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# Aortic valve disease

*Exploring methods, models, and mechanisms*

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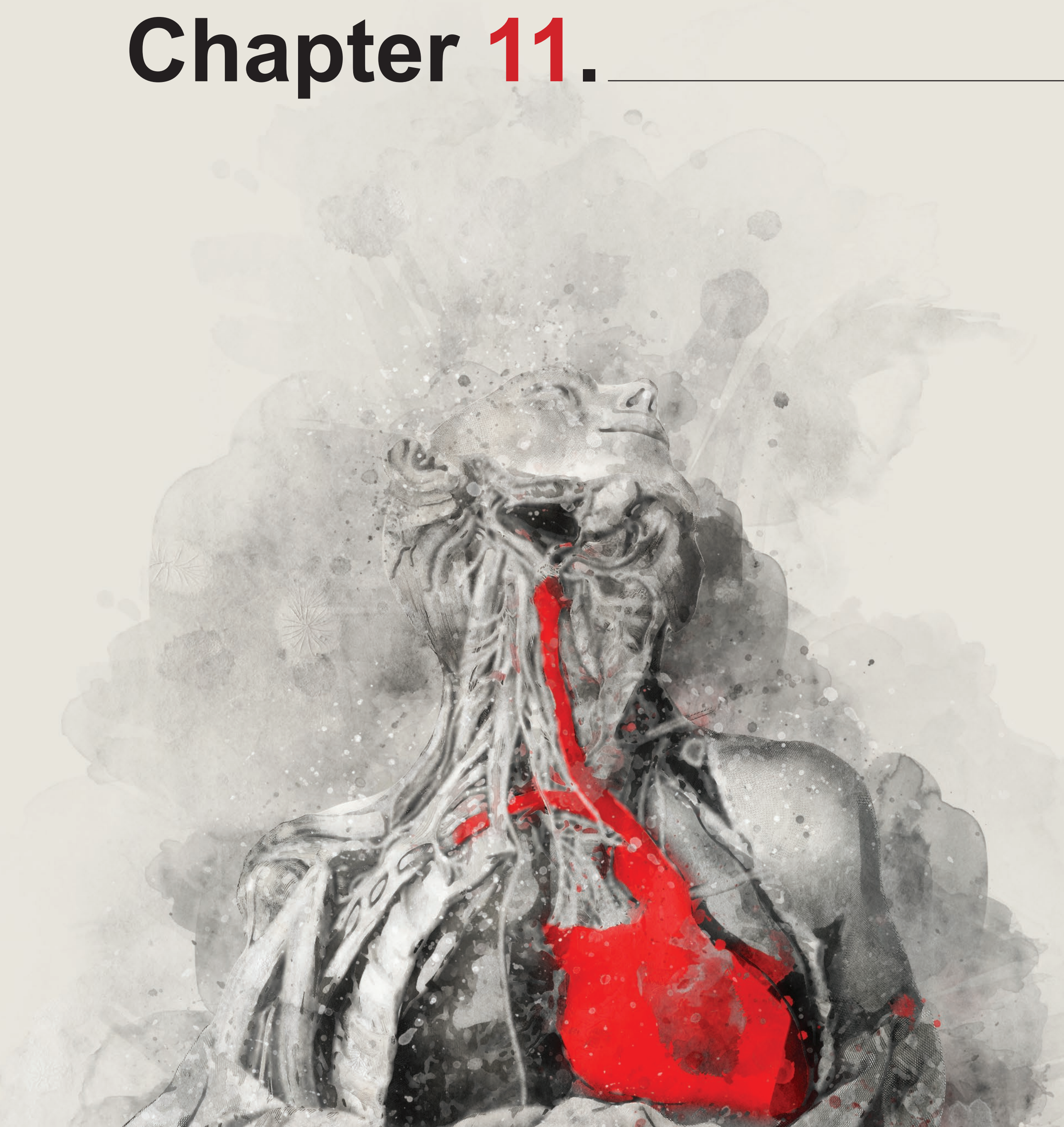
# Part three
## Final thoughts

