Diabetes-associated Proinflammatory Mediators: The Influence on Tie Receptor Signalling in Endothelial Cells.

PhD in Vascular Pathology awarded by De Montfort University

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Statement

The work published in this literature is the sole work of the author, unless indicated by acknowledgement or reference to published literature. The contents of the work have only been submitted for the degree of PhD in Vascular Pathology at De Montfort University.

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Abbreviations

Abbreviation	Definition
$\tilde{x}$	Median
ABIN-2	A20-binding inhibitor of NF-κB-2
AGE	Advanced Glycation End-Products
AKT	Serine/Threonine Protein Kinase
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Ang-3	Angiopoietin-3
Ang-4	Angiopoietin-4
ANOVA	Analysis of Variance
ApoE-/-	Apolipoprotein E Knockout
ARDS	Acute Respiratory Distress Syndrome
ASK1	Apoptosis Signal-Regulating Kinase 1
BMI	Body Mass Index
CCD	Coiled-coil domain
cDNA	Complementary DNA
COVID-19	SARS-Cov-2
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
CRDs	Cysteine-Rich Domains
CRP	C-Reactive Protein

Abbreviation	Definition
DMO	Diabetic Macular Oedema
Dok-R	Docking Protein
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas-Associated Death Domain
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FOXO1	Forkhead Box O1
FReD	Fibrinogen-related domain
GGT	Gama Glutamyl-Transferase
GLUT4	Glucose Transporter Type 4
GPCR	G-Protein-Coupled Receptor
Grb2	Growth Factor Receptor-Bound Protein 2
HbA_{1c}	Glycated Haemoglobin
HDMEC	Human Dermal Microvascular Endothelial Cells

Abbreviation	Definition
HMCEC	Human Microvascular Coronary Endothelial Cells
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule 1
IG	Immunoglobulin-like
IgG	Immunoglobulin G
IKK	IκB Kinase
IL	Interleukin
IL-1	Interleukin-1
IL-1R	Interleukin-1 Receptor
INF-γ	Interferon-γ
iNOS	Inducible Nitric Oxide Synthase
IQGAP1	IQ Motif Containing GTPase Activating Protein 1
IRAK	Interleukin-1 Receptor-Associated Kinases
JNK	c-Jun N-terminal Kinase
kDa	Kilo-Dalton

Abbreviation	Definition
LOX-1	Lectin-like Oxidised Low-Density Lipoprotein Receptor-1
LSGS	Low Serum Growth Supplement
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
mRNA	Messenger RNA
MYD88	Myeloid Differentiation Primary Gene 88
nAMD	Neovascular Age-related Macular Degeneration
Nck	Non-catalytic Kinase
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOS3	Nitric Oxide Synthase 3
NSAID	Non-Steroidal Anti-Inflammatory Drugs
$ONOO^-$	Peroxynitrate-Mediated Mechanism
PAI-1	Plasminogen Activator Inhibitor-1
PAK1	P21 Activating Kinase
PAMPs	Pattern Associated Molecular Patterns

Abbreviation	Definition
PBS	Phosphate Buffered Saline
PGI_2	Prostaglandin
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PTB	Phosphotyrosine Binding
PTF	Pentoxifylline
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RAGE	Receptor for Advanced Glycation End products
RAP1	Ras-Associated Protein-1
RIP	Receptor-Interacting Protein
RTK	Receptor Tyrosine Kinase
SCD	Super-clustering domain
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SGLT	Sodium-Glucose Co-Transporter
ShcA	SHC Adaptor Protein
Shh	Sonic Hedgehog

Abbreviation	Definition
Shp2/SHPTP2	Src homology-2 domain-containing protein tyrosine phosphatase-2
sTie-2	Soluble Tie-2
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Triton-X100
Tie	Tunica interna endothelial cell kinase
TIR	Toll/Interleukin Receptor
TNF	Tumour Necrosis Factor
TNFR1	Tumour Necrosis Factor Receptor 1
TNF-α	Tumour Necrosis Factor-α
TRADD	Tumor Necrosis Factor Receptor Type 1-Associated Death Domain
TRAF6	TNF Receptor-Associated Factor 6
TRPC	Transient Receptor Potential-Canonical
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VEPTP	Vascular Endothelial Protein Tyrosine Phosphatase
VSMC	Vascular Smooth Muscle Cells

Abstract

Vascular integrity is vital to maintain normal vascular function. Diabetes is a global issue which affects hundreds of millions of individuals, and if left untreated can lead to long-term vascular complications. Inflammatory mediators associated with diabetes, impact on the vasculature and can contribute to the development of atherosclerosis and coronary artery disease. Vascular stability is controlled by several signalling pathways, however, the Angiopoietin-1/Tie receptor pathway functions to serve multiple purposes that relate to the overall function of endothelial cells, in turn the function of the vasculature. Angiopoietins are a group of glycoproteins that bind exclusively to a family of receptor tyrosine kinase receptors known as Tie receptors. The functions of activated signalling, through Ang-1 binding to Tie-2, include vascular stability, vessel remodelling and enlargement, migration, and the mediation of anti-inflammatory processes. Ang-1 mediates its protective effect by activation the PI3K/AKT pathway. The ratio between the Tie receptors and their associated ligand, Ang-1, and Ang-2, are crucial regulators of the Ang-1/Tie-2/AKT signalling pathway that can cause various vascular diseases. Inflammatory mechanisms have been noted to have an impact on vascular dysfunctions, including the dysfunction in diabetic patients. IL-1β and TNF-α have been reported to be significant pro-inflammatory cytokines that affect the vasculature through the mechanisms of the immune response.

The aim of this study is to analyse the impacts of diabetes-associated inflammatory cytokines on the Ang-1/Tie signalling pathway. This was addressed by first profiling patient samples for cytokines and Ang-2, using RANDOX multiplex ELISA as well as traditional sandwich ELISA. The acute and chronic impact of IL-1β and TNF-α on Tie

receptor expression, and on Ang-1-induced PI3K/AKT activation, was examined in HUVECs using Western blot techniques. The functional impact of cytokine exposure on HUVECs was further analysed by performing a cell viability assay using a fluorescent LIVE/DEAD assay.

Profiling of TNF-α only showed an increase in type 2 diabetic patients. IL-6 analysis returned results of both type 1 and type 2 diabetic patients to have increase levels to that of the control. Ang-2 returned significant differences in between the volunteer groups. Type 1 diabetics were seen to have lower levels of Ang-2 and type 2 diabetics reported an increase in Ang-2 levels (*p = 0.039*).

Acute exposures to IL-β and TNF-α up until 3hours in HUVECs reported an increase in Tie-2 levels, with the addition of increase in Ang-1-induced Tie-2 phosphorylation. IL-1β further displayed increases in Ang-1-induced AKT activity. For results of acute exposures, the following time points displayed significance for IL-1β and TNF-α (IL-1β Tie-2 1hour *p = 0.045*; IL-1β Tie-2 2hour *p = 0.043*; IL-1β pTie-2 0.5hour *p = 0.001*; IL-1β pTie-2 4hour *p = 0.001*; TNF-α pAKT 1hour *p = 0.050*; TNF-α pAKT 4hour *p = 0.006*).

The chronic exposures of IL-1β up to 48hours displayed reduced Tie-2 levels, as well as decreases in AKT levels, however, significance was not observed. On the other hand, the activity of Ang-1-induced AKT was seen to show significant decreases in phosphorylation of AKT (3hour *p = 0.025*; 6hour *p = 0.014*; 24hour *p = 0.002*; 48hour *p = 0.002*). TNF-α displayed increase in Tie2 levels with decrease shown in AKT levels, though not significant. Similar to IL-1β, TNF-α display significant decreases in Ang-1-mediated AKT activity the longer the HUVECs were exposed to the cytokine (6hour *p = 0.033*; 12hour

p = 0.016; 24hour *p = 0.006*; 48hour *p < 0.001*). Both IL-1β and TNF-α displayed significant decreases in mature Tie-1 levels as well as a decrease in the ratio of mature: immature Tie-1 ($p \leq 0.05$).

The functional cell survival assay performed on HUVECs showed significant increase in cell death over the length of 24hours when incubated with IL-1β ($p = 0.007$). Even though not significant, HUVECs exposed to IL-1β in the presence of Ang-1 displayed an increase in cell death compared to cells treated with Ang-1 alone.

In conclusion, the increase in levels of TNF-α and IL-6 observed in certain diabetic patient samples, alongside the changes to the Tie receptors and Ang-1-induced AKT activity in HUVECs exposed to TNFα and IL-1β over a chronic period, suggests potential disruption to the Ang-1/Tie signalling pathway in disease conditions including diabetes and inflammatory diseases.

Publication and Presented Work

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Chapter 1: Introduction

1.1 Endothelial Function and Vascular Integrity

The human body relies on the vascular system for one of the vital survival functions, transport of oxygen around the body. The design of the vascular system is dependent on the normal function of endothelial cells (Pittman, 2011). Not only is the transport of oxygen a purpose of this system, but also the deliverance of nutrients, hormones, and other endogenous mediators to different parts of the body. The main three vessel types in the body are arteries; transporter of oxygenated blood away from the heart, veins; carrier of deoxygenated blood to the heart, and lastly capillaries; small vessels between arteries and veins that exchange gases, nutrients, and waste between the different tissues. Endothelial cells are a thin layer on the innermost part of blood vessels that are encompassed in connective tissue and smooth muscle (Alberts et al., 2002). While the endothelium is a monolayer, its ability to respond to both physical and chemical stress makes it quite a complex system. Homeostasis of this system relies controlling vascular tone, vessel wall inflammation and cellular adhesion (Deanfield, Halcox and Rabelink, 2007). Endothelial cells have mechanoreceptors that allow detection of shear stress from the flow of blood. This will allow the vessel to adapt the diameter and wall size to accommodate the blood flow (Jufri et al., 2015). This is resultant of biochemicals categorised as vasoactive mediators. Nitric oxide is an example of a mediator which causes the dilation of blood vessels through smooth muscle relaxation. On the contrary, endothelin-1 is a vasoconstrictor which will reduce the size of the vessel (Pearson, 2000; Alberts et al., 2002). The endothelium also links with the inflammatory response

allowing inflammatory related cells and chemokines to access sites of chemical or physical stress. Through stimulation of thrombin, inflammatory cytokines or bacterial endotoxins, adhesion molecules increase to allow leukocyte tethering before allowing it to migrate between the endothelial cells towards the site of infection/damage. Acute inflammation has two types of endothelial activation; type I is mediated by ligands binding to G-protein-coupled receptors (GPCR). Type I results in the synthesis of prostaglandin I_2 through arachidonic acid conversion via cyclooxygenase 1 (COX1) and nitric oxide production through nitric oxide synthase 3 activation (NOS3) (Pober and Sessa, 2007). Additionally, the activator of NOS3, calmodulin, also activates myosin-light-chain kinase to cascade in the attachment of actin filaments to tight and adheren junctions to promote the opening of the junctions to allow vascular leakage. Type II also occurs via GPCRs but become desensitised to prevent re-stimulation. The prostaglandin (PGI_2) is synthesised through COX2 however this process is stimulated via interleukin-1 (IL-1) or tumour necrosis factor (TNF) activation. In addition to this, actin filament reorganisation will open intracellular junctions to promote vascular leakage (Pober and Sessa, 2007).

Under chronic inflammation the activation of angiogenesis begins with the binding of ligands to receptor tyrosine kinases. One of the most well researched ligands of angiogenesis is vascular endothelial growth factor (VEGF). This process of this is to activate pathways like extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/ Serine/Threonine Protein Kinase (PI3K/AKT) to enhance translation of cell cycle proteins as well as promote cell survival. Angiopoietins are

another angiogenic protein that contributes to role of cell survival and endothelial cell function in inflammatory associated cases.

1.2 VEGF

Vascular Endothelial Growth Factor (VEGF) is a highly potent, pro-angiogenic factor which has been extensively studied in the field of angiogenesis. The role of VEGF begins during the embryonic stages of vessel development through differentiations of hemangioblasts to endothelial cells via vasculogenesis. Later in the human development, through processes of pregnancy, menstrual cycle and wound repair, the formations of new vessels from existing vasculature are impacted by VEGF through neoangiogenesis. While VEGF displays its role in the development of normal body function, it also plays a vital role in the development of pathological conditions including tumour growth and metastasis, inflammatory processes, ischaemic processes, and several other postnatal conditions. VEGF is a heterodimeric glycoprotein that is only 40kDa containing a cystine-knot motif and structure formed from bisulfide bonds. This glycoprotein binds to its associative tyrosine kinase receptor, VEGFR, on the extracellular domain. The activation through ligand binding causes phosphorylation of tyrosine residues which in turn activate a cascade of signalling mediation resulting in proliferation, survival, vasodilation permeability and migration (Melincovici et al., 2018). The primary VEGF effects of vasodilatory mechanisms stem from nitric oxide production. The autophosphorylation of the VEGFR activates the PI3K/Akt signalling cascade to increase intracellular calcium levels. The resulting calcium can lead to eNOS activation but also PI3K/Akt can also directly activate eNOS (Pandey et al., 2018). VEGF is noted to work complimentary to angiopoietins in the process to form mature blood vessels. VEGF

is essential for early-stage development of blood vessels to which the role of angiopoietin comes into its role in the maturation and remodelling of the vasculature. Both are vital in embryonic development as studies have shown knockouts of either signalling can cause fatalities (Thurston, 2002).

1.3 Angiopoietin

Angiopoietins are a glycoprotein family of ligands that primarily function to regulate the vasculature within the body (Brindle, Saharinen and Alitalo, 2006). These ligands have an affinity to a particular Receptor Tyrosine Kinase (RTK) known as Tunica Interna Endothelial cell kinase receptors (Tie) (Buhimschi et al., 2010). This family of ligands are comprised of four structurally similar proteins, Angiopoietin 1-4 (Suri et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The similarity stems through their structures starting from an N-terminal with a short amino region that serves to regulate clustering of the protein, this is also referred to as the super-clustering domain (SCD) (Davis et al., 2002; Barton et al., 2006). This domain continues to the coiled-coil motif, this large region also known as coiled-coil domain (CCD) is seen to be the active region that is responsible for the mediation of the homo-oligomerisation/hetero-oligomerisation of the ligand (Barton and Tzvetkova, 2005; Barton et al., 2006). A short linker region then connects the CCD to the C-Terminal fibrinogen-related domain (FReD). FReD is comprised of three sub-domains: A, B and P. In particular, the P sub-domain exclusively functions for receptor recognition and binding to the Tie receptor (see Figure 1) (Barton and Tzvetkova, 2005; Barton et al., 2006; Hansen et al., 2010). Of the family of four, Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are the most

common studied as well as the most biologically active (Uemura, 2010; Akwii et al., 2019). The balance between these two are pivotal to the integrity of the vasculature. The ratio of the ligands is associated with several pathophysiological conditions. These include cardiovascular disease, sepsis, and tumour angiogenesis. This particular ligand is produced by cells supporting the vasculature, including vascular smooth muscle cells and pericytes (Jeansson et al., 2011; Sequeira Lopez, 2016).

1.3.1 Angiopoietin 1

Angiopoietin 1 is reported to be the receptor-activating glycoprotein and thereby eliciting the vascular protective function of the Angiopoietin/Tie Receptor signalling pathway. This glycoprotein is produced by vascular smooth muscle cells to serve a paracrine function (Adamis and Berman, 2010). This protein is a 498 amino acid structure where initially on Ang-1 was revealed to be a 70kDa protein, however upon PNGase-F treatment, removal of residual sugars reduces the molecular weight to the expected 55kDa. This was additional confirmation of Ang-1 as a glycoprotein (Davis et al., 1996). The importance of the Ang-1/Tie-2 signalling pathway is seen in a study by Suri et al (1996), using a mouse model to observe the effects reducing Ang-1 production. The effects seen include embryonic angiogenic defects. The study also defined Ang-1 to exclusively bind to Tie-2 to perform the normal function of signalling. Ang-1 natively exists as trimeric, tetrameric and pentameric homo-oligomers that form larger multimeric clusters via cysteines within the amino terminal motif (Kim et al., 2005). Through the precise genetic engineering of the different multimers, it has been seen that the minimum order of the ligand must be a tetramer to provide the agonistic

response. Dimers and monomers of Ang-1 observe an antagonistic response (Davis et al., 2002). The binding of Ang-1 to Tie-2 is exclusive, it has been seen that when Tie-2 is dimerised with its orphan receptor Tie-1, it will partially inhibit binding to the Tie-2 receptor (Marron et al., 2007). While the response of Ang-1 is to promote vascular integrity, it has been shown that Ang-1 does not participate in the proliferation of endothelial cells, instead it is described as being anti-apoptotic resulting in the survival of the cells (Davis et al., 1996; Kim, Kim, So, et al., 2000). Ang-1 maintains quiescence and stability during embryonic development, however, the protective role on mature vasculature is almost exclusively observed during diseased states (Jeansson et al., 2011). The study by Jeansson et al (2011) demonstrated Ang-1 to be more effective under diabetic conditions. While Ang-1 plays its role in vascular protection, it also subjects itself to reducing monocyte/macrophage infiltration by depleting adhesion molecules responsible for leukocyte adhesion (Kim et al., 2001).

1.3.2 Angiopoietin 2

Angiopoietin-2 shares a 60% homology to its homolog Ang-1 with the main structural difference being the P domain that forms the receptor binding region. Ang-2 is seen as the antagonistic ligand with a binding specificity to Tie-2 only, however it is reported that under certain conditions Ang-2 can bind to the receptor and enable activation of the signalling cascade. The function has seen to be dependent on certain environmental factors, one such factor involves the levels of vascular endothelial protein tyrosine phosphatase (VEPTP). Ang-2 will activate Tie-2 when levels of VEPTP are low in the lymphatic vasculature. This ligand has a lower affinity in comparison to Ang-1, however

will perform the agonistic function in the absence of Ang-1 therefore and additional mechanism for the cell survival (Maisonpierre et al., 1997; Yuan et al., 2009; Souma et al., 2018). Ang-2 is an autocrine glycoprotein that is expressed in endothelial cells and stored within Weibel-Palade bodies. During the inflammatory response Ang-2 also has a paracrine action on leukocytes (Scholz, Plate and Reiss, 2015). This protein is natively found as trimers, tetramers, pentamers but rarely in higher order multimers. This protein has no reported minimum size for binding or function (Pietilä et al., 2012). The antagonist effect was displayed in mice infected with *Mycoplasma pulmonis*. Tie-2 phosphorylation was suppressed leading to an increase in forkhead box O1 (FOXO1) activation. FOXO1 activation initiates the procedure of cell apoptosis thus reducing vascular integrity (Yuan et al., 2009). There has also been reports of Ang-2 functioning to promote vascular sprouting in the absence of vascular endothelial growth factor (VEGF) (Holash et al., 1999).

1.3.3 Angiopoietin 3/4

Through Ang-1 and Ang-2 complementary DNA (cDNA) screening via low stringency hybridisation methods, mouse-Angiopoietin-3 (Ang-3) and human-Angiopoietin-4 (Ang-4) were discovered through the process. Both of these are interspecies orthologs characterised as the third protein to bind to Tie-2. Ang-3 shares a 45.1% and 44.7% homology with Ang-1 and Ang-2, respectively (Nishimura et al., 1999). However, Ang-3 and Ang-4 only share 65% homology (Lee et al., 2004). Additionally, like with Ang-2, Ang-3 expression is induced by hypoxia (Abdulmalek et al., 2001; Yamakawa et al., 2003; Yamakawa et al., 2004). The expression of the agonist Ang-4 varies among different

human tumours (Brown et al., 2000; Currie et al., 2001; Currie et al., 2002). The resemblances of Ang-3 and Ang-4, to Ang-2, are also familiar with the major form being a disulphide-linked dimer. This is suggested to be a result of the number and location of the cysteine residues (Valenzuela et al., 1999). Ang-3 is a potent agonist in mice whereas Ang-4 is a potent agonist in humans (Lee et al., 2004). Ang-3 serves an antagonist function whereby inhibiting Ang-1-induced phosphorylation of Tie-2 in humans, whereas Ang-4 induces phosphorylation (Valenzuela et al., 1999). Ang-3 and Ang-4 strongly induce survival and migration in their respective host. This is suggestive that Ang-4 could be an alternative to Ang-1 for the purpose of endothelial cell survival therapeutics (Lee et al., 2004).

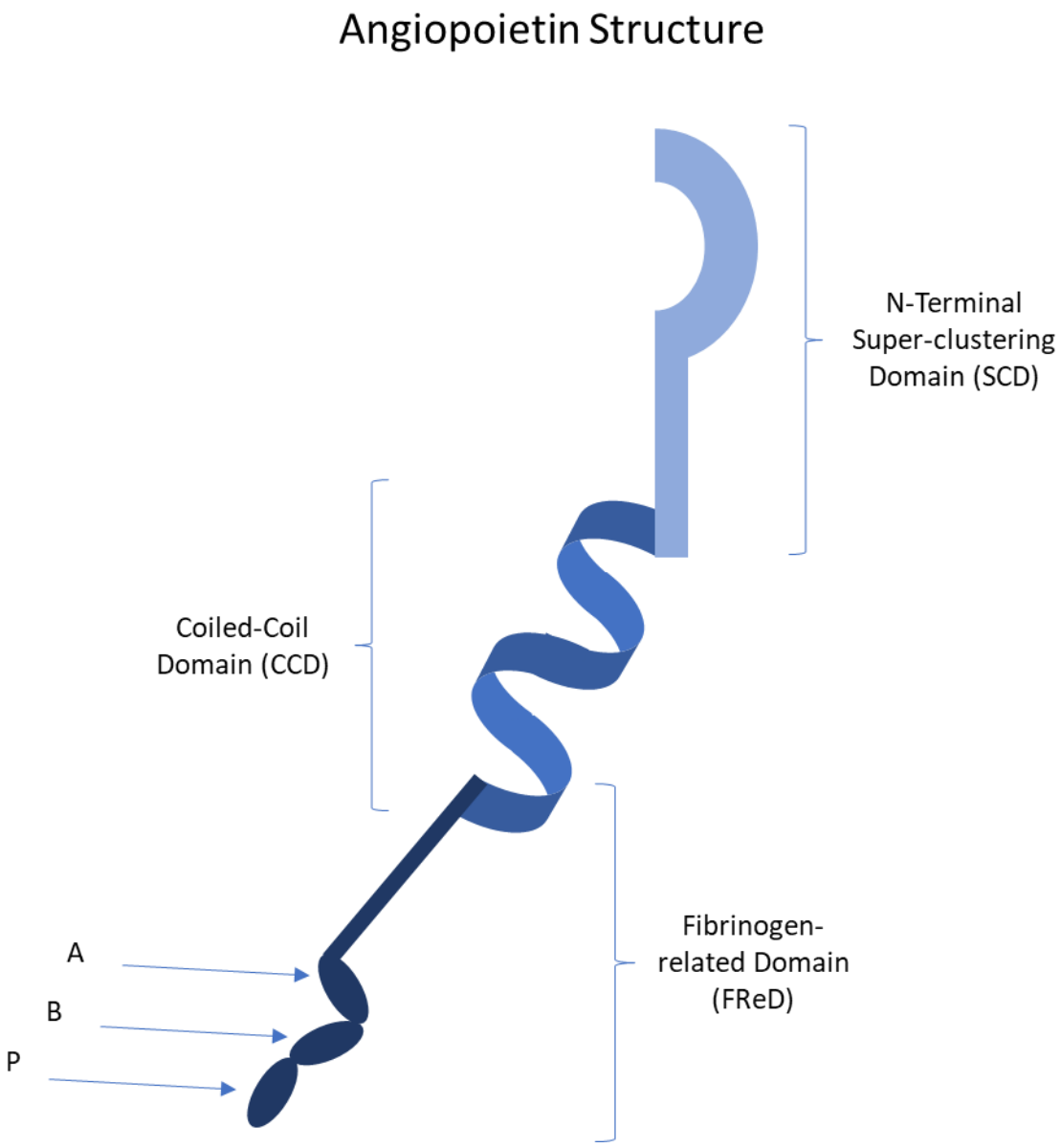


Figure 1. Angiopoietin Structure. *The diagram highlights the three main structures; N-Terminal Super-clustering Domain (SCD), Coiled-Coil Domain (CCD) and Fibrinogen-related Domain. Within the FReD domain the three sub-domains are noted as A, B and P.*

1.4 Tie Receptor

The cell membrane is encompassed in a variety of receptors, of which the RTK's form the largest group of membrane receptors (O'Connor and Adams, 2010). Among this group, the RTK Tie is predominantly expressed in endothelial but *in vivo* studies have found it to be expressed in particular hematopoietic cells (Mustonen and Alitalo, 1995). This sub-category of receptor is comprised of two receptor forms, Tie-1 and Tie-2, the former being known as the orphan receptor to Tie-2 (Mueller and Kontos, 2016). These two isoforms share a high degree of homology with each other, albeit some of the individual domains have a low percentage identity, the overall structure is very similar. As seen in Figure 2, the receptors are comprised of several domains on the extracellular region and a very atypical tyrosine kinase domain intracellularly. Towards the N-terminal section, three epidermal growth factor (EGF) repeats are sandwiched between two immunoglobulin-like (IG) domains, followed by three fibronectin type III repeats before the receptor spans through the lipid membrane of the cell (Sato et al., 1993; Macdonald et al., 2006).

1.4.1 Tie-1

Tie-1 is almost solely expressed in endothelial cells with expression being predominant during embryonic vascular development as well as during adult physiological and pathological angiogenesis (Kontos et al., 2002). While Tie-1 is known to be antagonistic, studies have indicated that the Tie-1 receptor is crucial for the normal development of embryonic vasculature. Tie-1-decficient mice did not survive late during gestation or immediately in the postnatal period. The cause of which was severe oedema and

haemorrhaging, hence suggesting that Tie-1 plays a pivotal role in the regulation of vascular integrity (Puri et al., 1995; Sato et al., 1995). In addition to these mice studies, those lacking in Tie-1 were also seen to have fewer numbers of endothelial cells, further adding to the evidence of Tie-1s supportive role in endothelial cells survival (Puri et al., 1995). Tie-1 has the capacity to undergo proteolytic cleavage of its ectodomain, hence forming a soluble Tie-1, in a tissue culture model, this is found in the tissue culture supernatant (Yabkowitz et al., 1997; Yabkowitz et al., 1999; Bilimoria and Singh, 2019). Simultaneously, the endodomain of Tie-1 can be seen to coimmunoprecipitate with Tie-2, further adding to Tie-1 modulating the signalling and function of Tie-2 (Marie B. Marron et al., 2000). Furthermore, Tie-1 has also been seen to be coimmunoprecipitated with the protein tyrosine phosphatase, Src homology-2 domain-containing protein tyrosine phosphatase-2 (Shp2), this being suggestive that Tie-1 may have the capacity to transduce signals in absence of ligand activation (M. B. Marron et al., 2000).

1.4.2 Tie-2

Tie-2 cloning was first reported in 1992 from a cDNA library of tunica interna of blood vessels (Dumont et al., 1992). Similarly, to Tie-1, the expression of this transmembrane receptor is highly sourced in the endothelium. Additionally, the deletion of the Tie-2 gene proved to have lethal effects in utero with oedema, haemorrhages and stunted vascular development (Dumont et al., 1994). The results of a gene deletion study, whereby mice had the Tie-2 gene deleted at mid-gestation or beyond, which inevitably resulted in subcutaneous oedema, hence providing evidence for the role of Tie-2 as a vascular stabiliser independent to developmental cardiovascular patterning defects

(Thomson et al., 2014; Souma et al., 2016). Another similarity to Tie-1, Tie-2 also is capable of proteolytic cleavage of its ectodomain to form a 75kDa soluble Tie-2 (sTie-2) protein, that has been coined as a ligand trap. This cleavage can be induced by stimulation with phorbol myristate acetate, however the mechanism regulating this process remains elusive. However, there is evidence of sTie-2 being elevated in certain diseases (Findley et al., 2007a; Alawo et al., 2017; Bilimoria and Singh, 2019).

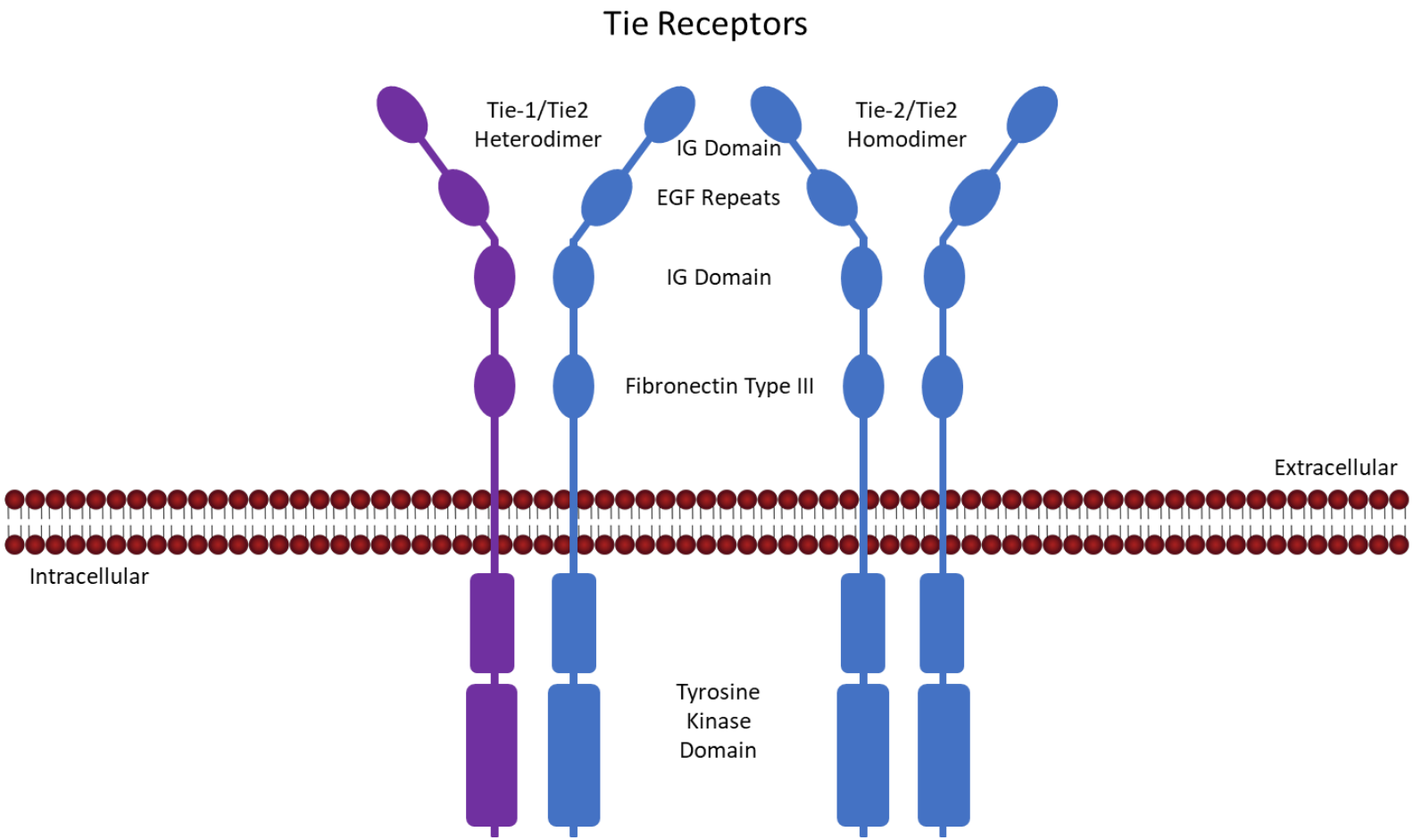


Figure 2. Tie Receptor Diagram. *The diagram depicts the dimerisation of the Tie receptors. The extracellular domain of the receptor contains an IG domain, EGF repeats, another IG domain, and a Fibronectin Type III domain. Intracellularly the receptor holds the Tyrosine Kinase domain.*

1.5 Angiopoietin1/Tie-2 Signalling

Upon binding of the tetramer Ang-1 ligand to the Tie-2 homodimer, the tyrosine kinase domain autophosphorylates to initiate a signalling cascade through downstream mediators leading to various vascular functions as shown in Figure 3 (Davis et al., 1996; Kim et al., 2005).

1.5.1 Vascular Integrity

Vascular integrity is the resultant effect of regulatory mechanisms designed to reduce vessel permeability. Intracellular junctions are responsible for mediating adhesion and communication between endothelial cells. These junctions are comprised of gap junctions, adheren junctions and tight junctions. Gap junctions are a communicative structure that allows for small molecules to pass between cells. Adheren junctions are crucial to contact inhibition of endothelial cell growth as well as paracellular permeability for circulating leukocytes. Tight junctions are the vital complex that provides the functional barrier through regulation of paracellular permeability and cell polarity. During vascular dysfunction, permeability is increased due to changes in the regulatory mechanisms of the cellular junctions (Bazzoni and Dejana, 2004; Murakami and Simons, 2009). Vasculature integrity and survival stems from the activation of the PI3K/AKT pathway via activation of the receptor through Ang-1 binding. The regulatory p85 subunit of PI3K is recruited to the receptors phosphorylated tyrosine residue 1102 to initiate the pathway (Brindle, Saharinen and Alitalo, 2006). AKT activation through PI3K activation results in the inhibition of forkhead transcription factor, FKHR. The normal function of FKHR is to promote apoptosis of endothelial cells through the

regulation of several genes associated vascular destabilisation and remodelling, including Ang-2. Thus inhibition leads to the survival of the endothelial cells and by extension the vasculature (Kontos et al., 1998; Kwak et al., 1999; Jones et al., 1999; Kim, Kim, So, et al., 2000; Papapetropoulos et al., 2000; Kwak et al., 2000).

1.5.2 Vessel Remodelling and Enlargement

One of the functions of Ang-1 relates to the remodelling and reorganisation of endothelial cells to form tubule-like structures, this is not seen in cells lacking Tie-2. This is further aided by the production of proteases including plasmin and matrix metalloproteases, but also the inhibition of metalloprotease-2 (Kim, Kim, Moon, et al., 2000). The PI3K pathway can also promote vessel remodelling and enlargement through endothelial nitric oxide synthase (eNOS) via SHC Adaptor (ShcA) protein. Focal adhesion kinase (FAK) can also produce the same result from PI3K activation (Jones et al., 1999). This function is also mediated via Erk1/2 stimulation involves an upstream adaptor, growth factor receptor-bound protein 2 (Grb2), to be recruited to the tyrosine residue 1102 on Tie-2. Additionally, tyrosine phosphatase and adaptor protein, SHPTP2, is also recruited to the phosphorylated Tie-2 to contribute to Erk1/2 stimulation via Grb2 recruitment (Brindle, Saharinen and Alitalo, 2006).

1.5.3 Migration

Ang-1 stimulated endothelial cell migration involves both PI3K and adaptor docking protein (Dok-R). The tyrosine domain will recruit Dok-R to elicit cell migration by phosphorylating the Dok-R protein to create an interacting site for Non-catalytic Kinase (Nck) and p21 activating kinase (PAK1). The recruitment of Dok-R is vital for migration

with the Phosphotyrosine Binding (PTB) domain interacting with the phosphorylated tyrosine residue 1108 on Tie-2, as well as the Pleckstrin Homology (PH) domain being linked with PI3K-dependent membrane localisation (Brindle, Saharinen and Alitalo, 2006). PAK1 not only functions to induce cell migration, it also causes cytoskeletal remodelling (Jones et al., 1999; Harfouche et al., 2003; Kichina et al., 2010).

1.5.4 Anti-inflammatory Mediation

Ang-1 reports indicate suppression of leukocyte adhesion to Human Umbilical Vein Endothelial Cells (HUVECs) by inhibiting numerous inflammatory-associated adhesion molecules. E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1 are all subject to decreased expression as a result of Ang-1 stimulation of Tie-2 (Brindle, Saharinen and Alitalo, 2006). Ang-1 also recruits A20-binding inhibitor of NF-κB-2 (ABIN-2) to the receptor. This function inhibits IκB kinases (IKKs) and consequently suppresses transcription factor Nuclear Factor-κB (NFκB) 70 (Van Huffel et al., 2001; Hughes, Marron and Brindle, 2003; Tadros et al., 2003). In addition to this anti-inflammatory function Ang-1 stimulation also leads to the activation of Ras-Related C3 Botulinum Toxin Substrate 1 (RAC1) via Ras-Associated Protein-1 (RAP1) or IQ Motif Containing GTPase Activating Protein 1 (IQGAP1) which promotes the stabilisation of cortical actin. This improves cell junction integrity as well as decreases endothelial cell permeability to prevent inflammatory agonists from acting on the endothelial cell barrier (Prasain and Stevens, 2009; Saharinen, Eklund and Alitalo, 2017).

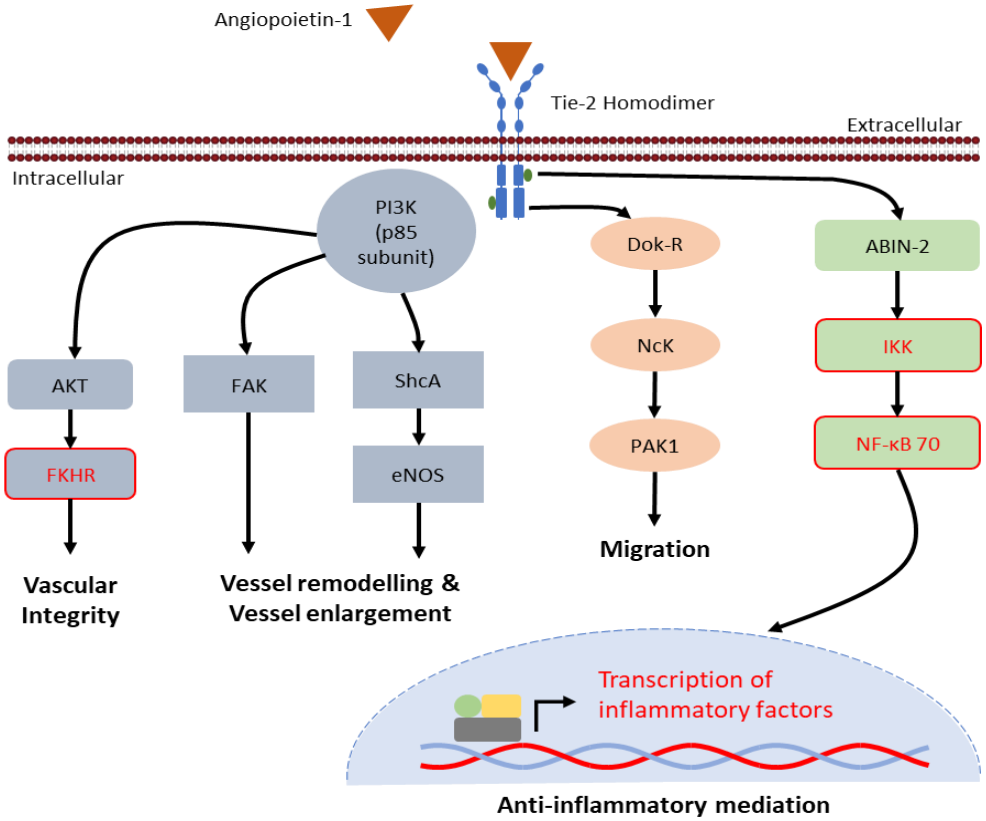


Figure 3. Ang-1/Tie-2 Signalling Pathway. *This pathway highlights the key outcomes of Ang-1 induction on the Tie-2 homodimer receptor. Vascular integrity is promoted through the inhibition of FKHR (highlighted in red), through the activation of the PI3K/AKT cascade. Vessel remodelling and Vessel enlargement are the result of PI3K activating mediator like FAK and eNOS. Migration relies on the activation of Dok-R to promote PAK1. The anti-inflammatory mediation is a result of inhibited transcription of inflammatory factors through ABIN-2 activation leading to the inhibition of IKK and NF-κB 70 (highlighted in red).*

1.6 Regulation of Ang-1/Tie-2 Signalling

The regulation of the ligands as well as the receptors, are key factors in disease development. There has been evidence to show that imbalances in the ligand ratio can prove to have disastrous effects and reduce the vascular protection in diseased individuals. Likewise, receptor ratio changes can be just as deleterious.

1.6.1 Ang-1/Ang-2 Imbalances and Therapeutic Connotations

When physiological levels of Ang-1 are reduced in comparison to Ang-2, this implicates further action of the antagonistic function of Ang-2 and hence supports the mechanism of vascular instability. Angiopoietin levels have been reported to be affected by diseased blood vessels (Sandhu et al., 2004; Post et al., 2008; Horio et al., 2014; Theelen et al., 2015). Plaques with a high microvascular density display increased levels of Ang-2 which results in a decrease in Ang-1:Ang-2 ratio (Post et al., 2008). Additional reports indicate that use of anti-Ang-2 antibodies can reduce the size of fatty streak formation in carotid artery plaques in mice (Theelen et al., 2015). Mice with induced myocardial ischemia from left coronary artery ligation, display an increase in Ang-2 and a reciprocal decrease in Ang-1 (Sandhu et al., 2004). ApoE$^{-/-}$ mice have an increased secretion of Ang-2 from atherosclerotic lesions. The increase in Ang-2 accelerates vascular inflammation by activating NF-κB-dependent proinflammatory cascades, thus increases migration of macrophages to the sites of atherosclerotic lesions. Ang-2-/- in these mice have also demonstrated a reduction of instability of the plaques (Horio et al., 2014).

There is a relationship between sepsis and endothelial injury whereby the endpoint of life-threatening infections is microvascular dysfunction. During inflammatory cytokine

stimulation, Ang-2 levels are increased resulting in the regulation of Tie-2 signalling. When infection takes place the increased Ang-2 alters the Ang balance in favour of increased Ang-2/Tie-2 hence contributing to the destabilisation of the endothelial vasculature. It is suggested that regardless of microbial aetiology, any disruption to the numerous mechanisms of Ang/Tie regulation with result in endothelial dysfunction and microvascular leak (Leligdowicz et al., 2018).

Pneumonia is an infection of the lungs which can go on to develop into sepsis. This promotes reduction in Ang-1 while the Tie-2 inhibition by Ang-2 is increased. There was evidence to show that angiopoietins have a potential to be used has biomarker and/or novel routes for therapy in combination with antibiotics to prevent acute lung injury (Gutbier et al., 2018). In recent years, biomarkers have been suggestive of the Ang/Tie axis. Within individuals of European ancestry, Ang-2 is associated with higher risks of acute respiratory distress syndrome (ARDS) and may also be a causal factor of ARDS development (Reilly et al., 2018). A Chinese study also found that Ang-2 was among several other proteins that were seen to be a promising biomarker for identifying patients who will develop severe sepsis as opposed to those who develop a low risk sepsis (Liu et al., 2018). This particular pattern is not specific to adult patients, however, is also seen in neonates, young infants, and children. The circulating serum Ang levels were unswervingly associated with clinically significant endpoint of sepsis, despite the fact that the endothelium was in an immature state. The mechanisms of sepsis and vascular leak were the same as the older populace (Wright et al., 2018). While the elevation in Ang-2 is further reported in another paediatric study, it has also been reported that the Ang-1 levels are elevated in children under 5 years or age. This

significant elevation of Ang-1 was also seen with the decrease in Ang-2/Ang-1 ratio across all the treatment groups within the study. It was also suggested that neither Ang impacts on basal human dermal microvascular endothelial cells (HDMECs) nor do they modulate any TNF mediated permeability (Pierce et al., 2019). Biomarkers have been widely assessed with ARDS outcome but also has been seen to be associated with mortality as a result of sepsis. The rising Ang-2 were associated with death (Calfee et al., 2012; Zinter et al., 2016).

