652	chr18:45359466-45457517	10551	Homo sapiens SMAD family member 2 (SMAD2), transcript variant 2, mRNA
SMAD4	NM_005359	chr18:48556583-48611411	8789	Homo sapiens SMAD family member 4 (SMAD4), mRNA
LTBP4	NM_001042544	chr19:41103141-41135725	5163	Homo sapiens latent transforming growth factor beta binding protein 4 (LTBP4), transcript variant 1, mRNA
TGFB1	NM_000660	chr19:41836812-41859831	2217	Homo sapiens transforming growth factor, beta 1 (TGFB1), mRNA
ADAMTS10	NM_030957	chr19:8645126-8675588	4237	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 10 (ADAMTS10), mRNA

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
MMADHC	NM_015702	chr2:150426147-150444330	1466	Homo sapiens methylmalonic aciduria (cobalamin deficiency) cblD type, with homocystinuria (MMAD)
ACVR1	NM_001105	chr2:158594043-158656005	3183	Homo sapiens activin A receptor, type I (ACVR1), transcript variant 2, mRNA
COL3A1	NM_000090	chr2:189839099-189877472	5490	Homo sapiens collagen, type III, alpha 1 (COL3A1), mRNA
COL5A2	NM_000393	chr2:189896641-190044605	6930	Homo sapiens collagen, type V, alpha 2 (COL5A2), mRNA
FN1	NM_212482	chr2:216225179-216300791	8815	Homo sapiens fibronectin 1 (FN1), transcript variant 1, mRNA
COL6A3	NM_004369	chr2:238232655-238322850	10599	Homo sapiens collagen, type VI, alpha 3 (COL6A3), transcript variant 1, mRNA
EMILIN1	NM_007046	chr2:27301435-27309265	3959	Homo sapiens elastin microfibril interfacer 1 (EMILIN1), mRNA
LTBP1	NM_206943	chr2:33172369-33624575	6168	Homo sapiens latent transforming growth factor beta binding protein 1 (LTBP1), transcript variant 1, mRNA
JAG1	NM_000214	chr20:10618332-10654694	5988	Homo sapiens jagged 1 (JAG1), mRNA
EMILIN3	NM_052846	chr20:39988606-39995498	3827	Homo sapiens elastin microfibril interfacer 3 (EMILIN3), mRNA
MMP9	NM_004994	chr20:44637547-44645200	2387	Homo sapiens matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
SLC2A10	NM_030777	chr20:45338279-45364985	4241	Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 10 (SLC2A10), mRNA
GATA5	NM_080473	chr20:61038553-61051026	2595	Homo sapiens GATA binding protein 5 (GATA5), mRNA
CBS	NM_000071	chr21:44473990-44492303	2609	Homo sapiens cystathionine-beta-synthase
COL6A1	NM_001848	chr21:47401663-47424963	4246	Homo sapiens collagen, type VI, alpha 1 (COL6A1), mRNA
COL6A2	NM_001849	chr21:47518033-47552763	3455	Homo sapiens collagen, type VI, alpha 2 (COL6A2), transcript variant 2C2, mRNA
UFD1L	NM_005659	chr22:19437464-19466738	1783	Homo sapiens ubiquitin fusion degradation 1 like (yeast) (UFD1L), transcript variant 1, mRNA
MAPK1	NM_002745	chr22:22113947-22221970	5916	Homo sapiens mitogen-activated protein kinase 1 (MAPK1), transcript variant 1, mRNA
VHL	NM_000551	chr3:10183319-10195354	4560	Homo sapiens von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase (VHL)

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
ZPLD1	NM_175056	chr3:102153859-102198685	3639	Homo sapiens zona pellucida-like domain containing 1 (ZPLD1), mRNA
MYLK	NM_053025	chr3:123331143-123603149	7852	Homo sapiens myosin light chain kinase (MYLK), transcript variant 1, mRNA
AGTR1	NM_031850	chr3:148447967-148459902	2426	Homo sapiens Renal tubular dysgenesis, Hypertension
PDCD10	NM_007217	chr3:167402096-167437945	1454	Homo sapiens programmed cell death 10
TGFBR2	NM_001024847	chr3:30647994-30735633	4704	Homo sapiens transforming growth factor, beta receptor II (70/80kDa) (TGFBR2), transcript variant 1, mRNA
FBN2	NM_001999	chr5:127593601-127873735	10724	Homo sapiens fibrillin 2 (FBN2), mRNA
NKX2-5	NM_004387	chr5:172659107-172662315	1961	Homo sapiens NK2 homeobox 5 (NKX2-5), transcript variant 1, mRNA
B4GALT7	NM_007255	chr5:177027119-177037346	1747	Homo sapiens xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 (B4GALT7), mRNA
ADAMTS2	NM_014244	chr5:178537852-178772431	6772	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 2 (ADAMTS2)
AGGF1	NM_018046	chr5:76326210-76361058	4514	Homo sapiens angiogenic factor with G patch and FHA domains 1 (AGGF1), mRNA
MTRR	NM_002454	chr5:7870908-7900171	3317	Homo sapiens 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
TNXB	NM_019105	chr6:32008932-32077151	13143	Homo sapiens tenascin XB (TNXB), transcript variant XB, mRNA
COL11A2	NM_080680	chr6:33130469-33160245	6425	Homo sapiens collagen, type XI, alpha 2 (COL11A2), transcript variant 1, mRNA
COL9A1	NM_001851	chr6:70925743-71012786	3805	Homo sapiens collagen, type IX, alpha 1 (COL9A1), transcript variant 1, mRNA
NOS3	NM_000603	chr7:150688144-150711687	4345	Homo sapiens nitric oxide synthase 3 (endothelial cell) (NOS3), transcript variant 1, mRNA
HOXA1	NM_005522	chr7:27132614-27135625	2561	Homo sapiens homeobox A1 (HOXA1), transcript variant 1, mRNA
CCM2	NM_001029835	chr7:45067233-45116069	2161	Homo sapiens cerebral cavernous malformation 2 (CCM2), transcript variant 1, mRNA
KRIT1	NM_194455	chr7:91830050-91871449	4762	Homo sapiens KRIT1, ankyrin repeat containing
COL1A2	NM_000089	chr7:94023873-94060544	5087	Homo sapiens collagen, type I, alpha 2 (COL1A2), mRNA
TGFBR1	NM_004612	chr9:101867412-101916473	6475	Homo sapiens transforming growth factor, beta receptor 1 (TGFBR1), transcript variant 1, mRNA

Gene	*ID*	*Chromosomal location*	*Gene lenght (bp)*	*Codified protein*
PTGS1	NM_001271368	chr9:125134095-125154823	5382	Homo sapiens prostaglandin-endoperoxide synthase 1
ENG	NM_000118	chr9:130578196-130616634	3201	Homo sapiens endoglin
COL5A1	NM_001278074	chr9:137533651-137736688	8455	Homo sapiens collagen, type V, alpha 1 (COL5A1), transcript variant 2, mRNA
NOTCH1	NM_017617	chr9:139388896-139440238	9309	Homo sapiens notch 1 (NOTCH1), mRNA
GNAQ	NM_002072	chr9:80335191-80646219	2215	Homo sapiens guanine nucleotide binding protein (G protein), q polypeptide (GNAQ), mRNA
AGTR2	NM_000686	chrX:115301958-115306225	2906	Homo sapiens angiotensin II receptor, type 2 (AGTR2), mRNA
FLNA	NM_001110556	chrX:153576900-153603006	8557	Homo sapiens filamin A, alpha (FLNA), transcript variant 2, mRNA
ELN	NM_000501	chr7 74027789-74069907	3480	Homo sapiens elastin

5.4 SEQUENCING GOLD STANDARD TECHNIQUES: THE SANGER METHOD

The Sanger method has an important role in detecting mutations and it is still used to confirm the mutations that may previously be discovered with roche 454 platform. Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. Developed by Frederick Sanger and colleagues in 1977, it was the most widely used sequencing method for approximately 25 years. The classical chain-termination method requires a single-stranded DNA template derived from the denaturation of a cloning vector, a DNA primer, a DNA polymerase, normal deoxynucleosidetriphosphates (dNTPs), modified di-deoxynucleosidetriphosphates (ddNTPs), the latter end for terminate DNA strand elongation. These chainterminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase extension of DNA when a modified ddNTP is incorporated. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while the four other nucleotides are ordinary ones. The deoxynucleotide is added in approximately 100-fold excess of the corresponding dideoxynucleotide (e.g. 0.5mM dATP:0.005mM ddATP)

allowing for enough fragments to be produced while still transcribing the complete sequence. Putting it in a more sensible order, four separate reactions are needed in this process to test all four ddNTPs. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments are heat denatured and separated by size using gel electrophoresis. In the original publication of 1977, the formation of base-paired loops of ssDNA was a cause of serious difficulty in resolving bands at some locations. This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C). The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image. In the image on the right, X-ray film was exposed to the gel, and the dark bands correspond to DNA fragments of different lengths. A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The relative positions of the different bands among the four lanes, from bottom to top, are then used to read the DNA sequence. Technical variations of chain-termination sequencing include tagging with nucleotides containing radioactive phosphorus for radiolabelling, or using a primer labeled at the 5' end with a fluorescent dye. Dye-primer sequencing facilitates reading in an optical system for faster and more economical analysis and automation. The later development by Leroy Hood and co-workers of fluorescently labeled ddNTPs and primers set the stage for automated, high-throughput DNA sequencing. Chaintermination methods have greatly simplified DNA sequencing. For example, chaintermination- based kits are commercially available that contain the reagents needed for sequencing, pre-aliquoted and ready to use. Limitations include non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequence, and DNA secondary structures affecting the fidelity of the sequence. *Dye-terminator sequencing* utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than four reactions as in the labelled primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which emit light at different wavelengths**.** Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing. Its limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has been addressed with the use of modified DNA polymerase enzyme systems

and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs". The dye-terminator sequencing method, along with automated high-throughput DNA sequence analyzers, is now being used for the vast majority of sequencing projects.Sequence ladder by radioactive sequencing compared to fluorescent peaks. DNA sequencers separate strands by size (or length) using capillary electrophoresis, they detect and record dye fluorescence, and output data as fluorescent peak trace chromatograms. Sequencing reactions (thermocycling and labelling), cleanup and re-suspension of samples in a buffer solution are performed separately, before loading samples onto the sequencer. A number of commercial and non-commercial software packages can trim low-quality DNA traces automatically. These programs score the quality of each peak and remove low-quality base peaks (which are generally located at the ends of the sequence). The accuracy of such algorithms is inferior to visual examination by a human operator, but is adequate for automated processing of large sequence data sets.

5.5 BIOINFORMATIC ANALYSIS OF DATA

While different sequencing technologies may use different initial raw data (e.g., imaging files or fluctuations in current), the eventual outputs are nucleotide base calls. Short strings of these bases, varying from dozens to hundreds of base pairs for each fragment, are combined together, often in a form of a FASTQ file [Dolled-Filhart MP et al., 2013]. From here, bioinformatics analysis of the sequence falls into three general steps:

(1) alignment, matching each of the short reads to positions on a reference genome

(2) variant calling

(3) filtering and annotation.

Alignment is the process of mapping short nucleotide reads to a reference genome. Because each of the millions of short reads must be compared to the 3 billion possible positions within the human genome, this computational step is not trivial. This step is thus computationally intense and time consuming. The Sequence Alignment/Map (SAM) and Binary Alignment/Map (BAM) formats are the standard file formats for storing NGS read alignments. There are various software programs, some commercially available and others freely available to the scientific community, that can be used to perform sequencing read alignment.

The most widely used sequence alignment algorithm may be the Smith-Waterman

algorithm that was first proposed by Smith and Waterman in 1981 and optimized by Gotoh in 1982. It performs local sequence alignment, which is designed especially for dissimilar sequences that are suspected to contain regions of similarity or similar sequence motifs within their larger sequence context. To determine similar regions between two nucleotide or protein sequences, the Smith-Waterman algorithm, instead of looking at the total sequence, compares segments of all possible lengths and optimizes the similarity measure. The Smith-Waterman finds the alignment in a more quantitative way by giving scores for matches and mismatches for every possible pair of residues. The scores are predefined in scoring matrices, such as PAM (point accepted mutation) and BLOSUM (blocks substitution matrix). In general, a positive score is assigned for a match, a negative score for a mismatch, and a negative score for a gap penalty.After alignment of the short reads to the reference genome, the next step in the bioinformatics process is variant calling. Since the short reads are already aligned, the sample genome can be compared to the reference genome and variants can then be identified. These variants may be responsible for disease, or they may simply be genomic noise without any functional effect. Variant call format (VCF) is the standardized generic format for storing sequence variation including SNPs, indels, larger structural variants and annotations. The computational challenges in SNP (variant) calling are due to the issues in identifying "true" variants versus alignment and/or sequencing errors. Yet the ability to detect SNPs with both high sensitivity and specificity is a key step in identifying sequence variants associated with disease, detection of rare variants, and assessment of allele frequencies in populations. The difficulty of variant calling is complicated by three factors: (1) the presence of indels, which represent a major source of false positive SNV identifications, especially if alignment algorithms do not perform gapped alignments; (2) errors from library preparation due to PCR artifacts and variable GC content in the short reads unless paired-end sequencing is utilized; and (3) variable quality scores, with higher error rates generally found at bases at the ends of reads. Therefore, the rate of false positive and false negative calls of SNVs and indels is a concern. A detailed review of SNPcalling algorithms and challenges recommends recalibration of per-base quality scores (e.g., GATK, SOAPsnp), use of an alignment algorithm with high sensitivity (e.g., Novoalign, Stampy), and SNP calling using Bayesian procedures or likelihood ratio tests and incorporation of linkage disequilibrium to improve SNP call accuracy. We provide an overview of some of the software packages for variant calling below.

5.5.1 Sorting Intolerant from Tolerant (SIFT)

The SIFT program uses "sequence homology to predict whether an amino acid substitution will affect protein function and hence, potentially alter phenotype. "Sorting Intolerant From Tolerant" (SIFT) prediction is based on conserved amino acid residues through different species using comparative sequencing analysis through PSIBLAST.

This relies on the presumption that amino acid residues that are essential for protein function should be evolutionally conserved by natural selection. To predict whether an amino acid substitution in a protein will affect protein function, SIFT considers the position at which the change occurred and the type of amino acid change. Given a protein sequence, SIFT chooses related proteins and obtains an alignment of these proteins with the query. Based on the amino acids appearing at each position in the alignment, SIFT calculates the probability that an amino acid at a position is tolerated conditional on the most frequent amino acid being tolerated. If this normalized value is less than a cutoff, the substitution is predicted to be deleterious (Figures 38 and 39) [Pauline C. Ng et al. 2003].

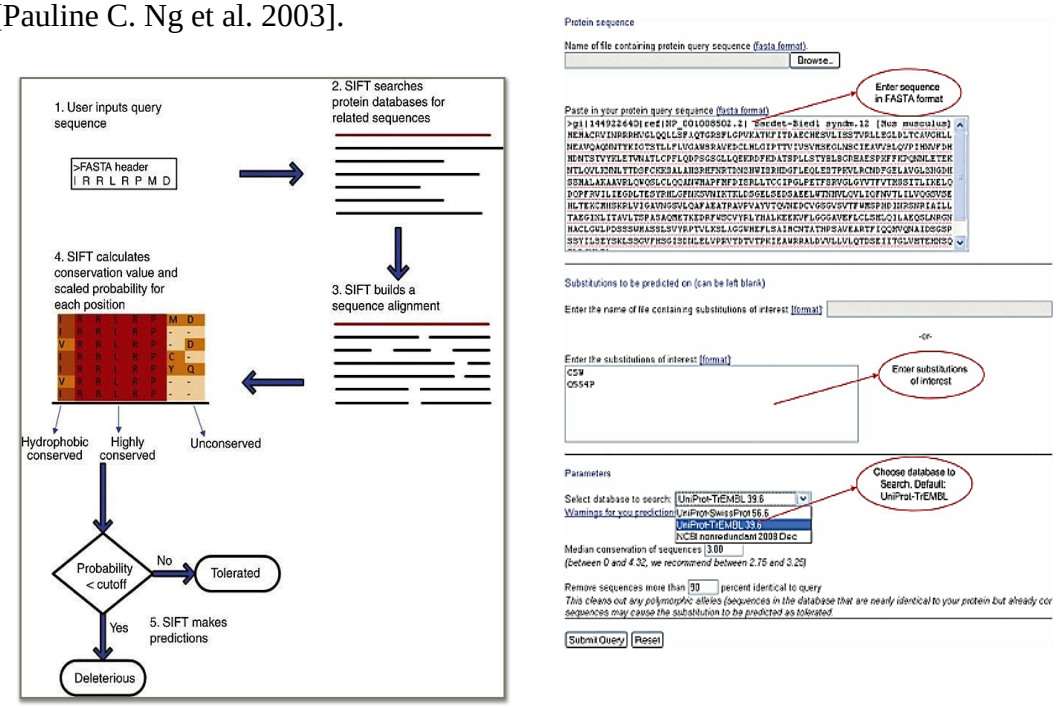


Fig 38. On the left Sorting Tolerant From Intolerant' (SIFT) algorithm flowchart for scoring individual amino acid substitutions (AASs) [Kumar et al. 2009]; on the right Protein sequence in FASTAand AASSformat, SIFT Home page http://sift.jcvi.org/www/SIFT_dbSNP.html

Protein ID	Substitution	dbSNP ID	Prediction	Score	Median Info	Number of Seqs at position
NP_001008498	V352I	rs27180025	TOLERATED	1.00	1.38	99
NP_001008499	Y219C	rs33644739	DAMAGING	0.00	1.90	98
NP_001008501	C192Y	rs32419104	TOLERATED	0.25	1.46	84
82934505	Q23L	rs13484479	DAMAGING *Warning! Low confidence.	0.00	4.32	1
82934725	K52E	rs26925385	TOLERATED	1.00	4.32	2
82934691	P52R	rs26956085	TOLERATED	1.00	4.23	101

* Low confidence means that the protein alignment does not have enough sequence diversity.

Click here to download the following table in tab separated format. You can open it using excel with delimiter set as TAB

If you received a warning that the sequences were not diverse enough, you can have SIFT choose more diverse sequences here.

Fig 39. Table showing the results and predictions on the damage caused by the investigated amino acid substitutions [http://sift.jcvi.org]

5.5.2 PolyPhen

PolyPhen/PolyPhen2 algorithm predicts the potential impact of AAS on the structure and function of human protein based on protein sequence, phylogenetic and structural information. An amino acid replacement might occur at a specific site where binding to other molecules or the formation of a secondary/tertiary structure is disrupted. Therefore, PolyPhen determines if the AAS is found at a site which is annotated as a disulfide bond, an active site, a binding site, or a specific motif such as transmembrane domain. Another function of PolyPhen is to compare the sequence and polymorphic regions of homologous proteins in the same family to identify AASs that are rare or never observed in the family. In addition, PolyPhen also maps of the substitution site to the known 3-dimensional protein structure to assess if an AAS has the potential to destroy protein structure via an alteration of, for example, the hydrophobic core of a protein, electrostatic interactions, or interactions with ligands or other molecules (Figures below).

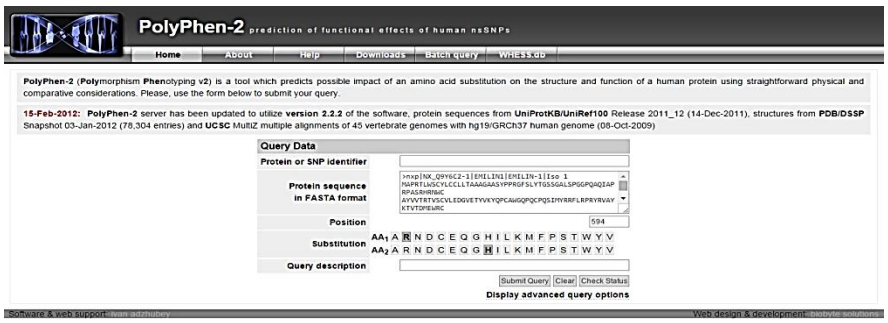


Fig 40. Polyphen-2 initial page [genetics.bwh.harvard.edu/pph2/]

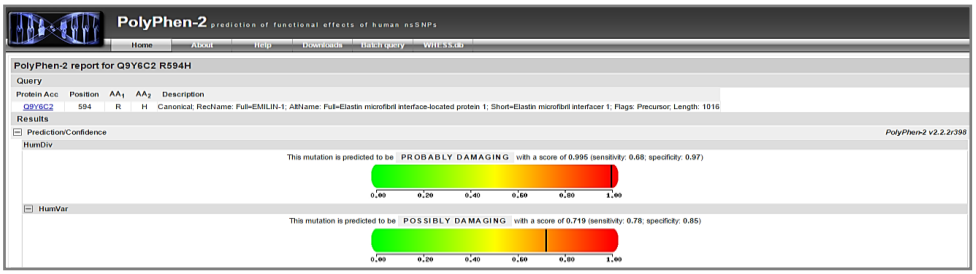


Fig 41. Screen detailing the results of the research of a single amino acid substitution or SNP; the colored bar shows the strength of any harmful effect of replacing the functionality of the protein

Fig 42. Screen showing the mutation and the adjacent sequences in different organisms, underlining if the amino acid of interest is conserved or not

5.5.3 Mutation Taster

MutationTaster is a free, web-based application for rapid evaluation of the disease-causing potential of DNA sequence alterations. MutationTaster integrates information from different biomedical databases and uses established analysis tools. Analyses comprise evolutionary conservation, splice-site changes, loss of protein features and changes that might affect the amount of mRNA. Test results are then evaluated by a naive Bayes classifier2, which predicts the disease potential. A typical query is completed in less than 0.3 seconds. Depending on the nature of the alteration, MutationTaster chooses between three different prediction models, which are either aimed at ‘silent’ synonymous or intronic alterations (without_aae), at alterations affecting a single amino acid (simple_aae) or at alterations causing complex changes in the amino acid sequence (complex_aae). To train the classifier, we generated a dataset with all available and suitable common polymorphisms and known diseasecausing mutations extracted from common databases and the literature. We cross-validated the classifier five times including all three prediction models and obtained an overall accuracy of 91.1 ± 0.1%. MutationTaster can be used via an intuitive web interface to

analyze single mutations as well as in batch mode. To streamline and to standardize the analysis of NGS data, Mutation Taster provides Perl scripts that can process data from all major platforms (Roche 454, Illumina Genome Analyzer and ABI SOLiD). MutationTaster hence allows the efficient filtering of NGS data for alterations with high disease-causing potential. Present limitations of the software comprise its inability to analyse insertion-deletions greater than 12 base pairs and alterations spanning an intron-exon border. Also, analysis of non-exonic alterations is restricted to Kozak consensus sequence, splice sites and poly(A) signal. We will add tests for other sequence motifs in the near future. MutationTaster is available at http://www.mutationtaster.org/.

