

DUBLIN CITY UNIVERSITY

PHD THESIS

The role of altered cyclic strain patterns on proliferation and apoptosis of vascular smooth muscle cells - Implications for in-stent restenosis

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Declaration

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Abstract

Currently, Percutaneous Transluminal Coronary Angioplasty (PTCA) followed by stent implantation is the frontline treatment in the management of coronary artery disease. To-date the main drawback of stent implantation is in-stent restenosis. Restenosis is a partial re-occlusion of the arterial wall predominantly due to vascular smooth muscle cells (SMC) migration from the media to the intima and subsequent SMC proliferation. A stent procedure dramatically alters the level of strains and stresses in the coronary artery and hence the mechanical environment of the SMC. We hypothesised that there exists a direct causal relationship between the level of strain and vascular SMC proliferation and apoptosis within the vessel wall. Cyclic strain can be seen as made by different independent components: mean strain, amplitude and frequency.

In this work, the role of each strain component (mean strain, amplitude and frequency) in controlling Bovine Aortic Smooth Muscle Cells (BASMC) proliferative and apoptotic capacity was investigated. Cyclic strain decreased SMC proliferation and increased apoptosis in a temporal manner. The mean cyclic strain had no significant effect on the proliferative and apoptotic behaviour of SMC whereas SMC behaviour was highly dependent on strain amplitude. This observation was further validated using human SMC.

The role of mean strain and strain amplitude was further investigated using a novel in-house phantom mock arterial system where BASMC were cultured inside a perfused stented Sylgard[®] mock artery under physiological levels of strain. Vascular SMC proliferation was significantly increased (+40%) and apoptosis decreased within the stented region in comparison to the more compliant upstream and downstream non-stented regions of the mock Sylgard[®] artery.

We therefore conclude that the decrease in strain amplitude experienced by vascular SMC within the stented region may be responsible for SMC accumulation due to enhanced proliferation and decreased apoptosis. This study provides important evidence for the use of more compliant stent designs to maintain the anti-proliferative effect of cyclic strain on vascular SMC and therefore reduce restenosis.

Chapter 1

Introduction

1.1 Cardiovascular diseases

Cardiovascular diseases are the main cause of death in the western world [1]. Arteriosclerosis, heart failure, stroke and aneurysm are among the most common cardiovascular diseases.

In 2006, the percentage of deaths resulting from cardiovascular diseases in Ireland was 35% compared to 50% in the 1980s [2]. This dramatic drop is partly due to the introduction and the improvement of medical devices. In the field of cardiovascular diseases the introduction of new medical devices, like stents, has been revolutionary. These devices represent a less-invasive alternative to by-pass surgery and their use is becoming increasingly the standard for the treatment of coronary artery diseases.

1.2 Coronary artery disease

Coronary artery disease (known also as ischemic heart disease or atherosclerotic heart disease) consists of the formation of an atheroma on the walls of a coronary artery. An atheroma is an accumulation of lipid-rich cells, cell debris, connective tissue and macrophages on the walls of a coronary artery forming an atherosclerotic plaque [3]. The wall of a coronary artery is composed by three layers: intima, media and adventitia, see fig 1.1. The intima is a thin layer of endothelial cells (ECs), the media is mostly composed of smooth muscle cells (SMC) and the adventitia has a more collagenous composition.





An atherosclerotic plaque (the atheroma) is usually much stiffer than the arterial wall. Due to its different mechanical properties the plaque can detach from the arterial wall creating a downstream clot in the blood flow. The plaque can also increase its volume to occlude the coronary artery. Both these conditions lead to a myocardial infarction due to ischemia of the heart muscle. Of the total number of mortal cardiovascular diseases in 2006 in Ireland, 51% were coronary artery diseases [2]. Different techniques have been developed to treat coronary heart diseases: bypass procedure, atherectomy or stent procedure.

Coronary artery by-pass grafting (CABG) consists of by-passing the occluded coronary artery using a vein or an artery from elsewhere in the body or a prosthetic artery. In the United States more than 500,000 CABG

procedures are performed every year [5]. This technique has a low death rate and associated complications but it also has many disadvantages [6]. It is highly invasive as it is usually carried out while the heart is stopped and therefore requires the use of a cardiopulmonary bypass module. It also involves considerable recovery time and it is therefore more commonly used for the most serious cases.

Atherectomy is rarely used and only in cases of complete or near complete vessel occlusion. It consists of the removal of the atherosclerotic plaque by means of a rotating blade mounted on a catheter [7].

Percutaneous Transluminal Coronary Angioplasty (PTCA) was used for the first time in 1977 and it was the first 'non-invasive' technique developed for the treatment of coronary artery diseases [8]. It consists of positioning an expandable balloon at the plaque site by means of a catheter. The balloon is expanded pressing against the atherosclerotic plaque and re-enlarging the arterial lumen. Thereafter the balloon is deflated and removed leaving an improved blood flow. This technique has offered a good alternative to bypass surgery, however, it has shown a high rate of re-occlusion (re-stenosis) due to elastic recoil of the arterial wall or formation of intimal hyperplasia (IH). Cardiovascular stents were introduced into the market to solve these complications.

1.3 Coronary stenting

The coronary stenting procedure is a further development of the percutaneous transluminal coronary angioplasty (PTCA) procedure and is used to restore immediate patency inside an occluded atherosclerotic coronary artery. A stent generally consists of a mesh metallic tube that can be mounted on the angioplasty inflatable balloon in a crimped state [6]. When the angioplasty catheter is placed at the coronary occlusion site the balloon is expanded along with the stent. Through this expansion, the stent assumes a tubular shape to buttress the atherosclerotic wall of the artery. Stent procedures cause injury to the artery walls creating

lacerations of the internal elastic lamina and of the media, see fig 1.2 [9]. These injuries are among the main reason for the long-term failure of stent interventions: i.e. restenosis. Restenosis is a partial re-occlusion of the arterial wall due to the formation of a proliferative neointima inside and around the stent struts (intimal hyperplasia). Between 10% and 50% of bare metal stent procedures result in in-stent restenosis [10]. To overcome this problem a new stent family was put on the market: drug eluting stents (DES). DES are covered with an antiproliferative drug to prevent in-stent restenosis. Different clinical trials (CYPHER, RAVEL, SIRIUS, TAXUS, ASPECT) have proven the efficacy of DES to reduce restenosis and many medical companies have already launched their DES models using antiproliferative drugs like Sirolimus, Paclitaxel and Taxus [11].

1.4 In stent restenosis

Although restenosis represents the major drawback for stent implantation [12], the origin of this disease and the factors that modulate its extension are largely unknown. Four phases have been identified in its development: thrombosis, inflammation, SMC migration and proliferation and remodelling [13]. Migration of smooth muscle cells from the media to the intima and their overproliferation are mainly responsible for the creation of the neo-intima.

Clinical trials have been carried out to evaluate the restenotic level associated with stents of different sizes, geometries, shapes or implanted at different pressures in order to improve stent design and deployment techniques. Correlations were established between stent-induced injury or stretch and the restenotic response [14, 15, 16, 17]. Vascular injury has been quantified using different variables such as the penetration of the stent-struts inside the arterial wall [14] or by an aggressiveness score [15] resulting from the product of the balloon/artery diameter ratio and the final inflation pressure of the balloon. However, balloon/artery diameter



Figure 1.2: Expansion of a stent inside an atherosclerotic plaque [4].

ratio and the final inflation pressure can also be associated with the stress and strain levels on the arterial wall after stent expansion. Stresses and strains can thus also be used as indicators of the restenotic level. Previous reports [16, 18] demonstrated that high levels of strains and stresses on stented artery walls can be used as determinants in restenosis development after injury. In fact, it was reported that the amount of stent-induced intimal hyperplasia (IH) was proportional to the circumferential vascular strain.

Arakawa *et al.* [16] found that the higher stretches on the arterial wall due to the expansion of a stent accelerates the IH formation in human coronary arteries. Furthermore, a higher level of stent-to-vessel CSA (cross sectional area) ratio after stenting resulted in an increased level of IH. Therefore they concluded in order to limit vascular injury, stent deployment requires an optimal rather than maximal lumen diameter. Stents should be rigid enough to prevent lumen loss from remodelling but not so rigid to cause excessive injury and stress to the vessel during vasoconstriction and thus to cause restenosis or neo-intima formation. A stent-to-vessel CSA ratio of 0.7 was found to be the optimum compromise between initial gain and IH formation. Essentially, they related their results to the strain pattern inside a stented artery.

Koyama *et al.* [18] carried out a detailed study on the relationship between vascular morphological changes after stenting and the magnitude of IH. They established that the changes in total vascular area before and after stenting rather than lumen or plaque area were better predictors of late in-stent neointimal growth. They also suggested that neo-intimal proliferation is more related to the stretch or injury of the adventitia than to the intimal side deformation.

The Arakawa and Koyama results stress the importance of the changes in mechanical environment of the arterial wall due to the expansion of a rigid metallic stent mesh inside the artery causing high levels of stress and strain.

The main limitation of their works was the lack of analysis of the specific strain and stress fields. An more insightful approach to the problem would be to investigate the different elements contributing to the strain in a stented artery, such as cyclic frequency, mean strain and cyclic strain amplitude. In the scientific literature there is a surprising lack of information on the contribution of individual strain/stress components on the restenotic process.

In vivo clinical trials have always been the main source of information for restenosis but they do not enable an easy separation of the different

elements of the stress and strain pattern. *In vitro* systems, on the other hand, enable accurate analysis to be carried out on the effect of mechanical forces on smooth muscle and endothelial cell behaviour. *In vitro* systems have the limitation of not being able to recreate the entire physiological environment but have the strong advantage of enabling control of arterial wall mechanical parameters such as mean strain/stress, amplitude or frequency in real-time.

It has been suggested that strain may be the most homeostatic parameter in the cardiovascular system [19]. A stent procedure dramatically changes the strain environment in an artery potentially compromising the homeostatic balance.

Furthermore, it is widely recognised that cells behave in different ways according to their mechanical environment [20]. It is also known that strain is a key factor in determining the proliferative and apoptotic capacity of vascular smooth muscle cells (SMC) [21, 22, 23]. Changes in SMC growth are critical to vascular remodelling and restenosis following injury. A key factor in restenosis development is the abnormal proliferation of SMC that contributes to the artery wall thickening [14]. To better understand the causes of restenosis, and to improve both bare metal stents and DES mechanical and pharmacological characteristics, a greater understanding of the influence of various levels of strains and stresses on cells in the vascular wall is required.

1.5 Objectives of the project

In vivo and under physiological conditions SMC experience a characteristic 1Hz biaxial cyclic strain waveform. The mean strain level varies depending on the artery and published literature reports a range of values [24, 25, 26, 27]. Although the exact *in vivo* strain value is difficult to determine what is known is that the mean strain value in arteries under physiological conditions is relatively low whilst the amplitude of the cyclic strain due to blood pressure is relatively high [26, 24]. Vernet et al

[28] reported a cyclic strain amplitude of 5-6% in the aorta of new Zealand white rabbits before stenting. After a stenting procedure these values are very different. In a stented artery the mean strain value increases up to 20% (but it can reach even 50%, depending on the location) and the amplitude can drop to 1.5%-2% [25, 28], see fig 1.3.



Figure 1.3: Change in the artery mechanical environment after stenting.

This work focuses on the study of the effects that these changes in the mechanical environment have on smooth muscle cells and their relationship with in-stent restenosis *in vitro*.

Two major hypotheses were formulated:

- 1. Changes in the mechanical environment of an artery following a stenting procedure drive the restenosis process
- 2. The enhanced proliferative and apoptotic response of SMC after stenting is dictated by the change in mechanical environment

In this project the relationship between the change in the strain pattern after stenting and SMC proliferation and apoptosis was investigated. SMC were exposed to cyclic strain recreating in vitro the strain pattern SMC experience in vivo and the results were interpreted in the light of restenosis development. In particular the following areas were analysed:

- 1. The effects of cyclic strain on the proliferative and apoptotic capacity of SMC were investigated.
- 2. The role of strain frequency in the proliferation and apoptosis of SMC was determined.
- 3. The role of mean strain vs cyclic strain amplitude on SMC activity was investigated.
- 4. The assessment of the effect of an antiproliferative drug (Sirolimus) on cyclically strained SMC was measured.
- 5. The results of all cell studies were interpreted to help improve future stent designs.

These goals were achieved by dividing the work in two major areas as is shown in the flowchart in fig 1.4. In the first part, tests were carried out to assess the influence of cyclic mean strain vs amplitude on the proliferation and apoptosis of SMC.

A *Flexercell*^(R) straining machine is a laboratory device able to strain cells under different strain conditions. This machine was calibrated and subsequently set to achieve cyclic strains patterns with different mean strain and amplitude values in order to analyse the influence of every strain component (frequency, amplitude and mean strain) on the proliferative and apoptotic behaviour of SMC. The *Flexercell*^(R) machine produces no shear stress.

In the second part of the work (see fig 1.4) the SMC activity in response to stenting was evaluated. A custom made culture chamber was inserted into a commercially available bioreactor tubing system $(CellMax^{\mathbb{R}})$. The core of the novel culture chamber is a perfused elastomeric mock coronary artery (MCA) on which SMC can be cultured

intraluminally and strained under cyclic pulsatile pressure. The influence of near physiological levels of strain and stresses on SMC can in such a way be assessed *in vitro*. The MCA can be stented and therefore the influence of an altered, stent induced, strain and stress environment on SMC activity could also be analysed.

The results from these two sets of tests have provided important new information for the development of future generations of stents and provide a useful pre-clinical device for evaluating SMC behaviour for different stent geometries and materials. In this context, a specific Medtronic drug-eluting stent (DES) was evaluated to study its mechanical effect and the drug elution profile on SMC behaviour.



Figure 1.4: FlowChart of the project structure

Chapter 2

Literature Review

2.1 Cardiovascular diseases

Cardiovascular diseases are the primary causes of death in western countries including Ireland [1, 2]. The American Heart Association has estimated the economic cost of the treatment of cardiovascular diseases in the United States in 2009 to be 475.3 billion of dollars, without considering the related human suffering and loss of lives [29]. More than 80% of deaths from cardiovascular diseases take place in low-and middle-income countries and are almost equally distributed between men and women [30].

Arteriosclerosis, heart failure, hypertension, stroke and aneurysms are the most common forms of cardiovascular diseases. Arteriosclerosis is a disease epitomised by hardening and the loss of elasticity of arteries [31]. It is often associated with hypertension, a chronic increase in blood pressure.

Heart failure occurs when the heart can not supply sufficient blood to meet the body's needs. The major causes for heart failure include myocardial infarction, myocardial ischaemia, hypertension, valvular heart disease and cardiomyopathy.

A stroke is the loss of brain functions due to a lack of blood supply to the brain. Thrombus formation, embolism or haemorrhage are the main factors leading to brain ischemia and thus stroke. Consequences of a stroke is partial inability of the brain to function preventing the patient from moving one or both limbs, speech impediment or sight limitations.

An aneurysm is a localised dilation of a blood vessel. The aetiology of the disease is still debated but it is associated with a weakening of the vessel wall. Most common aneurysms occur in arteries at the base of the brain (the circle of Willis) and in the aorta. The main problem related to aneurysms is that they are generally asymptomatic and can increase in size up to rupture without being detected leading to sudden death from haemorrhage.

Different risk factors have been associated with cardiovascular diseases. Among the most significant are age, smoking, obesity, lack of physical exercise and diet. A diet rich in high saturated fats, for instance, leads to accumulation of cholesterol in the blood which is insoluble and therefore can deposit on the arterial wall.

Smoking has been associated with 11% of total cardiovascular deaths, mainly manifesting as chronic obstructive pulmonary disease [32]. Inflammation and oxidative stress have been found to increase this effect [33].

Obesity is another major risk factor for cardiovascular disease. Excess free fatty acid liberation and reduced insulin action in peripheral tissues of the liver, adipose tissue and skeletal muscle, results in endothelial dysfunction and the onset of vascular disease [34].

These risk factors can be controlled in part by adopting a healthy lifestyle, balanced diet and physical activity. However there are other risk factors which cannot be controlled such as age, sex and genetic predisposition. Aging, for instance, is associated with increase in collagen content, cross-linking of the collagen, elastin fracture and reduction in the elastin content. All of these factors lead to the stiffening of the arterial walls and hypertension making aging the major risk factor for atherosclerosis [35].

2.2 Atherosclerosis

Atherosclerosis is the main (but not only) type of arteriosclerosis. It is a chronic inflammatory reaction of the body to the local accumulation of lipoproteins in the arteries. It develops in vessels of large to medium size such as coronary arteries, the aorta and carotid arteries, and especially in areas of the arterial wall which experience low shear stress. Besides the normal symptoms of the arteriosclerosis there is the formation of an atheroma, a stenosis within the artery which can cause obstructions to the blood flow [3]. Coronary occlusion of the artery lumen or plaque detachment (which may cause clots downstream in the artery) are the major factors responsible for myocardial infarctions [36].

The American Heart Association has developed an atherosclerotic plaque ranking on the basis of disease severity, see fig 2.1. A type V atherosclerotic plaque is considered the threshold for surgical intervention. In accordance with the American Heart Association a type V (fibrotic) atherosclerotic plaque consists of a necrotic core covered by a cap of collagen fibres and SMC [31]. An atherosclerotic plaque of type V is composed of different components: macrophages, lymphocytes, microvessels, collagen and smooth muscle cells (SMC), see fig 2.2. It is usually characterised by a fibrous cap covering a core of calcified areas, connective tissue and necrotic SMC.

The formation of fatty streaks, the first sign of an atherosclerotic plaque, results from the accumulation of cellular and extracellular materials between the endothelium and the vascular media. Furthermore, a damaged endothelium has been associated with atherosclerosis development due to an increase of endothelium permeability leading to low density lipoproteins (LDL) penetration inside the arterial wall [39]. In cases of binding between LDL molecules and proteoglycans, free radicals can induce the formation of oxidised LDLs (ox-LDL) which are monocyte chemoattractants. The constant accumulation of lipids along with the arrival of macrophages leads to the formation of lipid-laden foam cells which contribute extensively to the

Nomenclature and main histology	Sequences in progression	Main growth mechanism	Earliest onset	Clinical corre- lation
Type I (initial) lesion isolated macrophage foam cells	I		from	
Type II (fatty streak) lesion mainly intracellular lipid accumulation	II	growth mainly	decade	clinically silent
Type III (intermediate) lesion Type II changes & small extracellular lipid pools	Ť	lipid accumu- lation	from	
Type IV (atheroma) lesion Type II changes & core of extracellular lipid	IV		third decade	
Type V (fibroatheroma) lesion lipid core & fibrotic layer, or multiple lipid cores & fibrotic layers, or mainly calcific, or mainly fibrotic	V	accelerated smooth muscle and collagen increase	from fourth decade	clinically silent or overt
Type VI (complicated) lesion surface defect, hematoma-hemorrhage, thrombus	└→VI	thrombosis, hematoma		

Figure 2.1: American Heart Association (AHA) atherosclerosis ranking [37].

atherosclerotic swelling [31]. Furthermore foam cells contain coagulant initiators that lead to thrombus formation once in contact with the blood stream as may happen after a stenting procedure due to endothelium denudation.

Chronic inflammation, the enhancement of the endothelium permeability and accumulation of thrombotic/vasoconstrictor factors cause an increase in SMC recruitment from the media and migration to the endothelial layer [40]. SMC are also thought to produce inflammatory mediators, monocyte chemoattractants and to lead to lipid trapping of extracellular matrix proteins. SMC are also considered important to



Figure 2.2: Representation of the atherosclerotic plaque components: fibrous cap, lipid, calcium, cellular debris and macrophages turned into foam cells [38].

maintain plaque stability by producing a fibrous cap [36].

Plaques can be stable or vulnerable. A stable plaque is characterised by a high concentration of SMC and collagen with a limited number of pro-inflammatory lipid-laden cells. Stable plaques are thicker and more resistant to the blood flow than vulnerable plaques and generally protect the atheroma from the blood flow. Although a stable plaque occludes the arterial lumen more than a vulnerable plaque it constitutes a less serious clinical problem due to its stability [40].

Vulnerable plaques in contrast, are thinner and are richer in lipid-laden cells and macrophages. They have lower mechanical resistance and are prone to rupture especially in the plaque shoulder region where monocytes are more abundant [40]. Detached debris can form a clot when in contact with platelets and other complement factors [41]. Moving downstream where the lumen diameter is usually smaller the clot can form a thrombus and occlude the vessel [40].

Different techniques have been developed to treat atherosclerosis such as coronary artery by-pass grafting (CABG), percutaneous transluminal

coronary angioplasty (PTCA), bare metal stents (BMS) and drug eluting stents (DES). All of these techniques have both advantages and disadvantages and it is the responsibility of the surgeon to decide on the best treatment. Coronary artery by-pass grafting (CABG) has a low death rate and low complications. It is highly invasive, it requires a cardiopulmonary bypass module during heart arrest and involves considerable recovery time. The introduction of PTCA in 1977 and of combined BMS and DES revolutionised the treatment of heart diseases. It removed most of the disadvantages related to CABG, providing a non-invasive technique developed for the treatment of coronary artery diseases [8]. These techniques are considered today the standard for the treatment of coronary artery diseases leaving the use of the bypass surgery to the most serious cases. The main drawback to PTCA or the implant of BMS and DES is re-occlusion due to elastic recoil of the arterial wall or formation of intimal hyperplasia (IH): namely, restenosis.

2.3 Restenosis

During percutaneous transluminal coronary angioplasty (PTCA) an angioplasty balloon is expanded inside the coronary artery reaching pressure values even beyond 20 atm. The pressure and the friction of the balloon against the arterial wall generally cause endothelium denudation and occasionally plaque rupture. The exposure of the sub-endothelial layer of the arterial wall (made of collagen, elastin fibres and SMC) to platelets and complement factors triggers a thrombotic response that is the major cause of vessel re-occlusion in the first month after intervention. However several studies have established that after 1 month the thrombus in the artery is minimal and that migration and proliferation of SMC between 1 and 3 months following the intervention are the main reasons for restenosis formation [42]. SMC migration and proliferation is normally a physiological response to injury caused to the arterial wall. Restenosis is thus considered an overreaction of the normal healing process of the artery [43]. A direct proportional relationship has been reported between the depth of the injury inside the arterial wall and the amount of restenosis due to SMC proliferation. When vascular injury is limited to the endothelial layer a lower amount of neo-intima formation has been measured compared to cases of deeper injuries in the media layer [42]. This has led to the hypothesis that the restenotic process initiates in the media of the vessel. Another important element in restenosis following angioplasty intervention is elastic recoil, which can account for up to 50% of arterial reocclusion. The restenosis percentage due to elastic recoil depends on many factors such as plaque eccentricity and plaque burden. The expansion of an angioplastic balloon in a vessel with an eccentric atherosclerotic plaque can dilate the elastic healthy part of the artery instead of the stiff atheroma resulting in almost immediate vessel recoil after balloon removal. To overcome this problem and to guarantee the patency of the vessel endovascular stents were developed [5].

Endovascular coronary artery stents have the ability to reach luminal diameters beyond the capability of angioplasty balloons minimising complications such as restenosis and reducing symptoms such as angina. However re-occlusion after stent procedures (BMS) still accounts for up to 25% of treated patients [44]. The lower level of restenosis (between 30% and 50% with PTCA) reported with stent procedures is in part due to the greater initial gain that a stent procedure provides [44]. However restenosis remains the main limitation of stenting.

In contrast to the more short term response to the disruption of the arterial wall by an angioplasty balloon, in-stent restenosis is characterised by a prolonged inflammatory reaction to the chronic presence of a foreign body penetrating the arterial wall.

Animal (especially porcine) models have been employed in the study of restenosis. In animal preclinical tests the different stages of injury healing have shown to be remarkably similar to the human response with a distinct pattern of arterial injury and repair. However, the temporal response in animals and humans to healing is substantially different. The healing process after placement of a bare stainless steel stent in a human coronary artery can be five to six times longer than in pig or rabbits. In animals, peak neointimal growth is observed at 28 days, compared with 6-12 months in humans. The explanation for this difference could be the underlying atherosclerotic process in clinical trials while in animal models stents are usually placed inside healthy arteries without inflammation [45].

Many clinical trials have been carried out in the last 10 years to investigate which stimulus could be triggering the restenotic process and at what stage it acts. Stents of different shapes, materials and mechanical properties have been compared in order to identify it. Even if the in-stent restenosis trigger still remains unknown today, clinical trials have been a precious source of information for new stent designs and for the amelioration of surgical stent deployment. Angiography is the main technique to follow the development of restenosis during clinical trials. However, human material for histological or laboratory analysis is more difficult to obtain because it derives mostly from autopsy. For this reason most of the knowledge about restenosis and neointimal formation comes from intense study of animal injury models.

The development of in-stent restenosis has been monitored in porcine animal models and the process has been found to be comprised of four phases [13]:

- 1. Thrombotic response of the blood to the injury caused by the stent to the arterial wall.
- 2. Inflammatory reaction at the thrombotic sites by means of tissue infiltrating monocytes.
- 3. Migration of SMC from the vessel media to the intima and proliferation of SMC.
- 4. Arterial wall remodelling.

