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Tissue properties and collagen remodeling in heart valve tissue engineering

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Tissue properties and collagen remodeling in heart valve tissue engineering

PROEFSCHRIFT

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Daphne van Geemen

geboren te Woerden

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prof.dr. C.V.C. Bouten
en
prof.dr.ir. F.P.T. Baaijens

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dr. A. Driessen-Mol

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Summary

Tissue properties and collagen remodeling in heart valve tissue engineering

Valvular heart disease is a major health problem worldwide causing morbidity and mortality. Heart valve replacement is frequently applied to avoid serious cardiac, pulmonary, or systemic problems. However, the current replacements do not consist of living tissue and, consequently, cannot grow, repair, or remodel in response to changing functional demands. Heart valve tissue engineering (HVTE) seeks to overcome the shortcomings of the existing valve replacements by creating living autologous heart valves. One of the main challenges of HVTE is to control tissue formation, collagen remodeling and consequent tissue mechanical properties during the *in vitro* culture phase. Additionally, it is important to define benchmarks based on the target native heart valve tissues to compare with the tissue structure and mechanical properties of tissue-engineered (TE) heart valves. The aim of this thesis is to define benchmarks, understand and optimize tissue development and resulting tissue mechanical properties of TE heart valves, with special emphasis on collagen remodeling.

In order to provide insights into the evolution and maturation of the extracellular matrix and mechanical properties and to provide benchmarks for TE heart valves, matrix composition, maturation and mechanical properties of native human aortic and pulmonary heart valves were studied. It was observed that the matrix composition and the mechanical properties change with age and that a significant part of the mechanical behaviour of the human native heart valve leaflets is defined by the composition and maturation of the matrix.

Tissue (mechanical) properties of TE heart valves should be optimized towards the provided benchmarks during the *in vitro* culture phase. To this end, possible indicators of *in vitro* tissue outcome were determined to enable prediction of the properties of the autologous tissues cultured for individual patients. It was found that α-Smooth muscle actin (αSMA) might be such an indicator. In addition, interspecies differences in tissue (mechanical) properties were evaluated to determine whether ovine TE heart valves are representative of human TE heart valves as the ovine model is the prescribed animal model to evaluate heart valve replacements. This study suggested that the culture process of ovine tissue can be controlled, whereas the mechanical properties, and hence functionality, of tissues cultured with human cells are more difficult to predict, indicating once more the importance of early markers to predict tissue outcome.

As a further step towards clinical application and to circumvent the use of animal-derived medium components in the culture protocol, fetal bovine serum was replaced by human platelet lysate for the culture of autologous TE heart valve constructs. Although tissue composition and maturation were similar, mechanical properties were much lower for the tissues cultured in platelet lysate, most likely due to an increased production of matrix-degrading enzymes leading to an altered collagen architecture. Thus, collagen architecture, rather than collagen content alone, is dominant in defining the tissue mechanical properties.

To stimulate tissue formation and maturation towards the right collagen architecture for *in vivo* mechanical functionality, mechanical conditioning of the engineered tissue is commonly pursued. Previous studies indicated that intermittent conditioning, in which cyclic and static strain are alternated, is favoured to obtain mature tissues in a short time period. To unravel the underlying mechanism of intermittent conditioning, the effects of cyclic strain and static strain after cyclic strain were examined at gene expression level. This study indicated that a period of static strain is required for collagen synthesis and remodeling, while continuous cyclic strain shifts this balance towards collagen remodeling and maturation. These results imply that the mechanical conditioning protocol should change over time from intermittent conditioning to continuous cyclic strain to improve collagen maturation after its synthesis and, therewith, the mechanical properties of TE heart valves.

In summary, the results from this thesis suggest that in addition to collagen content and maturation, collagen organization is particularly important in defining the tissue mechanical properties. Thus, optimization of culture protocols should focus on obtaining the proper collagen architecture for creating mechanically functioning TE heart valves. Autologous culture of TE heart valves using human platelet lysate is not preferred, since it prevents the formation of a load-bearing organized collagen network. Mechanical conditioning protocols should start with intermittent conditioning, followed by continuous cyclic strain to enhance collagen maturation after its synthesis. Considering the interpatient variability in tissue outcome of tissues cultured with similar protocols, it must be noted that further refinement, or even personalization, of culture protocols might be necessary. To this end, markers of tissue outcome, such as αSMA, are necessary to predict and adapt culture protocols and, therewith, individual tissue outcome at an early stage during culture. Although these suggestions require additional (*in vivo*) study, the results of this thesis provide substantial insight on how to improve *in vitro* HVTE strategies to control tissue properties and collagen remodeling for optimization of TE heart valves towards their native counterparts.

Chapter 1

General introduction

Heart valve tissue engineering seeks to overcome the shortcomings of current valve replacements by creating living autologous heart valves that have the ability to grow, repair, and remodel. Despite previous research efforts, the (load-bearing) structure-function properties of current tissue-engineered heart valves do not resemble those of native valves and are not yet ideal for long-term *in vivo* functionality and a safe translation to the clinical practice. As collagen is the main load-bearing component of heart valves, the formation of aligned and cross-linked collagen architecture is essential to improve the load-bearing properties of tissue-engineered heart valves. Hence, tissue engineering protocols should be optimized to improve these properties. The aim of this work is to understand and optimize tissue development and resulting mechanical properties of tissue engineered heart valves, with special emphasis on collagen remodeling in the valve leaflets. The focus of this thesis is on the semilunar heart valves. Therefore, this chapter provides background information on native human semilunar heart valve development, heart valve replacements, including tissue-engineered heart valves, and optimizing collagen synthesis and remodeling during neo-tissue formation.

1.1 The semilunar heart valves

The human heart pumps blood throughout the body and has four valves that direct the blood flow in one direction. The valves open and close approximately 100.000 times a day and 3.7 billion times in a lifetime. The concomitant cyclic loads on the valves require durability, flexibility and strength of the tissue, as well as a high degree of remodeling capacity to cope with changes due to growth and loading conditions. The atrioventricular valves are located between the atria and the ventricles and prohibit the reverse flow during systole (contraction of the heart). The semilunar valves are located at the base of the pulmonary trunk (pulmonary valve) and the aorta (aortic valve). These valves prevent backflow of blood from the large arteries into the ventricles during diastole (relaxation of the heart).

The semilunar valves are called semilunar because of their half-moon shape in cross section (figure 1.1). They consist of three thin cup-like leaflets, or cusps, fixed to a fibrous thickening of the arterial wall or root, called the annulus. The points where two adjacent leaflets attach to the root are called commissures. Behind the semilunar valves the arterial wall bulges out to form the so-called sinuses of Valsalva. The nodule of Arantius is a fibrous section in the middle of the free edge of each leaflet. The coaptation of the three nodules ensures complete central closure of the valve. At the end of ventricular systole, blood flow reverses briefly toward the ventricles. Due to this reverse flow, the leaflets fill with blood and snap together.

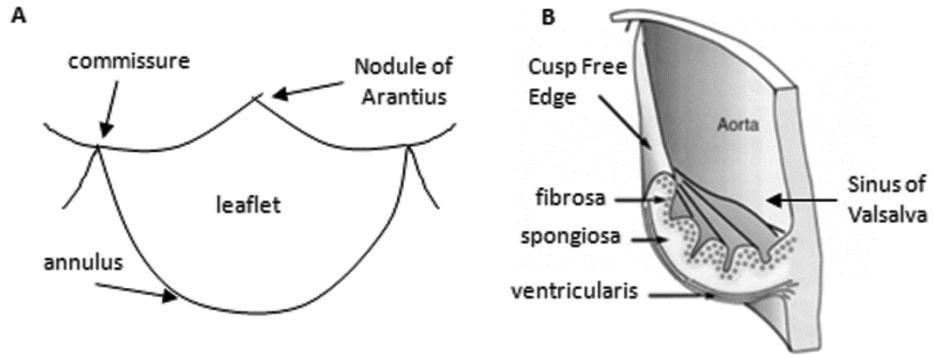

Figure 1.1 *Schematic overview of the anatomy of a semilunar valve leaflet. (A) Front-view of one leaflet indicating commissures, nodule of Arantius, and the annulus. (B) Schematic cross-section of the aortic heart valve indicating the three layers (fibrosa, spongiosa, and ventricularis), the cusp free edge, and the sinus of Valsalva (adapted from Vesely, 1998).*

1.1.1 Fetal semilunar heart valve development

The development of the human semilunar heart valves starts in the middle of the fifth week post gestation with the appearance of a small bulge (the formation of the cardiac cushions) at the outflow tract of the heart (Larsen, 2001). A critical step in the valvulogenesis of semilunar valves involves the transformation of endocardial cells with a quiescent epithelial phenotype into mesenchymal cells that invade the cardiac jelly (Barnett and Desgrosellier, 2003). This cell infiltration takes place at the downstream end of the heart tube, where the future valves will be located (Maron and Hutchins, 1974). The infiltrating cells digest the gel-like matrix to replace it with a denser matrix comprised of collagens and proteoglycans (Butcher and Markwald, 2007). Due to the continued mesenchymal cell expansion and extracellular matrix (ECM) deposition, the cushions extend into the lumen to maintain unidirectional blood flow (Moorman and Christoffels, 2003). Finally, by 9 weeks, the cushions become excavated from the arterial side inward to create the semilunar heart valves (Larsen, 2001; Butcher and Markwald, 2007).

Valvulogenesis of the heart valves takes place under pressures below 10 mmHg (1.3 kPa) and a heart rate that increases from 65 beats per minute (bmp) to approximately 100 bpm by the end of week 7 (Stock and Vacanti, 2001). Further maturation of the semilunar valves takes place under increasing heart rate, which reaches a maximum of 155-160 bpm by week 20 post gestation (Stock and Vacanti, 2001), and under increasing blood pressure. Prior to birth, the transvalvular pressures at the aortic and pulmonary sites are equal. The fetal systolic blood pressure increases linearly from 37 mmHg (4.9 kPa) at 20 weeks' gestation to 58 mmHg (7.7 kPa) at 40 weeks' gestation (Struijk *et al*., 2008). In addition, the valve diameter increases linearly with time (Tongprasert *et al*., 2011). The pulmonary valve is somewhat larger and increases in diameter slightly faster,

from 2.1 mm at 14 weeks' gestation till 10.1 mm at 40 weeks' gestation, than the aortic valve, which is increasing from 1.8 mm at 14 weeks' gestation to 8.9 mm at 40 weeks' gestation. Valve growth and physiologic parameters during fetal development are illustrated in figure 1.2A.

1.1.2 Postnatal semilunar heart valve development

After birth, the pressures in the systemic and pulmonary circulation change immediately. After establishment of respiration, the pulmonary arterial pressure rapidly decreases, while systemic pressure gradually increases (figure 1.2B and C). The mean pulmonary arterial pressure approaches 50% of the mean systemic pressure by the end of the first day and drops to more or less the adult level within the first 2 weeks of life (Gao and Raj, 2010). This means that the transvalvular pressures over the aortic valve are significantly higher than those over the pulmonary valve. This difference is established directly after birth and increases slightly during childhood. In the adult healthy heart, the transvalvular pressure at the pulmonary site is on average 10 mmHg (1.3 kPa), while the transvalvular pressure at the aortic site is approximately 80 mmHg (10.6 kPa) (Silverthorn and Garrison, 2004). The heart rate decreases from around 140 bmp to approximately 115 bmp in the first two years of life. Thereafter, the heart rate

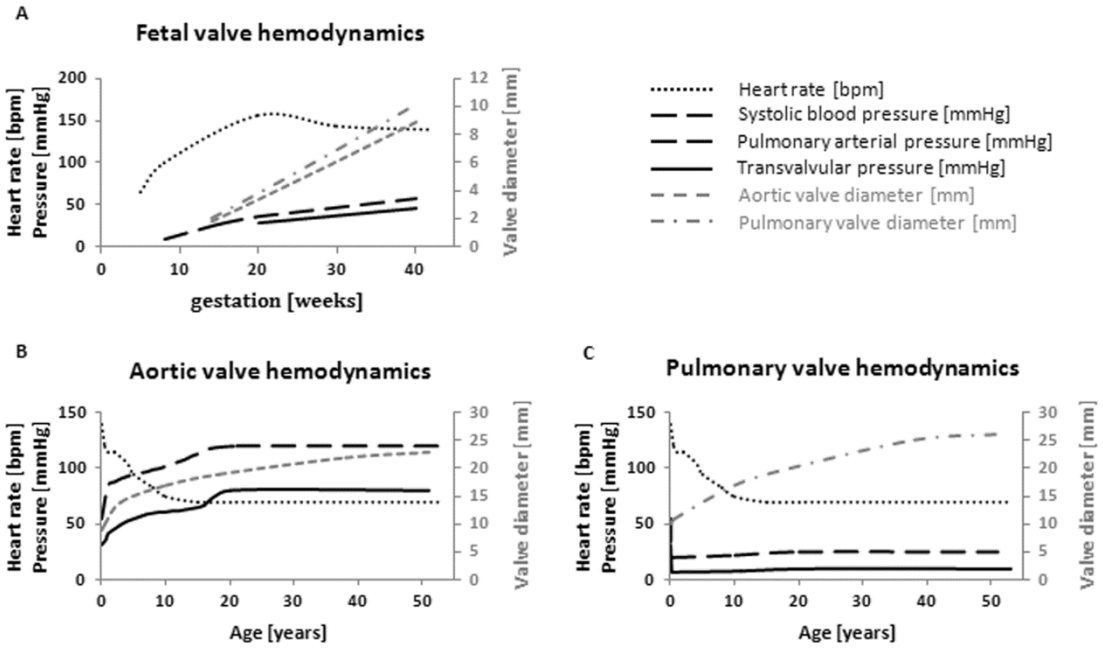

Figure 1.2: *Valve hemodynamics during fetal development (A) and during postnatal development in the aortic (B) and the pulmonary valve (C). In the fetal stages, valves are formed under increasing pressures and increasing heart rate. After birth, systemic and transvalvular pressures over the aortic valve are increasing during childhood, while the pulmonary arterial pressure and transvalvular pressure over the pulmonary valve are decreasing towards adult values directly after birth. Valve diameter is rapidly increasing during fetal development and the first years of life. Thereafter, valve diameter increases only slowly.*

gradually decreases to 70 bmp in adults.

Prior to birth, the morphology of the two semilunar valves is identical (Maron and Hutchins, 1974), but their postnatal structure and (mechanical) properties, as well as their precise development with time, are still unknown. The adult pulmonary valve leaflets, however, are more delicate than the aortic valve leaflets, pointing to a different development probably related to the lower pressure in the pulmonary circulation. The abrupt change in the neonatal circulation after birth, with increased pressures and higher oxygenation at the aortic valve, is associated with a greater number of activated cells (Aikawa *et al*., 2006) that likely respond to the higher local tissue stresses by altering cellular stiffness and collagen synthesis (Merryman *et al*., 2006). Due to an increased collagen expression (Hinton and Yutzey, 2011), the adult aortic valve is thicker than the adult pulmonary valve.

1.1.3 Morphology of semilunar heart valve leaflets

The heart valve leaflets mainly consist of two types of cells, valvular interstitial cells (VICs) and valvular endothelial cells, within an extracellular matrix (ECM). The endothelial cells form a single layer of cells lining the heart valve leaflet surface, the endothelium. This endothelium provides a protective, non-thrombogenic layer and plays an important role in many physiological functions, including leaflet surface permeability. The VICs have two distinct phenotypes: the normal/quiescent phenotype, which is classified as fibroblast-like cells, and the developing/remodeling/activated phenotype, classified as myofibroblast-like cells (Rabkin-Aikawa *et al*., 2004). The VICs in the fetal valves are activated myofibroblasts that mediate ECM remodeling. In the adult valve, VICs are mostly quiescent fibroblast-like cells and the ECM is well adapted to the environmental conditions. Nevertheless, the VICs can easily undergo phenotypic modulation from quiescent fibroblast-like cells to activated myofibroblasts to modulate the ECM under changing environmental conditions (Rabkin-Aikawa *et al*., 2004). When equilibrium is restored, the cells return to their quiescent state.

The ECM of the leaflets mainly consists of collagen, elastin, and proteoglycans. In cross-section, the leaflet is nicely structured into three layers: fibrosa, spongiosa, and ventricularis (figure 1.1B). Rabkin-Aikawa et al. (2004) described a lack of these distinguishable layers in the fetal valve leaflets, with predominant accumulation of proteoglycans, a weak staining for collagen, and almost no detected elastin (Rabkin-Aikawa *et al*., 2004). After birth, the three-layered structure becomes clearly defined (Gross and Kugel, 1931). In the adult leaflets, a dense collagen network, aligned in circumferential direction, is predominantly detected at the outflow side, the fibrosa. The fibrosa has corrugations, which produce a visible surface rippling in systole but disappear during diastole (Schoen and Levy, 1999) (figure 1.3). The collagen network in this layer bears the loading of the leaflet by transmitting it to the pulmonary artery or

aortic wall (Sauren *et al*., 1980). Elastin is mainly radially aligned at the inflow side, or ventricularis, of the leaflets. During systole, elastin restores the contracted configuration of the leaflet (Vesely, 1998). Near full closure of the valve, when the collagen has fully unfolded (figure 1.3), the load-bearing element shifts to collagen, and stress rises steadily while coaptation is maintained (Schoen and Levy, 1999). In the middle layer, the spongiosa, mainly proteoglycans, glycosaminoglycans (GAGs), and a few loosely connected fibrous proteins are found. The GAGs absorb water and swell to form a gel to absorb shocks during the valve cycle and accommodate the shear between the leaflet layers. This unique layered structure and architecture enables the leaflets to be extremely soft and pliable when unloaded and practically inextensible when pressure is applied.

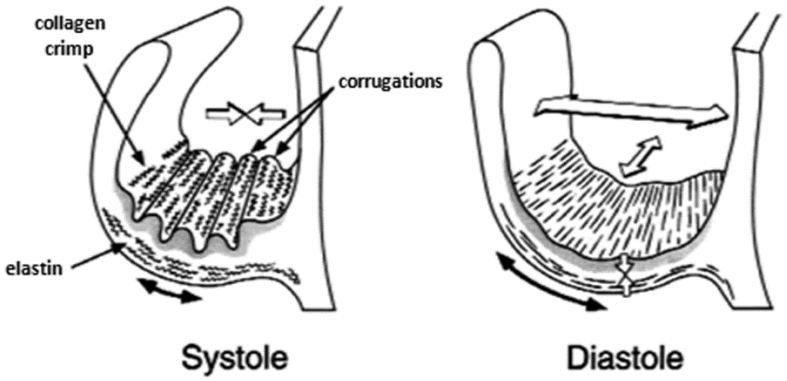

Figure 1.3: *Schematic representation of the collagen and elastin architecture in the leaflet during systole and diastole (adapted from Schoen and Levy, 1999). Collagen is circumferentially aligned in the fibrosa, while elastin is radially aligned in the ventriculars. During systole, the leaflet surface is rippled (corrugations), which disappear during diastole when collagen and elastin fibers are unfolded.*

1.2 Collagen

Collagens are the major components of the ECM and the main proteins responsible for the structural integrity of tissues providing resistance to tensile stress (Hulmes, 2002). It is hypothesized that a mature well-organized collagen network, resembling the native collagen architecture, is necessary to provide the structural and mechanical integrity for proper functioning of tissue-engineered heart valves.

Collagens are characterized by tandemly repeating Gly-X-Y amino acid triplets and have a unique triple helical structure. There are 27 types of collagens (Kavitha and Thampan, 2008), which are classified in 5 subfamilies based on their molecular and supramolecular structures. The collagens mainly present in the heart valve, typically type I, III and V, belong to the family of fibril-forming collagens. After collagen molecules are formed, they will assemble in the extracellular space into collagen fibrils, which

often aggregate into larger, cable-like bundles, referred to as collagen fibers (Alberts *et al*., 2002) (figure 1.4).

1.2.1 Collagen synthesis

In ECM-producing cells, such as (myo)fibroblasts, individual collagen polypeptide chains are synthesized on ribosomes and injected into the lumen of the endoplasmic reticulum (ER) as pro-α chains. In the ER, selected proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine residues. Some of these hydroxylysine residues are glycosylated. Thereafter, three pro-α chains combine to form a triple-stranded helix known as procollagen. The hydroxyl groups of hydroxylysine and hydroxyproline residues form interchain hydrogen bonds that help stabilize the triple-stranded helix. In the neutral pH environment of the ER, heat shock protein 47 (hsp47) is able to bind to the collagen chains and promotes correct formation of the procollagen by transporting it to the Golgi compartment. After successful transportation, hsp47 is shed from procollagen due to the reduced pH in the Golgi compartment (Dafforn *et al*., 2001). Next, procollagen is secreted into the extracelluar space, where the propeptides are removed to convert the procollagen molecule into an insoluble collagen molecule. The collagen molecules have the tendency to self-assemble to form collagen fibrils. The fibrils begin to form in the extracellular space close to the cell surface, often in deep invaginations of the plasma membrane. The underlying cortical cytoskeleton can

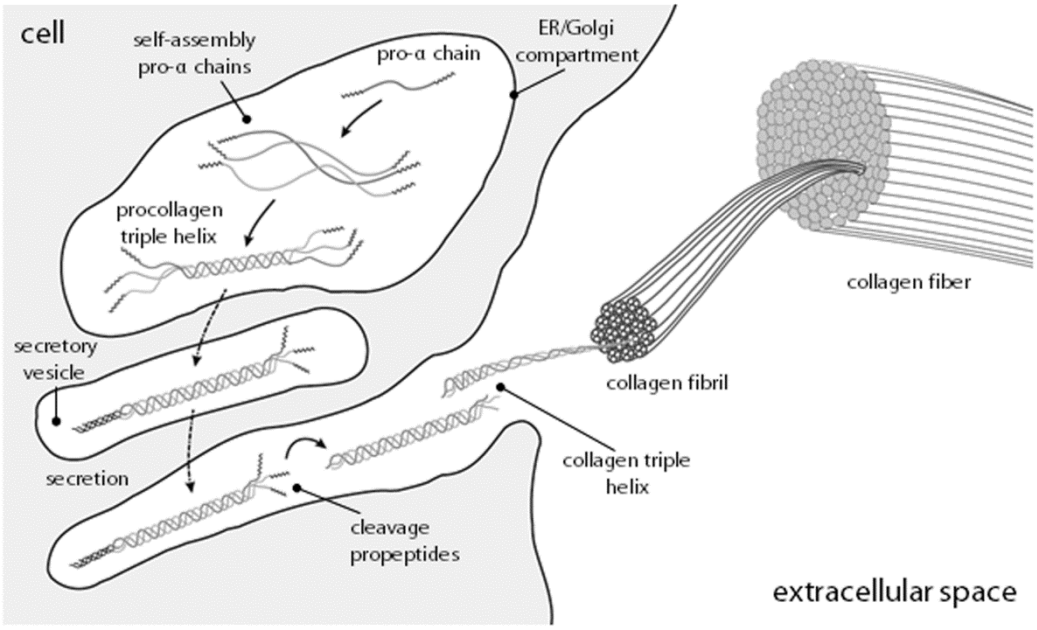

Figure 1.4: *The intra- and extracellular events in the formation of a collagen fibril. In the endoplasmatic reticulum three pro-α-chains are combined to form procollagen. This is secreted into the extracellular space, where the propeptides are removed. The removal of the propeptides will trigger the collagen molecules to self-assemble into collagen fibrils, which will eventually aggregate into a collagen fiber.*

influence the sites, rates, and orientation of fibril assembly (Alberts *et al*., 2002). Finally, the collagen fibrils will aggregate to form a collagen fiber (figure 1.4).

1.2.2 Collagen cross-linking

After the fibrils have formed in the extracellular space, they are strengthened and mechanically stabilized by the formation of covalent cross-links between lysine residues of the constituent collagen molecules (Kadler *et al*., 1996; Alberts *et al*., 2002). The first step in the cross-link formation is the deamination of certain lysine and hydroxylysine residues by the extracellular enzyme lysyl oxidase (LOX) to yield highly reactive aldehyde groups. These aldehydes react spontaneously to form di- and tri-functional covalent bonds with each other or with other lysine or hydroxylysine residues. These aldehyde-derived cross-links can be divided into two classes, one based on lysine aldehydes (allysine route), and the other on hydroxylysine aldehydes (hydroxyallysine route) (Eyre *et al*., 1984). Within the hydroxyallysine route, the cross-links can further mature into trivalent hydroxylysyl pyridinoline (HP) cross-links, which consist of three hydroxylysine residues, and lysyl pyridinoline (LP) cross-links, consisting of two residues of hydroxylysine and one lysine. In heart valves mainly HP cross-links are found, as these cross-links predominate in highly hydroxylated collagens, such as collagen type I (Bailey *et al*., 1998). These mature cross-links make the collagen fibers less susceptible to enzymatic degradation (Paul and Bailey, 2003). Moreover, as collagen cross-linking stabilizes the collagen fibril, the load-bearing capacity of collagen in heart valves is dependent on cross-link density (Balguid *et al*., 2007).

1.2.3 Collagen degradation

To regulate collagen turnover, collagen is prone to be degraded by proteolytic enzymes locally secreted by the cells. The most important collagen degrading enzymes are matrix metalloproteinases (MMPs), which form a subfamily of the metzincin superfamily of proteases. There are over 20 human MMPs that cleave practically all protein components of the ECM. The MMPs are divided into collagenases, gelatinases, stromelysins, and matrilysins. For degradation of collagen, collagenases (MMP1) and gelatinases (MMP2 and MMP9) are most important. Collagenase has the ability to cleave collagen fibrils to produce denatured collagen, while the breakdown of this denatured collagen occurs by gelatinase. MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), to control the MMP-mediated collagen degradation.

1.2.4 Collagen architecture & remodeling

The term collagen architecture refers to all aspects of collagen within the tissue, such as collagen content and type, collagen cross-link density and type, collagen orientation, length, and thickness of the collagen fibers. As mentioned above, the fibrosa of the heart valve leaflets is predominantly composed of circumferentially aligned, macroscopically crimped, densely packed collagen fibers, largely arranged parallel to the free edge of the leaflet (Schoen and Levy, 1999). Due to this architecture, the leaflets are highly anisotropic. Nonetheless, the postnatal maturation towards this specific collagen architecture and the resulting anisotropy, is not fully understood.

Changes in collagen architecture are referred to as collagen remodeling. Cells within a tissue can respond to local biochemical and mechanical stimuli by altering the collagen synthesis, degradation, and traction forces, leading to a different architecture to change the material properties of the tissue. This change can be achieved in response to regulation of MMP activity by the cells (Stamenkovic, 2003; Phillips and Bonassar, 2005), or by the secretion of ECM proteins and cross-linking of collagen. The collagen architecture and remodeling in tissue-engineered heart valves can be controlled by mechanical conditioning to create proper functioning heart valves that are strong enough for implantation. The optimal conditioning protocol, to create the best architecture for proper mechanical functioning, still needs to be determined.

1.3 Heart valve replacements

To avoid serious cardiac, pulmonary, or systemic malfunctioning due to valvular dysfunction, approximately 285,000 heart valve replacement surgeries are performed annually worldwide (Mikos *et al*., 2006). Currently, there are two types of commercially available heart valve replacements: mechanical and bioprosthetic valves. Mechanical valves are made from pyrolytic carbon or titanium coated with pyrolytic carbon. They can generally last a life-time, but life-long anticoagulation therapy is required to prevent thromboembolism. Therewith, patients with a mechanical valve have an increased risk of bleeding due to the required anticoagulation therapy.

Bioprosthetic valves are either of animal origin (xenograft) or can be harvested from a human donor (homograft). Porcine heart valves or valves formed from bovine pericardium are known as xenografts. They are treated with glutaraldehyde, which sterilizes the valve tissue and makes them biologically acceptable for the recipient (Bloomfield, 2002). Homografts are sterilized using an antibiotics solution and are cryopreserved or stored in a fixative (Bloomfield, 2002). The major advantage of these bioprosthetic valves is that there is no need for anticoagulation therapy. Nevertheless, clinically important degenerative changes, including calcification and collagen

breakdown, develop in most of the bioprosthetic valves over time, which limits their durability (Barnhart *et al*., 1982).

A shortcoming of all current heart valve replacements is that they do not consist of living tissue, and, consequently, do not adapt or remodel to changing circumstances. Thus, development and implantation of a living valve is of utmost importance, as a living valve can significantly improve life expectancy and quality of life (El-Hamamsy *et al*., 2010).

1.4 Heart valve tissue engineering

Heart valve tissue engineering aims to develop living autologous heart valves that have the ability to grow, repair, and remodel to function a lifetime *in vivo*. Tissue engineering (TE) was first described in 1993 by Langer and Vacanti as 'an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function' (Langer and Vacanti, 1993). Various TE approaches are being employed, either to develop the valve substitute *in vitro* or to use the regenerative potential of the body and develop the valve substitute *in situ*. The classical *in vitro* TE approach is based on the description of Langer and Vacanti, in which (autologous) cells are isolated and expanded in culture prior to seeding them onto a biodegradable scaffold (figure 1.5). Several cell sources, ranging from prenatally derived (stem) cells (Schmidt *et al*., 2005; Schmidt *et al*., 2006; Schmidt *et al*., 2007a; Weber *et al*., 2011c) to adult (stem) cells (Hoffman-Kim *et al*., 2005; Schmidt and Hoerstrup, 2006; Siepe *et al*., 2008; Schaefermeier *et al*., 2009; Schmidt *et al*., 2010; Apte *et al*., 2011), can be used to seed into the scaffolds. Various

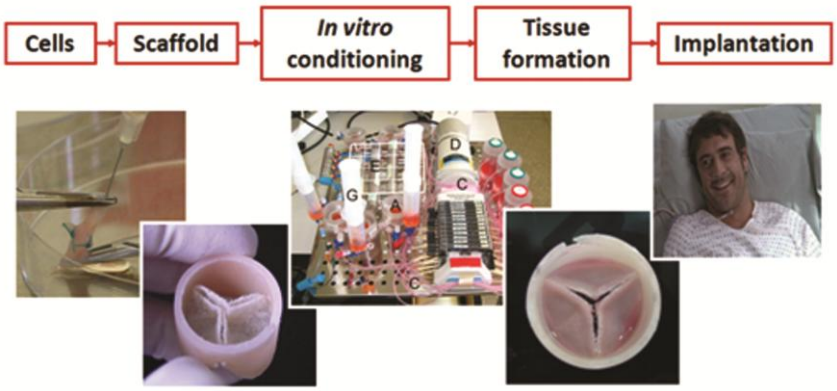

Figure 1.5: *The classical in vitro tissue engineering paradigm. Autologous cells are isolated, expanded, and seeded into a biodegradable heart valve shaped scaffold. Subsequently, the cell-scaffold construct is subjected to biochemical and mechanical stimuli to enhance tissue formation. Finally, a functional TE heart valve is implanted into the patient.*

scaffolds are being used for heart valve TE (e.g. reviewed by (Vesely, 2005; Mendelson and Schoen, 2006; Schmidt *et al*., 2007b; Mol *et al*., 2009; Apte *et al*., 2011; Bouten *et al*., 2011; Weber *et al*., 2011a)). After cell seeding, the cell-scaffold construct is usually subjected to environmental stimuli in a bioreactor to enhance ECM formation (figure 1.6). These stimuli can mediate cells to release cytokines or growth factors. Cells sense these (mechanical and biochemical) stimuli and control their extracellular environment by changing or remodeling their ECM until a functional (autologous) heart valve has grown that can be used for implantation.

For preclinical testing of the TE heart valves, an ovine model is the gold standard. The sheep has similar heart valves compared to humans in terms of mechanical properties and hemodynamic flow parameters (Rashid *et al*., 2004). In addition, sheep develop more rapidly than humans and changes which take years to develop in humans can develop within months in sheep (Barnhart *et al*., 1982). Finally, the sheep presents a 'worst-case-scenario' for calcification, due to its enhanced calcium metabolism (Ali *et al*., 1996; Hoerstrup *et al*., 2006). In 1995, the first successful replacement of a single autologous pulmonary TE leaflet was demonstrated in sheep (Shinoka *et al*., 1995). And in 2000, Sodian *et al.*, Stock *et al.*, and Hoerstrup *et al.* report functional tri-leaflet pulmonary TE heart valves implanted in sheep (Sodian *et al*., 2000; Stock *et al*., 2000; Hoerstrup *et al*., 2000a). Nevertheless, it is still unclear whether ovine TE heart valves are representative for the TE product obtained from human cells.