5.5.4 SNPs&Go

ghgSNPs&GO is a SVM (support vector machine)-based classifier consisting of a single SVM that takes in input protein sequence, profile and functional information (see figure below).

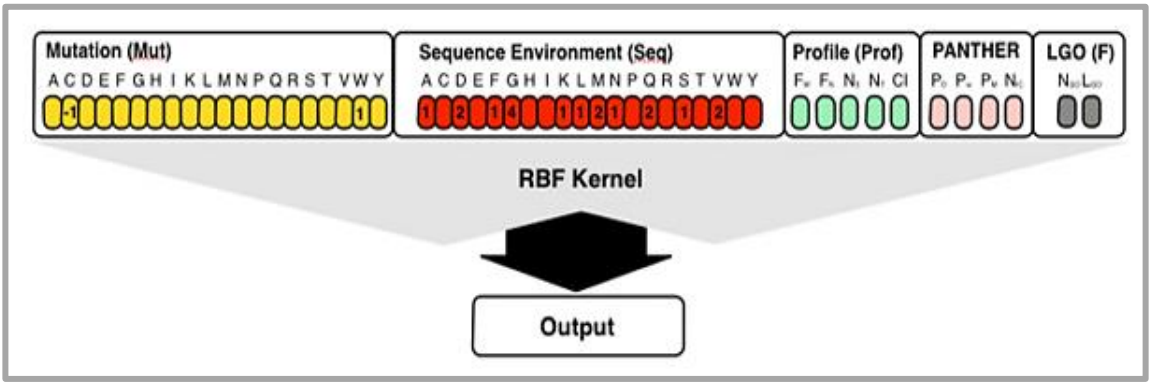
The SNPs&GO input is build according to the following steps:

- for a given mutation the substitution from the wild-type residue to the mutant is encoded in a 20 elects vector that have -1 in the position relative to the wild-type residue, 1 in the position relative to the mutant residues and 0 in the remaining 18 positions.
- a second 20 elements vector encoding for the sequence environment is build reporting the occurrence of the residues in a window of 19 residues around the mutated residue (Seq).
- For a given protein, its sequence profile features (Prof) are extracted from a BLAST search output From the output we evaluate both both the frequency of the wild type (Fi(WT)) and mutated (Fi(MUT)) residues at position i. NAL is the number of sequences in the alignment at a given position and the CI is the Conservation Index
- We consider in the input vector, four outputs of the PANTHER algorithm the predicted probability of deleterious mutation, the frequencies of the wild-type and mutated residue and the number of independent counts (NIC)
- The input is composed by the sum of log-odd scores for all the GO terms associated to the protein under consideration and their parents and the number of all the GO terms associated to the protein undergoing variation (LGO).

The server also implements PhD-SNP (*Predictor of human Deleterious Single Nucleotide Polymorphisms*) method that takes in input different subsets of SNPs&GO's input features. The PhD-SNP method takes in input the first 45 elements vector encoding for the sequence and profile information. Selecting the option "All methods" the prediction of PhD-SNP is calculated and included in the output.

SNPs&GO^{3d} is based a SVM-based classifier based on a single SVM that takes in input protein 3D structure, profile and functional information (see figure below). The SNPs&GO^{3d} input is build following the next steps:

- for a given mutation the substitution form the wild-type residue to the mutant is encoded in a 20 elemts vector that have -1 in the position relative to the wild-type residue, 1 in the position relative to the mutatnt residues and 0 in the remaining 18 positions.
- a second 20 elements vector encoding for the structural environment is build reporting the occurrence of the residues in a radius shell of 6 Ang around the C-alpha of the mutated residue (3D). One element encoding for the Relative Solvent Accessible area (RSA) of the mutated residue is calculated using DSSP algorithm .
- For a given protein, its sequence profile features (Prof) are extracted from a BLAST search output. From the output we evaluate both the frequency of the wild type (Fi(WT)) and mutated (Fi(MUT)) residues at position i. The NAL is the numeber is the number of sequences in the alignment at a given and position and the Conservation Index (CI).
- We consider in the input vector, four outputs of the PANTHER algorithm: the



predicted probability of deleterious mutation, the frequencies of the wild-type and mutated residue and the number of independent counts (NIC)

- The input is composed by the sum of log-odd scores for all the GO terms associated to the protein under and their parents and the number of all the GO terms associated to the protein under mutation (LGO).

The server also implements S3D-PROF method that take in input different subsets of SNPs&GO^{3d}'s input features. The S3D-PROD method takes in input the first 46 elements vector encoding for the structure and profile information. Selecting the option "All methods" the predictions of S3D-PROF and standard sequence-based SNPs&GO are calculated and included in the output.

5.6 STATISTICAL ANALYSES

Statistical analyses were performed using the SPSS package v19. Genotype distributions were compared between the different groups of patients by $\chi 2$ analysis. Categorical variables are expressed as frequencies and percentages. Unless otherwise indicated, data are given as median and inter-quartile range. Comparisons of continuous variables between or among different groups genotypes were performed by the nonparametric Mann-Whitney or Kruskal-Wallis test. Logistic regression analysis was used to estimate OR and 95% confidence intervals (CI) for categorical dependent variables, and linear regression analysis was used to estimate β coefficient and standard error for dependent continuous variables. P-values <0.05 were considered to indicate statistical significance.

CHAPTER 6

PART I

6.1. RESULTS

We analyzed a total of 75 consecutive Italian unrelated patients (47 men, median age 51 years) diagnosed with MFS according to the revised Ghent criteria [Loyes BL 2010] (Table 9). All patients underwent mutational screening for *FBN1* and *TGFBR1*and *TGFBR2* genes. Forty-seven patients (63%) carried a pathogenetic *FBN1*mutation, while no *TGFBR1* or *TGFBR2* pathogenetic mutations were detected in the 75 patients. In the 47 *FBN1*-positive patients, 48 pathogenetic mutations were identified: 31 (65%) missense, 7 (15%) premature stop codons, 3 (8%) frameshift, 5 (10%) splice site mutations, and 1 (2%) small deletion (Figure 44).

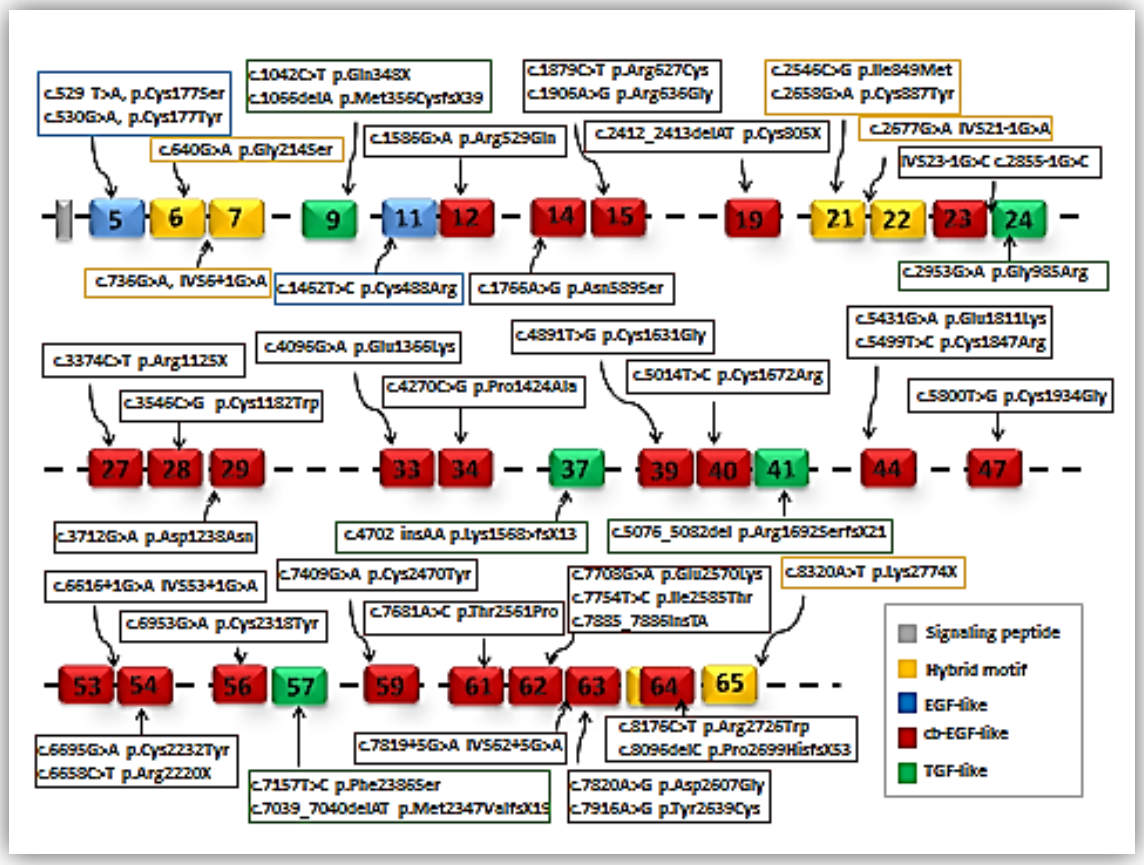


Fig. 44. Schematic representation of locations of 48 *FBN1* variants and symbol keys; exons are numbered in each box.

Patients with or without *FBN1* pathogenetic mutations were comparable for age, sex, prevalence of cardiovascular risk factors (hypertension, dyslipidemia, diabetes, smoking habit), and both for cardiovascular, ocular and systemic features contributing to MFS diagnosis.

Table 9 . Demographic and clinical characteristics of the analyzed 75 unrelated consecutive MFS patients

Features	MFS subjects (all) n = 75	MFS subjects with pathogenetic FBN1 mutation n = 47	MFS subjects without pathogenetic FBN1 mutation n = 28	*p*-Value
Age, years	34.5 (17-76)	33 (24-45)	43 (33-52)	0.060
Sex (Male) n (%)	47 (62.7)	28 (59.6)	19 (67.9)	0.790
Hypertension n (%)	10 (13.3)	5 (10.6)	5 (17.8)	0.486
Dyslipidemia n (%)	5 (6.6)	4 (8.5)	1 (3.5)	0.645
Smoking Habit n (%)	15 (20.0)	10 (21.3)	5 (17.9)	0.775
Diabetes n (%)	0 (0)	0 (0)	0 (0)	-
Aortic root diameter* (mm)*	40 (35-49)	40 (35-49)	42 (34-44)	0.843
Z-score	3.36 (2.08-6.38)	3.99 (1.78-7.14)	2.96 (2.10-4.65)	0.216
AORTIC ROOT CODE				
Z-score<2 n (%)	11 (14.7)	8 (17.0)	3 (10.7)	
Z-score≥2 n (%)	39 (52.0)	22 (46.8)	17 (60.7)	0.489
Aortic surgery n (%)	25 (33.3)	17 (36.2)	8 (28.6)	
Z-score≥2 or Aortic surgery n(%)	64 (85.3)	39 (83.0)	25 (89.3)	0.455
Systemic score	7 (5-10)	7 (5-10)	7 (4-8)	0.346
Systemic score ≥7 n (%)	48 (64.0)	30 (63.8)	18 (64.3)	1

All continuous variables are expressed as median (interquartile range); *=aortic diameter of patients who did not undergo aortic surgery

The direct sequencing of the coding and intronic adiacent regions of *TGFBR1* and *TGFBR2* gene, allowed the identification of 10 common polymorphisms in *TGFBR2* gene and 6 common polymorphisms in *TGFBR1* gene. Genotype distributions and minor allele frequencies (MAFs) for all the identified polymorphisms in the overall cohort of patients and in the two groups of patients are reported in Table 10. No statistically significant difference in genotype distribution between *FBN1* mutation positive and negative patients was observed for the 16 polymorphisms in the two TGF-ß receptors genes (Table 10).

Table 10. Genotype distribution of the *TGFBR1* and *TGFBR2* gene polymorphisms identified in MFS patients (overall cohort, MFS patients with or without pathogenetic *FBN1* mutations

Gene	Polymorphism	Genotype	MFS subjects (all) n = 75	MFS subjects with pathogenetic *FBN1* mutation n = 47	MFS subjects without pathogenetic *FBN1* mutation n = 28	*p*-Value
TGFBR1	rs192247094	TT	73 (97.3)	46 (97.9)	27 (96.4)	0.707
		TG+GG	2 (2.7)	1 (2.1)	1 (3.6)	
	rs334354	GG	55 (73.3)	34 (72.3)	21 (75.0)	0.801
		GA+AA	20 (26.7)	13 (27.7)	7 (25.0)	
	rs67687202	TCTTT/TCTTT	54 (72.0)	33 (70.2)	21 (75.0)	0.655
		TCTTT/-+-/-	21 (28.0)	14 (29.8)	7 (25.0)	
	c.1256-15del1T	T/T	62 (82.7)	42 (89.4)	20 (71.4)	0.047
		T/-+-/-	13 (17.3)	5 (10.6)	8 (28.6)	
	rs868	AA	58 (77.3)	35 (74.5)	23 (82.1)	0.443
		AG+GG	17 (22.7)	12 (25.5)	5 (17.9)	
	rs554578321	9A/9A	63 (84.0)	39 (83.0)	24 (85.7)	0.755
		9A/6A+6A/6A	12 (16.0)	8 (17.0)	4 (14.3)	
TGFBR2	rs1155705	AA	44 (58.7)	24 (51.1)	20 (71.4)	0.083
		AG+GG	31 (41.3)	23 (48.9)	8 (28.6)	
	rs11466512	TT	50 (66.7)	30 (63.8)	20 (71.4)	0.500
		TA+AA	25 (33.3)	17 (36.2)	8 (28.6)	
	rs113194608	CC	73 (97.3)	46 (97.9)	27 (96.4)	0.707
		CT+TT	2 (2.7)	1 (2.1)	1 (3.6)	
	rs35719192	GG	74 (98.7)	46 (97.9)	28 (100)	0.437
		GA+AA	1 (1.3)	1 (2.1)	0 (0)	
	rs377397188	GG	74 (98.7)	46 (97.9)	28 (100)	0.437
		GC+CC	1 (1.3)	1 (2.1)	0 (0)	
	rs2276767	CC	52 (69.3)	30 (63.8)	22 (78.6)	0.181
		CA+AA	23 (30.7)	17 (36.2)	6 (21.4)	
	ex7 3'UTR+78 T>C	TT	74 (98.7)	46 (97.9)	28 (100)	0.437
		TC+CC	1 (1.3)	1 (2.1)	0 (0)	
	rs149112005	GG	73 (97.3)	45 (95.7)	28 (100)	0.269
		GA+AA	2 (2.7)	2 (4.3)	0 (0)	
	rs143024112	AA	73 (97.3)	45 (95.7)	28 (100)	0.269
		AT+TT	2 (2.3)	2 (4.3)	0 (0)	
	c.383delA	A/A	64 (85.3)	43 (91.5)	21 (75.0)	0.051
	p.Lys128Ser fsX35	A/-+-/-	11 (14.7)	4 (8.5)	7 (25.0)	

In order to evaluate a possible role as modifiers of *TGFBR1* and *TGFBR2* genes on the clinical phenotype of MFS patients, the association of the 16 polymorphisms with cardiovascular manifestations was evaluated. In Table 11 and Table 12 the data on aortic root diameter, aortic Z-score, aortic surgery according to *TGFBR2* (Table 11) and *TGFBR1* (Table 12) genotypes are reported. Carriers of A allele of rs11466512 polymorphism showed significantly reduced Z-score with respect to homozygous wild-type MFS patients (p=0.032) (Table 11). Even if they did not reach the statistical significance, carriers of delA allele of c.383delA polymorphism showed significantly reduced Z-score and number of subjects undergoing aortic surgery with respect to homozygous wild-type MFS patients (p=0.083 and p=0.088, respectively) (Table 11), and carriers of A allele of rs2276767 polymorphism showed significantly increased Z-

score with respect to homozygous wild-type MFS patients (p=0.252) (Table 11). Carriers of delT allele of c.1256-15del1T polymorphism showed significantly reduced Z-score and subjects undergoing aortic surgery with respect to homozygous wild-type MFS patients (p=0.211 and p=0.049, respectively) (Table 12). At the linear regression analysis adjusted for the presence of *FBN1* mutations, TGFBR2rs11466512 only remained statistically associated [β=-1.82 (SE 0.88), p0.043]. At the logistic regression analysis, adjusted for the presence of *FBN1* mutations, with global aortic manifestations severity (aortic Z-score≥2 or aortic surgery) as dependent variable, none of the 4 polymorphisms were significantly associated (data not shown).

The protective effect of a genetic *TGFBR1* and *TGFBR2* score including rs11466512, c.383delA, and c.1256-15del1T polymorphisms (Score 1), and rs11466512, c.383delA, rs2276767, and c.1256-15del1T (Score 2) polymorphisms was also evaluated. MFS patients with 1 or more protective alleles included in Score 1 had statistical significant reduced aortic Z-scores [2.90 (IQR 1.65-4.43)] with respect to patients with no protective alleles [4.50 (IQR 2.58-7.32)] (p=0.018). The logistic regression analysis remains statistically significant also when adjusted for the presence of pathogenetic *FBN1* mutations [OR=0.213 (95%CI 0.054-0.843, p=0.028)]. Similarly, MFS patients with 2 or more protective alleles included in Score 2 had statistical significant reduced aortic Z-scores [2.20 (IQR 1.48-3.37)] with respect to patients with 1 or no protective alleles [4.20 (IQR 2.48-7.12)] (p=0.007). At the linear regression analysis adjusted for the presence of pathogenetic *FBN1* mutations, they both, genetic Score 1 or genetic Score 2 remained significantly associated with lower Z-scores (Table 13). Patients with severe aortic manifestations (aortic Z-score ≥2 or aortic surgery) showed a lower prevalence of subjects with 1 or more protective alleles included in genetic Score 1 (42.2%) than patients with no or milder cardiovascular involvement (72.7%, p=0.061). Patients with severe aortic manifestations (aortic Z-score ≥2 or aortic surgery) showed a significantly lower prevalence of subjects with 2 or more protective alleles included in genetic Score 2 (29.7%) than patients with no or milder cardiovascular involvement (63.6%, p=0.029). The protective effect of genetic Score 1 and Score 2 on global aortic manifestations severity (aortic Z-score ≥2 or aortic surgery) was also observed by logistic regression analysis even if Score 2 only reaches the full statistical significance (Table 13).

Table 11. Aortic root diameter, aortic Z-score, number of patients undergoing aortic surgery according to genotypes of the *TGFBR2* identified polymorphisms

Gene	Polymorphism	Genotype	MFS subjects (all) n = 75	Aortic root diameter median mm* (range)	*p*-value	*Z*-score median (percentiles) [n patients]	*p*-value	Patients undergoing Aortic surgery n (%)	*p*-value
TGFBR2	rs1155705	AA	44 (58.7)	41.9 (20-60)	0.592	3.6 (1.80-6.66) [32]	0.731	12 (27.3)	0.219
		AG+GG	31 (41.3)	40 (20-54)		3.2 (2.07-6.43) [18]		13 (41.9)	
	rs11466512	**TT**	**50 (66.7)**	**41.8 (20-60)**	**0.173**	**4.0 (2.39-7.25)[33]**	**0.032**	**17 (34)**	1
		TA+AA	**25 (33.3)**	**38 (20-54)**		**2.2 (1.33-4.77) [17]**		**8 (32)**	
	rs113194608	CC	73 (97.3)	40.5 (20-60)	1	3.5 (2.02-6.72) [48]	0.620	25 (34.2)	0.549
		CT+TT	2 (2.7)	40.5 (37-44)		2.8 (2.30-2.80) [2]		0(0)	
	rs35719192	GG	74 (98.7)	41 (20-60)	0.960	3.3 (2.05-6.55) [49]	0.880	25 (33.8)	1
		GA+AA	1 (1.3)	40 (40-40)		3.9 (3.94-3.94) [1]		0 (0)	
	rs377397188	GG	74 (98.7)	40.5 (20-60)	-	-	-	-	-
		GC+CC	1 (1.3)	-					
	rs2276767	**CC**	**52 (69.3)**	**39.5 (20-60)**	**0.234**	**3.3 (1.75-5.45) [36]**	**0.252**	**16 (30.8)**	0.597
		CA+AA	**23 (30.7)**	**44 (20-56)**		**4.9 (2.14-7.16) [14]**		**9 (39.1)**	
	ex7 3'UTR+78 T>C	TT	74 (98.7)	40.5 (20-60)	-	-	-	-	-
		TC+CC	1 (1.3)	-					
	rs149112005	GG	73 (97.3)	41 (20-60)	0.120	3.4 (2.05-6.55) [49]	1	24 (32.9)	1
		GA+AA	2 (2.7)	24 (24-24)		3.3 (3.35-3.35) [1]		1 (50)	
	rs143024112	AA	73 (97.3)	41 (20-60)	0.120	3.4 (2.05-6.55) [49]	1	24 (32.9)	1
		AT+TT	2 (2.3)	24 (24-24)		3.3 (3.35-3.35) [1]		1 (50)	
	c.383delA p.Lys128Ser fsX35	**A/A**	**64 (85.3)**	**40 (20-60)**	**0.933**	**3.9 (2.19-7.00) [40]**	**0.083**	**24 (37.5)**	**0.088**
		A/-+-/-	**11 (14.7)**	**41.9 (33-51)**		**2.1 (1.72-3.48) [10]**		**1 (9.1)**	

*=aortic diameter of patients who did not undergo aortic surgery

Table 12. Aortic root diameter, aortic Z-score, number of patients undergoing aortic surgery according to genotypes of the *TGFBR1* identified polymorphisms

