1

General introduction and Hypotheses

Cardiovascular diseases
1.1 Introduction

Cardiovascular Diseases: Congenital Heart Disease

In 1888 the French physician Etienne-Louis Arthur Fallot described a “tetrad” of congenital anatomical defects in a heart, which are now collectively referred to as tetralogy of Fallot (TF). TF is characterized by a (sub)valvular pulmonary stenosis, a ventricular septal defect (VSD), dextroposition of the aorta (overriding the VSD) and concomitant right ventricular hypertrophy (RVH)\(^1,2\) (Fig. 1.1). The right ventricular (RV) outflow tract obstruction can be more or less severe, depending on the degree of malformation as well as the extent of RVH. With an incidence of 1 per 2000 new-borns TF is a frequent cyanotic congenital heart malformation.

![Tetralogy of Fallot Diagram](image)

Figure 1.1 Diagrammatic representation of human tetralogy of Fallot (Modified from Stephenson et al.\(^3\))
The pulmonary stenosis in TF originates from the anterior displacement of the outlet septum resulting in narrowing of the sub-pulmonary outlet, often accompanied by stenosis of the pulmonary valve and hypoplasia of both the pulmonary annulus and pulmonary trunk. The pulmonary valve itself is often abnormal, dysplastic and immobile, causing an additional obstruction to pulmonary blood flow. The pulmonary valve may show fused valve leaflets giving dome-shaped, bicuspid, unicusp, imperforate or atretic valves, with in some patients rudimentary or complete absent leaflets, with only a ridge of dysplastic valve tissue. In more extreme forms, the aorta will be exclusively connected to the RV, representing TF with complete double outlet ventriculo-arterial connection.

In approximately 20-25% of the cases with TF the pulmonary stenosis is complete, the obliteration of the RV outflow tract results in pulmonary atresia with VSD (PA-VSD). This complicated form of TF is characterized by the extremely underdeveloped RV infundibulum with marked anterior and leftward displacement of the infundibular septum often fused with the anterior RV wall. Together with severe hypoplasia or absence of parts of the central pulmonary arteries, these patients depend on a patent ductus arteriosus or the presence of multiple systemic pulmonary collateral arteries (SPCAs) for their pulmonary circulation and survival. The SPCAs are large and distinct arteries, highly variable in number that usually arise from the aortic arch or the subclavian, carotid or even the coronary arteries.

Pathophysiology of TF

The RV outflow tract obstruction and the defect in the inter-ventricular septum lead to an equal systolic pressure in both ventricles and aorta, causing a varying degree of right to left shunting of the blood. Babies born with this congenital heart anomaly are usually referred to as blue babies as they have a diminished pulmonary flow and consequently insufficient blood oxygenation leading to hypoxemia, cyanosis and sometimes cyanotic spells. Clinical findings associated with severe, longstanding cyanosis include polycythemia (abnormal increase of red blood cells), difficulty in feeding, failure to gain weight, retarded growth and physical development, dyspnoea on exertion, and some times cerebral thrombosis. Nowadays clubbing of the fingers and toes, pulmonary haemorrhage, stroke and infections of the brain and severe hypoxic spells rarely develop.

In response to the increased pressure overload caused by pulmonary stenosis in combination with the VSD, the RV undergoes hypertrophic growth. This is an adaptive process wherein an increase in RV mass caused by expanding myocytes attempts to normalize the wall stress. This adaptive response can further lead to a proportionate increase in coronary flow and growth of the coronary vasculature and to structural changes in the myocardial architecture (myocardial remodeling).
Without surgical treatment, TF ultimately results in cyanotic complications and cardiac failure. Approximately 30% of neonates with TF die within the first year of life if untreated, 90% of the children would die before the age of 20 years and only 3% will reach the age of 40 years. Nearly 70% of patients with TF require an operation during their first year of life because of hypoxic spells or persistent hypoxemia defined as resting arterial oxygen saturation less than 70%. Generally the surgical approach involves primary repair in the first year of life by means of closing the VSD and relieving the RV outflow tract obstruction. Surgical repair thus aims at preventing complications due to long-standing RVH such as ventricular dysfunction and arrhythmia. Surgical treatment can be corrective in infants at a very young age, provided that the pulmonary arterial system is complete and well developed. If not, palliative surgery may improve the clinical situation and prepare for later correction. The actuarial survival rate, including hospital deaths, after corrective surgery for TF is reported to be 89-93% at 15 years. Midterm re-operations are reported in 2-19% of the patients, mostly because of residual pulmonary stenosis and to a lesser extent due to residual VSD. Re-operation for pulmonary valve replacement is reported to be performed in about 9% of the TF patients. The actuarial freedom from re-operation was 85-89% at 15 years.