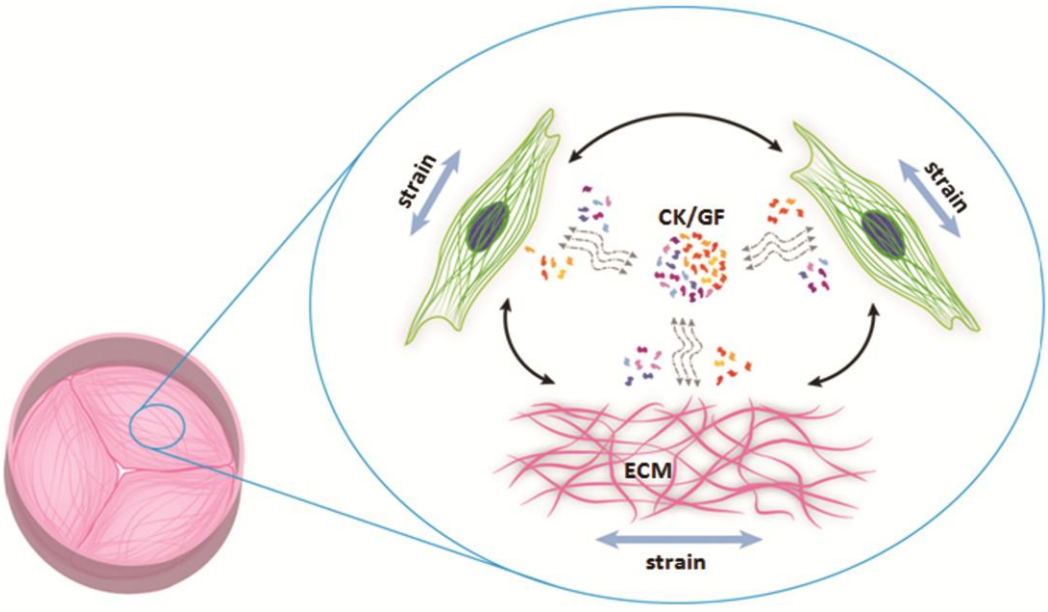

Figure 1.6: *Conditioning strategies during the in vitro culture period. The cell-scaffold construct is biochemically or mechanically conditioned. Cytokines (CK) or growth factors (GF), either added to the medium (exogenous) or secreted by the cells as a response to a conditioning regime (endogenous), will trigger the cells to enhance extracellular matrix (ECM) formation. In turn, the matrix can also influence the cells to remodel itself upon environmental stimuli.*

The *in situ* TE approach focuses on the direct implantation of a scaffold without an *in vitro* culture period. The 'smart' scaffold will attract endogenous cells, which populate the scaffold and result *in vivo* tissue formation that will take over the function of the degrading scaffold (Mol *et al*., 2009). *In situ* TE offers a quick, cheap, and on-demand approach. However, this TE approach is still in its infancy and, as cells are needed for the *in vivo* tissue formation, the main challenge is to understand the population process of the scaffold with autologous cells.

1.4.1 Mechanical conditioning

Mechanical stimulation plays a pivotal role in organogenesis during embryonic development. Therefore, it is not surprising that mechanical conditioning is used in heart valve TE to stimulate ECM production (Mol *et al*., 2003; Freed *et al*., 2006; Boerboom *et al*., 2008; Rubbens *et al*., 2009b; Rubbens *et al*., 2009c). Mechanical conditioning increases ECM formation (e.g. collagen content) and organization and, therewith, the mechanical properties of the engineered tissues (Hoerstrup *et al*., 2000a; Seliktar *et al*., 2003; Isenberg and Tranquillo, 2003; Mol *et al*., 2003; Ku *et al*., 2006; Syedain *et al*., 2008; Syedain and Tranquillo, 2011). The effects of mechanical conditioning are dependent upon the degree and the duration of these stimuli (Xing *et al*., 2004a; Xing *et al*., 2004b; Ku *et al*., 2006). Elevated cyclic stretch results in increased cell proliferation and apoptosis, increased collagenase and gelatinase activity, and also induces calcification in aortic valve leaflets (Balachandran *et al*., 2009; Lehmann *et al*., 2009). Thus, a proper mechanical conditioning protocol should be chosen with caution.

Continuous mechanical conditioning is often applied as it reflects a physiological loading condition. However, this regime might not be optimal to engineer heart valves. Cells tend to become insensitive to constant environmental stimuli. To overcome this, the magnitude of the stimulus can be changed to disrupt the adaptation response, and, thus, increasing the effect of the stimulus (Syedain *et al*., 2008). Another method to overcome cell adaptation is by application of intermittent mechanical conditioning. In an intermittent protocol, the mechanical loading is combined with rest periods to disturb the adaptation response. For heart valve TE, intermittent mechanical conditioning is favored as it is thought to balance the collagen production during the static conditioning (rest periods) and enhance cross-linking during the dynamic conditioning (Rubbens *et al*., 2009b). Nevertheless, the underlying mechano-regulatory mechanism is not yet known. Understanding of this mechanism is highly relevant to optimize the existing TE protocols to improve tissue structure-function properties of TE heart valves.

1.4.2 Biochemical conditioning

Biochemical conditioning involves the addition of stimulatory factors, e.g. cytokines or growth factors, either directly to the growth medium or by incorporation into the

scaffold material. Cytokines and growth factors are secreted by cells to function as mediators of cell communication and to stimulate cellular growth, proliferation, differentiation, and maturation. The addition of stimulatory factors to the growth medium only has a short-term effect and should, therefore, be repeated during culture. Alternatively, cytokines, growth factors or their functional derivatives can be incorporated into the scaffold material to obtain tethered or timed-release of these substances. This can also be beneficial for *in situ* TE, as in this approach the scaffold needs to attract cells and guide and control cell function (Mol *et al*., 2009; Bouten *et al*., 2011).

During *in vitro* heart valve TE, the culture medium can be supplemented with various growth factors (table 1.1). For example, hepatocyte growth factor (Ota *et al*., 2005; Huang *et al*., 2007), transforming growth factor beta (Long and Tranquillo, 2003; Stegemann and Nerem, 2003a; Stegemann and Nerem, 2003b; Narine *et al*., 2004; Appleton *et al*., 2009; Chiu *et al*., 2010), basic fibroblast growth factor (Bos *et al*., 1999; Hoerstrup *et al*., 2000b; Williams *et al*., 2006; Ramaswamy *et al*., 2010), epidermal growth factor (Appleton *et al*., 2009), platelet-derived growth factor (Stegemann and Nerem, 2003a; Stegemann and Nerem, 2003b; Appleton *et al*., 2009), and ascorbic acid (Hoerstrup *et al*., 2000b; Williams *et al*., 2006; Ramaswamy *et al*., 2010) to stimulate cell differentiation, proliferation, and ECM production.

Another way of biochemical conditioning is by changing medium conditions towards those required for fully autologous human heart valve culture, thus omitting all animal-derived substances in the medium. Human platelet lysate, for instance, contains a different subset of cytokines and growth factors as compared to fetal bovine serum (Riem Vis *et al*., 2010). As platelet lysate can be obtained autologous, it is favored over

Biochemical stiumuli	Effects
HGF	Early recellularization in tissue-engineered heart valves (Ota *et al.*, 2005; Huang *et al.*, 2007)
TGFβ	Differentiate fibroblasts towards myofibroblasts (Narine *et al.*, 2004; Chiu *et al.*, 2010)
	Increases αSMA expression (Stegemann and Nerem, 2003a & 2003b)
	Increases cell proliferation, migration, and invasion (Appleton *et al.*, 2009; Chiu *et al.*, 2010)
	Induces elastin production (when combined with insulin) (Long and Tranquillo, 2003)
	Increases collagen expression (when combined with EGF and bFGF) (Appleton *et al.*, 2009)
EGF	Increases collagen expression (when combined with TGFβ and bFGF) (Appleton *et al.*, 2009)
bFGF	Induces rapid endothelialization of tissue-engineered grafts (Bos *et al.*, 1999)
	Increases collagen production (Hoerstrup *et al.*, 2000b; Williams *et al.*, 2006; Ramaswamy *et al.*, 2010)
PDGF	Increases cell proliferation (Stegemann and Nerem, 2003a & 2003b; Appleton *et al.*, 2009)
Ascorbic acid	Increases collagen production (Hoerstrup *et al.*, 2000b; Williams *et al.*, 2006; Ramaswamy *et al.*, 2010)

Table 1.1: *Effects of biochemical conditioning in heart valve tissue engineering by supplementation of hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), or ascorbic acid.*

fetal bovine serum to create autologous TE heart valves and tissue formation can be stimulated by this different subset of cytokines and growth factors.

1.5 Outline

One of the main challenges of the *in vitro* heart valve tissue engineering approach is to control tissue formation and collagen remodeling, and hence tissue mechanical properties, during the *in vitro* culture phase. Moreover, it is important to find markers that predict whether tissue structure and mechanical properties of TE heart valves are good enough for implantation and long-term functioning in patients. Tissue (mechanical) properties of native human heart valves should be used as benchmarks for these TE heart valves. In addition, understanding of tissue development, collagen remodeling, and resulting tissue (mechanical) properties, is crucial for optimization of tissue culture protocols to create functional, load-bearing heart valves. Therefore, the aim of this work is to understand and optimize tissue development and resulting mechanical properties of TE heart valves, with special emphasis on collagen remodeling in the valve leaflets.

Measurements on human aortic and pulmonary valve tissue of different age groups in **chapter 2** give insight in the development of native tissue (mechanical) properties and collagen remodeling. This native data provide benchmarks for TE heart valves and the optimization of TE heart valves toward their native counterparts. In **chapter 3**, human and ovine heart valve tissue constructs were engineered to determine possible indicators of *in vitro* tissue outcome. In addition, interspecies differences in tissue mechanical properties were determined to evaluate whether ovine TE heart valves, which are now the gold standard in pre-clinical studies, are representative for human TE heart valves. In **chapter 4**, TE heart valve constructs were biochemically conditioned by changing the medium conditions towards autologous, human heart valve culture. Tissue architecture, collagen remodeling, and mechanical properties were determined in TE heart valve constructs cultured with different subsets of growth factors and cytokines (fetal bovine serum versus platelet lysate). In **chapter 5**, the effects of continuous cyclic strain and static strain after mechanical stimulation were analyzed and compared at the gene expression level in an attempt to further understand and predict mechanically-induced collagen remodeling in heart valves. Finally, **chapter 6** presents a general discussion and conclusion of the most important findings in this thesis

Chapter 2

Evolution of Matrix Composition and Mechanical Properties of Pediatric, Adolescent and Adult Human Aortic and Pulmonary Valves: Benchmarks for Tissue-Engineered Heart Valves

The contents of this chapter are based on D. van Geemen, A.L.F. Soares, A. Driessen-Mol, M. Janssen-van den Broek, A.J. van den Bogaerdt, A.J.J.C. Bogers, M.J. Goumans, F.P.T. Baaijens, and C.V.C. Bouten (2012) "*Evolution of Matrix Composition and Mechanical Properties of Pediatric, Adolescent and Adult Human Aortic and Pulmonary Valves: Benchmarks for Tissue-Engineered Heart Valves*" (in preparation)

2.1 Introduction

Annually, approximately 285,000 heart valve replacements are performed worldwide to avoid cardiac, pulmonary, or systemic problems due to valvular disease (Yacoub and Takkenberg, 2005; Pibarot and Dumesnil, 2009). Semilunar valves, in particular the aortic valve, are mostly affected. The current types of heart valve replacements enhance survival and quality-of-life of most patients, but have several limitations (Mendelson and Schoen, 2006). For example, life-long anticoagulation therapy to prevent thromboembolism is necessary for patients with a mechanical valve, while in most bioprosthetic valves degenerative changes, including calcification and collagen breakdown, develop with time, limiting their durability. The most important drawback of current heart valve replacements, however, is that they do not consist of living tissue and, therefore, do not have the ability to grow, repair, and remodel. Especially in pediatric and adolescent patients, multiple valve replacement operations to implant larger valves are needed to accommodate growth. Replacement of the diseased aortic valve by a living pulmonary valve autograft, common for pediatric and adolescent patients, is considered clinically effective in reconstruction of the aortic valve (Ross procedure) (Ross *et al*., 1992). Nevertheless, the pulmonary site still requires a prosthesis.

Heart valve tissue engineering (TE) seeks to overcome the current limitations of heart valve prostheses by creating a living autologous heart valve replacement that can grow and adapt in response to changing functional demands. Key parameters of functional autologous TE heart valves are: valve geometry, tissue morphology, cell type, extracellular matrix (ECM) composition and architecture, and mechanical properties, which may all change with the target age group for valve replacement. The functionality of native human heart valves represent the criteria for the desired characteristics of TE heart valves (Schoen, 2011). Thus, data from human native valves provides benchmarks for the TE heart valve parameters and the optimization of TE heart valves toward their native counterparts. Valvulogenesis and tissue morphogenesis of semilunar heart valves have been extensively studied, but these studies mainly focused on heart valves of animal origin (e.g. porcine heart valves) (Moretti and Whitehouse, 1963; Stephens and Grande-Allen, 2007; Stephens *et al*., 2008; Stephens *et al*., 2010). In addition, most studies with human valves concentrate on either fetal or adult valves (Gross and Kugel, 1931; Bashey *et al*., 1967; Maron and Hutchins, 1974; Vesely *et al*., 2000; McDonald *et al*., 2002; Stradins *et al*., 2004; Hinton and Yutzey, 2011), while studies on human pediatric and adolescent valves are sparsely available (Maron and Hutchins, 1974; Christie and Barratt-Boyes, 1995; Aikawa *et al*., 2006). Nevertheless, the evolution of structure-function properties from birth to adult has not been studied.

The above mentioned studies have demonstrated that the semilunar heart valve leaflets have a three-layered structure. The thin ventricularis, at the ventricular surface of the leaflet, is rich in radially aligned elastin. The fibrosa is located on the arterial side

of the leaflet, and is composed of a dense collagen network, predominantly arranged in the circumferential direction. The middle layer, spongiosa, is mainly composed of proteoglycans and a few loosely connected fibrous proteins. These layers become clearly defined in advancing postnatal age periods (Gross and Kugel, 1931). However, the age-related change in protein and layer composition remains largely unknown.

Prior to birth, the tissue morphology of the two semilunar valves has been described as identical (Maron and Hutchins, 1974). After birth, the pressures in the systemic and pulmonary circulation change immediately with a rapid decrease in the pulmonary arterial pressure and a gradual increase in the systemic pressure (figure 2.1). It is thought that the reduced pressure in the pulmonary circulation reflects in thinner valve leaflets in the pulmonary valves as compared to the aortic valves, while the abrupt change to the neonatal circulation immediately after birth has been associated with a greater number of activated cells in the aortic valves compared to the pulmonary valves (Aikawa *et al*., 2006). It has been proposed that the enhanced number of activated cells leads to a thicker adult aortic valve, which is probably due to increased collagen production and a thicker fibrosa layer (Hinton and Yutzey, 2011). To date, only two studies concentrated on the differences in mechanical properties between human adult aortic and pulmonary valves. In these studies, the observed differences between the aortic and pulmonary valves were minimal (Vesely *et al*., 2000; Stradins *et al*., 2004). Mechanical properties of human pediatric and adolescent aortic and pulmonary valves remain unexplored.

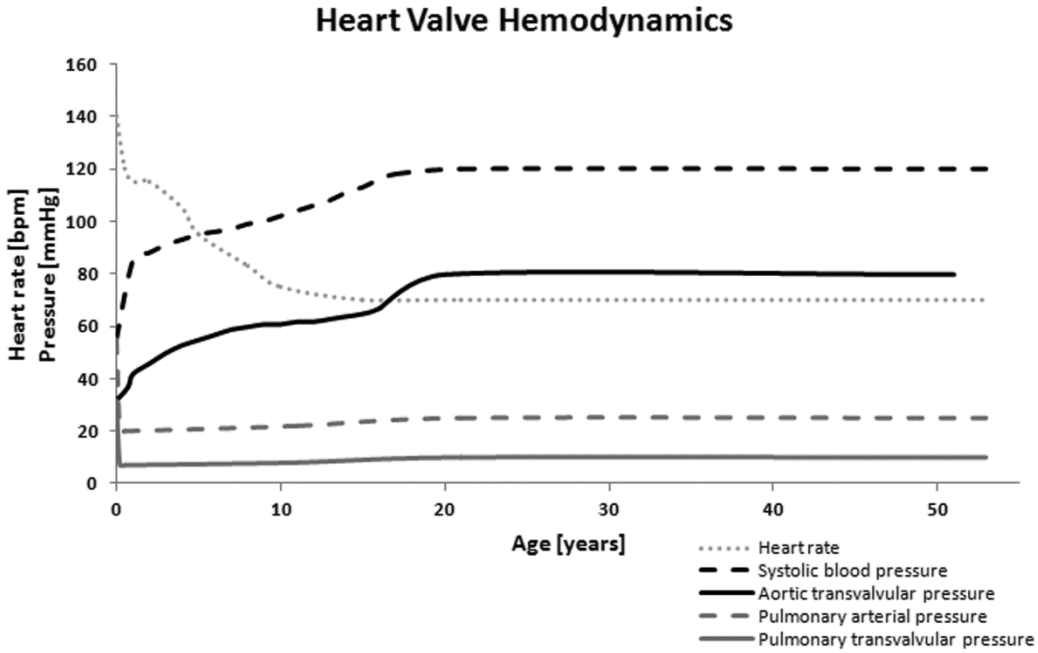

Figure 2.1: *Valve hemodynamics during postnatal life in the aortic and pulmonary valve. Systemic and transvalvular pressures over the aortic valve (black lines) increases during childhood, while the pulmonary arterial pressure and transvalvular pressure over the pulmonary valve (dark grey lines) decreases towards adult values directly after birth.*

The number of cells in both semilunar valves decreases progressively with advancing age (Gross and Kugel, 1931; Aikawa *et al*., 2006; Stephens and Grande-Allen, 2007). In addition, cell proliferation and apoptosis are higher in fetal valves, while the fetal cells show an activated myofibroblasts-like phenotype engaged in matrix remodeling rather than the quiescent fibroblast-like phenotype present in adults (Aikawa *et al*., 2006). There is no consensus in the literature about the ECM changes with age. During fetal life, collagen content is increasing, while collagen organization and maturation are assumed to take place only after birth, resulting in more aligned fibers in adult valves (Aikawa *et al*., 2006; Stephens and Grande-Allen, 2007). After birth, the sparsely available studies on aortic valves describe a modest decrease of the total collagen content in the aging adult human aortic valve (Bashey *et al*., 1967; McDonald *et al*., 2002), whereas other studies show an age-related increase in collagen content of porcine valves (Stephens and Grande-Allen, 2007; Stephens *et al*., 2010). One study showed that the elastin content was equal in different adult age groups (Bashey *et al*., 1967), while others found a dramatic increase in elastin with increasing age (McDonald *et al*., 2002; Aikawa *et al*., 2006). The proteoglycan content has not been reported to change in the aging adult aortic valves (McDonald *et al*., 2002). However, the composition of the proteoglycans in bovine and porcine heart valves does change with age (Moretti and Whitehouse, 1963; Stephens *et al*., 2008).

The mechanical properties of the semilunar valves also change with adult age. Up to 25 years of age, the radial stretch of the human aortic valve declines rapidly, then remains constant, and starts to decline slowly from the age of 40 years on (Christie and Barratt-Boyes, 1995). This age-related decrease in stretch is probably related to an increasing stiffness of the aortic leaflets with age (Stephens *et al*., 2010). These age-related changes in leaflet stiffness are probably the result of the increasing transvalvular pressures and associated decreasing aortic compliance (VanAuker, 2006; Stephens *et al*., 2010). This age-related increase in leaflet stiffness might also imply degeneration or calcification of the valve, as the stiffness of the mitral valve is increasing with degeneration and the severity of calcification (Imanaka *et al*., 2007), although, calcification is mostly observed in diseased or very old heart valves.

Although tissue properties of mainly human fetal and adult aortic heart valves have been extensively studied, the evolution of structure-function properties from young to old age is largely unknown. More importantly, there is no conclusive data on human pediatric and adolescent heart valves, while these are the target age groups are for tissue engineering. Thus, target values for pediatric and adolescent TE valve replacements are lacking and the question of ‘How good a pediatric living valve replacement should be’ cannot be answered. Furthermore, there is no consensus about structure-function differences between pulmonary and aortic valves, while the first human TE heart valve replacements are expected to take place at the pulmonary side, e.g. during a Ross operation.

This study is the first to assess tissue mechanical properties, ECM composition, and maturation of paired human aortic and pulmonary valves of different age groups (fetal, child, adolescent, and adult). We aimed to study the evolution of structure-function properties and valve remodeling to provide age-specific benchmarks for future tissue engineering therapies.

2.2 Materials & Methods

2.2.1 Tissue preparation

Fourteen sets of cryopreserved healthy human aortic and pulmonary valves (e.g. aortic and pulmonary valve of the same donor creating an n = 28) of different ages (fetal, child, adolescent, adult; table 2.1) were obtained from Dutch postmortem donors, giving permission for research. The post-natal valves, which were assessed to be unfit for implantation, were obtained from the Heart Valve Bank Rotterdam (Erasmus University Medical Center, Rotterdam, The Netherlands), while the fetal valves were obtained within a collaboration with the department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands. All valves were structurally and mechanically unaffected. The cause of death of the donors was not related to valvular disease or conditions known to precede valvular disease. In addition, observations from other groups demonstrated that the applied cryopreservation protocol for these valves did not affect structural integrity of collagen and elastin (Gerson *et al*., 2009) and mechanical properties (Virues Delgadillo *et al*., 2010), suggesting that the cryopreserved heart valves can be used to study the tissue (mechanical) properties.

The cryopreserved valves were stored at -80 °C and thawed just prior to ECM analysis and mechanical testing (described below). The valves were thawed according to the guidelines of the Heart Valve Bank Rotterdam. Briefly, the package containing the cryopreserved homograft was gently agitated in warm saline (± 40 °C) to dissolve ice-crystals and soften the graft. After thawing, the package containing the valve was opened and contents were deposited in a bowl. Cold phosphate buffered saline (PBS; Sigma) was gently added to allow the dimethyl sulfoxide (DMSO) to dilute from the tissue into the solution. The thawed valve was photographed (data not shown) and cut immediately according to a cutting scheme in preparation of the pre-defined analyses (figure 2.2). Samples for histology and biochemical assays were fixed in formalin or snap-frozen, respectively, within 24 hours, while mechanical testing was performed within 48 hours after thawing.

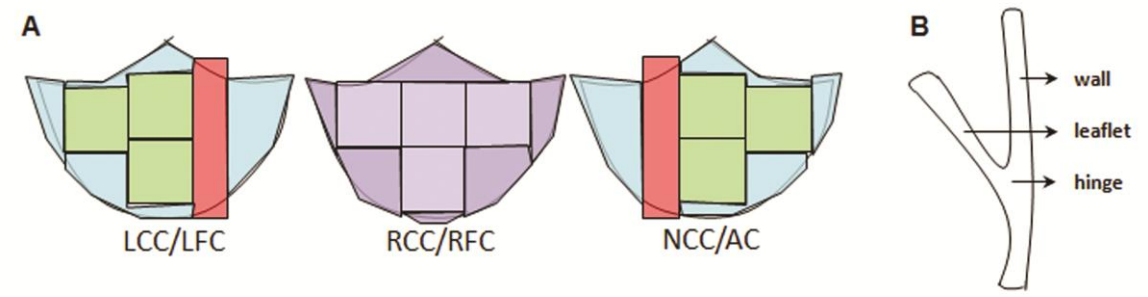

Figure 2.2: *(A) Schematic overview of the cutting scheme of one heart valve. The samples for the biaxial tensile tests are indicated in green. The parts for histology indicated are in red. The right coronary cusp (RCC, aortic valve) and right facing cusp (RFC, pulmonary valve) are used for indentation tests (purple). Afterwards, this leaflet is cut for biaxial tensile tests. Left-over tissue is indicated in blue and freeze-dried for biochemical assays. (B) Schematic cross-section of the heart valve for histology, which indicates the wall, leaflet and hinge regions. LCC: Left coronary cusp; LFC: Left facing cusp; NCC: non coronary cusp; AC: anterior cusp.*

2.2.2 Valve geometry and morphology

Valve geometry was characterized from measures of valve diameter, leaflet size (both radial and circumferential), and leaflet thickness. The annulus diameter and the morphological appearance (e.g. presence of fibrosis or artheroma) of the heart valve leaflets were provided by the Dutch Heart Valve Bank. The size of the right (coronary) leaflet was measured in circumferential and radial direction (figure 2.3A) to assess the dimensions of the leaflets. The thickness of the same leaflet was measured as part of the mechanical the indentation tests.

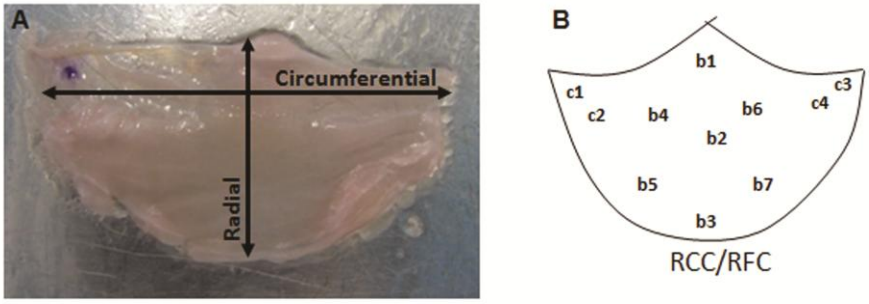

Figure 2.3: *(A) The size of the leaflets is measured in circumferential and radial direction. The measured position is indicated with the arrows. (B) Indentation tests are performed in the commisural (c1 – c4) and belly (b1 – b7) region of the right coronary cusp (RCC)/right facing cusp (RFC).*

2.2.3 Mechanical testing

Indentation tests (n = 26; table 2.1) and biaxial tensile tests (n = 12; table 2.1) were performed to study the mechanical properties of the leaflets. While the indentation tests provided insights into local mechanical properties, the biaxial tensile tests were performed to provide more global mechanical properties. Performing both tests will further provide information about the stiffness of the leaflets at low (indentation tests) and high (biaxial tensile tests) strains. In addition, biaxial tensile testing gives information on the anisotropic mechanical properties in both radial and circumferential direction of the leaflets.

2.2.3.1 Indentation tests

To characterize local tissue mechanical properties, spherical indentation tests were performed to the belly (approximately 7 indentations per leaflet) and the commissural (approximately 4 indentations per leaflet) region of the right (coronary) leaflet (figure 2.3B). For indentation tests, the fibrosa-side of the leaflet was place face-down and the tests were performed as described previously (Cox *et al*., 2008). Briefly, a spherical sapphire indenter (diameter 2 mm) was used to compress the tissue with a constant indentation speed of 0.01 mm/s to simulate quasistatic loading conditions. At the indented locations, one preconditioning cycle followed by two additional indentation tests were performed to measure the elastic modulus (E-modulus), which represented the slope at 20% indentation, and the thickness. A drop in the force signal was noticed when the indentor touched the leaflet. The height of the indentor at that moment corresponds with the thickness of the indented sample. Per leaflet, approximately 4 measurements in the commissure and 7 measurements in the belly were averaged to determine the mean thickness in the commissure and belly, respectively.

2.2.3.2 Biaxial tensile tests

The samples for the biaxial tensile tests were kept hydrated and were placed on aluminium foil to mount the samples in a BioTester 5000 device (CellScale, Canada) using a BioRakes mounting system with 0.7 mm thin space. The samples were then tested while submersed in PBS to mimic natural conditions. During testing, the samples were stretched to peak values (maximum of 78% strain) in the circumferential and radial direction. The stress-strain curves were obtained at a strain rate of the initial length per minute (l_0/min). The average biaxial stress-strain curves were derived by calculating the mean of the several stress-strain curves per donor. The E-modulus was defined as the slope of the linear part of the stress-strain curve. In addition, the extensibility was defined as the point where the fitted slope of the linear part of the stress-strain curve crosses the x-axis. The data were averaged per donor.

2.2.4 Cell phenotype, tissue composition and maturation

The leaflet parts for histology (leaflet, hinge region and part of the arterial wall; figure 2.2 and table 2.1) were fixed overnight in 3.7% formaldehyde in PBS, processed and subsequently embedded in paraffin. They were sectioned at 10 µm thickness and cellular phenotype and matrix composition were qualitatively studied with histology and immunofluorescent stainings. The leaflet parts for biochemical assays were lyophilized and afterwards digested in papain solution (100 mM phosphate buffer [pH=6.5], 5 mM L-cystein, 5 mM EDTA, and 125 – 140 µg papain per ml) to determine total cell number (DNA content), matrix composition (sulfated glycosaminoglycans (sGAG) and hydroxyproline content), and matrix maturation (collagen cross-links).

2.2.4.1 Qualitative analyses

The sections were studied by hematoxylin and eosin (H&E) staining for general tissue composition, Masson Trichrome (MTC kit, Sigma) for collagen visualization, Verhoeff-Van Gieson staining for collagen and elastin, and Safranin-O staining for proteoglycans. Additionally, matrix components and cellular phenotype were assessed with immunofluorescent stainings. After pretreatment with 6 M guanidine-HCl, 50 mM dithiothreitol, 20 mM Tris (pH 8.0) and washing with 20 mM Tris (pH 8.0) to enhance the antigen, elastin was analyzed with a polyclonal rabbit IgG antibody against elastin (abcam, 1:500 dilution). Collagen type I and type III antigens were retrieved by boiling in 10 mM Sodium citrate-HCl buffer (pH 6.0) and incubation in 0.04% pepsin buffer, respectively. Thereafter, collagen type I and III were determined with polyclonal rabbit IgG antibody against collagen type I (abcam, 1:250 dilution) and with polyclonal rabbit IgG antibody against collagen type III (abcam, 1:200 dilution). The alpha smooth muscle actin (αSMA) antigen was retrieved by boiling in 10 mM sodium citrate-HCl buffer (pH 6.0). A monoclonal IgG2a mouse anti-human antibody against αSMA (Sigma, 1:500) was used as phenotypic marker for valvular interstitial cells (VICs). αSMA positive cells were classified as active myofibroblasts, while αSMA negative cells were classified as quiescent fibroblasts (Rabkin-Aikawa *et al*., 2004). αSMA was visualized with a goat anti-mouse IgG2a Alexa 488 (Molecular Probes, 1:300 dilution), while the matrix components were visualized with donkey anti-rabbit Alexa 555 (Molecular Probes, 1:300 dilution). Additionally, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

2.2.4.2 Quantitative analyses

The amount of DNA (e.g. total cell number) in the leaflets was quantified using the Hoechst dye method (Cesarone *et al*., 1979) with a reference curve prepared of calf thymus DNA (Sigma). A modification of the assay described by Farndale *et al.* (Farndale *et al*., 1986) with shark cartilage chondroitin sulfate as a reference was used to measure the content of sGAG. To determine the hydroxyproline quantity, as a measure for the

collagen content, the assay according to Huszar *et al.* (Huszar *et al*., 1980) and a reference of trans-4-hydroxyproline (Sigma) was used. The number of mature collagen hydroxylysylpyridinoline (HP) cross-links, as a measure for tissue maturity, was measured in the digested samples using high-performance liquid chromatography as described previously (Bank *et al*., 1996; Robins *et al*., 1996; Bank *et al*., 1997). The number of HP cross-links was expressed per collagen triple helix. The cross-link analyses were performed in two series, in which several months were between the first and second series. The division of the valves over the two series can be found in table 2.1.

2.2.5 Data analyses

All data are presented as means and the standard error of the mean. The postnatal data were sub-divided in three age groups (table 2.1): child (0.7 – 11 years; n = 3), adolescent (18 – 22 years; n = 4), and adult (38 – 53 years; n = 6). Correlations between parameters and with age were analyzed with correlation matrices. We assumed that the data were normal distributed, because the valves were (randomly) obtained from the normal population. Therefore, significant differences between the age groups and aortic and pulmonary valves were studied with two-way ANOVA. A level of $p < 0.05$ was used to indicate significance. GraphPad Prism software (GraphPad Software, Inc, USA) was used for the statistical analyses.

Group	Age	Histology	Biochemical assays	Cross-links	Indentation tests	Biaxial tensile tests
fetal	21 wks	X				
Child	0.7 yr	X	X	2	X	X
	4 yr	X	X	1	X	
	11 yr	X	X	2	X	X
Adolescent	18.3 yr	X	X	1	X	
	18.6 yr	X	X	1	X	
	20 yr	X	X	2	X	X
	22 yr	X	X	1	X	
Adult	38 yr	X	X	2	X	X
	39 yr	X	X	2	X	X
	43 yr	X	X	1	X	
	48 yr	X	X	1	X	
	51 yr	X	X	2	X	X
	53 yr	X	X	1	X	

Table 2.1: *Overview of the composition of the age groups and the experiments performed on the heart valve leaflets (indicated with an 'X'). The cross-link analyses were performed in two separate series, indicated with either '1' or '2'.*

2.3 Results

2.3.1 Geometry and morphology of the human heart valve leaflets changes with age

For each age-group, the annulus of the heart valves increased with age (figure 2.4), from approximately 10 mm (aortic valve) or 11 mm (pulmonary valve) to roughly 23 mm (aortic valve) or 25 mm (pulmonary valve). The annulus of the pulmonary valve was slightly larger than the annulus of the aortic valve. In particular, in the pediatric group the annulus increases rapidly from approximately 10 mm to 17 mm. Thereafter, the annulus increases only slowly.

Both in the aortic and pulmonary valve, leaflet size increased in circumferential and radial direction with age (figure 2.5A, B) and this increase was similar for both valves. Also the circumferential-to-radial ratio increased slightly and significantly with age in both leaflets, from approximately 1.5 to 2.1 (data not shown), indicating that the leaflets grow more in the circumferential direction.

The thickness of the aortic valve leaflets was very heterogeneous, as reflected by the error bars (figure 2.5C). The aortic valve leaflets were thicker than the pulmonary valve leaflets. This difference was predominant in the belly. The commissural region of the aortic leaflets was thinner than the belly region. In the pulmonary valve leaflets, the thickness was slightly decreasing with age. This was not observed for the aortic valves.