Gene	Polymorphism	Genotype	MFS subjects (all) n = 75	Aortic root diameter median mm* (range)	*p*-value	*Z*-score median (percentiles) [n patients]	*p*-value	Patients undergoing aortic surgery n (%)	*p*-value
TGFBR1	rs192247094	TT	73 (97.3)	41 (20-60)	0.491	3.5 (2.11-6.72) [48]	0.153	25 (34.7)	0.549
		TG+GG	2 (2.7)	37.5 (37-38)		1.7 (1.48-1.74) [2]		0 (0)	
	rs334354	GG	55 (73.3)	41 (20-60)	0.415	3.8 (2.00-6.20) [35]	0.824	20 (36.4)	0.417
		GA+AA	20 (26.7)	39 (20-56)		3.3 (2.10-7.12) [15]		5 (25.0)	
	rs67687202	TCTTT/TCTTT	54 (72.0)	41.5 (20-60)	0.288	3.9 (2.07-6.37) [34]	0.603	20 (37.0)	0.414
		TCTTT/-+-/-	21 (28.0)	38.5 (20-56)		3.1 (1.87-6.74) [16]		5 (23.8)	
	c.1256-15del1T	**T/T**	**62 (82.7)**	**40 (20-60)**	**0.785**	**3.9 (2.14-6.93) [38]**	**0.211**	**24 (38.7)**	**0.049**
		T/-+-/-	**13 (17.3)**	**41.9 (33-51)**		**2.5 (1.85-3.86) [12]**		**1 (7.7)**	
	rs868	AA	58 (77.3)	40.5 (20-60)	0.785	3.5 (1.92-6.00) [38]	0.467	20 (34.5)	0.777
		AG+GG	17 (22.7)	40.4 (24-56)		3.4 (2.41-7.18) [12]		5 (29.4)	
	rs554578321	9A/9A	63 (84.0)	41 (20-60)	0.848	3.3 (2.10-5.94) [43]	0.763	20 (31.7)	0.519
		9A/6A+6A/6A	12 (16.0)	38 (25-56)		3.6 (1.00-7.59) [7]		5 (41.7)	

*=aortic diameter of patients who did not undergo aortic surgery

Table 13. Linear regression analysis with aortic root Z-score as dependent variable (A) and logistic regression analysis with aortic manifestations severity (aortic Z-score ≥2 or aortic surgery) as dependent variable (B)

A	***Linear Regression Analysis****		
	β	**SE (Standard Error)**	***p*-Value**
Score 1	-1.98	0.83	0.021
Score 2	-2.12	0.86	0.017
B	***Logistic Regression Analysis****		
		OR (95% IC)	***p*-Value**
Score 1	0.26	(0.06-1.09)	0.066
Score 2	0.21	(0.05-0.84)	0.028

*adjusted for presence of pathogenetic FBN1 mutation; Score 1 polymorphisms=rs11466512, c.383delA, and c.1256-15del1T; Score 2 polymorphisms=rs11466512, c.383delA, rs2276767, and c.1256-15del1T

6.2 DISCUSSION

Data reported in the first part of this PhD thesis are the result of the genetic analysis of *FBN1*, *TGFBR1* and *TGFBR2* genes in a cohort of 75 Italian unrelated MFS patients and contribute to define the role of TGF-β receptors genes genetic variants on MFS clinical phenotype determination and/or modulation. The major results of the study are the following: I) no pathogenetic mutations in *TGFBR1* and *TGFBR2* genes were identified in the cohort of MFS patients, in particular, in those MFS patients without *FBN1* pathogenetic mutations; II) four out of the 16 common genetic variants (polymorphisms) identified in *TGFBR1* or *TGFBR2* genes showed a significant association with the severity of aortic manifestations (aortic Z-score ≥2 or aortic surgery). In particular, for the latter issue, the effect of two *TGFBR1* and *TGFBR2* genetic scores (Score 1 including 3 polymorphisms and Score 2 including 4 polymorphisms) on cardiovascular severity was evaluated and found out to be protective.

The role of *TGFBR1* and *TGFBR2* genetic variants in MFS phenotype has been largely investigated during the last decades [Gao LG et al., 2010]. In fact, the evidence of an increased TGF-β signaling in MFS patients, together with the well established

association between TGF-β receptors (*TGFBR1* and *TGFBR2*) mutations and LDS, which shares a similar pattern of cardiovascular defects (TAA, mitral valve prolapse/regurgitation, and aortic dilatation with regurgitation) with MFS, gave rise to the hypothesis of an altered TGF-β signaling in determining cardiovascular manifestations (especially with regard to aneurysm formation and progression) in both syndromes, alongside other MFS-like conditions, as well as determining the complete phenotypes.

Existing literature shows discordant data resulting from *TGFBR1* and *TGFBR2* mutational screening in MFS patients so that the role of these genetic variants in the pathogenesis of the disease remains unclear [Mizuguchi T et al., 2004; Singh KK et al., 2006; Matyas G et al., 2006; Disabella I et al., 2005; Ki CS et al., 2005; Loeys BL et al., 2005]. In this study, no mutations were found neither in the 47 MFS patients in which a pathogenetic *FBN1* mutation was identified, nor in the 28 patients without a pathogenetic *FBN1* mutation. These data are consistent with the findings of two studies published in 2005: in the first, by Loeys and colleagues, 7 of 93 classic MFS patients without *FBN1* gene mutation had neither a *TGFBR1* nor a *TGFBR2* mutation [Loeys BL, 2005], and in the second, by Ki and colleagues, the authors failed to identify *TGFBR2* mutations in 29 MFS patients (24 subjects were negative for pathogenetic mutations in *FBN1*, the remaining 5 were not screened for the *FBN1* mutations) [Ki CS et al., 2005]. Our results further agree with the part of the experts in the field arguing that LDS represents a distinct syndrome, widely different from classical MFS concerning genes associated with.the disease. Our data are not in agreement with those of Mizuguchi and colleagues who, in 2004, identified *TGFBR2* and *TGFBR1* in a Japanese individual with MFS, in affected members of a French MFS family originally found not to be associated with *FBN1*, and in 4 unrelated probands leading to the hypothesis of the existence of a second locus for MFS, defined as MFS2 (3p25-p24.2) [Mizuguchi T et al., 2004]. The association between *TGFBR1/2* mutations and MFS-related disorders has been since then investigated by a large number of studies. In 2006, Matyàs and colleagues examined a cohort of 70 patients with MFS-related disorders, who were negative for *FBN1* mutations, and identified nine novel and one known heterozygous sequence variants in *TGFBR1* and *TGFBR2* genes, three of them being associated with MFS2 [Matyàs G et al., 2006]. During the same year, Disabella and colleagues identified two*TGFBR2* gene mutations in two adult patients diagnosed with MFS according to Ghent criteria who had a negative *FBN1* gene screening [Disabella E et al., 2006], Sakai and colleagues searched for *TGFBR1* and *TGFBR2* mutations in 41

unrelated patients fulfilling the diagnostic criteria of Ghent nosology or with the tentative diagnosis of MFS, in whom mutations in the *FBN1* coding region were not identified, and reported 3 *TGFBR2* mutations in 2 cases not fulfilling Ghent criteria, 2 *TGFBR2* in 2 patients diagnosed with MFS, 2 *TGFBR1* mutations in 2 cases not fulfilling Ghent criteria, and 2 *TGFBR1* in 2 patients diagnosed with MFS [Singh KK et al., 2006]; it should however be noticed, as the authors themselves pointed out in the paper, that three other patients, one with *TGFBR1* and two with *TGFBR2* mutations show manifestations of LDS in addition to features of classical MFS, an observation which might confirm the evidence of the broad clinical overlap among MFS1, MFS2, and LDS rather than implicate a large and specific contribution of TGF-β receptors genetic variants to the pathogenesis of classic MFS where no *FBN1*mutation is present. More recent studies move to the direction of a less relevant role of TGF-β receptor variants in classic MFS phenotype determination. Sakai and colleagues analyzed *FBN1*, *FBN2*, *TGFBR1*, and *TGFBR2* mutations by direct sequencing in 49 patients with MFS or suspected MFS and were able to identify TGF-β receptors variants only in the subgroup of patients not fulfilling the Ghent criteria [Sakai LY et al., 2006], thus not excluding MFS from the clinical spectrum of *TGFBR1/2* mutations, but suggesting their incidence in classical MFS to be lower than the 5% usually reported in literature (as TGF-β receptors abnormalities seem to be occasional). Adès and colleagues reported the same novel *TGFBR1* mutation in two patients fulfilling the Ghent criteria whose craniofacial features were, however, far more pronounced than in MFS, and one individual also had a cleft palate [Adès LC et al., 2006]. In light of that, the authors discussed in the paper that fulfillment of the Ghent criteria may not always differentiate clinically between MFS and Furlong syndrome (a marfanoid condition which is usually associated with craniosynostosis, delayed fontanelle closure, and hypertelorism, may include ptosis, cleft palate, and normal height, and is not associated with ectopia lentis), or between MFS and a primary TGF-β signaling pathway disorder. Stheneur and colleagues, in 2008, screened 457 probands suspected of being affected with Marfan syndrome or related disorders for the two receptors genes finding that yield of mutation detection within the two genes was very low: 4.8% for classical MFS, 4.6% for incomplete MFS and 1% for TAAD in the *TGFBR2* gene; 6.2%, 6.2% and 7% respectively in the *TGFBR1* gene [Stheneur C et al., 2008]. It should also be noted that MFS and incomplete MFS probands showed no significant differences for mutation detection rates in the *TGFBR2* gene suggesting this gene screening to have a yield that is limited for molecular diagnosis in MFS probands. In addition, considering system

involvement, 2 probands carrying a *TGFBR2* gene mutation had ectopia lentis while a major cardiac involvement was almost always present. The association between *TGFBR2* gene and Marfan-related syndromes has been also investigated by Chen and colleagues in 2010, who screened a total of 6 unrelated Chinese patients. Among them, two were diagnosed with LDS, two with MFS2, and two had involvement of one or two organ systems associated with MFS. The pathogenetic mutations identified were 3, 2 in LDS patients one in the MFS2 subject, while in the other three patients investigated, despite sharing a similar phenotype with the positive subjects, no mutation were detected [Chen J et al., 2010]. These results underlined the importance of a *TGFBR1* and *TGFBR2* screening for those patients presenting some features of LDS, or being non-*FBN1*-related MFS. In 2011, Baetens and colleagues applied massive parallel sequencing for *FBN1*, *TGFBR1* and *TGFBR2* to 87 MFS subjects satisfying the Ghent criteria that allowed them to identify 75 mutations in the first gene and no mutation in the two receptors [Baetenes M et al., 2011].

This number of data could lead us to reconsider the extent of *TGFBR1/2* mutations incidence in determining MFS classical phenotype not associated with *FBN1*mutations.
The definition of the clinical diagnosis, due to the objective difficulties attributable to the overlapping clinical manifestations in this phenotypic continuum, could be one of the reasons of the discordant data obtained on the association of *TGFBR1/2* mutations in MFS. Our data, obtained on the 75 MFS patients all diagnosed according to the revised Ghent criteria by the same expert clinician in the field of connective tissue disorders in the Regional Referral Center for Marfan syndrome and related disorders, suggest that *TGFBR1* and *TGFBR2* mutations play a minor role as major genetic determinants of MFS.
Nevertheless, these data do not allow us to exclude the potential role of an altered TGF-β signaling pathway in modulating the patients clinical phenotype, especially relating to cardiovascular manifestations, not necessarily assuming mutations in the receptors genes to be causative of MFS. It is although possible that other genes interacting with *TGFBR1/2* or acting in the same pathway are affected thus determining and/or modulating the extent of the cardiovascular involvement.
In fact, a significant amount of literature data supports the role of TGF-β1 in thoracic aortic aneurysm onset and progression in non-syndromic and syndromic (e.g. MFS) forms of the disease. Habashi and colleagues, in 2006, demonstrated the association between aortic aneurysm in a mouse model of MFS and increased TGF-β signaling and

the use of TGF-β antagonists such as TGF-β–neutralizing antibody or the angiotensin II type 1 receptor (AT1) blocker, losartan, could prevent its formation [Habashi JP, 2006]; Nataatmadja and colleagues showed in the same year an overexpression of TGF-β in MFS which have been associated with altered hyaluronan synthesis, increased apoptosis, impaired progenitor cell recruitment, and abnormal directional migration, factors that were likely to contribute to aneurysm development [Nataatmadja M, 2006].

Besides the consolidated evidence of the TGF-β involvement in ascending aortic dilatation formation, a number of studies have also assessed the association between single nucleotide polymorphisms (SNPs) in the TGF-β receptors and abdominal aortic aneurysm (AAA) but the findings have been inconsistent. Lucarini and colleagues examined one SNP in *TGFBR1* (9A6A) in a case-control study of 453 AAA subjects and failed to find any association [Lucarini L et al., 2009]; Golledge and colleagues explored the association between 58 SNPs in *TGFBR1*and *TGFBR2* within 1711 men, almost all of them carrying small AAAs (97% of total subjects) and reported a weak association between rs1078985 *TGFBR2* polymorphism and AAA, which, however, did not hold after adjustment for multiple testing [Golledge J et al., 2009]; in 2010, Baas and colleagues assessed 35 SNPs in the two receptors genes and reported the association of 12 *TGFBR1* and *TGFBR2* SNPs with AAA: 3 *TGFBR1* SNPs (rs10819634, rs1571590, and rs1626340) and 6 *TGFBR2* SNPs (rs764522, rs3087465, rs1036095, rs4522809, rs9831477,and rs1346907) confirmed their association with the disease in a combined case-control group of 1760 individuals [Baas AF et al., 2010]; Biros and colleagues, in 2011, conducted a meta-analysis using individual subject data from three independent case-control studies (more than 4500 individuals) and reported an association of two *TGFBR2* SNPs (rs764522 and rs1036095) with AAA [Biros E et al., 2011].

In the present study, despite the absence of pathological mutations, direct sequencing of the two genes allowed the identification of 16 common polymorphisms, 10 in *TGFBR2* and 6 in *TGFBR1* gene. Given the previous literature evidences suggesting a potential association between TGF-β receptor polymorphisms and aneurysm formation, we tested the genetic common variants identified with regard to the severity of cardiovascular manifestations in our cohort of MFS patients (aortic root diameter, aortic Z-score, aortic surgery). Three *TGFBR2* polymorphisms (rs11466512, rs2276767, c.383delA) and one *TGFBR1* polymorphism (c.1256-15del1T) were found to be significantly associated with reduced Z-scores and number of subjects undergoing aortic surgery.

rs11466512 SNP is an intronic variant (located in intron 3) of the *TGFBR2* gene. It has been previously found in a MFS proband (fulfilling the MFS diagnostic criteria), carrying a *FBN1* heterozygous nonsense mutation [Ma M, 2016]. In 2010, Santiago-Sim and colleagues hypothesized that formation of intracranial aneurysm (IA) and aortic aneurysm, which display similar pathologies including degeneration of ECM, destruction of elastic lamina and loss of media, might share common genetic mechanisms, such as mutations in TGF-β receptor genes. Mutational screening of 44 patients with familial IA for genes coding TGF-β and its receptors did not allow the authors to identify pathogenetic mutations, while rs11466512 SNP was found together with other variants [Santiago-Sim T et al., 2010]. Ruigrok and colleagues, in 2012, analyzed common genetic variants in *TGFBR1* and *TGFBR2*,using a tag single nucleotide polymorphisms approach, in a Dutch intracranial aneurysm case–control population (481 patients and 648 controls): rs2276767 *TGFBR2* SNP was found not to be associated with IA [Ruigrok YM et al., 2012]. In 2013, Jekarl and colleagues, found this variant to be associated with ossification of the posterior longitudinal ligament (OPLL). Assuming autoimmune disease, increased local angiogenesis and ectopic calcification to be concurrent features in OPLL, the authors suggested genetic variants beneath TGF-β and its receptors to participate in the pathogenesis of the disease, due to the association of TGF-β signaling with bone development and metabolism and the role of the cytokine as a mediator in calcification of the aortic valve, resulting in stenosis of the cups [Jekarl DW et al., 2013]. The authors found this polymorphism to be significantly associated with OPLL. This variant has also been found in 3 BAV patients who underwent mutational screening for *NOTCH1*, *GATA5*, *TGFBR1* and *TGFBR2* genes [Foffa I et al., 2013]. c.383delA *TGFBR2* polymorphism, located in exon 3, was also found in 8 patients [Foffa I et al., 2013]. In this paper, two novel *NOTCH1* mutations were identified; Notch1 signaling has a role in repressing Bmp2 (bone morphogenic protein-2) expression which is involved in aortic calcification and in the pathogenesis of OPLL [Nigam V and Srivastava D, 2009; Stapleton CJ et al.,, 2011]. Given the increasing evidence of an interaction between Notch1 pathway and TGF-β [Klüppel M and Wrana JL, 2005] and its role in vascular remodeling, they speculated on the existence of a complex interaction between these molecules and Bmp2 which, if altered, might lead to different consequences in distinct organs. *TGFBR1* and *TGFBR2* were analyzed by McKnight and colleagues in 2007 using WAVE™ dHPLC technology, leading to the identification, amongst others, of *TGFBR2* polymorphisms rs11466512 and c.383delA which were genotyped using TaqMan, Invader or

Pyrosequencing for their association with diabetic nephropathy in an Irish, type 1 diabetic case (n = 272) control (n = 367) collection, finding no significant differences in genotype or allele distributions between cases and control [McKnight AJ et al., 2007]. c.383delA *TGFBR2* polymorphism was also analyzed with direct sequencing in a study conducted by Tocharoentanaphol and colleagues, in 2008, along with other polymorphisms in 13 genes related to atherosclerosis comparing their allele frequencies distribution between Thai population (32 helathy individuals) and others (Caucasian, Han Chinese, Japanese and African) [Tocharoentanaphol C et al., 2008], showing a similar pattern. c.1256-15del1T *TGFBR1* genetic variant, located in intron 8, has not been previously described and associated with phenotypes. Even if no definite consensus on the effect of the 4 polymorphisms identified in our study exists, the above mentioned associations with different pathological picture underline the plausibility of their role in altering TGF-β signaling and then also cardiovascular manifestations in MFS patients.

Interestingly, both the genetic scores investigated (including 3, score 1, or four polymorphisms, score 2) were found to be significantly associated with a lower risk of cardiovascular manifestations also when the analysis were adjusted for the presence of pathogenetic *FBN1* mutations. In particular, the score 2 showed a statistically significant protective effect with respect to the global aortic manifestations severity (aortic Z-score ≥2 or aortic surgery) by logistic regression analysis.

We observed a protective role of the single polymorphisms and of their combination. As noted above, decades of intensive study in TGF-β pathway have led to controversial discoveries on the role of the cytokine in aneurysm development. TGF-β has an important role in the regulation of ECM formation and remodeling; ECM itself acts as a dynamic regulator of the cytokine bioavailability and signaling through the "canonical" and "non canonical" TGF-β pathways [Wheeler MB et al., 2014]. Still, the identity and interactions among downstream altered signaling pathways molecules, representing the result of mutations within different genes, as well as their effect on tissue integrity and dysfunction, are not entirely clarified [Cook JR et al., 2015]. As already mentioned, a number of studies agree on the evidence in according to which the TGF-β signaling elevation and its hyperactivity result in TAA progression in MFS, as a consequence of the original observation that either AT1r antagonism or TGF-β neutralization prevented aneurysm growth and medial degeneration in MFS mice [Neptune ER et al., 2003; Habashi JB et al., 2006; Holm TM et al., 2011]. To the best of our knowledge, we can expect mechanisms underlying TAA formation in MFS and related disorders to be quite

different from that leading to AAA, being MFS due (in most cases) to a monogenetic mutation in fibrillin, which is in contrast with AAA that's usually not the consequence of a single gene defect, but the result of multiple genetic and environmental factors combined together. Furthermore several studies suggested that increased TGF-β signaling is the cause of both AAA and TAA [Fukui D et al.,, 2003; Habashi JP et al., 2006], indicating a role of its genetic variants in aneurysm formation in general. However, subsequent investigations disclosed that TGF-β activity is critically required for protection against the development of AAA and dissection in C57BL/6 mice treated with Ang II, due to its role in controlling excessive monocyte/macrophage activation, inhibition of matrix degradation, and the preservation of medial smooth muscle cell survival [Wang Y et al., 2010]. A study conducted by Cook and colleagues in 2015 showed that, together with a beneficial effect of losartan, TGF-β neutralization either exacerbated or mitigated TAA formation depending on whether treatment was initiated before or after aneurysm formation, respectively, in mice with severe MFS. The authors speculate that, during the early stage of TAA formation in MFS mice, in response to increasing hemodynamic stress, the compromised aortic wall activates constitutive AT1r over-activation and protective TGF-β signaling, the latter leading, as the arterial dysfunction escalates, to medial degeneration and rupture of the vessel wall, in a negative feedback loop model in which TGF-β represents the key center.

Evidences on the overall complexity of the signaling are furthermore provided by recent studies showing how the inactivation of genes encoding both positive (*SMAD3* and *TGFB2*) and negative effectors (*SKI*) of this pathway lead to the development of aneurysmal diseases. In addition it has been demonstrated that, despite a decreased pathway activation resulting from heterozygous mutations in *TGFBR2* gene, normal levels of phosphorylated Smad2 were maintained, thus suggesting the presence of possible compensation mechanisms [Gallo EM et al., 2014]. Moreover, a possible linkage disequilibrium with other variants, not yet identified, in the regulatory regions or in other genes, which could activate processes compensating those leading to TGF-ß signalling activation, could not be longer excluded.

A major limitation of this work is the low number of subjects that determine the low statistical power of the study to definitely support the role of the *TGFBR1/2* polymorphisms in modulating the severity of cardiovascular manifestations in MFS patients.

