Kingston University London

The influence of Adenosine Deaminase 2 on Blood Outgrowth Endothelial Cells

A thesis submitted in partial fulfilment of the requirements of Kingston University for the award of Doctor of Philosophy

By

Loryn HALLIDAY

First Supervisor: Dr Francesca Arrigoni

Supervisory Team: Dr Nicholas Freestone and Professor Paul Brogan

School of Life Science, Pharmacy and Chemistry

August 2021

This page intentionally left blank

Declaration

I hereby declare that this thesis entitled, 'The influence of Adenosine Deaminase 2 on Blood Outgrowth Endothelial Cells', has been submitted exclusively for the degree of Doctor of Philosophy at Kingston University London and has not formed the foundation for any other award at any university or tertiary structure.

Loryn Halliday

August 2021

Acknowledgments

Without the help and continuous support of so many people, I would not have been able to complete this thesis. Firstly, I would like to thank Dr Francesca Arrigoni for your guidance and enthusiasm for research which made this process exciting. You have challenged me to think in different ways to become a better researcher and it is no exaggeration to say that I owe my science career to you. I will never forget your help in my hour of need. To Dr Nicholas Freestone, no matter how busy you are, you have always made time to support me. I have learnt so much from you and you have been a wonderful mentor throughout this process.

I would like to thank Prof. Paul Brogan whose extensive clinical and academic knowledge in paediatric vasculitis has greatly contributed to the project. I really appreciate both you, and everyone at the Institute of Child Health, who made me so welcome. Without the access to specialist equipment and resources this project would not have been possible. A special mention goes to Dr Patricia Hunter, Dr Ying Hong and Prof. Despina Eleftheriou whose individual expertise I learnt so much from.

I am very grateful to Prof. Jayne Mitchell at Imperial College London who supported my development as a researcher and gave me the opportunity to learn from members of her research group. Without her contribution of expertise in BOECs, I would not have been able to complete this project. Thank you to Dr Isra Marei and Dr Blerina Ahmetaj-Shala who patiently answered every question and went out of their way to support me.

At Kingston University I was extremely lucky to have Dr Lauren Mulcahy-Ryan and Dr Ali Ryan, who made a special effort to make me feel welcome and have been pivotal in my journey as a PhD student.

I am indebted to my wonderful lab partner, Natasha Luckhurst. Having someone to share this PhD experience with has made it so much better. You have pushed me and always been ready and waiting with a cup of tea! Your intellect, strength and solidarity have truly inspired me. I am so lucky to have been surrounded by brilliant people, who will be my friends for life: Ronni, Sinéad, Sharan, Jon, Nico and Amtul. Thank you for your love, kindness and the distractions. Emotionally and scientifically, you have supported me every step of the way.

I feel so appreciative to have worked in such a lovely environment every day. This is down to all those past and present in the IRTL, in particular: Farshad, Arshia, Katrina, Nauf, Hiba, Almas, Amanda, Sanaz, Arryan, Sooz and Gustavo.

To my oldest friends Katja, Gemma, Georgia, Katie, Vina and Tabby, you are so special to me. I take daily inspiration from each of you. Thank you for encouraging me to take a break, listening to me go on, and for your unwavering support through this important part of my life.

Thank you to my parents who have supported me emotionally and encouraged my pursuit of academic success. To my sisters, Róisín and Mairéad, who continuously teach me to see the lighter side of life and have never failed to believe in me, I am forever grateful. And, to Falco, who is always at my side and whose unfaltering encouragement, has got me through this challenging period of my life.

Presentations and abstracts

Dimethylarginine dimethylaminohydrolase levels in blood outgrowth endothelial cells. Oral presentation. Physiology 2021 (The Physiological Society). Virtual conference hosted from London, UK, July 2021.

Halliday, L., Freestone, N., and Arrigoni, F.