In all adult pulmonary and aortic valve leaflets, some degree of fibrosis was observed. Fibrosis was mostly found in the central belly region, but it was also observed in the

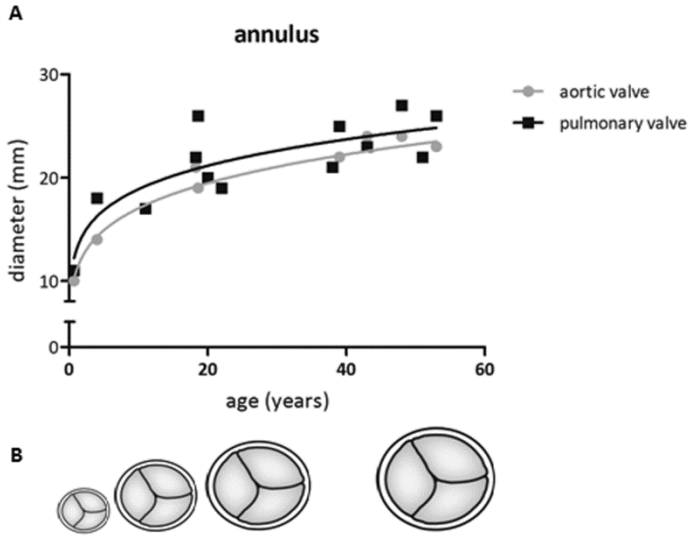

Figure 2.4: *Growth in annulus in the aortic (grey) and pulmonary (black) valve (A). In the first years of life, the diameter increases rapidly, thereafter, the diameter increases slowly. The pulmonary valve is slightly larger compared to the aortic valve. (B) Schematic representation of the growth in annulus.*

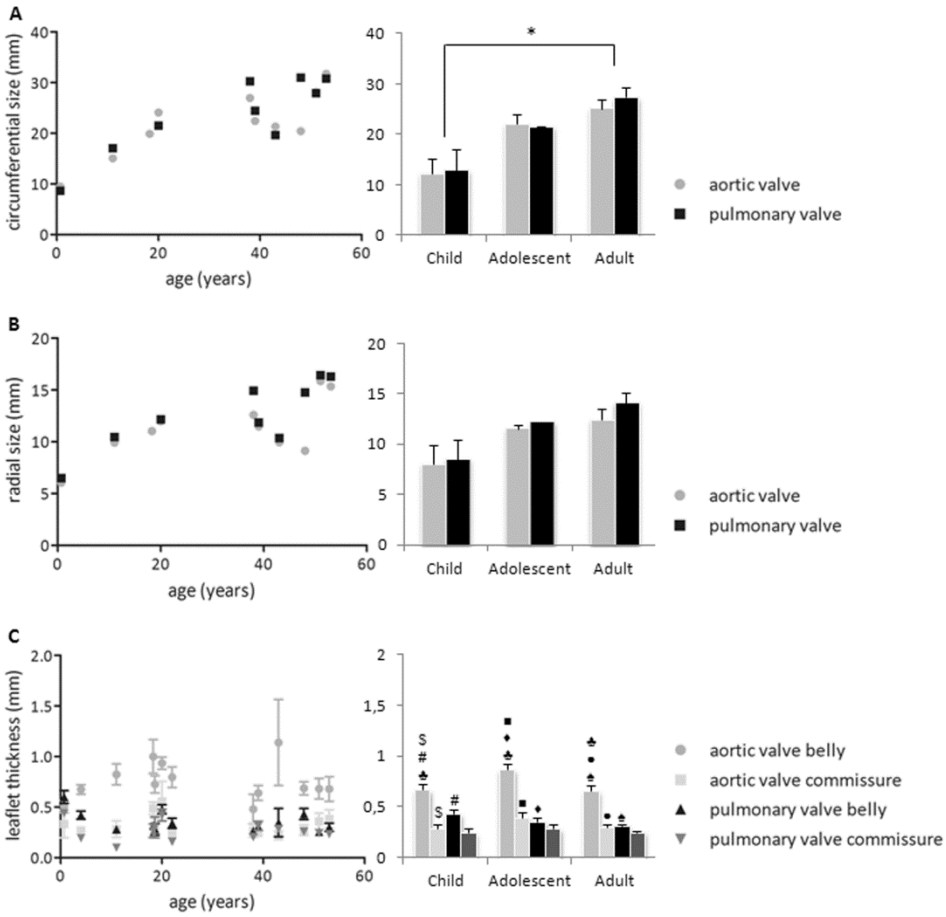

Figure 2.5: *Changes in leaflet geometry (left side: all data points for correlation analysis with age; right side: grouped data). (A) The size of the leaflet, measured in circumferential direction, is increasing with age ($p < 0.01$ in both aortic and pulmonary valve). (B) The size of leaflet measured in radial direction is also increasing with age ($p < 0.05$ in the aortic valve and $p < 0.01$ in the pulmonary valve). (C) The thickness of the leaflet is similar in all age groups. The aortic valve is slightly thicker compared to the pulmonary valve, especially in the belly.*
** ($p < 0.05$) indicates significant difference between age groups for both the aortic and pulmonary valve. Significant differences between groups ($p < 0.05$) are indicated by paired symbols.*

belly region attached to the annulus. Some adult valves showed atheroma spots at locations attached to the annulus. In the valves from the adolescent group also mild fibrosis was seen, though, not in all valves. In the pediatric valves no fibrosis was observed at all.

2.3.2 Three-layered structure becomes more pronounced with age

The three-layered structure was clearly visible in all studied aortic and pulmonary valves, even in the fetal valve (figure 2.6). Collagen was mainly found in the fibrosa according the Masson Trichrome staining and Verhoeff-Van Gieson staining.

Immunofluorescent staining for collagen type I and type III showed that in the fibrosa layer mainly collagen type I was present (figure 2.6), while collagen type III was observed throughout the leaflet (data not shown). Elastin was observed in the ventricularis with both Verhoeff-Van Gieson and the immunofluorescent staining. In the fetal valve, elastin was clearly observed with the elastin antibody staining, but not with the Verhoeff-Van Gieson (figures 2.6A, B). sGAG was observed in the spongiosa of the leaflet and in the

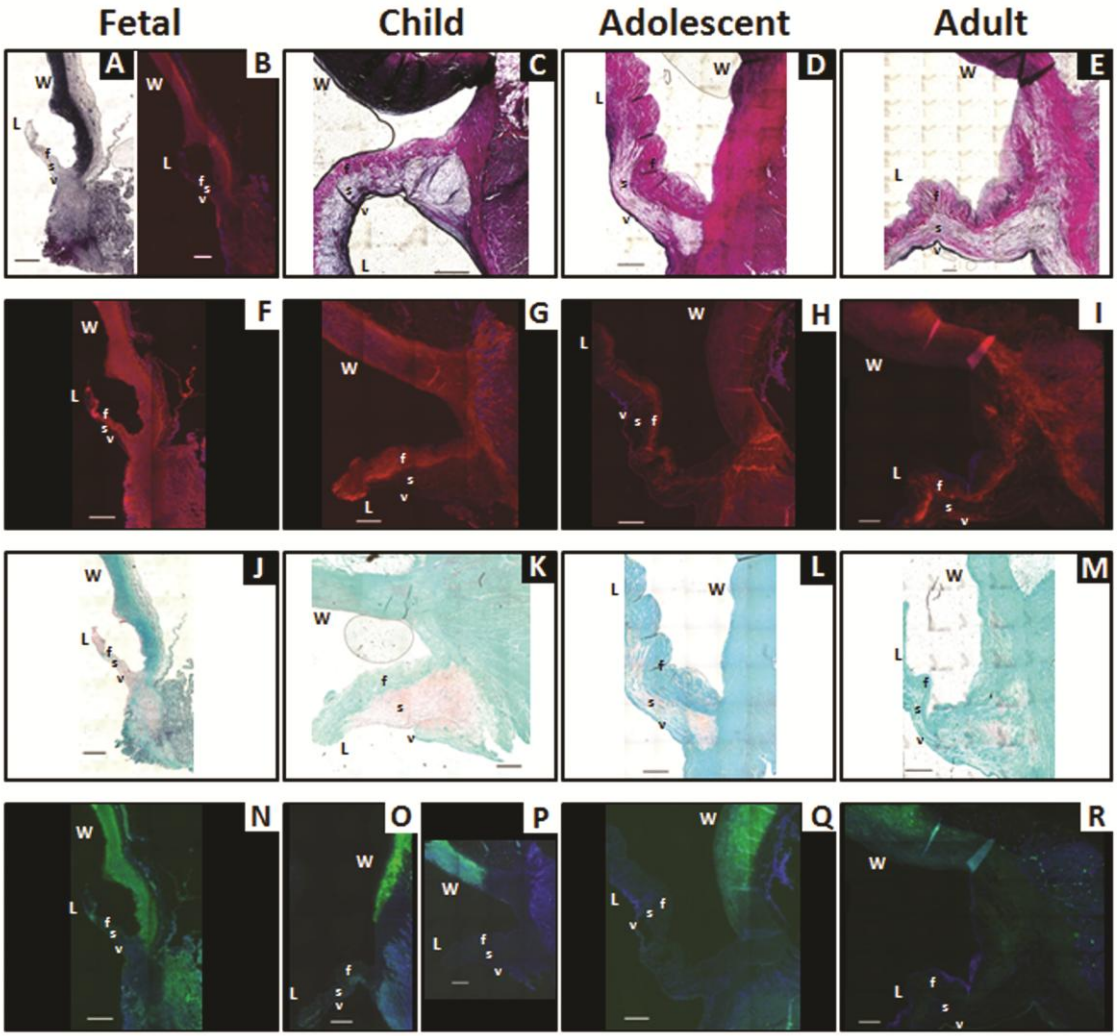

Figure 2.6: *Representative histological and immunofluorescent stainings on aortic valves. Since the differences between the aortic and pulmonary valve were minimal, the aortic valve was chosen to represent the observed findings. (A, C-E) Verhoeff-Van Gieson staining for collagen (red) and elastin (black) visualization. (B) Elastin was observed in the fetal valve with an immunofluorescent staining (red) with in blue cell nuclei. (F-I) Collagen type I immunofluorescent staining (red) with in blue cell nuclei showed that this type of collagen was predominant in the fibrosa. (J-M) Safranin-O staining for GAG visualisation (red/orange), which was mainly present in the spongiosa layer and the hinge region. (N-R) αSMA immunofluorescent staining (green) with in blue cell nuclei. In the leaflets of the fetal and 8 month old donor (N and O, respectively) αSMA-positive cells were observed, while in the older leaflets almost no αSMA-positive cells were observed. The scale bars indicated 500 µm. L: leaflet; W: wall; f: fibrosa; s: spongiosa; v: ventricularis*

hinge region of the valve. The three-layered structure became more pronounced with age. No distinct differences were observed between the pulmonary and aortic valve, however, the observations seem to be more pronounced in the aortic valve.

2.3.3 Cellular content and phenotype change rapidly after birth

According to the stainings (figure 2.6), cell content seemed to decrease with age. Interestingly, in the fetal and pediatric leaflets, cells were observed throughout all layers of the leaflet, while the cells in the adolescent and adult leaflets were mainly located in the spongiosa and ventricularis layers. Also the DNA content, as measured by biochemical assays, was slightly higher in the pediatric leaflets compared to the adolescent and adult leaflets (figure 2.7). Especially, the leaflets from the 8 month and 4 year old donors appeared to have more DNA than the other leaflets (> 11 year). No significant differences between the aortic and pulmonary valve were observed with respect to cell number.

αSMA-positive cells were only observed in the leaflets of the fetal and 8 month old donor. In the older valve leaflets (> 4 years old) almost no αSMA-positive cells were observed. In the fetal and pediatric valves, αSMA-positive cells were also observed in the hinge region and the root/arterial wall. In the adult valves, almost no αSMA-positive cells were found in the hinge region, though they were observed in the root/arterial wall.

2.3.4 Matrix composition is shifting towards more collagen

The biochemical assay data is presented in figure 2.7. The sGAG content decreased with age in both the pulmonary and aortic valve. Though, there are significant differences between both valves. In the pediatric aortic valves, more sGAG was observed than in the pulmonary valve. In the adult valves, the sGAG content was similar in both valves. Therefore, there was a larger decrease with age in the sGAG content of the aortic valve. The hydroxyproline content increased with age in the aortic valve. Also the hydroxyproline-to-sGAG ratio in both valves increased with age. In the pediatric aortic valve more sGAG compared to hydroxyproline was present, while in the adult valves more hydroxyproline compared to sGAG was present. In the pulmonary valve, in all age groups slightly more hydroxyproline compared to sGAG was present, which become more pronounced with age.

The results of the first and second series of collagen cross-link analyses did not correspond with each other (e.g. the values of the first series were all two times lower as compared to the values in the second series). Therefore, the results are described separately. In the first series, the tissue also matured with age in both the aortic and pulmonary valve leaflets, as the number of HP cross-links increased with increasing age

(figure 2.7D). However, this was not observed in the heart valve leaflets of the second series.

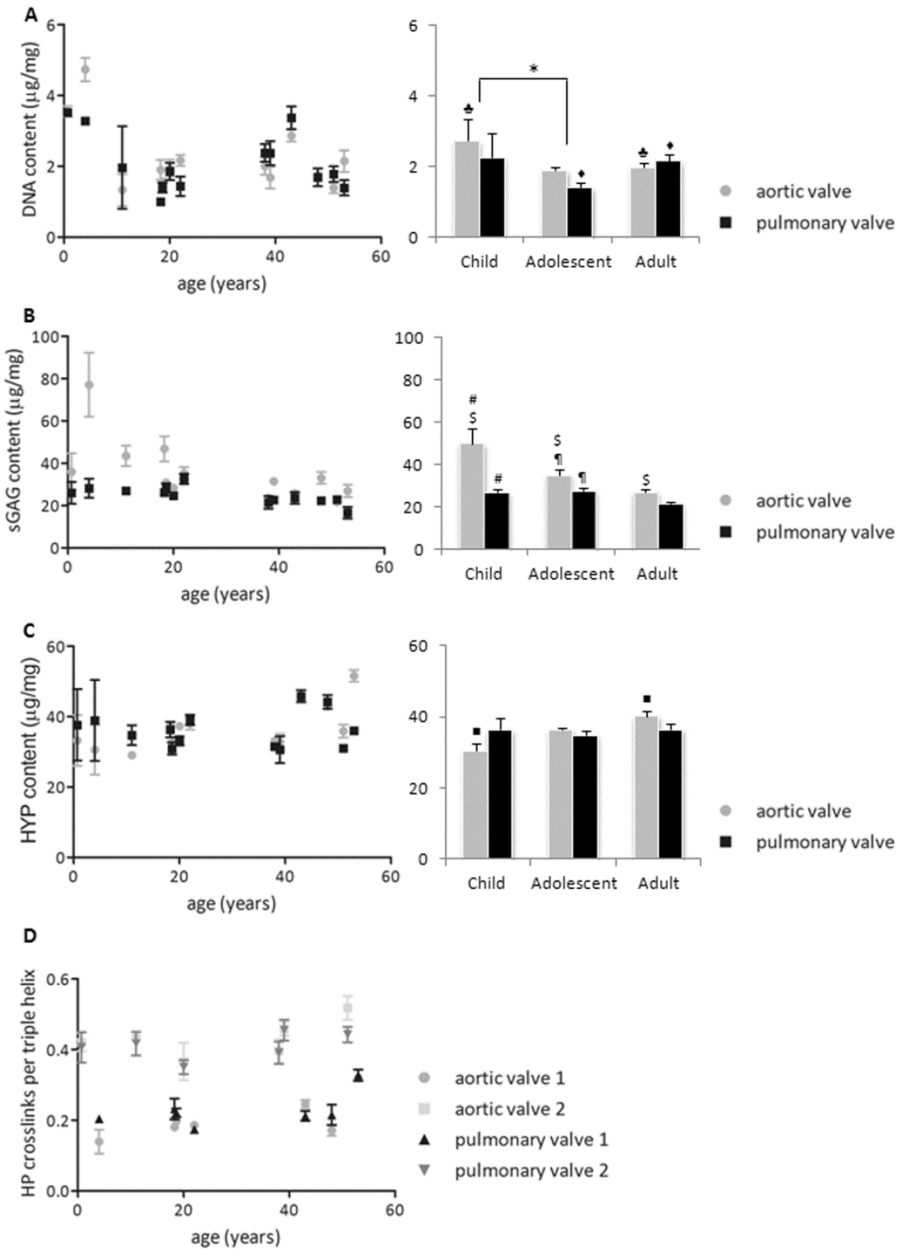

Figure 2.7: *Changes in DNA- (A), sGAG- (B), hydroxyproline content (HYP; C), and collagen cross-links (D) (left side: all data point for correlation analysis with age; right side: grouped data). DNA content is slightly, but significant, higher in children, especially in the first years of life (0 – 4 years), compared to the adolescents. sGAG content decreases with age ($p < 0.05$ in aortic valve and $p < 0.01$ in the pulmonary valve). Hydroxyproline content increases with age in the aortic valve ($p < 0.05$), but does not change with age in the pulmonary valve. The number of HP cross-links increases with age ($p < 0.05$ in both valves). * ($p < 0.05$) indicates significant difference between age groups for both the aortic and pulmonary valve. Significant differences between groups ($p < 0.05$) are indicated by paired symbols.*

2.3.5 Leaflets become stiffer with age

Mechanical properties of the heart valve leaflets were measured with indentation tests (figure 2.8) and biaxial tensile tests (figure 2.9). In the aortic valve leaflets, the E-modulus increased with age in the belly and commissure. In addition, in this valve the E-moduli in both circumferential and radial direction increased with age. In the pulmonary valve leaflets, the E-modulus increased only significantly in the belly. No age-related increase in circumferential and radial direction was observed in this valve. In both valves and at all ages, the E-moduli in the circumferential direction were higher than the E-moduli in radial direction.

The leaflets of the pediatric and adolescent were more extensible than the adult leaflets. The extensibility of the leaflets of the 8 month old donor was similar as the extensibility of the adult leaflets. This was observed in the aortic and pulmonary valve. The pulmonary leaflets of the 11 and 20 year old donors were more extensible than their aortic leaflets.

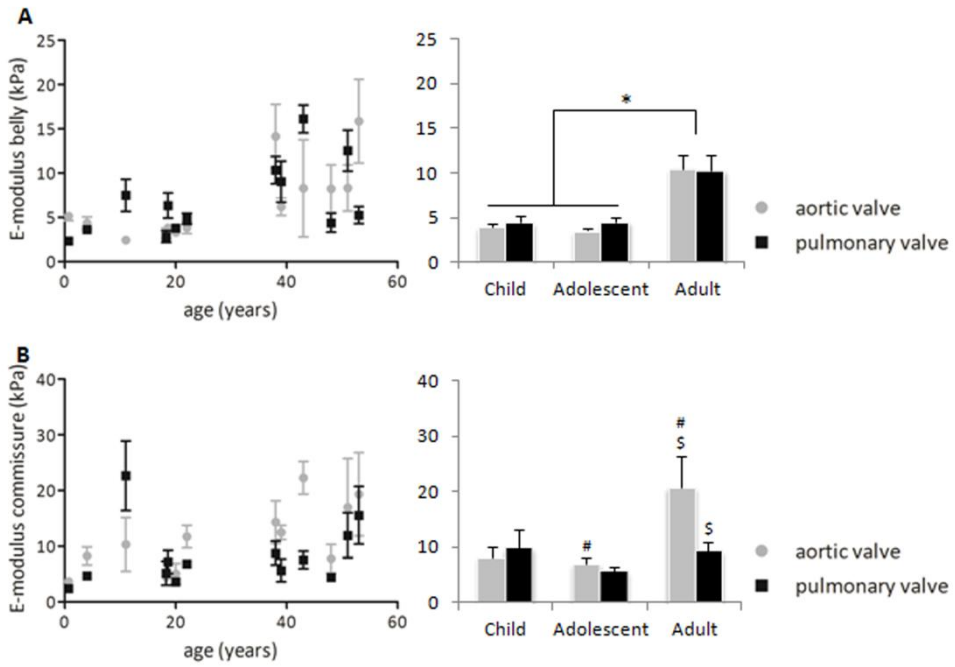

Figure 2.8: *Changes in elastic modulus (E-modulus) in the belly (A) and commissure (B) measured with indentation tests (left side: all data point for correlation analysis with age; right side: grouped data). In the belly, the E-modulus is only significantly increasing with age in the aortic valve ($p < 0.01$). In both valves, the E-modulus increases in particular from adolescent to adult. The E-modulus in the commissure is increasing with age ($p < 0.05$ in the aortic valve and $p < 0.01$ in the pulmonary valve). * ($p < 0.05$) indicates significant difference between age groups for both the aortic and pulmonary valve. Significant differences between groups ($p < 0.05$) are indicated by paired symbols.*

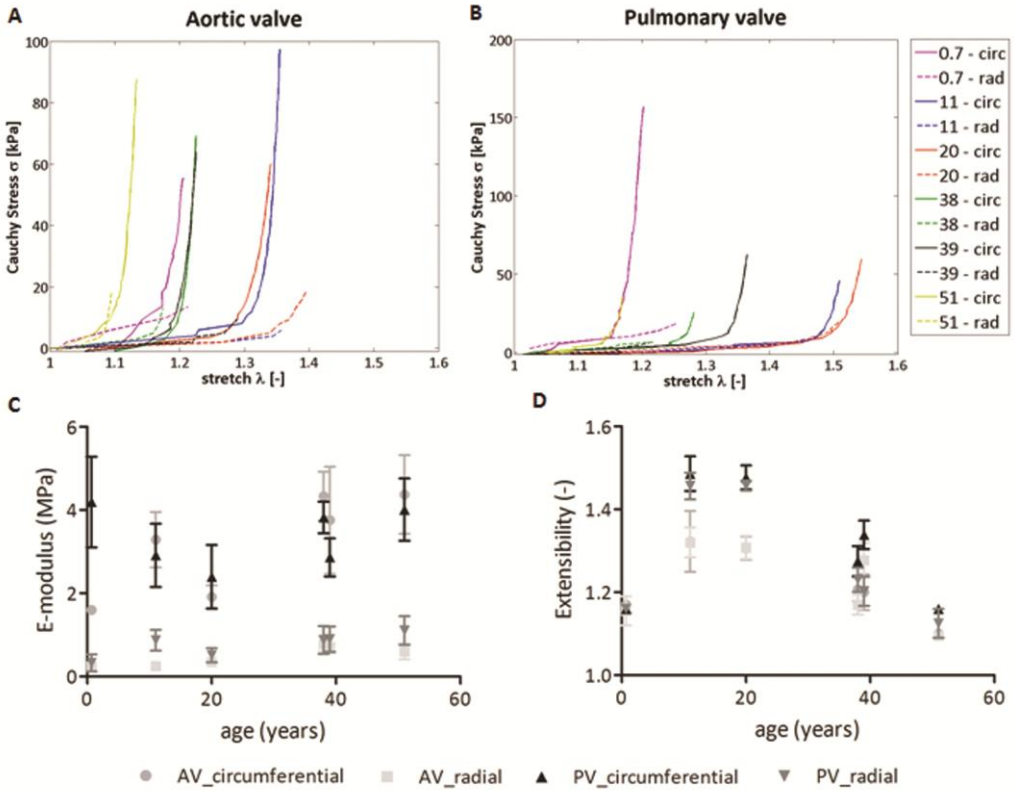

Figure 2.9: *The averaged stress-strain curves (A, B), E-modulus (C), and extensibility (D) of the biaxial tensile tests. The E-moduli in both circumferential and radial direction increase with age in the aortic valve ($p < 0.05$). In the pulmonary valve the E-modulus does not increase with age. The leaflets of the 11 and 20 years old donors are more extensible than the adult leaflets. In addition, in these young donors, the pulmonary leaflets were slightly more extensible than the aortic leaflets.*

2.3.6 Matrix composition and maturation are related to mechanical behavior

In the aortic valve, a positive correlation was observed between the hydroxyproline content and the E-modulus in the belly obtained with the indentation tests ($p < 0.05$). Thus, the E-modulus is increasing with increasing hydroxyproline content. An increase in the hydroxyproline content was also correlated with an increase in the number of cross-links in the first series ($p < 0.01$). Furthermore, in the first series the number of cross-links was positively correlated with the E-moduli in the belly ($p < 0.01$) and the commissure ($p < 0.05$) obtained with the indentation tests. In the second series, the number of cross-links was only positively correlated with the E-modulus in the commissure ($p < 0.05$). No other relevant correlations in the aortic valve were observed.

In the pulmonary valve, a negative correlation was observed between the sGAG content and the E-modulus in the commissure obtained with the indentation tests ($p < 0.05$), meaning that the E-modulus is decreasing with increasing GAG content. A correlation between increasing cross-link number obtained in the first series and

increasing E-modulus in the commissure obtained with indentation tests was observed in this valve as well ($p < 0.05$). In addition, an increase in E-modulus in circumferential direction was correlated to a decrease in extensibility in circumferential direction ($p < 0.01$). No other relevant correlations were observed in the pulmonary valve.

2.4 Discussion

This study describes for the first time the tissue mechanical properties, ECM composition and maturation of pairs of human aortic and pulmonary donor valve leaflets in different age groups to study structure-function properties and valve remodeling with age. Understanding of the native tissue properties can be used to optimize age-specific TE protocols to provide the desired characteristics of TE heart valves.

The annulus and leaflet size increased with age. The annulus increased rapidly during the first years of life, and increased only slowly from adolescence to adulthood. This can be due to growth or to a decrease in compliance causing dilatation of the annulus. Merryman (2010) suggested that at older ages the compliance decreases (Merryman, 2010), which might increase the diameter of the annulus with age. The thickness of the leaflets did not increase with age. However in all age groups, the belly was thicker than the commissure. In the adolescent and adult valve leaflets, this difference in thickness might be explained by the fibrosis observed in the leaflets, which was observed in all adult valve leaflets and in some of the adolescent heart valve leaflets. Fibrosis is associated with a disorganized collagen network and an increased collagen synthesis (Chen and Simmons, 2011). As we observed an increase in the hydroxyproline content of the heart valves with age, the hydroxyproline content and fibrosis might be related in these valve leaflets. Mazzone *et al.* (2004) observed dense fibrosis in all leaflets of aortic valves with calcified aortic stenosis (Mazzone *et al*., 2004), which suggests that fibrosis is involved in calcification. Fibrosis in the heart valves develops with age and is present in all adult heart valves, suggesting that valve degeneration starts at a relatively early age and might develop eventually in almost all heart valves, as valve degeneration is observed in more than 75% of the heart valves of 85 years and older (Lindroos *et al*., 1993).

Similar to other studies, our mechanical characterization indicated anisotropic mechanical behavior, e.g. the leaflets were stiffer in circumferential direction than in radial direction (Sauren *et al*., 1983; Christie and Barratt-Boyes, 1995; Leeson-Dietrich *et al*., 1995; Stradins *et al*., 2004; Balguid *et al*., 2007; Stephens *et al*., 2010). The anisotropy in the leaflets is caused by the highly aligned circumferential collagen fiber orientation at the free edge of the leaflets and a more hammock-like structure in the

belly. Next to this, the adult valve leaflets were less extensible than the pediatric and adolescent leaflets, which has also been described by others for porcine (Stephens *et al*., 2010) and human (Christie and Barratt-Boyes, 1995) aortic leaflets. In our study, also the pulmonary valve leaflets were studied. Stradins *et al.* (2004) compared the human aortic and pulmonary leaflets and showed similar mechanical properties in both valves (Stradins *et al*., 2004). In addition, Leeson-Dietrich (1995) described similar mechanical properties of the porcine aortic and pulmonary valves, with the exception of modulus that was significantly higher in the aortic valve (Leeson-Dietrich *et al*., 1995). Also in our study, the aortic and pulmonary leaflets had similar mechanical properties according to both mechanical tests. This suggests that the mechanical functionality of both valves is similar, although, the transvalvular pressures on the aortic valve are higher compared to the pressures on the pulmonary valve. This implies that the pulmonary valve is able to sustain the systemic pressures and can, thus, be used for replacing the diseased aortic valve (Ross procedure).

The number of cells increased from fetal to adolescent age, which was probably associated with an increased cell proliferation-to-apoptosis ratio in the young valves (Aikawa *et al*., 2006). Thereafter, the number of cells remained constant. Similar to the findings of Aikawa *et al.* (Aikawa *et al*., 2006), the cells in the leaflets of the fetal and 8 month old donor were αSMA-positive, suggesting an activated myofibroblast phenotype (Rabkin *et al*., 2002; Rabkin-Aikawa *et al*., 2004). Nevertheless, in the older aortic leaflets no αSMA-positive cells were observed, which suggests that the gradual increase in transvalvular pressure over the aortic valve has no effect on cell phenotype. Even so, in the fetal and pediatric valves, αSMA-positive cells also populate the hinge region, while in the adolescent and adults valves those cells are only observed in the arterial wall. This implies that the cells in the hinge region might dedifferentiate, undergo apoptosis, or migrate towards the arterial wall. Furthermore, no differences in αSMA-positive cells between the aortic and pulmonary valve were observed, suggesting that all studied valves were well adapted to either systemic or pulmonary pressures.

All studied leaflets, including the fetal leaflets, demonstrated a three-layered structure, with mainly collagen type I in the fibrosa, sGAG in the spongiosa, and elastin in the ventricularis. Aikawa *et al.* (2006) described that the three-layered structure with elastin in the ventricularis become apparent at 36 weeks of gestation (Aikawa *et al*., 2006), whereas our data demonstrated the presence of elastin already by week 21. This discrepancy might be due to the staining to visualize elastin. We studied the presence of elastin with two different stainings. In the fetal leaflets, elastin was hardly observed with the Verhoeff-Van Gieson staining, but was clearly present with the immunofluorescent straining, indicating a three-layered structure in fetal valves younger than 36 weeks of gestation.

Our data further showed a decrease in sGAG content with age, especially in the aortic valve leaflets. This is in contradiction with McDonald *et al.* (2002), who described no

significant change with age in the GAG content (McDonald *et al*., 2002). Though, they studied the GAG content in valves from donors aged 20 years or older with histological stainings. In our study, the highest sGAG content was observed in the pediatric group and only a slight decrease between adolescence and adulthood was found. In the aortic valve, the hydroxyproline content increased with age. This corresponds to the findings on porcine heart valves observed by Stephens and coworkers (Stephens and Grande-Allen, 2007; Stephens *et al*., 2010) and on human valves by Keller and Leutert (Keller and Leutert, 1994). In addition, the collagen-to-sGAG ratio in the leaflets shifted with age to more collagen compared to sGAG. Merryman (2010) hypothesized that the ECM stiffness increases with age (Merryman, 2010). This might be explained by this shift towards more collagen compared to sGAG in the heart valve leaflets, as in our study the E-modulus is increasing with increasing hydroxyproline content and decreasing sGAG content. The increase in stiffness with age might also be explained by the increase in collagen cross-links, as we also observed a positive correlation between the number of collagen cross-links and the E-modulus, which corresponds with previous work from our group (Balguid *et al*., 2007). Nevertheless, in this study, the results of the two series of cross-link analyses did not correspond with each other. Therefore, future studies are necessary to determine the effects of the collagen cross-links on the evolution of the native heart valves. In addition, as the results are based on three to six valves per age group, future studies with more valve may be needed to confirm the results in this study.

In our group, Korstmit *et al.* (2009) previously investigated the DNA, sGAG and hydroxyproline content of human TE heart valves created from myofibroblasts seeded into rapidly degrading PGA/P4HB scaffolds and cultured for 4 weeks in a bioreactor system (Kortsmit *et al*., 2009a; Kortsmit *et al*., 2009b). Despite an underestimation of one third in these values due to the scaffold contribution in the dry weight, the DNA content in their TE heart valves is similar to the DNA content of the native heart valves studied here. In addition, they found a sGAG content that resembles the sGAG content of the pulmonary valve and the sGAG content of the adult aortic valve. The TE hydroxyproline content is, however, much lower as compared to the native valves. In the aortic valve, the hydroxyproline content increased significantly with age, and even the native pediatric valve leaflets contain a higher hydroxyproline content. Also in the native pulmonary valve leaflets, the hydroxyproline content was higher than in the TE heart valves. In addition, the collagen-to-sGAG ratio is different in the TE heart valves. In these valves, there is more sGAG compared to collagen, while in the adult native heart valves, there is more collagen compared to sGAG. Since the ECM composition of the heart valve is related to maturation and mechanical properties, it might be suggested that TE protocols should be optimized to improve the hydroxyproline content and therewith the (mechanical) functionality of these heart valves. Nevertheless, the E-modulus of the TE valves cultured by Kortsmit and coworkers (Kortsmit *et al*., 2009a; Kortsmit *et al*., 2009b) were similar to the adult native valves in the radial direction. In

addition, the E-moduli in circumferential direction were only comparable to the pediatric and adolescent aortic heart valves, but not to the adult native valves. On the other hand, the TE heart valves cultured by Mol *et al.* (Mol *et al*., 2006) showed mechanical properties which were even stiffer compared to native valves. It is, thus, difficult to control the mechanical properties of the TE heart valves. In addition, stiff TE heart valves can be cultured, while the collagen content is not comparable to the native valves. This suggests that not only the matrix composition and maturation are important for defining the mechanical functionality. Another property important for the mechanical functionality is the collagen architecture (Sacks and Schoen, 2002; Lindeman *et al*., 2010). Thus, to increase the long-term *in vivo* functionality of the TE heart valves, not only the matrix composition and maturation should be optimized, also the collagen architecture to define the anisotropic properties of the leaflets should be studied in native valve leaflets to improve this in the TE heart valves.

In summary, for the first time, tissue mechanical properties, matrix composition and maturation of pairs of human aortic and pulmonary valve leaflets of different age groups (fetal, child, adolescent, adult) were studied. Both the aortic and pulmonary valve leaflets develop throughout life. Especially in the aortic valve leaflets, the extracellular matrix composition changes with age with increasing hydroxyproline content and decreasing GAG content, probably to support the changing hemodynamic conditions. Nevertheless, the differences between the aortic and pulmonary valve are minimal. The changes in the matrix composition and maturation influence the mechanical properties, as the elastic modulus increases with increasing hydroxyproline content, increasing cross-link number, and decreasing GAG content. The results in this study provide age-specific benchmarks for evaluating and optimizing future therapies such as tissue engineering of heart valves.