1.2 Myocardial hypertrophy

From the postnatal period to maturation the human heart undergoes a six-fold increase in heart mass. The growth is most rapid in the first postnatal months, when the total number of myocytes double and remain relatively constant thereafter. In this period the myocyte diameter increases from 5 μm during infancy to 14-16 μm in adults. Although the cardiac myocytes comprise one-third of the total cell number in the adult heart, they are responsible for 70 percent of the cardiac volume. The growth of the heart becomes slower with the age, were the RV growth ceases at approximately 50 years of age while the left ventricle slowly continues to increases in volume. The increase in myocardial mass is a result of myocyte hyperplasia (increase in cell number) and hypertrophy (increase in cell size). Myocyte hypertrophy can be either more eccentric, with longitudinal growth of the myocytes and normal wall thickness/dimension ratio, or concentric with increased wall thickness/heart dimension ratio. The physiological increase in heart size by eccentric hypertrophy can be a result of isotonic athletic training like running, whereas isometric exercise such as weight lifting stimulates concentric hypertrophy. The diameter of long-distance runners can grow up to a 14% greater maximum diameter and 80% greater mass in the left ventricle. The growth of the adult heart can be enhanced by the thyroid hormones, catecholamines and the renin-angiotensin system (RAS) hormones. Under normal physiological conditions the adult human cardiac myocytes are believed to be terminally differentiated and have lost their ability to proliferate. Although some mild concentric hypertrophy may develop in the left
ventricle as a consequence of age related decrease in sensibility of the peripheral vasculature. However, under pathological conditions, such as pressure and volume overload and heart failure, there are some indications that cardiomyocytes re-initiate myocyte differentiation. Under those circumstances the myocyte diameter can increase to more then 40 μm, depending on the degree of compensatory hypertrophy.

Pathological myocardial hypertrophy

In response to environmental and pathological conditions the heart may remodel to sustain the cardiac performance. Pathological myocardial cell hypertrophy is a physiological adaptive compensatory response, to increased haemodynamic overload, where the increase of cardiac mass serves to normalize wall stress and to permit normal cardiovascular function. Chronic haemodynamic overload will lead to cardiac remodeling with an increased risk of decompensated hypertrophy and finally result in cardiac failure. The decompensated hypertrophy is a result from a number of intrinsic and extrinsic mechanisms of the cardiac myocytes, including changes in cardiomyocytes contractility, in regulatory-calcium-cycling and structural proteins together with apoptosis, necrosis, inadequate growth secondary to altered signal transduction pathways and extracellular matrix remodeling. The myocyte dropout, due to myocardial cell death and hypertrophy, of the remaining viable myocytes has been proposed to account for impaired function and failure during ageing and hypertension. At the same time apoptosis is also of importance in contributing to the remodeling of the cardiac chambers and vascular geometry in response to pathologic stimuli.

Pathological conditions such as ventricular outflow tract obstruction, systemic or pulmonary arterial hypertension or aortic coarctation, may cause pressure overload and cardiac hypertrophy. This will increase the wall stress and result in concentric hypertrophy, with characteristic wall thickening. Pressure overload hypertrophy will activate early oncogenes and angiotensin II, mediated by the RAS-system and the responses of the cardiomyocytes to stretch. Volume overload, occurring during mitral or aortic regurgitation, however, may cause increased either diastolic wall stress or both systolic and diastolic wall stress and result in (left) ventricular hypertrophy.

Molecular markers of cardiac hypertrophy

Alterations in gene expression play an important role in the myocardial adaptation in response to increased work load. A battery of genes including immediate early genes (like proto-oncogenes, c-fos, c-jun and c-myc) and genes from the fetal period (like ANF, β-MHC and α-skeletal actin)
have been implicated in the cellular alterations leading to ventricular hypertrophy\textsuperscript{37-39}. The altered and increased cardiac protein synthesis, resulting in a hypertrophic phenotype, can be triggered by dynamic or static stretch of neonatal or adult cardiomyocytes\textsuperscript{40}. Increased stretch of myocytes activates the transcription of the early genes c-fos, c-jun and c-myc stimulating hyperplasia and (fetal) myocyte growth within minutes\textsuperscript{41}. Angiotensin II, growth factors and noradrenaline are known to activate these early genes\textsuperscript{23}.