# Chapter 11.



## General discussion

# General discussion

The aortic valve is situated in arguably the most mechanically demanding environment in the body; systematically and reproducibly recapitulating the dynamics involved in aortic valve homeostasis *in vitro* and *in vivo* has proven to be a difficult task. This is underlined by the absence of a pharmaceutical treatment for (fibro) calcific aortic valve disease (CAVD) and the absence of a viable prosthesis for aortic valve replacement. Nonetheless, much progress has been made over the last 15 years and the pathobiology of the aortic valve is gaining increased interest and appreciation. We underline the importance of the hemodynamic environment on aortic valve homeostasis in **chapters 2 and 3**, and show that altered hemodynamics alone can alter cell phenotype and miRNA expression in otherwise healthy aortic valves. In **chapter 4**, we shed some light on why post-radiation patients are more likely to develop aortic valve disease, develop it earlier, and undergo valve replacement more often than non-irradiated patients do. Progress in *in vitro* CAVD research is held back by the lack of a standardized biologically relevant culture model for aortic valve cells, as described in **chapter 6**. In order to address several shortcoming in *in vitro* research, we used a novel microwell platform for 3D co-culture of aortic valve interstitial cells (VICs) and valvular endothelial cells (VECs) in **chapter 7**. When all pharmaceutical approaches fail, investing in viable aortic valve prostheses seems a wise approach: in **chapter 8**, we implanted sheep with decellularized extracellular matrix (ECM) valve constructs to assess its use for aortic valve replacement. These studies emphasize the importance of the aortic valve microenvironment on valve homeostasis, regeneration, and disease.

Motivated by the outcome of the studies and ongoing research presented in this thesis, this chapter discusses the main findings within the context of improved and accelerated translation of basic research to clinical therapies.

*Modeling aortic valve disease*

The most important factor limiting the translation of basic research to the clinic is the lack of models that mimic human physiology, besides humans itself. Since the unethical use of human experimentation is regulated by adaptation of the Nuremberg Code and later the Declaration of Helsinki as recently as 1964, numerous alternatives for CAVD research have been developed, in order of biological relevance: large animal, small animal, *ex vivo* tissue, 3D cell culture, and lastly 2D cell culture and *in silico* models. Large animal models, like rabbit or pig, are able to recapitulate several important aspects of human CAVD including the hemodynamics, the tri-layered valve architecture and lipoprotein metabolism [1]. Small animals like mice however lack most of these similarities with the human aortic valve situation, but are a suitable platform for more mechanistic studies because genetic manipulation is relatively easy, and mice are small and cost effective. Only pigs however naturally develop CAVD with age, and all animal models require dietary or genetic interventions to induce or accelerate calcification. Valvulogenesis is studied in zebrafish and chick and mouse embryos. These models can give insight in congenital malformations like bicuspid valves, which are prone to premature calcification, and can clarify the role of reactivating developmental pathways in adult CAVD. Studies that aim to develop viable alternatives for aortic valve replacement on the other hand, generally view sheep as the animal model that best mimics the human physiology of the heart [2].

Animal studies are generally the final step before clinical research, however nearly all that is known about the mechanobiology of the aortic valve is based on *in vitro* and *ex vivo* findings. In fact, direct *in vivo* manipulation of the aortic valve hemodynamic environment, to assess the effect of e.g. shear stress on aortic homeostasis is rarely performed. In **chapters 2 and 3**, we hypothesized that aortic valve tissue obtained from patients supported by left ventricular assist devices (LVAD) would give great insight into the effect of severely altered blood flow dynamics in otherwise normal aortic valves. LVAD implantation significantly alters blood flow over the valve leaflet, and depending on the LVAD pump speed settings, the high velocity unidirectional laminar flow normally experienced by the ventricularis layer during systole nearly disappears, leading to lower wall shear stress. At the aortic side of the leaflet, retrograde blood flow from the LVAD outflow into the aortic sinuses can lead to exacerbated oscillatory flow. The reduced ventricular pressure and high backflow lead to decreased valve-open time or even continuous valve closure; in its closed position, the leaflets are exposed to the highest pressure and stretch stresses, which has been linked to increased matrix remodeling in expression of pro-inflammatory markers [3, 4].

Although such radical changes in hemodynamics are not observed outside the LVAD population, increases in blood pressure and reduction in valve opening associated with AS could induce alterations in shear stress magnitude and are thought to contribute to CAVD [5]. CAVD is typically initiated at the aortic side of the valve, which is exposed to low oscillatory shear stress [6] and these type of stresses have been hypothesized to induce aortic valve disease. *Ex vivo* and *in vitro* studies have indeed shown that altered shear stress can induce an inflammatory response at the fibrosa side of the leaflet [7, 8]. The ventricularis layer however appears to be relatively protected against alterations in flow [7]. This is possibly due to a combination of low stiffness of the ventricularis layer, ECM composition, preconditioning by unidirectional laminar flow, and general phenotypical differences between layer-specific cells [9-13].