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Chapter 3

Variation in Tissue Outcome of Ovine and Human Engineered Heart Valve Constructs: Relevance for Tissue Engineering

The contents of this chapter are based on D. van Geemen, A. Driessen-Mol, L.G.M. Grootzwagers, R.S. Soekhradj-Soechit, P.W. Riem Vis, F.P.T. Baaijens, and C.V.C. Bouten (2012) "*Variation in Tissue Outcome of Ovine and Human Engineered Heart Valve Constructs: Relevance for Tissue Engineering*" Regenerative Medicine, 7(1):59-70

3.1 Introduction

Valvular heart disease is a major health problem causing significant morbidity and mortality, worldwide (Lloyd-Jones *et al*., 2009). The prevalence of valvular diseases increases with age, ranging from 0.7% in the 18-44 year old group to 13.3% in the 75 years and older group (Nkomo *et al*., 2006; Mol *et al*., 2009). Therefore, with an expected shift to an older and larger population of the world, the social and economic burden of valvular heart diseases will continue to increase.

The main causes of valvular dysfunction are calcification of the leaflets, rheumatic fever, endocarditis, myxomatous degeneration, or congenital heart pathology, leading to stenosis or insufficiency of the valves. To avoid serious cardiac, pulmonary, or systemic problems, surgical valve repair or, more often, heart valve replacement is frequently applied. As a result, approximately 285,000 heart valve replacement surgeries are performed annually worldwide (Mikos *et al*., 2006).

Today's heart valve replacements (bioprosthetic and mechanical valves) enhance survival and quality of life of most patients, but have several limitations (Mendelson and Schoen, 2006). For example, bioprosthetic valves are prone to calcification and structural deterioration, while the application of mechanical valves requires lifelong anticoagulation therapy to control thromboembolism. The most important limitation is that these valve types do not consist of living tissue and, consequently, do not adapt or remodel to changing circumstances. Heart valve tissue engineering (TE) seeks to overcome the shortcomings of current valve replacements by creating living autologous heart valves that have the ability to grow, repair, and remodel. TE heart valves are ideal replacements for children and young adults, as these valves can grow and adapt to changing physiological environments after implantation. However, also for older adults a TE heart valve is preferred, as life expectancy and quality-of-life is improved when a living valve is implanted (El-Hamamsy *et al*., 2010).

Various TE approaches are being employed, either to develop the valve substitute *in vitro* or to use the regenerative potential of the body (*in situ*) for the tissue culture phase. Here, we concentrate on the classical *in vitro* TE approach. For autologous heart valve TE cells are isolated and expanded in culture prior to seeding them on a biodegradable carrier scaffold. This carrier can either be a hydrogel, decellularized scaffold, or a synthetic scaffold. Various cell sources, ranging from prenatally derived stem cells to adult (stem) cells, can be used to seed into the scaffolds. All these scaffolds have their own (dis)advantages, have different remodeling capacities, and result in different tissue quality. The different scaffolds for heart valve TE have been reviewed and discussed extensively in literature (see e.g. (Vesely, 2005; Mendelson and Schoen, 2006; Schmidt *et al*., 2007b; Mol *et al*., 2009; Apte *et al*., 2011; Bouten *et al*., 2011; Weber *et al*., 2011a)). The scaffold of our choice is a rapidly degrading synthetic scaffold

that allows for fully autologous tissue formation of high quality (Mol *et al*., 2006) and with good remodeling capacities in vivo (Hoerstrup *et al*., 2000a).

After cell seeding, the cell-scaffold construct is subjected to mechanical stimuli in a bioreactor to enhance extracellular matrix formation until a functional heart valve is grown that can be used for implantation (Hoerstrup *et al*., 2000a; Mol *et al*., 2006). For future clinical practice, it is foreseen that autologous cells from the diseased recipient itself will be used to culture TE heart valves. In addition, it is important to control the tissue culture process to predict tissue outcome and functionality prior to implantation.

For preclinical testing of TE heart valves, an ovine model is currently the gold standard. The sheep is the animal of choice for assessment of cardiac valves due to the resemblance of its heart valves to those of humans in terms of mechanical properties and heamodynamic flow parameters (Rashid *et al*., 2004). Furthermore, sheep develop more rapidly than humans. Therefore, bioprostheses implanted for a few months in juvenile sheep show changes comparable to those that take several years to develop in patients (Barnhart *et al*., 1982). In addition, the sheep is also a good animal model for calcification, as it presents a "worst-case-scenario" due to their enhanced calcium metabolism (Ali *et al*., 1996; Hoerstrup *et al*., 2006). However, it is still unclear whether ovine TE heart valves are indicative of the TE product obtained from human cells.

To our knowledge, most TE heart valves studies have focused on the results obtained using cells of one donor, and when multiple donors are used, the variation between the valves created from these subjects is not studied. In this study, heart valve tissue constructs were engineered with cells of multiple donors to (1) evaluate the level of variation in tissue outcome within species (intraspecies variation), (2) study the differences between species (interspecies differences), and (3) determine possible indicators of tissue outcome. To this end, we investigated the expression of certain protein of myofibroblasts from different sheep and patients and have prepared heart valve tissue constructs of the same cells. The mechanical properties and extracellular matrix (ECM) formation was analyzed after 4 weeks of culture as indicators of tissue outcome. Tissue outcome and cellular protein expression were correlated to identify early markers to predict tissue outcome.

3.2 Material & Methods

3.2.1 Cell culture

Human myofibroblasts were acquired from segments of saphenous vein obtained from 7 patients (5 male, 2 female, mean age 57.6 ± 11.7 years) undergoing coronary artery bypass surgery using a venous graft. Individual permission using standard informed consent procedures and prior approval of the ethics committee of the

University Medical Center Utrecht was obtained. Due to ethical reasons, cells from younger healthy humans could not be isolated. Ovine myofibroblasts were acquired from segments of jugular vein obtained from 8 adult female sheep (Swifter, approximately 2 years, 73.3 ± 3.0 kg) by approval of the animal ethics committee of the University Medical Center Utrecht according to local and national regulations. Cells were isolated and expanded using standard culture methods as previously described (Schnell *et al*., 2001; Mol *et al*., 2006). The expansion medium (2D medium) for myofibroblasts consisted of DMEM Advanced (Invitrogen, Breda, Netherlands]) supplemented with 1% Penicillin/Streptomycin (P/S; Lonza) and 1% GlutaMax (Gibco). The medium was further supplemented with 10% fetal bovine serum (FBS; Greiner Bio-one, Alphen a/d Rijn, Netherlands) for human myofibroblasts or 10% lamb serum (Gibco) for ovine myofibroblasts.

3.2.2 Engineered heart valve constructs

3.2.2.1 Scaffold preparation and sterilization

Rectangular scaffolds (25x5x1 mm), composed of rapidly degrading non-woven polyglycolic acid (PGA; thickness, 1.0 mm; specific gravity, 70 mg/cm3; Cellon, Bereldange, Luxembourg), were coated with poly-4-hydroxybutyrate (P4HB; obtained within a collaboration with prof. S. Hoerstrup, University Hospital Zurich) to provide structural integrity to the mesh (Hoerstrup *et al*., 2000a). After drying, the two outer 3-4 mm parts of the long axis of each scaffold strip were glued to stainless steel rings using a 20% solution of polyurethane (PU; DSM, Geleen, Netherlands) in tetrahydrofuran, leaving an 18×5 mm area for seeding. The solvent was allowed to evaporate overnight. The rings with the scaffold strips were placed in 6-well plates and in the vacuum stove overnight for further drying. The next day, the scaffolds were sterilized in 70% ethanol for 30 minutes and subsequently washed twice in phosphate buffered saline (PBS; Sigma). Hereafter, the scaffolds were placed in tissue engineering medium (TE medium) until cell seeding. For human constructs, TE medium consists of 2D medium, supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma), while for ovine scaffolds, TE medium, consists of DMEM Advanced (Invitrogen) supplemented with 1% Penicillin/Streptomycin (P/S; Lonza), 1% GlutaMax (Gibco), 2.5% lamb serum (Gibco), and L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma).

3.2.2.2 Cell seeding and Tissue culture

Passage 6-7 myofibroblasts were seeded in the constructs (n = 6 per sheep or patient) using fibrin as a cell carrier (Mol *et al*., 2005). In short, myofibroblasts were suspended in TE medium containing thrombin (10 IU/ml, Sigma). Subsequently, this cell suspension was mixed with an equal volume of TE medium containing fibrinogen (10 mg/ml, Sigma). This fibrin/cell suspension ($15*10^6$ cells/ml) was mixed until the onset of polymerization

of the gel, after which 90 µl was dripped onto each construct to seed $1.35*10^6$ cells per construct. To allow further firming of the fibrin gel, the seeded constructs were placed in an incubator at 37 °C and 5% CO_2 for 30 minutes. Hereafter, 6 ml TE medium was added to each construct and placed back into the incubator. The heart valve constructs were cultured for 4 weeks and TE medium was changed every 2-3 days.

3.2.3 Cell characterization

As markers of an early stage of the tissue engineering process we characterized the myofibroblasts. To characterize the ovine myofibroblasts of all sheep, immunofluorescence was performed on passage 6 ovine myofibroblasts, which were seeded on coverslips (n=3 per sheep) and cultured for 2-3 days. Cells were subsequently fixed in 3.7% paraformaldehyde and permeabilized in 0.1% Triton X-100 (Merck, Amsterdam, Netherlands). Afterwards, sections were incubated in 2% BSA (Roche) in PBS to block non-specific binding and, subsequently, incubated with the primary antibodies. The antibodies used were as follows: monoclonal IgM mouse anti-human antibody against vimentin (Abcam, Cambridge, UK) to indicate mesenchymal origin, monoclonal IgG2a mouse anti-human antibody against alpha smooth muscle actin (α-SMA, Sigma) and monoclonal IgG2b mouse anti-human antibody against non muscle myosin heavy chain (SMemb; Abcam) to indicate contractile properties of the cells, and monoclonal IgG2b mouse anti-human antibody against heat shock protein 47 (hsp47; Stressgen, Michigan, USA) to indicate the matrix forming capacities. The specific stainings were visualized with a goat anti-mouse IgG2a Alexa 488 (Invitrogen) for α-SMA, goat anti-mouse IgG2b Alexa 488 (Invitrogen) for SMemb and hsp47, and goat anti-mouse IgM Alexa 555 (Invitrogen) for vimentin. After an additional staining with 4',6-diamidino-2-phenylindole (DAPI) to stain cell nuclei, sections were mounted with mowiol (Calbiochem, San Diego, USA). Stained sections were analyzed and pictures (n=4 per coverslip) were taken randomly by fluorescent microscopy (Axiovert 200; Carl Zeiss, Sliedrecht, Netherlands). Hereafter, the ratio between the total number of myofibroblasts (as indicated by the DAPI staining) and the number of myofibroblasts positive for an individual marker was calculated with ImageJ software (Rasband,2011). To quantify the stainings, the percentage of myofibroblasts positive for a marker was calculated by dividing the number of positive cells by the total amount of cells.

The phenotype of the human cells of the same patients was characterized in a similar manner and described in a previous study (Riem Vis *et al*., 2010). Quantification of the sections was performed according above mentioned protocol.

3.2.4 Tissue properties

3.2.4.1 Mechanical testing

Mechanical properties of all ovine and human engineered heart valve constructs were determined after 4 weeks of culture by uniaxial tensile tests in longitudinal direction of the constructs (n = 4 per patient or sheep) using a tensile stage equipped with a 20N load cell (Kammrath-Weiss, Dortmund, Germany). Measurements were averaged per patient or sheep. The Digimatic Micrometer (Mitutoyo America Corporation, Aurora, USA) was used to measure the thickness of the constructs, while the width of the constructs was measured using a caliper. Stress–strain curves were obtained at a strain rate equal to the initial sample length (20 mm) per minute. The Cauchy stress was defined as the force divided by the cross-sectional area of the construct. The ultimate tensile strength (UTS) was represented by the maximum stress value of the curves while the slope of the linear part of the curve, hence at large strains, represented the elasticity modulus (E-modulus) of the tissue, as a measure for tissue stiffness.

3.2.4.2 Qualitative analysis of tissue composition

Tissue formation was analyzed qualitatively by histology. Engineered heart valve constructs (n = 2 per patient, and n = 1 per sheep) were fixed in 3.7% formaldehyde in PBS and subsequently embedded in paraffin. Samples were sectioned at 10 µm and studied by hematoxylin and eosin (H&E) staining for general tissue development and Masson Trichrome (MTC kit, Sigma) for collagen visualization. The stainings were analyzed and pictures were taken using a Zeiss light microscope (Carl Zeiss). To distinguish between juvenile and mature collagen fibers, picrosirius red staining (Puchtler *et al*., 1973; Junqueira *et al*., 1979) was performed. Pictures were taken by bright field and polarized light microscopy (Carl Zeiss).

Cell proliferation within the engineered heart valve constructs was analyzed with immunofluorescence. After pretreatment of boiling the paraffin slides in 10 mM citrate buffer, pH 6.0, the slides were incubated with a polyclonal IgG rabbit anti-human antibody against Ki-67 (Thermo Scientific; Immunologic, Duiven, The Netherlands). The specific stainings were visualized with a goat anti-rabbit IgG Alexa 488 (Invitrogen). After an additional staining with DAPI to stain cell nuclei, sections were mounted with mowiol (Calbiochem). Stained sections were visualized by fluorescent microscopy (Axiovert 200; Carl Zeiss).

3.2.4.3 Quantitative analysis of tissue composition

The total content of DNA per engineered heart valve construct, as an indication of cell number, and sulfated glycosoaminoglycans (GAGs) and hydroxyproline (HYP) was

determined from constructs previously used for the tensile tests (n = 4 per patient or sheep) to study matrix composition. Measurements were averaged per patient or sheep. Lyophilized samples were digested in papain solution (100 mM phosphate buffer (pH=6.5), 5 mM L-cystein, 5 mM EDTA and 125-140 µg papain per ml) 60°C for 16 hours. The Hoechst dye method (Cesarone *et al*., 1979), with a reference curve prepared of calf thymus DNA (Sigma), was used to determine the amount of DNA. The content of sulfated GAGs was determined on the basis of the protocol described by Farndale *et al*. (Farndale *et al*., 1986), and shark cartilage chondroitin sulfate was used as a reference (Sigma). In short, 40 µl of diluted sample, without addition of chondroitin AC lyase, chondroitin ABC lyase and keratanase, was pipetted into a 96-well plate in duplicate. Subsequently, 150 µl dimethylmethylene blue was added and absorbance was measured at 540 nm. To determine the HYP quantity, an assay, according to Huszar *et al*. (Huszar *et al*., 1980), and a reference of trans-4-hydroxyproline (Sigma) were used.

3.2.5 Statistics

Data on cell phenotype, quantitative analysis of tissue composition, and mechanical properties are provided as group mean ± standard deviation. To indicate intraspecies variability the range in measurements is provided, and this variability was analyzed using one-way ANOVA. Interspecies differences were analyzed using independent t-tests. In addition, linear regression analysis was used to investigate correlations between the independent parameters αSMA and HYP content, and HYP content and E-modulus. A level of $p < 0.05$ was used to indicate significance. We used SPSS 17 software for statistical analysis.

3.3 Results

Tissue constructs were successfully engineered for all 7 patients and for all 8 sheep. As mentioned, cell phenotype was studied prior to seeding of the scaffolds to characterize myofibroblast phenotype and to indicate relevant differences at an early stage of the tissue culture process. Four weeks later, mechanical properties and extracellular matrix composition were quantified as indicators of tissue outcome.

3.3.1 Cell characterization

Ovine myofibroblasts were characterized by immunofluorescence. Myofibroblasts have a phenotype with contractile and matrix forming capacities. This phenotype was further studied to find possible markers to predict the tissue outcome. Ovine myofibroblasts stained positive for vimentin, SMemb and hsp47. Almost 100% of the

myofibroblasts of all sheep stained positive for these markers (figure 3.1). No intersheep variability was observed for these markers. There was, however, a significant variation in αSMA positive cells between the sheep ($p < 0.01$). The percentage of αSMA positive cells ranged from 8.5% to 51%, with an average for the total group of ovine heart valve constructs of 22% ± 14%. Human myofibroblasts also stained almost 100% positive for vimentin, SMemb, and hsp47. Significant interpatient variations were found in αSMA ($p < 0.01$). The average percentage of αSMA positive cells for all patients was 65% ± 34%.

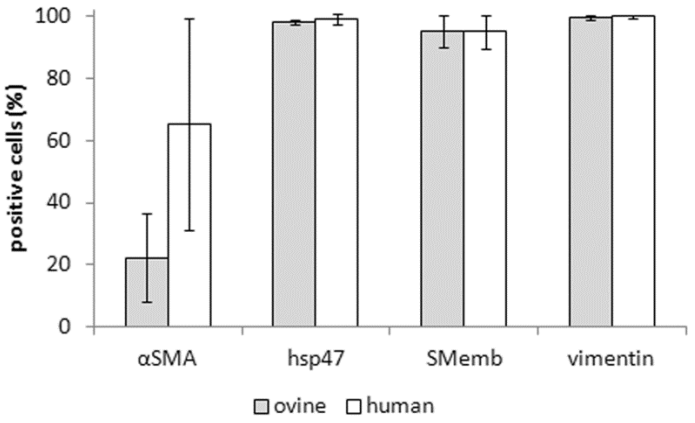

Figure 3.1: *Cell phenotype. Cellular phenotypes of ovine and human myofibroblasts were studied with immunofluorescent staining of αSMA, hsp47, SMemb and vimentin. In both ovine and human cells, only intraspecies differences in αSMA were observed. αSMA: α-smooth muscle actin; hsp47: heat shock protein 47; SMemb: nonmuscle myosin heavy chain.*

3.3.2 Tissue properties

3.3.2.1 Mechanical properties

The averaged stress-strain curves, tissue stiffness (E-modulus), and tissue strength (UTS) of heart valve constructs engineered from cells of both species are shown in figure 3.2 and table 3.1. The E-modulus ranged from 1.10 ± 0.17 MPa to 7.46 ± 0.82 MPa in the human tissue constructs (n=4 per patient). The E-modulus of ovine tissue constructs ranged from 3.76 ± 1.13 MPa to 6.61 ± 1.49 MPa (n=4 per sheep). The Ultimate Tensile Strength ranged from 0.25 ± 0.07 MPa to 1.37 ± 0.19 MPa in the human constructs and from 0.62 ± 0.24 MPa to 1.05 ± 0.18 MPa in the ovine constructs (n=4 per subject). No significant interspecies differences were observed. However, there were significant interpatient differences ($p < 0.01$), while there was no intersheep difference.

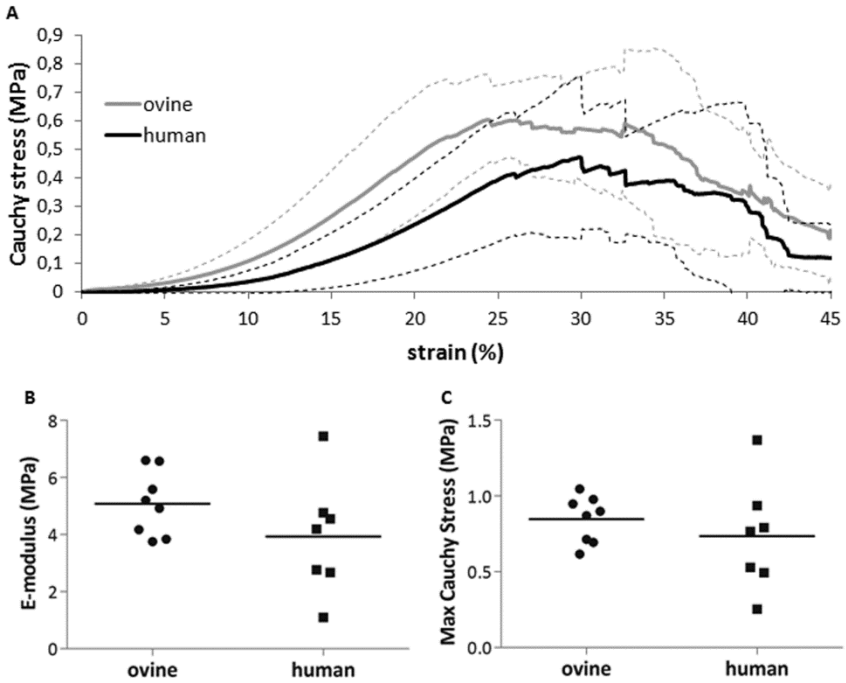

Figure 3.2: *Mechanical properties including (A) averaged stress-strain curves, (B) elasticity modulus and (C) ultimate tensile strength of the human and ovine tissue-engineered constructs. Solid lines represent averaged stress-strain curves, while the dotted lines indicate the standard deviation. No significant interspecies differences were observed; however, there were significant interpatient differences. E-modulus: Elasticity modulus; Max: Maximum.*

3.3.2.2 Qualitative tissue composition

Pictures of general tissue development (H&E), collagen deposition (MTC) and collagen fiber thickness (picrosirius red) are shown in figure 3.3. Only a slight variation in the general tissue development is observed when analyzing the H&E staining. Collagen is seen throughout the tissue as indicated by both MTC and picrosirius red staining. This is observed for all human and ovine tissue constructs. Noteworthy, the human tissue constructs show a more homogeneous tissue, while in the ovine tissue constructs a surface tissue layer is present. Interspecies differences were also observed when picrosirius red staining was analyzed with polarized light microscopy. The collagen fibers in the ovine tissue constructs were yellow-orange, while the fibers in the human constructs were orange-red. This might suggest that in the ovine constructs the collagen fibers were less mature then in the human tissue constructs.

Cell proliferation within the engineered heart valve constructs was analyzed with a Ki-67 immunofluorescent staining. This staining indicates that almost all cells are proliferating within the constructs (data not shown).

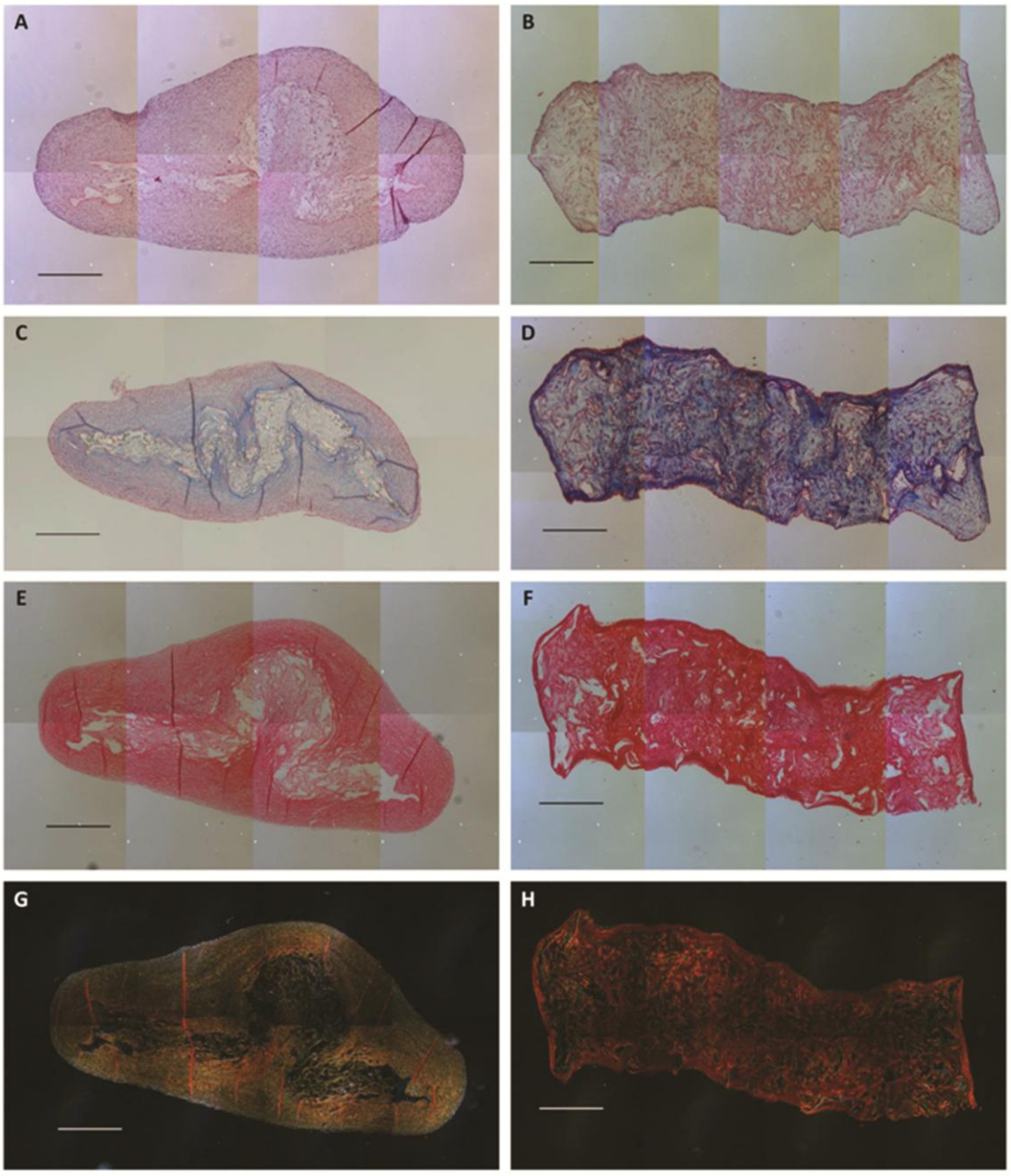

Figure 3.3: *Histology of tissue-engineered constructs cultured with ovine (left) or human (right) myofibroblasts. Representative pictures of tissue composition are displayed for (A, B) hematoxylin and eosin staining, (C, D) Masson Trichrome staining, and picrosirius red staining with (E, F) bright field and (G, H) polarized light. Scale bars indicate 500 µm. Ovine tissue constructs show a superficial tissue layer formation, while tissue is more homogeneously formed in human tissue constructs. This surface layer may have been formed due to higher proliferation rate of the ovine cells that also caused folding of the underlying tissues due to instrinsic compaction forces in this layer. These phenomena are most probably responsible for the discrepancies in construct shape. In addition, polarized light images of the picrosirius red staining suggest that the collagen fibers in the ovine constructs are less mature then the human tissue constructs.*

3.3.2.3 Quantitative tissue composition

The tissue composition of the engineered heart valve constructs was analyzed by DNA, GAG, and hydroxyproline content (figure 3.4 and table 3.1). The DNA- and GAG content were comparable between the subjects, but were significantly higher in ovine constructs compared to human constructs ($p < 0.01$). The DNA content, averaged for all patients, was 15.78 ± 2.63 µg/construct. The averaged DNA content of the ovine constructs was 42.35 ± 5.94 µg/construct. The mean GAG content in the human constructs was 126.6 ± 16.9 µg/construct, and in the ovine constructs the average GAG content was 199.3 ± 40.0 µg/construct. On the other hand, the variation in the hydroxyproline content between the patients was large; the HYP concentration ranged from 43.49 ± 1.73 µg/construct to 130.93 ±11.52 µg/construct. On the contrary, the variation between the constructs engineered from the myofibroblasts of the different sheep was small; the HYP concentration ranged from 26.78 ± 7.03 µg/construct to 61.61 ± 13.98 µg/construct.

The GAG and HYP content were also normalized for the DNA content. This shows that the amount of GAG and HYP per DNA in the human engineered heart valve constructs was significantly higher ($p < 0.01$) compared to ovine engineered heart valve constructs (figure 3.4D).

Linear regression analysis of our results showed that the collagen content (e.g. HYP content) in the engineered constructs increased with the number of αSMA positive cells before seeding (figure 3.5A). In both species this trend is observed, however, this correlation was only significant for the ovine cells and constructs.

Properties	Ovine	Human
E-modulus [MPa]	5.08 ± 1.13	3.94 ± 2.02
UTS [MPa]	0.85 ± 0.15	0.74 ± 0.36
DNA [µg per construct]	42.35 ± 5.94	15.78 ± 2.63*
sGAG [µg per construct]	199.25 ± 40.00	126.63 ± 16.90*
HYP [µg per construct]	45.65 ± 10.99	89.84 ± 34.83*
GAG/DNA [-]	4.82 ± 0.80	8.22 ± 1.40*
HYP/DNA [-]	1.12 ± 0.34	5.87 ± 2.51*

Table 3.1: *Tissue composition and mechanical properties of ovine and human tissue-engineered constructs to indicate differences between species. *Significant difference compared with ovine engineered constructs ($p < 0.01$). E-modulus: Elasticity modulus; GAG: Glycosaminoglycan; HYP: Hydroxyproline; UTS: Ultimate tensile strength.*

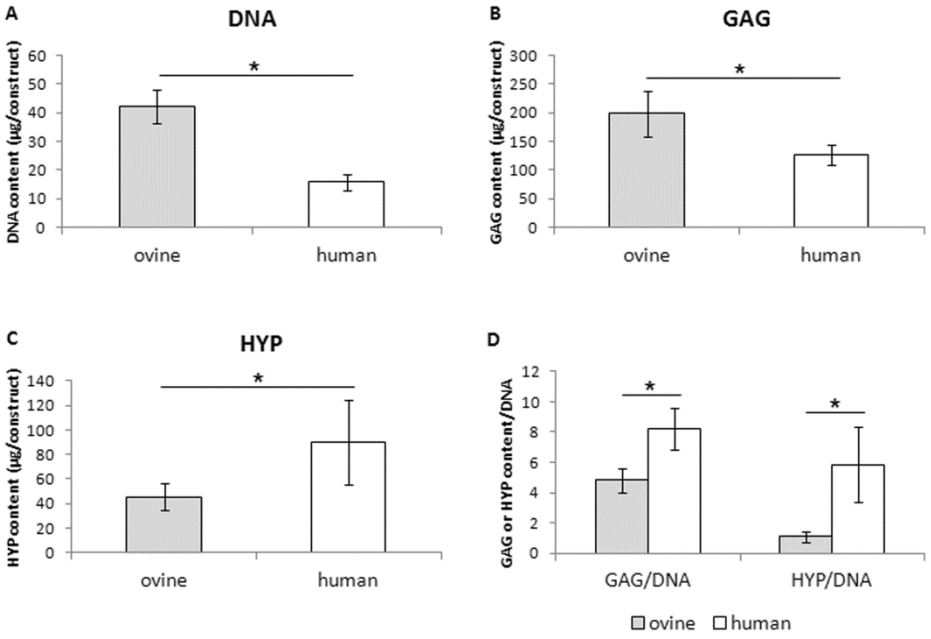

Figure 3.4: *Variability in cell number and extracellular matrix composition between ovine and human engineered constructs, studied by variation in (A) DNA, (B) GAG and (C) HYP content, respectively. In addition, (D) the GAG and HYP content is normalized for the DNA content. *p < 0.01. Significant interspecies differences are found in cell number and extracellular matrix composition. GAG: Glycosaminoglycan; HYP: Hydroxyproline.*

Also a significant relation between collagen content (e.g. HYP concentration) and E-modulus was observed with linear regression analysis. The tissue stiffness increased with increasing collagen content. This was seen in both ovine and human tissue constructs (figure 3.5B). No other significant correlations between cellular and tissue properties were found.

3.4 Discussion

In this study, we investigated cellular phenotype and tissue properties of ovine and human TE heart valve constructs, obtained using myofibroblasts from different sheep and patients, to (1) quantify the intraspecies variation in tissue outcome, (2) study interspecies differences, and (3) determine possible indicators of tissue outcome. Cellular phenotype was characterized and studied to indicate differences between subjects and species at an early stage of the tissue engineering process, whereas tissue properties were used to indicate tissue outcome and functionality after 4 weeks of culture when the tissue is generally strong enough to be implanted (Mol *et al*., 2006).