In conclusion, the data of this thesis debunk the incidence and the role of mutations in *TGFBR1* and *TGFBR2* genes as major determinants of MFS phenotype, while they

suggest a possible role in modulating the severity of cardiovascular manifestations in patients affected by this syndromic disorders. In addition to that, it would also be important to evaluate the role of these variants as possible modulators of the phenotype by including in the study a large number of subjects affected by syndromic or familiar TAA in which mutational screening of those genes associated or suspected to be associated with aneurysmal disease should be performed. The lack of this datum represents another limitation of the study and this further analysis should be taken into account in order to better define the role of the TGF-ß receptors polymorphisms as susceptibility factors for aneurysmal disease. These data finally suggest the usefulness of a genetic score based on *TGFBR1/2* polymorphic variants in framing their risk profile and emphasize the notion of a deep understanding of the genetic background in monogenic syndromic disorders together with a comprehensive clinical evaluation could allow to optimize patients follow-up and management

CHAPTER 7

PART II

7.1 RESULTS

In Table 14 demographic and clinical characteristics of the 88 BAV patients enrolled during the three years of the project are reported. Patients came to the attention of the "Tuscany Region Referral Center for the study and diagnosis of Marfan syndrome and related disorders" (Head Prof. Guglielmina Pepe), Department of Experimental and Clinical Medicine, University of Florence - SOD Atherothrombotic Diseases, Careggi Hospital. The Center allows a multidisciplinary clinical, instrumental, and genetic diagnosis through a structured path that provides patient management by a range of specialists (clinical pathologist, internist, cardiologist, ophthalmologist, orthopedist, medical geneticist, neurologist, neuroradiologist, molecular biologist).
In the two tables the clinical and demographic characteristics of 84 age and sex comparable MFS patients are also reported. The consecutive enrolled BAV patients have a median age of 42 years (range 18-72) and 25.3% were females. Concerning the cardiovascular characteristics, 78.1% of BAV patients show the type 1 morphology of the bicuspid valve. Forty-six point seven percent of BAV patients have Z-score ≥2 at the aortic root level. The median ascending aorta diameter were 38 mm (range 22-53 mm). Among the 23 BAV patients, who underwent *FBN1* Sanger mutational screening for differential diagnosis with respect to MFS, only one patients carried a pathogenetic mutation. Interestingly, BAV patients showed reduced (root-ascending aorta) delta values [0.001 (IQR -5.25-3.25)] with respect to MFS patients [9.00 (IQR 6.00-13.00)] and BAV/MFS patients [0.010 (IQR -2.75-14.75)] ($p<0.0001$) (Figure 45).

Even if patients with ≥7 points of systemic score [calculated according to revised Ghent criteria (Loeys BL et al., 2010)] were significantly lower in BAV patients than in MFS patients or BAV/MFS patients (Table 14), the analysis of systemic features (Table 14) showed that BAV patients have at least in part common systemic manifestations with

MFS patients furtherly complicating, together with the possible presence of aortic aneurysm, the differential diagnosis between MFS and BAV. The analysis of clinical variables among BAV, MFS and BAV/MFS patients underline some statistically significant differences both in cardiovascular and systemic characteristics that could help clinicians to perform correct clinical diagnosis in the case of overlapping manifestations. In particular, we developed a "Florence score" that included the following 5 variables – pectus carinatum, pes planus, wrist and thumb signs, myopia≥3OD, and mitral valve prolapse associated to MFS diagnosis. In fact, patients with ≥2 manifestations were significantly lower in BAV patients (11/79, 13.9%) than in MFS patients (68/84, 81.0%) and BAV/MFS (7/9, 77.7%) ($p<0.0001$).

Table 14. Demographic and clinical, in particular cardiovascular, characteristics of enrolled bicuspid aortic valve patients and age and sex comparable Marfan syndrome patients

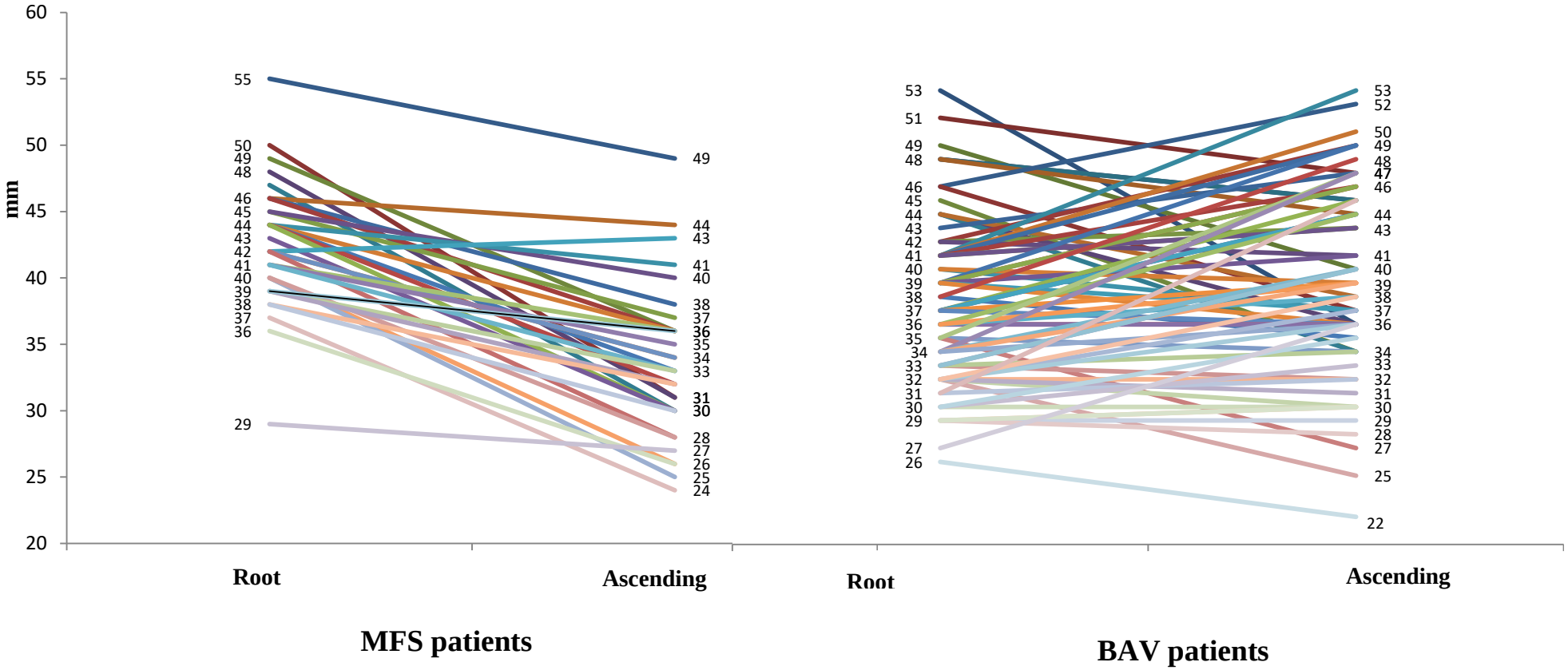
Features	BAV N=79	MFS N=84	BAV/MFS N=9	*p*-Value
Age, years	42 (18-72)	43 (18-69)	49 (29-54)	0.480
Sex n (%)	20/79 (25.3)	19/84 (22.6)	1/9 (11.1)	0.622
Height (cm)	176 (150-194)	182.5 (122-205)	180 (162-798)	<0.0001
Weight (kg)	74 (46-112)	74.5 (27-56)	79 (54-100)	0.370
Hypertension n (%)	30/78 (8.5)	7/84 (8.3)	4/9 (44.4)	<0.0001
Diabetes n (%)	2/78 (2.6)	0/84 (0)	0/9 (0)	0.299
Dyslipidemia n (%)	18/78 (23.1)	6/84 (7.1)	3/9 (33.3)	0.007
Smoking habit n (%)	10/62 (16.1)	8/47 (17)	1/5 (20)	0.972
Aortic root diameter (mm)	37.9 (26-53)	42 (27-56)	44 (40-52)	<0.0001
Ascending aortic diameter (mm)	38 (22-53)	33 (24-60)	42 (30-52)	0.002
Anulus (mm)	24 (17-29)	26 (19-32)	28 (27-32)	<0.0001
Aortic junction (mm)	32 (20-52)	33 (22-51)	42 (37-42)	0.047
Aortic arch (mm)	25 (18-45)	26 (17-45)	34 (26-40)	0.111
Aortic root Z-score	1.83 (-2.08-6.62)	3.31 (-1.41-8.92)	3.85 (2.07-7.03)	<0.0001
Z-score >2 n (%)	37/79 (46.7)	69/84 (82.1)	9/9 (100)	<0.0001
Aortic surgery n (%)	2/79 (2.5)	17/84 (20.2)	2/9 (22.2)	
EF	64 (54-76)	60 (43-80)	65 (54-74)	0.001
MVP n (%)	21/79 (26.6)	62/84 (73.8)	5/9 (55,6)	<0.0001
Valves type				
1 (R-L)	50/64 (78.1)	-	6/7 (85.7)	0.640
2 (R-N)	14/64 (21.9)	-	1/7 (14.3)	
***FBN1* mutation n**	1/23	24/41	1/2	

Abbreviations: EF=Ejection Fraction; MVP=mitral valve prolapse

Features	BAV	MFS	BAV/MFS	*p*-Value
Livedo reticularis n (%)	6/79 (7.8)	0/84 (0)	0/9 (0)	0.026
MVP				
1 MVP n (%)	21/79 (25.3)	60/84 (71.4)	4/9 (44.4)	
Surgery n (%)	1/79 (1.3)	2/84 (2.2)	1/9 (11.1)	<0.0001
PNX n (%)	3/79 (3.8)	7/84 (8.3)	0/9 (0)	0.638
Arched palate n (%)	37/79 (46.8)	64/84 (76.2)	6/9 (66.7)	0.001
Dolichocephaly				
1 (face) n (%)	30/79 (38)	16/84 (19)	4/9 (44.)	0.047
2 (neck) n (%)	9/79 (12.4)	5/84 (6)	1/9 (11.1)	
3 (both) n (%)	17/79 (21.5)	27/84 (32.1)	3/9 (33.3)	
Jaw				
Ipognathic n (%)	23/79 (29.1)	18/84 (21.4)	3/9 (33.3)	0.011
Retrognathic n (%)	7/79 (8.9)	7/84 (8.3)	2/9 (22.2)	
Ipo/Retro n (%)	29/79 (36.7)	15/84 (17.9)	2/9 (22.2)	
Striae n (%)	68/79 (86.1)	69/84 (82.1)	9/9 (100)	
PE n (%)	27/79 (34.2)	35/84 (41.7)	5/9 (55.6)	0.557
PC n (%)	14/79 (17.7)	36/84 (42.9)	4/9 (44.4)	0.002
Scoliosis >20° n(%)	9/79 (11.4)	41/84 (48.8)	1/9 (11.1)	
Surgery	-	6/84 (7.1)	-	<0.0001
Kyphosis n (%)	26/79 (32.9)	18/84 (21.4)	4/9 (44.4)	0.138
PP n (%)	8/79 (10.1)	41/84 (48.8)	4/9 (44.4)	<0.0001
Hindfoot valgus n (%)	25/79 (31.6)	31/76 (40.8)	8/9 (88.8)	<0.0001
Elbow valgus n (%)	14/79 (17.7)	30/84 (35.7)	4/9 (44.4)	0.020
Thumb sign n (%)	6/79 (7.6)	30/84 (35.7)	2/9 (22.2)	<0.0001
Wrist sign n (%)	3/79 (3.8)	29/84 (34.5)	1/9 (11.1)	<0.0001
Wrist AND thumb sign n (%)	2/79 (2.5)	23/84 (27.4)	1/9 (11.1)	<0.0001
Myopia n (%)	12/79 (15.2)	31/89 (37.3)	4/9 (44.4)	<0.0001
Ectopia lentis n (%)	1/75 (1.3)	30/83 (36.1)	1/9 (11.1)	<0.0001
Systemic Score*	7/79 (8.9)	59/84 (70.2)	9/9 (100)	<0.0001
Dural ectasia	6/46 (13)	38/54 (70.4)	1/4 (25)	<0.0001
ACE inhibitors n (%)	9/78 (11.5)	9/81 (11.1)	3/9 (33.3)	0.151
ARB n (%)	19/78 (24.4)	40/81 (49.4)	2/9 (22.2)	0.003
BB n (%)	29/78 (37.2)	48/79 (60.8)	6/9 (66.7)	0.007
Diuretics n (%)	2/78 (2.6)	4/82 (4.9)	1/9 (11.1)	0.427
Nitrates n (%)	0/78 (0)	0/82 (0)	9/9 (100)	
Ca antagonists n (%)	0/78 (0)	0/82 (0)	0/9 (0)	-

Abbreviations: MVP=Mitral Valve Prolapse; PNX=; PE=Pectus Excavatum; PC=Pectus Carenatum; PP=Pes Planus; *****=according to Revised Ghent criteria (Loeys Dietz 2010)

Fig 45. Representation of aortic root and thoracic ascending aorta diameters in BAV and MFS patients



Fifteen out of 79 BAV patients were analyzed by next generation sequencing (NGS). The NGS analysis available in the Center is a targeted sequencing of 91 genes known or with plausibility to be associated with connective tissue disorders (see "Materials and Methods" chapter for details). The demographic and clinical characteristics of the 15 sequenced patients were reported in Table 15. All analysed patients had specific indication to genetic diagnosis and the specialist requested the genetic characterization by NGS. All index subjects and, where available, related affected or not affected individuals underwent genetic counseling and signed a written informed consent for diagnostic and research purposes. Each of the subjects could give consent a) only to the analysis of genes closely related to the clinical diagnosis suspected/identified by the specialist or b) the entire panel of 91 genes, and could revoke such consent at any time as desired. The 15 patients gave the consent for the entire panel of genes.

In Table 15 are summarized, for each patients, the genetic variants with a minor allele frequency (MAF) <0.05 in the European populations (where available).

For all patients more than 1 rare genetic variants were identified (Table 15) ranging from 2 of patient P2 to 11 of P11. Even if we concentrate the attention on variants predicted to be probably damaging with the majority of in silico algorithms, the large part of patients (n=12) showed more than 1 rare genetic variants, ranging from 2 to 10 of P14 (Table 15).

The total number of genetic variants with MAF<0.05 in the 15 BAV patients was 78: 43 with a MAF<0.01 and 34 called damaging with at least 1 *in silico* prediction tool.

In two patients, genetic variants in *FBN1* gene were observed: P1 (p.Asn542Ser and p.Lys2460Arg) and P14 (p.Asp1689Asn).

Six patients showed genetic variants in genes previously suspected to be associated with non-syndromic BAV: P1 (p.Val1739Met *NOTCH1*), P2 (p.Val517Ile *AXIN1* benign according to all *in silico* prediction tools), P3 (p.Arg1279His *NOTCH1*), P5 (p.Asp495Glu *AXIN1* benign according to all *in silico* prediction tools), P6 (p.Pro131Leu *PDIA2*), P11 (p.Ala316Val *PDIA2* and p.Glu201Lys *UFD1L*).

Twelve genetic variants were identified in genes involved in TGF-β signalling: 5 *LTPB1*, 2 *LTBP2*, 1 *LTBP4*, 1 *SMAD3*, 1 *TGFBR3*, and 2 *TGFB1*. Genetic variants in genes coding TGF-β signalling components were identified in 9 out of 15 patients. Three patients showed 2 TGF-β signalling genetic vatiants (P1: p.Arg1330Gln *LTBP1* and p.Arg423Trp *TGFBR3*; P4: p.Ser1266Phe *LTBP1* and p.Asp519_Pro522del *LTBP4*; P11 p.Pro319Gln *LTBP2* and p.Thr263Ile *TGFB1*).

Seventeen genetic variants involved 9 genes coding collagens: *COL2A1*, *COL3A1*, *COL5A1*, *COL5A2*, *COL6A1*, *COL6A2*, *COL6A3*, *COL9A1*, *COL11A1* (Table 15).

In particular, seven genetic variants were identified in genes coding the three collagen VI chains (1 in *COL6A1*, 3 in *COL6A2*, and 3 in *COL6A3* gene). Two patients showed 2 mutations involving collagen chains: P10 (c.1770+4G>A *COL6A2* and p.Asp2831His *COL6A3*) and P12 (p.Pro460Ser *COL5A2* and p.Arg784His *COL6A2*).

Seven genetic variants involved genes coding different members of the ADAMTS family of metalloprotease (2 in *ADAMTS2* and 2 in *ADAMTS10* gene) and 3 in *ADAMTSL4* gene, a glycoprotein that share significant similarity with ADAMTS.

Table 15. Characteristics of genetic variants of 15 patients who underwent NGS analysis. Demographic and clinical characteristics of the 15 subjects.

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
P1								44	M	181/70	BAV/TAA/ nsCTD (1)	39/46	3.50	1	3
NOTCH1	NM_017617	c.[5215G>A]+[=] p.[Val1739Met]+[=]	rs377294245 MAF (EU)=N/A	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
FBN1	NM_000138	c.[1625G>A]+[=] p.[Asn542Ser]+[=]	MAF (EU)=N/A	TOLERATED	BENIGN	DISEASE	DISEASE CAUSING								
FBN1	NM_000138	c.[7379A>G]+[=] p.[Lys2460Arg]+[=]	rs144189837 MAF (EU)=N/A	TOLERATED	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
LTBP1	NM_206963	c.[3989G>A]+[=] p.[Arg1330Gln]+[=]	rs141080282 MAF (EU)=0.005	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
TGFBR3	NM_003243	c.[1267C>T]+[=] p.[Arg423Trp]+[=]	rs766001542	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
P2								43	F	165/57	BAV/TAA/ nsCTD (1)	36/35	2.21	1	3
EMILIN1	NM_007046	c.[1718G>A]+[=] p.[Arg594His]+[=]	rs200339477 MAF (EU)=N/A	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
AXIN1	NM_000 138	c.[1549G>A]+ [=] p.[Val517Ile]+ [=]	rs1498490 71 MAF(EU) =0.002	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
P3								46	F	165/61	BAV/dilAo/ PVM (2)	33/38	0.86	1	3
NOTCH1	NM_017 617	c.[3836G>A]+ [=] p.[Arg1279His]+[=]	rs6175154 3 MAF (EU)=0. 01	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
LTBP1	NM_206 943	c.[736G>A]+[=] p.[Glu246Lys] +[=]	rs3534072 6 MAF(EU) =0.018	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
P4								52	F	165/70	BAV/TAA* (1)				
COL6A3	NM_004 369	c.[9245C>G]+ [=] p.[Pro3082Arg]+[=]	rs1829769 77 MAF (EU)=0. 001	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
LTBP4	NM_001 042544	c.[1556_1567 del]+[=] p.[Asp519_Pro522del]+[=]	MAF(EU) =N/A	-	-	-	-								
ABCC6	NM_001 171	c.[2171G>A]+ [=] p.[Arg724Lys] +[=]	rs5807378 9 MAF(EU) =0.0109	TOLERATED	BENINGN	NEUTRAL	POLY-MORPHISM								
ABCC6	NM_001 171	c.[224A>G]+[=] p.[Ile742Val]+ [=]	rs5959313 3 MAF(EU) =0.01	TOLERATED	BENINGN	NEUTRAL	POLY-MORPHISM								
LTBP1	NM_206 943	c.[3797C>T]+ [=] p.[Ser1266Phe]+[=]	rs6175174 2 MAF(EU) =0.01	DAMAGING	PROBABLY DAMAGING	*	DISEASE CAUSING								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
P5								33	M	185/82	BAV/TAA/ nsCTD (1)	41/40	3.50	0	3
FGF8	NM_033163	c.[77C>T]+[=] p.[Pro26Leu]+[=]	rs137852660 MAF(EU) =0.001	DAMAGING	BENIGN	NEUTRAL	DISEASE CAUSING								
AXIN1	NM_003502	c.[1485C>G]+[=] p.[Asp495Glu]+[=]	rs146947903 MAF(EU) =0.008	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
AGGF1	NM_018046	c.[397G>A]+[=] p.[Glu133Lys]+[=]	rs34203073 MAF(EU) =0.02	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
ENG	NM_000118	c.[14C>T]+[=] p.[Thr5Met]+[=]	rs35400405 MAF(EU) =0.02	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHISM								
P6								44	F	164/61	BAV/MASS (1)	29/28	-0.58	0	5
MFAP2	NM_002403	c.[166C>T]+[=] p.[Arg56Trp]+[=]	rs759153602 MAF (EU)=N/A	DAMAGING	PROBABLY DAMAGING	DISEASE	POLY-MORPHYSM								
LTBP1	NM_206943	c.[3797C>T]+[=] p.[Ser1266Phe]+[=]	rs61751742 MAF(EU) =0.013	DAMAGING	PROBABLY DAMAGING	-	DISEASE CAUSING								
PDIA2	NM_006849	c.[392C>T]+[=] p.[Pro131Leu]+[=]	rs774230086 MAF(EU) =N/A	DAMAGING	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
FLNA	NM_001110556	c.[1286C>T]+[=] p.[Thr429Met]+[=]	rs36051194 MAF(EU)=0.02	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
P7								62	F	160/62	BAV/TAA* (1)	47/48	5.74	0	3
LRP1	NM_002332	c.[527T>A]+[=] p.[Val176Asp]+[=]	MAF(EU)=N/A	TOLERATED	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
PLOD1	NM_000302	c.[826A>C]+[=] p.[Ser276Arg]+[=]	MAF(EU)=N/A	TOLERATED	BENIGN	DISEASE	POLY-MORPHYSM								
P8								70	F	160/62	BAV/TAA* (1)	42/48	3.54	0	3
MYH11	NM_001040113	c.[5073C>G]+[=] p.[Ser1691Arg]+[=]	rs760908992 MAF(ExAC):A=0.00003/4	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
ADAMTSL4	NM_019032	c.[464G>C]+[=] p.[Gly155Ala]+[=]	MAF(EU)=N/A	DAMAGING	POSSIBLY DAMAGING	DISEASE	DISEASE CAUSING								
P9								36	F	162/54	BAV/FTAAD/nsCTD (1)	34/39	1.77	0	5
COL5A1	NM_001278074	c.[5306C>T]+[=] p.[Ser1769Phe]+[=]	MAF(EU)=N/A		PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
EMILIN1	NM_007046	c.[2707G>A]+[=] p.[Glu903Lys]+[=]	rs360457900 MAF(EU)=0.029	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
ADAMTS2	NM_014244	c.[2480G>A]+[=] p.[Arg827Gln]+[=]	rs35445112 MAF(EU)=0.035	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
LTBP2	NM_000428	c.[4769T>C]+[=] p.[Val590Ala]+[=]	rs139932140 MAF(EU)=0.014	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
CAPN2	NM_001748	c.[901+8C>G]+[=]	rs193257695 MAF(EU)=0.02	-	-	-	-								
P10								57	M	173/71	BAV(1)	37/33	0.7	0	3
COL2A1	NM_001844	c.[3950T>C]+[=] p.[Met1317Thr]+[=]	MAF(EU)=N/A	DAMAGING	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
ABCC6	NM_001171	c.[3190C>T]+[=] p.[p.Arg1064Trp]+[=]	rs41278174 MAF(EU)=0.03	DAMAGING	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING								
COL6A3	NM_004369	c.[8491G>C]+[=] p.[Asp2831His]+[=]	rs36104025 MAF(EU)=0.07	DAMAGING	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
LRP5	NM_002335	c.[1999G>A]+[=] p.[Val667Met]+[=]	rs4988321 MAF(EU)=0.04	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
MYH11	NM_001040113	c.[813T>C]+[=] p.[Tyr271=]	rs34341838 MAF(EU)=0.04	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
ADAMTS10	NM_030957	c.[2403+4A>G]+[=]	rs187565033 MAF(EU)=0.008	-	-	-	-								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
COL6A2	NM_001849	c.[1770+4G>A]+[=]	rs9981981 MAF(EU)=0.03	-	-	-	-								
P11								45	M	175/70	BAV (1)	36/43	1.74	0	4
COL11A1	NM_001854	c.[2921C>A]+[=] p.[Pro974Gln]+[=]	rs78046647 MAF(EU)=0.005	TOLERATED	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING								
COL3A1	NM_000090	c.[991C>T]+[=] p.[Gln331*]+[=]	MAF(EU)=N/A	-	-	-	-								
VHL	NM_000551	c.[74C>T]+[=] p.[Pro25Leu]+[=]	rs35460768 MAF(EU)=0.002	DAMAGING	BENIGN	DISEASE	POLY-MORPHYSM								
COL9A1	NM_001851	c.[2299A>G]+[=] p.[Met767Val]+[=]	rs6910140 MAF(EU)=0.01	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
COL2A1	NM_001844	c.[3949A>G]+[=] p.[Met1317Val]+[=]	MAF(EU)=N/A	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
LTBP2	NM_000428	c.[956C>A]+[=] p.[Pro319Gln]+[=]	rs2304707 MAF(EU)=0.04	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
PDIA2	NM_006849	c.[947C>T]+[=] p.[Ala316Val]+[=]	rs45459806 MAF(EU)=0.02	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
TGFB1	NM_000660	c.[788C>T]+[=] p.[Thr263Ile]+[=]	rs1800472 MAF(EU)=0.03	TOLERATED	BENIGN	DISEASE	POLY-MORPHYSM								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
COL6A1	NM_001848	c.[2042T>C]+[=] p.[Ile681Thr]+[=]	rs138884734 MAF(EU) = (-)	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
UFD1L	NM_005659	c.[601G>A]+[=] p.[Glu201Lys]+[=]	rs748367529 MAF(ExAC):T= 0.00004/4	TOLERATED	BENIGN	NEUTRAL	DISEASE CAUSING								
FLNA	NM_001110556	c.[1286C>T]+[=] p.[Thr429Met]+[=]	rs36051194 MAF(EU) =0.02	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
P12								43	M	179/80	BAV/MFS (2)	40/40	2.07	0	7
MTHFR	NM_005957	c.[1958C>T]+[=] p.[Thr653Met]+[=]	rs35737219 MAF(EU) =0.01	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
PLOD1	NM_000302	c.[1678A>G]+[=] p.[Ile560Val]+[=]	rs369696269 MAF(EU) = (-)	TOLERATED	BENIGN	NEUTRAL	DISEASE CAUSING								
COL11A1	NM_001854	c.[2043+8G>A]+[=]	rs2622875 MAF(EU) =0.02	-	-	-	-								
COL5A2	NM_000393	c.[1378C>T]+[=] p.[Pro460Ser]+[=]	rs35830636 MAF(EU)=0.03	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
FBN2	NM_001999	c.[1592G>C]+[=] p.[Gly531Ala]+[=]	rs34450503 MAF(EU) =(-)	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
LRP1	NM_002332	c.[6238G>A]+[=] p.[Asp2080Asn]+[=]	rs345772477 MAF(EU) =0.02	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
SMAD3	NM_005902	c.[870C>T]+[=] p.[Ile290=]+[=]	rs117185005 MAF(EU)=0.02	-	-	-	DISEASE CAUSING								
EMILIN2	NM_032048	c.[1455C>G]+[=] p.[Asp485Glu]+[=]	MAF(EU)=N/A	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
COL6A2	NM_001849	c.[1770+4G>A]+[=]	rs9981981 MAF(EU)=0.03	-	-	-	-								
COL6A2	NM_001849	c.[2351G>A]+[=] p.[Arg784His]+[=]	rs75120695 MAF(EU)=0.007	TOLERATED	BENIGN	NEUTRAL	DISEASE CAUSING								
P13								54	F	153/62	BAV/TAA/ MASS (1)	40/53	3.42	0	4
COL11A1	NM_001854	c.[3230C>T]+[=] p.[Pro1077Leu]+[=]	rs373734529 MAF(ExAC): A=0.00004/5	DAMAGING	POSSIBLY DAMAGING	NEUTRAL	DISEASE CAUSING								
ADAMTSL4	NM_019032	c.[130G>A]+[=] p.[Glu44Lys]+[=]	rs41317513 MAF(EU)=0.03	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
LTBP1	NM_206943	c.[3797C>T]+[=] p.[Ser1266Phe]+[=]	rs617517412 MAF(EU)=0.01	DAMAGING	PROBABLY DAMAGING	-	DISEASE CAUSING								
LRP5	NM_002335	c.[1999G>A]+[=] p.[Val667Met]+[=]	rs4988321 MAF(EU)=0.04	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
CBS	NM_000071	c.[253G>A]+[=] p.[Gly85Arg]+[=]	rs863223435 MAF(EU)=N/A	DAMAGING	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Root/ AscAo (mm)*	*Root Z-score*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
P14								40	F	160/62	BAV (1)	36/38	2.28	0	4
ADAMTSL 4	NM_019 032	c.[130G>A]+[=] p.[Glu44Lys]+ [=]	rs4131751 3 MAF(EU) =0.03	TOLERATED	PROBABLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
COL6A3	NM_004 369	c.[2195C>T]+ [=] p.[Thr732Met] +[=]	rs3707191 48 MAF(ExA C): A=0.0001 0/12	TOLERATED	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
AGTR1	NM_031 850	c.[730G>T]+[=] p.[Ala244Ser] +[=]	rs1272122 5 MAF(EU) =0.003	DAMAGING	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
KRIT1	NM_194 455	c.[815A>G]+[=] p.[Gln272Arg] +[=]	rs7655422 41 MAF(ExA C): C=0.0000 7/9 (ExAC)	TOLERATED	BENIGN	NEUTRAL	DISEASE CAUSING								
RET	NM_020 975	c.[2944C>T]+ [=] p.[Arg982Cys]+[=]	rs1715855 8 MAF(EU) =0.02	DAMAGING	PROBABLY DAMAGING	NEUTRAL	DISEASE CAUSING								
FBN1	NM_000 138	c.[5065G>A]+ [=] p.[Asp1689As n]+[=]	MAF(EU) =N/A	DAMAGING	POSSIBLY DAMAGING	DISEASE	DISEASE CAUSING								
ABCC6	NM_001 171	c.[1171A>G]+ [=] p.[Arg391Gly] +[=]	MAF(EU) =0.001	DAMAGING	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING								

