Determining the suitability of Human Umbilical Vein Endothelial Cells (HUVEC) and Endothelial Colony Forming Cells (ECFC) against Human Coronary Artery Endothelial Cells (HCAEC) as cell models for studying cardiovascular disease.

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Abstract

Cardiovascular diseases (CVD) are the most common cause of mortality globally and can be defined as disorders of the heart and blood vessels. CVD mortality rate and complications are on the rise, especially in developing countries. Age, obesity, smoking, diet, hypertension, genetics and diabetes are a few of the risk factors associated with CVD. Atherosclerosis is a significant contributor to CVD and is characterised by an accumulation of atherosclerotic plaque in the arteries, over time causing narrowing of the vessel lumen, limiting flow and causing tissue ischemia as well as acute occlusion due to atherothrombosis. Atherosclerosis initiates via dysfunction or damage of the endothelial cells (EC) that line the vessel wall. Endothelial dysfunction is amplified at branch points and curved sections in the vessel wall, exposed to disturbed blood flow patterns and limited in regions of arteries exposed to laminar flow, leading to focal development of disease. The protection in sections that experience laminar flow is predominantly driven through shear-regulated activation of *KLF2*, *KLF4* and *Nrf2* and suppression of *NFκB* activation in endothelial cells. In turn regulating the expression of over 1000 genes to modify endothelial behaviour and limit oxidative stress, inflammation and permeability. Huge amounts of research have been carried out to understand the processes, using a number of cell models to perform the research, including human coronary artery endothelial cells (HCAEC), which might be considered the gold standard, as well as human umbilical vein endothelial cells (HUVEC), because of their lower cost. This project assessed the relative gene expression of HCAECs and HUVECs cultured under identical conditions: static

culture, 'plaque promoting' oscillatory shear stress (±5 dynes/cm^2, 1Hz), or 'plaque-limiting' physiological laminar shear stress (15 dynes/cm^2). We concurrently evaluated the response of endothelial colony forming cells (ECFC), under the same conditions, evaluating whether these blood isolated cells can also be used as a model to investigate pathological processes involved in atherosclerosis from different patient groups. Our data concluded that KLF2 is significantly upregulated by laminar flow in HCAECs, whilst KLF4 is significantly upregulated by flow in all 3 cell types. The KLF2 and KLF4 responsive genes demonstrated a range of responses, with only eNOS showing significant upregulation in HCAECs by laminar flow. Additionally, *Nrf2* regulated genes showed the largest upregulation in HCAECs under laminar flow whereas little difference was seen in within the *NFκB* regulated genes. The results obtained from the project provide evidence that HCAEC remain the most suitable cell model, with HUVECs demonstrating potential with additional work however ECFCs are rendered as unsuitable, due to their inability to provide significant upregulation of atherosclerosis-relevant genes.

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1.0 Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of fatality word-wide and is a continuing major problem within the healthcare industry, (Jackson, 2011). World Health Organisation (WHO) have recently updated cardiovascular disease statistics in a bid to highlight the growing impact and concern they have on the community and healthcare facilities. Rheumatic heart disease, cerebalvascular disease and coronary heart disease, amongst many more, fall under the cardiovascular disease category, with cardiovascular disease generically identified as 'disorders of the heart and blood vessels', (World Health Organization, 2019). In 2019, WHO have reported that CVD is estimated to account for 31% of deaths globally, (World Health Organization, 2019) (Brennan et al., 2017), with 85% of these CVD deaths a consequence of heart attacks or strokes, particularly prevalent in low and middle-income countries (World Health Organization, 2019). The human body's vascular system's primary function is to supply every tissue with adequate oxygen and nutrients (Hahn and Schwartz, 2009). Impaired vascular function can lead to the onset of cardiovascular problems; such as atherosclerosis (Hahn and Schwartz, 2009).

1.2 Atherosclerosis

Atherosclerosis is a major cause of CVD and is initially asymptomatic, developing over decades. Atherosclerotic plaque formation within arteries can limit blood flow leading to tissue ischemia, for example, angina if in the heart or peripheral vascular disease, causing limb ischemia. Disruption of atherosclerotic plaques, by plaque rupture, plaque erosion or eruption of a calcified nodule, can trigger thrombosis and lead to acute occlusion, giving rise to acute ischemia, tissue damage, loss of organ function and death (White et al., 2016). If in the heart, it may trigger myocardial infarction, if affecting the blood supply to the brain, it can cause a stroke.

Atherosclerosis is a lipoprotein-driven, chronic inflammatory disease of the arterial wall involving progressive lesion formation and frequently causes narrowing of the arterial lumen (Weber and Noels, 2011). Atherosclerosis initiates at sites of disturbed flow, where reduced nitric oxide bioavailability allows smooth muscle cells to migrate and proliferate and form an intimal hyperplasia containing both smooth muscle cells and extracellular matrix (Hahn and Schwartz, 2009). Concurrent with this, the barrier function of the endothelium is also reduced, allowing more low-density lipoprotein to pass into the arterial wall. This becomes trapped within the neointima, where it oxidises over time, triggering the increase in expression of inflammatory cytokines (e.g. *CCL2*) and expression of adhesion molecules on the endothelium (Hahn and Schwartz, 2009). Uptake of ox-LDL by recruited monocyte-derived macrophage as well as smooth muscle cells, contributes to foam cell formation, further driving cytokine expression, inflammatory cell recruitment, endothelial dysfunction and plaque expansion (Spartalis et al., 2019).

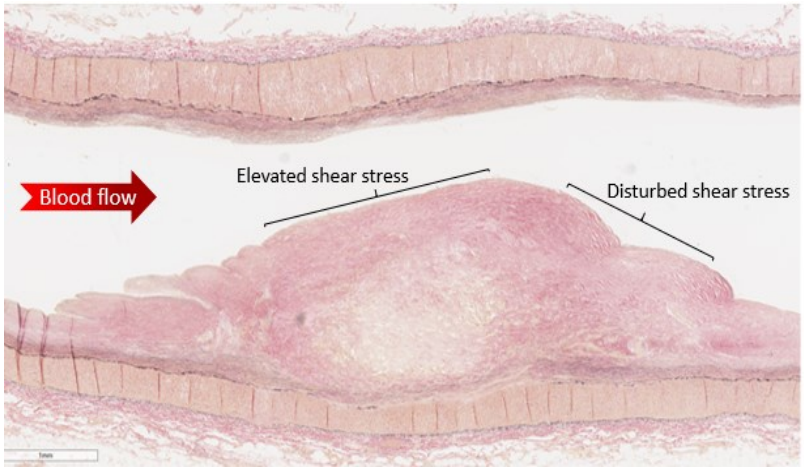


Figure 1: *Atherosclerotic plaque.* An example of atherosclerosis, plaque formation within an artery wall *in vivo*. This diagram demonstrates elevated sheer stress upstream to the blood flow and disturbed sheer stress on the downstream surface, both of which are crucial in the modification of endothelial cell behaviour. (White, Newby and Johnson., 2016).

Atherosclerosis develops focally at predicable sites within the arterial system, as demonstrated by figure 1. Bifurcation sites and regions of high curvature in arteries exposed to disturbed shear stress are predisposed to the onset of atherosclerosis (Dai et al., 2004). Regions exposed to low time-averaged wall shear stress and high oscillatory shear index/multidirectional flow strongly correlated to plaque formation (Hoogendoorn et al., 2019). The association between the focal nature and atherosclerosis and flow pattern demonstrates the key role that vascular endothelium plays in regulating the processes that drive atherosclerosis.

1.3 Vascular Endothelium

Defined as a monolayer of endothelial cells that line all blood vessels and lymphatics (Dimitris Tousoulis, 2019), the vascular endothelium is responsible for the regulation of vascular tone, thrombolysis and transport of molecules to the sub-endothelial space. This in turn regulates smooth muscle cell function, controlling proliferation and migration (Boulanger, 2016). Endothelial cells are mechanosensitive and are able to respond and react to different blood flow patterns, sensing the frictional force exerted upon their surface (shear stress) and cyclical stretch, converting these mechanical stimuli into a biological response (Baratchi et al., 2017, Charbonier et al., 2019).

1.4 Endothelial Cells and Mechano-sensing

Proteins belonging to the EC glycocalyx play a major role in transmitting shear stress to mechanoreceptors, which include integrins, ion channels and junctional receptors (Baratchi et al., 2017). Through syndecans connected to the cytoskeleton, or through glypicans connected to the plasma membrane, the glycocalyx can also transduce other shear sensors embedded in the plasma membrane, or focal adhesions and intracellular junction proteins via the cytoskeleton (Givens and Tzima, 2016).

The release and synthesis of pro and anti- anticoagulant factors, alongside growth factors, vasomotor regulators, Von Willebrand Factor and *TNF-a*, is dependent upon

mechanotransmission through EC ion channels. Ion channels play a key role when it comes to the sensing of shear stress in the vascular endothelium and when shear stress-dependant Ca^{2+} channels are stimulated, a rapid influx of Ca^{2+} is released into the EC cytoplasm, resulting in activation of endothelial nitric oxide synthase (*eNOS*) and NO (Baratchi et al., 2017) . In addition, transmission of force exerted by shear stress on the cell surface, to a tri-protein complex in intercellular adherent junctions, activates a phosphorylation signalling cascade downstream of platelet endothelial cell adhesion molecule-1 (*PECAM1*) (Baeyens and Schwartz, 2016). The cascade includes phosphorylation and activation of integrins that bind extracellular matrix (ECM) components and in turn activates Src-dependent signalling which eventually activate transcription factors *KLF2* and *KLF4*, via ERK5 modulating the expression of over 1000 genes in endothelial cells to mediate the laminar flow-induced quiescent atheroprotective phenotype (Dekker et al., 2006).

Mechanotransduction in EC through the endothelium is further regulated by focal adhesions, lying on the basal plasma membrane these protein complexes also drive integrin adhesion to the extra cellular matrix (ECM). Both EC and vascular smooth muscle cells are subjected to cyclic stretch by the pulsatile nature of blood flow. The literature is slightly misleading, with short-term exposure triggering an adaptive response, often interpreted as inflammatory (Ramella et al., 2019). EC exposed to 'short-term' cyclic stretch pathologically change their function, structure and morphology (Ramella et al., 2019). In contrast, *in vivo*, the cyclic strain is orientated 90° to shear stress, with laminar flow experienced along the long axis of the cell, with 3-5% cyclic strain across the cell's short axis. This enhances the quiescent, atheroprotective phenotype of endothelial cells (Lu and Kassab, 2011).

1.5 Nitric oxide

Under normal functional conditions *in vivo*, the endothelium is able to regulate the production of soluble gas vasodilator; nitric oxide (NO), which is continuously synthesized from L-arginine by calcium co-comodulant enzyme; endothelial nitric oxide synthase (*eNOS*) (Weber and Noels, 2011). The activity of *eNOS* is regulated by shear stress, particularly through Ca^{2+} signalling, but also through phosphorylation cascades, to modulate vessel tone of the large arteries, dependent on the blood supply needs of the distal tissue. The physiological functions of NO are crucial, acting directly on local ECs and underlying smooth muscle cells, participating in nitrosylation reactions to regulate local cell

growth and play a protective role as a consequence of vessel wall injury (Weber and Noels, 2011). Alongside supressing major inflammation regulator *NFκB*, NO also regulates platelet function and contributes to anti-thrombotic function of the endothelium. Platelet and endothelium-released NO inhibits platelet adhesion to the vessel wall by promoting the production of cyclic GMP (cGMP) therefore preventing thrombosis (Freedman and Loscalzo, 2003).

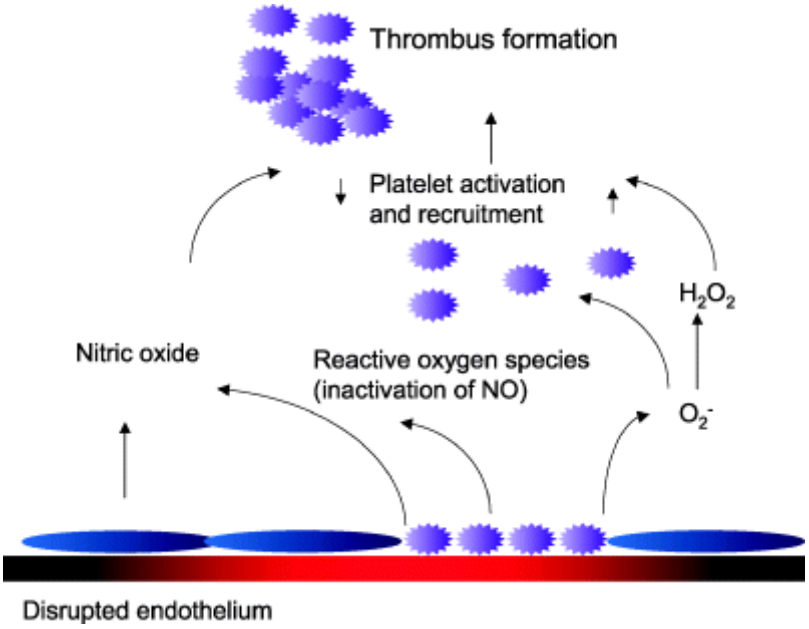


Figure 2: *Formation of a thrombus.* Healthy/ uninjured endothelium produces sufficient NO to prevent activation and adhesion of platelets, therefore preventing thrombus formation. When endothelium becomes damaged, platelet adhesion is increased, releasing prothrombotic substrates which promote the recruitment of additional platelets thereby contributing to thrombus formation (Freedman and Loscalzo, 2003).

1.6 Endothelial dysfunction

Disturbed flow doesn't activate the athero-protective signalling and gene expression pattern that is induced by laminar flow, priming the endothelium for inflammation and dysfunction (Warboys et al., 2011). The lack of the laminar flow-induced phenotype in endothelial cells increases the relative production of reactive oxygen species (*ROS*), priming the activation of *NFκB*. *eNOS* activity is reduced at sites of disturbed flow, coupled with quenching the NO produced by *ROS*, leading to a very much reduced bioavailability of NO (Warboys et al., 2011). The enhanced activation of *NFκB* increases the expression of adhesion molecules (particularly *VCAM1* and *ICAM1*) and the production of cytokines (e.g.

CCL2) stimulating monocyte adhesion. On a molecular scale, endothelial dysfunction is therefore defined as an increase in *ROS* production, reduced bioavailability of NO and the increase in adhesion molecule and cytokine expression (Charbonier et al., 2019). In a pathological state, *ROS* can negatively interact with endothelial cell ion channels and transcription factors, establishing alterations in lipid metabolism and heightening cell permeability potential, apoptosis, growth factor modifications, contributing to endothelium dysfunction (Carnevale et al., 2018). Endothelial dysfunction is recognized as a major contributor in the initiation of atherosclerosis and is highly associated with changes which create a diminished output in the production of the bioavailability of nitric oxide and an increase in oxygen free radical formation (Satta et al., 2017). Oxidation of lipoproteins and foam cell formation relate back to facilitation of vascular inflammation and are demonstrated to be a consequence of a reduced output of nitric oxide bioavailability also (Silva et al., 2012).

1.7 Oxidative Stress and Reactive Oxygen Species

Growing evidence supports the important and significant role that oxidative stress plays in atherosclerosis, alongside its important influence in disturbed blood flow pattern phenotype of endothelial cells (Charbonier et al., 2019). Oxidative stress can be defined as the imbalance in production of reactive oxygen species (*ROS*) and the anti-oxidant defence system, which favours the increase of the toxic product of oxygen metabolism, *ROS* (Howden, 2013, Satta et al., 2017) A large number of cardiovascular risk factors contribute to, or mediate their effect on the disease process through increasing endothelial dysfunction. Diabetes, obesity, smoking, and dyslipidaemias are not only all pro-atherogenic and risk factors for CVD, they also increase permeability dysfunction in endothelial cells through an imbalance in *ROS* production (Mundi et al., 2017). *ROS* are produced naturally within endothelial cells and smooth muscle cells, occurring within the vasculature, and their components include both free radical and non-radicals and participate in cell growth, death, inflammatory response and regulation of vascular tone (Carnevale et al., 2018). *ROS* are generated within the vessel wall from a variety of mechanisms including NADPH oxidase (Kattoor et al., 2017). NAPDH has shown crucial involvement to being a major source of *ROS* and elsewhere, extensive research has been recorded, showing a strong correlation between NAPDH and adhesion molecule expression, monocyte infiltration, SMC proliferation and superoxide anion generation

(Kattoor et al., 2017). Depletion of *eNOS* substrate, L-arginine, or cofactor tetrahydrobiopterin (BH4), may trigger *eNOS* uncoupling and *ROS* production further decreasing the bioavailability of NO and an increasing in O2 generation. An elevated production of *ROS*, or a failure to quench it, results in oxidative stress that damages DNA, RNA and proteins and promotes pathology and molecular aging.