Nonetheless, it is in this layer that we observed LVAD-induced pathologies including commissural fusion of the leaflets and pathological activation of VICs. This remodeling and differentiation however was not associated with calcific changes as observed in CAVD, which may suggest involvement of different pathways or a different timeline in LVAD-induced valve pathologies. The key in explaining the striking differences between LVAD- and CAVD-pathologies may lie in the initial inflammatory response. Both pathologies are likely initiated by shear stress-induced upregulation of endothelial cell adhesion molecules at the affected side, with subsequent monocyte and leukocyte infiltration, as shown after LVAD by CD68 and CD45 staining. While CAVD is associated with a pro-inflammatory macrophage M1-subtype [14], we show that most monocytes infiltrating the LVAD valves are of an anti-inflammatory alternatively activated subtype (M2). The exact role of macrophage subtypes in the aortic valve is not fully understood however. Interestingly, we and other authors [15] found that the normal adult aortic valve also contains unexpectedly high numbers of macrophages distributed throughout all three layers, ranging from 5-10% of the total valve interstitial cell population in our patient group. Counterintuitively, CD163+ cells, which are supposedly part of the M2-subset of CD68+ macrophages, formed a higher proportion of total valve cells than CD68+ macrophages did. The use of CD163 as a marker for M2 macrophages in general needs clarification as similar confusing outcome has been described by ourselves in **chapter 4**, and by others in different tissues [16]. Furthermore, the assumption of the existence of a macrophage that can be 100% assigned to either M1 or M2 subtype is not correct and neither is the use of a single marker to identify said subtypes. Nonetheless, these generally accepted methods of identifying macrophages implicate a role for these immune cells in normal valve homeostasis and shear stress-induced pathologies. It is indeed believed that recruiting monocytes is part of the normal valve homeostasis and that contribution of extracardiac cells is an age-dependent process [17]. Oba and colleagues however also found significant expression of M1-macrophage markers in healthy valves [15], which suggests that the ratio of M1/M2 is more important than the absolute numbers. The lack of inflammation and increased presence of myofibroblast-like cells after LVAD can also hint at another ability of macrophages, besides recruitment and activation of fibroblasts to the site of injury and secretion of pro-fibrotic TGFβ, namely: macrophage to myofibroblast transition (MMT). MMT is a phenomenon that has been described in several organs but has not yet been identified in the adult aortic valve. In studies on mouse and human chronic renal allograft injury and renal fibrosis, M2-macrophages were able to undergo transdifferentiation into a myofibroblast-like phenotype and contribute to interstitial fibrosis [18, 19]. Strikingly, at least half of the myofibroblast population in these fibrotic areas co-expressed macrophage markers, which means we cannot underestimate the contribution of macrophages to fibrotic disease. Rather unexpectedly, our study hints at a role for anti-inflammatory macrophages in valve homeostasis and shear stress-induced pathologies. Therefore, a better understanding of macrophage phenotype and function in the aortic valve may reveal novel targets for future therapeutic intervention of CAVD.





Implementation of circulating systemic factors, such as circulating bone marrow derived cells, into *in vitro* cell culture systems will be a great challenge. Inflammatory molecules of interest are generally supplemented to the culture media in an attempt to mimic the presence of immune cells in the valve, while actual co-culture of valve and immune cells is rarely performed. This poor representation of the native environment is inherent to *in vitro* research, which is reductionist in nature. In finding a suitable model for CAVD research, one must walk the fine line between biological relevance and experimental desires. More often however, judging by our results in **chapter 6**, native biology is not even considered, as over half of the VIC *in vitro* research on which we base our knowledge, has been performed in the crudest way possible: static monolayer culture on uncoated tissue culture polystyrene or glass surfaces. The shortcomings of 2D culture have been well noted, but one of the most impressive models, in terms of incorporating native physiology, remains a study by the group of Craig Simmons published in 2013 [20]. By using a bilayer membrane microfluidic device with VECs seeded on top of a porous membrane, and VICs embedded in a 3D matrix under the membrane, they were able to include 3D culture, co-culture, and exposure to shear stress in one model. Although the model would have benefitted from direct contact co-culture, and was only shown effective in short experiments, it is one of the few, if not the only published 3D VIC/VEC co-culture models that has been used for shear stress assessment. Surprisingly, no follow-up valve studies that cited this paper could be found seven years after publication. There are other ingenious studies, but in many cases, there are no additional papers using the same model. The reasons for this are unclear. One can speculate that these models were designed for a specific project that terminated with no follow-up, results could not be replicated by others, the design is too complex, expensive, labor-intensive, etc.