The benefit of increased expression of Ang-1 has been seen in mice where increased co-expression of Ang-1 and α5β1 integrin have improved vascular stability without having any adverse effects on the blood brain barrier. This is very important with regards to reducing the exacerbation of vascular permeability as a result of vascular remodelling required from ischemic injury recovery (Wang et al., 2019). This gives insight into the promising potentials of Ang-1 therapy. One such study reported that treatment with recombinant Ang-1 reduces haemorrhagic transformation as well as cerebral oedema when co-treated with tissue plasminogen activator (Kanazawa et al., 2017). Use of the protein itself has shown its benefits, however even a mimetic, vasculotide, has displayed therapeutic improvements on neuroprotection post ischemia. This mimetic works to decrease the expression of receptor for advanced glycation end products (RAGE) and TNFα, both of which are known to increase the permeability of the blood brain barrier (Venkat et al., 2018). Additionally, understanding the Ang/Tie axis allows for novel routes of therapy, as displayed by treatment with Vitamin D3 for the improvement on cerebral infarction. The study indicated that vitamin D3 had the capacity to promote angiogenesis and strengthen vessel integrity by causing an increase in VEGF and Ang-1

expression. This mechanism is a result of the Sonic Hedgehog (Shh) signalling pathway co-regulating the angiogenic factor expressions (Choudhry et al., 2014; Bao and Yu, 2018). Further evidence of the Shh pathway was given in study looking at Panaxatriol saponins, an extract of the Chinese herb Panax notoginseng, commonly used to treat ischemia. The extract up-regulates the Shh pathway and in turn elevate the angiogenic factor Ang-1 (Hui et al., 2017).

While it is often seen that Ang-2 is a good indicator for certain conditions, a study in mice found that Ang-2 was a significant mediator of cerebrovascular permeability. This in turn was corroborated by a human study which saw the stroke grade and size to be correlated with expression levels of Ang-2. It is suggested that the manipulation of Ang-2 on the Ang/Tie axis would provide therapeutic relief in ischemic stroke but also as it has a part in the regulation of the blood brain barrier, it could also be used to facilitate the delivery of a drug that is needed in the central nervous system for a neural condition (Gurnik et al., 2016).

The main areas that associate Ang/Tie to cancer are tumour angiogenesis and metastasis. Many forms of human cancer have shown elevations of Ang-2 whereby the source of Ang-2 has been primarily endothelial cells in glioblastomas and renal cell carcinoma specifically. A rat model gave evidence to show Ang-2 was connected to pericyte detachment and vascular regression, subsequently leading to the promotion of angiogenesis of the tumour (Scholz et al., 2016; Rautiola et al., 2016). Combination of Ang-2 blocking, and VEGF blocking sought to reduce angiogenesis of the tumour by reducing new vessel formation. Ang-1, despite its capacity to induce angiogenesis in

vascular remodelling, is not effective in reducing tumour growth but it does maintain tumour blood vessels to enable drug delivery (Saharinen, Eklund and Alitalo, 2017).

The idea of using Ang as a biomarker has been widely explored for different forms of cancer. Within gastric cancer, a phase III trial involving treatment of bevacizumab in addition to chemotherapy had assessed the role of Ang-2 as it is described as a vital propagator of tumour angiogenesis, metastasis, and resistance to antiangiogenic treatment. This study displayed Ang-2 to a novel prognostic marker of overall survival in advance gastric cancer with association to liver metastasis (Hacker et al., 2016). Ang-2 has also been demonstrated to be a useful serum tumor marker for liver cancer in a Chinese population. This study indicated the specificity of the marker by comparing the Ang-2 levels in liver cancer patients against chronic liver disease patients, showing that Ang-2 concentration is higher in the cancer patients (Chen et al., 2018). This is additionally supported by Pestana et al, whereby Ang-2 levels is a prognostic biomarker and potential diagnostic tool of hepatocellular carcinoma along with Ang-1 (Pestana et al., 2018). The association of bevacizumab to the Ang/Tie axis have been explain in a communication highlighting that Ang-1 and Tie-2 were correlated with improved progression-free survival in patients with ovarian cancer. Though the study mentioned states larger samples sizes are required to validate the results (Backen et al., 2014; Ciccolini et al., 2015). A similar outcome was seen in cervical cancer patients whereby serum Ang-1/Ang-2 ratio was increased against longer progression-free survival and overall survival (Yang et al., 2017). The use of the signalling axis can serve as a potential biomarker for the optimisation of therapeutics. One study suggests circulating Tie-2 should be monitored meticulously in order to optimise VEGF inhibitor treatment in

colorectal cancer (Jayson et al., 2018). Several studies have shown the implications of Ang-2 in lung cancer. Systematic reviews display a relationship between Ang-2 expression and non-small cell lung cancer prognosis. This was determined based on comparing tumour stages, lymphatic invasions and other relevant clinicopathological features, which then showed Ang-2 to be linked with poor prognosis for the patients (Xuan et al., 2016). Additionally, it could also be a useful prognostic marker in small cell lung cancer based on a study determining the efficacy of chemotherapy by measurement of Ang-2 levels (Zhang et al., 2015). The versatility of Ang as a biomarker for various cancers is very apparent. The nature of Ang being a consistent factor of tumour angiogenesis allows it to serve as an effective biomarker.

One of the most well-known chronic airway inflammatory conditions has connections with Ang. Asthma is associated with vessel remodelling in the lungs due to inflammatory mediators as a result of the condition (Murdoch and Lloyd, 2010). Angiogenesis is a part of remodelling of the vasculature in the airways and among the angiogenic factors released by mesenchymal and inflammatory cells is Ang. Serum Ang-1 has been reported to be elevated in asthma patients as well as Ang-1: Ang-2 ratio being correlated to lung function therefore suggestive that Ang could be a useful diagnostic marker of asthma. Further studies indicate the levels of Ang-1 and Ang-2 can indicate the exacerbation of asthma (Moon et al., 2014; Lee et al., 2016). One such study went further with analysis of severe asthma patient serum to further support the idea of Ang-2 being a biomarker of asthma (Makowska et al., 2016). In addition to Lee et al, a correspondence was submitted highlighting the results of serum Ang levels in children with stable asthma. There was no discernible difference between the stable asthma group and the healthy

control, however it was seen that Ang-1 and Ang-1: Ang-2 ratio was reduced in the asthmatic group. It has been suggested that the steroid treatment may work to control Ang-2 levels and lung function (Koksal and Ozbek, 2016; Jang, 2016). The diagnostics using angiogenic factors were assessed from exhaled breath condensate. While detection of Ang-2 was shown to be miniscule, the combined detection with VEGF may give evidence for pro-angiogenic action in mild asthma patients (Grzela et al., 2016).

Studies in asthmatic smokers displayed peculiar results whereby Ang-2 was shown to be elevated as a result of the inflammatory response, however the study also found Ang-1 to be elevated, therefore suggestive that Ang-1 is being produced for a protective response to control the Ang-2 levels. The study does mention there are several underlying inflammatory mechanisms should be explored to be able to optimise therapeutic strategies of asthma (Petta et al., 2015).

The therapeutic use of the Ang-1 protein was shown to greatly improve airway inflammation and reduce the airway remodelling. While these results were seen also in treatment with just aerosolised mesenchymal stem cells, co-treatment with Ang-1 also provided additional benefits including a reduction in proinflammatory gene expression. This study shows great promise, however remains in pre-clinical studies until safety, toxicity and tumorigenicity concerns are resolved (Halim et al., 2019). Use of a more potent form of Ang-1, COMP-Ang-1, was seen to benefit against mice with asthma, however the longevity of the disease could mean that chronic administration of COMP-Ang-1 could lead to unwanted airway angiogenesis (Cho et al., 2005; Moss, 2013).

Ang-2 elevation has been linked to pericyte dropout in diabetic retinopathy (Hammes et al., 2004; Park et al., 2014). This expression of Ang displays the same phenomenon in animals as it has in early diabetic retinopathy. However, suppression of retinopathy and retinal oedema in diabetic mice was seen when subjects were given injections of Ang-1 (Joussen et al., 2002). An interesting potential treatment for neovascular age-related macular degeneration (nAMD) and diabetic macular oedema (DMO) is a particular peptide derived from the non-collagenous domain of collagen IV by Asclepix Therapeutics. This is a novel therapy that aim to target Ang-2 in an unexpected dynamic. ATX107 works to utilise the high Ang-2 concentration and allow it to act as an agonist just as Ang-1 would in similar concentrations. Additionally, it also promotes VEGFR2 degradation hence reducing VEGF induced vascular leakage. This novel treatment is in preclinical development (Hussain et al., 2019).

Ang-2 levels and sTie-2 have been shown to have a link to certain markers within type 2 diabetic patients. Gamma glutamyl-transferase (GGT) is a marker of oxidative stress that is strongly related to macrovascular disease. This particular marker is seen to be able to predict serum Ang-2 levels. Additionally, glycated haemoglobin (HbA_{1c}), insulin levels and homeostasis model assessment of insulin resistance (HOMA-IR) are all strong predictors of sTie-2 in plasma. Thus, it is indicated that both Ang-2 and sTie-2 could be potentials in serving as new biomarkers for vasculopathy and long-term metabolic control, respectively (Rasul et al., 2011).

The role of angiopoietin is vastly diverse within diabetes, so much so it also assists with glucose homeostasis. Within the pancreas, Ang-1 is produced by β cells to support the

vasculature around the islets to ensure effective insulin uptake into the circulatory system. Ang-1 maintains the microenvironment of the islets. When deprived of Ang-1 inflammation and disruption of the islet vasculature led to impairment of the insulin secretory mechanism. This work is suggestive of a novel therapeutic route for the treatment of type 2 diabetes (Brissova et al., 2006; Park et al., 2019).

1.6.2 Tie-1/Tie-2 Ratio and Therapeutic Connotations

While the balance of ligands is crucial for receptor signalling, it is also very important to understand the balance of the receptors. One of the most important meditators produced by macrophages during atherosclerosis, is the pro-inflammatory cytokine TNF-α. This cytokine causes a time-dependent increase in the Tie-2:Tie-1 ratio by upregulating Tie-2 and suppressing Tie-1 in endothelial cell cultures (Zernecke and Weber, 2005). In addition to the suppression of Tie-1, TNF-α also induces proteolytic cleavage of the Tie-1 ectodomain (Korhonen et al., 2016). In contrast to TNF-α, VEGF is a multifunctional cytokine that has been shown to promote atherosclerosis. This particular cytokine has been reported to cause decrease in Tie-2:Tie-1 ratio by shedding the Tie-2 extracellular domain (Inoue et al., 1998; Findley et al., 2007b; Singh et al., 2012). The various cytokine influences on the Tie receptor alters the function on the vasculature in the diseased state. Similar changes in the Tie receptor ratio have also been seen in patients with other cardiovascular risk factors like obesity and dyslipidaemia. Serum samples of these patients have been analysed and show increased levels of soluble Tie-2 (sTie-2). sTie-2 is referred to as a ligand trap which will allow Ang-

1 to bind to it and reduce the bioavailability of Ang-1 to bind to full receptor Tie-2, thus reducing the signalling activity (Lieb et al., 2010; Alawo et al., 2017).

Atherosclerosis is also associated with disturbed blood flow in the vasculature as it can induce endothelial inflammation (Tabas, García-Cardeña and Owens, 2015). One such study suggested that non-laminar flow upregulates Tie-1 expression at the site of atheroma in mice models, however in contrast, in endothelial cell cultures normal laminal flow decreases Tie-1 expression. This result is also seen in apoE-deficient mice with the addition of showing a dose-dependent reduction in the atherosclerotic lesions of the distal aorta, thus displaying the significance of Tie-1 in the development of atheroma (Woo et al., 2011).

Physiological levels of Tie-2 receptor have also been reported to alter in diabetic mice (Chen and Stinnett, 2008). Diabetic mice had myocardial ischemia induced, which resulted in a significant reduction in Tie-2 and thus impair the Ang-1/Tie-2 signalling. Contrary evidence does show that increased glucose concentration reduces the activity of Tie-2 without reducing expression or increase internalisation of the receptor. This evidence was seen in macro- and microvascular human endothelial cells, Human Umbilical Vein Endothelial Cells (HUVEC) and Human Microvascular Coronary Endothelial Cells (HMCEC), respectively. The data shows that the downstream mediator PI3K/AKT phosphorylation is influenced by the elevated glucose, leading to a disruption of the vasoprotective action of Ang-1, thus propagating vascular diseases associated with diabetes (Singh, Brindle and Zammit, 2010).

1.7 Diabetes and associated inflammatory mediators.

1.7.1 Diabetes

Quality and Outcomes Frameworks is an incentive programme for General Practitioner surgeries in England, Wales, and Northern Ireland, with the task of recording prevalence of diabetes in people aged 17 and above. Scotland uses a Scottish Diabetic Survey to note the prevalence. Collectively the prevalence of diabetes in the UK is reported to be approximately 3.9 million (Diabetes UK, 2020). Diabetes mellitus specifically is a collective disease that revolves around the notion of a defect in insulin whether it be production or sensitivity. The main two categories are Type 1 and Type 2. Type 1 is an autoimmune disease whereby the body's own immune system destroys the β-cells in the Islets of Langerhans; therefore, insulin is not produced. Type 2 is more of an environmentally triggered disease where the body becomes insensitive to insulin and cannot utilise the insulin for the breakdown of glucose. The etiopathogenesis of environmental factors on type 2 diabetes involve factors like diet and exercise (Guthrie and Guthrie, 2004; Raman, 2016). Dietary triggers have been identified to have three major food types, which in vast quantities cause inflammation and promote diabetes. The main groups include refined cereal grains, omega-6 industrial seed oils and fructose (Raman, 2016). Within this umbrella there are many vascular complications caused by associated inflammation, including retinopathy, nephropathy and peripheral vascular disease (Fowler, 2008; Al-Ali et al., 2020). The other types of specific diabetes, for example, exocrine pancreatic diseases like pancreatitis and cystic fibrosis, endocrinopathies like Cushing's syndrome and pheochromocytoma, but also medically-

chemically induced by glucocorticoids, neuroleptics and pentamidine (Astrid Petersmann et al., 2019).

Diagnosis of diabetes is the clinical assessment of hyperglycaemia through blood glucose testing. Similarities in symptoms between type 1 and type 2 include polyuria and polydipsia, however similarities separate there where type 1 is generally an early onset condition that occur in paediatrics with symptoms being significant weight loss, low blood pressure and ketone bodies accumulating in the body resulting from diabetic ketoacidosis. These symptoms combined with fasting plasma glucose concentration ≥ 7.0mmol/l provide strong evidence of the condition. Urinalysis was widely conducted to aid in diagnosis, however since the advent of glucose meters, this practice has reduced. Urinalysis is performed to measure levels of glucose, ketones, and protein in the urine. The glucose in the urine is a direct result of elevated levels in the blood which passes through the kidney through the process of osmosis. Urine ketones are resultant of ketoacidosis, a process whereby the body itself uses fat stored instead of glucose as a source of energy for cellular metabolism. Triglycerides from adipose tissue undergo hydrolysis to break down into its key components, fatty acids, and glycerol. Hepatic metabolism of the fatty acids promotes the accumulation of ketone bodies from the elevated levels of acetyl coenzyme A in the tricarboxylic cycle being converted into β-hydroxybutyrate, acetate and acetoacetate (Nyenwe and Kitabchi, 2016; Dhatariya, 2019). Build-up of the ketone bodies will increase the overall pH internally but cycling around the body, the individual would experience an acetone smelling breath and ketones in the urine. The elevated glucose can also cause stress on the kidneys and cause nephrons to become damaged resulting in diabetic nephropathy.

Type 2 diabetic patients usually experience the opposite in some sense, whereby blood pressure is often high due to glucose creating a physiological change in the consistency of blood which causes the circulatory system to drive harder. This adds to the increased risk of atherosclerosis, the condition of plaque built up in the vasculature. Chronically increased pressures can damage the arterial wall creating an opportunity for the inflammatory response to occur and cause plaque formation at the site of immune action. While mortality directly resultant of diabetes is rare, it is the complications arising from diabetes that cause death. Atherosclerosis begins with damage on the endothelium wall recruiting macrophages to the site of injury in an inflammatory response. Within the lesions, macrophages engorge on cholesterol, changing them into foam cells which cluster together and form a hardened plaque. When left the plaque will continually build to narrow the lumen and increased pressure in that region can cause a rupture in the vessel, sending lipid-rich necrotic debris into the vasculature, potentially causing lesions in other location. This can all accumulate to eventually create a significant blockage in the vasculature which can be fatal (Lusis, 2000; Bäck et al., 2019). The inflammatory response is rather crucial in the development of this severe diabetic induced vascular condition. Engagement of the macrophages cause a release of a multitude of pro-inflammatory cytokines (Galkina and Ley, 2009).

1.7.2 Inflammatory Cytokines

The immune system is a complex system designed to protect an organism from foreign invaders and prevent diseases. Cytokines are an integral superfamily of proteins that allow for signalling between cells, essential for stimulating and regulating the immune

response. Cytokines are pleiotropic in functionality and are small soluble molecules that are produced by many cell types. These can be signalling cells in through autocrine functions or paracrine functions (Cameron and Kelvin, 2013). There are numerous cytokines all with varying roles within the body however there are a few cytokines that are noted to be the most potent of the family and have multifaceted functions. IL-1β, IL-6 and TNF-α are widely researched and there is an abundance of evidence around these cytokines as they mediate many inflammatory processes around the body (Carlson et al., 1999; Zhang and An, 2007; Taki et al., 2007; Umare et al., 2014).

1.7.2.1 Interleukin-1β

Originally the growing list of cytokines were to follow the interleukin system of nomenclature, however this partially adopted system bore confusion that interleukins were exclusively functional among leukocytes (Cameron and Kelvin, 2013). While the nomenclature of the system is widely accepted, it has remained mixed in the modern cytokine nomenclature system. Interleukin (IL) -1-related cytokines are comprised of IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA) and IL-18. Both IL-1α and IL-1β are primarily produced by mononuclear and epithelial cells when induced by injury and/or infection to promote the inflammatory response. These two proteins elicit fever and promote the expression of a variety of acute phase responses along with the activation of lymphocytes (Cameron and Kelvin, 2013). Knockout studies indicated that while both forms can induce fever, reduction in response was not seen when IL-1α was removed, therefore suggesting IL-1β can compensate the function of IL-1α, however this was not

applicable to IL-1β knockout despite the similarity in the two forms of IL-1 (Horai et al., 1998). One study found that injections with low nanogram doses of IL-1α or IL-1β caused detrimental toxicity to the subjects with experiences of fever, muscle and joint aches, fatigue, and gastrointestinal disturbance. This study highlighted the benefits of therapy to block IL-1 pathways (Dinarello, 1996). Additionally, in contrast to IL-1α, IL-1β is not found in cells of a healthy individual and its' function is dependent on a series of intracellular mechanism to propagate the inflammatory function. While the stimulus is generally microbial in origin, there are multiple cytokine that can induce the transcription of IL-1β. This being the rate limiting step in its production. (Dinarello et al., 1987). IL-1 mediates several inflammatory conditions. Events of acute ischemic diseases including the most common myocardial infarction (MI), stroke and kidney failure. With MI it is seen that after the initial attack and loss of cardiac muscles, the individual is at risk of a repeat infarction. IL-1 inductions are responsible in this as displayed through animal models showing improvement through IL blocking (Abbate et al., 2008; Abbate et al., 2010; Dinarello, Simon and Van Der Meer, 2012).

Physiologically, IL-1β is found as an inactive 31kDa precursor, pro-IL-1β. Induction of the precursor form is in response to pattern associated molecular patterns (PAMPs); however, this is known as the priming step and requires further stimuli. This only becomes the active form IL-1β when cleaved by caspase-1. Caspase-1 activation occurs via recruitment of inflammasomes (Lopez-Castejon and Brough, 2011; Afonina et al., 2015). These are a cluster of several components including adaptor molecules, cytosolic pattern recognition receptor and pro-caspase-1 (Schroder and Tschopp, 2010). Caspase-1-dependent processing of pro-IL-1β leads to the rapid secretion of IL-1β (Brough and

Rothwell, 2007). Once activated and bound to Immunoglobulin G (IgG) domain of IL-R1, recruitment of myeloid differentiation primary gene 88 (MYD88) to the Toll/Interleukin receptor (TIR) region further recruits Interleukin-1 receptor-associated kinases (IRAKs). This allows for the binding of IRAK1 and TNF receptor-associated factor 6 (TRAF6). Subsequently leading to the phosphorylation cascade of the IKK complex and IκBα. Activation of IκBα is accompanied by the polyubiquitination that degrade the protein to allow NFκB to dissociate and translocate to the nucleus to promote the expression of genes encoding for particular immune related response (Iwai, 2012).

Diabetes is a chronic condition that both type 1 and type 2 show very low concentrations of IL-1β in which its function as selective toxicity for pancreatic β-cells (Mandrup-Poulsen, Pickersgill and Donath, 2010). Evidence has shown that elevated glucose levels cause β-cells to produce IL-1β hence associating themselves in the destructive autoimmune function of recruitment of immune cells driven by IL-1β (Maedler et al., 2002; Donath and Shoelson, 2011). Additionally, deposits of islet amyloid can activate IL-1β production from the blood monocytes infiltrating to the deposition site hence propagating the loss of β-cells (Masters et al., 2010). Mouse models have shown caspase 1 inhibition and caspase 1 deficiency can reduce insulin resistance and improve insulin sensitivity, respectively (Stienstra et al., 2010). IL-1 in serum is seen to be elevated in newly diagnosed and previously diagnosed type 1 diabetic individuals with correlations to TNF-α, Interferon-γ (INF-γ) and IL-2 (Özer et al., 2003). This risk of this elevation was also displayed in a meta-analysis showing a strong association with the risk of type 2 diabetes (Liu et al., 2016)

The first line of therapy for inflammatory conditions would be use of aspirin or NSAIDs however are commonly associated with gastrointestinal complications and bleeding (Goldstein and Cryer, 2015). Glucocorticoids can also be prescribed for several autoimmune diseases however chronic use has known ramification of opportunistic infections and metabolic dysfunctions. If a disease is identified and being greatly dependent on IL-1β, specifically targeting IL-1β can prove highly responsive without the gastrointestinal side effect or metabolic disturbance (Dinarello, Simon and Van Der Meer, 2012). IL receptor is expressed in nearly all tissue and when antagonised it will inhibit IL-1α and IL-1β binding. Anakinra is a recombinant form of a naturally occurring antagonist and is the first selective IL-1Ra to be FDA approved. The Anakinra's mechanism involving direct binding to IL-1R competing against both IL-1α and IL-1β. Once bound it precedes to keep the receptor inactive and prevents the transduction of intracellular signalling (Baskar, Klein and Zeft, 2016). Additional receptor inhibitors are in development which display greater affinity and efficacy compared to Anakinra, these include chimeric IL-Ra-IL-1β (EBI-005) and MEDI-78998. Though developers of EBI-005, Eleven Biotherapeutics (now known as Sesen Bio), have stated from results of a phase III study, statistical differences were not seen between the treated group and the vehicle group however there as a significant improvement from the baseline (Eleven Biotherapeutics, 2015). MEDI-78998 is still in early development and remains licenced to AstraZeneca and MedImmune (Dinarello, Simon and Van Der Meer, 2012). Receptor targeting is one approach, the other is to neutralise IL-1 itself. Use of a soluble IL receptor will act as a ligand trap. FDA approved (Kapur and Bonk, 2009), Rilonacept is a molecule designed from the two extracellular chains of the IL-1R which are fused to

make a soluble trap for both Il-1α and IL-1β (Hoffman et al., 2008). In addition to this, anti-IL-1β antibodies have been shown to not only neutralise IL-1β but also reduce the production of the cytokine over a significant period of time (Gül et al., 2012).

1.7.2.2 Tumour Necrosis Factor-α

TNF is a small homotrimer, 17kDa in size and comprised of 157 amino acids. This is expressed as a 26kDa cell surface membrane protein and cleaved by TNF-α-converting enzyme to generate the active soluble protein (Horiuchi et al., 2010). This protein is produced by activated macrophages, T-lymphocytes, and natural killer (NK) cells. Like with IL-1β, TNF-α is involved in many inflammatory conditions. One of the main roles of TNF as the name would suggest, is to induce apoptosis. This protein serves to impact on the control of cell shape through the modulation of the cytoskeleton. However, it has been seen that the morphological function of TNF signalling is also applied outside of the immune response (Mathew et al., 2009). While TNF is best characterised for its ability to cause apoptosis in cells with aberrations, this is not the case for healthy cells, thus does not lead to healthy cell death when TNF levels are elevated (Kontoyiannis et al., 1999). The function outside of immunity is rather vital, especially in the development of secondary lymphoid organs, as this is partly a result of the migratory effect TNF has in follicular dendritic cells (Pasparakis et al., 2000; Mebius, 2003; Victoratos et al., 2006). Some of the TNF pro-apoptotic functions may have a role in other aspects of the immune response. Pro-inflammatory changes including increased vascular permeability and, neutrophil or fibroblast recruitment, in addition to osteoblast apoptosis, fibroblast cytolysis or epithelial-cell apoptosis are all resultant of TNF-α treatment, leading to

structural implications to the cytoskeleton and the cellular mechanisms involved in cytoskeletal regulation (Scanlon et al., 1989; Domnina et al., 2002; Domnina et al., 2004; Triplett and Pavalko, 2006). Within endothelial cells, TNF-α exerts its effect on many molecular mediators including p38 mitogen-activated protein kinases (MAPK), Protein Kinase C (PKC) and Rho kinase to elicit a permeability increase, cytoskeletal changes involving cell shape changes with additional increases in F-actin, formation of filopodia and membrane ruffles. Furthermore, TNF-α also acts on the G-protein signalling mediators in endothelial cells to disrupt the monolayer continuity (Brett et al., 1989; Koss et al., 2006; Triplett and Pavalko, 2006; Mckenzie and Ridley, 2007; Fong et al., 2015; Fong et al., 2019). The impacts on epithelial cells involve actin cytoskeletal reorganisation and enhanced cell migration through FAK mediation (Koukouritaki et al., 1999; Corredor et al., 2003).

TNFR1 is one of two receptors known to interact with TNF-α. This type of receptor is a single span transmembrane protein which has been characterised with cysteine-rich domains (CRDs) in the extracellular domain. Upon TNF-α binding, the death domain of the intracellular region recruits the binding of a Tumor necrosis factor receptor type 1-associated Death domain (TRADD) protein complex including receptor-interacting protein (RIP) and TRAF2. TRADD can also complex with Fas-associated death domain (FADD). Both of which can induce caspases to promote apoptosis. The TRADD, RIP and TRAF2 complex can also do this through activation of the c-Jun N-terminal Kinase (JNK) pathway, however the complex also has the capability to activate IKK and NF-κB to result in cell survival. The impact of TNFR1 on the Rho GTPases to result in cytoskeletal changes is linked via the interaction with the neurotrophin receptor p75, another type of

receptor from the TNFR-superfamily expressed primarily in neurons (Mathew et al., 2009).

There is evidence to suggest that TNF-α has a vital role in several dysfunctions. The protein has been seen to cause a disruption of microvascular and macrovascular circulation *in vitro* and *in vivo*. Increased expression of TNF-α as a result of advanced glycation end-products (AGE), lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) and NF-κB , consequent in endothelial dysfunction and other pathophysiological conditions (Zhang et al., 2009). TNF-α has the ability to regulate nitric oxide synthase (NOS) expression as well as activity and hence directly responsible for the production of nitric oxide (NO). While studies show TNF-α decreasing expression of one isoform of NOS, eNOS, it subsequently induces production of iNOS to contribute to the production of a peroxynitrate-mediated mechanism ($ONOO^-$) which further supplements to the nitrative stress on endothelial cells, thus impairing functionality of the vasculature (Macnaul and Hutchinson, 1993). TNF-α has well documented effects on lipid metabolism seen in sepsis but also there have been increases in the proinflammatory cytokine within adipose tissue in both animal and human studies. Additionally, similar studies have described it to be a causative factor in obesity-associated insulin resistance and pathogenesis of type 2 diabetes (Moller, 2000). There has also been a suggestion of a link between TNF-α and type 1 disease. A study of nonobese diabetic mice treated neonatally with TNF-α to result in an accelerated onset of type 1 diabetes, whereas the blockade of TNF-α resulted in the absenteeism of diabetes altogether (Lee et al., 2005).

The therapeutic effect of TNF-α manipulation has largely been based around cancer treatments due to its large involvement in the pathological processes. However, there have been certain therapeutic managements that have impacted on TNF-α. One study found that non-surgical periodontal therapy reduced levels of TNF-α in patients with poorly controlled diabetes. The decrease of TNF-α level removes the source of systemic inflammation however reduction of HbA1c levels was not significant without strict glycaemic control (Dağ et al., 2009). There has been a specific TNF-α antagonist which has displayed positive results in the improvement of glomerular disease (Navarro and Mora-Fernández, 2006). Anti-TNF-α antibodies administered in rats, reduce levels of proteinuria and histological lesions (Hruby et al., 1991). Additionally a TNF-α inhibitor SKF86002 shows great reduction in TNF-α production and improves renal function (Pan et al., 1996). There is a methylxanthine phosphodiesterase inhibitor known by pentoxifylline (PTF) which has been used to treat individuals with peripheral vascular disease (Ward and Clissold, 1987). It is reported that PTF reduces TNF-α mRNA accumulation and TNF-α gene transcription (Strieter et al., 1988). A study by Bilsborough et al, used PTF to reduce TNF-α in type 2 diabetes in order to assess the endothelial function in humans. However, the study proved to be inconclusive as the endothelium-dependant and -independent vascular functions (Bilsborough et al., 2002). While *in vitro* and animal studies conclude positive results with TNF-α reduction on improvement of endothelial function through the restoration of haemorrhage-induced nitric oxide production from endothelial cells (Wang et al., 1995).

1.7.2.3 Interleukin-6

The interleukin-6 cytokine belongs to the interleukin-6 family and is also comprised of IL-11, ciliary neurotrophic factor, oncostatin M, leukaemia inhibitory factor, cardiotrophin-like cytokine, cardiotrophin 1 and IL-27. These cytokines are grouped as one family due to the resulting receptor complex either contain two molecules of IL-6 or IL-11 or contain a single molecule of the other cytokines in the family. The complex formed is between the cytokine and the receptor subunit, gp130 (Rose-John, 2018). IL-6 specifically is a prototypical cytokine which is pleotropic in nature with a strong role in host defence. IL-6 binds to its specific receptor IL-6R. Upon complex associations with gp130, signals activate hepatocytes, immune-competent cells and haematological cells produce an acute immune response. The function of the response is to eradicate infectious agents and to heal tissues. Once stimulation of an infection or tissue damage has dissipated, synthesis of IL-6 is ceased and serum concentrations return to normal (Tanaka et al., 2016). Excessive production of IL-6 during prolonged infections or tissue damage can result in acute fatal complications or cause persistence in production of the cytokine to promote chronic inflammatory diseases (Kang, Tanaka and Kishimoto, 2015).

Actions of increased IL-6 in hepatocytes cause induction of acute phase proteins like C-reactive protein (CRP), complement C3, fibrinogen, haptoglobin, thrombopoietin, serum amyloid A, hepcidin and α1-antichymotrypsin. The vast variety of impacted proteins suggest the importance of IL-6 on signal mediation for acute phase responses (Tanaka, Narazaki and Kishimoto, 2018). Depending on location of production, IL-6 participates in a multitude of biological activities. Stromal cells in the bone marrow release IL-6 to

initiate fibroblast-like synoviocytes to produce receptor activator of NF-κB. This function is important for differentiation and activation of osteoclasts (Kotake et al., 1996). In vascular endothelial cells, release of IL-6 results in the increased IL-8, monocyte chemoattractant protein-1 (MCP-1), Intercellular Adhesion Molecule 1 (ICAM-1) and C5a receptors which results in VE-cadherin disassembly that increases permeability and interstitial oedema (Tanaka, Narazaki and Kishimoto, 2018).

IL-6 observations in serum have shown to correlate with obesity and type 2 diabetes development hence suggesting a causal link with metabolic disease development (Pradhan et al., 2001; Weiss et al., 2004). The role of IL-6 in metabolic control appears to be more complex when initially it was seen that it had a beneficial role when assessing mice with knockout IL-6 having developed mature-onset diabetes with increased leptin and insulin levels. Additionally, deletion of IL-6R also displayed insulin resistance indicating the protective role of IL-6 (Wunderlich et al., 2010; Matthews et al., 2010). However, evidence does indicate the induction of IL-6 through TNF-α stimulation in adipose tissue during obesity. Furthermore, in an IL-6 dependent manner, JNK1 activation in adipose tissue of mice led to insulin resistance in the liver (Schmidt-Arras and Rose-John, 2016). IL-6 has also been discovered to be an independent predictor of type 2 diabetes and associated cardiovascular events, giving rise to additional markers for disease prognosis (Spranger et al., 2003; Lowe et al., 2014). This was displayed in study in mice suggesting it to be a contributor to endothelial dysfunction in obesity and type 2 diabetes. The interactions of IL-6 are shown to exacerbate oxidative stress and reduce phosphorylation of eNOS which contributes to the endothelial dysfunction (Lee et al., 2017).

IL-6 directed therapy has been shown to work in many areas involving the neutralisation of IL-6 to treat multiple myeloma, however, use of an anti-IL6 antibody siltuximab (SYLVANT®) displayed contradictory results. Further to that study a receptor neutralising antibody was design tocilizumab (ACTEMRA®), which binds to IL-6R to prevent IL-6 binding and initiating a response (Schmidt-Arras and Rose-John, 2016). Within the study this greatly impacted on the therapeutic benefits on rheumatoid arthritis. Results of a mouse study also found that the blockade of IL-6R reduces insulin resistance and inhibits inflammasomes to protect against diabetic nephropathy (Wu et al., 2018).

1.8 Aims & Objectives.

While the study of inflammatory cytokines has been vastly researched, it is still unclear the impact certain inflammatory cytokines associated with diabetes, have on angiopoietin signalling in endothelial cells. Additionally, the influence these cytokines have on endothelial cells have yet to be described at a molecular level in terms of cell functionality.

The main aims of the project were:

1. To identify levels of IL-6 and TNF-α and Ang-2 in diabetes by performing ELISA on serum collected from blood samples of normal, type 1 and type 2 diabetic patients. Cytokine data was attained through the use of RANDOX multiplex ELISA array while Ang-2 results were collected using sandwich ELISA. The principle between the two techniques is the same however the RANDOX kit has the potential to analyse a multitude of cytokine and biomarkers simultaneously from

a single sample. Concentrations were calculated against a standard curve of each protein.

2. To examine the acute impacts of IL-1β and TNF-α on expression of Tie receptors and Ang-1 induced activation of the Tie-2 signalling pathway through AKT in a primary cell line (HUVECs), using techniques involving cell culture and direct stimulation of inflammatory cytokine which would be assessed through western blotting. The quantification of Tie-2 expression as well as phosphorylation of Tie-2 and AKT, would give evidence for the implication of cytokine stimulation in endothelial cells.
3. To examine the chronic impacts of IL-1β and TNF-α on expression of Tie receptors and Ang-1 induced activation of the Tie-2 signalling pathway through AKT in a primary cell line (HUVECs), using techniques involving cell culture and direct stimulation of inflammatory cytokine which would be assessed through western blotting. Quantification of Tie-1, Tie-2 and AKT expression as well as phosphorylated AKT expression would give evidence for the impacts the cytokines have on the protein expression of these vascular associated receptors and mediators.
4. To investigate the functional consequences of IL-1β and TNF-α stimulation on HUVECs. Using a technique to measure apoptosis, the effects of cytokine stimulation will have been calculated using fluorescent tagging of live and dead cells. The results should provide a scope of the physiological effects on the endothelial cells hence suggesting the overall effect on the vasculature.

Chapter 2: Materials and Methods

2.1 Consumables and Equipment

2.1.1 Materials

Reference Number	Material	Supplier	Catalogue number	Production Information
1	Gibco™ Medium 200	Fisher Scientific	10134873	Basal culture media comprised of essential and non-essential amino acids, vitamins, and other organic compounds. Media does not contain antibiotics, antimycotics, hormones, growth factors or proteins. This has also been buffered with HEPES and bicarbonate. The designed use is for plating and proliferation of large endothelial cell in an incubator with a 5% CO_2 atmosphere. Media must be supplemented with LSGS.
2	Gibco™ Low Serum Growth	Fisher Scientific	10616973	This is a 50X stock that is to be diluted in the Medium 200. Final concentrations in the supplemented

	Supplement (LSGS)			media are 2%v/v fetal bovine serum, 1µg/ml hydrocortisone, 10ng/ml human epidermal growth factor, 3ng/ml basic fibroblast growth factor and 10µg/ml heparin.
3	Gibco™ Fetal Bovine Serum, qualified, E.U.-approved, South America origin	Fisher Scientific	11573397	Undiluted serum with no buffers presents.
4	Gibco™ PBS, pH 7.4	Fisher Scientific	15374875	This solution is formulated without calcium and magnesium for the purpose of rinsing chelators from cultures before cell dissociation. This PBS is also phenol red-free and does not contain sodium pyruvate.

5	Recombinant Human IL-1 beta/IL-1F2 Protein	R&D Systems	201-LB-025	This is an *E. coli*-derived human IL-1 beta /IL-1F2 protein with a predicted molecular weight of 17kDa. The lyophilised stock was reconstituted at 25µg/ml in sterile PBS with 0.1% bovine serum albumin.
6	Recombinant Human TNF-alpha Protein	R&D Systems	210-TA-100	This is an *E. coli*-derived human TNF-alpha protein with a predicted molecular weight of 17.5 kDa. The lyophilised stock was reconstituted to 100µg/ml in sterile PBS with 0.1% bovine serum albumin.
7	Recombinant Human Angiopoietin-1 Protein	R&D Systems	923-AN-025	This protein is derived from a mouse myeloma cell line, NS0-derived Ser20-Phe498, with a C-terminal 6-His tag with an expected molecular weight of 56kDa. This lyophilised stock was reconstituted at 100µg/ml with 0.1% bovine serum albumin.
8	Amersham™ ECL™ Rainbow™	Sigma Aldrich	GERPN800E	Size range 12 – 225kDa.

	Marker - Full range			
9	Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Fisher Scientific	11832124	Size range 10 – 250kDa. Dye-stained proteins in 62.5mM Tris-H_3PO_4 (pH 7.5 at 25°C), 1mM EDTA, 2% SDS, 10mM DTT, 1mM NaN_3 and 33% glycerol.
10	COZY™ PRESTAINED PROTEIN LADDER	Clent Life Science	PRL0102	Size range 8 – 180kDa.
11	Invitrogen™ LIVE/DEAD™ Cell Imaging Kit (488/570)	Fisher Scientific	12353643	Based on cell-permeable dye for staining of live cells and cell-impermeable dye for staining of dead and dying cells. Live cell component produces intense, uniform green fluorescence in live cells (ex/em 488 nm/515nm). Live cells are distinguished by presence of

				ubiquitous intracellular esterase activity as determined by the enzymatic conversion of virtually nonfluorescent cell-permeant calcein AM to intensely fluorescent calcein, which is well-retained within live cells. Dead cell component produces predominantly nuclear red fluorescence (ex/em 570nm/602nm) in cells with compromised cell membranes, strong indicator of cell death and cytotoxicity. Red component is cell-impermeant and therefore only enters cells with damaged membranes. In dying and dead cells, bright red fluorescence is generated upon binding to DNA.
12	Invitrogen™ Human Angiopoietin-2 ELISA Kit	Fisher Scientific	10313773	Invitrogen™ Human Angiopoietin-2 ELISA Kit is a solid-phase ELISA designed to detect Ang-2 in serum, plasma, buffered solution, tissue culture medium and cell lysates. The kit

				comes with Hu Ang-2 Standard in lyophilised form containing 0.1% sodium azide, standard diluent buffer also containing 0.1% sodium azide, Hu Ang-2 antibody coated 96-well plate, incubation buffer with 0.05% sodium azide, anti-Hu Ang-2 biotin conjugate containing 0.1% sodium azide, streptavidin (100X), streptavidin diluent containing 3.3mM thymol, wash buffer (25X), stabilised chromogen (tetramethylbenzidine), stop solution and plate covers.
13	Gibco™ Trypsin-EDTA (0.5%), no phenol red	Fisher Scientific	10779413	Due to its digestive strength, trypsin solution is widely used for cell dissociation, routine cell culture passaging, and primary tissue dissociation. Concentrated forms of trypsin can be aseptically diluted to 1X using PBS. Made from trypsin powder, an irradiated mixture of proteases derived from porcine pancreas.

14	Metabolic Syndrome Array I	RANDOX	Unavailable	Multiplex testing kit, to measure concentrations of ferretin, interleukin-6, insulin, leptin, plasminogen activator inhibitor-1, resistin and TNFα, simultaneously from serum or plasma samples.

2.1.2 Equipment

Reference Letter	Equipment	Supplier	Catalogue number
a	Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-Rad	1658005
b	Mini Trans-Blot® Cell	Bio-Rad	1703930
c	GeneGnome Chemiluminescence imaging	Syngene	Discontinued Model
d	EVOS® FL Cell Imaging System	Thermo Fisher Scientific	Discontinued Model

e	GloMax® Microplate Reader	Discover	Promega	GM3000
f	RANDOX Investigation	Evidence	RANDOX	Unavailable

2.1.3 Solutions

#1. Activated 2X Sample Buffer:

4% SDS, 20% Glycerol, 120Mm Tris-Cl (pH 6.8), 0.004% Bromophenol Blue, 216mM DTT, 80mM Sodium Pervanadate

#2. Stacking Gel:

5% ProtoGel (30%; 37.5:1 acrylamide to bisacrylamide), 125mM Tris base, 3.47mM Sodium dodecyl sulphate, 4.38mM Ammonium persulphate, 0.1% *N*, *N*, *N*', *N*'-Tetramethylethylenediamine

#3. Resolving Gel:

10% ProtoGel (30%; 37.5:1 acrylamide to bisacrylamide), 370mM Tris base, 3.47mM Sodium dodecyl sulphate, 2.94mM Ammonium persulphate, 0.07% N, N, N', N'-Tetramethylethylenediamine

#4. Running Buffer:

24.8mM Tris base, 192mM Glycine, 3.47mM Sodium dodecyl sulphate

#5. Transfer Buffer:

24.8mM Tris base, 192mM Glycine; 10% Methanol

#6. Blocking Solution:

5% non-fat dried milk powder dissolved in TBS-T

#7. Tris-buffered saline with Triton X-100:

25mM Tris, 144mM NaCl, 0.1% Triton™ X-100 (Sigma Aldrich – T8787)

#8. Primary Antibody Solution:

A primary antibody diluted in blocking solution. See antibody list (2.5.4) for dilutions and additional information.

#9. Secondary Antibody Solution:

A secondary antibody diluted in blocking solution. See antibody list (see 2.5.5) for dilutions.