1.8 Atherogenesis

A reduction in the bioavailability of NO, coupled with *ROS* generation and an increase in growth factor exposure due to increased endothelial permeability, promotes proliferation and migration of smooth muscle cells (SMCs) (Kattoor et al., 2017; Chen et al., 2016). In healthy blood vessels SMCs remain in a quiescent state with a very low rate of proliferation. SMCs play a key role in the regulation of blood flow, blood pressure and maintenance of vascular tone. Their quiescent state is maintained through the expression of the proteins such as smooth muscle actin, smooth muscle-calponin and smooth muscle 22-alpha, (Chen et al., 2016). Reduced exposure to NO and increased growth factor exposure allows a few SMCs to undergo 'phenotypic switching' (Basatemur et al., 2019; Bennett, Sinha and Owens, 2016) This phenotypic switch is associated with an upregulation in secretion of pro-inflammatory mediators, tissue factor (TF), matrix metalloproteinase (MMP's) and inflammatory cytokines, resulting in migration to the intima, proliferation and production of extracellular matrix (ECM) (Jeremy et al., 1999).

Under normal circumstances, the endothelial barrier has highly selective permeability, minimising ingress of blood-borne factors, importantly low-density lipoproteins (LDLs). The amplification of endothelial dysfunction at sites of disturbed flow, reduces barrier function, increasing permeability as well as promoting an 'activated' EC state (Mudau et al., 2012). Increased rates of LDL entry at sites of disturbed flow increases the trapping of LDL within the intimal hyperplasia generated by SMC migration and proliferation. Trapped LDL becomes modified by oxidation and glycation reactions, promoting inflammatory signalling. Activated EC cells increase expression of monocyte adhesion molecules (*VCAM1 and ICAM1*) and chemoattractant cytokines e.g. TNFα, *CCL2* and CX2CL3 promoting immune cell rolling, tethering and firm adhesion, enabling trans-endothelial migration of monocytes to the sub-endothelium. Here, recruited monocytes transform into macrophages via cytokine stimulation where they take up oxLDL via scavenger receptors, transforming into foam cells if the amount is high. Continued high levels of ingress of LDL

at sites of disturbed flow creates a state of localized inflammation within the intima, encouraging further recruitment of immune cells in turn increasing producing of inflammatory cytokines, accelerating the formation of an atherosclerotic plaque (Baker et al., 2011).

1.9 Athero-protective phenotype of ECs

Unidirectional Laminar shear stress exerts its atheroprotective effect on the endothelium predominantly through the upregulation of Krupple-Like Factors *KLF2*/*KLF4*, activation of *Nrf2* (encoded by gene NFE2L2) (Howden, 2013; Satta et al., 2017) and a suppression of pro-inflammatory signalling via *NFκB* (Hahn and Schwartz, 2009; Satta et al., 2017; Warboys et al., 2011).

1.9.1 Krupple Like Factor 2/4

Krupple-like factors (KLF), a family of transcription factors, accumulate mainly in the cell nucleus and can act as either transcriptional activators or repressors, which function physiologically by either DNA-binding or interacting with their co-factors to express their effects (Jain et al., 2014). *KLF2* and *KLF4* collectively regulate over 1000 genes that control anti-inflammatory and anti-thrombotic properties of ECs, modulating barrier function of the vascular endothelium and EC quiescence (Theodorou and Boon, 2018). Upon induction of the KLFs through high shear stress, *KLF2* and *KLF4* translocate to the nucleus. One of their major co-factors for transcriptional activation is p300, which is also a co-factor for *NFκB*. (Jain et al., 2014). Competition for p300 between *KLF2*/4 and *NFκB* is one of the ways which laminar flow reduces *NFκB*-mediated gene expression, with the elevated expression in laminar flow favouring the upregulation of *KLF2*/4 responsive genes (Jain et al., 2014).

The group of protein kinases collectively known as MAP Kinases, induce cellular mechanisms such as apoptosis, proliferation and inflammation. Pathways activated by inflammatory cytokines include the JNK and p38 pathways to activate transcription factor AP1 and activating transcription factor 2 (ATF2) (Warboys et al., 2011). The MAPK and *NFκB* pathway are both clearly indicated in endothelial activation at regions of disturbed flow

however laminar shear-induced *KLF2* reduces MAPK's proinflammatory gene expression through inhibition of the nuclear localization of ATF2 (Fledderus et al., 2007).

KLF2 and *KLF4* upregulation by shear stress is predominantly mediated via a different MAP kinase extracellular signal-related kinase 5 (ERK5), promoting the induction of the athero-protective phenotype in arterial ECs (Dekker et al., 2006; Parmar et al., 2006). Laminar shear stress induces the phosphorylation of ERK5, which in turn increases the expression of both *KLF2* and *KLF4*, thereby coordinating the upregulation of over 1000 genes, with examples in table 1 (Dekker et al., 2006; Parmar et al., 2006). ERK5 possess a long COOH terminal, which is responsible for the activation of downstream transcription factors involved in anti-inflammatory processes, through transcriptional activity domains (Bera et al., 2014). Therefore, ERK5 activation is associated with increased quiescence, reduced apoptosis and migration, alongside reduction in the expression of adhesion molecules, *VCAM1* and *ICAM1* (Ramella et al., 2019). Studies using animal models have shown that ERK5-deificent mice have a short life span and die due to cardiovascular defects and angiogenic failure (Nithianandarajah-Jones et al., 2014).

Table 1: Target genes of KLFs

Target Genes of *KLF2*/4	**Gene Abbreviation**	**Function**
Thrombomodulin	*THMB*	Anti-thrombotic/ Anti-inflammatory
Nephroblastoma Overexpressed	*NOV*	
Peptidase Inhibitor 16	*PI16*	
Endothelial Nitric Oxide Synthase	*eNOS*	Regulates NO production/ Anti-inflammatory
Nitric Oxide	*NO*	Anti-inflammatory/ Vasodilator
Von Willebrand Factor	*VWF*	Haemostasis
Adrenomedullin	*ADM*	Vascular tone regulation
Nad(P)H Quinone Dehydrogenase 1	*NQO1*	Vascular tone regulation
Protein C	*Protein C*	Anti-thrombotic

In arterial endothelial cells, examples of genes responsive to *KLF2*/4 include; *eNOS*, *THMB*, peptidase inhibitor 16 (*PI16*) and neuroblastoma overexpressed (*NOV*). As previously mentioned, *eNOS* regulates the production of NO under laminar shear stress, promoting an anti-inflammatory and atheroprotective effect on the cell (Weber and Noels, 2011).

Expressed in all tissues and synthesized predominantly in endothelial cells, thrombomodulin is an integral transmembrane protein whose transcription is regulated by

shear stress through involvement of transcription factor; *KLF2* (Yau et al., 2015). Healthy vascular endothelium expresses various anti-coagulant properties, including *THMB*, which serves a purpose of down-regulating platelet mechanisms and thrombotic events, essentially preventing the formation of a thrombus (Martin et al., 2014). Extensive research has been conducted into the activation of thrombomodulin through laminar shear stress and it has been demonstrated to drive three anti-coagulant activities, including: catalysing thrombin-induced activation of protein C to activated protein C, binding with thrombin to prevent conversion of fibrinogen to fibrin and activation of platelets, fV, fVIII, fXI, and fXIII, and catalysing the inhibition of thrombin by anti-thrombin (Yau et al., 2015). Through the expression of thrombomodulin, endothelial cells can regulate protein c activation, conjunctively responsible for regulating thrombosis and decreasing inflammatory responses, reducing downstream effects of cell apoptosis in response to damage and injury (Yau et al., 2015). Thrombomodulin also holds direct anti-inflammatory activity where it reduces cytokine formation in the endothelium simultaneously decreasing leukocyte-endothelial cell adhesion (Esmon, 2003).

1.6.2 Nuclear Erythoid-2 Like Factor

First identified as a component involved in the regulation of oxidant and antioxidant gene expression by Moi et al in 1994, *Nrf2* is now recognized as a major regulator of the antioxidant/oxidant balance and directs cellular responses to oxidative and free radical stress (Satta et al., 2017). *Nrf2* coordinates the expression of many of the enzymes involved in the antioxidant defence that counteracts and protects the cell from damage(Howden, 2013; Satta et al., 2017). Sustained high-level activation of *Nrf2* may however be detrimental. The White lab have shown that overexpression of *Nrf2*, or regulated genes *OSGIN1*+2 cause cell detachment and may be a trigger of endothelial erosion and resultant thrombosis and infarction (Satta et al., 2019).

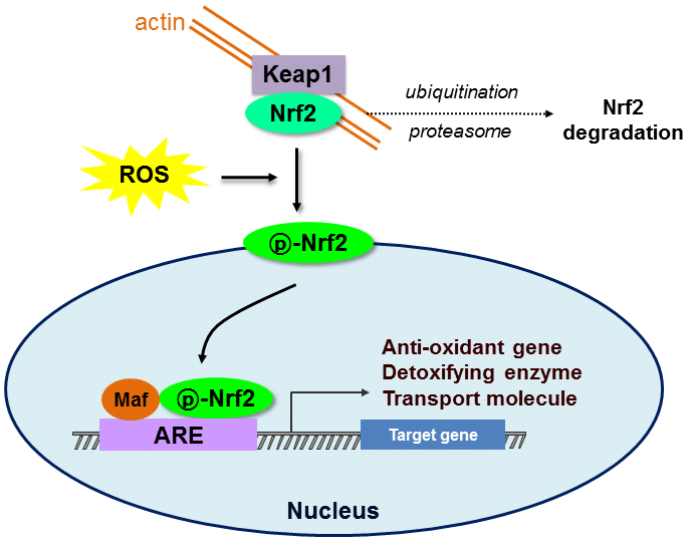


***Figure 3**: NRF2/KEAP1 Signalling Pathway*. Diagram of the *Nrf2* and Kelch-like-ECH associated protein pathway. We can see demonstrated here that under normal conditions *KEAP1* is suppressing the activation of *Nrf2*, *KEAP1* is therefore responsible for constant ubiquination of *Nrf2* within the proteasome of the cell. Alternatively, oxidative stress (*ROS*) causes inactivation of *KEAP1* resulting in phosphorylation of *Nrf2* and accumulation in the nucleus. Here, *Nrf2* heterodimerises with small MAFS (SMAFS) where it binds to antioxidant response element (ARE) activating appropriate genes (Oh and Jun, 2017).

Laminar shear stress activates *Nrf2* through two complementary mechanisms, firstly through lipid peroxides (Warabi et al., 2007) and secondly via a *COX-2* mediated pathway (Hosoya et al., 2005). Without activation, *Nrf2* is bound to its inhibitor *KEAP1*, which mediates its ubiquitination and degradation by *Cu13* (Mimura and Itoh, 2015; Warabi et al., 2007). The interaction between *KEAP1* and *Nrf2* can be electrophilically attacked, liberating *Nrf2*allowing translocation to the nucleus (Satta et al., 2017) Lipid peroxidation is strongly correlated to *Nrf2* activation, however it is unclear how shear stress leads to lipid peroxidation signalling (Warabi et al., 2007). Once in the nucleus, *Nrf2* heterodimerizes with small MAFs (sMAFs), and binds antioxidant regulatory element (ARE) (consensus binding sequence (A/G)TGACTCAGCA), this complex upregulates enzymes and proteins which have crucial functions in protecting the cell from toxicity and oxidative stress (Warabi et al., 2007) (Satta et al., 2017; Mimura and Itoh, 2015).

In addition, laminar flow-induced *KLF2* expression also enhances upregulation of antioxidant and detoxifying responses, through priming *Nrf2* activation. *KLF2* increases the nuclear translocation and accumulation of *Nrf2*, through an undefined mechanism,

synergising with the other mechanisms by which shear stress activates *Nrf2* to provide the atheroprotective effect of laminar flow (Fledderus et al., 2008; Satta et al., 2017). Not only does *KLF2* prime *Nrf2* for activation, it is also crucial for optimal activation of shear stress-mediated *Nrf2* binding to the ARE (Boon et al., 2008). In HCAECs, *Nrf2* regulate the expression of a number of genes examples of which are summarised in table 2.

Table 2: Target genes of Nrf2.

Target Genes of *Nrf2*	**Gene Abbreviations**	**Function**
Heme Oxygenase 1	*HMOX1*	Antioxidant generator/ degrades Heme
Oxidative Stress Induced Growth Inhibitor 1	*OSGIN1*	Antioxidant generator
Sulfiredoxin 1	*SRXN1*	Antioxidant response/ protection from oxidative stress
Nad(P)H Quinone Dehydrogenase 1	*NQO1*	Protection from oxidative stress
Matrix Metalloproteinase	*MMP1/3*	Degradation of extracellular matrix/ proteins
Hephaestin	*HEPH*	Metabolism/ Homeostasis of iron
Glutamate Cysteine Ligase	*GCLM*	Glutathione pathway/ Antioxidant
Glutamate Cysteine Ligase	*GCLC*	*GCLM* Catalytic Sub Unit

HMOX1 is thought to contribute to prevention of EC damage through the degradation of heme, the generation of anti-oxidants and the production of a vasodilator carbon monoxide (CO) (Kishimoto et al., 2019). Bilirubin is a by-product of *HMOX1* with a lipophilic property protecting EC membranes from cell damage as well as protecting LDL from oxidisation (Kishimoto et al., 2019). In response to *ROS* production, *Nrf2* activation in endothelial cells show an increase in heme-oxygenase 1 (*HMOX1*), nicotinamide adenine

dinucleotide phosphate quinone 1 (*NQO1*), oxidative stress induced growth inhibitor 1 (*OSGIN1*), glutathione (GSH) and glutamate cysteine ligase (*GCLM*), CO produced from *HMOX1* has shown reduction effects against intracellular *ROS* production through the inhibition of NAPDH oxidase (Kishimoto et al., 2019). Over oxidation causes inhibition of important peroxiredoxins, which are crucial to the reduction of *ROS*, such as hydrogen peroxide, through *Nrf2* these peroxiredoxins can be re-activated by regulating the gene expression of *SRXN1* (Kunnas et al., 2016). *Nrf2* mediated *OSGIN1* protects the cell from oxidative and inflammatory stress through MMF-pathways – induced by the *KEAP1*-cystienes interaction (Brennan et al., 2017).

1.6. 3 Suppression of Nuclear Factor KB

In the pathophysiology of atherosclerosis, *NFκB* is regarded as a key inflammatory regulator controlling proatherogenic processes including inflammation, cell proliferation and cell apoptosis, cytokines, cell adhesion molecules and chemokines (Baker et al., 2011).

NFκB activation is tightly regulated at several levels, from receptor activation, potentiation of the phosphorylation cascade and feedback through upregulation of inhibitor of KBα (*IκBα*) (Figure 4) (de Winther et al., 2005). *IκBα* has a binding site for *NFκB* in its promoter and is upregulated by *NFκB* signalling, providing a negative feedback loop that limits *NFκB* activation. Binding of *IκBα* to *NFκB* masks the nuclear localization signal, preventing translocation to the nucleus and activation of gene expression (Warboys et al., 2011). Upon activation of receptor signalling, phosphorylation of IKK phosphorylating *IκBα*, causing *IκBα*'s dissociation from *NFκB* and resulting in its ubiquination and degradation (Warboys et al., 2011; Kanters et al., 2003). DNA binding and transcription is also facilitated through phosphorylation of *NFκB* (Warboys et al., 2011).

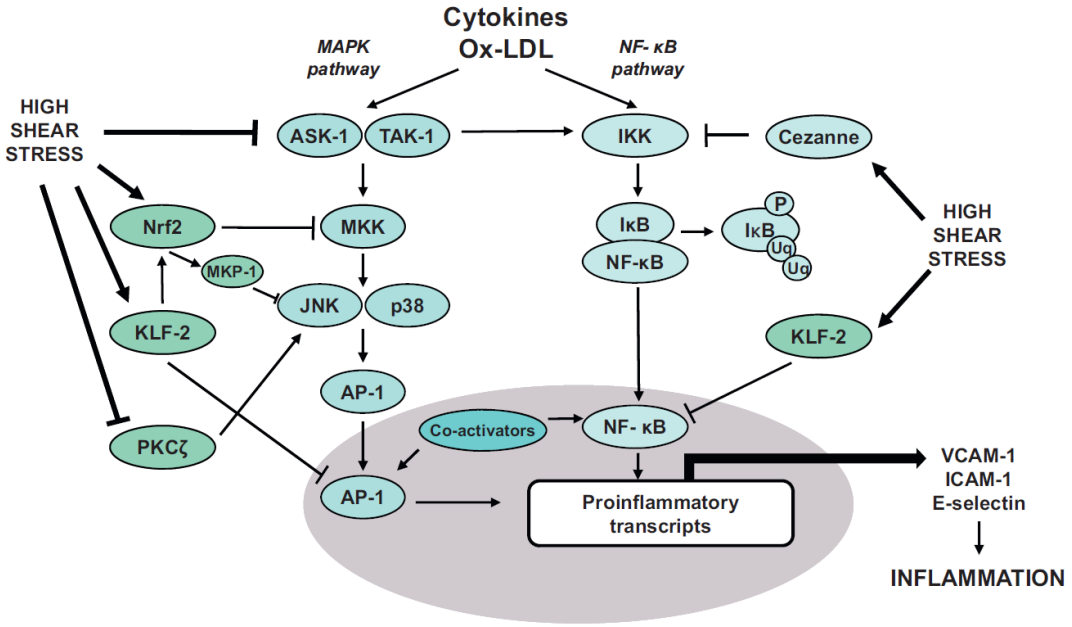


***Figure 4**: MAPK and NFκB pathways.* Activation of inflammation drivers; *VCAM1* and *ICAM1,* through the MAPK and *NFκB* pathways. *IκBα* is dissociated from *NFκB* through phosphorylation of IKK, *IκBα* is then subsequently degraded and transcription of inflammatory genes is regulated through *NFκB* s migration to the nucleus. the MAPK pathway demonstrates the MKK, JNK and p38 pathways, implicated in atherosclerosis (Warboys et al., 2011).