Mechanotransducers (e.g. tyrosine kinase-containing receptors and stretch-activated sarcomemmal ion channels) activate the process of mechanotransduction that in turn stimulates the intracellular growth pathways\textsuperscript{42}. Via this cytosolic pathway the expression of specific “hypertrophic” genes are upregulated\textsuperscript{43}. Insulin like growth factor (IGF), acidic and basic fibroblast growth factor (FGF-1 and FGF-2), platelet- derived growth factor (PDGF) and epidermal growth factor (EGF) are all growth factors that interact with those tyrosine-kinase receptors\textsuperscript{21}. Transforming growth factor β (TGF-β) however, is one of the factors that has a myocyte proliferation and growth inhibitory activity and is suppressed during myocyte hypertrophy, allowing myocytes to grow\textsuperscript{44}. Angiotensin II originated by the RAS-system, activates several protein kinases involved in cell growth and particularly in structural remodeling in cardiac hypertrophy and early postnatal development\textsuperscript{21,45,46}. Other hormones known to contribute to hypertrophy are; growth hormone which is involved in the myosin α to β-isofrom shift\textsuperscript{47} and thyroid hormone, induced during increased oxygen consumption and augmented cardiac workload, and considered to be an endocrine mediator of cardiac hypertrophy\textsuperscript{21,23}. The sympathetic nervous system is also thought to have a supporting regulatory role during pressure overload hypertrophy\textsuperscript{48}. Besides the net increased protein synthesis there is also a modestly increase of protein degradation in cardiac hypertrophy. This increased degradation, involves the lysosomal and cytosolic protease activation, which may play a crucial role in the variably altered geometry of the ventricles in response to pressure overload or volume overload, hypertrophy, regression, and atrophy\textsuperscript{49,50}. Normally the average half live of cardiac proteins is 5 days, regenerating the composition of the adult heart approximately every 3 weeks\textsuperscript{51,52}. During cardiac hypertrophy there is an net increase in protein synthesis accounting for the hypertrophic myocardial phenotype and increased mass\textsuperscript{51,52}.

Both myosin and myofibrillar ATPase activity are depressed in the hypotrophied myocardium. The loss of contractile protein may contribute to the impaired function as observed in cardiac muscle from failing human hearts\textsuperscript{53}. In small mammals there is a transcriptionally mediated shift from α- to β-myosin heavy chain (MHC) and from cardiac to skeletal actin isoform in response to pressure overload\textsuperscript{54,55}. Some studies report that this MHC shift\textsuperscript{56,57} is caused by significant loss of α-MHC expression rather then β-MHC up regulation\textsuperscript{12,58}. Because the α-form has a three to seven
fold greater ATPase activity than β-myosin, it is believed that the abundance in β-MHC increases the efficiency of force development by producing the same absolute muscle tension at a slower rate\textsuperscript{59}. In higher mammals and humans, decreased myosin ATPase activity of the hypertrophied heart is believed to be due to alterations in troponin isoform composition or post-translational generation of a lower molecular variant of β-MHC\textsuperscript{21,60}.

\textit{Myocardial hypertrophy in tetralogy of Fallot}

The characteristic pulmonary stenosis with VSD in TF, will lead to increased RV pressure overload. In an adaptive response to normalize the wall stress, the RV will hypertrophy with expansion of the cardiomyocytes. This will lead to RV remodeling and the development of myocardial fibrosis\textsuperscript{11,12,61}. Histopathological studies on TF myocardium report that the myocardial cell diameter in postoperative patients was reduced as compared to preoperative patients with TF, but postoperative cellular diameter was still larger than that in the age matched normal \textsuperscript{62,63}. These findings suggest that RVH after corrective surgery of TF can regress to some extent provided that residual pulmonary stenosis can be avoided.

1.3 Myocardial fibrosis

\textit{Interstitial fibrosis}

The normal myocardial cellular architecture consists of nicely arrayed cardiac myocytes comprising cardiac fibroblasts and endothelial cells in the interstitial space. In-between the myocytes there is a fibrous protein structure, the extracellular matrix, that contributes to the myocardial organization and architecture, supports transmission of the contractile force generated by cardiac myocytes, contributes to the passive stretch and serves to restore the ventricular myocytes to precontraction length. The extracellular matrix composition exists of collagenous proteins that account for proteins, for example collagen, fibronectin, glycoproteins, proteoglycans and elastin\textsuperscript{64}.

Throughout normal physiological myocardial growth there is a balance of hyperplasia of cardiac fibroblasts and connective tissue distribution\textsuperscript{37,65}. The collagen network, in the extra-cellular space of the myocardium, consists of collagen fibers, which provide the mechanical strength and stiffness of the myocardium\textsuperscript{66,67}. When the balance is disturbed, in case of pressure overload, the network gets damaged. As a result to the increased wall stress, the myocyte support is compromised allowing myocardial tissue expansion with disarray of myocytes\textsuperscript{68}. This, together with the increased tension on the fibers leads to a compensating excessive production of connective tissue, the development of fibrosis. This process may continue until the extracellular
matrix fibers together with the hypertrophied myocytes counterbalance the pressure overload and prevent (further) dilatation\textsuperscript{11-13,69}. Disproportionate accumulation of collagen, either reactive or reparative, has been observed during myocardial fibrosis. The increased accumulation of extracellular matrix proteins during myocardial fibrosis results in a decrease of compliance and declining contractile performance\textsuperscript{12,70,71}.