#10. Enhanced Chemiluminescence Solution:

100mM Tris-HCl (pH 8.5), 198µM p-coumaric acid, 1.25mM luminol, 0.01% H_2O_2

#11. Freeze Media:

78% Un-supplemented Medium 200, 12% Fetal Bovine Serum, 10% Dimethyl Sulfoxide

2.1.4 Primary Antibodies

Antibody	Vendor	Catalogue Number	Dilution	Additional Note
Human Tie-2 Antibody	Bio-techne (R&D Systems)	AF131	1:1000	
Phospho-Tie2 (Tyr992) Antibody	Cell Signaling Technology	4221S	1:850	Instead of milk powder in the blocking solution, use Bovine Serum Albumin
Akt (pan)(11E7) Rabbit mAb	Cell Signaling Technology	4685S	1:1000	Instead of milk powder in the blocking solution, use Bovine Serum Albumin
Human/Mouse/Rat Phospho-Akt (S473) Pan Specific Antibody	Bio-techne (R&D Systems)	AF887	1:1000	Instead of milk powder in the blocking solution, use Bovine Serum Albumin

Anti-TIE1 antibody	Abcam	Ab111547	1:1000
Human/Mouse/Rat beta-Actin Antibody	Bio-techne (R&D Systems)	MAB8929	1:2000

2.1.5 Secondary Antibodies

Antibody	Vendor	Catalogue Number	Dilution
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	7074S	1:2000
Goat IgG HRP-conjugated Antibody	Bio-techne (R&D Systems)	HAF017	1:2000
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling Technology	7076S	1:2000

2.2 Profiling Cytokines in Patient Serum Samples

This work has undergone ethical approval from De Montfort University Health and Life Sciences Research Ethics Committee with the approval reference FREC 855. This study stems from a larger collective project focused on analysing the effects of a combined exercise program on inflammatory and physiological parameters (Alsubaie, 2019; Chauhan, TBD). Patient details remain confidential with the research team, only the anonymised ID's and the respective multiplex ELISA data were shared with this project.

2.2.1 Sample Selection

The population of the study involved healthy subjects (non-diabetic), type 1 diabetic and type 2 diabetic. Volunteers within the 18-60-year age that also met the inclusion criteria were used. The exclusion criteria for the study are listed in Table 1 .

Table 1. List of exclusion criteria. *If volunteers were categorised with at least one of the exclusion criteria, the individual would not be able to participate in the study as these underlying conditions could impact the results as well as the exercise regime being detrimental to their health.*

Heart disease.	Bleeding.
Liver disease.	Epilepsy.
Kidney disease.	Acute exacerbated bronchial asthma.
Uncontrolled and very high blood pressure > 160/95 mmHg.	Osteoporosis.
Injury.	Arthritis.
Mentally ill.	Had recent surgery.
Medications which increase blood sugar e.g., steroids.	Using medication mask hypoglycaemic symptom e.g., beta blockers.

Power calculations were not conducted in this study as this was part of pilot study, hence statistically derived number of volunteers were not required at this stage. The pilot study was analysing the impacts of exercise on metabolic syndrome markers. This study was just focusing on then levels of IL-6 and TNF-α before the exercise regime took place, thus samples were collected pre-exercise. Of the 26 volunteers that took part in the study, only 21 samples were used for the purpose of this investigation on the account of TNF-α and IL-6 data being available. From the 21 samples, 19 samples had complete data sets.

2.2.2 Sample Collection

To maintain sterility and avoid cross-contamination, alcohol swabs and disposable latex-free tourniquets were used for venous blood collection. The blood was collected aseptically using a vacutainer system with a blood preparation tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant to appropriately store the blood and prevent degradation. Each blood sample was collected from the antecubital fossa as it is easily accessible, provides minimal discomfort to the volunteers and the area houses several large veins. Blood plasma was separated by chilled centrifugation at a setting of 3000rpm for 15 minutes while kept at a constant 4°C. Plasma was transferred to a labelled Eppendorf and stored at -80°C until required for analysis. This was performed by Dr Nawal Alsubaie and Dr Krishan Chauhan.

2.2.3 Multiplex ELISA for measuring IL-6 and TNFα

The Evidence Investigator™ from RANDOX (see 2.1.2 – f) is a revolutionary tool that allows for multiple ELISAs to processed at once using Biochip Array technology. The RANDOX biochip array, Metabolic Syndrome Array I (see 2.1.1 – 14), has the capacity to measure Ferritin, IL-6, Insulin, Leptin, PAI-1, Resistin and TNF-α. However, for the purpose of inflammatory cytokine profiling in this study only Interleukin-6 and Tumour Necrosis Factor α was measured.

Samples were thawed and all materials were equilibrated to room temperature before beginning the experiment. To the 9 vials of calibrator and 3 vials of the control 1ml of double deionise water was added and then left to roll on a tube roller for 30minutes. Meanwhile the 6 biochip carriers were attached to the handling tray before 200µl of assay diluent was pipetted into each well (54 wells). This was followed by the addition of 100µl of calibrator, control, and samples in order. The handling tray was tapped along each edge to aid in the mixing of the reagents. The handling tray was then placed on the base plate of a thermoshaker and then incubated for 1hour at 37°C at 370rpm, however prior, the manufacturers recommendation to equilibrate the thermoshaker at 37°C for 30minutes was addressed. The washing buffer provided needed to be diluted by a factor of 31.25 for use in the following step. Post incubation, the handling tray was removed from the thermoshaker and the reagents were discarded using a quick, flick of the handling tray over a sink. 2 quick wash cycles were conducted with wash buffer by vigorously tapping the sides for 20 seconds before discarding the reagents like before. 4 additional washes followed suit, with each one being 2minutes each. After the wash

buffer was discarded from the final wash, 300µl of conjugate was pipetted into each well and left to incubate at 37°C at 370rpm on a thermoshaker for 1hour. During this period, preparation of the working signal reagent EV40 was arranged, and the solution was then protected from light. Once incubation was complete the same washing protocol was repeated before removing the first carrier on the handling tray and then adding 250µl of EV40 to the carrier. This was then protected from the light for 2minutes before placed in the Evidence Investigator™. This was repeated for each of the other 5 biochips. The Evidence Investigator™ would automatically capture the images that would then be viewed on the computer.

2.2.4 Data Analysis of IL-6 and TNFα

Each sample was prepared in triplicates to be able to rule out any anomalies. The raw data was adjusted having removed the control values from each result. A plot of the standards was created, and the sample concentrations were calculated from the formula of the standard curve. A test of normality was conducted using SPSS to determine the nature of the result to then decide whether a parametric test would be required or a non-parametric test. Significance of the test would observe $p \leq 0.05$ to conclude results showing statistical significance.

2.2.5 Ang-2 ELISA

This experiment involved the use of a pre-coated Ang-2 ELISA plate kit (see 2.1.1 – 12). To begin, serum samples were thawed and mix gently to ensure components of the sample were evenly distributed. Sample concentrations should have been within the range of the standard curve, therefore based on recommendations by the

manufacturers the samples were diluted 10-fold in a standard diluent buffer. To create the standard curve, the standard solution also needed to be diluted in series. 11 standard dilutions were prepared ranging from 20000pg/ml to 0pg/ml (see appendix 2 for full dilution diagram). Likewise, with the sample, the standards were diluted with standard diluent buffer. Finally, before beginning the ELISA protocol, 1X Streptavidin-HRP solution from the 100X stock provided in the kit was prepared. Once all components were at room temperature and liquids were all mixed thoroughly, 25µl of Incubation Buffer was added to all wells except the chromogen blanks. Then 100µl of standards or samples were pipetted into the appropriate wells, again not having added anything to the chromogen blank. This was then followed by 50µl of Hu Ang-2 Biotin Conjugate solution in each well with the exception of the chromogen blank. The sides of the plate were gently tapped to mix the solutions in the wells, then covered and incubated at room temperature for 2hours. After the allotted time, the solution was aspirated, and the wells were washed 4 times with 1X Wash Buffer. 100µl of 1X Streptavidin-HRP solution was dispensed into each well except the chromogen blanks and covered before a further incubation for a further 30minutes. This was then followed by repeating the washing stage from the previous step. At this point 100µl of Stabilised Chromogen was added to each well and at this point visual observations of the plate indicated the solution turning blue. The plate was incubated for 30minutes in the dark, making sure the chromogen did not come into contact with any metals. After 30minutes 100µl of Stop solution was added to each well and the sides of the plate were tapped to mix the solutions, the blue solution had then changed to a yellow solution. Within 30minutes of

the Stop solution being added the plate was read in a plate reader (see 2.1.2 – e) with absorbance set to 450nm.

2.2.6 Data Analysis of Ang-2

Sample testing was performed in duplicates All the results had the background reading subtracted from them before plotting the standards to create a standard curve. The standard curve was then used to determine the concentrations in each sample, but each value needs to take into consideration of the dilution of the sample to start with. A test of normality would be conducted using SPSS to determine the nature of the result to then decide whether a parametric test would be required or a non-parametric test. Significance of the test would observe $p \leq 0.05$ to conclude results showing statistical significance.

2.3 Cell Culture

2.3.1 Primary Endothelial Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured using Gibco™ Medium 200 (see 2.1.1 – 1) supplemented with Gibco™ Low Serum Growth Supplement (LSGS) (see 2.1.1 – 2) and 5-12% of Gibco™ Fetal Bovine Serum (FBS) (see 2.1.1 – 3) depending on the pattern on growth in order to have the T-75 plates above 70% confluence. Cells were incubated at 37°C at 5% CO_2. Cells were passaged accordingly to grow enough cells for the use in each experiment. For experimental purpose, only cells between passage 2 and passage 6 were used. Cells after passage 6 often lose their ability to grow adequately

and cell viability reduces to below 50%. For each experiment, a confluent T-75 was transferred to a six well plate.

2.3.2 Cell Maintenance

During cell culture observing growth of the cells are vital for maintaining healthy cells for the purpose of experimentation and further growth and passage of the cell line. Key points to note during observations of the culture plate the cells are being grown in; media should remain clear; cloudiness of the media indicates large debris of dead cells or indicates infection from contamination through non-aseptic techniques. Under a light microscope, observation of the cell monolayer should be assessed, and the following questions should be answered:

- Is there a spread of cells around the whole plate?
- Are the cells close to each other or in clusters?
- Does the monolayer look like its folding on itself?
- Are the cells looking short and thin?

The cells should look as though they cover the entire surface of the plate, large areas indicate a lack of cells and poor technique with seeding of cells to the plate. Further to the cell need to appear close to each other and not over spread. Lack of contact between cells prevents optimal growth and proliferation as intercellular communications signal for the development of endothelial cells. Sometime over proliferation in one area of the plate and result in cells growing on top of each other which can affect growth and cause increases in apoptosis of cells as these cells grow whilst adhered and not in suspension.

Short and thin cells indicate increased stress either due to physical shock, infection or when they have been passaged too many times.

For HUVECs, switching between LSGS only supplemented Medium 200, 5% serum + LSGS supplemented Medium 200 and 10% serum + LSGS supplemented Medium 200, can be beneficial for optimal growth based on observation of the cells. 12% serum + LSGS supplemented Medium 200 is sometimes necessary to help boost the growth of the cells if they are proliferation slowly, however, should not be continually used throughout cell culture as it can result in cell stress and disassociation of the cells from the plate surface.

2.3.3 Freezing Cells

Freezing of cells allows for banks of cells to be created and therefore increase the amount and duration of projects while having to use just a single cell line starter. It is also useful to freeze down cells if they will not be used immediately, hence reducing a waste in consumables and time growing the cells. For freezing plates of cells must been grown to confluence. Plate should have media removed and washed with PBS before being trypsinised (see 2.1.2 – 13). Trypsin is added to the plate and left to incubate for 1minute at 37°C at 5% CO_2. Once it has been seen that all the cells have detached from the plate surface, equal volume of media is then added to prevent trypsin from causing cell apoptosis. If under a light microscope the cells are still attached, light taps to the plate can aid in dislodgment. The cell solution should then be transferred to a centrifuge tube and spun down at 1500rpm at 4°C for 5minutes. Supernatant should be discarded as cell pellet should be undisturbed. 1ml of fresh media should be used to gently mix with the cell pellet to create a 1ml stock solution of cells. A cell count (see 2.1.4) is then

conducted to calculate the volume required to freeze aliquots of 1million cell vials. The required volume is then added to freeze media (see 2.1.3 – #11) to make up 1ml in a vapour phase cryovial.

2.3.4 Cell Counting

The process of cell counting begins with preparing a 1ml solution of cells (as seen in 2.1.3). Mix well and aspirate 20µl of the solution to an Eppendorf. Equal volumes of trypan blue need to be added to the Eppendorf and mixed well. The resultant solution needs to be carefully loaded into a haemocytometer. Care must be taken not to overload the solution or underload, additionally avoid moving the coverslip on the haemocytometer. Figure 4 indicates the view of the haemocytometer under a light microscope. Cells within the four corner sections indicated are counted following the rule of only including cells that fall within each section and on the top and left line of the corner section. Once counted the sum of the four sections is applied to the following formula as *n*.

$$Average\ Number\ of\ Cells\ per\ ml = \frac{n}{2} \times 10000$$

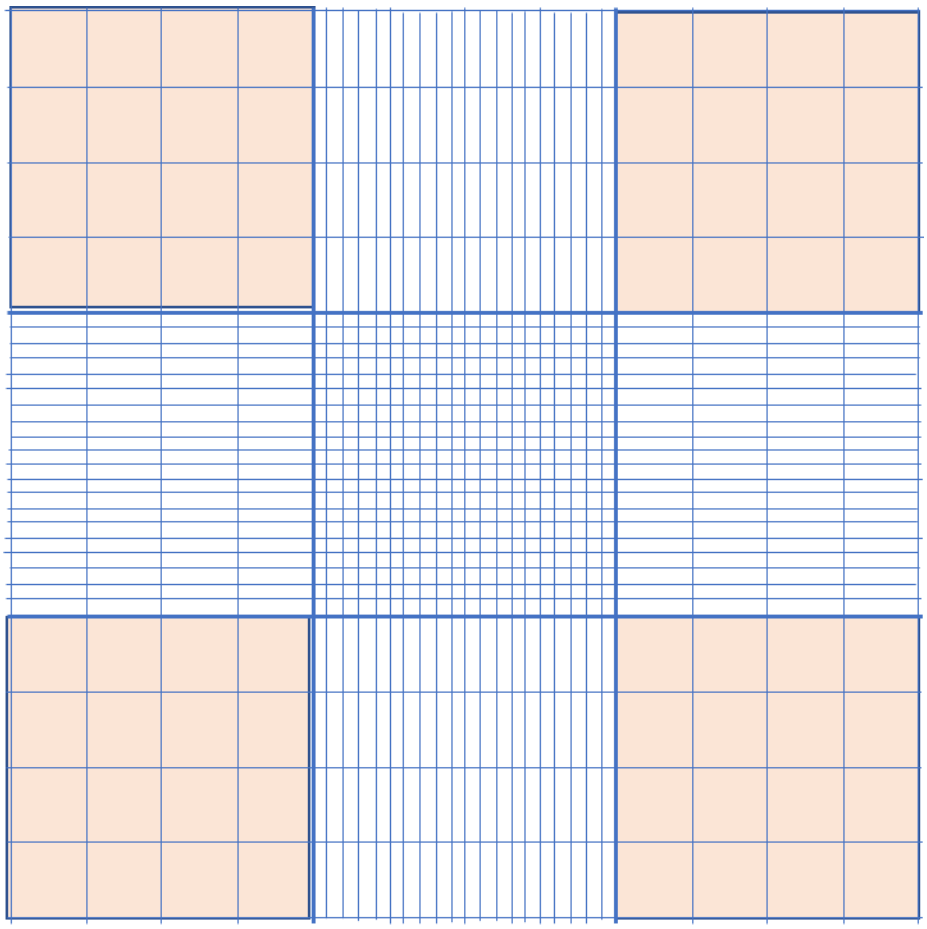


Figure 4. View of a Haemocytometer under a microscope. *The light orange sections in the corner are the sections in which the cells are counted to then be used in the calculations for cell count.*

2.4 Cell Stimulation

2.4.1 Optimisation of IL-1β

Preliminary studies were conducted to determine the concentrations required for cytokine to have an impact on the signalling pathway, but also to see when the response would plateau. The protocol follows the time course (see 2.4.2), except the time was set to a constant 10minutes with an additional 15minutes for Ang-1 stimulation. The concentrations tested were 10, 25, 50 and 100ng/ml, furthermore this was only

conducted with IL-1β. The trend showed Tie-2 expression to plateau at 25ng/ml however statistical significance was lacking. However, there was statistical significance for a decrease in phospho-Akt and the lowest point of activity at 25ng/ml (see appendix 1). To maintain control across the acute and chronic exposures of cytokines, both IL-1β and TNFα were used at final concentrations of 25ng/ml.

2.4.2 Acute Exposure of Cytokines on HUVECs

2.4.2.1 Interleukin-1β

HUVECs underwent stimulations in a 6-well plate having been transferred from a T-75. No cell counts were performed as the final experimental values would be relative based on a loading control. Figure 5 displays a table of time points and a diagram of the 6-well plate to indicate the samples that were collected from each time point. Prior to the start of the time course, each well was gently washed with warm Gibco™ PBS (see 2.1.1 – 4) and then added 1ml of Medium 200 with LSGS only, finally allowing it to have incubated for an hour before starting the time course. 25ng/ml Recombinant Human IL-1 β (see 2.1.1 – 5) was added to the respective well and then placed in the incubator to begin the timed experiment. At each respective time point the plate was removed from the incubator and IL-1β was added to the appropriate well at the given time point. This was repeated to the final time point at which Recombinant Human Angiopoietin-1 (100ng/ml) (see 2.1.1 – 7) was added in the last 15 minutes to the respective wells. To terminate the experiment, media was removed from each well and washed with cold PBS. 60µl of activated 2X sample buffer (see 2.1.3 – #1) was dispensed into each well, at which point the plates and subsequent samples were kept on ice. Each well was scraped,

and the contents were transferred to a marked Eppendorf. All the samples were sonicated at 20% amplification for 15 seconds before being heated on a dry heating block at 95°C for 5 minutes. Samples were centrifuged at 2000rpm for 1 minute. The samples could then be frozen or loaded on an SDS-PAGE for electrophoresis.

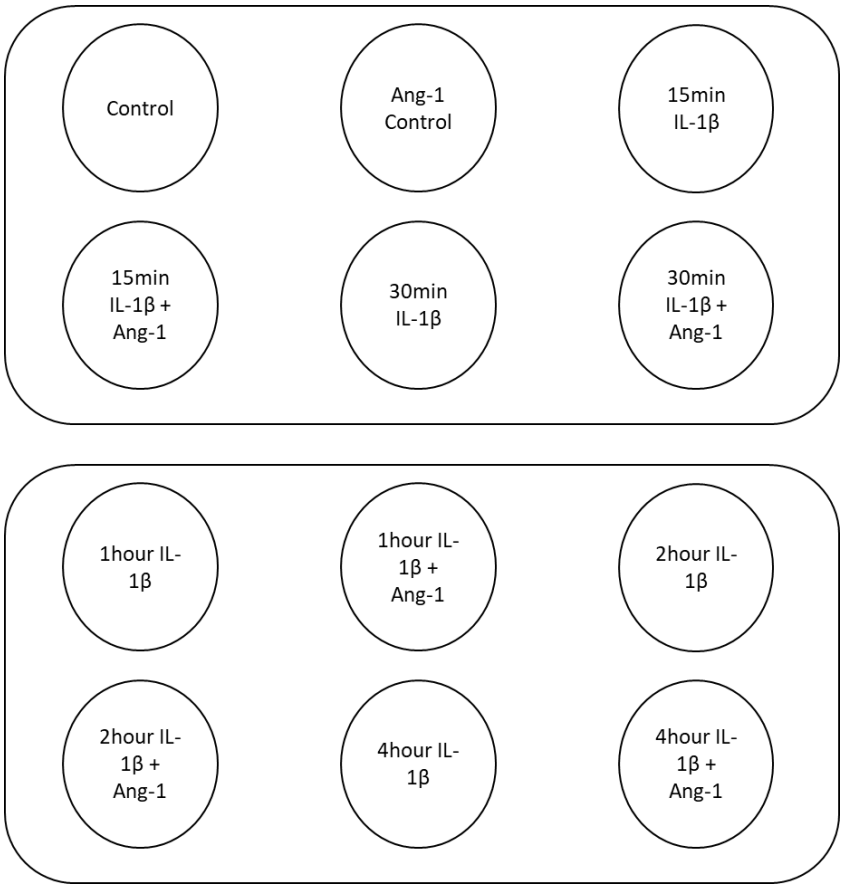


Figure 5. 6 well plates for IL-1β stimulations. *The diagram displays the time of IL-1β exposure and the presence or absence of Ang-1.*

2.4.2.2 Tumour Necrosis Factor α

HUVECs underwent stimulations in a 6-well plate having been transferred from a T-75. No cell counts were performed as the final experimental values would be relative based on a loading control. Figure 6 displays a table of time points and a diagram of the 6-well plate to indicate the samples that were collected from each time point. Prior to the start

of the time course, each well was gently washed with warm Gibco™ PBS (see 2.1.1 – 4) and then added 1ml of Medium 200 with LSGS only, finally allowing it to have incubated for an hour before starting the time course. 25ng/ml Recombinant Human TNF-α (see 2.1.1 – 6) was added to the respective well and then placed in the incubator to begin the timed experiment. At each respective time point the plate was removed from the incubator and TNFα was added to the appropriate well at the given time point. This was repeated to the final time point at which Recombinant Human Angiopoietin-1 (100ng/ml) (see 2.1.1 – 7) was added in the last 15 minutes to the respective wells. To terminate the experiment, media was removed from each well and washed with cold PBS. 60µl of activated 2X sample buffer (see 2.1.3 – #1) was dispensed into each well, at which point the plates and subsequent samples were kept on ice. Each well was scraped, and the contents were transferred to a marked Eppendorf. All the samples were sonicated at 20% amplification for 15 seconds before being heated on a dry heating block at 95°C for 5 minutes. Samples were centrifuged at 2000rpm for 1 minute. The samples could then be frozen or loaded on an SDS-PAGE for electrophoresis.

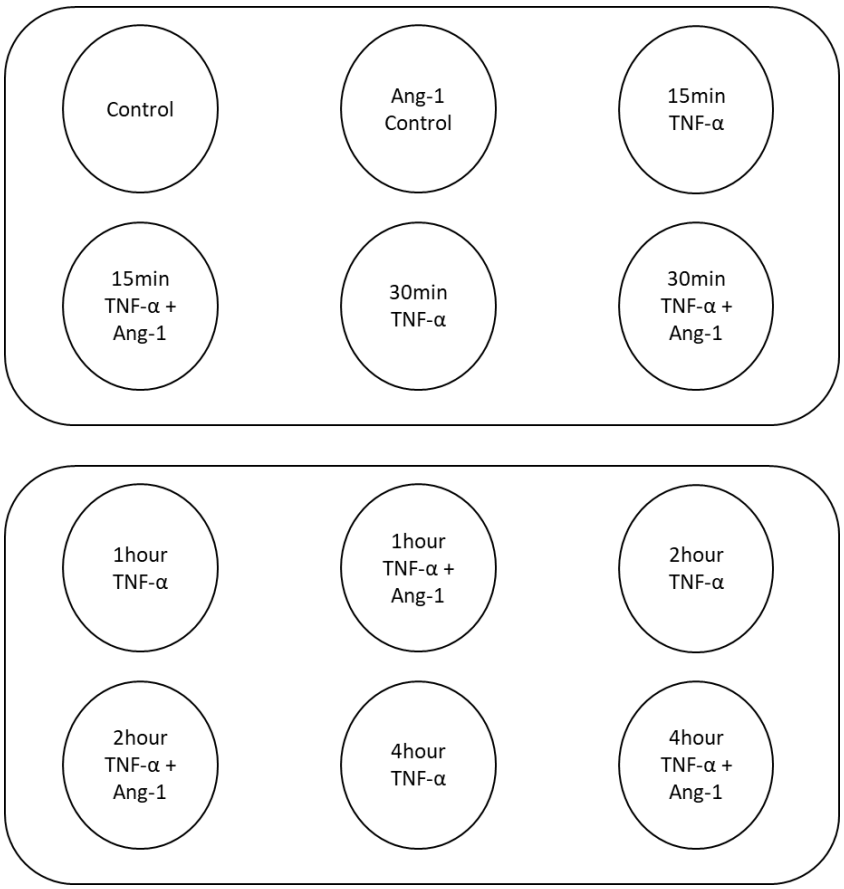


Figure 6. 6 well plates for TNF-α stimulations. *The diagram displays the time of TNF-α exposure and the presence or absence of Ang-1.*

2.4.3 Chronic Exposure of Cytokines on HUVECs

2.4.3.1 Interleukin-1β

HUVECs underwent stimulations in a 6-well plate having been transferred from a T-75. No cell counts were performed as the final experimental values would be relative based on a loading control. Figure 7 displays a table of time points and a diagram of the 6-well plate to indicate the samples that were collected from each time point. Prior to the start of the time course, each well was gently washed with warm Gibco™ PBS (see 2.1.1 – 4) and then added 1ml of Medium 200 with LSGS only, finally allowing it to have incubated

for an hour before starting the time course. 25ng/ml Recombinant Human IL-1β (see 2.1.1 – 5) was added to the respective well and then placed in the incubator to begin the timed experiment. At each respective time point the plate was removed from the incubator and IL-1β was added to the appropriate well at the given time point. This was repeated to the final time point at which Recombinant Human Angiopoietin-1 (100ng/ml) (see 2.1.1 – 7) was added in the last 15 minutes to the respective wells. To terminate the experiment, media was removed from each well and washed with cold PBS. 60µl of activated 2X sample buffer (see 2.1.3 – #1) was dispensed into each well, at which point the plates and subsequent samples were kept on ice. Each well was scraped, and the contents were transferred to a marked Eppendorf. All the samples were sonicated at 20% amplification for 15 seconds before being heated on a dry heating block at 95°C for 5 minutes. Samples were centrifuged at 2000rpm for 1 minute. The samples could then be frozen or loaded on an SDS-PAGE for electrophoresis.

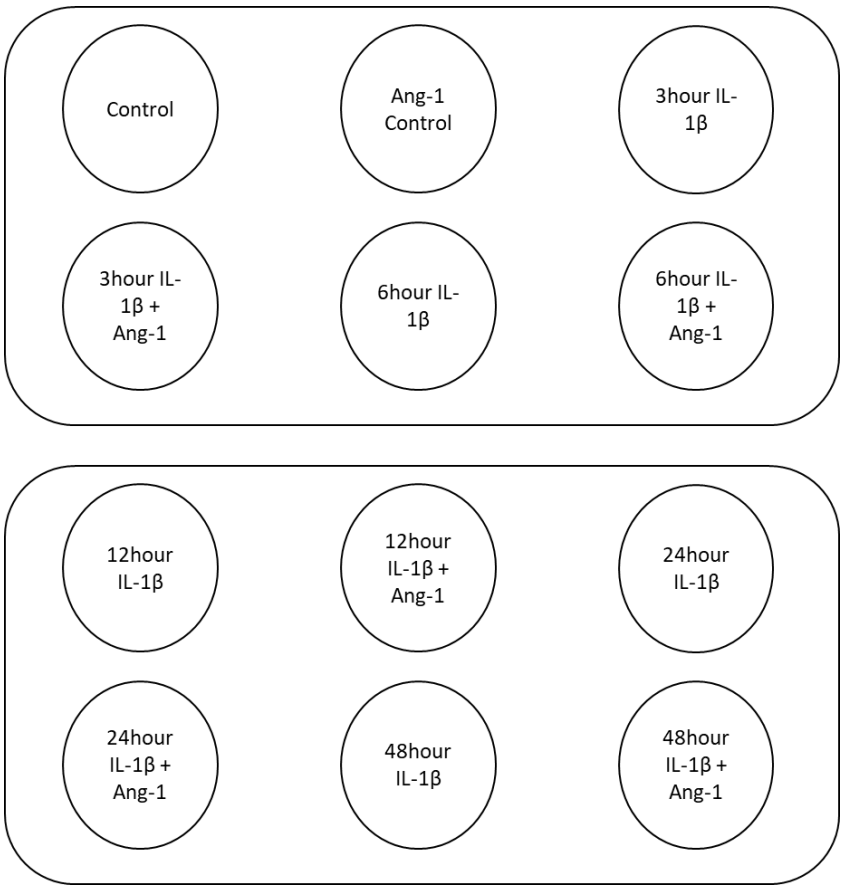


Figure 7. 6 well plates for IL-1β stimulations. *The diagram displays the time of IL-1β exposure and the presence or absence of Ang-1.*

2.4.3.2 Tumour Necrosis Factor α

HUVECs underwent stimulations in a 6-well plate having been transferred from a T-75. No cell counts were performed as the final experimental values would be relative based on a loading control. Figure 8 displays a table of time points and a diagram of the 6-well plate to indicate the samples that were collected from each time point. Prior to the start of the time course, each well was gently washed with warm Gibco™ PBS (see 2.1.1 – 4) and then added 1ml of Medium 200 with LSGS only, finally allowing it to have incubated for an hour before starting the time course. 25ng/ml Recombinant Human TNFα (see

2.1.1 – 6) was added to the respective well and then placed in the incubator to begin the timed experiment. At each respective time point the plate was removed from the incubator and TNFα was added to the appropriate well at the given time point. This was repeated to the final time point at which Recombinant Human Angiopoietin-1 (100ng/ml) (see 2.1.1 – 7) was added in the last 15 minutes to the respective wells. To terminate the experiment, media was removed from each well and washed with cold PBS. 60µl of activated 2X sample buffer (see 2.1.3 – #1) was dispensed into each well, at which point the plates and subsequent samples were kept on ice. Each well was scraped, and the contents were transferred to a marked Eppendorf. All the samples were sonicated at 20% amplification for 15 seconds before being heated on a dry heating block at 95°C for 5 minutes. Samples were centrifuged at 2000rpm for 1 minute. The samples could then be frozen or loaded on an SDS-PAGE for electrophoresis.

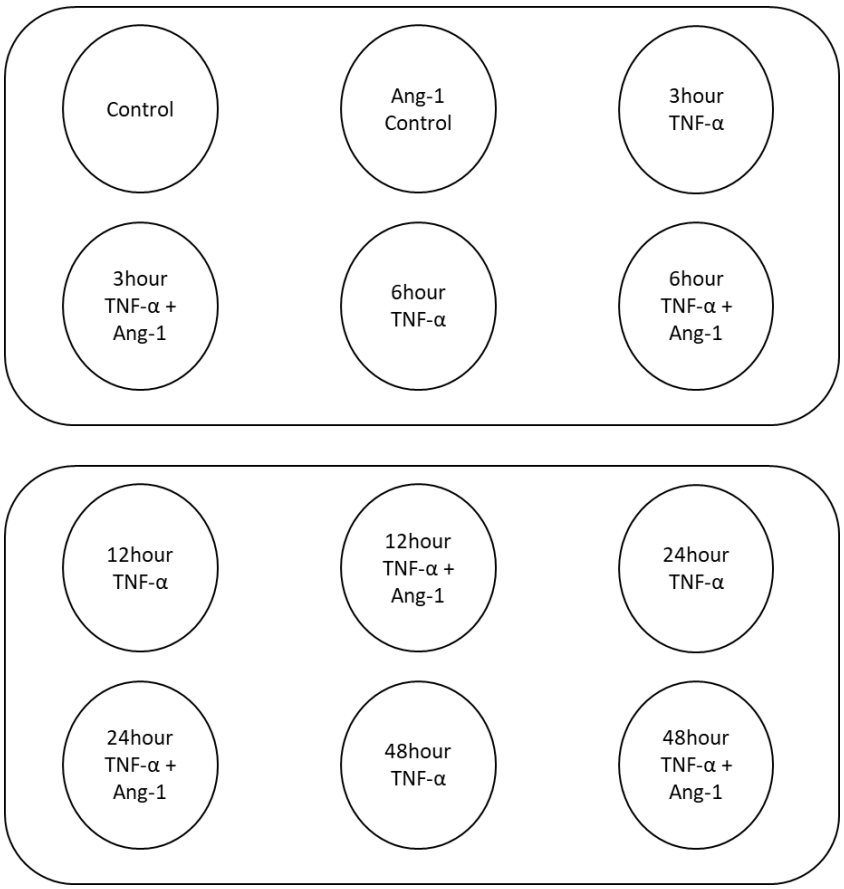


Figure 8. 6 well plates for TNF-α stimulations. *The diagram displays the time of TNF-α exposure and the presence or absence of Ang-1.*

2.4.4 Gel Electrophoresis & Western Blot

Gel Electrophoresis was conducted for the samples attained from cell stimulation using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (see 2.1.2 – a). The gels for this system were handcast with a 5% stacking (see 2.5.3 – #2) and a 10% resolving (see 2.1.3 – #3) section to the gel. The gels were cast 1mm thick with 10-wells. Each well was loaded with 30µl of the sample except for one well per gel which was loaded with 5µl of a protein ladder (during the course of this work multiple ladders were used) (see 2.1.1 – 8, 9, 10). As per the guidelines for running the gel, the tank was replete with a buffer

solution (see 2.1.3 – #4). Ensuring the tank was sealed, the apparatus was then connected to a power supply and left to run for approximately 1hour 45minutes at 120volts. The aim was for the samples to migrate and diffuse down toward the base of the gel. This was clearly visible due to the dye present in the sample buffer from the cell stimulation section.

Upon completion of the gel electrophoresis, the resolving gel was delicately extracted and placed in transfer buffer (see 2.1.3 – #5) The Mini Trans-Blot® Cell (see 2.1.2 – b) was set up, which required the layering of the gel and nitrocellulose membrane in the blotting cassette in a specific order as illustrated below:

- Foam
- Filter paper
- Gel
- Nitrocellulose membrane
- Filter paper
- Foam

The whole process was kept “wet” with the transfer buffer, and absolutely crucial that it was placed in the correct way with the electrodes also placed appropriately. After ensuring everything was in order, the tank was filled with transfer buffer and electrodes connected with a power pack and ran at 70volts for 1hour 30minutes. Once complete, the nitrocellulose membrane containing the transferred protein were either stored in a plastic wrap at 4°C or used immediately for antibody profiling.

2.4.5 Antibody Profiling

To measure the expression and activity levels of specific proteins, the nitrocellulose membrane was probed different antibodies. The membranes were first incubated in blocking solution (see 2.1.3 – #6) on a side-to-side rocker for 1hour minimum. The blocking process prevents nonspecific binding of antibodies to the membrane which would result is an increase in background signalling and prevents an accurate densitometry of the blot. After incubating in blocking solution, the membrane was briefly rinsed with TBS-T (see 2.1.3 – #7). The primary antibody solution (see 2.1.3 – #8) was then added to the blot and left to incubate at 4°C overnight on a side-to-side rocker. This will allow for enough time for the antibody to bind to the specific protein. Following this the primary antibody solution was discarded and then proceed with a series of washing steps. TBS-T was added to blots and left on the rocker for 10minutes to wash off the excess primary antibody solution. This set was repeated a further two more time for a total of three washes to ensure there was no excess primary antibody solution left on the blot. This is proceeded by the addition of a secondary antibody solution (see 2.1.3 – #9) to the membrane and incubated on the rocker at room temperature for 1hour minimum. The secondary antibody was removed and followed with three washes with TBS-T. After the final wash, an enhanced chemiluminescence solution (ECL) (see 2.1.3 – #10) was added to the blot and kept from remaining static for 1minute. The blot was removed from the solution and dried gently on a paper towel to remove excess ECL to reduce background noise during imaging. The blot was then processed using the GeneGnome Imager (see 2.1.2 – c). The captured digital image was saved as a .tiff file and the intensity of the protein bands were measured using ImageJ.

2.4.4 Data Analysis

The densitometry values were obtained from three independent experiments. Raw densitometric values were normalised against values of a house keeping protein (β-actin) for protein expression however, for protein activity, the phosphorylated value was normalised against the total protein. The final results were presented as relative values that represent a percentage in comparison to the control. The results were analysed for statistical significance using students T-test to with efforts to achieve a p-value of ≤0.05.

2.5 Live/Dead Functional Assay

2.5.1 Optimisation of Invitrogen™ LIVE/DEAD™ Cell Imaging Kit

While the majority of the protocol was followed from the manufacturer guidelines, initial usage of the kit, proved to be ineffective and caused cells to apoptose before images could be obtained after incubation. Initially after the cell stimulation, the wells were replete with PBS and the staining solution was added and left to incubate at room temperature for 15minutes. Following the change with un-supplemented Medium 200, it was found the incubation time was not long enough and the fluorescence was very weak, which push the idea to increase the incubation to 30minutes (see appendix 3).

2.5.2 Cell Stimulation

HUVECs underwent stimulations in a 6-well plate having been transferred from a T-75. A cell count was performed to ensure approximately 300,000 cells were seeded per well. Figure 9 displays a diagram of the 6-well plate to indicate the samples that were collected from each time point. Prior to the start of the time course, each well was gently

washed with warm Gibco™ PBS and then adding 1ml of Medium 200 with LSGS only then allowing it to incubate for an hour before starting the time course. 25ng/ml (final concentration) of Recombinant Human IL-1 β or Recombinant Human TNF-α was added to the respective well and then placed in the incubator to begin the timed experiment. At each respective time point the plate was removed from the incubator and IL-1β or TNF-α were added to the appropriate well at the given time point. This was repeated to the final time point at which Recombinant Human Angiopoietin-1 in the last 15 minutes to the respective wells. To terminate the stimulation, the media is removed, wells are washed briefly with warm PBS, then replete with un-supplemented Medium 200.

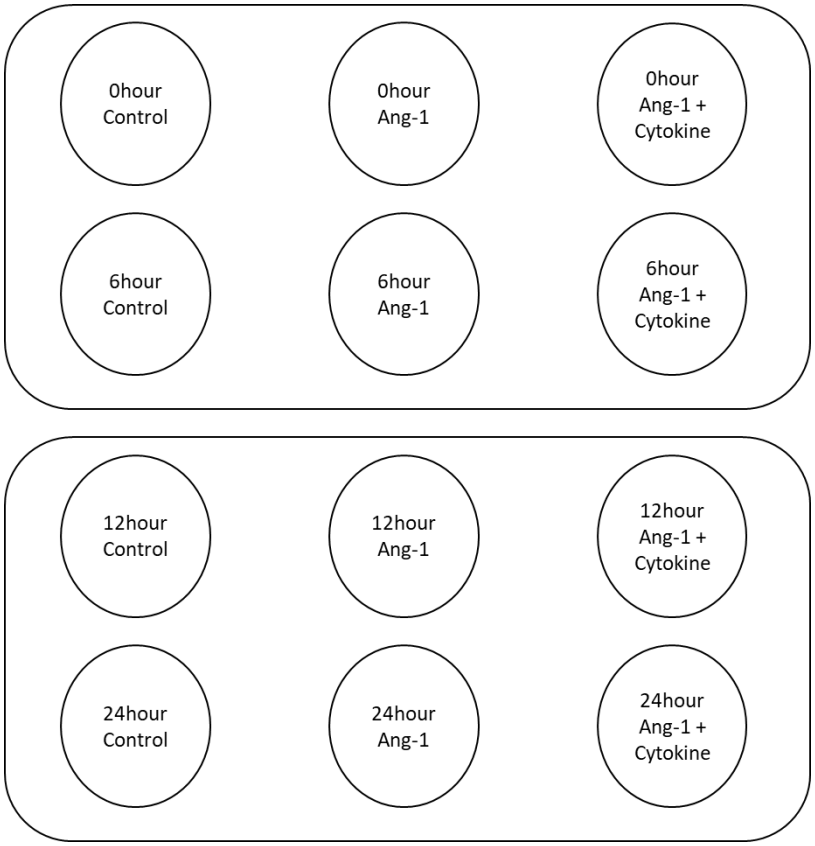


Figure 9. 6 well plates showing the contents of each sample for the cell viability stimulations. *First well in each row is the control of that specific time point and then proceeded by Ang-1 stimulated wells in the presence or absence of a cytokine.*

2.5.3 Assay

The Live/Dead solution (see 2.1.1 – 11) comes as a 1µM solution of Live Green and a lyophilised stock of Dead Red. A vial of 1ml Live Green is dispensed in a vial of 1µl Dead Red, once mixed, creates a 2X working solution of the Live/Dead solution. 165µl of the Live/Dead solution is added and left to incubate at room temperature for 30minutes. After 30minutes each well is imaged using an EVOS Microscope (see 2.1.2 – d) in three random location for the purpose of averaging the results. Each location is imaged with visible light, GFP light cube and RFP light cube[1].

2.5.4 Data analysis

Using cell counter tool in ImageJ, the number of cells in each image type was counted to obtain total cell count, Live cell count and Dead cell count. The percentage of live cells and dead cells were calculated and averaged for each sample. The following formula was used to give a percentage of live cells in and dead cells in each sample.

$$\%\ Live\ Cells = \frac{No.\,Live\ Cells}{No.\,Live\ Cells + No.\,Dead\ Cells} \times 100$$

$$\%\ Dead\ Cells = \frac{No.\,Dead\ Cells}{No.\,Live\ Cells + No.\,Dead\ Cells} \times 100$$

Statistical significance of the data was determined through Single Factor ANOVA with a p-value of 0.05.

[1] While the recommended light cube for the apoptosed cells is Texas Red, the microscope only had RFP, which when checked against the spectral compatibility with the fixable red stain, was seen to be rated fair.

Chapter 3: Profiling of Cytokines and Angiopoietin 2 in Diabetic Patient Samples

3.1 Introduction

Diabetes has many complications associated with it, including inflammatory associated vascular conditions. There are several inflammatory cytokines with relevance to diabetes including IL-1β, TNF-α, IL-6 and IL-10 (King, 2008; Tong et al., 2017). There has been evidence in both animals and humans to suggest cytokines have rather significant roles in diabetes. IL-6 has been suggested to decrease glycogen synthesis while TNF-α can limit the hepatic uptake of glucose. IL-6 is a multifunctional cytokine with a pathophysiological factor in type 2 diabetes. The elevated circulation of IL-6 is considered an independent predictor of type 2 diabetes. Evidence suggests its involvement in the inflammatory development, insulin resistance and also β-cell dysfunction. However, on the contrary, data supporting the positive impacts of IL-6 has begun to change the current view on IL-6. The evidence is beginning to show the anti-inflammatory effects as well as glucose metabolism improvement. The complex signalling cascade of IL-6 provides an explanation as to the pleotropic nature of this cytokine (Akbari and Hassan-Zadeh, 2018). The impacts of IL-6 on diabetes extend beyond the condition to other concurring conditions like obesity. The suggestion that the dysfunctions are a causation of each other indicate the role IL-6 displays in the body (Pradhan et al., 2001; Weiss et al., 2004). The complexity behind the pathophysiological role of IL-6 give evidence for both positive and negative effects of elevations of the

cytokine (Wunderlich et al., 2010; Matthews et al., 2010). Therapeutics behind IL-6 have been investigated to treatments in cancer however it has been seen that inhibition of IL-6 binding to its receptor can reduce insulin resistance and also inhibit inflammasomes to reduce propagation of diabetic nephropathy.

