



Supercritical CO₂ decellularized porcine pericardium is a promising material for an ascending aortic prosthesis

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B ACKGROUND: The current treatment of aneurysms and dissections is an operation in which the diseased part is replaced by a synthetic Dacron prosthesis. The elasticity of a Dacron prosthesis, however, is not even close to the elasticity of the aorta, resulting in a negative effect on the heamodynamics. Moreover, Dacron prostheses do not have the ability to grow with the body, which is especially a problem for young patients. A potentially better prosthetic material than Dacron is $scCO_2$ decellularized pericardium.

AIM: Our aim is to research whether porcine pericardium, decellularized with $scCO_2$, is suitable as a material for an ascending aortic prosthesis.

METHODS: To answer this question we came up with subquestions, which we answered with literature research and two experiments. We researched the influence of repetitive stress on pericardium, caused by the Abiomed pump for two hours. In our second experiment we researched whether there is a difference between the fulfillment of the Windkessel function by pericardium and Dacron.

RESULTS: The pericardial prosthesis has a 7 μ m (7%) decrease (not significant: p = 0.50 - 1.00, $\alpha = 0.05$) in thickness during Measurement 1 and a 30 μ m (33%) decrease (significant: p = 0.01 - 0.02, $\alpha = 0.05$) in thickness during Measurement 2. Experiment 2 showed that pericardium decreases the pulse pressure with approximately 30 mmHg, while Dacron increases the pulse pressure with approximately 29 mmHg at different prevailing pressures.

CONCLUSION: $scCO_2$ decellularized porcine pericardium is a promising alternative for a Dacron prosthesis. We therefore strongly recommend to do more research to show that the material meets all demands for an ascending aortic prosthesis.

1. Introduction

Annually, there are around a thousand hospitalizations for thoracic aneurysms in the Netherlands [1]. An aneurysm is easy to diagnose on a CT-scan or an MR angiogram as an enlarged portion of the ascending aorta [2, 3]. The first treatment after diagnosis is an attempt to control the aneurysm with antihypertensive medication. In addition, the patient could potentially stop smoking and could frequently go to medical checkups [4]. Aortic size depends on age and body size, but on average the normal diameter of the ascending aorta is 2.5 cm [2, 5]. Surgical repair of the aneurysm by a synthetic prosthesis is indicated when the aneurysm reaches a diameter of on average 5 - 5.5 cm [4]. At this point, the risk of a rupture is higher than the operation risk [6]. There is a very poor prognosis of an ascending aortic aneurysm: in the case of an aortic diameter between 5 and 6 cm, the chance of rupture is 20% per year [7]. The mortality of an operation on the ascending aorta is between 3% and 5% [8, 9].

1.1. Pathology

In contrast to an aneurysm of the abdominal aorta, an aneurysm of the ascending aorta is not often caused by atherosclerosis. However, Gasparovic et al. (2005) state that arteriosclerosis is the principal cause of ascending aortic aneurysms [10]. During the arteriosclerotic process





the tunica intima becomes fibrotic and in the tunica media smooth muscle cells and elastin are partially replaced by collagen. Because of this, the vessel wall stiffens and the Windkessel function cannot fully be fulfilled [11]. Precise mechanisms that are involved with causing an aneurysm of the ascending aorta are unknown, but an aneurysm is probably a consequence of a weak spot in the tunica media that is inflamed or degenerated [12, 13, 14]. Muscle tissue and elastic elements of the aortic wall are damaged, causing the vessel wall to loose its strength and elasticity and so it weakens [15]. This process is called idiopathic cystic medial degeneration.

Damaging of the muscle tissue and elastic elements normally occurs during aging, thus the incidence of an aneurysm increases with age [11]. In some patients, however, this process is accelerated, which leads to early formation of an aneurysm [14]. An aneurysm of the aorta is found in 10% of the autopsies [12].

Another indication for implanting an aortic prosthesis is a dissection, which is found in 0.25% of the autopsies and occurs most often in the ascending aorta. It arises on a weak spot on the lining of the aortic wall and is often related to degeneration of fibers of the tunica media, identical to an aneurysm. Moreover, there are indications for a genetic cause and that patients with specific syndromes, such as Marfan's syndrome, are predisposed to the development of a dissection [12]. A small tear in the tunica intima introduces blood to the tunica media where it piles up between the layers of the aortic wall, splitting the inner $\frac{2}{3}$ and the outer $\frac{1}{3}$ of the tunica media apart. In case of a dissection of the ascending aorta, it is necessary to operate. There is in fact a great risk that the dissection will extend toward the heart, causing a life threatening situation [9]. This is because a dissection can cause a rupture of the pericardium, causing a haemorrhage. It can also cause a cardiac arrest or an obstruction of the vessels to the head or other organs. Often the aortic valve is damaged [16]. Overall mortality of dissections nowadays is dropped to 20%, thanks to surgical treatment and control of hypertension. A treatment that only consists of medication results in a mortality of 50% in two weeks. In addition, a dissection could eventually lead to an aneurysm of the aorta [9].

1.2. Current prosthesis

During open heart surgery the diseased part of the aorta is removed and a prosthesis is sutured in the aorta as a replacement [17].

Synthetical Dacron, a thermoplastic polyethylene terephthalate, is a commonly used material for an aortic prosthesis at the moment [18]. Dacron is biocompatible with the human body and therefore there will be no calcification or rejection. The body reconstructs its own tissue in the Dacron prosthesis over time. Dacron prostheses are strong, they have a lifetime durability and they are collagen impregnated, so no blood can leak [19].

A disadvantage of the current aortic prosthesis is the influence it has on the haemodynamics in the aorta, due to the different mechanical properties compared with the human aorta. Elasticity of the Dacron prosthesis is much

more limited than the elasticity of the aortic wall in the circumferential direction [20]. Tremblay et al. (2009) investigated the difference in mechanical properties and showed that Dacron is 1.3 times stiffer than a normal ascending aorta under physiological stress [21]. Stiff materials have an insufficient ability to practice the Windkessel function, which influences the blood pressure [22]. Vardoulis et al. (2011) have demonstrated, by using a computer model of the aorta, that the closer a Dacron prosthesis is placed near the heart, the greater the impact is on the systolic pressure and pulse pressure. In case of an ascending aortic Dacron prosthesis, the systolic pressure increases 6% and the pulse pressure increases 21% compared with a healthy aorta. This is two times larger than in case of a descending aortic Dacron prosthesis [23]. Besides the low elasticity, there is another disadvantage. Dacron does not consist of human or animal tissue, but of synthetic material. Hence, it has a fixed form and size and cannot grow with the body. This is especially a problem for young patients in need of an aortic prosthesis.

1.3. Porcine pericardium as prosthesis material

An alternative material for an aortic prosthesis could be porcine pericardium. Pericardium consists of two layers: fibrous and serous pericardium. A pericardial prosthesis is made of fibrous porcine pericardium [7, 24]. Fibrous porcine pericardium is about 0.20 mm thick and consists of collagen type I and elastin fibers, which support the parietal pericardium and make it elastic [24, 25]. Extracellular matrix (ECM) of aortic tissue consists of type I and III collagen and elastin fibers [26]. Both ECM of pericardium as ECM of human aorta consist of collagen and elastin. For this reason, we suspect that decellularized porcine pericardium has the ability to fulfill the function of humane aorta.

Furthermore, Pires et al. (1999) proved that a layer of fibrous connective tissue is formed on the pericardial prosthesis when implanted in the aorta. This is favourable for ingrowth of the prosthesis. In addition, neoformation of elastin fibers occurs, which increases with duration of implantation [27]. Good ingrowth of the prosthesis and neoformation of elastin fibers are important advantages of pericardium as a material compared to the current synthetic material.

1.4. Current decellularization

As described above, pericardium seems like a good alternative for the currently used synthetic aortic prosthesis. In order to use donor pericardium, however, first cell nuclei and cell membranes need to be removed through decellularization. Donor cells can cause a serious rejection response of the body and a gradual deposition of calcium phosphate, causing calcification of the implanted tissue. During decellularization, most of the cell material is removed and only the ECM remains. Components of the ECM are generally well tolerated by patients, even by xenogeneic recipients [28]. The treated pericardium will therefore not be seen as foreign to the body, in contrary to Dacron.