Fibronectin and collagen type I and III are major components of the interstitial fibrillar network. They are predominantly synthesized by cardiac and vascular fibroblasts with a little expression by myocytes\textsuperscript{72}. The RAS-system, activated during pressure overload hypertrophy, activates fibroblast cell division enlarging the synthesis\textsuperscript{23}, where transcriptional upregulation of the encoding collagenous genes contribute to the myocardial fibrosis\textsuperscript{12}. Experimental\textsuperscript{73} and clinical data\textsuperscript{74} show that both fibronectin and collagen I and III are up-regulated during RVH\textsuperscript{75}. The TGF-\(\beta\) expression is also up-regulated during pressure overload cardiac hypertrophy. It is believed to be involved in the mechanisms that activate fibrosis by the stimulation of fibronectin and collagen expression in a variety of cell types and their incorporation in the extracellular matrix\textsuperscript{12,76}. Other factors that are thought to effect the collagenous synthesis by fibroblasts are PDGF, angiotensin and endothelin, although the exact mechanisms remains to be elucidated\textsuperscript{77}. Pressure overload hypertrophy, induced by aortic banding, showed a high increase of collagen I syntheses already after 2 days, followed by collagen III after one week, suggesting differential expression regulation\textsuperscript{77}. The higher collagen concentration is a result of a decreased collagen destruction and a higher collagen synthesis rate. The mRNA levels for collagen as well as for fibronectin were found to be increased in cardiac pressure-overload hypertrophy\textsuperscript{75}.

Under normal conditions there is a continuous expression and degradation of collagens by the adult cardiofibroblast\textsuperscript{77}. There is a rapid collagen turnover in the heart, were daily 5\% of the collagen fibers are renewed. Most mature cross-linked collagens are degraded extracellularly by members of the family of matrix metalloproteases (MMPs), whereas already a large fraction (60\%) of the newly synthesized collagens is degraded rapidly within 8 minutes intracellularly, in the lysosomes and endoplasmic reticulum\textsuperscript{78,79}. MMPs may play an important role in the tissue remodeling in both normal and pathological conditions. They belong to three main groups, classified on their substrate specificity. The MMPs involved in the myocardial remodeling are: the type IV collagenases (MMP-9 and MMP-2), the stromelysins (MMP-3) and the interstitial collagenases (MMP-1)\textsuperscript{80}. The MMPs are secreted as a proenzyme (zymogen) by a number of cells including fibroblasts, endothelial cells, smooth muscle cells and cardiomyocytes and can be activated by plasmin\textsuperscript{80,81}. The activity can be regulated by different cytokines and tissue inhibitors of MMPs (TIMPs)\textsuperscript{81} but the extracellular matrix turn over is mainly regulated by MMPs and the TIMPs\textsuperscript{82}. TIMPs can bind the active sites of the MMPs thereby blocking access to the collagen
An increase of the expression or activation of the MMPs or an decrease in of TIMPs leads to increased proteolytic activity in the extracellular space and increased extracellular remodeling including disregulated fibrillar collagen degradation, myofibril disarray and progressive myocyte loss. Increased levels of catecholamines, angiotensin II and endothelin may cause increased myocardial MMP levels in the remodeling heart whereas tumor necrosis factor-α (TNF-α) and ischemia are believed to increase the TIMP-1 and TIMP-4 levels respectively. From the four TIMPs known, TIMP-4 is the most cardiac specific one, although all 4 are active in the myocardium. An second role of TIMP 1 and 2 might be a fibroblast growth stimulation effect.

Peri-vascular fibrosis

During embryonic development, smooth muscle cells, endothelial cells and fibroblasts secrete extracellular matrix proteins in the peri-vascular area to form a supportive structure for the vascular wall. Under normal conditions, after birth, the vascular smooth muscle cells do not proliferate or develop contractile fibers but are kept in an quiescent state. When the smooth muscle cells are exposed to increased haemodynamic load, arterial injury, or physiological stress they will get activated, stimulate vascular remodeling, resulting in smooth muscle cell growth (due to hypertrophy) or mesenteric medial thickening (due to hyperplasia). Factors such as FGF, EGF and VEGF are known to stimulate the vascular smooth muscle hyperplasia.