TNFα research has also given evidence for the role of obesity-related insulin resistance and further pathologies of type 2 diabetes (Moller, 2000). TNFα displays a crucial role in the development of insulin resistance through the decrease in expression of glucose transporter type 4 (GLUT4). This transporter is insulin regulated and primarily located in adipocytes, cardiac muscle, and skeletal muscle. A reduction in this transporter would subsequently cause a lack of glucose to be transported into the cells (Huang and Czech, 2007; Guilherme et al., 2008; Olson, 2012). When considering type 1 diabetes, TNFα exerts its impact on pancreatic islets to induce apoptosis of β-cells and it is seen as one of the main stimuli of inflammation in the pancreas. The mechanism by which this occurs is through activation of NF-κB, a key regulator in the inflammatory process (Parkash, Chaudhry and Rhoten, 2005; Donath and Shoelson, 2011; Akash et al., 2012; Akash, Rehman and Chen, 2013; Rehman and Akash, 2016). Anti-TNFα therapeutics have carved a path in the treatment of insulin resistance, with significant results being shown in tissue-specific insulin resistance (Dominguez et al., 2005; Bernstein et al., 2006; Stanley et al., 2011)

While the field of markers has had extensive research in the areas of diabetes and vascular disease, studies on the co-association have been limited especially when comparing pro-inflammatory cytokine levels with Ang-2. Details of Ang-2 around type 2

diabetes have been covered in depth. The mounting evidence of Ang-2 regulation in certain disease has warranted further investigation especially in disease-associated vascular complications (Rasul et al., 2011; Shroff et al., 2013; El-Asrar et al., 2016; Schuldt et al., 2018). Ang-2 relationship with proinflammatory mediators in type 1 have been minimal.

To profile and test the association of IL-6, TNFα and Ang-2 in type 1 and type 2 diabetic patients, serum samples were subjected to different ELISA techniques as described in chapter 2.2. Both TNF-α and IL-6 results were attained from the RANDOX Metabolic Syndrome Array I kit (see 2.2.3). The Metabolic Syndrome Array I kit has the capability of detecting ferritin, IL-6, insulin, leptin, Plasminogen activator inhibitor-1 (PAI-1), resistin and TNF-α. In this study only TNF-α and IL-6 will be assessed to analysis the role these potent pro-inflammatory cytokines have of diabetes. The vascular specific protein was detected from the serum samples using a precoated ELISA plate with Ang-2 antibodies (see 2.2.5).

3.2 Results

3.2.1 Profile of TNF-α in Patient Samples

Figure 10 does not appear to display any noticeable trends, especially in samples from patients with type 1 diabetes, which appeared to have large variations in TNF-α concentrations. The median plots indicated in Figure 11 does show type 1 samples to have a lower concentration ($\tilde{x} = 3.74\ pg/ml$) compared to the control median ($\tilde{x} = 5.54\ pg/ml$). Median samples of type 2 were seen to be higher ($\tilde{x} = 6.0375\ pg/ml$)

than that of the control. Through Shapiro-Wilk test distribution of the data found to be not normal hence a non-parametric test was conducted to test for significance in the data. To analyse the differences between more than two sample groups of related data, Friedman test was the most appropriate. The *p-value* obtained from the Friedman test was 0.174. This indicates a lack in significance in the differences in the data between the groups. The interquartile range for the control and type 1 groups were substantial hence a suggestion to the insignificance of the data.

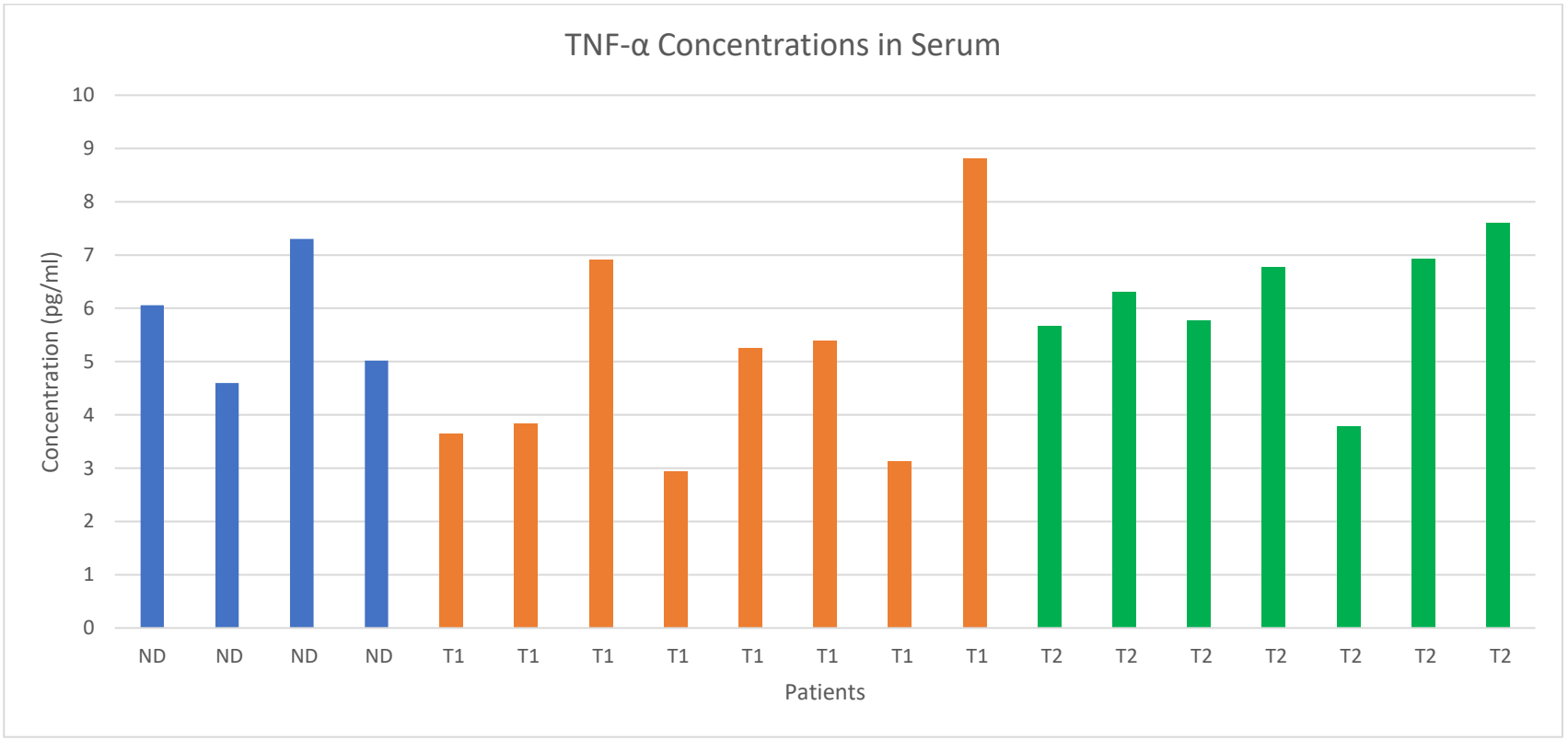


Figure 10. Graph indicating the TNF-α concentrations in each serum sample obtained from the RANDOX® Multiarray ELISA. *Results of the ELISA were processed using the RANDOX® software based on the standards obtained from the manufacturers calibrations. Plotted results are the averages of the triplicates for each patient. ND = Non-Diabetic Patients, T1 = Type 1 Diabetic Patients, T2 = Type 2 Diabetic Patient.*

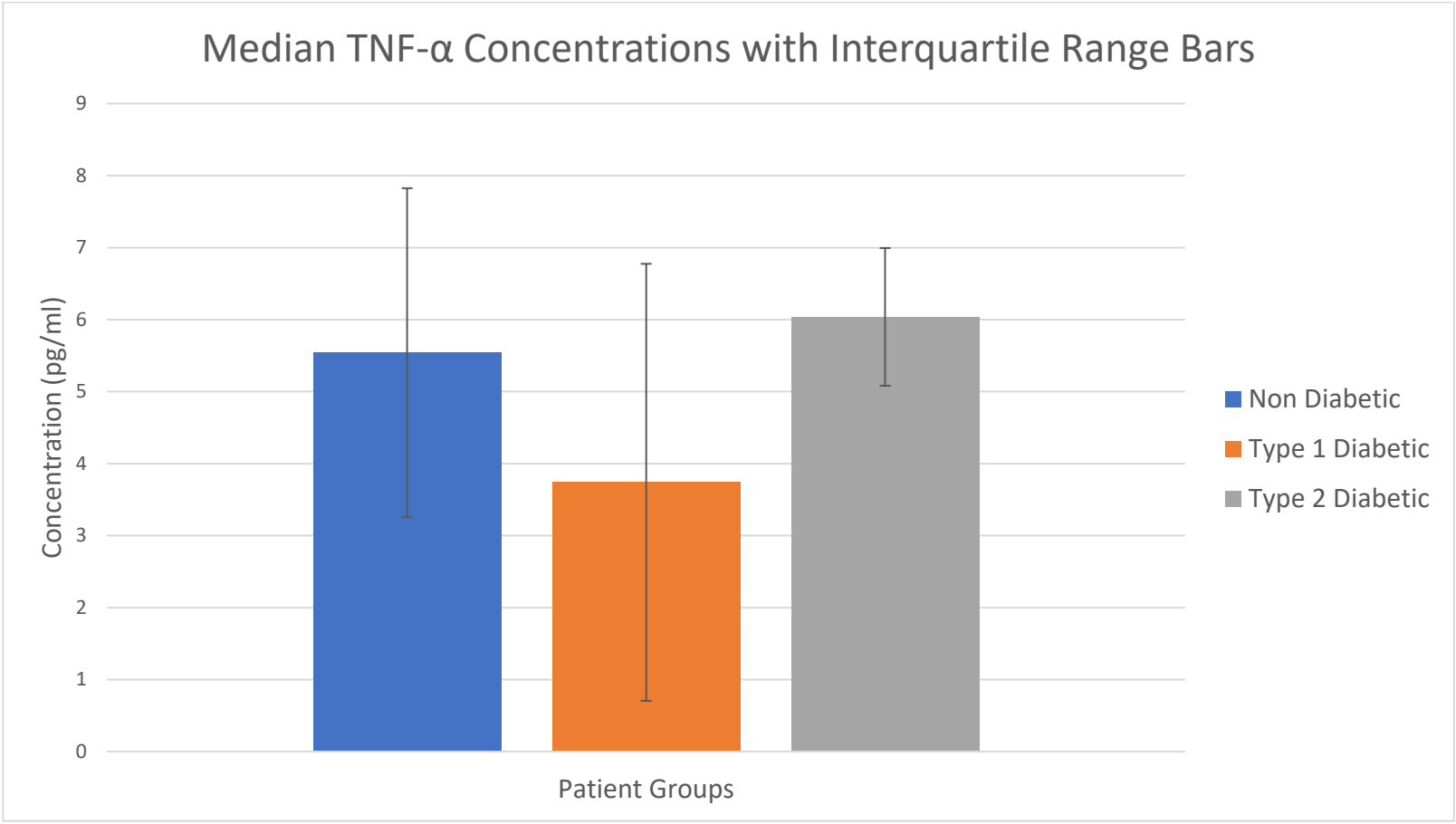


Figure 11. Median plot of TNF-α concentrations for each volunteer group. *Due to Shapiro-Wilk test noting non-normal distribution, data is plotted as median values with Interquartile Range bars.*

3.2.2 Profile of IL-6 in Patient Samples

From Figure 12, it can be seen that type 2 samples appear to have higher IL-6 serum levels compared to the control group, however the same pattern cannot be said about the type 1 samples. IL-6 concentrations show a median increase in both type 1 ($\tilde{x} = 0.95\ pg/ml$) and type 2 ($\tilde{x} = 1.62\ pg/ml$) in comparison to the non-diabetic control ($\tilde{x} = 0.725\ pg/ml$). Shapiro-Wilk test found the data to be not normally distributed hence Figure 13 is a plot of the median values of each volunteer group with interquartile range as the error bars. Due to distribution classification, Friedman test was the most appropriate statistical measure of differences in between the groups, however the test reported no significance with a *p-value = 0.174*.

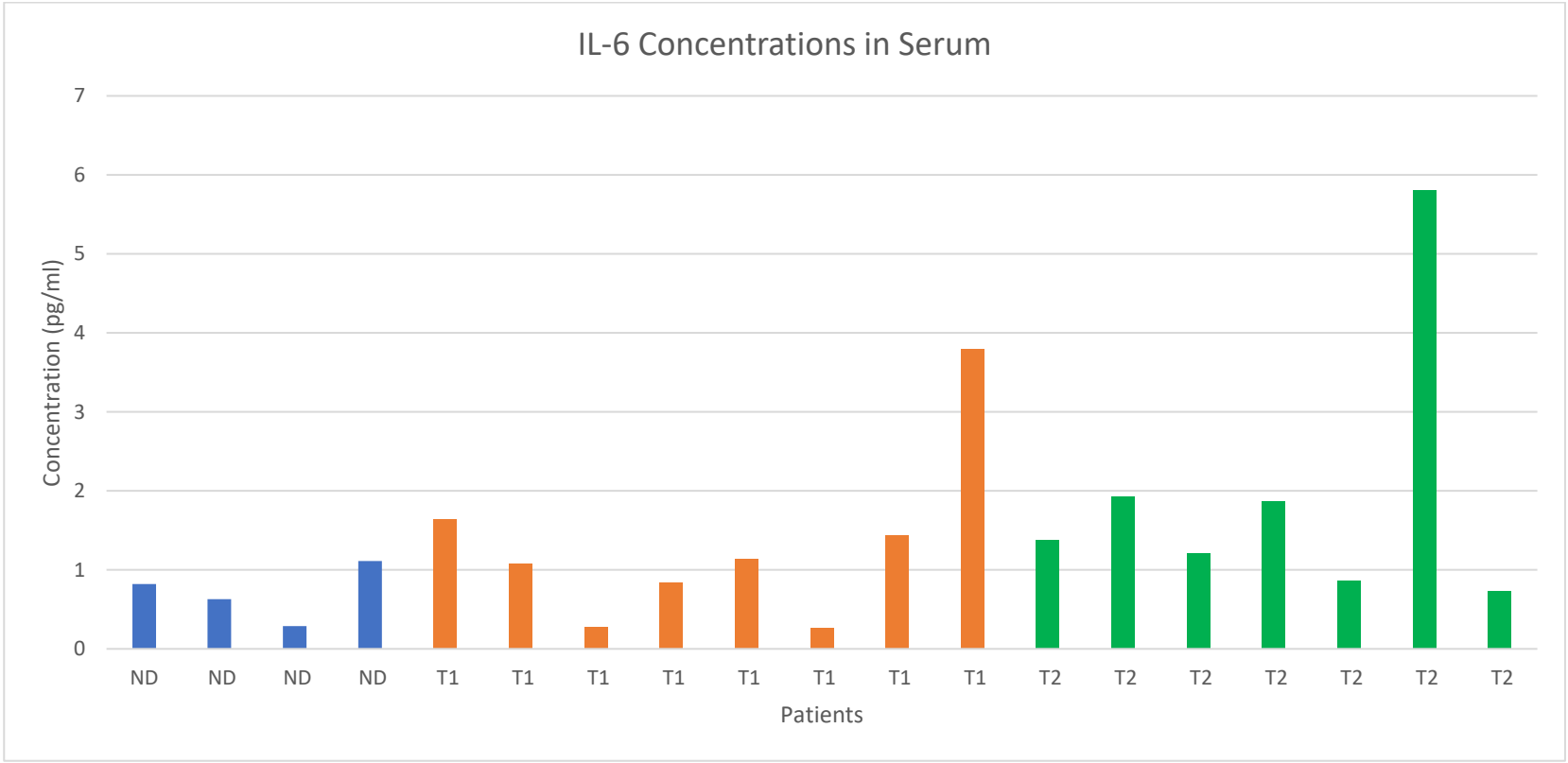


Figure 12. Graph indicating the IL-6 concentrations in each serum sample obtained from the RANDOX® Multiarray ELISA. *Results of the ELISA were processed using the RANDOX® software based on the standards obtained from the manufacturers calibrations. Plotted results are the averages of the triplicates for each patient. ND = Non-Diabetic Patients, T1 = Type 1 Diabetic Patients, T2 = Type 2 Diabetic Patient.*

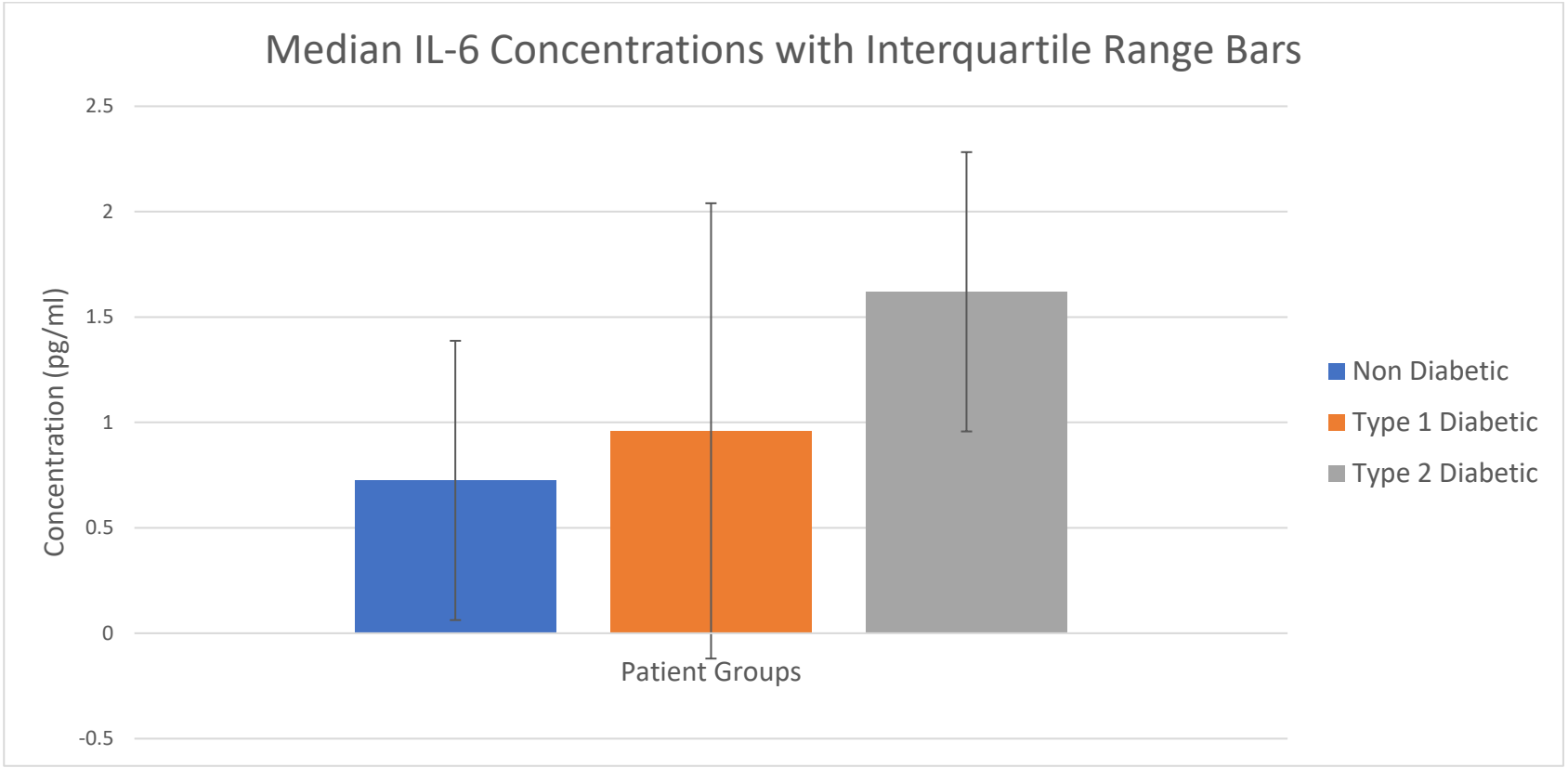


Figure 13. Median plot of IL-6 concentrations for each volunteer group. *Due to Shapiro-Wilk test noting non-normal distribution, data is plotted as median values with Interquartile Range bars.*

3.2.3 Profile of Ang-2 in Patient Samples

Figure 14 displays a slight visible trend where type 1 patients appear to have lower concentration of Ang-2 in serum, and type 2 patients have higher Ang-2 serum levels. The Ang-2 results differ to that of TNF-α and IL-6 in that results displayed statistical significance. The median concentration of the non-diabetic control was $\tilde{x} = 25.432\ \mu g/ml$, with a large median decrease in serum Ang-2 in type 1 diabetes with $\tilde{x} = 18.312\ \mu g/ml$. Type 2 displayed elevations in the Ang-2 concentration, showing $\tilde{x} = 31.254\ \mu g/ml$ (Figure 15). Normal distribution using Shapiro-Wilk found the data to not be normally distributed, hence performing Friedman test measured statistical differences in between the groups with *p-value* = 0.039. Though the interquartile range is large the difference between the groups remains significant.

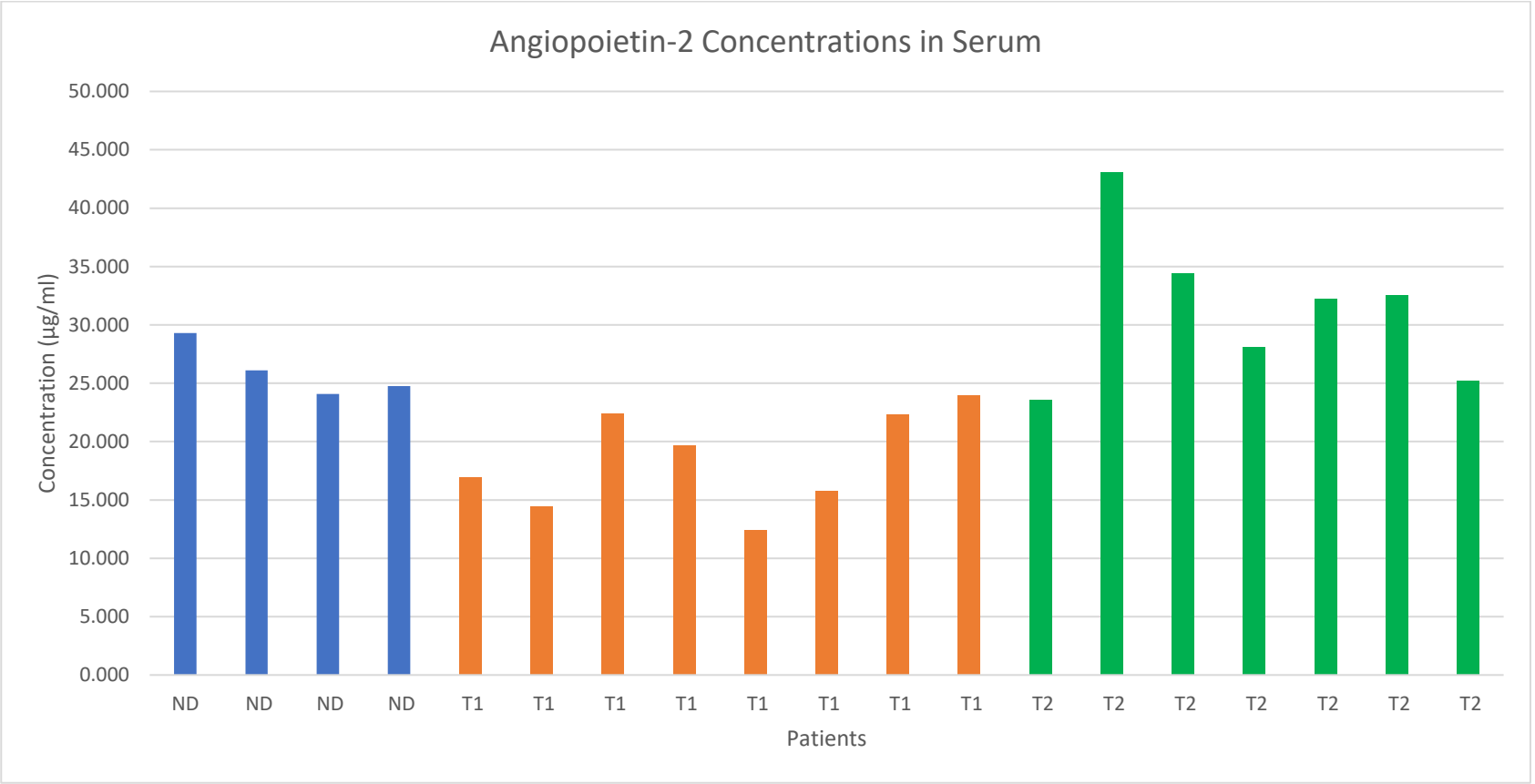


Figure 14. Graph indicating the Ang-2 concentrations in each serum sample conducted using traditional capture ELISA techniques. *Each concentration was obtained from a standard curve of TNF-a concentrations. The plotted results are averages of the duplicate testing conducted for each patient sample. ND = Non-Diabetic Patients, T1 = Type 1 Diabetic Patients, T2 = Type 2 Diabetic Patient.*

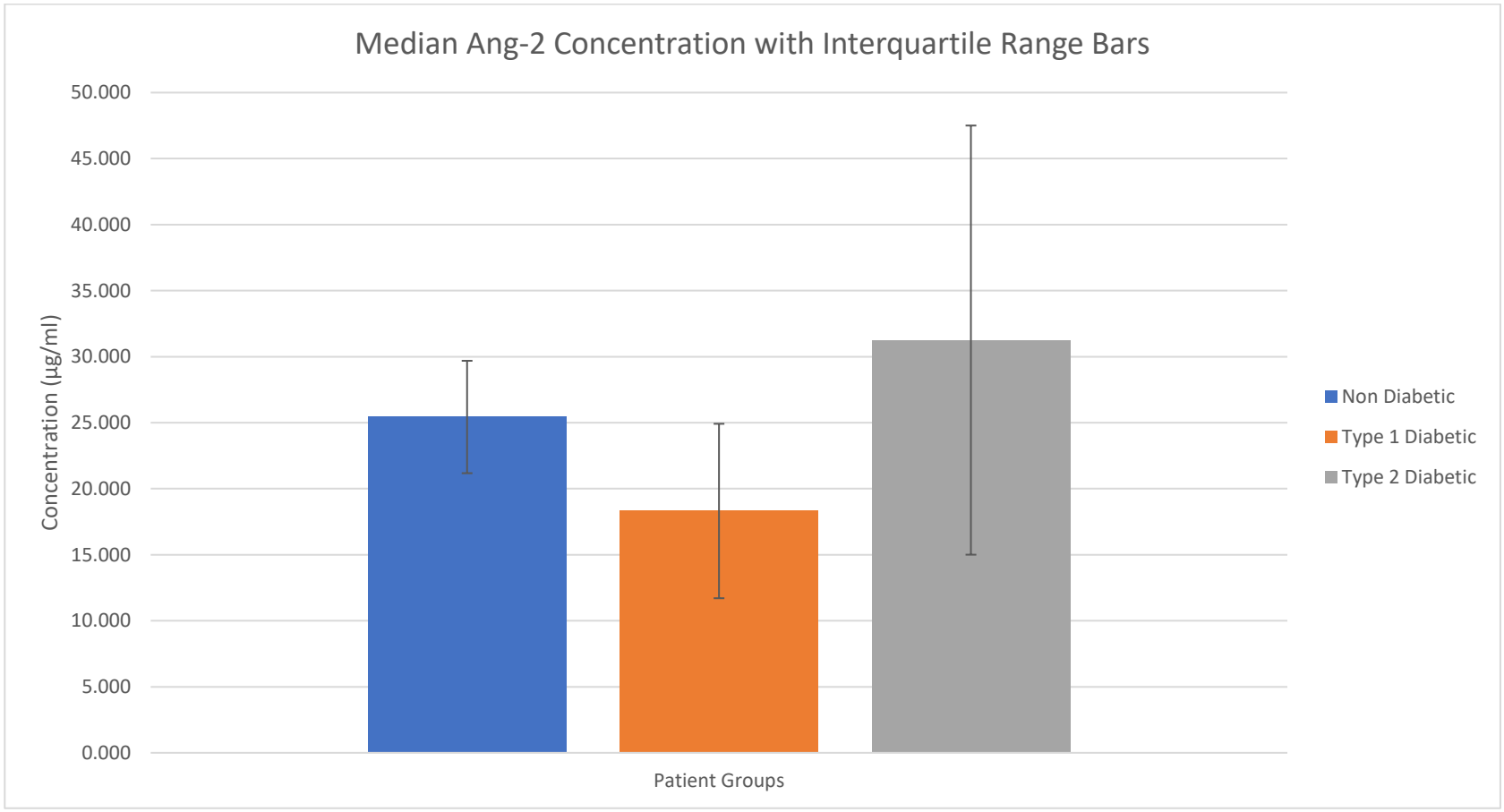


Figure 15. Median plot of Ang-2 concentrations for each volunteer group. *Due to Shapiro-Wilk test noting non-normal distribution, data is plotted as median values with Interquartile Range bars.*

3.3 Discussion

3.3.1 Type 1 Diabetes

Type 1 diabetic volunteers were found to have reduced serum levels of Ang-2 and TNF-α from the ELISA analysis. While only the Ang-2 is able to offer statistical significance, all the result provides further evidence toward the notion of the implication of diabetes on the protein prevalence in serum. The reduced levels are contradictory to the autoimmune nature of type 1 diabetes. Analysis of various studies assessing serum TNF-α concentrations found that the cytokine was elevated in type 1 patients irrespective of age, ethnicity and disease duration (Lechleitner et al., 2000; Qiao et al., 2017). It is expected that the activation of immune cells in this autoimmune disorder would result in increased levels of TNF-α. The lack in significance suggests this part of the data to be inconclusive which could be subject to error during experimentation. The reduction of TNF-α in serum has been seen in relation to the development of type 1 diabetes when patients are found to be in the pre-diabetic stage (Vitali et al., 2000), however this is not the case for the patients within this study as the patients were already diagnosed with the condition.

The IL-6 average supports the previous evidence of being elevated in the serum of patients with type 1 diabetes and microalbuminuria (Purohit et al., 2018). While the data was not statistically supported, the average provide additional evidence to the idea IL-6 increases in type 1 diabetes. IL-6 is another cytokine conversant in multitudes of biological processes hence making it difficult to fully understand its role in type 1 diabetes pathogenesis due to complexities (Rajendran et al., 2020). There have been

several studies to assess serum IL-6 in type 1 diabetic patients which found similar elevations to this study but in addition to the elevation it was reported that age, ethnicity and disease duration did not have any effect on the serum levels promoting type 1 diabetes (Y. L. Chen et al., 2017). While the study provided an unbiased meta-analysis of the publications surrounding the increased levels of IL-6 in type 1 patients, there was an undetermined factor of the complication of type 1 diabetes having an impact on the outcome.

The statistically significant Ang-2 decrease in the serum of type 1 diabetes is an opposing data set when compared to published literature. The lowered level were only seen in simvastatin-treated patients therefore implying the glycoprotein is present and function in the development of type 1 diabetes (Tuuminen, Sahanne and Loukovaara, 2014). The increased Ang-2 is also seen in adolescents with type 1 diabetes whether they experience microvascular complication or not (El-Asrar et al., 2016). Details around the serum levels of Ang-2 in relation to type 1 diabetes are very limited, and this study is the first to display decreased Ang-2 serum levels in type 1 diabetic patients.

Spearman Rank Correlation was also conducted to assess the relationship between Angiopoietin and the cytokines, the results displayed small rank coefficients with no statistical significance, suggesting no conclusion can be drawn about the relationship between angiopoietin and the cytokines in this sample set.

3.3.2 Type 2 Diabetes

The pattern for type 2 diabetes data was quite similar to type 1 diabetes. The TNF-α concentrations displayed some evidence of increased levels, however the lack of

significance from the Friedman test would mean the result would remain inconclusive. The multifaceted nature of TNF-α makes it a prime candidate for research into biomarkers and new avenues of therapy. It has been stated in literature that TNF-α elevations are seen in type 2 diabetic patients as well as patients experiencing type 2 diabetic nephropathy (Y. ling Chen et al., 2017). Additionally, studies have shown strong associations of type 2 diabetes risk with a multitude of inflammatory cytokines including TNF-α (Liu et al., 2016). The cytokine has large role in other co-factors of diabetes, including obesity as it has been reported that TNF-α is significantly elevated due to increased expression by adipocytes and inflammatory cells resultant to chronic inflammation (Alzamil, 2020).

IL-6 was also found to have a median increase, though did not show statistical relevance. This increase supports the evidence suggesting increased IL-6 is also strongly associated with risk of developing type 2 diabetes (Liu et al., 2016). Contrary to a cross-sectional study based on type 2 retinopathy, IL-6 was found to have significant decreases compared to the control and patients without retinopathy, hence suggesting the pathophysiology of retinopathy creates a mechanism to decrease expression of IL-6 (Afzal et al., 2014). However, several therapeutic studies target IL-6 in an effort to reduce serum levels to improve the diabetic condition. Therapeutics with metformin and metformin-insulin combination have seen a positive effect in type 2 diabetes with the addition reduction in IL-6 (Borowska et al., 2016). The treatments of diabetes with co-morbidities like obesity follow similar route however assess the condition using IL-6 as a marker. Canagliflozin is an SGLT inhibitor with superior HbA1c reducing properties. This additionally demonstrated decreases in serum IL-6 by 22%. This in turn creates a

positive outcome for insulin sensitivity and reduce cardiovascular risks (Garvey et al., 2018).

The result of the Ang-2 ELISA found significant increase in serum levels of type 2 diabetes. This was the expectation based on the profile of Ang-2 being predominant in conditions of chronic inflammation. A large study involving the complication of angiopathy found results of increased Ang-2 levels in patients with type 2 diabetes. Moreover, the number of micro/macro-vascular also resulted in further increases in Ang-2 levels (Li, Qian and Yu, 2015). The increases in Ang-2 are seen in many aspects of type 2 diabetic complications, including different stages of albuminuria. Patients with normal albuminuria were seen to have increase serum and urinary Ang-2 compared to healthy controls. In addition, the degree of albuminuria correlated positively with Ang-2 urinary levels. Furthermore, Ang-1 levels were seen to be decreased hence the ratio of angiopoietins would result in key clinical pathologies of vascular complications as a result of type 2 diabetes (Chen et al., 2015). The increased Ang-2 in serum was also observed in a cross-sectional study investigating the relationship between the glycoprotein and cardiovascular risk factors associated with type 2 diabetes. Significance was noted in hypertensive diabetic subjects and well as positive correlations being shown with factors including BMI, systolic blood pressure, fasting blood glucose, HbA1c and triglycerides (Siddiqui et al., 2018).

Spearman Rank Correlation was also conducted to assess the relationship between Angiopoietin and the cytokines, the results displayed small rank coefficients with no

statistical significance, suggesting no conclusion can be drawn about the relationship between angiopoietin and the cytokines in this sample set (data not shown).

3.4 Conclusion

The findings of this chapter indicate the involvement of cytokines in type 1 and type 2 diabetes, albeit with a lack of significance, however the literature supports the findings as the main premise of diabetes revolves around chronic targeting of inflammatory processes. Ang-2 elevation suggest diabetes would result in increased vascular instability as seen in conditions like retinopathy. However, the conditions and additional mechanisms could result in favour of endothelial survival as there was a decrease in Ang-2 for type 1 diabetics suggesting a mechanism of protection. The elevation of Ang-2 has been widely reported to inhibit Ang-1/Tie signalling in type 2 diabetic induced environment however the impact these cytokines have on Ang-1 signalling is of great interest in understanding their role on vascular function (Bilimoria and Singh, 2019).

Chapter 4: Acute Cytokine impact on Ang-1/Tie-2 Signalling

4.1 Introduction

Acute functions of inflammatory cytokine elicit a function of protection and healing as a result of some form of stress on tissue or infection. This short-lived inflammatory response is characterised as being active from minutes to days with the presence of immune cells and inflammatory protein mediators. Termination of the immune response occurs when the detrimental stimulus is removed and the inflammatory mediators have been squandered or inhibited (Zhang, 2008). Within the vasculature there are three stages of acute inflammation; vasodilation of the vessels to increase volume of blood flowing to the target region, increased permeability of the micro vessels to allow immune cells to pass between the vessel walls to target the area of stress or infection, and lastly vascular stasis to allow for the pooling of inflammatory mediators and immune cells to respond to the stimulus (Zhang, 2008). Endothelial cells experience cytoskeletal changes that disrupt junctions in venules and capillaries to allow for immune cells to reach the stimulus. This usually occurs 4-6hours post stimulation and lasts for several days. The most predominant mediators involved in the process include IL-1 and TNFα (Zhang, 2008).

IL-1 has been shown to act on cultured endothelial cells both directly and selectively to elicit two vital functions. The induction of IL-1 causes endothelial cells to biosynthesis and express tissue factor-like procoagulant activity and also increase the adhesiveness

of endothelial cells to allow for an increase in leukocytes and monocytes (Bevilacqua et al., 1985). IL-1β specifically promotes monocyte differentiation to M1-like macrophages and dendritic cells which also supports the differentiation of B-lymphocytes to plasma cells (Medzhitov, 2008; Miyake and Kaisho, 2014; Hotamisligil, 2017).

The pleotropic nature of TNFα gives it multiple responsibilities for many biological functions. Evidence has shown TNFα to be crucial in mediation of bacterial infections and acute inflammation. While results from inhibition of TNFα could not be concluded in acute inflammation is has been suggested that there could be undesirable implications. Reports of TNFα-mediated activation of NF-κB could lead to protection of certain cell populations from apoptosis during acute inflammation, however it should be noted that over-production of TNFα could be directly implicated in apoptotic injury (Ksontini, 1998).

The role of acute exposure of cytokines has not been extensively studied due to the primary role of acute inflammation being a protective and healing function of the body. To assess the implications of IL-1 and TNFα, recombinant IL-1β and TNFα will be used to stimulate HUVECs to see the impact on Tie-2 receptor expression and with the addition of Ang-1 the activity of the receptor and signalling mediators will be assessed using methods of gel electrophoresis and western blotting (see 2.4). IL-1β shares similar properties to IL-6, where the both have proinflammatory and anti-inflammatory properties (Scheller et al., 2011; Dinarello, 2018). Both are characterised alongside TNFα as being the main cytokines involved in the development of diabetic conditions

(Navarro-González and Mora-Fernández, 2008). IL-1β has been described to induce IL-6 through the PI3K/AKT pathway of NF-κB activation (Cahill and Rogers, 2008).

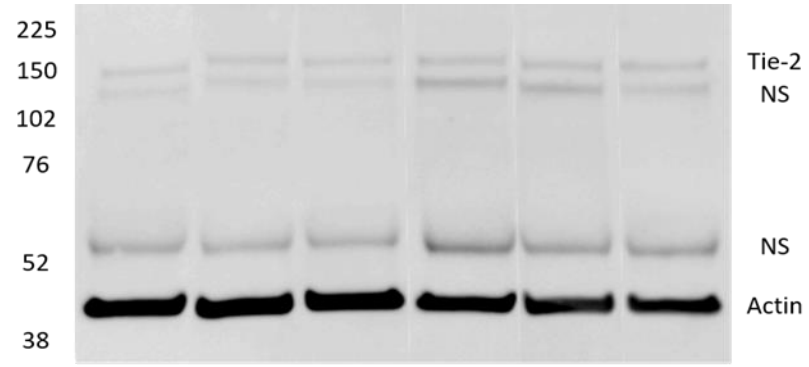
4.2 Results

4.2.1 Acute Impact of IL-1β and TNFα on Tie-2 Expression

To assess the impacts of IL-1β, HUVECs were treated with recombinant IL-1β (25ng/ml) at six time points (0hr, 0.25hr, 0.5hr, 1hr, 2hr and 4hr). The 4hour cut-off links with reference to the start of the delayed response in the acute inflammatory response as mentioned by Zhang (2008). Physiological levels were not used from the human study as previous experimentation found 25ng/ml of IL-1β to demonstrate the maximum response in Ang-1 signalling changes (see Appendix 1). Physiological levels are much smaller and would not be able to illicit a response in the cell model as the purpose of the method is to see where the interactions are occurring and what aspect of signalling is being effect (Alecu et al., 1998). Expression levels of Tie-2 were obtained using western blotting with the uses of a Tie-2 antibody (see 2.1.4 – Human Tie-2 Antibody). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for Tie-2.

Tie-2 blot displayed increasing expression (Figure 16) up to 2hour exposure of IL-1β in HUVECs with a 56.1% average increase in expression (Figure 17), then decreased as it approaches to 4hour time point to an average of 16.8% increase against the control. The significance in Tie-2 increases of 33.7% and 56.1% can be seen at 1hour and 2hour time

points, respectively, both with *p-values* ≤ 0.05 (*p = 0.045* and *p = 0.043*, respectively). The other time points lack the statistical significance due to large standard error mean.