Thus, although the pericardium is xenogeneic, the patient does not have to take immunosuppressive medicines and thereby does not suffer from the side effects of this, like renal failure. [7]. Then, the decellularized pericardium can be made into an aortic prosthesis in which cells of the recipient can grow after implantation [29].

One of the current methods to remove cells is with the help of surfactants. These are surface tension lowering substances with detergent characteristics, causing cells to be flushed away from the tissue effectively. However, it is difficult to remove these toxic surfactants completely from the tissue, because they have a high affinity with the ECM. To preserve the structure and thereby the mechanical properties of the ECM, it is often necessary to chemically crosslink the tissue with an aldehyde. This process is, however, related to calcification and encapsulation of the tissue after implantation [29]. Alternative decellularization techniques, where it is not necessary to use chemicals, could be helpful for solving these problems.

In 1996 a promising alternative were stentless prostheses, processed with glutaraldehyde and detoxified by the No-React process, developed by Shelhigh. The stentless protheses were made of a combination of porcine and bovine pericardium [30]. The Shelhigh BioConduit was implanted between 1998 and 2008 in 175 patients, with a mean age of 71.1 \pm 7.4 years. Although preoperative results were promising, the follow-up results were worrisome. There was a relatively high incidence of endocarditis [31]. The main reason for the U.S. Food and Drug Administration (FDA) to stop the Shelhigh BioConduit production and to recall all products was the poor sterility during manufacturing [32, 33].

1.5. Decellularization with supercritical CO₂

A new decellularization technique uses supercritical carbon dioxide (scCO₂). A supercritical substance has high diffusion speed and permeability, because it has properties of both liquids and gases. scCO₂ has good critical conditions at a temperature of 32° C and a pressure of 7.38 MPa, which ensures that the mechanical properties of the pericardium hardly change during the treatment [29].

 $scCO_2$ diffuses into a cell and causes changes in the cellular environment, such as lowering the pH and secretion of the cytosolic enzyme lactate dehydrogenase (LDH), which is an indication for mechanical degeneration [34]. At the end of the treatment, CO_2 returns to the gas phase and naturally diffuses out of the pericardium. Additionally, pericardium decellularized with $scCO_2$ can be stored dry, because no chemicals are needed for storing. The advantage of these processes is that no chemical substances remain in the pericardium. This means that tissue, which is decellularized with $scCO_2$, is safe for patients [29, 35].

Until now it is proved that pericardium, decellularized with $scCO_2$, does not shrink, that it is flexible and that it does not rupture within minutes under repetitive stress [36]. If decellularized pericardium will be used as an ascending aortic prosthesis, it is important that it is capable to withstand the pressures in this environment for a long period of time. Our research is therefore mainly focused on long-term measurements of $scCO_2$ decellularized pericardium.

1.6. Research questions

Our main research question is: 'How suitable is porcine pericardium, decellularized with supercritical CO_2 , as a material for an ascending aortic prosthesis?'

To answer this question it is important to take into account several aspects, which we phrase into subquestions. For example, it is important to know which mechanical forces and pressures are working on the ascending aorta to determine and measure whether the pericardium can handle these. We will research this by means of the subquestion: 'Which mechanical forces are working on the ascending aorta?' (Subquestion 1).

Pulsatile flow to which the aorta is exposed, can influence the inflammatory reaction and the cells that grow into the prosthesis. It is important to examine what this influence is, because this can have a direct influence on the extent of regeneration and eventual mechanical properties of the prosthesis. The subquestion therefore is: 'How does flow influence the proliferation of tissue?' (Subquestion 2).

ECM of the pericardium consists of collagen and elastin fibers, which are orientated in a certain way. It is important to find out what the best way of fiber orientation is for an ascending aortic prosthesis. The subquestion is: 'What is the effect of the orientation of collagen fibers on the mechanical properties of an aortic prosthesis?' (Subquestion 3).

We will answer the question 'How does pericardium, decellularized with $scCO_2$, change after long-lasting repetitive stress?' (Subquestion 4) by means of an experiment.

Eventually it is important to determine whether a prosthesis, made of porcine pericardium, decellularized with $scCO_2$, fulfills the Windkessel function in a better way than the current Dacron prosthesis. To answer this, we ask ourselves the question 'Can we show with our experimental set-up that pericardium, decellularized with $scCO_2$, fulfills the Windkessel function in a better way than a Dacron prosthesis?' (Subquestion 5). These subquestions enable us to answer our main question.

2. Methods

We decided to answer our first three questions with literature and the last two with experiments. In order to obtain information for the first three subquestions we used our study books, scientific articles and we even approached a teacher.

2.1. Experiments

To answer the fourth and fifth question we performed experiments. We sutured pericardium in a tubular shape with pledgets on the seam (Supplement, page 19). We made the tube of pericardium in such a way that the fibers were oriented in the best possible way according to literature, which is a predominantly circumferential orientation (Literature results: Subquestion 3, page 6). In both the experiment for the fourth as the experiment for the fifth question, we used a Dacron prosthesis as control group, because this is the current golden standard.







Figure 1: Experiment set-up, in which the Abiomed pump, mechanical heart, two blood pressure monitors and reservoir can be seen. Pericardium or Dacron can be placed between the tubes between the blood pressure monitors.

To determine the effect of long-term repetitive stress on the pericardium, we made a set-up with the Abiomed AB5000, a mechanical heart, a reservoir and tubes (Figure 1 and 14). We fixed the pericardium or Dacron between tubes, between the mechanical heart and the reservoir (Figure 18 and 19). From now on we refer to this experiment as Experiment 1.

To determine whether the pericardium fulfills the Windkessel function in a better way than the Dacron prosthesis, we added two blood pressure monitors: one before and one after the pericardium (Figure 1 and 14). From now on we refer to this experiment as Experiment 2.

Experiment 1 is a long-term experiment and we did two measurements of two hours each for both the pericardial tube as for the Dacron prosthesis. We initially wanted to do measurements of 24 hours, but due to technical difficulties we eventually could only do measurements of two hours (Discussion: Abiomed and hand pump, page 11). By means of this experiment we wanted to know whether the pericardium can handle the mechanical forces that work on the aorta. If the pericardium would not rupture, we wanted to know whether it stretches or not. That is why we measured the thickness of the pericardium before and after the experiment. We measured the thickness with a micrometer. The Dacron prosthesis is the control group, of which we measured the length and diameter before and after the two hour measurement with measuring tape. We checked the pressure before and after the pericardium roughly every 30 minutes, while Experiment 1 was running.

In Experiment 2 we did some short measurements, so we could see whether the Windkessel function was performed. To obtain these results, we used the Abiomed pump and the hand pump, that comes with the Abiomed. We pumped with the hand pump to determine the pressure before and after the pericardial tube and did the same for the Dacron

prosthesis. We called this 'Measurement 1'. Following, we placed a Hoffman clamp after the pericardium or Dacron to increase the pressure a bit and then determined the pressures before and after again. We used the Hoffman clamp to increase the pressure a bit further and repeated this until the tube could not be compressed any more. In that way we obtained Measurement 2, 3 and 4. We obtained Measurement 5 by using the Abiomed pump, without the Hoffman clamp. Since the Abiomed needs time to adjust itself to the set-up, we read the pressures after one or two minutes.

Finally, we analyzed the results of our experiments and compared them with literature. To compare the thickness of the pericardium before and after Experiment 1, we applied the Student's t-test with a significance level of 5% (Supplement, page 18). We did the same for the comparison between the length of the Dacron prosthesis before and after Experiment 1.

3. Literature results

3.1. Subquestion 1 - Mechanical forces

To determine the suitability of pericardium for a prosthesis of the ascending aorta, we first need to know which forces are normally working on the ascending aorta.

3.1.1. Fluid pressure and laminar flow

In fluids there is a pressure, which is the force exerted per unit area by the fluid, perpendicular to an (imaginary) plane. With a fluid in a tube, both height and flow have an influence on this fluid pressure, which is described by the law of Bernoulli (Equation 4, page 16). In blood, however, there is yet another factor influencing the fluid pressure.



The blood flow is in fact not constant over the entire cross section of the vessel. Due to its viscosity, the blood has a laminar flow, which means that the blood along the wall has a low speed that increases towards the center of the vessel. By replacing the flow rate of the blood particles by a mean flow rate (Equation 5, page 16), Bernoulli's law applies on the fluid pressure of the blood [37].

