

**Roundabout receptor 4 and its role in endothelial
barrier dysfunction following cardiac surgery**

Thesis presented for the degree of Doctor of Philosophy

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Declaration of Originality

I hereby declare that this submission is my own work, or if not, it is clearly stated and fully acknowledged. All information in this thesis has been obtain and presented in accordance with academic rules and ethical conduct.

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Abstract

Endothelial barrier dysfunction contributes to the systemic inflammatory response in patients undergoing cardiac surgery with surgery necessitating cardiopulmonary bypass. The bypass enables to perform complicated operations on static heart, but it comes with risks such as a damage to the endothelium which causes inflammation. Activation of endothelial cells by inflammatory mediators leads to changes in endothelial function. These changes include up-regulation of adhesion molecules, production and secretion of inflammatory mediators and chemo-attractant agents, as well as vasoactive compounds. These changes are central to the pathological processes leading to sepsis syndrome. The exaggerated release of pro- and anti-inflammatory cytokines leads to development of septic and vasoplegic syndromes which complicate recovery and can even cause a death.

Currently, there are no specific therapies that target the endothelium, but greater insight into the pathogenesis of endothelial barrier dysfunction could lead to new biomarkers and therapies. Roundabout receptor 4 could be a novel biomarker and target for specific pharmacological intervention. Roundabout receptor 4 was originally identified as an axon guidance molecule but is now known to mediate various endothelial processes including cell migration and proliferation. At the outset of this thesis a role for Roundabout receptor 4 and its Slit 2 glycoprotein ligand as regulators of endothelial barrier function were also beginning to emerge; but there was little evidence of how Roundabout receptor 4 expression profiles and function might differ between endothelial cells; or of implications of Roundabout receptor 4 detected in human plasma. In patients (n=48) undergoing cardiac surgery, standard biomarkers of endothelial activation significantly increased 24h after surgery; by contrast, Roundabout receptor 4 increased immediately post-operatively, as did another novel biomarker of endothelial dysfunction - Syndecan-1. Roundabout receptor 4 could be a useful early marker of endothelial dysfunction in this patient group. There were significant associations between Roundabout receptor 4 plasma levels and prolonged length of stay in intensive care and hospital.

Higher pre-operative plasma levels of Roundabout receptor 4 were also associated with the development of vasoplegia, a post-operative complication. Vasoplegia is caused by an imbalance of vasodilator and vasopressor mechanisms in the body, but the exact pathophysiology is not known. Vasoplegic patients are treated with high and prolonged doses of vasoconstrictor medications. This treatment can cause peripheral vasoconstriction and ischemia of limbs that can lead to metabolic acidosis and tissue necrosis.

In vitro, Robo4 external expression decreased in endothelial cells treated with inflammatory cytokines. Robo4 plasma levels increased after cardiac surgery with snCPB. These results suggest that Robo4 might be cleaved or secreted by endothelial cells exposed to inflammation and released into the bloodstream.

Roundabout receptor 4 expression was greater on human umbilical vein endothelial cells than on human pulmonary artery endothelial cells; and decreased following stimulation with tumour necrosis factor-alpha. In parallel, Slit2 increased barrier integrity in human umbilical vein endothelial cells but not in human pulmonary artery endothelial cells; difference could relate to differences in Roundabout receptor 4 expression levels, as Slit2 effects were inhibited with Roundabout receptor 4 short interfering RNA.

The finding from these investigations inform greater understanding of the important role of Robo4 as an endothelial biomarker and its role in barrier function.

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List of Abbreviations

Ang2	Angiopoietin-2
ANOVA	Analysis Of Variance
BHF	British Heart Foundation
BMMC	Bone marrow mononuclear cells
BMVEC	Brain microvascular endothelial cells
CABG	Coronary artery bypass graft
CPB	Cardiopulmonary bypass
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
EC	Endothelial cells
ESC	Embryonic stem cells
FADD	Fas-Associating Death Domain
fl-RAGE	Full-length membrane-bound RAGE
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H7N9 IAV	H7N9 influenza A virus
HBMEC	Human Brain Microvascular EC
HCAEC	Human coronary artery endothelial cells
HCMEC	Human cerebral microvascular endothelial cells
HDMEC	Human dermal microvascular endothelial cells
HEKC	Human embryonic kidney cells
HMGB1	High-mobility group box 1
HPAEC	Human pulmonary artery endothelial cells
HPMSC	Human placental multipotent mesenchymal stromal cells
HPTC	Human primary trophoblast cells
HREC	Human retinal endothelial cells
HREC	Human retinal endothelial cells
HRGEC	Human renal glomerular endothelial cells
HUVEC	Human umbilical vein endothelial cells

ICU	Intensive Care Unit
IL-1 β	Interleukin-1 beta
IL-10	Interleukin-10
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile range
LGC	Luteinized granulosa cells
LOS	Length of stay
MASMC	Mouse aortic smooth muscle cells
MCP-1	Monocyte Chemoattractant Protein 1
MFI	Median Fluorescence Intensity
MODS	Multiple organ dysfunction syndrome
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end-products
Robo	Roundabout receptor
SD	Standard Deviation
si-RNA	Short interfering Ribonucleic acid
SIRS	Systemic inflammatory response
snCPB	Surgery necessitating cardiopulmonary bypass
s-RAGE	Soluble RAGE
TEEI	Trans-endothelial electrical impedance
Th1	T-cell helper 1
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNF- α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion protein 1

VS	Vasoplegic syndrome
vWF	Von Willebrand Factor

List of Publications:

S Patel, **T Svermova**, A Burke-Gaffney, R. G. Bogle, 2018, DRUG-ELUTING BALLOONS WITH PROVISIONAL BAIL-OUT OR ADJUNCTIVE STENTING IN *DE NOVO* CORONARY ARTERY LESIONS (a systematic review and meta-analysis). Cardiovascular Diagnosis and Therapy, 2018;8(2):121-136. doi: 10.21037/cdt.2017.10.09

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A Burke-Gaffney, **T Svermova**, S Mumby, SJ Finney, TW Evans, 2014, RAISED PLASMA ROBO4 LEVELS ARE ASSOCIATED WITH CARDIAC SURGERY-ACQUIRED ACUTE KIDNEY INJURY. PLoS One. 2014 Oct 31;9(10):e111459. doi: 10.1371/journal.pone.0111459. eCollection 2014.

List of Presentations:

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T Svermova, Z Nikolakopoulou, JA Mitchell, A Burke-Gaffney 2015, INCREASED PLASMA SLIT2 LEVELS FOLLOWING CARDIAC SURGERY, The International Society for Applied Cardiovascular Biology European Meeting, Nuremberg, Germany, 2015

1. INTRODUCTION

1.1. Cardiovascular Disease

1.1.1. Context

British Heart Foundation (BHF), UK, describes cardiovascular disease (CVD) as a term covering all diseases affecting heart and blood circulation. These diseases include coronary heart disease, aortic diseases, peripheral arterial disease, angina, heart attack, heart failure, transient ischaemic attack and stroke. Over the last 20 years, prescriptions for medical therapy and also surgical interventions for CVD increased considerably. The work in this thesis was supported by the BHF and it investigates some of the complications that can prolong recovery from cardiac surgery; and proposes a potential molecular target – Roundabout receptor (Robo) 4 - that could be a future biomarker and focus for developing novel pharmacotherapies to limit post-cardiac surgery complications.

1.1.2. Epidemiology

CVD is the world's most common cause of death and its mortality rates are increasing. In 2013, over 17 million people died from CVD worldwide which was 31.5% of all deaths, compare to 12 million in 1990 which was 25.9% of all deaths. In Europe alone, there were around 4 million deaths in 2012 which was 45% of all deaths. More women than

men die from cardiovascular disease. In 2012 in Europe 2.2 million women and 1.8 million men died from CVD (Townsend et al, 2016). The main reasons for the increase in mortality from CVD is the growth of population, the ageing of population and the epidemiologic changes in disease. For example, in South Asia the increase in death from CVD between 1990 and 2013 was 97.4% which represents 1.7 million more deaths in 2013 than in 1990 in the region (Roth *et al*, 2015). On the other hand, the mortality rates in Central and Western Europe are declining (Roth *et al*, 2015; Bhatnagar et al, 2016). For example, in the UK the decrease in death from CVD, between 1980 and 2013, was 68% (Bhatnagar *et al*, 2016). In 1960s CVD was most common in high-income countries and almost unknown in low-income countries, while now it affects most people in low and middle-income countries. The World Health Organisation (WHO) has set a target to reduce CVD worldwide by 25% by 2025 (Yusuf et al, 2015). In the United Kingdom, CVD is the biggest killer of women (28% of all death) and second biggest killer of men (29% of all death). Most CVD death are caused by coronary heart disease (46% of CVD death) and stroke (26% of CVD death) (Bhatnagar et al, 2015). See Figure 1 for more details on CVD in context of other major causes of death, in men and women, in the United Kingdom and European region of 53 states as defined by the WHO.

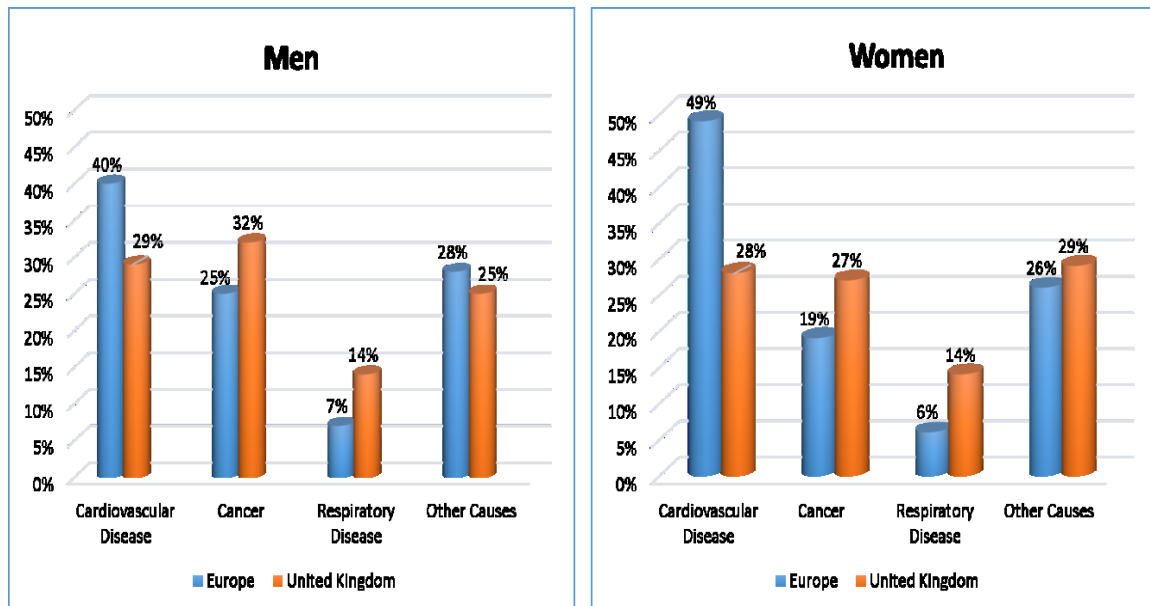


Figure.1. Major causes of death in Europe and the United Kingdom, among men and women in 2015/16. Adapted from data published by Bhatnagar et al, 2015 and Townsend et al, 2016.

1.1.3. Risk Factors

There are many risk factors leading to the development of CVD, such as high blood pressure, high cholesterol, diabetes and obesity. These are mostly results of bad diet (high salt intake, high animal products consumption and excessive alcohol drinking), smoking of tobacco, inactivity and stress (Yusuf et al, 2015). In addition, risk factors for valvular heart disease are infections, genetics (born with and/or run in family) and age as heart valves thicken and stiffen with age (lung et al, 2003).

1.1.4. Treatment and Surgery

CVD in the UK presents substantial financial burden for the National Health Service. In 2012 the cost of medical and surgical CVD treatment in England alone was almost £7billion. The cost is rising every year. There were more than 300 million medications prescribed for CVD in 2013 which was six time more than 30 years ago. (Bhatnagar et al, 2015). The most common CVD surgical intervention is coronary artery bypass graft (CABG). It aims to restore myocardial perfusion using arterial or venous grafts to bypass coronary occlusion or stenosis (Roncada et al, 2015). Another often performed procedure is repair or replacement of mitral or aortic valves. Where ever possible repair is preferred over replacement as it is less invasive and has better long-term survival prognosis of the repair (Chan et al, 2018). Both, CABG and valve corrections, are open-heart surgeries that usually require cardiopulmonary bypass (CPB).

1.2. Cardiopulmonary Bypass

1.2.1. Historical perspective

CPB allows surgeons to perform open heart surgery and it has advanced the field of cardiac interventions. It was successfully used for the first time in 1953 by John Gibbon, to repair an atrial septal defect (Gibbon, 1954). Nowadays, over 2000 operations using bypass are performed every 24 hours globally (Bhatnagar et al, 2015).

1.2.2. Basic principles

The CPB machine replaces the function of the heart and lungs during cardiac surgery. Its main purpose is to provide oxygenated blood to the body, when the heart is stopped during surgical intervention (Estafanous et al, 2001). The de-oxygenated blood is drained through venous cannula to the bypass machine, where it goes through a membrane oxygenator and then through arterial cannula back to the aorta (Estafanous et al, 2001). The bypass machine is highly complicated equipment that has a large number of monitors for checking the blood oxygen saturation, blood pressure, temperature, gases and electrolytes during cardiac surgery (Sarkar and Prabhu, 2017). A schematic overview of CPB can be seen in the Figure 2.

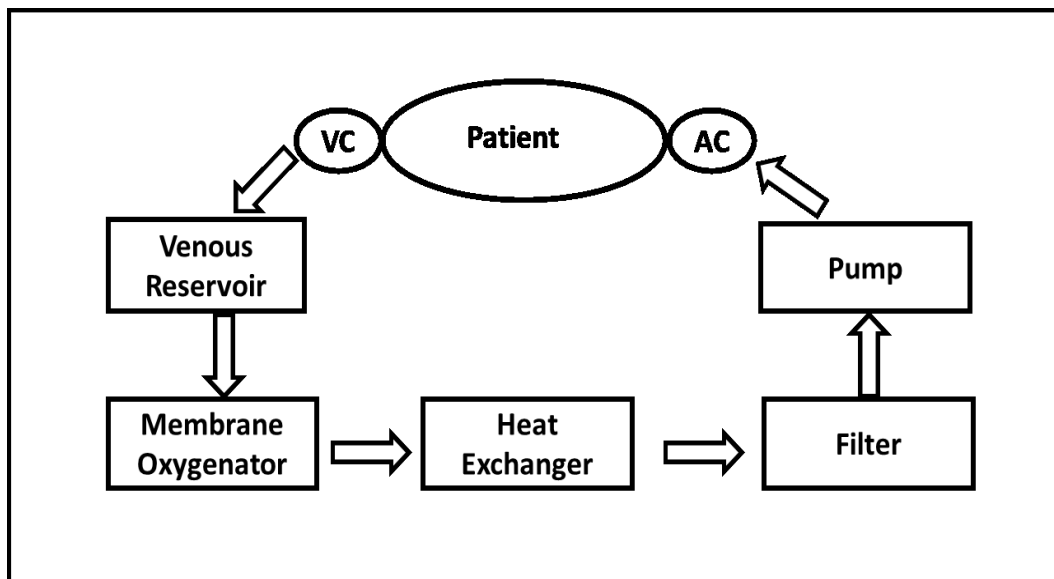


Figure 2. Schematic overview of cardiopulmonary bypass. VC= venous cannula, AC = arterial cannula.

1.2.3. Complications

A reliable bypass apparatus has to meet three main requirements – (1) to oxygenate blood, (2) to pump blood without damaging red blood cells, (3) to anticoagulate blood reversibly. In the 1950s the biggest problem was to find a safe way to oxygenate blood. In the 1960s commercial production of bypass machines with hard-shell bubble oxygenators started. In the 1980s bypass started using fibre micropore membrane oxygenators which are still used today. Since the 1980s a cooling technique is used on the heart to prevent tissue damage (Stoney, 2009). Over the years the CPB equipment has improved immensely, but use of CPB is still not without risks (Groom et al, 2005). These risks include platelet dysfunction (blood clots and bleeding) due to dilution of clotting factors, air embolism, cerebral injuries leading to stroke and respiratory distress leading to acute lung injury (Mekontso-Dessap et al, 2001). During CPB, the blood is exposed to foreign surfaces in the pump and tubing (Omar *et al*, 2015), as well as to physical injury caused by surgical manipulation or hemodynamic shear stress (Verrier and Morgan, 1998). This leads to activation of numerous enzyme pathways and production of inflammatory mediators that damage the endothelium (Omar *et al*, 2015). The release of inflammatory cytokines leads to inflammation, and can contribute to systemic inflammatory response syndrome (SIRS), Sepsis and Vasoplegic syndrome (Sarkar and Prabhu, 2017). The longer the bypass machine is used during an operation, the greater the risks (Estafanous et al, 2001). Another risk factor contributing to post-CPB complications is age. People live longer and undergo operations requiring CPB at later stage of their life. Almost 50% of heart surgery patients endure slight cerebral

complications; up to 5% suffer a stroke; increased risk of stroke is associated with age (Newman et al, 2001).

1.3. Systemic inflammatory response and Sepsis Syndrome

1.3.1. Epidemiology

About one-third of all hospital in-patients suffer from Systemic inflammatory response syndrome (SIRS). In Intensive care units (ICU) more than 50% of patients suffer from SIRS and in surgical ICU it is more than 80% patients that are affected by SIRS (Brun-Buisson C, 2000). The sepsis syndromes represent the leading cause of death in adult ICUs. Every year there are about 500,000 patients, in both Europe and the USA, affected by SIRS (Paterson and Webster, 2000). SIRS and sepsis can progress to septic shock and multiple organ dysfunction syndrome (MODS), as it is shown in Figure 3, which can cause organ failure and death. The mortality from severe sepsis is around 40%, the proportion of patients who die as a consequence of septic shock and MODS is 70% (Annane et al., 2005).

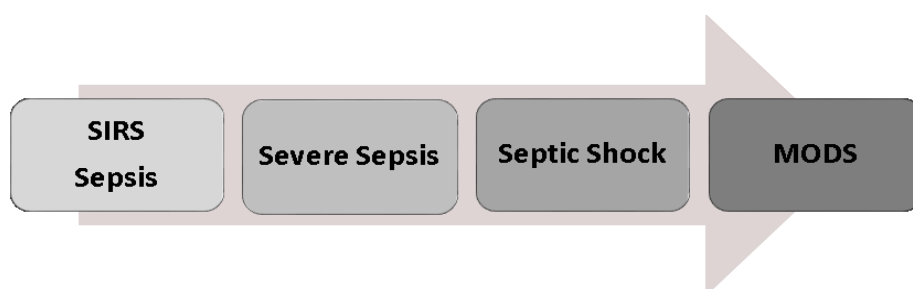


Figure 3. Schematic overview of Sepsis Syndrome. The progression from SIRS/early sepsis to multiple organ dysfunction syndrome.

1.3.2. Pathophysiology

The endothelium plays an active role in the inflammatory response to infection during sepsis. The immune system releases a number of pro- and anti-inflammatory cytokines that damage the endothelium by either disrupting the intracellular junctions or by changing the cytoskeletal structure of endothelial cells (Lee and Slutsky, 2010). Sepsis is a result of exaggerated inflammatory response to infection and is characterised by vasodilation, increased microvascular permeability and leukocyte accumulation (Peters et al, 2003). The immune response in sepsis has three phases: phase 1 = hypo-responsiveness that lasts only a short time; phase 2 = hyper-responsiveness when pro- and anti-inflammatory cytokines are released and leukocyte recruitment is increased. This cytokine storm leads to organ damage, such as acute kidney injury or acute lung injury. Phase 3 = hypo-function of immune system that increases chances of infection. This phase can last days or weeks (Hotchkiss and Karl, 2003).

1.3.3. Definition and Diagnosis

The sepsis syndrome was defined for the first time by American College of Chest Physicians and Society of Critical Care Medicine in 1991. The definition has been since revised two times. First time in 2001 by American College of Chest Physicians, Society of Critical Care Medicine, American Thoracic Society, Surgical Infection Society and European Society of Intensive Care Medicine at International Sepsis Definitions Conference; and second time in 2016 by a committee of 55 international experts

representing 25 international organizations (Rhodes et al, 2016). It is of note that the definition used throughout this thesis refers to the 2001 definition as this was current at the stage of this PhD project. SIRS can be caused by infection or by non-infectious insult, like surgery (Comstedt et al, 2009). The manifestation of SIRS and early sepsis are so similar that they cannot be clinically really distinguished (Dellinger et al, 2013). SIRS is diagnosed, if at least two of the following criteria are present:

1. Temperature (fever $>38^{\circ}\text{C}$ or hypothermia $<36^{\circ}\text{C}$)
2. Respiratory rate (>20 breath/min = tachypnea; or $\text{PaCO}_2 < 4.3\text{kPa}$)
3. Heart rate (>90 beats/min = tachycardia)
4. White blood cell count (leucocytosis $>12 \times 10^9/\text{l}$ or leucopenia $< 4 \times 10^9/\text{l}$)

The second revision in 2016, changed the definition of sepsis as organ dysfunction that is caused by a dysregulated host response to infection and is life-threatening. Organ dysfunction is then defined as an increase of two or more in the Sequential Organ Failure Assessment (SOFA) scoring system (Gul et al, 2017).

1.3.4. Therapy

The pathology of SIRS and its sequelae is still poorly understood and currently there is no available treatment to repair the damaged endothelium. At present, patients receive supportive treatment, such as fluid resuscitation with crystalloids and albumin to replenish lost fluids. Vasopressors, like norepinephrine and epinephrine, are used to maintain arterial pressure (Dellinger et al., 2013). Novel therapy strategies are needed.

1.4. Vasoplegic Syndrome

1.4.1. Epidemiology

Vasoplegic syndrome (VS), also known as vasodilatory shock, is a serious complication following cardiac surgery with the use of cardiopulmonary bypass (CPB). It occurs in about 9-44% of patients requiring surgery necessitating cardiopulmonary bypass (snCPB) (Omar *et al*, 2015).

1.4.2. Definition

Vasoplegia is characterized by severe and persistent hypotension, decreased systemic vascular resistance and arteriolar reactivity, and increased need for fluids and vasopressor agents, despite an adequate cardiac output (Mekontso-Dessap *et al*, 2001; Sharawy *et al*, 2014). Vasoplegic syndrome (VS) is diagnosed when an arterial pressure <50 mmHg, left atrial pressure <10 mmHg, right atrial pressure <5 mmHg, cardiac index >2.5 L min⁻¹ m⁻² and low systemic vascular resistance (<800 dyne s⁻¹ cm⁻⁵) (Shanmugam G, 2005). The exact pathophysiology of VS is not fully understood. It involves interactions between plasma proteins, platelets, leukocytes and endothelial cells (Omar *et al*, 2015) which leads to an imbalance of vasodilator and vasopressor mechanisms in the body (Fisher and Levin, 2010). This can result in insufficient tissue perfusion and metabolic acidosis (Omar *et al*, 2015). Factors contributing to the development of VS are endothelial injury and muscle hyperpolarization (Sharawy *et al*, 2014). Also, the duration of cardiac surgery is a risk factor, for every additional 30 minutes on bypass, the risk of

vasoplegia increases by 38% (Fisher and Levin, 2010). Patients who develop vasoplegia are at a greater risk of mortality and morbidity following snCPB (Fisher and Levin, 2010). Currently there are no biomarkers to predict development of post-snCPB vasoplegia.

1.4.3 Treatment

The prognosis of VS is very poor. Vasoplegic patients are treated with prolonged and high doses of vasoconstrictor medications. This treatment may have serious side effects. It can cause peripheral vasoconstriction and ischaemia of upper and lower limbs that can lead to metabolic acidosis and tissue necrosis. Vasoconstrictors that are used to treat VS are Phenylephrine, Norepinephrine, Vasopressin and Methylene Blue (Fisher and Levin, 2010).

1.5. Cytokines in SIRS and Sepsis

1.5.1. Cytokines introduction

Three cytokines involved in inflammation and sepsis, which are commonly used in experiments assessing endothelial barrier function, are IL-1 β , IL-6 and TNF- α . Treatment with IL-1 β increased the permeability of the endothelial barrier in vitro, in HUVEC and HRGEC (Du et al, 2014). Plasma levels of IL-1 β and IL-6 were higher in septic patients who died compare to those who survived (Chaundry et al, 2013). Shakoory and colleagues, who re-analysed data from a multi-center study from eleven countries across Europe and North America, showed that treatment with IL-1 receptor antagonist

IL-1Ra was associated with significant improvement in survival of patients with sepsis and concurrent hepatobiliary dysfunction and disseminated intravascular coagulation (Shakoory et al, 2016). An *in vivo* study in IL-6-knockout mice showed that the deletion of the IL-6 gene decreased lung inflammation in a model of acute lung injury. This study also showed that treatment with anti-IL-6 significantly reduced lung inflammation in a mouse model (Cuzzocrea et al, 1999). In 1985, Beutler and colleagues were the first to show that TNF-antiserum reduced mortality in mice infected by sepsis-causing endotoxin lipopolysaccharide (Beutler et al, 1985).

Cytokines are small proteins that act as inflammatory mediators. They are secreted by multiple cell types and have many and varied functions. Table 1 shows the cytokines' cell origin, inflammatory properties and main functions. Cytokines are often produced in cascades, when cytokine secreted by one cell sends a signal to another cell to produce another cytokine. The names of cytokines are often related to the names of cells that secrete them, for example cytokines secreted by monocytes are called monokines and those secreted by lymphocytes are called lymphokines (Zhang et al, 2007). Cytokines are released in response to activating stimuli. A stimulus can be endotoxin, ischemic-reperfusion injury or oxidative stress. Cytokines can have both, pro- and anti-inflammatory functions (Jaffer et al, 2010) as it can be seen in the summary of pro and anti-inflammatory cytokines involved in sepsis in Table 1. Cytokine profiles vary depending on the infecting microorganism (Gao et al, 2008).

1.5.2. Pro-inflammatory cytokines

Pro-inflammatory cytokines are mainly generated by macrophages (Zhang et al, 2007). They are involved in up-regulation of secondary cytokines and acute phase proteins, and in attracting inflammatory cells; examples of pro-inflammatory cytokines are IL-1 β , TNF- α and IL-6 (Chaundry et al, 2013).

1.5.2.1. Interleukin-1 beta (IL-1 β)

IL-1 β is also called catabolin (Chaundry et al, 2013). It is a pro-inflammatory cytokine that is produced in response to inflammatory agents, or microbial endotoxins. IL-1 β plays a central role in immune and inflammatory responses. It is mainly produced by macrophages and monocytes, but it is also released by non-immune cells like fibroblasts and endothelial cells (Zhang et al, 2007). High levels of IL-1 β were found in septic patients that have died (Chaundry et al, 2013). IL-1 β belongs to a group of pyrogenic cytokines. It mediates the development of fever during immune response to an insult (Schulte et al, 2013). Treatment with IL-1 β increases the permeability of endothelial barrier in vitro, in HUVEC and HRGEC (Du et al, 2014).

1.5.2.2. Tumor necrosis factor alpha (TNF- α)

TNF- α is a pro-inflammatory cytokine that is involved in many inflammatory diseases, infectious or non-infectious, like arthritis, atherosclerosis and sepsis (Jaffer et al, 2010). It is produced by immune cells like macrophages and lymphocytes, as well as non-

immune cells like fibroblasts and keratinocytes. TNF- α is known as an early regulator of immune response. It takes about 30 minutes after the incident for the TNF- α to be released from macrophages (Schulte et al, 2013). TNF- α binds to tumor necrosis factor receptors (TNFR) 1 and 2. TNFR1 and TNFR2 are cell surface receptors that mediate the inflammatory responses (Zhang et al, 2007). TNF- α uses a number of cell signalling pathways. The three main intra-cellular pathways are (1) Fas-Associating Death Domain (FADD), (2) TNF- α receptor-associated factor-Apoptosis signal-regulating kinase 1 (TRAF-ASK1) and (3) TNF- α receptor-associated factor 2- Death domain kinase receptor interacting protein (TRAF2-RIP) (Jaffer et al, 2010). TNF- α is also called pyrogenic cytokine because its main role is to induce a fever (Schulte et al, 2013). In vitro, TNF- α increases HUVEC permeability (Seynhaeve et al, 2006).

1.5.2.3. Interleukin-6 (IL-6)

IL-6, also called interferon – beta 2, is produced by different cell types, mainly by macrophages and T-cells. IL-6 has a dual-function as a pro-inflammatory and anti-inflammatory cytokine (Chaundry et al, 2013). It inhibits the release of IL-1 β and TNF- α and increases the release of anti-inflammatory cytokines like IL-10 and cortisol (Schulte et al, 2013). IL-6 enhances leukocyte recruitment in inflammation by up-regulating chemokine production and adhesion molecule expression (Romano et al, 1997). IL-6 is also known to stimulate Toll-like receptors to promote chemotaxis (Jaffer et al, 2010). Toll-like receptors (TLRs) activate NF- κ B signalling pathway and enhance the mRNA transcription of IL-6, TNF- α , and IL-1 β . TNF- α and IL-1 β also activate transcription factors

to produce IL-6 (Tanaka et al, 2014). IL-6, together with glucocorticoids, regulates acute phase response (APR) of the immune system to an inflammation. APR commence 12-48 hours after the start of inflammation (Vandevyver et al, 2014). Acute phase proteins (APP) are produced during APR (Cray et al, 2009). One of the best known APP is C-reactive protein that is used as a biomarker of inflammation (Pepys & Hirschfield, 2003). Another well-known APP is alpha-2-macroglobulin (A2MG). A2MG binds IL-6, and also IL-1b and TNF, and these bindings lead to a production of other molecules, like platelet activating factor, that have an important role in the body's responses to sepsis (Hocheplied et al, 2002). Cells that express membrane bound interleukin-6 receptor as well as signalling receptor protein gp130 are responsive to IL-6 through classical signalling. Cells that express only gp130 responded to IL-6 only if it is in a complex with soluble interleukin-6 receptor (IL-6/sIL-6R complex). The soluble interleukin-6 receptor is generated by proteolytic cleavage of the membrane bound precursor. Activation of cells by the IL-6/sIL-6R complex is called trans-signalling. All cells display gp130 on their surface, but only few cells express membrane bound interleukin-6 receptor. Generation of soluble form of the IL-6 receptor increases the spectrum of IL-6 target cells. The anti-inflammatory functions of IL-6 are mediated by classic signalling. The pro-inflammatory responses of IL-6 are mediated by trans-signalling (Scheller, 2011). IL-6 has an important role in many disorders, such as sepsis, cancer, cardiovascular and autoimmune diseases (Chaundry et al, 2013). Raised plasma levels of IL-6 are found in many conditions, such as burns, trauma (surgery) and sepsis (Schulte et al, 2013). In patients with rheumatoid arthritis, IL-6 induces excessive production of VEGF which leads to increased endothelial

permeability and angiogenesis (Nakahara et al, 2003). Higher plasma levels of IL-6 and TNF- α were found in patients suffering from infections caused by gram-negative microorganisms when compared to infections caused by gram-positive organisms (Gao et al, 2008). IL-6 is involved in the inflammatory response and is used as a biomarker of the acute phase of inflammation. Overproduction or continued production of IL-6 leading to high levels of IL-6 in patients with severe sepsis and septic shock is associated with poor survival rate (Srisangthong et al, 2013; Chaundry et al, 2013). Serum levels of IL-6 also reflect the severity of organ dysfunction in critically ill patients (Shimazui et al, 2017). However, the exact role of IL-6 in sepsis is not clear. It is still not known whether IL-6 is just a marker of inflammation or if it is an actual mediator of organ injury (Remick et al, 2005).

1.5.3. Anti-inflammatory cytokines

Sepsis develops when there is an imbalance between pro and anti-inflammatory responses to infection. Pro and anti-inflammatory cytokines regulate migration of immune cells to the site of infection to localize the infection, preventing it from becoming systemic. Dysregulation of cytokine release may lead to endothelial dysfunction. The role of anti-inflammatory cytokines is to balance and regulate the production and responses of pro-inflammatory cytokines and to prevent infection from becoming systemic (Zhang et al, 2007; Schulte et al, 2013). They help to prevent harmful effects of prolonged or excessive inflammatory reactions (Chaundry et al, 2013). An

uncontrolled inflammatory response can contribute to SIRS and sepsis. Examples of anti-inflammatory cytokines are IL-4 and IL-10 (Jaffer et al, 2010).

1.5.3.1. Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that is produced by immune cells such as monocytes, B and T-lymphocytes and macrophages (Schulte et al, 2013). IL-10 has an important role in regulating inflammatory responses in SIRS and sepsis. It works in two ways – (1) it suppresses the production of pro-inflammatory cytokines, for example TNF- α , IL-1, IL-6, IFN- γ , GM-CSF in cells; and (2) it neutralizes pro-inflammatory actions of released IL-1 and TNF- α by stimulating the production of IL-1Ra and sTNFRs (Schulte et al, 2013). It also up-regulates the expression of other anti-inflammatory cytokines (Zhang et al, 2007). IL-10 is one of the key cytokines in the development and progression of sepsis (Chaundry et al, 2013). Latifi and his colleagues used cecal ligation and puncture (CLP) mice model to investigate the role of IL-10 in sepsis. They demonstrated that IL-10 regulates the development of sepsis and controls the onset of septic shock and mortality in the CLP model (Latifi et al, 2002). It has been reported that higher plasma IL-10 concentration contribute to higher mortality of patients with sepsis. Li and colleagues studied the relationship between IL-10 and lymphocytes in sepsis and found out that IL-10/lymphocyte ratio was associated with mortality in septic patients (Li et al, 2017). IL-10 functions as a temporal regulator of the transition from early reversible sepsis to the late phase of irreversible septic shock (Latifi et al, 2002).

The continues production of IL-10 is thought to cause immune system impairment (immunosuppression) that leads to worsening of the patient's condition (Wu et al, 2009) and also exposing the patient to a possible secondary infection (Hotchkiss et al, 2017). Higher levels of IL-10 were found in patients with sepsis and MODS comparing to healthy subjects (Jaffer et al, 2010). It was found that higher levels of IL-10 were associated with increased mortality (Chaundry et al, 2013).

1.5.3.2. Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a small, 23kDa, monomeric glycoprotein that is secreted by many different types of cells such as T-cells, macrophages, fibroblasts, epithelial and endothelial cells and also tumour cells (Shi et al, 2006). GM-CSF is an important hematopoietic growth factor and cytokine. It has both, pro-inflammatory as well as anti-inflammatory properties, depending on the circumstances (Mathias et al, 2015)

In healthy people the circulating levels of GM-CSF are almost undetectable. High levels can be detected in local tissues, for example in the skin of allergic patients, or in lungs of asthmatic patients. The levels of GM-CSF normally increase in response to stimulation by cytokines, for example TNF- α , IL-1 β and IL- 6. Overexpression of GM-CSF causes severe inflammation. It is thought that GM-CSF plays a role in many inflammatory diseases such as rheumatoid arthritis, inflammatory lung and renal disorders (Shi et al, 2006). GM-CSF has been tested as a treatment in cancer, AIDS (Shi et al, 2006) and sepsis (Mathias et al, 2015). The time of the treatment is a very important factor. If given too early, it can worsen the tissue injury during the hyper-inflammatory phase, by increasing

the pro-inflammatory cascade of cytokines (Mathias et al, 2015). GM-CSF is one of the most investigated cytokines in sepsis. In critically ill patients it has shown to improve clinical markers, but it did not make a difference to survival rate (Mathias et al, 2015).

1.5.4. Chemokines MCP1 and IL-8 in Sepsis

1.5.4.1. Monocyte Chemoattractant Protein 1 (MCP-1)

The body's response to pathogens in sepsis is characterized by the migration and infiltration of leukocytes into the tissue, which is the initiating step of the inflammatory cascade. This response is mediated by MCP-1. MCP-1 is a soluble CC chemokine and pro-inflammatory mediator that regulates the progression of inflammation and the production of pro-inflammatory cytokines (Ramnath et al, 2008 ; Wanget al, 2018). It was first discovered in 1989 (Matsushima *et al*, 1989). MCP-1 is expressed by many cell types, for example fibroblasts, smooth muscle cells and endothelial cells (Barre et al, 2018). It gets upregulated by different stimuli, such as IL-1 β , TNF- α and IFN- γ (Panee, 2012). MCP-1 plasma levels were found to be elevated in patients with sepsis. During inflammation, a production of MCP-1 is needed to help the body to fight against infection, but overproduction of MCP-1 can lead to complications, for example in patients with sepsis it can cause an organ failure and/or multiple organ dysfunction. (Ramnath et al, 2008). The levels of MCP-1 were higher in septic patients who have died than those who have survived. The higher the levels of MCP-1, the higher the chances of death. MCP-1 measured in septic patients on first and third day of their stay in ICU

showed to be a reliable biomarker in predicting all-cause mortality in patients with sepsis, especially those with septic shock. (Barre et al, 2018)

1.5.4.2. Interleukin-8 (IL-8)

High levels of IL-8 were measured in blood samples of patients with sepsis and those levels were associated with death in septic patients (Chaundry et al, 2013). IL-8 is also called growth related oncogene – GRO/KC (Zhang et al, 2007). It is produced primarily by macrophages, but also by endothelial and epithelial cells (Chaundry et al, 2013). IL-8 is a pro-inflammatory cytokine belonging to a cysteine-X-cysteine (CXC) group of chemokines. (Chaundry et al, 2013). Chemokines induce chemotaxis in targeted cells, CXC chemokines activate neutrophils (Ramnath et al, 2008). IL-8 sends chemical signals to recruit neutrophils and granulocytes to target inflammation, because of this function IL-8 is also called neutrophil chemotactic factor (Chaundry et al, 2013).

1.6. RAGE, RAGE ligands and TLRs in Sepsis

1.6.1. Receptor for advanced glycation end-products (RAGE)

The receptor for advanced glycation end-products (RAGE) was first described by Neeper and colleagues in 1992 (Neeper et al., 1992). RAGE is an immunoglobulin-like cell surface multi-ligand pattern-recognition receptor (Chuah et al., 2013). It is found in two forms – (1) full-length membrane-bound (fl-RAGE or mRAGE); and (2) soluble (sRAGE or

esRAGE). Soluble RAGE is produced by alternative mRNA splicing and/or by proteolytic cleavage of fl-RAGE. Plasma sRAGE is composed of esRAGE, an endogenous splice variant of RAGE missing the transmembrane domain of the receptor (Yonekura et al, 2003). Full length RAGE has one V-type domain, two C-type domains, transmembrane domain and cytoplasmic tail. The V-domain is the site of the majority of the ligand binding. The cytoplasmic tail is involved in intracellular signalling mediating cell migration (Sparvero et al., 2009). In healthy adults, RAGE is highly expressed predominantly in lung and skin cells. The physiological reason for the high expression is unknown. RAGE is present at low levels in vascular tissue and increases during inflammation (Chuah et al., 2013). RAGE is associated with many inflammatory conditions such as cancer, neurodegeneration (Alzheimer's disease), metabolic disorders, diabetes (diabetic vascular complications), pulmonary and cardiovascular disorders (Sparvero et al., 2009). Levels of sRAGE also increase significantly after surgery necessitating cardiopulmonary bypass (snCPB), in patients with sepsis, acute pancreatic and pneumonia (Creagh-Brown et al., 2010; Creagh-Brown et al, 2013).

RAGE was originally identified as a receptor for the advanced glycation end-products (AGEs) which are results of non-enzymatic glycoxidation of macromolecules; their formation is greater at sites of inflammation and conditions associated with systemic or local oxidant stress (Schmidt et al., 2001). Binding of AGEs to RAGE has an important role in diabetic complications (Hsieh et al, 2004). It has since been found that RAGE binds a wide range of structurally diverse ligands such as HMGB1, S100 proteins and amyloid- β (Sparvero et al., 2009). Binding of RAGE and amyloid- β leads to oxidative stress which

is an important factor contributing to the initiation and progression of Alzheimer's disease (Hsieh et al, 2004). Binding of RAGE and S100 proteins, namely S100A8 and S100A9, causes a tissue activation that leads to chronic inflammation. A continual tissue activation and inflammation during the promotion phase of chemically induced carcinogenesis encourages tumor development (Gebhardt et al, 2008). Binding of RAGE and HMGB1 promotes inflammatory responses and has a major role in acute inflammation and sepsis (Sparvero et al, 2009).

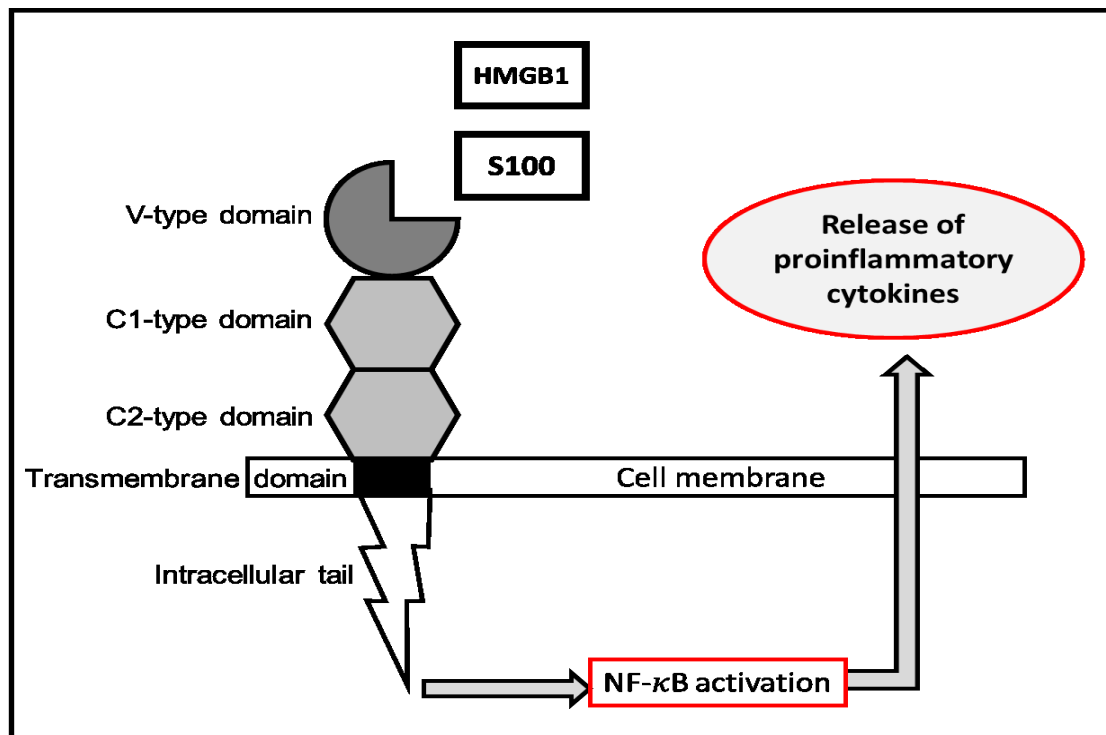


Figure 4. A diagram of RAGE and its ligands S100 proteins and HMGB1. Binding of S100 proteins and HMGB1 to RAGE activates the transcription factor NF-κB. This leads to RAGE upregulation and release of proinflammatory cytokines and recruitment of inflammatory cells leading to acute and chronic inflammation (Chuah *et al*, 2013)

1.6.2. S100 Proteins

The S100 protein family is a group of more than 20 different proteins and first of these was described by B.W. Moore in 1965 (Moore, 1965). The name “S100” came from their ability to dissolve in 100% saturated solution of ammonium sulphate. They are calcium-binding proteins, with some also binding to zinc and copper (Foell et al., 2007). S100 proteins form homodimer and heterodimer protein complexes: S100A is heterodimer with subunits α and β ; S100B is homodimer with two β subunits (Turker Yordan et al., 2011). S100 proteins have roles inside and outside cells. They are involved in calcium homeostasis, protein phosphorylation and enzyme activation. Binding of S100 proteins to RAGE is implicated in many different diseases – S100B in neurodegeneration, S100A, S100A4 and S100A6 in cancer, S100A8/A9 and S100A12 in inflammation (Hsieh et al., 2004). S100A8, S100A9 and S100A12 are naturally occurring in granulocytes suggesting they have a role in innate immunity. RAGE is believed to transduce pro-inflammatory signals in endothelial cells and cells of the immune system. High levels of S100A8, S100A9 (which often exist as a heterodimer S100A8/A9) and S100A12 are found at sites of inflammation (Foell et al., 2007). Huang and Tu set up a sepsis model in rats to investigate the mechanism of S100 family proteins in sepsis. The results showed that S100A8, S100A9, and S100A12 were significantly up-regulated in their rat sepsis model group compared to the control group. This suggests that S100 proteins have a role in the development of sepsis. (Huang and Tu, 2015). Zoelen and colleagues showed in their experiments that circulating levels of S100A8/A9 were higher in patients with sepsis and also in healthy humans injected with Lipopolysaccharide (LPS) (Zoelen et al, 2009).