Table 3: Target genes of NFκB.

Target Genes of *NFκB*	**Gene**	**Function**
Cytokines/Chemokines	*TNF-α*, IL-1, IL-2, IL-6, IL-8, IL-12	Inflammatory response and angiogenesis
Cell Adhesion Molecules	ICAM-1, VCAM-1, MMP's	Adhesion and invasion
Cyclins and Growth Factors	G-CSF, M-CSF, Cyclin D1	Cell Proliferation
Regulators of Apoptosis	IAPs, Bcl-XL	Cell Apoptosis and Regulation

MAPK and *NFκB* are both pathways which are equally implicated in inflammation and lesion development, since their regulation is notably differential between sites which are atheroprone and atheroprotective (Warboys et al., 2011). For example, the expression of cell adhesion molecules *ICAM1* and *VCAM1* are regulated both by *NFκB*, but also MAPK via phosphorylated ATF2 binding to their promoters, indicating the synergy between these pathways. *KLF2* reduces JNK activity, via inhibition of protein kinase C elipson, (Warboys et

al., 2011). As previously stated, *KLF2*/4 compete with *NFκB* for their shared activating co factor p300 (Jain et al., 2014). Therefore, laminar shear stress-induced upregulation of *KLF2* suppresses both *NFκB* and MAPK-driven inflammation by multiple pathways.

Endothelial research

Multiple cell types have been used to study the response of the endothelium to laminar and disturbed flow, including Human coronary artery endothelial cells (HCAEC), human aortic endothelial cells (HAoEC), Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAoEC). Additionally, the use of late outgrowth endothelial colony forming cells (ECFCs) and endothelial cells derived from induced pluripotent stem cells (IPS-ECs) offer the possibility of generating endothelial, or endothelial-like cells from the blood or tissue biopsies of patients, allowing influence of patient characteristics on endothelial behaviour to be more easily modelled. The potential relevance of each of these different endothelial cells to study the cell processes involved in initiating or driving atherosclerosis to be has not been investigated. A side-by-side comparison of each cell type to determine the suitability of each cell type as cell models, would allow the most suitable and cost-effective cell model to be identified. To date, no such comparative study has been performed.

Aims

The aim of this research project is to perform comparative tests on several different endothelial cell lines to identify the best and most cost-effective cell model to perform research into the endothelial-dependent processes that regulate atherosclerosis. For this study we have selected to compare HCAECs, HUVECs and ECFCs. HCAECs are considered the most relevant cell line for the study of atherosclerosis, because of their relevance to cardiovascular disease and acute coronary syndromes. However, HCAECs are expensive to buy originate from a single donor and are supplied at passage 2, limiting the number of passages possible, before they undergo replicative senescence. For this reason, many studies choose to use HUVECs, which are comparatively cheap (approx. £100 rather than £500 per batch). They are often purchased as pooled donors, reducing the effect of each donor's genetics and epigenetics on the responses being measured. ECFCs offer the ability to investigate endothelial function from patients with particular risk factors e.g. diabetes, hypertension, defined genetic mutations, allowing the effects of these traits on endothelial function to be investigated.

Analysis of gene expression of each of these cell types in identical media under identical culture conditions will identify whether there are significant differences between HCAECs and HUVECS or ECFCs and determine if they are all suitable models for atherosclerosis research.

2.0 Materials and methods

Cell culture

Ethical approval for project granted from Manchester met university through Ethos review. Separate ethical approval for collecting blood and generating ECFCs had already been established.

Endothelial Colony Forming Cells

Deriving ECFC

ECFC were derived fresh from the buffy coat of 3 different healthy donor's blood by a fully trained phlebotomist. All blood work was performed under the same sterile conditions as the other cells, along with the same materials.

The phlebotomist took approximately 60-70mls of blood from each donor and 4-6 falcon tubes were used (dependant on the volume of blood), were 20mls of Ficoll-Pique was added. The blood: PBS solution was then carefully layered on top. Prior to centrifugation of the blood solution a 'cloudy' layer can be seen, ECFC reside within this layer and approximately 12mls of this cloudy layer was added to a separate tube with an additional 3mls of PBS. The new ECFC/PBS solution was centrifuged again where a pellet was left behind, after removal of supernatant. Although the pellet is not always visible, it was further suspended in 9mls of endothelial cell media.

Culturing ECFC

900ul of collagen and 8.1mls of PBS were combined in a falcon tube and vortexed. Two sterile plastic 6-well plates were coated with 1ml of the solution along with 1ml of the pellet solution. The ECFC are able to bind to the collagen coating and another 2ml of endothelial cell media is added to each well, with 4ml in total in each well. Media was changed the day after the cells had been plated, using the same media, and every other day following from that.

Cell culture

Human Coronary Artery Endothelial Cells (HCAECs) and Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from PromoCell whilst Endothelial Colony Forming Cells (ECFCs) were derived from healthy blood donors, all cell types were used at a passage between 2-6. All cell types were cultured under identical conditions and grown in the same media for at least one passage, MV2 Endothelial Cell media supplemented with a 5ml aliquot of penicillin: streptomycin (PromoCell). All analysis was performed on ECs seeded onto gelatin coated glass slides to standardise the experiments. The aim was to perform all experiments on confluent monolayers of ECs. To achieve this, $2.5x10^5$ HCAECs or ECFCs, or $3.5x10^5$ HUVECs (due to their naturally smaller size) were seeded onto a gelatin coated slide, within a silicon gasket, which has a growth area of approximately $9.3cm^2$. ECs were cultured for 72 hours on the slides to allow complete confluency to be achieved. ECs were then cultured under static conditions, or flowed conditions using our established parallel plate flow apparatus to expose the ECs to 'athero-prone' oscillatory flow (OSS = ±5 dynes/cm2, 1 Hz) or 'athero-protective' laminar flow (LSS = 15 dynes/cm2). ECs were cultured under identical static, OSS or LSS conditions for 72 hours before analysis of gene expression.

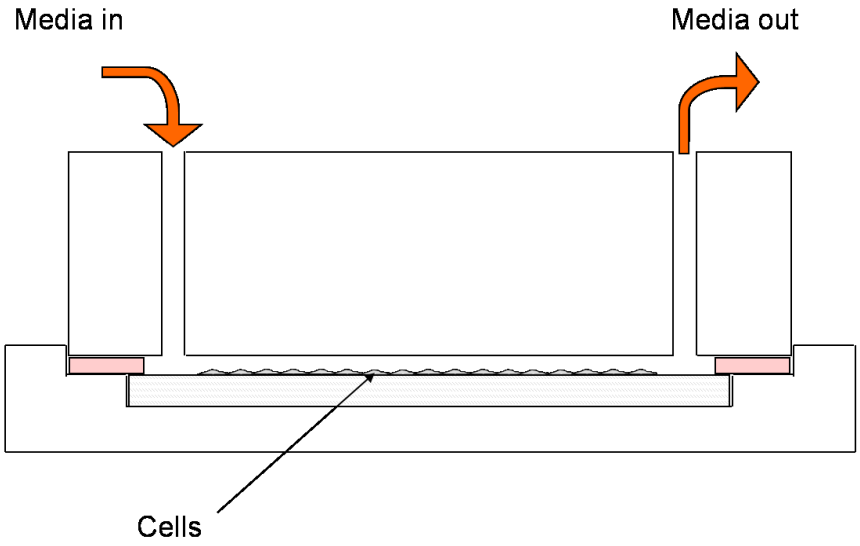


Figure 5: *Parallel flow plate.* Demonstrating the structure of the parallel flow plate where cells were placed under mimicked atheroprone and atheroprotective flow conditions.

Table 4: MV2 Endothelial Cell Media

Reagent	***Quantity***
Endothelial Cell Medium MV2	500 ml

Fetal Calf Serum	0.5 ml
Epidermal Growth Factor	5 ng
Basic Fibroblast Growth Factor	10 ng
Insulin Like Growth Factor	20 ng
Vascular Endothelial Growth Factor	0.5 ng
Ascorbic Acid	1 ul
Hydrocortisone	0.2 ug

Table 5: DMEM Complete

Reagent	***Volume***
DMEM	435 ML
FBS	50 ML
L-Glutamine	10ML
Penstrep	0.5 ML (1x)

Table 6: Reagents used for cell culture

Reagent	***Brand Name***	***Catalogue Number***
DMEM	Sigma	D6546
MV2 Endothelial Cell Media	PromoCell	C22221
Trypsin	Lonza	BE17-161E
Attachment Factor	Gibco	-
L-Glutamine	Sigma	G7513
Penicillin: Streptomycin (Penstrep)	Sigma	P0781
PBS	Lonza	17-516F

Cell Lysis and Obtaining Samples

Following exposure to identical culture conditions, the individual slides were washed in cold PBS on ice, and phase contrast images obtained at a magnification of X5 and X20, to allow comparison of morphology. Cells were then lysed in 300µl SDS lysis buffer [2% SDS; 50 mM Tris pH 6.8; 10% glycerol] and 150µl immediately transferred to RNA lysis buffer (Norgen) allowing both protein and RNA analysis form the same sample. Samples were stored at -80°C, until further use.

Table 7: Reagents used for cell lysis

Reagent	***Brand Name***	***Catalogue Number***
Double Distilled Water	-	-
Glycerol	-	-
Tris Base	Fisher Scientific	BP152-1
SDS		

RNA Isolation and PCR

Total RNA extraction was achieved using the Norgen Biotek Total RNA Purification kit according to the manufacturer's instructions. Briefly, lysates were passed through a DNA binding column to reduce the amount of contaminating genomic DNA in the purified RNA sample. Following this, lysates were then passed through an RNA binding column before being washed 3 times. The RNA was then eluted, quantified using a nanodrop and stored at -80°C for further analysis. One advantage of using the Norgen kit is that it purifies both long and short RNAs to facilitate both mRNA + lncRNA and also miRNA analysis.

Table 8: Reagents and materials used for RNA isolation

Reagent	***Brand Name***	***Catalogue Number***
Ethanol	-	-
Beta-Mercaptoethanol	Sigma	8.05740
Total RNA Purification kit	Norgen Biotek	48300
Reverse Transcription Kit	Qiagen	205311

Reverse Transcription

In preparation for Quantitative Polymerase-Chain Reaction (qPCR), cDNA was produced using the Qiagen Reverse Transcription Kit. RNA samples were thawed on ice along with the RT kit reagents. Whilst samples thawed, the following RT Master Mix were pre-prepared:

- 0.9ul of RT Enzyme per RNA sample
- 4ul of RT Mix per RNA sample

This master mix was then briefly mixed and stored on ice until further use. 1µl of genomic DNA (GDNA) Removal reagent was added to each individual sample, along with RNase-and DNase-free H_2O to a total volume of 15µl. Samples were incubated at a temperature of 45°C for 2 minutes, before being cooled to 4°C. 5ul of the RT Master Mix was added to the samples, with the reverse transcription being performed using the following 3 incubations:

1. Samples incubated at 25°C for 3 minutes
2. Samples incubated at 45°C for 10 minutes
3. Samples incubated at 85°C for 5 minutes – to inactivate the RT Enzyme

Quantitative PCR

Key differences in gene expression and signalling pathways were analysed by qPCR. Quantitative PCR was performed on a Bio-rad CFX Maestro machine. qPCR was performed using duplicate 10µl reactions:

Reagent	**Volume**
• SYBR Green	• 5ul
• DNase and RNase Free H2O	• 3.6ul
• 10µM Primer (forward and reverse)	• 0.4ul
• cDNA	• 1uL

For each primer set used, an optimisation experiment was performed where a gradient of different anneal temperatures was used. The optimal anneal temperature was subsequently used to assess comparative gene expression levels. The qPCR reactions were performed using a semi-skirted 96 well plate, where 9ul of Master Mix was added in duplicates using the EDPA Multichannel Pipette for consistent accuracy inside a PCR preparation hood to prevent contamination. 1ul of cDNA was added to the corresponding duplicate wells before being covered by StarSeal Advanced Polyolefin Film to prevent sample evaporation. The plate was transferred to the Bio-Rad CFX Maestro machine and ran at the appropriate settings, including SYBR, melt curves and the correct temperature according to the gene of interest being used.

Statistical Analysis

All analyses were performed on an n=3 where each n was obtained from a different batch of ECs from a different donor or donor pool. qPCR data underwent statistical analysis using a two-way analysis of variation test (2way A*NOV*A), with a Tukey post-hoc test using the GraphPad Prism 8 software. A P value < 0.05 was considered statistically significant. Graphs are presented with standard error of mean values (SEM).

3.0 Results

3.1 Primer optimisation

To analyse gene expression under the flow conditions we used qPCR. To ensure optimal annealing temperatures were used for the genes, gradient qPCRs were performed on a CFX Bio-rad Maestro for all of the primer sets used in this study.

Table 9: *Primer Details*. Primers used in QPCR alongside their crucial data including; Gene name, primer sequence, CT Value, temperatures and ideal temperature range for primer usage. Red writing depicts temperatures, which are no of use and green represents the optimal primer temperatures.

Gene	Sequence	CT values							Anneal Temp range
		58°C	60.8°	63.5°	66.9°	69.7°	71.2°	72°	
SRNX1	756F GCCAAGGTGCAGAGCCTCGT 757R GCGGGGATGGTCTCTCGCTG	**32.6**	**31.68**	**30.53**	**33.16**	**N/A**	**N/A**	**N/A**	58-63.5
IκBα	740F CGCCCAAGCACCCGGATACA 741R AACGTCAGACGCTGGCCTCC	**30.11**	**29.98**	**30.03**	**30.30**	**33.60**	**39.50**	**N/A**	58-66.9
VCAM1	409F GCCCGGCTGGCTTTGGAGGC 410R TGGTGACTCGCAGCCCGTAGTGC	**28.77**	**28.74**	**28.79**	**28.74**	**29.02**	**31.30**	**32.93**	58-69.7
THMB	232F CAACACACAGGGTGGCTTCG 233R GGCTGGACAGGCAGTCTGGT	**32.70**	**34.13**	**33.40**	**37.04**	**N/A**	**N/A**	**N/A**	58-63.5
CCL2	704F ATTCCCCAAGGGCTCGCTCAG 705R ACTTCTGCTTGGGGTCAGCACA	**28.43**	**28.38**	**28.25**	**28.37**	**30.55**	**35.61**	**N/A**	58-66.9
KLF4	369F TGGACCCCCTCTCAGCAATG 370R CTCTTGGTAATGGAGCGGCG	**30.65**	**30.12**	**29.35**	**31.05**	**N/A**	**N/A**	**N/A**	58-66.9
GCLM	754F GTCCTTGGAGTTGCACAGCTGGA 755R GGCATCACACAGCAGGAGGC	**25.79**	**26.11**	**25.92**	**26.42**	**28.36**	**33.91**	**38.12**	58-66.9
GAPDH	181F AGTCCATGCCATCACTGCCACC 182R CAGGAAATGAGCTTGACAAAGTGG	**23.14**	**23.17**	**23.31**	**25.19**	**35.98**	**N/A**	**N/A**	58-66.9
NOV	716F TGGTGCGGCCCTGTGAACAA 717R AGCGGCCATCACTGCAGACC	**30.45**	**30.78**	**30.38**	**29.85**	**30.30**	**30.26**	**30.80**	58-72
KLF2	218F GTGAGAAGCCCTACCACTGCAACT 219R CCGGTTCTCTGGGTCCAATAAATA	**28.51**	**28.45**	**28.18**	**29.91**	**N/A**	**N/A**	**N/A**	58-66.9
eNOS	204F GCCGGAACAGCACAAGAGT 205R GAGGATGCCAAGGCCGC 768R CCGTGGCCACTGCAGGATGTA	**31.58**	**30.88**	**30.30**	**34.58**	**N/A**	**N/A**	**N/A**	58-63.5

OSGIN1	746F GGGAGCCTGGCACTCCATCG 747R CCCGGCTGTTGCGAAGACCT	28.04	27.87	27.47	27.52	28.17	31.17	32.60	58-69.7
CX3CL1	718F CTGTCGTGGCTGCTCCGCTT 719R TCGGGTCGGCACAGAACAGC	24.02	23.69	23.41	23.36	23.23	23.63	24.17	58-72
PI16	611F ATGTGCGGCCACTACACGCA 612R CCTTCACGTTCCCCGGAGGC	28.27	28.06	27.22	27.03	28.48	32.35	35.75	58-69.7
RPLP0	998F GCAGCAGATCCGCATGTCCC 999R TCCCCCGGATATGAGGCAGCA	20.37	20.35	20.19	20.51	22.07	25.33	29.30	58-69.7
PI16	611F ATGTGCGGCCACTACACGCA 626R TCAGTCGCCCGGAAGGATGG	30.79	29.35	27.49	26.40	26.49	27.46	28.45	63.5-72

The melt curves of each of the primer sets were also analysed to ensure each primer set worked efficiently. For each primer set, an optimal annealing temperature was identified, allowing quantification of each gene product in the prepared cDNAs. Melt curves of each primer set were also recorded, demonstrating a dominant single peak for all primer sets, demonstrating only one desired amplicon was present. *THMB* and *eNOS* (Figure 9 and 15) demonstrate minor smaller peaks at all temperatures tested suggesting the presence of another size of amplicon. As each primer set spanned an intron, it is possible that this is a splice variant of the target gene, or may have been a contaminating amplicon for another gene. In both cases the area under the curve was considerably smaller than the large peak, suggesting a minimal effect on the total quantification of the amplicon.