When a viscous fluid, like blood, flows through a tube, it requires energy to compensate friction losses and thus to sustain flow. This energy is provided by the external environment of the tube through maintaining a pressure difference over the tube. The relationship between this pressure difference and the flow in a tube is described by the law of Poiseuille. Within this law it is assumed that fluid does not flow too fast and that the tube is horizontal with a constant cross-section:

$$\Phi = \frac{\Delta P}{R} \qquad \qquad \begin{array}{l} \Phi = \text{flow} \\ \Delta P = \text{pressure difference} \\ R = \text{flow resistance} \end{array} \tag{1}$$

$$R = \frac{8\mu L}{\pi r^4} \qquad \begin{array}{c} L = \text{length} \\ \mu = \text{Poisson ratio} \\ r = \text{radius} \end{array} \tag{2}$$

Flow through a tube is thus directly proportional to the pressure difference between the ends of the tube and inversely proportional to the flow resistance. Because the flow resistance is inversely proportional to the fourth power of the radius, the resistance changes sharply at relatively small changes of the radius. By adjusting the radius of arteries, the body can adapt blood flow to the needs of the respective organs in an efficient manner [38]. For a laminar flow through a tube of which the cross section and/or the height is not constant, a combination of the laws of Bernoulli and Poiseuille applies (Equation 7, page 16).

3.1.2. Tissue elasticity

Until now we have only considered pressure and flow in a rigid tube. Blood vessels, however, have the ability to adjust their radius due to their elasticity.

If the heart contracts, blood is pumped into the aorta. Pressure in the aorta increases and therewith radius and volume of the aorta also grow. During this change of radius of the aorta, the wall tension will change. In order to display how the wall tension changes, Hooke's law (Equation 8, page 16) and Laplace's law (Equation 10, page 17) need to be merged to form the following equation:

$$T = \frac{R - R_0}{R_0} E d \qquad \begin{array}{c} T = \text{ wall tension} \\ R_0 = \text{ radius before change} \\ R = \text{ radius after change} \\ E = \text{ Young's modulus} \\ d = \text{ wall thickness aorta} \end{array}$$
(3)

This equation, resulting from the assumption that elasticity is linear, only describes the reality well at low values of pressure. At higher values of pressure, collagen in the vessel wall will dominate the properties of elastin. Elasticity then enters the non-linear area. In this area deformations are not reversible, which is called plastic deformation. Upon removal of the force, the original form does not come back. An even further increase of the force ultimately results in a fracture [38].

3.1.3. Windkessel function of the aorta

In case of the aorta, high adaptability is necessary because of the high pulsating pressures it has to withstand. With an increasing ventricle pressure, aortic pressure also increases. The aorta then expands and becomes a blood reservoir. After the aortic valve closes, the aorta no longer fills with blood and supplies the vascular system with blood. Aortic pressure and volume thereby decrease, approximately according to an exponential decay. Hence, the aorta has the ability to expand with increasing pressure and when pressure decreases again, it goes back to its original form due to its elasticity. This property of the aorta makes sure that the rest of the vascular system does not have to carry such a heavy load. If the aorta would be a rigid tube, blood would be pulsing much stronger in the rest of the vascular system, resulting in a much heavier load. This important function of the aorta is known as the Windkessel function, which is described in the Windkessel model (Supplement, page 17) [37].

3.2. Subquestion 2 - Flow and proliferation

Pulsatile flow could influence proliferation of cells. Since there is pulsatile flow in the aorta, due to the heart rate and blood pressure, we look into the influence of pulsatile flow on proliferation of cells around the pericardial prosthesis.

3.2.1. Cell migration to the wound

When the prosthesis is inserted into the aorta, there will be a wound. Endothelial cells polarize and migrate to the wound to make the monolayer continuous again. This process builds upon a couple of cellular activities: [39, 40]

- border cells are induced for directed migration
- inner cells are modulated for autonomous random migration
- cell motion within the endothelium are coordinated

In response to various growth factors, fibroblasts also migrate to the wound, they proliferate and form a new ECM by excreting collagen and fibronectin. Possibly present stem cells can establish new blood vessels, after migrating to the wound [41].

3.2.2. A grated substrate

Franco et al. (2012) found out that the substrate affects the rate of proliferation of endothelial cells under flow. They did experiments with grated and flat substrates and looked at the effect of flow perpendicular to the wound. There is a significant increase in the speed of endothelial regeneration on substrates with gratings oriented in the direction of the flow, compared to flat substrates under perpendicular flow. A large wound does close on a grated substrate, but does not close on a flat substrate. This has a few reasons.





First, adherens junctions between border and inner cells are stabilized by the grated substrate. Because of this, inner cells migrate together with border cells to close the wound. On a flat substrate, adherens junctions are not stabilized, causing border cells to migrate individually, get loose and be flushed away by the flow. Second, cells on a grated substrate migrate further than cells on a flat substrate. This is because the motility signal is spread further into the monolayer, because of the better connection between the border and inner cells [42].

Robotti et al. (2014) have done a similar study. They did research into the endothelialization under supraphysiological stress. They observed that physiological flow and physiological wall shear stress (WSS) have no impact on the wound healing and proliferation. With a flat substrate, an endothelial layer with good integrity can be formed. Supraphysiological flow and supraphysiological WSS, however, have an adverse effect on wound healing and proliferation, resulting in a poorer integrity of the endothelial layer. A gridded embossed surface is needed in order to obtain a good integrity [43].

3.3. Subquestion 3 - Effect of fiber orientation

3.3.1. Components of the aortic wall

Three layers can be distinguished in the aortic wall: tunica intima, tunica media and tunica adventitia (Figure 2). The tunica intima is the inner layer and consists of a monolayer endothelial cells with some subendothelial tissue. The tunica media consists of smooth muscle cells, an elastic lamina and a network of collagen and elastin fibers. The elastic lamina splits the tunica media in a couple of layers that are reinforced by fibers. The tunica adventitia is the outer layer of the aorta that mainly consists of thick bundles of collagen fibers and is surrounded by connective tissue [44].



Figure 2: Layers of the artery wall, with ECM components [44]

ECM of the aortic wall consists mainly of type I and III collagen and elastin fibers, but also contains other components like smooth muscle cells, glycoproteins and glycosaminoglycans [45, 46]. In a healthy artery, mechanical properties under physiological pressures are mainly determined by the collagen and elastin fibers in the tunica media [44]. Under supraphysiological pressures, the tunica adventitia also plays a role [47]. Elastin determines stretchability and resilience of arteries and collagen determines stiffness and strength of arteries. This means that collagen fibers make sure that the artery wall can resist high pressures, making it the most important ECM component for mechanical properties. Furthermore, collagen plays an important role in the development of arterial diseases and degeneration of tissue. If collagen is disrupted, tensile strength of the aorta decreases and an aneurysm can develop more easily [46, 48, 49].

3.3.2. Mechanical properties

The aortic wall behaves as a two-phase material: collagen fibers, with high tensile strength and Youngs modulus, absorb most of the force under physiological pressure and the elastin network, with a low Youngs modulus, divides these forces uniform [50, 51].

The stress-strain curve of aortic tissue with collagen and elastin fibers can be divided into three phases (Figure 3). At the beginning of the strain (early phase), stress is mainly absorbed by elastin fibers, while the collagen fibers are still twisted. If strain becomes more fierce, collagen fibers unfold, causing them to activate. In the stress-strain curve this can be seen as a non-linear response (transition phase). Moreover, additional collagen fibers are recruited, which eventually absorb high strains (later phase) [47, 52].

Arteries do not behave according to Hook's law, because of the combination of elastic fibers and collagen fibers (Supplement, page 16). Collagen fibers are less elastic and reach their maximal length at a great extension, causing arteries to withstand strain better, the more they are extended [50].



Figure 3: Stress-strain correlation of collagen and elastin fibers of aortic tissue [52]







3.3.3. Fiber orientation aorta

During histological and microscopic studies, researchers found out that collagen fibers are mainly oriented circumferential in the tunica media (Figure 4) [46, 49, 53, 54, 55]. If there is a gradual increase in stresses on the tissue, the collagen fibers straighten and some reorganize in the direction of the applied stress (Figure 5) [49, 53].