Phase one, the thrombotic response is due to the inflated angioplasty balloon abrading the thromboresistant endothelium layer leaving deeper tissues in contact with the blood stream (see fig 2.3 A). The thrombosis causes an aggregation of platelets, fibrin and trapped erythrocytes which can constitute a scaffold for subsequent intimal hyperplasia [46]. Once activated platelets produce SMC mitogens which can stimulate the synthesis of extracellular matrix and the possible migration and proliferation of SMC. At first restenosis has been associated with the high



Figure 2.3: Phases and timing of the in-stent restenosis development [13]. SAM=surface-adherent monocytes, TIM=tissue-infiltrating monocytes

thrombotic response following stent implantation. In fact, metal endovascular stents are extremely thrombogenic. Anticoagulant treatment is necessary to maintain a level of subacute thrombosis below 5%. However this is achieved with the concurrent risk of peripheral haemorrhagic complications between 7 and 13%.

Rogers and Edelman compared the thrombotic response of two different stent materials in relation to the degree of restenosis [47]. New Zealand white rabbits were treated with stainless steal and polymer coated stents with the same design. The level of thrombosis was found to be significantly lower in the polymer coated stents. However, both stents had the same degree of chronic occlusive neointimal hyperplasia. In other tests the same authors found that stents of the same material but different geometries had different thrombosis rates that were correlated with neo-intima formation. From their tests, the authors concluded that there is a correlation between the stent material and the thrombotic response but nothing can be said about the influence of the material on intimal hyperplasia. They concluded that a distinction should be made between the effect of the foreign material on the arterial wall and the impact on vascular injury. The thrombotic response was instead found proportional to the level of the stent-induced injury and stent strut penetration: at the sites of greater injury a greater presence of platelets aggregations ("white thrombus") and fibrin-entrapped red blood cells ("red thrombus") were found whilst lesser injury would have been able to spare the artery wall [48].

In phase two, cell recruiters and mitogens present in the thrombi recruit endothelial cells to cover the thrombus surface within 3-4 days. Macrophages (tissue-infiltrating monocytes, TIM) and lymphocytes (surface-adherent leukocytes, SAM) are subsequently recruited and are able to migrate inside the newly formed thrombus beyond the endothelial layer to attach to the tightly stretched internal elastic membrane(see fig 2.3 B). These cells are chemoattractant and secrete growth factors and thereby facilitate further cell recruitment and hyperplasia. In fact the amount of monocytes on the thrombus is a good index of cell proliferation at a later stage [13].

The third phase involves predominantly cell proliferation. After 7 days from stent implantation (see fig 2.3 C) SMC and fibroblasts get activated when in contact with platelet-related mitogens and growth factors. This phase starts with the formation of a thin cap just beneath the endothelium. This cap grows with time whilst the early thrombotic mononuclear assembly is gradually absorbed. Evidence has shown that the growth is thicker at sites of greatest stent injury [14]. Activated SMC subsequently migrate from the media to the thrombotic bulk and start proliferating substituting the early thrombus neointimal mass with a more mature one composed of smooth muscle cells. In the media SMC normally exhibit a contractile phenotype. A dense network of fibrils in the cytoplasm prevents SMC from responding to mitogens and Under strong stimuli SMC can change phenotype and proliferating. become synthetic. During in-stent restenosis they change their phenotype from contractile to synthetic which leads to their enhanced proliferation. SMC Besides proliferation synthetic and fibroblasts produce proteoglycans. Proteoglycans can develop into bundles of collagen which stiffen the artery and increase the restenotic mass [17]. Rogers and Edelman found a correlation between the depth of the injury and the level of neo-intima formation. In fact a score index system was developed to describe the injury caused by a stent after its expansion and it was found to be correlated to the extent of neointima formation in a porcine model (see fig 2.4).

In white rabbit iliac arteries two different types of stents were deployed at a stent-to-artery diameter ratio of 1.2:1. There was a greater injury and neointimal thickening in the stent design with the same amount of metal but higher number of strut-strut intersections when evaluated using the same index scoring procedure [47]. The difference was explained in the way the applied force was transmitted to the artery. A greater number of strut-strut intersections resulted in an increase of the frequency of radial force application.

Animal and clinical trials have established that the extent of SMC proliferation following injury can be associated with the deformation of
Score	Injury						
0	Internal elastic lamina intact, endothelium denuded, media may						
	be compressed but not lacerated						
1	Internal elastic lamina lacerated, media typically compressed but						
	not lacerated						
2	Internal elastic lamina lacerated, media visibly lacerated,						
	external elatic lamina intact, but may be compressed						
3	External elastic lamina lacerated, large lacerations of media						
	extending through the external elastic lamina. Coil wire of stents						
	occasionally residing in adventitia.						

Figure 2.4: Score index for stent injury [49]

the arterial wall. A study of Arakawa *et al.* [16] in human coronary arteries found a correlation between higher vessel strains after stenting and the increase level of IH. Following 6 months after stenting higher stent-to-vessel cross sectional area (CSA) ratio resulted in more neointimal proliferation. A stent-to-vessel CSA ratio of 0.7 was optimum to minimise restenosis. Hence, stent deployment protocols may be improved to get optimal rather than maximal lumen diameters in order to minimise vascular injury. Therefore the expansion of a stent inside an artery can alter the SMC proliferative signals giving a potent stimulus for proliferation. However animal clinical trials have shown that the response of the vessel to stent-induced injury is limited to the necessity for the re-endothelialization of the lumen which lasts for 21-56 days in animals and up to 3 months in humans.

The final and fourth phase of neointimal formation is arterial remodelling: the artery changes in shape and dimension to adapt itself to the new strain imposed by the stent struts (see fig 2.3 D). The first deformation consists of elastic recoil following balloon inflation. This process is followed by an increase of collagen deposition and elastin destruction due to the presence of chronic inflammation [14]. Remodelling can usually appear in different shapes as it possible to see in fig 2.5. In perfect remodelling the expansion of the artery is enough to



Figure 2.5: Different remodelling types [41].

relocate the neointima at the sides of the vessel. When the arterial expansion is not sufficient to re-accommodate the whole neointima the lumen is not totally reopened. This is the case of favourable but not perfect remodelling. When there is no arterial expansion or the artery shrinks unfavourable remodelling has occurred [41]. Elastic recoil initially and later collagen deposition are the main causes for unfavourable remodelling. Stent procedures have in part solved the balloon angioplasty remodelling problem by forcing the artery to stay open minimising elastic recoil. However collagen deposition between the stent struts and fibrosis can produce draping and prolapse of tissues causing lumen narrowing.

The Edelman and Rogers model [13] of in-stent restenosis is well established and accepted by the majority of researchers and physicians. However, this theory has been recently challenged by new findings on the role of progenitor/stem cells (VPCs) [50, 51, 52, 53, 54, 55]. VPCs are primitive stem cells usually found in the bone marrow or in the blood stream. Under the stimulation of specific pathogenic factors it has been hypothesised that VPCs can participate in the regeneration, repair and remodelling of the injured arterial wall including atherogenesis and post-angioplasty restenosis [50]. It has been proven that VPCs are able to transform into mature vascular endothelial or smooth muscle cells [51]. The new theory maintains that VPCs can infiltrate into the arterial wall from the blood stream and once there can transform into SMC and accumulate as opposed to migrating from the media to the intima [54, 55]. The origin of VPCs is not totally clear. Some studies have shown a presence of these cells in bone marrow, peripheral blood and vascular adventitia. VPCs exhibits a extraordinary vitality with more than 70 population doublings in a 3-4-month time period, much quicker than the normal lifespan of regular somatic diploid cells [56].

This new theory is supported by some interesting discoveries. A close similarity was found between specific characteristics of SMC in the atherosclerotic plaque and SMC derived in the laboratory from VPCs harvested in the bone marrow, characteristics that are not usually present in normal medial SMC [57]. Other observations in favour of this new theory include rapid formation of neointima even without the presence of medial SMC [58]. These findings have led many researchers to think that at least a part of SMC in the neointimal layer may infiltrate the arterial wall from the blood lumen as opposed to from the media. Other evidence comes directly from clinical trials with bare or drug eluting stents. A recent study demonstrated a very poor effect of sirolimus-coated DES on the arterial medial SMC, a fact that has led researchers to believe SMC may originate from bone marrow-derived vascular progenitors at the balloon-injury site [59].

The adventitia has recently been considered to be possibly involved in atherosclerosis and restenosis development [60, 61]. For instance, fibroblasts have been shown to migrate from adventitia to the neointima following injury [62]. Hu *et al.* [63] found an abundance of progenitor cells in the adventitia whilst excluding their bone-marrow origin. When progenitors cells were added to the adventitia of irradiated vein grafts they differentiated to SMC and their presence was identified in the neointima of vein graft atherosclerotic lesions. Werner [52] hypothesised that progenitor cells in the adventitia act as a reservoir from which cells are drawn in cases of physiological repair or other mechanisms. Cells could remain in the adventitia in an undifferentiated and quiescent state until stimulated to differentiate and proliferate.

Other studies found a relation between dysfunction, impaired adhesion and reduced number of endothelial progenitor cells (EPCs) in the blood [64] and the presence of in-stent restenosis.

Because the early replacement of the endothelium after a stent procedure halts neointima formation, many groups have examined the role of EPCs. For instance, activation of the adventitia (a likely source of EPCs) during in stent restenosis has brought further credence to the hypothesis, so much so that EPC-seeded stents have been proposed as possible therapeutic devices for re-endothelialisation to prevent in-stent restenosis [65].

The change in mechanical conditions following stenting is therefore hypothesised to be a critical stimulus for progenitor stem cells to change phenotype and become activated leading to restenosis formation regardless of the origin of the precursors to the proliferative SMC. In any event, it is clear that the behaviour of SMC in response to the mechanical environment, irrespective of origin, is pivotal in the restenotic event.

2.4 Biomechanical environment in a stented artery

Proliferation and apoptosis of SMC are key factors in the development of restenosis. A stent implant creates dramatic and chronic changes of the mechanical environment for smooth muscle cells, including an acute reaction due to endothelium denudation during the procedure. In a stented artery the level of strains and stresses the SMC are exposed to is much higher than under physiological conditions. The consequences of such a level of stresses and strains have often been analysed by finite elements simulations [66, 67, 68]. However the *in vitro* response to mechanical stimuli on SMC inside a stented artery has never been studied.

The diameter of a coronary artery varies in general between 2 and 3 mm depending on the person and on the location in the coronary arterial tree [25]. In physiological conditions arteries are not symmetric and can have different wall thickness and geometries. Stents are standardised devices which force the artery into a symmetric configuration. Following stent expansion the coronary artery can enlarge between 20 and 50%, and in some cases even more [28, 69].

According to the literature [25, 70] when a coronary artery, is strained the stress-strain curve's first derivative increases dramatically due to the stiffening of the arterial wall, see fig 2.6. Holzapfel et al [70] evaluated by finite element analysis the level of stress a stented artery experiences for these deformations: maximum stress values reached as high as 300-400 kPa in the coronary intima.

These values represent an average of stresses inside the arterial wall: every cell will thus bear a value of stress and strain that could be greater or smaller than the average value, however the mean value can be used as point of reference.

Due to the presence of the expanded stent, SMC undergo high mean equi-biaxial strains and low oscillating strain (and stresses) due to the



Figure 2.6: Stress-strain relationship calculated by Holzapfel in the rat abdominal aorta for different axial strain percentages [70].

pulsatile flow of blood. Because SMC proliferation is the main cause of restenosis, an understanding of the SMC proliferative response in this altered strain environment can provide useful information for future stent design. In order to simulate the in-vivo levels of strains inside of a stented artery it is imperative to know the strain levels of a stented artery before and after a stenting procedure.

Vernhet *et al.* [28] measured the level of strain of an artery before and after stenting procedures in infrarenal aortas of New Zealand white male rabbits. Using a sonographic transducer they measured systolic blood pressure changes, blood-flow velocity, systolic diameter, and diameter changes of the artery before and after procedures with different stents: Wallstent, Palmaz stents, Jostent, see fig 2.7. Their values can be used as a reference for the strain level changes in a coronary artery after a stenting procedure. They found that a stent causes an average 21% expansion of the artery (increasing the mean strain SMC undergo) and a dramatic decrease of the oscillatory strain due to the pulsatile blood pressure (see

fig 2.7). From average strain amplitude of 4-5% before stenting, oscillatory strains dropped to between 1.4% and 2% after stent implantation. Taking these values as a reference point our study proposes to mimic the strain pattern SMC undergo before and after stenting *in vitro* and further investigate if the increase of mean strain and stress values or the decrease in strain amplitude is the basis for the proliferation of SMC.

2.5 The role of VSMC growth in intimal hyperplasia

Cell fate is the array of possible phenotypic outcomes a given cell may have over a life span. Cell proliferative and apoptotic capacity are among the most important determinants of cell fate. Many diseases are characterised by altered cell fates such as cancer, atherosclerosis and hypertension-related arteriosclerosis. All these diseases are characterised by altered cell growth which is the the balance between proliferation and apoptosis. Modification of the normal proliferative capacity of SMC is crucial in the progression of neointimal lesions. Restenosis is a hyperplastic response of vascular SMC following stent injury of the arterial wall. Apoptosis of SMC promotes vessel remodelling, coagulation and inflammation [71].

In restenosis SMC apoptosis reduces fibrous cap stability for the degradation of the ECM produced by the SMC increasing the chances of plaque detachment and consequent thrombosis with occlusion of the vessel [72].

In order to improve stent design and reduce restenosis, a better understanding and quantification under different conditions (stent geometry, material and deployment technique) of the proliferative and apoptotic capacity of SMC is necessary.







Figure 2.7: Comparative presentations of diameter (d), systolic diameter change (Δ d), blood pressure (P), and ECG, at level of stent, before and after stenting. Recordings show Wallstent (Schneider, Blach, Switzerland) (rabbit 2) (A), Palmaz stent (Johnson & Johnson, Warren, NJ) (rabbit 6) (B), and Jostent (Jomed, Rangendingen, Germany) (rabbit 12) (C). Note decrease in systolic diameter change (Δ d) after stenting in all instances [28].

2.5.1 VSMC proliferation

Cells in culture are characterised by a typical exponential growth function named doubling. Because every mother cell can split into two daughter cells, a cell population should double every generation. In reality this is not completely true due to an amount of cells that die at every passage (P0, P1, P2 passage is the cell generation change) from a generation to the next. The cell cycle is a sequence of events leading to cell replication. This process is sub-divided in two periods:

- 1. Inter-phases: period in which the cell accumulate nutrients and energy for its duplication
- 2. Mitosis phase: the cell splits into two daughter cells with identical nuclei.

The inter-phase is divided in three phases (fig 2.8):

- The G1 (G stands for Gap or Growth) phase starts at the end of the mitotic phase M until the beginning of DNA synthesis. During the M phase the biosynthetic activity of the cell is very low. In the G1 phase there is a strong recovery of cell activity as synthesis of enzymes necessary for the S phase takes place.
- 2. During the S phase there is the doubling of the cell DNA with replication of the chromosomes. RNA transcription is very low at this stage except for the histones production.
- 3. The G2 phase is characterised by protein synthesis especially for microtubules formation necessary during mitosis.
- 4. Another phase of this period is the G0 phase. It is an alternative to the G1 phase. It is also called "post-mitotic" to indicate its quiescent or senescent status in which most of the eukaryote cells spend most of their life-time. This phase is characteristic of fully differentiated cells that spend their time doing a specific function and are not

proliferating. Cellular senescence is instead due to DNA damage and is an alternative to apoptosis for cells that are not able to duplicate. The G0 phase time is very variable from cell type to cell type. Some cells like neurons enter the G0 phase and stay in this phase, possibly permanently. Other cell types like epithelial cells continue to proliferate without entering this phase. Vascular smooth muscle cells are usually in a fully differentiated state but under particular strain conditions can return to the G1 phase and restart proliferation.



Figure 2.8: Cell cycle phases: M=Mitosis, I=Interphase.

Vascular SMC proliferation has an important role in the formation of the atherosclerotic fibrous plaque and of in stent neo-intimal hyperplasia. This disease can find a therapeutic solution by inhibiting SMC proliferation with antiproliferative treatment. One of the most important protein related to cell division is the mTOR. The mTOR (mammalian target of Rapamycin (also known as Sirolimus)) is a protein of the family of the serine/threonine kinase. It has been associated to the regulates cell division, cell growth, cell survival and transcription. The mTOR activity is influenced by the presence of nutrients: when cells are not able to grow to normal size they will not undergo cell division [73]. Sirolimus is a well recognised antiproliferative drug that acts on the mTOR to inhibit SMC cell proliferation. The Sirolimus antiproliferative properties have prompted its use for drug eluting stents in order to curb SMC proliferation during restenosis. It is part of this work the evaluation of the effect of Sirolimus eluting stent on SMC proliferation and apoptosis.

2.5.2 VSMC apoptosis

Apoptosis is genetically programmed cell death for multicellular organisms and it is the means by which the body gets rid of injured, infected or damaged cells in a fast and organised manner. Apoptosis differentiates itself from the other form of cell death: necrosis. Apoptosis is a programmed and active cell death process characterised by changes in the cell membrane, loss of membrane asymmetry and attachment, blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. The organised apoptotic process leads to the membrane cleavage and the formation of apoptotic bodies that are destroyed by macrophages through phagocytosis. In such a way an inflammatory reaction is avoided because there is no release of cytosolic material outside of the cells. Apoptosis can be triggered by intrinsic or extrinsic signals through several pathways that generally converge into the caspase signal pathway. When the apoptotic process for a cell is triggered the cell does not need any other external signal to carry on the suicide program. SMC and EC apoptosis is regulated by an extensive variety of physical, biochemical, viral factors; mechanical forces, heat, radiation, oxidative stress, free radicals, cytokines, oxidised lipids (cholesterol), growth factors, bacteria, viruses and more [74]. The apoptotic process inside an atherosclerotic plaque is therefore a multivariable controlled process where all immune cells like macrophages and T- lymphocytes, as well as lipid-rich foam cells, play a crucial role. Cells of the immune system do not act directly on the SMC apoptotic process but rather release cytokines (such as INF- γ , FasL, TNF- α and IL-1) when they are activated by antigenic products like oxidised lipoproteins and heat shock protein-60, which act on SMC inducing proliferation or apoptosis. For instance, cytokines could prompt the activation of the caspase cascade leading to apoptosis of SMC and consequent weakening of the arterial wall [75, 76], see fig 2.9. A direct consequence would be the formation of an unstable atherosclerotic plaque.



Figure 2.9: Interplay of cells, cytokines and growth factors inside an atherosclerotic plaque. Macrophages activation activates T cells and consequently release of many cytokines and other factors having damaging effects on smooth muscle cells [77].

Necrosis is instead a non organised, sudden cell death due to a traumatic event induced by external stimuli. Necrosis can be caused by injury, infection, ischemia, cancer, or poisons. A typical necrotic situation is represented by myocardial infarction due to lack of oxygen within cardiac muscle. The sudden and disorganised necrotic cell death does not give the cell the chance to launch signals for the arrangement of the cell debris scavenging. This leads to cell swelling and membrane breakdown

with the release of cytosolic material into the extra-cellular space resulting in a local inflammatory response.

2.6 Biomechanical regulation of vascular SMC growth

Biomechanical stimuli have a strong influence on growth and adaptation of all the organ systems of the human body [78, 79, 80]. Alterations in the mechanical environment can influence the progression of disease, healing and remodelling. Arterial diseases such as atherosclerosis and intimal hyperplasia are well recognised to be influenced by fluid shear forces and vessel wall injuries [81, 82, 83, 84]. Atherosclerotic lesions are concentrated in critical points of the arterial tree (bifurcations and curved regions) where hemodynamic forces are more intense and blood flow disrupted [85]. A homeostatic strain level has been measured within the cardiovascular system [86]. Deviations from this strain level may therefore have very important implications for vascular growth, healing and remodelling and it has been suggested that strain may be the most homeostatic parameter in the cardiovascular system such that interventional strategies should always aim to maintain normal strain levels [19]. Many studies therefore have investigated the influence of mechanical forces, pressure or strain on cell activity with a view to determining the optimum medical device or drug for treatment of such diseases.

2.6.1 Stress and strain

Smooth muscle cells and endothelial cells undergo different kinds of stresses due to their different positions inside the arterial wall. Under physiological conditions endothelial cells are subjected to mechanical pulsatile forces of compression in the radial direction and tensile forces in the circumferential direction. Due to their position as a blood flow barrier they also undergo hemodynamic shear stress. Under physiological conditions smooth muscle cells are not in direct contact with the blood flow and experience minimal blood shear stress but are subjected to pressure from the pulsatile force of blood flow.

Many studies [81, 82, 83, 84] have been carried out to investigate the effect of pressure and shear stress on SMC and EC function with more recent biomedical studies utilising a more focused engineering approach in that they refer to circumferential, radial and axial stresses and strains, and not just physiological environments but also a broader range of loadings. In such a way it is possible to examine how vascular cells react under pathological conditions.

In a stented coronary artery the radial, circumferential and axial stresses and strains are altered in addition to the presence of severe injury to the arterial wall. Furthermore, during stent procedures the endothelial layer is denuded by the frictional force of the angioplasty balloon leaving SMC in direct contact with the blood flow.

These strains and stresses are, in many cases, involved in different biological and chemical pathways inducing various effects on SMC and EC; proliferation, apoptosis, change in phenotype, vascular adaptation, migration etc.

Cyclic strain can affect SMC ability to proliferate, differentiate, and produce ECM (extracellular matrix). SMC are usually present as a contractile-differentiated phenotype during their normal life cycle as part of a mature arterial wall whereas during vascular remodelling, they undergo phenotypic switching to a de-differentiated synthetic phenotype. SMC switch to the synthetic phenotype under particular conditions (atherosclerosis, vascular wall injuries), see fig 2.10.

Cyclic strain thus influences vascular SMC phenotype. The contractile phenotype is usually related to the *in vivo* differentiated and quiescent state of SMC where orientation is perpendicular to the stretch direction. In this state, differentiated-SMC marker proteins such as SM-1, SM-2, MLCK and desmin (phenotype) are produced whilst in the de-differentiated phenotype marker myosin A is produced and $\alpha - actin$



Replicative

Synthetic phenotype

Re-expression of fetal or embryonic genes

Figure 2.10: SMC change from "Contractile" to "Synthetic" phenotype due to injury [87].

is a marker for both SMC phenotypes [88]. It has been shown that cyclic strain *in vitro* can enhance differentiated ECM synthesis and remodelling and stimulate SMC proliferation and differentiation. Li *et al.* stretched rabbit vascular SMC from 5% to 15% at 30 cycles/min and obtained an increase in proliferation from 1.4 to 1.6 fold [21]. In contrast, other studies have found that cyclic strain, along with differentiation augmentation, reduces SMC proliferation and DNA synthesis independently from the associated extra cellular matrix [22].

Predel *et al.* [89] reported different cell behaviour according to the source of cells: proliferation of SMC from saphenous vein but no response under the same stimuli for SMC from the internal mammary artery. Other studies [90, 91] have found no SMC proliferation at all in response to strain or proliferation only in the presence of specific growth factors or proteins. SMC proliferate in response to specific growth factors

such as PDGF or Insulin Like Growth Factor (IGF-I) [21, 92]. These growth factors work in an autocrine manner and their production is stimulated by cyclic strain.

In contrast, Hipper and Isenberg [22] reported a completely different response. Their study showed a decrease in DNA synthesis following SMC exposure to cyclic strain (a strong indication of low proliferation level). This response was found to be proportional to the exposure time to the cyclic stretch and independent from the extracellular matrix. They thus hypothesised no autocrine pathway for SMC proliferation in response to cyclic strain.

McKnight and Frangos [93] found that SMC had a different reaction not just to strains and stress but also to different strain rates and for different cell orientation alignment.

Another important stimulus for SMC is the lumen blood pulse pressure. Blood pressure has been shown to have an important role in SMC proliferation and apoptosis directly or mediated by pressure induced fluid flux. It has previously been shown that the inhibition of the pulse pressure can cause intimal hyperplasia and stenosis [94]. Furthermore it has been reported that artery cuffing atrophies the vessel due to increase in apoptosis [95].

Birney *et al.* [96] demonstrated the effect of pulse-pressure on bovine aortic SMC (BASMC) apoptosis and reported a mitogen-activated-protein-kinase (MAPK) dependent pathway in these cells in response to pulse pressure. The pulse pressure changes produced by a transmural fluid flux inside a bioreactor co-culture system were responsible for an increase of capsase-3 activity (an apoptosis marker) in BASMC in a force and time dependent manner and independently from the presence of ECs [97]. No vascular EC apoptotic pathways were activated during the tests.

SMC differentiation and proliferation nevertheless is modulated not just by cyclic strain but also by growth factors, cytokines, and hormones and by ECM composition. All of these elements can be involved in the apoptosis and proliferation of vascular smooth muscle cells and they can interact with mechanical stimuli to give cells apoptotic or proliferative signals (see Rakesh and Agrawal review [77]). For instance, PDGF presence in culture medium along with cyclic strains can cause de-differentiation [23]. On the other hand, differentiation was observed when TGF- β was present. Differentiation also increases under cyclic strain when SMC are cultured upon type collagen I, laminin and pronectin proteins [98].

2.6.2 Shear stress

Assuming axisymmetry in the longitudinal direction of the artery, from the equilibrium equations the following is obtained:

$$\frac{1}{r}\frac{\partial}{\partial r}\left(rt_{rz}\right) = 0 \to t_{rz} = \frac{d}{r}$$

where d is a constant. This equation [87] points out that, in the presence of a lumen blood shear stress, d is not equal to zero at $r = R_i$ (artery inner radius) and t_{rz} (tangential stress) has a continuous monotonic behaviour at r increasing to $r = R_a$ (radius of the adventitia layer).