Introduction: Dimethylarginine dimethylaminohydrolase (DDAH) is a major endogenous regulatory enzyme controlling asymmetric dimethylarginine (ADMA) levels. ADMA, which inhibits nitric oxide synthase (NOS), is a mediator elevated in numerous disease states and several diseases associated with vascular dysfunction have been associated with aberrant levels of DDAH (Arrigoni et al., 2003; Palm et al., 2007). Investigating the endothelial cell (EC) function of individuals and assessing the nitric oxide (NO) pathway including the DDAH pathway, has been limited to invasive techniques and animal studies. Recent developments in culture techniques to extract progenitive endothelial cells from whole blood, known as blood outgrowth endothelial cells (BOEC) or endothelial colony-forming cells (ECFC), means that endothelial cells can be isolated in a non-invasive manner from a relatively small amount of donor's blood and represent an individual's epigenetic makeup (Paschalaki et al., 2013). We therefore sought to measure DDAH and NOS mRNA in these cells to see if this was a viable model for assessing the NO pathway.

Methods: The study was approved by the Kingston University Faculty Research Ethics Committee (Reference 1617/024). Blood outgrowth endothelial cells (BOECs) were isolated and cultured from male and female adult healthy donors (n=5). BOECs were isolated as previously described until the formation of a characteristic cobbled-shaped morphology (Ormiston et al., 2015). BOECs were confirmed to have classical EC surface marker expression (CD31, CD144, low CD34) using flow cytometry. Extracted BOECs and HUVECs (Sigma-Aldrich) were cultured under standard conditions and plated for 2-3 days, until confluent and treated under control or inflammatory conditions (TNF-α, 10ng/ml) for 24 hours. RNA was extracted using TRIzol™ Reagent (Invitrogen) and cDNA synthesis performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qRT-PCR was executed using primers for NOS2,

NOS3, DDAH1 and DDAH2 mRNA, with ACTB and GAPDH used as reference genes. Analysis used the 2–ΔΔCt method for quantification.

Results: NOS3 expression was identical between HUVEC and BOEC and decreased following inflammatory stimulation to an equivalent level in both cell types (BOEC p=0.0275 and HUVEC p=0.0367, n=5). NOS2 was not detected under quiescent conditions or following inflammatory stimuli.

DDAH2 mRNA levels were greater than DDAH1 in both BOEC and HUVEC (DDAH2>DDAH1 BOEC 15.2x p=0.0001, HUVEC 4.7x p=0.0248). Under quiescent and inflammatory conditions, DDAH1 gene expression did not differ between BOEC and HUVEC. However, there was 2.9x more DDAH2 gene expression in BOEC than HUVEC at rest (p=0.0489, n=5) and following incubation with TNF-α, DDAH2 mRNA increased in HUVEC so that the two cell types expressed equivalent amounts.

Conclusions: This is the first time that DDAH expression has been examined in BOEC. BOEC express more DDAH2 than HUVEC under resting conditions with both cell types expressing more DDAH2 mRNA than DDAH1. There was no difference in the expression of NOS3 in either EC type. DDAH2 is known to contribute to endothelial cell NO bioavailability, and as an endothelial cell model, these cells may provide better insight into an individual's endothelial health than HUVEC.

This work was funded by Animal Free Research UK.

Keywords: BOEC; endothelial colony–forming cells; ECFC; DDAH; Nitric oxide; NO.

References

Arrigoni FI et al. (2003). Circulation 107(8):1195-201.

Palm F et al. (2007). Am J Physiol Heart Circ Physiol 293: H3227–H3245.

Paschalaki KE et al. (2013). Stem Cells 31(12): 2813–2826.

Ormiston ML et al. (2015). J Vis Exp. 106: 53384.

ADA2 expression and activity in blood outgrowth endothelial cells.

Abstract. 3rd International Conference on Deficiency of ADA2. Virtual conference hosted from Washington, USA, November 2020.

Halliday, L., Brogan, P., Freestone, N., and Arrigoni, F.

Introduction: Autosomal recessive loss-of-function mutations in ADA2 causes deficiency of adenosine deaminase 2 (DADA2), a monogenetic systematic vasculitis, sometimes also associated with immune deficiency or marrow failure. Children with a deficiency in ADA2 (DADA2) exhibit; inflammation, endothelial damage, and elevated plasma adenosine levels. ADA2 is normally expressed in leucocytes; expression and activity of ADA2 in endothelial cells (EC) remains uncertain and may depend the origin of EC studied. We hypothesised that ADA2 may be expressed by, and functionally important to, EC biology.