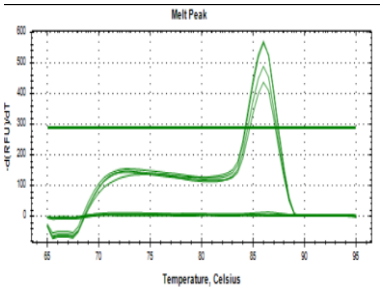


Figure 6: Melt curve for SRXN1 primer.

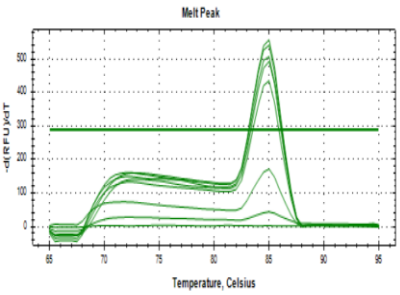


Figure 7: Melt curve for IκBα primer.

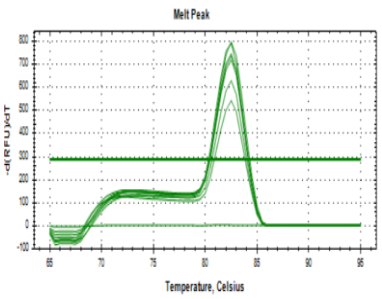


Figure 8: Melt curve for VCAM-1 primer.

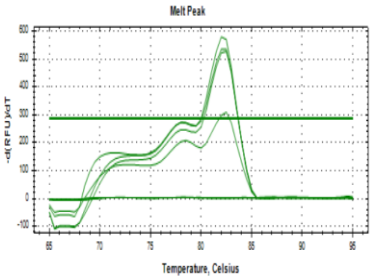


Figure 9: Melt curve for THMB primer. primer.

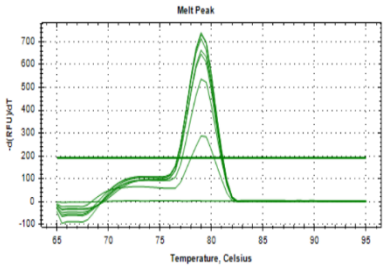


Figure 10: Melt curve for CCL2 primer.

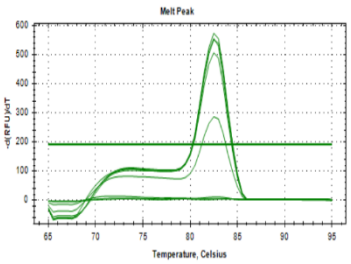


Figure 11: Melt curve for E-selectin

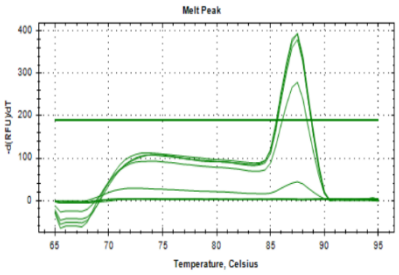


Figure 12: Melt curve for KLF4 primer.

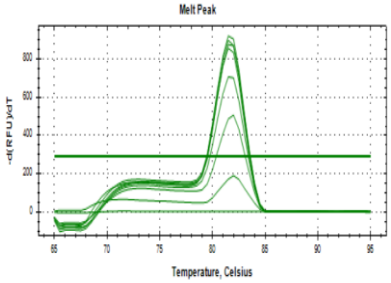


Figure 13: Melt curve for GCLM primer.

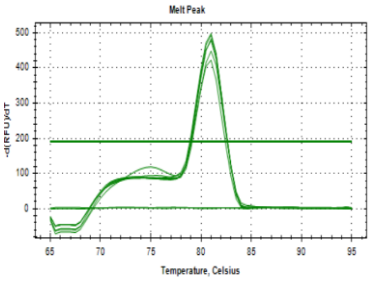


Figure 14: Melt curve for GAPDH.

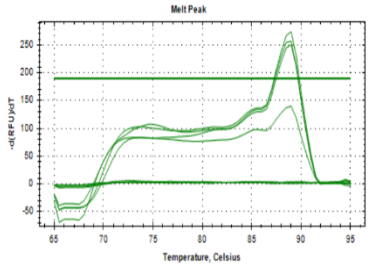


Figure 15: Melt curve for eNOS primer. primer.

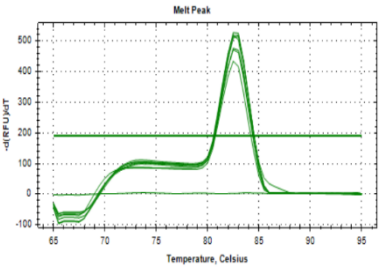


Figure 16: Melt curve for NOV primer.

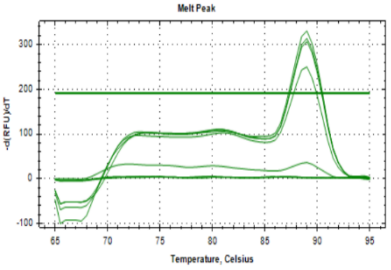


Figure 17: Melt curve for KFL2

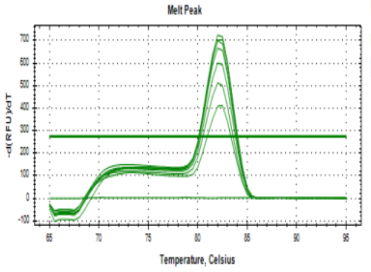


Figure 18: Melt curve for PI16 primer

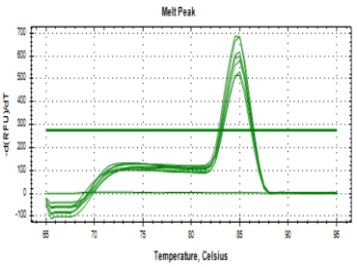


Figure 19: Melt curve for PI16 primer.

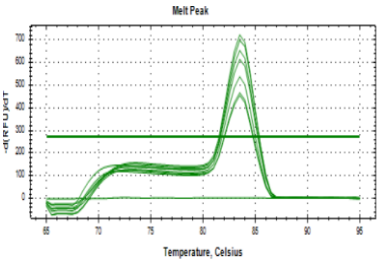


Figure 20: Melt curve for RPLP0 primer.

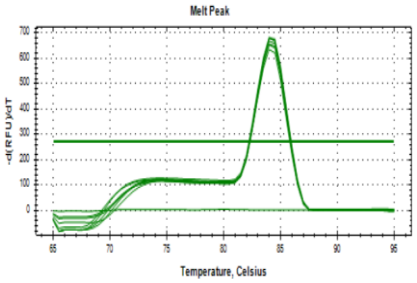


Figure 21: Melt curve for CX3CL1 primer.

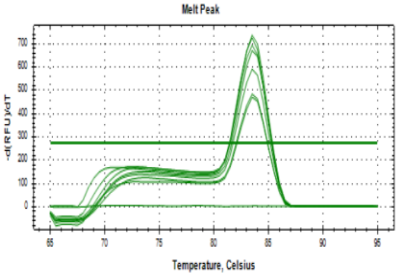


Figure 22: Melt curve for OSGIN1 primer.

Figures 6-22: *Melt curves generated for each primer set used in the analysis of gene expression.*

3.2 Cell morphology

Each individual batch of cells, cultured and grown from a 1ml vial, were imaged using the laboratory microscope at a 10X magnification, whilst seeded onto their glass slides prior to being dissembled from the parallel flow plates. Cell morphology was assessed to determine whether cells aligned to their allocated flow conditions. Each cell batch's shape, size and structure were analysed and example morphologies are demonstrated in figure 23, figure 24 and figure 25.

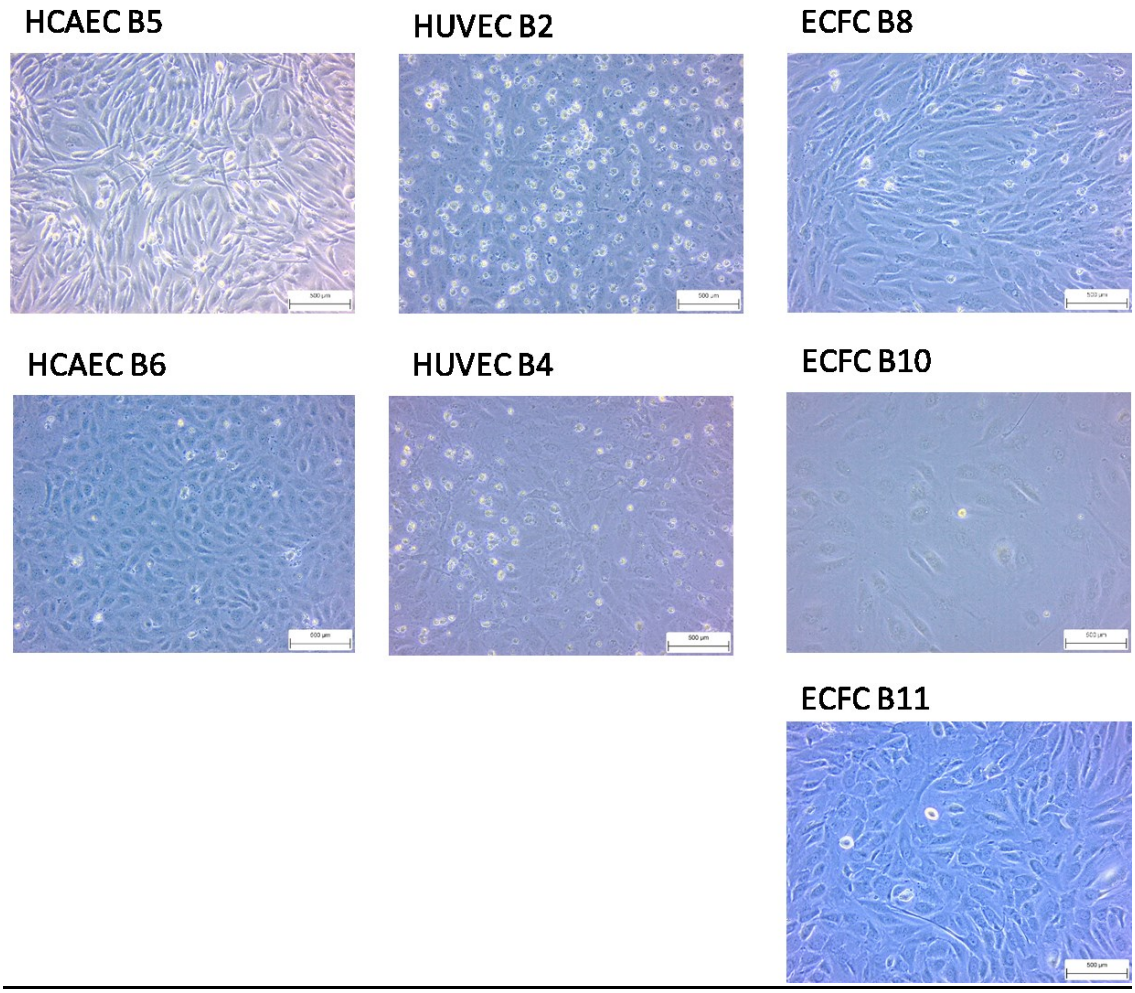


Figure 23: *Cells cultured under static culture conditions*. Cells under static culture appear to demonstrate a mostly elongated morphology. Cells under static culture are similar to those under oscillatory shear as figures 23 and 24 both show a lack of uniform along with a random orientation. ECFC batch 10 (B10) were of a lesser confluency than B11 and B8 under static culture. HCACE B7 and HUVEC B5 under static culture images missing due to loss of imagery on laboratory microscope.

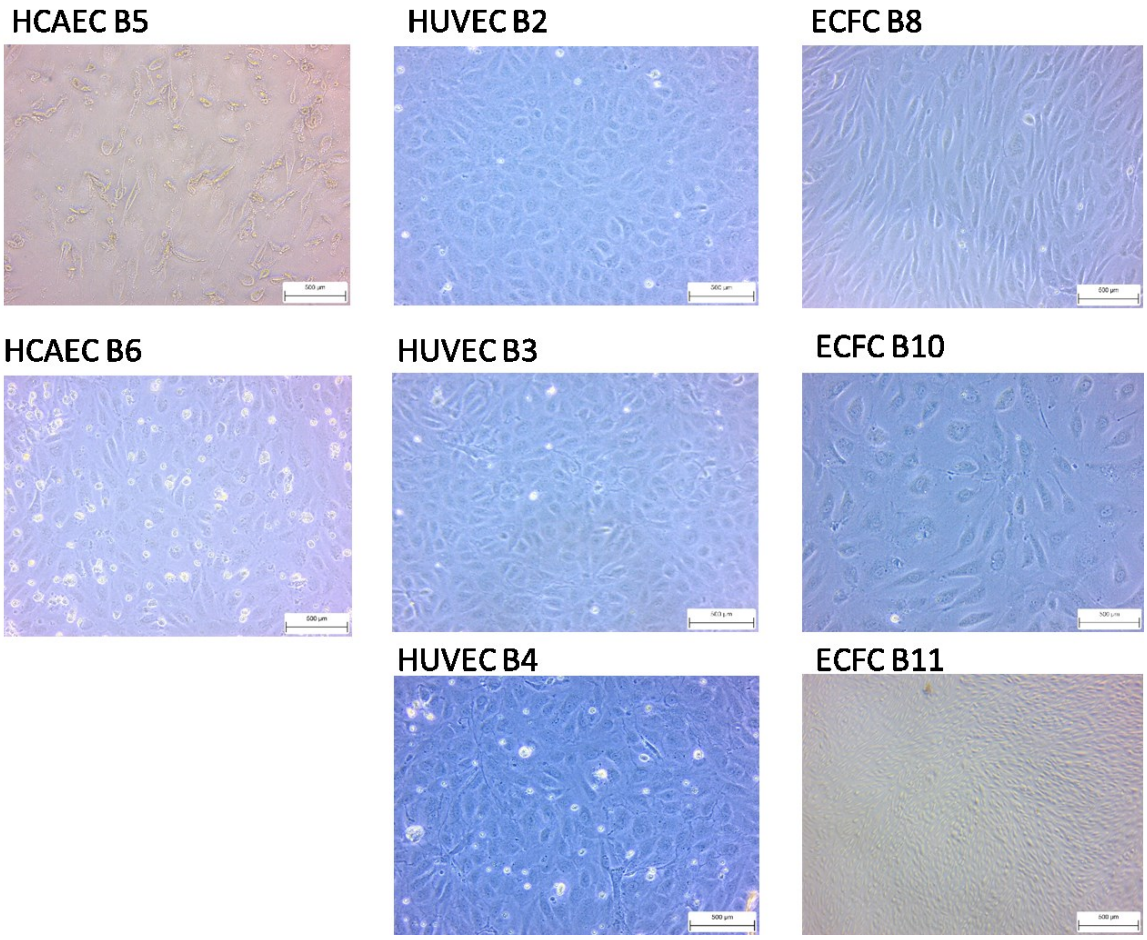


***Figure 24**: Cells under oscillatory culture conditions.* Cells here show a polygonal morphology and are non-aligned, exhibiting a 'lack of organisation', this is a typical morphology demonstrated in cells exposed to disturbed shear. ECFC B10 are a lot less confluent than B11 and B8. HCAEC B7 under oscillatory culture image missing due to loss of imagery on laboratory microscope.