1.6.3. HMGB1 (Amphoterin)

Another RAGE ligand is High-mobility group box 1 (HMGB1). This is a small protein that has both, intracellular and extracellular functions. Intracellularly, it acts as a non-histone DNA-binding protein and mediates gene transcription in nucleus (Sims et al., 2010). HMGB1 is released from cells either by active secretion or by passive secretion from necrotic, but not apoptotic, cells. Extracellularly, it acts as a cytokine and stimulates production of inflammatory mediators, such as TNF- α and nitric oxide. HMGB1 exerts these pro-inflammatory effects by binding to RAGE (Kokkola R, 2005) and induces endothelial permeability through RAGE and Src family tyrosine kinase pathway. It is not understood which of the nine known members of the Src family of tyrosine kinases is involved in HMBG-1 induced EC permeability (Huang et al., 2012). HMGB1 has a role in cancer, autoimmune diseases , acute and chronic inflammation (Sims et al., 2010), also patients with severe sepsis and septic shock have high levels of HMGB-1 in the circulation (Huang et al., 2012).

1.6.4. Toll-like Receptors

There is another group of receptors that plays an important role in the tissue injury and inflammation, called Toll-like receptors (TLRs). It has been proposed that RAGE and TLRs regulate immune and inflammatory responses together (Ibrahim et al., 2013). Both RAGE and TLRs are pattern recognition receptors (PRRs). They recognise pathogen-associated molecular patterns (PAMPs) and through binding to them activate pro-inflammatory transcription factors such as nuclear factor kappa B (NF- κ B), interferon

response factor-3 (IRF-3) and activator protein-1 (AP-1). Different infectious microorganisms activate different TLRs and through them different transcription factors to start immune responses leading to inflammation and sepsis (Gao et al., 2008). There are eleven known TLRs, numbered TLR1-TLR11. They are found in the cytoplasm, the cell membranes and in body fluids. TLR2 and TLR4 are expressed extracellularly and TLR9 intracellularly (Takeda and Akira, 2005). TLR1, TLR2, TLR4, TLR5 and TLR6 recognize bacteria. TLR3, TLR7 and TLR8 recognize viruses. TLR9 recognises both bacteria and viruses (Tsujimoto et al, 2008). Takeuchi and colleagues showed in their mice model that TLR2 and TLR4 recognize different bacterial cell wall components. TLR2 recognizes gram-positive and TLR4 gram-negative bacteria (Takeuchi et al, 1999). In inflammation and sepsis Toll-like receptors activate NF- κ B signalling pathway and enhance the mRNA transcription and synthesis of pro-inflammatory cytokines like IL-6, TNF- α , and IL-1 β (Tanaka et al, 2014). Tsujimoto and colleagues measured levels of TLR-2 and TLR-4 in patients with sepsis and found out that the levels of both TLRs were significantly higher in septic patients compare to healthy controls (Tsujimoto et al, 2008).

1.7. Endothelial biomarkers

1.7.1. Von Willebrand Factor

Von Willebrand Factor (VWF) has an important role in vascular inflammation that can progress to systemic inflammation and sepsis. VWF is involved in both, hemostatic and inflammatory pathway enabling adhesion and recruitment of both platelets and leukocytes in inflamed tissue. By these actions vWF modulates vascular permeability

and oedema formation and may promote atherosclerotic plaque formation and inflammation (Gragnano et al, 2017).

Von Willebrand Factor protein has been named after a Finish professor E. A. von Willebrand who discovered a new type of bleeding disorder, later called von Willebrand disease. Von Willebrand Factor is a large multimer glycoprotein of different sizes, from 2 to 100 monomers and up to 20,000kDa, which carries clotting factor VIII and maintains a balance between bleeding and thrombosis (Meyer et al, 2012; Gragnano et al, 2017). It is produced mainly by endothelial cells and to lesser extent by megakaryocytes – precursors of platelets (Ware et al, 2004; Kawecki et al, 2017).

VWF is released into the circulation in response to activation or injury to endothelium, such as during inflammation (Ware et al, 2004; Kawecki et al, 2017). In response to inflammatory stimuli endothelial cells release vWF which then recruits leukocytes and platelets to inflammation sites and promotes their adhesion. This changes the vascular permeability and leads to tissue oedema (Kawecki et al, 2017). Von Willebrand Factor is a well-establish biomarker of endothelial damage. Plasma levels of vWF increase in sepsis due to acute perturbation of the endothelium (Mourik et al, 1999). High levels of VWF, that are thought to be caused by endothelial cell activation, are a predisposing factor for post-cardiac surgery vasoplegia (Kortekaas et al, 2013).

1.7.2. Angiopoietin – 2

Angiopoietin 2 (Ang-2) is a secreted glycoprotein and endothelium specific growth factor that has an important role in angiogenesis (cancer) and endothelial barrier dysfunction (sepsis) (Siner et al, 2009; Clajus et al, 2012). There are three angiopoietins in the human body – Ang-1, Ang-2 and Ang-4 (Orfanos et al, 2007). Ang-1 and Ang-2 compete for binding to tyrosine kinase receptor Tie2 that is expressed on endothelial cells. In the healthy state Ang-1 binds to Tie2 and activates PI3K/Akt pathway in endothelial cells; in the unhealthy state, such as sepsis, Ang-2 binds to Tie2 and disrupts the Ang-1/Tie2 signalling pathways. This makes the endothelium susceptible to cytokines causing inflammation and SIRS (Yuan et al, 2009; Clajus et al, 2012). Ang-2 plays an important role in organ dysfunction in patients suffering from sepsis. It is involved in endothelial cells damage leading to vascular dysfunction and sepsis; and in epithelial cells damage causing acute lung injury and acute respiratory distress syndrome (Siner et al, 2009; Heijden, 2008). Clajus and colleagues measured serum levels of Ang-2 in 25 adult patients undergoing cardiac surgery with CPB. The release of Ang-2 correlated with the duration of CPB and aortic cross-clamp time. Ang-2 levels at 12 h post-CPB predicted the length of ICU stay, duration of mechanical ventilation and mortality (Clajus et al, 2012).

1.7.3. Syndecan-1 – Marker of the endothelial glycocalyx

1.7.3.1. Context

The luminal side of endothelium is covered by carbohydrate-rich gel-like layer called glycocalyx. It is also known as endothelial glycocalyx layer (EGL) or endothelial surface layer (ESL) (Rehm et al, 2007). The existence of non-cellular layer on endothelium was first proposed by J.F. Danielli in 1940 (Danielli, 1940). Twenty six years later, it was visualised for the first time by J.H. Luft using ruthenium red staining and electron microscopy (Luft, 1966). Glycocalyx thickness depends on the blood vessel diameter. A study by Vink and Duling revealed that glycocalyx in muscle capillaries is about 0.5µm thick (Vink and Duling, 1996). Other studies showed that glycocalyx in small arteries is 2-3µm (Haaren et al, 2003) and in large arteries is around 4.5 µm thick (Megens, 2007). It forms a luminal mesh that enables binding of plasma proteins (Ushiyama et al, 2016).

1.7.3.2. Glycocalyx Structure

The main glycocalyx constituents are membrane-bound proteoglycans: syndecans and glypicans. Syndecans are attached to the EC membrane via single-span transmembrane protein and can act as a receptor. Glypicans are attached to the EC membrane via glycosyl-phosphatidylinositol (GPI) anchor (Schott et al, 2016). Proteoglycans form a backbone for various glycosaminoglycan attachments (Schott et al, 2016). These attachments are: heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan. Syndecans and glypicans can have various types of

glycosaminoglycan attachments and these bindings can change in response to different stimuli and circumstances. The most common proteoglycans are those with heparan sulfate attachments, they represent about 50-90% of the total number of proteoglycans in glycocalyx. The second common are proteoglycans with chondroitin sulfate attachments. The ratio of proteoglycans with heparan sulfate and with chondroitin sulfate are present in vascular endothelium in ratio 4:1. The glycosaminoglycan hyaluronan presented in the glycocalyx is not bound to a proteoglycan (Reitsma et al, 2007). Other glycocalyx components are secreted glycosaminoglycans (e.g. hyaluronan) and proteoglycans (e.g. perlecan) (Schott et al, 2016). The schematic representation of glycocalyx and its components can be seen in Figure 5.

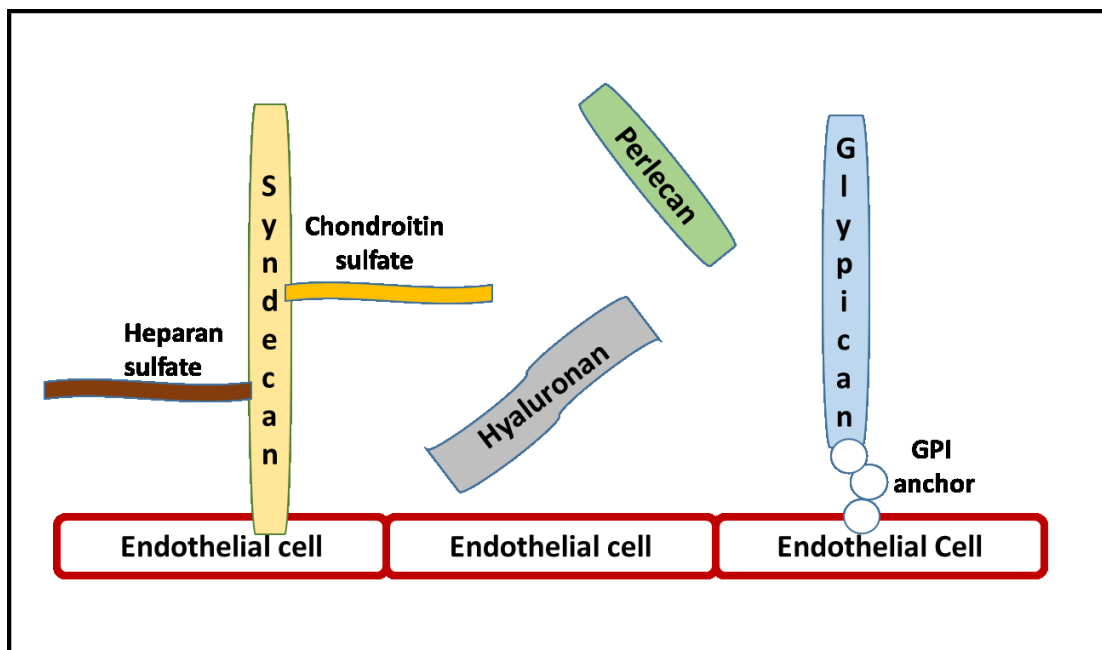


Figure 5. Schematic representation of glycocalyx and its components.

1.7.3.3. Glycocalyx Function and Dysfunction

Glycocalyx plays an important role in coagulation, endothelial barrier permeability and inflammation (Johansson et al, 2011). It shields endothelial cells from direct contact with circulating blood cells and regulates the adhesion of these cells. It also acts as a shear stress sensor and regulator of mechanotransduction (Schott et al, 2016). EGL is delicate and can be easily damaged by trauma, haemorrhagic shock, hypovolemia, ischemia and reperfusion (Schott et al, 2016). Damage to the glycocalyx impairs endothelial barrier function and allows adhesion of platelets and leukocytes (Rehm et al, 2007). Animal studies showed that when glycocalyx is damaged, its components (syndecan-1 and heparin sulphate) are shed into blood circulation. In human studies, patients who underwent ischemia and reperfusion during cardiac surgery had higher levels of syndecan-1 and heparin sulphate in plasma, suggesting they suffered glycocalyx damage as a consequence of the intervention during surgery (Rehm et al, 2007). High levels of syndecan-1 in patients who suffered a trauma were associated with increased mortality (Johansson et al, 2011).

1.8. Endothelial Barrier

1.8.1. Endothelial Barrier Function

The endothelium is a monolayer of closely connected endothelial cells lining the inner surface of every blood vessel, from the largest arteries to the smallest capillaries. These endothelial cells are called vascular endothelial cells and they have an important role in

the function of every organ in the body (Rajendran et al., 2013). Endothelial cells, called lymphatic endothelial cells, line all lymphatic vessels (Vandenbroucke et al., 2008). All the endothelial cells in the human body weigh in total 1000 grams and endothelium covers a total surface area of 4000 to 7000 square meters (Johansson et al, 2011). The endothelium creates a semi-permeable barrier to separate circulating blood from vascular smooth muscle and surrounding tissue. Its main function is to co-ordinate exchange of fluids and solutes, nutrients and metabolic waste, across the vessel wall. It is also involved in the transit of white blood cells in and out of the bloodstream, and it prevents pathogens and toxins in the circulation from entering into tissue (Yuan and Rigor, 2010). It also mediates immunological and inflammatory processes (Esper et al, 2006). The endothelium is an active organ that is involved in many processes such as: regulation of vascular tone; growth, proliferation and angiogenesis; thrombosis and thrombolysis (Rajendran et al., 2013). It releases substances in response to chemical or physical stimuli and produces agonistic and antagonistic substances that help to preserve homeostasis (Esper et al, 2006). Tissue growth and blood vessel repair would not be possible without endothelial cells. They are able to adjust their numbers to suit a requirement. It has positive (growth and repair) as well as negative (cancer angiogenesis) consequences for the body (Yuan and Rigor, 2010).

1.8.2. Endothelial Barrier Dysfunction

Activation of endothelial cells by cytokines and other inflammatory mediators leads to changes in endothelial function. These changes include up-regulation of adhesion

molecules, production and secretion of inflammatory mediators and chemo-attractant agents as well as vasoactive compounds. These changes are central in the pathological processes leading to sepsis syndrome (Hovinga et al, 2007). Endothelial barrier dysfunction contributes to SIRS and sepsis syndrome. In some cases, it can lead to an organ failure that complicates recovery from cardiac surgery requiring CPB. Endothelial barrier dysfunction is characterised by leakage of protein-rich plasma into surrounding tissue, leading to tissue swelling (oedema). The oedema presses on tissue and disturbs fluid homeostasis and tissue oxygenation leading to organ dysfunction (Yuan and Rigor, 2010). Damage to endothelium can be caused by infection, inflammation, trauma, burns, acute pancreatitis, exposure to drugs or toxins, like those found in cigarette smoking. Endothelium dysfunction is also a cause, or consequence, of many diseases, such as: hypertension, coronary artery disease, chronic heart failure, stroke, chronic kidney failure, diabetes, (Rajendran et al., 2013); multiple sclerosis, epilepsy, cancer and sepsis (Yuan and Rigor, 2010). The mechanisms by which cardiopulmonary bypass causes endothelial permeability are still not fully understood. Koning and colleagues showed in their experiments that CPB-induced changes in endothelial barrier function were caused by disturbed cell–cell interactions (Koning et al, 2016).

1.8.3. Endothelial Transport Routes

Transport of molecules and ions across endothelium requires passage either through the cells (transcellular route) or between the cells (paracellular route). Intracellular transport (transcellular route), also known as transcytosis, is facilitated by a vesicular

system that transports macromolecules, such as plasma proteins (Vandenbroucke et al., 2008). Paracellular transport is accommodated by opening and closing of junctions between cells and is used by molecules smaller than 3nm. Endothelial cells are connected through tight, gap and adherens junctions. Tight junctions (TJ) control the passage of ions and solutes; gap junctions (GJ) control small molecular weight molecules; adherens junctions (AJ) support cells' communication and maturation (Dejana et al., 2009). Within these three junctions are transmembrane adhesive proteins which, through intracellular signalling, promote tight cell-to-cell adhesion and cell communication. The adhesive proteins in TJ are claudins, occludin, nectin and junctional adhesion molecules (JAMs); in GJ connexins; and in AJ nectin and VE-cadherin. There are other adhesive proteins located in the intercellular cleft, like platelet endothelial cell adhesion molecule-1 (PECAM-1), cluster of differentiation 146 (S-endo-1/CD146), cluster of differentiation 99 (CD99) and protocadherin 12 (Liebner et al., 2006). Most of these proteins are common in other types of cells, but VE-cadherin and claudin-5 are endothelial cells-specific (Wallez and Huber, 2008).

1.9. Endothelial Cell Heterogeneity

1.9.1. Context

There are three main types of cells found in the blood – erythrocytes (involved in oxygen and carbon dioxide exchange), leukocytes (involved in immune responses) and thrombocytes (involved in hemostasis). These cells are carried around the body through

blood vessels. Different types of blood vessels have different structures, but they all share the same general features. Arteries and arterioles have thicker walls than veins and venules, because they carry blood under greater pressure. What they all have in common is the inner lining with endothelial cells. Because of their position, endothelial cells come in contact and interact with all blood cells. They are instrumental in leukocyte adhesion during inflammation and sepsis (Rajendran et al, 2013). Slit2 through binding to Robo4/1 receptors have an effect on endothelial cells, but can also interact with circulating hematopoietic cells such as platelets, and inflammatory cells. Disruptions in Slit2–Robo signalling can cause vascular injury by a number of different mechanisms, for example by recruiting inflammatory cells or by enabling platelets adhesion and spreading (Yuen and Robinson, 2013). It has been shown that endothelial cells that come from different vascular beds differ in structure and function (Ribatti et al, 2002). The differences are largely due to extracellular environment, for example sheer stress, pH, oxygenation, hormones, growth factors and other biochemical and biomechanical cues. Endothelial cells can also undergo site-specific epigenetic modification through DNA methylation, histone modification and chromatin remodelling. These changes are reversible and may change due to disease or ageing (Aird, 2006). Endothelial cells lining arteries are exposed to higher and faster blood flow than those residing in veins and capillaries. The arterial endothelial cells are mainly responsible for aiding vasomotor tone of vessel walls. Endothelial cells lining veins are not exposed to such a high flow as arterial endothelium and are therefore more suitable for leukocyte trafficking. Endothelial cells lining capillaries are mainly involved in exchange of nutrients, waste

and gases, because they are separated, by only a thin layer of extracellular matrix, from a tissue of an underlying organ (Ribatti et al, 2002; Aird, 2006).

1.9.2. Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) are isolated from umbilical cords. The isolation process is quite straightforward and yields HUVEC without contamination of other cell types. The cells are relatively easy to grow in cell culture and represent a good model for studying the endothelium in sepsis and inflammation. They have been used in medical research for more than forty years. HUVEC were isolated for the first time in 1963 by Yuji Maruyama (Japan) and they were first time successfully cultured in 1973 by Eric A. Jaffe (USA). HUVEC is a good cellular model commonly used for physiological studies of the endothelium (Apostolova et al, 2011). Schildberger and her colleagues developed and characterized a cell culture model of sepsis. They used LPS to induce endothelial activation in HUVEC and measured nuclear factor-kB (NF-kB) activity, expression of adhesion molecules, and release of cytokines involved in sepsis - TNF- α , IL-1b, IL-6, IL-8 and IL-10 (Schildberger et al, 2010)

1.9.3. Human pulmonary artery endothelial cells

Human pulmonary artery endothelial cells (HPAEC) are isolated from human pulmonary arteries. These arteries carry deoxygenated blood from the heart to the lungs. HPAEC play an important role in vascular homeostasis and gas exchange in the lungs. Human

PAEC are not easy to isolate and culture, therefore animal (bovine, rodent or murine derived) PAEC are more often used in research projects. The lung tissue is very complex with mix of many different cells and the successful isolation is also dependent on fast access to the lung tissue (Comhair et al, 2012). HPAEC were first time isolated in 1978 by the British-American scientist Una Ryan (Ryan et al, 1978).

1.9.4. Human Coronary Artery Endothelial Cells

Human Coronary Artery Endothelial Cells (HCAEC) are isolated from coronary arteries. These cells are exposed to fast blood flow and high levels of sheer stress (Aird et al, 2006). The arterial endothelial cells play an important role in the maintenance of vascular tone, as they release and produce substances, which ensure a balance between vasodilation and vasoconstriction in the cardiovascular system. They are also involved in the regulation of arterial blood flow and cardiac functions. (Levy-Ontman et al, 2017).

1.10. Roundabout Receptors and Slit Proteins

1.10.1 Roundabout Receptors

There are four members of the roundabout family of receptors - Robo1, Robo2, Robo3 and Robo4. The Robo1, Robo2 and Robo3 have a similar structure of five immunoglobulin-like (Ig) motifs, three fibronectin (FN) type 3 motifs, a transmembrane domain, and four cytoplasmic motifs CC0-CC3. Robo4 is smaller and has only two Ig-like

motifs, two FN3 motifs, a transmembrane domain and two cytoplasmic motifs CC0 and CC2 (Yadav and Narayan, 2014) as can be seen in Figure 6.

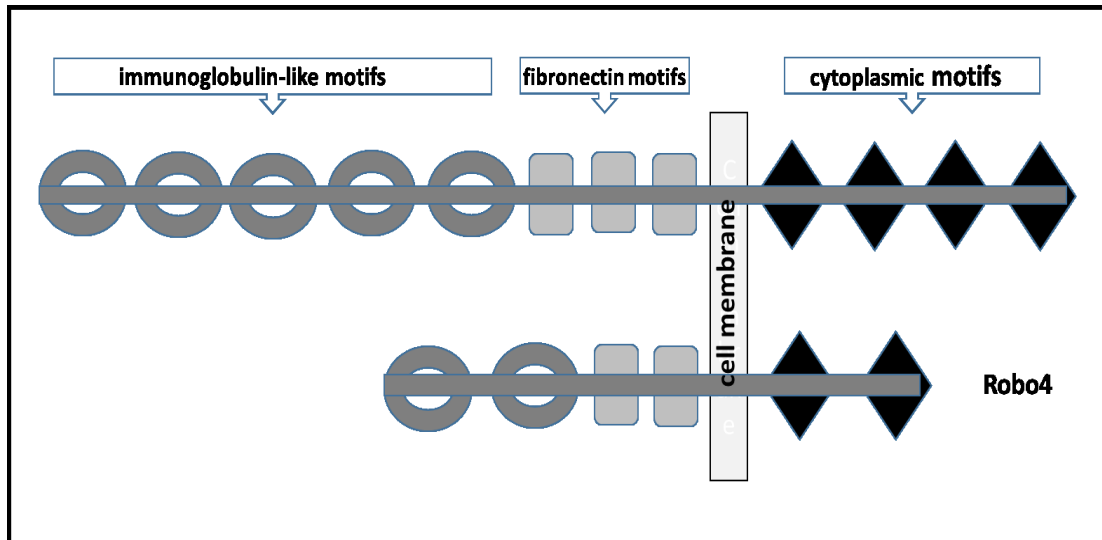


Figure 6. Schematic representation of Robo receptors. At the top are the larger Robo1, Robo2 and Robo3, at the bottom the short Robo4.

Robo receptors were first described in 1993 (Seeger et al., 1993) and in 1999, Slit proteins were identified as their ligands (Brose et al., 1999). The immunoglobulin-like (IG) domains of Robo and the leucine-rich repeat (LRR) region of Slit were identified to be important for Robo-Slit signalling (Battye et al, 2001). Robo-Slit binding is known to regulate cell migration, proliferation, apoptosis, adhesion and angiogenesis. It has an important role in lung, kidney and nervous system development; and also in inflammation, sepsis, cancer (Yadav and Narayan, 2014)) and reproduction (Dickinson and Duncan, 2010).

1.10.2. Slit Proteins

The Slit family consists of three members: Slit1, Slit2 and Slit3. Slit-1 is mainly expressed in the nervous system; whereas, Slit2 and Slit3 are also expressed throughout the body (Hohenester et al., 2006). Slits are large glycoproteins secreted, initially, as full length proteins of approximately 200kDa. The full length protein is subsequently proteolytically cleaved into N-terminal or C-terminal fragments in a region EGF1-2 and EGF5-6 (Bacharvet et al., 2001). All three Slits in vertebrates have the same structure – four leucine-rich repeat domains (LRR) D1-D4, six epidermal growth factor (EGF)-like domains, a laminin G-like domain, three EGF-like domains and C-terminal cysteine knot domain (Dickinson and Duncan, 2010). Slit-2 is a ligand for all four Robo receptors and binding to Robo1 and Robo4 regulates angiogenesis and vascular permeability. Binding to Robo-1 promotes angiogenesis, binding to Robo4 blocks VEGF-induced angiogenesis and vascular permeability (Acevedo et al., 2008).

1.10.3. Robo and Slit in Inflammation

VE-cadherin is a protein found in adherens junctions between endothelial cells. Its role is to maintain the physiological function of endothelial barrier (Dejana et al., 2009). In inflammation, the release of cytokines disrupts the endothelial barrier through displacement of VE-cadherin, causing hyper-permeability. Slit2-Robo4 interaction

prevents dissociation of p120-catenin from VE-cadherin; promotes its localisation in the cell junction and protects the endothelial barrier integrity (London et al., 2010), see Figure 7.

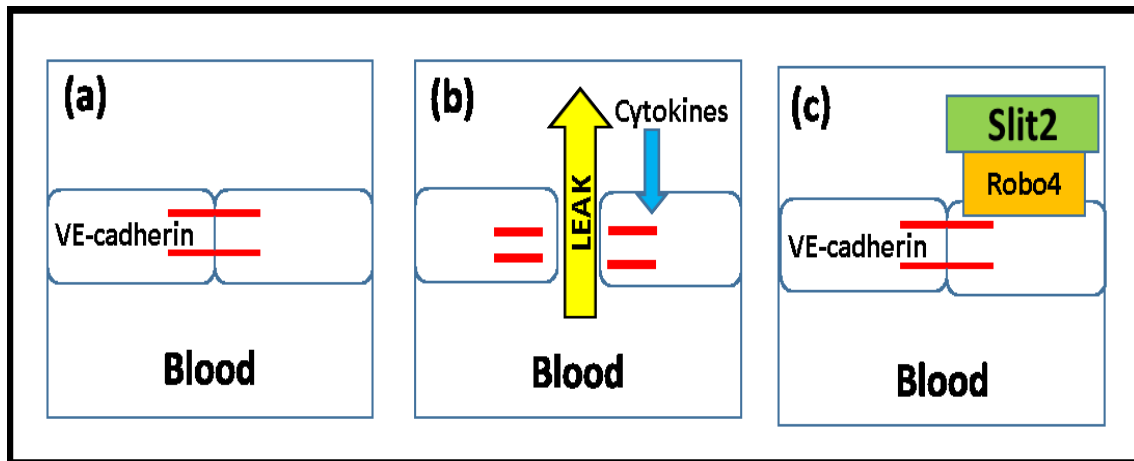


Figure 7. Robo4 and Slit2 in Inflammation. (a) Under normal conditions, endothelial barrier is semi-permeable. (b) Disruption of endothelium by inflammatory stimuli causes vascular leak. (c) Enhancement of VE-cadherin by Robo4-Slit2 binding repairs the damaged endothelium.

Higher levels of Robo4 were measured in plasma samples of cardiac surgery patients (Burke-Gaffney et al, 2014), but the mechanism for Robo4 release is not known.

1.11. Thesis Hypothesis and Aims

1.11.1. Thesis Hypothesis

The work described in this thesis was undertaken to test the hypothesis that release of endothelial barrier protective protein Robo4, following inflammation produced by cardiopulmonary bypass, may be associated with post-operative complications; and that targeting Robo4 could prevent endothelial barrier dysfunction, *in vitro*.

1.11.2. Thesis Aims

The overall purpose of this project was two-fold: first, to investigate plasma Robo4 as a potential biomarker for complications associated with cardiac surgery requiring CPB, particularly those associated with endothelial barrier dysfunction; second, to investigate Robo4 receptor expression on endothelial cells and consequences of inhibiting Robo4 on endothelial barrier function. As there are 2 distinct but complementary components of this thesis the results are presented in two sections: section one refers to Robo4 and associated inflammatory mediators in patient studies; and section two, concerns all *in vitro* experiments- investigations into expression and function of Robo4 on endothelial cells.

In section one, particular emphasis was on associations between plasma Robo4 levels before and after cardiac surgery and indices of inflammation (endothelial biomarkers, inflammatory cytokines and RAGE/RAGE ligand-S100 protein); differences in patients

with severe systemic inflammatory response and vasoplegic shock syndromes; and association with ICU and hospital stay length of stay.

In section two, the overall aim was to produce an expression profile of Robo4 on endothelial cells in comparison to other markers, in particular Robo1, and changes associated with inflammation; and also to investigate the consequences of targeting Robo receptors pharmacologically, with Robo ligand Slit2; and genetically, using Robo4 knockdown, on endothelial barrier integrity.

The specific aims of this thesis were therefore to:

- Determine whether plasma Robo4 levels were raised following cardiac surgery requiring CPB
- Determine whether when these patients were divided into sub-groups – patients with and without SIRS, with and without sepsis or with and without vasoplegic syndrome, Robo4 levels were higher in syndromic patients.
- Carry out similar analyses (as in aims 1 and 2) for inflammatory markers sRAGE and RAGE ligand -S100A8/A9.

- Investigate associations between Robo4 and inflammatory markers sRAGE and S100A8/A9; another plasma marker of endothelial damage syndecan-1; and indices of morbidity, specifically length of stay in ICU.
- Determine expression and release of Robo4 associate with HUVEC; and how the expression profile compared to other endothelial cells (HPAEC and HCAEC); and following TNF- α exposure. HUVEC are endothelial cells that are easily and inexpensively cultured and were therefore used in many published endothelial barrier function studies. However, due to heterogeneity between endothelial cells, differences in Robo4 expression and function between endothelial cells are predicted.
- Determine whether there is sufficient RAGE expressed on HUVEC so that RAGE ligands (e.g. S100 proteins) could be used as inflammatory stimuli to alter endothelial Robo4 expression and function. An original intention of this thesis was to explore the link between RAGE and Robo4 expression and function.
- Establish an *in vitro* model of vascular barrier function using cultured endothelial cells with electrical impedance as the readout of barrier function.
- Investigate the consequences on endothelial barrier function of targeting Robo4 pharmacologically with Slit2 ligand; and genetically, using Robo4 'knockdown' techniques.

2. METHODS AND MATERIALS

2.1. General Methods

2.1.1. Centrifugation

Centrifugation is a technique used to separate particles from a solution. The particles (for example cells) are suspended in a solution (for example growth medium) and placed in a centrifuge tube. The tube is then placed in a rotor and spun. The speed at which samples are spun differ depending on the fragility of particles, for example cells are spun at around 200 RCF, plasma samples around 3000 RPM and urine samples at 10 000 RPM. The RCF (relative centrifugal force) is the amount of acceleration (force) applied to the sample. The RPM (revolutions per minute) is used as a measure of rotational speed. The centrifuges used in this PhD project were general purpose centrifuge Rotina 46R made by Hettich, Germany, and small bench top centrifuge M240R made by Boeco, Germany.

2.1.2. Light Microscopy

Light or optical microscopy is a technique that allows us to look at specimens that are not visible to naked eye, for example cells. It uses focused light together with objective and ocular lenses to enlarge a specimen, so it can be viewed. A very first simple microscope was put together by Hans and Zacharias Jansen, Dutch spectacle makers, in the late 16th century.

The microscope used in this PhD project was Olympus CK40 with objective lenses x4, x10, x40 and ocular lens x10, giving the maximal final magnification of x400. It also had

a camera that enabled to take pictures of observed samples (see Figure 10 for an example of confluent HUVEC). The advantage of light microscope is that live cells can be observed in their environment without the need of staining.

2.2. Cell Culture

2.2.1. Cell counting

In this project, cells were manually counted using a haemocytometer (see Figure 8) and light microscope Olympus CK40 with x10 and x40 objectives.

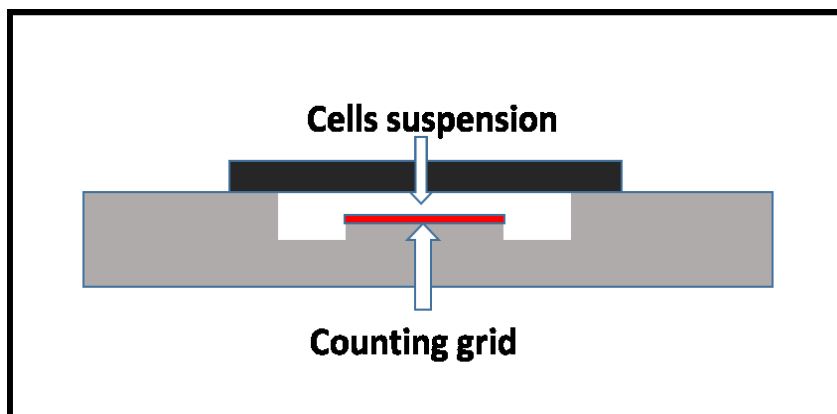


Figure 8. Schematic overview of Haemocytometer. The diagram shows side view of haemocytometer. The cover glass and mounting support creates a chamber where the sample is placed. The counting grid is used to count the cells.

The cells were counted within four large squares 1x1mm (as seen in Figure 9). Each large square was divided into 16 small squares, 0.05x0.05mm, for ease of calculation. The cytometer was covered by a special coverslip glass which created an area of known volume, 100nl per 1mm³.

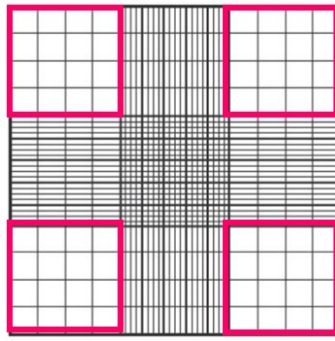


Figure 9. Haemocytometer gridlines. A diagram showing four large squares (16 small squares each) within which the cells are counted.

The total number of cells was estimated using a formula:

$$\text{Total cells per ml} = \frac{\text{total cells counted}}{4} \times \text{dilution factor} \times 10,000$$

2.2.2. Culture and Subculture Protocol (Lonza)

HUVEC were purchased from Lonza as cryopreserved cells (passage 1); and cultured and sub-cultured up to passage 5 in flasks T25, in complete growth medium containing 2% Foetal Bovine Serum (FBS), 0.4% Human Fibroblast Growth Factor-B (rhFGF-B), 0.1% Human Epidermal Growth Factor (rhRGF), 0.1% Vascular Endothelial Growth Factor (VEGF), 0.1% Gentamicin Sulfate Amphotericin-B (GA-1000), 0.1% R3 Insulin-like Growth Factor-1 (R³-IGF-1), 0.1% Ascorbic Acid, 0.1% Heparin and 0.04% Hydrocortisone. Hepes Buffered Saline Solution (Hepes-BSS) contained 30mM Hepes. Trypsin concentration in the Trypsin/EDTA (Ethylenediaminetetraacetic acid) was 0.25 ng/ml. The proportions

and concentrations of Trypsin/EDTA and the composition of trypsin neutralizing solution (TNS) is proprietary of Lonza, who would not reveal the details to me.

The culture and subculture protocol is outlined below:

1. Subculture when cells are 70-80% confluent (see Figure 10)
2. Remove the medium (pipette off)
3. Wash the cells: pipette 5ml of HEPES-BSS into flask, swirl and pipette off.
4. Remove the cells: pipette in 2ml trypsin/EDTA and rock the flask to cover all cells.
5. Put under microscope and wait until 90% of cells are rounded up
6. Gently tap against the palm of your hand, tap every 30sec until cells have detached
7. Neutralize the trypsin: pipette in 4ml TNS
8. Pipette the solution (cells/trypsin/EDTA/TNS) into a falcon centrifuge tube
9. Pipette in 2ml HEPES-BSS, swirl and add to the falcon centrifuge tube
10. Spin for 5mins at 220xg (RCF), 20°C (slow break – speed 5)
11. Remove the supernatant and break the pellet with pipette.
12. Add 2-3ml of medium to the pellet and put 10µl on a slide to count the cells
13. Calculate cells and add 5000 cells per cm² (T25 = 125 000 cells)
14. Add 5ml of warm medium to T25 flask (1ml of medium per 5cm²)

15. Put into a 37°C humidified incubator with 5% CO₂

16. Change medium (37°C warm) the next day and then every other day thereafter.

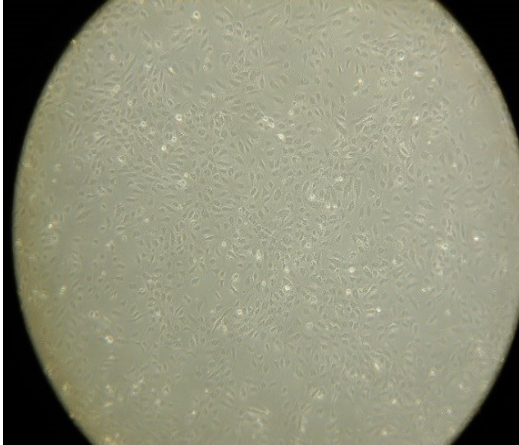


Figure 10. A picture of confluent endothelial cells (HUVEC, Lonza). These cells were observed under light microscope during this PhD project. The final magnification was x100.

2.2.3. Culture and Subculture Protocol (PromoCell)

HUVEC, HPAEC and HCAEC were purchased from Promocell as cryopreserved cells (passage 1); and cultured and sub-cultured up to passage 5 in flasks T25 in complete growth medium containing 2% FBS and 0.1% of each: hFGF, hEGF, R3 IGF-1, VEGF, AA, HC and Heparin. HEPES buffered Balanced Salt Solution contained 30 mM HEPES, D-Glucose, NaCl, KCl, Na-Phosphate and Phenol Red. Trypsin/EDTA ratio was 0.04%/0.03%. Trypsin neutralizing solution (TNS) contained 0.05% trypsin inhibitor from soybean and 0.1% Bovine Serum Albumin (BSA).

The culture and subculture protocol is outlined below:

1. Aspirate medium from T25 flask
2. Add 2.5ml of Hepes solution and wash the cells
3. Replace Hepes with 2.5ml of Trypsin in EDTA solution
4. Gently tap the flask to help cells to detach
5. Add 2.5ml of Trypsin Neutralizing solution
6. Transfer to a tube and centrifuge at 220xg (RCF) for 3 minutes
7. Remove supernatant and re-suspend cells in 1 ml of medium
8. Count the cells
9. Add 5,000-10,000 cells per cm² to T25 flask with 5ml growth medium
10. Put into a 37°C humidified incubator with 5% CO₂
11. Change medium (37°C warm) every 2-3 days

2.2.4. Cryopreservation Protocol

The protocol for cryopreserving HUVEC and HPAEC and HCAEC is outlined below:

1. Freezing solution: Medium (8ml) +Fetal Bovine Serum (1ml) +Dimethyl Sulfoxide (1ml)
2. Detach and count the cells
3. Re-spin the cells and remove the supernatant.
4. Re-suspend the pellet in freezing solution at 1-2 million cells per 1ml
5. Put 1ml of cells in freezing solution into cryo-freezing vials
7. Place vials to a freezing container filled with 250ml of propanol-2 (99.5%) to ensure controlled rate of cooling $-1^{\circ}\text{C}/\text{minute}$ and store in -70°C overnight
8. Move vials into liquid nitrogen (-200°C) for long term storage
9. To take-up the cells from liquid nitrogen:
 - a) Defrost the cells in water bath (37°C)
 - b) Add 5ml of warm HUVEC medium to T25 flask
 - c) Add the cells and place in the incubator
 - d) Change medium the next day and then every other day thereafter.

2.2.5. MTT Assay – Cell viability assay

2.2.5.1. MTT – Basic principle

MTT assay was developed by Tim Mosmann in 1983 (Mosmann, 1983). It is a quantitative colorimetric assay to assess cytotoxicity and cell viability. Its principle is a conversion of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into formazan. MTT is cleaved by cells that are alive and metabolically active, not by dead cells (Ramnath et al, 2008). MTT powder is dissolved in Dulbecco's modified eagle's medium (DMEM) to produce yellow solution. When added to a cell monolayer it gets converted it into formazan which is then dissolved in dimethyl sulfoxide (DMSO) to produce blue solution. Concentration of colour is measured by spectrophotometer at 550-570nm (Socker et al, 2012).

2.2.5.2. MTT – Protocol

100ul of MTT solution is pipetted into each well of 96-well plate. The plate is left to incubate at 37C for 15 minutes. The MTT solution is removed and the plate is left to air dry for at least 45 minutes. Then 100ul of DMSO is added to each well and the plate is placed on a shaker until the content of each well is fully dissolved. In the end absorbance of each well is measured by spectrophotometer at 550-570nm.

MTT – Protocol Summary

Reagents:

MTT solution (1mg/ml in DMEM)

DMEM (Dulbecco's modified eagle's medium)

DMSO (Dimethyl sulfoxide)

Protocol:

100ul of MTT to each well

↓

Leave for 15 minutes in incubator (37°C)

↓

Remove MTT solution

↓

Leave for 45 minutes to air dry

↓

100ul of DMSO to each well

↓

Place on a shaker until fully dissolved

↓

Read absorbance at 550nm

Figure 11. MTT cell viability Protocol

2.3. Specific Assays

2.3.1. ELISA - Basic Principal

ELISA (Enzyme-linked immunosorbent assay) is a colorimetric assay technique used to detect a specific antigen, for example protein, peptide or hormone. The method was developed in 1970s to replace radioimmunoassays. There are four types of ELISA: (1) Indirect, (2) Direct, (3) Sandwich and (4) Competitive.

All ELISAs used in this thesis were Sandwich ELISAs. The sandwich ELISA is most commonly performed in 96-well polystyrene plates. The plates are coated with a specific capture antibody and when the investigated sample is added, the antigen of interest binds to the capture antibody. All unbound substances are washed off. A biotin labelled detection antibody then binds to the antigen creating a “sandwich” of an antigen held between two antibodies. The next step is to add streptavidin-HRP (horseradish peroxidase) complex that binds to biotin-conjugated detection antibody. The last step is to add 3,3',5,5'-tetramethylbenzidine (TMB) developing substrate that binds to HRP enzyme label and produces a colour reaction that is measured by spectrophotometer. The intensity of colour is in proportion to the amount of the antigen bound to the antibodies. All the steps of the assay can be seen in Figure 12. Figures 13 shows ELISA's two last colour-producing steps.