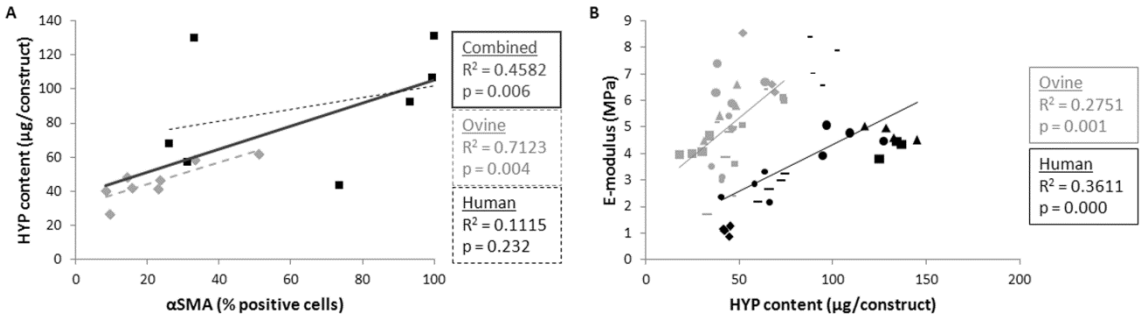

Figure 3.5: *Correlation between composition and mechanical properties of engineered heart valve constructs. (A) The relationship between αSMA and collagen content, analyzed with linear regression. Ovine results are indicated as the grey symbols, while the black symbols represent the human results. Collagen content in the engineered constructs increases with the number of αSMA-positive cells before seeding (indicated with the bold black line for the combined ovine and human results). This relation is, however, only significant in the ovine model. (B) Relation between collagen content and E-modulus of ovine (grey) and human (black) engineered constructs was indicated by linear regression analysis. The different symbols represent the different subjects (e.g. patients or sheep). The E-modulus increases with the collagen content. αSMA: α-smooth muscle actin; E-modulus: Elasticity modulus; HYP: Hydroxyproline.*

3.4.1 Intraspecies variation in tissue outcome

In this study, a large variation in tissue properties and matrix composition was seen in the heart valve constructs engineered from human cells. Especially, with respect to the mechanical properties and collagen content of the samples, large differences between constructs, engineered from myofibroblasts of different patients, were found. Nevertheless, the observed variation in human TE outcome in the present study may well be representative of the normal variation in heart valve properties (also related to personal characteristics, such as age, gender, and disease history). Balguid et al (2007) studied the mechanical properties of the leaflets of nine normal native human aortic valves (Balguid *et al*., 2007). In the circumferential direction of the leaflets they measured an E-modulus of 15.6 ± 6.4 MPa and an UTS of 2.6 ± 1.2 MPa, and in radial direction an E-modulus of 2.0 ± 1.5 MPa and an UTS of 0.42 ± 0.24 MPa. Standard deviations were large, indicating large intraspecies variability within healthy native valve tissue (48.9 ± 11.4 years). Variation in mechanical properties of human TE heart valves was also depicted by Kortsmit and colleagues (2009) (Kortsmit, 2009). The TE heart valves they cultured with myofibroblasts from one patient had an E-modulus in the circumferential direction of 2.19 ± 0.90 MPa and in radial direction of 0.90 ± 0.36 MPa, while the E-moduli of TE heart valves cultured under similar conditions with myofibroblasts of a second patient were significantly lower (circumferential direction: 0.32 ± 0.10 MPa; radial direction: 0.27 ± 0.03 MPa). Kortsmit assumed that these differences were related to the different cell sources (e.g. cells from different patients)

utilized in the experiments (Kortsmit, 2009), although differences may have also been arisen from the fact that the tissues were not cultured under the same circumstances (i.e. at the same time). Notwithstanding the cause of the variation in tissue outcome, Kortsmit's study implies that for proper prediction of TE valve outcome for clinical applications, human cells and cells from multiple donors should indeed be used.

In our study, human or ovine tissue constructs were cultured at the same time, under similar conditions. Therefore, the interpatient variability is not explained by possible reproducibility problems. However, the difficulty to reproduce TE experiments at different time points remains an issue. In future clinical practice it will also be difficult to reproduce implantation procedures, as all patients are different (e.g. different age, gender, disease history, etc), and all circumstances will be different (e.g. different hospital, surgeon, etc). This implies that the variation in outcome may increase even more.

3.4.2 Interspecies differences

Tissue outcome was different between species. Ovine cells are more proliferative compared to human cells. The Ki-67 cell proliferation immunofluorescent staining indicated that cells within the construct engineered from cells of both species are still proliferating after 4 weeks of culture (data not shown). This corresponds with the DNA content we found after 4 weeks of tissue culture. Per human cell 6.5 pg of DNA can be found (Dolezel *et al*., 2003). As the sheep genome is of similar size to the human genome (Buchanan *et al*., 1993), also roughly 6.5 pg DNA per ovine cell is present. This suggests that the ovine myofibroblasts tripled from about $1.35 * 10^6$ cells (e.g. the number of cells seeded per constructs) to approximately $6.5 * 10^6$ cells after 4 weeks of culture. In the human constructs the myofibroblasts proliferated to approximately $2.4 * 10^6$ cells. Thus, even with a lower serum concentration the ovine cells proliferated faster in the tissues than the human cells. Other aspects that may affect cell proliferation, such as rate of scaffold degradation and resulting scaffold stiffness did not differ between species. Therefore, the differences may have been caused by the species differences, or perhaps due to age differences. Despite these differences, the data suggest that even for the used patient group proliferation is good and suffices to produce tissue. Additional studies should elaborate on the effects of age and/or various co-morbidities on 48myofibroblasts proliferation when used as a source for autologous heart valve TE in different patient groups.

Furthermore, the ovine constructs contain more cells and GAG, but have a smaller hydroxyproline content when compared to the human constructs. In addition, as mentioned, the human cells are not as proliferative as the ovine cells. However, the human cells are more synthetic as they produce higher amounts of GAG and hydroxyproline per DNA. The GAG production per DNA in the human constructs is

almost twice as high when compared to the ovine construct, while the hydroxyproline production per DNA in the human constructs is five times higher than in the ovine constructs.

Unexpectedly, ovine constructs appear to be stiffer then human constructs with the same hydroxyproline content. This might be explained by for instance the amount of cross-links, a marker for tissue maturity (Balguid *et al*., 2007). However, this is probably not the case in this study as the picrosirius red staining indicates that the collagen fibers in ovine tissue are not as mature compared to the fibers in the human constructs. Therefore, the stiffness of the tissue is probably not only correlated to the collagen content, but possibly by the total matrix composition and architecture. In the future, additional studies to investigate the total tissue composition and architecture are needed.

Another difference between the species is that mechanical properties and tissue composition of all ovine TE constructs are comparable, while there is variation in these properties for the human TE constructs. Our results suggest that the culture process and tissue outcome of healthy (ovine) tissues can be controlled, whereas the composition and mechanical properties of tissues originating from patient material shows more variation and are hence more difficult to predict. In addition, this variation in human heart valve constructs is difficult to predict from ovine results, and it is, therefore, hard to translate these results to the clinic.

In this study, different culture medium compositions were used for the culture of ovine and human constructs. In order to do animal studies, a species-specific serum (i.e. lamb serum) was chosen. Also for future clinical implementation is the usage of FBS undesired. In previous studies, we have investigated the possibility of platelet lysate to replace FBS (Riem Vis *et al*., 2010; Geemen *et al*., 2011). However, it appeared that platelet lysate could not serve as an alternative as it reduced the tissue mechanical properties (Geemen *et al*., 2011). Serum is, thus, required for mechanically functionally heart valve tissue engineering. A very recent study shows that human serum, indeed, increases tissue mechanical properties and, therefore, might be a good alternative to replace FBS (Riem Vis *et al*., 2011). Additionally, in our study different serum concentrations were used to culture the ovine and human constructs. In a recent review, Mol et al (2009) described that the outcome of ovine tissue-engineered valves was dramatically different from their human equivalents when similar culture conditions were used (Mol *et al*., 2009). Therefore, medium composition was changed to enable a more homogenous tissue formation (like in the human constructs). These changes in culture conditions are also showing that the translation from ovine model to the clinical practice is not straightforward.

A limitation of our study is that the healthy (ovine) cells were young cells, while the patient cells originated from older (human) material. Thus, except from species differences, variations in cell and tissue properties may have been caused by the age

difference of the cell sources. Therefore, it was also interesting to study the cellular and tissue properties of cells isolated from older sheep. However, due to availability, this was not feasible in this study, and, hence, remains a subject for future studies. In addition, variation may have been caused by the disease, although in our patient group all patients suffered from the same cardiovascular disease. Though, disease history and co-morbidities may not have been the same, which can lead to additional interpatient variations. Due to ethical considerations, it is, however, not possible to isolate venous myofibroblasts from healthy humans. Another limitation is that ovine and human cells are isolated from a different vessel type. Due to practical reasons, ovine cells were isolated from the jugular vein, while the human cells were isolated from the saphenous vein. However, Grenier et al (2003) found no major differences in proliferative activity of cells isolated from the jugular or saphenous vein in a canine model (Grenier *et al*., 2003). Hence, the ovine jugular vein was chosen as healthy cell source. Although the jugular and saphenous vein contain equally well-suited cells for heart valve TE, our data indicate that translation of TE results from ovine (cell) models alone to the clinical practice is not sufficient. Although this may be recognized by the field (Hjortnaes *et al*., 2009; Schoen, 2011), it should be noted that interpatient variability contributes significantly to TE outcome, and this cannot be assessed using an ovine model.

3.4.3 Possible indicators of tissue outcome

Considering the variations in tissue outcome observed in the present study, there is a need to predict the properties of autologous tissues cultured for individual patients for future clinical application of autologous heart valve tissue engineering. Especially, it is important to find markers to determine the integrity of ECM components, as this is the principal determinant of the durability of heart valves (Sacks *et al*., 2009). During tissue culture, it should be possible to study specific ECM property markers in the culture medium. For instance, collagen synthesis and degradation markers can be studied with enzyme-linked immunosorbent assays (ELISAs). Furthermore, it is already possible to on-line monitor and control the evolution of mechanical properties during the tissue culture of heart valves (Kortsmit *et al*., 2009a). Quality criteria that characterize the tissue engineered heart valves as “good enough” for clinical use should be established (Hjortnaes *et al*., 2009). As the need exists to find means to accurately evaluate the functionality of the tissue engineered constructs before implantation, also earlier indicators (e.g. before tissue culture) of tissue outcome should be determined. Among others, protein and gene expression of the cells before seeding can be studied (Mendelson and Schoen, 2006). Though, gene expression is hard to directly correlate to tissue properties, and, hence, may not be suitable as a marker to predict tissue outcome. Therefore, in this study, we determined early indicators for tissue outcome at the protein level. Only differences in αSMA expression in both ovine and human myofibroblasts were found. αSMA is a marker of contractility of myofibroblasts, which

are typically positive for αSMA (Eyden, 2008). The local cell environment appears to be responsible for inducing the myofibroblast phenotype (Ehrlich *et al*., 2006). For instance, there is high expression of αSMA when cells experience mechano-tension from pulling at the surrounding matrix and neighboring cells, while the need for the expression of αSMA is lost when the cells are relieved of mechano-tension (Ehrlich *et al*., 2006). Venous myofibroblasts typically used in heart valve TE express αSMA, synthesize abundant ECM for functional tissue growth, and are characterized as a developing/remodeling/ activated phenotype (Rabkin-Aikawa *et al*., 2004; Aikawa *et al*., 2006). Also in our present study the myofibroblasts showed a developing/remodeling/activated phenotype (e.g. αSMA positive) prior to seeding. 2D studies of Merryman and co-workers (2006) showed a correlation between αSMA and the collagen biosynthetic protein hsp47 (Merryman *et al*., 2006). Higher expression of αSMA suggests that the cells are adapted for the stresses imposed on them, while higher hsp47 expressions are necessary to synthesize larger amounts of collagen (Merryman *et al*., 2006). In this study, we showed that the number of αSMA positive ovine myofibroblasts before seeding indeed positively correlates with the collagen content of the tissue constructs engineered from these cells. In addition, the stiffness of the tissue increases with increasing hydroxyproline content. Thus, from the current results there is indirect evidence that constructs seeded with more αSMA positive cells – and hence with a more remodeling/activated phenotype (Rabkin-Aikawa *et al*., 2004) – become stiffer. Hence, variations in αSMA may be used as an early marker to predict differences in tissue outcome related to collagen content and maybe even tissue mechanical properties. As variations in engineered tissue outcome for future clinical practice is expected to be higher than demonstrated for the constructs in the present study, it is extremely important to find early indicators of tissue outcome, such as αSMA. To evaluate αSMA as a predictive marker of tissue outcome, future studies should therefore focus on testing the predictive value of this marker. Ideally, such studies should include tissue constructs engineered with cells with a controllable degree of αSMA expression. While to our knowledge, it is not yet possible to control αSMA expression, to create for instance a 0% or 100% positive αSMA cell construct, ongoing studies in our group investigate relationships between αSMA, tissue mechanical properties, tissue structure and compaction (Vlimmeren *et al*., 2011) to control the TE process, understand variability, and predict tissue outcome.

3.5 Conclusion

The culture process and tissue outcome of tissue engineered from ovine cells can be controlled, whereas the mechanical properties of tissue originating from human material are difficult to predict. Additionally, αSMA is a potential predictor of tissue mechanical properties. The collagen content in the TE construct increased with the

number of αSMA positive cells before seeding. In addition, tissue stiffness increases with the collagen content. Therefore, the search for early indicators of tissue outcome to predict the variation in tissue culture for individual patients is of utmost importance for future clinical application of autologous heart valve tissue engineering. Moreover, this search should be performed with human cells of multiple donors, as the translation from the ovine model is not yet straightforward.

Acknowledgements

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Chapter 4

Decreased Mechanical Properties of Heart Valve Tissue Constructs Cultured in Platelet Lysate as Compared to Fetal Bovine Serum

The contents of this chapter are based on D. van Geemen, P.W. Riem Vis, R.S. Soekhradj-Soechit, J.P.G. Sluijter, M. de Liefde-van Beest, J. Kluin, and C.V.C. Bouten (2011) "*Decreased Mechanical Properties of Heart Valve Tissue Constructs Cultured in Platelet Lysate as Compared to Fetal Bovine Serum*" Tissue Engineering Part C, 17(5):607-617

4.1 Introduction

Autologous heart valve tissue engineering (TE) is an emerging strategy for future heart valve replacements (Schoen and Levy, 1999; Taylor, 2007). Currently, heart valve replacements enhance survival and the quality of life, but have several limitations (Mendelson and Schoen, 2006). Most importantly, these valves do not consist of living tissue and, therefore, will not grow. Heart valve TE seeks to overcome these limitations by creating a heart valve that has the ability to grow, repair and remodel.

In general, the heart valve TE strategy involves isolation of autologous cells, followed by *ex vivo* expansion and seeding of these cells on biodegradable scaffolds of synthetic or natural origin. The cell-scaffold constructs are then subjected to mechanical triggers in a bioreactor to stimulate extracellular matrix formation until a strong and functional load-bearing heart halve is grown that can be used for implantation. *Ex vivo* expansion of cells and the culture of the TE valve require a source of nutrients and growth factors in basal culture media. Fetal Bovine Serum (FBS) is usually selected for this purpose. However, several studies have shown that cells are able to take up animal-derived proteins from FBS and present these antigens after implantation (Spees *et al*., 2004). In some studies, this has lead to immune responses against the implanted cells and thus failure of the treatment (Selvaggi *et al*., 1997; Horwitz *et al*., 2002; Martin *et al*., 2005; Mannello and Tonti, 2007). Therefore, there is an ongoing search for alternative and preferable autologous sources of nutrients and growth factors.

One of the alternatives for FBS is human platelet-lysate (PL). Platelets contain granules that are rich in several interleukins and growth factors, including EGF, TGF-β and bFGF, which, *in vivo*, are released at sites of injury when proliferation of cells and remodeling of matrix is required (Weibrich *et al*., 2002; Eppley *et al*., 2006; Rozman and Bolta, 2007). PL is formed by forced release of growth factors in human serum and is believed to induce proliferation and extracellular matrix (ECM) remodeling in tissue regeneration and repair. In addition, it can be obtained autologously. Furthermore, unlike platelet rich plasma, platelet numbers are largely reduced in PL due to centrifugation prior to addition to the medium, and it does not need to be activated by thrombin. However, to prevent clotting, heparin should be added to PL enriched medium. Nevertheless, PL has been shown to be a promising substitute for FBS when applied for autologous culture of adult stem cells. PL is able to promote mesenchymal stem cell expansion (Doucet *et al*., 2005; Capelli *et al*., 2007), while multilineage differentiation is retained (Doucet *et al*., 2005; Capelli *et al*., 2007; Schallmoser *et al*., 2007). Furthermore, at the tissue level, PL shows promising results regarding bone TE applications (Kasten *et al*., 2008; Prins *et al*., 2009).

For autologous valve TE, it is desired that optimal tissue properties can be achieved in the shortest time possible. Hence, cultured cells should have a high duplication rate to reduce culture time, produce high amounts of ECM proteins and are able to remodel

produced matrix to optimize fiber arrangements. In a previous study, we have shown that venous derived mesenchymal cells (myofibroblasts), frequently used for heart valve TE, meet these primary criteria when cultured in PL (Riem Vis *et al*., 2010).

However, for the culture of load-bearing heart valves, not only the amount of cells and matrix are important to ensure strong tissue. The formation of strong tissue depends on a delicate balance between formation of ECM and degradation of newly formed fibers. As collagen is the main load-bearing component of functional heart valves, the collagen in engineered valves should be good enough to withstand hemodynamic forces upon implantation. Therefore, the aim of this study was to examine tissue formation, functionality, and mechanical properties of engineered heart valve constructs cultured in PL as an alternative for FBS.

4.2 Materials & Methods

An established 3D tissue model, consisting of human myofibroblasts seeded onto a biodegradable scaffold was used to study mechanical properties and tissue composition. For this purpose we harvested human myofibroblasts (MFs) of 9 patients. These cells were expanded up to passage 7 and for each patient 10 constructs were engineered per condition, e.g. PL or FBS. These tissue constructs were cultured for 4 weeks and, hereafter, mechanical properties and tissue composition of the constructs were analyzed, quantitatively and qualitatively.

4.2.1 Culturing myofibroblasts

4.2.1.1 Cell isolation

Segments of vena saphena magna (±3 cm) were obtained from 9 patients undergoing coronary artery bypass surgery using a venous graft. Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht was obtained, and tissue was further treated anonymously, as described previously (Schnell *et al*., 2001; Riem Vis *et al*., 2010). Briefly, venous segments were transferred to the laboratory in serum-free medium (Dulbecco's modified Eagle's medium-advanced [Gibco; Invitrogen, Breda, Netherlands], 2mM GlutaMax [Gibco], and 10 μg/mL gentamycin [Lonza, Verviers, Belgium]), wherein the adventitia was removed from the medial/intimal layer. Subsequently, the vessel was washed in antibiotics solution (phosphate-buffered saline [PBS; Sigma, Venlo, Netherlands], 2.5 μg/mL AmphotericinB [Biochrom; VWR, Amsterdam, Netherlands], and 200 μg/mL Gentamycin [Gibco]).

The lumen of the vessel was incubated in endothelial cell medium with collagenase (EBM2 [Lonza], EGM2 single quots [Lonza], 20% FBS [HyClone; Perbio, Etten-Leur, Netherlands], and 2 mg/mL collagenase A [Roche, Almere, Netherlands]), after which endothelial cells were scraped off with a cell scraper.

Tissue segments were cut into small squares (2x2 mm) and plated on culture plates with the lumen faced down, receiving either FBS medium (serum-free medium + 10% FBS) or PL medium (serum-free medium with 5% PL and 10 U/mL heparin [LEO Pharma, Breda, Netherlands]). Human platelets in serum were obtained from the hospital blood bank, pooled from five donors with similar blood type and rhesusfactor, and buffered with citrate-phosphate dextrose. The pooled plasma bags were estimated to contain a platelet count of 10^6 platelets per mm^3. Moreover, the presence of several growth factors and cytokines were screened in our previous study (Riem Vis *et al*., 2010). PL was frozen in aliquots at -80°C, thawed, and centrifuged (8 min 900 rcf) prior to addition to the culture medium, as described by others (Weibrich *et al*., 2002; Schallmoser *et al*., 2007). Cells were expanded using standard culture methods as previously described (Schnell *et al*., 2001; Mol *et al*., 2006). The expansion medium (2D medium) for MFs consisted of DMEM Advanced [Invitrogen, Breda, Netherlands] supplemented with 1% Penicillin/Streptomycin (P/S; Lonza) and 1% GlutaMax (Gibco). The medium was further supplemented with 10% FBS (Greiner Bio-one, Alphen a/d Rijn, Netherlands) or 5% PL and 0.2% heparin (LEO Pharma) for the FBS and PL groups respectively.

4.2.1.2 Scaffold preparation and sterilization

Rectangular scaffolds (30x6x1 mm) composed of rapidly degrading non-woven polyglycolic acid (PGA; thickness, 1.0 mm; specific gravity, 70 mg/cm3; Cellon, Bereldange, Luxembourg), were coated with poly-4-hydroxybutyrate (P4HB; provided by Symetis Inc., Zürich, Switzerland) to provide structural integrity to the mesh. After drying overnight, the two outer 5 mm of the long axis of each construct were glued to stainless steel rings using a 20% solution of polyurethane (PU; DSM, Geleen, Netherlands) in tetrahydrofuran, leaving a 20×6 mm area for TE. The solvent was allowed to evaporate overnight.

The rings with the scaffold strips were placed in 6-well plates and sterilized in 70% ethanol for 30 minutes and, subsequently, washed twice in PBS (Sigma, Venlo, Netherlands). Hereafter, the strips were placed in TE medium (2D medium, supplemented with L-ascorbic acid 2-phosphate [0.25 mg/ml; Sigma]) overnight.

4.2.1.3 Cell seeding

MFs were seeded in the constructs (n = 10 per group) using fibrin as a cell carrier, as previously described by Mol *et al.* (Mol *et al*., 2005). In short, MFs were suspended in TE medium, without FBS or PL, containing thrombin (10 IU/ml; Sigma). Subsequently, this cell suspension was mixed with an equal volume of TE medium, without FBS or PL, containing fibrinogen (10 mg/ml; Sigma). This fibrin/cell suspension ($20*10^6$ cells/ml) was mixed until onset of polymerization of the gel after which 180 µl was dripped onto the strip. To allow further firming of the fibrin gel, 6-well plates were placed in an incubator at 37 °C and 5% CO_2 for 30 minutes. Hereafter, the wells were filled with 6 ml TE medium, with PL or FBS, and placed back into the incubator. The constructs were cultured for 4 weeks and TE medium was changed every 2-3 days.

4.2.2 Mechanical testing

Mechanical properties were determined after 4 weeks of culture by uniaxial tensile testing in longitudinal direction of the engineered constructs (n = 4 per patient and culture condition) using a tensile stage equipped with a 20N load cell (Kammrath-Weiss, Dortmund, Germany). Measurements were averaged per culture condition.

A Digimatic Micrometer (Mitutoyo America Corporation, Aurora, USA) was used to measure the thickness of the strips prior to tensile testing. Stress–strain curves were obtained at a strain rate equal to the initial sample length (20 mm) per minute. The Cauchy stress was defined as the force divided by the cross-sectional area. The ultimate tensile strength (UTS; e.g. the maximal Cauchy stress) was determined from the curves while the slope of the linear part of the curve represented the elasticity modulus (E-modulus) of the tissue.

4.2.3 Qualitative tissue composition

4.2.3.1 Histology

Tissue formation was analyzed qualitatively by histology. Tissue constructs were fixed in 3.7% formaldehyde in PBS and subsequently embedded in paraffin. Samples were sectioned at 10 µm and stained with hematoxylin and eosin (H&E) for general tissue development and Masson Trichrome (MTC kit, Sigma) for collagen visualization. The stainings were analyzed using a Zeiss light microscopy (Carl Zeiss, Sliedrecht, Netherlands). To distinguish between juvenile and mature collagen fibers, Picrosirius Red Staining (Puchtler *et al*., 1973; Junqueira *et al*., 1979) was performed and examined by means of crossed polar microscopy (Carl Zeiss).

4.2.3.2 Immunofluorescence

Immunofluorescence was performed on paraffin sections. The used antibodies and their corresponding secondary antibodies are depicted in table 1. Sections were deparaffinized and antigen was retrieved by incubation in either boiled TRIS-EDTA buffer for 20 minutes or in 0.04% pepsin buffer for 8 minutes. Afterwards, sections were incubated in 1% BSA (Roche) in PBS to block non-specific binding. Prior to overnight incubation (4 °C) with the primary antibodies (table 4.1), the sections were permeabilized with 1% Triton-X-100 (Merck, Amsterdam, Netherlands) in PBS. The specific stainings were visualized with fluorescent secondary antibodies (table 1). After an additional staining with DAPI to stain cell nuclei, sections were mounted with mowiol (Calbiochem, San Diego, USA). Stained sections were analyzed and pictures were taken by means of fluorescent microscopy (Axiovert 200, Carl Zeiss).

Primary antibody			Corresponding secondary antibody		
collagen type I	IgG1	Sigma	Alexa 488	IgG1	Invitrogen
collagen type III	IgG1	Sigma	Alexa 488	IgG1	Invitrogen
αSMA	IgG2a	Sigma	Alexa 488	IgG2a	Invitrogen
vimentin	IgM	Abcam	Alexa 555	IgM	Invitrogen
Hsp47	IgG2b	Stressgen	Alexa 488	IgG2b	Invitrogen
Desmin	IgG1	DAKO	Alexa 555	IgG1	Invitrogen
SMemb	IgG2b	Abcam	Alexa 488	IgG2b	Invitrogen

Table 4.1: *Antibodies used in this study. All primary antibodies were monoclonal mouse anti-human antibodies. All secondary antibodies were goat anti-mouse antibodies. Only in case of monoclonal IgG1 mouse anti-human antibody against collagen type III, the antigen was retrieved by incubation in pepsin buffer. For all other antibodies, the antigen was retrieved by incubation in TRIS-EDTA buffer.*

4.2.4 Quantitative tissue composition

The total content of DNA (as an indication of cell number), sulfated glycosoaminoglycans (GAGs) and hydroxyproline (HYP) was determined on constructs previously used for the tensile tests. Lyophilized samples were digested in papain solution (100 mM phosphate buffer (pH=6.5), 5 mM L-cystein, 5 mM EDTA and 125-140 µg papain per ml) 60°C for 16 hours. The Hoechst dye method (Cesarone *et al*., 1979) with a reference curve prepared of calf thymus DNA (Sigma) was used to determine the amount of DNA. The content of sulfated GAGs was determined on the basis of the protocol described by Farndale *et al*. (1986) (Farndale *et al*., 1986) and shark cartilage chondroitin sulfate was used as a reference (Sigma). In short, 40 µl of diluted sample, without addition of chondroitin AC lyase, chondroitin ABC lyase and keratanase, was

pipetted into a 96-wells plate in duplicate. Hereafter, 150 µl dimethylmethylene blue was added and absorbance was measured at 540 nm. To determine the HYP quantity, an assay according to Huszar *et al*. (Huszar *et al*., 1980) and a reference of trans-4-hydroxyproline (Sigma) was used. The number of mature collagen hydroxylysylpyridinoline (HP) cross-links, as a measure for tissue maturity, was measured in the same hydrolyzed samples using high-performance liquid chromatography as described previously (Bank *et al*., 1996; Robins *et al*., 1996; Bank *et al*., 1997). The number of HP cross-links was expressed per collagen triple helix (TH).

4.2.5 Collagen remodeling

4.2.5.1 Medium analysis

Concentrations of the remodeling enzymes matrix metalloproteinases (MMP)-1, MMP-2, and procollagen type I C-peptide (PIP; a marker for collagen I synthesis) were determined on medium samples after 4 weeks of culturing. ELISAs were performed according to the recommendations from the supplier. MMP-1 and MMP-2 concentrations were quantified by immunoassays for human MMP-1 and MMP-2 protein (RayBiotech; Tebu-bio, Heerhugowaard, Netherlands). PIP was determined using a procollagen type I C-peptide ELISA kit (Takara Bio, Otsu Shiga, Japan).

4.2.5.2 Zymography

Zymography analysis was performed on medium conditioned by cells in tissue constructs, corrected for the amount of DNA. Similar amounts of medium from patients that produced tissue in both PL and FBS was pooled, to give an averaged overview of protease expression. To reduce aspecific signals from abundant serum proteins, albumin and IgG-fractions were removed using the Aurum Serum Protein Mini Kit (Bio-Rad, Veenendaal, Netherlands), according to the manufacturer's instructions. The samples were prepared with Laemmli buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol and 0.004% Bromophenol blue) and loaded on 10-15% poly-acrylamide gels containing 2 mg/mL gelatin or 4 mg/mL casein, as described previously (Sluijter *et al*., 2004). After running, gels were incubated overnight at 37°C in Brij-solution (50 mM tris-HCL 7.4, 10 mM $CaCl_2$, 0.05% Brij35 (w/v dilute 600x) and stained with Coomassie Blue (25% MeOH, 15% HAc and 0.1% Coomassie Blue [Polysciences, Inc., Eppelheim, Germany]). Analysis and quantification of total (active and inactive) protease secretion was performed using the ChemiDoc XRS system (Bio-Rad) and QuantityOne software (Bio-Rad).

4.2.6 Statistics

Data on quantitative analysis of tissue composition, mechanical properties and collagen remodeling are provided as mean ± standard deviation. We used SPSS 17 software for statistical analysis. Paired t-tests were used to test differences in tissue outcome for tissues cultured in FBS or PL. A p-value < 0.05 was considered significant.

4.3 Results

Tissue constructs were engineered from myofibroblasts of 9 different patients. In the tissue constructs of two patients cultured with FBS a bacterial infection was found. The constructs cultured with the cells of these patients were, therefore, excluded from this study. The results of the constructs of the remaining seven patients are described below.

4.3.1 Biomechanical properties

The averaged stress-strain curves are shown in figure 4.1, and tissue stiffness (E-modulus) and strength (UTS) are shown in figure 4.2 and table 4.2. The E-modulus ranges from 1.10 ± 0.17 MPa to 7.46 ± 0.82 MPa in the tissue constructs cultured in FBS medium, while the constructs cultured in PL medium have an E-modulus that ranges from 0.46 ± 0.26 MPa to 2.83 ± 0.36 MPa (figure 4.2A). The Ultimate Tensile Strength ranges from 0.25 ± 0.07 MPa to 1.37 ± 0.19 MPa in the constructs cultured in FBS medium and from 0.12 ± 0.09 MPa till 0.52 ± 0.09 MPa in the constructs cultured in PL medium (figure 4.2B). There are differences in E-modulus and UTS between constructs of different patients. However, the constructs of all patients were significantly stiffer

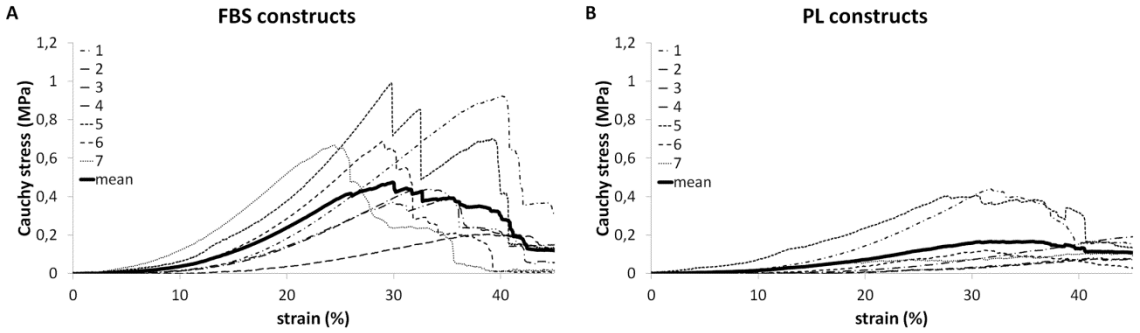

Figure 4.1: *Stress-strain curves of the tissue-engineered constructs cultured in media supplemented with FBS (A) or PL (B). The stress-strain curves of the constructs of the different patients are indicated with the different lines. The dark black line indicates the average-stress-strain curve of all constructs cultured in FBS (A) or PL (B). FBS, fetal bovine serum; PL, platelet-lysate.*

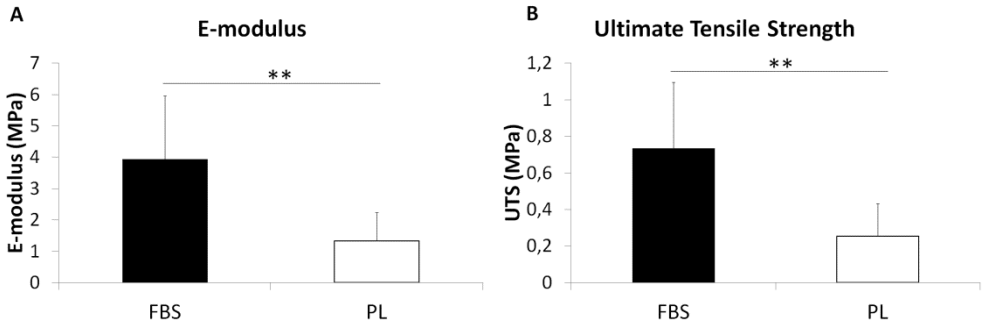

Figure 4.2: *Mechanical properties, including E-modulus (A) and ultimate tensile strength (B). **(p < 0.01) represents significant difference between constructs cultured in FBS medium and PL medium. Tissue constructs engineered in FBS medium are stiffer and stronger than constructs cultured in PL medium.*

(higher E-modulus) and stronger (higher UTS) when cultured in medium supplemented with FBS ($p < 0.01$).