The aorta is exposed to high blood pressures and high circumferential (elastical) strains. Given this fact, it is reasonable that the fibers are mostly oriented circumferentially. Like this, the aorta can absorb pulsating pressure waves and prevent the aortic wall to stretch too far [46]. According to Ottani et al. (2001) the fiber direction is always an indication for the tensile stresses that are prevalent in the tissue, because fiber reorganization is the most effective way to optimize the strength without raising the mass or adjusting the metabolism [56]. When looked at multiple cross sections in different planes, it seems that there is not just a circumferential orientation, but also a small longitudinal movement of the fibers. Hence, the collagen fibers actually run in the shape of a helix with a small angle of inclination [55, 56]. This special structure makes the tunica media very strong and resilient and makes it capable of resisting stresses in both circumferential and longitudinal direction [44].

Since the fibers are oriented in a certain way, the mechanical properties of the aorta depend on the direction of the force. Wu et al. (2015) proved that the ultimate tensile strength (UTS) and later-phase elasticity modulus are twice as big in the circumferential direction than in the longitudinal direction and that the early-phase elasticity modulus does not differ between the circumferential and longitudinal direction [52]. Thus, the early-phase elasticity modulus, that is determined by elastin fibers, is not influenced by the direction of the force. In contrast the later-phase elasticity modulus, which is determined by collagen fibers, is dependent of the direction of the force. This is probably caused by the circumferential orientation of the collagen fibers.

Pichamuthu et al. (2013) also proved that circumferential oriented samples were stronger and had a higher stiffness than samples that were longitudinal oriented [48].

4. Experiment results

4.1. Subquestion 4 - Long-lasting repetitive stress

Pericardium was able to withstand two hours of repetitive stress without rupturing completely. Results of the longterm experiments are shown in Table 1 and 2. Length and diameter of Dacron and thickness of the pericardium before and after the two-hour measurements can be seen in these tables.

During the experiment, water is dripping out of the Dacron. This also happened during the experiments with pericardium, where water at certain points began to leak out of holes near the suture and near the edges of the tubes.

After two hours, the length of Dacron is increased 2 mm (Table 1). The diameter did not change. At the end of Measurement 1 and 2 of the pericardium, the thickness decreased (Table 2). The difference in thickness of the pericardium before and after Measurement 1 is 7 μ m (7%). For Measurement 2 the same pericardium is used and after this measurement the thickness is decreased with 30 μ m (33%) compared to the thickness before Measurement 2.

4.2. Subquestion 5 - Windkessel function

The results of the short measurements can be seen in Table 3 and 4. They show systolic and diastolic pressures at two positions, before and after the Dacron or pericardium and the pressure difference between these two.

Systolic pressure increases and diastolic pressure decreases after the Dacron prosthesis, regardless of the pressure before it (Table 3). This is the case with both the hand pump and the Abiomed pump. The mean increase in the systolic pressure is +19 mmHg, the mean decrease in the diastolic pressure is -10 mmHg.

In case of the pericardium, it can be seen that the systolic pressure decreases and that the diastolic pressure remains stable after the pericardial tube, regardless of the pressure before it (Table 4). This is the case with both the hand pump and the Abiomed pump. The mean decrease in systolic pressure is 30 mmHg.

In Figure 6 the difference between the pressure before and after the prostheses is plotted against the pressure before the prostheses. It can be seen that the higher the systolic pressure is before the pericardium, the less negative the pressure difference. However, it can also be seen here that the higher the systolic pressure is before the Dacron prosthesis, the more positive the pressure difference.

The pressure difference of the pericardium stays equal when the diastolic pressure increases. With Dacron, however, the pressure difference becomes less negative when the diastolic pressure increases.

5. Discussion

5.1. Subquestion 1 - Mechanical forces

The ascending aorta has to deal with fluid pressure, wall tension and the Windkessel effect. These factors are interrelated and thus they are complex requirements for a prosthesis to satisfy. If the prosthesis is not elastic enough to withstand physiological blood pressures, the wall tension has risen to the yield point of the material. At this point, the area of elasticity becomes an area of plasticity. If fluid pressure in the prosthesis exceeds this point, any deformation of the prosthesis will be permanent. Thereby the prosthesis loses its elasticity, which means that it cannot fulfill the Windkessel function anymore. This is the same process as with the formation of an aneurysm, which was the initial problem. For the prosthesis to be a good replacement for the ascending aorta it therefore has to be able to withstand physiological blood pressures and wall tension in the ascending aorta. In addition, the prosthesis has to be able to fulfill the Windkessel function, because otherwise







Figure 4: (a) Circumferential cross section of the aortic wall, on which the circumferential arrangement of collagen bundles can be seen [53], (b) Polarized light microscopy (PLM) image of the tunica media of the aorta. Horizontal is the circumferential direction, vertical the longitudinal direction [46], (c) PLM image of the tunica media of the aorta. Mainly the circumferential orientation can be seen. Horizontal is the circumferential direction, vertical the longitudinal direction [54], (d) Second-harmonic imaging microscopy image of a section of the tunica media of the aortic wall. Collagen fibers are accentuated. Collagen bundles are layered and are oriented in the circumferential direction. Horizontal is the circumferential direction, vertical the longitudinal direction. [49]



Figure 5: Longitudinal cross section of the aortic wall. If there is a higher load (b-i) in the longitudinal direction, collagen fibers increasingly reorientate in the longitudinal direction [53]





	$\begin{vmatrix} \text{Length (mm),} \\ \text{mean} \pm \text{SD} \end{vmatrix}$	Diameter (mm), mean \pm SD	Pressure (mmHg), mean \pm SD	Cardiac output (L/min), mean \pm SD	Heart frequency $(/\min)$, mean \pm SD					
Before After	$ \begin{array}{r} 126 \pm 0 \\ 128 \pm 1 \end{array} $	28 ± 0 28 ± 0	$\frac{183/28 \pm 9/11}{198/22 \pm 7/12}$	4.8 ± 0.1	53 ± 1					

 Table 1: Two hour measurement of Dacron

 Table 2: Two hour measurements of pericardium

	Measurement	$\begin{vmatrix} \text{Thickness } (\mu \mathbf{m}), \\ \text{mean } \pm \mathbf{SD} \end{vmatrix}$	Pressure (mmHg), mean \pm SD	Cardiac output (L/min), mean \pm SD	Heart frequency $(/\min)$, mean \pm SD
Before After	1	$ \begin{array}{r} 98 \pm 34 \\ 91 \pm 19 \end{array} $	$\frac{158/13 \pm 8/4}{139/11 \pm 2/9}$	5.1 ± 0.2	57 ± 1.7
Before After	2	$ \begin{array}{c c} 91 \pm 19 \\ 61 \pm 7 \end{array} $	$\frac{197/40 \pm 4/14}{168/40 \pm 3/8}$	5.0 ± 0.1	55 ± 0.9

Table 3: Pressure before and after the Dacron prosthesis. Measurements 1 to 4 are executed with the hand pump. During Measurement 1 no Hoffman clamp is used. During measurement 2 to 4, the Hoffman clamp is tightened sequentially, by which the pressure was increased. Measurement 5 is done with the Abiomed pump for comparison.

	Measurement 1	Measurement 2	Measurement 3	Measurement 4	Measurement 5 $$
Pressure before (mmHg)	175/65	180/70	195/80	240/105	240/75
Pressure after (mmHg)	190/55	190/55	215/75	260/100	260/60
Pressure difference (mmHg)	+25/-10	+10/-15	+20/-5	+20/-5	+20/-15

Table 4: Pressure before and after the pericardium. Measurements 1 to 4 are executed with the hand pump. During Measurement 1no Hoffman clamp is used. During measurement 2 to 4, the Hoffman clamp is tightened sequentially, by which thepressure was increased. Measurement 5 is done with the Abiomed pump for comparison.

	Measurement 1	Measurement 2	Measurement 3	Measurement 4	Measurement 5
Pressure before (mmHg)	155/30	165/45	195/60	240/80	155/40
Pressure after (mmHg)	125/30	130/45	165/60	220/80	120/40
Pressure difference (mmHg)	-30/0	-35/0	-30/0	-20/0	-35/0



Figure 6: The difference between the pressure before and after the prostheses is plotted against the pressure before the prostheses



the cardiac system and the prosthesis itself would have to deal with supraphysiological pressures.

5.2. Subquestion 2 - Flow and proliferation

Since a prosthesis is not completely the same as a human aorta, there is a chance that there will be a supraphysiological flow. If this is the case, wounds, made by implanting the prosthesis, heal faster when the aortic prosthesis has a grated surface. Moreover, adjacent endothelial cells grow into the prosthesis faster when the prosthesis has a grated substrate and a good integrity is obtained.