Two types of sandwich ELISA kits were used in this project: Quantikine with antibody coated plates (Robo4, Slit2, vWF, sRAGE, Ang-2, S100A8/A9), and DuoSet without pre-coated plates (GM-CSF, Syndecan-1).

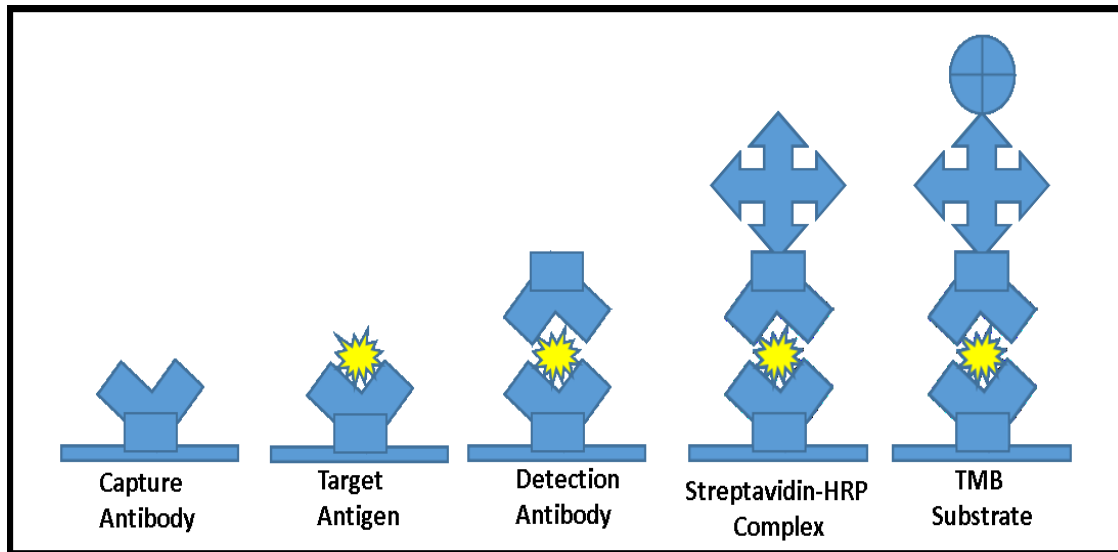


Figure 12. Diagram showing the individual steps of sandwich ELISA. It is called sandwich, because the target antigen is “sandwiched” between capture and detection antibodies.

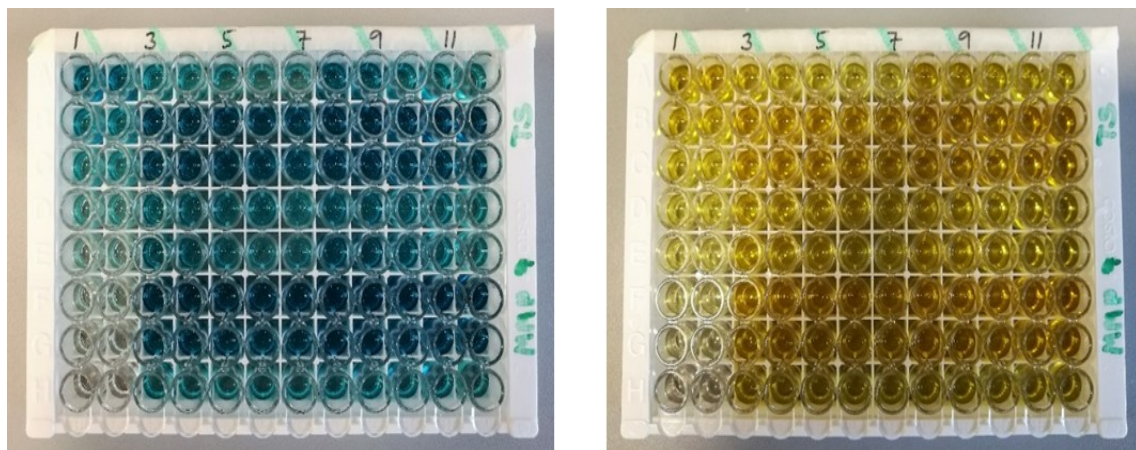


Figure 13. ELISA in progress. Adding of TMB substrate produces a blue colour reaction (an image on the left). Adding of stop solution (acid) turns the blue colour into yellow (an image on the right). The result of the reaction between TMB and acid enables the measurement of colour absorption by a spectrophotometer.

2.3.1.2. ELISA Quantikine protocol

Reagents supplied:

Antibody coated plate 1x96microwells

Wash buffer (1:25)

Biotin antibody (1:100) and diluent

Standard (4000pg/ml) and diluent

HRP-avidin (1:100) and diluent

TMB substrate (A:B=1:1)

Stop solution

Protocol:

100ul of standard or sample per well → Incubate 2hr at 37°C.

↓

Remove the liquid from each well – do not wash!

↓

100ul of Biotin-antibody per well → Incubate 1hr at 37°C

↓

Wash 3 times with wash buffer

↓

100ul of HRP-avidin per well → Incubate 1hr at 37°C

↓

Wash 5 times with wash buffer

↓

100ul of substrate → Incubate 30mins at 37°C

↓

50ul of Stop solution → Read absorbance at 450nm/550nm

Figure 14. An example of a Quantikine ELISA protocol (Robo4)

Plasma samples and standards are pipetted at 100µl per well in duplicates and left to incubate for 2 hours at 37°C. The samples are then removed from the plate and 100µl of biotin antibody is added to each well and left to incubate for 1 hour at 37°C. After the incubation the plate is washed three times with wash buffer. 100µl of HRP-avidin is added to each well and left to incubate for 1 hour at 37°C. After the incubation the plate is washed five times with wash buffer. Then 100µl of TMB substrate is added and left to incubate for 30 minutes at 37°C. The reaction is stopped by adding 50µl of stop solution and absorbance is read at 450nm/550nm.

2.3.1.3. ELISA DuoSet protocol

100µl of capture antibody is pipetted per each well and left to incubate over night at room temperature. In the morning the antibody is washed off three times with wash buffer and 300 µl of reagent diluent is added per well. After 1 hour of incubating it at room temperature the plate is washed three times with wash buffer. Plasma samples and standards are pipetted at 100µl per well in duplicates and left to incubate for 2 hours at room temperature. After the incubation the plate is washed three times with wash buffer. 100µl of detection antibody is added and left to incubate for 2 hours at room temperature. After the incubation the plate is washed three times with wash buffer. 100µl of HRP-avidin is added to each well and left to incubate for 20 minutes at room temperature in the dark. After the incubation the plate is washed three times with wash buffer. Then 100µl of TMB substrate is added and left to incubate for 20 minutes at

room temperature in the dark. The reaction is stopped by adding 50µl of stop solution and absorbance is read at 450nm/550nm.

Reagents: (supplied in Ancillary Set, R&D Systems, Cat. No. DY008)

Plate 1x96 micro wells
Wash Buffer (1:25)
Reagent Diluent (1:10)
TMB substrate (A:B = 1:1)
Stop solution (2N H₂SO₄)

Reagents: (supplied in GM-CSF Duo Set, R&D Systems, Cat. No. DY215)

Capture antibody (2ug/ml)
Detection antibody (0.5ug/ml)
Standard (1000pg/ml)
HRP-Streptavidin (1:40)

Protocol:

100ul of capture antibody per well – incubate overnight at RT – Wash 3x

↓

300ul of reagent diluent per well – incubate 1h at RT – Wash 3x

↓

100ul of standard or sample per well – incubate 2h at RT – Wash 3x

↓

100ul of detection antibody per well – incubate 2h at RT – Wash 3x

↓

100ul of HRP-Streptavidin per well – incubate 20mins at dark – Wash 3x

↓

100ul of substrate per well – incubate 20mins at dark

↓

50ul of stop solution and read absorbance at 450nm/550nm

Figure 15. An example of a DuoSet ELISA protocol (GM-CSF)

2.3.2. Flow Cytometry

2.3.2.1 Flow Cytometry - Basic principle

Flow cytometry is a cell analysing technique using fluorescent antibodies conjugated to fluoro-chromes. This technique was invented in the 1960s in the USA and became commercially available in the early 1970s. The flow cytometer is composed of three main systems - the fluidics, optics, and electronics. The fluidics system transports sample in a single-file stream of cells through the optical system in a high speed of thousands cells per second. The optics system is composed of light sources, lenses and lasers. When a single cell passes by the laser beam it causes light to scatter as a forward or side angle scatter, see an example of scatter graph in Figure 16. Forward scattered light measures the sizes of cells. The larger the cell, the stronger forward scatter signal. Side scattered light measures the granularity of the cells in its path. The more complex (granular) the cell, the higher the scatter signal. The electronics systems allows to control the cytometer and analysing the data. A flow cytometer is often mistaken for a fluorescence activated cell sorter (FACS). A flow cytometer does not perform cell sorting. It works with fluorescently labelled cells in a single-cell suspension. Cells stained by fluorescence-tagged antibodies emit fluorescent light after excitation by a compatible wavelength laser of flow cytometer (Herzenberg et al, 2002). The measured data are expressed as Percentage of Cells and Median Fluorescence Intensity (MFI). The Percentage of Cells shows us how many cells in the tested cell suspension expressed our protein of interest. MFI shows how much (the median) of the protein of interest was in each of the tested cell. The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit was used to determine the

viability of the cells in every single sample in all flow cytometry experiments. The gates were then set in a way that all dead cells were excluded from the final results.

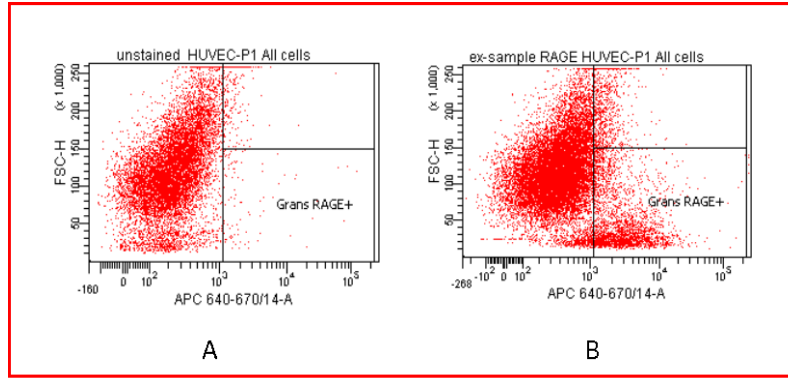


Figure 16. Flow Cytometry scatter graphs. Scatter graphs of (A) control unstained, and (B) sample stained with RAGE antibody. Graph (A) shows the gates being set on unstained HUVEC (control). Graph (B) shows the cells that stained negative (left to the gate) and positive (right to the gate) for extracellular RAGE. Untreated, basal HUVEC showed that some cells stained positively for RAGE on the extracellular surface when compared with unstained cells.

2.3.2.2 Flow Cytometry – Protocol

The endothelial cells were cultured, split, counted and re-suspended in PBS/EDTA. The cell suspension (50ul) was transferred to eppendorf tubes in density of 200,000 cells/tube. Life/dead marker (Fixable Violet Life and Dead Stain Kit, Life Technologies, Oregon, USA) was added to all tubes, except un-stained controls, to assess the number of live cells in each sample. Fluorescent antibodies for external expression were added. After 30 minutes of being left on ice, the samples were washed with PBS, spinned and fixed. Before adding fluorescent antibodies for internal expression, the cells' membrane was permeabilized. After the permeabilization, the cells were stained by fluorescent

antibodies and left in a dark for 25 minutes. After that, all samples for external and internal expression were spun and the supernatants were replaced with PBS/EDTA. The samples were then measured at the Imperial College flow cytometry facility, using Flow cytometer BD LSRFortessa developed by BD Biosciences. The data were analysed using the integration software of BD LSRFortessa, called BD FACSDiva.

Flow Cytometry – Protocol Summary

1. 50ul of cell suspension (200,000cells/tube) in PBS/EDTA
2. Mix 2ul of life-dead + 50ul PBS/EDTA, then add 1ul of the mix to samples
3. Add external fluorescent antibodies, tap-mix and leave on ice for 30mins
4. Add 1ml of PBS/EDTA to all tubes and spin at 400xg (RCF), 4°C for 10mins
5. Tip off PBS/EDTA – leave about 100ul and tap-mix
6. Add 1ml of FIX buffer to all – invert 10x to mix
7. Leave in the dark in room temperature for 15mins
8. Spin intracellular tubes 600xg (RCF), 4°C for 5mins and remove supernatant
9. Add 1ml of cold PERM – gently invert x10 and leave on ice in dark for 30mins
10. Spin 1500xg (RCF), 4°C for 5mins
11. Remove supernatants, tap-mix and add internal fluorescent antibodies
12. Leave on ice in the dark for 25mins
13. Add 1ml PBS/EDTA and spin all tubes (internal and external) 1500g, 4°C for 5mins
14. Remove supernatants and add 300ul of PBS/EDTA
15. Transfer to FACS tubes and keep on the ice in the dark until read on Flow cytometer

Figure 17. A summary of Flow cytometry protocol for staining cells

2.3.3. Short interfering RNA knockdown

2.3.3.1 Short interfering RNA knockdown - Basic principle

Short interfering RNA (siRNA) is used to silence (knockdown) a protein-coding gene for a short period of time, about 48-96 hours. It is synthetically produced RNA that targets a specific mRNA for degradation. The siRNA transfects a cell as a double stranded structure. One of the strands is degraded and the other strand forms RNA-induced silencing complex (RISC). This complex binds to targeted mRNA sequence which is degraded. This causes temporary silencing of the targeted gene (Petri and Meister, 2013).

2.3.3.2 Short interfering RNA knockdown – Protocol

1. Wash cells twice in serum-free (SF) medium and leave for 90 minutes
2. Prepare transfection mix (for 1 well of 96-well plate)
3. Solution A = 19.6ul of SF medium plus 0.4ul DharmaFECT → incubate for 5min
4. Solution B = 19.9ul of serum free medium plus 0.1ul of stock (20uM) siRNA
5. Mix solution A + solution B and incubate for 20 mins at RT
6. Add 160ul of SF medium and 40ul of transfection mix per well and incubate for 5hrs
7. Aspirate off transfection medium and add 2ml of full medium (supplements + serum).
8. Incubate for 72h and then use for experiments

2.3.4. Trans-endothelial electrical impedance (TEEI)

2.3.4.1 TEEI - Basic principle

Electrical impedance is a measure of the total opposition that a circuit presents to electrical current. Two instruments developed by ACEA Biosciences Inc., iCELLigence

RTCA and xCELLigence RTCA, were used in this PhD project to measure transendothelial electrical impedance (TEEI). The RTCA stands for “real time cell analysis”, because it allows monitoring of the experiments and reading of the data in real time. There are some differences between these two instruments, see Table 1, but the principle is same for both, see Figure 18.

Instrument	Number of cradles	Number of wells per plate	Surface area per well	Max liquid volume	Wireless
iCELL	2	8	64 mm ²	840ul	yes
xCELL	3	16	20 mm ²	280ul	no

Table 1. iCELLigence and xCELLigence specifications. The specifications for iCELLigence (iCELL) and xCELLigence (xCELL) instruments by ACEA Biosciences Inc.

The iCELLigence instrument has two cradles which enables two experiments to run independently of one another. Each cradle takes one 8-well plate. The xCELLigence instrument has three cradles which enables three experiments to run independently of one another. Each cradle takes one 16-well plate, into which cells are usually seeded in duplicates. Gold microelectrodes are fused to the bottom surface of a plate that sits

between the negative and positive terminals. The cell culture medium acts as an electrically conductive solution through which an electrical current of 22 mV travels. The electrons leave the negative terminal, pass through the conductive solution, and then complete the circuit by depositing onto the positive terminal. The cells attached to the bottom of the well impede the flow of electrons as it can be seen in the Figure 18.

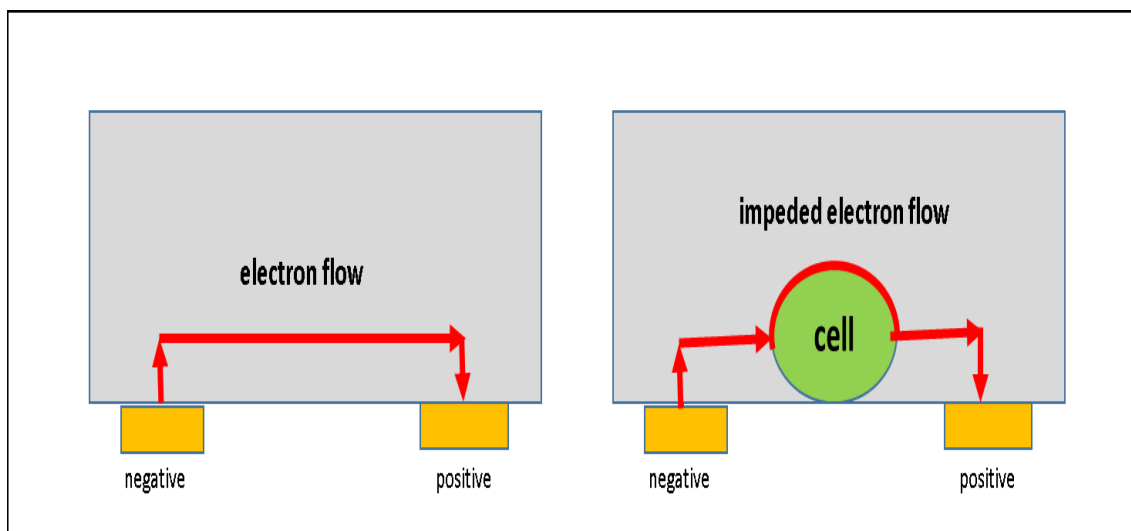


Figure 18. The principle of the ACEA instruments. A view of a single well is shown, with and without a cell. Before the cells are added, electric current flows freely through culture medium completing the circuit between the electrodes. After the cells are added and start attaching to the surface, the electrical current flow is impeded.

A parameter, called Cell Index (CI), is used to express the electrical impedance caused by the attached cells. $CI = \text{impedance at time point } n - \text{impedance in the absence of cells} / \text{nominal impedance value}$. The Cell index does not have a unit. The CI increases as the cells adhere and proliferate as can be seen in Figure 19. Cell seeding density with an optimal CI has to be established before experiments commence.

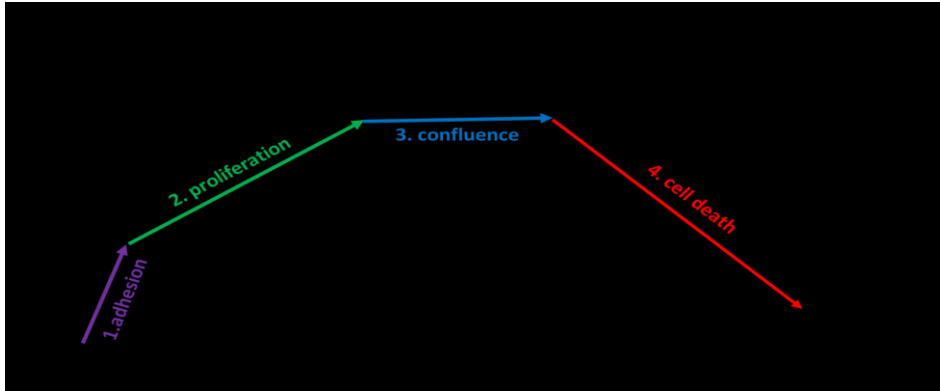


Figure 19. Measuring TEEI on xCELLigence. This figure shows four stages during an endothelial barrier experiment. 1. A quick increase of TEEI due to cell adhesion. 2. Slow increase of TEEI due to cell proliferation. 3. Plateau in TEEI due to cell confluence. 4. Slow decrease of TEEI due to cell detachment (cell death).

2.3.4.2 TEEI – Protocol

50µl of medium is pipetted per well and incubated at room temperature for 30 minutes. 150µ of cell suspension is added to well in duplicates. The cell density is 6,000 cells per well for HUVEC and 12,000 cells per well for HPAEC. The plate with cells is incubated at room temperature for 30 minutes and then placed in xCelligence instrument and run at 37°C. Next day the medium is refreshed by changing 100µl of it. The cells are left to proliferate until they reach confluence showed as plateau. At the start of plateau 40µl of medium is removed and 20µl of Slit2 (final concentration of 30nM) is added and left to incubate for 1 hour at 37°C. Then 20ul of TNF-α (final concentration of 0.1ng/ml) is added and the experiment is left to run at 37°C until the cells start dying, i.e. leave the plateau.

Reagents:

Treatment: TNF- α 0.1ng/ml; Slt2 30nM

Seeding density/well: 6000cells (HUVEC), 12000cells (PAEC)

Protocol:

Place xCELLigence Instrument Into an Incubator (37°C)



Add 50ul of medium Into 16-well plate and Incubate at RT for 30mins



Calibrate the plate with medium In xCELLigence Instrument



Add 150ul of cell suspension to plate and Incubate at RT for 30mins



Place plate In xCELLigence Instrument and start experiment



Change half medium (100ul) the next day



When plateau starts, add treatment:



Remove 40ul of medium and add 20ul Slt2 (final conc. 30nM)



After 60mins add 20ul of TNF- α (final conc. 0.1ng/ml)

Figure 20. Protocol for measuring TEEI using xCelligence instrument.

2.4. Clinical Methods

2.4.1. Study Population

Patients undergoing cardiac surgery for valve replacement and/or coronary artery bypass grafting requiring CPB at the Royal Brompton Hospital in London, were recruited over two years period, between 2013 and 2015.

2.4.2. Ethics Approval and Patients Consent

Patients signed written informed consent to allow the use of their blood samples for the research with ethic's number 10/H5004/9, approved by the Research Ethics Committee for the Royal Brompton and Harefield NHS Foundation Trust.

2.4.3. Sample Collection

Blood samples (10 ml) were collected via a peripheral venous line in patients into a collecting tube with citrate, just before the start of the surgery and then immediately after CPB, and at 2 hours and 24 hours post-CPB. The blood was centrifuged at 1000g for 15 minutes to separate plasma which was then stored at -80°C for further analysis.

2.4.4. SIRS, Septic and Vasoplegic Samples

Patient samples were divided into three groups (1) with and without SIRS, (2) with and without Sepsis and (3) with and without Vasoplegia. Patients with SIRS were those who had least two of the following criteria: fever $>38^{\circ}\text{C}$ or hypothermia $<36^{\circ}\text{C}$, respiratory rate >20 breath/min or $\text{PaCO}_2 < 4.3\text{kPa}$, heart rate >90 beats/min and leucocytosis $>12 \times 10^9/\text{l}$ or leucopenia $< 4 \times 10^9/\text{l}$. Patients with Sepsis were those who needed an administration of antibiotics for four or more days. Patients with Vasoplegia were those who had a mean arterial pressure <50 mmHg, left atrial pressure <10 mmHg, right atrial pressure <5 mmHg, cardiac index $>2.5 \text{ L min}^{-1} \text{ m}^{-2}$ and low systemic vascular resistance ($<800 \text{ dyne s}^{-1} \text{ cm}^{-5}$).

2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software, version 5.02 (GraphPad Software, San Diego, USA). Clinical data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric tests, and expressed as median interquartile range (IQR). Cell culture data were statistically analysed by ANOVA, Kruskal-Wallis and Mann-Whitney non-parametric tests and where appropriate by t-test. Data are presented as mean $\pm$ standard deviation (SD). Significance is shown in graphs by asterisks: $p < 0.05$ is expressed as one asterisk, $p < 0.01$ is expressed as two asterisks and $p < 0.001$ is expressed as three asterisks, in accordance with the analysing software GraphPad Prism.

2.6. Materials

2.6.1. Cell Culture:

HUVEC pooled	Lonza, GmbH, Köln, Germany
Endothelial Cells Growth Medium Bullet Kit	Lonza, GmbH, Köln, Germany
Reagent Pack TM (Trypsin, TNS, Hepes)	Lonza, GmbH, Köln, Germany
HUVEC pooled and single-donor	PromoCell GmbH, Heidelberg, Germany
HUVEC VEGF-pre screened	PromoCell GmbH, Heidelberg, Germany
Endothelial Cells Growth Medium Kit 2	PromoCell GmbH, Heidelberg, Germany
Sub-culturing pack (Trypsin, TNS, Hepes)	PromoCell GmbH, Heidelberg, Germany
HPAEC	PromoCell GmbH, Heidelberg, Germany
HCAEC	PromoCell GmbH, Heidelberg, Germany
Cell Culture Nunc TM EasyFlask TM 25 & 75cm ²	ThermoFisher Scientific, Waltham, USA
Cellstar Falcon Tubes 15 and 50ml	Grenier Bio-One, Kremsmunster, Austria
2-propanol	Sigma-Aldrich, St Louis, USA

2.6.2. Cytokines and Antibodies:

Recombinant Human TNF- α	R&D Systems, Minneapolis, USA
Recombinant Human IL-1 β	R&D Systems, Minneapolis, USA
Recombinant Human IL6	R&D Systems, Minneapolis, USA
Recombinant Human Slit2-N	PeptoTech, Rocky Hill, USA
Fixable Violet Life and Dead Stain Kit	Life Technologies, Oregon, USA
RAGE antibody	Insight Biotech, Santa Cruz, USA
Lightning-Link [®] Allophycocyanin (APC)	Innova Bioscience, Cambridge, UK

TLR2 (TL2.1) Alexa Fluor 647 Monoclonal Ab	Imgenex, Santa Cruz, USA
TLR4 (HTA125) PE Monoclonal Ab	Imgenex, Santa Cruz, USA
TLR9/CD289 PE Monoclonal Ab	Imgenex, San Diego, USA
ROBO1, Rabbit, Anti-Robo Polyclon Ab, FITC	Bioss, Woburn, USA
ROBO4, Mouse, PE-conjugated Ab	R&D Systems, Minneapolis, USA
ArC™ Amine Reactive Compensation Bead Kit	Life Technologies, Oregon, USA
AbC™ Total Antibody Compensation Bead Kit	Life Technologies, Oregon, USA
Human Robo4 siRNA	Dharmacon, Lafayette, USA
Slit2 Monoclonal Ab	ThermoFisher Scientific, Waltham, USA
Recombinant Human EN-RAGE Protein	R&D Systems, Minneapolis, USA
Recombinant Human Robo4 Protein	Sino Biological, Beijing, China

2.6.3. ELISA kits:

Robo4 ELISA	Cusabio, Wuhan, China
vWF Zymutest (vWF) ELISA	Hyphen, BioMed, Neuville, France
Angiopoietin-2 ELISA	R&D Systems, Minneapolis, USA
S 100A8/A9 (Calprotectin) ELISA	Buhlmann, Schönenbuch, Switzerland,
RAGE (Quantikine) ELISA	R&D Systems, Minneapolis, USA
GM-CSF (Duo Set) ELISA	R&D Systems, Minneapolis, USA
Duo Set ELISA Ancillary Reagent Kit	R&D Systems, Minneapolis, USA
Syndecan-1 (Duo Set) ELISA	R&D Systems, Minneapolis, USA

2.6.4. Chemicals and Reagents:

Perm Buffer	BD Biosciences, San Diego, USA
Fix Buffer	BD Biosciences, San Diego, USA
Phosphate-buffered saline (PBS)	Sigma-Aldrich, St Louis, USA
Sterile Water	Sigma-Aldrich, St Louis, USA
E-Plate View 16	ACEA, Biosciences, San Diego, USA
E Plate L8	ACEA, Biosciences, San Diego, USA

3. RESULTS AND DISCUSSION

3.1. Section 1. Robo4 in SIRS, Sepsis and Vasoplegia: Implications for cardiac surgery

3.1.1. Context

At the start of this project Burke-Gaffney *et al* published a study demonstrating that plasma levels of Robo4 increased after cardiac surgery in adult patients. Robo4 levels correlated with endothelial damage markers Ang-2 and vWF and were higher in patients with acute kidney injury (Burke-Gaffney et al, 2014). This finding established endothelial Robo4 as a novel plasma biomarker in this patient group. Patients undergoing cardiac surgery with CPB are therefore at risk of developing endothelial dysfunction and capillary leak leading to-tissue oedema. Post-operative oedema is a clinical marker of SIRS/sepsis and can contribute to organ failure (lung and kidney); which prolongs ICU and hospital stay and prolongs long term recovery (Creagh-Brown et al, 2010). Whilst endothelial hyperpermeability is implicated in these conditions, the mechanisms involved have not yet been fully elucidated. Specific pharmacological interventions targeting endothelial hyperpermeability are lacking and new drugs that could enhance endothelial barrier function are urgently required. Endothelial-expressed Robo4, in conjunction with its Slit2 ligand, has emerged as a novel molecule that could act as a biomarker for endothelial dysfunction associated with cardiac surgery and CPB; and also as a potential novel strategy to protect the endothelial barrier during cardiac surgery using cardiopulmonary bypass. Therefore, the overall aim of the first results section of this thesis was to explore further the potential of Robo4 as a plasma biomarker in this patient group; whilst, the aim of the subsequent, in vitro, section of the thesis was to

investigate the potential of endothelial Robo4 as a therapeutic target for reducing endothelial barrier dysfunction, in cell culture models.

To explore the potential of Robo4 as a plasma biomarker in patients undergoing cardiac surgery with CPB it was considered necessary to assess Robo4 levels in the context of other plasma markers: well-established endothelial biomarkers- Ang-2 and vWF (see section 1.7.1. and 1.7.2 in introduction); a novel marker of endothelial dysfunction – syndecan-1 (see section 1.7.3. in introduction); and also biomarkers of inflammation previously researched by this lab (Creagh-Brown et al, 2013) - RAGE and S100 proteins (see section 1.6.1. and 1.6.2 in introduction)

3.1.2. Specific Hypothesis, Aims and Objectives

The hypothesis tested in the first results section was that inflammation-induced increase in plasma levels of endothelial barrier protective molecule, Robo4, in patients undergoing cardiac surgery with CPB indicates post-operative complications and prolonged recovery.

The specific aims of this prospective study of 48 patients were therefore to:

1. Determine whether plasma Robo4 levels were raised following cardiac surgery requiring CPB
2. Determine whether when these patients were divided into sub-groups – patients with and without SIRS, with and without sepsis or with and without vasoplegic syndrome, Robo4 levels were higher in syndromic patients.

3. Carry out similar analyses (as in aims 1 and 2) for inflammatory markers sRAGE and RAGE ligand -S100A8/A9.
4. Investigate associations between Robo4 and inflammatory markers sRAGE and S100A8/A9; another plasma marker of endothelial damage syndecan-1; and indices of morbidity, specifically length of stay in ICU.

The specific objectives to address these aims were to:

- Measure plasma levels of Robo4 (and its ligand Slit2) pre, immediately after CPB and at 2h and 24h after CPB- subsequently designated pre, post-operation (op); 2h post op and 24h post op.
- Measure plasma levels of Syndecan-1 at pre, post, 2h and at 24h post-operatively.
- Measure plasma levels of RAGE and its ligand S100A8/A9 pre, post, 2 and at 24h post-operatively.
- Measure plasma levels of Ang-2 and VWF (in 10 patients only due to assay costs) at pre, post, 2h and at 24h post-operatively.
- Compare Robo4, sRAGE, S100A8/A9 in patients with and without SIRS, sepsis and vasoplegia
- Correlate Robo4 with other plasma biomarkers (sRAGE, S100A8/A9 and syndecan-1) and with length of stay in ICU.

3.1.3. Methods

3.1.3.1. Sample collection

The cohort of plasma samples from 48 patients undergoing cardiac surgery for valve replacement and/or coronary artery bypass grafting requiring CPB at the Royal Brompton Hospital in London, were collected between 2013 and 2015. Blood samples (10 ml) were collected via a peripheral venous line in patients, into a collection tube with citrate, just before the start of the surgery and then immediately after CPB, and at 2 hours and 24 hours post-CPB. The blood was centrifuged at 1000g for 15 minutes to separate plasma which was then stored at -80°C for further analysis. Before the operation, patients signed written informed consent to allow the use of their blood samples for the research with ethic's number 10/H5004/9, approved by the Research Ethics Committee for the Royal Brompton and Harefield NHS Foundation Trust. Biomarkers were measured in plasma samples using enzyme-linked immunosorbent assays (ELISA) – see section 2.3.1. in the general methods. The detection range of ELISA used to measure plasma levels can be seen in Table 2.

Biomarker	Detection Range
Angiopoietin-2	46.9 – 3000 pg/ml
Von Willebrand factor	0 – 150 %vWF
Syndecan-1	8.0 – 256 ng/ml
Robo4	62.5 – 4000 pg/ml
RAGE	78.0 – 5000 pg/ml
S100A8/A9	0.4 – 24.0 µg/ml

Table 2. The detection range of ELISA (plasma). The detection range of ELISA kits for measuring human plasma biomarkers.

3.1.3.2. Patient sub-group analysis

Patient samples were divided into three groups (1) with and without SIRS, (2) with and without Sepsis and (3) with and without Vasoplegia. Patients with SIRS were those who had least two of the following criteria: fever $>38^{\circ}\text{C}$ or hypothermia $<36^{\circ}\text{C}$, respiratory rate >20 breath/min or $\text{PaCO}_2 < 4.3\text{kPa}$, heart rate >90 beats/min and leucocytosis $>12 \times 10^9/\text{l}$ or leucopenia $< 4 \times 10^9/\text{l}$. Patients with Sepsis were those who needed an administration of antibiotics for four or more days. Patients with Vasoplegia were those who had an arterial pressure <50 mmHg, left atrial pressure <10 mmHg, right atrial pressure <5 mmHg, cardiac index $>2.5 \text{ L min}^{-1} \text{ m}^{-2}$ and low systemic vascular resistance ($<800 \text{ dyne s}^{-1} \text{ cm}^{-5}$).

3.1.4. Results

3.1.4.1. Patients' demographics

Personal information about the patients – age, gender, BMI - were collected before the start of surgery. Any additional relevant information was collected during their stay at the hospital in accordance with the Ethics approval number 10/H5004/9. All collected information can be seen in Table 3 on the next page.

Pre-operative characteristics		Percentage of patients
Age, mean (SD) - years	64 (12)	
Gender F/M	9/39	
Body mass index, median (IQR) kg/m ²	26.5 (23.5-30.6)	
Operative variables		
CPB time median* (IQR) - minutes	119 (90 – 161)	
Valve surgery (MVR/AVR)	23 (12/11)	47.9%
Coronary artery bypass graft (CABG)	22	45.8%
CABG with valve surgery	3	6.25%
Post-operative outcomes		
ICU length of stay, median (IQR) hours	23.9 (21.8 – 43.9)	
Hospital length of stay, median (IQR) days	8 (7-10.75)	
SIRS Yes/No	37/11	77.1%
Sepsis Yes/No	18/30	37.5%
Vasoplegia Yes/No	16/32	33.3%
Hospital mortality (%)	2	4.2%

Table 3. The patients' demographics. A table of pre-operative, operative and post-operative variables of study patients in cohort 2 (n=48). Notes: * time from the start of anaesthesia to the end of bypass; MVR = Mitral valve replacement; AVR = Aortic valve replacement.

3.1.4.2. Plasma levels of Angiopoietin-2 increase 24h after surgery

Preoperative levels of Ang-2 were 1.48 (1.22-3.19) ng/ml, immediately post-operatively the levels were 1.87 (1.39-4.11) ng/ml, 2 hours post-operatively the levels were 2.29 (1.61-4.22) ng/ml and 24 hours post-operatively the levels were 6.97 (5.68-13.91) ng/ml. The increase in Ang2 levels at 24 hours post-operatively was significantly ($p<0.001$) higher than preoperative levels, also significantly ($p<0.01$) higher than post-operatively and 2 hours post-operatively levels ($p<0.05$). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric tests and are expressed as median (IQR) ng/ml.

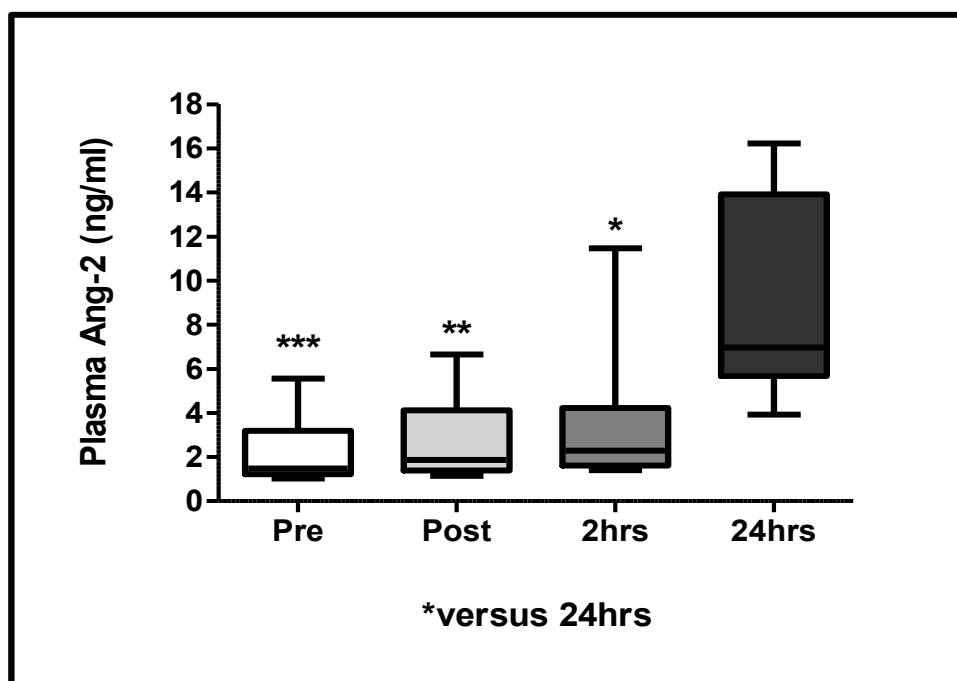


Figure 21. Plasma levels of Angiopoietin-2. Levels of Ang-2 were measured in all four time-points: pre, post, 2hrs post and 24hrs post snCPB. Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml (n=10); $p < 0.001$ is expressed as three asterisks, $p < 0.01$ as two asterisks and $p < 0.05$ as one asterisk.

3.1.4.3. Plasma levels of Von Willebrand Factor increase 24h after surgery

Preoperative levels of vWF were 73.5 (44.2-112.3) %vWF, immediately post-operatively the levels were 117.0 (104.9-147.0) %vWF, 2 hours post-operatively the levels were 140.9 (104.7-167.8) %vWF and 24 hours post-operatively the levels were 169.1 (139.9-205.4) %vWF. Levels of vWF have significantly ($p < 0.001$) increased 24hrs after the snCPB compare to preoperative levels. Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric tests and are expressed as median (IQR) ng/ml.

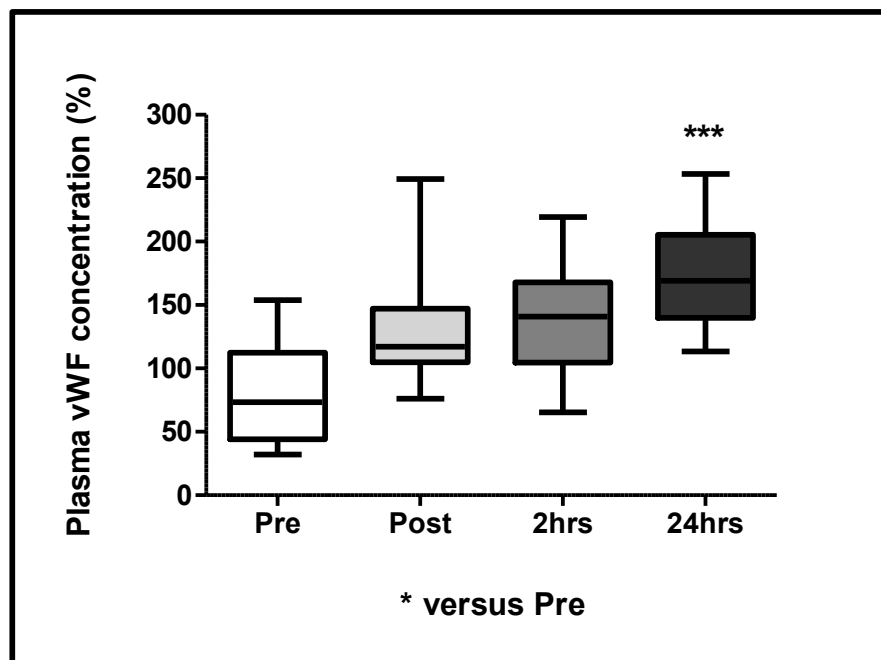


Figure 22. Plasma levels of Von Willebrand Factor. Levels of VWF were measured in all four time-points: pre, post, 2hrs post and 24hrs post snCPB. Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml ($n=10$); $p < 0.001$ is expressed as three asterisks

3.1.4.4. Plasma levels of Syndecan-1 increase immediately after surgery

Preoperative levels of Syndecan-1 were 41.29 (21.22-65.75) ng/ml, immediately post-operatively the levels were 61.65 (44.83-122.80) ng/ml, 2 hours post-operatively the levels were 98.63 (55.17 – 143.40) ng/ml and 24 hours post-operatively the levels were 71.24 (43.92-147.50) ng/ml. Levels of Syndecan-1 have significantly ($p < 0.05$) increased immediately after the snCPB compare to preoperative levels and stayed significantly ($p < 0.001$) raised at 2 hours post-operatively. At 24hrs post-operatively the levels decreased, but were still significantly ($p < 0.05$) higher compare to preoperative levels.

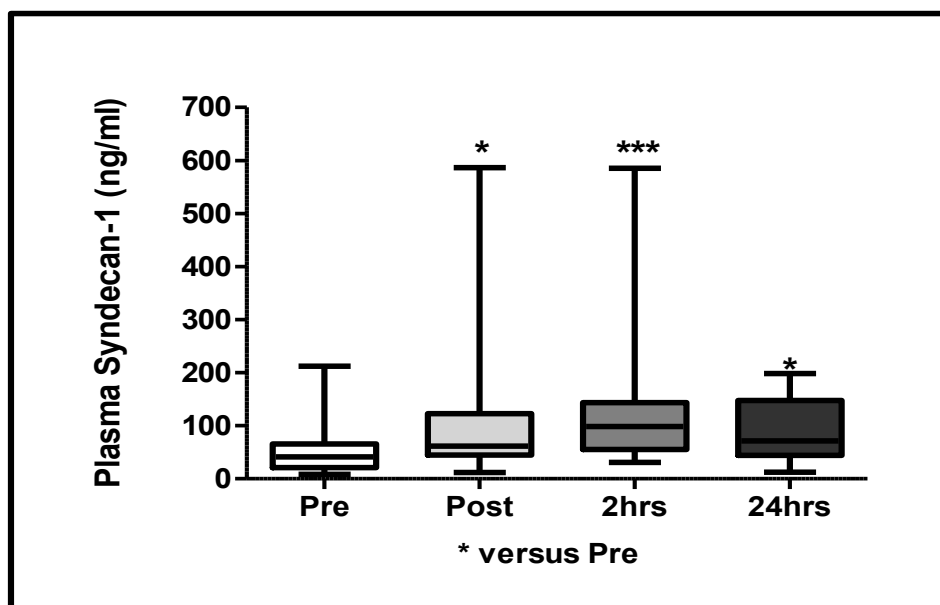


Figure 23. Plasma levels of Syndecan-1. Levels of Syndecan-1 were measured in all four time-points: pre (n=41), post (n=40), 2hrs post (n=32) and 24hrs post snCPB (n=42). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml; $p < 0.001$ is expressed as three asterisks; $p < 0.05$ is expressed as one asterisk.