Extracellular matrix proteins

In the myocardium the myocytes represent only one-third of the number of cells, but over two-third of their volume, whereas the number of fibroblasts, which produces most of the collagen fibers represent as much as two-third of the cells. Four from the five collagen subtypes in the heart, type I, III, V and VI are produced by cardiac fibroblasts and type IV by other cell types including cardiac myocytes. Collagen type I represents 80% of the total cardiac collagen content and is the major collagenous product of cardiac fibroblasts, which is important for the cardiac mechanical strength and stiffness. Collagen type III, important for tissue elasticity, represents about 12% of total cardiac collagen content. Collagen type I and III are widely distributed in-between myocytes and among muscle fibers. Collagen types IV, V and VI constitute approximately 10-12% of total cardiac collagen content. The collagen fibers have several functions, (i) one is to provide a scaffolding that supports muscle cells and blood vessels, (ii) to act as a lateral connection between cells and muscle bundles to control architecture while co-ordinating the delivery of force from the myocytes to the ventricle, (iii) to provide myocardial stiffness by tensile properties and resilience to resist myocardial
deformation, maintain shape and wall thickness, and to prevent ventricular aneurysm and rupture. Fibronectin, a glycoprotein located in the extracellular matrix of most tissue, serves as a bridge between cardiac myocytes and interstitial collagen mesh network. It influences diverse processes including cell growth, adhesion, migration, and wound repair. During the development of cardiac hypertrophy due to pressure overload, fetal fibronectin has been shown to accumulate in rats. Fibronectin binds collagen and modulates the collagen fibrillogenesis, resulting in higher collagen type I concentration and a lower collagen type III concentration. The fibronectin mRNA-splicing variant encoding the EIIIA segment is an isoform, expressed during wound healing, embryogenesis and pressure-overload hypertrophy. The structural properties of the EIIIA isoform of fibronectin are not well understood but it is postulated that this isoform may be important in the deposition of the extracellular matrix.

**Fibrosis in tetralogy of Fallot**

Progressive myocardial fibrosis due to cardiac hypertrophy is an important cause of myocardial dysfunction, clinical deterioration and cardiac death. Morphometrical studies on TF revealed that the RV is affected with enhanced fibrosis associated with increased myocytes diameter and myocardial disarray. A post mortem analysis study of myocardium of TF patients showed normal fibrosis levels in the left ventricle and abnormal increased levels in the RV of patients older than 1 year with an increase in outflow tract and subendocardium. The collagen concentration of the RV is about 30\% higher than the left ventricle, because the RV myocytes are smaller. Within the RV the highest concentrations are found in the outflow tract and subendocardium. Changes in the cardiac extracellular matrix proteins composition have also been observed in other congenital heart disease, however in most of the types they were relatively small.

**1.4 Myocardial angiogenesis**

**Vasculogenesis and angiogenesis**

Angiogenesis is generally used to describe the formation of new blood vessels from pre-existing vessels. This involves not only the formation of capillaries, but also the formation of small and large blood vessels. The central physiological role of angiogenesis is to ensure a proper blood flow through the tissue. It is a multi-step process involving endothelial cell activation, migration, proliferation, tube formation and maturation. In contrast, vasculogenesis concerns the differentiation of a primitive network of new vessels during embryogenesis. The tissue glucose and oxygen concentrations are important environmental factors as pro-angiogenic stimuli. Besides
endothelial cells several other cell types like vascular smooth muscle cells, pericytes and fibroblasts and non-cellular structures, such as the basal lamina and the extracellular matrix are involved\textsuperscript{104,105}.

\textit{Development of pre-mature vasculature}

During embryogenesis when the number of cells is increasing within an avascular tissue, there is an increased demand for oxygen which can not be met by the oxygen diffusion. The hypoxic state will trigger vessel formation by a process referred to as vasculogenesis. Undifferentiated precursor cells (angioblasts) will differentiate to endothelial cells that assemble into a vascular labyrinth. The endothelial cells will differentiate and proliferate to form a premature tubular network\textsuperscript{106}. This network includes some of the major vessels in the embryo, such as the aorta and major veins, as well as a honey comb-like plexus connecting these major vessels. Vascular endothelial growth factor (VEGF) is one of the first growth factors to be induced in this process. It stimulates the formation of an unstable immature primary vasculature. In the next process, referred to as angiogenic remodeling, the initial primitive network of premature vessel is modified, through both sprouting and vessel enlargement, to form the interconnecting branching patterns characteristic for a stable mature complex vascular network. During this process, vessel walls, premature as well as mature, and endothelial cells integrate tightly with supporting cells (such as smooth muscle cells and pericytes) and surrounding matrix, influenced by VEGF, Angiopoietin 1 (Ang-1) and ephrin B2. The endothelial cells are kept in a quiescence state by Ang-1. Once the endothelial cells become quiescent they can survive for years. Expression of Ang-2 (like in pathological conditions) can destabilize the vessels and bring them in regression. If at the same time VEGF is co-expressed, VEGF can induce angiogenic sprouting in the destabilized vessel\textsuperscript{105,107}.