Patient/ Gene	*Ref Seq*	*Variant description*	*dbSNP code MAF*	*In silico analysis*				*Age*	*Sex*	*Height (cm)/ weight (kg)*	*Diagnosis /(BAV type)*	*Ao (mm)*	*AscAo (mm)*	*Calcium*	*Systemic score*
				SIFT	**POLYPHEN**	**SNP&GO**	**MUTATION TASTER**								
EMILIN2	NM_032048	c.[671G>A]+[=] p.[Arg224Gln]+[=]	rs34307586 MAF(EU)=0.03	DAMAGING	BENIGN	NEUTRAL	POLY-MORPHYSM								
TGFB1	NM_000660	c.[788C>T]+[=] p.[Thr263Ile]+[=]	rs1800472 MAF(EU)=0.03	TOLERATED	BENIGN	DISEASE	POLY-MORPHYSM								
COL6A2	NM_001849	c.[2608G>A]+[=] p.[Asp870Asn]+[=]	rs145785230 MAF(ExAC): A=0.00009/5	TOLERATED	POSSIBLY DAMAGING	NEUTRAL	POLY-MORPHYSM								
P15									M	177/94	BAV/TAA/ nsCTD (1)	51/47	6.78	0	3
CAPN2	NM_001748	c.[523G>A]+[=] p.[Glu175Lys]+[=]	MAF(EU)=N/A	TOLERATED	PROBABLY DAMAGING	DISEASE	DISEASE CAUSING	52							
FBN2	NM_001999	c.[203C>T]+[=] p.[Ala68Val]+[=]	rs62390671 MAF(EU)=0.04	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								
ADAMTS2	NM_014244	c.[2480G>A]+[=] p.[Arg827Gln]+[=]	rs35445112 MAF(EU)=0.035	TOLERATED	BENIGN	NEUTRAL	POLY-MORPHYSM								

Abbreviations: AscAo=Ascending Aorta; BAV=bicuspid aortic valve; *=patients who underwent surgery

In order to evaluate the association of the genetic variants identified in the 15 sequenced patients, where 1) the family members were available, 2) they aimed to contribute to the genetic diagnosis in the proband and/or to research on genetic bases of BAV, and 3) gave their written informed consent, we enrolled affected and not affected members of the families. At present we have the availability of 15 families and we are collecting clinical data and performed blood withdrawal for genetic analysis. In 3 out of 15 we have large part of the family and we proceeded in evaluating the impact of the genetic variants identified in the probands.

P1_ NGS targeted approach identified in the proband (II-1) 5 rare heterozygous variants (MAF<0.01) (Table) in 4 different genes: *FBN1* (p.Asn542Ser and p.Lys2460Arg), *NOTCH1* (p.Val1739Met), *LTBP1* (p.Arg1330Gln) and *TGFBR3* (p.Arg423Trp). These variants were confirmed through Sanger technology (Figure 46).

Characteristics of identified variants, and results from in silico prediction of the pathogenetic effect were reported in Table 15. Variants detected in the proband were also investigated in his first-degree relatives (Figure 47). *NOTCH1* and *LTBP1* mutations were present in proband's mother (I-1) and brother (II-2). Concerning *FBN1* gene, both p.Asn542Ser and p.Lys2460Arg variants were inherited in cis by the mother (I-1), and not found in any of other family members. Finally, the *TGFBR3* variant was transmitted by the father to the offspring (Figure 47).

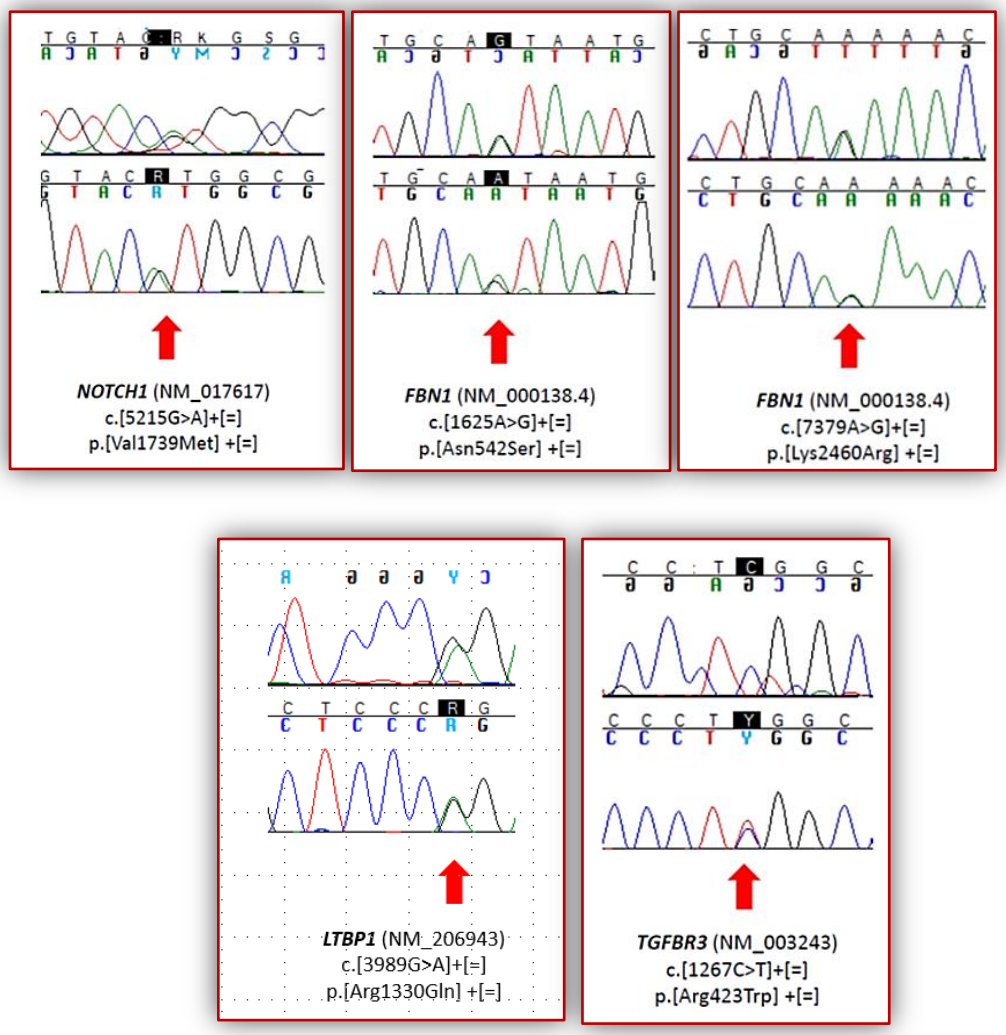


Fig 46. Sanger electropherograms showing 5 rare heterozygous variants identified in Family 1 proband

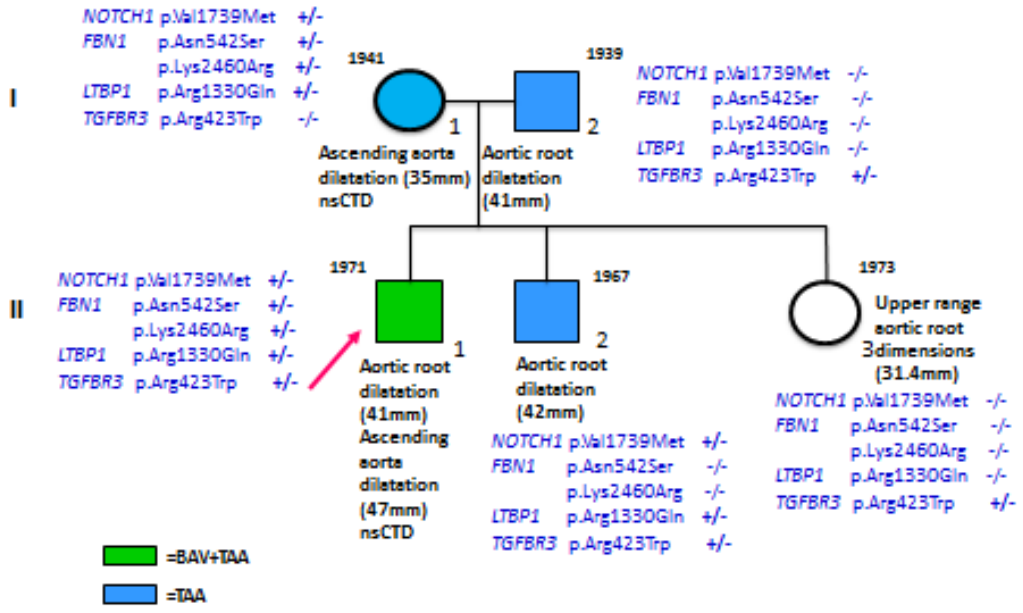


Fig 47. Family 1 pedigree with the relative years of birth

In the proband we also identified 139 common genetic variants (39 non-synonimous) in the coding regions (Table 16)

Table 16. Genetic variants (missense) identified in patient II-1

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
PLOD1 **NM_000302**	c.[295G>A]+[=] p.[Ala99Thr]+[=]	rs7551175 MAF(EU)=0.146	T	B		
EMILIN3 **NM_052846**	c.[1832C>G]+[=] p.[Ala611Gly]+[=]	rs61739314 MAF(EU)=0.029	T	PD	N	P
EMILIN3 **NM_052846**	c.[1595G>A]+[=] p.[Ser532Asn]+[=]	rs2235592 MAF(EU)=0.213	T	B		
LTBP1 **NM_206943**	c.[389G>A]+[=] p.[Arg1330Gn]+[=]	rs141080282 MAF(EU)=0.005	T	PD	N/A	DC
LTBP3 **NM_00113014**	c.[1313C>T]+[=] p.[Ala438Val]+[=]	rs11545200 MAF(EU)=0.069	T	PD		
MYH11 **NM_00104011**	c.[3721G>A]+[=] p.[Ala1241Thr]+[=]	rs16967494 MAF(EU)=0.288	D	B		
MTHFR **NM_005957**	c.[665C>T]+[=] p.[Ala222Val]+[=]	rs1801133 MAF(EU)=0.364	D	PD		

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MTHFR **NM_005957**	c.[1286A>C]+[=] p.[Glu429Ala]+[=]	rs1801131 MAF(EU)=0.313	T	B		
LRP5 **NM_002335**	c.[3989C>T]+[=] p.[Ala1330Val]+[=]	rs3736228 MAF(EU)=0.134	T	B		
ADAMTSL4 **NM_019032**	c.[577G>C]+[=] p.[Ala193Pro]+[=]	rs41317515 MAF(EU)=0.533	T	B		
MTR **NM_000254**	c.[2756A>G]+[=] p.[Asp919Gly]+[=]	rs1805087 MAF(EU)=0.161	T	B		
NOS3 **NM_000603**	c.[894T>G]+[=] p.[Asp298Glu]+[=]	rs1799983 MAF(EU)=0.344	T	B		
PDIA2 **NM_006849**	c.[553G>A]+[=] p.[Glu185Lys]+[=]	rs419949 MAF(EU)=0.184	T	B		
PDIA2 **NM_006849**	c.[1163G>A]+[=] p.[Arg388Gln]+[=]	rs400037 MAF(EU)=0.190	T	B		
PDIA2 **NM_006849**	c.[857C>T]+[=] p.[Thr286Met]+[=]	rs2685127 MAF(EU)=0.118	D	PD		
COL2A1 **NM_001844**	c.[4213G>A]+[=] p.[Gly1405Ser]+[=]	rs2070739 MAF(EU)=0.096	D	PD		
COL2A1 **NM_001844**	c.[3991G>A]+[=] p.[Val1331Ile]+[=]	rs12721427 MAF(EU)=0.071	T	B		
ABCC6 **NM_001171**	c.[1896C>A]+[=] p.[His632Gln]+[=]	rs8058694 MAF(EU)=0.486	T	B		
ABCC6 **NM_001171**	c.[3803G>A]+[=] p.[Arg1268Gln]+[=]	rs2238472 MAF(EU)=0.301	T	B		
ABCC6 **NM_001171**	c.[1841T>C]+[=] p.[Val614Ala]+[=]	rs12931472 MAF(EU)=0.500	T	B		
ADAMTS17 **NM_139057**	c.[1445T>C]+[=] p.[Met482Thr]+[=]	rs28567966 MAF(EU)=0.144	T	B		
ADAMTS17 **NM_139057**	c.[3281A>G]+[=] p.[Asn1094Ser]+[=]	rs2573652 MAF(EU)=0.335	T	B		
ADAMTS17 **NM_139057**	c.[647C>T]+[=] p.[Ser216Leu]+[=]	rs7496668 MAF(EU)=0.348	T	B		

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MYLK **NM_953025**	c.[62C>A]+[=] p.[Pro21His]+[=]	rs28497577 MAF(EU)=0.085	T	B		
COL11A1 **NM_001854**	c.[3986C>T]+[=] p.[Pro1323Leu]+[=]	rs3753841 MAF(EU)=0.387	D	B		
COL9A1 **NM_001851**	c.[1862A>G]+[=] p.[Gln621Arg]+[=]	rs1135056 MAF(EU)=0.427	T	B		
COL9A1 **NM_001851**	c.[1015T>C]+[=] p.[Ser339Pro]+[=]	rs592121 MAF(EU)=0.363	T	B		
FN1 **NM_212482**	c.[44A>T]+[=] p.[Gln15Leu]+[=]	rs1250259 MAF(EU)=0.232	T	B		
MMP9 **NM_004994**	c.[836A>G]+[=] p.[Gln279Arg]+[=]	rs17576 MAF(EU)=0.380	T	B		
COL6A2 **NM_001849**	c.[2039G>A]+[2039G>A] p.[Arg680His]+[Arg680His]	rs1042917 MAF(EU)=0.509	D	PD		
COL6A1 **NM_001848**	c.[2549G>A]+[=] p.[Arg850His]+[=]	rs1053312 MAF(EU)=0.272	D	B		
MTRR **NM_002454**	c.[524C>T]+[=] p.[Ser175Leu]+[=]	rs1532268 MAF(EU)=0.336	T	B		
LTBP4 **NM_001042544**	c.[2458A>G]+[=] p.[Thr820Ala]+[=]	rs1051303 MAF(EU)=0.452	T	B		
LTBP4 **NM_001042544**	c.[3422C>T]+[=] p.[Thr1141Met]+[=]	rs10880 MAF(EU)=0.392	T	B		
LTBP4 **NM_001042544**	c.[2359A>G]+[=] p.[Thr787Ala]+[=]	rs1131620 MAF(EU)=0.452	T	B		
LTBP4 **NM_001042544**	c.[580G>A]+[=] p[Val194Ile]+[=]	rs2303279 MAF(EU)=0.239	T	B		
ADAMST10 **NM_030957**	c.[401G>C]+[=] p.[Ser134Thr]+[=]	rs7255721 MAF(EU)=0.295	T	B		
COL6A3 **NM_004369**	c.[9206C>T]+[9206C>T] p.[Thr3069Ile]+[Thr3069Ile]	rs1131296 MAF(EU)=0.404	T	B		
COL3A1 **NM_000090**	c.[2092G>A]+[=] p.[Ala698Thr]+[=]	rs1800255 MAF(EU)=0.262	T	B		

T=tolerated, D=disease causing B=benign, PD=probably/possibly damaging N=neutral, DS=disease P=polymorphism, DC=disease causing

P2_After NGS analysis, we identified 39 non-synonymous genetic variants in subject II-5 of the second family. Among the 39 missense genetic variants, 2 were rare (Table 15). These two heterozygous mutations affected *EMILIN1* and *AXIN1* gene (Table 15); the first mutation in *EMILIN1* gene determines a nucleotide substitution from guanine to adenine at position 1781 of the coding sequence (exon 4) and then an amino acid change from arginine to histidine at 594 amino acid position; the second mutation in *AXIN1* gene determines a nucleotide substitution from guanine to adenine at position 1549 of the coding sequence (exon 5) and then an amino acid change from valine to isoleucine at 517 amino acid position. Both mutations were confirmed by direct Sanger sequencing (Figure. 48)

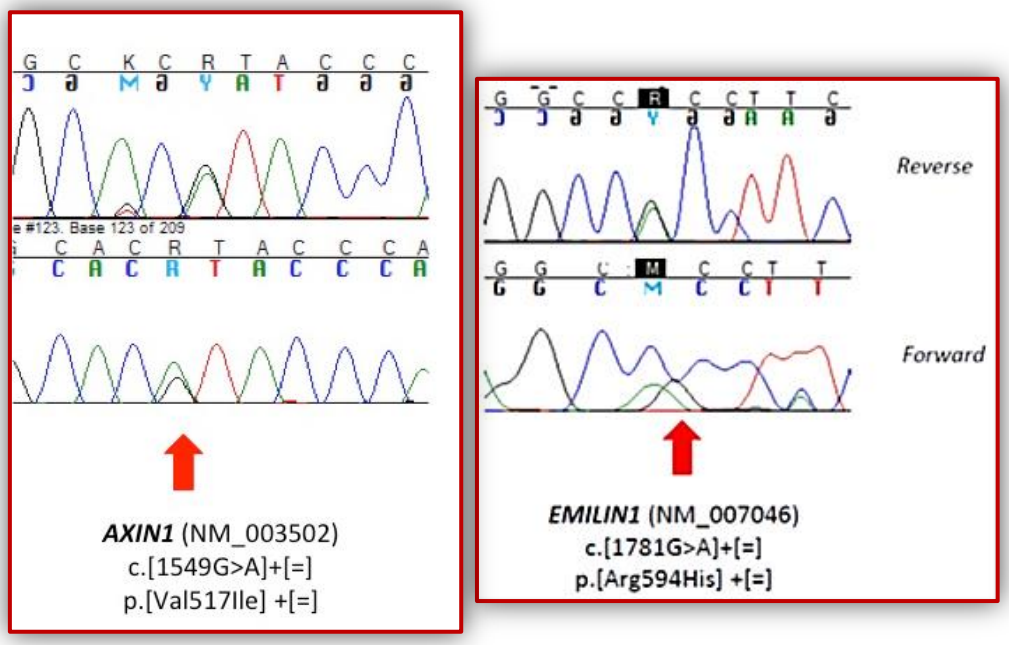


Fig 48. Sanger electropherograms showing 2 rare heterozygous variants identified in subject II5 of Family 2

The two genetic variants were investigated his first-degree relatives; *EMILIN1* mutation was identified in the mother of the proband (I-4), in two aunts (I-1 e I-2), and in her brothers (II-2 e II-6). *AXIN1* mutation wasn't detected in any of the proband's family members (Figure 50).