There are two main consequences to this observation: there should be perivascular supports to offset t_{rz} , SMC probably undergo shear strain and stresses similar to ECs, see fig 2.11. These stresses have a very low order of magnitude compared with the other stresses and play a less relevant biomechanical role but they may be important in activating biochemical pathways. Furthermore SMC undergo another kind of shear stress that is interstitial to the arterial wall. Previous studies [87] have shown that even though this stress is in the order of 0.1 Pa it is responsible for one quarter of the PGI2 release as occurs for ECs. These studies [87] have also shown that shear stress is involved in gene expression of SMC. Papadaki *et al.* described the production of nitric oxide syntheses (NOS) (flow mediator for the NO production) under shear stresses between 0.1 and 2.5 Pa. Lee *et*



Figure 2.11: Schematic representation of the shear stress and strain for SMC in the media of the arterial wall.

al. have also shown an alignment of SMC perpendicular to the blood flow *in vitro* that has been shown to also happen *in vivo* [21].

2.7 Mechanotransduction

Vascular SMC have the ability to convert mechanical signals into a biochemical response. SMC have specific receptors on the cell membrane that mediate the attachment between the cell and the tissues surrounding it. The action of stress or strain on SMC could perturb the cell surface or alter receptor conformations leading to signalling pathways activation which could modify cell fate [99]. Integrins, G-protein and G protein-coupled receptors, tyrosine kinase receptors and ion channels are among the most important mechanosensors regulating the response of SMC to strain .

2.7.1 Integrins

Integrins are responsible for the attachment of a cell to the extracellular matrix (ECM) by coupling the ECM to the cytoskeleton inside the cell. Integrins have covalently bound subunits, α and β and they consist of a

single transmembrane domain, a large extracellular (EC) domain and a small cytoplasmic domain. The EC domain can bind to different ECM ligands such as fibronectin, vitronectin, laminin and collagen [100]. There are fifteen α and eight β different types of subunits. Heterodimerization of the different α and β subunits allows the formation of twenty different combinations of integrin receptors.

Clustering with other bound integrins results in the formation of highly organized intracellular complexes, known as focal adhesions. Focal adhesions are biochemical signaling hubs with different biological functions including the transmission of mechanical and regulatory signals.

 β 1 and β 3 integrins have been shown to have a role in adhesion and migration of vascular smooth muscle cells [101, 102], and are sensitive to mechanical strain in VSMCs [103].

G protein-coupled receptors and tyrosine kinase receptors have a unidirectional nature. Integrins are instead bidirectional signal transmitters with "outside-in" signalling involving interaction of integrins with the extracellular matrix (ECM) rather than with soluble ligands [104].

2.7.2 Ion Channels

Following cyclic strain, ion channels such as Ca^{2+} and Na^+ channels increase their frequency of opening with a consequent increase in intracellular Ca^{2+} , Na^+ and other divalent cations. This may have implications for vascular remodelling where changes in cell fate of vascular SMC occur. Increased cytosolic levels of calcium stimulates quiescent cells to enter into the cell cycle leading to cell proliferation [105].

2.7.3 **Receptor Tyrosine Kinsases**

Receptor tyrosine kinases (RTKs) are high affinity cell surface receptors. They include the vascular endothelial growth factor (VEGF), platelet-derived growth factors (PDGF), and insulin receptor families. Mechanical strain increases both vascular VEGF expression and phosphorylate the VEGF receptor in vascular SMC [106]. PDGF receptors α and β are reported to be up-regulated by mechanical stress. When PDGF binds to the correspondent plasma membrane receptor it leads to the auto-phosphorylation of the tyrosine residues in the PDGF receptor kinase domain. Several phosphorylation events follow as well as the activation of MAPK cascades [107].

2.7.4 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are activated by an external signal such as a ligand or cyclic strain. When a ligand binds to the GPCR it causes a conformational change in the GPCR which allows it to act as a guanine nucleotide exchange factor (GEF). The GPCR can then activate an associated G-protein by exchanging its bound GDP for a GTP. Small guanine nucleotide binding proteins (small G proteins) or GTPases include five families of Ras, Rho, Rab, Sar1/ADP ribosylation factor and Ran, which oscillate between inactive GDP-bound G-proteins and active GTP-bound G proteins. Growth factors, cytokines and G protein-coupled receptors allow activation of G proteins, and GDP association inhibitors and GTP-ase activating proteins cause the small G protein to remain in its inactive GDP-bound state. G-proteins have important downstream effects in vascular SMC. Ras promotes cell proliferation through activation of the Raf-MEK-ERK cascade [108]. RhoA increases sensitivity of vascular SMC through activation of ROCK (Rho kinase) [109] and reduced p21Cip1 or p27Kip1 results in Rho-induced cell proliferation in the vasculature of hypertensive rats [110].

2.8 Bioreactor development for mechanical characterisation of vascular cell activity

The role of the physical and mechanical environment on cell fate is widely recognised [111]. To control the physical, mechanical and chemical conditions in which cells grow many types of bioreactors have been developed in recent years. The general idea of a bioreactor is to mimic *in vitro* the physiological and/or pathological conditions cells experience in vivo *in vivo*.

"A bioreactor may be defined as a system that simulates physiological environments for the creation, physical conditioning, and testing of cells, tissues, precursors, support structures, and organs *in vitro*" [112].

The simplest bioreactors are flasks and dishes normally used for cell culture. The introduction of tissue engineering and mechanotransduction led to the necessity for more sophisticated devices. There are two main goals of a bioreactor: maximise gas and nutrient transfer and to apply the physical forces to cell constructs simulating the forces that cells experience *in vivo* [113]. Physical forces in the cardiovascular system can be divided into three groups:

- 1. Shear stress: the tangential force generated by the friction of the blood flow on the vessel walls
- 2. Circumferential stress due to the expansion of the vessel under the blood pressure
- 3. Compression as a direct consequence of the radial pressure on cells in the vessel wall.

The most recent bioreactors are able to control the mechanical or electromechanical environment of cells and tissues. According to McFetridge *et al.* [113] the design of a good bioreactor should follow the following characteristics:

1. Control of the chemical environment; e.g. CO₂, PO₂, nutrients, pH

- 2. Control of the physical environment; e.g. pulsed flow rate and frequency, and pressure
- 3. Maintenance of sterility
- 4. Accessibility, e.g. removal and addition of culture/growth components
- 5. Materials compatibility, e.g. cell-media exposure to all components within the system
- 6. Maintenance of long-term cell culture
- 7. Reproducibility of 'standardised' reactor design
- 8. Ability to monitor and control the development of tissue engineered constructs or cell growth
- 9. Simplicity in design and implementation

The most critical requirement is to maintain a sterile environment for cells without compromising the user friendly handling of the system. In the design of a bioreactor, the complexity of the system is important as the risk for the device becoming contaminated is higher the more complex the system. Bioreactors are often composed of different parts that must be assembled under a biosafety cabinet such as the placement of a scaffold or the connection of a cable for data acquisition. The design of earlier systems was complex with many cables to maintain a constant real-time control on all the variables. As a consequence these systems were more liable to contamination. The trend in recent years is the design of user-friendly bioreactors that can be easily handled by an operator instead of complex devices which risk contamination. For instance, a complex bioreactor can be designed to keep parameters such as temperature and CO_2 constant. However the solution adopted in most cases is to fit the entire system inside a standard humified (100% humidity) incubator at $37^{\circ}C/5\%$ CO₂. Gasses can be regulated by means of specific ventilation filters which equalise the gas concentration inside the bioreactor with the gas concentration of the incubator.

The complexity of the physiological environment prevents designs from mimicking in full the complete representation of the physical and mechanical environment in vivo. Biological signalling is extremely complicated and difficult to control. However a bioreactor enables control of some of these variables in vitro. Among the mechanical variables, pulsatile forces, pressure, flow rate, shear stress, frequency, stroke rate and stroke volume are extremely important design considerations [112]. Among biochemical factors that can be controlled are nutrients/cells by-products distribution and their fluid dynamics. The pH level of the medium depends on the concentration of these products together with the temperature and CO_2 levels. Even if there is no current standard design many elements are common to many bioreactors:

- 1. A reservoir for cell culture medium
- 2. A pump for inducing a pulsatile flow
- 3. One way valves for the creation of a heartbeat pressure waveform
- 4. A culture chamber for cell seeding
- 5. A compliance resistance chamber for regulating the medium flow

So far, the majority of bioreactors have been built to provide a specific stimulus for cells such as strain or shear stress depending on the goal of the researchers. Petri dishes and T-flasks are the most trivial systems. Cells are in a static environment and gas and nutrient diffusion is provided by diffusion. Temperature and CO_2 are regulated by the incubator in which they are placed and air exchange is controlled with specific ventilation filters. Cells do not undergo any mechanical or physical stimulation. Chemical stimulation could be dictated by pH changes due to medium consumption. Among the multitudes of different

bioreactors it is possible to distinguish some bioreactor types according to the mechanical stimulus they apply to cells such as dynamic and biomimetic system.

2.8.1 Dynamic system

Dynamic bioreactors work with rotating wall vessels (RWV). RWV combine the advantages of cell culture in low shear stress conditions with a microgravity environment. RWV were first developed by NASA. These systems were later developed in different forms such as the slow lateral turning vessel (STLV), the rotating wall perfused vessel (RWPV) and the rotating wall perfused vessel (RWPV). Some of these systems are now commercially available like the STLV (Synthecon, Houston, TX). They usually consist of two concentric cylinders with the inner cylinder functioning as a gas exchange membrane and the external one rotating on its axe creating a microgravity environment. The space between the two cylinders contains culture medium (around 100ml) and up to 12 tissue constructs. The rotation of inner and outer cylinders generates centrifugal and drag forces which create (with the acting gravitational force) a microgravity environment due to dynamic equilibrium. Cell/scaffold constructs move in the interspace between the two cylinders in a freefall manner. The system is operated horizontally with a solid-body rotation at rates of 15-40 rpm. Cells cultured in these bioreactors at low rates of shear stress $(0.8 dyne/cm^2)$ have shown the production of more superior tissue engineered constructs in structure and functionality [114]. The main drawback for RWV bioreactors is the non-uniform distribution of cells due to continuous collisions of the scaffolds with the bioreactor wall causing cell damage and disrupting matrix production and cell attachment.

2.8.2 **Biomimetic systems**

Biomimetic systems mimic the *in vivo* environmental conditions of cells. In the cardiovascular sector bioreactors have been especially used to simulate pulsatile physical forces that vessels or cardiac valves experience *in vivo*. The benefit of these systems is the more accurate reproducibility of the physiological or pathological conditions. In tissue engineering applications biomimetic systems have proven to be superior to static culture systems for cell penetration and differentiation inside the pores of a scaffold. Niklason *et al.* [115] developed a perfusion system able to provide intraluminal pulsatile flow to four reactors. Each reactor contains one scaffold. A pump exerts a variable stroke volume of 0-10ml per stroke and a pressure of 27030 mmHg. The bioreactor can work at a range of pulse rates from 60-165 beats/min, see fig 2.12.



Figure 2.12: Schematic illustration of a pulsatile bioreactor. The pulsatile movement of the medium through the four constructs stretch the cell constructs [116].

Other bioreactors have been designed to impart cyclic strain to cell

constructs. Seliktar [116] created a bioreactor in which silicon tubes were perfused by pneumatic-controlled medium to created cyclic strains up to 10%. Cells were seeded on a construct around the silicon tube which was able to transmit to them the cyclic strain impulse. The overall system can fit inside a standard CO_2 incubator at 37°C and cells were strained between 4 and 8 days at a frequency of 1Hz.

In the cardiovascular research area, bioreactors have been built to recreate the characteristic pressure heartbeat waveform of arteries in order to mechanically stimulate cells: just pressure in the case of rigid scaffolds or both pressure and strain in the case of compliant vessels. The typical shape of the heartbeat waveform can be recreated by placing a bioreactor, a pulsatile pump, one way check valves and a compliance chamber in series in the circuit [113]. In fig 2.13 every component of the bioreactor circuit contributes to the formation of the heartbeat waveform. The basic pressure waveform of a peristaltic pump is transformed by the compliance chamber which reduces the amount of noise due to the vibrations of the mechanical movement of the pump head. The one way valve prevents back flow similar to the heart valves *in vivo* giving the waveform its final conformation [113].

2.9 Drug eluting stents

Coronary stents are employed in more than 90% of percutaneous coronary interventions (PCI). They have been tested to reduce early complications and late restenosis (restenosis occurring > 1 year after stent implantation) as compared to conventional balloon angioplasty. Bare metal stents (BMS) reduce restenosis from 40% to 20% as compared with angioplasty alone. However stents have failed to reach acceptable restenosis rates, in particular, in challenging patient cohorts such as those with diabetes or in specific conditions with small or tortuous vessels, venous grafts, or in long and/or heavily calcified lesions. Recurrent restenosis is also more common after percutaneous treatment of in-stent



Figure 2.13: Creation of an heartbeat pressure waveform. (A), basic characteristic pressure waveform of a peristaltic pump; (B), the addition of a compliance chamber removes much of the noise of the system and improves the shape of the waveform; (C), the introduction of a one-way check valve into the flow system after the peristaltic pump prevent back flow giving the final heartbeat pressure waveform [113].

restenosis, with a consequential clinical and economic impact on health-care systems.

In order to prevent restenosis stents of different geometries and materials have been tested in clinical trials, but none have proven to be effective. Many different biological mechanisms contribute to restenosis. Drugs targeting only one pathway for a short time may have poor efficacy. Systemically administered drugs (antiplatelet agents, anticoagulants, calcium-channel blockers, cholesterol-lowering agents, antioxidants) have provided no real satisfactory results in human trials, even if they had beneficial results in animal models. The difference in results between human and animal tests has been explained by the insufficient drug concentration at the injury site or lack of chronic dosing [117].

Intracoronary radiation has recently emerged as a promising modality to control IH development. Radiation is ineffective for preventing restenosis in *de-novo* lesions, whereas brachytherapy (radiotherapy where the radioactive source is placed close to the lesion) is effective in reducing recurrent restenosis. However radiation is cytotoxic and follow-up has revealed alarming sequelae such as edge restenosis and late thrombosis giving concerns about the effect of radiation in the long term [118].

The parallel between the uncontrolled proliferation during neo-intima formation and benign tumour cell growth has led to the concept of using immunosuppressive agents to treat IH. Drug eluting stents (DES) have thus emerged as a solution to obtain time-controlled local release of an immunosuppressive drug at the site of the lesion. DES have the advantage of providing a local pharmacological response to restenosis without the disadvantages of the systemic toxicity of the drug [119]. DES surfaces are covered with bio-active agents which are locally released onto the tissues surrounding the stent struts. These agents can be slowly released through polymeric primers attached to the stent struts. Alternatively, a carrier can be employed to surround and release them into the blood. In this case the carrier can be strut-adherent and thus coating the stent or it can span (strut-spanning) the struts [117]. A combinatorial action of the drug, the polymer, and elution kinetics determines the safety and the efficacy of this kind of treatment.

Many pharmaceutical agents are under investigation for the treatment of restenosis together with different stent models, see fig 2.14. The most common are antiproliferative drugs previously used in cancer treatment or as immuno-suppressants in organ transplant. Among the most promising is Sirolimus. Sirolimus is able to block the progression of the cell-cycle by expressing different cytokines. The first randomised double-blind trial was the RAVEL study [120]. It compared a sirolimus coated stent with a standard uncoated stent spanning over 238 patients with single coronary lesions in 19 hospitals. Patients with complex coronary lesions were not considered. 6 months following the implant procedure the angiographic rate of restenosis was 26.6% in the uncoated stent group and 0% among DES. There were no reported cases of subacute thrombosis. After two years there was still no significant neo-intima presence. These data are very promising. However, preliminary and toxic effects of the drug or an inflammatory late response due to the polymeric primer could cause damage to the distal vascular bed or simply delay IH formation. Furthermore the RAVEL study did not take into consideration more problematic cases with complex coronary lesions.

A second clinical trial named SIRIUS [121] was carried out on 1100 randomised patients to test the long-term safety in complex coronary lesions of treatment with rapamycin-coated or uncoated stents. A considerable reduction of in-stent restenosis (3.2% drug-stent vs 35.4% uncoated stent) and in segment restenosis (8.9% vs 36.3%) was measured without differences in adverse effects.

Therefore DES remain very promising and there is great enthusiasm and anticipation for DES of the next generation. The main drawback for these devices is their cost. The implant of a DES can cost four times a bare metal stent leading to a debate on the advantage of drug eluting stents compared to the economic burden of restenosis such as hospitalisation and increase of medications and diagnostic testing [122]. The development of new DES is time consuming and clinical trials are very expensive. It is also for this reason that one of the goals of this work is the development of an *in vitro* preclinical testing technique which could be used to optimise the biomechanical and pharmacological characteristics of intravascular stent designs. Such a system would give early indications on the design of a new stent to reduce restenosis without the need for expensive clinical trials.

Study and reference	Drug	Follow up	Antiplatelet treatment*	Definition of percutaneous revascularisation†	Angiographic binary restenosis definition	Death
Trials in the sirolimus RAVEL ³⁸	(and analogues) subgroup Sirolimus	l year	Clopidogrel or ticlopidine	TLR: clinically driven or evidence of restenosis	In-target lesion including proximal and distal edge; et 6 months	Any
SIRIUS ³⁹	Sirolimus	l year	Clopidogrel 3 months	TLR: clinically driven and restenosis >70%	In segment zone including margins 5 mm distal and proximal: at 8 months	Any
E-SIRIUS [∞]	Sirolimus	9 months	Clopidogrel or tidopidine 2 months	TLR: clinically driven	In lesion including 5 mm proximal and distal edge; at 8 months	Any
C-SIRIUS41 42	Sirolimus	l year	Clopidogrel 2 months	TLR: clinically driven including stenosis >70%	In-stent lesion; at 8 months	Any
FUTURE I∕II ⁴⁵	Everolimus	6 months	NA	TLR	In segment; at 6 months	Any
SES-SMART59	Sirolimus	8 months	Clopidogrel 2 months	TLR	In segment; at 8 months	Any
SCANDSTENT ⁴⁴	Sirolimus	l year	NA	TLR	In-stent lesion; at 6 months	Any
DIABETES43	Sirolimus	9 months	NA	TLR	In segment; at 9 months	Any
ENDEAVOR II10	Biolimus	l year	NA	TVR		Any
Trials in the paclitaxel	(and analogues) subgroup	,				'
SCORE ⁴⁶	QP2 is 7-hexanoyltaxol	l year	Ticlopidine or clopidogrel 1–6 months	TLR	In stent; at 6 months	Cardiac death
TAXUS I ⁴⁷⁴⁸	Slow release polymer based paclitaxel	1 year	Clopidogrel 6 months	TVR: indication not reported, only Q wave MI	In-stent lesion (edges excluded); at 6 months	Any
ASPECT ⁵²	Non-polymer based paclitaxel low or high dose	6 months	Clopidogrel or ticlopidine 1 or 6 months, or cilostazol	TLR: ischaemia driven	Including proximal and distal references; at 4–6 months	Any
TAXUS II ^{48 49}	Slow or moderate release polymer based paclitaxel	1 year	Clopidogrel (or ticlopidine) 6 months	TVR: indication not reported	In total analysis segment including 5 mm proximal and distal edge; at 6 months	Cardiac death
TAXUS IV ^{48 60}	Slow release polymer based paclitaxel	9 months	Clopidogrel for 6 months	TVR: ischaemia driven	In analysis segment including proximal and distal edge: at 9 months	Cardiac death
ELUTES ⁵⁵	Non-polymer based	1 year	Clopidogrel 3 months	TLR: clinically driven	In stent; at 6 months	Any
DELIVER ^{53 54}	Non-polymer based paclitaxel	1 year	Clopidogrel 3 months	TVR	In segment including 5 mm proximal and distal; at 8 months	Cardiac death
PATENCY ⁵⁶	Non-polymer based paclitaxel	9 months	Clopidogrel 3 month	TLR	In stent? At 9 months	Any
TAXUS V ⁶¹	Slow release polymer based paclitaxel	9 months	NA	TVR: indication not reported	In stent? At 9 months	Cardiac death
TAXUS VI62	Moderate release	9 months	NA	TVR: indication not reported	In segment? At 9 months	Cardiac death

Figure 2.14: Summary of the results of different clinical trials with drug eluting stents [123].

2.9.1 Sirolimus

One of the goals of this project is to ascertain the performance of the antiproliferative drug Sirolimus (known also as Rapamycin) on SMC growth. Sirolimus (see fig 2.15) is an immunosuppressive drug that is usually employed in organ transplant to block the activation of T- and B-cells by inhibiting the response to (Interleukin -2) IL-2 [124]. Interleukin-2 is a pro-proliferation signal for T and B cells. Due to its antiproliferative effect Sirolimus is amongst the most frequently used drugs in DES.



Figure 2.15: Rapamycin structure.

Sirolimus is a macrolide antibiotic ("-mycin") that was first discovered as a product of the bacterium Streptomyces hygroscopicus in a soil sample from an island called Rapa Nui, better known as Easter Island. It has been shown in different works [125, 126] that Sirolimus can affect SMC migration and proliferation. They have demonstrated that Sirolimus can inhibit both *in vitro* and *in vivo* SMC proliferation [125] by stopping the cell cycle progression from G1 to S associated with reduction in cyclin-dependant kinase (CDK) activity. Sirolimus binds to cytosolic protein FK-binding-protein-12 (FKBP12). The the Sirolimus-FKBP12 complex inhibits the mammalian target of Rapamycin(mTOR) pathway through depression of PP2A (a protein phosphatase), resulting in inhibition of lymphocyte proliferation.

Further work [126] noticed that Sirolimus is effective on SMC

migration. Important factors regulating cell migration is Sirolimus concentration and exposure times. In vitro, using a modified Boyden chamber, it has been shown [28] that a Sirolimus dose as low as 1-2ng/ml has an inhibitory effect on rat and human SMC migration induced by PDGF-BB at least for 72 hours. Subsequently cell viability was so decreased that it was not possible to carry out an assessment of the cells. High Sirolimus concentrations for short periods of time had no effect on the rat aortic SMC (RASMC) migration, suggesting that cells need to stay in a Sirolimus-rich medium for at least 6 hours before they are migration sensitive. In vivo, it has been demonstrated [126] on Yorkshire albino pigs that high Sirolimus doses $(9mgkg^{-1}d^{-1}$ for 7 days) and prolonged exposure inhibit migration diminishing intima thickening by 98% but low doses $(2mgkg^{-1}d^{-1}$ for 2 days) and low exposure times have been shown to have no effect on alloimmune injury and a hyperplasia reduction of just 45%. The effect on SMC migration persists on SMC after two weeks of drug exposure suggesting that, in vivo, one week treatment may be sufficient to influence SMC migration for up to three weeks post intervention . Fig 2.16 shows the decrease of rat and human SMC migration after 48 hours due to the effect of different Sirolimus concentrations as a percentage of the control sample (0% Sirolimus concentration). Picture A shows also the effect on migration of FK506 compared to Sirolimus: FK506 has no visible effect on migration.

Both *in vivo* and *in vitro* histological analyses have shown no difference between Sirolimus treated and untreated cells as shown in fig 2.17. Nevertheless, not all cells can be considered sensitive to Sirolimus as findings have shown that from 10% to 20% of aortic SMC are Sirolimus resistant.

The mTOR transduces proliferative signals mediated through the signalling pathways of phosphatidylinositol-3-kinase (PI3K) and the protein kinase B (Akt), activating downstream protein kinases that are required for both ribosomal biosynthesis and translation of key mRNAs of proteins required for G1 to S phase traverse. By targeting mTOR, the immunosuppressant and antiproliferative agent Sirolimus (RAP) inhibits



Figure 2.16: (A) The effect on migration of FK506 compared to Sirolimus on rat SMC: FK506 has no visible effect on migration. B) The effect on migration of Sirolimus on human SMC [126].



Figure 2.17: SMCs treated with and without Sirolimus: there is no evidence of histological differences [127].

those signals required for cell cycle progression, growth, and proliferation [128], see fig 2.18.

Tests done by Marx *et al.* [125] have shown that Sirolimus as low as 1 ng/ml can inhibit RASMC proliferation. The proliferation inhibition, due to the presence of Sirolimus, persisted at least through 7 days (168 hours) of cell culture. In human aortic smooth muscle cell proliferation inhibition was decreased by 50% after 72 hours. In contrast, FK520



Figure 2.18: The RAP-sensitive signal transduction pathway. RAP can bind the FK506 binding protein-12 (FKBP- 12). The RAP-FKBP12 binding mTOR, inhibits its kinase activity. In turn mTOR inhibits the phosphorylation and activation of the downstream translational regulators, 4E-BP1/ PHAS-1 and p70s6k. These downstream effects decrease the translation of mRNA of may specific proteins involved in the G1 to S phase traverse [128].

produces a small increase of cell growth compared with the control. Cell viability for control, Sirolimus-treated, and FK520-treated cells was > 99%, see fig 2.19.

The antiproliferative and anti-migratory effects of Sirolimus have indicated its potential suitablity for use as a drug to coat stents in stent-angioplasty interventions.