3.1.4.5. Plasma levels of Robo4 increase immediately after surgery

Preoperative levels of Robo4 were 4.77 (3.76-6.43) ng/ml, immediately post-operatively the levels were 7.67 (5.62-9.17) ng/ml, 2 hours post-operatively the levels were 5.66 (4.53-7.15) ng/ml and 24 hours post-operatively the levels were 5.86 (4.35-6.95) ng/ml. Levels of Robo4 have significantly ($p < 0.001$) increased immediately after the snCPB compared to preoperative levels. At 2 hours post-operatively the Robo4 levels decreased significantly ($p < 0.01$) compare to immediately after the op levels. At 24 hours post-operatively the Robo4 levels were similar to 2 hours post-operatively.

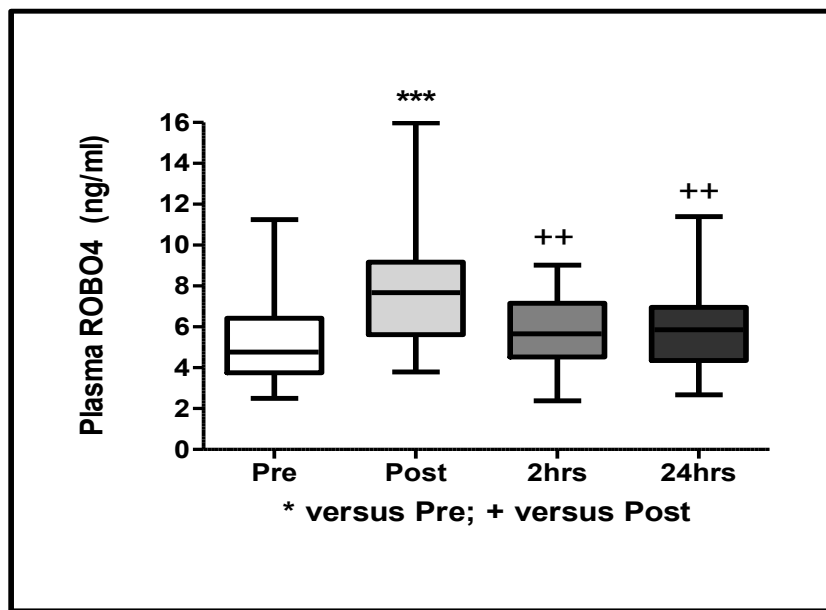


Figure 24. Plasma levels of Robo4. Levels of Robo4 were measured in all four time-points: pre (n=48), post (n=47), 2hrs post (n=37) and 24hrs post snCPB (n=47). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml; *compared to pre-operative levels ($p < 0.001$ is expressed as three asterisks***); +compared to post-operative levels ($p < 0.01$ is expressed as two asterisks ++).

3.1.4.6. Plasma levels of Robo4 in patients with and without SIRS are similar

Preoperative levels of Robo4 in patients who developed SIRS were 4.92 (3.66-6.76) ng/ml, immediately post-operatively the levels were 6.94 (5.38-9.23) ng/ml, 2 hours post-operatively the levels were 5.67 (4.26-7.32) ng/ml and 24 hours post-operatively the levels were 5.71 (4.19-6.86) ng/ml. Preoperative levels of Robo4 in patients who did not develop SIRS were 4.24 (3.89-6.43) ng/ml, immediately post-operatively the levels were 7.95 (5.76-8.77) ng/ml, 2 hours post-operatively the levels were 5.62 (5.60-6.63) ng/ml and 24 hours post-operatively the levels were 6.02 (4.70-7.70) ng/ml. There was no significant difference in any time points between patients with and without SIRS.

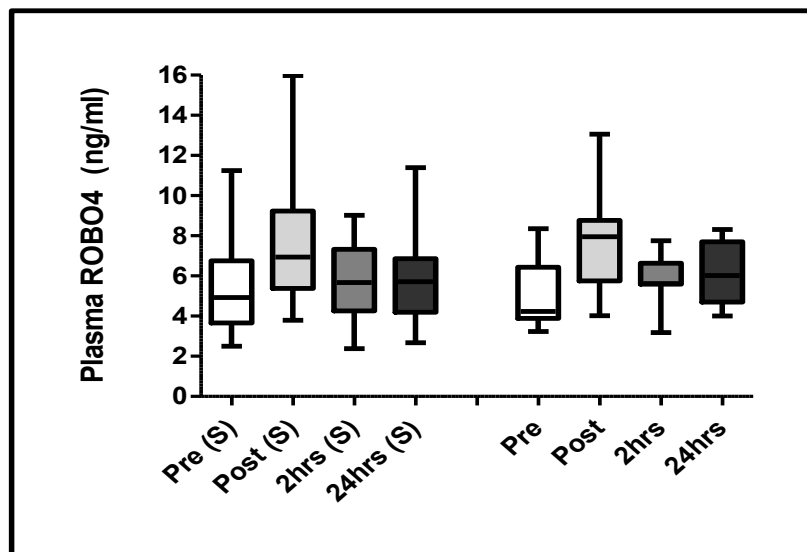


Figure 25. Plasma Robo4 in patients with and without SIRS. Patients with SIRS (S) – Pre (n=37), Post (n=36), 2hrs Post (n=28), 24 hrs Post (n=31); patients without SIRS - Pre (n=11), Post (n=11), 2hrs Post (n=8), 24 hrs Post (n=8). There was no significant difference in any time points between patients with and without SIRS. Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.7. Plasma levels of Robo4 in patients with and without Sepsis are similar

Preoperative levels of Robo4 in patients who developed Sepsis were 5.46 (3.99-7.35) ng/ml, immediately post-operatively the levels were 8.01 (6.38-10.09) ng/ml, 2 hours post-operatively the levels were 5.96 (5.12-7.37) ng/ml and 24 hours post-operatively the levels were 5.79 (4.35-6.70) ng/ml. Preoperative levels of Robo4 in patients who did not develop Sepsis were 4.92 (4.65-7.50) ng/ml, immediately post-operatively the levels were 8.20 (6.75-9.88) ng/ml, 2 hours post-operatively the levels were 6.68 (5.83-7.38) ng/ml and 24 hours post-operatively the levels were 6.87 (5.98-7.21) ng/ml. There was no significant difference in any time points between patients with and without Sepsis.

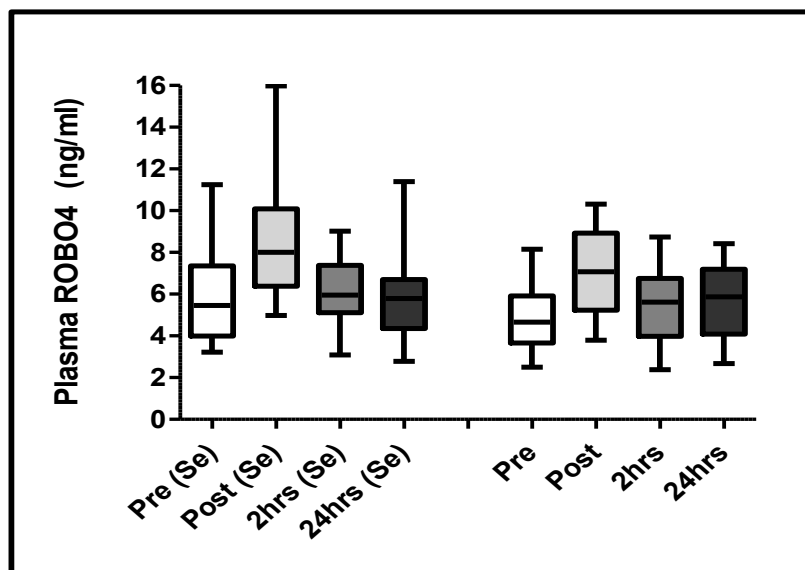


Figure 26. Plasma Robo4 in patients with and without Sepsis. Patients with sepsis (Se) – Pre (n=18), Post (n=18), 2hrs Post (n=13), 24 hrs Post (n=14); patients without sepsis - Pre (n=30), Post (n=28), 2hrs Post (n=24), 24 hrs Post (n=25). There was no significant difference in any time points between patients with and without sepsis. Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.8. Plasma levels of Robo4 in patients with and without Vasoplegia

Preoperative levels of Robo4 in patients who developed Vasoplegia were 7.13 (4.71-8.09) ng/ml, immediately post-operatively the levels were 8.77 (8.07-11.72) ng/ml, 2 hours post-operatively the levels were 7.30 (5.59-7.47) ng/ml and 24 hours post-operatively the levels were 6.26 (4.43-7.20) ng/ml. Preoperative levels of Robo4 in patients who did not developed Vasoplegia were 4.23 (3.63-5.44) ng/ml, immediately post-operatively the levels were 6.42 (5.29-8.03) ng/ml, 2 hours post-operatively the levels were 5.61 (4.02-6.51) ng/ml and 24 hours post-operatively the levels were 5.73 (4.31-6.76)ng/ml.). There was significant ($P<0.05$) difference in preoperative values between patients with and without Vasoplegia. Patients who developed Vasoplegia after the operation had significantly higher preoperative Robo4 levels than patient who did not developed Vasoplegia after the operation.

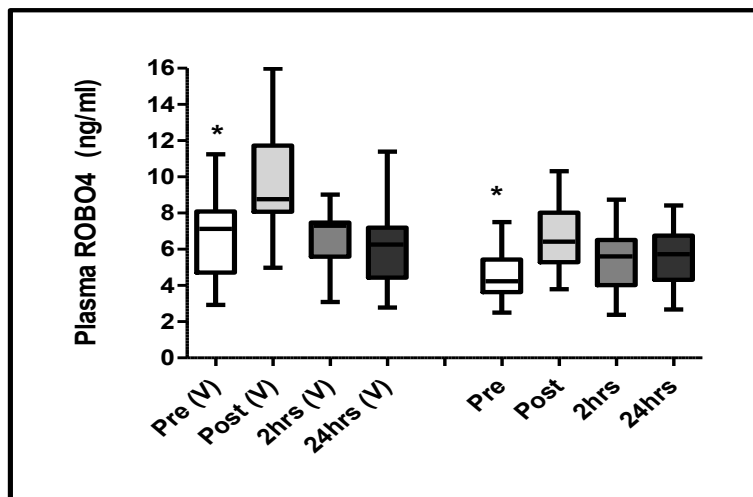


Figure 27. Plasma Robo4 in patients with and without Vasoplegia. Patients with Vasoplegia (V) – Pre (n=16), Post (n=15), 2hrs Post (n=11), 24 hrs Post (n=12); patients without Vasoplegia - Pre (n=32), Post (n=31), 2hrs Post (n=26), 24 hrs Post (n=27). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.9. Plasma levels of sRAGE increase immediately after surgery

Preoperative levels of sRAGE were 1.29 (0.78-2.11) ng/ml, immediately post-operatively the levels were 3.24 (2.18-4.37) ng/ml, 2 hours post-operatively the levels were 1.90 (1.27-2.49) ng/ml and 24 hours post-operatively the levels were 0.69 (0.49-0.91)ng/ml.

The levels of sRAGE have significantly ($p<0.001$) increased immediately after the snCPB and then significantly ($p<0.01$) decreased at 2hrs post-operatively. At 24 hours post-operatively the sRAGE levels were significantly ($p<0.001$) lower compare to all other time post (preoperative, post and 2 hours post-operatively).

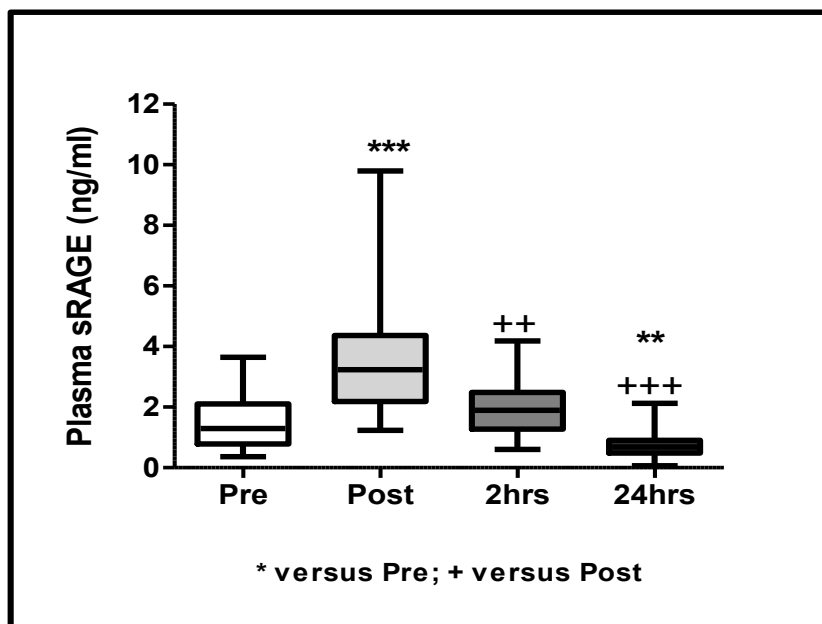


Figure 28. Plasma levels of sRAGE. Levels of sRAGE were measured in all four time-points: pre (n=48), post (n=47), 2hrs post (n=37) and 24hrs post snCPB (n=38). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml; *compared to pre-operative levels ($p < 0.001$ is expressed as three asterisks***, $p < 0.01$ is expressed as two asterisks**); +compared to post-operative levels ($p < 0.001$ is expressed as three asterisks +++, $p < 0.01$ is expressed as two asterisks ++).

3.1.4.10. Plasma levels of sRAGE in patients with and without SIRS are similar

Preoperative levels of RAGE in patients who developed SIRS were 1.31 (0.75-2.12) ng/ml, immediately post-operatively the levels were 3.03 (1.98-4.26) ng/ml, 2 hours post-operatively the levels were 2.13 (1.21-2.52) ng/ml and 24 hours post-operatively the levels were 0.71 (0.46-0.90) ng/ml. Preoperative levels of RAGE in patients who did not developed SIRS were 1.25 (1.07-1.95) ng/ml, immediately post-operatively the levels were 3.44 (2.42-4.37) ng/ml, 2 hours post-operatively the levels were 1.89 (1.54-2.30) ng/ml and 24 hours post-operatively the levels were 0.73 (0.51-1.02) ng/ml. There was no significant difference in any time points between patients with and without SIRS.

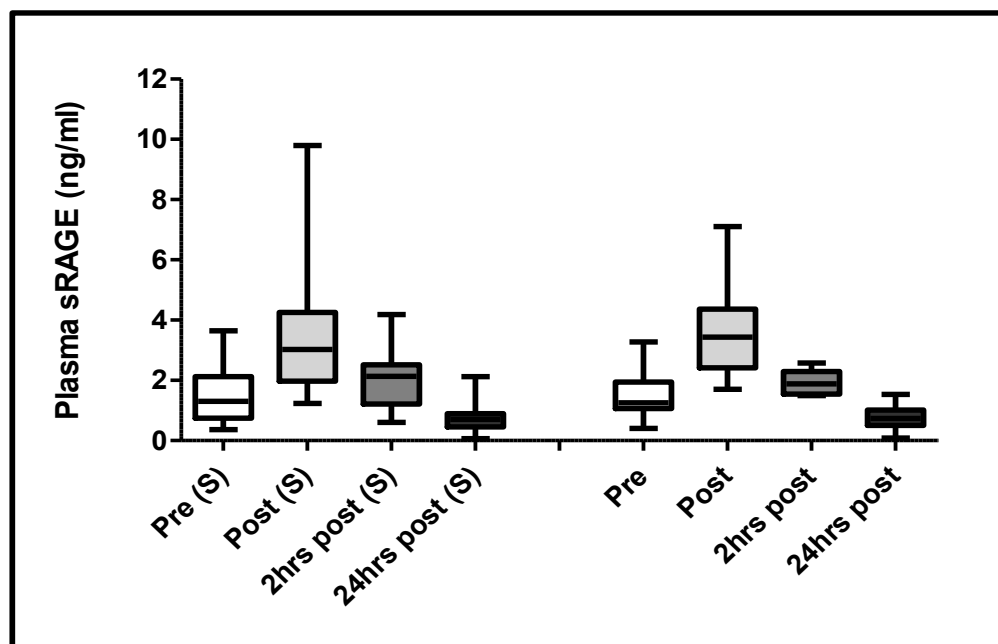


Figure 29. Plasma sRAGE in patients with and without SIRS. Patients with SIRS – Pre (n=37), Post (n=36), 2hrs Post (n=28), 24 hrs Post (n=31); patients without SIRS - Pre (n=11), Post (n=11), 2hrs Post (n=8), 24 hrs Post (n=8). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.11. Plasma levels of sRAGE in patients with and without Sepsis are similar

Preoperative levels of RAGE in patients who developed Sepsis were 2.07 (0.96-2.44) ng/ml, immediately post-operatively the levels were 3.27 (2.08-5.77) ng/ml, 2 hours post-operatively the levels were 1.83 (1.02-2.46) ng/ml and 24 hours post-operatively the levels were 0.72 (0.40-0.96) ng/ml. Preoperative levels of RAGE in patients who did not develop Sepsis were 1.18 (0.75-1.64) ng/ml, immediately post-operatively the levels were 3.19 (2.34-3.81) ng/ml, 2 hours post-operatively the levels were 2.06 (1.50-2.49) ng/ml and 24 hours post-operatively the levels were 0.71 (0.51-0.86) ng/ml. There was no significant difference in any time points between patients with and without Sepsis.

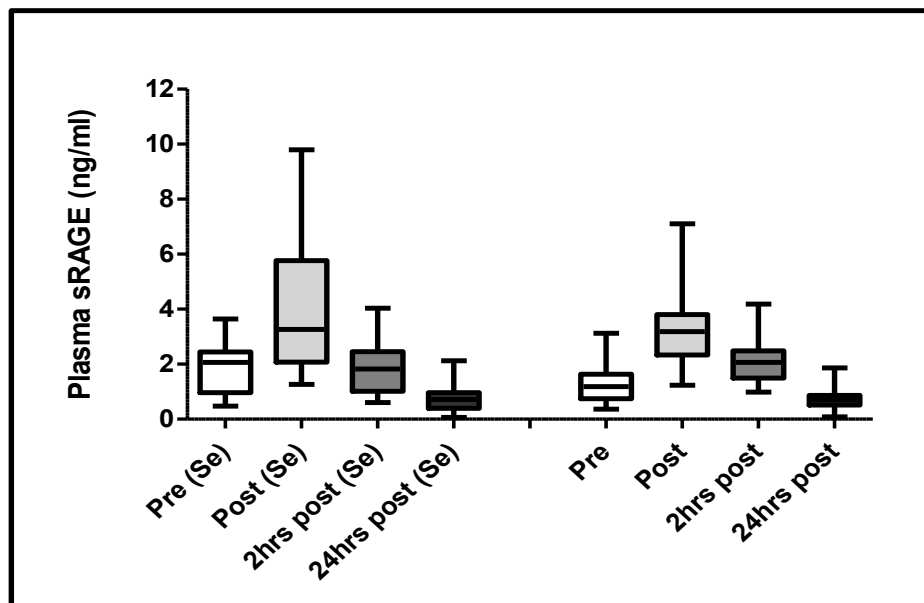


Figure 30. Plasma sRAGE in patients with and without sepsis. Patients with sepsis – Pre (n=18), Post (n=18), 2hrs Post (n=13), 24 hrs Post (n=14); patients without sepsis - Pre (n=30), Post (n=28), 2hrs Post (n=24), 24 hrs Post (n=25). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.12. Plasma levels of sRAGE in patients with and without Vasoplegia are similar

Preoperative levels of RAGE in patients who developed Vasoplegia were 1.86 (0.76-2.91) ng/ml, immediately post-operatively the levels were 3.29 (2.46-5.62) ng/ml, 2 hours post-operatively the levels were 1.83 (1.20-2.56) ng/ml and 24 hours post-operatively the levels were 0.81 (0.44-0.99) ng/ml. Preoperative levels of RAGE in patients who did not developed Vasoplegia were 1.24 (0.78-1.85) ng/ml, immediately post-operatively the levels were 3.24 (2.18-3.74) ng/ml, 2 hours post-operatively the levels were 2.06 (1.45-2.48) ng/ml and 24 hours post-operatively the levels were 0.67 (0.50-0.90)ng/ml.). There was no significant difference in any time points between patients with and without Vasoplegia.

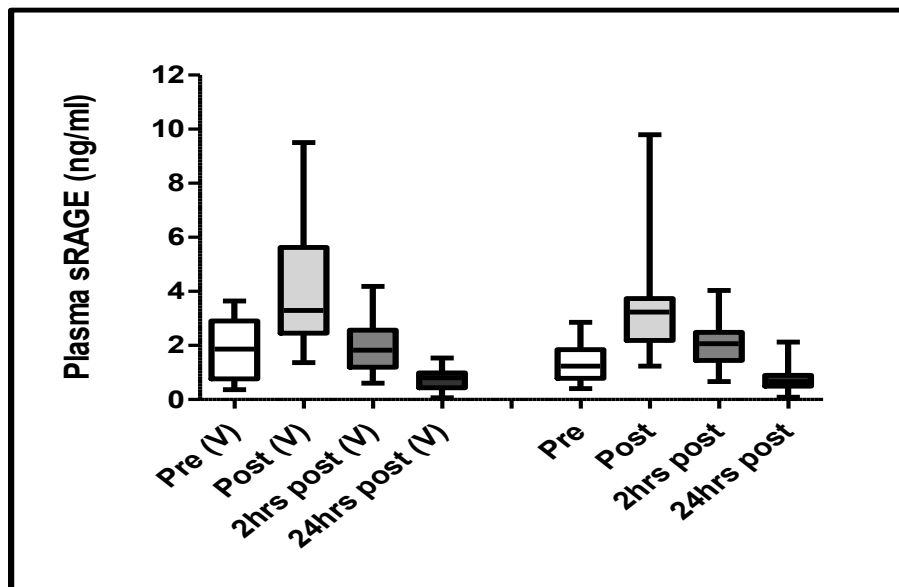


Figure 31. Plasma sRAGE in patients with and without Vasoplegia. Patients with Vasoplegia – Pre (n=16), Post (n=15), 2hrs Post (n=11), 24 hrs Post (n=12); patients without Vasoplegia - Pre (n=32), Post (n=31), 2hrs Post (n=26), 24 hrs Post (n=27). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml.

3.1.4.13. Plasma levels of S100A8/9 increase after cardiac surgery

Preoperative levels of S100A8/A9 were 1.20 (0.60-2.03) µg/ml, immediately post-operatively the levels were 4.98 (3.80-7.80) µg/ml, 2 hours post-operatively the levels were 5.18 (2.85-7.79) µg/ml and 24 hours post-operatively the levels were 2.78 (1.70-4.40) µg/ml.

Levels of S100A8/9 have significantly ($p < 0.001$) risen immediately after the snCPB and stayed at similar level at 2hrs post-operatively time-point. The levels dropped significantly 24hrs post snCPB compare to immediately post-operatively levels, however they were still significantly higher than preoperative levels.

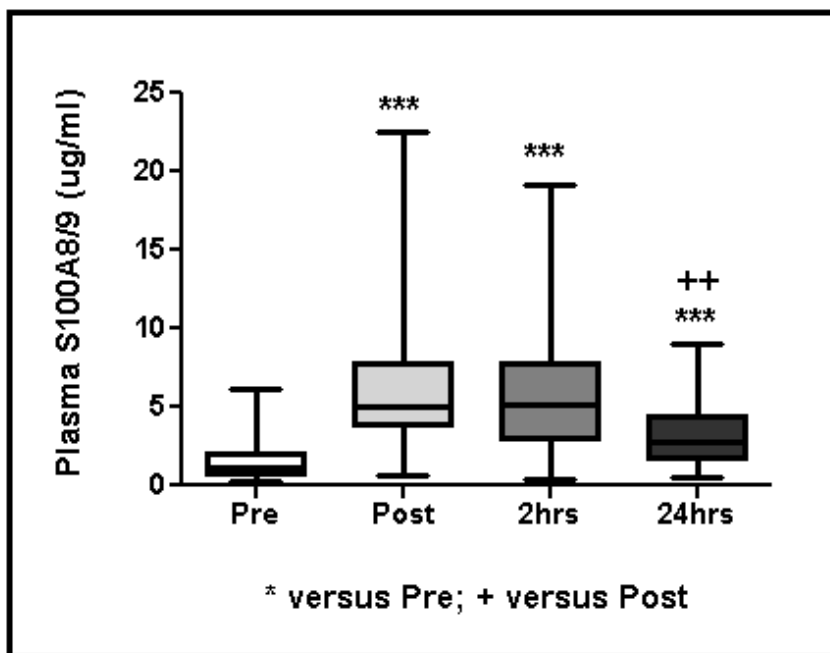


Figure 32. Plasma levels of S100A8/9. Levels of S100A8/A9 were measured in all four time-points: pre (n=48), post (n=47), 2hrs post (n=38) and 24hrs post snCPB (n=37). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml; $p < 0.001$ is expressed as three asterisks; $p < 0.01$ is expressed as two asterisks.

3.1.4.14. Plasma levels of S100A8/9 in patients with and without SIRS are similar

Preoperative levels of S100A8/A9 in patients who developed SIRS were 1.29 (0.67-2.02) $\mu\text{g/ml}$, immediately post-operatively the levels were 4.46 (3.22-7.91) $\mu\text{g/ml}$, 2 hours post-operatively the levels were 5.37 (3.14-10.44) $\mu\text{g/ml}$ and 24 hours post-operatively the levels were 2.82 (1.62-4.94) $\mu\text{g/ml}$. Preoperative levels of S100A8/A9 in patients who did not developed SIRS were 0.62 (0.54-2.97) $\mu\text{g/ml}$, immediately post-operatively the levels were 6.87 (4.81-10.68) $\mu\text{g/ml}$, 2 hours post-operatively the levels were 5.87 (2.90-7.13) $\mu\text{g/ml}$ and 24 hours post-operatively the levels were 3.53 (2.07-4.40) $\mu\text{g/ml}$. There was no significant difference in any time points between patients with and without Sepsis.

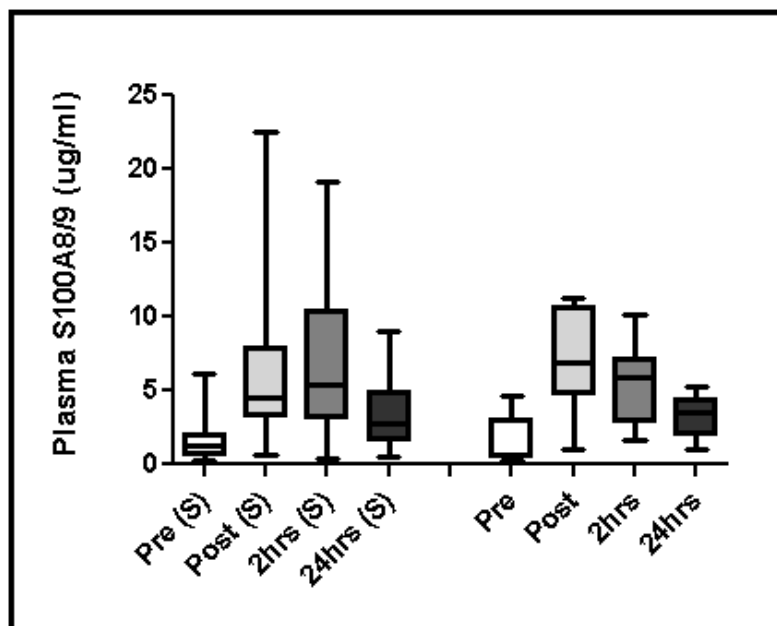


Figure 33. Plasma S100A8/9 in patients with and without SIRS. Patients with SIRS – Pre (n=37), Post (n=36), 2hrs Post (n=28), 24 hrs Post (n=31); patients without SIRS - Pre (n=11), Post (n=11), 2hrs Post (n=8), 24 hrs Post (n=8). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) $\mu\text{g/ml}$.

3.1.4.15. Plasma levels of S100A8/9 in patients with and without Sepsis are similar

Preoperative levels of S100A8/A9 in patients who developed Sepsis were 0.95 (0.33-1.57) $\mu\text{g/ml}$, immediately post-operatively the levels were 5.26 (3.21-12.91) $\mu\text{g/ml}$, 2 hours post-operatively the levels were 4.98 (2.53-12.82) $\mu\text{g/ml}$ and 24 hours post-operatively the levels were 2.43 (1.41-3.35) $\mu\text{g/ml}$. Preoperative levels of S100A8/A9 in patients who did not developed Sepsis were 1.38 (0.63-2.24) $\mu\text{g/ml}$, immediately post-operatively the levels were 5.01 (3.88-7.22) $\mu\text{g/ml}$, 2 hours post-operatively the levels were 5.97 (3.65-7.63) $\mu\text{g/ml}$ and 24 hours post-operatively the levels were 3.75 (2.53-4.80) $\mu\text{g/ml}$. There was no significant difference in any time points between patients with and without Sepsis.

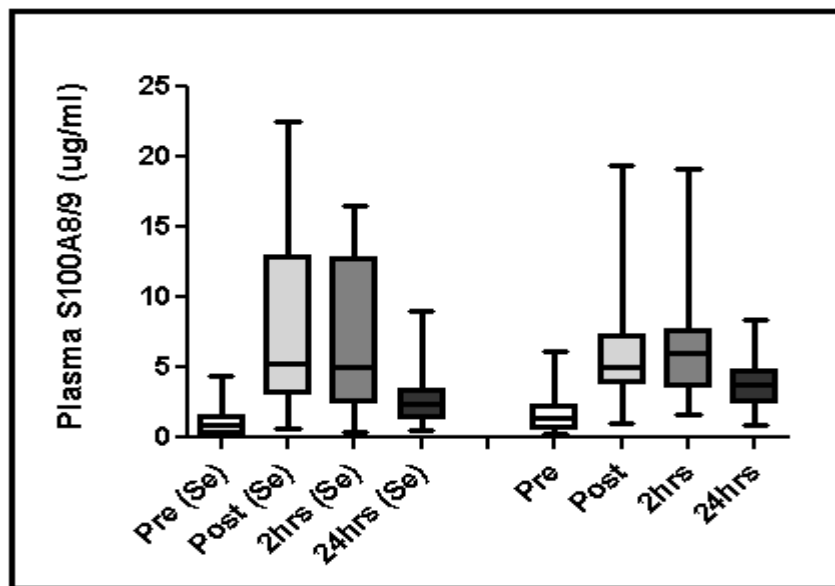


Figure 34. Plasma S100A8/9 in patients with and without sepsis. Patients with sepsis – Pre (n=18), Post (n=18), 2hrs Post (n=13), 24 hrs Post (n=14); patients without sepsis - Pre (n=30), Post (n=28), 2hrs Post (n=24), 24 hrs Post (n=25). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) $\mu\text{g/ml}$.

3.1.4.16. Plasma levels of S100A8/9 in patients with and without VS are similar

Preoperative levels of S100A8/A9 in patients who developed Vasoplegia were 1.26 (0.87-1.88) ng/ml, immediately post-operatively the levels were 7.9 (4.46-14.43) ng/ml, 2 hours post-operatively the levels were 7.76 (3.22-16.07) ng/ml and 24 hours post-operatively the levels were 4.88 (1.54-6.13) ng/ml. Preoperative levels of S100A8/A9 in patients who did not developed Vasoplegia were 1.19 (0.57-2.08) ng/ml, immediately post-operatively the levels were 4.24 (3.17-7.20) ng/ml, 2 hours post-operatively the levels were 5.18 (2.66-7.21) ng/ml and 24 hours post-operatively the levels were 2.88 (2.34-4.31) ng/ml. There was no significant difference in any time points between patients with and without Vasoplegia.

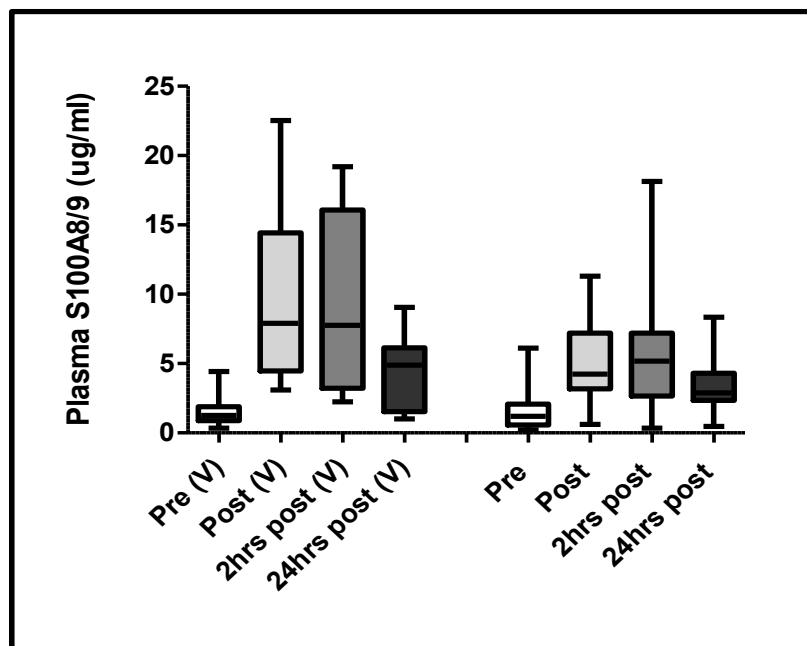


Figure 35. Plasma S100A8/9 in patients with and without Vasoplegia. Patients with Vasoplegia – Pre (n=16), Post (n=15), 2hrs Post (n=11), 24 hrs Post (n=12); patients without Vasoplegia - Pre (n=32), Post (n=31), 2hrs Post (n=26), 24 hrs Post (n=27). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) $\mu\text{g/ml}$.

3.1.4.17. Association between Robo4 and RAGE and S100A8/A9

The association between Robo4 (all time points) with plasma levels of sRAGE or S100A8/A9 suggested a weak, but highly significant ($p < 0.05$), association between these pathways in general. However, no association between Robo4 and sRAGE or S100A8/A9, immediately post-bypass, were found - suggesting that activation of RAGE is not specifically linked to shedding of Robo4 in this context.

	Time-point	Spearman r	Correlation	P value	Total
Robo4 vs RAGE	Pre-CPB	0.06361	No	0.6675	n=48
Robo4 vs RAGE	Post-CPB	0.0644	No	0.6638	n=48
Robo4 vs RAGE	2 hrs post-CPB	0.2563	Yes -weak	0.1257	n=37
Robo4 vs RAGE	24hrs post-CPB	0.01154	No	0.9444	n=39
Robo4 vs RAGE	All	0.2258	Yes – weak	0.0031	n=170

Table 4. Correlation between Robo4 and RAGE at all time-points.

	Time-point	Spearman r	Correlation	P value	Total
Robo4 vs S100A8/A9	Pre-CPB	0.1321	No	0.3707	n= 48
Robo4 vs S100A8/A9	Post-CPB	0.0690	No	0.6763	n=47
Robo4 vs S100A8/A9	2 hrs post-CPB	0.07183	No	0.6727	n=37
Robo4 vs S100A8/A9	24hrs post-CPB	0.04534	No	0.7840	n=39
Robo4 vs S100A8/A9	All	0.2671	Yes – weak	0.0004	n=170

Table 5. Correlation between Robo4 and S100A8/A9 at all time-points.

3.1.4.18. Association between Robo4 and Syndecan-1

The association between Robo4 (all time points) with plasma levels of Syndecan-1 (all time points) suggested a weak, but highly significant ($p < 0.05$), association between these two components of endothelial barrier.

	Time-point	Spearman r	Correlation	P value	Total
Robo4 vs Syndecan-1	Pre-CPB	0.1503	No	0.3481	n=41
Robo4 vs Syndecan-1	Post-CPB	0.0741	No	0.654	n=39
Robo4 vs Syndecan-1	2 hrs post-CPB	0.2754	Yes - weak	0.1337	n=31
Robo4 vs Syndecan-1	24hrs post-CPB	0.005013	No	0.9779	n=33
Robo4 vs Syndecan-1	All	0.1910	Yes – weak	0.0214	n=145

Table 6. Correlation between Robo4 and Syndecan-1 at all the time-points.

3.1.4.19. Association between Robo4 and ICU and hospital length of stay

There is a significant ($p < 0.05$) moderate association between Robo4 levels at post-operatively and hospital LOS; and between Robo4 levels at post-operatively and ICU LOS. However there is no difference when grouped as with and without SIRS; and with and without sepsis.

	Time-point	Spearman r	Correlation	P value	Total
Robo4 vs hospital stay	Post-CPB	0.3442	Yes - moderate	0.0191	n=46
Robo4 vs ICU stay	Post-CPB	0.3752	Yes - moderate	0.0241	n=36

Table 7. Correlation between Robo4 at post-operatively time-point and hospital LOS and ICU LOS.

3.1.5. Discussion

3.1.5.1. Endothelial damage biomarkers Ang2, vWF and Syndecan-1

Angiopoietin-2 is a secreted glycoprotein and endothelium specific growth factor that is used as a biomarker of endothelial damage (Siner et al, 2009). In this study, Ang2 levels at immediately post-operatively and at 2hrs post-operatively were similar to preoperative levels and increased significantly 24hrs post-operatively. Similar results were recorded by Clajus and colleagues in their study of 25 adult patients undergoing heart surgery with CPB. They suggested that Ang2 is a likely mediator of endothelial barrier dysfunction in patients after cardiac surgery with CPB because the binding of Ang-2 to Tie2 receptor makes the endothelium susceptible to cytokines causing inflammation and subsequently SIRS (Clajus et al, 2012). Another well-characterised biomarker of endothelial damage is von Willebrand Factor. In this study the levels of vWF started increasing immediately post-operatively, with even higher increase at 2 hrs post-operatively and reaching significant increase at 24 hrs post-operatively. These findings are similar to published studies on patients undergoing cardiac surgery with CPB (Tsang et al, 1998; Panagiotopoulos et al, 2010). Syndecan-1 is a main constituent of glycocalyx that covers the luminal side of endothelium. When glycocalyx gets damaged, Syndecan-1 is shed into blood circulation. Patients who underwent ischemia and reperfusion during cardiac surgery and suffered endothelial damage as a consequence, showed higher levels of Syndecan-1 in plasma (Rehm et al, 2007). Plasma levels of Syndecan-1 measured in this study have significantly increased immediately post-operatively and stayed significantly raised at 2 hours post-operatively. At 24hrs post-

operatively the levels decreased, but were still significantly higher compared to preoperative levels. These results confirm that Syndecan-1 is shed after a trauma caused by cardiac surgery with CPB. Johansson and colleagues found that high levels of syndecan-1 in patients who suffered a trauma were associated with increased mortality (Johansson et al, 2011). An association with mortality could not have been assessed in this study, as only two patients (4%) died after the cardiac surgery.

3.1.5.2. Robo4 in SIRS, Sepsis and Vasoplegia

This study investigated the hypothesis that plasma levels of Robo4 could be a clinically useful biomarker of an endothelial damage after cardiac surgery. The levels of Robo4 increased significantly immediately after operation - suggesting endothelial damage - and returned to the baseline levels 24 hours after operation. It is of note that compared with two well-recognised markers of endothelial dysfunction, Ang2 and vWF, the kinetics of Robo4 release were more rapid. Indeed, as we and others have shown, both the initial increase and subsequent resolution – returning to basal levels by 24h - occur more rapidly (Burke-Gaffney et al., 2014). It could be that the rapidity of Robo4 appearance in plasma points to rapid cleavage off the protein from the cell membrane, followed by quick clearance. A previous study detected Robo4 in serum samples from patients with advanced lung cancer (Coleman et al., 2010), but did not investigate mechanisms responsible. Robo4 would be a better biomarker of endothelial damage than Ang-2 because its levels increase significantly immediately after surgery, while Ang-2 levels take 24 hours to increase significantly when compared to pre-surgery levels. It

is not known yet what function the release of Robo4 has. It is possible that Robo4, being on the outside of endothelial cell, embedded in its membrane, gets cleaved off the membrane together with glycocalyx components, such as syndecan-1, and is therefore the first measurable marker of endothelial damage. The reason for Robo4 appearing in plasma sooner than Ang2 and vWF might be due to the fact that Robo4 is embedded in EC membrane and it may be shed/cleaved from the membrane while VWF and Ang2 are released from cells and this probably takes longer. Our findings suggest that Robo4 might be cleaved or secreted by endothelial cells exposed to inflammation and release into the blood stream. Rapid removal of Robo4 from the cell surface could possibly act as a first, fast response to surgical trauma, i.e. to release the protective check on barrier function. Alternatively, it is simply a bystander effect, and Robo4 is a marker of endothelial damage occurring during cardiac surgery requiring CPB. There is a significant moderate association between Robo4 levels at post-operatively and ICU length of stay. However there is no difference when grouped as with and without SIRS; and with and without sepsis.

Robo4 could also be used as a pre-operative indicator of patients in risk of developing post-CPB vasoplegia. Patients who developed vasoplegia after the operation had significantly higher pre-operative Robo4 levels than patients who did not develop vasoplegia after the operation. However, it has not been possible, in this study, to assess whether the higher pre-operative levels of Robo4 were or were not linked to some pre-existing conditions, as we did not have available information on patients' co-morbidities. It has been shown that Robo4 increases in some chronic inflammatory conditions, such

as arthritis, or in pathological angiogenesis during tumour development in cancer (Zhao et al, 2016), or in patients with diabetes mellitus. It is possible that cardiac surgery patients with underlying conditions might have higher levels of Robo4 before the operation and perhaps it was the combination of pre-existing condition together with the cardiac surgery that lead to vasoplegia. Regardless of the reason for the high pre-operative levels of Robo4 was, Robo4 shown to be a marker of post-surgery development of vasoplegia.

3.1.5.3 RAGE and S100A8/9 in SIRS, Sepsis and Vasoplegia

RAGE is an immunoglobulin-like cell surface multi-ligand pattern-recognition receptor that binds a wide range of structurally diverse ligands and one of them is S100A8/A9 (Sparvero et al., 2009). RAGE is present at low levels in vascular tissue and increases during inflammation (Chuah et al., 2013). In this study, plasma levels of RAGE have significantly increased immediately post-operatively and then significantly decreased at 2hrs post-operatively. At 24 hours post-operatively the RAGE levels were at similar levels as baseline (preoperative). Levels of S100A8/9 – RAGE ligand - have significantly risen immediately after the snCPB and stayed at similar levels at 2hrs post-operatively. The levels at 24hrs post-operatively dropped significantly compare to immediately post-operatively levels, however they were still significantly higher than preoperative levels. These results are similar to those published by Creagh-Brown in 2013. Whilst levels of sRAGE and S100A8/A9 increased post-surgery, there were no differences between patients with or without SIRS, sepsis or vasoplegia. Jabaudon et al, also identify RAGE as

a marker of an organ damage – acute lung injury – and in their study the levels of RAGE also were not different in patients with and without sepsis (Jabaudon et al, 2011). Matsumoto with colleagues showed, in their study of 24 patients with sepsis, that serum levels of RAGE increased with the severity of sepsis. Their results suggest that circulating RAGE reflect RAGE signalling pathway activity, which induce the excessive inflammatory response involved in endothelial injury in sepsis. During inflammation and sepsis PAMPs bind to RAGE and induce synthesis of IL-6, VCAM-1 and PAI-1 through nuclear translocation of NF- κ B. DAMPs bind to RAGE and aggravate the inflammatory responses through augmentation of RAGE signalling. Matsumoto et al propose that RAGE may be useful as a biomarker for sepsis (Matsumoto et al, 2015). In this thesis, we wanted to explore a possible link between RAGE and Robo4 given that both were endothelial receptors and both risen immediately after the surgery. Unfortunately, there was not enough RAGE expression on endothelial cells that were being used in this study to be able to investigate interactions between RAGE activation and Robo4 expression and function.