In **chapter 7**, we propose an aortic valve culture model with similar capabilities but with a much simpler setup, and importantly, high throughput potential. We believe that 3D culture, co-culture with VECs and exposure to mechanical stimulation are minimal requirements for an effective aortic valve *in vitro* model, as these factors play crucial roles in regulating native aortic valve homeostasis.



The substrate to begin with, is the material in direct contact with the cells and its biomechanical properties largely define cell biology. In general, stiffer substrates induce higher stress and more αSMA expression in VICs, as is seen in the native aortic valve's collagen-rich fibrosa layer, which is the stiffest of the three valve layers and most

affected in disease [21]. Given the great mechanosensitive capacities of aortic valve cells, replication of these subtle microenvironmental changes is essential for obtaining reliable, biologically relevant results and should be the starting point for any *in vitro* platform for CAVD research. The use of substrates in cell culture however introduces several confounding factors that prevent further translation to the clinic.

Substrate is no factor in our microwell-cultured spheroids, as spheroids are composed only of cells and the matrix they secrete. Consequently, the cells will produce the matrix that is appropriate for the situation. Although still a work in progress, we demonstrate first signs of collagen deposition at day 7 in osteogenic medium (which includes ascorbic acid to induce collagen synthesis). Production of other key ECM constituents like elastin and proteoglycans will have to be determined. The only other VIC spheroid culture model in literature [22] demonstrated that VICs indeed express most of the relevant ECM proteins (after stimulation with ascorbic acid), which judging from the histology, looks to be layered in architecture. After 22 days in culture, the outer third of the spheroid was composed of circumferentially aligned collagen fibers, while polysaccharides such as GAGs were found immediately under this layer, in effect mimicking the fibrosa and spongiosa layers, respectively. Biochemical analysis also demonstrated a gradual increase of elastin content over time. It would be interesting to see where this elastin is located within the spheroid. In reality, there would be no appropriate location for a ventricularis layer in a spheroid. If it were in the outer layer, it would form a hybrid fibrosa/ventricularis layer. If it were to follow the apparent layering as seen in histology, the ventricularis would be located in the spheroid core, unlike the native ventricularis. Ideally, the outer layer of one hemisphere would contain mostly elastin while the other hemisphere is rich in collagen, but this seems an unlikely scenario. One theoretical way of achieving hemispheroid polarity is by microfluidic superfusion, where a laminar flow is directed over the spheroids, and the shape of the microwells causes turbulent flow in the bottom of the well. It is however more likely that the whole spheroid would experience turbulent flow. This is of course purely speculative and practically impossibly due to the free-floating nature of the spheroids.

ECM proteins can also be supplemented to spheroid culture media to improve self-assembly, or by taking this a step further and completely embedding your cells in a substrate, you have your typical scaffold dependent 3D culture model. Both natural and synthetic scaffolds materials like collagen, gelatin-based hydrogels, and PEG-based gels are generally used for VIC 3D culture. These constructs are tuned to resemble the mechanical functions of the whole valve or of specific valve layers, like single fibrosa and spongiosa layer models [23, 24]. Recently, functional integration of multiple leaflet layer mechanics into a single dual-layered 3D GelMA/HAMA hydrogel demonstrated the formation of layer-specific microcalcification and ECM degradation [25]. Replicating the stiffness of the native valve is essential for proper VIC function, but it is difficult to obtain relevant measurements in native aortic valves. Stiffness in native valve tissue is highly anisotropic, owing to the mainly circumferential orientation of collagen. Canine aortic valve leaflets e.g. have been shown *in vivo* to elongate by about 10% in the circumferential direction and about 30% in the radial direction during the cardiac cycle [26]. Stiffness of aortic valve tissue is usually in the 10-30 kPa range but is highly dependent on the direction of measurement, the layer that is measured, the technique that is used, the age of the tissue donor, and tissue storage method. Overall, the fibrosa layer is stiffer than the ventricularis, which in turn is stiffer than the spongiosa, and old valves are stiffer than young valves, but all are considerably heterogeneous [10, 25, 27-29]. Thus, a general approximation of overall leaflet stiffness can be achieved *in vitro*, but challenges regarding the ideal chemical composition or topography of the substrate, degradation rate, and toxicity of degradation products have not been solved. The optimal substrate for VIC culture therefore remains to be determined.