Clinical trials of Sirolimus coated stents has shown its effectiveness in inhibiting neointimal formation in 30 patients with stable and unstable angina. In a clinical trial conducted by Sousa *et al.*[129] two different kinds of Sirolimus-coated stents were prepared: fast-release coated stents with drug release within 15 days and slow-release drug-coated stent with drug release greater than 28 days. Half of the patients received the fast-release formulation and half received the slow release formulation. Just three patients had > 15% intimal hyperplasia at 4 months and no patient had > 50% vessel narrowing. Edge restenosis or stent thrombosis



Figure 2.19: Inhibition induced by Sirolimus, as a function of time, in rat aortic smooth muscle cell (RASMC) proliferation compared with FK520. The behaviour for human aortic smooth muscle cells (HASMC) at 72 hours is shown in the inset. Control cells were treated with no drug compared to the RASM and HASMC for which 100 ng/mL of Sirolimus or 100 ng/mL FK520 were used [125].

was not present and at eight months follow-up reported no major event. For both fast-release and slow-release formulations similar results were obtained although preliminary results indicated а fast-release formulation can be more effective to curb excessive proliferation and migration of vascular SMC after coronary intervention. In an ultrasound-measured study [130] the intimal hyperplasia reduction in patients treated with Sirolimus-coated stents persisted at longer follow-up (6 months in 13 patients treated in Rotterdam and 12 months in 27 patients treated in Sao Paulo). These data suggest Sirolimus may provide protection against intimal hyperplasia after stent implantation in coronary arteries, see fig 2.20.

The FKBP12 related immunosuppressant drug FK506 (tacrolimus) also binds to FKBP12, but this complex inhibits the phosphatase calcineurin. FK506-FKBP12 has thus no antiproliferative or antimigratory activity in vascular SMC. Sirolimus-FKBP12 has no activity against calcineurin; rather, it inhibits a kinase called the target of Rapamycin



Figure 2.20: Antiproliferative, Anti-migratory, anti-hyperplasia effect of Sirolimus-Rapamycin: A) Sirolimus inhibits rat and human (inset) vascular SMC proliferation *in vitro* B) Sirolimus inhibits vascular SMC migration *in vitro*. C) Sirolimus inhibits intimal hyperplasia in a porcine coronary artery after angioplasty(PTCA model). D) Sirolimus-coated stent inhibits intimal hyperplasia, as demonstrated by intravascular ultrasound after 4 months of follow-up [130].

(TOR), which is a component in a pathway that regulates cell cycle progression. The finding that Sirolimus inhibits multiple fundamental regulators of cell cycle progression in vascular SMC suggested that it might have utility in the prevention of diseases linked to vascular SMC proliferation. Clinical trials [8] have shown that DES covered with Sirolimus are extremely promising in the treatment of in-stent restenosis in comparison with bare metal stents and in comparison with DES covered with other drugs. Hoffmann *et al.*[15] for instance found a mean reduction of intimal hyperplasia thickness up to 90% when a Sirolimus DES was used compared to a bare metal stent. Even with different DES covered with other drugs, Sirolimus has shown a better level of intimal
hyperplasia containment, see fig 2.21.



Figure 2.21: Intimal hyperplasia thickness for a DES covered with different drugs [15].

2.10 Summary

Bare metal stents have been able to restore immediate patency in coronary arteries representing an important improvement of CABG [42]. Despite the great effort made by the industry, in-stent restenosis still represents a strong limitation for the further development of this medical device intervention. Clinical trials have emphasised the importance of the role of vascular smooth muscle cells in the development and regulation of the restenotic process [13]. SMC migration from media to intima and their overproliferation creates the neo-intima hyperplasia at the basis of the re occlusion of the arterial lumen. It is also known from the literature that the mechanical environment plays a decisive role in SMC fate, regulating SMC proliferation and apoptosis [82, 83, 84, 86]. Several studies have found a correlation between the altered strain environment and the level of restenosis [16, 18]. In their paper Vernhet *et al.* [28] have quantified this

alteration in mechanical environment. A stent procedure resulted in an increase of mean strain up to 21% and a reduction of the cyclic strain amplitude to 1/1.5%. The final outcome is therefore a great increase in the mean strain and a sharp drop of the cyclic strain amplitude.

Since mechanical strain is a key factor in determining cell fate we hypothesise that these changes in the mechanical environment may drive the restenosis process and the proliferative and apoptotic response of SMC.

The aim of this project is to investigate these hypotheses. In particular this work focuses on the separation and analysis of the different components which form the pattern of cyclic strain: mean strain, amplitude and frequency. In many clinical trials high restenosis rates have often been associated to high stress and strain on the arterial wall after the expansion of a rigid stent. However, strain and stress were treated generically without investigating to which component of the strain (mean strain or amplitude) the uncontrolled SMC growth was related.

Frequency is another variable defining cyclic strain which could modulate SMC proliferation and apoptosis. A stent expansion modifies the haemodynamics of the blood leading to local perturbation of the pressure [131]. There is therefore the possibility of irregularity in the strain frequency in the stented area of the artery. A relationship has already been found between the strain frequency and SMC phenotypic alteration [132] which could be related to the contractile-synthetic phenotype change during in-stent restenosis. It is also important to investigate the role of frequency on SMC proliferation and apoptosis, even for the possible implications in patients with conditions of altered cyclic strain frequency such as bradycardia and tachycardia.

A clarification of the involvement of mechanical strain in the proliferation and apoptosis of vascular SMC is crucial for the design of a new generation of stents with no or limited restenosis. Different stent geometries have already been associated with different levels of strain and stress and to different restenosis rates. Knowledge of the influence of changes in strain after stenting on SMC proliferation and ultimately on the restenosis rate would give fundamental indications for the design of new stents.

In this work SMC were exposed to cyclic strain at different mean strains and amplitudes and the contribution of the main strain components (mean strain, amplitude and frequency) to SMC proliferation and apoptosis evaluated. This was achieved by means of the Flexercell[®], a laboratory straining machine able to stretch cells at different values without shear stress.

Clinical trials are long and very expensive. Their outcomes are often difficult to read and translate into new stent design guidelines. In the second part of this work, a preclinical testing device to quantitatively evaluate the proliferation and apoptosis of SMC was developed and used to confirm the results obtained by means of the Flexercell[®] machine under shear stress conditions. A perfused stented mock coronary artery inside a custom-made bioreactor was used to test the effect of bare metal stents on the level of proliferation and apoptosis of SMC in the stent region, upstream and downstream. Such a technique can enable early indications on stent design to be provided without the need for expensive clinical trials.

Drug eluting stents have also emerged as an effective treatment for atherosclerotic lesions with lower rates of restenosis compared to drug eluting stents [11]. The development of drug eluting stents is still ongoing through different clinical trials. The eluting drug, geometry and implant technique emerge as the key variables to reduce or eliminate restenosis. The pre-clinical testing technique developed in this work has provided a useful means to test stents with these different characteristics in order to improve stent design.

Chapter 3

Materials and Methods

3.1 Introduction

A Flexercell[®] straining machine was employed in this work to strain SMC at different amplitudes, mean strains and frequencies.

In order to have confidence in the results the reliability of the Flexercell[®] system is fundamental. Data from the scientific literature suggesting the unreliability of the factory calibration and differences between the desired strains (inputs) and the recorded strain outputs of the machine suggested the necessity to recalibrate it. A new calibration method was developed to obtain the desired strain values. The role of strain in the proliferation and apoptosis of SMC was assessed along with the roles of strain frequency, cyclic mean strain and amplitude.

Given that the ultimate goal of the project is to study the response of SMC to altered levels of strain induced by a stent, the Flexercell[®] system was inadequate for this purpose. The machine is limited to straining cells on a flat elastic membrane. This is a different situation from a stented-artery where SMC are arranged around the tubular shape of the coronary artery in a shear environment after the denudation of the endothelium following angioplasty balloon expansion. A novel bioreactor was therefore built to investigate the role of the strain pattern (high mean strain and low oscillation) on the proliferation and apoptosis

of SMC in different areas of a cell seeded stented mock coronary artery (MCA).

New generation stents are drug-eluting-stents (DES). They are covered with an antiproliferative drug to slow down SMC proliferation. The novel bioreactor was used to compare SMC proliferation in DES to BMS. The overall scheme of the work can be seen in fig 3.1.



Figure 3.1: Flow chart of the project.

3.2 Materials

All materials purchased were of the highest purity commercially available and were of cell culture standard where applicable. For a complete list of the materials used in this work, refer to Appendix A.

3.3 Tissue Culture

Cell culture activities were carried out in a clean and sterile Bio air 2000MAC laminar flow cabinet. Cells were visualized with an Olympus CK30 phase contrast microscope. All the tests using cells were carried out at least three times.

3.3.1 Culture of Bovine Aortic Smooth Muscle Cells (BASMC)

The main cell line used in this study were bovine aortic smooth muscle cells (BASMC), purchased from the Coriell Institute (New Jersey, USA), Cat No. AG08504. The cells were cultured in RPMI-1640 medium, supplemented with 10% foetal bovine serum (FBS), and 1% penicillin / streptomycin (P/S), and maintained in a 37°C humidified atmosphere of 5% CO_2 /95% air using a Hera water jacketed cell culture incubator. The cells were cultured between passage 5 and 15 in 175cm², 75cm², 25cm² flasks and were routinely fed every 3-4 days. Sub-culturing of cells was carried out at 80-90% confluency by trypsinisation. Phosphate-buffered saline (PBS) was used to wash cells before incubation at 37°C for 5 minutes with 2x Trypsin/Ethlyenediamine Tetracetic Acid (EDTA), diluted from the 10x stock solution. RPMI-1640 medium containing 10% bovine foetal serum (BFS), and 1% penicillin/streptomycin (P/S) was then added to the cells to stop the trypsinisation process.

3.3.2 Culture of Human Iliac Smooth Muscle Cells (HSMC)

HSMC were purchased from the Coriell Institute (New Jersey, USA), Cat No. AG11548. The cells were grown in PromoCell[®] smooth muscle cell growth medium 2, supplemented with 5% foetal calf serum (FCS), 0.5ng/ml of epidermal growth factor, 2ng/ml of basic fibroblast growth factor and 5ng/ml of insulin. Cells were maintained at 37°C, 5% *CO*₂

/95% air in a humidified atmosphere using a Hera water jacketed cell culture incubator. The cells were cultured between passages 2-7 in 75cm² and 25cm² flasks and were routinely fed every 2 days. Sub-culturing of the cells was carried out at 80-90% confluency by trypsinisation. Cells were washed in sterile phosphate-buffered saline (PBS) and incubated in 0.25x Trypsin/Ethlyenediamine Tetracetic Acid (EDTA), diluted from the 10x stock solution with PBS for 5 minutes at 37°C. An equal volume of growth medium 2 containing 5% foetal calf serum (FCS), grow factors and 1% penicillin / streptomycin (P/S) was then added to the cells to stop the trypsinisation process.

3.3.3 Cryogenic Cell Storage and Recovery of Cells

The long-term maintenance of BASMC was performed in a liquid nitrogen cryofreezer unit. Cells to be frozen were trypsinised from the flask, spun down in serum-containing media for 5 min at 1000 rpm, and the pellet was resuspended in 20% (v/v)FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v). 1 ml of suspension was then transferred to sterile cryovials and frozen at -80°C at a rate of 1°C/minute using a Nalgene cryo-freezing container. To recover cells from longterm storage, cells were thawed quickly at 37°C and resuspended in 5 ml of growth medium. A cell pellet was thus obtained by centrifugation at 1000 rpm for 5 minutes and it was resuspended in fresh medium and transferred to a culture flask. The following day cells were washed in PBS and fresh culture medium was added.

3.4 Cell activity Assays

3.4.1 6-well-plates cell counting

After washing cells in PBS, 6-well plates were washed twice in PBS and cells treated with 2x Trypsin / Ethlyenediamine Tetracetic Acid (EDTA) solution in PBS for 5 minutes at 37°C. The number of cells in at least three

different wells were counted by means of a Sigma Brightline Haemacytometer.

3.4.2 FACS analysis

Proliferation

Carboxyfluorescein diacetate succinimidyl esters (CFDA SE) are colourless and non fluorescent reagents that can passively diffuse into cells. When their acetate groups are cleaved by intracellular esterases they become highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines bringing the formation of fluorescent conjugates which are well-retained and can be fixed with aldehyde fixatives. Unconjugated reagent and by-products diffuse passively to the extracellular medium and can be washed away. A dye-protein adducts form in labelled cells which is kept by the cells during development, meiosis, and in vivo tracing. After cell division daughter cells inherit the label. Transfer to adjacent cells is not possible. CFDA SE have been detected in lymphocytes up to eight weeks after injection into mice, and in viable hepatocytes even 20 days after intrahepatic transplantation by fluorescence microscopy [133], see fig 3.2.

For proliferation analysis, BASMC were washed once with PBS and 1 ml of 5 M carboxy-fluorescein diacetate succinimidyl ester (CFDA, prepared in PBS) was added to each well for 5 minutes at 37°C. Following incubation, CFDA was replaced with fresh media. After applying strain to the cells they were harvested by trypsinisation/centrifugation and washed twice with 1 ml ice-cold PBS (containing 0.1% BSA). Cells were then placed on ice pending flow cytometry analysis. The concentration of the dye in the cells, which is inversely proportional to the rate of proliferation, was measured by flow cytometry (Becton Dickinson FACSCaliber) at an excitation peak of 492 nm and emission peak of 517 nm.



Figure 3.2: Read-out of a flowcytometry machine(FACS) when culturing cells using CFDA SE. The shift between the two curve represents the proliferation of the treated cells (dash curve) compared to the control (continuous curve)

Apoptosis

Phosphatidylserine (PS) is a phospholipid usually kept on the cytosolic side of the cell membrane. During apoptosis the cell membrane loses its phospholipid asymmetry and phosphatidylserines (PS) transit from the inner leaflet of the phospholipid bilayer to the cell surface providing a simple mechanism for discerning cells undergoing apoptosis.

Annexin V (Mr 36 kDa) is a member of the annexin family of calciumdependent phospholipid binding proteins. It shows high affinity for PS-containing phospholipid bilayers. Annexin V can detect PS groups just when they are exposed on the extracellular face of a cell membrane binding with an affinity of approximately 7 nM providing a convenient tool for detection of apoptotic cells. Changes in cell membrane phospholipid asymmetry can be visualized using a fluorochrome conjugate of annexin V, see fig. 3.3.



Figure 3.3: Read-out of a flowcytometry machine (FACS) when culturing cells using Annexin V for measuring apoptosis. Depending on the quadrant in which a cell falls it can be viable, early apoptotic, late apoptic or necrotic.

For apoptosis analysis, cells were washed once in PBS, harvested by trypsinisation, pelleted by centrifugation (1000 rpm for 5 min), washed in 1 ml ice-cold PBS (containing 0.1% BSA), and resuspended by gentle pipetting. Cells were again pelleted by centrifugation and re-suspended in 200 l of 1x Annexin-binding buffer. Propidium Iodide (0.4 l from 100 g/ml working solution) and $1\mu l$ AlexaFluor 488 Annexin V were added to the cell suspension and incubated at room temperature for 15 min. Cells were then placed on ice pending flow cytometry analysis. Apoptotic cells were then counted by flow cytometry (Becton Dickinson FACSCaliber), measuring the fluorescence emission at 530 nm and > 575 nm.

3.4.3 Cell counting in the mock coronary artery

After washing in PBS, mock coronary arteries were cut into three sections of the same length. One section was taken upstream of the flow, one section at the stent region and another section downstream. The three sections were dipped inside three 1X PBS trypsin solutions of 200 μl . The number of cells upstream, downstream and in the stent region were counted by means of a haematocytometer.

3.4.4 Immunocytochemistry

Proliferation

Mock coronary arteries were washed in PBS and fixed in 3% formaldehyde for 15 minutes. Their membranes were made permeable with a 0.2% Triton X-100 PBS solution applied for 5 minutes. Cell nuclei stained with 1/2000dilution DAPI solution (4', were а 6-diamidino-2-phenylindole) for 3 minutes. Then, mock coronary arteries were cut in three sections of equal length. One section upstream of the flow, one section in the stent region and another section downstream. The three sections were longitudinally opened with a scalpel and flattened onto microscope slides for analysis with a fluorescence microscope (Olympus DP-50). Digital pictures of the different regions were taken and the number of cells counted in a constant square region of 300X300 pixels in the upstream, stented and downstream regions and the cell number evaluated.

Apoptosis

Cells were washed in PBS and incubated in Annexin V $(30\mu l/ml)$ and propidium iodide for 30 minutes at room temperature. Cells were washed in PBS and visualised using the Olympus DP-50 fluorescent microscope (excitation 460-490 nm, emission 515-565 nm). Each Mock coronary artery was then washed in PBS and incubated in a solution of $20\mu M$ of DAPI with $5\mu l$ of Annexin V. Again the mock vessels were cut into three sections which were longitudinally opened with a scalpel and flattened on microscope slides for analysis with a fluorescence microscope (Olympus DP-50). Digital pictures of the different regions were taken using the annexin V green 488 nm and the DAPI blue 500 nm fluorescence filters and the images overlapped. The number of apoptotic cells out of the overall population was evaluated by calculating the percentage of cells with a green dye out of the total population with the DAPI blue staining.

α -actin

BASMC were tested for α -actin. Cells were washed twice in PBS and fixed with 3% Formaldehyde for 15 minutes at room temperature. Cells were washed again twice in PBS and treated in 0.2% Triton X-100 solution of PBS for 5 minutes at room temperature. After washing twice in PBS, a 5% milk solution in PBS was used for 30 minutes as a blocking solution for cell membranes. Cells were then incubated with α -actin in 5% blocking solution for 1 hour and later washed again twice in PBS. Cells were then incubated with annexin V in 5% blocking solution for half hour and then covered with tin foil. BASMC were subsequently analysed under a fluorescent microscope.

$GSK-3\beta$

Cells were washed in PBS and fixed with 0.3% formaldehyde for 15 min at room temperature. Cells were washed twice in PBS and permeabilised with 0.025% Triton-X-PBS for 5 min. Cells were washed twice in PBS. Cells were blocked in 5% BSA- PBS-1% Tween for 1 hr at room temperature and incubated overnight with a phospho(ser9) -GSK-3 β primary antibody (1/50 dilution in 5% BSA- PBS-1% Tween, Cell Signal) at 4°C. Cells were washed twice for 15 min in PBS-1%Tween and incubated in an anti-rabbit Alexafluor 488 secondary antibody (1/400 dilution in 5% BSA- PBS-1% Tween, Invitrogen) for 30 minutes in the dark. Following two more 15 min washes in PBS, cells were visualised using the Olympus DP-50 fluorescent microscope (excitation 460-490 nm, emission 515-565 nm).

Statistical analysis

Results were expressed as mean \pm SEM. Comparison between control versus treated cells were made with an unpaired Student's t-test. Statistical analysis and significance was established at p \leq 0.05.

3.5 The Flexercell[®] Tension Plus System

One of the most commonly used systems to apply controlled mechanical stretch to cells to determine its influence is the FX-4000TM system (Flexercell[®] Tension Plus System, Flexcell Corp, McKeesport, PA, USA). This biological laboratory machine enables controlled stretching of a cultured monolayer of cells and it has been used extensively to understand the effect of cyclic mechanical loading on smooth muscle cells and endothelial cells with particular emphasis on simulating the conditions that these cells experience in diseased vessels [134]. The machine can be used to investigate cellular responses to uniaxial and biaxial stretching of various magnitudes, waveforms and frequencies.

3.5.1 Flexercell[®] Set-up

Cells are cultured onto compliant silicone membranes at the bottom of the specially designed 6-well-plates. This model gives an in situ mechanical representation of the strain SMC experience *in vivo*. The Flexercell[®] FX-4000TM is composed of a computer (and related software) and two electronic control units to regulate in real time the pressure inside two Flexercell[®] baseplates. The baseplates, each consisting of four 6-well-plates, are maintained at 37°C inside a 5%-*CO*₂ incubator for cell culture. The FX-4000TM system also includes an external vacuum pump. The pump creates a vacuum deforming the membranes around cylindrical Delrin[®] loading posts to generate equibiaxial strains on the membrane, see fig 3.4.

The controller regulates the pressure created by the vacuum pump



Figure 3.4: Equibiaxial deformation of the Flexcell membranes over the loading posts [135].

inside the Flexercell[®] plate to create different pressure waveforms. Different stretch waveforms can be applied: constant, triangular, quadratic, sinusoidal and heartbeat. For each waveform the membrane stretch is calculated by the Flexcell International software on the basis of the pressure-deformation equation set by the factory calibration. According to the manual the Flexercell[®] machine can achieve maximum biaxial stretch of 20%. The operator can view the programmed stretch of the membrane via the software as displayed in real time on the computer screen. However, during each test protocol no direct feedback of the actual membrane stretch is provided to the controller. Data from the scientific literature and differences between inputs and outputs of the machine suggested the necessity to recalibrate it.

3.5.2 Flexercell[®] calibration and optimization

To ensure the reliability of the strain system independently of the different frequencies, mean strains and amplitude, it was necessary to quantify the strain field of the Flexercell[®] 6-well-plates for different waveforms at various frequencies, and to compare the results with the Flexercell[®] software inputs and outputs. In cases where the output did not match the desired strain level, a method to calibrate the machine in-house was applied using new pressure-stretch equations. This new calibration method enabled the Flexercell[®] machine to be used to set-up cell experiments more accurately. In Appendix B the novel calibration technique is described and all the results are presented.

3.5.3 Frequency

Using the newly calibrated Flexercell[®], tests to establish the influence of cyclic strain on SMC growth (proliferation and apoptosis) were carried out. At the same time the role of cyclic strain frequency in SMC proliferation and apoptosis was investigated. Bovine aortic smooth muscle cells (BASMC) were used in these tests.

BASMC were cultured and trypsinised using the techniques already described and seeded on Flexercell[®] 6-well-plates at a concentration of 1 X 10⁵ cells per well. Cells were left to adhere to the silicon elastic membrane in full (10% serum) medium overnight. The medium was subsequently changed to low serum (0.2%) medium for 48 hours quiescence to allow all cell cycles to be reset to the G_0 phase. In case of proliferation analysis a Vybrant CFDA-SE was used to mark smooth muscle cells with a florescence dye. Cells were counted by means of a haemocytometer and the 6-well-plates were placed on two different base plates of the straining machine.

Using the newly calibrated Flexercell[®], a heartbeat waveform at a physiological strain of 5% and frequencies of 1 Hz and 0.5 Hz were applied for the two different baseplates. Every 24, 48 and 96 hours a 6-well-plate was removed from the straining machine and left unstrained

in the same incubator to maintain the same environmental conditions of the straining cells. After 96 hours the level of proliferation or apoptosis was measured by means of a FACS (Fluorescence Activated Cell Sorting) flowcytometric machine. In all the 6-well-plates the level of proliferation was also assessed by cell counting. Results were compared to cells cultured on static 6-well-plates (0Hz). In the case of analysis of apoptosis, cells were marked with a Vybrant Alexa Fluor 488 Annexin V and propidium iodide prior to the analysis. A proliferation test was repeated with confluent BASMC and cells counted with a haemocytometer.

3.5.4 Mean strain and strain amplitude

The expansion of a comparably rigid stent inside a coronary artery strains and thus stiffens the arterial wall. A direct consequence of the stent expansion is the increase in mean strain and a decrease in the cyclic strain amplitude. The mechanical environment of SMC changes from one of low mean strain/high strain amplitude to a condition of high mean strain/low strain amplitude.

The difference in SMC proliferation and apoptosis when cells were strained mimicking the change in strain pattern before and after stenting was measured. In order to better understand the restenotic process it is important to distinguish and separate the effect of the mean strain from the effect of cyclic strain amplitude on SMC proliferation and apoptosis.

BASMC were cultured, induced to quiescence and dyed with CFDA-SE using the techniques previously described. Cells were trypsinised and seeded on 6-well-plates. Two sets of different tests were carried out where BASMC were strained with a calibrated Flexercell Tension Plus FX-4000T system using the following patterns:

- 1. Different mean strains (5% and 10%) and the same sinusoidal cyclic amplitude (2%)
- 2. Strain at a physiological amplitude of 6% with a mean strain of 5% $(5\% \pm 3\%)$ and strain at a pathological pattern of high mean strain

(10%) and low amplitude (2%) mimicking the mechanical change after stent expansion.

In both cases, after 48 hours of straining BASMC were washed twice in PBS and treated with 2x Trypsin / Ethlyenediamine Tetracetic Acid (EDTA) solution in PBS for 5 minutes at 37°C. Vybrant CFDA-SE dye and Alexa Fluor 488 Annexin V and propidium iodide were used to determine cell proliferation and apoptosis, respectively, using FACS analysis. Cell counts were also performed using a hemocytometer. The same protocol was repeated with human iliac artery SMC to confirm the results and cells counted with a hemocytometer.

3.6 Bioreactor

The Flexercell[®] machine is an important instrument to measure cell reaction to cyclic stretch. However it does not allow cells to be cultured in environment with both strain and shear stress conditions. an Furthermore a cyclic strained monodimensional layer of cells is a coarse approximation of the complex geometrical and biological environment of a stented coronary artery. For this reason a novel bioreactor was developed, capable of mimicking the strain and the flow SMC experience *in vivo* before and after a stent procedure. A culture chamber was designed for this goal to fit into a CellMax[®] bioreactor tubing system (Spectrum Laboratories, Rancho Dominguez, CA), see fig 3.5. The CellMax[®] capillary system (fig 3.5 and fig 3.6) is a commercially available bioreactor suitable for long-term cell culture. It simulates in vitro the heartbeat pressure waveform that cells experience *in vivo* by means of a pulsatile pump.