5.3. Subquestion 3 - Effect of fiber orientation

Since tissue has the largest strength and elasticity modulus in the direction in which collagen and elastic fibers are oriented, the fibers are naturally oriented in the direction where most of the stresses occur. In a healthy aorta, the fibers are therefore mainly oriented in the circumferential direction with a small longitudinal movement, generating the shape of a helix. We think it is useful to orient fibers of the ECM of the decellularized pericardium just like in a healthy aorta. We therefore choose to orient the fibers of the pericardial tubes for our experiments in a mainly circumferential direction.

The more collagen fibers are oriented in a circumferential way, the stiffer the tube becomes. A stiff tube would have a negative effect on the Windkessel function, which is something we want to avoid at all times. However, we think that a mainly circumferential orientation of the fibers in a pericardial prosthesis, would not have such a negative effect on the Windkessel function. Fibers in pericardium are not all oriented in the same way, thus a mainly circumferential direction in a tube still includes fibers in other directions.

ECM of pericardium and aorta both consist of collagen type I and elastin fibers. The aorta additionally consists of type III collagen. Type III collagen in the aortic wall increases flexibility and provides most of the tensile strength of the aorta [57, 58]. Type III collagen also has an important function in the normal fibrillogenesis of type I collagen [59]. In patients with a disease due to genetic mutations in the encoding for type III collagen, a rupture or aneurysm of the aorta often occurs [58, 59, 60]. This means type III collagen has an important function in the aorta. A prosthesis for the ascendings aorta made of scCO₂ decellularized pericardium might therefore be insufficient.

5.4. Subquestion 4 - Long-lasting repetitive stress

After two hours of repetitive stress, the length of the Dacron prosthesis is increased 2 mm and the diameter did not change. During the experiment systolic pressure was higher and diastolic pressure lower than in the physiological situation. Based on our results, the difference in length of the Dacron prosthesis before and after the experiment is not significant, p = 0.10-0.20 and $\alpha = 0.05$ (Supplement, page 18). Therefore we conclude that Dacron can handle two hours of repetitive stress and that our

set-up can be used to test pericardium. We measured the length of the Dacron only one time in the right way (Supplement, page 19). In order to reduce the risk of measurement errors, it would be better to test and measure the Dacron several times.

Pericardium was also able to withstand two hours of repetitive stress. Both measurements of the pericardium show a decrease in thickness. Bases on our results, the decrease in thickness of 7 μ m during Measurement 1 is not significant, p = 0.50-1.00 (Supplement, page 18). On the contrary, we conclude the decrease in thickness of 30 μ m during Measurement 2 is significant, p = 0.01-0.02 and $\alpha = 0.05$. During the second measurement, with the same piece of pericardium, the decrease in thickness was much greater than during the first measurement. During Measurement 2, the mean systolic pressure was a lot higher than during Measurement 1. This might be the cause of the greater reduction in thickness of the pericardium during Measurement 2.

If the decrease in thickness is dependent on the duration of the repetitive stress, this would imply that the prostheses will become thinner and thinner when pericardium is implanted for several years and eventually fail. The pericardium will, however, become stronger when cells grow into the material. Therefore, more research is needed to show what influence is bigger (Recommendations, page 12)

During both measurements of the pericardium, systolic pressure was higher and diastolic pressure lower than in a physiological situation. This could have resulted in an overestimation of the reduction in thickness of the pericardium during our two hour measurement. However, there is no overestimation when a prosthesis is implanted in patients with hypertension and supraphysiological pressures. In these patients, the pericardium will become thinner like in our experiments. We did, however, show that the pericardium can withstand such high pressures for two hours.

For measuring the thickness of pericardium we used a micrometer. Because pericardium is a soft material, this measuring instrument compresses the pericardium. Therefore, we can only indicate the relative decrease in thickness and not the absolute results of this measurements.

5.5. Subquestion 5 - Windkessel function

Windkessel function of the aorta mainly depends on the elasticity of the vessel. An elastic aorta, with a great Windkessel effect, damps the fluctuation in blood pressure. As a result, at any given value of the mean arterial pressure the systolic pressure decreases and the diastolic pressure increases. This means that the pulse pressure decreases. A decline in the Windkessel function, as a consequence of a stiffer aorta, leads to an increase in the systolic pressure and a decrease in diastolic blood pressure, relative to a healthy aorta. The pulse pressure thus becomes higher [61].

Since Dacron creates an increase in the systolic blood pressure and a decrease in the diastolic blood pressure, this indicates that it acts like a stiff tube and that it cannot exert a proper Windkessel function. As opposed to Dacron, pericardium creates a decrease in the systolic blood pressure, while the diastolic pressure remains unchanged.



Pericardium thus creates a decrease in pulse pressure. From this experiment, we can indeed conclude that pericardium fulfills the Windkessel function in a better way than a Dacron prosthesis.

Our results show a relationship between the systolic pressure before the pericardium and the pressure difference, which is caused by the pericardium. The higher the systolic pressure before the pericardium, the lower the pressure difference. This means that the pericardium fulfills the Windkessel function best at low systolic pressures and the higher the systolic pressure, the less the Windkessel effect. The latter also applies to Dacron.

We think that it is a logical consequence that the Windkessel function is fulfilled less when the systolic pressure increases, because the vessel is more filled under a higher pressure. As a consequence, it can expand less and pressure will thus be absorbed less. This effect will be more under a higher pressure. We think that the curve first rises quickly and then flattens out. Therefore, we do not think there is a complete linear relationship between the systolic pressure before the pericardium and the pressure difference. Moreover, we just have four measuring points, which show a little part of the graph. It could well be that the linearity does not exist by lower or higher pressures. Therefore, it is difficult to predict the pressures by 120 mmHg, the physiological value.

The results of the pericardium could, however, be influenced by leakage, with an average of 7.5 L/h. We see a decrease in systolic pressure after compared to before the pericardium, from which we draw our conclusion. Water leakage through the holes of the wall of the pericardium could, however, cause a decrease in the systolic pressure too. We do not believe that the reduction in systolic pressure is only caused by the leakage, because we expect that the leakage per minute is too small to have provided for a pressure drop of an average of 30 mmHg in a couple of minutes. We therefore conclude that the decrease in systolic pressure might be overestimated in our results, but that it is not only caused by leakage. In addition, the diastolic pressure after the pericardium is equal to the diastolic pressure before the pericardium in all of our measurements. If there would not be any leakage of the pericardium, the diastolic pressure would increase after the pericardium relative to before the pericardium. The difference between diastolic pressure before and after the pericardium is thus underestimated in our results.

In summary, if there is no leakage, there is still a decrease in systolic pressure due to the pericardial prosthesis and an increase in diastolic pressure due to the pericardial prosthesis. This still leads to the conclusion that pericardium provides for a better Windkessel function than Dacron.

5.6. Set-up and tubes

We experienced a couple of difficulties with building our set-up. For example, it would be desirable to test the pericardial tube with an average aortic diameter. However, this was not easy to realize. The diameters of the inlet and outlet of the mechanical heart and reservoir, which we have at our disposal, have fixed values. The outputs of the mechanical heart have a diameter of $\frac{1}{2}$ inch (1.27 cm). If

we would use a pericardial tube with a diameter larger than 1.27 cm, there would be a pressure drop in the pericardial tube. Then we would not fully be testing the influence of repetitive stress on the pericardium. This is why we chose to use the same diameter as the outlets of the mechanical heart and the reservoir for the pericardium and tubing.

The outlets of the reservoir have a diameter of $\frac{3}{8}$ inch (0.95 cm), which still results in a pressure difference with the other tubes, with a diameter of 1.27 cm. However, we assumed that this does not have a great influence on the pericardium.

The Dacron prosthesis we used as a control group, has a diameter of 3 cm. We were aware that this would result in a pressure drop in the Dacron prosthesis and thus the Dacron would not be enduring the same pressures as the pericardial prosthesis. However, we assumed that narrowing the diameter of the Dacron prosthesis would lead to bigger problems. We would then in fact have to cut the Dacron and suture it at a smaller diameter, resulting in leakage problems, which in turn would still provide a pressure drop in the Dacron prosthesis. Moreover, the mechanical properties of the Dacron prosthesis would decline by interrupting the woven structure.