Objective: Our aim was to study ADA2 mRNA expression and enzymatic activity in ECs derived from peripheral blood mononuclear cells (PBMCs) in comparison to human umbilical vein endothelial cells (HUVEC), and THP-1s (human monocytic cell line). In addition, examine the impact of exogenous ADA2 on EC growth and migration in vitro.

Methods: Blood outgrowth endothelial cells (BOECs) were isolated and cultured from adult healthy donors (n=5). Briefly, PBMCs were extracted from whole blood, cultured in EBM-2 media on human collagen coated plates for 7-21 days, until the formation of colonies with characteristic cobbled-shaped morphology. BOECs were confirmed to have classical EC surface marker expression (CD31, CD144, low CD34) using flow cytometry. BOECs, HUVECs and THP-1s were cultured under standard conditions and plated for 2-3 days, until confluent. qRT-PCR was executed using primers for adenosine receptors and ADA2 mRNA, with ACTB used as a reference gene. ADA2 enzyme activity of the cell supernatants was measured using an ADA assay kit (Diazyme), with the addition of a specific ADA1 inhibitor, Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 100nM. Proliferation was assessed using a colorimetric BrdU ELISA assay and migration was quantified by a wound healing assay, after cells were treated with increasing concentrations of adenosine (from 10nM to 100μM) and ADA2.

Results: When compared to THP-1 cells, low mRNA expression of ADA2 in both EC types was observed (n=3, BOEC 2.163+/-1.7, HUVEC 1.011+/-0.1 and THP 1 12.41+/-2.1). BOEC expression of adenosine receptors was A2BR>A2AR, and HUVEC expression was A2AR>A2BR.

BOEC showed significantly greater ADA2 activity (1.409+/-0.223 U/L, n=12, p=0.002) than HUVEC, (0.009+/-0.008 U/L, n=12) but still significantly less than activity observed in THP-1 cells (5.456+/-0.246 U/L, n=3, p=0.0043).

Treatment of both BOECs and HUVECs with exogenous recombinant ADA2 decreased both growth rate (IC50=4.14 U/L, n=5) and migration (BOEC -18 +/-7.4%, at 18 hours, n=5, p <0.05, and HUVEC -26.54+/-7.083, n=5, p=0.0028) in a dose dependent manner (IC50=9.7 U/L, n=5). However, with extreme physiological concentrations of adenosine (5x10-7M) this effect was reversed, and proliferation increased. In the presence of the adenylate cyclase inhibitor, SQ22536, migration effects were reversed, but this was a phenomenon only observed in BOEC.

Conclusions: Despite low mRNA expression of ADA2 in both EC phenotypes, ADA2 activity was found in BOEC-derived supernatants.

Exogenously added ADA2 inhibited proliferation and migration of BOECs, but promoted growth under extreme physiological adenosine concentrations (as found in mild hypoxia or DADA2 patients).

ADA2 inhibition of BOEC migration was prevented by an adenylate cyclase inhibitor, suggesting the mechanism is cAMP pathway dependent.

BOECs are ECs utilised in the circulating repair of the vascular endothelium. This work demonstrates an ADA2-dependent mechanism by which BOECs locally influence growth and repair, highlighting another means by which a deficiency of ADA2 may lead to vascular dysfunction.

Keywords: BOEC; DADA2; ADA2; Adenosine

Adenosine deaminase 2 restores the endothelial glycocalyx of blood outgrowth endothelial cells.

Poster presentation. Future Physiology 2019 (The Physiological Society). Liverpool, UK, December 2019.

Halliday, L., Esposito, G., Brogan, P., Freestone, N., and Arrigoni, F.

Background: Glycosaminoglycans (GAGs) are polysaccharide chains that contribute to the formation of a mesh-like layer on the surface of the endothelium: the glycocalyx. The integrity of this layer is vital for endothelial cell homeostasis and its disruption leads to endothelial dysfunction. Endothelial damage associated with certain systematic vasculitides has recently been linked to autosomal recessive loss-of-function mutations in the ADA2 gene. Direct effects of the ADA2 protein on the endothelium are unclear, although ADA2 is known to have a GAG binding site. We isolated blood outgrowth endothelial cells (BOECs) in order to study the repair potential of ADA2 on the endothelial glycocalyx, in healthy volunteers.