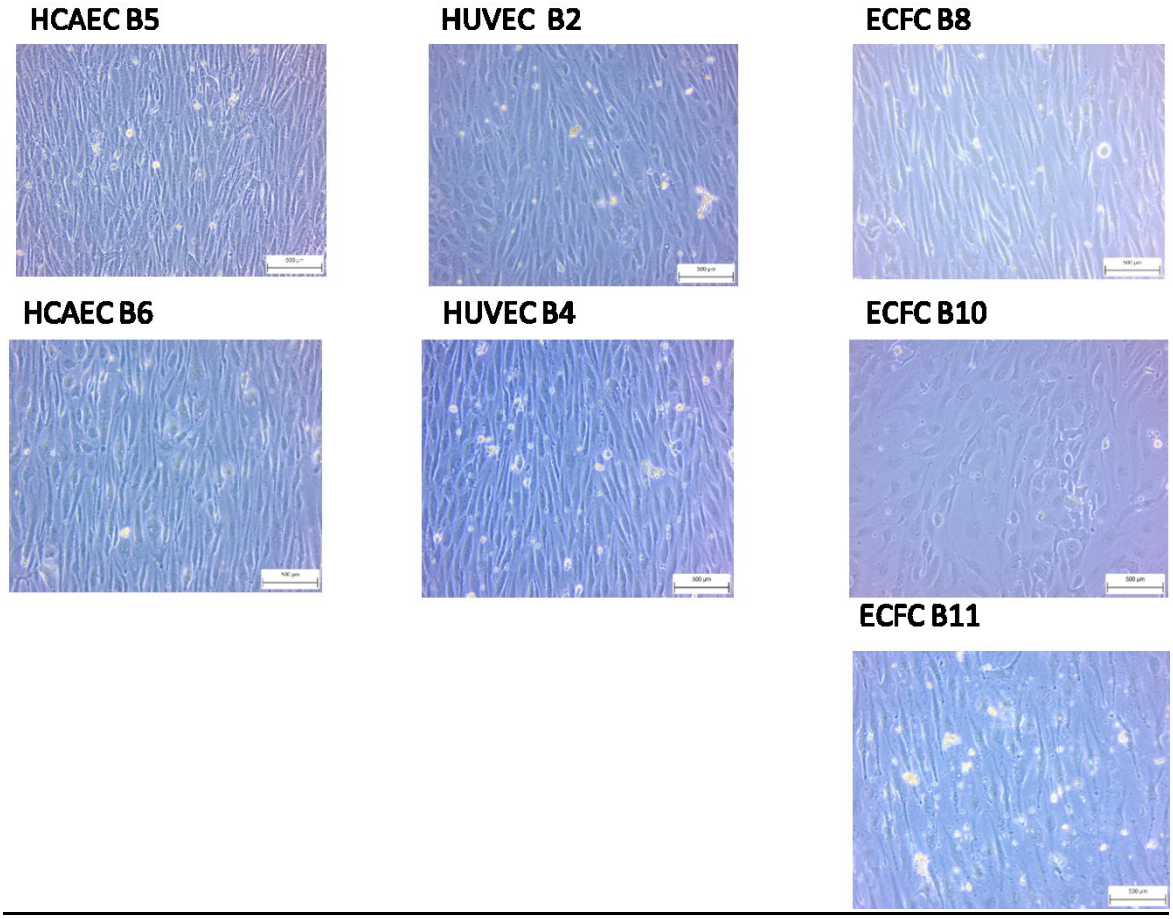


Figure 25: *Cells under laminar culture conditions.* Cells show a longitudinal morphology, with a clear uniformed orientation. This demonstrates that the cells have aligned to the definite direction of laminar flow, as expected, with ECFC B10 showing a slightly less confluent monolayer. HCAEC B7 and HUVEC B2 under laminar culture images missing due to loss of imagery on laboratory microscope.

Conclusion

HCAECs, HUVECs and ECFCs all aligned to their allocated flow conditions accordingly, correlating to the biomechanosensitivty of vascular endothelial cells. Although HUVECs are of a naturally smaller nature, morphological and alignment variation can be disregarded, as they follow the same morphological and alingment patterns as HCAECs and ECFCs. Batch to batch variation in ECFCs is demonstrated in B10, with a visibly lesser confluency under LSS, OSS and static culture, compared to ECFC B8, B11, HCAEC batches and HUVECs, whereas minimal variation can be seen between HUVECs and HCAECs. Under all conditions, ECFCs demonstrate a more variable nature, under laminar culture ECFC batches are generally of lesser quantity and under oscillatory culture, ECFC B8 exhibit a disturbed flow organisatoin although, their mophology appears more elongated when compared to HUVEC and HCAEC batches.

3.3 KLF Regulated Genes

The first identification of a KLF family member was made in 1993 where it was discovered in erythroid cells (Subramaniam M et al., 2010) with 18 known KLFs to date. Although all are regulators of gene expression and regulators of vascular homeostasis, they do not all share the same biological functions and are distributed amongst different tissues (Subramaniam M et al., 2010). *KLF2* and *KLF4* are members of the KLF family that express cytoprotective effects against vascular diseases and switching on approximately 1000 genes, *KLF2* is a flow-regulated integrator expressed in atheroprone regions of the human coronary arteries and induces anti-inflammatory (*eNOS*) and anti-thrombogenic (*THMB*) properties, along with *NOV* and *PI16*, whilst simultaneously inhibiting opposing factors (*VCAM1*) (Parmar et al., 2005). *KLF4* acts in concert with *KLF2* to promote the athero-protective phenotype in endothelial cells (Parmar et al., 2005). We therefore assessed the expression and regulation of *KLF2* and *KLF4* and a number of key *KLF2*/4 regulated genes in HCAECs, HUVECs and ECFCs under static, OSS and LSS culture conditions.

3.3.1 KLF2

No significant upregulation of *KLF2* by laminar flow was observed in ECFCs and HUVECs, compared to oscillatory shear stress or static culture, whereas in HCAECs *KLF2* expression was significantly upregulated by laminar shear stress with a 13-fold change in LSS against oscillatory shear stress (P=0.028) and a 10-fold change against static culture (P=0.028) (Fig 3.1, n=3). In relative terms, no significant differences were observed between the different cell types, indicating all three cell types express a similar level of *KLF2* mRNA. Therefore, HCAECs show a significant regulation of *KLF2* by flow, however all HCAECs, HUVECs and ECFCs all express a similar level of *KLF2* in identical culture conditions.

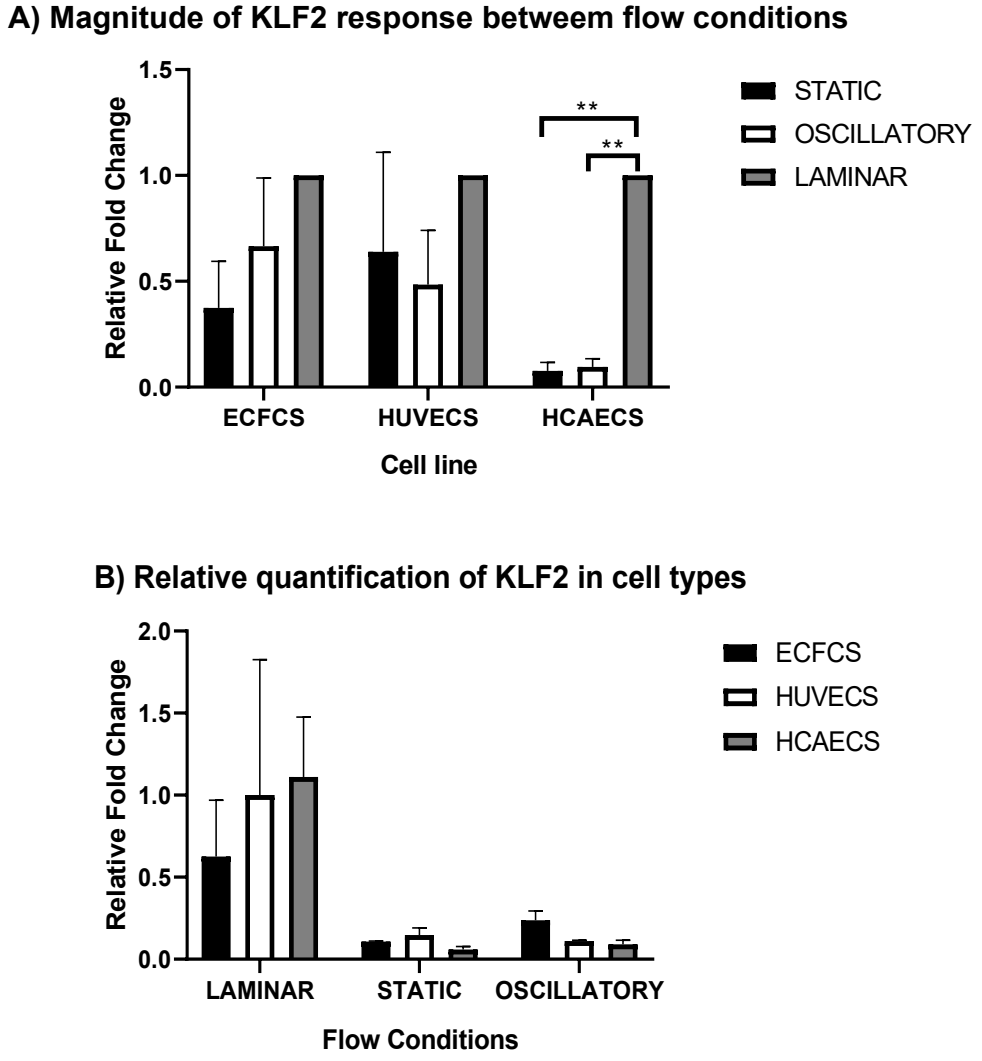


Figure 26: KLF2. A) The fold change of *KLF2* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAEC *KLF2* expression was significantly upregulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$), whereas no significant difference was detected in HUVEC or ECFC. B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types. No significant differences were found between any of the cell types under identical culture conditions. Data analysed using a 2-Way A*NOV*A, n=3, *$p<0.05$, **$P<0.01$, *** $p<0.001$.

3.3.2 KLF4

Significant upregulation of *KLF4* by laminar flow was observed in HCAEC, HUVEC and ECFC, compared to oscillatory and static culture. In HCAEC, *KLF4* expression was significantly upregulated by laminar shear with a 28-fold change in LSS against oscillatory shear and a 27-fold change against static culture. In HUVECS, *KLF4* expression was significantly upregulated by laminar shear with an 11-fold change in LSS against oscillatory shear and a 13-fold change against static culture. The fold induction of *KLF4* by laminar flow was not significantly different between the 3 cell types. Relative to HCAECs, both HUVECs and ECFCs expressed similar levels of *KLF4* in all 3 culture conditions, with HUVECs expressing less *KLF4* compared to ECFCs in OSS. Therefore, HCAECs, HUVECs and ECFCs all show a significant upregulation of *KLF4* by laminar flow; In addition, they all express similar levels of *KLF4* in identical culture conditions compared to HCAECs.

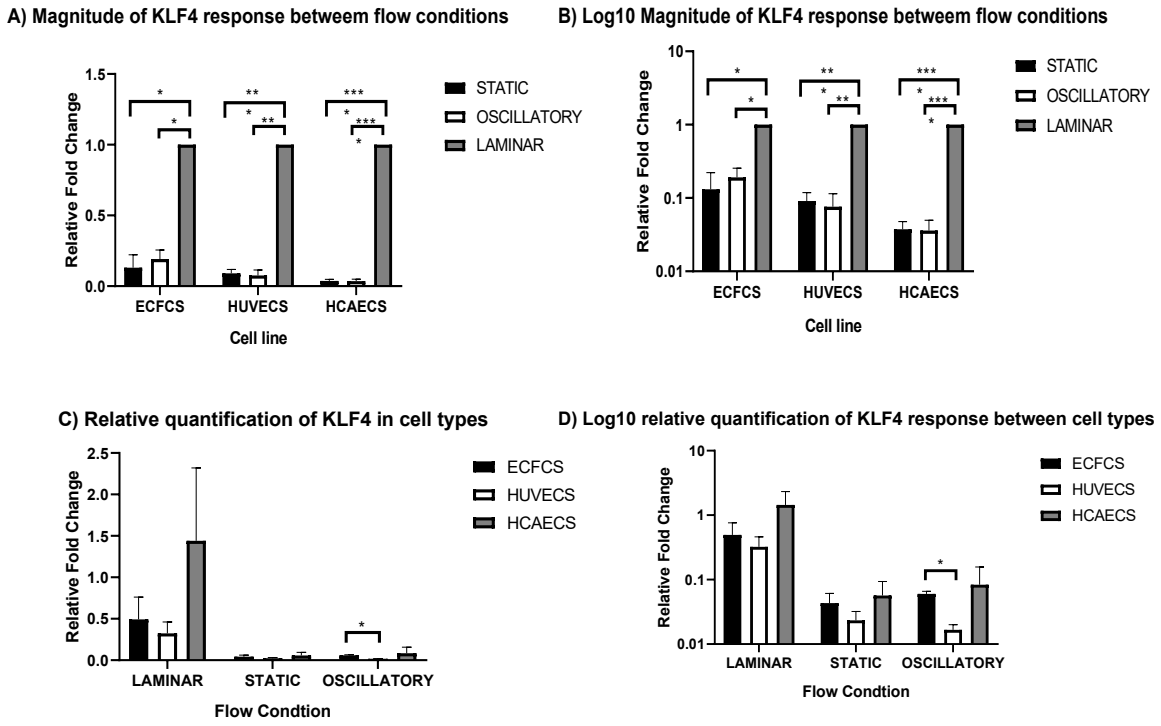


Figure 27: KLF4. A) The fold change of *KLF4* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented on a Log_{10} Scale graph). In all

cell types *KLF4* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions (P<0.05). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log_{10} Scale Y axis). HUVECs expressed significantly less *KLF4* compared to ECFCs under identical oscillatory shear stress conditions. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.3.4 NOV

Significant upregulation of *NOV* by laminar flow was observed in HUVECs and HCAECs, compared to oscillatory and static culture. The changes in *NOV* expression in ECFCs with laminar flow did not achieve significance. In HCAECs, *NOV* expression was significantly upregulated by laminar flow shear with a 132-fold change in LSS against oscillatory shear and a 123-fold change under static culture. HUVECs expressed a 39-fold in oscillatory shear against LSS and a 21-fold change under static culture. ECFCs expressed significantly more *NOV* when cultured under OSS, compared to HUVECs (P=0.0150) and HCAECs (P=0.0237). Therefore, HUVEC and HCAEC show a significant upregulation to *NOV* by flow, while ECFCs did not significantly upregulate *NOV* with laminar flow. However, they all express similar levels of *NOV* in both laminar and static culture conditions.

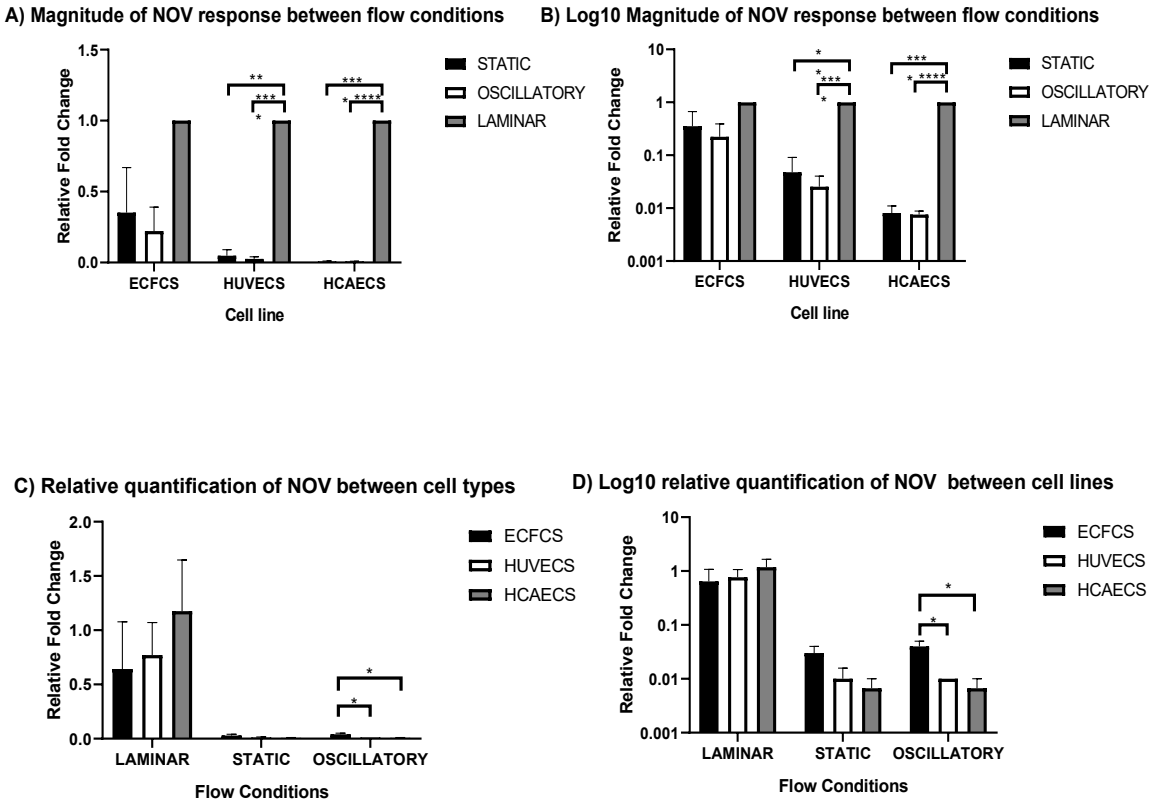
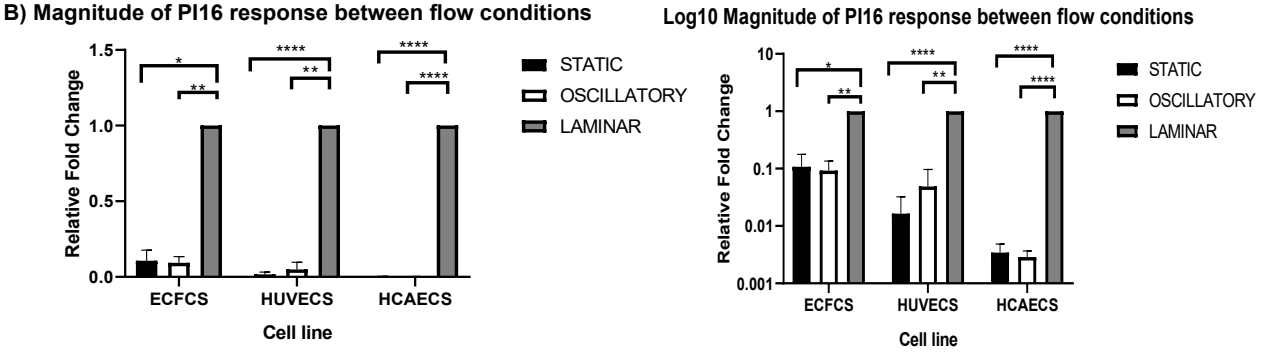


Figure 28: NOV. A) The fold change of *NOV* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented on a Log_{10} Scale Y axis). In HUVEC and HCAEC, *NOV* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions (P<0.05). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log_{10} Scale Y axis). All 3 cell types expressed similar levels of *NOV* under static and Laminar flow conditions, however ECFCs expressed significantly more *NOV* under OSS. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.3.5 PI16

The expression of *PI16* was significantly upregulated by laminar flow in HCAECs, HUVECs and ECFCs, compared to oscillatory and static culture. No detectable expression was demonstrated in HUVECs under static conditions or HCAECs under oscillatory flow. In relative terms, no significant differences were observed between the different cell types, indicating all three cell types express a similar level of *PI16* mRNA. Therefore, HCAECs, HUVECs and ECFCs all show a significant upregulation to *PI16* by flow, with similar level of *PI16* expression under identical culture conditions.