Another item for discussion is the stiffness of the tubes we used in our set-up. The tubes in our set-up are many times stiffer than the environment in which a prosthesis in the human body would have to function. In our set-up, the only parts that have the ability to perform the Windkessel function are the Dacron or pericardial prosthesis, while this is not the case in the human body. An aortic prosthesis in our set-up thus has to deal with much higher forces than in the human body.

5.7. Abiomed and hand pump

We have also encountered some problems with the Abiomed pump, which we had at our disposal. The Abiomed is designed to take over the function of the heart in a patient, and to adjust itself if needed. We could not set the heart rate or flow the way we wanted, because the pump then adjusts itself again. In addition, we think that the pressure sensors do not function as they should, which sometimes results in a high pressure alarm while there is no high pressure at all. This high pressure alarm in turn causes the pump to lower its frequency and volume. The Abiomed pump is thus highly inconstant, which led to the technical difficulties.

Due to the technical difficulties we had with the pump, we eventually were forced to do measurements of two hours. With longer periods of testing and physiologic pressures, we would have obtained more information about long-term implantation of a pericardial prosthesis.

For Experiment 2 we use the hand pump. The frequency and force with which we pump, have an influence on the pressure. With high frequencies, diastolic pressure is higher than with lower frequencies, because the system simply does not have the time to restore lower pressures. We tried to limit this influence by making sure that the same person pumped during the measurements for Experiment 2. This person tried to keep the same frequency and power for at least every measurement for a certain tested prosthesis. In





addition, we do not compare the pressures of the various prostheses of Experiment 1 with each other as being absolute numbers, but only compare an increase or decrease in pressures within one measurement.

5.8. Leakage

We notice that over time water starts to leak trough the wall of the Dacron prosthesis. The Dacron prosthesis we talked about in the section 'Experiment results' (page 7) leaked only 0,09 L/h, but with another Dacron prosthesis we tested, we saw that leakage quadrupled in a second measurement of two hours. We believe that this occurs because the coating on the inside of the Dacron prosthesis, which prevents leakage through the wall, is not made to remain during a long period of implantation. This ensures that cells can migrate through the wall of the prosthesis. Once the coating disappears during our experiments, the wall of the Dacron prosthesis begins to leak due to the lower viscosity of water as opposed to the viscosity of blood and due to the absence of an inflammatory response and of coagulation factors.

With the pericardial tube we had to deal with much greater leakage. The pericardial tube we talked about in the Experiment results (page 7), leaked 5.92 L/h in the first measurement and 9.03 L/h in the second measurement. Water leaked through holes created by suturing and through holes near the edges of the tubes, caused by friction between pericardium and tube. The increase in leakage is caused by further tearing of the holes. We think that the holes near the tubes would not be a problem for implantation of the pericardium, because that kind of friction does not exist in a physiological environment. We also think that the leakage near the sutures will not be a problem in the physiological environment, again because blood instead of water flows through the prosthesis. Blood has a higher viscosity than water, which could mean that blood will leak through the wall less quickly than water. Moreover, blood has thrombocytes and coagulation factors, which have the ability to close small holes.

There has not been done a lot of research yet about pericardium, which is in this way decellularized with $scCO_2$.

6. Recommendations

6.1. Improvements current set-up and experiments

For future research, we would recommend using a pump that can be set as desired. It has to be a pulsating pump, that pumps with a frequency of at least 60 beats per minute and that, ideally, can be set at very high frequencies so that, in a given time, a longer period of implantation can be simulated (accelerated testing).

A pump that does not adjust itself, is also useful for future research because it enables measurements longer than two hours. With longer measurements one can determine if the decrease in thickness of the pericardium is dependent on the duration of the repetitive stress and usage for several years can be simulated. A two hour measurement is not long enough to assess whether the prosthesis is safe enough to be implanted in a patient for several years. For biological heart valve prostheses, the FDA has established guidelines for durability tests. They indicate 200 million cycles as the the minimum durability requirement for flexible valves, which simulates approximately five years of use. In addition, the back pressure during testing must be 125 mmHg [62, 63]. Our advice for future research is to stick to these guidelines, because a prosthesis for the ascending aorta should also be able to withstand at least five years of repetitive stress.

In order to simulate physiological situation better, we recommend the use of wider tubes with a diameter of an average aorta, allowing the pericardial tube to also be made with that diameter. In addition, the pericardium can be compared better with the Dacron. A further requirement for this arrangement to work is that the other materials, such as the reservoir, must have the same input and output diameter as these wider tubes. This comes down to an average aortic diameter for the whole set-up.

To improve the simulation of the physiological situation even more, it would be very useful to research how a pericardial prosthesis functions in an elastic environment, similar to the environment of the ascending aorta. The piece of pericardium would ideally be placed in between aortic tissue. This would also give more information about whether or not the pericardium fulfills the Windkessel function when implanted in the aorta.

The disadvantage of the micrometer, is that it might compress the pericardium and as a result might measure a smaller thickness than the actual thickness. In order to measure the actual thickness in the future, it would be better to use a measuring instrument that does not compress the material. This says more about the real thickness and whether this thickness is save enough for implantation.

In our experiments we encountered some problems with leakage. It would be better to reduce leakage in the future, because it enables researchers to draw more useful conclusions. Leakage at the connection between the pericardium and the tubes can be solved partially or completely by reducing the friction. With the tubes we use, it might help to file them, making them less sharp. Using a fluid with a viscosity closer to blood could also reduce leakage, because the fluid is then less likely to leak through the holes at the sutures. A fluid with coagulation factors would be even better because those would seal the holes at the sutures, resulting in much less leakage. Ideally, researchers will use blood as fluid in the set-up.

6.2. Additional topics for research

For future research it would be very useful to examine if the decrease in thickness of the pericardium is dependent on the prevailing pressures. Future researches should test pericardium for a certain period of time at various pressures. The results of this experiment will show if the pericardium is suitable to function at physiological pressures or even at supraphysiological pressures.

To be able to say whether the pericardium is suitable for implantation, it is not only important whether it becomes



thinner by prevailing pressures, but also the extend to which ingrowth of cells can take place at the prevailing pressures after implantation. It is necessary to examine whether the thinning of the pericardium is in equilibrium with the strengthening of the prosthesis by the ingrowth of cells. An important part of this future research is whether it is possible for cells to grow into a tissue, in which this cell type has never grown before. Aortic tissue contains for instance smooth muscle cells and type III collagen, which are not present in pericardial tissue.

In the discussion we mentioned that we did not take into account that ECM of a human aorta also contains type III collagen, while ECM of pericardium does not contain this type of collagen. In order to investigate whether the absence of type III collagen in the pericardium is of great influence for it to function as an aortic prosthesis, we recommend to compare $scCO_2$ decellularized pericardium with $scCO_2$ decellularized aorta in a similar R&D setting. If it appears that collagen type III is crucial for an aortic prosthesis, it should be examined as discussed above whether it is possible for type III collagen to grow into the pericardium after implantation.

We conclude from literature that a mainly circumferential orientation of fibers would be the best way for us to make a tube out of pericardium, but as far as we know there are no experiments that prove the best orientation of fibers for a pericardial aortic prosthesis. A comparison of longitudinal orientation and circumferential orientation in an experiment could prove or reject our conclusion.

Furthermore, we think it would be useful to investigate if an addition of some kind of grating would make the pericardium more suitable as a material for an aortic prosthesis. Future researchers can add a grating on the inside of the pericardial tube and/or include a coating with growth factors such as VEGF. Experiments with these factors in an environment with relevant cells will demonstrate whether any of these factors, or both, have an additional beneficial effect on the proliferation of tissue.

7. Conclusion

The main question we worked with is: 'How suitable is porcine pericardium, decellularized with supercritical CO_2 , as a material for an ascending aortic prosthesis?' With our experiments we proved that porcine pericardium can withstand repetitive stress for two hours and the thickness remains the same under physiological pressures. However, it becomes thinner under supraphysiological pressures, indicating that further research is required to determine the importance of the consequences (Recommendations, page 12). Pericardium seems to fulfill the Windkessel function in a better way than Dacron, so this is a promising outcome. In addition, tensile strength has proven to be sufficient and porcine pericardium, decellularized with supercritical CO_2 , does not evoke unwanted reactions in the human body. In short, porcine pericardium, decellularized using supercritical CO_2 , is a promising material and we strongly recommend to do more research to prove whether it can be used as a prosthesis for the ascending aorta.