For many time points the confidence intervals are quite small, but in the post-operative samples across a range of molecules measured (Robo4, sRAGE and S100A8/A9), the upper confidence interval is high

3.1.6. Limitations

The blood sample collection has not been without its difficulties. Almost all pre-operative, immediately post-operative and 24 hours post-operative samples (n=47-n=48) were collected. However, the time-point at 2 hours after operation proved to be a difficult one. Only 37 blood samples were collected. There were two main reasons, firstly, it was a time-point at which patients were already alert but feeling uncomfortable after the heart surgery and declined to give blood sample. This was within their right as they could withdraw their consent at any time during the study. The second, less common reason was when a clinician would not sign consent due to the medical condition of the patient. Before every sample collection a clinician's signature was required.

It might have been beneficial to the study, to measure Robo4 and other markers of endothelial damage in patients who underwent cardiac surgery without the need for CPB. Results as such would make it clearer whether the endothelial damage was caused by the cardiopulmonary bypass or the surgical procedure *per se*.

It might have also been useful to collect data on co-morbidities, such as diabetes mellitus (DM), as this would provide more information on elevated Robo4 levels in pre-operative patients. It is known that some conditions, such as the above mention DM, increase levels of Robo4.

The final limitation of this study was that at the time of this project there was not available reliable ELISA kit to measure Slit2 in human plasma. The ELISA kit had a narrow range of detection, so some time-points of our plasma samples had to be diluted to fit

the standard curve, while other time-points were too low to fit the curve even undiluted. The ELISA could not detect the very low levels of Slit2 at preoperative and 24h post-operatively. For the immediately post-operatively time-point the plasma needed to be diluted, but different dilutions produced different results. There was only a small number of samples in which Slit2 levels could be measured. These results are in the Appendix section of this thesis. Slit2 is a Robo4 ligand regulate together the endothelial function and dysfunction. To be able to measure levels of Slit2 in patients who underwent trauma caused by cardiac surgery with snCPB would have given more insight into how these two proteins work together in vivo.

3.1.7. Summary

Robo4 is a novel molecule involved in regulating endothelial function. This study presents results of increased levels of Robo4 in plasma of patients undergoing cardiac surgery with CPB. These patients are in increased risk of endothelial injury which can potentially lead to organ damage. The increased plasma levels of Robo4 immediately post-operatively suggest that Robo4 could be a valuable early marker of endothelial damage in patients undergoing a cardiac surgery with CPB. Robo4 could also be used as an indicator of patients in risk of developing post-CPB vasoplegia. Patients who developed vasoplegia after the operation had significantly higher preoperative Robo4 levels than patient who did not developed vasoplegia after the operation.

In conclusion, Robo4 is a promising biomarker for vasoplegia and prolonged hospital and ICU stay following snCPB, and these results suggest that protection of endothelium could be a worthy therapeutic target in these patients. This is the subject of investigation in the second, in vitro, section of this thesis.

3.2. Section 2. Expression and function of Robo4 in vitro

3.2.1. Context

At the start of this thesis the literature suggested that Robo4, originally described as a molecule involved in axon guidance, could be detected in the supernatant of cultured human dermal microvascular endothelial cells (HDMEC) and HUVEC (Huminięcki et al., 2002). A subsequent study showed a protective effect of Slit2 on murine lung endothelial cells barrier function impaired by VEGF-165. The results showed that Slit2-Robo4 binding suppressed the hyperpermeability caused by VEGF-165 (Jones et al, 2008). Other in vitro experiments by the same group showed that Slit2 reversed LPS, TNF- α and IL-1 induced permeability increase in mouse lung microvascular endothelial cells (London et al, 2010). Whilst the simplest explanation for these findings was that Slit2 activates Robo4, it had been shown previously that Slit2 did not directly bind Robo4 and the involvement of other Robo receptors - in particular Robo1 - was postulated (Sheldon et al, 2009).

Having established in the first results section of this thesis that plasma levels of Robo4 were associated with complications related to increased endothelial permeability i.e. vasoplegic shock, the overall aim of this in vitro section was to investigate the potential of Robo4 as a therapeutic target for reducing endothelial barrier dysfunction in endothelial cell culture models. To explore the potential of Robo4 as a possible future therapeutic target, it was considered necessary to establish the profile of Robo4 expression associated with cultured endothelial cells; changes in response to

inflammatory stimuli; and the contribution of Robo4 to preventing endothelial barrier dysfunction, in vitro.

3.2.2. Specific Hypothesis, Aims and Objectives

The hypothesis tested in the second results section was that endothelial cells express Robo4 and that Robo4 prevents endothelial barrier dysfunction.

The specific aims were therefore to:

1. Determine expression and release of Robo4 associate with HUVEC; and how the expression profile compared to other endothelial cells (HPAEC and HCAEC, cells readily available in the lab); and following TNF- α exposure. HUVEC are endothelial cells that are easily and inexpensively cultured and were therefore used in many endothelial barrier studies. However, since the literature suggested considerable heterogeneity between endothelial cells, differences in Robo4 expression and function between endothelial cells are predicted.
2. Determine whether there is sufficient RAGE expressed on HUVEC so that RAGE ligands (e.g. S100 proteins) could be used as inflammatory stimuli to alter endothelial Robo4 expression and function. An original intention of this thesis was to explore the link between RAGE and Robo4 expression and function.
3. Establish an in vitro model of vascular barrier function using cultured endothelial cells with electrical impedance as the readout of barrier function. An original

intention of this thesis was to use leakage of labelled macromolecules across endothelial monolayers as an assay of barrier dysfunction but electrical impedance –measured in real-time-proved to be a more superior model.

4. Investigate the consequences on endothelial barrier function of targeting Robo4 pharmacologically with Slit2 ligand; and genetically, using Robo4 'knockdown' techniques

The specific objectives to address these aims were to:

- Measure extracellular and intracellular expression of Robo4 on HUVEC, HPAEC and HCAEC, untreated and treated with TNF- α .
- Measure extracellular and intracellular expression of Robo1 on HUVEC and HPAEC, untreated and treated with TNF- α .
- Measure expression of extracellular and intracellular expression of Slit2 in HUVEC, untreated and treated with TNF- α .
- Measure extracellular and/or intracellular expression of RAGE in HUVEC, untreated and treated with TNF- α .
- Measure extracellular and/or intracellular expression of TLR-2, 4, 9 in HUVEC.
- Measure release of soluble Robo4 into culture media from HUVEC activated with inflammatory cytokines (TNF α , IL-6, IL-1 β) and Slit2.

- Measure release of soluble Robo4 into culture media from PAEC activated with inflammatory cytokines (TNF α , IL-6, IL-1 β) and Slit2.
- Assess bioactivity of Slit2 on release of GM-CSF from HUVEC (as Slit2 previously shown to prevent GM-CSF release – Zhang et al, 2007).
- Establish appropriate seeding densities for HUVEC and PAEC for endothelial barrier integrity assay.
- Establish the concentration-dependent effects of TNF- α . and Slit2 on HUVEC barrier integrity.
- Establish effects of TNF- α . + Slit2 on HUVEC and HPAEC barrier integrity.
- Show by FACS analysis knockdown of Robo4 in HUVEC (and PAEC) using siRNA to Robo4
- Investigate whether Robo siRNA reverses the effects of Slit2 on endothelial barrier dysfunction.

3.2.3. Methods

(a) Pooled human umbilical vein endothelial cells (HUVEC) were obtained from Lonza GmbH (Köln, Germany) and cultured in Lonza EGM2 complete medium containing 2% foetal bovine serum (FBS), 0.4% Human Fibroblast Growth Factor-B (hFGF), 0.1% Human Epidermal Growth Factor (hEGF), 0.1% R3 Insulin-like Growth Factor-1 (R3 IGF-1), 0.1% Vascular Endothelial Growth Factor (VEGF), 0.1% Ascorbic Acid (AA), 0.1% Gentamicin Sulfate Amphotericin-B, 0.1% Heparin and 0.04% Hydrocortisone (HC) according to the supplier's instructions. Cells were incubated at 37°C and 5% CO₂.

(b) Pooled, (c) single-donor and (d) VEGF pre-screened HUVEC from PromoCell GmbH (Heidelberg, Germany) were cultured in PromoCell EGM2 complete medium containing 2% FBS and 0.1% of each: hFGF, hEGF, R3 IGF-1, VEGF, AA, HC and Heparin. Cells were incubated at 37°C and 5% CO₂. Cells specification for four lots of HUVEC from Lonza and PromoCell are shown in Table 8. HUVEC from both suppliers were sub-cultured and used in experiments up to passage 5.

	Gender	Race	Doubling Time	Lot number
Lonza pooled	M/F mixed	B, C, C,	18h	189151
PromoCell pooled	M/F/F	C, C, C	15.5h	3092301
PromoCell single	M	C	14.9h	3111301
PromoCell pre-screened	M	C	15.4	2012601.1

Table 8. Endothelial cells Specifications. Pooled HUVEC from Lonza and pooled, single and VEGF pre-screened HUVEC from PromoCell. M=male, F=female, B=black, C= Caucasian.

(1) Flow cytometry was used to measure expression of all the receptors and ligands investigated in this study. The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit was used to determine the viability of the cells in every single sample in all flow cytometry experiments. The gates were then set in a way that all dead cells were excluded from the final results.

(2) An iCELLigence and xCELLigence real-time cell analysis instruments were used to measure trans-endothelial electrical impedance (TEEI) on endothelial cells treated with inflammatory cytokine TNF- α and Slit2, a ligand for Robo4.

(3) ELISA was used to measure the release of GM-CSF. The detail descriptions of all these methods are in the main method section of this thesis. The detection range of ELISA used for cell culture experiments are in Table 9.

Biomarker	Detection Range
GM-CSF	15.6 – 1000 pg/ml
Robo4	62.5 – 4000 pg/ml

Table 9. The detection range of ELISA (cell culture). The ELISA kits detection range of markers measured in cell culture supernatants.

3.2.4. Results

3.2.4.1 Expression of RAGE and TLRs

3.2.4.1.1 Expression of RAGE, TLR2, TLR4 and TLR9 in HUVEC

When four batches of HUVEC were compared (n=3 independent experiments for each cell lot), there was not significant difference in the number of cell expressing RAGE between the lots: 4.5% $\pm$ 1.46, Lonza pooled cells; 3.2% $\pm$ 1.65, PromoCell pooled; 4.3% $\pm$ 0.99, PromoCell single donor; and 3.9% $\pm$ 1.71, PromoCell pre-screened cells. Similarly, internal RAGE expression was not significantly different for each cell lot: 89.7% $\pm$ 13.47, Lonza pooled cells; 98.6% $\pm$ 1.11, PromoCell pooled; 98.7% $\pm$ 1.05, PromoCell single donor; and 99.3% $\pm$ 0.55, PromoCell, pre-screened. However, there was a significant difference ($p < 0.05$) in the levels of intensity of expression of RAGE (as determined by median fluorescent intensity [MFI]) in positive cells between Lonza HUVEC and PromoCell HUVEC, both intracellularly and extracellularly. The intracellular MFI of RAGE in positive Lonza pooled was 4872.3 ($\pm$ 591.35) compared to 1476.3 ($\pm$ 226.8) PromoCell pooled; 1557.7 ($\pm$ 353.88) PromoCell single and 1761.3 ($\pm$ 211.06) PromoCell pre-screened cells. The extracellular MFI of RAGE on positive Lonza pooled HUVEC was 1661.7 ($\pm$ 578.54) compared to 381.7 ($\pm$ 12.10) PromoCell pooled, 376.3 ($\pm$ 6.11) PromoCell single and 381.3 ($\pm$ 5.86) PromoCell pre-screened cells.

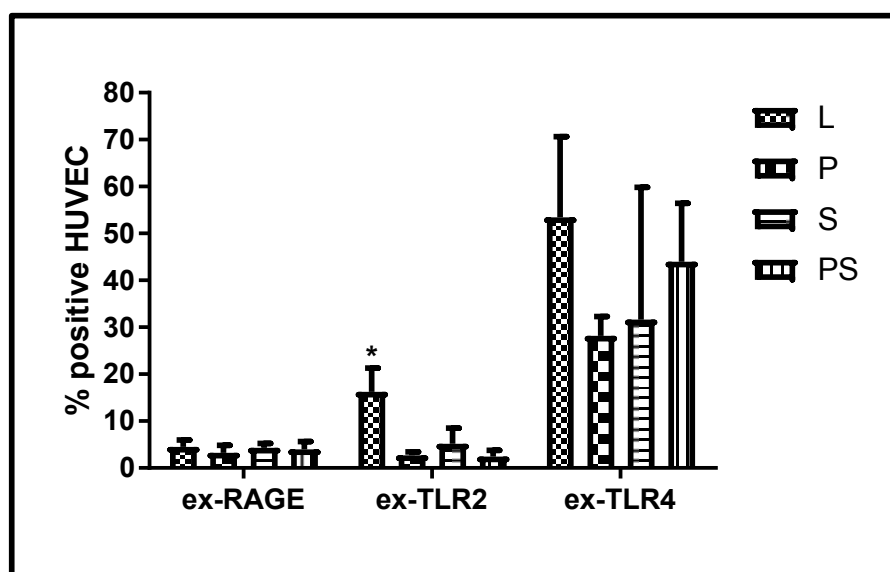


Figure 36. External expression of RAGE, TLR2 and TLR4. Measured in four types of HUVEC: pooled from Lonza (L); pooled (P) single (S), and pre-screened (PS) from PromoCell (n=3; mean with SD). Graph shows percentage of cells that tested positive for extracellular RAGE, TLR2 and TLR4. Data were analysed by non-parametric Kruskal-Wallis and are expressed as mean $\pm$ SD. (p <0.05 is expressed as one asterisk).

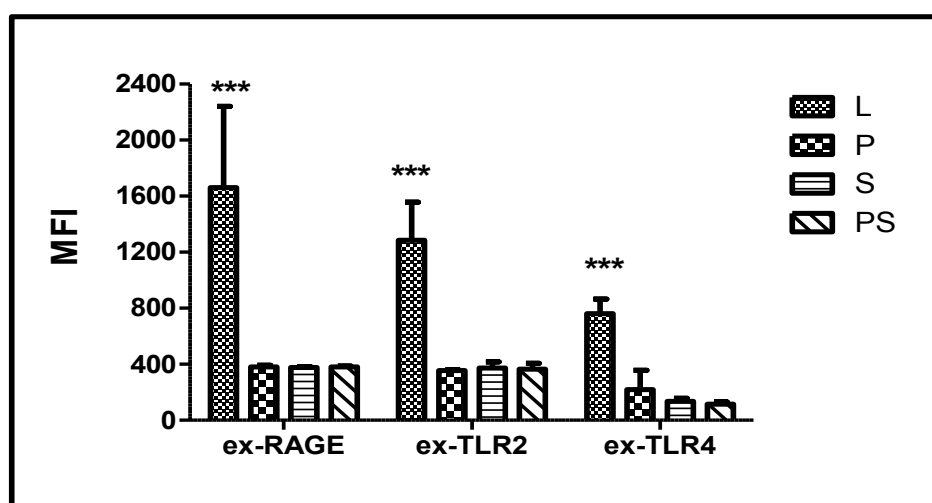


Figure 37. Median Fluorescence Intensity of external RAGE, TLR2 and TLR4. Measured in four types of HUVEC: pooled from Lonza; pooled, single and VEGF pre-screened from PromoCell (n=3; mean $\pm$ SD). (p <0.001 is expressed as three asterisks)

The number of cells staining positive for internal TLR9 was similar in all four batches (97.1%±3.32, Lonza pooled; 100% in PromoCell pooled; 100% in PromoCell single; 99.8%±0.26 in PromoCell pre-screened cells). By contrast, the numbers of cells expressing TLR2 externally in Lonza pooled HUVEC was significantly higher ($p<0.05$) in comparison to the other three cell types (pooled, single and pre-screened cells) from PromoCell. The number of cells expressing TLR2 in Lonza pooled was 16.2%±5.09 vs. PromoCell pooled 2.7%±0.72; in PromoCell single, 5.2%±3.33; and in PromoCell pre-screened 2.4%±1.39. There was no significant difference in the number of cells expressing TLR4 in all four cell lots: 53.5%±17.14, in Lonza pooled; 19.0%±16.12, in PromoCell pooled; 31.6%±28.23 in PromoCell, single; and 43.9%±12.50, in PromoCell pre-screened see.

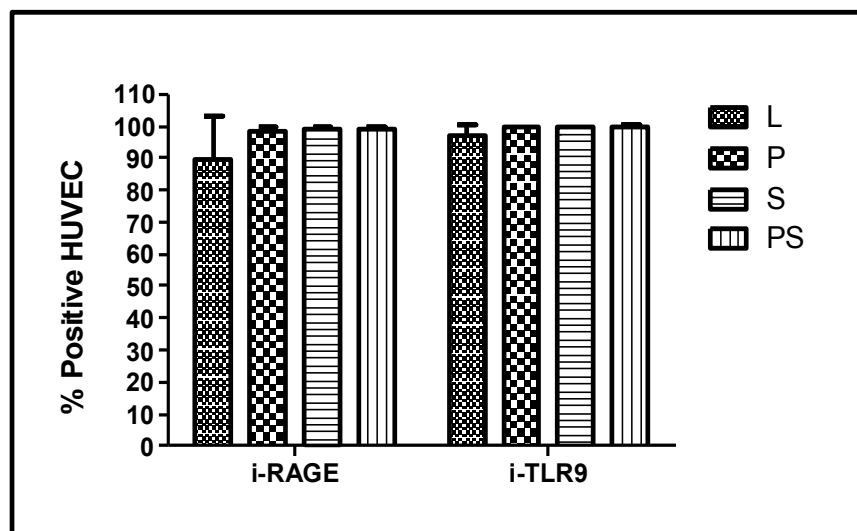


Figure 38. Expression of intracellular RAGE (i-RAGE) and intracellular TLR9 (i-TLR9). Measured in four types of HUVEC: pooled from Lonza (L); pooled (P) single (S), and pre-screened (PS) from PromoCell (n=3; mean with SD). Graph shows percentage of cells that tested positive for intracellular RAGE and TLR9.

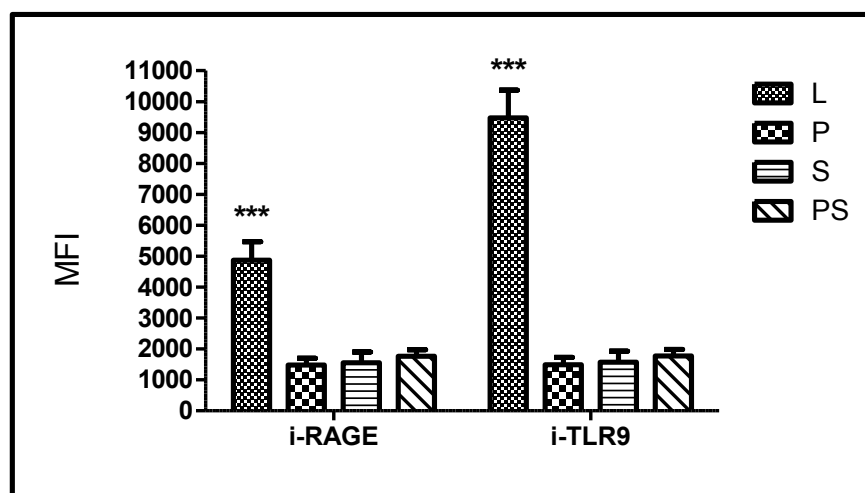


Figure 39. Median Fluorescence Intensity of intracellular RAGE (i-RAGE) and intracellular TLR9 (i-TLR9). Measured in four types of HUVEC: pooled from Lonza; pooled, single and VEGF pre-screened from PromoCell (n=3; mean with SD). Graph shows intercellular results. There is a significant difference ($p < 0.05$) between RAGE MFI on pooled Lonza HUVEC and pooled Promocell HUVEC, in intracellular expression.

3.2.4.1.2 Expression of RAGE in HUVEC treated with TNF- α

The number of cells expressing RAGE externally was $3.07\% \pm 1.12$. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing RAGE externally, increased to $4.00\% \pm 0.26$. The percentage of cells expressing RAGE externally have not changed significantly after the treatment.

The levels of intensity of RAGE expression per cell, determined by median fluorescent intensity (MFI) was 432.3 ± 43.9 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of RAGE decreased to 390.7 ± 51.1 . The MFI has not changed significantly after the treatment.

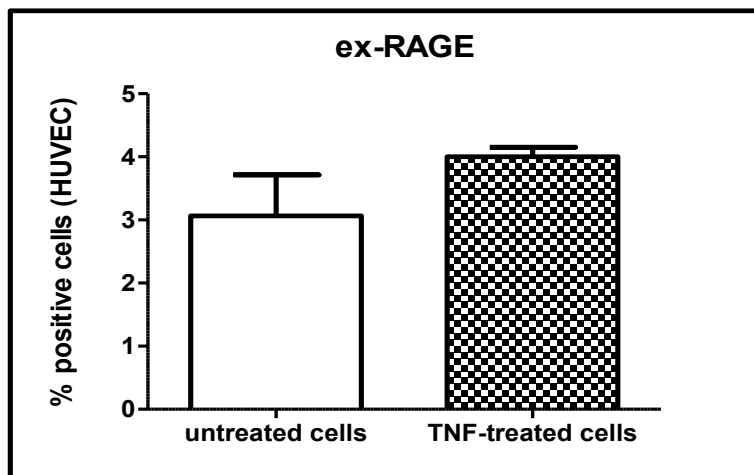


Figure 40. External RAGE expression on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=3; mean with SD). Data were analysed by non-parametric Mann-Whitney test. The percentage of cells expressing RAGE externally have not changed significantly after the treatment.

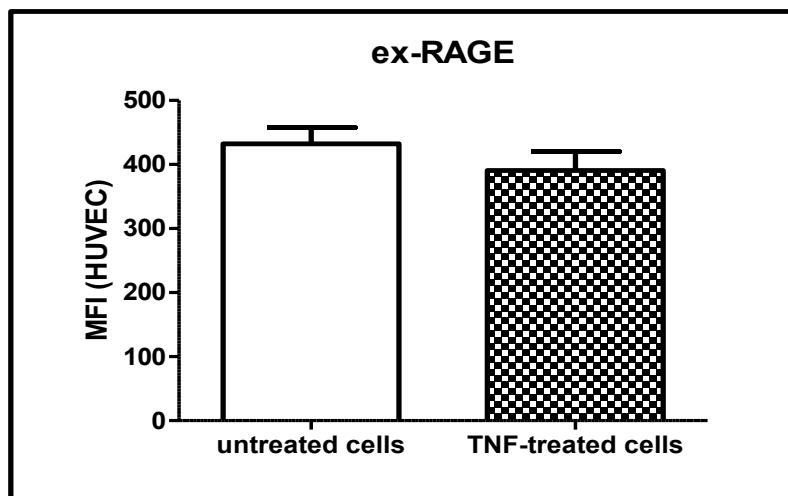


Figure 41. Median fluorescence intensity of external RAGE on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=3; mean with SD). Data were analysed by non-parametric Mann-Whitney test. The MFI, intensity of RAGE expression per cell, have not changed significantly after the treatment.

The number of cells expressing RAGE internally was 95.93 ± 6.18 . After the treatment with $\text{TNF-}\alpha$ 10ng/ml for 23 hours, the number of cells expressing RAGE internally, increased to $99.93\% \pm 0.06$. The percentage of cells expressing RAGE externally have not changed significantly after the treatment.

The levels of intensity of RAGE expression per cell, determined by median fluorescent intensity (MFI) was 1321.0 ± 475.0 in untreated cells. After the treatment with $\text{TNF-}\alpha$ 10ng/ml for 23 hours the levels of intensity of RAGE increased to 1531.3 ± 275.6 . The MFI has not changed significantly after the treatment.

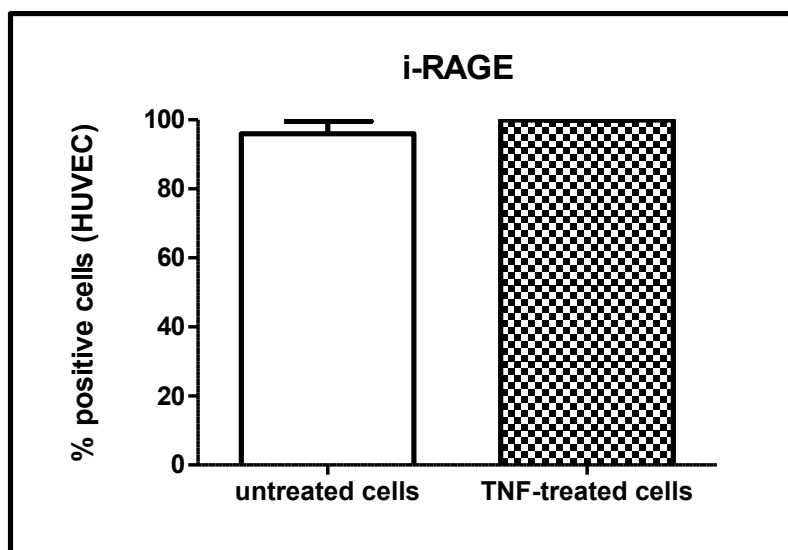


Figure 42. Internal RAGE expression in HUVEC. Cell were treated with $\text{TNF-}\alpha$. 10ng/ml for 23hrs (n=3; mean with SD). Data were analysed by non-parametric Mann-Whitney test. The percentage of cells expressing RAGE internally have not changed significantly after the treatment.

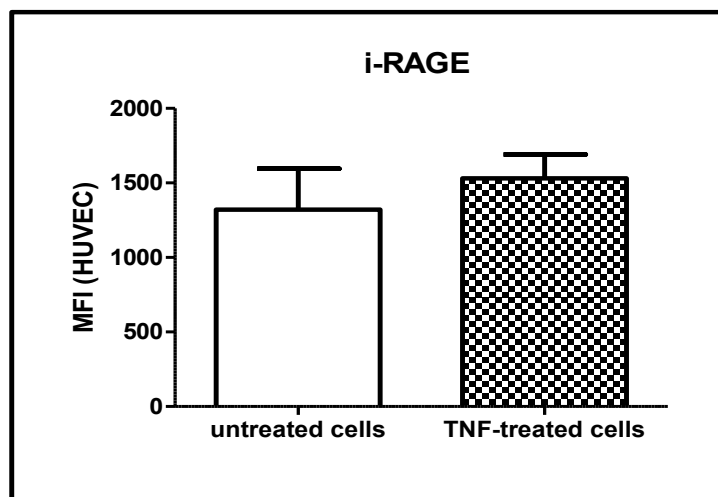


Figure 43. Median fluorescence intensity of internal RAGE in HUVEC. Cells were treated with TNF- α 10ng/ml for 23hrs (n=3; mean with SD). Data were analysed by non-parametric Mann-Whitney test. The MFI, intensity of RAGE expression per cell, have not changed significantly after the treatment.

3.2.4.2 Expression of Robo4

3.2.4.2.1 Expression of Robo4 in HUVEC treated with TNF- α

The number of cells expressing Robo4 externally was $95.16\% \pm 2.79$. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Robo4 externally, decreased to $64.07\% \pm 20.97$. The expression of extracellular Robo4 have significantly ($p=0.0135$) decreased in cells treated with TNF- α .

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 950.6 ± 241.9 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Robo4 increased to 1087.7 ± 420.0 . The MFI has not changed significantly after the treatment.

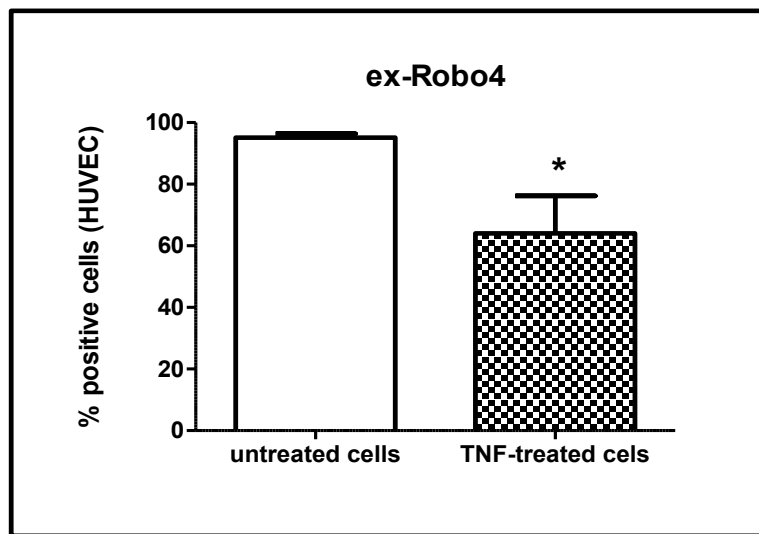


Figure 44. External Robo4 expression on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and are expressed as mean $\pm$ SD.

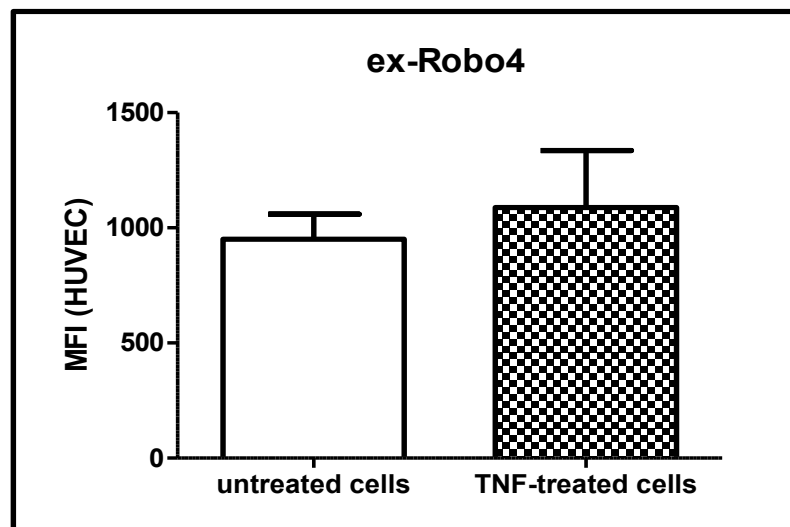


Figure 45. Median Fluorescence Intensity of external Robo4 expression on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and are expressed as mean $\pm$ SD.

The number of cells expressing Robo4 internally was $34.30\% \pm 12.71$. After the treatment with $\text{TNF-}\alpha$ 10ng/ml for 23 hours, the number of cells expressing Robo4 internally, decreased to $29.67\% \pm 5.95$. The expression of intracellular Robo4 has not changed significantly after the treatment.

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 12433.8 ± 2126.9 in untreated cells. After the treatment with $\text{TNF-}\alpha$ 10ng/ml for 23 hours the levels of intensity of Robo4 increased to 15168.7 ± 922.5 . The MFI has not changed significantly after the treatment.

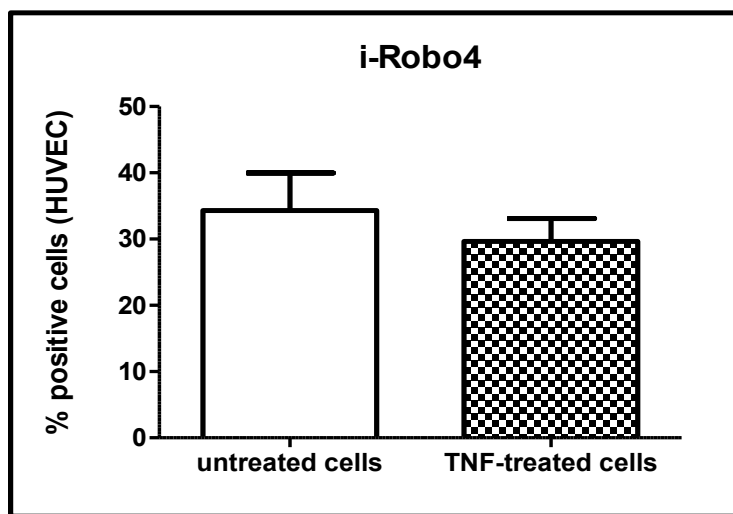


Figure 46. Internal Robo4 expression in HUVEC. Cell were treated with $\text{TNF-}\alpha$. 10ng/ml for 23hrs ($n=5$ for untreated cells and $n=3$ for $\text{TNF-}\alpha$. treated cells). Data were analysed by non-parametric Mann-Whitney test and are expressed as mean $\pm$ SD. The number of cells expressing Robo4 internally have not changed significantly after the $\text{TNF-}\alpha$ treatment.

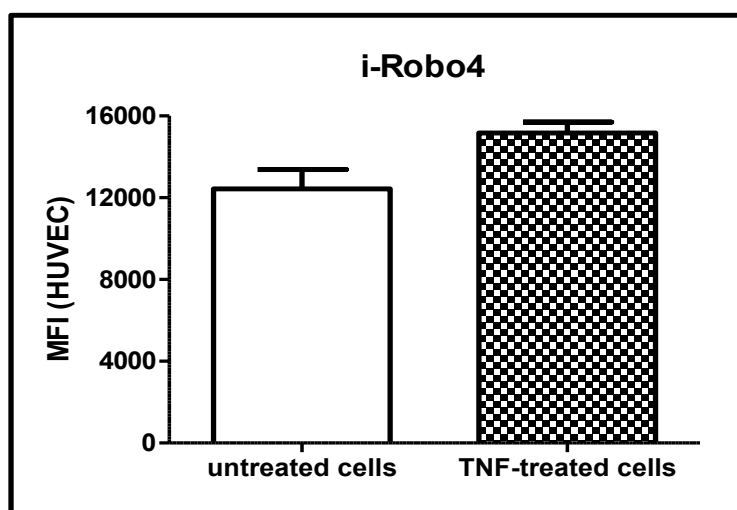


Figure 47. Median Fluorescence Intensity of internal Robo4 expression in HUVEC. Cells were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells).). Data were analysed by non-parametric Mann-Whitney test and are expressed as mean $\pm$ SD. The MFI of Robo4 internally have not changed significantly after the TNF- α treatment.

3.2.4.2.2. Expression of Robo4 on HPAEC treated with TNF- α

The number of cells expressing Robo4 externally was 58.0% $\pm$ 8.12. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Robo4 externally, decreased to 29.35% $\pm$ 6.41. The expression of extracellular Robo4 have significantly (p=0.0286) decreased in cells treated with TNF- α .

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 2719.0 $\pm$ 189.5 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Robo4 decreased to 1693.0 $\pm$ 89.1. The MFI has changed significantly (p=0.0202) after the TNF- α treatment.

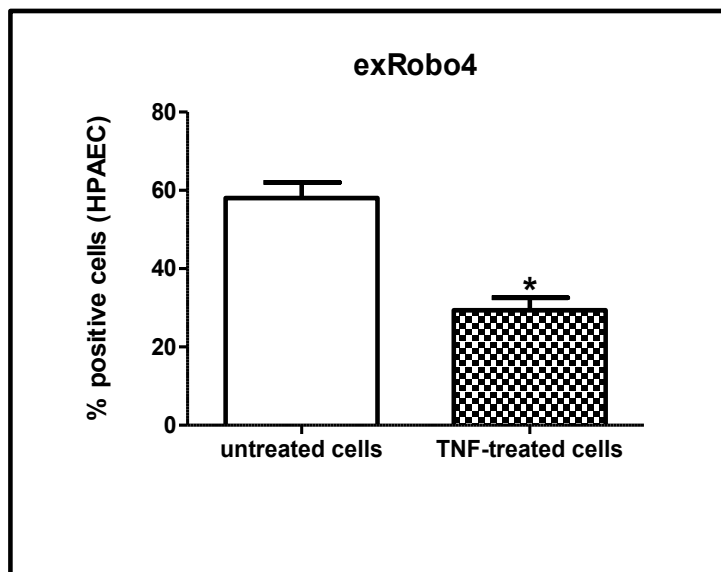


Figure 48. External Robo4 expression on HPAEC. Cells were treated with TNF- α 10ng/ml for 23hrs (n=4). Data were analysed by non-parametric Mann-Whitney test and are expressed as mean $\pm$ SD. The expression of extracellular ROBO4 has significantly (p=0.0286) decreased in cells treated with TNF- α .

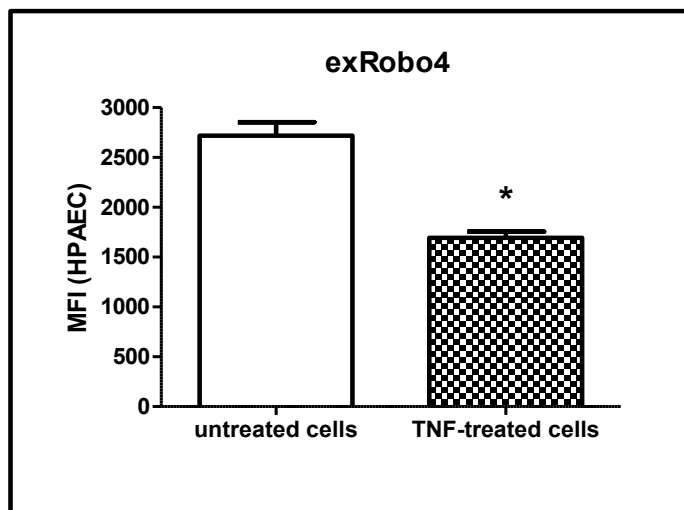


Figure 49. Median Fluorescence Intensity of external Robo4 expression on HPAEC. Cells were treated with TNF- α 10ng/ml for 23hrs (n=2). Data were analysed by t-test and are expressed as mean $\pm$ SD. The MFI of Robo4 externally has significantly (p=0.0286) decreased in cells treated with TNF- α .

3.2.4.2.3. Expression of Robo4 on HCAEC treated with TNF- α

The number of cells expressing Robo4 externally was 80.13% $\pm$ 13.83. After the treatment with TNF- α 0.1ng/ml for 1 hour, the number of cells expressing Robo4 externally, decreased to 68.00% $\pm$ 7.07. The expression of extracellular Robo4 has not changed significantly after the treatment.

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 609.7 $\pm$ 103.5 in untreated cells. After the treatment with TNF- α 0.1ng/ml for 1 hour the levels of intensity of Robo4 increased to 620.5 $\pm$ 10.6. The MFI has not changed significantly after the treatment.

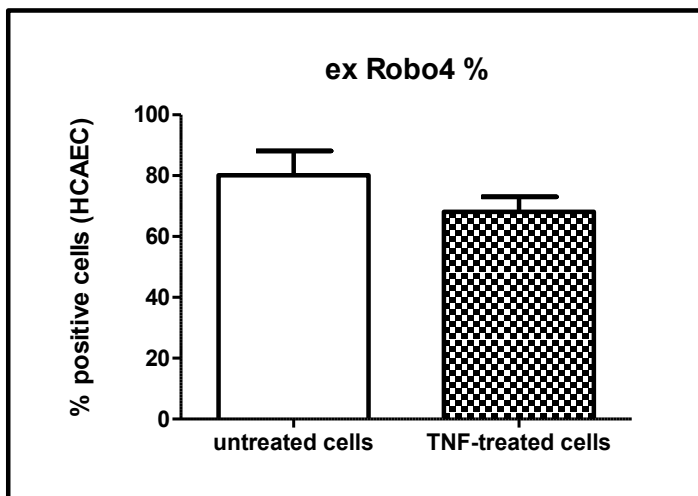


Figure 50. External Robo4 expression on HCAEC. Cell were treated with TNF- α . 0.1ng/ml for 1 hour (untreated cells n=3; TNF- α .-treated cells n=2). The number of cells expressing Robo4 externally have not changed significantly after the TNF- α treatment. Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

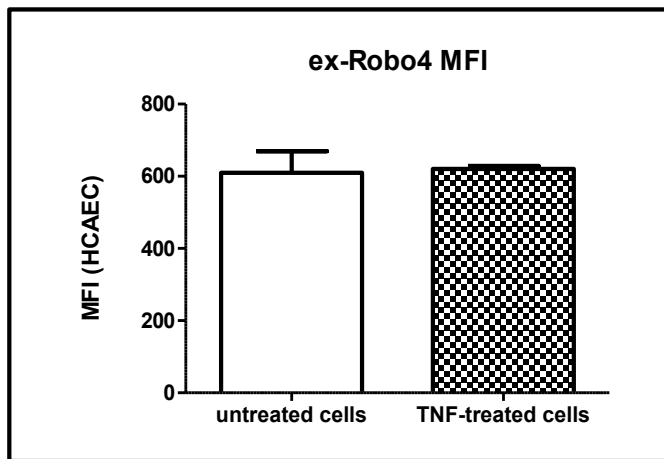


Figure 51. Median Fluorescence Intensity of external Robo4 expression on HCAEC.

Cells were treated with TNF- α 0.1ng/ml for 1 hour (untreated cells n=3; TNF- α -treated cells n=2). The MFI of external Robo4 has not changed significantly after the TNF- α treatment. Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

3.2.4.3 Expression of Robo1

3.2.4.3.1 Expression of Robo1 in HUVEC treated with TNF- α

The number of cells expressing Robo1 externally was 7.15% $\pm$ 2.41. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Robo1 externally, increased to 16.17% $\pm$ 7.35. The percentage of cells expressing Robo1 externally have not changed significantly ($p=0.057$) after the treatment.

The levels of intensity of Robo1 expression per cell, determined by median fluorescent intensity (MFI) was 1948.0 $\pm$ 456.2 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Robo1 decreased to 1761.7 $\pm$ 748.6. The MFI has not changed significantly after the treatment.

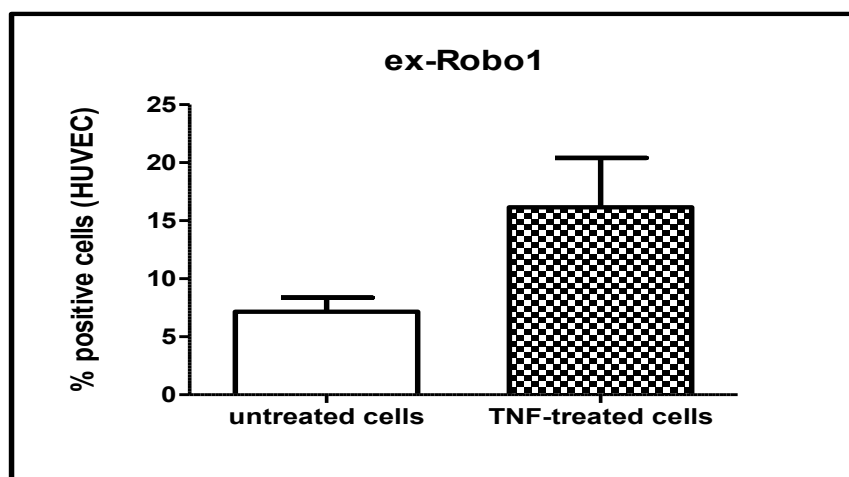


Figure 52. External Robo1 expression on HUVEC. Cell were treated with TNF- α 10ng/ml for 23hrs (n=4 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

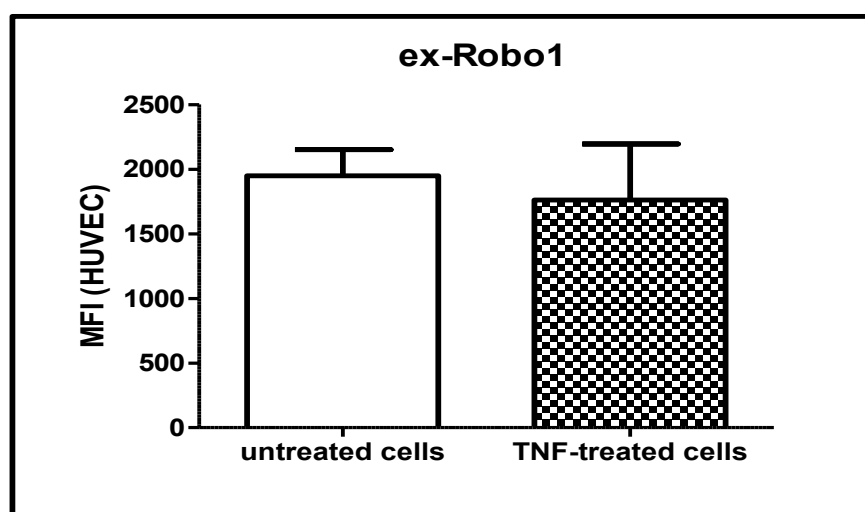


Figure 53. Median Fluorescence Intensity of external Robo1 expression on HUVEC. Cell were treated with TNF- α 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

The number of cells expressing Robo1 internally was 100% $\pm$ 0. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Robo1 internally stayed the same 100 % $\pm$ 0.