Futhermore, subject II5 showed 117 synonimous SNP and 61 intronic SNPs in the exon flanking intronic regions (Table 17).

Table 17. Genetic variants (missense) identified in patient II-5

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MMP9 **NM_004994**	c.[1721G>C]+[=] p.[Arg574Pro]+[=]	rs2250889 MAF(EU)=0.046	T	B		
MMP9 **NM_004994**	c.[836A>G]+[=] p.[Gln279Arg]+[=]	rs17576 MAF(EU)=0.381	T	B		
MMP9 **NM_004994**	c.[2003G>A]+ c.[=] p.[Arg668Gln] p.[=]	rs17577 MAF(EU) =0.175	T	B		
ELN **NM_000501**	c.[1264G>A]+[=] p.[Gly422Ser]+[=]	rs2071307 MAF(EU)=0.420	T	B		
MYLK **NM_053025**	c.[62C>A]+[=] p.[Pro21His]+[=]	rs28497577 MAF(EU)=0.085	T	B		
ADAMTSL4 **NM_019032**	c.[577G>C]+[=] p.[ALa193Thr]+[=]	rs41317515 MAF(EU)=0.466	T	B		
ADAMTS17 **NM_139057**	c.[3281A>G]+[=] p.[Asn1094Ser]+[=]	rs2573652 MAF(EU)=0.335	T	B		
ADAMTS17 **NM_139057**	c.[1445T>C]+[=] p.[Met842Thr]+[=]	rs28567966 MAF(EU)=0.144	T	B		
ADAMTS17 **NM_139057**	c.[1337C>T]+[=] p.[Thr446Ile]+[=]	rs72755233 MAF(EU)=0.104	T	B		
CAPN2 **NM_01748**	c.[1702A>C]+[=] p.[Lys490Gln]+[=]	rs17599 MAF(EU)=0.242	T	B		
AGT **NM_000029**	c.[803T>C]+[=] p.[Met268Thr]+[=]	rs699 MAF(EU)=0.412	T	B		
MTHFR **NM_005957**	c.[665C>T]+ c.[=] p.[Ala222Val]+ p.[=]	rs1801133 MAF(EU)=0.365	D	PD		
MTRR **NM_002454**	c.[1049A>G]+ c.[=] p.[Lys350Arg]+ p.[=]	rs162036 MAF(EU)=0.136	T	B		

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MTRR **NM_002454**	c.[1783C>T]+ [=] p.[His595Tyr]+ [=]	rs10380 MAF(EU)=0.110	D	B		
COL3A1 **NM_000090**	c.[2092G>A]+[=] p.[Ala698Thr]+[=]	rs1800255 MAF(EU)=0.262	D	B		
FN1 **NM_212482**	c.[44A>T]+ [=] p.[Gln15Leu]+ [=]	rs1250259 MAF(EU)=0.233	T	B		
COL6A3 **NM_004369**	c.[9206C>T]+[=] p.[Thr3069Ile]+[=]	rs1131296 MAF(EU)=0.404	T	B		
COL6A3 **NM_004369**	c.[8962A>G]+[=] p.[Met2988Val]+[=]	rs11690358 MAF(EU)=0.112	T	B		
AGGF1 **NM_018046**	c.[2092C>A]+[=] p.[Pro698Thr]+[=]	rs34400049 MAF(EU)=0.295	T	B		
ADAMTS2 **NM_014244**	c.[3529C>T]+[=] p.[Pro1177Ser]+[=]	rs1054480 MAF(EU)=0.295	T	PD		
COL9A1 **NM_001851**	c.[1862A>G]+[=] p.[Gln621Arg]+[=]	rs1135056 MAF(EU)=0.427	T	B		
COL9A1 **NM_001851**	c.[1015T>C]+[=] p.[Ser339Pro]+[=]	rs592121 MAF(EU)=0.363	T	B		
CCM2 **NM_001029835**	c.[220G>A]+[=] p.[Val74Ile]+[=]	rs2107732 MAF(EU)=0.102	T	B		
COL1A2 **NM_000089**	c.[1645C>G]+[=] p.[Pro549Ala]+[=]	rs42524 MAF(EU)=0.240	T	B		
ABCC6 **NM_001171**	c.[1896C>A]+[=] p.[His632Gln]+[=]	rs8058694 MAF(EU)=0.486	T	B		
ABCC6 **NM_001171**	c.[1841T>C]+[=] p.[Val614Ala]+[=]	rs12931472 MAF(EU)=0.500	T	B		
LTBP4 **NM_001042544**	c.[580G>A]+[=] p.[Val194Ile]+[=]	rs2303729 MAF(EU)=0.464	T	B		
LTBP4 **NM_001042544**	c.[2359A>G]+[=] p.[Thr787Ala]+[=]	rs1131620 MAF(EU)=0.452	T	B		

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
LTBP4 **NM_001042544**	c.[2458A>G]+[=] p.[Thr820Ala]+[=]	rs1051303 MAF(EU)=0.452	T	B		
LTBP4 **NM_001042544**	c.[3422C>T]+[=] p.[Thr1141Met]+[=]	rs10880 MAF(EU)=0.392	T	B		
EMILIN3 **NM_052846**	c.[1595G>A]+[=] p.[Ser532Asn]+[=]	rs2235592 MAF(EU)=0.213	T	B		
COL6A1 **NM_001848**	c.[2549G>A]+[=] p.[Arg850His]+[=]	rs1053312 MAF(EU)=0.272	T	B		
COL6A1 **NM_001848**	c.[2669C>T]+[=] p.[Ser890Leu]+[=]	rs13051496 MAF(EU)=0.260	T	B		
COL6A2 **NM_001849**	c.[2039G>A]+[=] p.[Arg680His]+[=]	rs1042917 MAF(EU)=0.490	D	PD		

T=tolerated, D=disease causing B=benign, PD=probably/possibly damaging N=neutral, DS=disease P=polymorphism, DC=disease causing

P3_NGS approach allowed the identification of 33 heterozygous genetic variants in another subject of the second family (II-4), 2 of which were rare (MAF<0.01). *NOTCH1* mutation determines a nucleotide substitution from guanine to adenine in position 3836 of the coding sequence (exon 23) and an amino acid change from arginine to histidine in position 1279 of the protein. The second variant was identified in the *LTBP1* and determines a nucleotide substitution from guanine to adenine in position 736 of the coding sequence (exon 3) and an amino acid change from arginine to histidine in position 246. Both mutations were confirmed by Sanger direct sequencing (Figure 49).

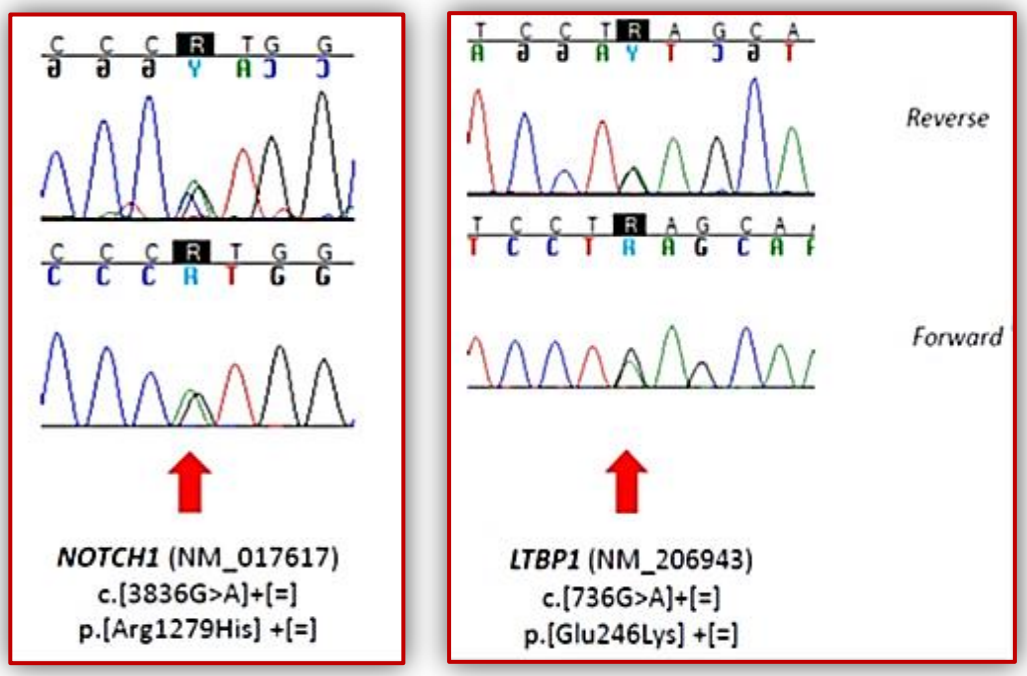


Fig 49. Sanger electropherograms showing 2 rare heterozygous variants identified in subject II4 of Family 2

Both mutations were then investigated in the subject's first-degree relatives. *NOTCH1* mutation was identified in the subject's mother (I-4), in the aunts (I-1, I-2, I-3) and in her brothers (II-2 e II-3). *LTBP1* mutation has been detected in the mother (I-4), two aunts (I-2, I-3) and brothers of the proband (II-2 e II-3) (Figure 49).

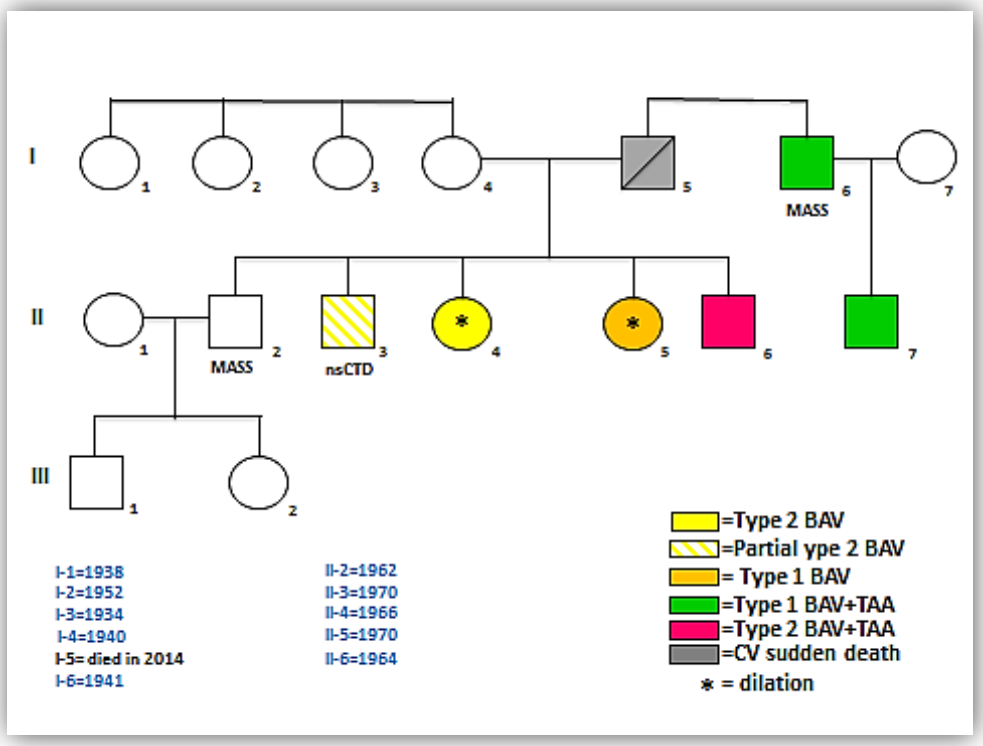


Fig 50. Family 2 pedigree with the relative years of birth

Futhermore, the subject II4 showed 167 synonimous SNP and 26 intronic SNPs in the exon flanking intronic regions (Table 18)

Table 18. Genetic variants (missense) identified in patient II-4

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MTRR **NM_002454**	c.[1783C>T]+[=] p.[His595Tyr]+[=]	rs10380 MAF(EU)=0.110	T	B		
MTRR **NM_002454**	c.[1049A>G]+ [=] p.[Lys350Arg]+ [=]	rs162036 MAF(EU)=0.136	T	B		
LTBP4 **NM_001042544**	c.[2458A>G]+ [=] p.[Thr820Ala]+ [=]	rs1051303 MAF(EU)=0.452	T	B		
LTBP4 **NM_001042544**	c.[3422C>T]+ [=] p.[Thr1141Met]+ [=]	rs10880 MAF(EU)=0.392	T	B		

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
LTBP4 **NM_001042544**	c.[580G>A]+[=] p.[Val194Ile]+[=]	rs2303729 MAF(EU)=0.464	T	B		
LTBP4 **NM_001042544**	c.[2359A>G]+[=] p.[Thr87Ala]+ [=]	rs1131620 MAF(EU)=0.452	T	PD		
COL6A1 **NM_001848**	c.[2549G>A]+[=] p.[Arg850His]+[=]	rs1053312 MAF(EU)=0.272	T	B		
COL6A1 **NM_001848**	c.[2669C>T]+[=] p.[Ser890Leu]+[=]	rs13051496 MAF(EU)=0.260	T	B		
COL6A2 **NM_001849**	c.[2039G>A]+[=] p.[Arg680His]+[=]	rs1042917 MAF(EU)=0.510	T	PD		
COL6A3 **NM_004369**	c.[9206C>T]+[=] p.[Thr3069Ile]+[=]	rs1131296 MAF(EU)=0.404	T	B		
COL6A3 **NM_004369**	c.[8962A>G]+[=] p.[Met2988Val]+[=]	rs11690358 MAF(EU)=0.112	T	B		
CCM2 **NM_001029835**	c.[421G>A]+[=] p.[Val141Ile]+[=]	rs11552377 MAF(EU)=0.174	T	B		
COL11A1 **NM_001854**	c.[4603T>C]+[=] p.[Ser1535Pro]+[=]	rs1676486 MAF(EU)=0.170	T	B		
MMP9 **NM_004994**	c.[836A>G]+[=] p.[Gln279Arg]+[=]	rs17576 MAF(EU)=0.381	T	B		
MMP9 **NM_004994**	c.[2003G>A]+[=] p.[Arg668Gln]+[=]	rs17577 MAF(EU)=0.175	T	B		
MMP9 **NM_004994**	c.[1721G>C]+[=] p.[Arg574Pro]+[=]	rs2250889 MAF(EU)=0.046	T	B		
CAPN2 **NM_001748**	c.[1702A>C]+[=] p.[Lys568Gln]+[=]	rs17599 MAF(EU)=0.242	D	B		
MTHFR **NM_005957**	c.[665C>T]+ [=] p.[Ala222Val]+[=]	rs1801133 MAF(EU)=0.365	D	PD		
TGFB1 **NM_000660**	c.[29C>T]+[=] p.[Pro10Leu]+[=]	rs1800470 MAF(EU)=0.382	T	B		

Gene RefSeq	Variant description	dbSNP code MAF	SIFT	Poly-phen	SNP &go	Mutation Taster
EMILIN3 **NM_052846**	c.[1595G>A]+[=] p.[Ser532Asn]+[=]	rs2235592 MAF(EU)=0.213	T	B		
LRP6 **NM_002336**	c.[3184G>A]+[=] p.[Val1062Ile]+[=]	rs2302685 MAF(EU)=0.170	T	B		
ADAMTS17 **NM_139057**	c.[3281A>G]+[=] p.[Asn1094Ser]+[=]	rs2573652 MAF(EU)=0.335	T	B		
ADAMTS17 **NM_139057**	c.[1445T>C]+[=] p.[Met482Thr]+[=]	rs28567966 MAF(EU)=0.144	T	B		
ADAMTS17 **NM_139057**	c.[1337C>T]+[=] p.[Thr446Ile]+[=]	rs72755233 MAF(EU)=0.104	T	B		
AGGF1 **NM_018046**	c.[2092C>A]+ [2092C>A] p.[Pro698Thr]+ [Pro698Thr]	rs34400049 MAF(EU)=0.295	T	B		
ADAMTS2 **NM_014244**	c.[733G>A]+ [733G>A] p.[Val245Ile]+ [Val245Ile]	rs398829 MAF(EU)=0.276	T	B		
ADAMTSL4 **NM_019032**	c.[577G>C]+[=] p.[Ala193Pro]+[=]	rs41317515 MAF(EU)=0.466	T	B		
PDIA2 **NM_006849**	c.[553G>A]+[=] p.[Glu185Lys]+[=]	rs419949 MAF(EU)=0.184	T	B		
COL1A2 **NM_000089**	c[.1645C>G]+[=] p[.Pro549Ala]+[=]	rs42524 MAF(EU)=0.240	D	B		
COL9A1 **NM_001851**	c.[1015T>C]+[=] p.[Ser339Pro]+[=]	rs592121 MAF(EU)=0.363	T	B		
AGT **NM_000029**	c.[803T>C]+[=] p.[Met268Thr]+[=]	rs699 MAF(EU) =0.411	T	B		

T=tolerated, D=disease causing B=benign, PD=probably/possibly damaging N=neutral, DS=disease P=polymorphism, DC=disease causing

P4_In the proband of family 3, subject II-8, 23 non synonymous mutations were detected (Table 19). Six rare heterozygous mutations were identified (MAF≤0.05) (Table 15). Rare variants (MAF<0.01) were identified in *LTBP4*, *COL6A3*, *LTBP4* and *ABCC6* genes and they were confirmed by Sanger direct sequencing. *LTBP4* gene

mutation (exon 12) is a 12 nucleotide in frame deletion which determines the deletion of 4 amino acids in the protein sequence (p.Asp519_Pro522del). *COL6A3* gene mutation determines a nucleotide substitution from cytosine to guanine in position 9245 of the coding sequence (exon 41) and an amino acid change from proline to arginine in position 3082 of the protein. *LTBP1* mutation determines a nucleotide substitution from cytosine to timine in position 3797 of the coding sequence (exon 12) and an amino acid change from serine to phenilalanine in position 1266 of the protein. Two genetic variants were detected in *ABCC6*, the first one determining a nucleotide substitution from guanine to adenine in position 2171 of the coding sequence (exon 41) and an amino acid change from arginine to lysine in position 724 of the protein, and the second one determining nucleotide substitution from adenine to guanine in position 2224 of the coding sequence (exon 17) and an amino acid change from isoleucine to valine in position 742 of the protein (exon 17). All variants were confirmed by Sanger direct sequencing. (Figures 51 and 52)