Methods: BOECs were isolated and cultured from healthy donors. Firstly, peripheral blood mononuclear cells (PBMCs) were extracted from whole blood, cultured in EBM-2 media on collagen-coated plates, for 7-21 days, until colonies formed with characteristic cobbled-shaped morphology. BOECs were confirmed, using flow cytometry, to have classical endothelial cell surface marker expression (CD31, CD144, low CD34). BOECs (n=3) were cultured on chamber slides for 2-3 days until confluent and then incubated with GAG degradative enzymes for 2 hours; confirmed by staining. Cells were then treated with either ADA2 (10 U/L) or untreated and allowed to recover for a further 18 hours. The samples were then fixed, blocked and stained with primary antibodies specific for the GAGs; Heparan sulfate (HS) and Chrondroitin sulfate (CS). The slides were stained with FITC secondary antibodies and Hoechst nuclear stain. Samples were analysed by confocal microscopy, and data acquired over 3 random fields was evaluated using Image J software.

Results: 100% of BOECs expressed both HS and CS, with GAG coverage over the entire cell. Following enzymatic treatment, HS and CS expression was removed. Incubation with ADA2 for 18 hours restored the original GAG coverage. Over this time, untreated BOECs (media

alone), expressed significantly less GAGs than with ADA2 ($p<0.001$) and were restored to only 52% (+/- 3.6% SEM) of their initial coverage. It took a further 18 hours before untreated BOECs recovered their full glycocalyx expression.

Conclusions: Glycocalyx removal precedes endothelial damage. This is the first time that the promotion of glycocalyx and GAG repair by an exogenous enzyme, ADA2, has been reported. ADA2 accelerated BOEC glycocalyx recovery following GAG degradation and BOECs widely expressed the two most commonly expressed GAGs. This data provides a suggested mechanism by which ADA2 protects against endothelial damage. Therefore, in ADA2-deficient patients, this may contribute to the endothelial damage and ensuing inflammation and vasculitis that is observed.

Keywords: ADA2, BOEC; Glycosaminoglycans; Glycocalyx

Halliday, L., Brogan, P., Freestone, N., and Arrigoni, F. (2020). **Selected Abstracts from Pharmacology 2019: Influence of adenosine deaminase2, on the angiogenic function of blood outgrowth endothelial cells.** British Journal of Pharmacology 177, 2579.

Background and Purpose: Adenosine deaminase 2 (ADA2), usually released from circulating leukocytes, has recently become a therapeutic target in treating children with systematic vasculitis due to autosomal recessive loss-of-function mutations in the *ADA2* gene (formerly known as *CECR1*). Children with a deficiency in ADA2 (DADA2) exhibit inflammation, endothelial damage, and elevated plasma adenosine levels, although the direct influence of ADA2 on the endothelium is unclear.

Experimental Approach: Our aim was to investigate the influence ofADA2 in the presence of its substrate, adenosine, on the proliferation and migration of blood outgrowth endothelial cells (BOECs), which are endothelial progenitors isolated and cultured from healthy donors (n= 5). PBMCs were extracted from whole blood, cultured in EBM-2media on collagen-coated plates for 7–21 days, until the formation of colonies with characteristic cobbled-shaped morphology. BOECs were confirmed to have classical endothelial cell surface marker expression (CD31, CD144, low CD34) using flow cytometry. BOECs were then treated with increasing concentrations of adenosine (from 10nM to100μM) with and without ADA2 (10 U/L−1) for 24 hr before being assessed for changes in cell proliferation (Colormetric BrdU ELISA) or cell migration (scratch wound assay).

Key Results: ADA2 treatment of BOEC decreased both growth rate of BOECs and migration (−18 ± 7.4%, at 18hr, n=5, P< .05). Adenosine influenced endothelial proliferation in a dose-dependent manner increasing BOEC cell proliferation at lower concentrations (10nM–0.5μM) and reduced proliferation at higher concentrations (1–100μm). ADA2 did not influence the proliferation of BOEC in the presence of adenosine, except at 0.5μM, where it created a significant spike in endothelial cell growth (+65 ± 17.4%,n=5,P< .01).