Time (h)	0	0.25	0.5	1	2	4
IL-1β	-	+	+	+	+	+
p-value		0.377	0.247	0.045	0.043	0.287

Figure 16. Levels of Tie-2 expression HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using Tie-2 and β-actin. Though Tie-2 is reported as a 126kDa molecule, due to glycosylation is seen to be between 140-160kDa. β-actin is reported as a 42kDa molecule. NS indicates non-specific bands.*

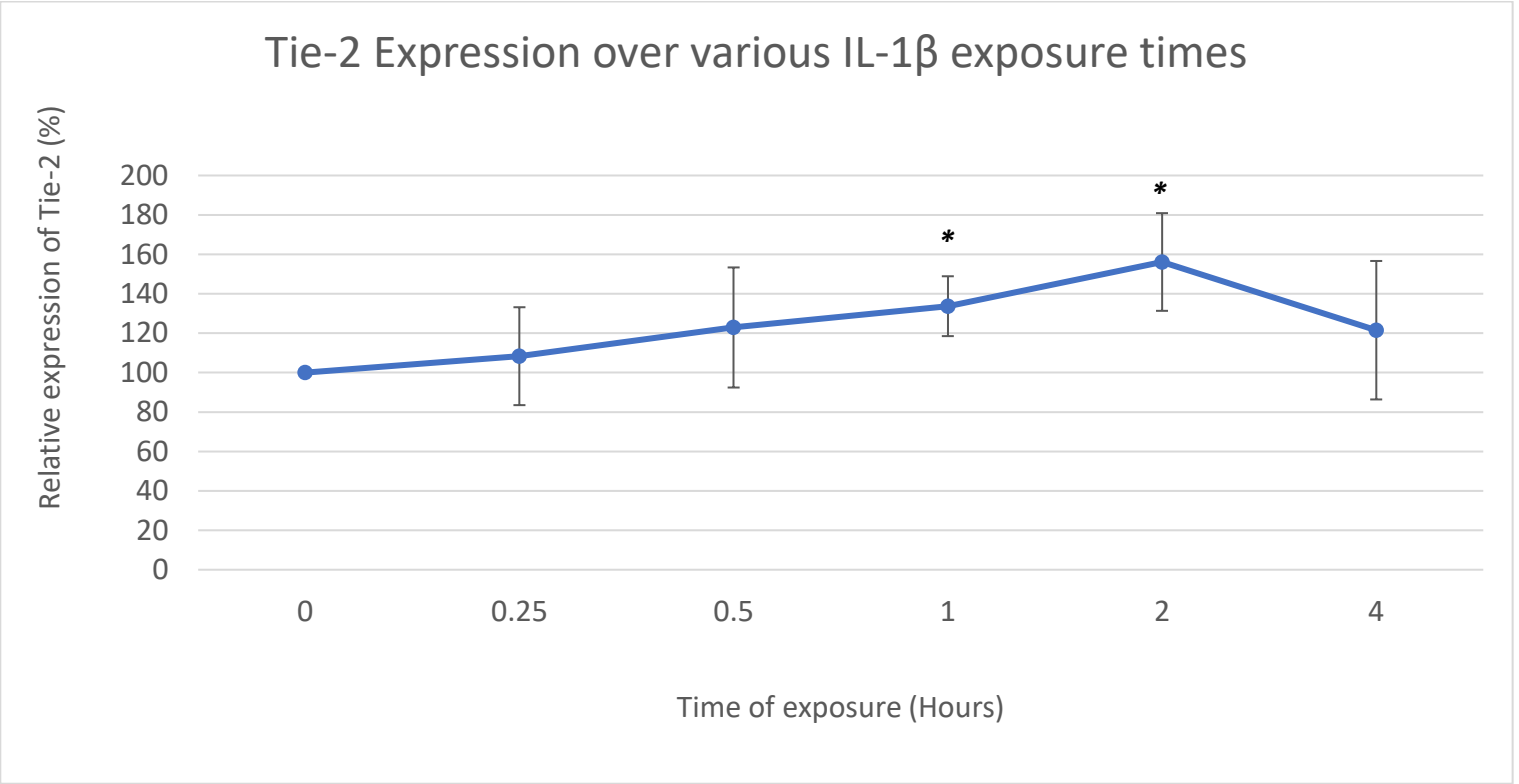


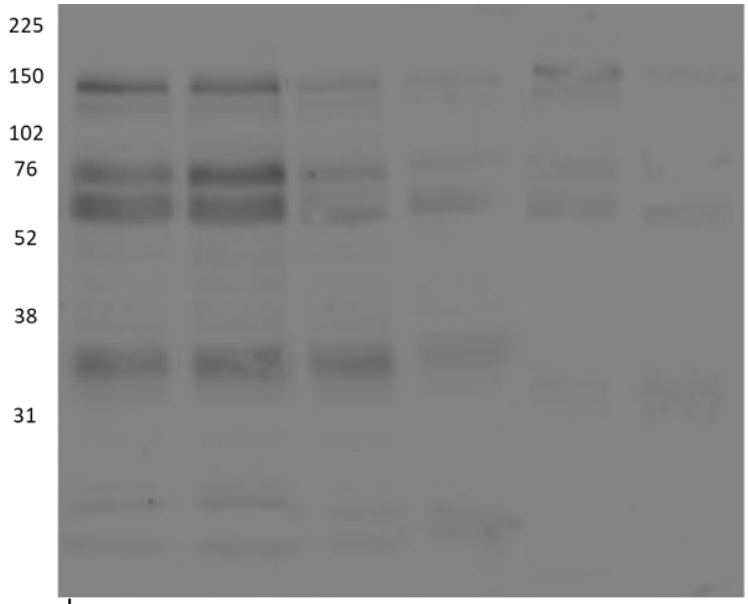
Figure 17. Graph indicating the percentage Tie-2 expression values when exposed to IL-1β over varying time points. *The levels of Tie-2 expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of Tie-2. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*

Similar experiments were performed to assess the impacts of TNFα on Tie-2 expression in HUVECs. Cells were treated with recombinant TNFα (25ng/ml) at six time points (0hr, 0.25hr, 0.5hr, 1hr, 2hr and 4hr). However, during experimentation there were a number of issues around the TNFα stimulation analysis of Tie-2 expression. During cell culture, there was a reoccurring period of infections of cells being grown. After narrowing down the source of infection and finally growing enough cells for the acute stimulation, none of the antibody images for Tie-2 and Actin displayed any bands and hence was excluded from this study. Published work by Singh et al (2012) displayed the impacts of TNFα stimulation on HUVECs over a time course of up to 24hours. The acute time points did not display significance however the results are suggestive of TNFα causing increases in Tie-2 expression the longer the stimulation is applied.

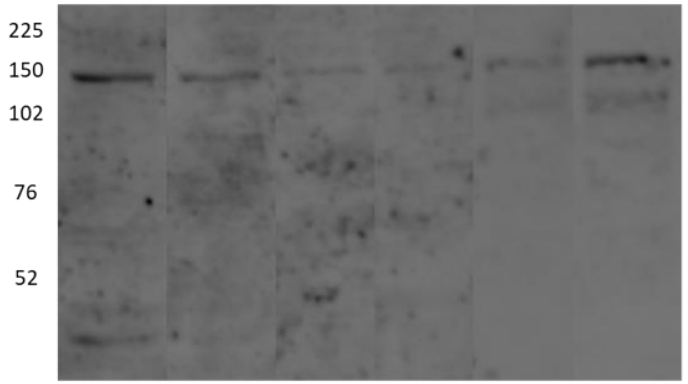
4.2.2 Acute Impact of IL-1β and TNFα on Ang-1 induced Tie-2 Activity

The influence on IL-1β and TNFα was assessed through stimulation of each cytokine in separate experiments. Each sample was stimulated with the addition of Ang-1 (100ng/ml) for 15minutes before the end of each time point. The Ang1 concentration was based on a similar study which assessed the impacts of glucose and fatty acids on Ang1 induced signalling (Singh, Brindle and Zammit, 2010). Cell culture studies do not use physiological concentrations as the response would not display any results due to the miniscule concentrations that are seen in human studies (Kim, Kim, So, et al., 2000; Schreitmüller et al., 2012). HUVECs were treated with recombinant TNFα (25ng/ml) or recombinant IL-1β (25ng/ml) at six time points (0hr, 0.25hr, 0.5hr, 1hr, 2hr and 4hr). Ang-1-induced activity of Tie-2 was measured by normalising the phosphorylated Tie-2 levels with normal Tie-2 and plotting the average relative percentage phospho-Tie-2 levels from three independent experiments (Figure 19, Figure 21). Appendix 4 highlights Ang-1 functioning to activate Tie2 and cause phosphorylation. Blot and graph indicate a trend of an initial increase and then decrease towards the end of the time course. The increase in Ang-1 induced Tie2 activity was seen to be 4-fold at 1hour of exposure to IL-1β, however the large standard error associated with this time point gives no statistical significance. The increase at 30minutes by 2-fold is supported by a statistical significance with a *p-value* = 0.001. At the 2hour time point a decrease in the activity is seen with the largest decrease shown at 4hours with an 85.3% decline in activity compared to the control. Significance is reported with a *p-value* = 0.001. The trend observed when cells

were treated with TNFα, was the same as IL-1β. The increase peaks at 1hour with an approximately 2-fold increase in activity compared to the control, at which point the activity decreases to and elevation of 7.2% at the final 4hour time point. All results of the TNFα exposure on Tie-2 activity raised no statistical significance from the students' T-test.



pTie-2 (Y992)
NS



Tie-2
NS

Time (h)	0	0.25	0.5	1	2	4
IL-1β	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.236	0.001	0.186	0.288	0.001

Time (h)	0	0.25	0.5	1	2	4
IL-1β	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.236	0.001	0.186	0.288	0.001

Figure 18. Ang-1-induced Tie-2 activity in HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pTie-2 and Tie-2. Tie-2 is reported as a 126kDa molecule, due to glycosylation is seen to be between 140-160kDa. pTie2 is also seen at 140-160kDa. NS indicates non-specific bands.*

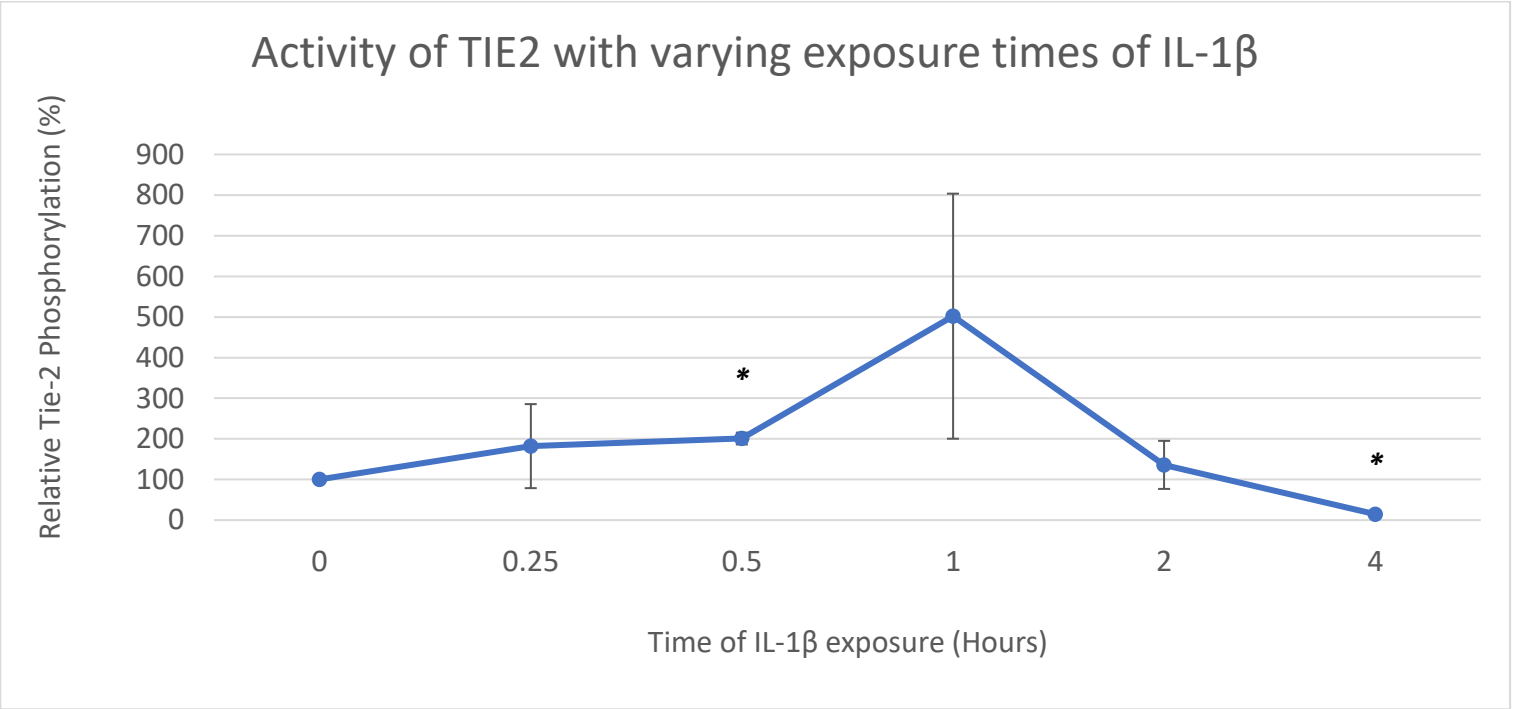
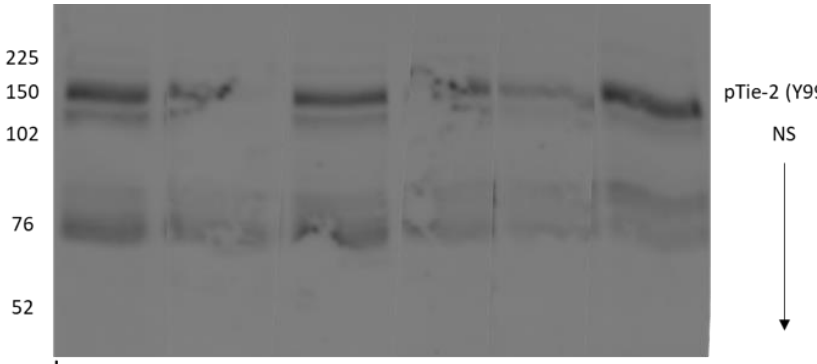
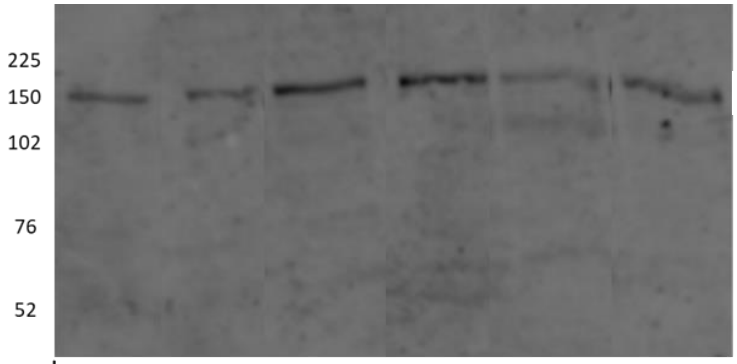


Figure 19. Graph indicating the percentage pTie-2 expression values when exposed to IL-1β over varying time points. *The levels of pTie-2 expression were normalised against normal Tie-2 densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of Tie-2. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*



Time (h)	0	0.25	0.5	1	2	4
TNFα	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		N/A	0.294	0.281	0.119	N/A



Time (h)	0	0.25	0.5	1	2	4
TNFα	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		N/A	0.294	0.281	0.119	N/A

Figure 20. Ang-1-induced Tie-2 activity in HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pTie-2 and Tie-2. Tie-2 is reported as a 126kDa molecule, due to glycosylation is seen to be between 140-160kDa. pTie2 is also seen at 140-160kDa. NS indicates non-specific bands.*

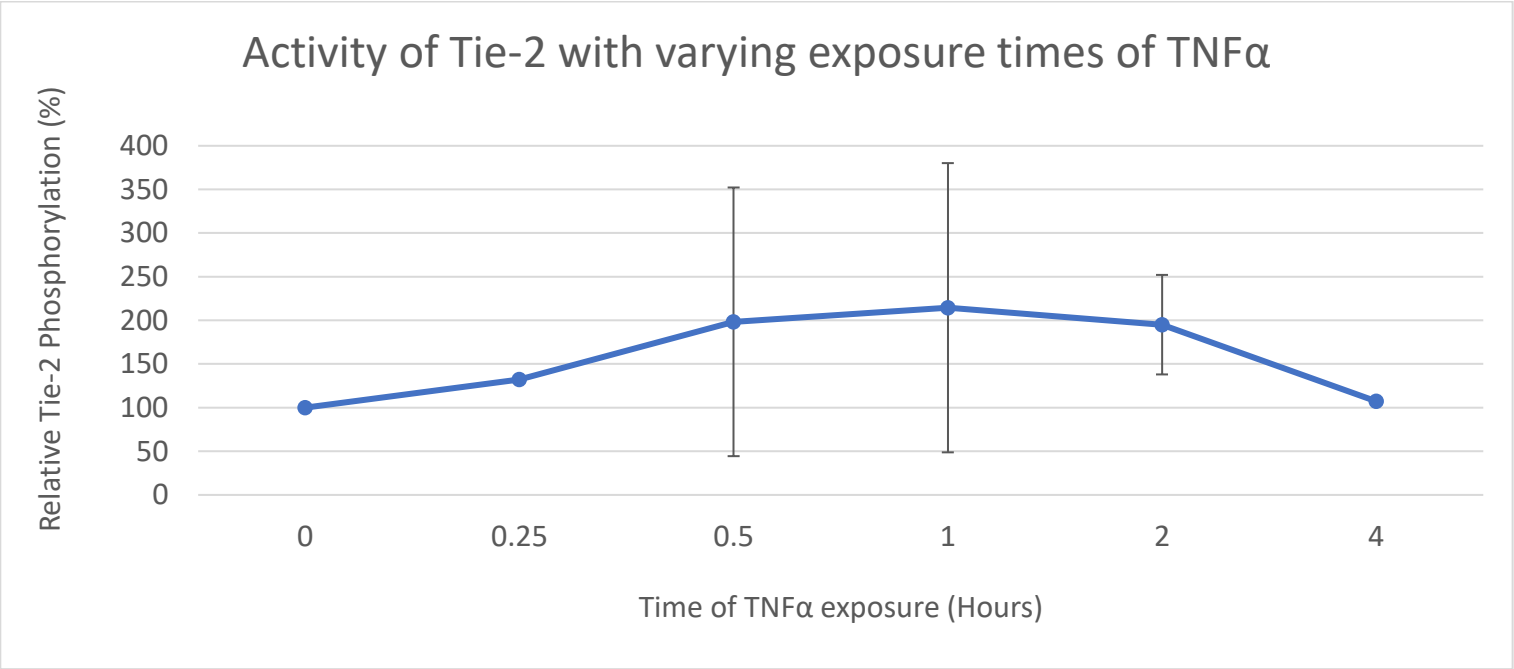


Figure 21. Graph indicating the percentage Tie-2 expression values when exposed to TNF-α over varying time points. *The levels of pTie-2 expression were normalised against normal Tie-2 densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of Tie-2. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*

4.2.3 Acute Impact of IL-1β and TNF-α on Ang-1-induced AKT Activity

PI3K/AKT is a crucial mediator in the Ang-1/Tie-2 signalling pathway as it functions for survival of endothelial cells. To assess the impacts of cytokine exposure on the vital signalling mediator. Each sample was stimulated with the addition of Ang-1 for 15minutes before the end of each time point, HUVECs were treated with recombinant TNFα (25ng/ml) or recombinant IL-1β (25ng/ml) at six time points (0hr, 0.25hr, 0.5hr, 1hr, 2hr and 4hr). The Ang-1-induced activity of AKT was measured by quantifying the phosphorylated AKT against the total AKT in each sample. Appendix 4 highlights Ang-1 functioning to activate Akt and cause phosphorylation beyond the basal activity. Results of IL-1β stimulation display an increasing trend in activated AKT over the time course, with the highest increase in activity of 78.5% at 2hours. While the data point at 1hour conveys a 28.3% decrease in activity against the control, none of the time points share any statistical significance with the control with all *p-values* > 0.05, as a result of large standard errors. TNFα shows a similar pattern of increasing activity, but only up to 30minutes with an increase of 44.8% however at 1hour, there is a decrease of 38.5% against the control. A continued decrease in AKT activity is seen up to the 4hour time point which was recorded with the lowest average decrease in activity of 55.6%. The students' T-test returned values of significance at 1hour and 4hour time points, with *p = 0.050 and p = 0.006*, respectively.

Time (h)	0	0.25	0.5	1	2	4
IL-1β	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.421	0.096	0.217	0.179	0.420

Time (h)	0	0.25	0.5	1	2	4
IL-1β	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.421	0.096	0.217	0.179	0.420

Figure 22. Ang-1-induced AKT activity in HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pAKT and AKT. AKT is reported as a 60kDa molecule. pAKT is also seen at 60kDa.*

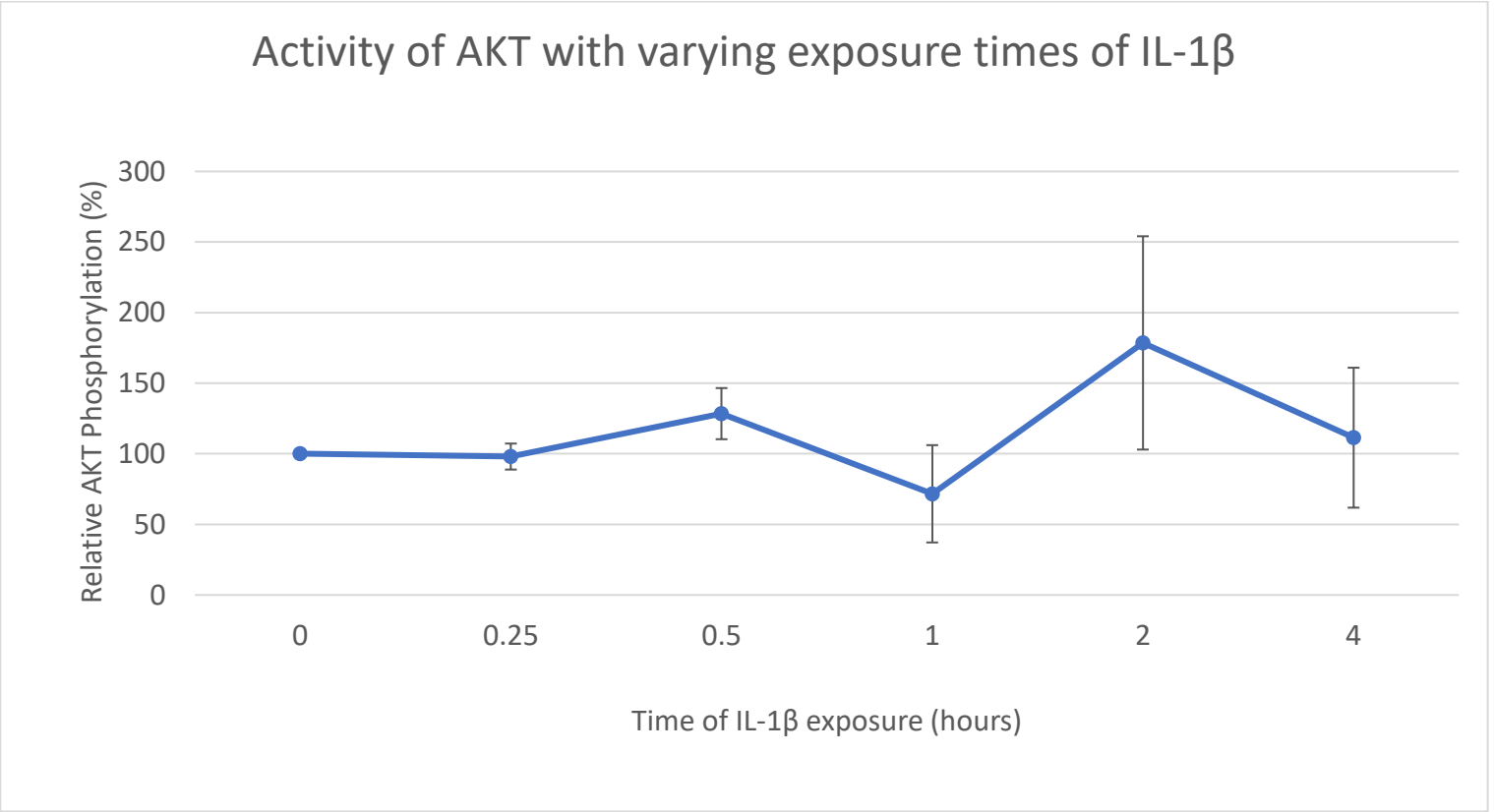
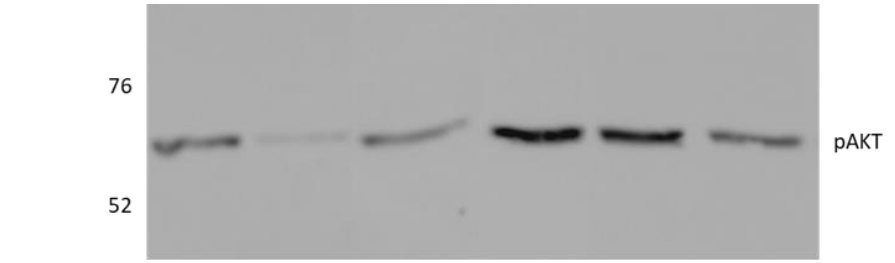
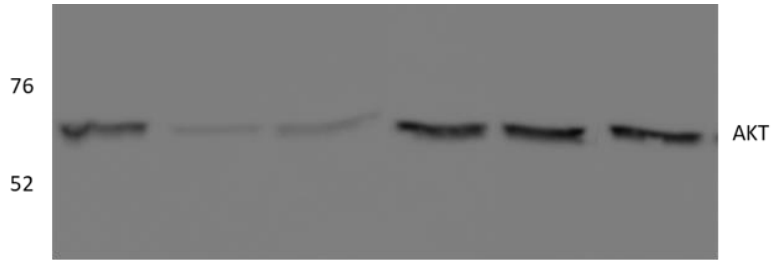


Figure 23. Graph indicating the percentage pAKT expression values when exposed to IL-1β over varying time points. *The levels of pAKT expression were normalised against normal AKT densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*



Time (h)	0	0.25	0.5	1	2	4
TNFα	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.427	0.269	0.050	0.143	0.006



Time (h)	0	0.25	0.5	1	2	4
TNFα	-	+	+	+	+	+
ANG1	+	+	+	+	+	+
p-value		0.427	0.269	0.050	0.143	0.006

Figure 24. Ang-1-induced AKT activity in HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pAKT and AKT. AKT is reported as a 60kDa molecule. pAKT is also seen at 60kDa.*

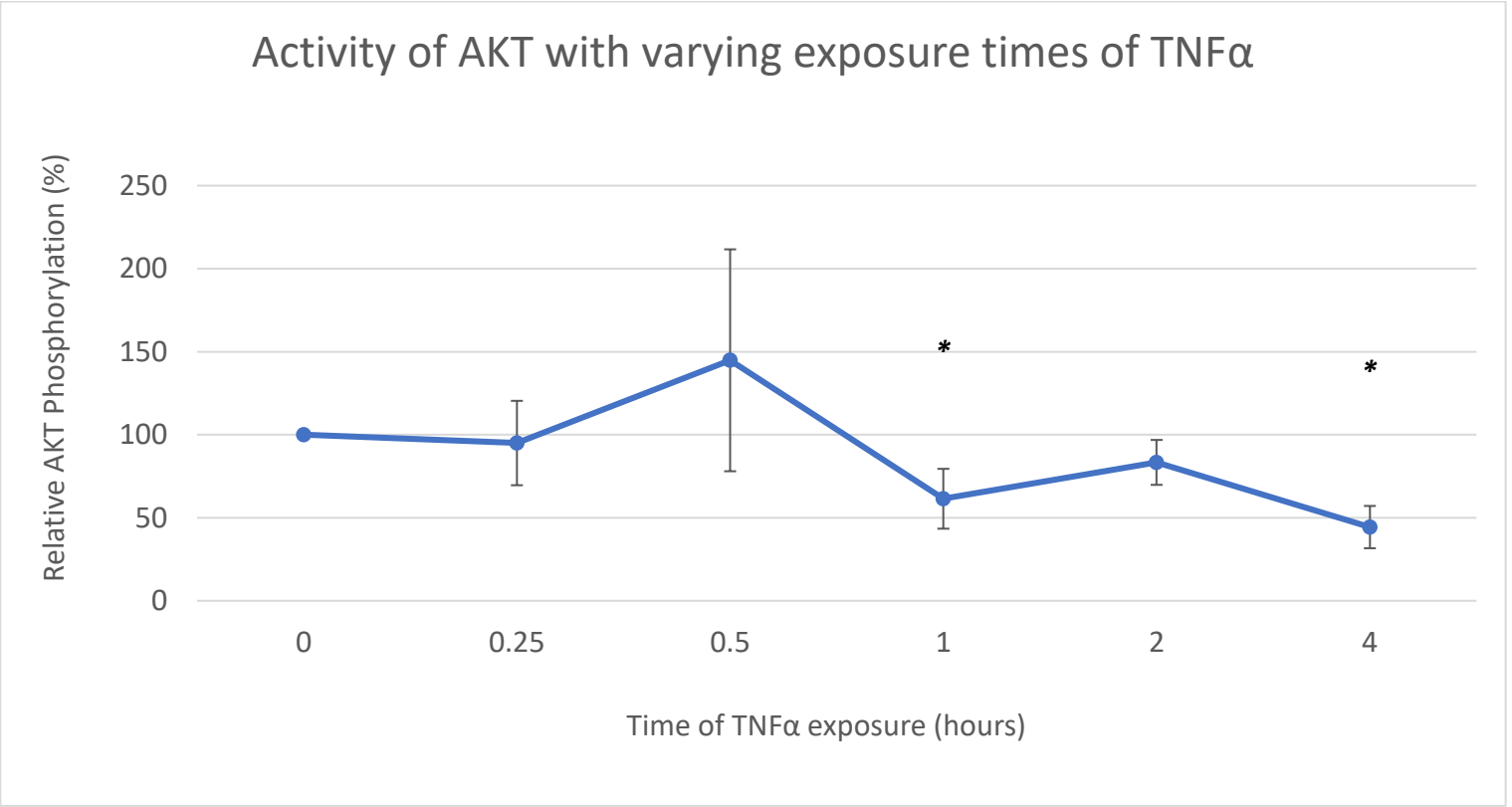


Figure 25. Graph indicating the percentage pAKT expression values when exposed to TNF-α over varying time points. *The levels of pAKT expression were normalised against normal AKT densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*

4.3 Discussion

4.3.1 Implication of IL-1β on Ang/Tie Signalling

From the result (see 4.2), IL-1β has some significant impacts on Tie-2 expression and activity. The stimulation of IL-1β (see 4.2.1) results in an increase in Tie-2 expression, with significance around the 1hour and 2hour time points (p = 0.045 and p = 0.043, respectively). This increase in Tie-2 expression is suggestive of a mechanism of vascular protection with an acute response to IL-1β. On approach to the 4hour time point, while the result of the decreasing trend in the expression do not show statistical significance, the reduction in expression is suggestive of the acute inflammatory response as explained by Zhang (2008), wherein a delay in the response is reported to be 4-6hours post stimulation of an inflammatory cytokine. This point then results in the cytoskeletal changes in in the endothelial cells, along with disruptions in the junctions around venules and capillaries (Zhang, 2008). This is further supported by evidence from section 4.2.2, whereby the activity is following a similar pattern of increase up to 1hour, which suggests with increased expressions, there is an increased in Ang-1 responsiveness. But on approach to the 4hour time point as with the expression decreasing, the activity decreases below the control value to 14.7% with a p = 0.001. This would indicate not on only a decrease activity due to decreased expression but also a regulatory mechanism to inhibit activity to begin the immune response. Furthermore, the pattern of behaviour with AKT activity appeared inverse to Tie-2, with a decrease in activity at 1hour and then an increase up to 4hours. While the result did not share any statistical significance from the T-test, it is worth noting that IL-1β also signals through PI3K/AKT pathways which

could explain the inverse action resulting from IL-1 signalling mechanisms (Sizemore, Leung and Stark, 1999; Xiao et al., 2016).

4.3.2 Implication of TNFα on Ang/Tie Signalling

TNFα has some varying impacts to the signalling of the receptor and downstream mediators. TNFα initially shows an increase in in phosphorylation of Tie-2 with the highest activity of approximately 2-fold increase compared to the control at 1hour. This increase reduces back down to near control level by 4hours (7.2% against the control). This gives a similar pattern to IL-1β showing initial endothelial protective mechanisms but then the mechanism of the inflammatory response set in to reduce activity. Zhang (2008) categorises TNFα as one of the inflammatory cytokines that initiates the delayed response 4-6hours post stimulus (Zhang, 2008). While the results of TNFα exposure on Tie-2 activity did not report values of significance, the similarities in increases and decreases in Tie-2 activity with IL-1β are noteworthy as this gives evidence of acute proinflammatory cytokine activity on endothelial cells. The acute exposure impacts on AKT phosphorylation do report significant results. With initial increases up to 30minutes of an increase of 44.8%, at 1hour there is a decrease below control to only 61.5% of the control activity. This decrease against the control remained though the remaining time points and the lowest recorded activity was 44.4% of the control activity which was seen at 4hours. This is controversial to Li et al (2018) as their study gives evidence of the upregulation of the PI3K/AKT pathway in rats. However, this study measure the impacts within a shorter time period compared to their values obtained after 14days of

treatments (Li et al., 2018). The role of TNFα was also reported to induce PI3K/AKT for cell survival in cancer models (Gu et al., 2006).

4.4 Conclusion

The overall analysis of acute exposures with inflammatory cytokines on the Ang-1/Tie-2 signalling pathway, display novel evidence first reported from this study. The immediate reaction of cytokine stimulus on endothelial cells activates the Ang-1/Tie-2 signalling cascade with increase in expression and activity before the mechanics of the acute inflammatory response sets in. The contradictory results of the AKT activity would need further assessment, although only TNFα displayed significant results, the similarities in the trend gives a good understanding in the signalling impact immediately post stimulation. The trend of the results suggests further mechanisms of other signalling pathways acting on the PI3K/AKT pathway due to is versatility in cellular signalling.

This chapter highlights the impact of the short exposure time of the cytokines on Ang/Tie signalling. The pattern of Tie-2 decrease at 4hours gives precedence for assessment of the chronic exposure of proinflammatory cytokines on endothelial cells with the understanding that there should continue to be a decrease in Tie-2 and its activity.

Chapter 5: Chronic Cytokine Exposure on Endothelial Cells

5.1 Introduction

Prolonged exposure of cytokines is associated with numerous pathophysiological conditions that impede on human life from minor inflammation to severe complications leading to fatalities. The multifaceted nature of inflammatory cytokines means it plays a role in many different disorders. The inflammatory response in the vasculature involves complex interactions between immune cells and the cells associated with the vasculature including endothelial cells, vascular smooth muscle cells and the extracellular matrix. The recruitment of cytokines has consequential effects on the vasculature which when exposure is prolonged can lead to complication including vascular dysfunction, atherosclerosis, aneurysms and also hypertension (Sprague and Khalil, 2009). Cytokines themselves stimulate the proliferation and differentiation of immune cells, with suggestion that they have a capacity to induce vascular cell growth and migration (Sprague and Khalil, 2009). There are specific functional effects of cytokines on vascular cells, including mechanisms controlling vascular tone as well as structural changes to the vasculature in both the vessel wall construction and extracellular matrix (Sprague and Khalil, 2009). While under normal conditions this may be viewed as protective, over expression and/or prolonged stimulation can result in serious consequences. Such example is seen in the growth of rat aortic VSMCs promoted

by IL-1 through the production of endogenous platelet-derived growth factor. However, this very mechanism under abnormal conditions can cause dysfunctional growth of VSMC that results in early stages of atherogenesis (Ikeda et al., 1990). During the process of vascular injury and atherogenesis, smooth muscle cells migrate from the media to the intima, a crucial process of vessel wall remodelling. Migration is seen to be stimulated by a number of cytokines including IL-1β and TNFα. The mechanism involves activation of signalling cascades that trigger cytoskeletal remodelling and changes of cell adhesion to the matrix (Gerthoffer, 2007).

TNFα is an initiator of apoptosis in numerous cell types. With recruitment of caspase-8 and autocatalytic activation, hierarchal activation of caspase results in the culminating initiation of caspase-3 which is reported to result in apoptosis of endothelial cells (Muzio et al., 1998; Madge and Pober, 2001). Adhesion of immune cells to endothelial cells are promoted through cytokine stimulation causing an increase in vascular permeability. TNFα exerts its effect through expression of transient receptor potential channel homologues in vascular endothelial cells. The resultant effect of Transient Receptor Potential-Canonical (TRPC) expression is the permeability of endothelial cells (Sprague and Khalil, 2009). The induction of TNFα-induced TRPC expression in endothelial cells is vital mechanism of Ca^{+2} influx and elevation of endothelial cell permeability (Paria et al., 2004). This proinflammatory cytokine is reported to cause time-dependent increases in Tie-2:Tie-1 ratio through upregulation of Tie-2 with an additional suppression in Tie-1 expression (Zernecke and Weber, 2005). In addition to suppression of expression, Tie-1 has also been shown to be proteolytically cleaved of its ectodomain (Korhonen et al., 2016).

Understanding the long-term impacts of proinflammatory cytokines on Ang-1/Tie-2 Signalling and regulation of receptor functions can provide insight into the functional impact on endothelial cells and by extension the implication on the vasculature. Identification of key signalling changes can provide novel therapeutic targets. To provide evidence of IL-1β and TNF-α influences, HUVECs were stimulated with the inflammatory cytokines either in the presence or absence of Ang-1 to obtain date for expression changes as well as Ang-1-induced activity changes. Lysates obtained from the cell stimulations were separated using SDS-PAGE electrophoresis and transferred to nitrocellulose membrane for protein detection using methods of western blotting (see 2.4). As a result of data obtained in chapter 4, it was important to additionally analyse the chronic impact of IL and TNF had on Tie-1 in regulating Ang-1 signalling pathway. The regulatory mechanism of Tie-1 has a pivotal role on vascular integrity therefore understanding the impact on chronic exposures on Tie-1 will provide further evidence on the changes in signalling as a result of cytokine exposure.

5.2 Results

5.2.1 Chronic Impact of IL-1β and TNF-α on Tie-2 Expression

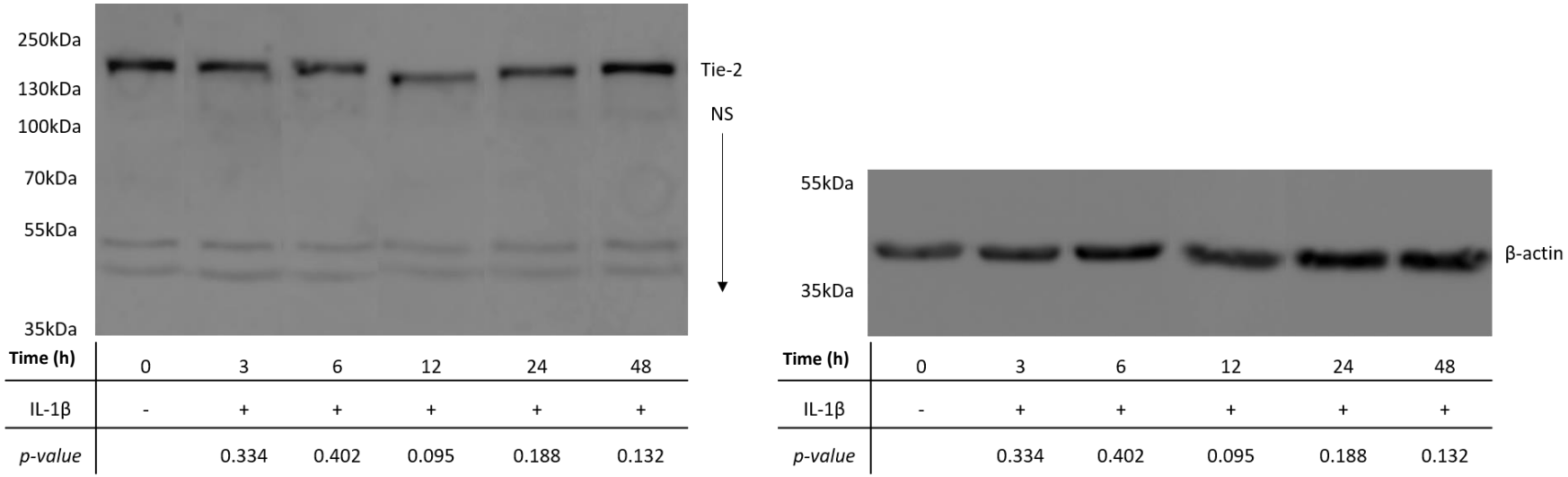
To assess the impacts of IL-1β, HUVECs were treated with recombinant IL-1β (25ng/ml) at six time points (0hr, 3hr, 6hr, 12hr, 24hr and 48hr). The 48hour cut-off relates to preliminary results indicating expression levels of Tie2 at 20hour to be elevated, hence this would further add to understanding of chronic exposure and assess changes beyond the 20hour exposure (Bilimoria and Singh, 2017). Expression levels of Tie-2 were obtained from western blots using Tie-2 antibody (see 2.1.4 – Human Tie-2 Antibody).

Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for Tie-2.

A general decrease in Tie-2 levels was observed as shown in Figure 26. The lowest decrease in expression was noted at 12hours with a decrease to 75.4% expression in comparison to the control (Figure 27). The levels at 24 and 48hour then seemed to plateau. Even though there was a decrease in Tie-2 levels observed, the T-test performed showed no significant changes to the level of Tie-2 at the different time points compared to control.

Similar experiments were performed to assess the impact of TNFα on Tie-2 expression in HUVECs. Cells were treated with recombinant TNFα (25ng/ml) at six time points (0hr, 3hr, 6hr, 12hr, 24hr and 48hr). Expression levels of Tie-2 were obtained from western blots probed with Tie-2 antibody (see 2.1.4 – Human Tie-2 Antibody). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for Tie-2.

Blot and graph (Figure 28, Figure 29) display and increasing trend, however at 12hour there was a sharp decrease in expression before elevating above the control. The initial increase at the 6hour time point is seen to be 35.6% elevation compared with the control. At 12hour the sharp decrease in expression is 66.9% of the control. The rise at 24hours reaches the highest expression of Tie-2 at 44.9% above control. The final recorded expression of 30.2% increase at 48hours. Again, statistical significance was not observed at any time point compared to the control.