The CellMax[®] serial pump can create a medium flow waveform similar to the stroke of the heart. Two pins push against the flexible and thin silicon tube simulating the heart stroke. The pump works by means of an electronic controller that allows 8 different speeds to be set. Different speeds correspond to different flow rates and stroke



Figure 3.5: The CellMax[®] capillary system: the original cartridge was removed and substituted for a custom made culture chamber [136].



Figure 3.6: Medium Directional Flow(Oxygenation Tubing not shown) [136].

frequencies. There are also different pulsatile pin lengths which correspond to different maximum pressure intensities. Depending on the length of the pins it is possible to obtain different flow rates for different speeds as shown in fig 3.7.



Figure 3.7: Flow rate / Speed / pin setting for the pulsatile pump. These values correspond to the original CellMax cartridge [136].

The tubing system carries the culture medium from a medium reservoir to the culture original cartridge inlet and back from the culture cartridge outlet to the medium reservoir. A series of loops of the tubing inside the CellMax[®] module system gives time for the medium to oxygenate before reaching the cartridge as a result of the special silicon material of the tube. The thin silicon wall of the tubing allows positive gas-exchange between the external environment and the inner medium along the long loops of tubing around the CellMax[®] module. The medium flow passes through two valves positioned before and after the cartridge preventing backflow: it acts like a real heart valve generating fluid dynamics similar to those *in vivo*. The original cartridge is the core of the CellMax[®] system: it consists of 50 capillary tubes on which cells can be cultured under shear stress conditions both intraluminally and extraluminally. The CellMax[®] pumping system can have four cartridges running simultaneously by placing them with the related tubing systems in four different compartments. Parallel tests can therefore be carried out using one cartridge as a control.

For the goals of this project such a system presents some strong

limitations:

- 1. The rigidity of the capillary tube inside the cartridge does not mimic the strains SMC are exposed to *in vivo*.
- 2. The capillary tubes are much smaller than a coronary artery.
- 3. It is not possible to remove the capillaries to enable analysis of the cells in the tube.
- 4. It is not possible to insert a coronary stent and therefore to determine the response of SMC to changes in the mechanical environment due to the presence of a stent.

For these reasons a novel culture chamber for the bioreactor was designed to fit inside the CellMax[®] capillary system instead of the original cartridge.

3.6.1 The novel vascular bioreactor for stenting

In fig 3.8 the overall scheme describing how the novel bioreactor functions is shown. A serial pump system moves the medium from a reservoir to the culture chamber. The serial pump along with the two one-way valves and the compliance chamber contribute to the creation of a heartbeat pressure waveform that propagates through the tubing system straining the MCA in the culture chamber. The new culture chamber consists of a biocompatible Plexiglas[®] open box with an inlet and an outlet for medium perfusion of the MCA, see fig 3.9 and for more detail appendix C.

A transparent cover closes the chamber on the top and it can be fixed in place with a series of screws. Sealing is guaranteed by a deformable elastomeric gasket (RTV 102, Radionics) that is placed inside a groove all around the chamber. Two additional inlets on the lateral side are used for extraluminal medium replacement and for an air-exchange hydrophobic filter. The chamber was designed to hold a mock artery with mechanical



Figure 3.8: Scheme for the assembly of the bioreactor.



Figure 3.9: The culture chamber without the cover.

properties similar to the mechanical properties of a real coronary artery. When the medium passes through the culture chamber, it generates cyclic strains in the mock coronary artery that are near the physiological amplitude of 6% [28]. Specific tests were carried out to measure the mock artery strain pattern when the CellMax[®] serial pump pulsatile rate was close to the physiological frequency. The system was set-up in working conditions and the diametral distension of the MCA was recorded by means of a video-extensometer positioned above the culture chamber as was done for the Flexercell[®] calibration. Static pressure was modified by changing the height of the medium resevoir compared to the culture chamber. Static and dynamic pressure level were also set by tuning the downstream area of the tube with a precision clamp until the strain and pressure pattern were near the physiological level. The MCA strain during the time was regulated to mirror the heartbeat pressure waveform imposed by the serial pump as shown in fig 3.10.



Figure 3.10: Recorded cyclic strain of the mock coronary artery

SMC can be cultured on the mock artery intraluminally and strained with the same pattern they experience *in vivo*. Furthermore the bioreactor allows the placement of a stent inside the MCA such that the effect of the strain on SMC in physiological and pathological conditions can be compared, see fig 3.11. The values of strain in a stented artery were measured by means of the videoextensometer upstream, in the stent region and downstream. Four different culture chambers can be used simultaneously and the results of the level of proliferation and apoptosis of SMC in parallel studies can be compared. In fig 3.11 a stent has been placed inside the mock artery to show the functioning of the system as a pre-clinical testing device for stents.



Figure 3.11: Stent implanted inside a mock coronary artery

Bare metal and drug eluting stents were provided by our industrial partner in this project, namely Medtronic AVE. The effect on SMC proliferation of specific sirolimus-coated stents was determined using the new bioreactor as a preclinical testing device for the evaluation of the Endeavour stent (DES Medtronic Driver stent) on the restenotic process.

3.6.2 Mock coronary artery construction

The mock coronary arteries were manufactured from transparent Sylgard[®] 184 with 3 mm inner diameter, 0.4 mm constant wall thickness and with a length of 4 mm. Sylgard[®] is a silicon elastomer that can be easily created in a laboratory by mixing a viscous base with a liquid curing agent. The base/curing agent mixing ratio determines the mechanical properties of the material (see fig 3.12): the increase of the base/curing agent ratio decreased the Young's modulus of the material.

A detailed description of the process to create mock coronary arteries is described in appendix D. Briefly, by altering the ratio of base to crosslinking agent it is possible to create Sylgard[®] mock coronary arteries



Figure 3.12: Elastic moduli determined by tensile test for PDMS substrata. From company datasheet [137].

with mechanical properties close to the mechanical properties of real coronary arteries. Previous work [138] showed that a base/cure agent ratio of 16:1 was the closest to the properties of porcine carotid arteries in the circumferential direction, see fig 3.13.

Mock coronary arteries were manufactured using a custom made device consisting of a mandrel of 3 mm of diameter surrounded coaxially by a plastic tube with internal diameter of 3.8 mm. This system was inserted into a special rig for Sylgard[®] to be sucked between the coaxial mandrel and elastic tube. The external plastic tube fit perfectly inside an other external rig that prevented it from further deformation in the oven and the mandrel from moving, possibly affecting the coaxiality. The whole system was subsequently placed in a 120°C oven overnight to cure the elastomer. Once the elastomer was cured the plastic tube was cut and peeled off. The cured Sylgard[®] was then detached from the mandrel by injecting acetone with a needle between the mandrel and the elastomer.

SMC do not adhere naturally to the Sylgard[®] surface due to its hydrophobic nature. To convert the Sylgard[®] surface from hydrophobic to hydrophilic the tube was dipped inside a 70% sulphuric acid solution several times for a total time of 60 seconds. In case of tests requiring the



Figure 3.13: Comparison between the mechanical properties of porcine coronary arteries and mock coronary arteries with different base/cure agent ratios [138].

use of several MCA for comparison of different results, all MCA were treated simultaneously, for the same amount of time with the same sulphuric acid solution to be sure of obtaining the treatment and therefore comparable results. The Sylgard[®] tube was subsequently sterilised in an autoclave. More details about MCA preparation can be found in Appendix D.

3.6.3 Evaluation of SMC adhesion on flat Sylgard[®] sheets and tubes

To test the effect of the sulphuric acid treatment, tests were carried out on flat Sylgard[®] sheets to test cell attachment. Cells were cultured to 80/90% confluence and seeded on autoclaved flat Sylgard[®] layers treated with/without sulphuric acid and with/without fibronectin. After 72 hours of culture the cells were fixed in 3% formaldehyde and their membranes made permeable with 0.2% Triton X-100 to let the nuclei be stained with DAPI (4', 6-diamidino-2-phenylindole). The amount of

adherent cells was assessed. A second series of tests were carried out to evaluate the strength of the attachment between the cells and Sylgard[®] when a physiological shear stress was applied. Cells were seeded on the elastomer treated with sulphuric acid and fibronectin. The Sylgard[®] was then subjected to a physiological shear stress of 10 dynes/cm² for 24 hours and the DAPI staining results compared to a control layer that had not been subjected to shear stress.

3.6.4 Seeding of BASMC on mock coronary arteries

Mock coronary arteries (MCAs) were prepared with the use of the already described rig. Mock arteries were cut to a length of 5 cm to fit inside the novel bioreactor. Special female luer slip connectors (Value Plastic [®], Fort Collins, CO) were attached to the artery ends to connect with the related male luer connectors on the bioreactor culture chamber, see fig 3.11. Mock coronary arteries were treated in 70% sulphuric acid, washed in distilled water and sterilised in an autoclave at 120°C. The MCAs were then moved to normal 15ml tubes with a sterile solution of fibronectin in PBS $(8\mu g/ml)$ by means of sterile tweezers. The 15ml tubes were inserted into a rotating device for 3 hours to obtain a uniform deposition of fibronectin on the MCAs' surfaces. The friction between the female luer locks and the 15ml tube guaranteed a 360 degree rotation of the mock coronary arteries and thus, a uniform distribution of fibronectin. BASMC were trypsinised from a normal flask and seeded on the treated mock coronary arteries overnight inside a rotating 15ml tubes. BASMC were left for 48 hours in quiescence to strengthen their attachment with the underlying membrane. In case of the use of several MCA for comparison of different results, all MCA were treated simultaneously, for the same amount of time with the same sulphuric acid/fibronectin solutions and they were seeded with the same BASMC concentrations in order to obtain comparable results.

3.6.5 Mock coronary artery stenting

Some MCAs were stented to study the proliferation and apoptosis of BASMC when mechanical conditions were altered by this medical device. Driver BMS and DES were provided by Medtronic AVE. Driver stents are cobalt alloy stents with an internal diameter of 3 mm. They have a nominal and burst pressure of 9 and 16 atm, respectively. The elution profile and the description of the drug coating of DES was provided by Medtronic and can be seen in appendix E.

BMS were first crimped on an angioplasty balloon catheter of 9 mm length. The stents were sterilised in an autoclave. Subsequently they were deployed inside the mock coronary arteries inside a sterile controlled laminar flow cabinet by means of a Basix 25 anioplasty inflation syringe (Merit Medical Systems, South Jordan, Utah) expanding the angioplasty balloon.

DES were provided by Medtronic already in sterile conditions but not in a crimped state. The DES were crimped inside the laminar flow cabinet before being deployed. DES were inserted first into a sterile mock coronary artery on the angioplasty balloon and crimped on it using the mock artery as a barrier to avoid direct contact between the non-sterile crimping tool and the stent. DES were then deployed into the mock coronary arteries as previously with BMS.

3.6.6 Preparation and assembly of the Bioreactor

Ethanol first and sterile PBS later were run for 48 hours in the tubing system of the bioreactor in order to sterilise the device. The culture chamber was sterilised in parts in an autoclave and connected to the tubing system under a controlled laminar airflow cabinet. Under a laminar flow-controlled cabinet the culture chamber was connected to the rest of the tubing system with luer locks (Value Plastic[®], Fort Collins, CO) as it is possible to see in fig 3.14. Every time the female and male luer locks were joined inside the laminar they were first rubbed with an ethanol soaked tissue to avoid contamination. All the connectors are

resistant to the autoclave temperature and made of biocompatible clear polycarbonate. The medium reservoir was filled with 100ml of RPMI 1640 media supplemented with 10% foetal bovine serum and antibiotics (primocin[®] $100\mu g/ml$, Amaxa, MD, USA).





Mock coronary arteries previously seeded with BASMC and stented were placed into the culture chamber by means of sterile tweezers. Care was taken to eliminate air bubbles from the MCA and the tubing system to avoid pressure drops inside it. Luer slips mounted inside the culture chamber enabled easy attachment and detachment of MCAs. The extraluminal space of the bioreactor was filled with PBS with 1% antibiotic. At this stage the culture chamber was sealed with the cover by means of a series of screws. A hydrophobic air filter was connected to one of the two lateral connectors for air exchange.

These operations were repeated as many times as the number of mock coronary arteries to be analysed. The CellMax[®] serial pump system has four compartments for four culture chambers, see fig 3.14. The overall system was connected to the serial pump. The pump speed was kept at 1 for 24 hours and subsequently changed to 2 to let BASMC adapt to the

new shear stress conditions. At least two bioreactor units were used, one for the stented artery and one for a non-stented artery or DES artery.

3.6.7 Cell harvest

After 7 days in culture inside the bioreactor the proliferation of BASMC was evaluated. The bioreactor culture chamber was opened and the mock coronary artery removed by means of a tweezers. The medium reservoir was analysed to confirm that there was no fungi or bacteria contamination. The presence of cells in the medium was also evaluated to exclude detachment from the coronary artery. The level of proliferation and apoptosis was assessed upstream, in the stented region and downstream.

3.7 SMC response to Sirolimus in static conditions

Before analysing the effect of sirolimus-covered DES on the proliferation and apoptosis of SMC inside a perfused mock coronary artery the effect of the antiproliferative drug was tested on unstrained cells for different Sirolimus concentrations in the culture medium.

3.7.1 Cell count of SMC for different DMSO concentrations

To test the effect of the Sirolimus on BASMC, Dimethyl-sulfoxide (DMSO) was used to dilute the drug in the culture medium. However the concentration of DMSO in the medium affects cell proliferation. A simple test was carried out to evaluate the maximum level of DMSO cells were able to withstand inside a 6-well-plate without dying or affecting the proliferation process. Different quantities of DMSO were added to 2 ml

wells of culture medium and the number of cells was counted after 48 hours by means of a haemocytometer.

3.7.2 Cell count and FACS analysis of SMC for different Sirolimus doses

To evaluate the Sirolimus activity a first set of tests was carried out to assess the dose-response relation of this drug on SMC culture plates. The drug was tested at different concentrations on BASMC. Sirolimus was kept in the freezer at -20° C in a stock solution of 1mg per ml of DMSO. BASMC were cultured on T-75 and T-175 flask in full serum medium using passages between 10 and 15. BASMC were trypsinised and seeded on 6-well-plates at a concentration of 50X10⁴ cells/well and left to adhere overnight. Full serum medium was substituted for 48 hours with low serum (0.2%) medium for cell quiescence to ensure that cell cycles were synchronized and brought to the G_0 phase. Full serum medium was subsequently restored and a Vybrant CFDA-SE proliferation dye was applied to the cells. Cells were left to rest overnight. A sample of the stock solution of Sirolimus was thawed and diluted several times to obtain different levels of Sirolimus/DMSO concentrations: 100ng/ml, 50ng/ml, 10ng/ml, 5ng/ml, 1ng/ml, and 0.1 ng/ml. $3\mu l$ of DMSO with/without the drug and at the different concentrations, was added to 3 mls of full serum medium of cultured BASMC. Cells were left in the $37^{\circ}C$ 5% CO_2 for 72 or 120 hours and later FACS analysis and cell counting were performed to assess cell proliferation.

3.7.3 Drug Eluting Stents

The previously described novel bioreactor was used as a preclinical testing device to compare DES and bare metal stents in terms of proliferation of SMC in the upstream, stented and downstream area of the mock coronary artery. Two bioreactors were run in parallel in the equal conditions of pressure and strain and placed in the same incubator using the unique pumping system of the CellMax[®] bioreactor. After 7 days in culture the two mock coronary arteries were removed from the bioreactor and treated as previously described usng SMC staining with DAPI and Annexin V for the analysis of their proliferative and apoptotic behaviour, respectively.

Chapter 4

Results 1: SMC growth in response to strain

4.1 Introduction

The influence of mechanical strain and stress on cell behaviour is well known, i.e. mechanotransduction. Vascular smooth muscle cell (SMC) dysfunction plays a critical role in many cardiovascular diseases such as arteriosclerosis, atherosclerosis and aneurysms. In particular, uncontrolled proliferation of SMC is crucial to the progression of in-stent In this work, we hypothesised that the change in the restenosis. mechanical environment after a stent procedure could contribute directly to the restenotic process and, in particular, alterations in the strain pattern experienced by SMC could influence the proliferative and apoptotic capacity of vascular SMC. Although many studies have tried to elucidate the behaviour of SMC under varying strain conditions, no clear indication regarding the role of strain on SMC proliferation and apoptosis has been revealed. In particular, to the author's knowledge there has never been any specific study to investigate the restenotic SMC proliferative and apoptotic behaviour in relation to changes in the mechanical environment after stent expansion, which leads to an increase of the mean strain and a drop in cyclic strain amplitude. In this chapter, the results of tests on SMC exposed to cyclic strain at different mean strains and amplitudes are presented. SMC were cultured in a non-perfused environment and strained using the Flexercell[®]strain rig on special 6-well- elastomer plates. To obtain precise strains for different frequencies, mean strain and amplitude the Flexercell[®] was calibrated using a novel technique, see appendix B.

4.2 Frequency

The calibration results were employed to obtain precise heartbeat waveforms to strain BASMC for 24, 48 and 96 hours at three different frequencies (0Hz, 1Hz and 0.5Hz) with an amplitude of 5%. The strain was recorded with a videoextensometer and confirmes a heartbeat waveform with an amplitude of 5% and a frequency of 0.5Hz, see fig 4.1. Similar strains were confirmed for the other frequencies.



Figure 4.1: Measurement of the heartbeat waveform on the 6-well-plate membranes during Flexercell[®] calibration.

Cell proliferation was evaluated by means of cell counting and FACS Apoptosis was evaluated by means of FACS analysis. analysis. Sub-confluent BASMC were strained for 24, 48 and 96 hours before cell counting revealed a temporal decrease in cell number which was proportional to the duration of strain and to the applied frequency. Compared to the static control, SMC number decreased by 23% and 55%, respectively, for the 1Hz and 0.5Hz cases after 96 hours of applied strain, see fig 4.2. These results were further confirmed by FACS analysis. In the fig 4.3a, 4.3b and 4.3c the position of every curve along the abscissa corresponds to the level of proliferation of the cells. The more the curve is positioned at lower abscissa values (to the left) the greater the level of proliferation of the correspondent group of cells. After 96 hours a greater level of proliferation was recorded for cells in static culture. А significantly higher proliferative level was recorded for cells strained at the physiological frequency of 1Hz compared to 0.5Hz. Cell counting for different times of applied strain was repeated for BASMC in confluent conditions. In this case, BASMC decreased by 27% and 43%, respectively, for the 1Hz and 0.5Hz cases after 96 hours of applied strain. Cyclic strain decreased SMC proliferation in a temporal manner. Moreover, when cells were exposed to strain at the lower frequency, SMC proliferation was inhibited to a greater extent, see fig 4.2 and fig 4.3.

In parallel cultures using FACS analysis, the level of SMC apoptosis following exposure to cyclic strain was increased in a temporal manner. Fig 4.5 shows the percentage of apoptotic cells for different straining times and frequencies. After 96 hours of strain, there was 61% more apoptotic cells at 0.5Hz compared to 1Hz. Compared to the static control, the level of apoptosis after 96 hours increased by 226% and 425% when strained at 1Hz and 0.5Hz, respectively. Therefore, cyclic strain has a pro-apoptotic effect that is time dependent. When cells were exposed to the higher frequency, the pro-apoptotic effect of strain on BASMC was lower. Cyclic strain therefore has a temporal antiproliferative and pro-apoptotic effect on SMC *in vitro*. When cells are subjected to the physiological frequency conditions, cell growth was increased and

apoptotic activity reduced when compared to cells strained at the non physiological frequency.

4.3 Mean strain

The effect of a change in mean cyclic strain on the proliferation and apoptosis of SMC was evaluated by cell counting after BASMC were exposed to cyclic strain for 48 hours with the same amplitude (2%) but at different mean strains (5% and 10%). There was a 24% and 23% decrease in BASMC growth following exposure to different mean strains when compared to static controls with no significant difference evident between the two groups of strained cells, see fig 4.6. The data also confirmed an increase in proliferation (+32%) when SMC were not strained when compared to strain and showed that mean cyclic strain had no significant influence on SMC proliferation. The FACS analysis confirmed the same level of proliferation under varying mean strains of similar amplitude. The anti-proliferative effect of strain was represented by the rightward shift between the red curve, representing the static cell culture, and the other two curves, see fig 4.7.

Similar results were obtained for the same test when human iliac SMC were employed. SMC number decreased by 30% and 28%, respectively when compared to the static control with no significant difference observed between proliferation of the strained cells following exposure to varying mean strains of similar amplitude, see fig 4.8.

Complementary results were found using FACS analysis when apoptosis was evaluated for the same test. There was no significant difference in the percentage of apoptotic cells between the two groups of strained cells as it is shown in figure 4.9. A pro-apoptotic effect of strain was observed of 47% and 54%, for 5% and 10% mean strain, respectively when compared to the static control.

4.4 Amplitude

When BASMC were exposed to low mean strain (5%) and high amplitude (6%) versus high mean strain (11%) and low amplitude (2%) the proliferative and apoptotic capacity of BASMC was highly dependent on the amplitude of the cyclic strain. A low strain amplitude (2% vs 6%) promoted 22% increase in cell proliferation (p < 0.05). A decrease in cell number was found even at the lower amplitude of 2% compared to the static control but it was not significant, see fig 4.10.

These results were further confirmed in a parallel test by FACS analysis, as shown in fig 4.11. The shift between the green and the violet curve represents the difference in proliferation among the two groups of cells treated at different amplitudes. The same test was repeated when human iliac SMC were strained for 72 hours with similar results, see fig 4.12. A 66% increase in proliferation was measured for low strain amplitude compared to the high strain amplitude. Using the same protocol in parallel studies the level of apoptosis was measured in BASMC. The exposure of BASMC to a greater amplitude resulted in 94% greater cell apoptosis compared to the low strain amplitude, see fig 4.13.


Figure 4.2: Cyclic strain has an anti-proliferative effect on BASMC that is time and frequency dependant. BASMC were seeded at a concentration of 1 X 10⁵ cells per well in a non confluent state. BASMC were strained for 0, 24, 48 and 96 hours with a 5% heartbeat waveform (see fig 4.1) at 0, 0.5 (black) and 1Hz (red). Cells were counted after 96 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. *p < 0.05, compared to 1Hz for the same time of applied strain.**p < 0.05 compared to 0 hours.



Figure 4.3: Cyclic strain has an anti-proliferative effect on BASMC that is time and frequency dependant: flowcytometric analysis. BASMC were strained for 24(a), 48(b) and 96(c) hours with a 5% heartbeat waveform (see fig 4.1 at 0Hz(red), 0.5Hz(pink) and 1Hz(green). The Vybrant CFDA-SE dye was utilized to determine cell proliferation using FACS analysis. The shift between curves represents the relative difference in proliferation.



Figure 4.4: Cyclic strain has an anti-proliferative effect on BASMC that is time and frequency dependant. Confluent seeded cells. BASMC were strained for 0, 24, 48 and 96 hours with a 5% heartbeat waveform at 0, 0.5 (black) and 1Hz (red). Cells were strained when they had reached at least 90% confluency. Cells were counted after 96 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. *p < 0.05 compared to 0 hours and to 0.5Hz at 96 hours.**p < 0.05 compared to 0 hours and between 0.5 and 1Hz.



Figure 4.5: Cyclic strain has pro-apoptotic effect on BASMC that is time and frequency dependant: flowcytometric analysis. BASMC were strained for 24, 48 and 96 hours with a 5% heartbeat waveform at 0Hz, 0.5Hz and 1Hz. The Vybrant Alexa Fluor 488 Annexin V and propidium iodide were utilized to determine cell apoptosis using FACS analysis, n=3. *p < 0.05, **p < 0.05 compared to 0 hours.



Figure 4.6: **SMC proliferation is independent from the cyclic mean strain.** BASMC were strained with a sinusoidal curve at two different mean strain, 5% and 10% but at the same amplitude of 2%. Cells were counted after 48 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. *p < 0.05.



Figure 4.7: **SMC proliferation is independent from the cyclic mean strain: flowcytometric analysis.** BASMC were strained for 48 hours with a sinusoidal curve at two different mean strain, 5% and 10% but at the same amplitude of 2%. The Vybrant CFDA-SE dye was utilized to determine cell proliferation using FACS analysis.



Figure 4.8: SMC proliferation is independent from the cyclic mean strain: human SMC. Human iliac SMC were strained with a sinusoidal curve at two different mean strain, 5% and 10% but at the same amplitude of 2%. Cells were counted after 72 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=1. *p < 0.05.



Figure 4.9: Cyclic mean strain has no significant effect on the apoptosis of SMC: flowcytometric analysis. BASMC were strained with a sinusoidal curve at two different mean strain, 5% and 10% but at the same amplitude of 2%. The Vybrant Alexa Fluor 488 Annexin V and propidium iodide were utilized to determine cell apoptosis using FACS analysis, n=3.



Figure 4.10: Amplitude rather than mean strain determines the proliferative capacity of SMC. BASMC were strained at $5\% \pm 3\%$ and $11\% \pm 1\%$ and the lvel of proliferation evaluated after 48 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. * p < 0.05 compared to the static and $11\% \pm 1\%$ strain.



Figure 4.11: **Amplitude rather than mean strain determines the proliferative capacity of SMC: FACS analysis.** BASMC were strained at $5\% \pm 3\%$ and $11\% \pm 1\%$ and the lvel of proliferation evaluated after 48 hours of applied strain using FACS analysis. The Vybrant CFDA-SE dye was utilized to determine cell proliferation using FACS analysis.