More sophisticated substrate-based models layer VECs on top of 3D cultured VICs to study the interplay between these cell types [20, 30, 31]. Direct co-culture, where VICs and VECs are in direct contact with each other is the preferable method, as it allows for processes like endoMT and VEC migration to occur as it does in the native valve. To facilitate the extraction of cell type specific expression profiles, indirect co-culture is also used in which a porous membrane separates the VECs from the VICs for easy isolation [20]. Introduction of VIC/VEC co-culture significantly improved biological relevance of obtained results, as VECs have been shown to regulate normal VIC phenotype and remodeling [32] and exert protective effects on VICs through the secretion of nitric oxide [12]. Co-culture with VECs is therefore a crucial factor to implement in cell culture of VICs if your goal is to improve translation of *in vitro* results to the clinic. Our microwell platform seems to be particularly suited for co-culture as demonstrated by the ease of self-sorting and site-appropriate migration of VECs from a random VIC/VEC mix.

If 3D environment and co-culture are so important, why not use *ex vivo* whole tissue? After all, *ex vivo* tissue fits both requirements as it maintains native ECM architecture and VEC layer and it is a better surrogate than any laboratory modeled construction. Biomechanical and cell-matrix interaction studies indeed greatly benefit from *ex vivo* whole tissue as demonstrated by the collective work of the group of professor Yoganathan [3, 7, 33-37]. The tissue used in these experiments ranges from complete aortic roots to small tissue strips from the belly of individual leaflets. Tissue can additionally be stripped from endothelial cells so a pure VIC response can be evaluated. In many ways, *ex vivo* fills the gap between *in vitro* and *in vivo* research. *Ex vivo* studies however are often short in duration; a median culture duration of 4 days was seen in 39 studies. *In vitro* VICs are

cultured nearly twice as long on average. Extended *ex vivo* culture of valve leaflets with mechanical stimulation is possible up to 14 days and calcification was detected using alizarin red and von Kossa staining [35]. *In vitro* calcification studies typically last 21 days for optimal detection of calcification. Limitations of the *ex vivo* approach include donor variation, difficulty to obtain large number of tissues for multiple experiments, no tunable substrate properties, increased complexity, low-throughput, and higher change of microbial contamination.

A lifetime of fluctuating pressure, shear stress, bending stress, and stretch cannot be ignored when culturing aortic valve cells. As mentioned before, not only is exposure to these stresses a necessity for normal valve homeostasis, these factors are also understood to be major driving forces behind aortic valve disease [37-39]. This topic is excellently reviewed by Gould and colleagues [40]. It is therefore alarming to see that <10% of all published *in vitro* VIC studies exposed cultured cells to mechanical stimulation (**chapter 6**). Mechanical stresses directly affect valve cells, but can also interact with other microenvironmental cues that regulate cell function. For example, *ex vivo* interaction has been observed between cyclic stretch and exposure to TGFβ, where individual treatments had moderate effect on VIC activation while simultaneous exposure resulted in a profound synergistic effect [41]. This exemplifies how deeply intertwined factors leading to CAVD are and how excluding any of these variables from VIC culture can lead to results that have little biological relevance, but on the other hand do lead to better functional understanding.

*In vitro* and *in vivo* simulation of pathological processes is far from standardized [42]. There are countless methods to induce calcification *in vitro* and in animal models, but not all are clinically relevant. For example, *in vitro* stimulation of VIC with high phosphate concentrations induces a different type of calcification than stimulation with standard osteogenic medium does. These methods are often indiscriminately used but are significantly different. The latter depends on TNAP (tissue-nonspecific alkaline phosphatase) for hydrolysis of β-glycerophosphate into inorganic phosphate for mineralization. The significance of TNAP in native valve calcification is not clear but both TNAP-dependent and independent pathways may play a role [43, 44]. Native calcification is thought to arise from hyperphosphatemia or chronic inflammation, which involve distinct mechanisms, or any combination of these and other undiscovered mechanisms. Besides proper *in vitro* model design, appropriate choice and evaluation of assays is crucial to yield significant insight into valve disease as reviewed by Bowler and Merryman [45]. On-chip imaging using histological and immunohistochemical stainings allows for high throughput morphological and phenotypic screening as seen in our microwell system (live/dead assay) and by others using the same model [46].