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
p-value		0.334	0.402	0.095	0.188	0.132

Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
p-value		0.334	0.402	0.095	0.188	0.132

Figure 26. Levels of Tie-2 expression HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using Tie-2 and β-actin. Though Tie-2 is reported as a 126kDa molecule, due to glycosylation is seen to be between 140-160kDa. β-actin is reported as a 42kDa molecule. NS indicates non-specific bands.*

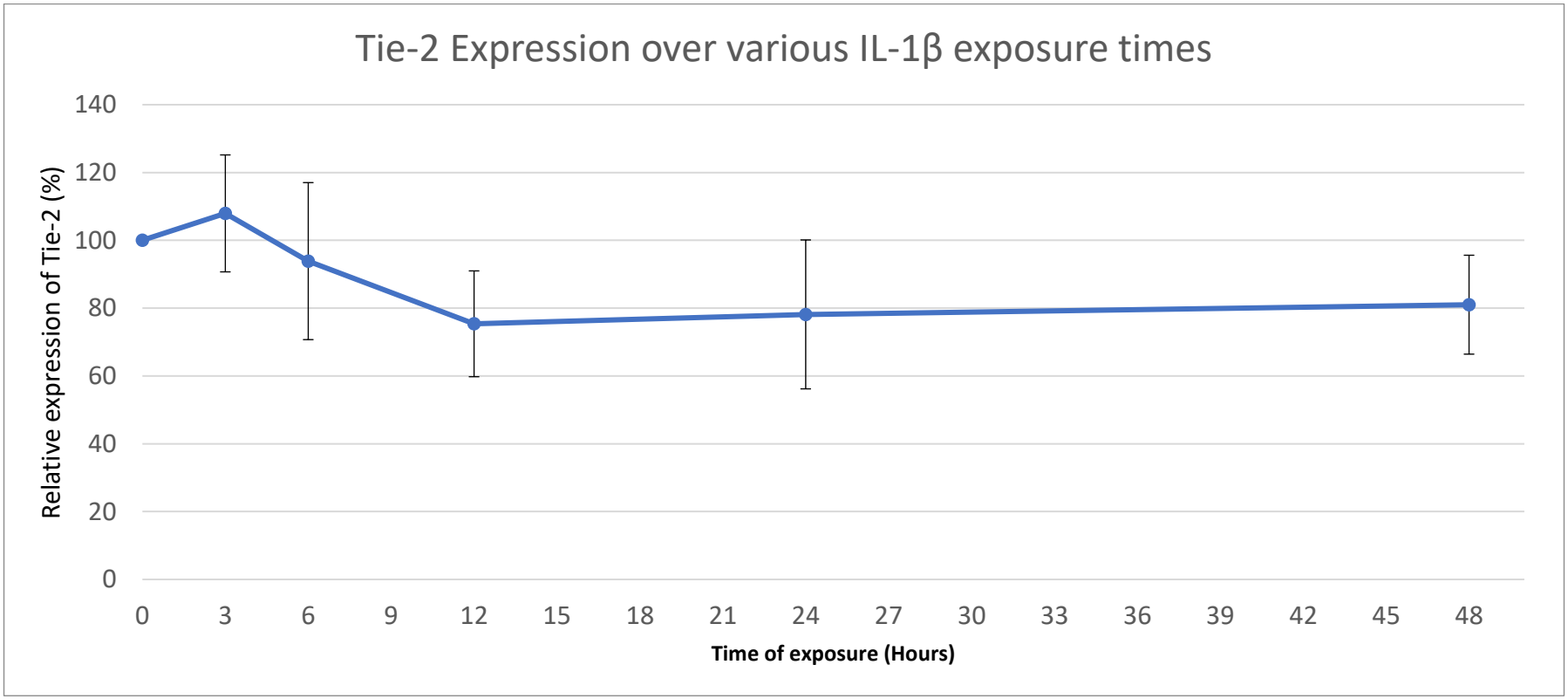
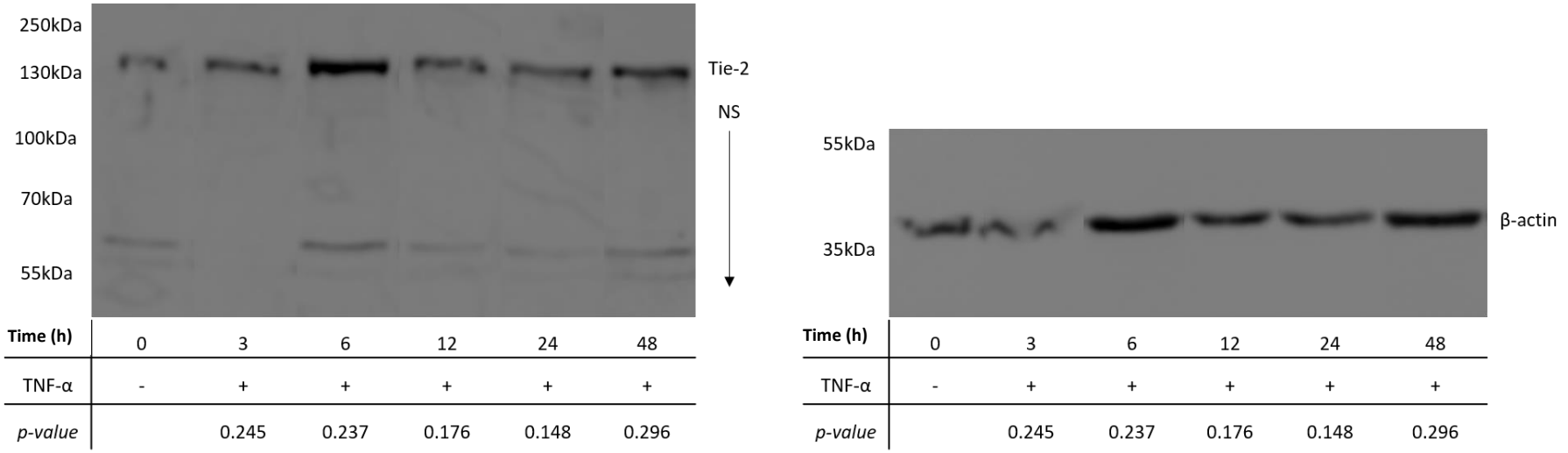


Figure 27. Graph indicating the percentage Tie-2 expression values when exposed to IL-1β over varying time points. *The levels of Tie-2 expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of Tie-2. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*



Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
p-value		0.245	0.237	0.176	0.148	0.296

Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
p-value		0.245	0.237	0.176	0.148	0.296

Figure 28. Levels of Tie-2 expression HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using Tie-2 and β-actin. Though Tie-2 is reported as a 126kDa molecule, due to glycosylation is seen to be between 140-160kDa. β-actin is reported as a 42kDa molecule. NS indicates non-specific bands.*

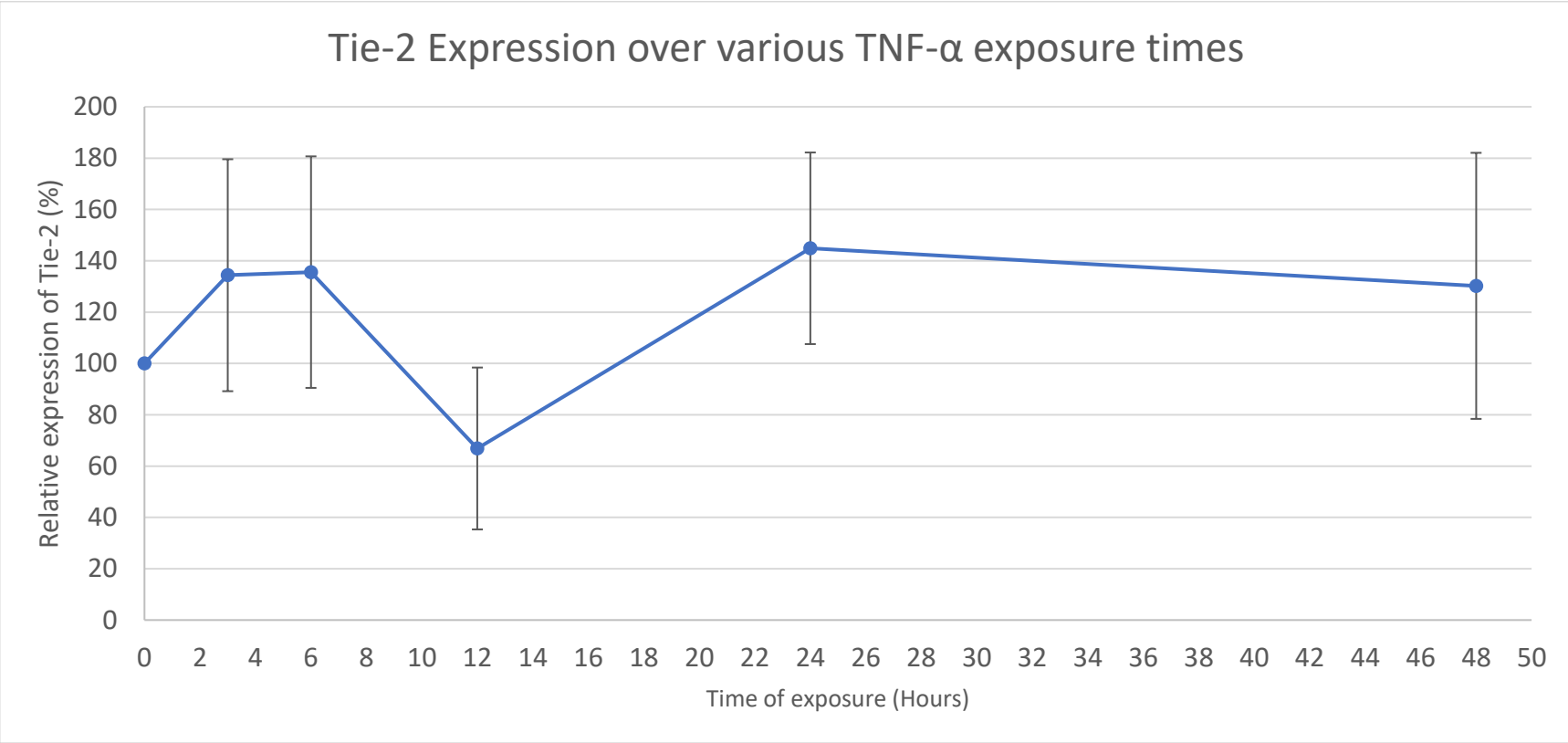


Figure 29. Graph indicating the percentage Tie-2 expression values when exposed to TNF-α over varying time points. *The levels of Tie-2 expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of Tie-2. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*

5.2.2 Chronic Impact of IL-1β and TNF-α on AKT Expression

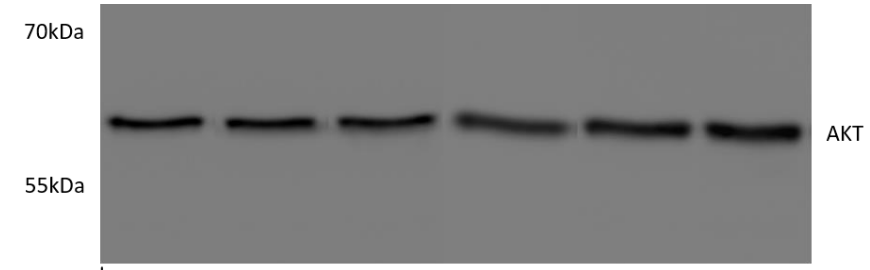
The assessment of AKT expression was conducted on HUVECs exposed to recombinant IL-1β (25ng/ml) at six time points (0hr, 3hr, 6hr, 12hr, 24hr and 48hr). Expression levels of AKT were obtained by probing western blots with AKT antibody (see 2.1.4 – Akt (pan)(11E7) Rabbit mAb). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for AKT.

Figure 30 and Figure 31 displays a decrease in the level of AKT over the course of the IL-1β treatment. The influence of IL-1β on AKT expression shows a gradual decrease in AKT expression with the lowest expression level at 48hours with a 20.2% decrease in expression. The 48hour time point is the only sample point of the IL-1β exposure to show a significant decrease in AKT expression with T-test displaying a *p-value = 0.041*.

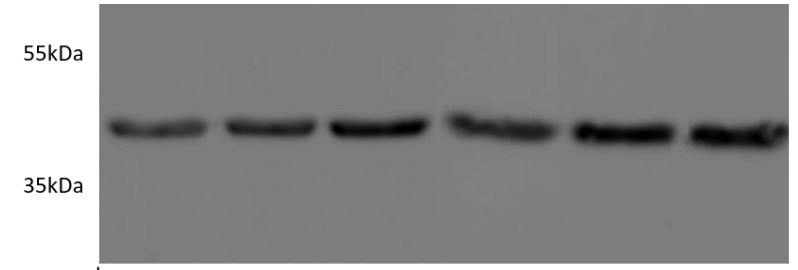
TNF-α stimulation were conducted in a similar manner with HUVEC exposure to recombinant TNF-α (25ng/ml) at six different time points (0hr, 3hr, 6hr, 12hr, 24hr, 48hr). Expression levels of AKT were obtained using western blots probed with AKT antibody (see 2.1.4 – Akt (pan)(11E7) Rabbit mAb). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for AKT.

Blot and graph (Figure 32, Figure 33) show an overall decreased expression of AKT expression from TNF-α stimulation with the exception of a dramatic spike at 6hour. The 6hour spike was a 48.6% increase in AKT in contrast to the control. The lowest expression level was seen at 12hour with a decrease of 40.6% in AKT expression. A

gradual return to normal expression was indicated with the final 48hour time point displaying a 17.2% decrease in expression of AKT compared to the control at 0hour. No values of statistical significance were obtained from T-test of TNF-α influence on AKT expression.



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
p-value		0.432	0.300	0.250	0.055	0.041



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
p-value		0.432	0.300	0.250	0.055	0.041

Figure 30. Levels of AKT expression HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using AKT and β-actin. AKT is reported as a 60kDa molecules. β-actin is reported as a 42kDa molecule.*

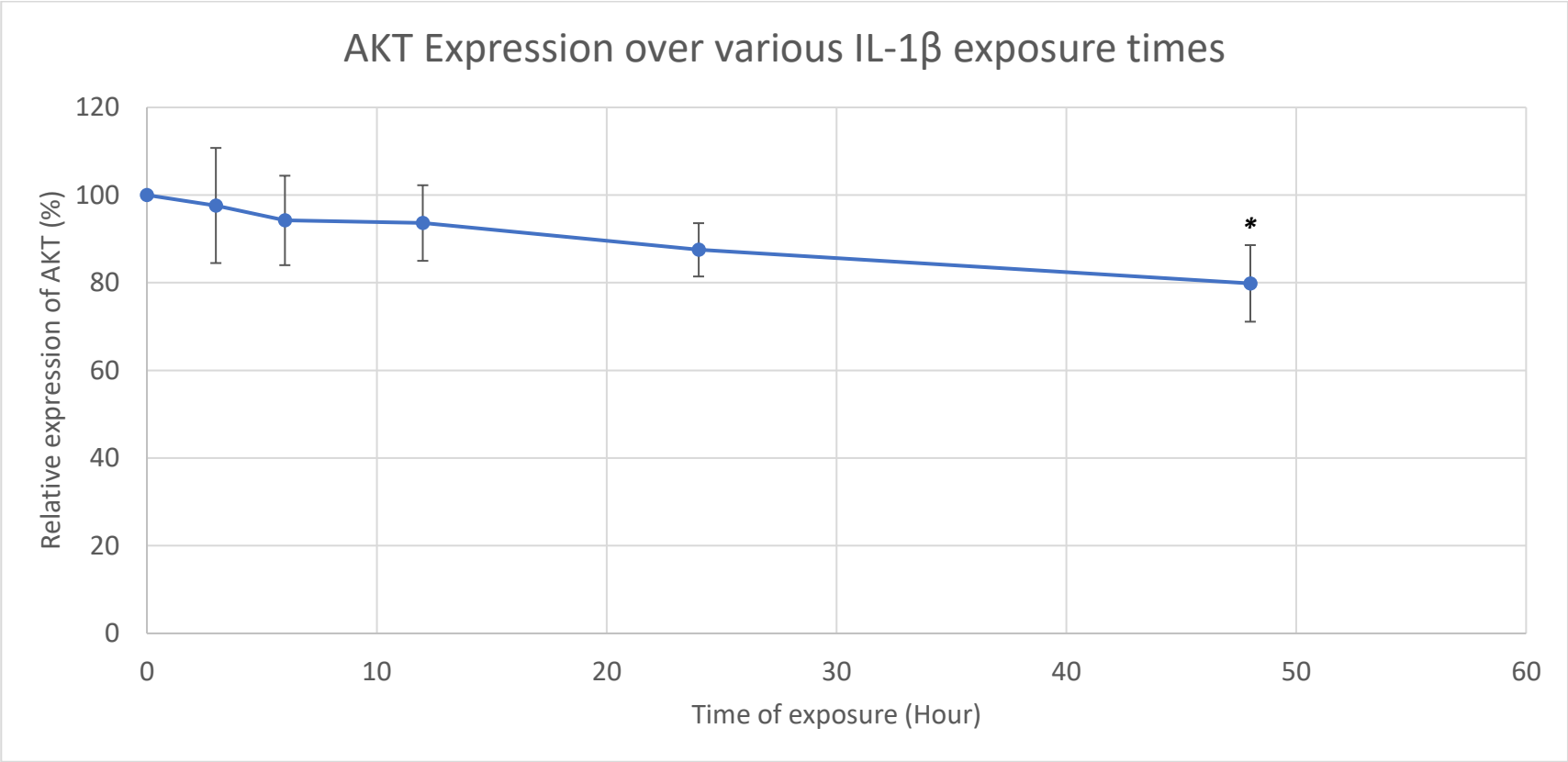
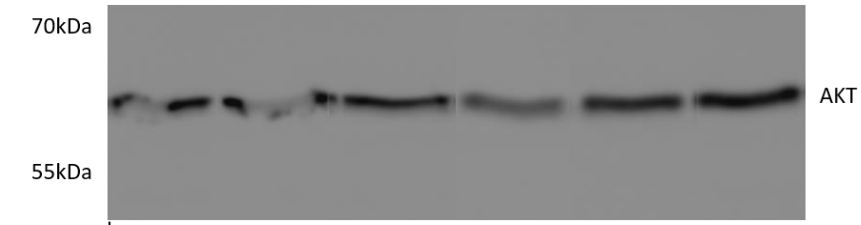
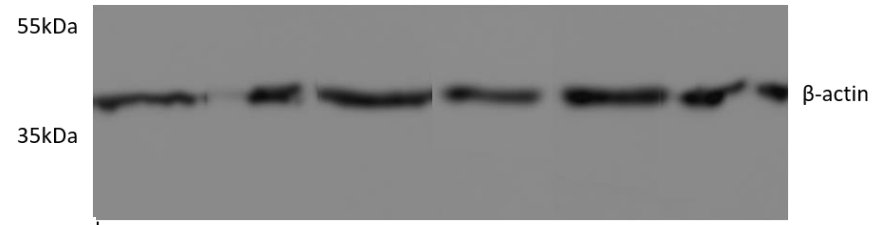


Figure 31. Graph indicating the percentage AKT expression values when exposed to IL-1β over varying time points. *The levels of AKT expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*



Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
p-value		0.125	0.328	0.064	0.169	0.332



Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
p-value		0.125	0.328	0.064	0.169	0.332

Figure 32. Levels of AKT expression HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using AKT and β-actin. AKT is reported as a 60kDa molecules. β-actin is reported as a 42kDa molecule.*

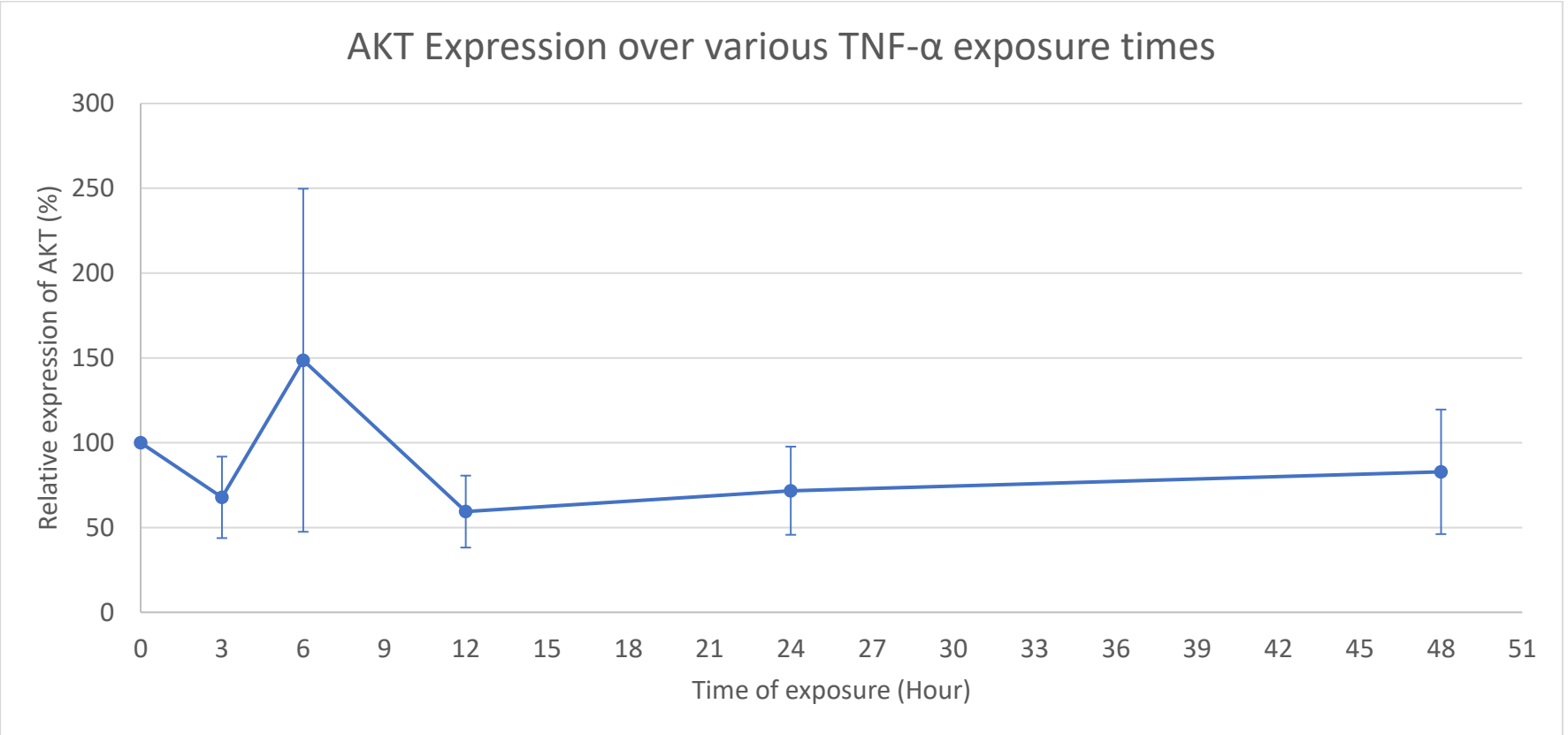


Figure 33. Graph indicating the percentage AKT expression values when exposed to TNF-α over varying time points. *The levels of AKT expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*

5.2.3 Chronic Impact of IL-1β and TNF-α on Ang-1-induced AKT Activity

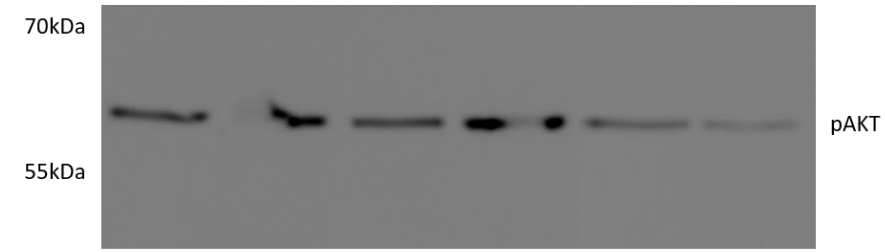
The assessment of Ang-1-induced AKT activity was conducted on HUVECs exposed to recombinant IL-1β (25ng/ml) at six time points (0hr, 3hr, 6hr, 12hr, 24hr and 48hr). Levels of phosphorylated AKT (pAKT) were obtained from western blots using pAKT antibody (see 2.1.4 – Human/Mouse/Rat Phospho-Akt (S473) Pan Specific Antibody). Expression was normalised against densitometric values of AKT (see 2.1.4 – Akt (pan)(11E7) Rabbit mAb) to give a relative percentage activity of AKT.

Blot and graph of IL-1β exposure on Ang-1-mediated AKT activity is shown in Figure 34, Figure 35, respectively. A decrease trend in AKT activity over the course of exposure of IL-1β was observed. There was with the exception of the 12hour time point showing an increase in Ang-1-AKT activity. Up to the 6hour time point, the activity of AKT decreases by 38.3% before an increase of 7.8% compared to the control at 12hours. After the 12hour time point, a decrease in AKT activity is seen through to the 48hour time point where the lowest recorded activity of AKT was noted to have a 73.4% decrease. Values of statistical significance was reported at 3hour, 6hour, 24hour and 48hour ($p = 0.025$, $p = 0.014$, $p = 0.002$ and $p = 0.002$, respectively).

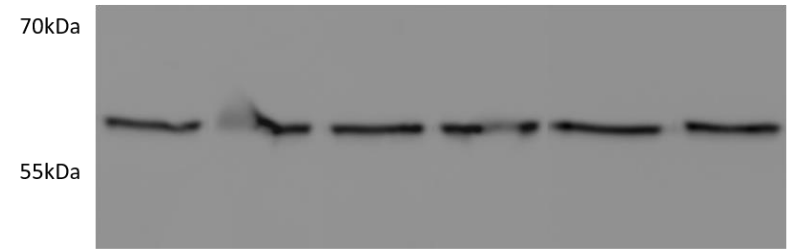
The impact of TNF-α on Ang-1-induced AKT activity was measured in a similar manner with HUVECs exposure to recombinant TNF-α (25ng/ml) at six different time points (0hr, 3hr, 6hr, 12hr, 24hr, 48hr). Blots were probed with pAKT antibody (see 2.1.4 –

Human/Mouse/Rat Phospho-Akt (S473) Pan Specific Antibody). Expression was normalised against densitometric values of AKT (see 2.1.4 – Akt (pan)(11E7) Rabbit mAb) to give a relative percentage activity of AKT.

The activity of AKT was seen to decrease from blot and graph shown on Figure 36 and Figure 37. A steady decrease is seen up to 12hours with a decrease of 63.0%, which is then followed by a slight elevation to 47.1% activity compared to the control in the 24hour sample. There is a decrease that follows up to the 48hour time point with the lowest activity of 26.1% compared to the control at 0hour. T-test values of the data set reports statistical significance at 6hour, 12hour, 24hour and 48hour ($p = 0.033$, $p = 0.016$, $p = 0.006$ and $p < 0.001$, respectively).



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
Ang-1	+	+	+	+	+	+
p-value		0.025	0.014	0.130	0.002	0.002



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
Ang-1	+	+	+	+	+	+
p-value		0.025	0.014	0.130	0.002	0.002

Figure 34. Ang-1-induced AKT activity in HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pAKT and AKT. AKT is reported as a 60kDa molecule. pAKT is also seen at 60kDa.*

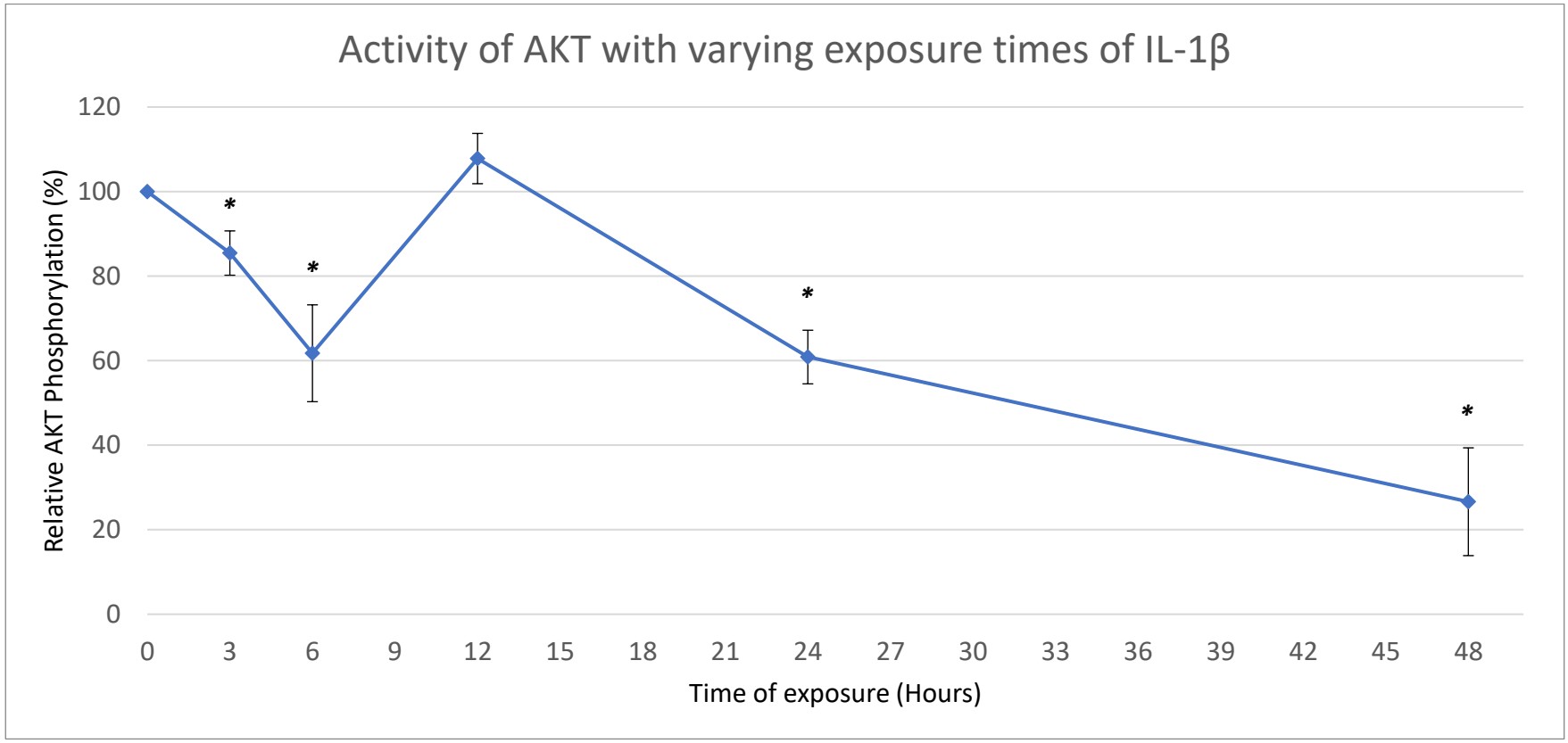
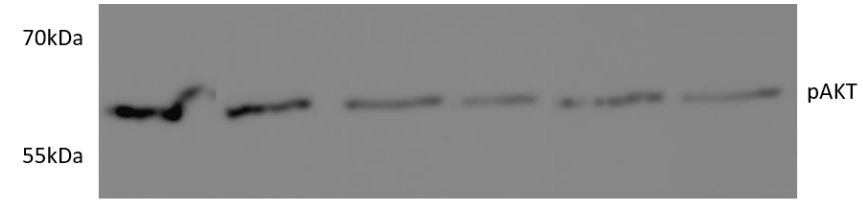
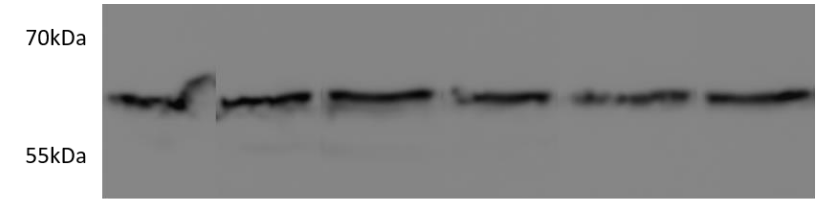


Figure 35. Graph indicating the percentage pAKT expression values when exposed to IL-1β over varying time points. *The levels of pAKT expression were normalised against normal AKT densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*



Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
Ang-1	+	+	+	+	+	+
p-value		0.285	0.033	0.016	0.006	0.000



Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
Ang-1	+	+	+	+	+	+
p-value		0.285	0.033	0.016	0.006	0.000

Figure 36. Ang-1-induced AKT activity in HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using pAKT and AKT. AKT is reported as a 60kDa molecule. pAKT is also seen at 60kDa.*

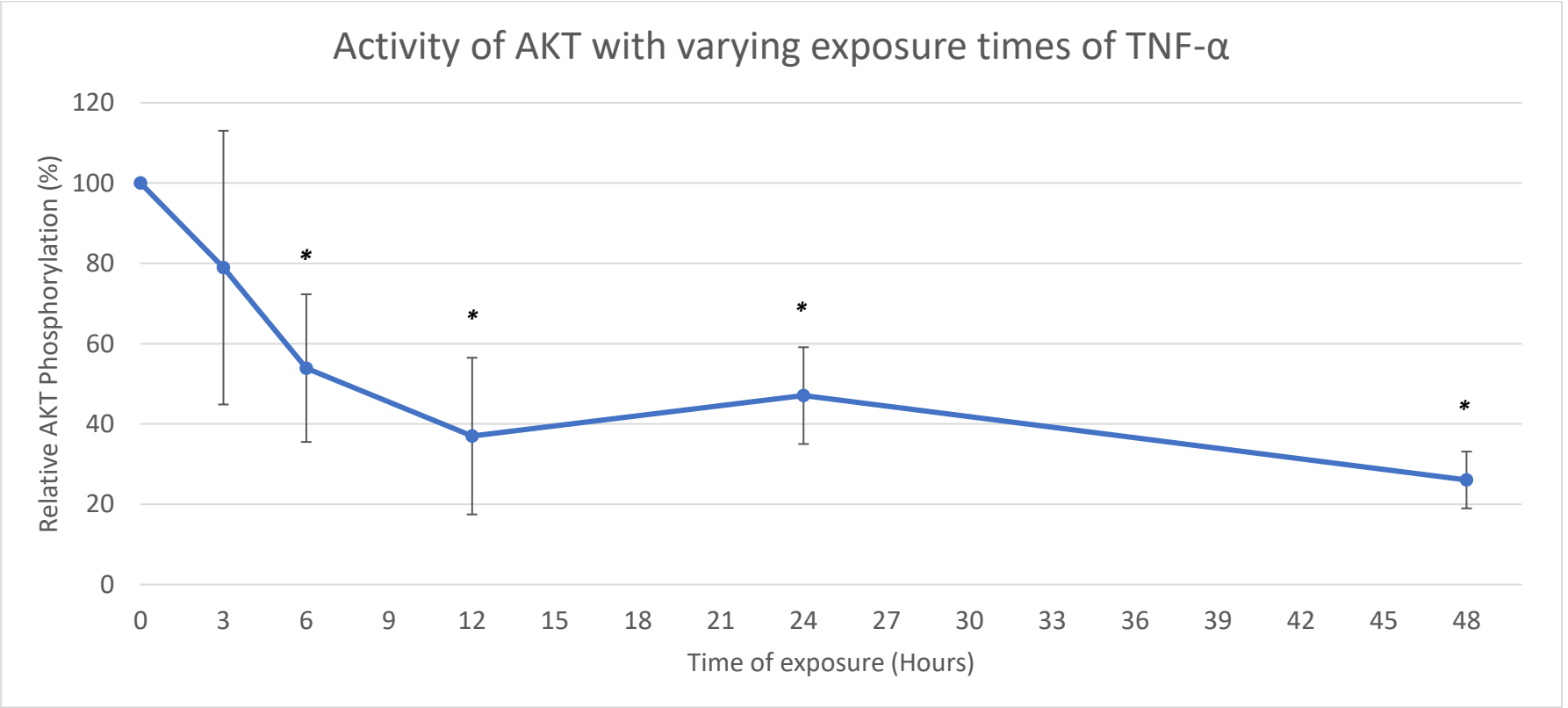


Figure 37. Graph indicating the percentage pAKT expression values when exposed to TNF-α over varying time points. *The levels of pAKT expression were normalised against normal AKT densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % activity of AKT. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*

5.2.4 Chronic Impact of IL-1β and TNF-α on Tie-1 Expression

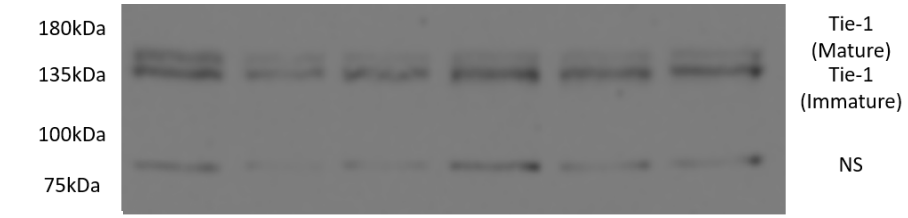
To assess the impacts of IL-1β on Tie-1 expression, HUVECs were treated with recombinant IL-1β (25ng/ml) at six time points (0hr, 3hr, 6hr, 12hr, 24hr and 48hr). Expression levels of Tie-1 were obtained using western blotting with the use of a Tie-1 antibody (see 2.1.4 – Anti-TIE1 antibody). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for Tie-1.

From the blots (Figure 38) the top mature Tie-1 band shows decreased expression over the course of time exposed to IL-1β. The immature band also decreased but then increases. In comparison to the control the mature band is decreased with IL-1β exposure. The graph (Figure 39) displays the decrease in mature Tie-1 expression with the lowest expression recorded at 6hours exposure to IL-1β with an 86.5% decrease in expression. The values of expression slowly increase up to 48hours showing a decrease in expression of 67.7%. All time points were seen to show statistically significant differences in the expression in comparison to the control at 0hour ($p = 0.004$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.003$, respective to each time point). The ratio of mature: immature Tie-1 (Figure 40) displays a similar trend to mature expression whereby there is a dramatic decrease in the ratio followed by a continued reduction in mature: immature Tie-1 ratio. The lowest recorded ratio was at 6hour with a mature: immature ratio of 0.26. The value increased slightly at 12hours but remained below 0.4 up to 48hours. Every time point for the mature: immature ratio displayed statistical

significance with *p = 0.008, p = 0.001, p < 0.001, p < 0.001* and *p = 0.002*, respective of each time point for the experiment.

TNF-α experimentation was conducted in a similar process with HUVEC exposure to recombinant TNF-α (25ng/ml) at six different time points (0hr, 3hr, 6hr, 12hr, 24hr, 48hr). Expression levels of Tie-1 were obtained using western blotting with the use of a Tie-1 antibody (see 2.1.4 – Anti-TIE1 antibody). Expression was normalised against densitometric values of β-Actin (see 2.1.4 – Human/Mouse/Rat beta-Actin Antibody) to give a relative expression value for Tie-1.

Blot (Figure 41) displays a decrease in overall Tie-1 expression over the time course with TNF-α stimulations, with graphs (Figure 42, Figure 43) showing the same trend. The mature Tie-1 graph notes an 81.7% decrease in mature receptor expression before increasing to a 68.1% reduction in mature Tie-1 at 12hour compared to the control at 0hour. The expression further drops at 24hours to the lowest recorded decrease in mature Tie-1 expression of 91.2%. Statistical significance through T-test indicate significance for all time point in comparison to the control at 0hour (*p = 0.001, p = 0.001, p = 0.012, p < 0.001* and *p = 0.016*, respective to each time point. The mature: immature Tie-1 ratio graph showed the same trend with the exception of the 12hour time point displaying an increase in the ratio from the 6hour time point to 0.86. At 24hours the ratio drops to the lowest recorded point of 0.30 and then back up to 0.45. Statistical significance is seen at 6hour, 24hour and 48hour (*p = 0.001, p = 0.002* and *p = 0.048*, respectively.



Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
Tie-1 Expression *p-value*		0.004	0.000	0.000	0.000	0.003
Mature Immature Ratio *p-value*		0.008	0.001	0.000	0.000	0.002

Time (h)	0	3	6	12	24	48
IL-1β	-	+	+	+	+	+
Tie-1 Expression *p-value*		0.004	0.000	0.000	0.000	0.003
Mature Immature Ratio *p-value*		0.008	0.001	0.000	0.000	0.002

Figure 38. Levels of Mature Tie-1 expression and Mature/Immature Tie-1 Ratio in HUVECs exposed to IL-1β. *HUVECs were treated with 25ng/ml of IL-1β at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using Tie-1 and β-actin. Tie-1 is reported to have two bands, an upper 145kDa and a lower 145kDa. β-actin is reported as a 42kDa molecule.*

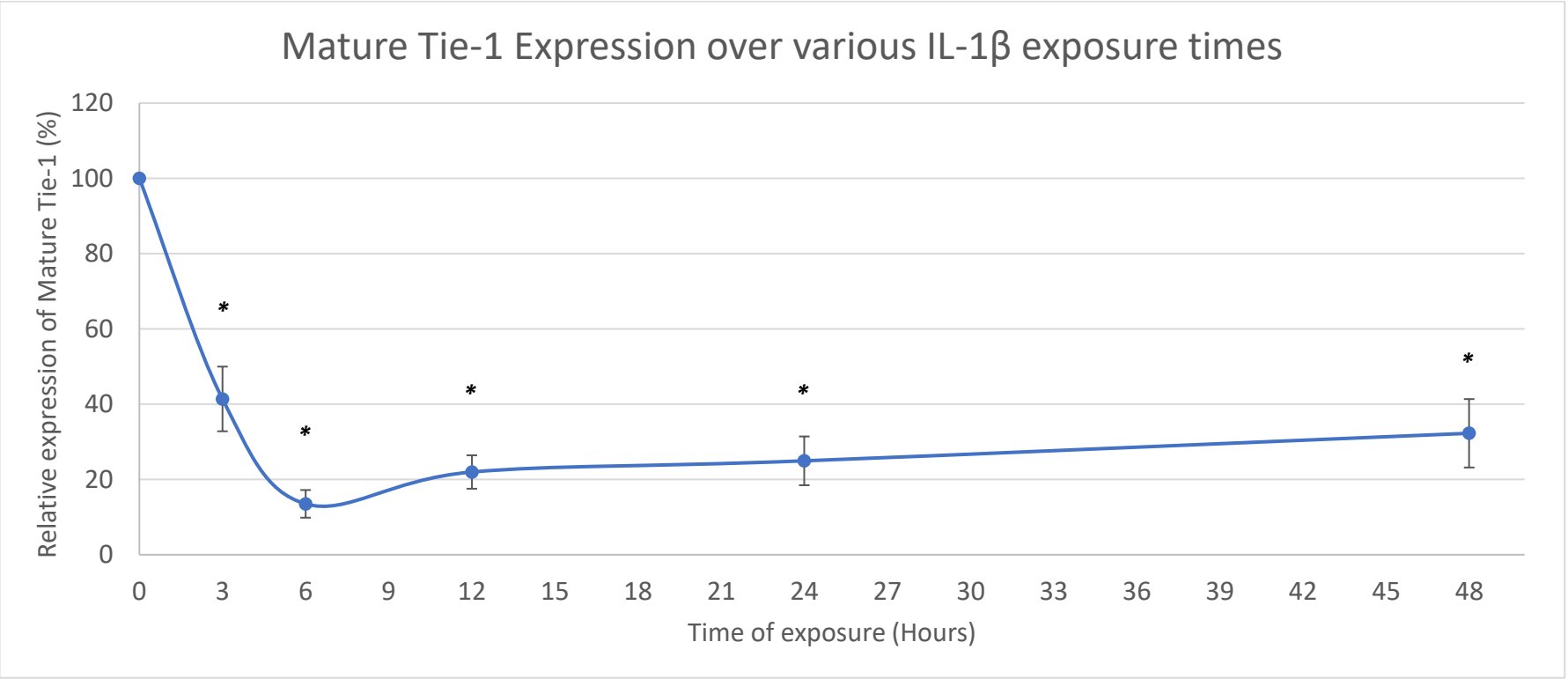


Figure 39. Graph indicating the percentage Mature Tie-1 expression values when exposed to IL-1β over varying time points. *The levels of Mature Tie-1 expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of Mature Tie-1. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*

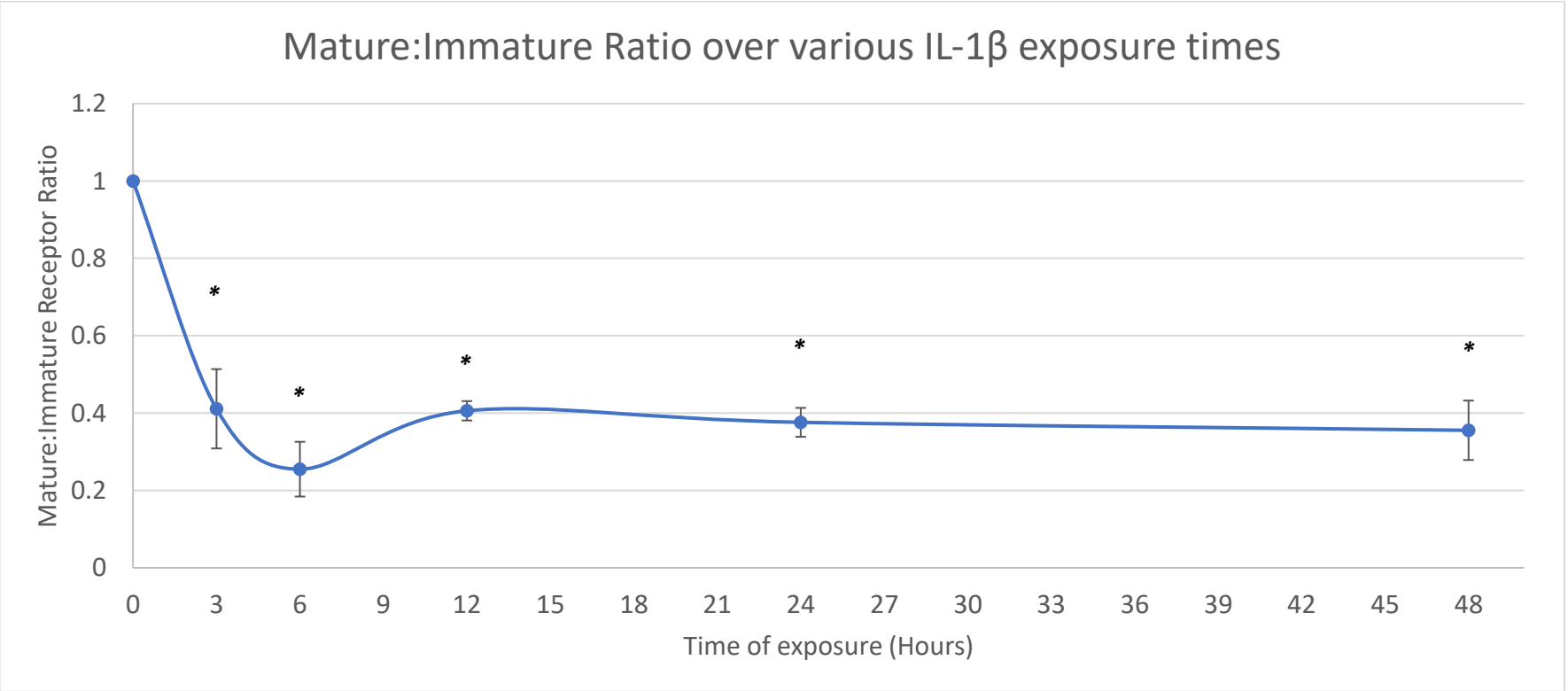
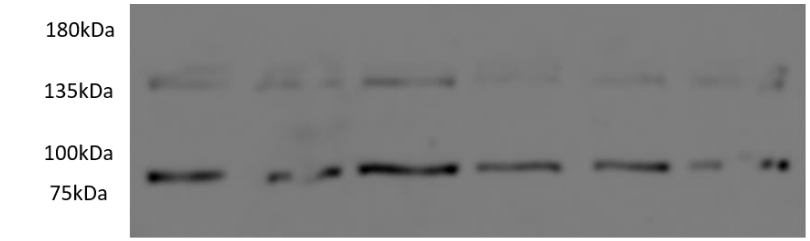
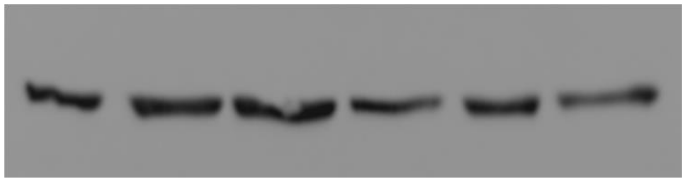