Figure 4.12: **Amplitude rather than mean strain determines the proliferative capacity of SMC: human SMC.** Human iliac SMC were strained at $5\% \pm 3\%$ and $11\% \pm 1\%$ and the lvel of proliferation evaluated after 72 hours of applied strain using an hematocytometer. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. *p < 0.05 compared to the static control and $11\% \pm 1\%$.



Figure 4.13: Amplitude rather than mean strain determines the apoptotic capacity of SMC. BASMC were strained at $5\% \pm 3\%$ and $11\% \pm 1\%$ and apoptosis was evaluated after 48 hours of applied strain using FACS analysis. The Vybrant Alexa Fluor 488 Annexin V and propidium iodide were utilized to determine cell apoptosis using FACS analysis. Results are expressed as the average of the number of cells of three wells of a 6-well-plate, n=3. *p < 0.05.

4.5 Discussion

Dysfunction in SMC cell fate has been related to several cardiovascular diseases such as atherosclerosis, aneurysms and in-stent restenosis. According to the widely accepted model of in-stent restenosis, SMC migrate from the media to intima where they over-proliferate and subsequently accumulate between the endothelium and the media creating a neointimal layer that subsequently occludes the artery [13]. The origin and the factors that contribute to the abnormal behaviour of SMC are still source of much debate among researchers. An alternative model where the infiltration of SMC from bone-marrow derived progenitor stem cells has recently been proposed whereby under certain conditions, progenitors cells mature into proliferating SMC within the neointima [50, 55]. Regardless of the model, the crucial role of SMC proliferation in the development of cardiovascular diseases is indisputable.

In the last few years, mechanotransduction, and in particular, cyclic strain has emerged as a key player in regulating the vascular SMC cell fate. It has been suggested that strain may be the most homeostatic regulator of the cardiovascular system where cyclic strain influences SMC phenotype, proliferation, apoptosis and protein expression [19].

Cyclic strain is composed of several components such as mean strain, amplitude and frequency. Previous studies in the literature have tended not to consider these specific components when analysing the mechanotransduction within vascular cells. A more accurate analysis requires consideration of the contribution of these different components.

The present study analysed the change in SMC proliferation and apoptosis following alterations in the mechanical strain environment of a vessel analogous to a stent procedure to understand the factors modulating restenosis. The different contributors of cyclic strain such as mean strain, amplitude and frequency were analysed. The results from these tests provide important information for the improvement of stent design and deployment, however, they also provide insights into a broader array of diseases such as hypertension or atherosclerosis development.

In an artery SMC are organised in helicoidal shape with a low pitch angle [87]. Under these conditions, in the straight sections of arteries, SMC undergo uniaxial strain in the circumferential direction. Hence, the strain environment should involve stretching of SMC uniaxially. Many cardiovascular diseases, however, such as aneurysms and atherosclerosis are associated with a disarray in the initial organisation of SMC leading to an equibiaxial strain environment. Furthermore, cardiovascular disease often occurs at branch points or bifurcations in arteries where mechanical strain can be considered isotropic, even under physiological conditions. For this reason much of the work carried out in mechanotransduction involves biaxial strain of SMC.

The effect of mechanical stimuli on SMC behaviour has been studied previously. However, little is known about the influence of cyclic strain on proliferation and apoptosis of vascular SMC. *In vivo* studies which have been carried out in relation to in-stent restenosis and intimal hyperplasia have shown SMC proliferation is related to pulse pressure alterations [94]. Other studies have related SMC proliferation to the high strain levels absorbed by SMC after stent procedures [21, 13]. Furthermore artery cuffing has been shown to increase apoptosis leading to atrophy of the vessel [95]. While cyclic strain may determine SMC proliferative and apoptotic behaviour, there is controversy in the literature about the pro-proliferative or antiproliferative effect of cyclic strain on SMC.

Some authors reported an increase in SMC proliferation [21, 139] whereas other authors have not noticed any proliferation following exposure of SMC to cyclic strain. Predel *et al.* [89] experienced different cell behaviour depending on the cell source: proliferation of SMC from saphenous vein but no response under the same stimuli for arterial SMC from the internal mammary artery. Other studies [90, 91] have shown that SMC proliferation occurs when specific growth factors or proteins are present such as PDGF or Insulin Like Growth Factor (IGF-I) [21, 92].

These growth factors are stimulated by cyclic strain and they work in an autocrine manner.

Hipper and Isenberg [22] have shown the opposite response. Following exposure of SMC to cyclic strain they recorded a decrease in DNA synthesis (indicator of low proliferation level) proportional to the exposure time and level of cyclic strain and independent of the extracellular matrix. They hypothesized that there is no autocrine pathway for SMC proliferation following strain. Similar results were found by Morrow *et al.* [140] who demonstrated decreased proliferation and increased apoptosis following exposure of SMC to biaxial cyclic strain.

The response of SMC can also depend on the binding protein used for cell attachment to the stretching substrate. Kim *et al.* [141] found an increase in proliferation when SMC were coated on fibronectin while Reusch *et al.* [88] found laminin or collagen type I did not increase SMC proliferation.

Most of the previous studies did not measure proliferation but estimated cell growth using molecules such as [3H]-thymidine which is an index of DNA synthesis in the cell as a prelude to cell proliferation. In reality, mitosis is quite a complex process in which cell splitting is among the last events to occur in the cell. These systems cannot be considered a full measure of proliferation but an index of possible proliferation. For a precise estimation of the number of cells, cell counting and objective techniques such as flow cytometry are more suitable. The Flexercell[®] unit used in this study has been used for most of these mechanotransduction experiments published to date. As elucidated in this work (see appendix B), the calibration of this Flexercell[®] straining machine should be checked regularly to ensure accurate strain. It is possible that incorrectly calibrated Flexercell[®] units have led to some skewed results.

More importantly however most of the previous studies did not differentiate between the different components of the strain such as amplitude, mean strain and frequency. This distinction is particularly relevant in such conditions as in-stent restenosis or atherosclerosis where a stiffening of the arterial wall causes a drop in the cyclic strain amplitude and, in the case of stenting, where there is a significant increase in mean strain.

The present study clearly identifies a correlation between cyclic strain and SMC proliferative and apoptotic behaviour. Cyclic strain has both a time and frequency dependent antiproliferative and proapoptotic effect on SMC. The antiproliferative and pro-apoptotic effect was found to be amplitude rather than mean strain dependant. To the author's knowledge this is the first work to determine the role of cyclic strain independently of the mean strain in proliferation and apoptosis of vascular SMC.

These results may have an important role in the generation of SMC proliferation related conditions such as in-stent restenosis or atherosclerosis. Our data also indicates a relationship between apoptosis and mechanical stimuli. Cyclic strain increases the level of apoptosis *in vitro* of SMC in a time dependent manner. When SMC are strained close to physiological conditions, the apoptotic level is decreased independently of exposure time.

The overall results of this study clearly define a model for in-stent restenosis. The paradigm associated with restenosis where high mean strain levels but low strain amplitude occurs in the stented area is examined. From the results of this chapter, it is clear that the amplitude rather than the mean strain regulates the antiproliferative and pro-apoptotic effect of strain. The drop in strain amplitude in a coronary artery after a stent procedure would remove the antiproliferative effect of cyclic strain and thereby result in an engulfment of the arterial wall, ultimately explaining intimal hyperplasia.

Frequency is another component of the cyclic strain that has been poorly analysed. SMC frequency dependency is of major importance especially in new emerging technologies such as tissue engineering.

4.5.1 Frequency

As it has been seen from the literature, fluid pulsatility, shear stress and cyclic strain influence smooth muscle cell activity. The results in this chapter show that SMC proliferation and apoptosis are also frequency dependent. In fact, the data suggest that SMC under cyclic strain conditions exhibit greater proliferation and lower levels of apoptosis when they are strained close to the physiological frequency. They also suggest that a non physiological frequency can change the homeostasis of the arterial wall giving rise to anomalies. As the last phase of the restenosis process is remodelling which is strain and stress dependent, these data suggest that remodelling may also be frequency dependent.

Previous work from Balcells *et al.* [142] on the response of endothelial cells to different flow frequencies led to very similar results. Balcells et al. tested the level of proliferation of endothelial cells from different animals and organs for a range of different frequencies. Endothelial cells showed a peak in proliferation corresponding to the physiological frequency at which these cells were normally exposed to *in vivo*. They concluded that an 'optimal' frequency exists at which endothelial cells proliferate and maintain healthy growth. A strong correlation can be made between this work and Balcells' study due to the synergy in which SMC and EC work. Further tests should be carried out but the greater level of proliferation and lower apoptosis found in the present work when SMC were strained at the physiological frequency of 1Hz compared to the non physiological frequency of 0.5Hz indicates that an optimal proliferative straining frequency may also exist for SMC. Balcells et al. associated different flow frequencies instead of cyclic strain to EC proliferation. No flow or shear stress was applied to SMC in our work. Balcells et al. imparted different flow frequencies after seeding cells into a rigid perfused cylindrical scaffold preventing stretching of EC. It is therefore possible to hypothesise that the response of EC in the study by Balcells et al. could have been dictated by the flow pressure causing cyclic compression deformation on EC seeded on the scaffold.

McKnight and Frangos [93] found a correlation between strain rate and ERK1/2 phosphorylation whereby a slow cyclic strain rate produced no dephosphorylation or even ERK1/2 dephosphorylation in SMC in an orientation dependent manner. High strain rates produced an increase in ERK1/2 phosphorylation. ERK1/2 is a member of the MAPK family that responds to extracellular signalling with the intracellular regulation of cell proliferation and apoptosis.

In conclusion, these results show a time dependent antiproliferative and pro-apoptotic effect of the amplitude of cyclic strain in SMC. Mean strain showed no significant effect on SMC growth behaviour suggesting that in cases of in-stent restenosis the final diameter of the expanded stent does not influence the proliferative and apoptotic capacity of SMC. Straining frequency modulated SMC growth. When SMC were subjected close to the physiological frequency condition, the growth was increased and the apoptosis reduced.

Although strain was applied in a 2-D environment and provided valuable information about the behaviour of SMC, previous studies have found that SMC can behave very differently when strained in three dimensions [143]. A 3-D culture system can better mimic the appropriate mechanical environment experienced by SMC *in vivo*. Using the Flexercell[®] straining unit the effects of cyclic strain on SMC could be isolated from other important factors such as shear stress and pulse pressure. However, for development of a complete model of in-stent restenosis, a novel bioreactor was built where the influence of more than one physiological force could be applied to SMC *in vitro*.

Chapter 5

Results 2: SMC growth in response to stenting

The Flexercell[®] machine is an important tool which has been extensively used to characterise cells under various strain conditions. However it does not provide the three dimensional environment in which cells are perfused *in vivo*.

For this reason a bioreactor was developed in this project. Cells were cultured inside perfused mock coronary arteries (MCA) under cyclic strain conditions. The novel perfused bioreactor was subsequently used as a preclinical testing device for the evaluation of the level of SMC proliferation and apoptosis in a comparatively stiff stented region in relation to the compliant non-stented upstream and downstream regions.

The proliferative and apoptotic response of SMC to an altered mechanical environment following a stent procedure is presented in this chapter.

Drug-eluting-stents (DES) are the newest commercially available generation of stents. They are covered with an antiproliferative drug which slows down SMC proliferation. Various clinical trials have tested the efficacy of several different drugs on SMC growth following stenting. Among them, Sirolimus has emerged as one of the best candidates for retarding the progression of in-stent restenosis. The response of BASMC to different Sirolimus doses and exposure times was also measured. In addition, sirolimus coated DESs were tested in the novel perfused bioreactor and the antiproliferative effect of the drug was quantified under cyclic strain and physiological flow conditions.

5.1 Cell adhesion on the Mock Coronary Artery (MCA)

5.1.1 Sylgard[®] surface treatment

In order to optimise cell attachment to the MCA, the Sylgard[®] surface was treated with 80% sulphuric acid solution and/or fibronectin. BASMC were seeded on the sylgard[®] surfaces and after 12 hours they were fixed in formaldeyde and stained with DAPI. Cells were counted in a standard region of 300X300 pixels under a fluorescent microscope with magnification 4X.

Where the Sylgard[®] surface was untreated with sulphuric acid solution, only 19 cells were counted, see fig 5.1a. When Sylgard[®] was treated with the sulphuric acid solution only, 21 cells were found in the selected region, see fig 5.1b. The use of fibronectin increased the number of BASMC to 28, see fig 5.1c, whilst treatment with sulphuric acid followed by fibronectin resulted in maximum cell adhesion whereby 35 BASMC attached to the Sylgard[®]surface, see fig 5.1d. Fibronectin supported good cell adhesion but the treatment with the sulphuric acid increased the cell attachment further.

5.1.2 Shear stress resistance

Resistance of adherent BASMC on the Sylgard[®] surface to shear stress was tested under 10 dynes / cm² shear stress conditions. Comparison of fluorescent images of DAPI stained BASMC after 12 hours under shear stress conditions and static conditions showed no significant difference in BASMC number, see fig 5.2a and 5.2b.

5.2 $\alpha - actin$ staining

BASMC were regularly stained with $\alpha - actin$ to check any possible phenotype modifications. Cells can change phenotype depending on the stimuli from the external environment. An elastomeric substrate such as sylgard[®] could prompt cells to change their phenotype. BASMC seeded and adherent on sylgard[®] stained positive for $\alpha - actin$, confirming that BASMC maintain their phenotype once cultured on the sylgard[®] surface, see 5.3.

5.3 Proliferation of BASMC: Immunocytochemistry

BASMC were strained in stented and unstented MCAs under cyclic strain conditions. Following 4 days in culture, images of cell attachment were taken using a fluorescence microscope in the upstream, stented and downstream regions of the stented MCA after DAPI staining. The number of BASMC in regions of 300X300 pixels were counted in the three different sections of the MCA, see fig see fig 5.4. There was a $156 \pm 1.98\%$ (n=3) and $38 \pm 4.93\%$ (n=3) increase in proliferation was measured in the stented region when compared to the upstream and downstream regions of the stented MCA, respectively, see fig 5.5. Time t=0 refers to the number of BASMC following static culture and before the cyclic strain conditions were applied. A significant increase in cell number of $67 \pm 3.98\%$ (n=3) was measured between the upstream region of the stent and the cell number upstream before strain was applied. When cell counting was performed in a stented MCA and in parallel in a non-stented MCA, a significant difference in proliferation was measured. In the stented region of the MCA a $386 \pm 23.3\%$ (n=3) increase in proliferation was measured compared to an artery without an implanted stent. A small but statistically significant difference was also measured between SMC proliferation in the non-stented MCA after 4 days in culture compared with time t=0, see fig 5.6.

5.4 Proliferation of BASMC: Cell counting

SMC proliferation was also assessed in different regions of the mock coronary artery using trypsinisation and cell counting with an hematocytometer. The results confirmed those observed with the fluorescence microscope images of the three different regions. In the stented region the number of cells was $234 \pm 0.24\%$ (n=3) greater than in the upstream region and $41 \pm 0.46\%$ (n=3) greater than in the downstream region, see fig 5.7.

5.5 Apoptosis of BASMC: Immunocytochemistry

Using Annexin V and DAPI staining, the percentage of apoptotic cells was counted in the different regions of the MCA. Apoptotic cell membranes appear green under the fluorescence microscope while all cell nuclei show a characteristic DAPI blue thus enabling the percentage of apoptotic cells to be quantified. After 4 days in culture the level of apoptosis in the stented artery was $84 \pm 0.70\%$ (n=3) lower than in the non-stented artery, see figs 5.8b and 5.8d. Apoptosis in the stented region was $75 \pm 0.95\%$ (n=3) and $56 \pm 0.04\%$ (n=3) lower than the upstream and downstream regions, respectively, see figs 5.8 and 5.9.

5.6 GSK-3β

GSK-3 β has a critical role in many cell processes, particularly cell proliferation and apoptosis. GSK-3 β has been shown to be anti-proliferative in many contexts. In SMC, VLDLs and endothelin-1 promote proliferation through GSK-3 β [144, 145]. GSK-3 β has also been

shown to induce apoptosis in response to hypoxia, DNA damage, heat shock and growth factor removal [146]. Immunocytochemistry of GSK β expression in SMC clearly identified a difference in GSK-3 β expression between the compliant non-stented area of the MCA and the rigid stented area of the MCA and the negative control (without the presence of the secondary antibody), see fig 5.10.

5.7 Effect of Sirolimus on SMC: Static conditions

5.7.1 Effect of different drug solvent (DMSO) concentrations on SMC growth

Sirolimus, as with most drugs, needs to be dissolved in a suitable buffer before it can be delivered to cells. The buffer used in this study is DMSO. Prior to using DMSO, however, it was necessary to ensure that DMSO itself did not have any significant impact on the growth of SMC. To ensure that DMSO concentrations used had minimal influence on SMC growth, the influence of various concentrations of DMSO was investigated. A DMSO concentration lower than 5μ l per 2mls of medium (0.25%) did not have a significant impact on cell growth, see fig 5.11.

5.7.2 Effect of Sirolimus on SMC: Different doses

After 72 hours and 120 hours of SMC culture in sirolimus-rich medium a significant decrease in cell number (30% and 38% respectively) was recorded for drug concentrations greater than 10ng/ml, see fig 5.12. A dose response curve was calculated (fig 5.13) and the half maximal inhibitory concentration (IC_{50}) of the drug was 5.62 ng/ml. These data were further confirmed using FACS to determine the antiproliferative effect of the drug. Increasing the concentration of Sirolimus increased the antiproliferative effect of the drug exponentially, see fig 5.14.

To evaluate if the changes in proliferation were affected by metabolic rates and if the effect of the drug was persistent following a change in the cell medium, a test was carried out whereby 100ng/ml of drug was added to 2 different cell cultures and after 72 hours of cell culture the medium was changed in only one cell culture. After 120 hours from the beginning of the test, FACS analysis was carried out to test for proliferation. There was no significant difference between the cells in which the medium was changed and the cells with the original medium, see fig 5.15. These tests suggest that the Sirolimus effect is dose and time dependent but that the drug can continue with effect even if the medium is changed.

5.8 Drug Eluting Stents (DES): Proliferation

The novel MCA bioreactor was also used to compare the level of proliferation of BASMC when DES and Bare Metal Stents (BMS) of the same design were deployed under the same conditions inside parallel MCAs. Statistically significant differences in the level of proliferation was measured in the stented and downstream regions between DES and BMS. A $38 \pm 3\%$ and $19 \pm 28.8\%$ decrease in cell number was measured in the DES stented and downstream regions compared with the same regions of a BMS stented MCA, respectively. No statistically significant difference was noted in the SMC activity in the upstream regions of the stented MCAs, see fig 5.16 and 5.17.

5.9 Drug Eluting Stents (DES): Apoptosis

Apoptosis, like proliferation, was measured in the different regions of MCAs. No significant difference was measured between BMS and DES in terms of the apoptotic capacity of BASMC in the three regions.



Figure 5.1: **BASMC seeded on the Sylgard**[®] **surface treated with sulphuric acid and/or with fibronectin.** Cells were DAPI stained and counted under a fluorescence microscope. Treatment with fibronectin and sulphuric acid resulted in maximum cell attachment, fig 5.1d.



(a) Cells seeded with shear stress

(b) Cells seeded without shear stress

Figure 5.2: **BASMC adherence on Sylgard**[®]. (a) in the presence of a shear stress of 10 dynes/cm² and (b) in nstatic conditions



Figure 5.3: BASMC seeded on Sylgard[®] stained positive for DAPI and $\alpha - actin$.

Medium Flow



Figure 5.4: **SMC proliferation in the different regions of the mock coronary artery: histocytochemistry.** BASMC were cultured in the stented MCA in the perfused bioreactor for 96 hours under cyclic strain conditions. BASMC were analysed using a fluorescence microscope following DAPI staining.



Figure 5.5: SMC proliferation in the different regions of a mock coronary artery: cell count results. SMC proliferation in the different regions of a mock coronary artery is inversely proportional to the relative level of strain (CS = cyclic strain). BASMC were analysed using a fluorescence microscope following DAPI staining and the number of cells in a constant area of 300X300 pixels were counted in the upstream, stented and downstream regions of the MCAs, n=3, *p < 0.05.



Figure 5.6: **Proliferation in a MCA without a stent.** Proliferation in the MCA without a stent implanted is very limited when compared to the stented vessel and time t=0, n=3, *p < 0.05.



Figure 5.7: SMC proliferation in the different regions of a mock coronary artery: count with an hemacytometer. SMC proliferation in the different regions of a mock coronary artery is inversely proportional to the relative level of strain. Mock coronary arteries were cut into three regions of equal lengh corresponding to the upstream, stented and downstream area and BASMC were trypsinised and counted with a haematocytometer, n=3, *p < 0.05.



Figure 5.8: **SMC apoptosis in different regions of a stented and non-stented mock coronary artery.** All cell nuclei were stained with DAPI and show the typical blue stain under the fluorescent microscope. Apoptotic cells can be recognised by their green membranes following staining with Annexin V.



Figure 5.9: **SMC apoptosis in the different regions of the mock coronary artery.** SMC apoptosis in the different regions of the mock coronary artery showing apoptosis is proportional to the relative level of cyclic strain. BASMC seeded MCAs were placed inside the bioreactor where they were perfused for 96 hours under cyclic strain conditions. MCAs were subsequently removed from the bioreactor, cut longitudinally and the BASMC were then stained with DAPI and Annexin V and analysed under a fluorescence microscope.



(a) Unstented area

(b) Stented area



(c) Control without secondary antibody

Figure 5.10: **GSK-3** β expression in green is higher in the non-stented region of the MCA where cyclic strain is higher. GSK-3 β expression is an indicator for lower levels of SMC proliferation.



Figure 5.11: **Viable cells for defferent DMSO concentrations.** Different DMSO concentrations were tried to find the right concentration for drug dilution without compromising cell viability.



Figure 5.12: **BASMC count after 72 and 120 hours of culture in different Sirolimus concentrations.**



Figure 5.13: Sirolimus dose-response curve after 120 hours of BASMC culture.


Figure 5.14: **Sirolimus effect on SMC using FACS analysis.** Proliferation of BASMC for different Sirolimus concentrations quantified using FACS. Red= Control Green=0.1ng/ml Violet=1ng/ml Light blue=5ng/ml Orange=10ng/ml Yellow=50ng/ml Dark Blue= 100ng/ml.



Figure 5.15: **Effect of metabolism on Sirolimus effect.** Comparison of BASMC cultures with Sirolimus, where the medium was changed after 72 hours (green) and where the medium was maintained unchanged for 120 hours.



Figure 5.16: **Comparison of BASMC proliferation in a DES vs BMS.** DES and BMS were tested in the novel bioreactor at the same conditions and the level of proliferation in the three regions evaluated, n=3, * ANOVA (p < 0.05).



(a) Bare Metal Stent

(b) Drug eluting stent

Figure 5.17: **SMC proliferation in the stented region of two MCAs for a BMS and a DES.** The greater number of SMC in the BMS compared to the DES is related to the antiproliferative effect of Sirolimus.







(d) Stented Region - DES



Figure 5.18: **SMC apoptosis in the regions of two MCAs for a BMS and a DES.** No difference was measured in SMC apoptosis between BMS and DES.

5.10 Discussion

In this chapter the activity of BASMC has been quatified in a three dimensional straining environment of the perfused bioreactor. The important differences in the response of SMC grown in a 2-D and a 3-D environments were examined. In a 3-D environment, SMC generally express less smooth muscle markers and the level of proliferation and migration are usually lower [143].

Bioreactors have been widely used is tissue engineering for mechanical characterisation of biological constructs. The bioreactor developed in this work was used as a preclinical testing device for the evaluation of proliferation and apoptosis of SMC on mock coronary arteries stented with both bare metal and drug eluting stents. To the author's knowledge it is the first time in which a bioreactor has been used to evaluate the response of SMC to stenting. This is one of the first cases of deployment of a stent inside a model artery and, to the author's knowledge the first in which proliferation and apoptosis of SMC were analysed [147]. Similar studies by Cardinal et al. in 2006 deployed a stent into a tissue-engineered vessel to study cell response [148]. However, such a system used a complex tissue-engineered vessel as a cell substrate and therefore did not allow easy isolation of the different biological contributors to cells' behaviour. Furthermore, Cardinal et al. used a rigid, non-compliant ePTFE scaffold preventing deformations. More recent studies carried out by Punchard et al [149] examined the orientation, proliferation and apoptosis of endothelial cells and related markers. The advantage of these bioreactor systems is they provide cells simultaneously with the major mechanical forces and stimuli in vivo: pressure, shear stress and circumferential strain. In the current study, implantation of a stent inside the mock coronary artery provided important new information on the response of SMC to the altered mechanical strain environment as a result of the stent.

Punchard et al [149] analysed the response of endothelial cells to stent placement and they found a local realignment of cells due to the altered haemodynamic forces. Furthermore, the expression of inflammatory genes and ICAM-1, VCAM-1 was increased. During a stent procedure, the endothelium is generally removed by the friction of the angioplasty balloon against the arterial wall. The underlying SMC are thus exposed to the blood. For this reason, only SMC were considered in the present work.

The results presented in this chapter on the activity of BASMC cultured under cyclic strain conditions in a perfused bioreactor confirm the results in Chapter 4.

The increase in SMC number in the stented regions of the MCAs compared to the downstream and upstream sections of the artery reinforce the hypothesis that cyclic strain has an antiproliferative effect. Furthemore higher cell numbers were present in the downstream region compared to the upstream section of the MCA. Cyclic stain, measured using the video extensometer, was higher in the upstream region when compared with the downstream region and the stented region had the lowest cyclic strain . In the stented area there is a very high mean strain (24%) but the amplitude of the cyclic strain was very low (1%). In the upstream region a near physiological level of cyclic strain (5 ± 3%) was measured and the number of cells counted in the 300x300 pixel area after 4 days in culture was comparable to that measured at time t=0 (before straining). These results confirm that the level of proliferation is inversely proportional to the strain amplitude.