*In situ* tissue engineering and the use of decellularized scaffolds to create a living aortic valve replacement deals with much of the same and additional problems as encountered in modeling *in vitro* valve cell culture. The *in situ* approach for tissue engineering is technically challenging but clinically attractive and relies on the capacity of circulating host cells to populate and remodel the implanted biodegradable scaffold into a living valve replacement. Tissue formation in implants is mainly an inflammatory-driven process (foreign body response) that is dependent on scaffold properties and can be guided towards functional tissue regeneration by implementation of biochemical and biophysical cues [47-49]. Regeneration of such implants is dependent on circulating VIC-surrogates like fibrocytes, endothelial progenitor cells or CD34+ progenitor cells that are chemo-attracted by macrophages. Skewing the macrophage response towards M2 polarization resulted in better regeneration [50].

While synthetic heart valve scaffolds are not yet applied in the clinic, biological xenograft valve replacement has been used for over half a century (porcine and bovine pericardium). Xenografts are an interesting option because these tissues maintain their highly organized native 3D matrix structure, which is hard to replicate in the laboratory. Furthermore, there is a limited supply of human-derived allografts. Although the clinically used prostheses are decellularized and glutaraldehyde-treated to remove donor cells and antigens (α-gal epitope), and stabilize the tissue, premature calcification is the most frequent cause of failure, significantly limiting durability [51, 52]. Premature dysfunction in xenografts is multifactorial in nature and thought to be accelerated by both biomaterial and host factors and include tissue origin, tissue processing, implant position, mechanical stresses (conduit design), host age, and the host inflammatory response [53].

To eliminate glutaraldehyde-induced calcification, and to create a viable environment that allows active remodeling by engrafted cells, CorMatrix (CorMatrix Cardiovascular, Inc, Roswell, Ga) developed a proprietary method to process porcine small intestinal submucosa ECM sheets for use in the cardiovascular environment. Initial results in porcine models and clinical cardiac repair were encouraging and soon surgeons applied the material for heart valve repair with good short-term outcome. Mid- and long-term results were significantly less positive and showed high rates of structural failure and need for reoperation as summarized by Mosala Nezhad and colleagues [54]. Our study in sheep reveals a disturbing picture of what happens to a CorMatrix valved construct implanted in the pulmonary position (**Chapter 8**). Similar to mid- to long-term clinical outcome, our sheep model displayed a high rate of valve failure. This was caused by a high degree of chronic inflammation and failure to remodel, emphasizing previously raised words of caution against the use of CorMatrix in high-pressure areas of the cardiovascular system.

*In situ* regeneration and repair of implanted biomaterials in highly dynamic environments like the heart valves is a complex and multifactorial process that is still poorly understood. In our study, inadequate removal of xenogenic material may have contributed to an inflammatory environment incompatible with constructive tissue remodeling. For decellularized xenografts to be a viable tissue engineered alternative for valve replacement, novel methods of antigen removal with preservation of ECM integrity are required.

It was a Dutch pathologist by the name of A.J. Scholte, who first described carcinoid heart disease nearly 90 years ago, when he perceptively noticed thickened tricuspid valve leaflets in a patient with carcinoid tumor [55, 56]. Carcinoid heart disease is described in **chapter 9**. Briefly, carcinoid heart disease is a rare complication of metastatic neuroendocrine tumors that is characterized by plaque-like deposits of fibrous tissue on right-sided valves. The mechanisms leading to carcinoid heart disease in the native leaflet have not been sufficiently characterized yet [57]. Increased serum serotonin (5-HT) levels induce increased proliferation, TGFβ expression and ECM production in right-sided heart valves. That left-sided valves could also be affected by serotonin is evident from the fen-phen disaster, in which patients were treated with high dose appetite-suppressant drugs for >3 months [58]. The drugs caused rapidly increased serum serotonin levels and was quickly associated with an increased risk of carcinoid-like valve pathologies through 5-HT2B receptor agonism, leading to withdrawal from the market in 1997. Interestingly, TGFβ-induced aortic VIC activation can be inhibited by blocking the 5-HT2B receptor [59], presenting a possible target for pharmaceutical intervention [57]. Further evidence for a role of serotonin in the aortic valve comes from an *ex vivo* study on porcine aortic valves. The authors showed that physiological cyclic stretch alone was sufficient to upregulate 5-HT2 receptor gene expression, underlining that native aortic VICs are sensitive to serotonin [36]. These results additionally emphasize the importance of incorporating mechanical stimulation in *in vitro* culture models.