During angiogenic sprouting, new vessels (sprouts) from existing vessels are formed into a previously avascular tissue. In some cases it seems as if mature vessels must first be destabilized to allow subsequent sprouting. Angiogenic sprouting is responsible for the vascularization of certain tissues during normal development, such as the neural tube or the retina, and for most new vessel formation in the adult\textsuperscript{106,107}. To supply all rapidly growing myocytes of oxygen after birth, the capillary length and surface increases 23 times faster than the myocardial mass, however the capillary diameter decreases, limiting the increasing capillary volume\textsuperscript{23}. Destabilization of the vessel can apparently lead to vascular regression. The survival of the established vasculature, in the adults, depends on the quiescent state of the endothelial cells which have prolonged life times (several years). A continuous quantity of stabilizing factors is needed to keep the endothelial and smooth muscle cells in a quiescent state. An imbalance of survival and apoptotic factors could contribute to the regression of terminal myocardial vessels in pathological conditions of
hypertension and diabetes. The formation of new vessels in adults will only occur by angiogenesis\textsuperscript{104}. Under pathological conditions or during wound healing, when the quiescent vascular equilibrium is disrupted, autocrine induction or up-regulation of Ang-2ur or Ang-2 can reinitiate (angiogenic) vascular remodeling and angiogenic sprouting. In the presence of VEGF new vessels will be formed, but without VEGF vessels destabilize and go into regression\textsuperscript{105}. Although sometimes also adult vasculogenesis occurs, it only leads to immature poorly functional vasculature\textsuperscript{107}.

\textit{Angiogenic stimuli and mechanisms}

Angiogenesis is a complex process \textsuperscript{104,105} and maintained by multiple endogenous pro-angiogenic and anti-angiogenic factors. In many cases, the activity of factors depends on their local concentration and/or the microenvironment\textsuperscript{104,105}. The different angiogenic factors must be expressed in perfect harmony, on a complementary and co-ordinated manner to form functional vessels and to overcome vascular regression\textsuperscript{108}. Besides, many other non-vascular endothelium-specific growth factors like PDGF, TGF-\(\beta\) and many other gene products ranging from transcription factors to members of the Notch family have been shown to be crucial for proper vessel formation\textsuperscript{107,109,110}.

Among the angiogenic growth factors acting on the vascular endothelium, are the 5 members of the VEGF family, four members of the angiopoietin family and at least one family of the large ephrin family\textsuperscript{105}. VEGF is the most critical and specific vascular growth factor in the vascular formation, required for the initiation of immature vessels by vasculogenesis or angiogenic sprouting. During hypoxia its half-life is prolonged from 30 minutes to 8 hours\textsuperscript{123}. Ang-1 and ephrin B2 are needed for the further remodeling and maturation of the immature vasculature. In this process ephrin B2 is important in the distinction to develop arterial or venous vessels. Ang-1 is important to maintain the quiescence and stable state of the vasculature. Disruption of the stabilizing Ang-1 signal will reinitiate vascular remodeling in the adult (e.g. in tumor)\textsuperscript{105}. Autocrine production of Ang-2 (the Ang-1 natural antagonist) by endothelium cells will destabilize the vessels. VEGFs, angiopoietins and ephrin B2 apparently are not capable to trigger the entire process of vascular remodeling in adults individually\textsuperscript{105}.

Many of the angiogenic factors involved in the remodeling and establishment of functional blood vessels are regulated by paracrine signals from the receptor tyrosine kinases family\textsuperscript{106} as listed in table 1.1.
Table 1.1. Overview of tyrosine kinase receptors and their ligands, involved in angiogenesis

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<th>VEGF Receptor family</th>
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<td>Tie1</td>
<td>EphA2</td>
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<td>VEGFR-2 (KDR/Flk-1)</td>
<td>Tie2</td>
<td>EphB4</td>
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<tr>
<td>VEGFR-3 (Flt-4)</td>
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Receptor:

Ligand:

Other factors involved in angiogenesis are the acidic (FGF-1) and basic (FGF-2) fibroblast growth factors, which stimulate the endothelial cell growth. They are expressed by fibroblasts in response to hypoxia. FGF-1 stimulates the branching of the myocardial arteries and stimulates the formation of functional vessels by preventing the regression. In combination with vasoconstricting growth promoters, FGF-2 is an important endogenous activator of collateral vessel development in ischemic myocardium. During ischemia VEGF expression is up-regulated and triggers the release of stored FGF-2. The vasoconstricting growth promoters, such as angiotensin II, thrombin, thromboxane, prostaglandins, serotonin and endothelin, are less potent then peptide growth factors and they are believed to enhance other stimulators of vascular smooth muscle cell growth. In quiescent vasculature they are found to stimulate FGF-2 expression. TGF-β is one of the regulators that inhibit vascular smooth muscle cell growth but stimulates the synthesis of the extracellular matrix. At low concentrations TGF-β still can stimulate vascular growth, by increasing autocrine PDGF synthesis, however higher concentrations reduces the PDGF receptor expression, interrupting the autocrine effect. Thyroid hormones are involved in the capillary elongation or splitting of pre-existing vessels, increasing the capillary supply.