4.3.2 Qualitative tissue analysis

Pictures of general tissue development (H&E), collagen deposition (MTC), and collagen fiber thickness (picrosirius red) are shown in figure 4.3. Although there is a slight variation between the constructs of the different patients, the general tissue development, as seen in the H&E staining, between cardiovascular constructs cultured in FBS or PL is similar. Collagen is seen throughout the tissue; however it is more

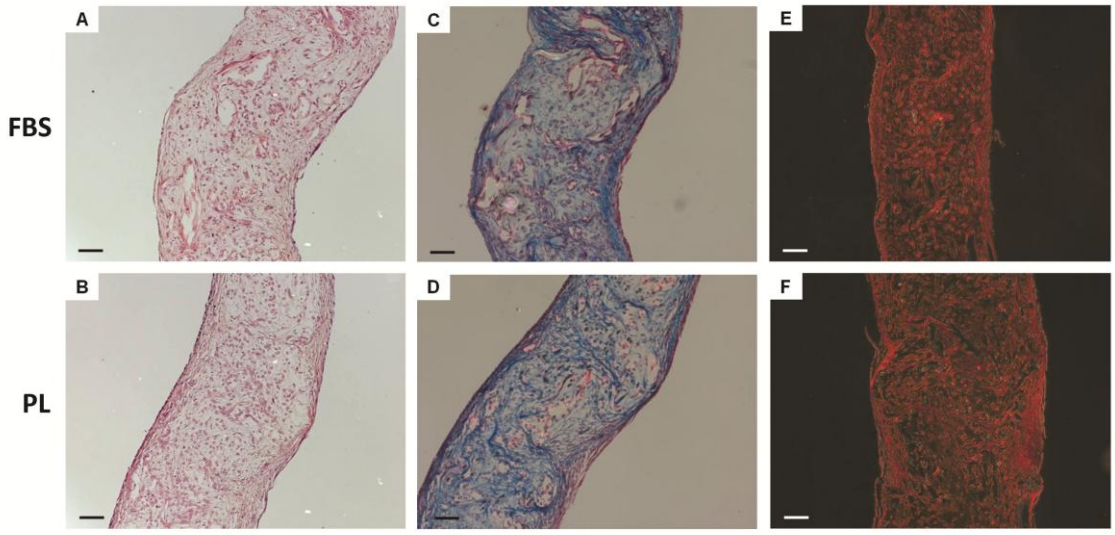

Figure 4.3: *Histology of tissue-engineered constructs cultured in FBS (A, C, E) and PL (B, D, F) medium. Representative pictures for tissue composition are displayed. (A, B) Hematoxylin and eosin staining, (C, D) Masson Trichrome Staining, and (E, F) picrosirius red staining. Original magnification was 10x. Scale bars indicate 100 µm.*

abundant at the surface layers. This is observed for all tissue constructs.

The phenotype of the myofibroblasts in the cardiovascular constructs was analyzed with immunofluorescence (figure 4.4). In all engineered constructs, cells stain positively for α-smooth muscle actin (αSMA), vimentin, heat shock protein 47 (hsp47), and nonmuscle myosin heavy chain (SMemb), but not for desmin. No consistent difference between FBS and PL is observed, though, there are differences between the constructs of different patients.

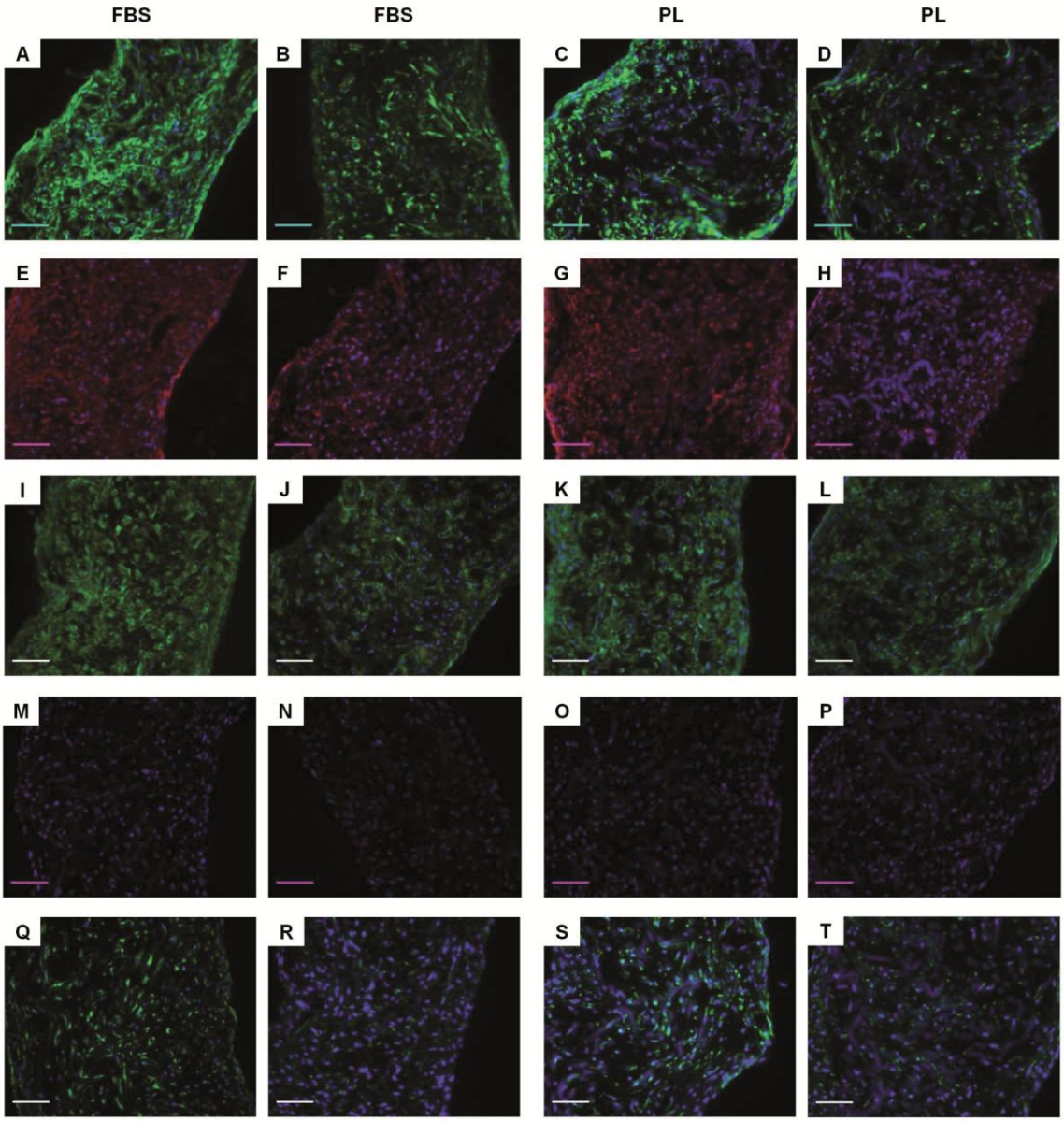

Figure 4.4: *Immunofluorescent staining of tissue-engineered constructs in both media. Results for phenotyping of two patients are displayed to indicate differences between patients. (A-D) α-smooth muscle actin, (E-H) vimentin, (I-L) nonmuscle myosin heavy chain (SMemb), (M-P) desmin, and (Q-T) heat shock protein 47. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI). Original magnification was 20x. Scale bars indicate 100 µm.*

To distinguish between collagen type I and collagen type III, the engineered constructs were analyzed by immunofluorescent staining (figure 4.5). Within PL and FBS groups large inter-subject variations were observed. These were not related to collagen type. Typically, if collagen type I was abundant, collagen type III was also and vice versa. Between FBS and PL groups no consistent differences in collagen production could be recognized.

Some scaffold remnants, which were still present in all tissue engineered constructs, were also stained with the DAPI staining (figure 4.4 and 4.5). In figure 5 such remnants are indicated with an arrow to illustrate the distinction between the remnants and the nuclei.

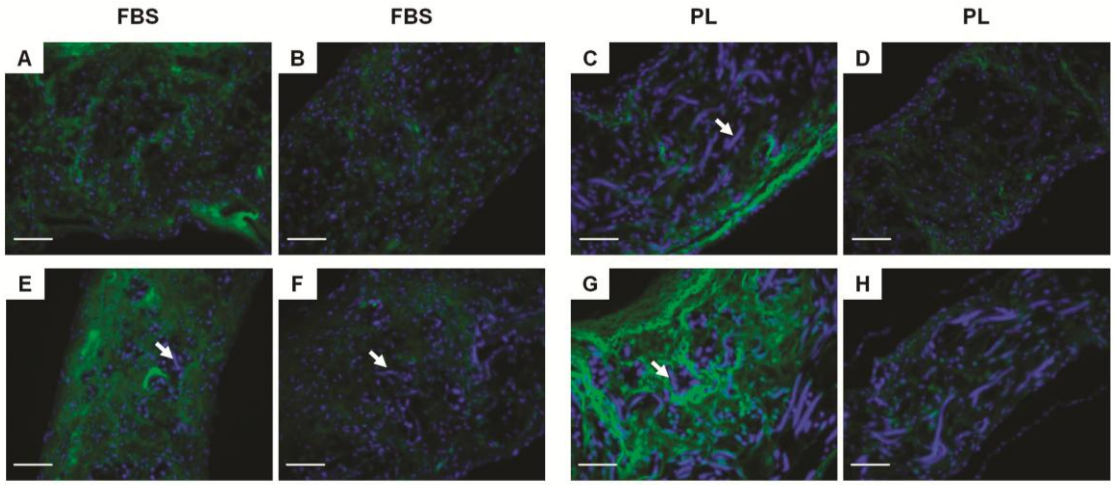

Figure 4.5: *Immunofluorescence of tissue-engineered constructs cultured in FBS (A, B, E, F) or PL (C, D, G, H) medium. Results of collagen type I and collagen type III immunofluorescent staining of two patients are displayed. (A-D) Collagen type I and (E-H) collagen type III. Nuclei are stained with DAPI. Original magnification was 20x. Scale bars indicate 100 µm. The white arrows indicate scaffold remnants.*

4.3.3 Quantitative tissue analysis

Tissue composition of the engineered constructs was quantified by DNA, GAG, and hydroxyproline analysis, and the amount of collagen crosslinks (table 4.2). The DNA per tissue construct was comparable for the constructs of all patients in the PL and FBS groups, and did not differ between the groups. Where averaged DNA content was 15.78 ± 2.63 µg/tissue strip for the FBS group, and 19.27 ± 3.09 µg/tissue strip for the PL group. This difference is not significant. Also no differences were observed in the GAG content, HYP concentration, and the number of HP cross-links per triple helix. Although there were no differences in HYP content between the PL and FBS groups, there was a slight variation between the constructs of the different patients. These results correspond with the Masson Trichrome, picrosirius red staining, and immunofluorescent stainings.

4.3.4 Collagen remodeling capacity

The effects of tissue culture in either FBS or PL on remodeling markers are shown in figure 6 and table 2. Collagen synthesis, indicated by PIP concentration measurements, was similar in tissue cultured in FBS or PL. The PIP concentration ranges from 18133 – 45100 ng/ml in the culture medium with FBS, and from 19712 – 55985 ng/ml in the culture medium supplemented with PL (figure 4.6A). For collagen degradation/remodeling the concentration of matrix metalloproteinases was measured. As indicated by ELISA analysis, MMP-1 and MMP-2 were elevated in tissue culture supplemented with PL (MMP-1: 155 ± 107 ng/ml; MMP-2: 10.1 ± 1.9 ng/ml) when compared to culture medium with FBS (MMP-1: 49 ± 44 ng/ml; MMP-2: 3.4 ± 0.5 ng/ml). These variations were also observed in the zymography analysis, that indicate the contribution of activated MMPs. Figure 4.6D shows a higher activated MMP-2 and MMP-9 in tissue culture medium supplemented with PL.

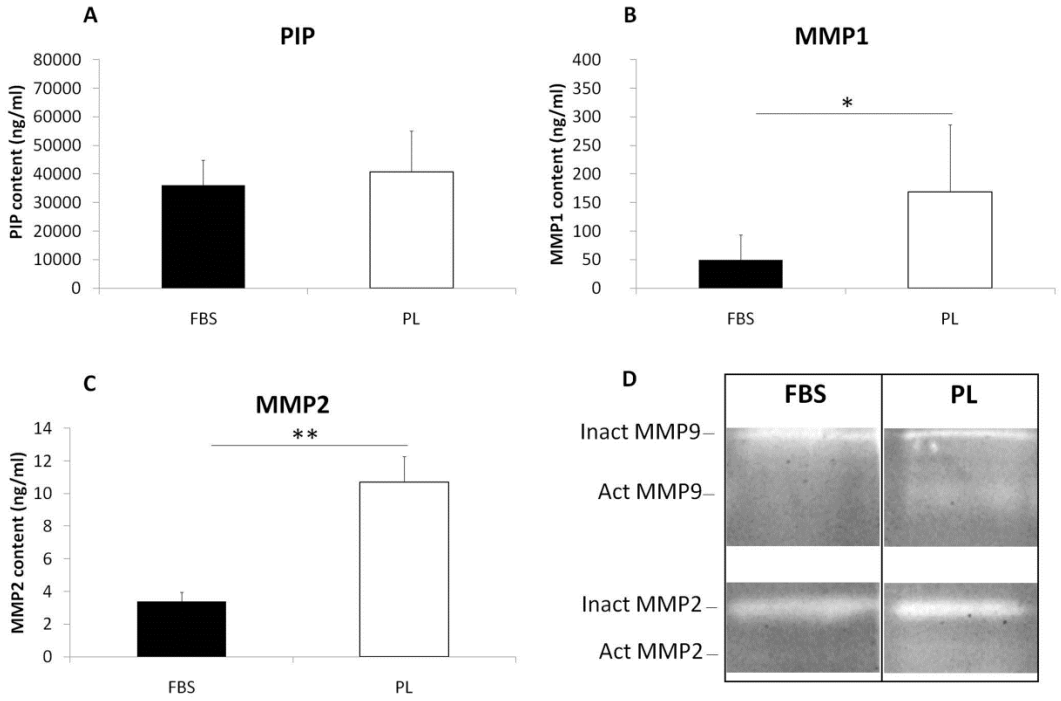

Figure 4.6: *Concentrations of the remodeling markers PIP (A), MMP-1 (B), and MMP-2 (C) in culture medium. *(p < 0.05) and **(p < 0.01) indicate significant differences between tissue-engineered constructs cultured in FBS medium and PL medium. Higher levels of MMP-1 and MMP-2 are observed in PL medium. (D) Zymography results for total MMP-2 and MMP-9 expression. More active and inactive MMP-2 and MMP-9 are present in the culture medium of constructs cultured with PL. MMP, matrix metalloproteinases; PIP, procollagen type I C-peptide.*

Tissue properties	FBS constructs	PL constructs
E-modulus (MPa)	3.94 ± 2.02	1.33 ± 0.90[a]
UTS (MPa)	0.74 ± 0.36	0.25 ± 0.18[a]
Thickness (mm)	0.74 ± 0.07	0.79 ± 0.07
Width (mm)	4.08 ± 0.42	4.33 ± 0.51[b]
DNA (µg/strip)	15.78 ± 2.63	19.27 ± 3.09
GAG (µg/strip)	126.63 ± 16.90	121.69 ± 22.45
HYP (µg/strip)	89.84 ± 34.83	88.42 ± 23.35
HP/TH (-)	0.17 ± 0.05	0.17 ± 0.06
PIP (ng/ml)	36032 ± 8844	40767 ± 14257
MMP-1 (ng/ml)	49 ± 44	155 ± 107[b]
MMP-2 (ng/ml)	3.4 ± 0.5	10.1 ± 1.9[a]

Table 4.2: *Mechanical properties and tissue composition of engineered constructs cultured in media supplemented with fetal bovine serum or platelet-lysate. Superscript letters indicate a difference compared to the FBS group: [a]$p < 0.01$ and [b]$p < 0.05$. FBS, fetal bovine serum; GAG, glycosaminoglycan; PL, platelet-lysate; UTS, ultimate tensile strength; HP, hydroxylysylpyridinoline; TH, triple helix; MMP, matrix metalloproteinases; PIP, procollagen type I C-peptide.*

4.4 Discussion

In our previous study, published in April 2010 (Riem Vis *et al*., 2010), we studied in 2D whether PL could serve as an alternative for FBS in human heart valve TE. This study showed promising results with respect to the expansion and matrix production and remodeling potential of cells cultured in PL as compared to FBS. In the present study, we, therefore, aimed to verify whether PL can serve as an alternative for FBS in 3D tissue engineered heart valve constructs. Engineered constructs, consisting of PGA/P4HB scaffolds seeded with myofibroblasts of 7 different patients, were cultured in TE-medium supplemented with either PL or FBS and mainly tested for tissue composition and mechanical properties. Contrary to the 2D studies, however, this 3D study indicates that, despite similarities in matrix production of tissues cultured in PL and FBS, tissue mechanical properties are drastically reduced after culture in PL. In addition E-moduli and ultimate tensile stress of PL-constructs reached values of about 2 times lower than those found for native heart valve leaflets in the radial direction (Balguid *et al*., 2007), whereas tissue mechanical properties of the constructs cultured in FBS-rich medium more closely resembled those of native valve leaflets.

Tissue mechanical properties strongly depend on ECM formation and maturation. Haut et al (1992) described that the tensile modulus was positively correlated with the

content of insoluble collagen in the canine tendon (Haut *et al*., 1992). Elbjeirami and co-workers (2003) found that the tensile strength and elastic modulus was increased in TE constructs, in which the ECM was enzymatically cross-linked (Elbjeirami *et al*., 2003). Moreover, Balguid and colleagues (2007) showed that there is a significant correlation of collagen cross-linking with tissue stiffness in circumferential direction in native heart valves (Balguid *et al*., 2007). Thus, in determining the mechanical properties of tissue constructs, not only collagen formation is of interest, but especially collagen cross-links appear important. Nevertheless, despite similar collagen content and a similar number of HP cross-links, which are the main type of collagen cross-links present in cardiovascular tissue, the tensile properties of constructs cultured in FBS were higher than those cultured in PL. Therefore, these structural properties alone do not explain the difference in mechanical properties found in this study.

Another factor that specifies tissue biomechanical properties is the collagen architecture (Baaijens *et al*., 2010). Lindeman et al (2010) described that mechanical properties of vessels are strongly influenced by collagen microarchitecture and that perturbations in the collagen network may lead to mechanical failure (Lindeman *et al*., 2010). In addition, Guidry and Grinnell (1987) reported that heparin modulates the organization of hydrated collagen gels (Guidry and Grinnell, 1987). Control collagen gels were composed of a uniform network of interlocking fibrils, while this network was disrupted in heparin-containing gels (Guidry and Grinnell, 1987). Heparin must be added to PL medium, to prevent coagulation of the platelets in the medium. Heparin, in combination with the increased matrix remodeling abilities, can alter the collagen architecture of the engineered constructs. To verify the collagen architecture of the constructs cultured in the present study, additional tissue constructs were engineered in PL or FBS medium and collagen fiber organization was visualized by whole mount collagen type I immunofluorescent staining. An inverted Zeiss Axiovert 200 microscope (Carl Zeiss) coupled to an LSM 510 Meta (Carl Zeiss) laser scanning microscope was used to visualize collagen type I organization. The collagen architecture in the TE constructs cultured in PL was observed to be different from that of the constructs cultured in FBS (figure 4.7), in a blinded procedure using 3 independent observers. It was concluded that the collagen architecture was less dense in the PL group as compared to the FBS group. Furthermore, in the PL-constructs the collagen fibers appeared to be shorter. This difference of the collagen network in PL is probably caused by enhanced collagen degradation by MMPs in the PL constructs and might indeed explain the decreased mechanical properties of the PL constructs as compared to the FBS constructs. Additional studies, incorporating a more quantitative approach including image analysis, are required to verify this relation between tissue architecture, MMP activity, and tissue mechanical properties in PL constructs.

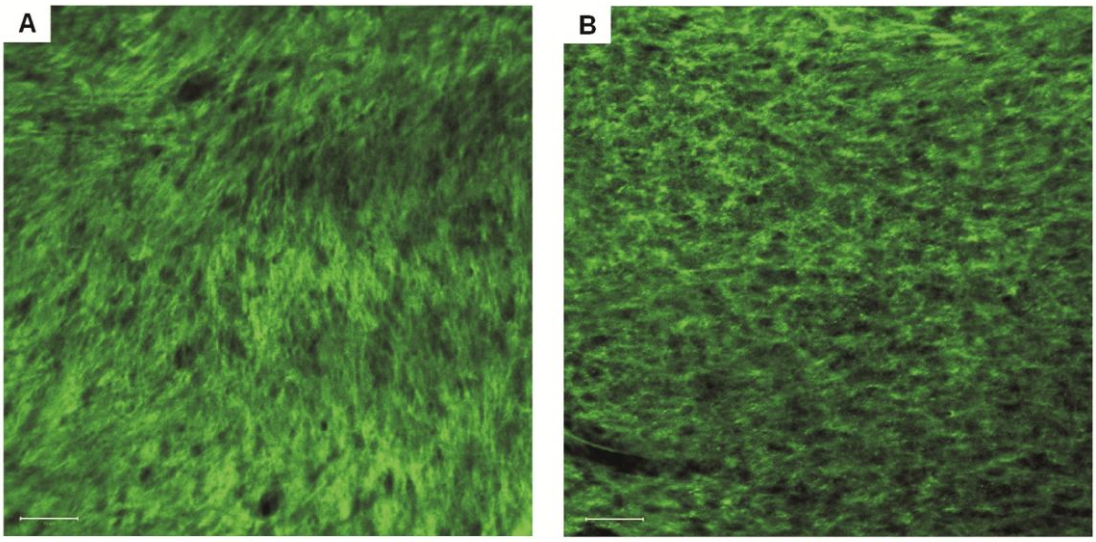

Figure 4.7: *Whole mount collagen type I immunofluorescent staining of engineered constructs cultured in FBS (A) or PL (B). The collagen network in the PL group seems less dense with shorter fibers. Original magnification was 20x. Scale bars indicate 50 µm.*

A last factor that might have affected the difference in the mechanical properties of the engineered constructs is the contribution of the scaffold material. Due to degradation of the material used in our studies, the mechanical properties of the cell-scaffold constructs will rapidly change with time. We have previously tested these properties for tissue constructs and bare scaffolds kept in FBS (Balguid *et al*., 2009a; Rubbens *et al*., 2009b). Initially, tissue mechanical behavior is linear in nature and resembles that of the bare scaffold, indicating a large contribution of scaffold material to total mechanical properties. However, with time the scaffold degrades and tissue is being formed, leading to a rapid increase in mechanical properties (E-modulus, UTS) and the typical non-linear stress-strain behavior of native cardiovascular tissues after 3 to 4 weeks of culture. Although scaffold degradation in PL in the present study may have been different from that in FBS, it is unlikely that this affected ultimate tissue mechanical properties after 4 weeks. For both culture conditions only scaffold remnants are present at this point in time, unlikely to significantly contribute to total tensile mechanical properties. Furthermore, by this time, both PL and FBS constructs have reached the non-linear stress-strain behavior typical of 'native' tissue without artificial material.

The composition and quality of the tissue are subject to change during tissue remodeling due to mechanical or biochemical environmental stimuli. In this study, the biochemical triggers were different for the PL and FBS groups. Superarray and western blot analyses, performed in our previous study (Riem Vis *et al*., 2010), showed generally higher expression of proteins involved in tissue repair and remodeling in PL-medium. PL medium contains higher concentrations of heparin-binding epidermal growth factor (HB-

EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and total transforming growth factor-beta (TGF-β) when compared to FBS. Furthermore, the levels of interleukins, IL17 and IL23, and proteins involved in tissue repair and matrix remodeling, MIP1a and uPA, are higher in PL than FBS. This suggests that the matrix remodeling capacities of tissues cultured in PL might be higher than of tissue cultured in FBS. Therefore, the level of collagen remodeling markers (MMP-1, MMP-2, and PIP) secreted in these tissues was also investigated in this study. MMP levels were higher in the PL group, which might be explained by the high levels of IL17, IL23, MIP1a and uPA in PL-medium (DiPietro *et al*., 1998; Langowski *et al*., 2006; Qiu *et al*., 2009). These proteins are also involved in the wound healing process (Stadelmann *et al*., 1998; Werner and Grose, 2003). Moreover, MMPs are crucial in the inflammatory and remodeling phases of wound healing (Utz *et al*., 2010). This suggests that the tissue formed in PL has similar properties as the scar tissue formed during wound healing. Characteristic of scar tissue is a disorganized collagen network and high remodeling properties, both resulting in initial weaker tissue. A hypothesis could be that, with time, tissue cultured in PL can remodel to stronger and more organized tissue.

Our main reason to test the use of PL was to define an autologous culture medium for future clinical application of heart valve tissue engineering. PL can be obtained autologously, offering the potential to culture fully autologous tissue engineered valves in the future. Though, we used pooled PL from several human subjects instead of autologous PL in the present study, we believe to have provided proof of principle for the use of PL in heart valve tissue engineering and sufficient data to conclude that culture of heart valve tissue constructs in PL medium results in tissue with poor mechanical properties.

In conclusion, at the cellular (2D) level PL might be a promising substitute for FBS; however, at the 3D tissue level, FBS induces a more stable and organized ECM, relevant for improved mechanical properties. Hence, our results indicate that serum is required for mechanically functionally heart valve tissue engineering, though this might preferably be obtained autologously. Future studies should point out if autologous serum can be used as an appropriate substitute for FBS in 3D tissue culture.

Acknowlegdgments

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Chapter 5

Alternating Mechanical Conditioning Strategies to Optimize Tissue Properties in Human Heart Valve Tissue Engineering

The contents of this chapter are based on D. van Geemen, A. Driessen-Mol, F.P.T. Baaijens, and C.V.C. Bouten (2012) "*Alternating Mechanical Conditioning Strategies to Optimize Tissue Properties in Human Heart Valve Tissue Engineering*" (submitted)

5.1 Introduction

The classical heart valve tissue engineering (TE) approach involves the *in vitro* culture of cell-seeded biodegradable scaffolds in bioreactor systems to trigger the cells to form tissue. Several strategies, including biochemical stimulation (e.g. growth factor supplementation) and mechanical conditioning (e.g. cyclic straining), have been developed to enhance the structural and mechanical properties of TE heart valves. Especially, mechanical conditioning in heart valve TE is used to enhance the collagen architecture and the mechanical properties of these TE heart valves. As mechanical conditioning plays an important role in the valvulogenesis during embryonic development (Butcher and Markwald, 2007), it is not surprising that a mechanical stimulus is also required for engineering tissues where mechanical function is critical, such as in heart valves (Freed *et al*., 2006; Butcher and Markwald, 2007; Butcher *et al*., 2008).

The effect of mechanical conditioning on *in vitro* 2D cell behavior is extensively studied, as is for instance reviewed by Wang and Thampatty (Wang and Thampatty, 2006). Within the heart valve TE field, several groups have studied the effects of mechanical conditioning to enhance the structural and mechanical properties of 3D engineered tissues. They demonstrated that mechanical conditioning improves the mechanical properties of engineered tissue through an increased extracellular matrix (ECM) production and organization (Hoerstrup *et al*., 2000a; Seliktar *et al*., 2003; Isenberg and Tranquillo, 2003; Mol *et al*., 2003; Ku *et al*., 2006; Syedain *et al*., 2008; Syedain and Tranquillo, 2011). The net effects were, however, dependent on the degree and the duration of the applied strain (Xing *et al*., 2004a; Xing *et al*., 2004b; Ku *et al*., 2006). Elevated cyclic strain levels resulted in increased cell proliferation and apoptosis, increased collagenase and gelatinase activity, and induced calcification in aortic valve leaflets (Balachandran *et al*., 2009; Lehmann *et al*., 2009). The latter effect was probably induced by an increased production of bone morphogenic proteins upon mechanical conditioning (Balachandran *et al*., 2010), indicating the delicate balancing of mechanical conditioning protocols to prevent pathological conditions.

Previous studies from our group demonstrated that continuous cyclic strain inhibits collagen formation in an early phase of heart valve TE (Rubbens *et al*., 2009c), and does not affect the total amount of collagen in the tissue when compared to static strain (i.e. constrained tissue culture with traction forces exerted by the cells within the tissue) (Balguid *et al*., 2007). However, the maturation of the tissue was improved by continuous cyclic strain due to enhanced cross-link densities and collagen fiber alignment. Tissue culture with intermittent conditioning (i.e. alternating periods of cyclic and static strain) rather than continuous cyclic strain accelerated the production of collagen in the first weeks of the culture period, cross-links, and collagen fiber alignment (Rubbens *et al*., 2009a; Rubbens *et al*., 2009b). These studies indicate that intermittent conditioning is favored over continuous cyclic strain.

Rubbens and co-workers hypothesized that intermittent conditioning balances the collagen production during static strain and enhances cross-links and alignment during cyclic strain (Rubbens *et al*., 2009a; Rubbens *et al*., 2009b). Thus, with intermittent conditioning more mature tissues are engineered. However, the underlying mechanism for this hypothesis is not clear. One hypothesis is that the cells need the static strain periods to adequately respond to the mechanical stimulus. To study the underlying mechano-regulatory mechanism of intermittent conditioning, we need to know how the cells within a TE construct respond to mechanical conditioning. One way to investigate this is by determining gene expression levels during and following cyclic strain. Understanding the mechano-regulatory mechanisms of strain-induced tissue development will benefit heart valve tissue engineering by providing conditioning protocols for optimized collagen production, organization, remodeling, and maturation within the tissues, which is one of the primary objectives in the development of functional TE replacements for load-bearing tissues (Webb *et al*., 2006).

Here, we aim to study the (immediate) effects of (continuous) cyclic strain at an early stage of heart valve TE and to study the effect of static strain after cyclic strain to obtain insights in the underlying mechanism of intermittent conditioning. After one week of static culture, TE constructs, consisting of human vascular-derived cells seeded onto rapidly degrading PGA/P4HB scaffolds, were cyclically strained for 3 hours (n=18). Next, the constructs were either subjected to continuous cyclic strain (n=9) or to static strain (n=9). Expression levels of TGFβ and genes involved in collagen synthesis, remodeling, and maturation were studied at different time points upon the start of cyclic strain. The immediate effect of cyclic strain was studied after 3 hours of cyclic straining, while the effect of continuous cyclic strain and the effect of static strain after cyclic strain were studied up to 24 hours upon the onset of mechanical conditioning.

5.2 Materials & Methods

5.2.1 Cell isolation and expansion

Vascular-derived cells were harvested from a piece of the human vena saphena magna obtained with consent from an anonymous donor and according to the Dutch guidelines for secondary use of materials. Cells were isolated and expanded using standard culture methods (Schnell *et al*., 2001; Mol *et al*., 2006) in a humidified atmosphere containing 5% CO2 at 37°C. These cells have previously been characterized as myofibroblasts with expression of vimentin, but not desmin, and a subpopulation of the cells expressed α-smooth muscle actin (αSMA) (Mol *et al*., 2006; van Geemen *et al*., 2012b). Culture medium consisted of advanced Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Breda, The Netherlands), supplemented with 10% Fetal Bovine Serum (FBS; Greiner Bio

one, Alphen a/d Rijn, The Netherlands), 1% GlutaMax (Gibco), and 1% penicillin/streptomycin (Lonza, Basel, Switzerland).

5.2.2 Engineered heart valve constructs

Rectangular scaffolds (24x4x1 mm), composed of rapidly degrading non-woven polyglycolic acid (PGA; thickness, 1.0 mm; specific gravity, 70 mg/cm3; Cellon, Bereldange, Luxembourg), were coated with poly-4-hydroxybutyrate (P4HB; obtained within a collaboration with prof. S.P. Hoerstrup, University Hospital Zurich) to provide structural integrity to the mesh (Hoerstrup *et al*., 2000a). After drying, the bottom surface of the scaffolds was reinforced with a non-toxic elastic silicone layer (Silastic MDX4-4210; Dow Corning, Midland, MI; thickness 0.5 mm), enabling precisely controlled cyclic deformation of the constructs up to several weeks (Boerboom *et al*., 2008). Three scaffolds per well were attached in longitudinal direction to the flexible membranes of six-well plates (Flexcell Int., Dunn Labortechnik, Germany) using Silastic MDX4-4210. By attaching the scaffolds at their outer ends, the tissues were constrained in the longitudinal direction, serving as static strain.

The scaffolds were vacuum-dried for 48 hours, followed by sterilization. First, the scaffolds were exposed for one hour to ultraviolet light, followed by 30 minutes incubation in an antibiotics solution (10% penicillin/streptomycin (Lonza) and 0,5% Fungin (Cayla - InvivoGen Europe, Toulouse, France) in sterile Phosphate Buffered Saline (PBS; Sigma, Zwijndrecht, The Netherlands)) and two times 15 minutes incubation in 70% ethanol. A day before seeding, tissue engineering medium (TE medium; culture medium supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma)) was added to facilitate cell attachment. The scaffolds were seeded with passage 7 vascular-derived cells using fibrin as a cell carrier (Mol *et al*., 2005). In short, the vascular-derived cells were suspended in TE medium containing thrombin (10 IU/ml, Sigma). Subsequently, this cell suspension was mixed with an equal volume of TE medium containing fibrinogen (10 mg/ml, Sigma). This fibrin/cell suspension ($15*10^6$ cells/ml) was mixed until the onset of polymerization of the gel to seed the cells into the scaffold. Medium was changed every 2-3 days.

5.2.3 Experimental design

The experimental design is illustrated in figure 5.1. After one week of culture under static strain to start tissue formation, the tissues in six wells were sacrificed to serve as control (t=0). The remaining wells with engineered heart valve constructs were subjected to uniaxial cyclic strain for 3 or 24 hours using a Flexercell FX-4000T straining device (Flexcell) to study (A) the immediate effects of cyclic strain (after 3 hours) and the effects of (B) continuous cyclic strain or (C) static strain after cyclic strain. A strain magnitude of 4% and a frequency of 1 Hz were chosen as a suitable strain condition

based on previous studies (Boerboom *et al*., 2008; Rubbens *et al*., 2009b). The samples (n = 3 wells per time point) were sacrificed at 1.5, 3, 4.5, 6, 9, and 24 hours after the onset of cyclic strain by snap freezing and were processed for gene expression analysis. The constructs in one well were pooled to serve as n = 1. The experiments were performed three times resulting in n = 18 for each time point within 0 – 3 hours, to study the immediate effect of cyclic strain, and n = 9 for each time point within 3 – 24 hours, to study the effects of both continuous cyclic strain and static strain after cyclic strain.