*Future perspective*

Eventually, radiation associated valve disease, as described in **chapter 4**, will be even rarer than it is today (in the absence of nuclear events). Irradiation techniques continue to improve and this has already led to significantly reduced radiation doses and volumes, thereby preventing unwanted side effects. At the cellular level, sublethal radiation induces DNA breaks, formation of reactive oxygen species (ROS) and an inflammatory state. ROS persist long after exposure to radiation and this can lead to a lingering low level chronic inflammation that hampers effective healing [60]. Furthermore, latent TGFβ that is sequestered in the ECM, which is a prominent feature of aortic valves, can be cleaved and activated by ionizing radiation, inducing a fibrotic response [61, 62]. Currently, only one publication assessed the *in vitro* effect of radiation on human VICs [63]. 10 Gy radiation significantly increased the expression of osteogenic factors BMP-2, OPN, ALP, and Runx 24 hours after irradiation. Unfortunately, only one dose of radiation was used, which is a significant limitation given the fact that radiation most likely has dose-dependent effects. Radiation has several interesting effects on tissues depending on the received dose. It is hypothesized that a dose threshold exists, below which radiation produces a protective effect against subsequent higher doses. This response is believed to be a generalized response to low stress and is strongly tissue-type dependent [64]. The existence of such a threshold could have important clinical implications if e.g. healthy tissue near the tumor (i.e. the heart), located inside the radiation window, could be pre-irradiated with low doses to induce protective effects during the actual high dose therapy. Unsurprisingly, it has been suggested that monolayer culture may not accurately model important aspects of radiation-induced pathologies, including cell-cell and cell-matrix interactions [65]. The spheroid co-culture model therefore is an excellent candidate to elucidate *in vitro* if this threshold is also present in the aortic valve. Although the first sentence of this paragraph is largely true, it is especially fitting that, when discussing the future perspective of this topic, manned spaceflight may actually cause a future rise in radiation associated valve disease. NASA has labeled space-radiation-induced cardiovascular disease as a significant risk for astronauts on long exploration missions [66].

At some point, VIC/VEC spheroids may be used as modular tissues for the clinical biofabrication of macroscale tissues. This alternative use of spheroids could also lead to new physiologically relevant 3D platforms, though at the cost of high throughput options. Feasibility of this modular approach was demonstrated in GelMA embedded VIC spheroids [67], which show fusion of individual spheroids into larger assemblies (up to 1,400 µm diameter) in a 7 day culture, while retaining some similarities with native valve tissue. Longer culture of these larger microtissues will undoubtedly show that this is far beyond the limitations of oxygen and nutrient diffusion. The first step towards biological relevance would involve the creation of a fused spheroid sheet with a thickness of about two to three spheroids (400-600 µm) to mimic native valve thickness. Two confluent T175 flasks with VICs could yield enough cells for a large (1x1cm) sheet, indicating the need for large numbers of cells for such a set-up. Spheroids could be introduced into a non-adhesive mold with specified thickness. Tissue liquidity [68] and the self-sorting capacity of VICs and VECs as observed in **chapter 7**, should theoretically create a single flat aortic valve tissue-like cell sheet with countless options for further experimentation. Preconditioning in a bioreactor to induce tissue formation can determine if these constructs develop any mechanical strength. This is interesting from a tissue engineering perspective because typical 3D hydrogel culture results in inherently weak material that bears no resemblance to native valve tissue and is in its

current form of no interest for *in vivo* application.

Extrusion based 3D bioprinting is another exciting new technique for spatial patterning that is completely compatible with spheroid culture [69]. Rather than a flat surface as described before, 3D bioprinting can use bioink (spheroids) to write on layers of biopaper (hydrogel or other scaffold types that serves as a glue) to create a stacked 3D structure of an aortic valve conduit, as was demonstrated for isolated valvular and vascular cells in alginate/gelatin hydrogels [70]. With advantages as low cost, flexibility, scalability, and compatibility with automation, bioprinting is an attractive and future-proof alternative for current 3D culture models.