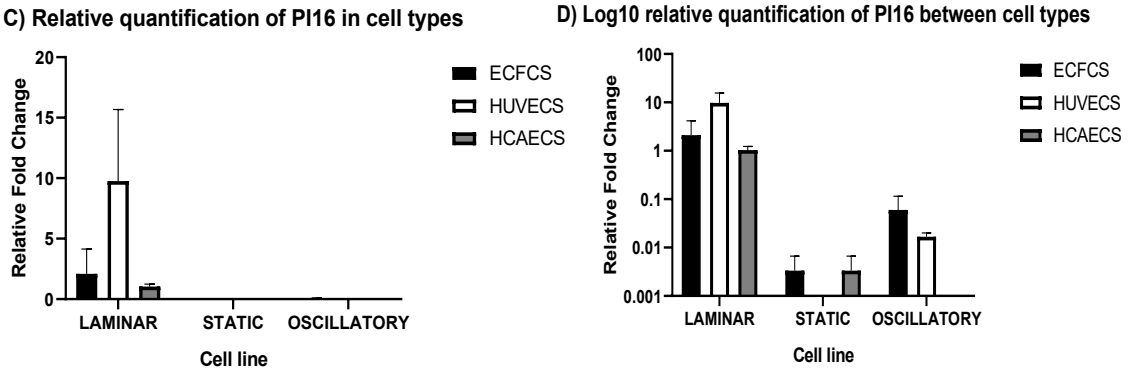


Figure 29: *PI16*. *A) The fold change of PI16 mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented on a Log_{10} Scale Y Axis). In HCAECs, HUVECs and ECFCs, PI16 expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions (P<0.05). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log_{10} Scale Y axis). No Significant differences were found between any of the cell types under identical flow conditions. Data analysed using a 2-Way ANOVA, n=3, *p<0.05, **P<0.01, *** p<0.001.*

3.3.6 eNOS

Significant upregulation of *eNOS* by laminar flow was observed in HCAECs, However the changes in expression in HUVECs and ECFCs did not reach significance. In HCAECs, *eNOS* expression was significantly upregulated by laminar shear with a 14-fold change in LSS against oscillatory conditions and a 9-fold change under static culture. In relative terms, no significant differences were observed between the different cell types, indicating they all express similar level if *eNOS* mRNA. Therefore, HCAECs show a significant upregulation to *eNOS* by flow; however, they all express similar levels of *eNOS* mRNA in identical culture conditions. As seen in figure 30 B),

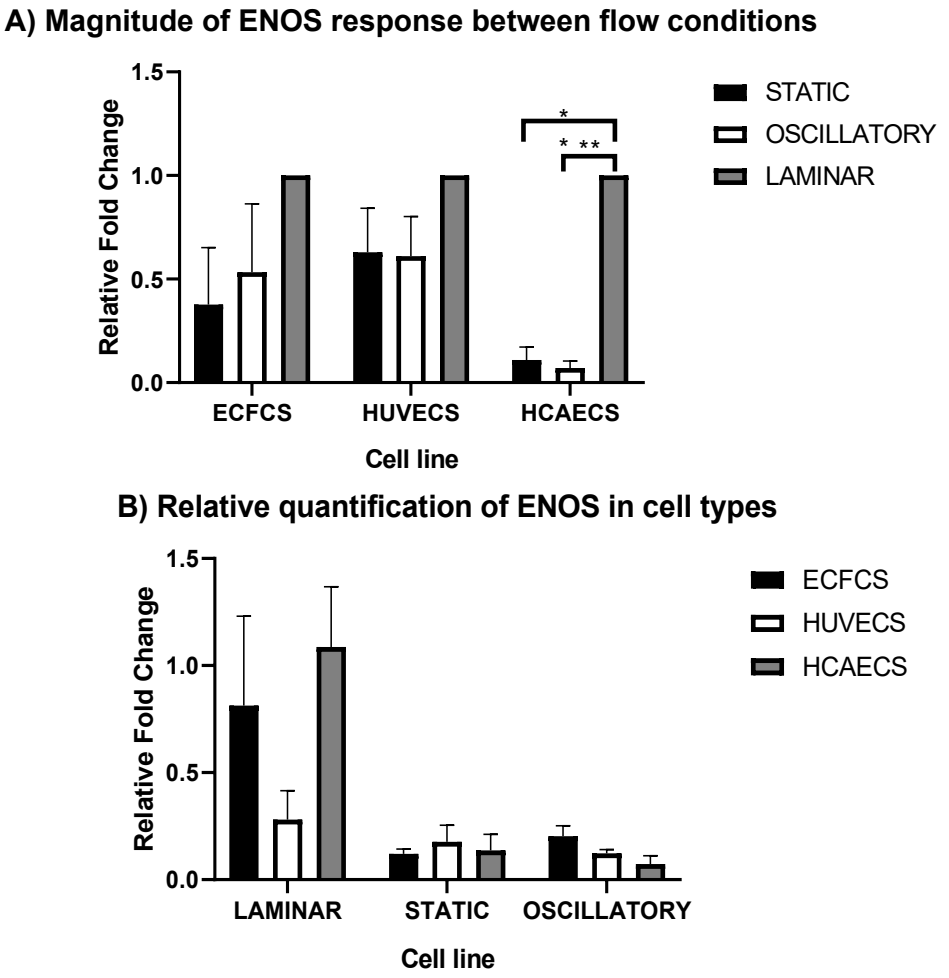


Figure 30: eNOS. A) The fold change of *eNOS* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAEC, *eNOS* expression was significantly upregulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types. No significant differences were found between any of the cell types under identical flow condition. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001

3.3.7 THMB

Significant upregulation of *THMB* by laminar flow was observed in HCAECs and HUVECs between OSS and LSS. In ECFCs, no regulation by laminar flow was observed. In HUVECs and HCAECs, *THMB* expression was significantly upregulated by laminar shear (19-fold and 10-fold). *THMB* showed high levels of expression in static culture of HUVECs and HCAECs, highlighting the difference between the gene expression profiles of ECs cultured under static and OSS conditions. In relative terms, no significant differences were observed

between the different cell types under identical culture conditions. Therefore, HCAECs and HUVECs show a significant regulation of *THMB* by flow, while ECFCs did not, however no significant differences in expression levels of *THMB* were observed under identical culture conditions between the three cell types.

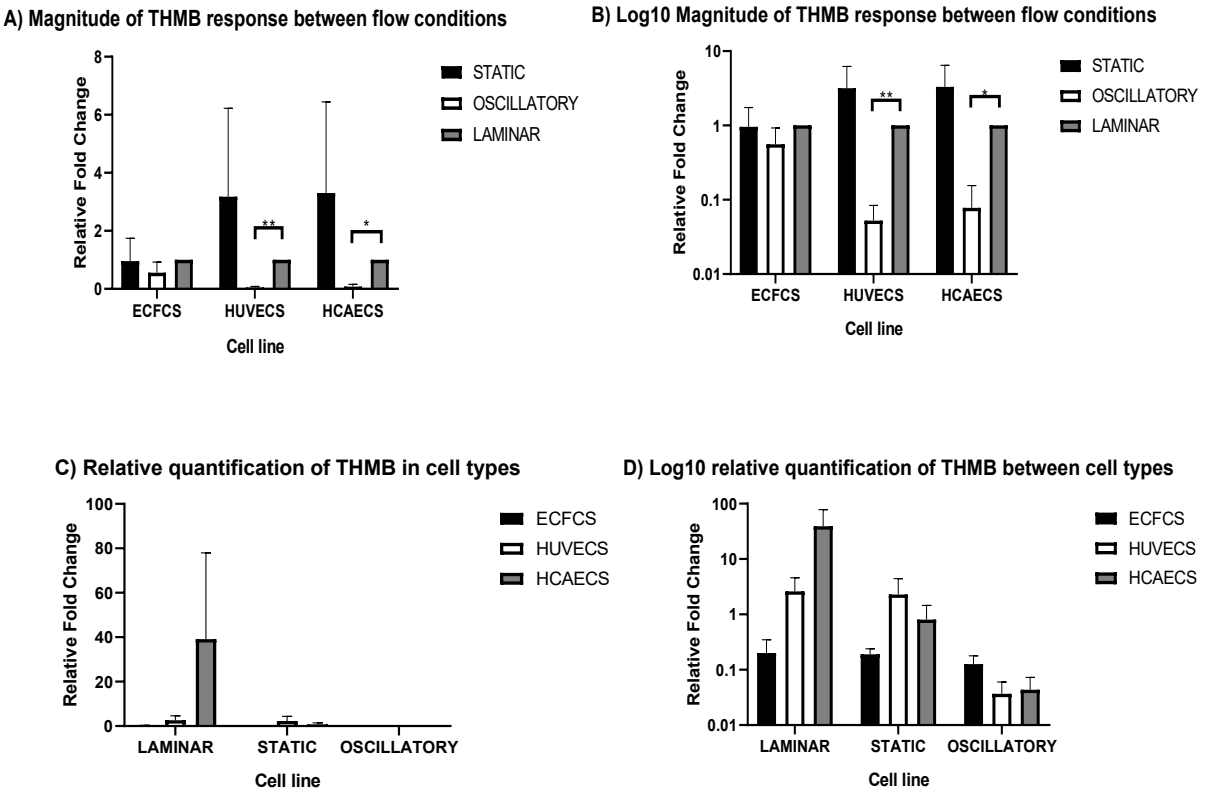


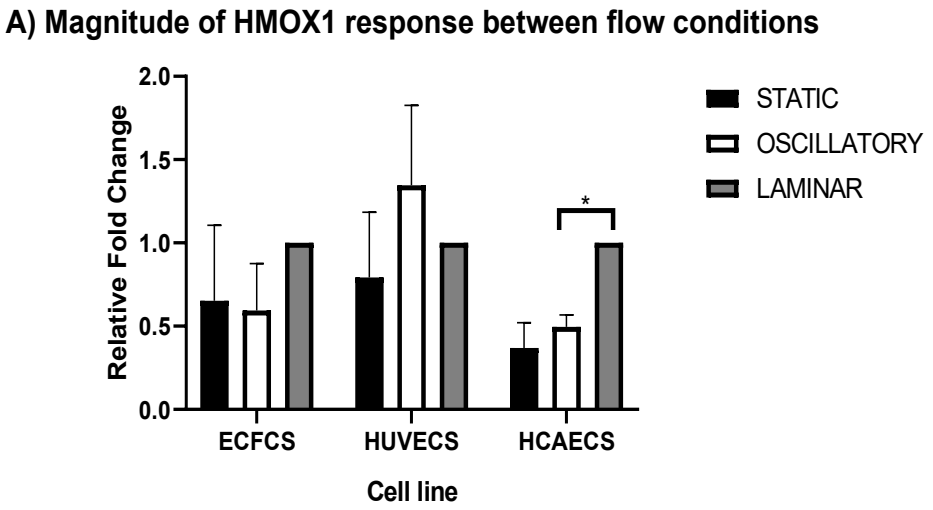
***Figure 31: THMB*.** A) The fold change of *THMB* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented with Log_{10} Scale Y axis). In HUVEC and HCAEC, *THMB* expression was significantly regulated by laminar flow, compared to culture in oscillatory conditions ($P<0.05$). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log_{10} Scale Y axis). No Significant difference were found between any of the cell types under identical culture conditions. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.4 NRF2 Regulated Genes

Nrf2 regulates a number of genes which serve to protect against antioxidant stress include *HMOX1*, NAPDH and oxidoreductase-1 (*NQO1*), *Nrf2* activity is enhanced by *KLF2* and *Nrf2* provides an element of the athero-protective phenotype induced by laminar shear stress (Satta et al., 2017, Nayak et al., 2011). Therefore, we assayed a number of *Nrf2*-regulated genes in HCAECs, HUVECs and ECFCs cultured under identical conditions to identify if shear stress regulates the activity of this important transcription factor.

3.4.1 HMOX1

Significant upregulation of *HMOX1* by laminar flow was observed in HCAEC, but not HUVECs or ECFCs. In HCAECs, *HMOX1* expression was significantly upregulated by laminar shear with a 2-fold change in LSS against oscillatory conditions and a 3-fold change under static culture. In relative terms, ECFCs expressed significantly more *HMOX1* under oscillatory conditions compared to HUVECs (P=0.0491). Therefore, HCAEC show a significant upregulation of *HMOX1* by flow; however, the level of expression was not significantly different, except between ECFCs and HUVECS under oscillatory shear culture conditions.



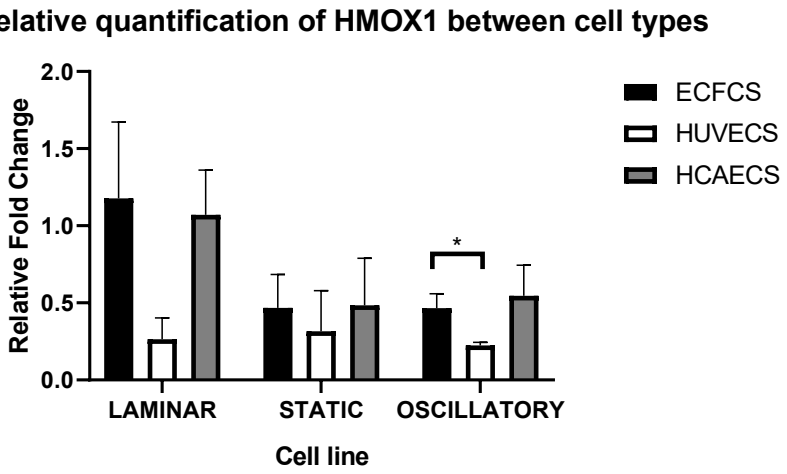


Figure 32: HMOX1. A) The fold change of *HMOX1* mRNA expression for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAEC, *HMOX1* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions (P<0.05). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types. Significant differences were found between ECFCs and HUVECs under identical oscillatory conditions. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.4.2 OSGIN1

A significant upregulation of *OSGIN1* by laminar flow was observed in HCAECs, HUVECs and ECFCs. In HCAECs, *OSGIN1* expression was significantly regulated by laminar shear with a 4-fold change in LSS against oscillatory conditions and a 7-fold change under static culture. In HUVECs, *OSGIN1* expression had a 5-fold change in LSS against oscillatory conditions, with a 3-fold change under static culture. ECFCs had a 2-fold change in LSS against oscillatory whilst under static culture, they had a 3-fold change. In relative terms, no significant differences were found between any of the cell types, indicating they all express a similar level of *OSGIN1* mRNA. Therefore, HCAECs show significant upregulation of OSGIN mRNA by flow, HUVECs are significantly upregulated in LSS against oscillatory shear and ECFCs in LSS against static culture. However, all cell types express a similar level of *OSGIN1* under identical culture conditions.