Besides hypoxia, capillary growth in the heart can be stimulated by moderate athletic training, as found in normal young but not in adult animal hearts, leading to growth of the arterioles and coronary vessels. The increased blood flow is stimulating mechanical factors like shear stress and wall tension, which are stimuli that initiate vessel growth. Increased shear stress leads to endothelial release of angiogenic prostaglandins and nitric oxide, whereas increased wall tension...
initiate smooth muscle cell and endothelial cell proliferation with the release of growth factors. Shear stress is also one of the factors known to be involved in vascular remodeling, where it can disrupt the peri-vascular matrix and releases plasminogen activator and MMPs activating a process that enables cell migration and proliferation\textsuperscript{21}. In the same manner, a lower heart rate is believed to enhance capillary growth. During the diastole, the capillary diameter is larger than during systole increasing the wall tension. A lower heart rate will enhance the diastolic period thereby increasing the capillary wall stress and tension. Besides higher blood pressures and velocity, shear stress can also be increased by increased haematocrit\textsuperscript{23,112}.

\textit{Angiogenic factors}

VEGF is known to be a critical vascular regulator. Although hypoxia can induce VEGF, it is not necessarily inducing a useful angiogenic response. A very precise concentration of other factors is needed to avoid vascular disorganization and induction of immature and leaky vessels\textsuperscript{105}. Alliteraions in VEGF expression during embryonic development can lead to profound abnormalities or early postnatal death\textsuperscript{113,114}. The VEGF-family consists of the 4 VEGF homologues (VEGF-A, VEGF-B, VEGF-C or VEGF-related protein (VRP) and VEGF-D) and placental growth factor (PIGF)\textsuperscript{115}. The different members of the VEGF family have overlapping abilities to interact with a set of cell-surface receptors such as VEGFR-1 (also known as flt-1), VEGFR-2 (also known as Flk-1 or KDR) and VEGFR-3 (also known as Flt-4). The VEGF receptors are closely related to receptor tyrosine kinase. Angiogenesis and the initiation of the permeability of blood vessels are regulated by VEGF via its receptors VEGFR-1 and VEGFR-2\textsuperscript{105}. VEGF-A, one of the most potent and crucial regulatory factors during myocardial angiogenesis\textsuperscript{105}, is induced in a wide range of cells under conditions of hypoxia\textsuperscript{116} and hyperglycemia\textsuperscript{117} and in the presence of some (growth) factors\textsuperscript{118} like phorbol ester, adenosine, TGF-\beta, PDGF and TNF-\alpha\textsuperscript{105}. It is a ligand for the endothelial cell surface receptors, flt-1 and KDR\textsuperscript{119}. VEGF-KDR binding activates a cascade that promote angiogenesis by promoting vascular endothelium cell proliferation, inducing vascular leaking and permeability but also by enhancing the glucose transport\textsuperscript{120}. Although several isoforms (VEGF\textsubscript{120}, VEGF\textsubscript{121} and VEGF\textsubscript{165}) of this member are known, the exact functions still have to be elucidated\textsuperscript{107}. It is known that VEGF-A is involved in the formation of the vascular lumen, when the endothelial cells assemble to solid cords. Intercalation or thinning of endothelial cells and fusion of pre-existing vessels, influenced by VEGF-A, allow vessels to increase their diameter and length\textsuperscript{107}. VEGF-B is believed to play a role in the in the coronary vascularization and growth. Loss of VEGF-B during embryogenesis reduces the heart size\textsuperscript{121}, whereas VEGF-C, a ligand for VEGFR3, is involved in embryonic and adult pathological angiogenesis. VEGFR-3 is also expressed on lymphatic vessels where it
regulates, via VEGF-C, the lymphangiogenic activity\textsuperscript{122-125}. Hardly anything is known about the physiological role of VEGF-D\textsuperscript{107}. Also from the growth factor PIGF only a little is known, although there are some indications that it might have a limiting role in adult vascular remodeling\textsuperscript{107}. From the VEGF receptors, VEGFR-2 seems to mediate the major growth and permeability whereas VEGFR-1 has a negative role by acting as a decoy receptor or by suppressing signaling through VEGFR-2. VEGF affects embryonic, neonatal and pathological angiogenesis via its receptor VEGFR-2\textsuperscript{126}. The exact involvement of VEGFR-1 signaling during (pathological) angiogenesis remains undetermined\textsuperscript{127}. Although VEGF does not seem necessary for the vascular maintenance in adults, disruption of VEGF expression mimics VEGFR-2 knock out, resulting in only a very few proliferated endothelial cells and complete absence of vasculature\textsuperscript{126,128,129}.