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Supplement

A. Additional information subquestions

A.1. Subquestion 1: Which mechanical forces are working on the ascending aorta?

Bernoulli's law [37]

$$P + \rho gh + \frac{1}{2}\rho v^2 = constant \qquad \qquad \begin{array}{l} \rho = density \text{ of the liquid} \\ g = gravitational acceleration \\ h = height \text{ in the tube} \\ v = flow rate of liquid particles \end{array}$$
(4)

Laminar flow through a tube

If a viscous fluid flows through a tube, a thin layer of fluid adheres to the wall, so that there is no flow along the wall. If friction forces in the fluid are not too large, layers are formed which slide along one another with each having a uniform velocity. This is known as laminar flow. For a fluid with laminar flow v in Bernoulli's law, will be replaced by the average flow within a cross section [37]:

$$\bar{v} = \Phi/O$$
 $\begin{array}{l} \Phi = \text{flow} \\
O = \text{cross section} \end{array}$
(5)

Reynolds number

Flow should increase linearly with driving pressure if resistance is constant. At high flow rates, however, flow rises less steeply and is no longer proportional to δP . From this point on, blood flow is turbulent, which results in energy loss. Reynolds number (*Re*) is a parameter that determines when flow becomes turbulent and is a dimensionless quantity:

$$Re = \frac{2r\bar{v}\rho}{\mu} \qquad r = \text{inner radius of tube} \\ \mu = \text{viscosity of fluid}$$
(6)

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Below an Re of 2000 blood flow is laminar and above an Re of 3000 blood flow is mostly turbulent [38].

Poiseuille's law

For a laminar flow through a tube of which cross section and/or height are not constant, a combination of the laws of Bernoulli and Poiseuille applies [37]:

$$P_{2} = P_{1} - R_{12}\Phi - \left(\rho g \Delta h + \frac{1}{2}\rho \Delta(v^{2})\right) \qquad \begin{array}{c} P_{1} = \text{ pressure at cross section 1} \\ P_{1} = \text{ pressure at cross section 1} \\ R_{12} = \text{ hydrodynamic resistance} \\ \text{between cross sections 1 and 2} \end{array}$$
(7)

Hooke's law

The extent to which an object deforms under influence of a force depends on the shape of the object and the elasticity of the material from which the object is made [38].

Young's modulus

Young's modulus is the force per unit area which is necessary to stretch a rod to its double length. Hooke's law no longer applies when non-linear effects occur due to large deformations, and E is then dependent on the force [38].





Poisson ratio

An increase in length, in general, will be accompanied with a reduction of the transverse dimension a. This phenomenon is called transverse contraction or Poisson. It has been found experimentally that the following equation applies to the change in the transverse dimension Δa [37]:

$$\frac{\Delta a}{a} = -\mu \frac{\Delta l}{l} \qquad \qquad \mu = \text{Poisson ratio} \tag{9}$$

Laplace's law

In a vessel within the body, filled with fluid, internal pressure (P_i) is larger than the pressure outside (P_o) . Because of the overpressure, $P = P_i - P_o$, the wall of the vessel is stretched and in the wall a tension arises. This tension reaches equilibrium with the forces that are caused by the overpressure. The relationship between the radius of a blood vessel and the pressure inside it can be described by Laplace's law for a cylinder:

$$T = PR \tag{10}$$

Wall tension is the force per unit length, measured in the longitudinal direction of the blood vessel [38].

Windkessel model

The easiest way to make the Windkessel function of the aorta into a model is to consider the heart as a pump that raises the volume of an elastic vessel (aorta) with the stroke volume 70 times per minute. Between strokes the vessel will slowly empty. Capillaries may be considered as a resistance to flow R and the elasticity of the aorta wall as (static) compliance C. Compliance describes the relationship between increase in pressure, and increase in volume in the vessel.

$$\Delta P = \frac{\Delta V}{C} \qquad \qquad \Delta P = \text{pressure difference} \\ \Delta V = \text{volume difference} \\ C = \text{compliance}$$
(11)

Under the assumption that this process repeats itself every stroke, we now determine a few laws, based on this model. Assuming that addition of the stroke volume to the blood volume aorta takes no time, the pressure in the aorta rises to;

$$P_s = P_d + \frac{V_s}{C} \qquad \begin{array}{c} P_s = \text{systolic pressure} \\ P_d = \text{diastolic pressure} \\ V_s = \text{systolic volume} \end{array}$$
(12)

After the addition of the stroke volume to the aorta, the aortic valves closes. The aorta will now provide the rest of the circulatory system with blood. Thus blood slowly leaks out of the aorta, causing pressure to decrease with a certain flow. This course of the pressure is shown by the following equation;

$$P(t) = (P_d + \frac{V_s}{C})e^{\frac{-t}{\tau}} \qquad P(t) = \text{blood pressure at certain time} \\ t = \text{time in seconds} \\ \tau = \text{RC}$$
(13)

After a stroke (of T seconds) the following equations can be derived from a state of equilibrium, in which diastolic pressure P_d and systolic pressure P_s have fixed values;

$$P_s = V_s / C \frac{1}{1 - e^{-T/\tau}} \tag{14}$$

$$P_d = V_s / C \frac{e^{-T/\tau}}{1 - e^{-T/\tau}}$$
(15)

In this model only variations in stroke volume or compliance give change to the pulse pressure $(P_{pulse} = P_s - P_d)$. But in reality all kind of complications occur, that cause the model to be incomplete. It did not take into account that regulation occurs, that the filling of the aorta takes some time, that the whole is affected by the mass of the blood and that the elasticity of arteries, especially in the elderly, strongly depends on the pressure (stiffer at higher pressure). Despite these limitations, the model provides a way to discuss the effect of changes in the cardiac blood pressure system [37].





A.2. Subquestion 4: How does pericardium, decellularized with scCO₂, change after long-lasting repetitive stress?

A.2.1. Statistical analysis

We measured the thickness of the pericardium at several places before and after two hour Measurement 1 & 2 (Table 5 and 6). To compare the thickness before and after the measurements, we used a Student's t-test:

- 1. We test $H_0: \mu_A = \mu_B$ against $H_1: \mu_A \neq \mu_B$
- 2. $T = \frac{\overline{A} \overline{B}}{S\sqrt{\frac{1}{n} + \frac{1}{m}}}$, where \overline{A} is the sample mean from a sample A of size n and with mean μ_A
- 3. $S^2 = \frac{n-1}{n+m-2}S_A^2 + \frac{m-1}{n+m-2}S_B^2$, where S_A is the sample variance of sample A
- 4. Next, we compare T with the critical value c that corresponds to the chosen significance level α of 5%. The critical value c can be found using a Table of values from Student's t-distribution.
- 5. Null hypothesis H_0 is rejected for too small or too large values of T: $T \leq -c$ or $T \geq c$, in favor of the alternative hypothesis H_1
- 6. When the critical value is not exceeded and the null hypothesis is not rejected, it means that there is no significant difference between the thickness before and after the measurement.

When the critical value is exceeded and the null hypothesis is rejected, it means that there is a significant difference between the thickness before and after the measurement.

For the difference in thickness at Measurement 1 of pericardium, test statistic T = 0.306 and the critical value c = 2.571 (significance level $\alpha = 5\%$). The critical value is not exceeded, thus the null hypothesis is not rejected. This means we can not show a significant difference between the thickness before and after the measurement based on our research (p-value between 0.50 and 1.00), at the chosen significance level of 0.05.

For the difference in thickness at Measurement 2 of pericardium, test statistic T = 3.219 and the critical value c = 2.306 (significance level $\alpha = 5\%$). The critical value is exceeded, thus the null hypothesis is rejected. This means we show a significant difference between the thickness before and after the measurement based on our research (p-value between 0.01 and 0.02), at the chosen significance level of 0.05.

We used the same statistical test to compare the length of the Dacron prosthesis before and after the 2 hour measurement. We measured this length at two points (Table 7).

For the difference in length of the Dacron prosthesis, test statistic T = -2 and the critical value c = 4.303 (significance level $\alpha = 5\%$). The critical value is not exceeded, thus the null hypothesis is not rejected. This means we can not show a significant difference between the length before and after the measurement based on our research (p-value between 0.10 and 0.20), at the chosen significance level of 0.05.