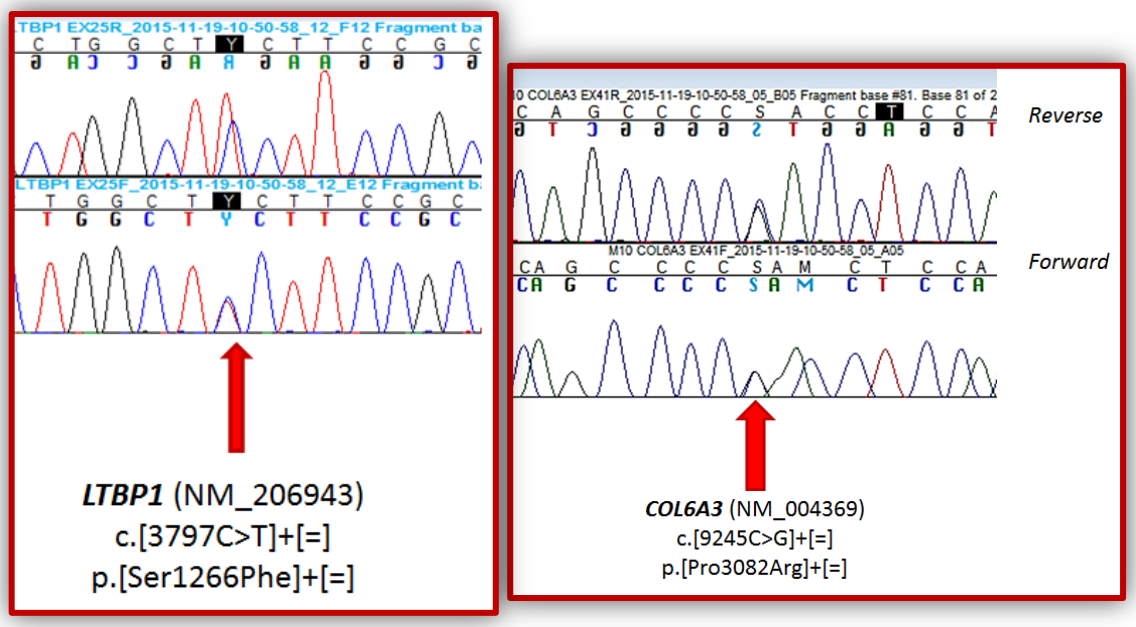
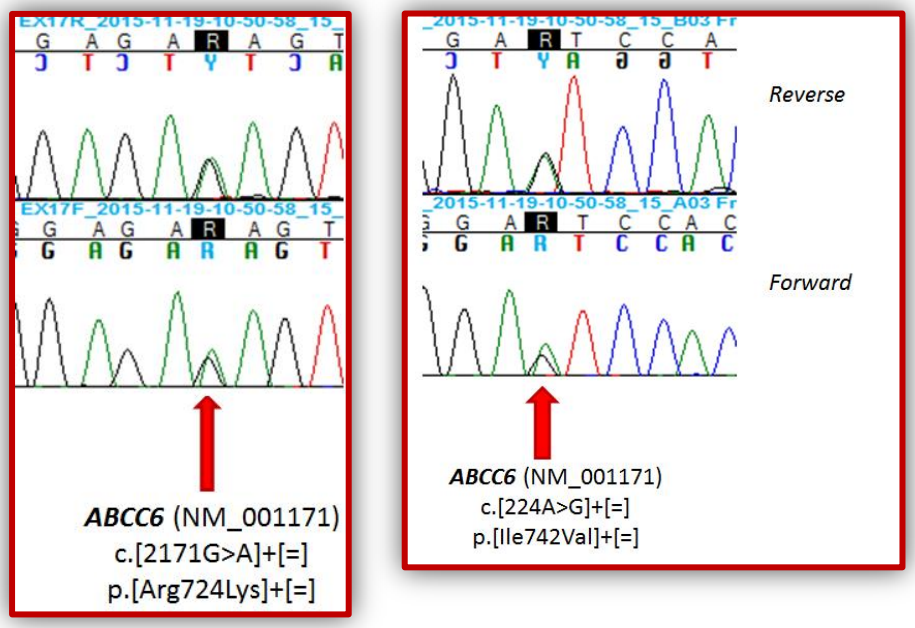


Fig 51. Sanger electropherograms showing *LTBP1* and *COL6A3* rare heterozygous variants identified in subject II-8 of Family 3



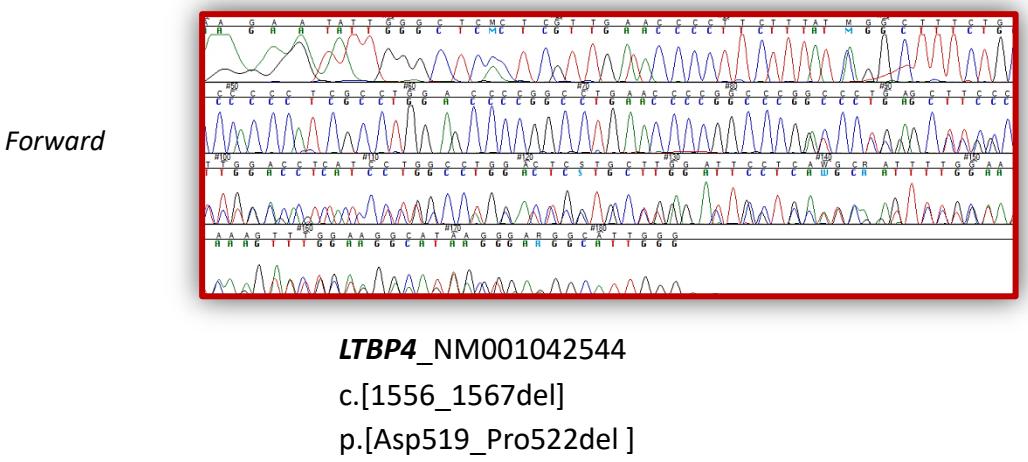


Fig 52. Sanger electropherograms showing *ABCC6* and *LTBP4* rare heterozygous variants identified in subject II-8 of Family 3

Rare variants were also investigated in the first-degree relatives of the proband. *LTB4* deletion was identified in the proband's sister (II-12); *COL6A3* mutation was detected in two nephews (III-8 and III-9) and in a sister (II-12); *LTBP1* mutation was identified in two proband' sisters (II-5 and II-12) and in two nephews (III-8 and III-9); both *ABCC6* mutations were detected in proband' sisters (II-5 and II-12) and in three nephews (III-1, III-8 and III-9) (Figure 53)

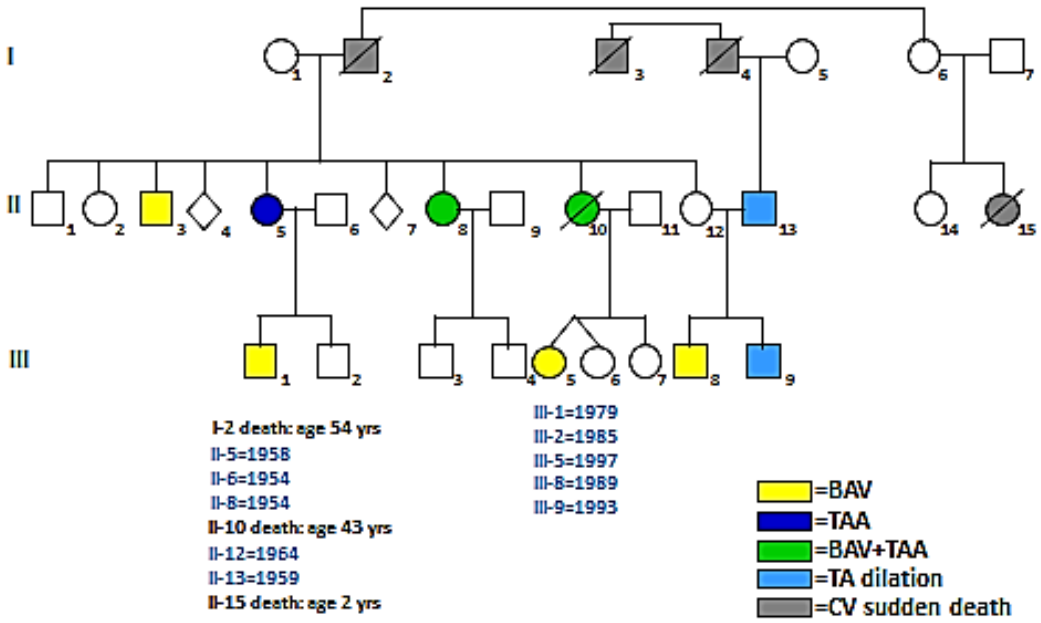


Fig 53. Family 3 pedigree with the relative years of birth

Table 19. Genetic variants (missense) identified in patient II-8 of family 3

Gene RefSeq	*Variant description*	*dbSNP code MAF*	*SIFT*	*Poly-phen*	*SNP &go*	*Mutation Taster*
MTRR **NM_002454**	c.[524C>T]+[=] p.[Ser175Leu]+[=]	rs1532268	T=0.3360	T	B	
MYH11 **NM_001040113**	c.[3721G>A]+[=] p.[Ala1241Thr]+[=]	rs16967494	T=0.2882	D	B	
MMP9 **NM_004994**	c.[836A>G]+[=] p.[Gln279Arg]+[=]	rs17576	G=0.3806	T	B	
CAPN2 **NM_001748**	c.[1702A>C]+[=] p.[Lys568Gln]+[=]	rs17599	C=0.2425	D	B	
NOS3 **NM_000603**	c.[894T>G]+[=] p.[Asp298Glu]+[=]	rs1799983	T=0.3439	T	B	
COL3A1 **NM_000090**	c.[2092G>A]+[=] p.[Ala698Thr]+[=]	rs1800255	A=0.2624	T	B	

Gene RefSeq	Variant description	dbSNP code MAF	SIFT	Poly-phen	SNP &go	Mutation Taster
MTHFR **NM_005957**	c.[665C>T]+[=] p.[Ala222Val]+ [=]	rs1801133	A=0.3648	D	PD	
COL2A1 **NM_001844**	c.[4213G>A]+[=] p.[Gly1405Ser]+[=]	rs2070739	T=0.0964	T	PD	
ELN **NM_000501**	c.[1264G>A]+[=] p.[Gly422Ser]+[=]	rs2071307	A=0.4205	T	PD	
ADAMTS2 **NM_014244**	c.[722G>A]+[=] p.[Arg241His]+[=]	rs11750821	T=0.1043	D	B	
COL11A1 **NM_001854**	c.[3968C>T]+[=] p.[Pro1323Leu]+[=]	rs3753841	A=0.6123	T	B	
COL9A1 **NM_001851**	c.[1015T>C]+[=] p.[Ser339Pro]+[=]	rs592121	G=0.3628	T	B	

T=tolerated, D=disease causing B=benign, PD=probably/possibly damaging N=neutral, DS=disease P=polymorphism, DC=disease causing

7.2 DISCUSSION

The major findings of this second part of the PhD thesis were 1) the demonstration that, even if there are several overlapping cardiovascular and systemic manifestations between bicuspid aortic valve and the most severe disorders of MFS, the evaluation of four variables easily diagnosable by clinicians not specialized in connective tissue disorders or medical genetics could allow a better distinction of patients affected by BAV or patients affected by MFS or BAV/MFS; 2) the strong suggestion that BAV, usually defined with an autosomal dominant pattern of inheritance with incomplete penetrance, is in the majority of cases a multifactorial genetic trait in which several genetic susceptibility and environmental factors interacting together determine the onset and complications of the disease.

Aortic dilation (mainly involving the ascending aorta) is one of the most common non-valvular findings in BAV occurring in 35-80% of adult patients [De Cario R et al.,2014; Nistri S et al., 2005; Cecconi M et al., 2006; Michelena HI et al., 2011; Della Corte A et

al., 2007]. It is mainly asymptomatic and has been reported in some cases to precede aortic dissection or rupture. The considerable incidence of aortic dilation in BAV patients suggests a correlation between the aortic disease and the congenital BAV malformation. Earlier studies, conducted by Larson and Edwards [Larson EW et al., 1984], reported a 9-fold increased risk of aortic dissection in BAV patients and an 18-fold higher risk in unicommissural aortic valve compared with controls. These findings suggested that the valve malformation may act as an independent risk factor for aortic dissection. However, the pathogenesis, as well as the cellular and molecular mechanisms underlying the development and progression of aortic dilation in BAV, is not clearly understood and remains controversial. Due to the high prevalence of isolated BAV, the perceived burden of thoracic aortic complications, namely aneurysm and dissection, has led many authors to an early indication to prophylactic aortic surgery in individuals with BAV in comparison with subjects with three-leaflet aortic valve [Hiratzka LF et al., 2010; Svensson LG et al., 2011]. This position has been also supported by the presence of multiple reported similarities between BAV and MFS [Nistri S et al., 2012]. Recently, however, two large, long-term, retrospective cohort studies of BAV patients [Michelena HI et al., 2011; Tzemos N et al., 2008], while confirming the absolute increased risk of aortic dissection in BAV in comparison with the general population, reported a low number of events. These findings have raised concerns [Sundt TM 3rd et al., 2010; Roberts WC et al., 2011] on the application of surgical criteria adopted in MFS patients to those with BAV and thoracic aortic aneurysm (TAA), resulting in more conservative and individualized recommendations for the repair of the aortic sinuses or replacement of the ascending aorta in BAV patients by the 2014 AHA/ACC valvular heart disease guidelines [Nishimura RA et al., 2014]. Recently, Isselbacher and coworkers (2016) demonstrated how patients with recurrent aortic dissection were more likely to have MFS (21.5% versus 3.1%) but not BAV (3.6% versus 3.2%); moreover, they observed that MFS diagnosis independently predicted recurrent aortic dissection with an hazard ratio of 8.6 (95% CI 5.8–12.8). The authors concluded that among those suffering acute aortic dissection, 5% have a history of a prior aortic dissection and that recurrent aortic dissection is strongly associated with Marfan syndrome.

In 2012, our group demonstrated a 4-fold increase in the prevalence of BAV in a large cohort of unrelated MFS patients with respect to the general population screened by echocardiography [Basso C et al., 2004]. Indeed, out of the 257 unrelated MFS patients,

BAV was unequivocally identified in 12 (4.7%) MFS patients [Nistri S et al., 2012]. Only 3 patients accepted to undergo mutation screening analysis [Nistri S et al., 2012]. Previously described mutations were detected in 2 out of 3 patients [Attanasio M et al., 2008], they involved conserved cysteine residues and are thought to be causative because they alter fibrillin-1 structure. These findings are consistent with data showing decreased FBN1 mRNA or protein content in a subgroup of BAV patients [Fedak PW et al., 2003], which suggest that *FBN1* may be one of the genes associated with BAV. Moreover, the association between the two disorders seems to cause a more severe involvement of the aorta with a higher percentage of TAA requiring surgery. Interestingly, Z score was>2 in all our adult patients [Loeys BL et al., 2010]. Moreover, ectopia lentis, dural ectasia, myopia and striae distense were common in our MFS/BAV, while they presented mostly minor skeleton criterion [De Backer J et al., 2006]. Noteworthy, all 12 MFS/BAV patients satisfied the clinical diagnostic criteria for MFS also according to the newly revised Ghent criteria [Loeys BL et al., 2010]. Although a reduced FBN1 content has been reported in the aortic media of BAV patients [Fedak PW et al., 2003], it has been stated that in multiple clinical disorders associated with FBN1 alterations there is no propensity for congenital aortic valve malformations [De Paepe et al., 1996]. Our data challenged this consideration, prompting the need for the mutation screening analysis of BAV patients not fulfilling the clinical criteria for MFS. Moreover, from the practical point of view, the paper of Nistri et al. [Nistri S et al., 2012] underscored the need to optimize the visualization of aortic valve morphology in MFS patients and the importance of comprehensive clinical evaluation of BAV patients to detect clinical features suggestive for inherited connective tissue disorders. Subsequently, our group performed a study aimed to screen for *FBN1* mutations in ten patients with BAV and thoracic aortic dilation, not fulfilling the clinical criteria for MFS [Pepe G et al., 2014]. Mutation analysis was performed on 8 of the 10 patients and *FBN1* mutations were detected in two. This was the first study reporting pathogenetic *FBN1* mutations in patients with BAV and aortic dilation/aneurysm in whom MFS and other more severe type 1 fibrillinopathies were clinically excluded [Loeys BL et al., 2010]. The detection of *FBN1* point mutations in patients with BAV/TAA without MFS, adds to the striking clinical heterogeneity of both BAV and type I fibrillinopathies showing that aortic dilation/aneurysm may develop in a subgroup of BAV patients as a manifestation of an inherited connective tissue disorder. Noteworthy, the two patients carrying the mutations were male, one displayed a family history of TAA, another had MVP, without systemic features which were otherwise prevalent in the remaining

patients. Moreover, both had aortic aneurysm size attaining the threshold for surgery according to old American and current European Guidelines [Bonow RA et al. 2006; Vahanian A et al., 2007], notwithstanding the young age, with the largest diameter localized at the level of the sinuses of Valsalva, a pattern of aortic dilation, specifically addressed as "root phenotype" by Della Corte et al [Della Corte A et al., 2007], and considered to be an independent predictor of fast progression [Della Corte A et al., 2014]. Interestingly, 8 out of our 10 patients displayed this phenotype, in association with a certain degree of systemic features typical of connective tissue disorders [De Cario R et al., 2014]. These findings call for greater focus on the BAV-related cardiovascular abnormalities rather than on the MFS-like systemic features, which may well coexist and warrant investigation in BAV patients in general, but are not associated with the *FBN1* mutations identified in the study. On the other hand, these *FBN1* mutations do not completely fulfil the definition of the major criterion for MFS according to the revised Ghent criteria since they have never been detected in MFS patients with TAA [Pepe G et al., 2014]. Therefore, our two BAV/TAA patients did not achieve the diagnosis of MFS.

Data of this thesis on a cohort of 79 consecutive BAV patients confirmed that patients have a strong male predominance (~75%) and come to the attention of the cardiologists and have BAV diagnosis at a median age for 42 years. This datum underline the utility in this moment of the patients clinical history for a differential diagnosis of the aortopathy that eventually accompanies the BAV diagnosis. In fact the identification of the presence of BAV in patients with diagnosis of MFS, as well as other related connective tissue disorders, results in a different management of patients, in particular concerning the timing of aortic surgery. This issue is particular important due to the fact that 1) in agreement with our and literature previous data, 46.7% of BAV patients in this study have Z-score ≥2 at the aortic root level; and 2) even if patients with ≥7 points of systemic score [calculated according to revised Ghent criteria (Loeys BL et al., 2010)] were significantly lower in BAV patients than in MFS patients or BAV/MFS patients, the analysis of systemic features showed that BAV patients have at least in part common systemic manifestations with MFS patients furtherly complicating, together with the possible presence of aortic aneurysm, the differential diagnosis between MFS and BAV.

The analysis of clinical variables among BAV, MFS and BAV/MFS patients underline some statistically significant differences both in cardiovascular and systemic

characteristics that could help clinicians to perform correct clinical diagnosis in the case of overlapping manifestations. At this purpose, we developed a Florence BAV-MFS score that included the following 5 variables - pectus carinatum, pes planus, wrist and thumb signs, myopia≥3OD, and mitral valve prolapse - able to identify BAV patients at risk of having or develop MFS diagnosis. We observed, in fact, that patients with ≥2 manifestations out of the 5 considered were significantly lower in BAV patients (11/79, 13.9%) than in BAV/MFS (7/9, 77.7%) or MFS patients (68/84, 81.0%). The five variable included in the Florence BAV-MFS score are easily assessable and diagnosable in a clinical contest not specialized in MFS and related disorders and could allow to refer the patient to a Referral Center for connective tissue disorders.

An original data of the study was the observation that BAV patients showed reduced (root-ascending aorta) delta values [0.001 (IQR -5.25-3.25)] with respect to MFS patients [9.00 (IQR 6.00-13.00)] and BAV/MFS patients [0.010 (IQR -2.75-14.75)]. This datum, besides the suggestion of differential physiopathological mechanisms, could be a further variable to be considered for differential diagnosis between BAV with TAA and MFS with TAA patients. Unfortunately, due to the fact that data on the two levels of the aorta, in particular thoracic ascending aorta diameters, were not available for all patients, we cannot test and eventually introduce this variable in the Florence BAV-MFS score.

The targeted NGS approach, allowing the sequencing of coding region and adiacent regions of consensus for exon splicing of 91 genes (associated with MFS and related disorders, bicuspid aortic valve, aortopathies and aortcic wall remodeling), on the 15 BAV patients identified 78 genetic variants with MAF<0.05 in the European populations (where available): 43 with a MAF<0.01 and 34 called damaging with at least 1 *in silico* prediction tool.

Of note is that for all patients more than 1 low frequency genetic variants were identified ranging from 2 of patient P2 to 11 of P11. Even when we focus the attention on variants predicted to be probably damaging with the majority of *in silico* algorithms, the large part of patients (n=12) showed more than 1 low frequency genetic variants, ranging from 2 to 10 of P14. This datum suggests that different genetic susceptibility factors, instead of a major pathogenetic variant in a single gene, might be responsible for the BAV phenotype and for the variability of the phenotype among different affected subjects. This datum is in agreement and strengthens a previous datum by Bonachea et al. that, using a targeted (97 genes in large part different from our panel of

genes) combinatorial next-generation sequencing approach for the study of bicuspid aortic valve, 1) identified a large number of putative disease-causing variants, including variants in 26 genes not previously associated with human BAV, and 2) found more than 1 genetic variant in the large part of index cases investigated.

In two patients, genetic variants in *FBN1* gene were observed: P1 (p.Asn542Ser and p.Lys2460Arg) and P14 (p.Asp1689Asn). The identification of the pathogenetic variants in the *FBN1* gene in patients with an aortic root dilatation and Z-score ≥2 [Loeys BL et al., 2010] determine the diagnosis of MFS and than the coexistance of BAV and MFS phenotypes in the same patient. The diagnosis of MFS, allows as previously underlined, a different estimation of dissection risk and patients management indicating once more the crucial role of genetic diagnosis in BAV patients suggestive of MFS.

Six patients showed genetic variants in genes previously suspected to be associated with non-syndromic BAV: P1 (p.Val1739Met *NOTCH1*), P2 (p.Val517Ile *AXIN1* benign according to all *in silico* prediction tools), P3 (p.Arg1279His *NOTCH1*), P5 (p.Asp495Glu *AXIN1* benign according to all *in silico* prediction tools), P6 (p.Pro131Leu *PDIA2*), P11 (p.Ala316Val *PDIA2* and p.Glu201Lys *UFD1L*). For some of these genetic variants the report in dbSNP even if at low frequency together with the benign prediction mitigate the major role in BAV; nevertheless, for the other, no definitive association with BAV can be sustained in absence of familial segregation or functional data.

In 11 sequenced BAV patients, this investigation identified rare and low frequency genetic variants in genes not previously known to be involved in human BAV; such variants are considered hypothesis-generating and these genes merit further testing in replication cohorts.

Twelve genetic variants were identified in genes involved in TGF-β signalling: 5 *LTPB1*, 2 *LTBP2*, 1 *LTBP4*, 1 *SMAD3*, 1 *TGFBR3*, and 2 *TGFB1*. Genetic variants in genes coding TGF-β signalling components were identified in 9 out of 15 patients. Three patients showed 2 TGF-β signalling genetic vatiants (P1: p.Arg1330Gln *LTBP1* and p.Arg423Trp *TGFBR3*; P4: p.Ser1266Phe *LTBP1* and p.Asp519_Pro522del *LTBP4*; P11 p.Pro319Gln *LTBP2* and p.Thr263Ile *TGFB1*). TGFβ is a crucial factor of vascular remodeling, the impaired signaling of which can alter the structure and composition of the extracellular matrix. Paloschi and coworkers in 2015 showed that TGFβ activation

during aneurysm formation is muted in patients with BAV, possibly as a result of an increased TGF-β sequestration in the extracellular space. In agreement with our genomic data, Rocchiccioli and coworkers (2016), by secretome analysis, found that in BAV aneurysms 21 out of 38 differentially modulated proteins were involved in TGF-ß signaling further confirming its crucial role of in TAA development, especially when associated to BAV. In particular, among the differentially expressed TGFß -related factors, they found that the LTBP4 exhibited significant reduced expression, confirmed by gene expression analysis, in BAV specimens [Rocchiccioli S et al., 2016].
It is well known that TGF-ß is stored in the ECM as a small latent complex (SLC) in an inactive form bound to latent TGF-ß binding proteins (LTBPs) [Shi M et al., 2011]. LTBPs comprise a family of four ECM proteins, LTBPs-1 to -4, that are structurally similar to fibrillins and for this reason they may exert similar biological functions. A deficit of fibrillin-1 is associated with MFS [Pepe G et al., 2016] and it is accompanied by inappropriate activation of TGF-ß, perhaps due to faulty interaction of the SLC with the ECM In particular, the lack of fibrillin-1 microfibrils is believed to disrupt LTBPs incorporation into the matrix and may result in the activation of TGF-ß.