The level of proliferation was very low in the non-stented MCA, greater than the number of BASMC at time t=0 but significantly lower than that measured in the stented MCA. The proliferation in the non-stented MCA was even lower than the upstream region of the stented artery. These results suggest a homeostatic role for cyclic strain in preventing SMC proliferation. Cyclic strain may prevent SMC from proliferating excessively, maintaining an optimum balance between proliferation and apoptosis to maintain SMC numbers, whilst a drop in cyclic strain amplitude could be responsible for uncontrolled proliferation.

Parallel and complementary results were observed when SMC apoptosis was measured in the stented and non-stented MCAs. The cyclic strain promoted a pro-apoptotic response in BASMC, such that, apoptosis was notably greater in the upstream and downstream regions when compared to the stented section of the MCA. In the case of the non stented MCA, higher numbers of apoptotic cells were observed when compared to all regions of the stented mock coronary artery.

From these data, several observations with regards to SMC growth and the response to physiological flow conditions can be made. SMC tended to align perpendicularly to the flow in the upstream and downstream sections of the mock coronary arteries while in the stented area no preferred direction was evident. This behaviour is similar to that reported by Punchard *et al.* [149] for endothelial cells, where EC were also found to align perpendicularly to the principal strain direction. In fact several research groups have observed that SMC align in the direction of the applied strain when cyclically strained compared to a disorganised arrangement under static culture conditions [150, 151].

In addition to the 2-D cell culture experiments, the bioreactor system clearly further validates a model of restenosis development centred on SMC proliferation and apoptosis, with cyclic strain amplitude as the critical regulator. The bioreactor system was used to further test this hypothesis by evaluating Sirolimus coated drug eluting stents. When implanted, the DES stents caused a diminished level of proliferation in the stented and downstream regions of the stented MCAs when compared to the BMS. No difference was observed in the proliferation of cells in the upstream region of the BMS and DES stented MCAs. This difference in behaviour between upstream and downstream is most likely attributed to the flow and direction of the perfusing medium causing drug detachment from the stent struts and carrying it downstream. Sirolimus was found to only have an antiproliferative effect on SMC but had no effect on SMC apoptosis. This is not surprising given the means by which Sirolimus acts to influence cell activity.

Clinical trials have been used for the evaluation of the efficacy of DES

in terms of restenosis development. Clinical trials are very expensive and feedback is both limited and requires a long timescale. The bioreactor system presented here provides a cost and time effective means to compare different stent designs, BMS or DES, providing an early indication of the likely degree of SMC proliferation and apoptosis following stenting and therefore enabling better strategies for prevention of in-stent restenosis.

To better understand the effect of Sirolimus alone on SMC actiivty, the drug was administered at different doses to the cultured SMC. Cell counts and FACS analyses confirmed the antiproliferative effect of Sirolimus on the BASMC. The tests also confirmed that Sirolimus acts in a dose dependent and time dependent manner. The inhibitory effect of the drug was noted in concentrations as low as 0.1ng/ml after 120 hours. A change in culture medium had no effect on Sirolimus action suggesting that that the drug effect persists for at least 48 hours in cell culture. Sirolimus smooth muscle cell inhibition was 30% after 72 hours and 36% after 120 hours when a concentration of 100ng/ml was used. These results differ from those found by Marx et al.[125] where an inhibition of 50% after 72 hours was reported. This may be explained by the fact that Marx et al. used human cells instead of bovine aortic smooth muscle cells and also used a different cell culture protocol. The half life of Sirolimus was measured to be 57-63 hours which would explain the small increase (30% to 36%) in the inhibitory effect of the drug from 72 to 120 hours.

Chapter 6

Discussion

In stent restenosis is the main drawback following stent procedures. It has been identified as a sequence of events which includes thrombosis, inflammation and vascular remodelling but which is predominantly due over-proliferation of SMC and consequently the creation of a to neointimal layer [13]. In order to improve stent design and reduce restenosis, it is important to investigate the factors which contribute the most to neointima formation. Altered SMC fate decisions, including proliferation, migration and apoptosis, are critical in many cardiovascular diseases such as atherosclerosis, hypertension and in-stent restenosis. The cardiovascular mechanical environment regulates the fate decision of SMC [111] and unphysiological or pathological mechanical conditions may therefore lead to altered SMC fate decisions [152]. Common examples of biological responses to mechanical environment changes are the stiffening of arteries during hypertension, dilatation of an artery due to an aneurysm and intimal hyperplasia following stenting. Under normal physiological conditions SMC are under constant cyclic strain in the media of coronary arteries due to the pulsatile nature of blood flow. When an intravascular coronary stent is introduced to restore patency to a stenosed atherosclerotic vessel, the arterial wall is expanded. As a result of the stress stiffening non-linearity of arterial tissue, arterial expansion results in increased vessel stiffness. In addition, when an artery is expanded and buttressed open by a rigid metal stent, the amplitude of the cyclic strain is significantly reduced in the stiff stented artery [28]. In the introduction of this work two major hypotheses were proposed:

- 1. Changes in the mechanical environment of an artery following a stenting procedure drive the restenosis process
- 2. The enhanced proliferative and apoptotic response of SMC after stenting is dictated by the change in mechanical environment

The changes in the mechanical environment of an artery as a result of stenting investigated in the present study were limited to the documented increase in the mean strain and reduction in cyclic strain amplitude observed due to arterial expansion and the presence of a metal stent with high radial strength [28]. Although mean strain had no effect on the growth of SMC, reduced cyclic strain amplitude was found to increase both BASMC and human SMC proliferation and reduce This finding supports our hypotheses, however, other apoptosis. alterations in the mechanical environment also occur during stenting and the limitations of the present work therefore needs some consideration. During stent placement, an angioplasty balloon is required to expand the artery and plastically deform the stent into its expanded state. The angioplasty process denudes the artery of its protective endothelial layer [47] exposing the subendothelial medial layer to fluid shear stresses. To represent the endothelial denudation following stenting, the in vitro system designed and used in the present study did not include endothelial cells and therefore neglected their influence. Re-endothelialisation does occur following stenting, however, and this was not considered in the present model. An in vitro system, by its nature, cannot replicate an *in vivo* system exactly, but it can offer insights into specific *in vivo* processes. The present study shows that once SMC are no longer protected by the endothelium that their growth can be dictated by the mechanical environment and in particular the cyclic strain amplitude that they are exposed to. It should be noted, however, that

although SMC proliferation is a major contributor in in-stent restenosis, thrombosis, inflammation and vessel remodelling are also known contributors to the re-occlusion of stented vessels. These processes were not considered in the cell experiments or the bioreactor system used in the present work and this enabled the contribution of SMC proliferation in the restenosis process to be quantified more clearly. Future studies could however, ascertain the relative contribution of thrombosis, inflammation and vessel remodelling in the restenotic process.

Clinical trials are the most complete method of analysing stents and the restenosis process. They maintain the complexity of the biological environment of SMC, however, in vivo systems prevent parametric studies because of the difficulty of controlling variables and measuring data in real time. Furthermore, they can be very lengthy and prohibitively costly. Clinical trials have however compared the restenosis outcome for different stents in order to isolate the factors which trigger or regulate restenosis development. Stent design has been recognised as a key contributor in restenosis [153] but no correlation has been found between the choice of stent material and intimal hyperplasia. Although different thrombotic reactions were measured for stents of different materials, the neointimal thickness following stent implantations was reported to be independent of the stent material chosen [13]. Vascular injury has been proposed as the primary stimulus for neointimal formation. A correlation has been reported between the depth of injury induced by individual stent struts to an artery and the subsequent restenosis [13]. Similarly, the degree of mechanical stretch induced in the arterial wall by a stent has also been linked to the degree of restenosis formation in a clinical trial on human coronary arteries [16]. Syeda *et al.* [154] confirmed the findings of Arakawa et al. [16] in an angioplasty clinical trial when they reported that lumen gain achieved by plaque compression did not induce a restenotic response whiles lumen gain achieved by inducing arterial wall stretch resulted in a significantly higher incidence of target lesion revascularization. The investigations of and Syeda et al. [154] support the hypothesis that Arakawa *et al.*

restenosis is not driven by the presence of a foreign body, the stent itself, but rather the mechanical environment changes induced in the arterial wall by the presence of the intravascular device or vascular therapy. The correlation between high values of stent-induced stretch in the arterial wall and the restenotic rate has led many stent designers to focus on reducing the stress and strain in stented vessels in an attempt to address the problem of restenosis [67, 155].

However, as elucidated in [28] the increased strain in a stented vessel also leads to reduction in cyclic strain amplitude. It is therefore necessary to consider the relative contribution of the different components of strain such as mean strain, amplitude and frequency on the development of intimal hyperplasia and this study is the first to attempt this task by focussing on SMC activity in response to these strain parameters.

6.1 Amplitude and mean strain

The most agreed biological model of in-stent restenosis development includes a phase whereby the migration of SMC from the media to the intima and SMC over proliferation is the key contributor to the development of intimal hyperplasia [13]. To determine if changes in vessel strain induced by a stent could contribute to this critical phase of in-stent restenosis, SMC were tested using a Flexercell[®] straining The level of machine under different mean strains and amplitudes. proliferation and apoptosis was measured. Although numerous previous studies have investigated the influence of strain on SMC activity [156], these is still an ongoing debate on the effect of strain on SMC proliferation. Some authors provide evidence of a pro-proliferative cyclic strain contribution [21] while others report a decrease in cellular proliferation with increase cyclic strain [22]. The results of the experimental studies presented here indicate that mean strain has no direct effect in SMC proliferation and apoptosis while cyclic amplitude regulates the proliferation and apoptosis level. Stent expansion was also

simulated inside mock coronary arteries in which SMC were seeded. Increased proliferation was measured between the struts of the stent compared to the upstream and downstream regions and the antiproliferative effect of the strain was found to be proportional to the cyclic strain amplitude. The results suggest that the amplitude of cyclic strain has a role in regulating the restenotic rate leading to the recommendation for the use and design of more compliant stents to maintain the antiproliferative effect of cyclic strain. In this work the initial stent lumen gain did not affect the proliferative or apoptotic behaviour of SMC and therefore stents which achieve a high lumen gain but which maintain near physiological cyclic strain in the stented region of the vessel may be more optimal for restenosis prevention. In fact, clinical trials have confirmed that more compliant stents can lower restenotic rates [153] although the reason for this outcome could not be explained. This distinction between the contribution of mean strain and strain amplitude on SMC activity, for the first time, provides a greater understanding of the in-stent restenosis process and may lead to changes in the way in which stents are designed, see fig 6.1.

It may also explain the dichotomy that exists in the available literature with regards to the influence of strain on SMC activity.

6.2 Phenotype influence

In a normal artery SMC express a contractile phenotype with a low proliferative rate [87]. During in stent restenosis (and atherosclerotic plaque formation) SMC change phenotype from contractile to synthetic and overproliferate creating intimal hyperplasia [87]. Tests *in vitro* have shown that cyclic strain can influence SMC phenotype [90, 157]. An early study of Birukov *et al.* [90] showed a serum independent increase of h-caldesmon following biaxial cyclic strain while Tock *et al.* [157] used alpha-actin as a marker of SMC differentiation reporting an increased expression under biaxial cyclic strain. Kanda and Matsuda [151] reported



Figure 6.1: Amplitude rather than mean strain regulates SMC proliferation. More compliant stents designed to maintain near physiological levels of cyclic strain amplitude in an artery may therefore prevent the progression of in-stent restenosis.

an increase in myofilaments, extracellular filamentous material and dense bodies when they compared cells exposed to strain and static cells. Furthermore Niklason *et al.* [115] showed that mechanical strain increases the SMC differentiation marker MHC. In restenosis development, cyclic strain may therefore not simply regulate SMC proliferation but also act as a trigger for SMC phenotype changes from contractile to synthetic leading to SMC overproliferation. Further tests could be carried out in future to specifically analyse the phenotypic changes of SMC as a result of different strain conditions, and in particular, in response to cyclic strain amplitude and mean strain independently.

6.3 Stem cells

In recent years another theory has emerged to describe the in-stent restenosis process. Some researchers have suggested that progenitor bone-marrow stem cells would be present in the circulatory system and could infiltrate the arterial wall in response to vessel injury. Under certain

conditions, progenitor stem cells can easily change phenotype to become synthetic SMC leading to in-stent restenosis. Recent studies showed that mechanical strain can affect SMC phenotype and proliferation. Park et al. [98] reported a reduced expression of the phenotypic SMC markers alpha-actin and SM22 in human bone marrow mesenchymial stem cells (MSC) when they were biaxially strained at 10% and 1Hz frequency. On the contrary they reported a transient increase in these markers when cells were uniaxially strained. Moreover uniaxial, but not equiaxial, strain produced a transient increase in collagen I expression. Hamilton et al. [158] found that cyclic strain decreased proliferation in rat bone marrow progenitor cells and it increased alpha-actin and SM-22alpha markers. These studies suggest that uniaxial strain may stimulate MSC to change to a differentiated contractile phenotype while biaxial strain conditions maintain MSC in a more synthetic phenotype. Although phenotypic changes of SMC was not the focus of the present work, the observed proliferation dependency of SMC on the strain environment provides some support for the possibility that cyclic strain amplitude maintains SMC in a contractile phenotype. Future work could analyse the influence of cyclic strain amplitude and mean strain on MSC to ascertain if differentiating between the individual strain components could provide even greater insights into the activity and phenotypic changes of these cells.

6.4 **Tissue engineering applications**

Tissue engineering is a new exciting field aiming to fully engineer *in vitro* vessels of small dimensions which can withstand hemodynamic forces *in vivo*. The main limitation preventing the use of these constructs is the lack of strength of the generated tissues [159].

Understanding the regulation of SMC growth could provide a basis for the construction of more viable tissue-engineered vascular grafts. Hemodynamic forces promote vascular cellular differentiation in tissue

engineered constructs resulting in superior mechanical properties compared to constructs which are cultured in static conditions [115, 160]. Cyclic strain, for instance, has been shown to enhance extracellular matrix remodelling and SMC differentiation [90, 141]. As previously discussed, however, there still remains some debate on the action of cyclic strain on SMC proliferation. The possibility to manipulate cell differentiation, proliferation and extracellular matrix production is critical for the development of new tissue constructs. Vascular cells are normally seeded and grown on tubular scaffolds inside bioreactors which are able to provide cells with the necessary mechanical stimulation. They are therefore under cyclic strain, a condition which is composed of two major components: amplitude and mean strain. In this work the independence of SMC proliferation and apoptosis from mean strain and the antiproliferative and pro-apoptotic effect of cyclic strain has been established. In vascular tissue engineering the goal is to obtain "working cells" in the contractile phenotype. However, for the development of engineered vascular constructs it could be necessary to modulate SMC growth and phenotype at different times points. It can therefore be hypothesised that cyclic strain could be used as an engineering tool to switch on and off the proliferation of SMC in tissue engineered It would, for instance, be possible to change the mean constructs. pressure inside cell seeded scaffolds to increase the mean strain and reduce the strain amplitude thereby encouraging cell proliferation. Furthermore in this work, the frequency dependency of the proliferative and apoptotic behaviour of SMC was also established. Cyclic strain frequency can therefore also be used to modulate proliferation and apoptosis of SMC with the strong possibility of a "optimum" state corresponding to the physiological frequency. This provides further insights for regulating vascular tissue growth in tissue engineered constructs.

6.5 Drug eluting stents evaluation

Drug eluting stents are effective against the development of in-stent restenosis. The effect of the different drugs has previously been analysed *in vitro* and there are numerous clinical trials with DES. However, to the author's knowledge the reaction of vascular cells to DES has never been examined under controlled cyclic strain conditions *in vitro*. As largely discussed, cyclic strain can modify the behaviour of vascular cells and cell signalling. It is therefore important to evaluate the antiproliferative effect of a drug under these strain conditions. In this work we assessed the effect of Sirolimus on BASMC. Sirolimus inhibits SMC proliferation by stopping the cell cycle progression from G1 to S associated with reduction in cyclin-dependant kinase (CDK) activity [125]. Sirolimus binds to the cytosolic protein FK-binding-protein-12 (FKBP12). The Sirolimus-FKBP12 complex inhibits the mammalian target of Rapamycin(mTOR) pathway through depression of PP2A (a protein phosphatase), resulting in inhibition of lymphocyte proliferation. The antiproliferative effect of Sirolimus was found to be greater on SMC in the stented region of the mock coronary artery compared to the downstream region. This may be explained by considering the influence of the flow og cell culture media which would most likely flush away and dilute the drug in the downstream region, while at the stent level there would be a constant supply from the primer. In the upstream region the drug had no influence given that it was diluted in the cell culture medium. The bioreactor tests carried out in this study on DES lasted for 7 days which corresponds to a timepoint when 80% of the drug would be released from the stent according to the drug elution profile of the stent, see appendix E. Medical device companies producing DES are interested in this type of analysis as it provides data and insights into the drug elution profile of the stents and its efficacy without the immediate need for expensive clinical trials.

6.6 Insights into other vascular diseases

Atherosclerosis is associated with changes in the mechanical properties of an artery and it is often linked with hypertension [35] where due to increased physiological pressure conditions the vessel would experience lower cyclic strain. It is therefore possible that even in atherosclerosis development the change in the strain environment could contribute to its formation. Thacher et. al [161] analysed the effect on both shear stress and reduction of cyclic strain on atherosclerosis development. Whilst they found that both shear stress and reduced compliance triggered endothelial dysfunction, they noted that only reduced compliance influenced SMC and caused dedifferentiation of the cells. Similar insights may be gained in understanding the aetiology of other diseases. Pulmonary hypertension, for instance, is associated with hardening of the arterial wall. It is also known that SMC and fibroblast migration and proliferation are responsible for neointima formation in this disease [162, 163]. Neointimal formation in pulmonary arteries may be explained by overproliferation of SMC due to the reduced cyclic strain amplitude in the hypertensive vessels. Gambillara et al. [164] perfused porcine carotid arteries using an ex vivo arterial support system. They experienced lower levels of SMC actin and desmin expressions in arteries exposed to reduced cyclic strain, where the arteries were wrapped with an external band to mimic systemic hypertension. On the other hand, higher levels of matrix metalloproteinase-2 (MMP-2) and increase in Ki67 expression were measured suggesting matrix degradation and the onset of cellular proliferation. Gambillara *et al.* stressed the importance of cyclic stretch in the maintenance of a differentiated and fully functional phenotype for SMC and for the regulation of SMC migration, proliferation, and matrix turnover. This is a further confirmation of the role of cyclic strain in the regulation of SMC behaviour through mechanotransduction.

Chapter 7

Conclusions

7.1 Main findings

- Cyclic strain has a temporal antiproliferative and pro-apoptotic effect on BASMC *in vitro*.
- Amplitude rather than mean strain determines the proliferative and apoptotic behaviour of BASMC. Tests carried out using human SMC further confirmed these results. SMC overproliferation which gives rise to neointimal formation following stenting procedures could be as a result of the reduced amplitude of the cyclic strain in the stented region of the vessel. The study results suggest that future generation stents should aim to maintain the vessel cyclic strain at near physiological levels.
- BASMC proliferation and apoptosis is frequency dependent. When cells are subjected close to the physiological frequency condition, cell growth is increased and apoptotic activity of these cells is reduced in comparison to cells strained at non physiological frequencies.
- The newly designed preclinical testing device for the evaluation of the proliferation and apoptosis of vascular SMC in the presence and absence of a stent was suitable for mimicking strains and stresses experienced by SMC in coronary arteries *in vivo*. The bioreactor

includes a Sylgard[®] mock coronary artery with mechanical properties similar to the real coronary artery.

- The bioreactor was used to test endovascular stents inside mock coronary arteries. The antiproliferative and pro-apoptotic effect of cyclic strain was confirmed by the analysis of BASMC in the stented mock coronary artery. SMC activity in the stented mock coronary artery was also found to be mean strain independent.
- The antiproliferative effect of Sirolimus on SMC was confirmed with the intensity of the inhibitory action found to be 30% after 72 hours exposure to the drug. An IC_{50} of 5.62 ng/ml was also measured.
- Sirolimus coated DES stents were analysed using the novel bioreactor under simulated physiological conditions. Sirolimus had an antiproliferative effect on BASMC in the stented area and in the downstream region of the vessel in comparison with BMS. No difference in BASMC proliferation was found in the upstream region. Comparison of SMC apoptosis levels in the BMS and DES showed that Sirolimus did not influence SMC apoptosis.

7.2 Future work

The results obtained from this study constitute a significant insight into the in-stent restenosis mechanism. The system developed in this work could, however, be used as the basis for further research in the field of in stent restenosis and also to provide insights into other diseases such as atherosclerosis, hypertension and aneurysm development. In addition, the insights gained into the response of SMC to their mechanical environment could be used to develop new vascular therapies or tissue engineered vessels. The following summarises some of the key areas into which the work to-date could be developed to provide further valuable insights in cardiovascular research:

- To provide further insights into the means by which the SMC activity is governed by strain in arteries, an analysis of SMC phenotypic changes at different cyclic strain amplitudes and mean strain could be carried out. The phenotypic changes following a stent procedure in mock coronary arteries could also be quantified in the bioreactor.
- A further development would be the use of a more sophisticated mock coronary artery. More sophisticated scaffolds from tissue engineering could be used. The bioreactor enables cell seeding both intraluminally and extraluminally. Using porous scaffolds suitable for tissue engineering blood vessels it would be possible to analyse SMC migration in different regions of the mock stented artery (upstream, downstream and in the stented region) by seeding cells on the external surface of the artery and monitoring their advancement towards the lumen using histological analyses. Similar tests have been carried out with a Boyden chamber by Poon textitet al. [126]. The advantage of the novel bioreactor is that cell migration could be analysed in a more complex environment where all three key mechanical forces which exist *in vivo* (shear stress, cyclic strain and pressure) would be present.
- Progenitor stem cells could be investigated to determine their role in neointima formation and their response to different cyclic strain amplitudes and mean strains.
- Real stented coronary arteries (i.e. porcine arteries) could be investigated using the bioreactor and histological studies used to quantify SMC proliferation, migration and phenotype change.
- Given the cyclic strain effect on SMC proliferation and apoptosis, the bioreactor could also be used to identify specific genes or proteins associated with the SMC proliferative and apoptotic reaction to cyclic strain. This could open the way for the

development of new target specific drugs and the development of new drug eluting stents.

• Stent design has been related to in-stent restenosis rate. The bioreactor could be used as a preclinical testing device to compare the level of SMC proliferation for different stent designs. Clinical trials will always be indispensable for evaluation of stent performances, however, this bioreactor could find application in early stage stent design. Old stent designs which have already a well documented clinical trial history could be used for validation and the system. The system could then provide invaluable preclinical test data which would cost effectively expedite the development of new stent designs.

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Appendices

Appendix A

Materials

Applied Biosystems (Warrington, UK)Silencer Select GSK-3 β siRNA

 $\frac{Axxora (Nottingham, UK)}{Anti GSK3\beta monoclonal IgG}$

BD Biosciences (Oxford, UK) BD FACSflowTM FACS Caliber Flowcytometer

Coriell Institute (New Jersey) Cryopreserved Bovine Aortic Smooth Muscle Cells (Passage 2) Cryopreserved Human Iliac Smooth Muscle Cells (Passage 2)

 $\frac{\text{Cell Signal (Beverley, MA)}}{\text{Anti-phospho-GSK3}\beta \text{ rabbit monoclonal IgG}}$

Dunn Labortechnik GmBH (Asbach, Germany) 6-well Bioflex® plates

 $\frac{\mbox{Flexcell International Corp. (Hillsborough, NC)}}{\mbox{Flexercell} \ensuremath{\mathbb{R}}\xspace{1.5mm} \mbox{Tension Plus}^{\mbox{TM}}\mbox{-}\mbox{FX-4000T}^{\mbox{TM}}\mbox{-}\mbox{system}}$

Invitrogen (Groningen, Netherlands)

Anti rabbit IgG - Alexafluor 488 conjugate VybrantTM Apoptosis Assay Kit # 2 VybrantTM CFDA SE Cell Tracer Kit

Merit Medical Systems (South Jordan, Utah))

Basix 25 anioplasty inflation syringe

Sarstedt (Drinagh, Wexford, Ireland)

1.5 ml eppendorf tubes
T25 tissue culture flasks
T75 tissue culture flasks
T175 tissue culture flasks
6-well tissue culture plates
5,10 and 25ml serological pipettes
15 and 50ml falcone tubes
cryovials

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY) Kodak 1D image analysis software

Sigma Chemical Company (Poole, Dorset, England)

Anti α -SMC actin Bovine Serum Albumin Brightline Haemocytometer DMSO EDTA Foetal Calf Serum (GSK3 β inhibitor) Methanol PBS tablets Penicillin-Streptomycin (100x) RPMI-164040 (GSK3 β inhibitor) Tween 20 Triton X-100 Trypsin-EDTA solution (10x)

Spectrum Laboratories

Cellmax Plus System CellMaxStarted kit Reservoir Cap, 33 mm

Thermofisher Scientific (Langenselbold, Germany)

White 96-well plates

Value Plastic [®] (Fort Collins, CO)

Female Luer Thread 1/8" ID Male luer with 5/16" thread Hex 1/4-28UNF

Appendix **B**

Flexercell[®] calibration

B.1 Introduction

One of the most commonly used systems to apply controlled mechanical stretch to cells to determine its influence is the FX-4000TM system (Flexercell[®] Tension Plus System, Flexcell Corp, McKeesport, PA, USA). Cells are cultured onto compliant silicone membranes at the bottom of the specially designed 6-well-plates. This model gives an in situ mechanical representation of the strain SMC experience *in vivo*.