Angiopoietins are the ligands for the Tie receptors (Tie1 and Tie2) which are like the VEGF receptors expressed within the vascular endothelium\textsuperscript{105}. The 4 members of the angiopoietin family (based on their homology with Ang-1) all bind primarily to Tie 2 receptor\textsuperscript{105,130}. Tie2 is widely expressed in cardiovascular tissue\textsuperscript{131,132}. There is some evidence that the Tie2 cascade regulates the extracellular recruitment of stromal cells required to encase and thereby stabilize primitive endothelial tubes\textsuperscript{133,134}. Ang-1 binding to endothelial Tie2 receptor is necessary to maintain the quiescence state in adult vasculature, via a maximum stimulation of the interaction between endothelial and peri endothelial cells, their surrounding support cells and matrix\textsuperscript{105}. It further protects the vasculature against vascular permeability and leakage induced by VEGF or inflammatory agents\textsuperscript{105}. Ang-1 alone fails to induce endothelial proliferation\textsuperscript{105,108,135}, however in presence of VEGF unprecedented hyper-vascularization will occur resulting in an increased number and size of the vessels\textsuperscript{136}. Further Ang-1 is believed to be a chemotactic for endothelial cells. Ang-2 binding to the Tie2 receptor can either activate or antagonize the Tie2 receptor\textsuperscript{137}. At least in presence of VEGF, Ang-2 will enhanced the sprouting of capillary vessels by antagonizing the stabilization effect of Ang-1. Ang-2 itself cannot induce angiogenesis but is indirectly involved in Angiotensin II-mediated angiogenesis by enhancing the angiogenic activity of VEGF\textsuperscript{138}. Angiotensin II can stimulate the remodeling of the heart and vessels via cell growth-promoting effects\textsuperscript{139}. By binding to Angiotensin II receptors AT1 and AT2, which are expressed in large amounts on the vascular endothelial cells in the myocardium\textsuperscript{140}, Angiotensin II can selectively induce and up-regulate VEGF and Ang-2 expression in cardiac vascular endothelial cells\textsuperscript{141}.

The ephrin receptors tyrosine kinases belong to the largest known family of growth factor receptors\textsuperscript{108}. Ephrin-B2 and its EphB4 receptor are believed to play a key role during vascular development, especially in establishing arterial venous identity and in fusing arterial and venous
vessels at their junctions\textsuperscript{105}. During adult angiogenic sprouting the expression of ephrin-B2 is found to be strongly up-regulated in the endothelium of new vessels. Ephrin-B2 is not only required during the earliest stages of arterial/venous determination, but may also be important during the development of arteries by regulating interactions between endothelial and smooth muscle cells involved in the formation of the arterial muscular walls\textsuperscript{105}.

\textit{Angiogenesis in tetralogy of Fallot}

In TF patients the RV myocytes hypertrophy as an adaptive response to the pulmonary stenosis in combination with the VSD, to normalize RV wall tension. This will trigger a response to improve myocardial perfusion by the formation of new capillaries and by the enlargement of pre-existing collateral vessels to reduce the increased capillary-myocyte diffusion distance, to prevent hypoxia and decreased nutrient delivery\textsuperscript{12,13,142}. But also the mechanical factors such as stretch, shear stress, and increased wall tension, which occur in TF due RV pressure and volume overload may be stimuli for angiogenesis during cardiac hypertrophy\textsuperscript{12,13,142}. Experimental animal studies with induced RVH due to pressure overload, revealed increased blood flow per unit myocardial mass at rest, without an increase in minimum coronary resistance\textsuperscript{143}. During volume overload RVH however, normal flow values per unit myocardial mass at rest are measured, with normal or mild increases minimum coronary resistance and normal or mildly decreased coronary reserve\textsuperscript{144}.

From previous studies on the myocardial vascularization in TF patients it is known that the overall myocardial capillary density in the infundibulum of the right ventricular outflow tract of (1-7 years old) TF patients was reported to be similar to normal capillary density (1648±123 vs. 1782±203 capillaries/mm\textsuperscript{2})\textsuperscript{145}. Also the capillary domain (cross-sectional tissue area nearest to that capillary) and the capillary tissue supply volume are not affected in TF. However the capillary segment length (distance between two branching points) was found to be increased\textsuperscript{145} whereas the capillary radius was decreases, from 2.8 to 2.4 \textmu m\textsuperscript{146}. There are also some indications that the capillary supply per myocyte is proportional to the increased myocyte size, keeping the capillary per myocyte ratio similar\textsuperscript{147}. The elimination of the \textit{hypertrophic} stimuli in TF are believed to reverse the RVH and the associated coronary circulatory abnormalities\textsuperscript{148}.

\section*{1.5 Hypotheses}

Different surgical interventions nowadays are applied to TF patients already at an early age to correct the cardiac malformations. It is, however, not yet established whether early intervention will result in (complete) regression of RVH and restore normal cardiac performance. The
underlying molecular mechanisms in the development and regression of RVH at different stages in the treatment of human TF still remains to be elucidated. The molecular and cellular characteristics of the RV myocardium at corrective and redo surgery are thought to correlate with the clinical condition of the patient and with cardiac prognosis. When experimental data and clinical parameters can be correlated more information will become available to enhance our knowledge in assessing the timing for surgery and eventually postoperative cardiac prognosis. Our working hypothesis is that molecular and cellular characteristics of the RV myocardium at corrective or redo surgery may provide information, relevant for the assessment of the clinical condition of the patient and important for cardiac prognosis.

1.6 References:


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