The levels of intensity of Robo1 expression per cell, determined by median fluorescent intensity (MFI) was 7855.6 $\pm$ 1216.7 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Robo1 increased to 9295.7 $\pm$ 611.6. The MFI has not changed significantly after the treatment.

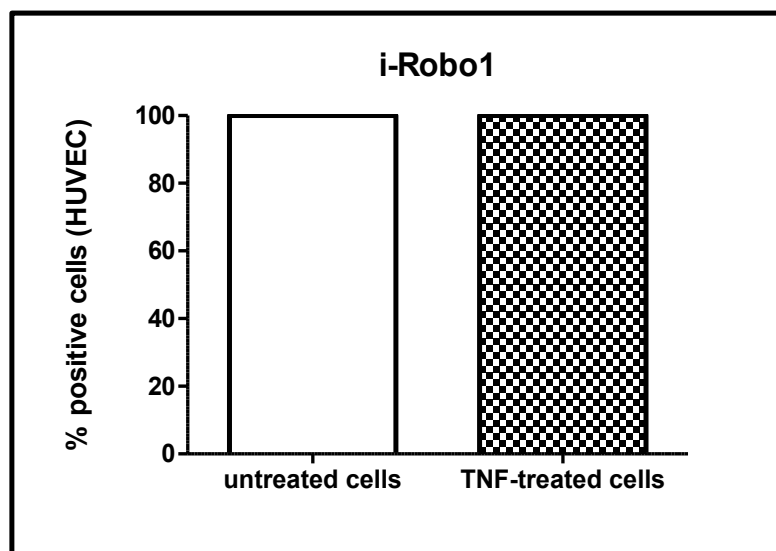


Figure 54. Internal Robo1 expression in HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

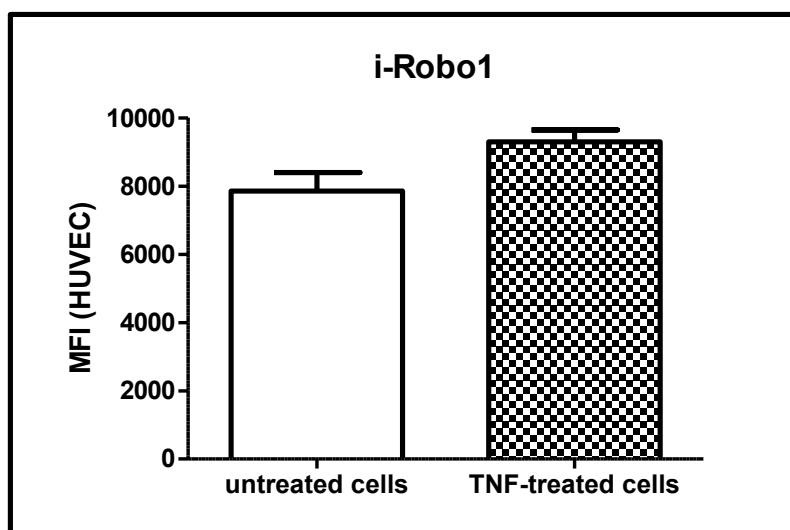


Figure 55. Median Fluorescence Intensity of internal Robo1 expression in HUVEC. Cells were treated with TNF- α 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

3.2.4.3.2. Expression of Robo1 on HPAEC treated with TNF- α

The number of cells expressing Robo1 externally was $74.80\% \pm 7.1$. After the treatment with TNF- α 10ng/ml for 23hrs, the number of cells expressing Robo1 externally, increased to $93.10\% \pm 1.00$. The expression of extracellular Robo1 has not changed significantly after the treatment. The levels of intensity of Robo1 expression per cell, determined by median fluorescent intensity (MFI) was 1988 ± 207.9 in untreated cells. After the treatment with TNF- α 10ng/ml for 23hrs, the levels of intensity of Robo1 increased to 2719.0 ± 189.5 . The MFI has not changed significantly after the treatment.

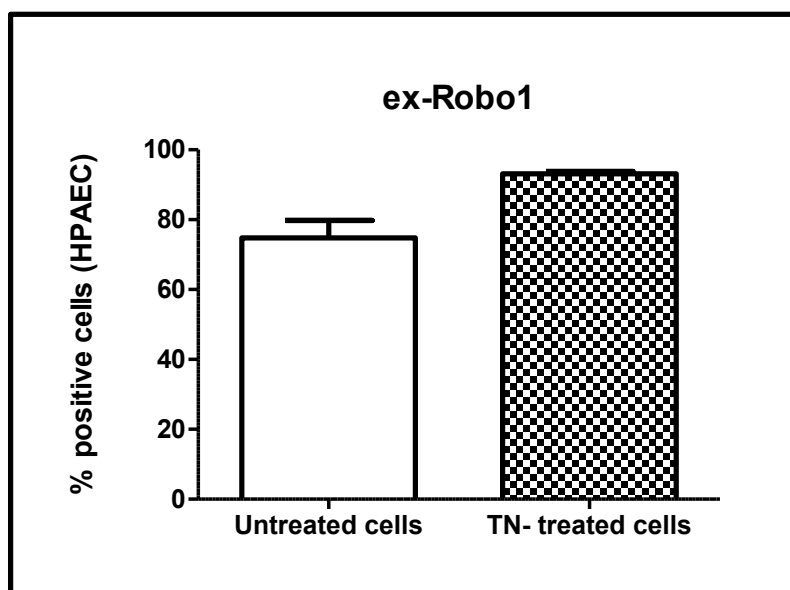


Figure 56. External Robo1 expression on HPAEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=2). Data were analysed by t- test and are expressed as mean $\pm$ SD. The expression of extracellular Robo1has not changed significantly after the TNF- α treatment.

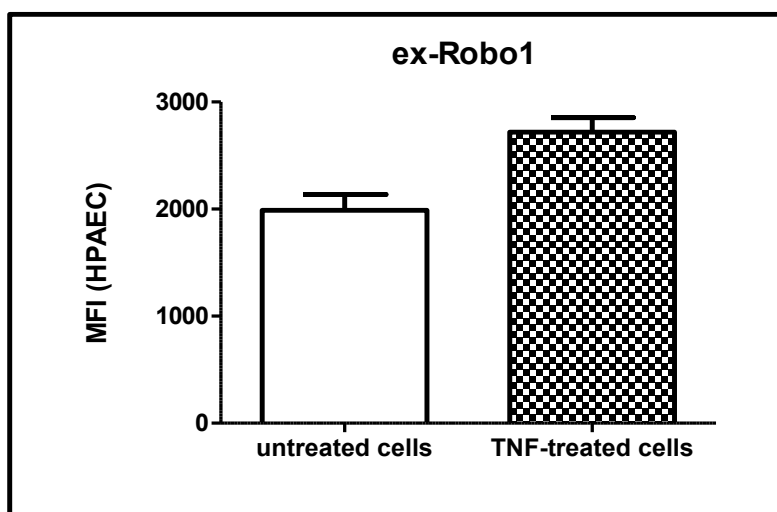


Figure 57. Median Fluorescence Intensity of external Robo1 expression on HPAEC. Cells were treated with TNF- α . 10ng/ml for 23hrs (n=2). Data were analysed by t- test and are expressed as mean $\pm$ SD. The MFI of Robo1 externally has not changed significantly after the TNF- α treatment.

3.2.4.4 Expression of Slit2 in HUVEC treated with TNF- α

The number of cells expressing Sli2 externally was $93.26\% \pm 5.14$. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Sli2 externally, increased to $94.83\% \pm 3.82$. The percentage of cells expressing Slit2 externally have not changed significantly after the treatment.

The levels of intensity of Slit2 expression per cell, determined by median fluorescent intensity (MFI) was 974.0 ± 217.4 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Slit2 increased to 1064.3 ± 428.8 . The MFI has not changed significantly after the treatment.

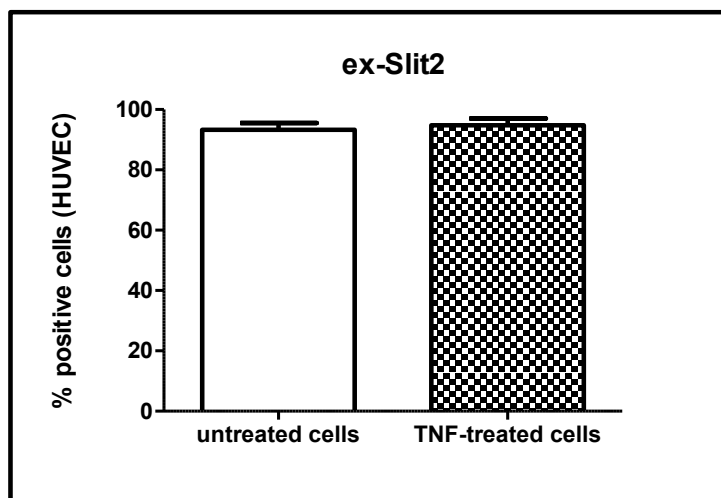


Figure 58. External Slit2 expression on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs. (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

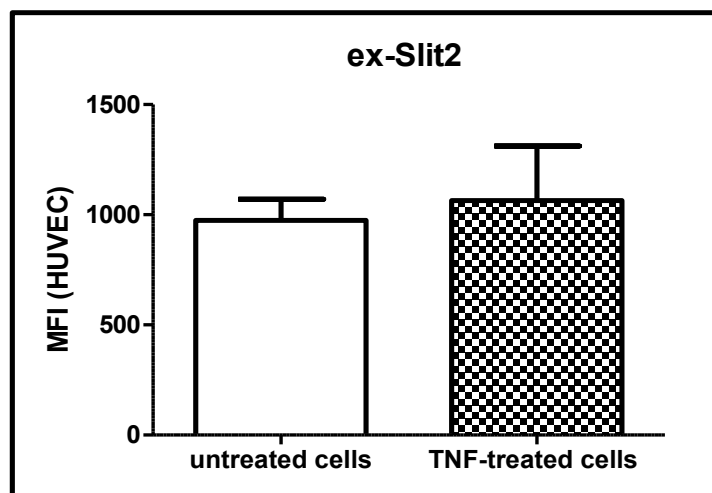


Figure 59. Median Fluorescence Intensity of external Sli2 expression on HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD

The number of cells expressing Sli2 internally was $99.76\% \pm 0.21$. After the treatment with TNF- α 10ng/ml for 23 hours, the number of cells expressing Sli2 internally, increased to $99.83\% \pm 0.15$. The percentage of cells expressing Slit2 internally have not changed significantly after the treatment.

The levels of intensity of Slit2 expression per cell, determined by median fluorescent intensity (MFI) was 7843.6 ± 1206.2 in untreated cells. After the treatment with TNF- α 10ng/ml for 23 hours the levels of intensity of Slit2 increased to 9298.0 ± 594.8 . The MFI has not changed significantly after the treatment.

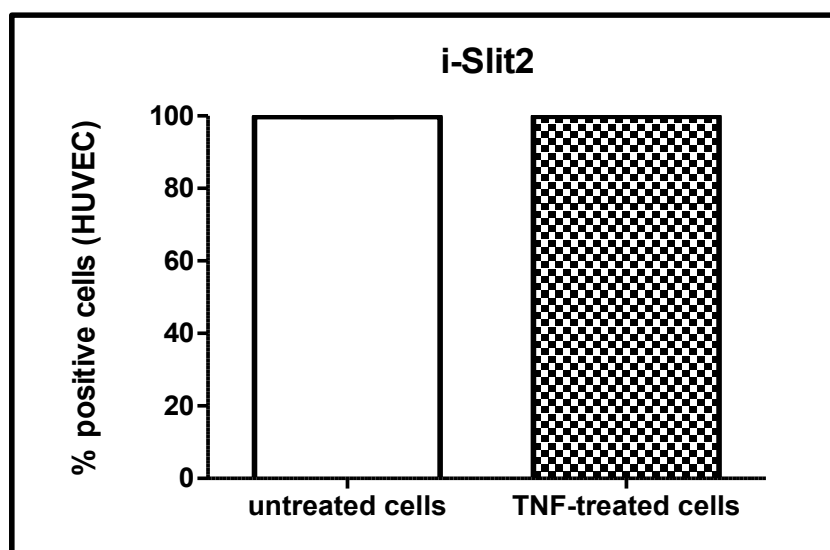


Figure 60. Internal Slit2 expression in HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs. (n=5 for untreated cells and n=3 for TNF- α . treated cells Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

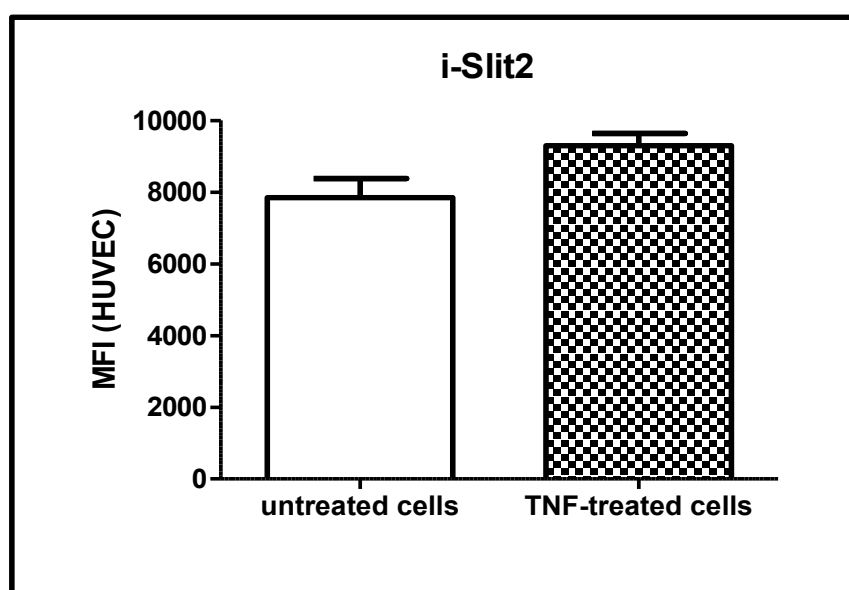


Figure 61. Median Fluorescence Intensity of internal Slit2 expression in HUVEC. Cell were treated with TNF- α . 10ng/ml for 23hrs (n=5 for untreated cells and n=3 for TNF- α . treated cells). Data were analysed by non-parametric Mann-Whitney test and expressed as mean $\pm$ SD.

3.2.4.5. Release of Robo4

3.2.4.5.1. Robo4 release in HUVEC treated with IL-1 β

The Robo4 release from untreated cells (6 hours) was 1.25 ± 0.35 ng/ml ($n=3$); the Robo4 release from cells treated with IL-1 β 10 ng/ml for 6 hours was 1.82 ± 0.63 ng/ml ($n=3$). This increase was not statistically significant. The Robo4 release from untreated cells (12 hours) was 1.38 ± 0.64 ng/ml ($n=3$); the Robo4 release from cells treated with IL-1 β 10 ng/ml for 12 hours was 1.50 ± 0.55 ng/ml ($n=3$). This increase was not statistically significant. The Robo4 release from untreated cells (24 hours) was 2.41 ± 0.39 ng/ml ($n=3$); the Robo4 release from cells treated with IL-1 β 10 ng/ml for 24 hours was 2.76 ± 0.36 ng/ml ($n=3$). This increase was not statistically significant.

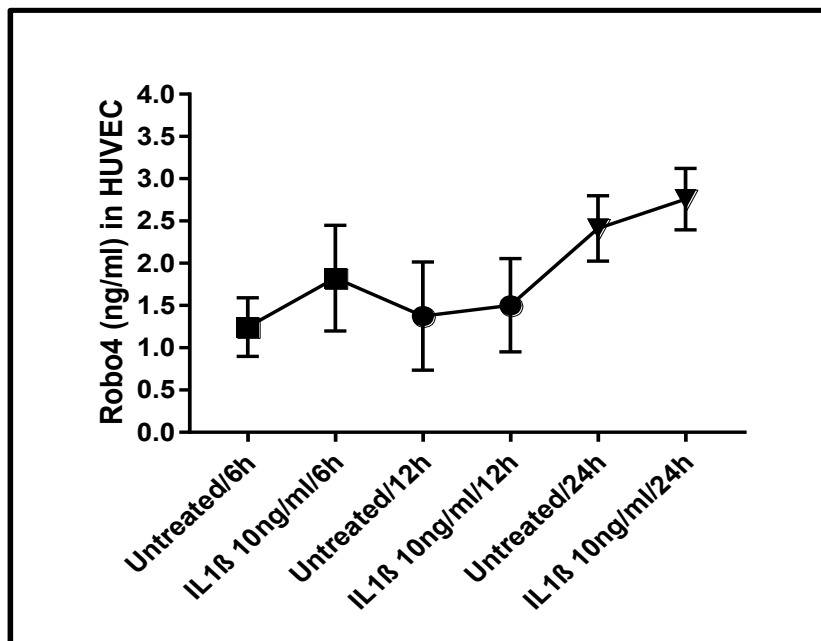


Figure 62. Robo4 release measured in HUVEC treated with IL-1 β . Cells were treated with IL-1 β 10 ng/ml for 6, 12 and 24 hours ($n=3$). The release of Robo4 was measured by ELISA. Data were analysed by non-parametric Kruskal-Wallis test and expressed as mean $\pm$ SD.

3.2.4.5.2. Robo4 release in HPAEC treated with IL-1 β

The Robo4 release from untreated cells (6 hours) was 1.61 ± 0.54 ng/ml ($n=2$); the Robo4 release from cells treated with IL-1 β 10ng/ml for 6 hours was 1.71 ± 0.14 ng/ml ($n=2$). The Robo4 release from untreated cells (12 hours) was 1.81 ± 0.16 ng/ml ($n=2$); the Robo4 release from cells treated with IL-1 β 10ng/ml for 12 hours was 2.46 ± 0.14 ng/ml ($n=2$).

The Robo4 release from untreated cells (24 hours) was 2.95 ± 0.26 ng/ml ($n=2$); the Robo4 release from cells treated with IL-1 β 10ng/ml for 24 hours was 3.32 ± 0.05 ng/ml ($n=2$).

Due to a small number of replicates ($n=2$) it was not possible to statistically analyse these results. The results show increasing trend of Robo4 release with lengths of treatment.

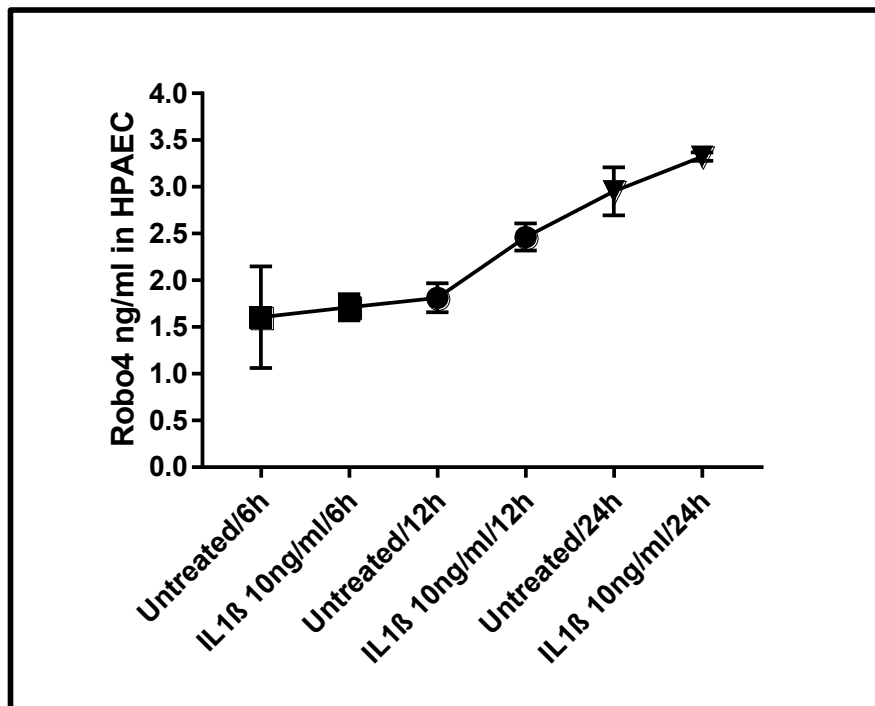


Figure 63. Robo4 release measured in HPAEC treated with IL-1 β . Cells were treated with IL-1 β 10ng/ml for 6, 12 and 24 hours ($n=2$). The release of Robo4 was measured by ELISA. The data are expressed as mean $\pm$ SD.

3.2.4.5.3. Robo4 release in HUVEC treated with IL-6

The Robo4 release from untreated cells (6 hours) was 1.96 ± 0.33 ng/ml ($n=3$); the Robo4 release from cells treated with IL-6 10ng/ml for 6 hours was 1.58 ± 0.64 ng/ml ($n=3$). This decrease was not statistically significant. The Robo4 release from untreated cells (12 hours) was 0.84 ± 0.31 ng/ml ($n=3$); the Robo4 release from cells treated with IL-6 10ng/ml for 12 hours was 1.13 ± 0.30 ng/ml ($n=3$). This increase was not statistically significant. The Robo4 release from untreated cells (24 hours) was 2.49 ± 0.60 ng/ml ($n=3$); the Robo4 release from cells treated with IL-6 10ng/ml for 24 hours was 2.46 ± 0.36 ng/ml ($n=3$). This decrease was not statistically significant.

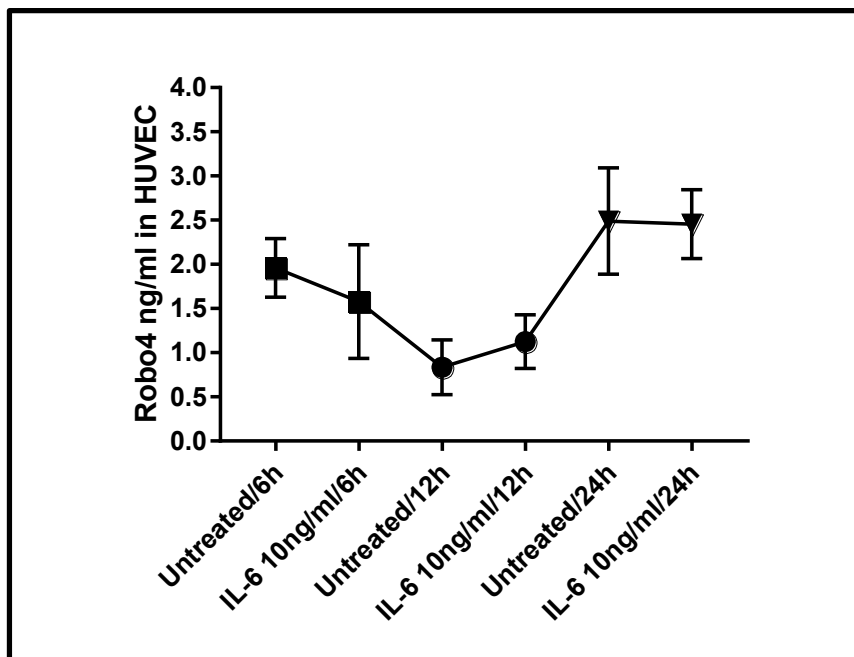


Figure 64. Robo4 release measured in HUVEC treated with IL-6. Cells were treated with IL-6 10ng/ml for 6, 12 and 24 hours ($n=3$). The release of Robo4 was measured by ELISA. The data were statistically analysed by non-parametric Kruskal-Wallis test and expressed as mean $\pm$ SD.

3.2.4.5.4. Robo4 release in HPAEC treated with IL-6

The Robo4 release from untreated cells (6 hours) was 1.91 ± 0.21 ng/ml ($n=2$); the Robo4 release from cells treated with IL-6 10ng/ml for 6 hours was 2.14 ± 0.36 ng/ml ($n=2$).

The Robo4 release from untreated cells (12 hours) was 2.45 ± 0.00 ng/ml ($n=2$); the Robo4 release from cells treated with IL-6 10ng/ml for 12 hours was 2.51 ± 0.07 ng/ml ($n=2$).

The Robo4 release from untreated cells (24 hours) was 3.04 ± 0.30 ng/ml ($n=2$); the Robo4 release from cells treated with IL-6 10ng/ml for 24 hours was 3.26 ± 0.34 ng/ml ($n=2$).

Due to a small number of replicates ($n=2$) it was not possible to statistically analyse these results. The results show increasing trend of Robo4 release with lengths of treatment.

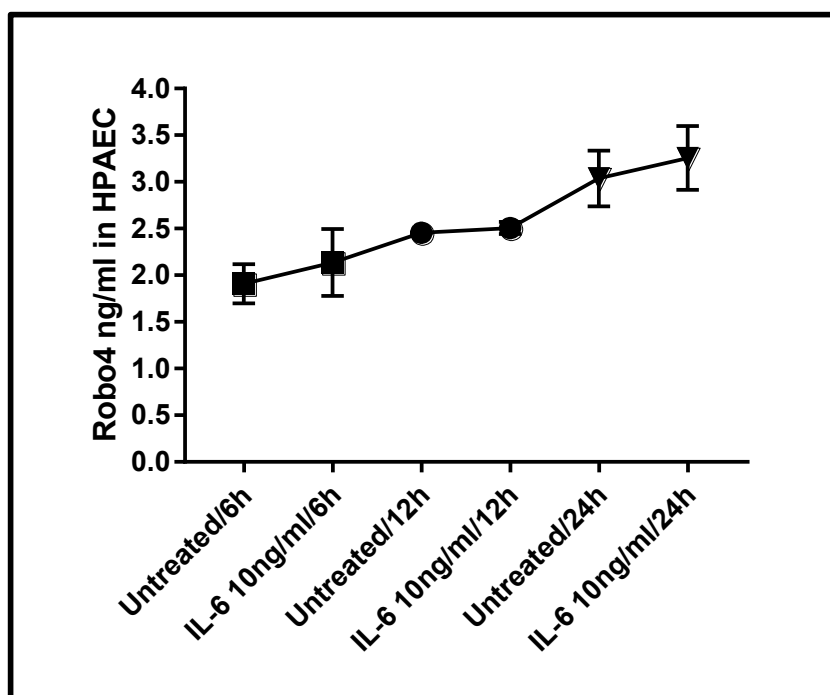


Figure 65. Robo4 release measured in HPAEC treated with IL-6. Cells were treated with IL-6 10ng/ml for 6, 12 and 24 hours ($n=2$). The release of Robo4 was measured by ELISA. The data are expressed as mean $\pm$ SD.

3.2.4.5.5. Robo4 release in HUVEC treated with TNF- α and Slit2

The Robo4 release from untreated cells (3 hours) was 1.20 ± 0.11 ng/ml ($n=3$); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 1.96 ± 0.54 ng/ml ($n=3$). This increase was not statistically significant.

The Robo4 release from untreated cells (6 hours) was 1.30 ± 0.11 ng/ml ($n=3$); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 1.15 ± 0.06 ng/ml ($n=3$). This decrease was not statistically significant.

The Robo4 release from untreated cells (12 hours) was 1.31 ± 0.23 ng/ml ($n=3$); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 1.74 ± 0.64 ng/ml ($n=3$). This increase was not statistically significant.

The Robo4 release from cells treated with TNF- α 0.1ng/ml for 3 hours was 1.29 ± 0.17 ng/ml ($n=3$); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 3 hours was 1.61 ± 0.73 ng/ml ($n=3$). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 0.1ng/ml for 6 hours was 1.58 ± 0.11 ng/ml ($n=3$); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 6 hours was 1.57 ± 0.65 ng/ml ($n=3$). This decrease was not statistically significant. The Robo4 release from cells treated with TNF- α 0.1ng/ml for 12 hours was 1.55 ± 0.14 ng/ml ($n=3$); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 12 hours was 2.06 ± 0.78 ng/ml ($n=3$). This increase was not statistically significant.

The Robo4 release from cells treated with TNF- α 1ng/ml for 3 hours was 1.59 ± 0.29 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 3 hours was 1.48 ± 0.61 ng/ml (n=3). This decrease was not statistically significant. The Robo4 release from cells treated with TNF- α 1ng/ml for 6 hours was 1.38 ± 0.25 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 6 hours was 1.99 ± 0.56 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 1ng/ml for 12 hours was 2.00 ± 0.43 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 12 hours was 2.61 ± 0.29 ng/ml (n=3). This increase was not statistically significant.

The Robo4 release from cells treated with TNF- α 10ng/ml for 3 hours was 1.38 ± 0.21 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 3 hours was 1.72 ± 0.42 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 10ng/ml for 6 hours was 1.38 ± 0.19 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 6 hours was 1.95 ± 0.51 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 10ng/ml for 12 hours was 1.88 ± 0.22 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 12 hours was 2.69 ± 0.94 ng/ml (n=3). This increase was not statistically significant.

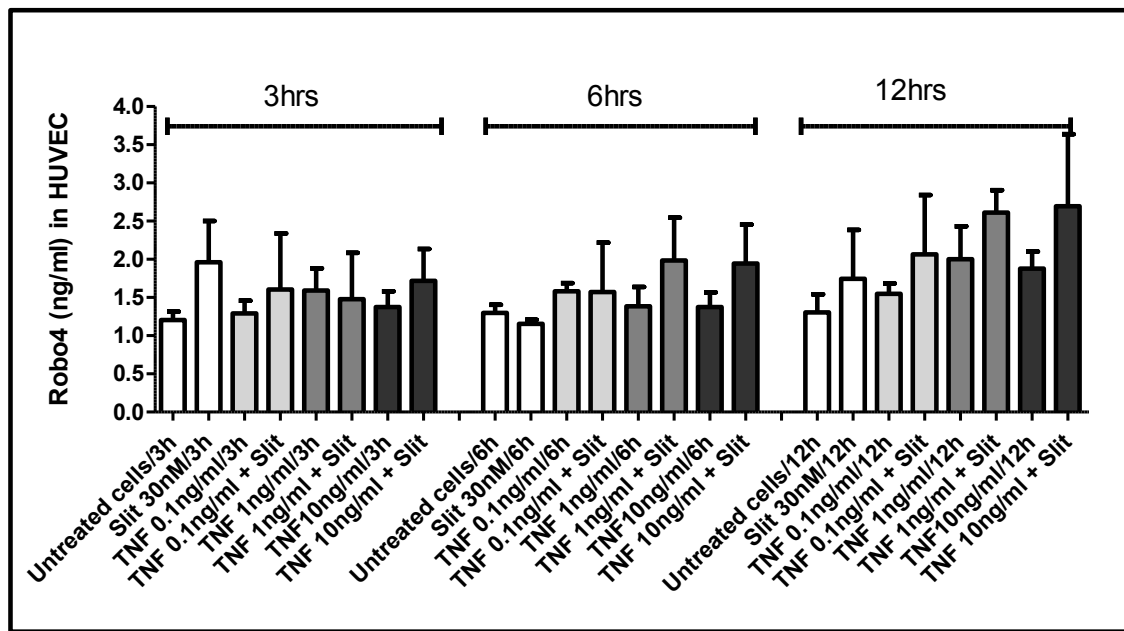


Figure 66. Robo4 release measured in HUVEC treated with TNF- α . Endothelial cells were treated with TNF- α 0.1, 1.0 and 10ng/ml for 3, 6 and 9 hours (n=3). The release of Robo4 was measured by ELISA. The data were statistically analysed by non-parametric Kruskal-Wallis test and expressed as mean $\pm$ SD. None of the results was statistically significant.

3.2.4.5.6. Robo4 release in HPAEC treated with TNF- α and Slit2

The Robo4 release from untreated cells (3 hours) was 1.25 ± 0.26 ng/ml (n=3); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 2.50 ± 0.48 ng/ml (n=3). This increase was statistically significant ($p < 0.05$). The Robo4 release from untreated cells (6 hours) was 1.06 ± 0.36 ng/ml (n=3); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 1.51 ± 0.51 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from untreated cells (12 hours) was 1.17 ± 0.05 ng/ml (n=3); the Robo4 release from cells treated with Slit2 30nM for 3 hours was 2.03 ± 0.52 ng/ml (n=3). This increase was not statistically significant.

The Robo4 release from cells treated with TNF- α 0.1ng/ml for 3 hours was 1.01 ± 0.27 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 3 hours was 1.30 ± 0.17 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 0.1ng/ml for 6 hours was 1.13 ± 0.25 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 6 hours was 1.09 ± 0.21 ng/ml (n=3). This decrease was not statistically significant. The Robo4 release from cells treated with TNF- α 0.1ng/ml for 12 hours was 1.32 ± 0.30 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 0.1ng/ml for 12 hours was 1.35 ± 0.16 ng/ml (n=3). This increase was not statistically significant.

The Robo4 release from cells treated with TNF- α 1ng/ml for 3 hours was 1.12 ± 0.33 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 3 hours was 1.20 ± 0.12 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 1ng/ml for 6 hours was 1.12 ± 0.19 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 6 hours was 1.08 ± 0.12 ng/ml (n=3). This decrease was not statistically significant. The Robo4 release from cells treated with TNF- α 1ng/ml for 12 hours was 1.55 ± 0.07 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 1ng/ml for 12 hours was 1.55 ± 0.10 ng/ml (n=3). There was no difference between these two treatments.

The Robo4 release from cells treated with TNF- α 10ng/ml for 3 hours was 1.14 ± 0.19 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 3 hours was 1.25 ± 0.48 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 10ng/ml for 6 hours was 1.05 ± 0.18 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 6 hours was 1.35 ± 0.09 ng/ml (n=3). This increase was not statistically significant. The Robo4 release from cells treated with TNF- α 10ng/ml for 12 hours was 1.73 ± 0.32 ng/ml (n=3); the Robo4 release from cells pre-incubated with Slit2 30nM for 60 minutes and then treated with TNF- α 10ng/ml for 12 hours was 2.20 ± 0.24 ng/ml (n=3). This increase was not statistically significant.

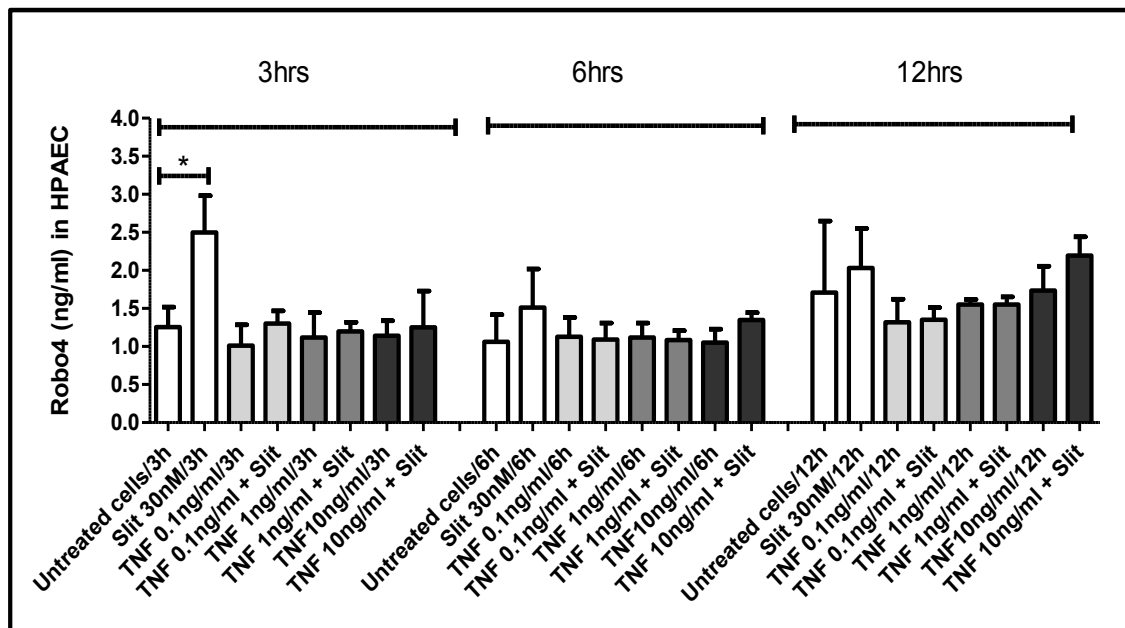


Figure 67. Robo4 release measured in HPAEC treated with TNF- α . Cells were treated with TNF- α 0.1, 1.0 and 10ng/ml for 3, 6 and 9 hours (n=3). The release of Robo4 was measured by ELISA. The data were statistically analysed by non-parametric Kruskal-Wallis test and expressed as mean $\pm$ SD. None of the results was statistically significant.

3.2.4.6. GM-CSF release in HUVEC treated with TNF- α and Slit2

The cells treated with TNF α 1ng/ml were set as a control (100%). The inhibitory effect of Slit2 pre-incubation for 1 hour was expressed as % of control. The cells pre-incubated for 1 hour with 30nM Slit2N, before TNF- α treatment 1ng/ml was added for 17 hours (n=9) were 86.97 $\pm$ 12.73% of control. This decrease was not statistically significant.

The cells treated with TNF α 10ng/ml were set as a control (100%). The inhibitory effect of Slit2 pre-incubation for 1 hour was expressed as % of control. The cells pre-incubated for 1 hour with 30nM Slit2N, before TNF- α treatment 10ng/ml was added for 17 hours (n=9) were 76.61 $\pm$ 11.71% of control. This decrease was statistically significant ($p < 0.001$).

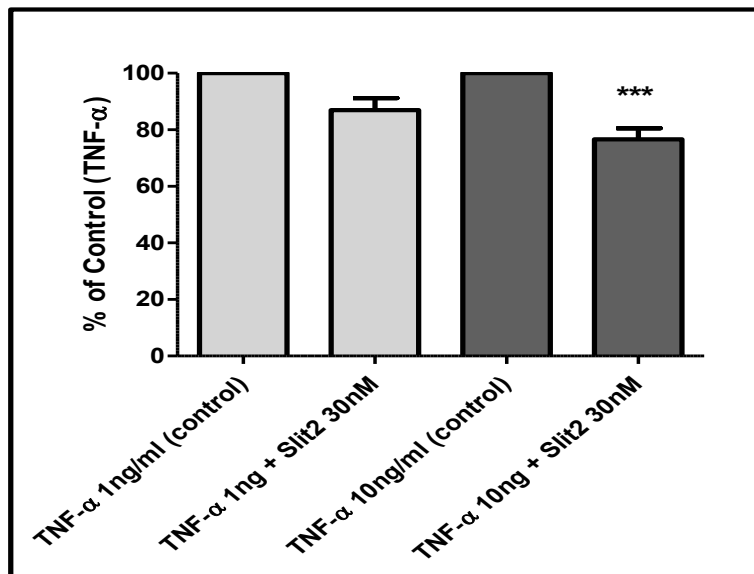


Figure 68. GM-CSF release from HUVEC treated with TNF- α and Slit2. HUVEC were pre-incubated for 1 hour with 30nM Slit2N, before TNF- α treatment 1ng/ml or 10ng/ml was added for 17 hours (n=9). The results were normalised, TNF- α was 100% and inhibition with Slit2N calculated as a percentage of TNF- α -induced release. The data were statistically analysed by non-parametric Kruskal-Wallis test and expressed as mean $\pm$ SD % of control.

3.2.4.7. Measuring TEEI

3.2.4.7.1. HUVEC seeding density

TEEI was measured in wells seeded with 5 000, 10 000, 20 000 and 40 000 cells. Seeding density of 20,000 cells per well was chosen for further experiments.

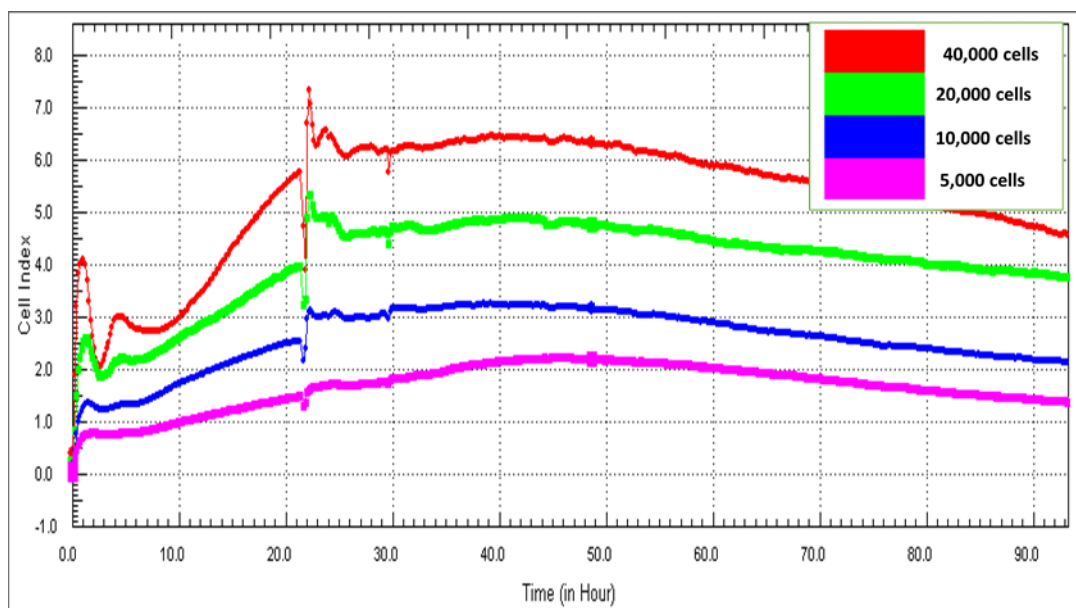


Figure 69. HUVEC seeding density. This graph is a real-time graph produced by RTCA iCELLigence System.