Previous works have been carried out to quantify the strain field in the Flexercell[®] Tension Plus System [165, 166]. Vande Geest *et al.* [165] focused on static conditions and one dynamic test protocol at 1 Hz whilst Woodell *et al.* [166] did look at the influence of frequency but only for the sine waveform and the strains of the membrane were not measured in real time. To the author's knowledge no study to-date has investigated the influence of frequency and waveform or proposed a method to calibrate the device in-house. The first step of the project was thus to assure the reliability of the strain system when this straining machine was used at different frequencies and different waveforms.

The first goal of this work was to quantify the strain field of the Flexercell[®] 6-well-plates for heartbeat, square and static waveforms at various frequencies, and to compare the results with the Flexercell[®]

software inputs and outputs. In cases where the output did not match the desired strain level, a method to calibrate the machine in-house was applied using new pressure-stretch equations. Ultimately, the results and methods presented here should enable us to use the Flexercell[®] machine to set-up cell experiments more accurately. It will also enable comparison of results from a range of different research centres to be compared with greater confidence.

B.2 Quantification of the strain field of the Flexercell[®] Tension Plus System

B.2.1 Experimental in-situ membrane strain measurement

Using a tripod, a video extensometer camera (MESSPHYSIK Materials Testing, Frstenfeld, Austria) was placed above the Flexercell[®] baseplate and linked to the video-extensometer computer system for image acquisition. The camera and the Flexercell[®] baseplate were set up in a horizontal position by means of a spirit level, see fig B.1.



Figure B.1: Flexercell[®] well showing the membrane with markers for strain measurement (left) and the apparatus and set-up for image acquisition (right): tripod with camera pointing to the baseplate where 4 6-well plates are placed.

Video-extensometer calibration was carried out using a calibration tool (provided by the video-extensometer company) positioned at the

same distance from the camera lens as the silicon membrane. Four marks were drawn in the centre of the membrane (see fig B.1) at a distance of 3mm from the centre by means of a permanent marker using a template. Four 6-well-plates were positioned on the Flexercell® plate using the recommended lubricant between the membrane and Delrin[®] supports. Several different pressure curves were run and the stretch of different 6-well-plate membranes was measured by tracking the four markers in real-time. The camera was set up to record a series of frames every 0.05 seconds during the deformation of the membrane for a few stretch cycles. The frames were analyzed by means of the video extensometer software to measure the maximum values of the strain amplitude at the peak strain level. Two parallel marks were used to measure the axial stretch and the other two orthogonal marks to measure the transverse stretch. A stretch cycle chosen randomly among the first 5-10 was thus analysed. In the case of constant deformation the measurements were taken after around 10 seconds from the beginning of the membrane extension. This procedure was applied to skip the first stretch cycles and the first seconds of constant deformation to allow the Flexercell[®] apparatus to reach a dynamic equilibrium. Different types of strain profiles were selected: constant, square and heartbeat deformation waveforms. Amplitudes between 0-5%, 0-10%, 0-15% and 0-20% were set and the stretch values measured by the video-extensometer. The tests were always carried out using just one Flexercell[®] baseplate of the two available in our lab. Two types of tests were carried out: calibration tests with new 6-well-plates to compare the actual silicone membrane extension with the Flexercell® output and input, whilst a second set of tests were carried out to compare the membrane deformation after 24 and 48 hours of cyclic stretch on the Flexercell[®] system.

B.2.2 Experimental ex-situ membrane strain measurement

Tests with a Zwick tensile testing machine to measure the viscoelastic properties of the membrane silicone material were also carried out.

Dog-bone shaped strips of the silicone membrane (thickness 0.5mm) were cut with a central width of 4mm by means of a custom made cutter and positioned on the Zwick testing machine (Zwick/Roell GmbH Group). Tests to failure, hysteresis cyclic tests and relaxation tests were carried out to evaluate the viscoelastic properties of the material. The failure tests were carried out by a crosshead position controlled speed of 3 mm/s using a preload of 0.01 N and recording the elongation by means of a video extensometer. Cyclic hysteresis test were carried out whereby the silicon membrane was stretched and relaxed at a crosshead position controlled speed corresponding to the average deformation of the silicon membrane in the Flexercell[®] for frequencies of 0.1 Hz, 0.5 Hz and 1 Hz and 10% strain, corresponding to 0.16, 0.8 and 1.6 mm/s. Relaxation tests were carried out by reaching a fixed stretch of 20% at a speed of 5 mm/s and recording the force on the sample for 30 minutes. All tests were carried out on three different plates, for three different frequencies: 0.1 Hz, 0.5 Hz and 1 Hz. The tests to measure the changes in the deformation behaviour after 24 and 48 hours were carried out using one plate but taking measurements from three different wells of the same 6-well-plate.

B.2.3 Evaluation of the new pressure-stretch equation

A new pressure-stretch equation was determined by firstly calculating the Flexercell[®] pressure values corresponding to the actual stretch using the Flexercell[®] factory set pressure-stretch equation. The new pressure-stretch equation was determined by curve fitting to these points.

B.2.4 Measured strain for various heartbeat waveforms

The mean of three measured values in the axial and transverse direction was determined for different stretch input values when a heartbeat waveform was imposed. The average value between the axial and transverse directions and the Flexercell[®] outputs were also determined and have been plotted in fig B.2.



Figure B.2: Measurements for different software stretch inputs of the Flexercell[®] response when using the heart beat waveform. Axial and transverse represent the stretch in two orthogonal directions on the Flexercell[®] membrane, the average the mean value between them and the output the Flexercell[®] computer output. Values are means \pm standard deviation.

As can be seen from the results, significant differences were observed between the strain values input, the software output and the actual measured strains. The measured values were almost always lower than the input values, yet they were generally higher than that output on the Flexercell[®] screen. The influence of the frequency of the waveform is evident given that for the same programmed strain amplitude, the actual strain increases with lower frequency. In fact, most of the measured values exceed the required strain value at the lowest frequency (0.1 Hz) with the exception of the highest strain amplitude (20%). There were no significant differences between the axial and the transverse values but a general increase in the standard deviation was observed with increasing strain amplitudes. When the straining machine is used for a long period of time it is also important to know if the straining pattern is maintained constant during the time. Fig B.3 illustrates the behaviour of the silicon membrane before and after 24 hours and 48 hours of cyclic strain at 1 Hz. A change in the straining behaviour of the silicon membrane can be seen, with the membrane becoming more compliant during the test, especially after 48 hours of stretching. The membrane becomes more and more and more compliant as the chosen level of strain is increased. As expected, given that there is no feedback within the Flexercell[®] system, the output remained constant.



Figure B.3: Measurements for different stretch inputs after 0, 24 and 48 hours of heartbeat cyclic stretch at 1Hz. Axial and transversal represent the stretch in two orthogonal directions on the Flexercell[®] membrane, the average the mean value between them and the output the Flexercell[®] computer output. Values are means \pm standard deviation.



Figure B.4: Measurements for different software stretch inputs of the Flexercell[®] response when using the square waveform. Axial and transverse represent the stretch in two orthogonal directions on the Flexercell[®] membrane, the average is the mean value between them, and the output is the Flexercell[®] computer output. Values are means \pm standard deviation.

B.2.5 Measured strain for various squared waveforms

Fig B.4 shows the mean of three measured values in the axial and transverse direction when the square waveform was imposed for different amplitudes. The average value between the axial and transversal directions and the Flexercell[®] machine outputs are also shown.

As can be seen, there are significant differences between the input values and the measured ones. The measured values are almost always lower than the expected values and different to the output of the Flexercell[®]. The measured stretch values are greater or almost equal to the output for all of the imposed strains. The difference between the output values and the measured values becomes more evident for lower frequencies. For higher values of stretch, however, the output values are



Figure B.5: Test to failure of the Flexercell[®] silicon membrane with the Zwick testing machine. A preload of 0.01N was applied.

closer to the measured values. The measured strains do not reach the expected theoretical ones for the frequency of 1Hz but they exceed them for lower frequencies at 10% and 15% stretch inputs and for 20% strain at 0.1 Hz. Higher strains and lower frequencies, in general, result in higher levels of standard deviations. There is, however, a very good match between the axial and transverse strains, even if, for high levels of stretch, higher standard deviations were measured. Fig B.5 shows the behaviour of the silicon membrane when stretched to failure at a strain speed of 5mm/s showing a linear behaviour up to rupture. Fig B.6 shows the relaxation test when the strain is brought up to 20% at a rapid speed of 5mm/s crosshead movement and maintained for 30 minutes. The stress drops from 395 kPa to 366 kPa or about 6.5%.



Figure B.6: Relaxation test when the membrane is kept strained at 20% stretch.

B.2.6 Tests imposing constant strains as input

Fig B.7 shows the measurements taken for different inputs in a series of constant stretching tests. The difference between the inputs and the measured values is also evident.



Figure B.7: Measurement of the stretch for different constant input values in the FX-4000TM software. Axial and transversal represent the stretch in two orthogonal directions on the Flexercell[®] membrane, the average is the mean value between them and the output is the Flexercell[®] computer output. Values are means \pm standard deviation.

B.2.7 Ex-situ membrane material properties

Fig B.8 shows the hysteresis cycles of stretch and relaxation of the silicon membrane when strained up to 10% stretch at different position controlled speeds (0.16, 0.8 and 1.6mm/s) simulating the three deformation speeds inside the Flexercell[®] for the three different frequencies of 0.1, 0.5 and 1.6Hz. As it is possible to observe the three

curves, corresponding to the three different frequencies, are almost totally overlapping and the hysteresis areas are keeping constant. These tests confirm a small level of viscoelasticity of the silicon membrane that it is however greater than the value measured by previous works (Vande Geest *et al.*[165]). Such a viscoelasticity level is thus not completely negligible.



Figure B.8: Hysteresis curves for different Flexercell[®] speed simulating the different frequencies inside the Flexercell[®].

B.2.8 Calculation of the new pressure-strain equation

Fig B.9 shows the new pressure-strain equation for a heartbeat waveform at a frequency of 1Hz curve compared with the factory curve along with the two related equations. It is possible to notice a slight but significant difference due to the underestimation of the Flexercell[®] output of the real strain as it can be seen in fig B.2. Along with the new equation the software and the Flexercell[®] controllers also require the inverse of the equation to pass from strain values to pressure values calculated to be:

$$p_{NEW} = 0.0024011192\epsilon_{NEW}^3 - 0.1899484146\epsilon_{NEW}^2 + 6.1317917385\epsilon_{NEW}$$

where p is the pressure values inside the Flexercell[®] baseplate and ϵ_{NEW} is the membrane strain. Tests with the new equations showed a match between the output values and the measured strains which were used for subsequent tests.



Figure B.9: Curve fitting to the pressure-strain points calculated using the video extensometer measurements.

B.3 Flexercell[®] calibration

The aim of this part of the work was to calibrate the FX-4000TM Flexercell[®] system in order to achieve accurate and reliable static and

This goal was achieved by measuring the dynamic cyclic strains. maximum peak strain and analysing the change in the membrane deformation before and after 24 hours and 48 hours of cyclic strain for different strain amplitudes. Three kinds of tests were carried out. In the first session of tests we compared the strain measured values to the Flexercell® outputs and inputs when a heartbeat, a squared and a constant waveform were applied. A second series of tests was carried out to evaluate the change of the strain behaviour of the FX-4000TM silicon membrane when undergoing cyclic heartbeat strains at 5%, 10%, 15% and 20% strain inputs for 24 and 48 hours. The last series of tests was carried out using a Zwick Testing machine to evaluate the mechanical properties of the 6-well-plate silicon membrane. The FX-4000TM manual (Flexercell[®] Tension Plus System, Flexcell International Company) states that the Flexercell[®] machine is able to reach, in dynamic conditions, a strain up to 12% at a maximum frequency of 1Hz, 17.5% at a maximum frequency of 0.5Hz and 20% at maximum frequency of 0.1Hz. As it is possible to notice from the graphs from fig B.2 to fig B.7 of our tests we measured very notable differences between the measured strains and the inputs. There are also strong differences between inputs, outputs and measured values when the amplitude is maintained constant and the frequency is changed: test at 1Hz, 0.5Hz and 0.1Hz were conducted. Previous works to evaluate the FX-4000TM performances (Vande Geest et al.[165], Woodell et al. [166]) found differences between the Flexercell® input-output and the measured values even if they limited their study to a small number of cases.

Vande Geest *et al.* compared the official data provided by Flexcell Corporation with static and dynamic (1Hz) experimental data, and with the calculated data of a finite element simulation of the mechanical behaviour of the silicon membrane. It is difficult to evaluate the Vande Geest *et al.* tests because the waveform type used by the author is not specified and the Flexercell[®] output values are not shown. Furthermore Vande Geest *et al.* used different values of pressures-strain from those in this study. However, the strain values measured by Vande Geest *et al.*

were found to be greater than those measured in our laboratory and closer to the input values than ours although a remarkable difference is still present especially at higher pressures. The static values we measured are also different from the values measured by Vande Geest, with values greater than the theoretical values.

Woodell et al carried out different tests measuring the Flexercell[®] membrane strain in a similar way and compared these values to the Flexercell[®] output values. In dynamic conditions they limited their study to the measurement strain caused by a sine wave of 10% programmed elongation at a frequency of 0.1Hz. For these settings they found a remarkable difference between the calculated membrane elongation values (around 4% over the time) and the Flexercell[®] output (around 11%). The other calibration values measured by Woodell et al. were at constant strains over the time between sine waves cyclic strain sessions of 10% and 20% strain. In these cases the differences between the Flexercell® output values and the measured values were sometimes greater than 5%. The major difference between the results of Woodell et al and our results is that we have found strains greater than the output values although always lower than the input values. Even if Woodell et al used a sine wave instead of a heart beat waveform and their tests were limited in number they confirm the necessity to calibrate the machine for every waveform type. This need arises from the viscoelastic properties of the system (baseplate + gel + membrane) that creates a time dependency of the system mechanical characteristics. The viscoelasticity of the silicone membrane and of the anti-friction gel between the membrane and the loading posts can play a part in the difference between measured and output values. Because of the viscoelasticity the force to be applied on the membrane to obtain a determined strain amplitude is usually greater than that expected in dynamic conditions. This becomes more evident at higher amplitudes and higher frequencies. Furthermore, higher differences between axial and transversal strains were noticed for higher amplitudes due to the lack of time for the membrane and the lubricant to distend properly during a duty cycle. The viscoelasticity of

the system is also confirmed by the fact that for higher frequencies and amplitudes it has been noticed that the minimum value of strain is not always zero but can vary between 0% and 1% showing incomplete membrane (and lubricant) relaxation once the negative vacuum pressure is zeroed. Our tests showed a limited viscosity of the 6-well-plates membrane. Thus, the lubricant viscoelasticity is the probable cause for viscoelasticity. Fig B.3 shows a difference in the mechanical characteristic of the silicon membrane after 24 and 48 hours of strain: the results show increase compliance after a long period of cyclic strain especially for higher amplitudes. Also a few strain cycles can induce changes in the membrane compliance, different levels of measured strain were observed when the membrane was preconditioned by a few strain cycles to higher strain levels.

It is evident that the frequency has a strong influence on the FX-4000TM performance. It is thus necessary to set up new pressure-strain equations for every frequency value to match the strain output values with the real strains undergone by the 6-well-plates membranes. In our case we found a new pressure-stretch equation by fitting the measured stretch values on a curve and calculating the parametric equation to insert in the Flexercell[®] software. It also appears evident that the cyclic stretching time has an effect on the mechanical characteristics of the silicon membrane. This is more evident for higher stretch values compared to low stretch values. This effect should be taken into account especially for high amplitude tests carried out for long periods of time. It has thus been possible to recalibrate the Flexercell[®] system for a specific pattern: a heartbeat waveform at a 1Hz frequency. The new calibration curve has been obtained by fitting the maximum values of the stretch measurement taken for the same frequency and different amplitudes. A different calibration method was set for a specific frequency and pattern which was not valid for other waveforms. The calibration method used at the Flexercell[®] factory assumed that the stretch values for a determined vacuum pressure inside the 6-wells-plate would be reliable for different waveform patterns. Our tests showed this is not always true. Our calibration method has the disadvantage that it does not guarantee the correspondence between the output value and the real stretch undergone by the membrane at every instant but it guarantees the same maximum value that is the most significant one for most of the experiments for which the Flexercell[®] machine is used. Taking into account that there could be differences from one straining machine to another due to fabrication process variations, working environment differences, the absolute values measured in this study could be different from those measured by the Flexcell Corporation and this could be a possible limitation of this study. Another limitation of this study is that just one of the two available platforms was used every time. The use of more than one baseplate at the same time could change the stretch values.

Appendix C

Culture chamber CAD drawing



Figure C.1: Pro-Engineering drawing of the bioreactor chamber.

Appendix D

Mock coronary artery construction process

D.1 Introduction

A simple way to obtain sylgard[®] mock arteries of 3 mm internal diameter and 0.4 mm constant wall thickness, suitable for mechanotransduction of cells will be described.

D.2 Material & Methods

Sylgard[®] is a silicon elastomer that can be easily created in a laboratory by mixing a viscous base with a liquid cure agent. The base/cure agent mixing ratio determines the mechanical properties of the material: an increase of the base/cure agent ratio increases the Young's modulus of the material. Mechanical tests on bone-shaped samples of porcine coronary arteries and sylgard[®] were performed. They established that a base/cure ratio of 16:1 is the most suitable to mimic a natural coronary artery, see fig D.1.



Figure D.1: Stress-strain relationship of bone-shaped sample of sylgard[®] cured at different base-cross linker ratios.

D.2.1 Sylgard Preparation

The base and curing agent were weighed on a laboratory scale and ratios of 10:1, 11:1, 14:1 and 16:1 were prepared. The base and curing agent were mixed for 30 seconds in a small container. During this process air bubbles formed inside the mixture. To remove all air bubbles from the mixture, the container was placed in a Nalgene vacuum desiccator which was attached to a vacuum pump. Once all of the air bubbles were removed the Sylgard[®] material was moulded into flat sheets and cured in an oven at 120 C for 1 hour. To study the effect of curing time, Sylgard[®] with a base to curing ratio of 16:1 was retained in the oven for two different time periods, 1 hour and 16 hours. In addition Sylgard[®] with a base to curing ratio of 16:1 was moulded into a custom built rig for mock coronary artery preparation and cured for 16 hours at 120 C.

D.2.2 Mechanical Tests

Uniaxial tensile tests were carried out on dogbone-shaped samples obtained from the Sylgard[®] prepared in flat sheets and tubes using a displacement controlled tensile testing machine (Zwick Material Testing, Germany) and utilizing a 20 N load cell. This enabled comparison between the mechanical properties of Sylgard[®] and coronary arteries. In this way the influence of fabrication parameters, such as base to curing agent ratio and curing time could be investigated to generate Sylgard[®] which best matched coronary artery properties. The samples were marked and a precise video extensometer (MESSPHYSIK Materials Testing, Austria) was utilised to measure the strain. A strain rate of 1 mm/sec was used and the preload was chosen as 0.001 N as the criterion for the testing machine to initiate recording the strain. Samples were preconditioned by five cycles of extension to 10% strain and then loaded to failure.

D.2.3 Mock Coronary Artery Rig Design and Functionality

A simple rig was designed in Pro/ENGINEER (Parametric Technology Corporation, Needham, Massachusetts, USA) to create mock arteries with an inner diameter of 3 mm and outer diameter of 3.8 mm, see fig D.2. The rig was designed such that idealised coronary artery geometries could be manufactured consistently and with no artefacts or damage to the Sylgard[®] tubes. The construction rig consisted of two stainless steel end spacers within which a polished stainless steel inner mandrel was placed. The stainless steel mandrel measured 3 mm in diameter, see fig D.3 and fig D.4. Both end spacers were tapered and slots were cut on the tapered ends to allow for transfer of fluid through these predefined channels at either end of the mandrel. Plastic outer moulds were manufactured from PTFE shrink tubing such that they had an inner diameter of 3.8 mm, see fig D.5. To do this 6 mm shrink tubing (Tyco Electronics, Kessel, Belgium) was heated onto a 3.8 mm diameter polished stainless steel pin using a heat gun at 150C. The pin was shaped like a hook at one end to enable the

release of the mould. This 3.8 mm PTFE cylindrical tubing was then used to form the outer shell of the Sylgard[®] mould and was placed over the end spacers as shown in fig D.3. A non stick coating was also sprayed onto the stainless steel pin prior to heat shrinking the PTFE tubing. This made it easier to release the heat shrink tubing once it had moulded to the diameter of the pin. The mock artery mould was constructed by inserting the 3 mm stainless steel mandrel inside the PTFE shell and fixing both ends inside the stainless steel end spacers such that both cylinders were concentric. The end spacers were specially designed to fix the mandrel in the centre such that the resulting mock artery had a uniform thickness along its length. The plastic mould was cut such that it was longer than the mandrel and covered the four holes cut in the end spacers. This ensured that the elastomer was able to pass through these holes to fill out the space between the inner mandrel and the outer PTFE tubing. PTFE isolating tape was used to avoid air infiltration between the plastic mould and the holders.

The elastomer was inoculated into the rig by suction of a vacuum pump linked to one end of the rig with a pneumatic connector. Fluid sylgard[®] came out from the holder into the coaxial space by means of four small holes created on it (fig D.5) and it was let run until the whole coaxial space was filled (fig D.6). At this stage the rig was kept overnight inside an oven for curing at 120°C. The maintenance of the coaxial symmetry during this phase was safeguarded by an external mould. The two parts of the mould were fixed with screws to avoid opening in the oven, see fig D.2. The sylgard[®] datasheet suggests keeping the polymer for one hour at 100°C for curing. This temperature is inadequate in this case due to the presence of a plastic tube around the polymer that prevents the heat from diffusing homogenously. In fact, it was noticed that the level of curing was lower in the centre of the mock artery if the sylgard[®] manual instructions were followed. The heat diffuses more from the open inlet and outlet than from the surrounding mould. The mock artery was thus kept in the oven overnight at a temperature of 120°C. It is important at this stage not to close the holder intakes to allow



Figure D.2: Pro-Engineering assembly of the rig for the production of mock coronary arteries

the elastomer to expand longitudinally during curing.



Figure D.3: Image of mandrel linked to the holders and surrounded by the coaxial tube.

Once the polymer was cured the rig was taken out of the oven and let cool down for 30 minutes at room temperature. The mould was disassembled and the two holders removed leaving the coaxial tube



Figure D.4: Assembled rig, ready for the elastomer injection.

formed by the tubular sandwich: mandrel + cured mock artery + plastic tube. The external plastic tube was removed by unwinding it with a spiral movement taking care not to break the underlying mock artery, see fig D.7. Subsequently the mock coronary artery was removed from the mandrel by injecting acetone between it and mandrel with a needle, see fig D.8. At this stage care must be taken not to pierce the elastomer with the syringe needle when injecting the acetone. The mock artery was delicately slipped off the mandrel using the fingers to distribute acetone all along the length of the mandrel. The mock artery was then washed and conserved in distilled water until use.

Once the mock coronary artery was removed it was possible to reuse the rig. However it was necessary to clean the holders from the residual cured sylgard. This was easily done with the help of alcohol and a needle to clean the nozzles. It is important at this stage not to scratch the channels of the holders. Micro-furrows can alter the fluid-dynamics of the system and lead to the formation of bubbles during the Sylgard flow.

BASMC were subsequently seeded onto a Sylgard[®] mock coronary artery and left in culture for 72 hours. Results were analysed using DAPI staining. In order to analyse stained cells under the florescence microscope, the mock coronary artery was opened longitudinally with a



Figure D.5: Detail of the rig with in evidence one of the holes for the release of the uncured Sylgard[®] between the mandrel and the plastic tube. The polymer reaches the mandrel area through four channels grooved into the holder.

scalpel, the coronary artery flattened between two microscope glass slips and positioned under the microscope lens. Similar tests were carried out on BASMC seeded mock arteries mounted into the bioreactor. The Sylgard[®] surface was treated with 70% sulphuric acid and covered with fibronectin. Cells were analysed after 24 hours with DAPI staining.


Figure D.6: The vacuum pump is linked to the rig by means of a tube. The pump suction moves the polymer through the rig from a Sylgard container.



Figure D.7: Removal of the plastic mould from the mock coronary artery. The mandrel is in grey.



Figure D.8: Injection of acetone between the mandrel and the Sylgard mock coronary artery.

Appendix E

Sirolimus coated stents from Medtronic

Sirolimus-coated Driver[®] drug eluting stents were specially coated for this project in the Maastricht (Netherlands) Medtronic research center. The stents were provided sterile and weighed before and after coating in order to determine the quantity of drug on the struts of the stent. Before the drug coating, stents were covered with a polymeric primer, Poly(ethoxyethyl methacrylate) as a carrier for the drug.

Along with the data regarding the stents' weight and dimensions, Medtronic provided the Sirolimus[®] elution profile *in vitro* at 37°C in static conditions.



Figure E.1: SEM images of primed and bare metal stents with and without drug coating, from Medtronic Maastricht



Figure E.2: Sirolimus elution profile in vitro