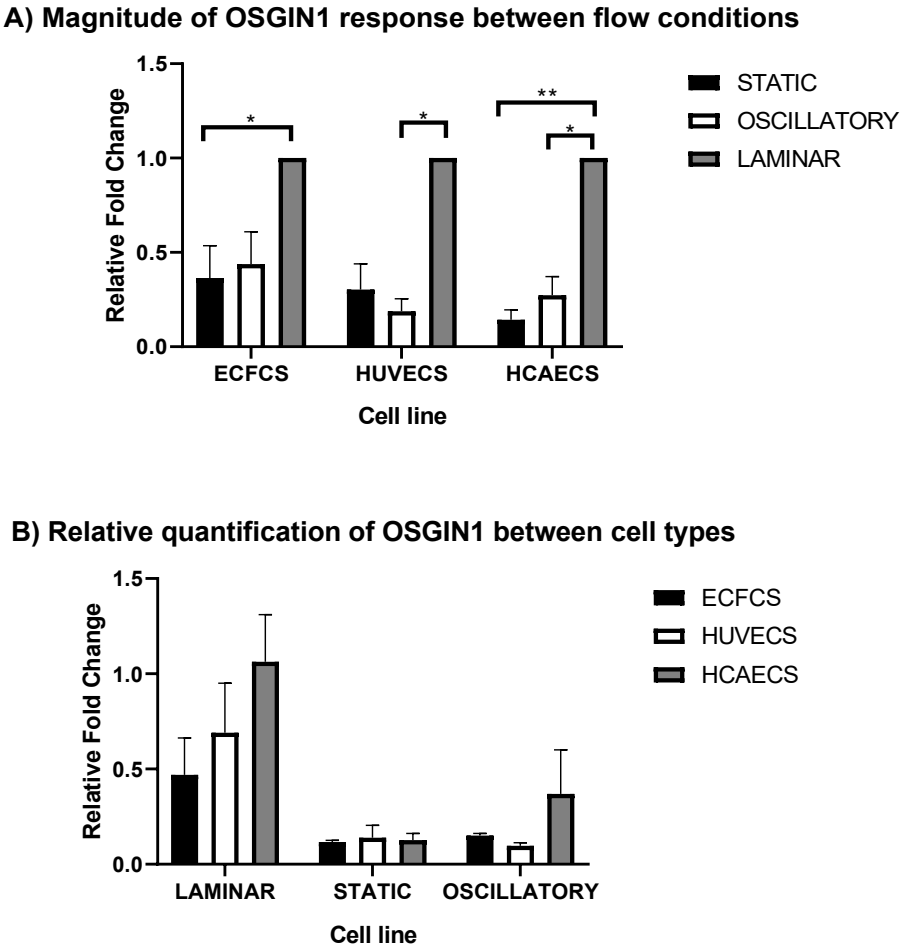


Figure 33: OSGIN1. A) The fold change of *OSGIN1* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAECs, HUVECs and ECFCs, *OSGIN1* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types. No Significant difference were found between any of the cell types under identical flow condition. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.4.5 SRXN1

A significant upregulation of *SRXN1* by laminar flow was observed in HCAEC but not HUVECs or ECFCs. In HCAECs, *SRXN1* expression was significantly regulated by laminar shear with an 18-fold change in LSS against oscillatory conditions and a 64-fold change under static culture. In relative terms, significant differences were found between ECFCs and HCAECs

under static culture, where ECFCs expressed significantly more *SRXN1* then HCAECs, P=0.0396. Therefore, HCAECs show a significant regulation of *SRXN1* by flow; however, all cell types express a similar level of *SRXN1* under laminar and oscillatory conditions, with ECFCs expressing more *SRXN1* than HCAECs under static culture.

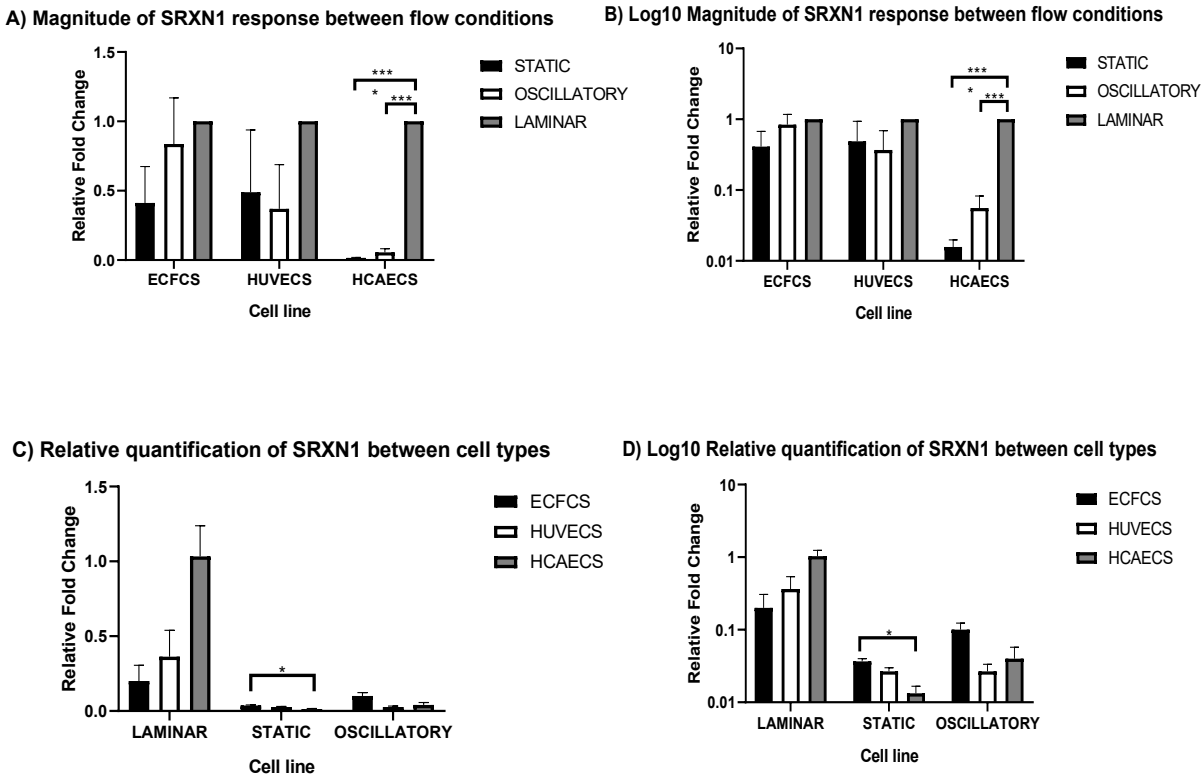


Figure 34: SRXN1. A) The fold change of *SRXN1* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented on a Log_{10} Scale Y axis). In HCAEC, *SRXN1* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log_{10} Scale Y axis). Significant differences were found between ECFC and HCAEC under static shear conditions. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.4.6 GCLM

A significant upregulation of *GCLM* by laminar flow was observed in HCAEC but not HUVECs or ECFCs. In HCAECs, *GCLM* expression was significantly regulated by laminar shear with a

4-fold change in LSS against static culture. In relative terms, no significant differences were found between any of the cell types, indicating they all express a similar level of *GCLM* mRNA. Therefore, HCAECs show a significant regulation of *GCLM* in LSS against static culture; however, all cell types express a similar level of *GCLM* under all culture conditions.

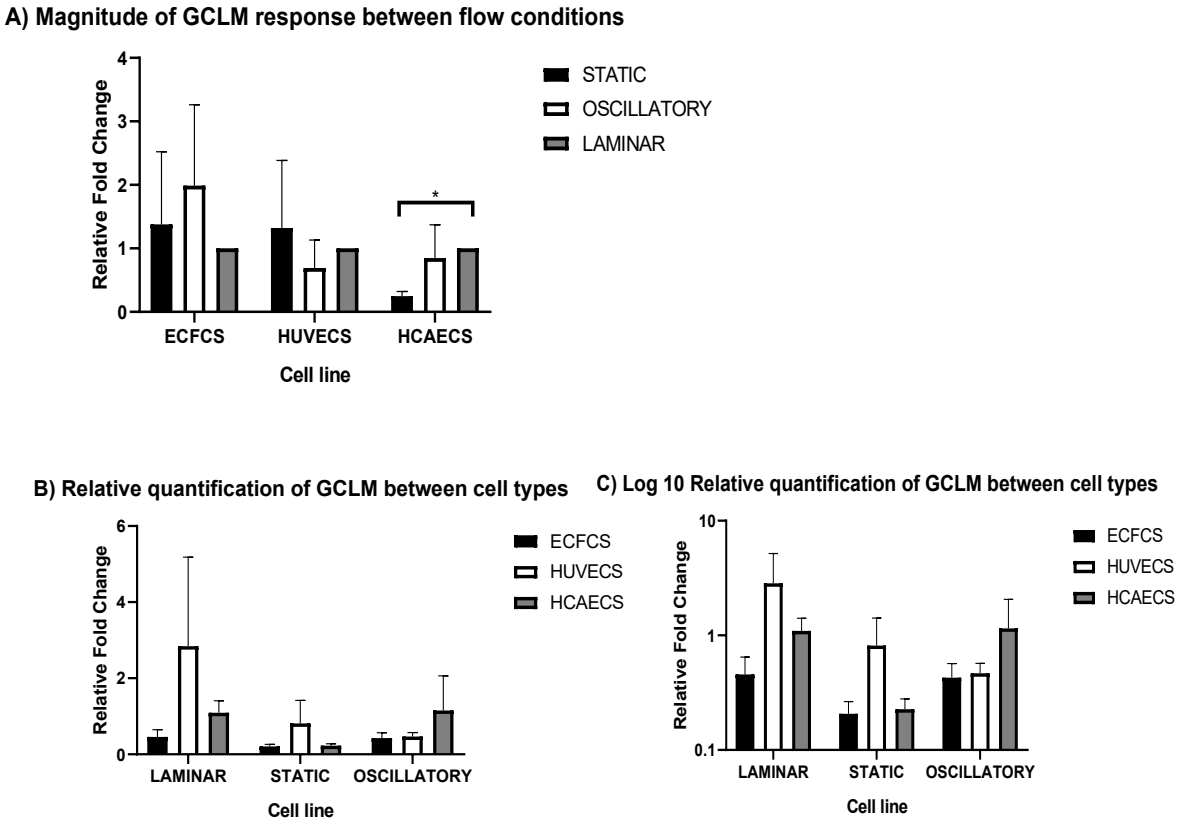


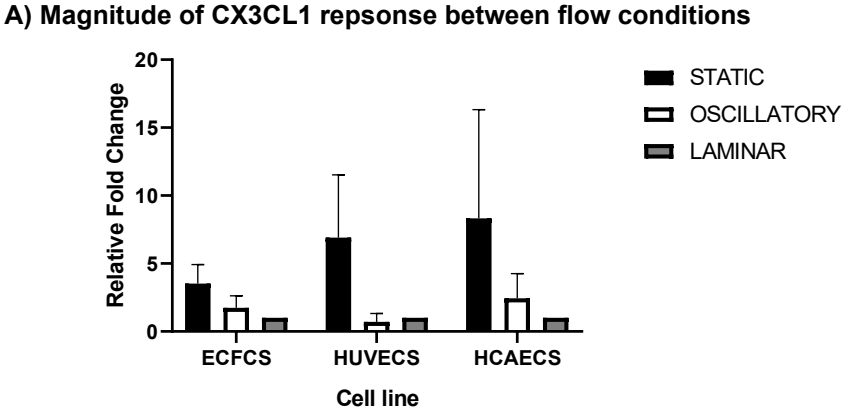
Figure 35: GCLM. A) The fold change of *GCLM* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAEC, *GCLM* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (C, displayed with Log_{10} Scale Y axis). No Significant difference were found between any of the cell types under identical flow condition. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

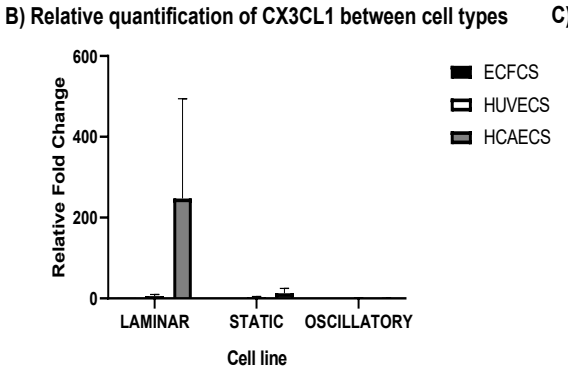
3.5 NFκB Regulated Genes

Responsible for the induction of proinflammatory genes and leukocyte recruitment, promoting atherosclerosis, *NFκB* is a tightly regulated that regulates the expression of genes such as; *VCAM1* and *ICAM1* (Warboys et al., 2011). Therefore, we assayed the expression of *NFκB*-regulated genes in HCAECs, HUVECs and ECFCs under identical culture conditions, to determine if they express similar levels of *NFκB*-regulated genes.

3.5.1 CX3CL1

Significant upregulation of *CX3CL1* by laminar flow was not observed in HCAEC, HUVEC or ECFC. In relative terms, no significant difference was found between the cell types, indicating they all express similar levels of *CX3CL1*. Therefore, all cell types are unresponsive to *CX3CL1* under flow, with none of them showing a significant upregulation and no significant differences were found between the cell types under each flow condition, therefore cells all express similar levels of *CX3CL1*.





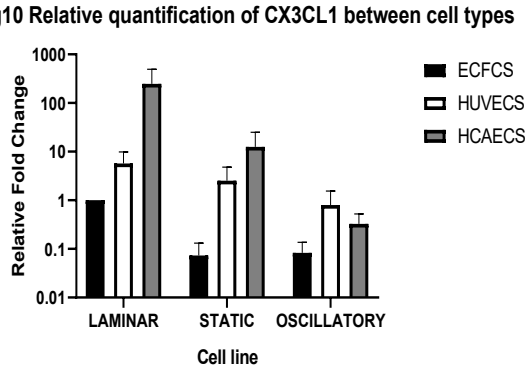
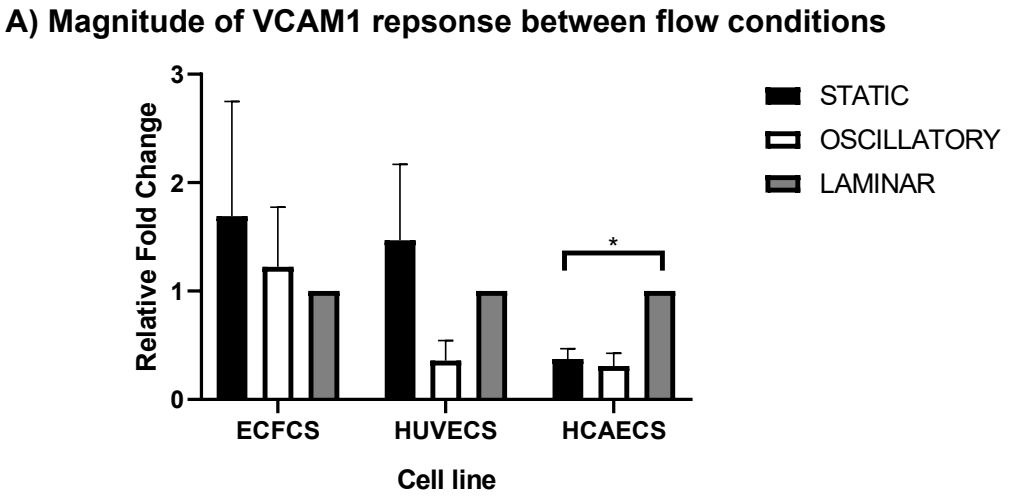


Figure 36: CX3CL1. A) The fold change of *CX3CL1* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. No significant regulation of *CX3CL1* was observed in any of the cell types, compared to culture in static or oscillatory conditions (P<0.05). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (C, Displayed with Log10 Scale Y axis). No Significant differences were found between any of the cell types under identical flow condition. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.5.2 VCAM1

Significant upregulation of *VCAM1* by flow was observed in HCAECs. In HCAECs, *VCAM1* expression was significantly regulated by laminar shear with a 5-fold change in LSS against static culture. In relative terms, no significant differences were found between the cell types under the identical flow conditions. Therefore, HCAEC show a significant regulation of *VCAM1* in LSS under static culture; however, HCAECs, HUVECs and ECFCs all express a similar level of *VCAM1* under all identical culture conditions.