3.2.4.7.2. HUVEC treated with IL-1 β , IL-6 and TNF- α

The data were obtained over a 5-hour period after the IL-1 β treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The IL-1 β treatment is showed as a change in TEEI compared to control (% of control). The treatment with IL-1 β 0.1ng/ml lowered the impedance to 82.13 ± 1.60 %of control. The treatment with IL-1 β 1ng/ml lowered the impedance to 62.69 ± 4.96 % of control. The treatment with IL-1 β

10ng/ml lowered the impedance to 60.33 $\pm$ 5.81% of control. The IL-1 β 10ng/ml treatment caused a significant ($p<0.05$) decrease in TEEI compare to control. The treatment with IL-6 0.1ng/ml increased the impedance to 100.50 $\pm$ 1.02 % of control. The treatment with IL-6 1ng/ml lowered the impedance to 98.55 $\pm$ 0.88% of control. The treatment with IL-6 10ng/ml lowered the impedance to 96.85 $\pm$ 0.65of control. None of the treatments caused statistically significant change. The treatment with TNF- α 0.1ng/ml lowered the impedance to 84.65 $\pm$ 4.72% of control. The treatment with TNF- α 1ng/ml lowered the impedance 75.37 $\pm$ 3.54% of control. The treatment with TNF- α 10ng/ml lowered the impedance to 70.43 $\pm$ 4.22% of control. The TNF- α 10ng/ml treatment caused a significant ($p<0.05$) decrease in TEEI compare to control as seen in Table 10.

Concentration	IL1- β % of Control	IL-6 % of Control	TNF- α % of Control
0.0ng/ml	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
0.1ng/ml	82.13 $\pm$ 1.60	100.50 $\pm$ 1.02	84.65 $\pm$ 4.72
1.0ng/ml	62.69 $\pm$ 4.96	98.55 $\pm$ 0.88	75.37 $\pm$ 3.54
10ng/ml	60.33 $\pm$ 5.81	96.85 $\pm$ 0.65	70.43 $\pm$ 4.22

Table 10. IL-1 β , IL-6 and TNF- α dose-dependent responses. TEEI measurements of HUVEC monolayer treated with IL-1 β , IL-6 and TNF- α 0.1, 1.0 and 10 ng/ml on xCELLigence. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control. Data are presented as mean $\pm$ SD % of control (n=3) and statistically analysed using Kruskal-Wallis non-parametric test.

3.2.4.7.3. TNF- α dose-dependent response

The data were obtained over a 5-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The TNF- α treatment is shown as a change in TEEI compared to control. The treatment with TNF- α 0.1ng/ml lowered the impedance to $90.97 \pm 1.84\%$ of control. The treatment with TNF- α 1ng/ml lowered the impedance to $82.74 \pm 0.27\%$ of control. The treatment with TNF- α 10ng/ml lowered the impedance to $76.27 \pm 1.33\%$ of control. None of the treatment caused statistically significant change. TNF- α concentration of 0.1ng/ml was chosen for further experiments.

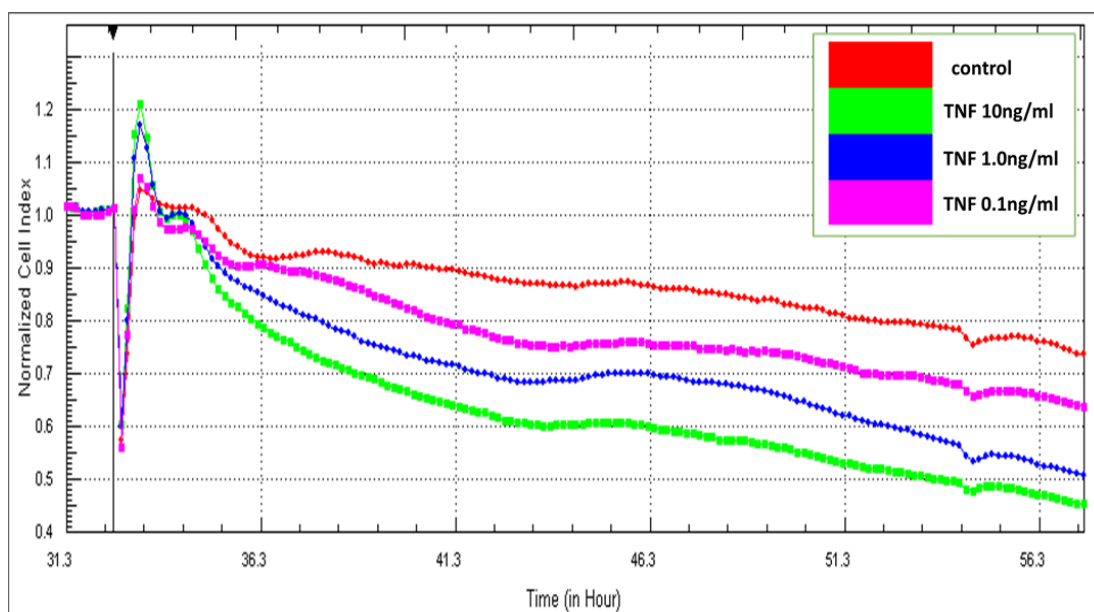


Figure 70. **TNF- α dose-dependent response.** The graph is representative (n=1) of the real-time graph produced by RTCA iCELLigence System and shows the difference in treatment as % of control.

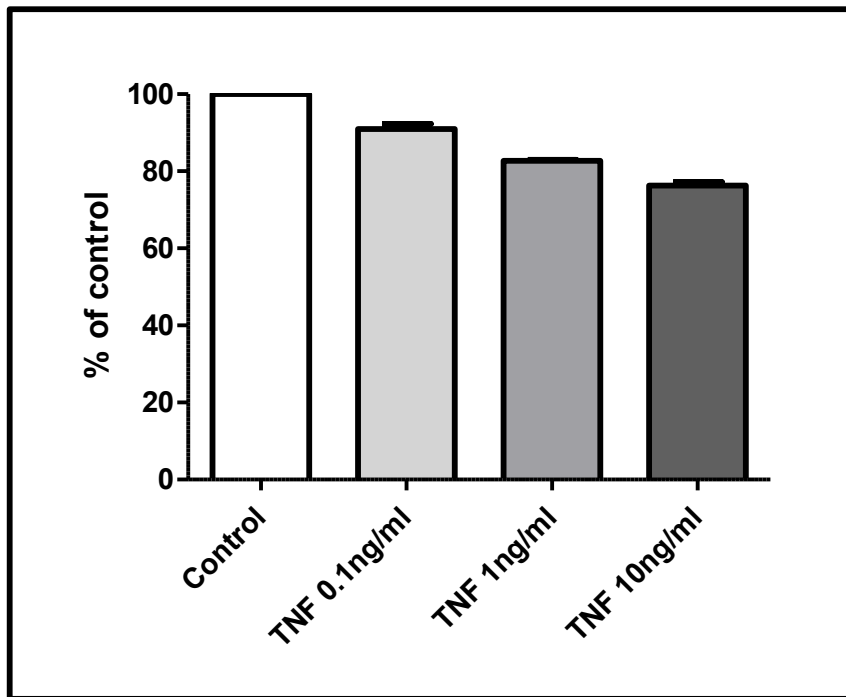


Figure 71. **TNF- α dose-dependent response.** TEEI measurements of HUVEC monolayer treated with TNF- α 0.1, 1 and 10 ng/ml on iCell. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control. Data are presented as mean $\pm$ SD % of control (n=2).

3.2.4.7.4. Slit2 dose-dependent response

The data were obtained over a 5-hour period after the Slit2 treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The Slit2 treatment is shown as a change in TEEI compared to control. The treatment with Slit2 10nM increased the impedance to $100.13 \pm 0.66\%$ of control. The treatment with Slit2 30nM increased the impedance to $105.68 \pm 6.06\%$ of control. The treatment with Slit2 100nM increased the impedance to $113.06 \pm 9.43\%$ of control. None of the treatment caused statistically significant change. Slit2 30nM was chosen for further experiments.

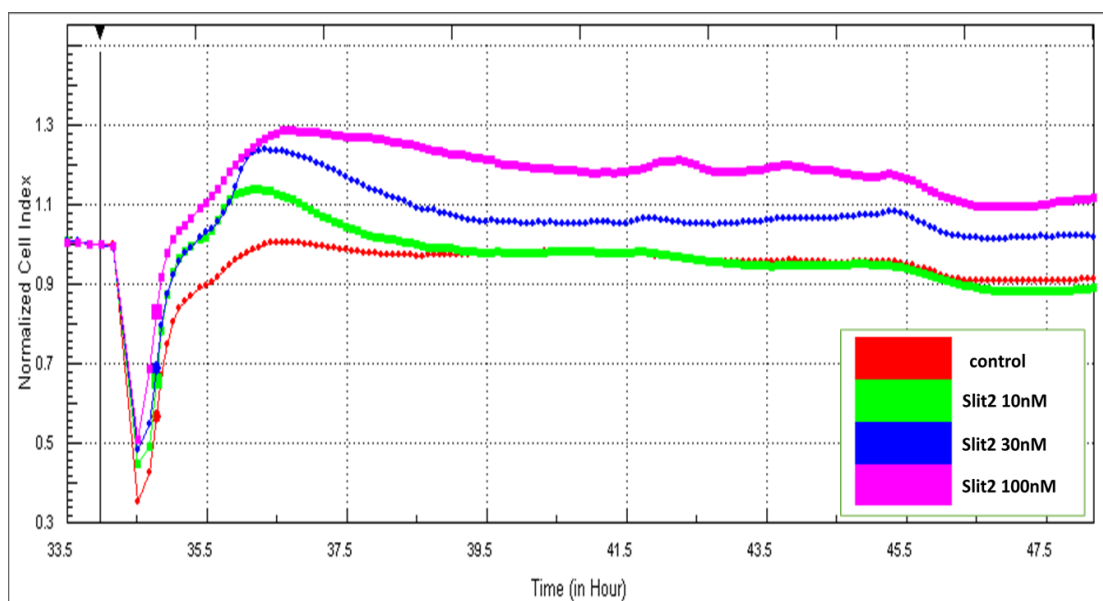


Figure 72. **Slit2 dose-dependent response.** TEEI measurements of HUVEC monolayer treated with Slit2 100nM, 30nM and 10nM. The graph is representative (n=1) of the real-time graph produced by RTCA iCELLigence System and shows the difference in treatment as % of control.

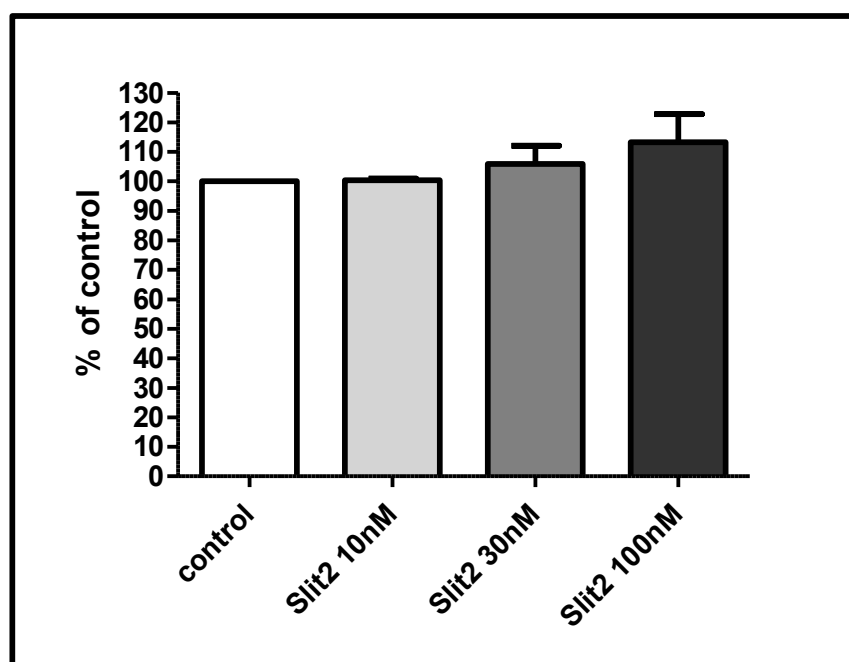


Figure 73. **Slit2 dose-dependent response.** TEEI measurements of HUVEC monolayer treated with Slit2 10nM, 30nM and 100nM. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control. Data are presented as mean $\pm$ SD % of control (n=3) and statistically analysed using Kruskal-Wallis non-parametric test.

3.2.4.7.5. HUVEC treated with TNF- α and Slit2

When the cells reached a plateau they were treated with Slit2 30nM for 1 hour, after which TNF- α 0.1ng/ml was added. The data were obtained over a 5-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The Slit2 and TNF- α treatments are showed as a change in TEEI compared to control. The treatment with Slit2 30nM alone increased the impedance to $113.59 \pm 6.31\%$ of control. The treatment with TNF- α 0.1ng/ml alone decreased the impedance to $88.65 \pm 9.05\%$ of control. The treatment with Slit2 30nM and TNF- α 0.1ng/ml increased the impedance to $93.44 \pm 8.59\%$ of control. None of the treatment caused statistically significant change as analysed by Kruskal-Wallis non-parametric test.

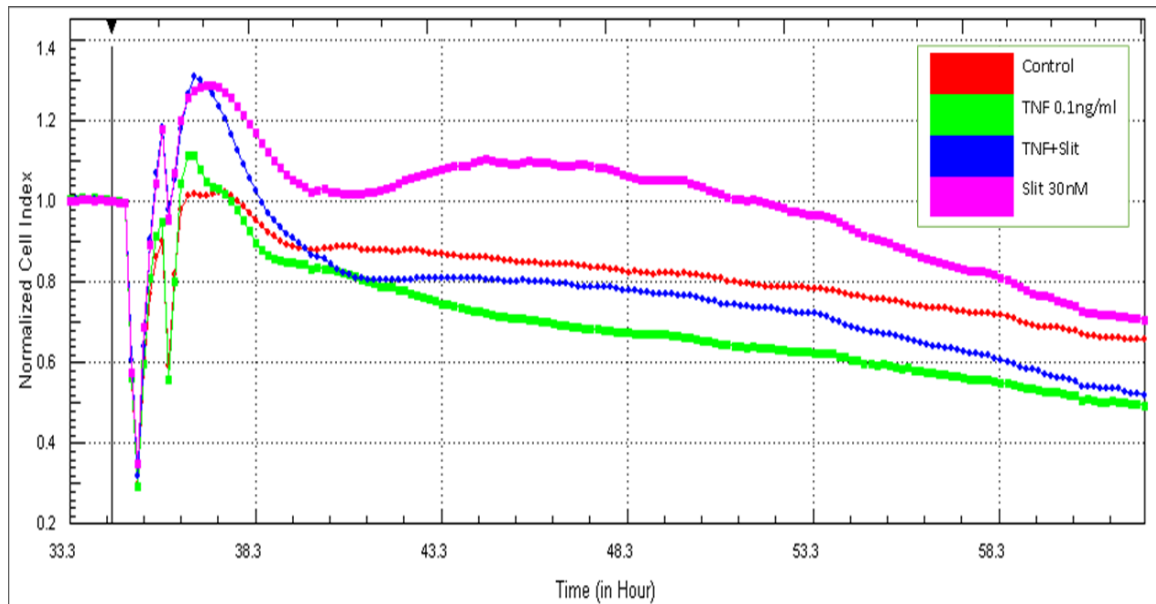


Figure 74. **TEEI measurements of HUVEC treated with TNF- α and Slit2.** HUVEC monolayer was treated with TNF- α 0.1ng/ml and Slit2 30nM. The graph is representative (n=1) of the real-time graph produced by RTCA iCELLigence System and shows the difference in treatment as % of control.

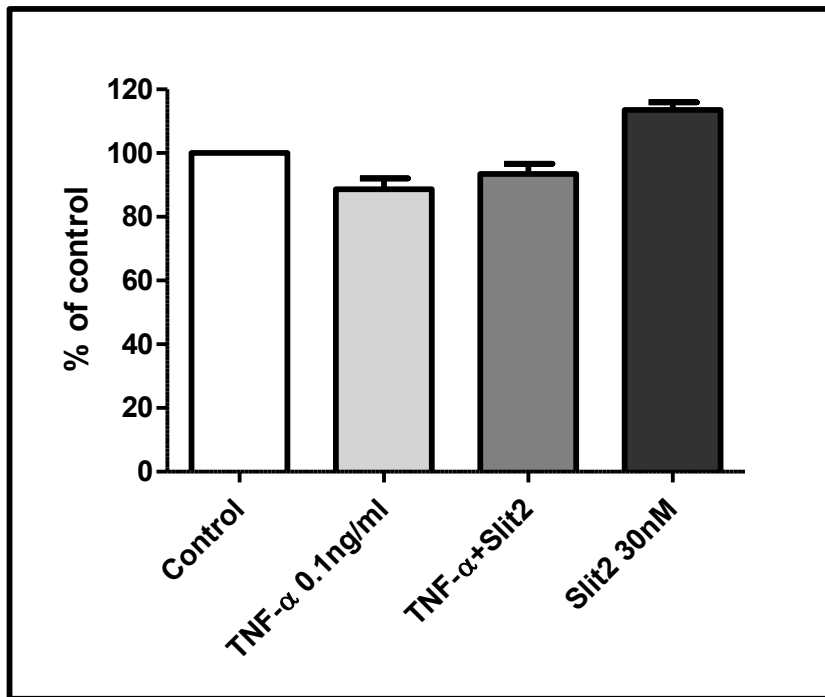


Figure 75. **TEEI measurements of HUVEC treated with TNF- α and Slit2.** TEEI measurements of HUVEC monolayer treated with TNF- α 0.1ng/ml and Slit2 30nM. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control. Data are presented as mean $\pm$ SD % of control (n=7) and statistically analysed using Kruskal-Wallis non-parametric test.

3.2.4.7.6. HPAEC seeding density

TEEI was measured in wells seeded with 4 000, 6 000, 8 000 and 12 000 cells as it can be seen in Figure 76. Seeding density of 12,000 cells per well was chosen.

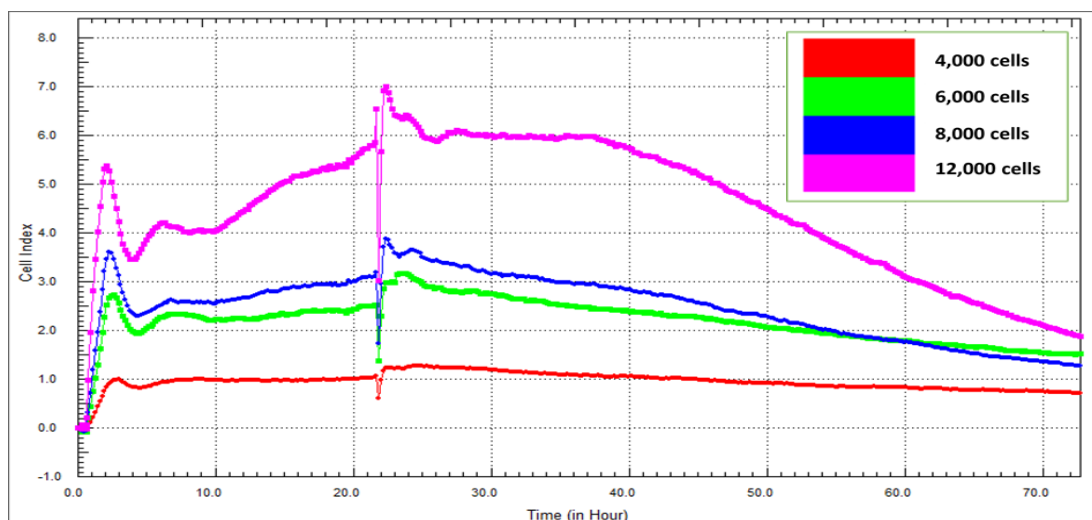


Figure 76. **HPAEC seeding density.** This graph is a real-time graph produced by RTCA xCELLigence System.

3.2.4.7.7. HPAEC treated with TNF- α and Slit2

When the cells reached a plateau they were treated with Slit2 30nM for 1 hour, after which TNF- α 0.1ng/ml was added. The data were obtained over a 5-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The Slit2 and TNF- α treatments are showed as a change in TEEI compared to control. The treatment with Slit2 30nM alone decreased the impedance to $95.86 \pm 6.42\%$ of control. The treatment with TNF- α 0.1ng/ml alone decreased the impedance to $66.16 \pm 9.04\%$ of control which was, compare to the control, statistically significant

($p>0.05$) as analysed by one-way ANOVA. The treatment with Slit2 30nM and TNF- α 0.1ng/ml decreased the impedance to $67.52\pm9.86\%$ of control.

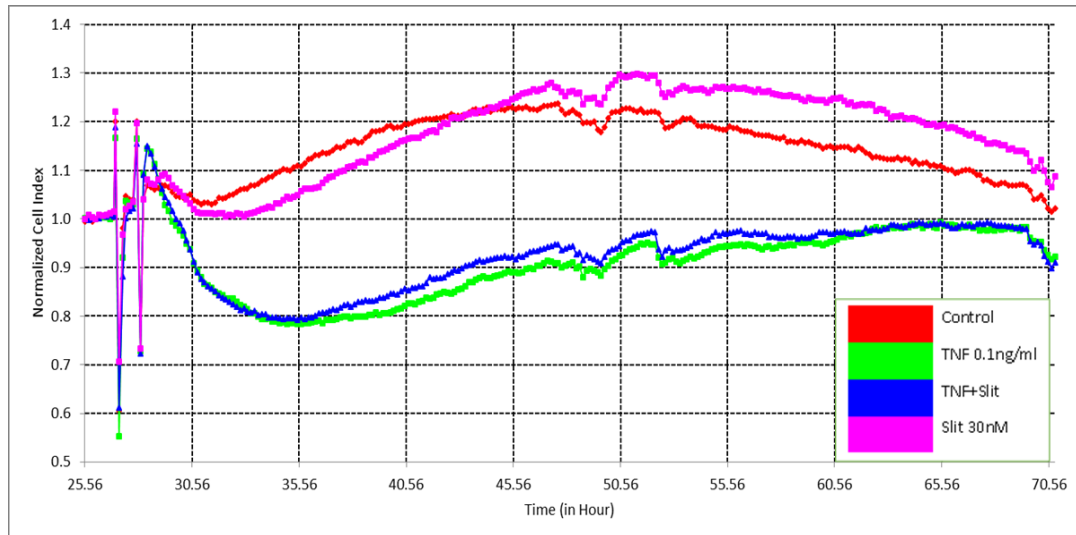


Figure 77. **TEEI measurements of HPAEC treated with TNF- α and Slit2.** HPAEC monolayer was treated with TNF- α 0.1ng/ml and Slit2 30nM. The graph is representative ($n=1$) of the real-time graph produced by RTCA xCELLigence System and shows the difference in treatment as % of control.

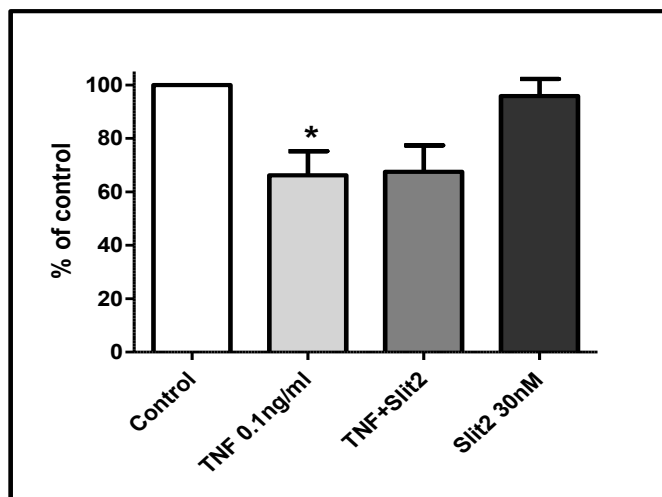


Figure 78. **TEEI measurements of HPAEC treated with TNF- α and Slit2.** HPAEC monolayer was treated with TNF- α 0.1ng/ml and Slit2 30nM. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control. Data are presented as mean $\pm$ SD % of control ($n=3$) and statistically analysed using one-way ANOVA.

3.3.4.8. siRNA knockdown of Robo4

3.2.4.8.1. siRNA knockdown of Robo4 expression in HUVEC

The number of cells expressing Robo4 externally was 55.43% $\pm$ 20.20. After the si-RNA knockdown treatment, the number of cells expressing Robo4 externally, decreased to 9.67% $\pm$ 3.29. The expression of extracellular Robo4 has significantly ($p=0.0498$) decreased in si-RNA knocked down cells.

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 2190.0 $\pm$ 215.0 in untreated cells. After the treatment the levels of intensity of Robo4 decreased to 1981.0 $\pm$ 1001.8.

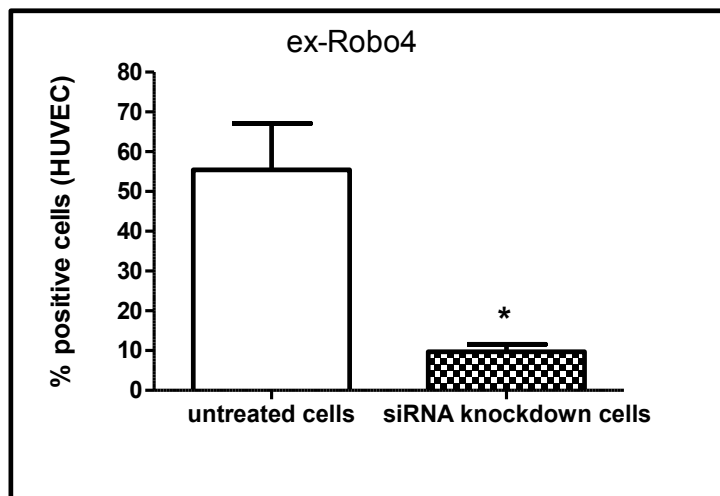


Figure 79. Si-RNA knockdown of Robo4 expression on HUVEC. Cells were transfected with si-RNA knockdown reagent for 20 minutes and then incubated for 72 hours. The expression of external Robo4 has decreased significantly ($p=0.0498$) after the treatment. The data were analysed by t-test and are expressed as mean $\pm$ SD ($n=3$).

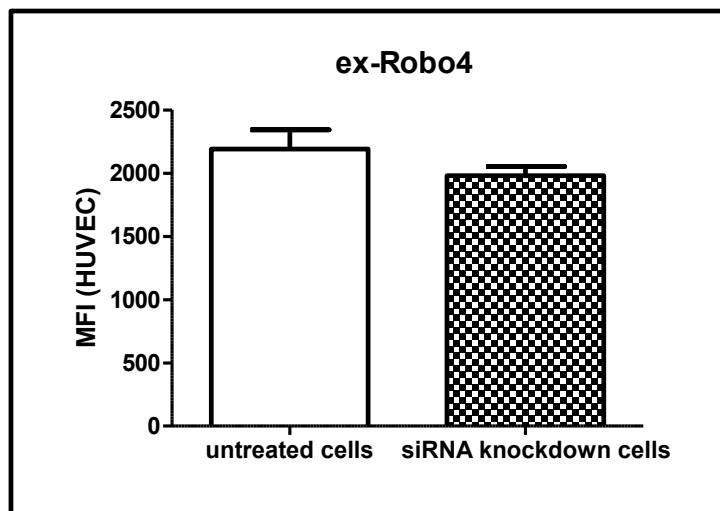


Figure 80. Median Fluorescence Intensity of Si-RNA knockdown Robo4 expression on HUVEC. Cells were transfected with si-RNA knockdown reagent for 20 minutes and then incubated for 72 hours. The MFI of external Robo4 has decreased from 2190 to 1981 after the si-RNA knockdown treatment. The data are expressed as mean $\pm$ SD (n=2).

3.2.4.8.2. siRNA knockdown of Robo4 expression in HPAEC

The number of cells expressing Robo4 externally was 61.95% $\pm$ 14.35. After the si-RNA knockdown treatment, the number of cells expressing Robo4 externally, decreased to 22.65% $\pm$ 5.02.

The levels of intensity of Robo4 expression per cell, determined by median fluorescent intensity (MFI) was 3513.5 $\pm$ 430.6 in untreated cells. After the treatment the levels of intensity of Robo4 decreased to 2712.0 $\pm$ 99.0.

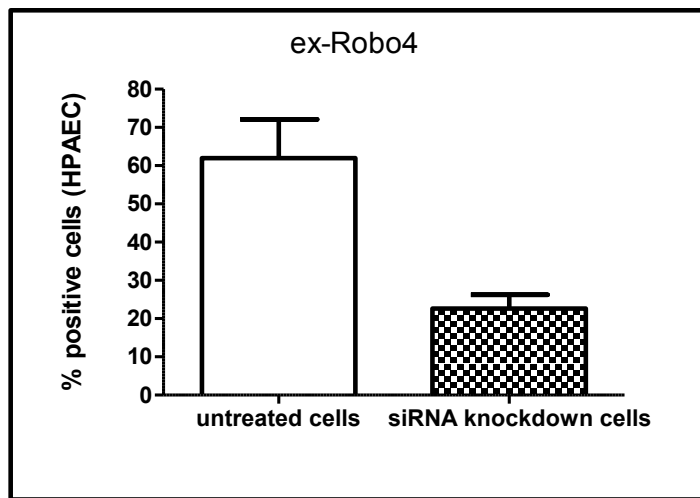


Figure 81. Si-RNA knockdown of Robo4 expression on HPAEC. Cells were transfected with si-RNA knockdown reagent for 20 minutes and then incubated for 72 hours. The number of cells expressing Robo4 externally has decreased after the treatment from 62% to 23%. The data were are expressed as mean $\pm$ SD (n=2).

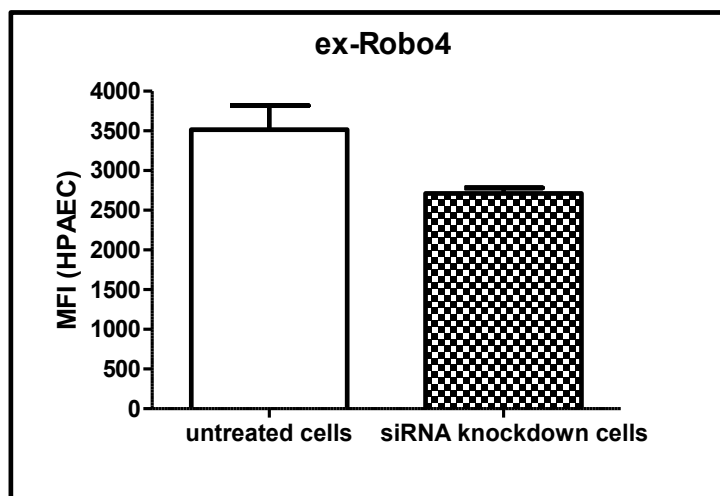


Figure 82. Median Fluorescence Intensity of Si-RNA knockdown Robo4 expression on HPAEC. Cells were transfected with si-RNA knockdown reagent for 20 minutes and then incubated for 72 hours. The MFI of external Robo4 has decreased from 3513 to 2712 after the si-RNA knockdown treatment. The data are expressed as mean $\pm$ SD (n=2).

3.2.4.8.3. si-RNA Robo4 knockdown in HUVEC treated with TNF- α and Slit2

HUVEC were seeded into xCell wells and incubated at 37°C. After 22 hours the cells were transfected with si-RNA knockdown reagent for 5 hours and after medium change left to grow until they reached a plateau. When the cells reached a plateau they were treated with Slit2 30nM for 1 hour, after which TNF- α 0.1ng/ml was added. The data were obtained over a 5-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die).

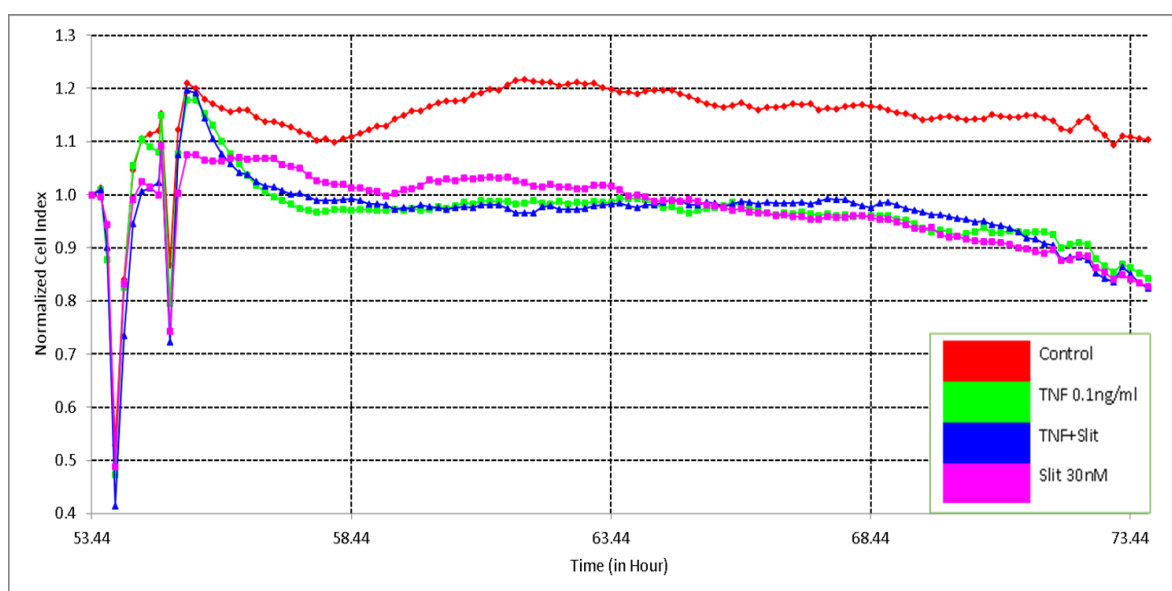


Figure 83. **si-RNA Robo4 knockdown in HUVEC treated with TNF- α and Slit2.** TEEI measurements of si-RNA Robo4 knockdown HUVEC monolayer treated with TNF- α 0.1ng/ml and Slit2 30nM. The graph is representative (n=1) of the real-time graph produced by RTCA xCELLigence System and shows the difference in treatment as % of control.

The untreated cells were set as a control (100%). The Slit2 and TNF- α treatments are showed as a change in TEEI compared to control. The treatment with TNF- α 0.1ng/ml alone decreased the impedance to 82.24% of control. The treatment with Slit2 30nM and TNF- α 0.1ng/ml decreased the impedance to 81.95% of control. The treatment with Slit2 30nM alone decreased the impedance to 83.92 % of control. Flow cytometry was used to show that KD did not kill my cells. The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit was used to determine the viability of the cells. The gates were then set in a way that all dead cells were excluded from the final results. Unfortunately, it was not possible to do more than n=1 experiment, because the Slit2 used throughout the project was changed by the manufacturer and was no longer bioactive in my experiments.

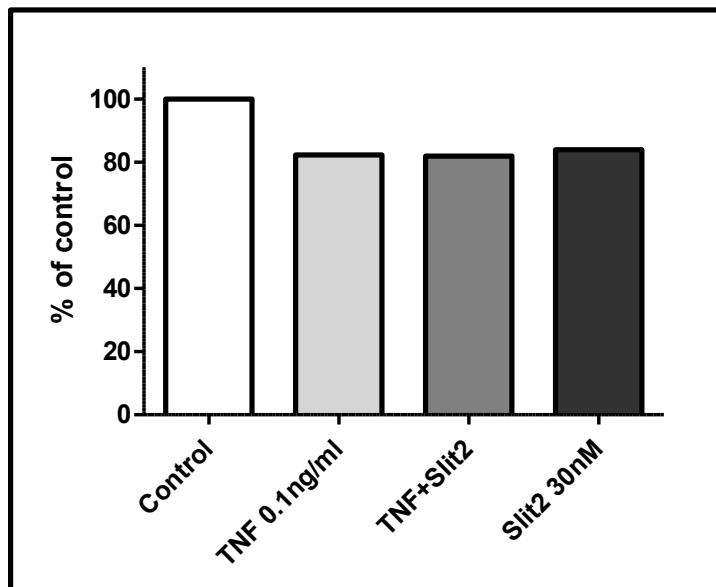
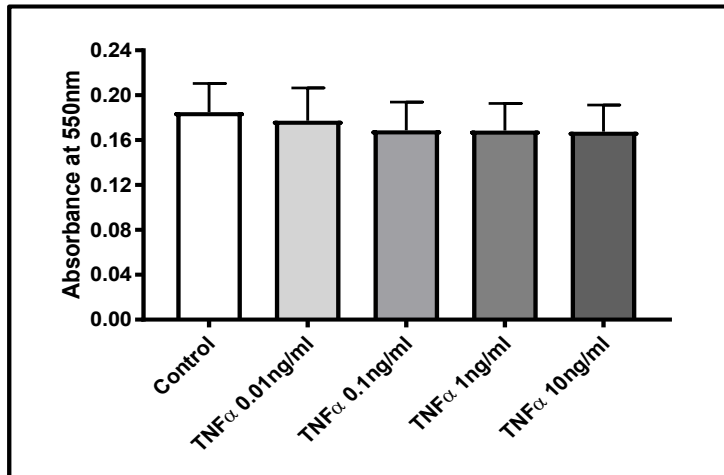


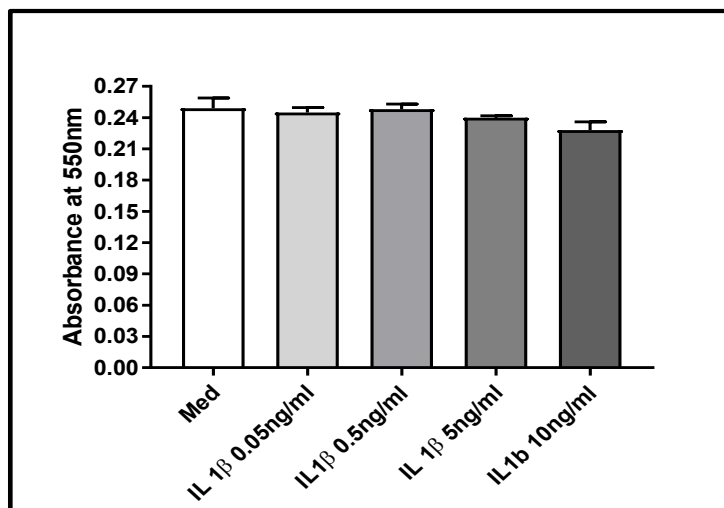
Figure 84. **si-RNA Robo4 knockdown in HUVEC treated with TNF- α and Slit2.** TEEI measurements of si-RNA Robo4 knockdown HUVEC monolayer treated with TNF- α 0.1ng/ml and Slit2 30nM. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control (n=1).

3.4.4.8.4. Treatment toxicity

All treatments were tested for their toxicity to cells using MTT method. The treatments used in this project did not cause a significant damage to treated cells when compared to untreated control.



MTT for HUVEC treated with different concentrations of TNF-α for 24 hours. The treatment with TNF-α concentration 0.01, 0.1, 1 and 10ng/ml (n=9) have not caused a significant damage to the cells.



MTT for HUVEC treated with different concentrations of IL-1β for 24 hours. The treatment with IL-1β concentration 0.05, 0.5, 5 and 10ng/ml (n=3) have not caused a significant damage to the cells.

3.2.5. Discussion

3.2.5.1 Expression of RAGE and TLRs

Initially, the aim of this thesis was to determine whether there was sufficient RAGE expression on HUVEC to enable the use of these endothelial cells in studies to investigate interactions between RAGE activation and Robo4 expression and function. The first step was to establish a reliable method that measures RAGE expression in/on endothelial cells. Flow cytometry method was employed to compare four different lots of HUVEC from two different suppliers. Extracellular expression on untreated cultured HUVEC was low, in range between 3.2% and 4.5% in three independent experiments for each cell lot. Gong and colleagues used flow cytometry to measure RAGE expression on the surface of circulating endothelial cells in healthy children. Their results showed that 17.7% of EC expressed RAGE extracellularly (Gong et al., 2012). Their RAGE expression results were higher than our results. This suggests either inherent differences between circulating endothelial cells and HUVEC; or perhaps a difference related to the age of the endothelial cells donor vs. HUVEC. Our umbilical vein cells were much younger compared to their cells. Given our understanding that RAGE expression can increase with age, this might be a plausible explanation.

The second step was to choose which cells should be used for the on-going project. The extracellular RAGE expression on four tested batches of HUVEC was similar. However, intensity (MFI) was greater in pooled HUVEC from Lonza. These cells were subsequently selected for the barrier integrity studies investigating Robo receptors. However, they were not suitable to be used for RAGE study, as less than 5% of them expressed the

receptor externally. When the cells were treated with TNF- α , the RAGE expression did not change significantly.

The cells were also tested for TLRs expression. Toll-like receptors (TLRs) play an important role in the tissue injury and inflammation. It has been proposed that RAGE and TLRs regulate immune and inflammatory responses together (Ibrahim et al., 2013). Both RAGE and TLRs are pattern recognition receptors (PRRs). They recognise pathogen-associated molecular patterns (PAMPs) and through binding to them, start immune responses leading to inflammation (Gao et al., 2008). RAGE and TLRs also share common ligands, such as HMGB and S100A8/9, and co-operate in the host immune responses. RAGE also interacts with intracellular adaptors TIRAP and MyD88 which are used by TLRs to activate downstream signalling pathways (Ibrahim et al, 2013). RAGE co-operation with TLRs intensifies inflammatory signalling. It is not known if RAGE and TLR interact directly at the receptor level, but it is possible as both are able to form homodimers and heterodimers (Gasiorowski et al, 2018).

The TLRs expression was similar to RAGE expression; where MFI was significantly higher in cells from Lonza than PromoCell. Our findings were consistent with other published data. In particular, Faure et al showed that vascular endothelial cells express more TLR4 than TLR2 (Faure et al., 2001). Likewise, our pooled HUVEC (Lonza) expressed significantly higher ($p < 0.005$) TLR4 than TLR2.

3.2.5.2. Expression of Robo receptors and their ligand Slit2

Robo4 expression on endothelial cells was originally identified by bioinformatic data mining, and then validated by immunohistochemistry tests (Huminiński et al., 2002).

Once the method, Flow cytometry, was established in this study as suitable to measure expressions on and in endothelial cells, Robo4 external and internal expression in HUVEC was measured. The results showed that most cells, around 95%, expressed Robo4 externally. In inflammation, the release of cytokines, such as TNF- α , disrupts the endothelial barrier through displacement of VE-cadherin, causing hyper-permeability. Slit2-Robo4 interaction prevents dissociation of p120-catenin from VE-cadherin; promotes its localisation in the cell junction and protects the endothelial barrier integrity (London et al., 2010). Treating the EC with pro-inflammatory cytokine TNF- α caused significant loss of external Robo4; only 64% of cells expressed the receptor after the treatment. The mechanisms of how TNF- α decreases Robo4 expression on EC is not known. We hypothesise that the Robo4 gets shed or cleaved by/or in response to TNF- α , possibly through the nuclear factor (NF)- κ B activation. This shed Robo4 is then detectable in blood circulation. This fits in with our findings in plasma samples where the levels of Robo4 after operation and suspected endothelial barrier damaged, increased. There have been investigations done into Robo1, but not Robo4, shedding. Recently it was shown that extracellular juxta-membrane region is involved in Robo1 shedding. Seki and colleagues found that Robo1 cleavage site in HEK293 cells is Gln888–Gln889 at the extracellular juxta-membrane region (Seki et al, 2010). It is possible that a similar mechanism might exist for Robo4.

For comparison, external Robo4 was measured in HPAEC and HCAEC. The number of HCAEC expressing Robo4 was 80% and treatment with TNF- α decreased the expression to 68% of cells. Robo4 was externally expressed on 58% HPAEC and TNF- α treatment lowered the expression significantly to 29%. We based the high expression of external Robo4 at the top of importance for our endothelial model, as we were to investigate the role of Robo4 in endothelial function and dysfunction. In HUVEC almost 95% of cells expressed the receptor of our interest. The high expression, together with the fact that HUVEC was used in many published endothelial studies, was a deciding factor for using HUVEC as our endothelial model.