Seventeen genetic variants involved 9 genes coding collagens: *COL2A1*, *COL3A1*, *COL5A1*, *COL5A2*, *COL6A1*, *COL6A2*, *COL6A3*, *COL9A1*, *COL11A1*. In particular, seven genetic variants were identified in genes coding the three collagen VI chains (1 in *COL6A1*, 3 in *COL6A2*, and 3 in *COL6A3* gene). No data are available on the role of collagens in BAV. Urbonavicius et al. (2010) showed by proyteomic approach an alteration of collagen a-3 (VI) chain expression in abdominal aortic aneurysm. Recently, several genes encoding for ECM components, including collagen a-3(VI), were found to be down-regulated in aortic dissection [Weis-Muller BT et al., 2006]. Most collagen in the aortic wall is of type I and type III. Elevated level of circulating amino terminal propeptide of type III procollagen has recently been found to be associated with size, expansion rate of AAA, and the need for later repair [Eugster T et al., 2005]. Nevertheless, collagens are microfibrillar components of the ECM with an important structural role in the matrix organization and mediates cell-matrix interactions; therefore, the involvement of different collagen genes could be conceivable.
Seven genetic variants involved genes coding for different members of the ADAMTS family of metalloprotease (2 in *ADAMTS2* and 2 in *ADAMTS10* gene) and 3 in *ADAMTSL4* gene, a glycoprotein that share significant similarity with ADAMTS. Disintegrin-like and Metalloproteinase with Thrombospondin Motifs (ADAMTS)

proteins that are fundamentally located in the extracellular matrix (ECM) have critical roles on different cellular processes by altering the ECM architecture. It is known that expression of some members of these proteinases increases in aneurismal and dissectional aortic tissue [Güneş MF et al., 2016]. Ren and coworkers (2013) showed that *ADAMTS-1* and *ADAMTS-4* levels are elevated in TAA and dissections.
The limitation of the task to confirm the association of known genes or demonstrate association of novel genes with BAV with just the study of the probands could be, at least in part, overcome by the sequencing analysis with the same technological approach of larger cohorts of BAV patients and controls by enrichment computational analysis [Hu H et al., 2013].

Even with the well known difficulties in enrolling large families with well clinically evaluated affected and not affected individuals, familial segregation analysis remains the gold standard approach in order to confirm or exclude.

During this PhD we started the enrollment of families (at present 12 families). For three families we have the sufficient number of subject to proceed with the evaluation of the genetic variants identified in the proband.

Family 1

The proband (II-1) is a 45-year-old Caucasian normotensive male (height 181 cm, weight 70 Kg). He presented with mild pectus excavatum and chest asymmetry, retrognathia, dolichocephaly, inguinal hernia, skin striae not associated with marked weight changes, Beighton score 4/9. Echocardiography of the aorta documented the presence of a BAV with fusion of the right and left coronary cusps and a raphe and no calcification, dilatation of the aortic root (41mm; Z-score 2.56), and of ascending aorta (47 mm), non-typical mild mitral valve prolapse. MRI of the lumbo-sacral region did not show the presence of dural ectasia. No ocular clinical manifestations were observed in II-1. No family history of MFS or other connective tissue disorders, and of aortic dissection or aortic/mitral valve replacement or aortic aneurysm was reported by the proband.

The clinical evaluation of the first degree family members of II-1 was performed.

The mother (I-1) is a 75-year-old Caucasian normotensive female (height 164 cm, weight 54 Kg). She presents a positive (≥7) systemic feature score including myopia >3DO, mild pectus asymmetry and pectus carinatum deformity, mild thoracolumbar

kyphosis, reduced elbow extension, hindfoot deformity. Echocardiography of the aorta documented normal size of the aortic root diameters at the sinuses of Valsalva within normal range (31 mm; Z-score -0.74), ascending aorta dilatation (35 mm), and mild mitral and tricuspid valve prolapse.

The father (I-2) is a 76-year-old Caucasian hypertensive male (height 177 cm, weight 89 Kg). He displays a systemic features' score of 4 for the presence of pectus carinatum deformity, pectus excavatum and thoracolumbar kyphosis. Echocardiography of the I-2 aorta documented the presence of aortic root at the sinuses of Valsalva dilatation (41 mm; Z-score 1.19) with a normal size of the ascending aorta (32 mm).

The brother (II-2) of the proband is a 49-year-old normotensive Caucasian male (height 183 cm, weight 105 Kg) with pectus excavatum, pes planus, striae distensae. He presented dilated aortic root (42 mm; Z-score 2.13), and normal size of the ascending aorta (30 mm).

The sister (II-3) of the proband is a 43-year-old normotensive Caucasian female (height 170 cm, weight 57 Kg) with severe scoliosis and osteoporosis. She presented normal aortic root (31 mm; Z-score 0.23), and ascending aorta (25 mm).

BAV was present only in the proband out of the 5 family members evaluated. In history taking of each member of the family there was no evidence of either occurrence of BAV or aortic surgery or sudden death.

This NGS design utilizing targeted sequencing allowed us, in the present study, to identify 5 rare, non-synonymous exonic variants predicted in large part to be damaging by *in silico* analysis, in a patient affected by complex phenotype with BAV, aortic root and thoracic ascending aorta dilatation, and connective features evocative for Marfan syndrome. Traditional Sanger sequencing methods confirmed that these variants in *NOTCH1*, *FBN1*, *LTBP1*and *TGFBR3* genes were inherited, in various combinations, from the parents, one of which (mother) with a phenotype, upon closer observation, attributable to Marfan syndrome. In fact, she presented the two different variants found in *FBN1* gene. The two mutations in the *FBN1* are: c.1625 A>G (p.Asn542Ser) in the exon 13 and c.7379A>G (p.Lys2460Arg) in the exon 59. The first determines an aminoacid change in the calcium-binding epidermal growth factor-like domain 4 (cbEGF4), whereas the second one induces an aminoacid change in the cbEGF38 domain. Fibrillin-1 cbEGF domains are known to mediate fibrillin assembly into

microfibrils, and in turn the biomechanical properties of the microfibrillar network [Handford PA et al., 2000]. Actually, both nucleotide substitutions do not affect highly conserved aminoacids of the cbEGF domain; nevertheless some of *silico* analysis tools used to evaluate mutations pathogenicity, showed a deleterious effect for both c.1625 A>G (FATHMM and Mutation Taster) and c.7379A>G (Polyphen, FATHMM and Mutation Taster). Concerning c.7379A>G variant, although it has been hypothesized its possible effect on splicing process by generating a cryptic splice site, *in vitro* studies did not evidence its influence on mRNA processing. This variant, reported in dbSNP database (rs144189837) with MAF≤0.0002 in both "ESP_Cohort_populations" and "ExAc_Aggregated_populations", has been previously identified in patients with features of Marfan syndrome [Robinson DO et al., 2012; Howarth R et al., 2007] and in thoracic aortic aneurysm with a Marfan-like phenotype [Campens L et al., 2015].

No information was available in literature or mutation databases for the c.1625 A>G variant. The sequencing analysis of exons 13 and 59, aimed to investigate the presence of *FBN1* mutations in the parents, showed that both variants were inherited *in cis* by proband's mother (I-1). These two mutations were already identified together (not specified whether *in trans* or *in cis* due to lack of parents analysis) in our lab in further two patients: a) one who underwent surgery for TAD type A and aortic valve substitution. The dissection included from the common carotid artery to the iliac artery. He displayed also mild myopia, striae distensae, altered lower/upper body segment ratio, radicular cysts. They were the only two mutations identified by Sanger sequencing of *FBN1* in this patient; b) in the other case with severe classic MFS diagnosed on the presence of ectopia lentis, TAA and systemic features with score 10 including myopia, >3DO, pectus carinatum, scoliosis, wrist and thumb signs, pes planus/hindfoot deformity. In this case we also found a further *FBN1* mutation (c.6850C>A; exon 55). According to the last revised Ghent criteria for MFS diagnosis, the identification of *FBN1* pathogenetic mutation in the proband, exhibiting aortic root dilatation (Z-score >2), allowed to diagnose MFS. Moreover, the identification of *FBN1* mutations in proband's mother also permitted to diagnose her as potential Marfan patient, even if with a less severe phenotype. According to international guidelines , the definition of MFS diagnosis in the proband determines the indication for an earlier aortic surgery. Moreover, in this patient, due to the presence of aortic root and ascending aorta dilatation, and genetic variants in *FBN1* and other potentially modifier

genes, this issue seems of particular relevance. In fact, in this patient, further potentially patogenetic variants for aortopathy were identified.

NOTCH1 mutation is a nucleotide substitution from guanine to adenine at position 5215 in the coding gene sequence (c.5215G>A, exon 28), responsible for valine to methionine aminoacid substitution at codon 1739 (p.Val1739Met), in the transmembrane domain. Actually, NOTCH1 is a transmembrane receptor, characterized by the presence of an extracellular domain, composed of 36 tandem epidermal growth factor (EGF)-like repeats and 3 cysteine-rich Notch/LIN-12 repeats, of an intracellular domain, in which an "ankyrin repeats" domain is recognizable, and of a transmembrane domain [Garg V et al., 2005]. The intracellular domain, thereby releasing from the cell membrane after cleavage events induced by ligand-receptor interaction, acts as a transcription factor, able to modulate expression of target genes [Kopan R et al., 2009], thus contributing to aortic valve alterations development. As cleavage of the intracellular domain occurs at the transmembrane domain [Deatharage CL et al., 2015], it could be conceivable that modifications of this domain may result in an altered signal transduction. *In silico* evaluation of pathogenicity revealed a possible damaging effect by Polyphen, FATHMM and Mutation Taster algorithms. This variant has been described in dbSNP database (rs377294245), with a minor allele frequency (MAF) of 0.00009 in the ExAc_Aggregated_Populations and has not been previously described in literature in BAV patients. Nonetheless other *NOTCH1* mutations have been associated with BAV manifestations (http://www.hgmd.cf.ac.uk/ac/all.php).

We have therefore identified this same variant in proband's mother (I-1) and brother (II-2), who, however, do not exhibit bicuspid aortic valve. Nevertheless, due to the incomplete penetrance of BAV trait, the role of *NOTCH1* as BAV causative gene cannot be longer excluded.

The c.3989G>A variant at *LTBP1* gene, also identified in the I-1 and in II-2 is predicted to be deleterious by Polyphen and Mutation Taster algorithm, and it might contribute to further modulate the phenotypic expression of the disease. LTBP1, a member of the LTBPs family, is known to have dual function: as structural component of the extracellular matrix and as modulator of TGF-beta availability essential for tissue formation and homeostasis [Todorovic V et al., 2012; Massam-Wu T et al., 2010].

A mutation in the exon 8 of *TGFBR3* gene was also found (c.1267C>T), with lying in the extracellular region of the protein and that cause aminoacid substitution from

arginine to tryptophan at codon 423 (p.Arg423Trp). The *TGFBR3* gene encodes for the TGF-ß type III receptor, a membrane proteoglycan often occurring as a co-receptor with other TGF-ß receptor superfamily members [Brown CB et al., 1999]. Previous data from literature indicated the contribution of *TGFBR1* and *TGFBR2* genes in thoracic aortic aneurysms and dissections [Tran-Fadulu V et al., 2009; Isselbacher EM et al., 2016]. Only one study showed a *TGFBR2* mutation in a patient with BAV and TAA [Girdauskas E et al., 2011]. No mutations in *TGFBR1* and *TGFBR2* were identified in 11 unrelated Italian patients with familial BAV [Foffa I et al., 2013], as well as in previous further two studies on BAV patients [Loscalzo ML et al., 2007; Arrington CB et al., 2008]. Interestingly, both father (I-2) and brother (II-2), exhibiting aortic root dilatation, and sister (II-3) of proband, in whom upper range of normal aortic root dimensions were observed, showed *TGFBR3* mutation, thus suggesting a co-segregation of *TGFBR3* variant with the "aortic root dilatation" phenotype. Furthermore, beyond these rare genetic variants, other polymorphisms such as *MTHFR* p.Ala222Val (heterozygous in II-1) associated with severe cardiovascular manifestations in MFS patients or abdominal aortic aneurysm [Giusti B et al., 2003; Brunelli T et al., 2000; McColgan P et al., 2009] or *NOS3*p.Glu298Asp (heterozygous in II-1) associated with hypertension [Jáchymová M et al., 2001] and AAA [Fatini C et al., 2005] could interact to modulate the clinical phenotype.

The presence of BAV in only one member of the family did not allow the definition of the contribution of these mutations on aortic valve defect.

Family 2

Subjects II5 and II4 were analysed by NGS. II5 is a 45 year old Caucasian female with BAV type 1 and mild aortic root dilatation at the sinuses of Valsalva and mild thoracic ascending aorta dilatation. Her father died for cardiovascular sudden death at the age of 74 years. The family reported a mild thoracic aorta dilatation. No information is available on the presence of bicuspid aortic valve in the father. Her mother (75 year old) has tricuspid aortic valve and normal thoracic aortic diameters. Subject II5 has 1 sister (II4, 49 yrs) and 3 brothers (II2, 53 yrs; II3, 45 yrs; II6, 51 yrs). II4 has BAV type 2 and mild thoracic ascending aorta dilatation. II2 has a MASS phenotype and a tricuspid aortic valve. II3 has a partial BAV type 2 and non specific connective tissue disorders (nsCTD). II6 has BAV type 2 and thoracic ascending aorta aneurysm. The uncle of the

proband (I6, 74 year old) has BAV type 1 and thoracic ascending aorta aneurysm. He underwent aortic valve substitution (mechanical heart valve prosthase St Jude 25).

Due to the fact that subjects II5 and II4 had different types of BAV morphology we decided to sequence the two subjects. Usually the BAV types characterized subjects of the same family. Recently, Hui and coworkers (2016) reported 2 pairs of male monozygotic twins with discordant aortic valve morphology. These 2 cases highlight the gap in knowledge of the genetic underpinnings of BAV. Genetic testing in 2 pairs of monozygotic twins with discordant phenotypes suggests that in addition to gene inheritance, epigenetic effects, somatic mutations, and environmental factors may play a more important role than previously suspected. Hui et al (2016) suggested that because understanding of the molecular mechanisms of thoracic aortic disease has outspaced that of BAV, these cases highlight the need for ongoing investigation of the pathogenesis of BAV and its associated syndromes.

The sequencing of the two sisters of the Family 2 showed that the two subjects did not share rare or low frequency genetic variants in the 91 genes analysed.

NGS approach allowed the identification of the following variants in subject II-4, affected by BAV type 2:

- the heterozygous genetic variant c.3836G>A in exon 23 of *NOTCH1* gene, determining the substitution from asparagine to histidine in position 1279, (p.Arg1279His). Even if this variant involves a gene known to be associated with BAV phenotype, its potential pathogenicity results questionable because almost all applied *in silico* prediction tools (SIFT, Polyphen, SNP&GO), describe it as benign. This variant is reported in dbSNP database (rs61751543) with a MAF=0.01;

- the heterozygous genetic variant c.736G>A in exon 3 of *LTBP1* gene, determining the substitution from glutamic acid to lysine in position 246 of the protein (p.Glu246Lys), is described in dbSNP (rs35340726) with a MAF in the european population around 1-2%; all *in silico* prediction tools classify this variant as benign.

These variants were not detected in the (subject II-5),. Subject II-5 who has BAV type 1, sister of proband II-4, carried different variants in two different genes, *EMILIN1* and *AXIN1*:

- in exon 4 of *EMILIN1* gene, the heterozygous variant c.1781G>A was identified, determining the substitution from arginine to histidine in position 594 of the protein (p.Arg594His). This variant exists in dbSNP (rs200339477) with an associated MAF of 0.0003 in the "ExAc_Aggregated_Populations" cohort. No data are available concerning MAF associated to this variant in European population. The prediction of pathogenicity is discordant according to the different applied *in silico* tools: SIFT e SNP&GO define this variant as benign, while Polyphen and Mutation Taster highlight its potential pathogenetic effect. Literature data report that deficiency of Emilin1 (Emilin1$^{-/-}$) in mouse aortic valve tissue results in early postnatal cell-matrix defects in aortic valve tissue, including elastic fiber fragmentation, that progress to latent aortic valve disease and premature death. The Emilin1$^{-/-}$ aortic valve also displays early aberrant provisional angiogenesis and late neovascularization. In addition, Emilin1$^{-/-}$ aortic valves were found to be characterized by early valve interstitial cell activation and proliferation and late myofibroblast-like cell activation and fibrosis [Munjal C et al., 2014]. Recent data in humans suggest a potential association between *EMILIN1* gene with an autosomal dominant hereditary connective tissue disease with a not better defined clinical picture [Capuano A et al., 2016];

- in exon 5 of *AXIN1* gene the heterozygous variant c.1549G>A was identified, determining the substitution from valine to isoleucine in position 517 of the protein (p.Val517Ile). This variant exists in dbSNP (rs149849071) with an associated MAF in the European population of 0,002. All applied *in silico* prediction tools define it as benign.

Mutational segregation analysis performed on the family members of subjects II-4 and II-5 did not allow us to define a clear association between these variants and a specific clinical phenotype. In fact, some of these variants have been identified in seemingly healthy subjects of the family (I-1, I-2, I-3, I-4), while none of them has been detected in the brother of the father presenting BAV and AAT.

Family 3

The proband (II-8) is a 61-year-old caucasian with BAV type 1 who underwent aortic valve substitution (mechanical heart valve prostheses). Her brother (II-3) has BAV type 1. Her sister (II-5) presents mild ascending thoracic aorta dilatation (36 mm) and she has a son (III-1) with BAV type 1. Subject II-10's daughter (III-5) has type 1 BAV.

Another proband's sister (II-12), seemingly healthy), has two sons (III-8 e III-9) with BAV and thoracic aorta dilatation respectively.

NGS approach allowed the identification of the following rare variants:

- in exon 12 of *LTBP4* gene a 12 nucleotide in frame deletion was identified determining the deletion of 4 amino acids in the protein sequence (p.Asp519_Pro522del). This variant was never described before and it was also identified in a proband' sister who seems healthy (II-12);

- *COL6A3* gene variant c.9245C>G (exon 41) determines and an amino acid change from proline to arginine in position 3082 of the protein. This variant exists in dbSNP (rs182976977) and has an associated MAF in the European population of 0.001. The potential pathogenicity of this variant appears to be questionable as almost all applied *in silico* prediction tools (SIFT, Mutation Taster, SNP&GO), describe it as benign. The same variant was identified also in a proband' sister who seems healthy (II-12) and in two nephews (III-8 and III-9) presenting BAV and thoracic aorta dilatation respectively;

- In exon 25 of LTBP1 gene the genetic variant c.2224A>G determining an amino acid change from serine to phenilalanine in position 1266 of the protein . This variant exists in dbSNP (rs 61751752) and has an associated MAF in the European population of 0.01 Almost all *in silico* prediction tools highlight its potential pathogenicity (SIFT, PolyPhen, SNP&Go). The same variant was also identified in two sisters of the proband (II-5 and II-12), presenting TAA the first and an apparently healthy phenotype the latter, and in two nephews (III-5 and III-8) both affected by BAV;

- in exon 17 of *ABCC6* gene, two rare genetic variants were identified, c.2171G>A and c.2224A>G determining the amino acide changes from arginine to lysine in position 724 the first and from isoleucine to valine in position 742 of the protein the latter. Both mutations exist in dbSNP (rs58073789 and rs59593133 respectively) with an associated MAF in the European population around 1%. Both variants were classified as benign according to all the applied in silico prediction tools. The same variants were also identified in in two sisters of the proband (II-5 and II-12), presenting TAA the first and an apparently healthy phenotype the latter, in three nephews (III-1, III-5 and III-8) all of them affected by BAV.

Mutational segregation analysis performed on the family members of subjects II-8 and did not allow us to define a clear association between the 4 variants and a specific

clinical phenotype BAV or TAA observed in the family. In fact, 1) some of these variants have been identified in apparently healthy subjects of the family, but this issue could be of limited importance due to the known reduced penetrance of the disease, and 2) more importantly none of the genetic variants identified in the proband were detected in all the BAV or TAA affected subjects.

In conclusion, data obtained in these first 3 analyzed families suggest that no major pathogenetic mutations in genes previously associated with BAV as well as in novel genes of the 91 included in our NGS panel are associated as major genetic determinants with this clinical phenotype in its isolated form as well as BAV/TAA. This datum together with those obtained in our 15 BAV patients and other previously commented literature data suggest that BAV often perceived as monogenic or oligogenic disorder might be declined as a complex multifactorial genetic trait due to the interaction of individual genetic profile, epigenetic effects, somatic mutations, and environmental factors.

A limitation of our study is represented by the use of a targeted NGS approach, in which a limited number of genes was considered. A whole exome sequencing approach (WES) could permit to achieve more information concerning the genetic profile of the patient and clarify whether other genes may be underlying BAV.

Our results underline the complexity of clinical and genetic diagnosis also in traits considered monogenic, but in which clinical manifestations can overlap due to both contribution of the same genes to different phenotypes as well as presence of comorbidities determined by different genes. Moreover, this work of thesis highlights the usefulness of high-throughput sequencing technologies to improve diagnosis in a scenario in which an increasing number of genetic traits need to be considered polygenic/multifactorial. Target gene capture/deep sequencing approach can greatly improve the genetic diagnosis/management of patients with BAV. This study once more demonstrates the power of NGS in confirming and expanding clinical phenotypes/genotypes of the extremely heterogeneous conditions, assisting in genetic counseling and in carrier detection as well as providing therapeutic options.

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