2D monolayer culture will never disappear, but there should only be one (or two) valid reason(s) for this: (1) to validate results previously obtained in 2D culture in new 3D models. Also (2), monolayer expansion of VICs is the only method of obtaining large numbers of cells for experiments, even if the experiment is later performed in 3D. 2D culture is so well established however, and one cannot deny decades of collective work, which above all is the basis of most of our basic insights into aortic valve disease. The large body of literature provides a relatively standardized framework for comparison on which future 3D research may be based. Nonetheless, acceleration of aortic valve research and increasing our understanding of biologically relevant mechanisms is only possible if we move towards more physiologically relevant *in vitro* models.

*Conclusion*

Ultimately, the almost countless number of factors that can influence the phenotype of aortic valve cells make understanding the molecular and cellular mechanisms of normal and diseased aortic valves extremely challenging. Additionally, the intimate connection between these factors adds a completely new level of complexity. We have discussed the aortic valve architecture with layer-specific biomechanical properties, the role of VECs, the heterogeneous cell population that inhabits the leaflet interior, and the contribution of extracardiac cells to valve homeostasis. We further discussed that the highly dynamic environment that exerts physiological cyclic pressure, shear stress, bending stress, and axial stretch upon the valve leaflets in a side-specific manner is necessary for proper valve function. The apparent critical role of cell-cell and cell-environment interactions in native valves however is often disregarded when investigating aortic valve disease; continuing down the same path will delay scientific progress and result in findings that are not relevant *in vivo*.

The development of an *in vitro* model representative for such a diverse environment and that is suitable for all experimental desires is unrealistic and unnecessary. Although a perfect *in vitro* cell culture system would be of unimaginable value, it would be too complex for widespread introduction in such a small field. The perfect *in vitro* model is essentially a modeled 1:1 copy of the human aortic root including its VICs, VECs, other cell types, layered ECM, and biomechanical properties. The perfect model has baseline healthy valve properties but is highly tunable. It includes a circulatory system connected to other organs, glands and tissues to simulate the effect of systemic factors and circulating cells. Different models would be needed for e.g. young and old patients, large and small people, males and females, and bi- and tricuspid valves and other geometries. Because of its accuracy, it would take decades of culture and stimulation before calcification starts to appear, which is impractical. On the other hand, it would give unprecedented insight into the initiation of human CAVD, an area with many unknowns. The existence of this functional valve model would also immediately solve the quest for a viable prosthesis for aortic valve replacement.

Although most of the previous paragraph is science fiction, impressive progress is made in all required areas. With rapidly developing concepts as organ-, body-, and disease-on-a-chip, and 3D bioprinting, it is just a matter of time before someone is willing to put all separate components together. Compared to the scenario above however, significant miniaturization and simplification is inevitable for practical application. The challenge is to use a model that is true to the *in vivo* situation, but maintains the ability for controlled manipulation of variables of interest and the easy isolation of different cells types for separate analyses. The rapidly increasing popularity of 3D bioprinting and the any-thing-on-a-chip approach suggests that we are approaching a reasonable compromise between biological relevance and controlled manipulation of variables.

In a realistic approach, researchers would adhere to several minimal requirements for *in vitro* aortic valve research to ensure reliably, clinically translatable, results. Briefly it comes down to this: Beside the tested variables, one should always maximize imitation of the native valve cell microenvironment. This includes incorporation of 3D culture, co-culture with VECs, and exposure to mechanical stress. In practice this would mean for example that unless you study the effect of substrate properties, valve cells should be cultured in and on a substrate that best resembles the layered leaflet architecture, which is to be determined. These models are preferably scalable and compatible with high throughput systems. At the cost of convenience, (although automation is a blessing) we gain increased significance of results.

Most models will have calcification as endpoint. As a first attempt to standardize cardiovascular calcification models, several requirements have been proposed which will help to better interpret and compare findings [42]: One must take into account all

variables described in the preceding paragraphs and address the results and limitations of the model in view of its clinical relevance. Thereby forcing the researcher to reflect upon the model they wish to use and presenting the reader with results that are better to interpret. At the end of the day, *in vitro* environments are only an imitation of reality, and no rational VIC can be fooled into believing that his life inside 'the matrix' is anything but a simulation.

A rational, well-thought-out approach for future aortic valve *in vitro* models will increase our levels of understanding and should lead to accelerated identification of novel pharmacological targets for preventive and curative therapies in the treatment of aortic valve disease. The high rate of technological advancement continuously opens new doors, which motivates and enables cardiovascular researchers to keep on moving the field forward.





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