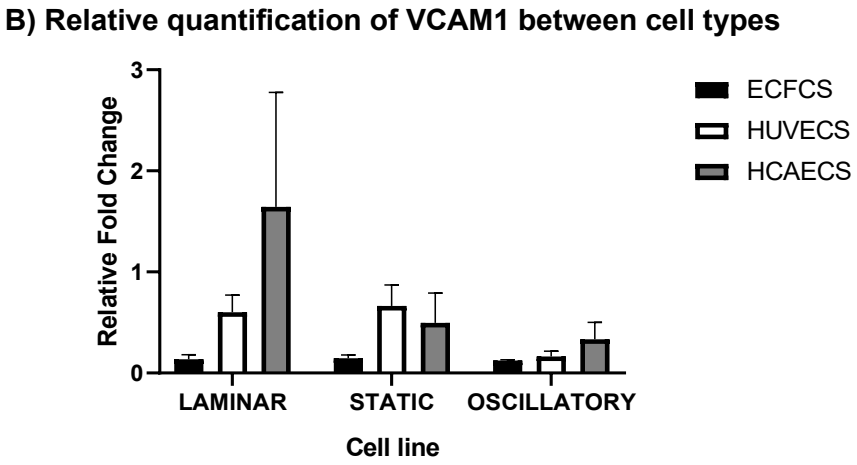
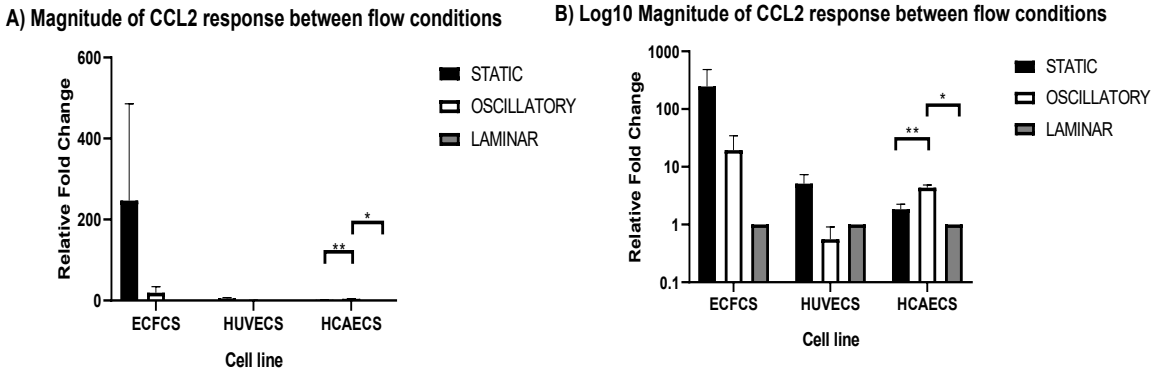


Figure 37: VCAM1. A) The fold change of *VCAM1* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow. In HCAEC, *VCAM1* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions (P<0.05). B) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types. No significant differences were found between any of the cell types under identical flow conditions. Data analysed using a 2-Way A*NOV*A, n=3, *p<0.05, **P<0.01, *** p<0.001.

3.5.4 CCL2

Significant downregulation of *CCL2* by laminar flow was observed in HCAECs, with a 4-fold change, but not in HUVEC or ECFCs. In HCAECs, CCL2 expression was reduced 4-fold in laminar shear compared to oscillatory shear. Therefore, HCAECs show a significant regulation of *CCL2* by flow; however, all cell types express a similar level of *CCL2* under the identical culture conditions.



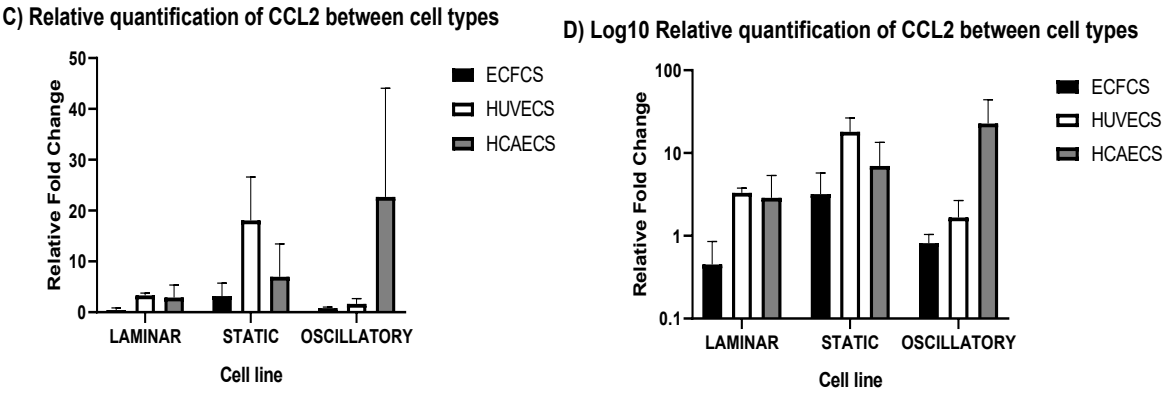


Figure 38: CCL2. A) The fold change of *CCL2* mRNA for each cell line under static, oscillatory and laminar flow conditions for 72 hours, relative to the amount quantified in each cell type cultured under laminar flow (B, same data presented on a Log10 Scale graph). In HCAEC, *CCL2* expression was significantly regulated by laminar flow, compared to culture in static or oscillatory conditions ($P<0.05$). C) Data displayed relative to HCAEC cultured under laminar flow, to allow comparison of the relative quantification of mRNA between cell types (D, displayed with Log10 Scale Y axis). No significant differences were found between any of the cell types under identical culture conditions. Data analysed using a 2-Way A*NOV*A, n=3, *$p<0.05$, **$P<0.01$, *** $p<0.001$.

4.0 Discussion

Through the utilization of modern laboratory techniques and technologies, this project aimed to determine whether HUVECs and ECFCs would be suitable for cardiovascular research, as an alternative to the more expensive HCAECs. Using identical media and conditions, confluent monolayers of HCAECs, HUVECs and ECFCs were cultured for a 72-hour period under static, oscillatory or laminar flow conditions. Each replicate was seeded from a single flask onto gelatin-coated slides and then cultured in parallel under the different conditions, to ensure that the only difference was the shear environment. Images of the cell types were taken along with collection of protein and RNA samples to carry out molecular analysis techniques. The gene expression from each cell line was then examined by qPCR using identical materials and methods, and performing the qPCRs for each gene within the same reaction, to ensure that there was uniformity in assessing the expression across each cell type.

4.1 Utility of cell types in cardiovascular research

HCAECs might be considered the gold standard for research into cardiovascular disease because they are obtained from the anatomical site associated with the most significant impact of atherosclerosis. HCAECs originate from a different embryonic origin compared to other arterial cells, potentially making their use in studies into the processes that regulate atherosclerosis even more important. The major drawback from using HCAECs is the price, making them unaffordable in some laboratories. It also reduces the ability to study HCAECs from multiple donors with particular risk factors (e.g. smokers or diabetics, or male v female donors) to examine the genetic or epigenetic effects of these risk factors on disease processes. HCAEC have been reported to have a greater susceptibility to inflammation compared to HUVECs as indicated by a research study performed by Lakota et al., (Lakota et al., 2009). Whilst little to no research has been conducted into ECFC appropriacy in disease modelling, one beneficial aspect of the cell line is their accessibility. ECFC are derived from the buffy coat layer in the blood, where they can be cultured and grown to desirable quantity as well as being derived from the of blood multiple patient groups, including those who are affected by diseases characterized by inflammation (Edwards et al., 2018). For example, ECFCs from patients with type 2 diabetes maintain a diseased phenotype have reduced angiogenic capacity and mitochondrial dysfunction and changes in energy metabolism compared to ECFCs from non-diabetic donors (Edwards et al., 2018). In atherosclerosis, it has been suggested that a high ECFC count may exert an atheroprotective effect in response to vascular damage, where they migrate to the site of injury and begin repair through the actions of endothelial cell replenishment and atherogenesis (Edwards et al., 2018).

4.2 Molecular regulation of atherosclerosis

In all aspects of atherosclerosis, blood flow is crucial for either pathogenesis of the disease or protection. Shear stress can be defined as a 'frictional force per unit area from flowing blood' acting upon the endothelium to control the morphology, structure and biochemical changes in EC lining the artery (Hahn and Schwartz, 2009). Atherosclerosis develops at bifurcation sites in the arteries, where oscillatory blood flow can mediate the foundations for disease development and progression (Hahn and Schwartz, 2009). Lesions found in these areas contain lipids, leukocytes and migrated smooth muscle cells which can

collectively lead to cholesterol crystal and calcification-containing necrotic cores; offering much greater and severe consequences (Hahn and Schwartz, 2009).

Laminar shear stress induces pathways mediated by transcription factors *KLF2*/4 and *Nrf2*, driving an atheroprotective response through the upregulation of anti-inflammatory and antioxidant genes (Fledderus et al., 2008; Satta et al., 2017). Although *Nrf2* is recognised as an atheroprotective pathway through the regulation of the oxidant/ antioxidant system, it may also contribute to the pathogenesis of endothelial erosion, if very high-level activation of *Nrf2* is sustained (Satta et al., 2019). *NFκB* is a pathway induced by disturbed shear stress, driving a proinflammatory response through genes such as *ICAM1*, *VCAM1* and cytokines such as *TNF-α*, with coordinated regulation of the MAPK pathway (Warboys et al., 2011). The drive seen in the inflammatory response correlates with the development of atherogenesis, favouring detrimental cellular effects including poor alignment to flow (Hahn and Schwartz, 2009).

4.2.1 Krupple-Like Factor Gene Regulation

The data presented within this study shows that laminar flow significantly upregulates both *KLF2* and *KLF4* in HCAEC, resulting in an increase of expression of all of the genes known to be regulated by *KLF2*/4 in endothelial cells. This universal response was not observed for HUVECs and ECFCs, which demonstrated variable regulation of *KLF2* +4 and their regulated genes by laminar flow. HUVECs and ECFCs did not significantly increase the expression of *KLF2* under laminar flow conditions, however there was a significant and similar upregulation of *KLF4* in all 3 cell types by laminar flow. Similarly, the KLF-regulated genes appeared to be regulated by flow on a continuum between the *KLF4* and *KLF2* pattern of regulation. *PI16* demonstrated a very large upregulation by laminar flow in all cell types, similar to *KLF4*. *NOV* demonstrated a large upregulation in HCAECs and HUVECs with laminar flow, as well as *THMB*. The expression of *eNOS* almost exactly mirrored that of *KLF2*. This might suggest the relative importance that either *KLF2* or *KLF4* plays in the regulation of each genes across the different cell types.

An observation made solely in *THMB* provides evidence that, contrary to popular belief, static culture is not the same as oscillatory culture and should not be used as an alternative model for oscillatory shear. In HCAECs and HUVECs, the expression of *THMB* was

significantly upregulated under static shear stress against oscillatory shear stress, underlining the differential expression pattern in static and OSS culture.

4.2.2 Nuclear Erythroid like Factor 2 Regulation

We demonstrated that there were also differences between HCAECs, HUVECs and ECFCs in the shear-regulation of known *Nrf2*-regulated genes. HCAEC demonstrated a significant upregulation of *HMOX1*, *OSGIN1*, *SRXN1* and *GCLM*, the relevant *Nrf2*-regulated genes, by laminar sheer stress. HUVECs and ECFCs both only demonstrated a significant upregulation of *OSGIN1* with laminar shear stress, from the *Nrf2*-regulated genes tested. HUVEC and ECFC present with a large variation across *Nrf2* regulated genes as well as *NFκB*, suggesting a large donor effect on the regulation of these genes. Increasing the number of donors tested might help determine if laminar shear stress significantly regulates these genes in HUVECs and ECFCs.

4.2.3 Nuclear Factor Kappa B Regulation

Data presented from our research on the *NFκB* regulated genes shows, HCAECs, HUVECs and ECFCs all demonstrate differences. In HCAECs, CCL2 demonstrated a downregulation by laminar flow, whilst in HUVECS, no significant regulation of the *NFκB* regulated genes by flow was observed (*CX3CL1, VCAM1* and *CCL2)*. TNF- α is a crucial proinflammatory cytokine which drives inflammation pathways including *NFκB* and can rapidly induce the transcription of genes involved in inflammation and cell proliferation and differentiation (Gupta et al., 2005). Introducing TNF-α in a separate study may be useful in determining further response of *NFκB* genes, due to the influence of TNF-α , in HUVECs and ECFCs. Data sets from both studies can then be used in a side by side comparison to note the effects of introduction of TNF-α.

4.3 Cell morphology

The mechanosensitive abilities of vascular endothelial cells are demonstrated through the correct morphological qualities and flow alignment in HCAECs, HUVECs and ECFCs. ECFCs appear less responsive to tissue culture, as their confluency varies from batch to batch and is visibly less between cell types. These variations may be due to donor differences or the general nature of ECFCs, the variation in ECFCs, compared to HUVECs and HCAECs, across atherosclerotic relevant genes that we tested are possibly linked to the variation demonstrated in the cell morphology.

4.4 Conclusion

Our data concludes that HCAECs are the most responsive cell type in the KLF, *Nrf2* and *NFκB* pathways, with significant upregulation of most genes in response to flow, with the only exception being *CX3CL1*, and therefore remain the most appropriate cell type to model atherosclerosis. HUVECs mostly demonstrate a significant upregulation of the genes collectively and therefore would be an ideal cell model, however further work with a larger N number should be done to confirm this. ECFCs can now be addressed as the most unresponsive cell type, with few significant upregulations to genes by flow and can be deemed inappropriate cell models for atherosclerosis research.

5.0 Future Work

There are many ways in which this set of experiments could be enhanced. Primarily, increasing the number of batches of cells used would allow the detection of smaller differences in gene expression to be observed. Due to time and money constraints we were limited to an n=3 for each cell type, with each replicate being derived from a different donor. This had a large effect on the ability to detect small changes in shear-regulated gene expression. The large variation seen between donors was a major limiting factor in this study. Similarly, additional types of analyses could be employed, including next generation sequencing to perform an unbiased assessment of gene expression could have been performed, rather than the limited assessment of key shear-regulated genes performed in this study. Widening the measurement of gene expression to include the quantification of changes in protein expression would account for potential regulation by microRNAs, which might regulate translation in a cell type-dependent manner.

An aspect not covered in this research project is the potential use of induced pluripotent stem cells (IPS). IPS are reprogrammed into a state of embryonic-like pluripotency, and then differentiated into a variety of human cells (Stemcell.ucla.edu, 2019). Following the generation of IPS cells, a new opportunity arose in the field of cardiovascular research and recent advances into stem cells has offered great potential for the use of IPS in cardiac research and regenerative medicine (Martins, Vunjak-novakovic and Reis, 2014). The nature of IPS cells residence, skin and blood from adult somatic cells, means ethical issues are not of any concern (Martins, Vunjak-novakovic and Reis, 2014). Additionally, IPS cells

offer no implications with transplantation rejection, since the cells themselves are derived from the patient's own blood or skin, offering more beneficial advantages in research and regenerative medicine (Martins, Vunjak-novakovic and Reis, 2014). IPS can be differentiated into endothelial cells, and offers the potential to examine EC function from patients with known genetic susceptibility to cardiovascular disease. Functional assessment of IPS-ECs would have been an interesting extension to the experiments performed her.

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7.0 Appendix

Blood donation participant information sheet for derivation of ECFCs.

Manchester Metropolitan University

Participant Information Sheet

Version 2; 14th February, 2017.

Title of Study: Investigating endothelial cell repair responses in an inflammatory environment.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Damage to the lining of blood vessels (endothelial dysfunction) underlies many diseases associated with inflammation and a high cardiovascular disease risk eg Type II Diabetes, Systemic Lupus Erythematosus (an auto-immune disease) and atherosclerosis (clogging or hardening of the blood vessels).

Cells and microvesicles (small particles released from blood cells) circulating in the blood are thought to play an important role in the health and maintenance of this delicate lining of the blood vessels, which become damaged during disease. The molecular and cellular mechanisms by which these cells and microvesicles influence blood vessel repair processes in disease are poorly understood.

The purpose of this project is to investigate the interaction between these damaged cells, microvesicles, inflammation and repair using a disease model that we can study in the laboratory.

Cells and blood plasma microvesicles can be harvested from the blood. We can grow the cells in the laboratory and subject them to various treatments to recapitulate disease conditions. We can investigate the function of these cells and microvesicles and examine the molecules and proteins that are altered in disease-like conditions compared to untreated cells.

This study is an early step in the complicated process of identifying markers in human patients who have endothelial dysfunction in diseases including type II diabetes and hardening of the arteries, which will be done in a complimentary project. This model will allow us to estimate the future development of disease and allow us to develop new therapeutic targets and biomarkers of disease.

We would like to invite you to help us identify these proteins and molecules, which are involved in this disease process by allowing us to take a sample of your blood, so that we may in the future prevent some of the vascular disease that amounts to one of the leading causes of death in the western world.

What will I have to do if I take part?

If you agree to take part you would be asked to donate a 60 ml sample of blood. This is a simple procedure carried out by a trained phlebotomist in our department and should take no more than 10 minutes. We will assign a number to your sample and your name will be kept confidential.

What are the possible risks of taking part?

You may experience a sharp scratch sensation for a second when the phlebotomist takes your blood via a small needle. The blood will be taken whist sitting in a comfortable, private and hygienic environment. Let the phlebotomist know if you have ever experienced fainting when giving blood previously.

Do I have to take part?

No, taking part is voluntary. If you would prefer not to take part, you do not have to give a reason. If you do decide to take part, we will ask you to sign a consent form and give you a copy of this information sheet and consent form to keep. If you decide to take part, you are still free to withdraw at any time.

What will happen to the results of the research study?

The results of the study will be published in scientific journals, presented as scientific conferences and at public engagement events. Your name will not be identified.

Thank you very much for considering taking part in our research.

If you wish to obtain advice about this research you may contact:

Dr Fiona Wilkinson

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Professor Yvonne Alexander

Tel: 0161 2475428

Healthcare Science

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