Figure 40. Graph indicating the ratio of Mature: Immature Tie-1 expression values when exposed to IL-1β over varying time points. *The levels of Mature Tie-1 and Immature Tie-1 were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as the ratio of Mature: Immature Tie-1. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*



Tie-1 (Mature)
Tie-1 (Immature)
NS



β-actin

Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
Tie-1 Expression *p-value*		0.001	0.001	0.012	0.000	0.016
Mature Immature Ratio *p-value*		0.150	0.001	0.392	0.002	0.048

Time (h)	0	3	6	12	24	48
TNF-α	-	+	+	+	+	+
Tie-1 Expression *p-value*		0.001	0.001	0.012	0.000	0.016
Mature Immature Ratio *p-value*		0.150	0.001	0.392	0.002	0.048

Figure 41. Levels of Mature Tie-1 expression and Mature/Immature Tie-1 Ratio in HUVECs exposed to TNF-α. *HUVECs were treated with 25ng/ml of TNF-α at different time points as indicated. Cells were lysed in lysis buffer and proteins separated and transferred to nitrocellulose membrane and subjected to Western blot using Tie-1 and β-actin. Tie-1 is reported to have two bands, an upper 145kDa and a lower 145kDa. β-actin is reported as a 42kDa molecule.*

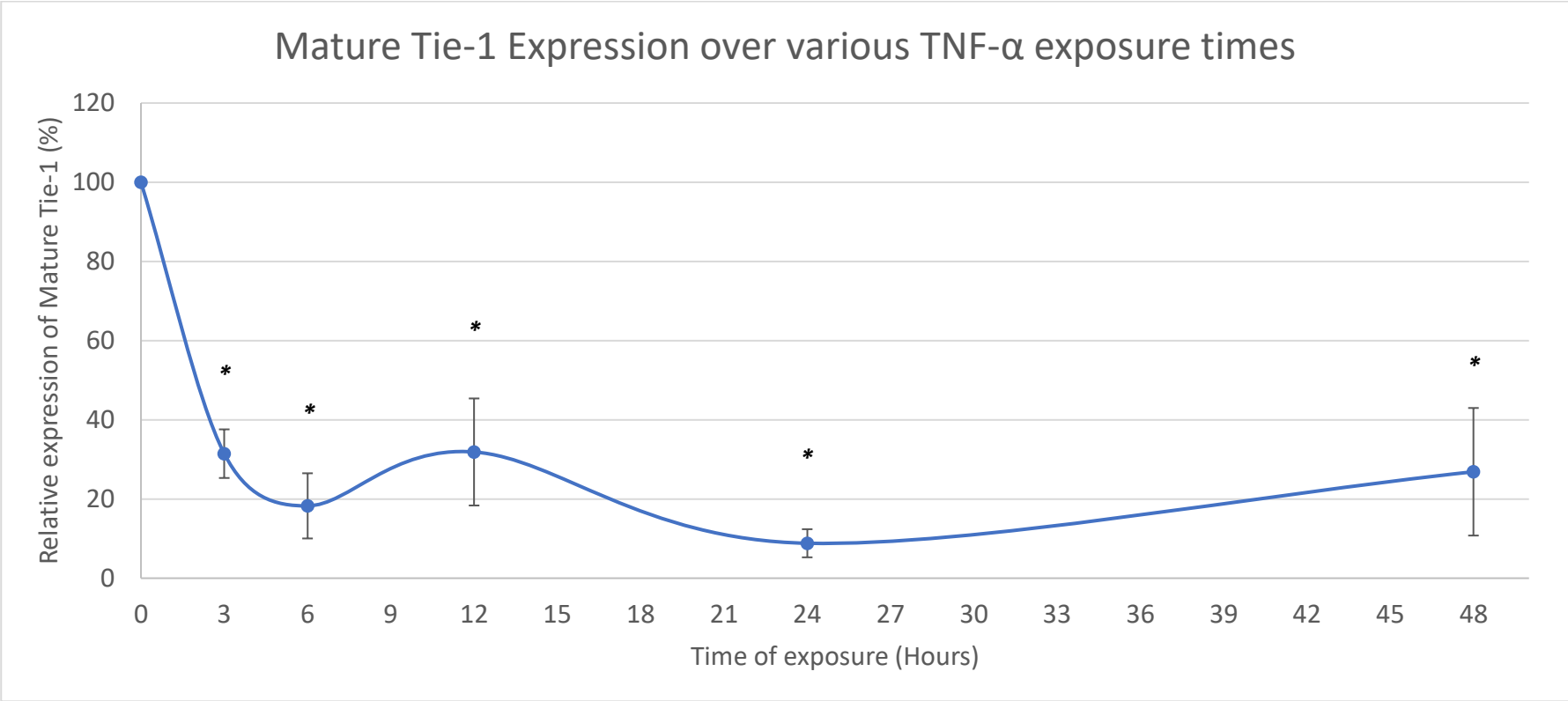


Figure 42. Graph indicating the percentage Mature Tie-1 expression values when exposed to TNF-α over varying time points. *The levels of Mature Tie-1 expression were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as % expression of Mature Tie-1. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, $P \leq 0.05$, have been noted on the graph as *.*

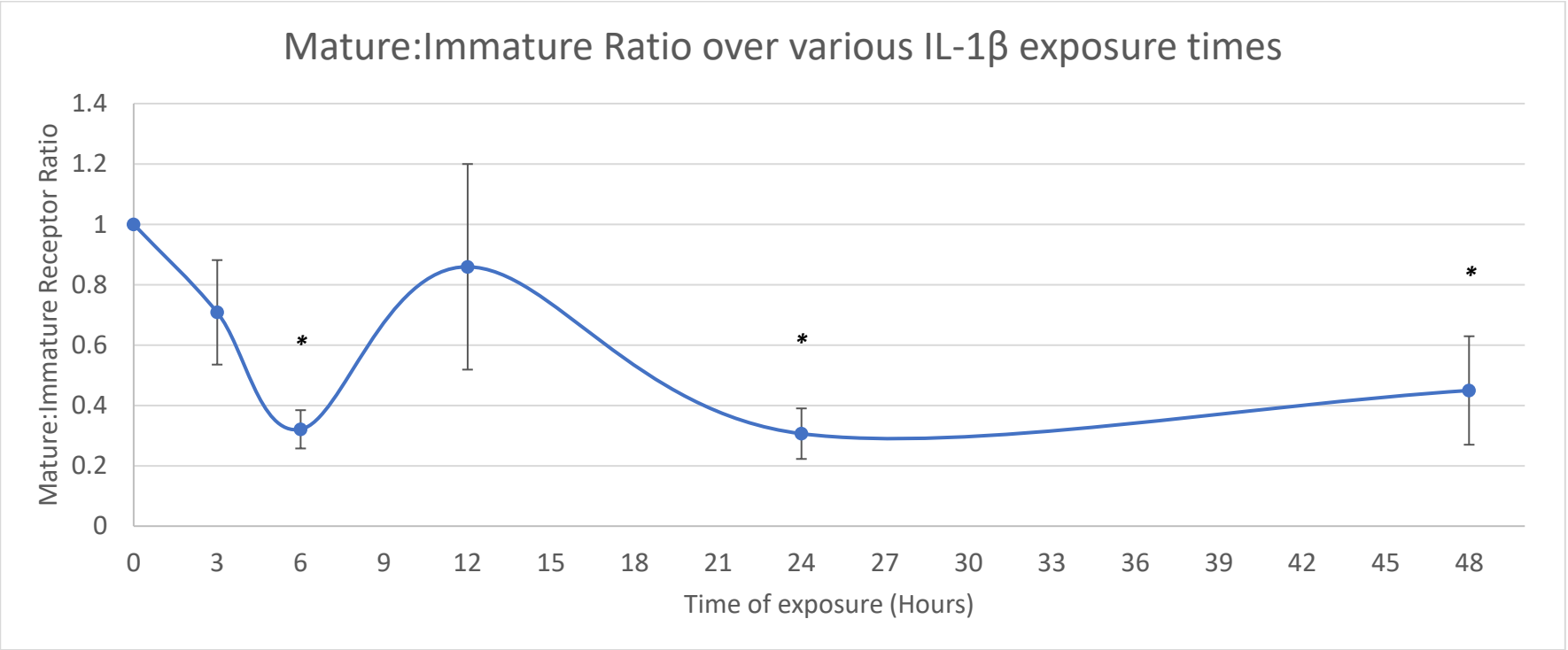


Figure 43. Graph indicating the ratio of Mature: Immature Tie-1 expression values when exposed to TNF-α over varying time points. *The levels of Mature Tie-1 and Immature Tie-1 were normalised against β-actin densitometry. The plotted results are averages of the three independent repeats of the experiment and presented as the ratio of Mature: Immature Tie-1. SEM bars are indicated on each time point. Statistically significant values obtained from T-test, P ≤ 0.05, have been noted on the graph as *.*

5.3 Discussion

5.3.1 Implication of IL-1β on Ang-1/Tie Signalling

IL-1β has displayed evidence of reduction in protein expression and activity associated with Ang-1 signalling cascade. While significance was not reported in the expression of Tie-2, the trend followed the same pattern as AKT expression whereby over the 48hours there is a decrease in the receptor Tie2 and signalling mediator AKT. Only the 48hour time point for AKT displayed significance ($p = 0.041$, vs Control). The results of Tie-2 expression further support chapter 4 with this initial acute exposure to IL-1β showing an increase in Tie-2 expression is seen in 5.2.1 with the 3hour value showing an increase and then decreasing at 6hour. The increase in Tie-2 during acute exposure would suggest cellular protection, however, the chronic exposure would indicate a reduction in Tie-2 regulated endothelial cell survival. With AKT reduction this further supports the notion of a reduction in cell survival as the PI3K/AKT pathway is vital in the role of apoptosis. With the exception of the anomaly seen at 12hours for AKT activity, the significant decreases in AKT phosphorylation add to the evidence for reduced Ang-1-induced Tie-2 signalling as a result of decreased expression of the receptor and signalling mediators. These results contradict earlier works by Bilimoria and Singh (2017), which found increases in the Tie-2 receptor expression and further increases in phosphorylation of AKT. The limited time points of that study may not have given a true representation of the impacts of IL-1β stimulation of HUVECs.

The expression of Tie-1 displayed significant decreases throughout the time course with all $p\text{-values} \leq 0.05$. Tie-1 serves its role as the Tie-2 regulator but is seen to have a pivotal

role in the integrity of the vasculature (Puri et al., 1995; Sato et al., 1995). The decrease in mature Tie-1 expression, being a fully glycosylated membrane protein, whereas an increase in the immature Tie-1 expression suggests that the Tie-1 receptor remains intracellularly being partially glycosylated (Marron et al., 2007). The significant decrease in the ratio of mature: immature could be suggestive of a mechanism of IL-1β stimulation which could inhibit the full glycosylation of Tie-1 and processing to cell surface. The reduction in Tie-1 has been reported to have beneficial impacts on tumorigenesis however can result in vascular dysfunction in atherosclerotic models (Woo et al., 2011; La Porta et al., 2018).

5.3.2 Implication of TNF-α on Ang-1/Tie Signalling

The exposure of TNF-α displayed mixed changes in the signalling mediation. The expression of Tie-2 was inverse to the trend displayed by IL-1β. The increase in Tie-2 expression suggests mechanism of increasing vascular integrity. Although a substantial decrease is seen at 12hours the lack in significance as a result of large standard error indicates this to be a potential anomaly however as all the time points do not display significance, the finding of Tie-2 expression as a result of TNF-α stimulation cannot be confidently concluded. In a similar study involving TNF-α stimulation up to 24hours, authors also reported an increase in Tie-2 expression over the time course (Singh et al., 2012).

Contrary to Tie-2 expression, expression of AKT is decreased as a consequence of TNF-α stimulations. The reduction in AKT would result in a decrease the resultant signal of the PI3K/AKT pathway hence reduce the vascular integrity and promote apoptosis of

endothelial cells. The increase seen at 6hours is considered an anomaly based on the large standard error. However, like with Tie-2, a lack of statistical significance across all time points provides great uncertainty over the data.

Although AKT expression lacked significance, results of phospho-AKT display result of interest that may support the decrease in AKT. Activity of AKT is the measure of phosphorylated AKT against the total AKT present. The level of AKT activity reduced over time with significance being report for all time points with exception of 3hours ($p \leq 0.05$). Based on the function of the PI3K/AKT being a promote of vessel integrity, the understanding around TNF-α pro-apoptotic functions would support the decrease seen in AKT activation (Domnina et al., 2004).

Like with the decrease in AKT expression and activity, expression of Tie-1 follows the same trend. Stimulations of TNF-α result in a reduction of Tie-1 expression. Specifically, the mature, fully glycosylated, surface receptor protein is seen to be reduced in expression over prolonged exposure to TNF-α. With statistical significance for all the time points, it can be stated that the decrease is significant in comparison to the control. In addition to the mature Tie-1 measurement, the immature Tie-1 was also measured. This immature form is intracellular and awaiting further glycosylation before translocating to the cell surface. The ratio of the two provides further evidence whether Tie-1 as a whole is being suppressed from expression or if mechanisms prevent the full processing of Tie-1 to cell surface. The findings of TNF-α stimulation on the ratio indicate a decrease in the mature: immature ratio with significance for 6hour, 24hour and 48hour. The large standard error for the 12hour time point gives reason to conclude it

as an anomaly. Blots displayed in a similar study showing the decrease in the mature Tie-1 support this study and on further inspection of the blots, a similar pattern can been seen for the immature receptor expression (Singh et al., 2012).

5.4 Conclusion

Chronic exposure of inflammatory cytokines on the Ang-1/Tie-2 has displayed results pertaining to the promotion of vascular instability and endothelial apoptosis. When viewing the acute date alongside the chronic data, it can be seen that the results support each other with the 2hour and 4hour time point from chapter 4 and the 3hour time point from this chapter. Both IL-1β and TNF-α lacked significance in the data sets, however, while the expression of AKT was both reduced for each cytokine the results of Tie-2 expression were opposite to each other with IL-1β displaying a decrease in expression and TNF-α showing an increase. With the reduction in AKT expression the same was seen in the protein's activity. Assessment of Tie-1 expression also indicated decrease in expression of mature receptor expression, however with the expression of immature Tie-1 the ratio between the two for both stimulatory cytokines show the incomplete glycosylation of the orphan receptor.

Further assessment of Tie-2 would need to be investigate along with the analysis of the phosphorylation of the receptor to attain a better understanding of where the changes in AKT are potentially being influenced. Additionally, it would prove beneficial to analyse the proteolytically cleaved Tie-1 protein to assess whether the reduction in Tie-1 can be associated with cleavage of the receptor ectodomain.

This chapter has highlighted the impact of chronic exposure to proinflammatory cytokines and has addressed the reduction in AKT signalling. Having understood the consequent of PI3K/AKT signalling to promote vascular integrity through cell survival, the implications of the cytokine would result in the apoptosis of endothelial cells. This could be assessed through the use of immunocytochemistry to assess cell viability through the analysis of cell apoptosis.

Chapter 6: Functional Study

6.1 Introduction

Endothelial cell integrity is crucial for vascular homeostasis and normal function of bodily organs. Injury or dysfunction is commonly associated with pathogenesis of different vascular diseases. While apoptosis is vital mechanism of development and homeostasis, the role of apoptosis will contribute to the detriment cause by conditions including cancer, atherosclerosis and also neurodegenerative disease (Winn and Harlan, 2005). Within atherosclerosis the apoptosis of smooth muscle cells and macrophages have been noted in the pathogenesis of the condition (Geng and Libby, 2002). It has also been seen that endothelial cells have been observed to apoptose in human atherosclerotic plaques (Tricot et al., 2000).

The process of atherosclerosis involves a complex network of cytokine and chemokine signalling. The release of the cytokines in atherosclerotic lesions have multiple function on cellular functions. TNF-α is a multifaceted cytokine, capable of activating cell survival or cellular apoptosis. Studies have shown this simultaneous mechanism where endothelial cells undergo apoptosis while vascular smooth muscle cells are promoted to proliferate (Rastogi et al., 2012). TNF super family induced apoptosis requires bind to its specific receptor to promote oligotrimerisation of the receptors (Madge and Pober, 2001; Chen et al., 2001; Choy et al., 2001). An aggregation of the death domain containing protein TRADD which in turn binds to FADD. The resultant consequence is the activation of procaspase-8 and apoptosis signal-regulating kinase 1 (ASK1) (Ichijo, 1998; Ashkenazi, 1998; Chen, 2002).

IL-1β is also a perpetuator of endothelial cell apoptosis. The pro-apoptotic nature of IL-1β was reported to induce apoptosis of endothelial cells to propagate diabetic retinopathy. Retinopathy is an ocular complication resultant of diabetes which involves the apoptosis of retinal capillary cells. Genes that promote transcription factors like NF-κB are expression through induction of IL-1β. This activation accelerates apoptosis and under hyperglycaemia, the results are exacerbated (Kowluru and Odenbach, 2004).

The exposure of cytokines has displayed changes in the signalling pathway of Ang/Tie. Reports have indicated changes in the pathway through chronic exposures of TNF-α causing increases in the Tie-1:Tie-1 ratio (Singh et al., 2012). Data from chapter 5 has presented evidence of these change with the addition of IL-1β induced action. The result saw a decrease in AKT phosphorylation which highlights a significant impact on the cellular action of endothelial cells, due to the PI3K/AKT cascade to have significant role in endothelial cell survival. Following this assessment, the AKT reduction was then assessed with the use of immunocytochemistry techniques to analyse the extent of apoptosis cause by cytokine stimulation. HUVECs were stimulated with either TNF-α or IL-1β for a set time period before being subject to the LIVE/DEAD cell assay (see 2.1.1 – 11). Analysis of fluorescent images would indicate the extent of Ang-1 induced cell survival when HUVECs are exposed to the cytokines over varying lengths of time.

6.2 Results

6.2.1 Chronic Impact of IL-1β Stimulation on Ang-1-induced Cell Survival

In order to measure the impacts of IL-1β on Ang-1-induced cell survival, HUVECs were exposed to Ang-1in the presence or absence of IL-1β over four time points (0hr, 6hr, 12hr and 24hr). Time points were cut off at 24hours as results from chapter 5 indicated a plateau in expression of Tie-2 and Tie-1 as well as the activity and expression of AKT, hence did not warrant the need to assess further time points beyond 24hours. Initial experimentation using the manufacturers recommendations relayed unusable data as conditions resulted in complete cell death in all samples as described in 2.5.1. The revised protocol as described in 2.5.2 and 2.5.3 was the method used for this experiment. Post-stimulation, cells were subjected to the LIVE/DEAD assay (see 2.1.1 – 11). After incubation, the cells were viewed using fluorescent microscopy to capture digital images of green fluoresced cells (GFP) and red fluoresced cells (RFP). Three random section of each sample well was imaged to obtain an average live and dead percentage for each sample. Each sample set was repeated in triplicated however data for the third repeat was discarded as a result of infection causing obscure results.

As expected from Figure 44, Figure 45, Figure 46 and Figure 47, the fluorescent images displayed over time an increase in the number of dead cells exposed to IL-1β, more so apparent at 12hour and 24hour. Figure 48 displays the average dead cell percentages with the trend also showing increased cell death. Against the control samples, Ang-1

appears to show reduced cell death after 6hour up until 24hour stimulation, however contrary to this, with the addition of IL-1β, there is an increase in the number of dead cells. The *p-value* obtained from a Two-Way ANOVA ($p = 0.007$) gives statistical significance in the variance in number of dead cells as time of exposure is increased in each treatment groups. However, $p > 0.05$ from the ANOVA test found that there was no significance in the variance of the results between each treatment group.

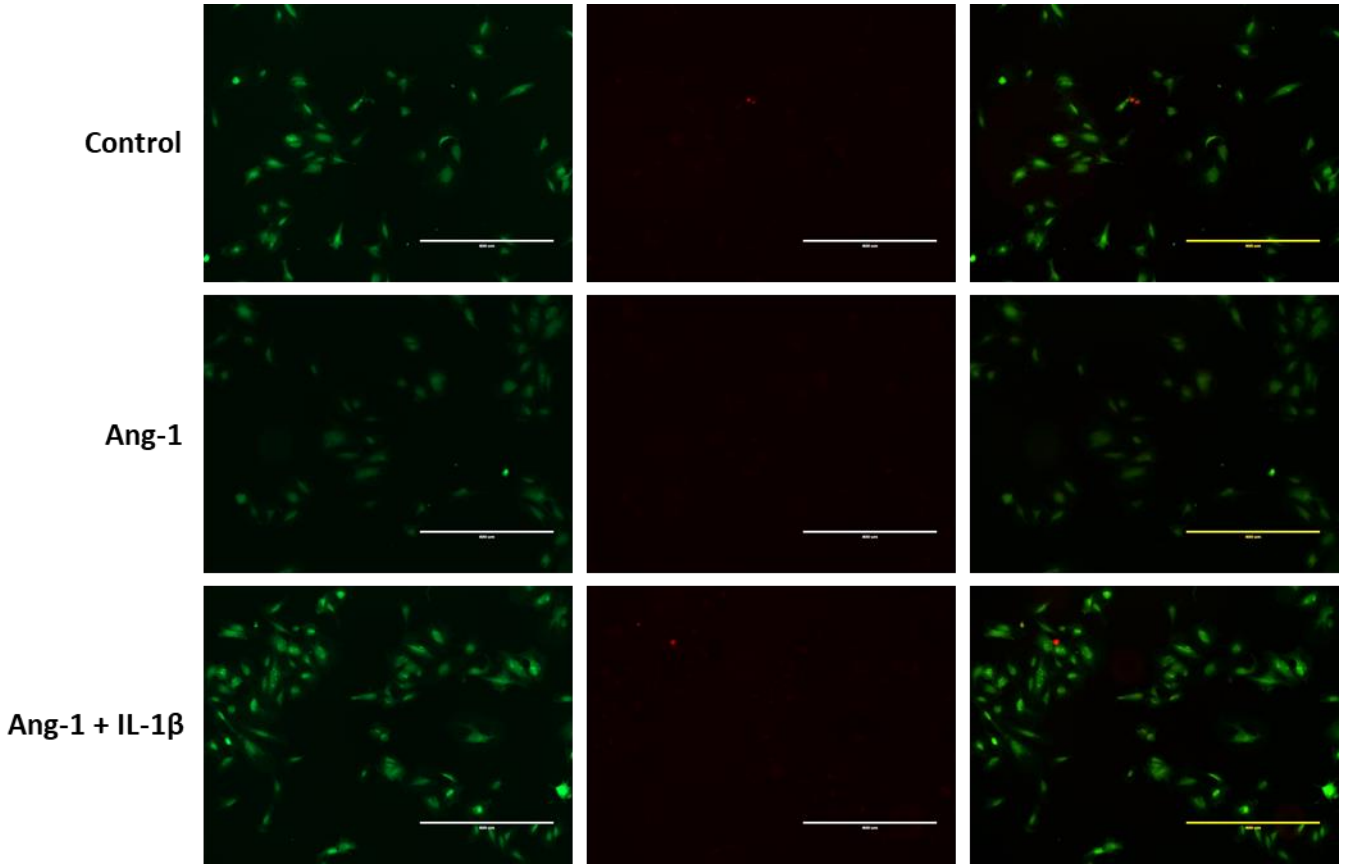


Figure 44. Fluorescent images of HUVECs at 0hour showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 before subjection to the Live/Dead assay. Ang-1 + IL-1β – HUVECs were stimulated with Ang-1 for 15minutes before adding IL-1β (25ng/ml) and immediately terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

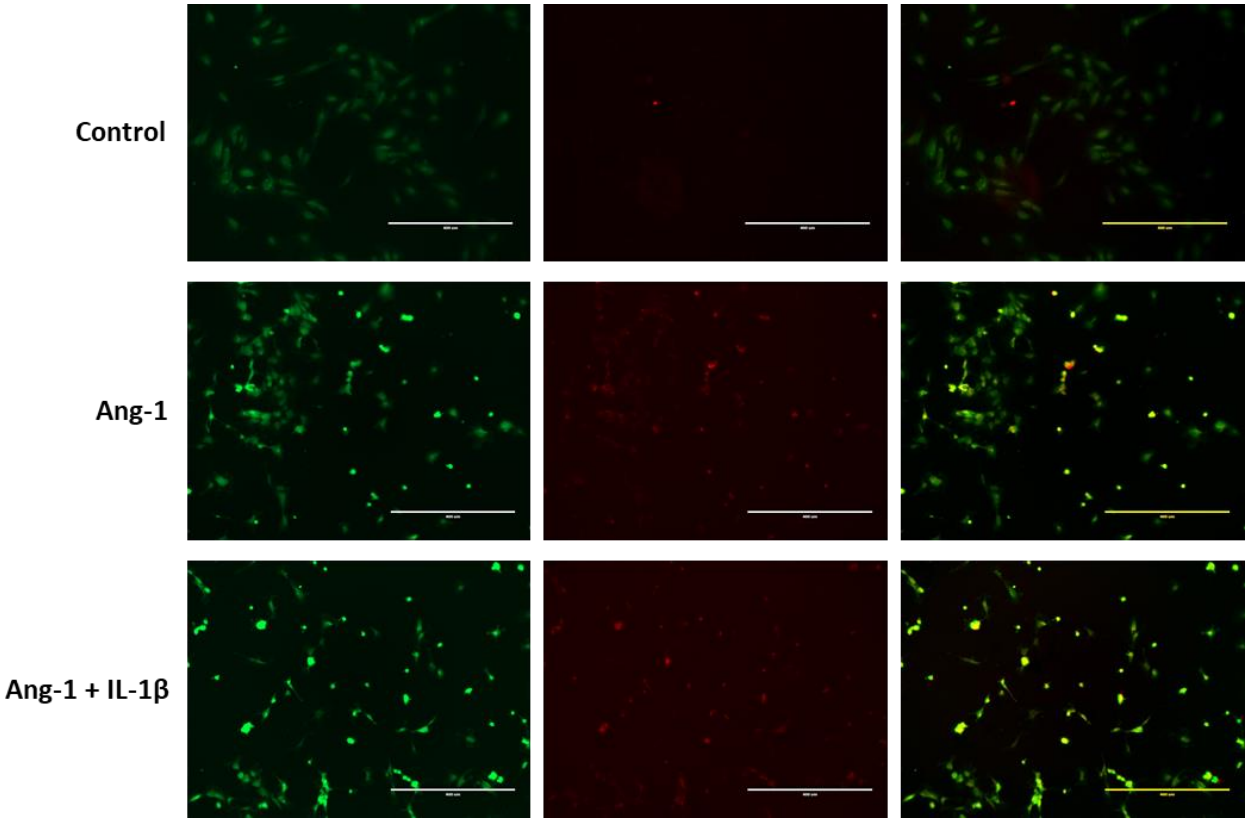


Figure 45. Fluorescent images of HUVECs at 6hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 6hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 5hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + IL-1β – HUVECs were stimulated with IL-1β (25ng/ml) for 5hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

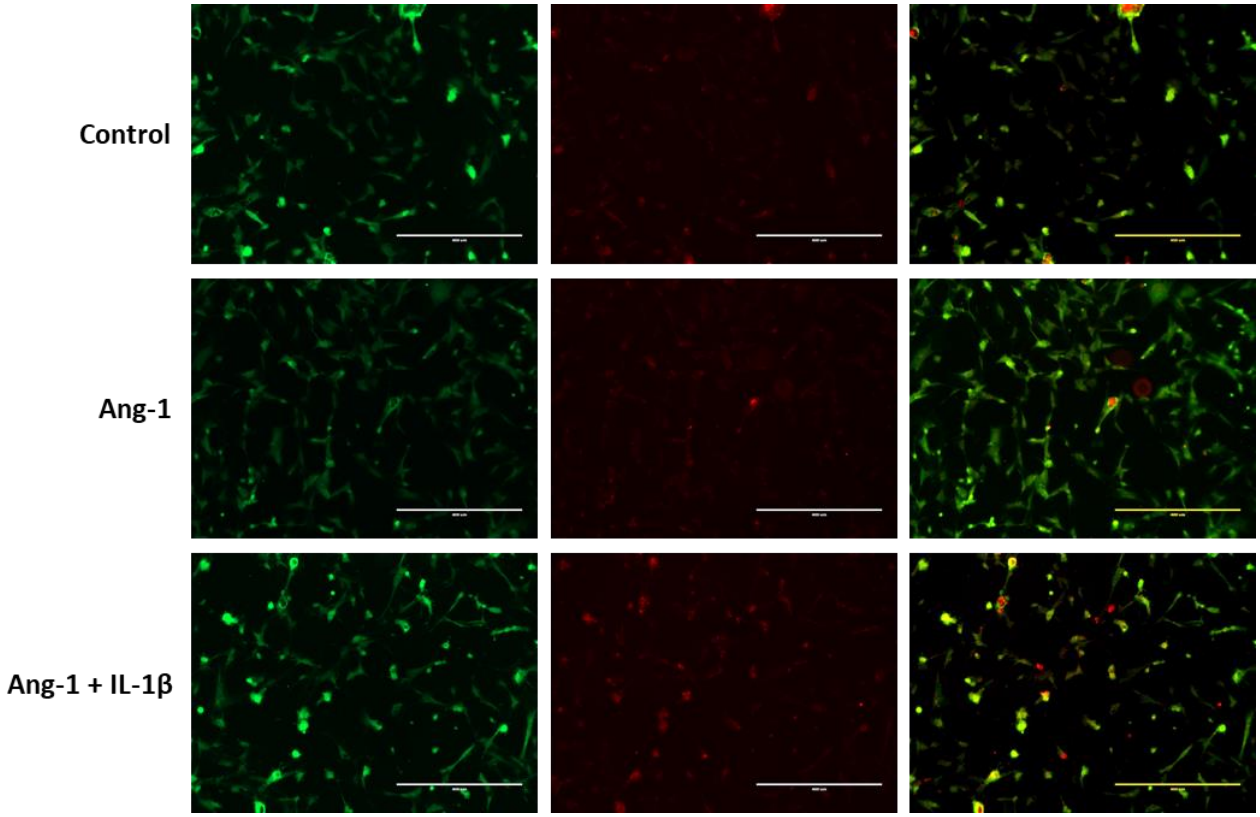


Figure 46. Fluorescent images of HUVECs at 12hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 12hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 11hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + IL-1β – HUVECs were stimulated with IL-1β (25ng/ml) for 11hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

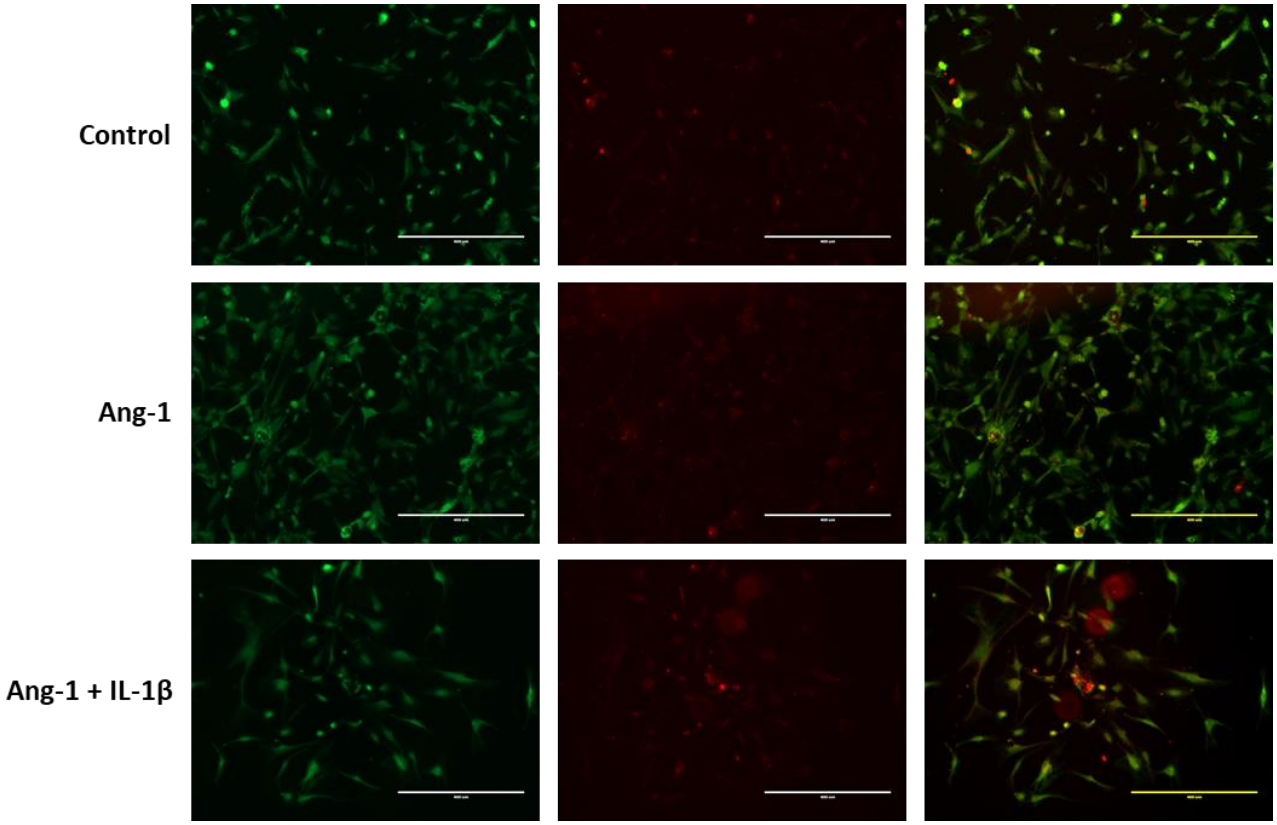


Figure 47. Fluorescent images of HUVECs at 24hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 24hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 23hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + IL-1β – HUVECs were stimulated with IL-1β (25ng/ml) for 23hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

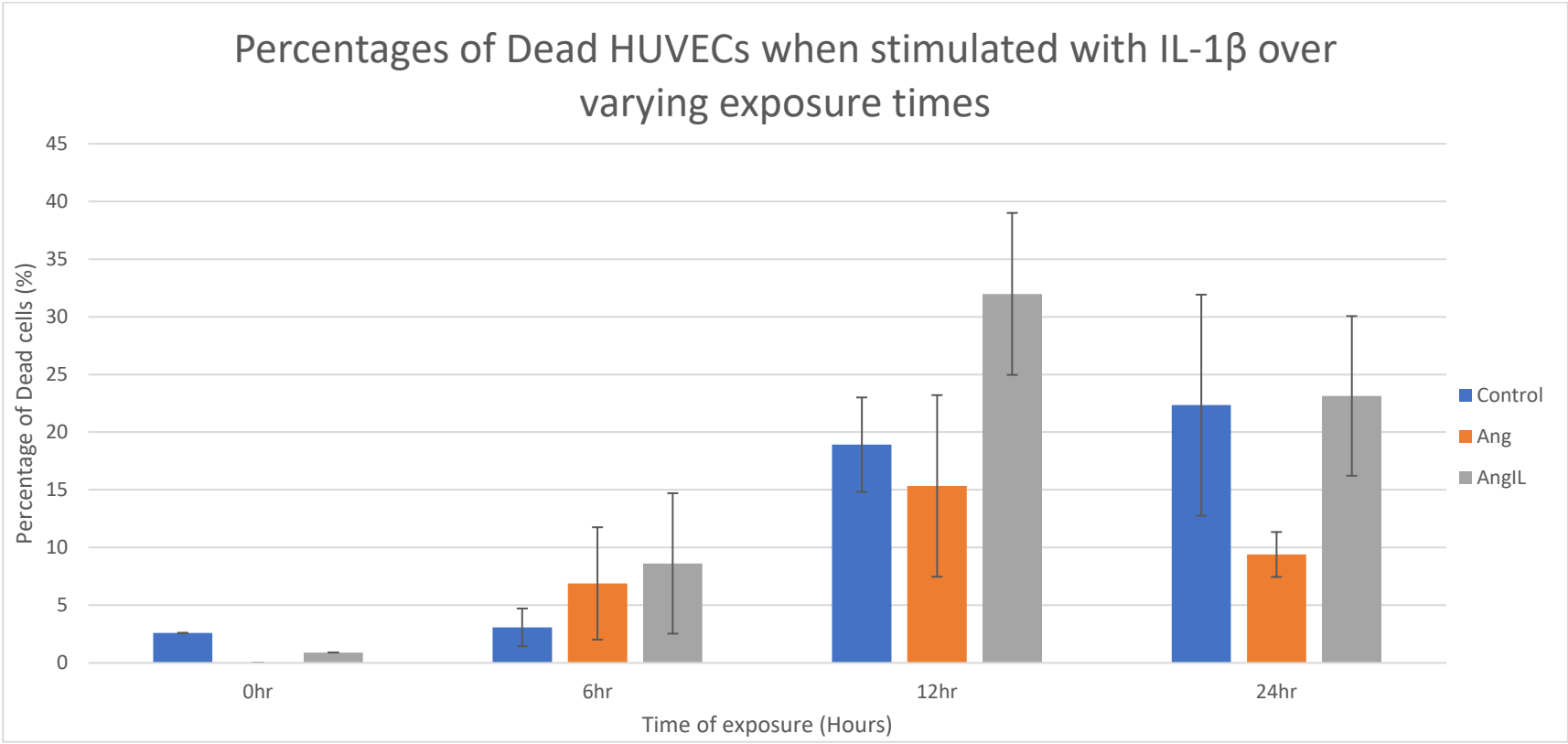


Figure 48. Graph indicating the percentage of dead cells at different IL-1β exposure times. *Cell counts were conducted of the live cell fluorescent images as well as the dead cell fluorescent images. A percentage of dead cells in each sample was calculated. Each sample was analysed in three random sections of the well, all three were averaged for each experiment. The plotted results are an average of two independent repeats of the experiment and are presented as the percentage of live cells of the total cells in each sample. SEM bars indicated for each sample group.*

6.2.2 Chronic Impact of TNF-α Stimulation on Ang-1-induced Cell Survival

In order to measure the impact of TNF-α on Ang-1-induced cell survival, HUVECs were exposed to Ang-1 in the presence or absence of TNF-α over four time points (0hr, 6hr, 12hr and 24hr). The protocol as described in 2.5.2 and 2.5.3 was the method used to conduct this experiment. Post stimulation, cells were subjected to the LIVE/DEAD assay (see 2.1.1 – 11). After incubation, the cells were viewed using fluorescent microscopy to capture digital images of green fluoresced cells (GFP) and red fluoresced cells (RFP). Three random section of each sample well was imaged to obtain an average live and dead percentage for each sample. Each sample set was repeated in triplicated however data for the third repeat was discarded as a result of infection causing obscure results.

Figure 49, Figure 50, Figure 51 and Figure 52 show images of cells exposed with Ang-1 in the absence or presence of TNF-α at the time points 0hour, 6hour, 12hour and 24hour, respectively. Figure 53 shows average percentage of each sample with a trend showing inconsistent patterns between the different time points. While against the control, TNF-α stimulations showed increased HUVEC apoptosis over the time course, Ang-1 treated cells also displayed increase in cell death compared to the control. The variance in the number of dead cells over the various time points was found to be statistically significant through ANOVA testing with a *p-value = 0.038*. The variation between the different treatment groups did not amount to values of significance with $p > 0.05$.