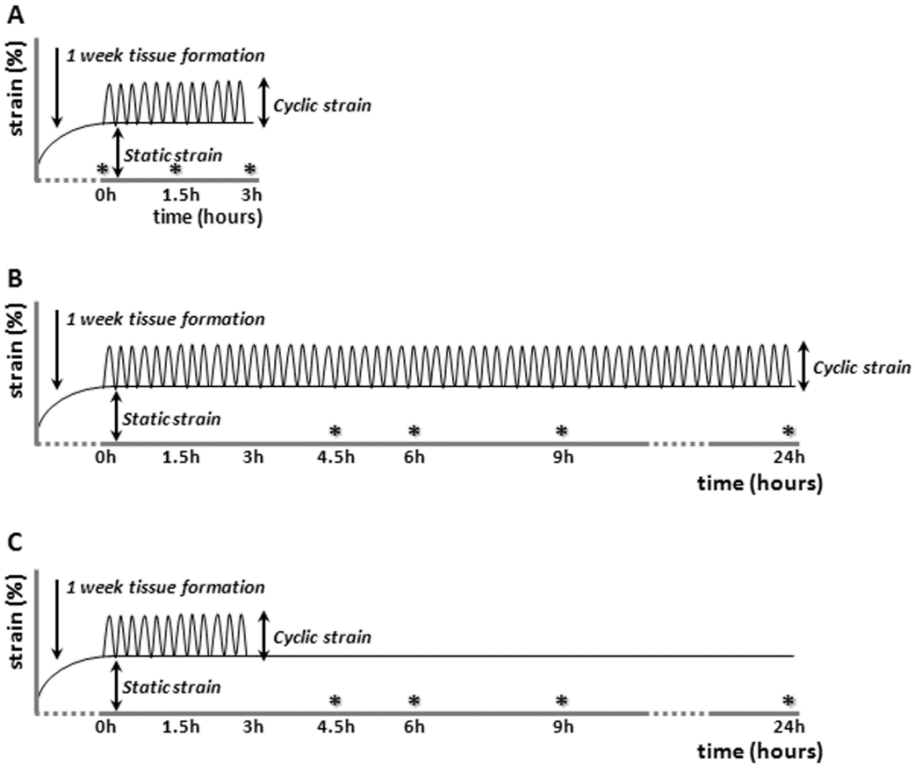

Figure 5.1: *Schematic overview of the experimental design. After one week of static strain to enhance tissue formation, the tissue-engineered (TE) constructs are cyclically strained (4% at 1 Hz) for 3 hours to study the immediate effect of cyclic strain (A). Thereafter, the TE constructs are subjected either to continuous cyclic strain (B) or static strain (C) to study the effect of ongoing cyclic strain and static strain after cyclic strain, respectively. The asterisks indicate the time points at which the TE constructs are sacrificed for gene expression analysis.*

5.2.4 Strain validation

The strain fields at the surface of the engineered constructs were validated using digital image correlation in a similar manner as described before (Boerboom *et al*., 2008). In short, after one week of static strain, a random dot pattern was sprayed onto the engineered heart valve constructs. The constructs (three per well) were subjected to uniaxial cyclic strain (4%, 1 Hz). During cyclic strain, images of the deformed state were recorded at 60 frames per second using a color high-speed camera (MotionScope M5C; IDT, Tallahassee, USA). The strain fields in the recorded images were analyzed using Aramis DIC software (Gom mbh., Germany).

5.2.5 Gene expression analysis

The snap frozen constructs were homogenized with a micro-dismembrator and lysed with β-mecaptoethanol in RLT buffer to minimize RNA degradation. RNA was isolated with the Qiagen RNeasy extraction kit according manufacturer's protocol (Qiagen, Venlo, The Netherlands). The concentration of RNA was determined spectrophotometrically (NanoDrop ND1000, Isogen Life Science, IJsselstein, The Netherlands). Subsequently, synthesis of cDNA was carried out with 500 ng of RNA in a 25 µl reaction volume consisting of dNTPs (Invitrogen), random primers (Promega, Madison, WI), DTT (Invitrogen), M-MLV (Invitrogen), M-MLV buffer (Invitrogen) and ddH_2O. Control reactions without M-MLV (-RT) were performed to screen for genomic DNA contamination.

Gene expression levels of genes involved in collagen synthesis (table 5.1; collagen type I (α1), collagen type III (α1), collagen type V (α1), alpha smooth muscle actin (αSMA), and decorin), collagen remodeling (table 5.1; matrix metalloproteinase (MMP)-1, MMP2), collagen maturation (table 5.1; periostin and the cross-link enzymes procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2) and lysyl oxidase (LOX)), and transforming growth factor β1 (TGFβ1) were measured. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were chosen as reference genes, as determined by the geNorm algorithm described by Vandesompele *et al.* (Vandesompele *et al*., 2002). A customized 384-well PCR array with the above mentioned genes and reference genes (SABiosciences, Qiagen) was used to perform real time PCR (RT-PCR). The PCR reaction (CFX384 Touch Real-Time PCR Detection System, Bio-Rad, Veenendaal, The Netherlands) was performed by adding SYBR Green Supermix (Bio-rad), ddH_2O, and cDNA to the customized PCR array. The temperature profile was 3 min at 95°C, 40 x (20 sec at 95°C, 20 sec at 60°C, 30 sec at 72°C), 1 min at 95°C, 1 min at 65°C, followed by a melting curve analysis.

Group	Genes	References
Synthesis	Collagen type I (α1) Collagen type III (α1) Collagen type V (α1) α Smooth muscle actin (αSMA) Decorin	Peacock et al. (2008) Votteler et al. (2010) Geemen et al. (2012) Reed et al. (2002)
Remodeling	Matrix metalloproteinase 1 (MMP1) Matrix metalloproteinase 2 (MMP2)	Stamenkovic (2003)
Maturation	Periostin Procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2) Lysyl oxidase (LOX)	Norris et al. (2009) Snider et al. (2008) Balguid et al. (2007)

Table 5.1: *Representation of the different genes involved in the groups 'collagen synthesis', 'collagen remodeling', and 'collagen maturation'.*

5.2.6 Data analysis

Relative expression levels were normalized to the geometric mean of both reference genes and the control group (t=0) and provided as mean ± standard error of the mean. Correlations between TGFβ, collagen synthesis, collagen remodeling, and collagen maturation genes and the effects of conditioning in time were analyzed with correlation matrices. A level of $p < 0.05$ was used to indicate significance. The strength of a correlation within and between groups of genes for collagen synthesis, remodeling, maturation and TGFβ were scored by their level of significance and the number of correlations involved in one group. The correlation strength was expressed as percentage of maximal correlation strength. When this correlation strength exceeded 50%, the groups of genes were considered to linked, indicative of coordinated expression within or between the groups of genes. The degree of coordinated expression of one or more groups of genes is expressed as ++ (75-100% correlation strength), + (50-75% correlation strength, and – (<50% correlation strength). GraphPad Prism software (GraphPad Software, Inc, USA) was used for the statistical analysis.

5.3 Results

5.3.1 Strain validation

The strain fields showed an inhomogeneous distribution during cyclic strain throughout each construct, but with a similar strain distribution in each of the three constructs in one well (figure 5.2). The average measured strains were approximately 4%. Therefore, we considered the strain application to all constructs in one well reproducible, enabling to study the effects of mechanical conditioning on gene expression.

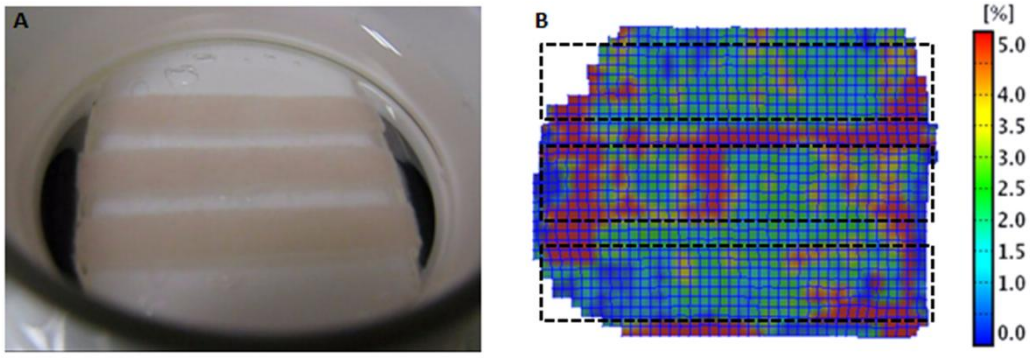

Figure 5.2: *(A) Three tissue-engineered (TE) constructs within one well of a six-well plate. (B) Strain distribution during cyclic strain throughout the TE constructs. The TE constructs are indicated by the black dotted lines. The strain fields during cyclic strain are inhomogeneously distributed throughout the TE constructs, but similarly in all three TE constructs within one well. An average of 4% strain was measured.*

5.3.2 Immediate effect of cyclic strain

An overview of the normalized gene expression levels is provided in figure 5.3. All significant correlations between the genes and with time during the first 3 hours of cyclic strain are indicated in the correlation matrix in figure 5.4A. Gene expression of collagen type I, type III, type V, αSMA, decorin, TGFβ1, and periostin are all downregulated in time. MMP2, PLOD2, and LOX expression are not affected by time and MMP1 gene expression is upregulated in time. The correlation strengths between the groups of genes and time during the first 3 hours of cyclic strain are shown in table 5.2A. The synthesis genes are downregulated in time. The synthesis genes are positively correlated to each other, indicative of coordinated collagen synthesis, while the remodeling and the maturation genes are not correlated to each other during the first 3 hours of cyclic strain. TGFβ correlated with the synthesis genes, but not with the remodeling and maturation genes.

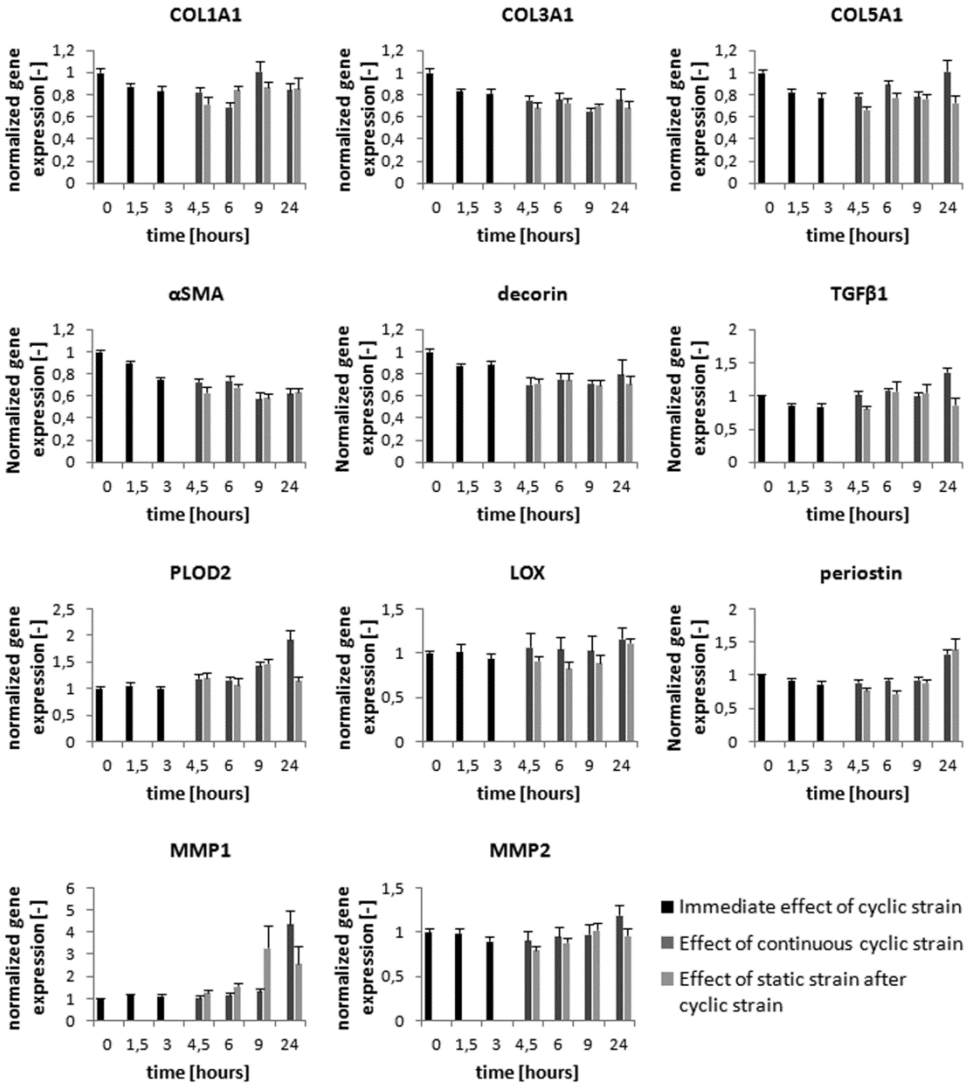

Figure 5.3: *Normalized gene expression levels of the genes involved in collagen synthesis (COL1A1, COL3A1, COL5A1, αSMA, decorin), remodeling (MMP1, MMP2), maturation (PLOD2, LOX, periostin), and TGFβ1.*

Effect of continuous cyclic strain

The normalized gene expression levels are indicated in figure 5.3 and the significant correlations between all genes and with time during continuous cyclic strain are indicated in figure 5.4B. Collagen type V, MMP1, MMP2, TGFβ, PLOD2, and periostin gene expression increases with time, while αSMA expression decreases with time. Collagen type I, type III, LOX, and decorin expression are not affected by time. The remodeling and maturation genes are upregulated with time (table 5.2B). During continuous cyclic strain, the remodeling genes and maturation genes were strongly correlated within and between the groups of genes, indicative for simultaneous coordinated collagen remodeling and maturation (table 5.2B). TGFβ expression was correlated to both collagen remodeling and collagen maturation.

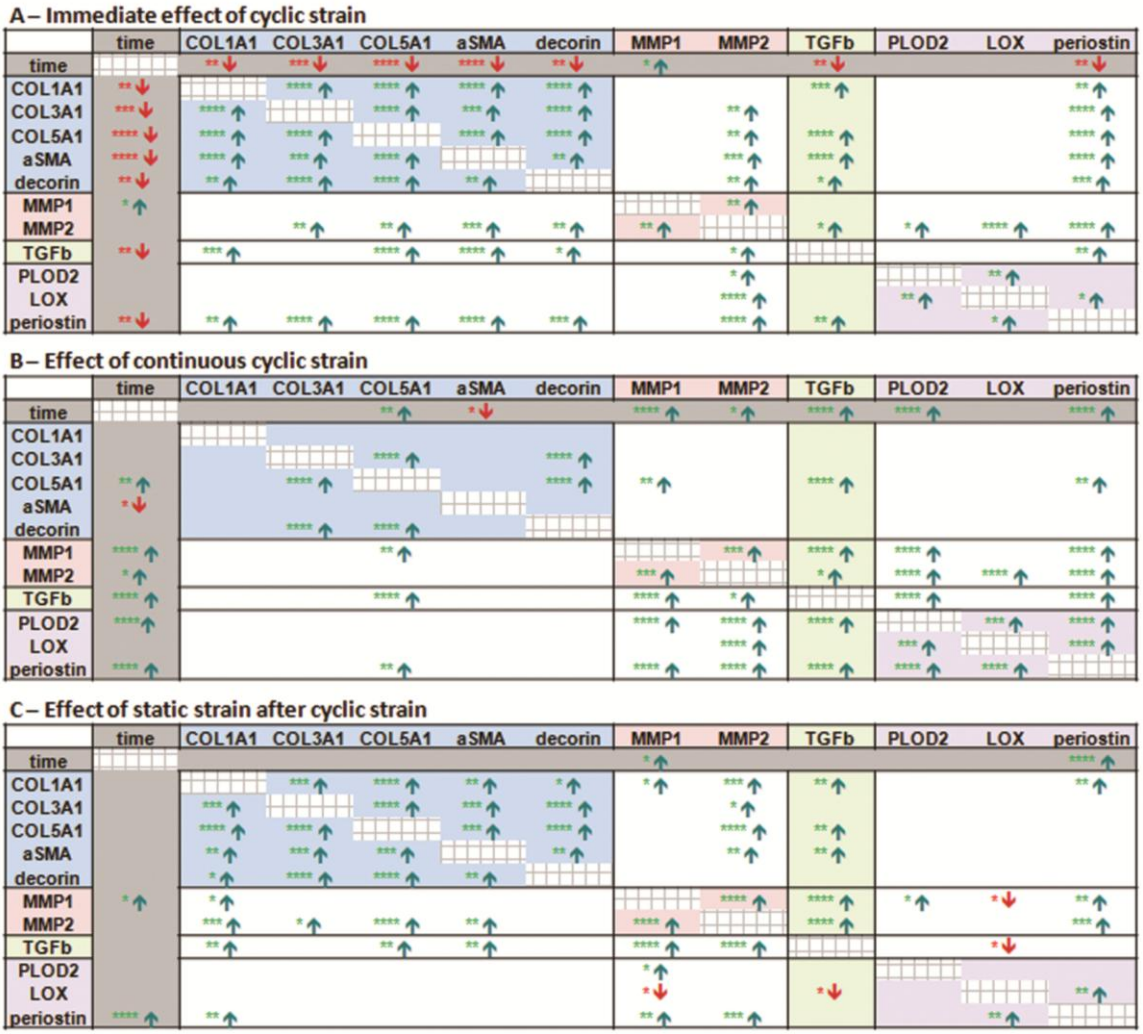

Figure 5.4: *Correlations with time and between all genes during the first 3 hours of cyclic strain (A), further in time with continuous cyclic strain (B) and during static strain after cyclic strain (C). The collagen synthesis genes are in blue, collagen remodeling genes in pink, collagen maturation genes in purple, and TGFβ in green. Correlation of the genes with time is indicated in grey. The green arrows specify a positive correlation, while the red arrows indicate a negative correlation. The p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.*

A – Immediate effect of cyclic strain				
	Time	**Synthesis**	**Remodeling**	**Maturation**
Synthesis	++ (↓)	++ (↑)	-	-
Remodeling	-	-	-	-
Maturation	-	-	-	-
TGFβ	+ (↓)	+ (↑)	-	-

B – Effect of continuous cyclic strain				
	Time	**Synthesis**	**Remodeling**	**Maturation**
Synthesis	-	-	-	-
Remodeling	+ (↑)	-	++ (↑)	++ (↑)
Maturation	+ (↑)	-	++ (↑)	++ (↑)
TGFβ	++ (↑)	-	+ (↑)	+ (↑)

C – Effect of static strain after cyclic strain				
	Time	**Synthesis**	**Remodeling**	**Maturation**
Synthesis	-	++ (↑)	-	-
Remodeling	-	-	++ (↑)	-
Maturation	-	-	-	-
TGFβ	-	-	++ (↑)	-

Table 5.2: *Correlation strengths of groups of genes during the first 3 hours of cyclic strain (A), further in time with continuous cyclic strain (B), and with static strain after cyclic strain (C). Collagen synthesis and TGFβ are downregulated in the first 3 hours of cyclic strain. Further in time, collagen remodeling and maturation are upregulated with continuous cyclic strain. When static strain is applied after cyclic strain, expression was affected with time. TGFβ is only upregulated further in time with continuous cyclic strain. In the first 3 hours of cyclic strain, collagen synthesis is coordinated and TGFβ expression is linked to collagen synthesis. Further in time, collagen remodeling and maturation are highly coordinated and TGFβ expression is linked to both. During static strain after cyclic strain, collagen synthesis and remodeling are coordinated and TGFβ expression is linked to collagen remodeling.*

5.3.4 Effect of static strain after cyclic strain

In figure 5.3 the normalized gene expression levels are provided and in figure 5.4C the significant correlation between all genes and time during static strain after cyclic strain are indicated. MMP1 and periostin expression increased in time, while all other genes are not affected by time. During static strain, the synthesis genes and remodeling

genes are correlated to each other, indicative for coordinated collagen synthesis and remodeling (table 5.2C). The maturation genes are not related, and also no relations between collagen synthesis, remodeling, and maturation are observed. TGFβ expression is correlated to collagen remodeling during static strain after cyclic strain.

5.4 Discussion

It is hypothesized that intermittent conditioning improves the maturity of the engineered tissue by balancing collagen production during static strain and enhancing collagen cross-links and alignment during cyclic strain (Rubbens *et al*., 2009a; Rubbens *et al*., 2009b). However, the underlying regulatory mechanism for this hypothesis is not clear. It might be that the cells need the static strain period to (adequately) respond to the mechanical stimulus. Therefore, the aim of this study was to determine the immediate effect of cyclic strain and the effect of continuous cyclic strain versus the effect of static strain after cyclic strain on collagen matrix-related gene expression to obtain insights in the mechano-regulatory mechanism of intermittent conditioning.

Although physiological stains in the native heart valve are higher (Thubrikar *et al*., 1980), previous studies in our group have indicated that 4% strain is optimal for engineering heart valve constructs (Boerboom *et al*., 2008). Therefore, in this study TE constructs were cyclically strained with 4% strain at 1 Hz and the expression of several genes involved in collagen synthesis, remodeling, and maturation was studied at various time points up to 24 hours after cyclic strain and compared to expression levels at t = 0.

Collagen type I, type III, type V, αSMA, and decorin were studied representing genes involved in collagen synthesis. These collagens were chosen as these are the major types of collagen in heart valves (Peacock *et al*., 2008; Votteler *et al*., 2010). αSMA was chosen as important marker for the myofibroblast phenotype (Hinz *et al*., 2001; Eyden, 2008). Vascular derived myofibroblasts, typically used in heart valve TE, express αSMA, synthesize abundant ECM for functional tissue growth, and are characterized as a developing/remodeling/activated phenotype (Rabkin-Aikawa *et al*., 2004; Aikawa *et al*., 2006). In our previous work, we described a positive correlation between αSMA and collagen (van Geemen *et al*., 2012a), therefore, αSMA was chosen as part of the collagen synthesis group of genes. Decorin, one of the proteoglycans found in heart valves, is part of the collagen synthesis group of genes, as it ‘decorates’ the surface of collagen fibril, which is essential in collagen fiber formation (Reed and Iozzo, 2002). MMP1 and MMP2 were studied as markers for collagen remodeling. MMPs play an important role in collagen remodeling by controlling cellular interactions with and in response to their environment (Stamenkovic, 2003). MMP1 has the ability to cleave collagen fibrils to produce denatured collagen. In turn, the breakdown of denatured collagen occurs through MMP2. Hereby, MMPs promote collagen turnover and remodeling. PLOD2, LOX,

and periostin were studied representing genes involved in collagen maturation. Collagen cross-links play an important role in tissue maturation and are crucial for providing mechanical integrity (Balguid *et al*., 2007) PLOD2 and LOX are enzymes involved in collagen cross-linking. PLOD2 is specific for collagen cross-linking, while LOX is also associated with elastin cross-linking (Kagan, 2000; Bank and van, V, 2002). The cross-links are formed following hydroxylation of the telopeptides of collagen. PLOD2 has been identified as a telopeptide lysyl hydroxylase, which is responsible for this hydroxylation process (van der Slot *et al*., 2003). Periostin regulates type I collagen accumulation and maturation (Norris *et al*., 2009), and is involved in ECM maturation and stabilization (Snider *et al*., 2008). TGFβ is involved in matrix production during development, wound healing, and a variety of pathological fibrotic disorders (Frazier *et al*., 1996; Lawrence, 1996; Branton and Kopp, 1999). Additionally, TGFβ is a primary inducer of fibroblast-to-myofibroblast differentiation (Desmouliere *et al*., 1993; Ronnov-Jessen and Petersen, 1993). Furthermore, TGFβ is released in response to mechanical load to stimulate cell proliferation, protein synthesis, and matrix orientation to strengthen TE constructs (Bishop and Lindahl, 1999; Webb *et al*., 2006). As TGFβ is involved in collagen synthesis, remodeling, and maturation, this gene was not categorized in one of the above-mentioned groups (e.g. synthesis, remodeling, maturation), but analyzed separately.

Although, several studies describe an increase in collagen synthesis after mechanical stimulation (reviewed by Bishop and Lindhal (Bishop and Lindahl, 1999)), we observed that the expression of collagen synthesis related genes is downregulated in time as an immediate effect of cyclic strain. A slight decrease in collagen gene expression (Kuo and Tuan, 2008) and decorin expression (Lee *et al*., 2001a) after one day of cyclic strain was also observed previously. Lee *et al.* suggested that the strain induced decorin decrease could promote reorganization of the collagen network by loosening of the ECM, as decorin binds to collagen and regulate collagen synthesis (Lee *et al*., 2001a). This implies that a correlation between all collagen synthesis related genes might be necessary for an improved ECM architecture. During the first three hours of cyclic strain, the expression of collagen synthesis related genes are indeed linked, which indicates coordinated collagen synthesis regulation. When the cyclic strain is continued, the collagen synthesis related genes are not correlated to each other anymore, while they remained correlated when static strain is applied after 3 hours of cyclic strain. This suggests that static strain after cyclic strain is required for coordinated collagen synthesis regulation.

The expression of collagen remodeling genes is not affected in the first three hours of cyclic strain and also not during static strain after cyclic strain. When cyclic strain is continued, the expression of these genes is upregulated in time. The expression of collagen remodeling related genes are correlated during continuous cyclic strain and during the static strain after cyclic strain, indicating coordinated collagen remodeling regulation. Balachandran *et al.*, Kuo and Tuan, and Powel *et al.* also observed an

increase in MMP1 after mechanical stimulation (Kuo and Tuan, 2008; Balachandran *et al*., 2009; Powell *et al*., 2010). The strain induced increase in MMP expression will lead to local degradation of the collagen fibrils as cells reorganize the ECM network (O'Callaghan and Williams, 2000). Therefore, it is preferred that collagen remodeling is accompanied by collagen synthesis and/or maturation to prevent the remodeling shift towards collagen degradation only and subsequent tissue deterioration. Our results indicate that during the static strain after cyclic strain, collagen synthesis is coordinated, while during continuous cyclic strain collagen remodeling and collagen maturation are coordinated.

The expression of the collagen maturation related genes is not affected during the first three hours of cyclic strain. When the constructs are continuous cyclically strained, the expression of these genes is upregulated in time and these genes are correlated to each other, suggesting coordinated collagen maturation during continuous cyclic strain. However, during static strain after cyclic strain, the genes are not linked and the expression of these genes is not affected in time. Previous studies in our group also showed that cyclic strain is required to increase collagen cross-link formation (Balguid *et al*., 2007; Rubbens *et al*., 2009b). In addition, Rios *et al.* and Wen *et al.* observed that periostin increased by cyclic strain (Rios *et al*., 2008; Wen *et al*., 2010). This suggests that continuous cyclic strain is necessary for collagen maturation, since these genes are only upregulated and correlated during continuous cyclic strain.

TGFβ is involved in collagen synthesis, remodeling, and maturation. In response to mechanical conditioning, TGFβ is released to stimulate cell proliferation, protein synthesis, and matrix orientation (Bishop and Lindahl, 1999; Webb *et al*., 2006). In 2D, fibroblasts secrete TGFβ even after 15 minutes of cyclic strain (Skutek *et al*., 2001). However, in this 3D study TGFβ expression decreased during the first 3 hours of cyclic strain and correlated with collagen synthesis in this early conditioning phase. During continuous cyclic strain, TGFβ was subsequently upregulated in time and correlated to collagen remodeling and maturation. During static strain after cyclic strain, TGFβ was only correlated to collagen remodeling. These results suggest that TGFβ indeed regulates collagen synthesis, remodeling, and maturation depending on the phases of mechanical conditioning.

Already in 1999, Chiquet described that very little is known about the pathway from a mechanical stimulus to alter ECM gene expression (Chiquet, 1999). However, hardly any additional knowledge is obtained since then. Only a couple studies are available that link the extracellular signal-regulated kinase (ERK) signaling pathway to cyclic strain (Papakrivopoulou *et al*., 2004; Syedain *et al*., 2008). Nevertheless, the exact role of ERK is not known. Additionally, in 2008, Butcher *et al.* stated that it is important to study how gene expression levels are changed with mechanical conditioning, as this might reflect how cells react upon a stimulus (Butcher *et al*., 2008). In this study, gene expression results were used to determine the short-term effects of (continuous) cyclic

strain and the effect of static strain after cyclic strain to unravel the underlying regulatory mechanism of intermittent conditioning. Our gene expression results show that the immediate effects of cyclic strain differ from the effects of continuous cyclic strain. Continuous cyclic strain is demonstrated to be important for collagen remodeling and maturation, while during short-term cyclic strain only collagen synthesis is coordinated. Static strain after cyclic strain is important for the coordination of collagen synthesis and remodeling, but is not preferred for collagen maturation. These results imply that during intermittent conditioning, static strain after cyclic strain is necessary for collagen synthesis and remodeling, and that continuous cyclic strain is needed for shifting the balance from collagen synthesis and remodeling to collagen remodeling and maturation. This might suggest that the mechanical conditioning protocol should change from intermittent conditioning (for coordinated collagen synthesis and remodeling) towards continuous cyclic strain (for coordinated collagen remodeling and maturation) during the culture period of TE heart valves to first allow for sufficient collagen synthesis, where after collagen maturation is stimulated to improve the resulting tissue properties. Future studies are necessary to determine the optimal length of static strain and the effects of subsequent a cyclic strain trigger after static strain before the current TE protocols can be optimized. Furthermore, the results obtained in this study should be verified at the protein level to see if matrix composition, maturation, and architecture and, therewith, the mechanical properties will improve. In addition, the underlying mechano-regulatory pathways are still not completely known. Most studies investigate strain induced effects on cell behavior and tissue formation on trial-and-error basis, while understanding of the mechano-regulatory pathways can result in precisely controlled mechanical conditioning regimes. Nevertheless, the method in this study provides insights into the mechano-regulatory mechanism of intermittent conditioning and how to optimize the current conditioning protocols to optimize the tissue properties in heart valve tissue engineering.

Acknowledgments

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Chapter 6

General discussion

Worldwide, valvular heart disease is a major health problem causing morbidity and mortality (Lloyd-Jones *et al*., 2009). To avoid serious cardiac, pulmonary, or systemic problems, heart valve replacement is frequently applied, resulting in approximately 285,000 heart valve replacements annually worldwide (Mikos *et al*., 2006). Existing heart valve replacements do not consist of living tissue and, as a consequence, cannot grow, repair, or remodel in response to changing functional demands. This is a major limitation, suggesting that the development and implantation of a living valve is of utmost importance. Especially, since a living valve can significantly improve life expectancy and quality of life (El-Hamamsy *et al*., 2010), living properties are key to the development of new, durable heart valve prostheses. Tissue engineering (TE) seeks to overcome this limitation by creating living autologous heart valves. One of the main challenges of TE is to control tissue formation and collagen remodeling, and hence tissue mechanical properties, during the *in vitro* culture phase. Moreover, it is important to find benchmarks that predict whether tissue structure and mechanical properties of TE heart valves are good enough for implantation and long-term functioning in patients. Tissue (mechanical) properties of native human valves should be used as such benchmarks. The formation of a proper collagen architecture is essential to improve the load-bearing properties of TE heart valves. The aim of this thesis is to understand and optimize tissue development and resulting tissue mechanical properties of TE heart valves, with special emphasis on collagen remodeling in the heart valve leaflets. The main findings of the present thesis will be summarized and discussed in this chapter.

6.1 Main findings of the thesis

To evaluate and optimize TE heart valves, matrix composition, maturation and mechanical properties of native human aortic and pulmonary heart valves were studied (chapter 2). This native data gives insight in the evolution and maturation of the matrix and mechanical properties of the valvular tissue and provides benchmarks for TE heart valve leaflets. The matrix composition of the aortic and pulmonary valves changes with age. In particular in the aortic valve the hydroxyproline content increases with age, while the glycosaminoglycan (sGAG) content decreases with age. The valves also become stiffer with age, according to an increasing E-modulus with age as measured with indentation and biaxial tensile tests. For the aortic valve leaflets, this increase in E-modulus, calculated from the indentation tests, correlates to the increase in hydroxyproline content and the increase in collagen cross-links. In the pulmonary valve, the increase in E-modulus correlates with the decrease in sGAG content and the increase in collagen cross-links. Thus, a significant part of the mechanical behavior of human native semilunar heart valves leaflets is defined by the composition and maturation of the matrix. This suggests that long-term *in vivo* functionality of TE heart valves leaflets

might be optimized by improving matrix composition and maturation towards those of native heart valves. Nevertheless, as long as the TE leaflets can perform their mechanical function and withstand the transvalvular pressures at the time of implantation, these properties could also be reached by remodeling of the living implant towards native tissue *in vivo*. In addition, the tissue (mechanical) properties of the valvular root and the development of the sinuses are expected to be important in defining the functionality of the TE heart valves. Future studies are, therefore, necessary to study the *in vivo* remodeling of TE heart valve leaflets, wall and sinuses towards their native counterparts.

To optimize the load-bearing properties at time of implantation and to minimize the time required for *in vivo* remodeling towards native heart valves after implantation, we need to improve tissue composition, maturation and mechanical properties of the TE heart valves already during the *in vitro* culture process. Therefore, we tried to determine possible indicators of *in vitro* tissue outcome and to evaluate the interspecies differences in tissue mechanical properties to determine whether ovine TE heart valves are representative for human TE heart valves (chapter 3). To this end, we engineered TE constructs using cells from different sheep (n=8) and patients (n=7). After 4 weeks of tissue culture, tissue properties of all ovine TE constructs were comparable, while there was variation in the properties of the human TE constructs, in particular for the mechanical properties and collagen content. When comparing ovine and human TE constructs, only interspecies differences in the matrix composition were observed. Ovine TE constructs contained more DNA, more sGAG, and less hydroxyproline compared to the human TE constructs, while mechanical properties were similar. The results obtained in this study suggest that the culture process of ovine tissues can be controlled, whereas the mechanical properties, and hence functionality, of tissues originating from human material are more difficult to predict due to the interpatient variability. On-line evaluation of tissue properties during culture or early markers to predict the properties of autologous tissues cultured for individual patients are, therefore, of utmost importance for future clinical application of autologous heart valve tissue engineering. αSMA might be such an indicator. The number of αSMA-positive cells before seeding was positively correlated with the hydroxyproline content of the TE constructs cultured from these cells. In addition, the stiffness of the TE constructs increased with increasing hydroxyproline content. Thus, there is indirect evidence that TE constructs seeded with more αSMA-positive cells – and hence a more remodeling/activated phenotype (Rabkin-Aikawa *et al*., 2004) – become stiffer. Therefore, variations in αSMA may be used as an early marker to predict differences in tissue outcome related to collagen content and maybe even tissue mechanical properties.