Only about a third of the tested HUVEC expressed Robo4 internally and treatment with TNF- α did not change this expression significantly. All HUVEC (100%) expressed Robo1 internally. The downstream effects of Slit/Robo signalling depends on both, the extracellular and intracellular environments. Robo1 has four intracellular domains CC0, CC1, CC2 and CC3, and Robo4 has two domains CC0 and CC2. Robo1 may serve as a co-receptor for Robo4 to transduce the Slit signal. The Robo1 intracellular domain confers a downstream signal by recruiting various factors and has a number of phosphorylatable sites present within the intracellular domains (Ballerd and Hinck, 2012).

External expression of Robo1 on HUVEC was only 7%. TNF- α treatment caused an increase of Robo1 expression to 16%. The results show that Robo1 does not get shed in response to pro-inflammatory cytokine in the same way as Robo4. Contrary, the treatment encourages the endothelial cells to express more Robo1. For comparison, Robo1 expression was measured on HPAEC. Much higher number of cells (74%)

expressed Robo1 externally. The treatment with TNF- α showed, as it did in HUVEC, an increase of Robo1 expression (93%).

Slit2 was externally expressed on a large number of HUVEC (93%). Internal expression of Slit2 was even higher at 99%. A treatment with TNF- α did not change the expression of Slit2 in/on HUVEC.

3.2.5.3. Release of Robo4

Firstly, HUVEC and HPAEC were treated with IL-1 β 10ng/ml for 6, 12 and 24 hours. After the treatment the medium was collected and levels of released Robo4 were measured by ELISA. The cytokine caused an increase in levels of Robo4. The longer the treatment was left on, the higher the release of Robo4 was. At 6 hours the release from HUVEC was 1.8ng/ml, at 24hrs the release was 2.8ng/ml. At 6 hours the release from HPAEC was 1.7ng/ml, at 24hrs the release was 3.3ng/ml. Secondly, HUVEC and HPAEC were treated with IL-6 for 6, 12 and 24 hours and levels of Robo4 released into medium were measured. The longer the treatment was left on, the higher the release of Robo4 was. At 6 hours the release from HUVEC was 1.6ng/ml, at 24hrs the release was 2.5ng/ml. At 6 hours the release from HPAEC was 2.1ng/ml, at 24hrs the release was 3.3ng/ml. The Robo4 release in response to IL-1 β and IL-6 was similar in both cell types and was not connected to Robo4 expression on cells – in HUVEC 95% cells expressed Robo4 externally, in HPAEC only 58% cells expressed Robo4 externally. Thirdly, HUVEC and HPAEC were treated with TNF- α and Slit2 at the same time to see if Slit2 makes a difference to Robo4 release. In both cell types Slit2 increased Robo4 release from

untreated cells, as well as cells treated with different concentrations of TNF- α at 3, 6 and 12hrs. The results suggest that there might be a mechanism by which Slit2 encourages Robo4 release. It has been suggested that Robo1/Slit binding might lead to Robo1 shedding. There have been investigations into Robo1, but not Robo4, shedding. Recently it was shown that extracellular juxta-membrane region is involved in Robo1 shedding. Seki and colleagues found that Robo1 cleavage site in HEK293 cells is Gln888–Gln889 at the extracellular juxta-membrane region (Seki et al, 2010). It is possible that a similar mechanism could exist for Robo4.

3.2.5.4. Release of GM-CSF

It was essential to test that Slit2, used in this project, was bioactive. This was done by measuring Granulocyte macrophage-colony stimulating factor (GM-CSF) release from HUVEC treated with TNF- α and Slit2. GM-CSF is produced by many types of cells, including endothelial cells, in response to inflammatory stimuli. Lenhoff and Olofsson published data on GM-CSF secretion by HUVEC in response to stimulation with TNF- α and other cytokines (Lenhoff and Olofsson, 1996). In both of our experiments with TNF- α 1ng/ml and 10ng/ml, HUVEC secreted GM-CSF. Simultaneous incubation with Slit2 reduced significantly ($p < 0.05$) the GM-CSF release. However, the inhibition by Slit2 30nM on TNF- α 1ng/ml was less than on TNF- α 10ng/ml; whilst this finding is not what might be expected, the results were consistent in all nine experiments. A possible explanation is that higher concentrations of TNF- α might enhance Slit2 binding or signalling, perhaps as part of a feedback mechanism to protect the endothelium. Indeed,

previous experiments in our lab showed that TNF- α caused an increase in Slit2 mRNA in HUVEC (Chowdhury et al., 2014). Zhao and colleagues used lipopolysaccharide (LPS) to induce inflammation in HUVEC to study Slit2/Robo binding. They found that Slit2 treatment inhibited LPS-induced inflammation, but also upregulated Slit2 and primary microRNA (mir-218-1) expression and downregulated Robo1 expression. Their findings suggest that there is a positive-feedback regulation of Slit2 expression, and Slit2 treatment can regulate Robo1 receptor expression through miR-218 (Zhao et al, 2014).

3.2.5.5 Establishing *in vitro* model

It was necessary to establish a reliable *in vitro* method of assessing endothelial barrier integrity. Two different approaches were employed: (1) measuring passage of molecules (horseradish peroxidase) through endothelial barrier using permeability assay; and (2) measuring trans-endothelial electrical impedance on a confluent endothelial monolayer.

3.2.5.5.1 The permeability assay: The effects of TNF- α and Slit2

For the first method, permeability assay, a protocol from a group at the University of Utah led by Professor Dean Y. Li (Jones et al., 2008) was adapted for this project. Whilst there are no data published on TNF- α /Slit2 treatment on HUVEC, Jones and his colleagues showed an effect of Slit2 on murine lung endothelial cells barrier damaged by VEGF-165. Their results showed that Slit2-Robo4 binding suppressed the hyper-permeability (*in vitro* and *in vivo*) caused by VEGF-165 (Jones et al, 2008). Also, *in vitro*

experiments by the same group showed that Slit2 reversed LPS, TNF- α . and IL-1 β induced permeability increases in mouse lung microvascular endothelial cells (London et al., 2010). By contrast, in our experiment, treatment with 10nM Slit2 did not show a significant reduction in permeability of TNF- α -treated cells. However, because of the variation (20%) between our experiments, it is not possible to say whether this lack of effect was due to experimental limitations, or whether differences in cell type (HUVEC vs lung microvascular endothelial cells) in some way contributed. It was decided not to use this methodology in this project.

3.2.5.5.2. Trans-endothelial electrical impedance: The effects of TNF- α and Slit2

The second method, suitable to assess endothelial barrier, was measuring trans-endothelial electrical impedance (TEEI). This was done using the real-time cell electronic sensing system iCELLigence and/or xCELLigence from ACEA. Biosciences, Inc. Electrical impedance increases as the cells grow and attach to the sensor device (plates with gold microelectrodes). The electric current is very weak (22 mV) and does not harm the cells (Sener, 2017). This method was used on HUVEC by Li et al, who investigated endothelial damage caused by high levels of glucose (Li et al., 2006). By using iCELLigence measurements, we were able to show that 0.1ng/ml TNF- α caused a decrease in impedance across HUVEC monolayer; whereas 30nM Slit2 increased impedance, i.e. increased the barrier function of these cells. These results suggest a possible role of Slit2 in limiting the damage caused by TNF- α . According to Jones and his colleagues Slit2 binds to Robo4 and blocks VEGFR2 signalling pathways, and by this prevents EC monolayer

damage and consequent vascular leak. The exact signalling pathways of Robo4 and Slit2 in endothelial permeability are currently unknown. Slit-2 binds to all four Robo receptors. Binding to Robo-1 promotes angiogenesis, binding to Robo4 blocks VEGF-induced angiogenesis and vascular permeability and repairs the damaged endothelium (Acevedo et al., 2008; Jones et al, 2008). However, Robo4 lacks a Slit2 binding site (Enomoto et al, 2015). It had been shown previously that Slit2 did not directly bind Robo4 and the involvement of other Robo receptors - in particular Robo1 - was postulated (Sheldon et al, 2009). A role for ADP-ribosylation factor 6 (Arf6) was also suggested in Slit2/Robo signalling. Jones et al showed in their experiments that Slit2 blocks Arf6 activation in response to VEGF-165 (permeability-inducing receptors) to regulate vascular leak (Jones et al, 2009). We also observed an effect of 30nM Slit2 on the basal endothelial monolayer. These results could suggest, either that Slit2 encourages the growth of endothelial cells; and/or that the cells junctions are 'tightened', possibly through maintenance/mobilisation of VE-cadherin to cell junctions. The same experiments with TNF- α and Slit2 were performed on HPAEC monolayer using xCELLigence. Those results showed that, same as in HUVEC, TNF- α decreased TEER across HPAEC monolayer. However, unlike with HUVEC, Slit2 did not increase electrical impedance. It would seem that Slit2 has a different role in HUVEC compared to HPAEC. It has been shown that endothelial cells that are derived from different vascular beds differ in structure and function (Aird, 2006).

3.2.5.6. siRNA knockdown of Robo4

Small (or short) interfering RNA (siRNA) was used to temporary silence protein coding for Robo4 gene. A siRNA protocol was established in our department for HCAEC and it was adapted for the use on HUVEC and HPAEC. Flow cytometry was used to assess the effect of the gene knockdown. The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit was used to determine the viability of the cells and the gates were then set in a way that all dead cells were excluded from the final results. The results showed a significant knockdown from 55.4% to 9.7% cells expressing Robo4 externally on HUVEC; and from 62.0% to 22.7% cells expressing Robo4 externally on HPAEC. When the experiment with TNF- α and Slit2 treatment on HUVEC monolayer was repeated with siRNA knockdown HUVEC, it showed that the Slit2 was not able to increase electrical impedance as it did in cells which were not siRNA knockdown. This proved the importance of Robo4 in repair of damaged endothelial barrier by Slit2.

3.2.5.7. HUVEC treated with IL-1 β , IL-6 and TNF- α

Three cytokines commonly used in experiments assessing endothelial barrier function, IL-1 β , IL-6 and TNF- α , were tested for their effect on HUVEC monolayer. IL-6 has a dual-function as a pro-inflammatory and anti-inflammatory cytokine (Chaundry et al, 2013). It inhibits the release of IL-1 and TNF- α and increases the release of anti-inflammatory cytokines like IL-10 and cortisol (Schulte et al, 2013). In our experiments IL-6 showed to damage the endothelial barrier the least. Even the highest concentration of 10ng/ml decreased impedance by only 1.5%. On the other hand, IL-1 β which is a strong pyrogenic

pro-inflammatory cytokine (Schulte et al, 2013), caused the most damage to the endothelial barrier. The highest concentration of 10ng/ml lowered electrical impedance by almost 40%. TNF- α , which is a pyrogenic pro-inflammatory cytokine, caused the decrease in impedance by almost 30% at its highest concentration of 10ng/ml.

3.2.6. Limitations

It was with regret that I was not able to repeat TEEI measurements of si-RNA Robo4 knockdown HUVEC monolayer treated with TNF- α and Slit2. I had only n=1 for this important experiment which showed that Slit2 was not able to increase electrical impedance in si-RNA Robo4 knockdown cells and demonstrated the importance of Robo4 in repair of damaged endothelial barrier by Slit2. Recombinant human Slit2-N produced by a company PeproTech Inc (USA) was used for all *in vitro* experiments on endothelial cells in this project. Towards the very end of the project the company replaced Lot #1110 465 with Lot #1013 465 and they also changed the buffer in which to reconstitute the protein. Unfortunately, after the change the Slit2 was no longer bioactive in the barrier integrity assay and therefore it was not possible to finish all experiments as planned.

In addition, if there was more time and money, it would have been most interesting to run experiments with glycocalyx components syndecan-1 and heparan sulfate in our established model of endothelial barrier. There are still some controversies about Robo4/Slit2 binding, especially as there is no apparent binding site on Robo4 to bind Slit2.

Robo1 is implied to be instrumental in Robo4/Slit2 binding as well as syndecan-1 and heparan sulfate which might act as secondary receptors to enhance Robo4/Slit2 binding.

3.2.7. Summary

Robo4 external expression decreased in endothelial cells treated with TNF- α . Plasma levels of Robo4 increased post-surgery. Our findings suggest that Robo4 might be cleaved or secreted by endothelial cells exposed to inflammation and released into the bloodstream. Rapid removal of Robo4 from the cell surface could possibly act as a first, fast response to trauma, i.e. to release the protective check on barrier function. Slit2 increased barrier integrity of endothelial cells, *in vitro*, although effects on TNF α -treated cells were less apparent. Whilst Slit2 is a potential novel therapeutic agent for protecting endothelial barrier function in human disease, its efficacy could be compromised by concomitant down-regulation of Robo4, under inflammatory conditions. The shedding of Robo4 means that there is less Robo4 available for Slit2 to bind to and this might contribute to difficulties in the repair of the endothelial damage.

4. THESIS SUMMARY

4.1. Thesis Discussion

The aim of this thesis was to advance our understanding about the protective mechanisms associated with endothelial barrier function and how these mechanisms are altered as a consequence of cardiac surgery with snCPB.

Endothelial barrier dysfunction contributes to the systemic inflammatory response in patients undergoing cardiac surgery with cardiopulmonary bypass. The bypass enables to perform complicated operations on static heart, but it comes with risks such as a damage to the endothelium which causes inflammation. Activation of endothelial cells by inflammatory mediators leads to changes in endothelial function. These changes include up-regulation of adhesion molecules, production and secretion of inflammatory mediators and chemo-attractant agents, as well as vasoactive compounds. These changes are central in the pathological processes leading to sepsis syndrome. The exaggerated release of pro- and anti-inflammatory cytokines leads to development of septic and vasoplegic syndromes which complicate recovery and can even cause a death. The aim was to learn what could be done to prevent endothelial barrier dysfunction and therefore improve post-surgery recovery and long term outcome and quality of life. Currently, there are no specific therapies that target the endothelium, but greater insight into the pathogenesis of endothelial barrier dysfunction could lead to new biomarkers and therapies.

Roundabout receptor 4 could be a novel biomarker and target for specific pharmacological intervention. Roundabout receptor 4 was originally identified as an axon guidance molecule but is now known to mediate various endothelial processes including cell migration and proliferation. At the outset of this thesis a role for Roundabout receptor 4 and its Slit 2 glycoprotein ligand as regulators of endothelial barrier function were also beginning to emerge; but there was little evidence of how Roundabout receptor 4 expression profiles and function might differ between endothelial cells; or of implications of Roundabout receptor 4 detected in human plasma.

The aim was to investigate the potential of Robo4 as a plasma biomarker of endothelial damage in a group of 48 patients undergoing cardiac surgery with snCPB in the Royal Brompton Hospital in London. To be able to explore the potential of Robo4 as a biomarker, it was necessary to assess its plasma levels in context of other biomarkers of endothelial damage – well established markers Ang-2 (Siner et al, 2009) and vWF (Tsang et al, 1998); and a novel endothelial markers Syndecan-1; and other markers of inflammation, RAGE and S100A8/A9 that were previously research by this research lab (Creagh-Brown et al, 2013; Rasiah et al, 2016). The standard biomarkers of endothelial activation, Ang2 and vWF, significantly increased 24h after surgery. These results were consistent with previously published studies on cardiac surgery patients (Clajus et al, 2012; Panagiotopoulos et al, 2010). By contrast, Roundabout receptor 4 increased immediately post-operatively, as did another novel biomarker of endothelial

dysfunction - Syndecan-1, RAGE and S100A8/A9 and return to basal levels by 24h post-operatively. These results suggest that Robo4 could be a useful early marker of endothelial dysfunction in this patient group. The raised post-operatively levels of Robo4 also significantly correlated with the overall length of stay in hospital as well as the ICU lengths of stay.

To investigate the results further, the patients were grouped as with and without SIRS, Sepsis and Vasoplegia to assess whether Robo4, RAGE and S100A8/A9, levels were higher in patients who developed these syndromes. There was no significant difference between patients who did and did not develop SIRS, Sepsis or Vasoplegia for RAGE and S100A8/A9 results. Robo4 plasma levels were also similar in patients with and without SIRS and Sepsis. However, there was a significant difference in preoperative levels of Robo4 in patients who did and did not developed Vasoplegia. Higher pre-operative plasma levels of Roundabout receptor 4 were also associated with the development of vasoplegia, a post-operative complication. Vasoplegia is caused by an imbalance of vasodilator and vasopressor mechanisms in the body, but the exact pathophysiology is not known. Vasoplegic patients are treated with high and prolonged doses of vasoconstrictor medications. This treatment can cause peripheral vasoconstriction and ischemia of limbs that can lead to metabolic acidosis and tissue necrosis. These results suggest that Robo4 could be a potentially useful pre-operative indicator of patients in risk of developing post-operatively Vasoplegia.

In the second section of this thesis, investigations into Robo4 and its ligand Slit2 *in vitro* were carried out to investigate their effects on endothelial barrier disruption in response to inflammatory stimuli, and whether Robo4 could become a potential therapeutic target for reducing endothelial barrier dysfunction. HUVEC were chosen for the *in vitro* studies because it was published that Robo4 was detected in HUVEC supernatant (Huminiecki et al, 2002), so we knew that the receptor was present in/on HUVEC. Firstly the expression of EC receptors Robo4, Robo1, RAGE, TLR2, TLR4 and TLR 9 were measured and their expression on HUVEC was compared to expression on HPAEC and HCAEC. The results showed that there was a high expression of Robo4 on endothelial cells – 95% of HUVEC, 80% of HCAEC and 58% of HPAEC expressed Robo4. Unfortunately the expression of RAGE was very low. Only between 3.2-4.5% of EC expressed the receptor, therefore, the original aim to explore a possible links and interactions between RAGE and Robo4 in inflammation could not have been investigated in this thesis.

All three types of tested EC were treated with inflammatory stimuli TNF- α , and the results showed that the Robo4 expression decreased after the treatment, showing that this inflammatory cytokine clearly was detrimental to Robo4. HUVEC Robo4 expression decreased from 95% to 64%, HCAEC from 80% to 68% and HPAEC from 58% to 29%. Other cytokines involved in inflammation and sepsis were also used to investigate what damage they would cause to Robo4. Cytokines IL-1 β and IL-6 were used to treat HUVEC and HPAEC and then Robo4 levels were measured in cell supernatants. The Robo4 release in response to IL-1 β and IL-6 treatment was similar in both types of EC. Next,

HUVEC and HPAEC were treated with TNF- α and Slit2 (Robo4 ligand) to see if Slit2 caused or prevented Robo4 release. In both types of EC Slit2 increased Robo4 release from untreated cells as well as cells treated with TNF- α , suggesting that there might be a mechanism by which Slit2 encourages the release of Robo4.

To further investigate the effect on Slit 2 on endothelial barrier, an *in vitro* model of assessing barrier integrity was established – measuring the electrical impedance across the endothelium. The results showed that TNF- α caused a decrease in impedance across HUVEC monolayer, and Slit2 increased impedance, i.e. increased the barrier function of these cells. These results suggest a role of Slit2 in limiting the damage caused by TNF- α . We also observed that Slit2 increased impedance on basal endothelial monolayer. These results might suggest, either that Slit2 encourages the growth of endothelial cells; and/or that the cells junctions are ‘tightened’, possibly through maintenance/mobilisation of VE-cadherin to cell junctions. For comparison, the same experiment was done on HPAEC and showed that TNF- α also caused a decrease in impedance, but Slit2 did not increase the impedance as it did in HUVEC. This suggest that there might be a different role for Slit2 in endothelial damage in different cell types. It has been previously shown that endothelial cells that are derived from different vascular beds differ in structure and function (Aird, 2006).

To confirm a role of Robo4/Slit2 in endothelial barrier integrity, siRNA knock down method was used to silence protein coding for Robo4 gene in HUVEC and a same

experiment with TNF- α and Slit2 was performed. This time, Slit2 was not able to increase the electrical impedance, i.e. was not able to repair the damaged endothelial barrier as it did before, because of the lack of Robo4.

In summary, Robo4 external expression decreased in EC treated with inflammatory cytokines. Robo4 plasma levels increased after cardiac surgery with snCPB. These results suggest that Robo4 might be cleaved off or secreted by endothelial cells exposed to inflammatory cytokines and released into the bloodstream. Given that there was a significant increase in plasma levels immediately post-operatively, it implies that Robo4 might act as a first, fast response to trauma, i.e. to release the protective check on barrier function. It might have been beneficial to the study, to measure Robo4 and other markers of endothelial damage in patients who underwent cardiac surgery without the need of a cardiopulmonary bypass. Results as such would make it clearer whether the endothelial damage was caused by the cardiopulmonary bypass or the surgical procedure *per se*. Robo4 ligand Slit2 increased barrier integrity of endothelial cells, *in vitro*. Our results of Slit2 levels in plasma (not presented in this study) showed, in a very small number of samples, an increase immediately post-operatively suggesting that these two, Robo4 and Slit2, work together to decrease the endothelium barrier damage in patients undergoing cardiac surgery with snCPB. The finding from these investigations inform greater understanding of the important role of Robo4 as an endothelial biomarker and its role in barrier function.

4.2. Thesis Conclusions

These investigations provide evidence supporting the role of Robo4 as a biomarker of endothelial dysfunction following cardiac surgery with cardiopulmonary bypass and as a possible therapeutic target to reduce the consequences of endothelial barrier dysfunction. Whilst these are important implications for patients under-going cardiac surgery world-wide, they also have ramifications well beyond this field and are likely to impact of other conditions and syndromes associated with endothelial barrier dysfunction. Further investigations are required to gain an even greater understanding of the role of Robo4, both cell associated and soluble forms, in diseases linked to endothelial dysfunction, before pharmacological agents that could promote barrier function could be considered in these conditions.

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6. APPENDIX

6.1. Ethics Approval

Royal Brompton & Harefield
NHS Foundation Trust

Research Office
Level 2, Chelsea Wing
Royal Brompton Hospital
Sydney Street
London SW3 6NP

9th May 2012

Biomedical Research Unit (BRU) Advanced Lung Disease (ALD) Biobank Research Project

Dr Gregor Quinlan
Senior Research Fellow
Critical Care and Sleep and Ventilation
National Heart and Lung Institute
Sydney Street
London

Dear Dr Quinlan

Project Title: Ageing and the maladapted innate immune response: implications for recovery from Cardiac Surgery
RB&HFT reference: MG10

Thank you for informing the R&D office about your study. We note that this study has been approved by the Respiratory Biomedical Research Unit (BRU) Heads of Consortia and will therefore be included under the Ethical Approval given to the BRU Advanced Lung Disease (ALD) Biobank (NRES reference 10/H0504/9).

Your project is using biological specimens collected by or stored within the BRU ALD Biobank and we are satisfied that you have met all the necessary requirements of the Human Tissue Act (2004) and the Human Tissue Authority codes of practices. You should ensure that Good Laboratory Practice and associated Health and Safety rules are followed in the use of these specimens.

The following documents concerning your project have been received:

Number	Document title	Date
1	BRU Registration Form	17/03/10
2	Biobank Access Form 1	02/08/10
3	Lay Summary	

You are required to keep the Research Office updated with any changes to your study (e.g. Specimens leaving the Trusts HTA licensed sites or changes to the Research Team).

Yours sincerely


Angela Cooper
Associate Director of Research

6.2. Summary of pro and anti-inflammatory cytokines involved in sepsis.

Cytokine	Cell Origin	Inflammatory Properties	Main Function
IL-1 β	Monocytes, macrophages	Pro-inflammatory	Fever, coagulation, hematopoiesis, apoptosis
IL-6	T-cells, macrophages, endothelial cells	Pro and anti-inflammatory	Acute phase response, fever, hematopoiesis,
IL-8	Macrophages, Endothelial and Epithelial Cells	Pro-inflammatory	Chemotaxis
IL10	T-cells, B-cells, monocytes	Anti-inflammatory	Inhibiting the release of pro-inflammatory cytokines
TNF- α	Macrophages, lymphocytes, fibroblasts, NK cells, T-cells	Pro-inflammatory	Fever, coagulation, cachexia, apoptosis
GM-CSF	T-cells, endothelial cells, mast cells, macrophages, fibroblasts,	Pro and anti-inflammatory	Cell growth, monocyte and granulocyte development
MCP-1	Fibroblasts, smooth muscle cells, endothelial cells	Pro-inflammatory	Leukocytes infiltration, chemotaxis

Table. Summary of pro and anti-inflammatory cytokines involved in sepsis. The table was adapted from Schulte et al, 2013 and Chaudhry et al, 2013 and lists cytokines that are involved in the development of septic syndrome.

6.3. Summary of the literature on Robo and Slit in endothelial permeability

Table 2 summa the seminal papers concerning Robo4 and/or Slit2 – published between 2015 and 2018. It shows that Robo4 and/or Slit2 are extensively studied in many conditions associated with endothelial dysfunction, such as diabetic retinopathy, brain trauma, inflammation and cancer.

Medical condition	Model	Results	Study/year
Inflammation	Animal study : mouse model Cell culture: HUVEC, U937	Robo4 regulates cytokine production in inflammation. Robo4-Unc5B signalling promotes IL-6 production by EC and monocytes.	Shirakura et al, 2018 (Japan)
Diabetic retinopathy	Cell culture: HREC	Hyperglycaemia-induced upregulation of Robo4 is mediated by enhanced transcription of transcription factor SP1.	Xie et al, 2017 (China)
Surgical brain injury	Animal study: rat model	Slit2 administered 1 h before injury increased BBB junction proteins expression (occludin and claudin 3), reduced BBB permeability, and decreased neurodeficits 24 h post-SBI	Sherchan et al, 2017 (USA)
Diabetic cerebral neovascularisation	Animal study: rat model Cell culture: BMVEC	Robo4 overexpression inhibited VEGF-induced angiogenic signalling and decreases cerebral neovascularisation caused by diabetes.	Abdelsaid et al, 2017 (USA)
Inflammation	Animal study : mouse model Cell culture: HUVEC , HCAEC	TNF α increased Robo4 expression in mice and in human primary ECs via NF- κ B activation (through 2753 and 2220 NF- κ B-binding motifs)	Tanaka et al, 2017 (Japan)
Ovarian hyperstimulation syndrome	Cell culture: HUVEC and LGC	Stimulation of HUVECs with Slit2 increased CDH5 (cadherin 5) and CLDN5 (claudin 5) decreased permeability. Stimulation of HUVEC with VEGF suppressed CDH5 and CLDN5 gene expression and increased permeability.	Bekes et al, 2017 (Germany)
Mechanical strain	Cell culture: ESC-derived embryoid bodies (CCE line)	Treatment of embryoid bodies with 10% static mechanical strain increased CD31-positive vascular structures and the angiogenesis guidance molecules plexinB1, ephrin B2, neuropilin1, semaphorin 4D and Robo4 ; as well as VEGF, fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor-BB.	Sharifpanah et al, 2017 (Germany)

Astroglial Scar	Animal study: rat model	Slit2 and Robos (Robo1, 2 and 4) expression was weak at the acute phase of astroglial scar, so it is unlikely that they mediate astroglial proliferation in response to ischemic insult. Slit2 and Robos were strongly expressed in astrocytes forming the glial scar (later stage), this indicates Slit2/Robos role in astroglial hyperthrophy and glial scar formation.	Jin et al, 2017 (South Korea)
Diabetic retinopathy (DR)	Animal study: mouse model	Robo4 inhibits angiogenesis and vessel permeability in DR independently of its cytoplasmic domain.	Zhang et al, 2017 (USA)
Avian influenza H7N9 IAV	Animal study: mice model	Cytokines produced in response to H7N9 IAV disrupt the endothelial barrier resulting in vascular leak and build-up of fluid in the alveolar space. 1 to 3 dpi, the expression of Slit2 and Robo4 in infected group was lower than in control. There might be a mechanism of IAVs to inhibit the Slit2–Robo4 signalling pathway. After 3dpi there was an increase of Slit2 and Robo4 expression. Binding of Slit2 with Robo4 enhances vascular barrier function by enhancing VE-cadherin.	Yu et al, 2016 (China)
Diabetic retinopathy (DR)	Cell culture: HREC	Under hyperglycemic conditions Robo4 expression increased. Under hypoxic conditions Robo4 expression decreased. Under combined hypoxic and hyperglycemic conditions Robo4 expression decreased.	Xie et al, 2016 (China)
Proliferation and migration	Cell culture: MASMC	The mRNA and protein expressions of Slit2, Slit3, Robo1 and Robo4 were detected in MASMC. Exogenous Slit3 promotes proliferation and migration of MASMC in vitro.	Ni et al, 2016 (China)

Carotid artery injury	Animal study: Rat carotid artery balloon injury model	There was a time-dependent up-regulation of mRNAs encoding Slit2, Slit3, Robo1, Robo2, and Robo4 in injured carotid artery. The early up-regulation of Robo4 may be driven by the acute inflammatory response and the increased expression of cell adhesion molecules; the later up-regulation of Robo4 may be driven by the re-endothelialization process.	Liu et al, 2016 (USA)
Cancer (Liver, Kidney, Lung)	Serum from patients with hepatocellular, renal cell and non-small cell lung cancer	During anti-angiogenic treatment pro-angiogenic cytokines are upregulated. Levels of CEC and CEP are increased and levels of TEM (Robo4 , ECSCR, Clec14) are decreased. Loss of vascular stabilization factors (Robo4) could promote therapy resistance.	Pircher et al, 2016 (Austria)
Placental angiogenesis	Cell culture: HUVEC , HPMSC	Expression levels of Robo1 and Robo4 in EC decreased with increasing doses of Slit2 . Slit2 secreted by HPMSC was an important modulator for Robo1/4 mediated migration and angiogenesis.	Chen et al, 2016 (Taiwan)
Ischemic Brain Injury	Animal study: murine model of transient forebrain ischemia	Slit2 was induced in reactive astrocytes in the CA1 region of the hippocampus following ischemic injury. Slit2/Robo4 signalling could be a contributing factor in transformation of astrocytes to the glial scar in response to ischemic injury.	Park et al, 2016 (South Korea)
Cancer (Breast)	Animal study: mice model Cell culture: breast cancer cell line E0.1 and E0.2	SecinH3, a small molecule drug which deactivates ARF6 downstream of Robo4, can enhance Robo4 signalling and thus inhibit breast cancer growth and metastasis. Small doses of SecinH3 would be better treatment strategy than large doses of Slit2 .	Zhao et al, 2015 (USA)

Migration	Cell culture: HUVEC	Slit2 /Robo1 signalling intrinsically exerts a migratory effect on HUVEC, and Robo4 regulates the effect. The migration of HUVECs therefore depends on the Robo1/Robo4 ratio. (Robo4 lacks Slit2 binding site.)	Enomoto et al, 2015 (Japan)
Herpetic Stromal Keratitis	Animal study: mice model	Herpes simplex virus type 1 (HSV-1) causes immunoinflammatory lesion stromal keratitis. Robo4 administration enhances the anti-angiogenic host feedback mechanism to reduce HSV-1 induced angiogenesis. Slit2 does not bind to Robo4 . Extracellular domains of Robo4 interact with the vascular-specific axon guidance receptor UNC5 β to counteract VEGF signalling.	Gimenez et al, 2015 (USA)
Gene regulation	Cell culture: HCAEC	Activator protein-1 (AP-1) is a transcription factor consisting of proteins belonging to c-Jun and c-Fos families. AP-1 binds to a TPA response element (TGA (C/G) TCA) by forming either Jun/Jun complex or Jun/Fos complex. AP-1 complexes c-Jun/c-Jun and c-Jun/Fra-1 regulate Robo4 gene expression. AP-1 binding could be regulated by DNA methylation and contribute to Robo4 promoter activation during EC differentiation from mesodermal cells.	Okada et al, 2015 (Japan)
Fibroblast Migration	Cell culture: HEK293FT	Slit2-Robo4 -srGAP2 signalling network is a key regulator of contact inhibition of locomotion in fibroblasts.	Fritz et al, 2015 (Switzerland)
Cancer (Bladder)	Animal study: mice model. Cell culture: human bladder cancer T24 cells.	In cancer tissue and cancer T24 cells, Robo1 expression was higher than Robo4 ; Robo2 and Robo3 were not expressed. In mice, tumour growth was smaller in Robo1 group than in Robo4	Li et al, 2015 (China)

	Tissue: human bladder carcinoma tissue	group. Intra-tumoural injections of Robo1 and Robo4 inhibited the tumour growth.	
Ovary Development	Animal study: hens' model. Culture: follicles from chicken ovaries.	In cultured follicles the expression levels of Slit1, 2, 3 and Robo1, 2, 3, 4 genes were hormonally regulated by activin A and/or inhibin A. Chicken Slit/Robo pathways differ from that of mammals.	Qin et al, 2015 (China)
Cancer (Lung)	Cell Culture: HUVEC and conditioned medium from lung adenocarcinoma cells	There are two splicing variants of Slit2 at exon 15, Slit2-WT and Slit2-deltaE15. Non-tumor lung cells express Slit2-deltaE15. Lung tumor cells express Slit2-WT. During tumor development, the expression of Slit2 switches from Slit2-deltaE15 to Slit2-WT. Slit2-deltaE15, but not Slit2-WT, inhibits growth of tumor cells.	Yang et al, 2015 (Taiwan)
Cancer (Brain)	Human glioma tissue and normal brain tissue	Robo4 was significantly upregulated in glioma tissues compared with normal brain tissues. Robo4 expression was co-localized with CD34 expression. High expression of Robo4 in glioma EC was directly associated with greater microvessel density in gliomas. Survival of patients with Robo4 high expression was shorter than that of patients with Robo4 low expression.	Cai et al, 2015 (China)
Diabetic retinopathy	Cell culture: HREC, HDMEC; Tissue: FVM from type 2 DM patients	Robo4 is positively regulated by HIF-1a in HDMEC in vitro (under normoxic and hypoxic conditions). Overexpressed HIF-1a is responsible for the upregulation of Robo4 expression.	Tian et al, 2015 (China)
Cancer (Leukaemia)	Cell culture: BMMC; Tissue: Bone marrow biopsy	Robo4 expression was elevated in Acute myeloid leukaemia patients. Patients with higher Robo4 expression had shorter disease-free survival and overall survival.	Chen et al, 2015 (Taiwan)

Transplantation	Cell culture: lungs, kidney, or bone marrow mice EC	Vascular EC actively promote Hematopoietic stem cells (HSC) translocation across vessel walls into the bone marrow (BM) space. Vascular barrier formation is supported by Robo4 and can be manipulated by VEGF or histamine. Tipping this balance in either direction would favour HSC location to either the blood for HSC harvesting or to the BM to promote HSC engraftment upon transplantation.	Smith-Berdan et al, 2015 (USA)
Inflammation in pregnancy	Cell culture: HPTC; Tissue: Human placenta	Obese pregnancies are characterised by increased inflammation and lower levels of Slit2. Pro-inflammatory mediators IL-1b, TNF- α and LPS decreased Slit2 expression in HPTC. Slit2 mediates its anti-inflammatory effects through binding to Robo1 that is expressed in placental trophoblast cells.	Lim et al, 2015 (Australia)
Cancer (Lung)	Cell culture: 293T and Lewis lung carcinoma cells; Animals: mouse cancer model	Antibodies targeting VEGFR2 or Robo4 both retard tumour growth and angiogenesis. Antibodies to VEGFR2 caused extensive toxicity; those to Robo4 were without adverse effects. Limitations: human and mouse IgG subclasses are not evolutionary homologues and may not function in the same way.	Zhuang et al, 2015 (UK)
Cancer (Brain)	Cell culture: HBMEC; Tissue: Human glioma tissues	Slit2/Robo4 inhibits VEGF-165-mediated angiogenesis by inhibiting the activity of VEGFR2-mediated PI3K/AKT and FAK signal pathways. Slit2/Robo1 inhibits glioma cell migration and invasion by inactivation of Cdc42-GTP.	Cai et al, 2015 (China)

Cancer (Brain)	Cell culture: HCMEC/D3, human astrocytes and human brain vascular pericytes; Tissue: Human gliomal tissues	Slit2/Robo4 reduces blood-tumor barrier permeability by down-regulating the expression of p-Src, p-Erk1/2, and MMP-9 and upregulating EC tight junction proteins (occludin, ZO-1 and claudin-5) expression and distribution. The Src/Erk1/2/MMP-9 signal pathways are involved in this process.	Cai et al, 2015 (China)
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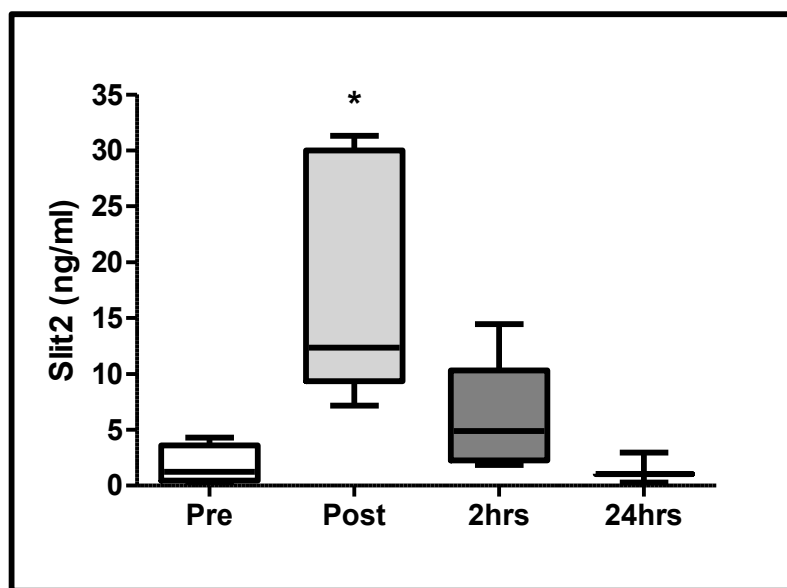
Table. Summary of the literature on Robo and Slit in endothelial permeability published between 2015 and 2018.

Abbreviations used: EC = endothelial cells; HREC = Human retinal EC; BMVEC = Brain microvascular EC; HUVEC = Human umbilical vein EC; HCAEC = Human coronary artery EC; LGC = Luteinized granulosa cells; HREC = Human retinal EC; MASM = mouse aortic smooth muscle cells; HPMSC = Human placental multipotent mesenchymal stromal cells; HDMEC = human dermal microvascular EC; HPTC = Human Primary Trophoblast Cells; HBMEC = Human Brain Microvascular EC; HCMEC = Human cerebral microvascular EC, ESC = embryonic stem cells; BMMC = Bone marrow mononuclear cells; HEK = Human Embryonic Kidney cells; H7N9 IAV = H7N9 influenza A virus;

6.4. Plasma levels of Slit2 increase immediately after surgery

Preoperative levels of Slit2 were 1.23 (0.45-3.60) ng/ml, immediately post-operatively the levels were 12.34 (9.35-29.99) ng/ml, 2 hours post-operatively the levels were 4.88 (2.25-10.30) ng/ml and 24 hours post-operatively the levels were 1.02 (0.27-2.96) ng/ml.

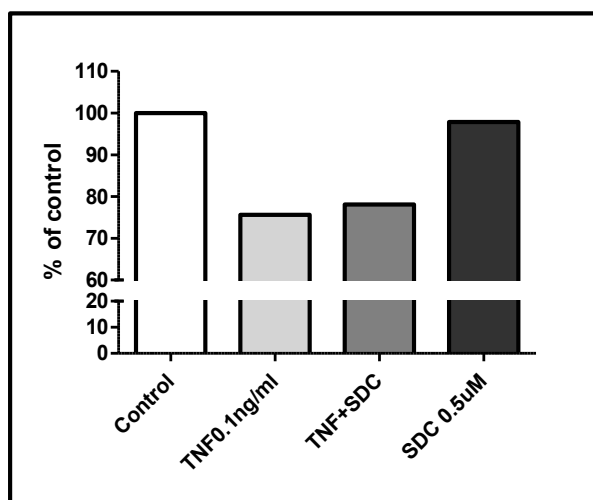
Levels of Slit2 have significantly ($p < 0.05$) increased immediately after the operation compare to preoperative levels. At 2 hours post-operatively the Slit2 levels decreased although there were still higher than at preoperative. At 24 hours post-operatively the Slit2 decreased significantly ($p < 0.05$) compare to immediately post-operatively.



Plasma levels of Slit2. Levels of Slit2 were measured in all four time-points: pre ($n=6$), post ($n=6$), 2hrs post ($n=5$) and 24hrs post snCPB ($n=3$). Data were statistically analysed by ANOVA and Kruskal-Wallis non-parametric test and are expressed as median (IQR) ng/ml; $p < 0.001$ is expressed as three asterisks; $p < 0.01$ is expressed as two asterisks.

6.5. HUVEC treated with TNF- α and Syndecan-1

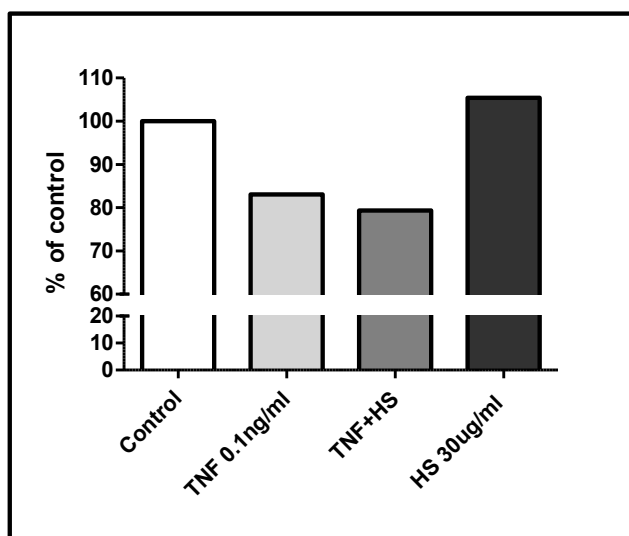
When the cells reached a plateau they were treated with syndecan-1 0.5 μ M for 1 hour, after which TNF- α 0.1ng/ml was added. The data were obtained over a 7.5-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The syndecan-1 and TNF- α treatments are showed as a change in TEEI compared to control (% of control). The treatment with syndecan-1 0.5 μ M alone decreased the impedance to 97.89% of control. The treatment with TNF- α 0.1ng/ml alone decreased the impedance to 75.61% of control. The treatment with syndecan-1 0.5 μ M and TNF- α 0.1ng/ml increased the impedance to 78.11% of control.



TEEI measurements of HUVEC monolayer treated with TNF- α 0.1ng/ml and Syndecan1 0.5 μ M. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control (n=1) over 7.5 hours, measured by RTCA xCELLigence System.

6.6. HUVEC treated with TNF- α and Heparan Sulfate

When the cells reached a plateau they were treated with Heparan Sulfate (HS) 30ug/ml for 1 hour, after which TNF- α 0.1ng/ml was added. The data were obtained over a 6-hour period after the TNF- α treatment, when the untreated cells (control) were in plateau, a state of little or no change, before starting to lift off (die). The untreated cells were set as a control (100%). The HS and TNF- α treatments are showed as a change in TEEI compared to control (% of control). The treatment with HS 30ug/ml alone increased the impedance to 105.38% of control. The treatment with TNF- α 0.1ng/ml alone decreased the impedance to 83.07% of control. The treatment with HS 30ug/ml and TNF- α 0.1ng/ml decreased the impedance to 79.32% of control.



TEEI measurements of HUVEC monolayer treated with TNF- α 0.1ng/ml and Heparan Sulphate (HS) 30ug/ml. The Cell Index was normalised just before the treatment was added. The graph shows the difference in treatment as % of control (n=1) over 6 hours, measured by RTCA xCELLigence System.