J	1	6		5		
					Mean \pm SD	p-value
A: Thickness before (μm)	145	85	65		98 ± 34	0.50 - 1.00
B: Thickness after (μm)	110	102.5	92.5	60	91 ± 19	

 Table 5: Thickness of the pericardium before and after two hour Measurement 1

Table 6: Thickness of the	he pericardium	before and	$after \ two$	hour	Measurement	2
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						Mean \pm SD	p-value
A: Thickness before (μm) B: Thickness after (μm)	$\begin{array}{c} 110 \\ 675 \end{array}$	$102.5 \\ 70$	$92.5 \\ 57.5$	60 60	50	$ \begin{array}{c} 91 \pm 19 \\ 61 \pm 7 \end{array} $	0.01 - 0.02

Table 7: Length of the Dacron prosthesis before and after the two hour Measurement

			$ Mean \pm SD $	p-value
A: Length before (mm) B: Length after (mm)	126 129	$\begin{array}{c} 126 \\ 127 \end{array}$	$ \begin{array}{r} 126 \pm 0 \\ 128 \pm 1 \end{array} $	0.10 - 0.20





A.2.2. Measuring the length of Dacron

We measured Dacron only one time in the right way. The first times we measured Dacron in its ribbed form. It is, however, not a requirement for the Dacron to keep this ribbed form. Normally, the ribs disappear over time, due to physiological pressures. In order to research whether the material stretches, we measured the length of the Dacron prosthesis the last time in its stretched form, before and after the experiments.

B. Experiment

B.1. Suturing

Since we had to create a tubular shape out of the pericardium, our supervisor F.R. Halfwerk taught us how to suture the pericardium. He told us that the running locked suture is the best technique to close the pericardium water-tight (Figure 7). Because the suture is locked, the tension is distributed over the surface evenly. The first times we tried this suture we did a single running locked suture on a skinpad (Figure 8). Thereafter we made a double locked suture on a tire, so we could see whether the sutured tire is water-tight (Figure 9). To check this, we held it under running water out of the tap. Hardly any water spurted out the suture holes. When we closed one end partly with our hands, more water spurted out of the holes.



Figure 7: Running locked suture [64]



Figure 8: Selfmade running locked suture on a skinpad



Figure 9: Selfmade double running locked suture on a tire

The first pericardium was sutured in the shape of a tube while the ends overlapped each other 12 We used a 4-0 Prolene[®] suture to make a double running locked suture (Figure 10). When we placed it in the set-up and pumped with the hand pump water came out of the suture holes. Because of this we decided to suture a new pericardium using a smaller suture, 5-0 Prolene[®]. Thereby we used a single running locked suture, so there would be less suture holes (Figure 11). In this second tube of pericardium water also leaked through the suture holes, so we had to find another solution. The thread is lasered in the needle, so the thread is smaller than the needle. When we suture, we make larger holes than we fill with the thread, which is why water has a way out.

To solve this problem we brainstormed for different options. We thought about options like making the liquid more viscous, gluing the holes or using a clamp instead of suturing. Finally we chose to combine pledgets on the suture and a different way of folding (Figure 13). By this way of folding the ends were put together outside the circle of the tube 12. Thus the tube was not perfect round anymore. We thought the advantage of this is that the suturing gets less tension, so the holes tear out less quickly. Two pledgets were used, one at each side of were the pericardium ends came together. The pledgets close the suture holes and at the same time make sure that the thread does not tear through the pericardium. Because we used the pledgets and a different way of folding we used a double running suture, advised by our supervisor J.G. Grandjean. Because of the new way of folding it was harder to suture the pericardium tube closely to the tubes, which is necessary because otherwise water leaks away.







Figure 10: Double running locked suture on a pericardium



Figure 11: Single running locked suture on a pericardium



Figure 12: We initially folded and sutured the pericardium like the left tube. To reduce the leakage we folded and sutured the pericardium like the right tube.

B.2. Set-up

In this paragraph, the set-up to test the pericardium will be described (Figure 14). For this set-up the following materials were used:

- Abiomed pump
- Mechanical heart
- Reservoir
- Tubes with a diameter of $\frac{3}{8}$ inch (=1 cm)
- Tubes with a diameter of $\frac{1}{2}$ inch (=1.3 cm)
- Tube connectors
- Connectors for Dacron prosthesis (Figure 16)
- Hoffman clamp (Figure 15)
- Pressure monitor
- Measuring instrument that translates water pressure into air pressure
- Dacron prosthesis
- Porcine pericardium
- Suture material
- Inner bicycle tire
- Balloons
- Cable tie
- Duct tape

In the hospital, the Abiomed pump was used to take over the heart function of a patient. This pump acts like a heart and is therefore very suitable to simulate the physiological situation. A disadvantage, however, is the age of the used pump. The pump is from 2005 and has not been used very much the last few years. Due to the aged battery of the Abiomed, we could not take it out of the electic point, which lead to some logistic difficulties.







Figure 13: Pledgets with a running suture on a pericardium



Figure 14: Set-up, in which the Abiomed pump, mechanical heart, two blood pressure monitors and reservoir can be seen. The pericardium is placed between the tubes between the blood pressure monitors

The mechanical heart translates air pressure to water pressure. The heart, pericardium, measuring instrument that translates water pressure to air pressure and the blood pressure monitor have to be on the same height. When this is not the case, we will not measure the real pressure in the system because of a head of water. The heart is located lower than the reservoir, which has two reasons. First, the mechanical heart has to be be full of water all of the time while the Abiomed pump is pumping, because it will crack when it fills up with air. Second, we want to create a diastolic pressure, which is created by the head of water.

Dacron is fixed to the tubes with a hand made connector, inner bicycle tire and cable ties (Figure 17 and 18). To test the pericardium, it is sutured with a double running suture into a tubular shape with a diameter of 1.3 cm. The pericardium is also fixed to the tube with inner bicycle tire, cable ties and duct tape (Figure 13 and 19).

B.3. Experiment 1

To acquire the results of this experiment the Abiomed pumped for two hours. We wanted to do measurements of 24 hours, but due to technical difficulties we did two Dacron measurements of nine hours. Then we had more technical difficulties, causing us to limit our measurements to two hours. We read the pressure before and after the pericardium or Dacron roughly every half an hour.

Sometimes the Abiomed pump gave an alarm, but this interrupted the measurement for no more than three minutes. That is why we saw this as negligible. It also happened that we had to shove the pericardium further on the tubes because of a hole in the pericardium. These holes were probably caused by friction between the pericardium and the tube, not because the pericardium could not resist the mechanical forces. It took a couple of minutes to untie, shove and fix the pericardium on the tubes again. By adding these minutes up at the end, we made sure that the pericardium and Dacron were tested for two hours.







Figure 15: Hoffman clamp [65]



Figure 16: Connectors for Dacron prosthesis, made in the workplace of Carré



Figure 17: Dacron fixed to the tubes



Figure 18: Schematical representation of how Dacron is fixed to the tubes, longitudinal cross section



Figure 19: Schematical representation of how pericardium is fixed to the tubes, longitudinal cross section





By means of this experiment we wanted to know whether the pericardium can handle the mechanical forces that work on the aorta. If the pericardium would not tear, we wanted to know whether it stretches or not. That is why we measured the thickness of the pericardium. Thickness is measured with a micrometer. The Dacron prosthesis is the control group, so we measured the length and diameter before and after a measurement. The length and diameter were measured with measuring tape.

We did not measure the same dimensions of the pericardium and Dacron. The thickness of Dacron cannot be measured because it is corded. Length and diameter of pericardium cannot be measured, because it is sutured around tubes. The pericardium would damage if we would take it off the tubes, so that is why we did not do that. Moreover, thickness, length and diameter are all dimensions with which can be seen whether the material is stretched.

B.4. Experiment 2

We pumped 30 times with the hand pump to determine the pressure before and 30 times to determine the pressure after the pericardium and Dacron. The pressure on the blood pressure monitor was read by two researchers to minimize the risk of failure.

B.5. Footage

As clarification a couple of videos can be seen if you click on the following link: https://www.dropbox.com/sh/jjxkxtgfcfwrpkr/AACOduaCrxZzBpx_GD46hFfga?dl=0. Here is a video of our set-up, but also videos in which we zoom in on the Dacron and pericardial prostheses in the set-up.