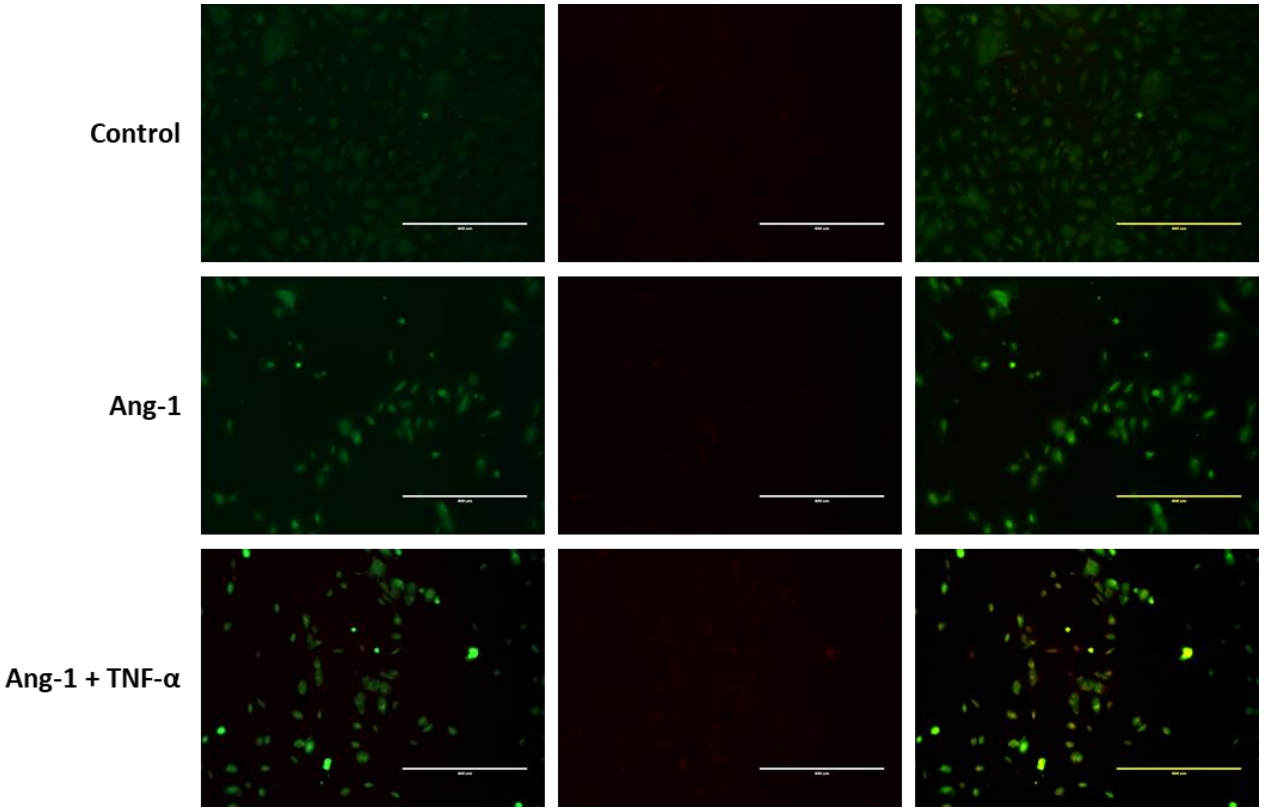


Figure 49. Fluorescent images of HUVECs at 0hour showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 before subjection to the Live/Dead assay. Ang-1 + TNF-α – HUVECs were stimulated with Ang-1 for 15minutes before adding TNF-α (25ng/ml) and immediately terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

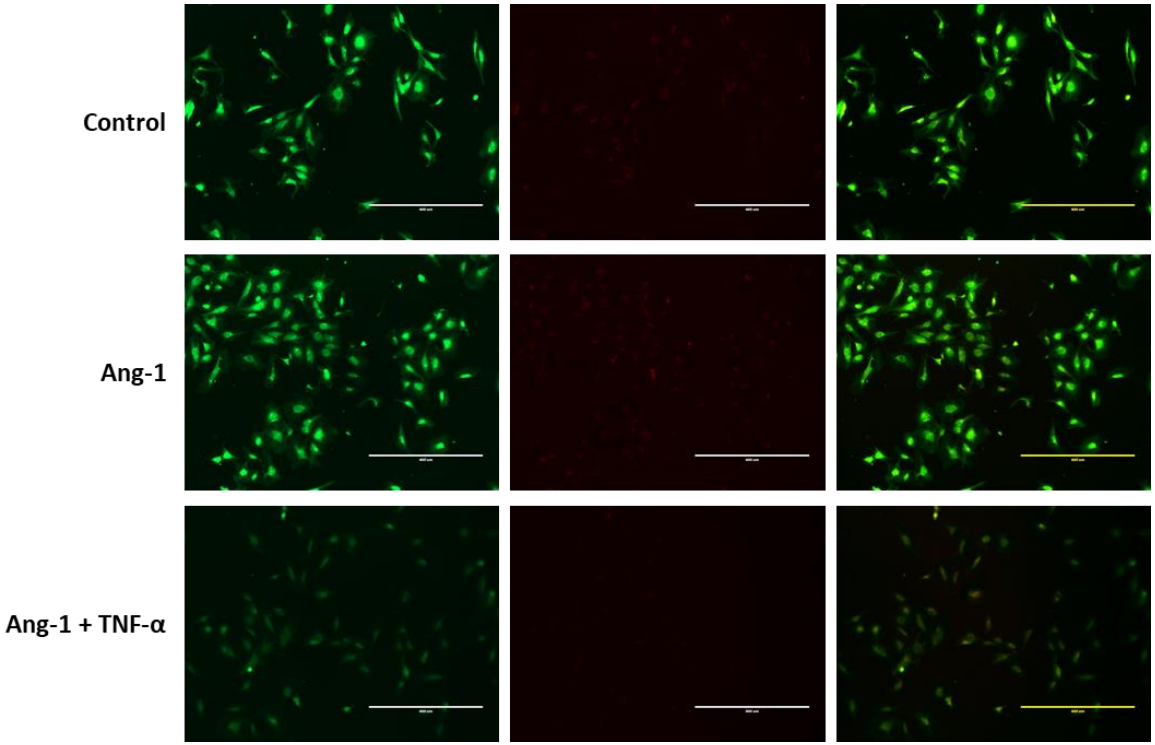


Figure 50. Fluorescent images of HUVECs at 6hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 6hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 5hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + TNF-α – HUVECs were stimulated with TNF-α (25ng/ml) for 5hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

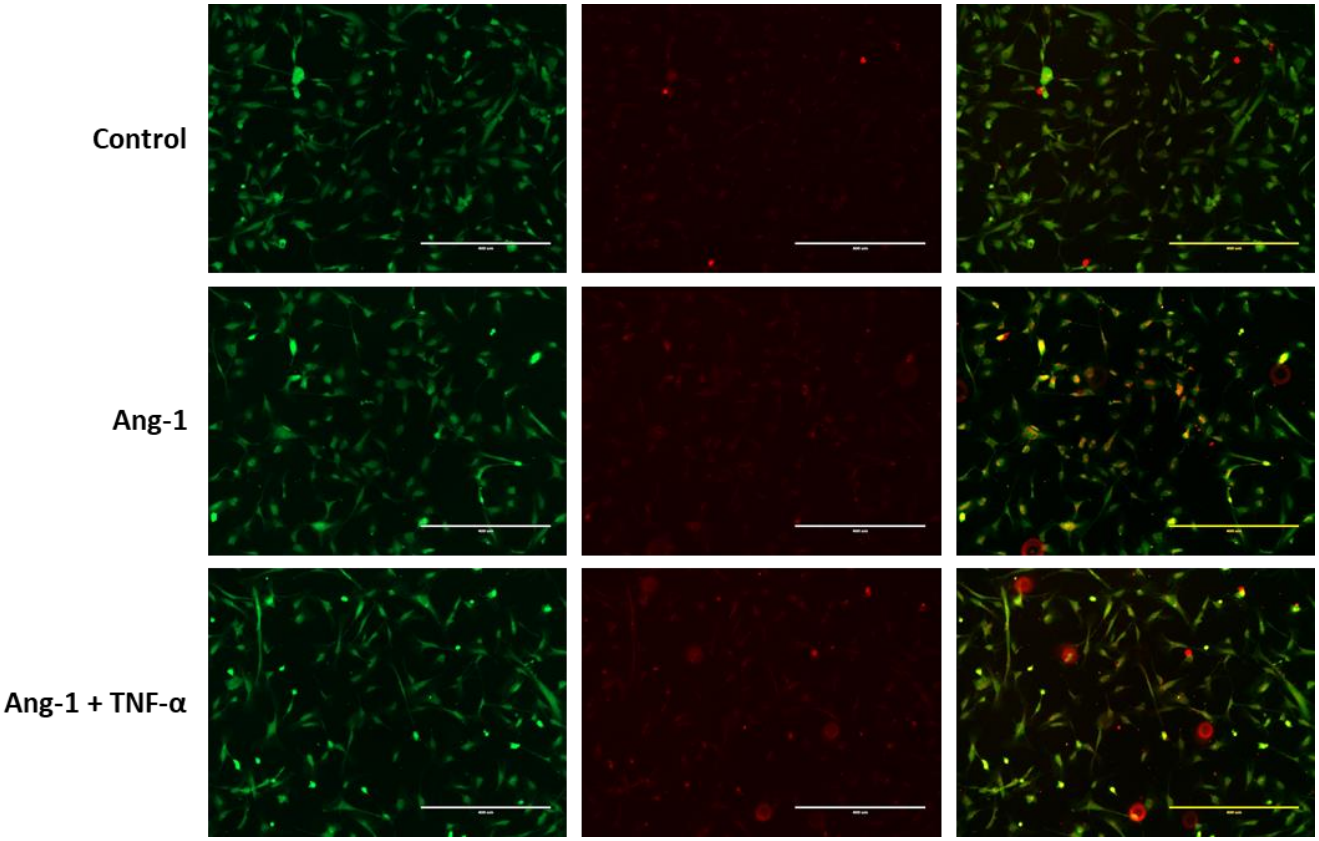


Figure 51. Fluorescent images of HUVECs at 12hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 12hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 11hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + TNF-α – HUVECs were stimulated with TNF-α (25ng/ml) for 11hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

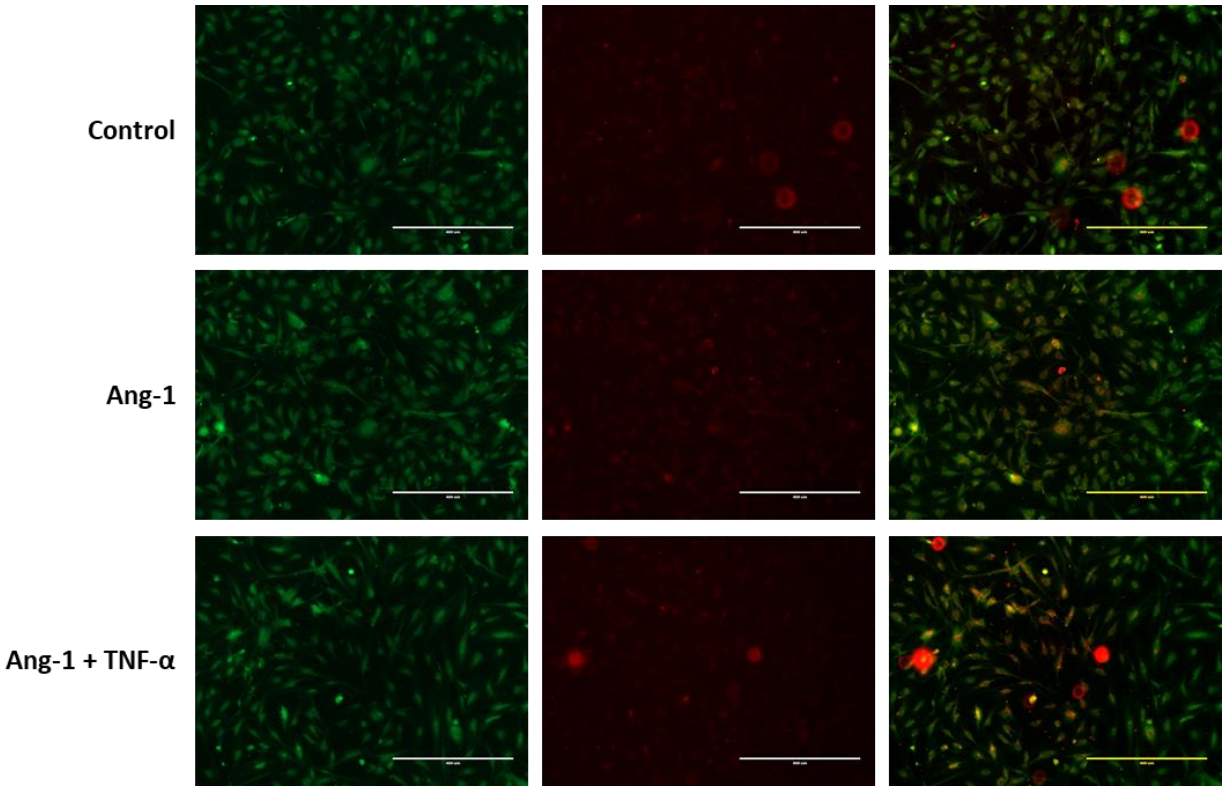


Figure 52. Fluorescent images of HUVECs at 24hours showing Live, Dead and Overlay. *Control – HUVECs were only subjected to the Live/Dead assay 24hours incubation without stimuli. Ang-1 – Cells were stimulated for 15minutes with recombinant Ang-1 after incubation for 23hours and 45minutes without stimuli before subjection to the Live/Dead assay. Ang-1 + TNF-α – HUVECs were stimulated with TNF-α (25ng/ml) for 23hours 45 minutes, then Ang-1 for 15minutes before terminating the stimulation and continuing with the Live/Dead assay. All images show a scale of 400µm.*

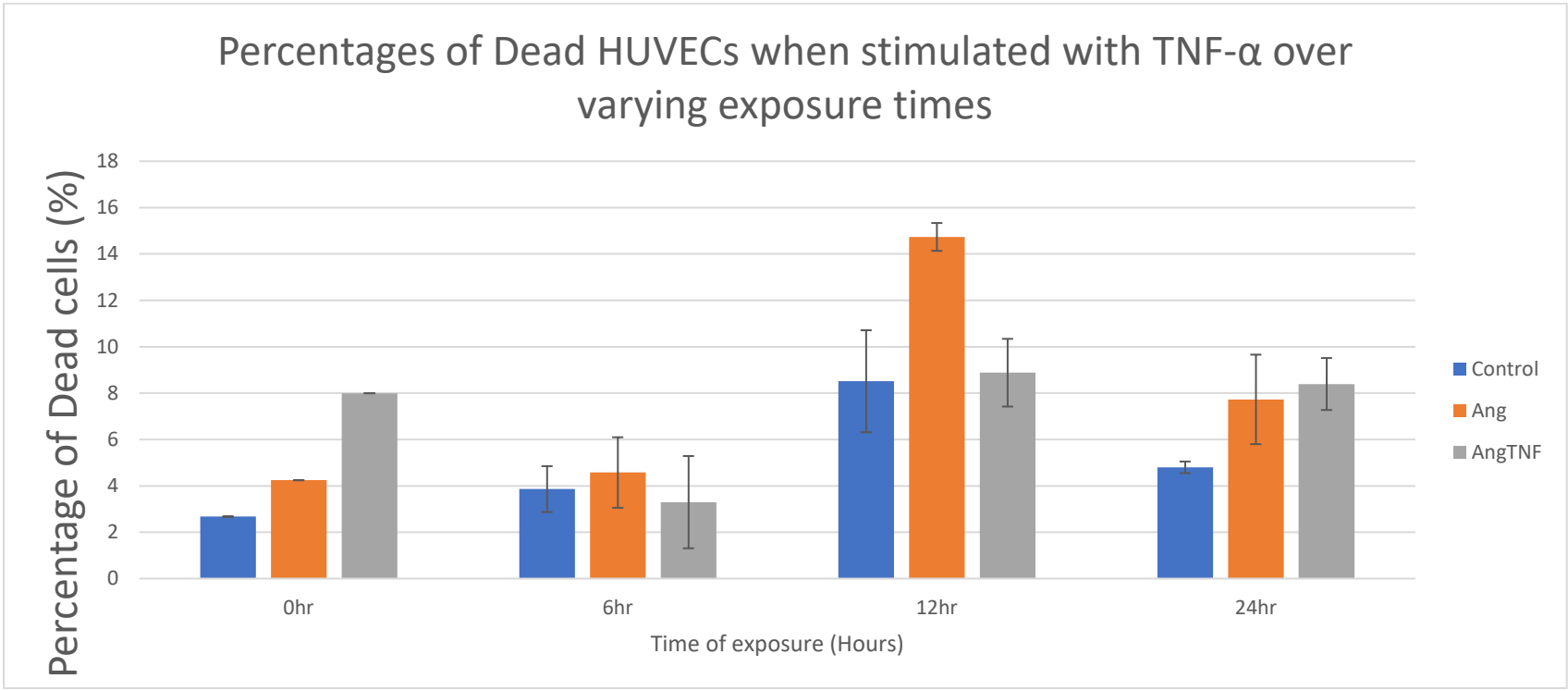


Figure 53. Graph indicating the percentage of dead cells at different TNF-α exposure times. *Cell counts were conducted of the live cell fluorescent images as well as the dead cell fluorescent images. A percentage of dead cells in each sample was calculated. Each sample was analysed in three random sections of the well, all three were averaged for each experiment. The plotted results are an average of two independent repeats of the experiment and are presented as the percentage of live cells of the total cells in each sample. SEM bars indicated for each sample set.*

6.3 Discussion

6.3.1 Implication of IL-1β Stimulation on Cell Survival

The results of IL-1β stimulation on HUVECs gave evidence of increased cellular apoptosis over prolonged incubation with the proinflammatory cytokine. Though significance in results was not seen between the sample types, a pattern was seen with Ang-1 displaying cellular protection over the longer time points compared with the control. The addition of IL-1β resulted in increased apoptosis compared with Ang-1 only stimulation. From this it can been seen that the function of Ang-1 is impacted negatively over chronic exposures to IL-1β which compliments the data observed in chapter 5, where Ang-1-induced AKT activity was significantly reduced under IL-1β conditions. The reduction in protein expression and signalling activity of Ang/Tie pathway, with focus on the PI3K/AKT cascade, is indictive of mechanisms of cell instability and apoptosis. The complimentary pattern supports evidence of IL-1β pro-inflammatory mechanism of disruption to vessel stability and apoptosis of endothelial cells (Kowluru and Odenbach, 2004).

6.3.2 Implication of TNF-α Stimulation on Cell Survival

TNF-α stimulations were seen to have inconsistent albeit instant implications to the number of cells that underwent apoptosis over the time course. The inconsistency in Ang-1 only results may indicate errors in experimentation as the expected impact of Ang-1 action is to promote cell stability and survival (Brindle, Saharinen and Alitalo,

2006). Over time course increases in cell death were seen among all samples and variance between the time points was significant with ANOVA test displaying $p = 0.038$. This could be a result of the recombinant Ang-1 not being active or the cells had experienced stress prior to the experimentation. This could be due to the force of rinsing the samples pre-stimulation, though care was taken to avoid this, the sensitive nature of HUVECs makes them vulnerable to mechanical stresses. Due to the inconsistency with the data, the experiment did not support the observations mad in chapter 5 where chronic TNF-α reduced Ang-1 AKT activity in HUVECs.

6.4 Conclusion

Assessment of the functional implications of cytokine stimulations further supports the impacts on cell signalling. Changes in signalling mechanisms will ultimately affect the functions in the cell unless additional mechanisms act on functionality. Over the time course, especially of IL-1β, more so at 12hour and 24hour, displayed increases in cell death which supports the evidence provided in chapter 5. The plateaued changes in Tie receptor expression and AKT signalling is suggestive of endothelial cell instability due to chronic exposure of cytokines, hence providing insight into the impacts of certain chronic diseases.

The results having lacked significance suggests further repeats would need to be conducted to add strength to the findings. The exposure of cytokines has impacts on many aspects of signalling, hence further functional assays could be conducted to gain a wider scope on cell functionality. The PI3K/AKT pathway also serves a role in vessel

remodelling, which can be assessed through tube formation analysis observing cells in a 3D-Matrix gel.

Chapter 7: Final Conclusion

7.1 Experimental Summary

The main focus of the research has been to assess the impacts of pro-inflammatory cytokines linked with diabetes on the signalling of the Ang-1/Tie signalling pathway. This study focused on the level of cytokines and vascular-associated Ang-2 in patient serum of diabetic individuals to understand the profile of these proteins. With the use of RANDOX multiplex ELISA, IL-6 and TNF-α profiles were analysed from serum samples through the use of this advanced ELISA technique. IL-6 displayed increases in the serum levels in both type 1 and type 2 diabetic patients, however the lack in significance from the non-parametric statistical tool, Friedman test, left the cytokine profile inconclusive. Additionally, TNF-α displayed a decrease in serum concentrations in type 1 diabetics, but an increase in type 2 diabetics, however the Friedman analysis also returned no significance in the data. Ang-2 data through standard sandwich ELISA of the patients presented statistically significant differences in serum concentrations between the volunteer groups. Type 1 diabetics were seen to have lower levels of Ang-2 compared to the control. The opposite was seen in type 2 diabetics with an increase in Ang-2 levels. Elevations in Ang-2 are noted to have regulatory mechanisms for Ang-1/Tie-2 signalling.

The diabetic condition is a chronic disease with lasting effects. With cytokines playing a vital role in the propagation and development of the disease it is important to understand the acute implications on the Tie receptor signalling before observing the chronic impacts. Acute inflammatory response is the process of an individual having the ability to fight infections. IL-1β and TNF-α impacts were examined to assess the impact

on the Tie2 signalling pathway. Techniques of HUVEC cell culture and western blotting allowed for the analysis of Tie2 levels and activity along with activity of AKT. The levels of Tie2 were found to be elevated during the short exposure to IL-1β. While the findings did not display statistical significance, similar findings were reported by Singh *et al* (2012) with TNF-α stimulations during acute exposure. The activity of Tie2 through phospho-Tie2 analysis presents results showing acute increases in Tie2 phosphorylation in response to IL-1β and TNF-α. The findings from AKT activity had opposite results between IL-1β and TNF-α. IL-1β presented results of increasing AKT activity whereas TNF-α showed a decrease in activity of AKT. The overall finding of acute cytokine exposure displays tangible implications to the Ang/Tie signalling pathway.

The results of acute exposure provided insights into changes in receptor functionality which added to the exploration of the chronic impacts on cytokine exposures to understand the relevance to prolonged inflammation and how conditions like diabetes impacts on the vasculature. Within this study the addition of Tie1 analysis was pertinent as it serves a vital role in the regulation of Ang/Tie signalling. The results of chronic inflammatory cytokine exposure gave rise to evidence highlighting the potential instabilities in vascular structures and apoptosis of endothelial cells. Protein expression of Tie2 and AKT returned inconclusive data however findings from AKT activity and Tie1 receptor levels contain statistically significant data. Mature Tie1 levels displayed significant decreases in expression along with the ratio of mature receptors to immature receptors. The importance of the immature Tie1 receptor is suggestive that Tie1 is being prevented from complete glycosylation to traffic to the cellular membrane. Activity of AKT was also influenced with significant decreases over the time course. The effects of

chronic exposure to cytokines are suggestive of a prolonged reduction in vascular protection leading to functional detriments to endothelial cells related to inflammatory related diseases.

With evidence of Ang/Tie signalling being influenced as a result of cytokine stimulation, the effects would be apparent through functional analysis of HUVECs. AKT data previously displaying decreased activity would indicate a decrease in cell survival through the PI3K/AKT cascade. The evidence suggest increases cell apoptosis over continued exposure to IL-1β and TNF-α. Using techniques of immunocytochemistry, analysis of HUVEC apoptosis was conducted with the fluorescent tagging of Calcein, a marker of live cells, and an ethidium dimer to highlight the dead cells or those undergoing apoptosis. While results did not present significance, the images captured did display increased cell death the longer the cells were exposed to either IL-1β or TNF-α. This supports the findings of AKT activity reduction and therefore enhance the evidence suggesting pro-inflammatory cytokines increase cell instability and apoptosis which would escalate to the dysfunction of the vasculature resulting from chronic inflammation as a result of conditions like diabetes.

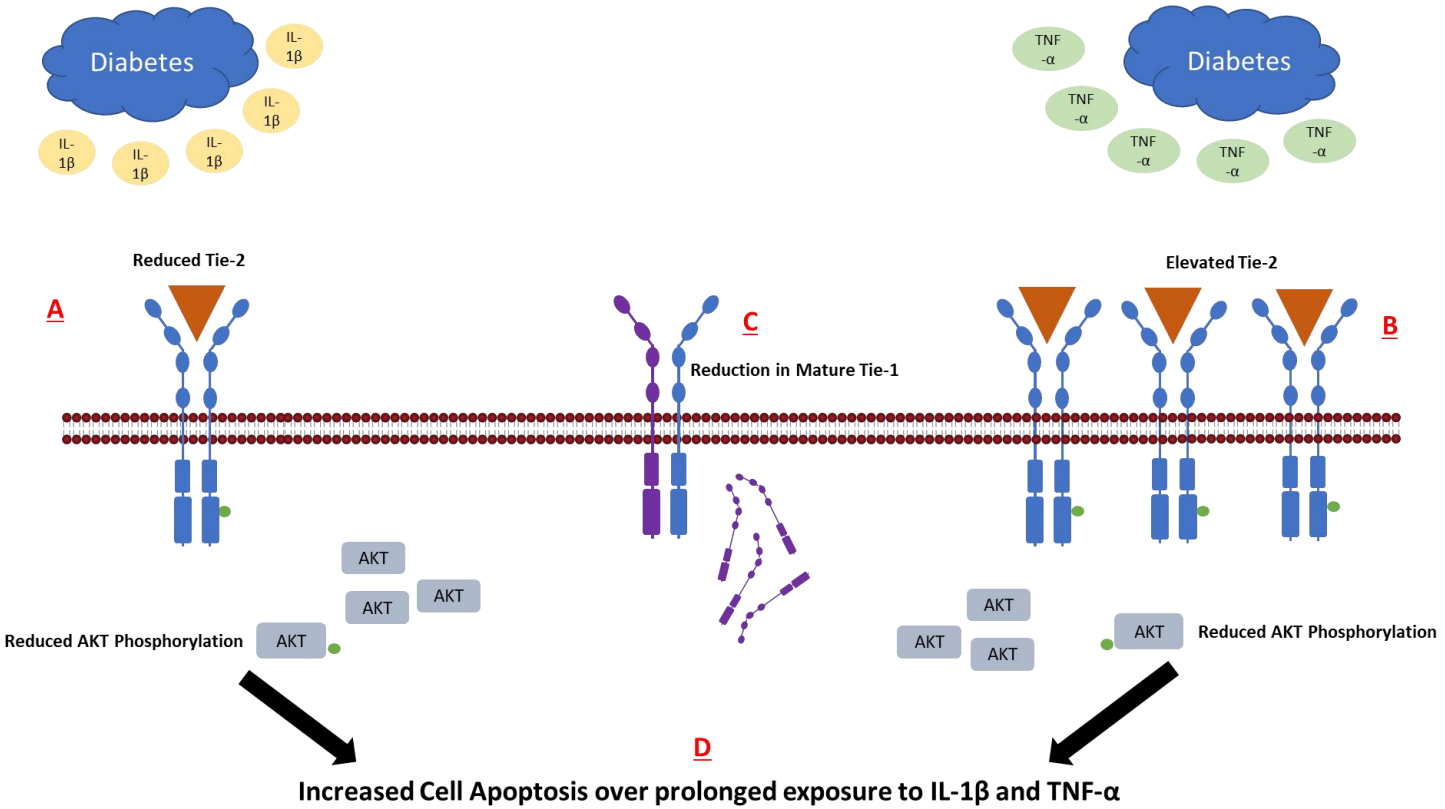


Figure 54. Graphical summary of the findings from this Thesis. *This diagram highlights the main findings from the experimental study of the project. A: In the presence of IL-1ß, levels of Tie-2 were found to be reduced. B: In the presence of TNF-α, levels of Tie-2 were seen to be increased. C: In both IL-1ß and TNF-α exposure, mature levels of Tie-1 were reduced with the ratio with immature intracellular Tie-1 decreasing. D: Increased cell apoptosis was seen over time in the stimulated groups as a result of decreased AKT phosphorylation.*

7.2 Limitations and Future Work

Limitations of this study included mainly incomplete data due to complications experienced in the laboratory. This was largely part of chapter 4 and 5. HUVECs are a well-known primary cell line, however working with this cell line over the course of the study the cells were found to be highly sensitive to changes and required specialised media in order for growth and maintenance. With various cell culture issues, maintenance of cells proved to be problematic, as did experimentation. In order to add to the study and strengthen the findings, repeats would need to be conducted to increase reliability and decrease error attained in the data. This was planned however due to impacts of COVID-19, it was not possible to return to the laboratory to complete this in the time frame of the PhD. The serum analysis from chapter 3 would have benefitted from a larger sample size and to have equal populates in each group so not to have a skew in the data.

This thesis provides a solid foundation in highlighting the impacts of inflammatory cytokine on endothelial cells. There are a number of areas that could be targeted for future work. The profiling of patient serum was just a small part of the numerous markers, cytokines, and protein levels measurement. Soluble Tie2 (sTie2) is known as a ligand trap and is responsible for the reduction in Ang-1 binding to cell membrane Tie2. Studies have indicated this to be apparent and hence would provide additional support to the data (Alawo et al., 2017). During this project, sTie2 analysis was conducted using sandwich ELISA, however due to calibration error in the standard curve, the results could not be analysed. Funding limitations inevitably meant the experiment could not be

repeated. As the signalling pathways controls many function of endothelial cells, additional functional assays could be performed to further highlight and provide further evidence of cytokine impacts. A tube formation assay was planned, and preliminary experimentation was conducted with an aim to investigate the impacts of TNF-α exposure over varying time point and measuring the implication on endothelial cell tube formation in a 3D-gel matrix. Due to funding and pressures on time this was not continued as results from that project suggested additional optimisations to be conducted. While this study looked at cellular function and protein levels, it would have been greatly supported by exploring the RNA expression data to give context to whether the changes in protein level are occurring at RNA level. This was part of the original plan for this thesis, with RNA samples from stimulated HUVECs having already been collected, however COVID-19 set in motion a series of lockdowns and samples remain preserved for future RT-PCR experimentation.

7.3 Final Summary

In summary this thesis has presented significant novel evidence of Ang/Tie signalling activity changes as a result of IL-1β and TNF-α exposures over prolonged periods of time demonstrating the detrimental effects of inflammatory mediators associated with Diabetes. Understanding the profile of these cytokines and vascular associated Ang-2 highlights the potential changes occurring in diabetic patient. The changes in the signalling mediation of the Ang/Tie pathway demonstrate the chronic impacts of pro-inflammatory cytokines. The modelling of cytokine impacts *in vitro* allows for a greater understanding of the mechanisms involved and can provide evidence for potential

targets of novel therapies or biomarkers of certain conditions. Highlighting the functional impacts provides a scope on the effect of chronic inflammation on the vasculature.

Table 2. Highlight of the key finding from each of the experimental chapters.

Chapter	Title	Findings
3	Profiling of Cytokines and Angiopoietin-2 in Diabetic Patient Samples.	• Indication of cytokine changes in Type 1 Diabetic and Type 2 Diabetic. • Ang-2 levels seen to increase in Type 2 Diabetics, Ang-2 decrease in Type 1 Diabetics.
4	Acute Cytokine Impact on Ang-1/Tie-2 Signalling.	• Suggestion of acute expose to IL-1β and TNF-α cause elevations in Tie-2 levels as well as activity of the receptor.
5	Chronic Cytokine Exposure on Endothelial Cells.	• No significant impact on Tie-2 levels from chronic cytokine exposure. • Evidence showing decreases in AKT with a subsequent significant decrease in AKT activity. • Significant data to show a decrease in Tie-1 receptor levels as well as the ratio between the mature and immature forms of the receptor.

6	Functional Study.	• The protective nature of Ang-1 is reduced in endothelial cells when exposed to IL-1β, therefore increasing cell apoptosis. • TNF-α showed increases in cell death over time but were not significant in relation to Ang-1.

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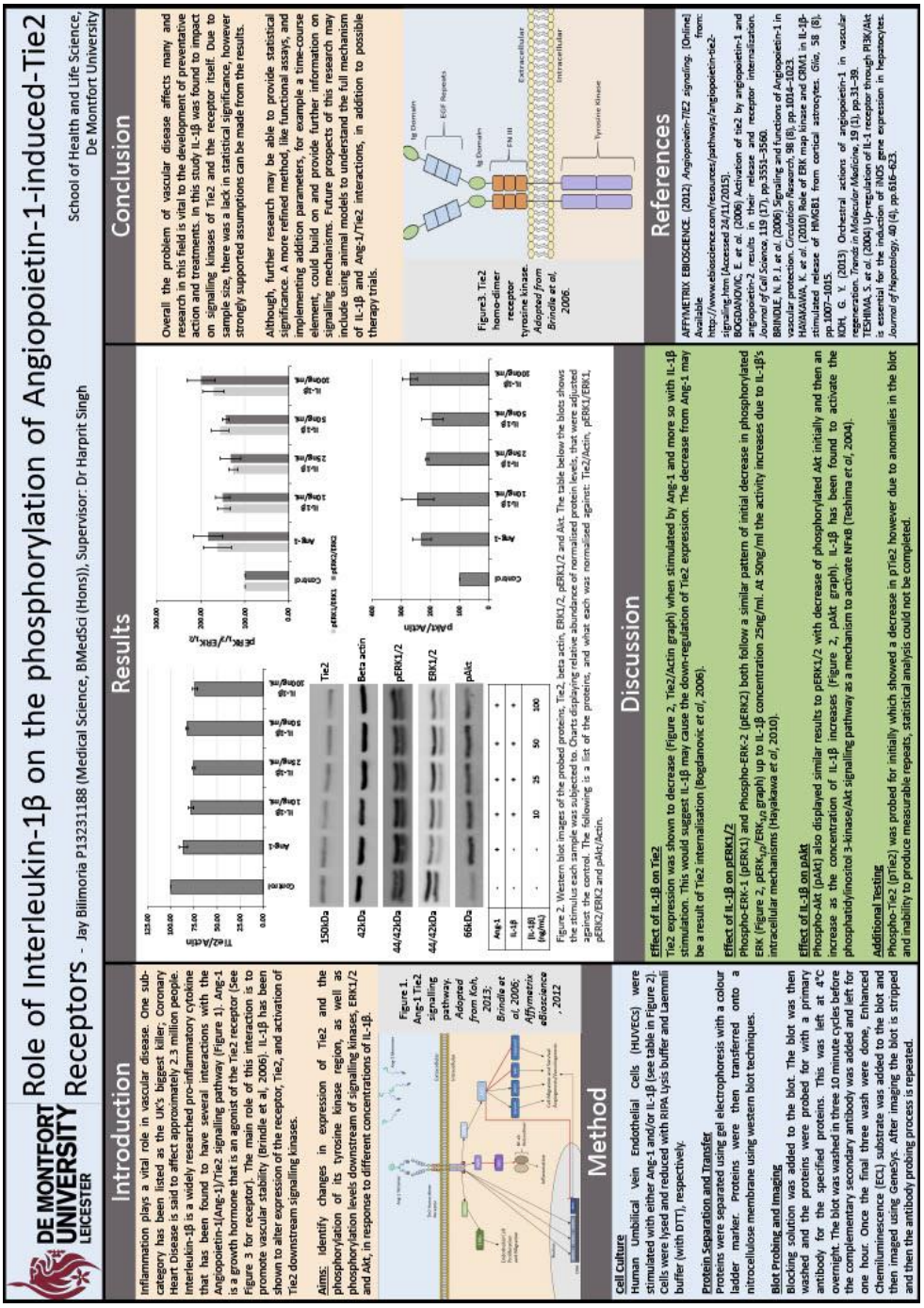
Appendices

Appendix 1 – Preliminary Study Optimising IL-β concentrations.


DE MONTFORT UNIVERSITY LEICESTER
Role of Interleukin-1β on the phosphorylation of Angiopoietin-1-induced-Tie2 Receptors
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Introduction
Inflammation plays a vital role in vascular disease. One sub-category has been listed as the UK's biggest killer; Coronary Heart Disease is said to affect approximately 2.3 million people. Interleukin-1β is a widely researched pro-inflammatory cytokine that has been found to have several interactions with the Angiopoietin-1(Ang-1)/Tie2 signalling pathway (Figure 1). Ang-1 is a growth hormone that is an agonist of the Tie2 receptor (See Figure 3 for receptor). The main role of this interaction is to promote vascular stability (Brindle et al, 2006). IL-1β has been shown to alter expression of the receptor, Tie2, and activation of Tie2 downstream signalling kinases.
Aims: Identify changes in expression of Tie2 and the phosphorylation of its tyrosine kinase region, as well as phosphorylation levels downstream of signalling kinases, ERK1/2 and Akt, in response to different concentrations of IL-1β.
Figure 1. Ang-1 Tie2 signalling pathway. Adopted from Koh, 2013; Brindle et al, 2006; Affymetrix eBioscience, 2012
Method
Cell Culture
Human Umbilical Vein Endothelial Cells (HUVECs) were stimulated with either Ang-1 and/or IL-1β (see table in Figure 2). Cells were lysed and reduced with RIPA lysis buffer and Laemmli buffer (with DTT), respectively.
Protein Separation and Transfer
Proteins were separated using gel electrophoresis with a colour ladder marker. Proteins were then transferred onto a nitrocellulose membrane using western blot techniques.
Blot Probing and Imaging
Blocking solution was added to the blot. The blot was then washed and the proteins were probed for with a primary antibody for the specified proteins. This was left at 4°C overnight. The blot was washed in three 10 minute cycles before the complementary secondary antibody was added and left for one hour. Once the final three wash were done, Enhanced Chemiluminescence (ECL) substrate was added to the blot and then imaged using GeneSys. After imaging the blot is stripped and then the antibody probing process is repeated.
Results
Tie2/Actin
PERK1/2/ERK1/2
Control
Ang-1
IL-1β 10ng/ml
IL-1β 25ng/ml
IL-1β 50ng/ml
IL-1β 100ng/ml
Tie2
Beta actin
150kDa
42kDa
pERK1/2
ERK1/2
44/42kDa
pAkt/Actin
pAkt
66kDa
Ang1
IL-1β
IL-1β (ng/mL)
10
25
50
100
Figure 2. Western blot images of the probed proteins, Tie2, beta actin, ERK1/2, pERK1/2 and Akt. The table below the blots shows the stimuli each sample was subjected to. Charts displaying relative abundance of normalised protein levels that were adjusted against the control. The following is a list of the proteins, and what each was normalised against: Tie2/Actin, pERK1/ERK1, pERK2/ERK2 and pAkt/Actin.
Discussion
Effect of IL-1β on Tie2
Tie2 expression was shown to decrease (Figure 2, Tie2/Actin graph) when stimulated by Ang-1 and more so with IL-1β stimulation. This would suggest IL-1β may cause the down-regulation of Tie2 expression. The decrease from Ang-1 may be a result of Tie2 internalisation (Bogdanovic et al, 2006).
Effect of IL-1β on pERK1/2
Phospho-ERK-1 (pERK1) and Phospho-ERK-2 (pERK2) both follow a similar pattern of initial decrease in phosphorylated ERK (Figure 2, $pERK_{1/2}/ERK_{1/2}$ graph) up to IL-1β concentration 25ng/ml. At 50ng/ml the activity increases due to IL-1β's intracellular mechanisms (Hayakawa et al, 2010).
Effect of IL-1β on pAkt
Phospho-Akt (pAkt) also displayed similar results to pERK1/2 with decrease of phosphorylated Akt initially and then an increase as the concentration of IL-1β increases (Figure 2, pAkt graph). IL-1β has been found to activate the phosphatidylinositol 3-kinase/Akt signalling pathway as a mechanism to activate NFkB (Teshima et al, 2004).
Additional Testing
Phospho-Tie2 (pTie2) was probed for initially which showed a decrease in pTie2 however due to anomalies in the blot and inability to produce measurable repeats, statistical analysis could not be completed.
Conclusion
Overall the problem of vascular disease affects many and research in this field is vital to the development of preventative action and treatments. In this study IL-1β was found to impact on signalling kinases of Tie2 and the receptor itself. Due to sample size, there was a lack in statistical significance, however strongly supported assumptions can be made from the results.
Although, further research may be able to provide statistical significance. A more refined method, like functional assays, and implementing addition parameters, for example a time-course element, could build on and provide further information on signalling mechanisms. Future prospects of this research may include using animal models to understand the full mechanism of IL-1β and Ang-1/Tie2 interactions, in addition to possible therapy trials.
Ig Domain
EGF Repeats
Ig Domain
Fib III
Extracellular
Intracellular
Tyrosine Kinase
Figure3. Tie2 homo-dimer receptor tyrosine kinase. Adopted from Brindle et al, 2006.
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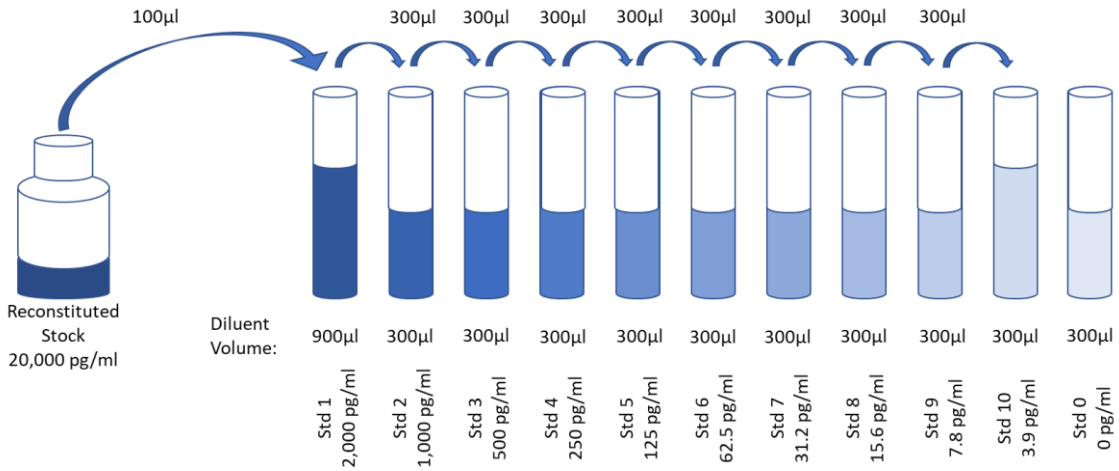
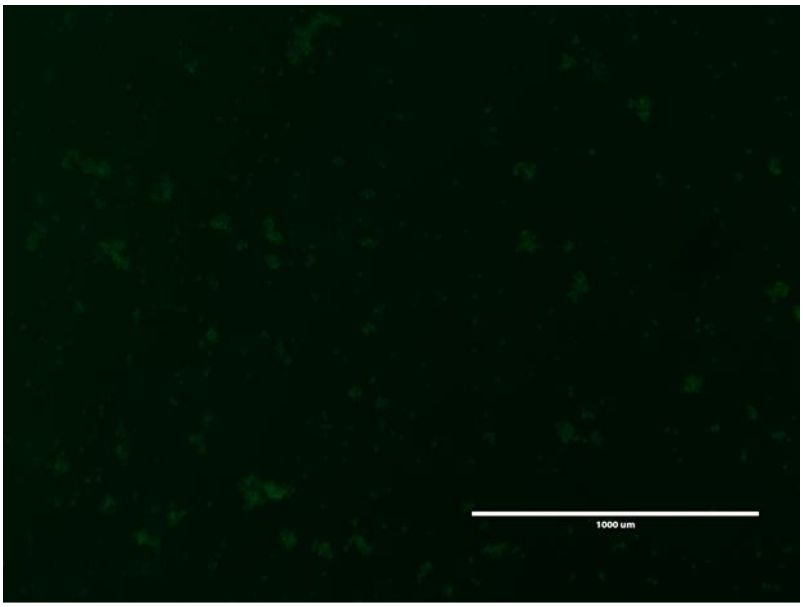
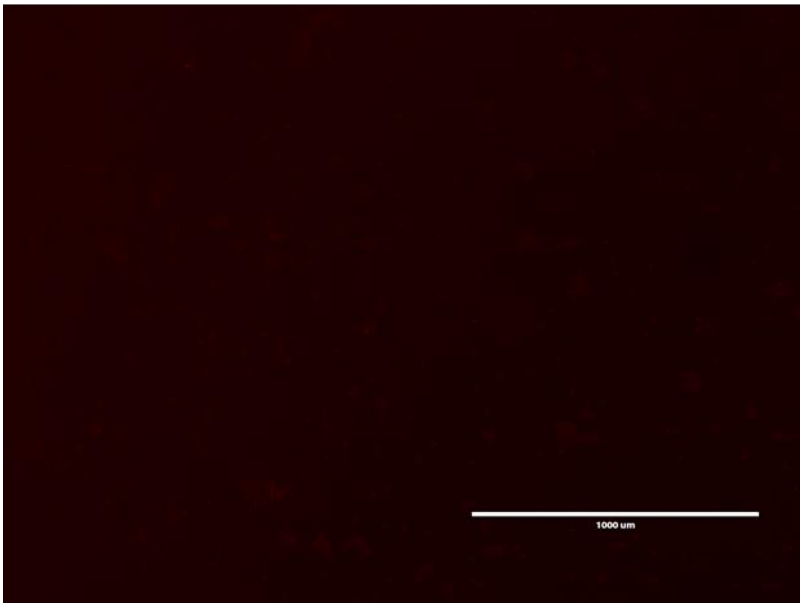
Appendix 2 – Serial Dilution to create the standards for the Ang-2 ELISA.



Appendix 3 – Images of the Cell Viability assay following only the manufacturers protocol.





Appendix 4 – Vehicle Controls demonstrating Ang-1 functioning to impose activity in Tie2 and Akt.