As a further step to clinical application and to reduce animal-derived medium components in the culture protocol, fetal bovine serum was replaced with human platelet lysate for the culture of autologous TE heart valve constructs (chapter 4).

Platelet lystate has a different subset of growth factors and chemokines to stimulate tissue formation compared to fetal bovine serum. Previous 2D studies showed promising results, in terms of matrix production and remodeling capacity, for the culture of heart valve constructs in medium supplemented with human platelet lysate (Riem Vis *et al*., 2010). Nevertheless, chapter 4 showed that these 2D studies do not predict the outcome of our 3D studies. Although, tissue composition (sGAG and hydroxyproline content) and maturation (number of collagen cross-links) were similar in TE constructs cultured with fetal bovine serum or human platelet lysate, the mechanical properties of the TE constructs cultured in human platelet lysate were less and probably not sufficient enough for *in vivo* functionality. Collagen synthesis was similar in both groups, however, collagen remodeling towards degradation was higher in the platelet lysate group. This has probably led to an altered collagen architecture, which was not as dense and with shorter fibers as the collagen architecture observed in the TE constructs cultured in fetal bovine serum. Thus, collagen architecture, rather than collagen content alone, is dominant in defining the tissue mechanical properties. In our search for the optimal autologous culture conditions, systematic studies on medium composition are necessary, obviously in combination with other external cues, such as mechanical conditioning.

Mechanical conditioning can be used to stimulate tissue formation and maturation towards a proper collagen architecture for *in vivo* mechanical functionality. Previous studies indicated that an intermittent conditioning protocol, in which cyclic strain is alternated with static strain, is favorable over continuous cyclic strain in obtaining strong, mature tissues in a short time period. It is assumed that this intermitted protocol enhances collagen synthesis during static strain and induces collagen cross-link formation during cyclic strain (Rubbens *et al*., 2009b). To unravel the underlying mechanism of intermittent conditioning, the effects of continuous cyclic strain and static strain after cyclic strain were determined at the gene expression level (chapter 5). Continuous cyclic strain appeared relevant for collagen remodeling and maturation, but not for collagen synthesis. Static strain after cyclic strain resulted in coordinated collagen synthesis and remodeling, but was not preferred for coordinated collagen maturation. The results of this study suggest that when designing mechanical conditioning protocols for improved tissue properties, a period of static strain is required for collagen synthesis and remodeling, while continuous cyclic strain is shifting this balance towards collagen remodeling and maturation. In addition, the obtained results suggest that the mechanical conditioning protocol should change during culture from intermittent conditioning towards continuous cyclic strain in time to improve collagen maturation after its synthesis and, therewith, the mechanical tissue properties of tissue-engineered heart valves.

Overall, the results from this thesis suggest that collagen organization, rather than collagen content and maturation, is the main determinant of tissue mechanical

properties. Thus, optimization of culture protocols should focus on obtaining the appropriate architecture (i.e. collagen content, maturation, and organization) for creating mechanically functioning TE heart valves. Autologous culture of TE heart valves using platelet lysate as a serum replacement is not useful, since it precludes the formation of a strong, load-bearing valve with organized, long collagen fibers, even under (static) strain. With respect to the culture medium, additional studies for the design of an autologous medium or defined synthetic medium are therefore required.

Our gene expression studies indicate that mechanical conditioning protocols to achieve the right collagen architecture should start with intermittent conditioning followed by continuous cyclic strain to enhance collagen maturation after its synthesis. Although this should be verified at the protein and tissue level, it is expected that such protocols will improve matrix architecture and resulting tissue mechanical properties *in vitro* and stimulate remodeling towards native-like heart valves *in vivo*. Considering the inter-patient variability in tissue outcome of TE constructs cultured with similar protocols, however, it must be noted that a further refinement or even personalization of culture protocols might be necessary to take into account individual differences. To this end, markers of tissue outcome are required to predict and modulate individual tissue outcome at an early stage of the culture protocol. αSMA might be an interesting candidate marker for this purpose and a first step towards patient-specific TE.

6.2 In-depth optimization towards native valves

The results obtained in this thesis provide insights to improve the matrix and mechanical properties of TE heart valves. Nevertheless, there are additional aspects that should be studied as well to further understand and improve the remodeling of TE heart valves toward native valves.

6.2.1 Lessons from valvulogenesis and valve development

Several findings in this thesis suggest that the current TE heart valve leaflets have characteristics specific of fetal heart valves, rather than postnatal heart valves. First, approximately half of the cells within the human TE constructs were αSMA-positive (chapter 3), indicating an developing/activated/remodeling phenotype, which has also been observed in fetal valves (Rabkin-Aikawa *et al*., 2004). In native heart valves, only in the leaflets of a very young donor (8 months old) αSMA-positive cells were present (chapter 2). These young native valves are likely to be in a remodeling stage, possibly due to changes in transvalvular pressures or the rapid valvular growth after birth (figure 6.1). Second, the collagen content in TE constructs (chapter 3) or TE heart valves cultured in our group (Mol *et al*., 2006; Kortsmit *et al*., 2009a; Kortsmit *et al*., 2009b)

was much lower compared to native valves. Even the collagen content of the pediatric native heart valves was higher than that of the cultured valves. Third, fetal valves predominantly consist of proteoglycans and sGAG (Aikawa *et al*., 2006), which was also the case for our TE constructs and valves, where more sGAG compared to collagen was observed. Finally, the number of collagen cross-links in the TE constructs was below the native range, which was in correspondence with previous observations in our group (Balguid *et al*., 2007; Balguid *et al*., 2009b). Therefore, the question arises if lessons from valvulogenesis and postnatal valvular development can be used to optimize TE heart valves towards their native counterparts.

Valvulogenesis starts under low pressures and a low heart rate (Stock and Vacanti, 2001). Further maturation of the heart valves takes place under increasing heart rate (Stock and Vacanti, 2001) and increasing transvalvular pressures (Struijk *et al*., 2008). During the same period, the collagen content in fetal valves increases rapidly (Aikawa *et al*., 2006). Merryman *et al.* described that adult valvular interstitial cells respond to local tissue stress by altering cellular stiffness and collagen synthesis, suggesting that transvalvular pressure and collagen synthesis are positively correlated (Merryman *et al*., 2006). However, the increase in collagen content is only related to an increase in transvalvular pressure during the stages of valvulogenesis and postnatal development until adolescence. In the aortic valve, the pressures are increasing until adolescence and in the same time-span the collagen content increases and the sGAG content decreases. In de pulmonary valve, the pressures decrease immediately after birth and remain constant throughout life. This is reflected in a constant collagen content throughout life and a constant sGAG content until adolescence. Thereafter, sGAG content decreases in the pulmonary valve and the collagen increases further in the aortic valve, while the pressures remains constant throughout adult life (figure 6.1). Also a change in E-modulus occurs after adolescence in both the aortic and pulmonary valve. Moreover, the trend in the sGAG decrease and the E-modulus increase during adulthood is more or less similar in the aortic and pulmonary valve. These observations imply that the hemodynamic conditions do not influence the matrix composition and mechanical properties of the healthy heart valves after adolescence, suggesting that ageing, rather than development, plays a role in defining these tissue properties during adulthood.

Thus, a gradual increase in pressure during the culture of TE heart valves, as is part of our current heart valve TE conditioning protocols, should be beneficial for the development of TE heart valves towards native valves. Nevertheless, the load-bearing properties at time of implantation of the current TE heart valves are not yet optimal. In addition, as we would like to minimize the time required for *in vivo* remodeling towards native heart valves after implantation and, thus, optimize the tissue properties already during the *in vitro* culture time, maybe we should use insights from the valve remodeling under pathological circumstances and use non-physiologic mechanical conditioning protocols. For instance, the tissue properties might be optimized by changing the

mechanical conditioning regime from intermittent conditioning to enhance collagen synthesis towards continuous cyclic strain to improve collagen remodeling and maturation (chapter 5).

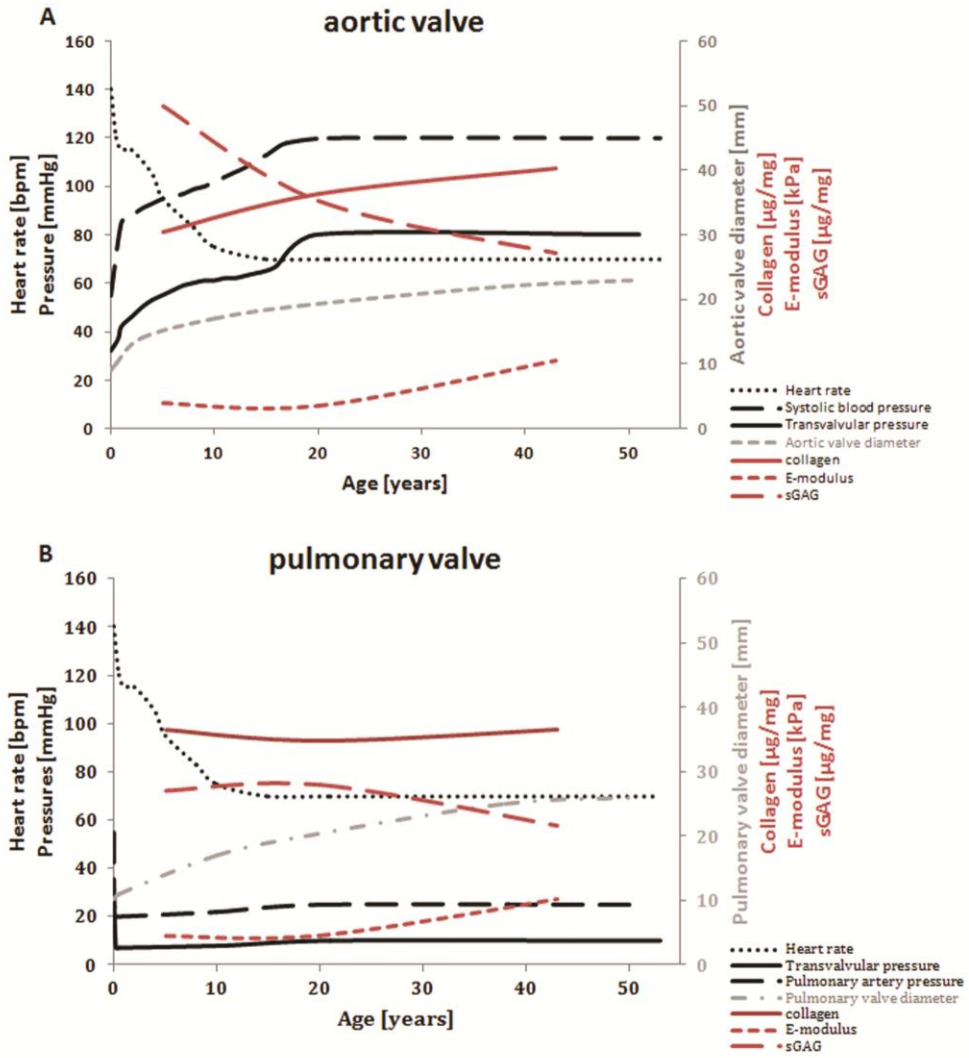

Figure 6.1: *Valve hemodynamics (black), diameter (grey) and matrix composition and mechanical properties (red) during postnatal development in the aortic (A) and pulmonary valve (B). Systolic and transvalvular pressures over the aortic valve increase until adolescence. The pulmonary artery and transvalvular pressure over the pulmonary valve decrease rapidly after birth and remain constant throughout life. Until adolescence, these hemodynamic conditions seem to influence the matrix composition. This is probably not the case during adult life; since the matrix composition changes and the stiffness of the leaflets increases (in both aortic and pulmonary valves) while the hemodynamic conditions remain constant. This suggests that ageing, rather than development, plays a role in defining the tissue properties during adulthood.*

6.2.2 Creating an anisotropic collagen architecture

The mechanical properties of heart valves are anisotropic due to a highly aligned circumferential collagen fiber orientation at the free edge of the leaflets and a more hammock-like structure in the belly. This complex fiber architecture allows higher tissue compliance in radial direction than in circumferential direction (Schoen, 1997), which is essential for its lifelong function. Collagen fibril orientation and thickness varies locally within the fibrosa layer, due to inhomogeneous mechanical demands across the leaflets (Balguid *et al*., 2008). For instance, higher local stresses, i.e. due to applied loading, are associated with larger collagen fibrils (Balguid *et al*., 2008). Perturbations in the collagen architecture might contribute to mechanical failure of bioprosthetic heart valves (Sacks and Schoen, 2002) and blood vessels (Lindeman *et al*., 2010). Thus, an appropriate collagen architecture might accommodate the pressures directly after implantation and ensure long-term mechanical functioning of TE heart valves. Mechanical conditioning protocols or tuning the scaffold to induce a collagen alignment during *in vitro* culture can optimize the TE heart valve architecture.

In tissues, cells have a pivotal role in the maintenance of the collagen architecture. They synthesize collagen and proteolytic enzymes and exert traction forces on the matrix, which likely arrange the synthesized collagen fibers. The collagen orientation may be influenced by contact guidance, in which cells orient themselves in alignment with topographical cues from the substrate or other neighboring cells (Manwaring *et al*., 2004; Neidert and Tranquillo, 2006). In addition, cells and collagen can align in the direction of the applied strain (Lee *et al*., 2008; Nguyen *et al*., 2009). Hence, *in vitro* mechanical conditioning is crucial to enhance collagen orientation and alignment and, therewith, the anisotropic architecture in TE constructs. In chapter 5, we showed that cells with TE constructs react upon mechanical conditioning by influencing collagen synthesis, remodeling, and maturation. Furthermore, previous studies in our group quantified that mechanical conditioning enhanced collagen alignment in TE constructs (Rubbens *et al*., 2009a) and improved the collagen architecture in TE heart valves, leading to anisotropic behavior (Mol *et al*., 2006). Even so, the TE heart valves are not yet sufficient for long-term *in vivo* functionality. Therefore, future studies should determine the collagen organization of the complete leaflet of native heart valves as a benchmark, which can be used to evaluate and optimize collagen fiber organization and hence anisotropy of TE heart valve leaflets.

6.2.3 Focus on the elastic matrix

To improve architecture and tissue mechanical properties, collagen was the primary focus of this thesis, as collagen is the main load-bearing component of the heart valves leaflets. The elastic matrix, the other major structural component in heart valves, acts as a ‘housekeeper’ that restores the collagen fiber configuration to its original state

between successive loading cycles, and is, hence, important for valve function to prevent fatigue of the collagen matrix (Vesely, 1998). In addition, damage to the elastic matrix will alter the mechanical properties by reducing the extensibility and increasing the stiffness of heart valves (Lee *et al*., 2001b), suggesting a role for the elastic matrix in the mechanical functionality of heart valves. Although, efforts within heart valve TE mainly focus on collagen formation, the elastic matrix is recognized as the missing link in cardiovascular TE (Patel *et al*., 2006) and might be necessary to further mimic native mechanical behavior to increase long-term *in vivo* functionality (Mol *et al*., 2009). In *in vitro* TE, the elastic matrix can be enhanced by biochemical conditioning (Long and Tranquillo, 2003; Williams *et al*., 2006; Luo *et al*., 2006; Bashur *et al*., 2012) as well as by mechanical conditioning (Isenberg and Tranquillo, 2003; Gupta and Grande-Allen, 2006; Bing *et al*., 2011; Bashur *et al*., 2012). However, most of these studies are performed with animal or neonatal human cells, while it is still unknown whether all adult human cells can synthesize an elastic matrix. Due to the patient-to-patient differences it might be difficult to synthesize a proper elastic matrix with the cells of all patients. Therefore, these studies should be performed with the cells of multiple donors. In addition, it should be investigated which biochemical or mechanical conditioning protocol leads to an organized elastic network in TE heart valves to improve the tissue mechanical functionality towards native heart valves.

6.3 The future of autologous *in vitro* heart valve tissue engineering

Autologous *in vitro* heart valve TE is a promising strategy to overcome the shortcomings of the current heart valve replacements by creating a living valve substitute. Nevertheless, it is a time consuming and expensive procedure. First, a piece of a vessel needs to be harvested from the patient to isolate cells, which will eventually synthesize the tissue of the TE heart valve. These cells are expanded for several weeks to obtain a sufficient amount of cells to populate a biodegradable scaffold. Next, this cell-scaffold construct is subjected to *in vitro* environmental stimuli for several weeks to enhance tissue formation. When successful, the resulting TE heart valve can be implanted to replace the diseased heart valve. Due to the patient-to-patient variation, patient specific protocols to culture the autologous TE heart valve are assumed to be necessary (see chapter 3). Nevertheless, it will probably take almost two months before an autologous TE heart valve is ready for implantation. This strategy prevents the application of TE heart valves for immediate replacements. These drawbacks limit the future potential of (autologous) TE heart valves and forces researchers to search for alternatives. The relatively new *in situ* TE approach might be a clinically attractive alternative by offering an off-the-shelf availability of TE heart valves. The main challenge is to attract cells into the scaffold or preformed matrix for *in situ* tissue formation and/or

maintenance. Recent studies in our group showed that decellularized TE heart valves might have the potential to offer an off-the-shelf product (Dijkman *et al*., 2012b). In addition, our group recently developed a mesofluidics platform to study the cell-scaffold interactions under hemodynamic conditions *in vitro*, and to screen and develop potential scaffolds for future *in situ* TE approaches (Smits *et al*., 2012). Nevertheless, this approach is still in its infancy and future studies are necessary to elucidate all aspects related to *in situ* TE. Therefore, (autologous) *in vitro* TE heart valves are more likely to be implanted in patients in the near future. In addition, and perhaps more important, the autologous TE strategies serve as the ideal model for (engineered) tissue development and, hence, as a validation model of new TE strategies, such as *in situ* TE. In addition, such a model provides insight into, for instance, 3D cell behavior, tissue formation and remodeling in healthy tissues as well as in different pathologies.

6.3.1 Interpatient variation

It is difficult to predict the *in vitro* tissue outcome of TE constructs cultured with cells from patients due to the large interpatient variation in mechanical properties and tissue composition (chapter 3). Nevertheless, this observed variation likely represent the normal variation in tissue properties, since in chapter 2 we observed a similar variation in the tissue properties of healthy native heart valves. A variation in tissue (mechanical) properties in native heart valves (Balguid *et al*., 2007) and TE heart valves (Kortsmit, 2009) was in our group previously observed. Additionally, in other native and TE cardiovascular tissues, such as blood vessels, an interpatient variability was observed (Konig *et al*., 2009; Dahl *et al*., 2011). This suggests that it is better to study the effects of autologous heart valve TE with cells from multiple donors. On the other hand, in chapter 4 we observed that all TE constructs cultured in fetal bovine serum were stronger and stiffer than the TE constructs cultured in human platelet lysate, thus, the effects of a conditioning regime or stimulus are similar in all donors. Nevertheless, these effects might be influenced by all sorts of co-morbidities, such as diabetes. Therefore, it is better to study the effects of a stimulus or conditioning regime with the cells of multiple donors.

6.3.2 Use of cells

Cells play an essential role in the tissue formation of TE heart valves. They synthesize and secrete matrix proteins, produce proteolytic enzymes, cytokines, and growth factors to remodel the matrix, and exert traction forces on the matrix to arrange its architecture. *In vivo*, the cells within a TE construct are thought to induce an inflammatory reaction by attracting monocytes and macrophages. Roh and coworkers (2010) hypothesize that these infiltrating monocytes produce cytokines and growth factors, which are necessary for cell proliferation and migration and an appropriate

tissue remodeling of the TE constructs towards native structures (Roh *et al*., 2010). The cells used in this thesis are vascular-derived cells and proved to be useful for heart valve TE (Schnell *et al*., 2001; Mol *et al*., 2006). Nevertheless, due to the traction forces and the presence of these cells, they account for approximately 85% of the total tissue retraction (Vlimmeren *et al*., 2012). When the cells are activated, they will even increase the tissue retraction by another 45% (Vlimmeren *et al*., 2012). The cellular activity is probably responsible for the mild to moderate valvular regurgitation observed in *in vivo* studies with TE heart valves cultured with different cell types (Hoerstrup *et al*., 2000a; Sutherland *et al*., 2005; Flanagan *et al*., 2009; Gottlieb *et al*., 2010; Schmidt *et al*., 2010; Syedain *et al*., 2011; Weber *et al*., 2011b).

One method to decrease the tissue retraction in TE heart valves is to decellularize the TE heart valves. In decellularized TE constructs only 15% tissue retraction, caused by passive matrix stress, is observed (Vlimmeren *et al*., 2012). In addition, *in vitro* tests with decellularized TE heart valves show almost no leaflet retraction after 24 hours of exposure to pulmonary conditions (Dijkman *et al*., 2012b). Moreover, the decellularization process did not alter the collagen structure or tissue strength of decellularized TE heart valves (Dijkman *et al*., 2012b) and other engineered cardiovascular structures, such as TE vessels (Dahl *et al*., 2011). Implanted decellularized TE vessels show promising *in vivo* results up to 6 months after implantation as they show cellular infiltration and do not show substantial intimal hyperplasia, dilatation, and calcification (Dahl *et al*., 2011). Implanted decellularized TE heart valves also showed cellular infiltration and excellent *in vivo* performance up to 8 weeks (Dijkman *et al*., 2012a). Nevertheless, after 16 and 24 weeks a reduction in leaflet size, leading to regurgitation, was observed (Dijkman *et al*., 2012a). Additional studies are, therefore, necessary to get more insights into tissue retraction, to search for possible other, less active, cell types, and to elucidate whether cells are needed at the time of implantation.

6.3.3 Tissue models

To fully understand how tissues develop and function in health or disease, it is important to get insights into the cell and tissue behavior within a 3D environment. *In vitro* tissue engineering can serve as such a 3D model. For instance, Choe *et al.* described a TE model of human bronchial mucosa to study physiological and pathophysiological airway remodeling, transepithelial transport, and inflammatory cell interactions with the mucosa (Choe *et al*., 2006). In addition, Song *et al.* showed that engineered heart tissue could serve as a platform for *in vitro* functional screens to provide mechanistic insights into strategies to cure diabetic myopathy (Song *et al*., 2011). Our TE constructs might provide insights into, for example, cell behavior in a 3D environment, cell-matrix interactions, and tissue formation. Apart from this, they might be used as an *in vitro* tissue model of wound healing and cardiovascular pathologies. For instance, our TE constructs cultured in platelet lysate may serve as a tissue model for the remodeling

phase of the wound healing process. Several growth factors (e.g. vascular endothelial growth factor, transforming growth factor-beta, basic fibroblast growth factor), interleukins (e.g. IL17 and IL23), and proteins involved in collagen remodeling (e.g. MMPs, macrophage inflammatory protein-1 alpha, and urokinase) are abundantly present in platelet lysate (Riem Vis *et al*., 2010) and are also involved in the wound healing process (Stadelmann *et al*., 1998; Werner and Grose, 2003). The tissue formed during wound healing, has a disorganized collagen network with increased remodeling capacities, which results in initial weaker tissue. This is similar to the TE constructs cultured in platelet lysate. Therefore, the TE constructs cultured in platelet lysate can be used to get insight into tissue remodeling during the wound healing process. Understanding of this remodeling process towards healthy tissue regeneration might even be used to optimize heart valve TE strategies. Alternatively, the collagen network in the TE constructs cultured in platelet lysate consisted of shorter fibers and was not as dense as the network observed in TE constructs in fetal bovine serum (chapter 4). A similar disrupted collagen architecture was observed in pathologies, such as for vascular aneurysms (Lindeman *et al*., 2010), suggesting that our TE construct might serve as a disease model for studying the development of the collagen architecture and mechanical properties in aneurysms by just changing the culture medium.

The use of engineered tissues as disease models is expected to aid in the reduction and refinement of animal models. Animal models are expensive and should be minimized due to ethical consideration. In addition, due to the complexity of the *in vivo* environment and the heterogeneity of native tissues is it difficult to distinguish whether the observed effects are direct or indirect. *In vitro* models can provide real-time information about disease mechanisms and in a higher throughput fashion, with better control of the experimental conditions than in animal experiments.

6.4 Conclusion

The main challenge of this thesis was to understand and optimize tissue development and tissue mechanical properties of TE heart valves, with special emphasis on the collagen remodeling in the heart valve leaflets. Tissue properties of human native heart valves were determined to define bench-marks for the TE heart valves and their optimization towards their native counterparts. Matrix composition, maturation, and architecture are all important in defining the mechanical functionality of the tissue. Changes in one of these properties have a direct effect on the mechanical functionality. For instance, this thesis showed that changing culture medium conditions towards autologous heart valve culture resulted in similar matrix composition and maturation compared to the control group. Nevertheless, the collagen architecture was altered by changing the culture medium conditions, resulting in different mechanical properties.

Thus, TE strategies to optimize TE heart valves towards native heart valves should focus on improving all three aspects (e.g. tissue composition, maturation, and architecture) and include both biochemical as well as mechanical environmental cues. With respect to these cues, indications from valvulogenesis, postnatal valve development and (engineered) tissue and disease models suggest that mechanical conditioning is an interesting method to improve these aspects. In this thesis, we concluded that static strain after a period of cyclic strain is favored for collagen synthesis, while continuous cyclic strain is needed for shifting the balance towards collagen maturation. This suggests that the conditioning protocol should change with time, starting with the synthesis of collagen and followed by mechanical cues that stimulate collagen maturation. The results of this thesis bring us one step closer in understanding the mechano-regulatory mechanism of tissue formation and maturation. Moreover, they provide insights in how to improve *in vitro* heart valve tissue engineering strategies to control the tissue properties and collagen remodeling in TE heart valves and to optimize them towards their native counterparts.

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Samenvatting

Een zieke hartklep wordt vaak vervangen om problemen aan hart, longen en lichaam te verkomen. Helaas bestaan de huidige hartklepprothesen niet uit levend weefsel, waardoor ze niet kunnen groeien of zich aan kunnen passen. Tissue engineering, of weefselkweek, is een veelbelovende techniek om een levende, lichaamseigen hartklepprothese te maken. Een belangrijke uitdaging daarbij is om de weefselontwikkeling, de collageenremodellering en de resulterende mechanische weefseleigenschappen tijdens de kweek te sturen. Om de geschiktheid van getissue-engineerde (TE) hartkleppen te bepalen is het verder van belang om de weefselstructuur en mechanische eigenschappen te kunnen vergelijken met benchmarks, gebaseerd op de eigenschappen van natieve hartkleppen. Het doel van dit proefschrift is om deze benchmarks te bepalen en om de weefselontwikkeling en de resulterende mechanische weefseleigenschappen van de TE hartkleppen te begrijpen en te optimaliseren.

Om inzicht te krijgen in de evolutie en maturatie van de extracellulaire matrix en de mechanische eigenschappen en om benchmarks voor TE hartkleppen te bepalen, werden samenstelling en maturatie van de matrix en de mechanische eigenschappen van natieve humane aorta- en pulmonaalkleppen onderzocht. Deze studie toonde aan dat de mechanische eigenschappen van natieve humane hartkleppen veranderen bij toenemende leeftijd en bovendien voor een significant deel bepaald worden door de samenstelling en de maturatie van de extracellulaire matrix.

Voor het verbeteren van de (mechanische) weefseleigenschappen van TE hartkleppen is het van belang dat deze eigenschappen tijdens de kweek voor individuele patiënten voorspeld kunnen worden. Een studie naar mogelijke indicatoren van de *in vitro* weefseleigenschappen toonde aan dat α-smooth muscle actine (αSMA) een veelbelovende indicator is. In deze studie werden tevens de verschillen in (mechanische) weefseleigenschappen tussen schaap en humane TE weefsels geëvalueerd om te onderzoeken of eigenschappen van schapen TE hartkleppen representatief en voorspellend zijn voor humane TE hartkleppen. Dit hebben we gedaan, omdat het schaap het voorgeschreven diermodel is om hartklepprothesen te onderzoeken. Uit de resultaten kunnen we suggereren dat het kweekproces voor weefsels gekweekt met schapen cellen beter in de hand gehouden kan worden, terwijl de mechanische eigenschappen, en daarmee de functionaliteit, van de weefsels gekweekt van materiaal van patiënten moeilijker is om te voorspellen. Dit laat nogmaals het belang zien van de identificatie van vroege markers om de weefseluitkomst te voorspellen.

Voor de translatie richting klinische toepassing en om het gebruik van dierlijke producten in het kweekmedium te voorkomen, werd het kweekserum vervangen voor humaan plaatjeslysaat, zodat een autologe TE hartklep gekweekt kan worden. Hoewel

de weefselsamenstelling en -maturatie hetzelfde waren, bleken de mechanische eigenschappen van het weefsel gekweekt in humaan plaatjeslysaat veel slechter. Een mogelijke oorzaak hiervoor was de verhoogde productie van matrix degradatie enzymen in plaatjeslysaat die heeft geleid tot een andere collageen architectuur. Hieruit blijkt dat niet alleen de *hoeveelheid* collageen, maar vooral de collageen*architectuur*, van belang is voor de uiteindelijke mechanische weefseleigenschappen van TE weefsel.

Een manier om de collageenarchitectuur en -maturatie van TE weefsels te bevorderen is om de weefsels tijdens het kweekproces mechanisch te belasten. Eerdere studies uit onze groep hebben laten zien dat intermittent belasting, waarbij cyclische en statische rek worden afgewisseld, de voorkeur heeft voor het verkrijgen van volgroeid weefsel in een korte tijd. Om het onderliggende mechanisme van intermittent belasting te bestuderen, werden in dit proefschrift de effecten van cyclische rek en statische rek na cyclische rek onderzocht op genexpressie niveau. Een periode van statische rek blijkt nodig te zijn voor de collageensynthese en remodelering, terwijl het continu cyclisch rekken belangrijk is om de balans te verschuiven naar collageenremodelering en maturatie. De resultaten suggereren dat het mechanisch belastingsprotocol tijdens de kweek moet overgaan van intermittent belasting in continue cyclische rek om de collageenmaturatie te verbeteren na de synthese van collageen. Daarmee zouden ook de mechanische eigenschappen van de TE hartkleppen geoptimaliseerd kunnen worden.

Samengevat laten de resultaten in dit proefschrift zien dat naast de hoeveelheid en maturatie van collageen, de collageenorganisatie in het TE weefsel van belang is voor de mechanische eigenschappen. Het optimaliseren van kweekprotocollen zou zich daarom moeten richten op het verkrijgen van een juiste collageenarchitectuur om functionele TE hartkleppen te creëren. Het gebruik van humaan plaatjeslysaat om autoloog te kunnen kweken is niet gewenst, omdat dit de vorming van een georganiseerd collageennetwerk tegen gaat. Het mechanisch belastingsprotocol zou moeten starten met intermittent belasting en tijdens de kweek over moeten gaan naar continue cyclische rek om de collageenmaturatie te bevorderen. Door de grote verschillen in weefseluitkomst bij gebruik van cellen van verschillende patiënten is het van belang om de kweekprotocollen verder te verfijnen of misschien wel patiënt-specifiek te maken. Daarbij zijn markers om de weefseluitkomst te voorspellen, zoals αSMA, noodzakelijk om de kweekprotocollen per patiënt aan te kunnen passen. Hoewel aanvullend (*in vivo*) onderzoek gewenst is om deze suggesties te toetsen, geven de resultaten in dit proefschrift richtlijnen voor de aanpassing van *in vitro* strategieën voor optimalisatie van weefseleigenschappen en collageenremodelering van TE hartkleppen richting de eigenschappen van natieve hartkleppen.

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Daphne, april 2012

Curriculum vitae

Daphne van Geemen is geboren op 26 juni 1983 te Woerden. In 2002 behaalde zij haar VWO diploma aan het Minkema College in Woerden. Aansluitend studeerde zij Biomedische Wetenschappen aan de Universiteit Utrecht. Als onderdeel van haar master, heeft zij 9 maanden stage gelopen in het Hubrecht Laboratorium in Utrecht. Hier heeft ze onderzoek gedaan naar het ontwikkelen van een zebravis model voor het Noonan en LEOPARD syndroom. Vervolgens heeft ze 6 maanden stage gelopen bij de Orthopaedie afdeling in het Universitair Medisch Centrum Utrecht, waar ze de cel overleving en differentiatie in fotopolymeriserende hydrogelen voor bot tissue engineering heeft onderzocht. In 2007 behaalde zij haar master diploma in de richting 'Biology of Disease'. In 2008 is zij begonnen met haar promotieonderzoek binnen de vakgroep Soft Tissue Biomechanics & Engineering aan de faculteit Biomedische Technologie van de Technische Universiteit Eindhoven. Als onderdeel van haar promotieonderzoek, werkte zij de eerste twee jaar deeltijds bij de afdeling cardio-thoracale chirurgie aan het Universitair Medisch Centrum Utrecht. Haar promotieonderzoek heeft geresulteerd in dit proefschrift.

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