Conclusion and Implications: This is the first time that the influence of adenosine and ADA2 has been studied on these cells. We demonstrated that physiological concentrations of ADA2 alone inhibited both endothelial cell proliferation and migration of BOECs; however, in the presence of adenosine, these changes were absent. In addition, the combination of

physiological levels of ADA2 in the presence of0.5-µM adenosine created significant increases in BOEC proliferation. We believe that these data strongly provide evidence of one mechanism by which ADA2, in the presence of adenosine, can promote endothelial health and repair and therefore in ADA2-deficient, DADA2 patients, might contribute to the endothelial damage and ensuing inflammation and vasculitis that is observed.

Adenosine deaminase 2 promotes growth and migration of blood outgrowth endothelial cells, in the presence of adenosine.

Poster Presentation. First Symposium on Synthesis & Drug Discovery. Kingston University London, UK, November 2019.

Halliday, L., Brogan, P., Freestone, N., and Arrigoni, F.

Introduction: Adenosine deaminase 2 (ADA2), usually released from circulating leukocytes, has recently become a therapeutic target in treating children with systematic vasculitis due to autosomal recessive loss-of-function mutations in the *ADA2* gene (formally known as *CECR1*). Children with a deficiency in ADA2 (DADA2) exhibit; inflammation, endothelial damage and elevated plasma adenosine levels although, the direct influence of ADA2 on the endothelium is unclear.

Method: Our aim was to investigate the influence of ADA2 in the presence of its substrate, adenosine, on the proliferation and migration of blood outgrowth endothelial cells (BOECs), which are endothelial progenitors isolated and cultured from healthy donors (n=5). PBMCs were extracted from whole blood, cultured in EBM-2 media on collagen-coated plates for 7-21 days, until the formation of colonies with characteristic cobbled-shaped morphology. BOECs were confirmed to have classical endothelial cell surface marker expression (CD31, CD144, low CD34) using flow cytometry. BOECs were then treated with increasing concentrations of adenosine (from 10nM to 100µM) with and without ADA2 (10 U/L) for 24 hours before being assessed for changes in cell proliferation (Colormetric BrdU ELISA) or cell migration (scratch wound assay).

Results: ADA2 treatment of BOEC decreased both growth rate of BOECs and migration (-18 +/-7.4%, at 18 hours, n=5, p <0.05). Adenosine influenced endothelial proliferation in a dose-dependent manner increasing BOEC cell proliferation at lower concentrations (10nM-0.5µM) and reduced proliferation at higher concentrations 1µm-100µm. ADA2 did not influence the proliferation of BOEC in the presence of adenosine, except at 0.5µM, where it created a significant spike in endothelial cell growth (+65 +/- 17.4% n=5, p <0.01).

Conclusions: This is the first time that the influence of adenosine and ADA2 has been studied on these cells. We demonstrated that physiological concentrations of ADA2 alone

inhibited both endothelial cell proliferation and migration of BOECs, however in the presence of adenosine, these changes were absent. In addition, the combination of physiological levels of ADA2 in the presence of 0.5μM adenosine created significant increases in BOEC proliferation.

We believe that this data strongly provides evidence of one mechanism by which ADA2, in the presence of adenosine, can promote endothelial health and repair and therefore in ADA2-deficient, DADA2 patients, might contribute to the endothelial damage and ensuing inflammation and vasculitis that is observed.

Keywords: BOEC; DADA2; ADA2; Adenosine

List of Acronyms

5-HT: 5-hydroxytryptamine

ACE: Angiotensin-converting enzyme

ACMG: American College of Medical Genetics and Genomics

ACTB: Actin beta

ADA: Adenosine deaminase

ADGFs: ADA-related growth factors

ADMA: Asymmetric dimethylarginine

Ado: Adenosine

ADP: Adenosine diphosphate

AMP: Association for Molecular Pathology

AR: Adenosine receptor

ATP: Adenosine triphosphate

BMP: Bone morphogenetic protein

BOEC: Blood outgrowth endothelial cells

bp: Base pairs

cAMP: cyclic adenosine monophosphate

CAMs: cell adhesion molecules

cDNA: Complementary DNA

CEC: Circulating endothelial cells

cGMP: Cyclic guanosine monophosphate

COPD: Chronic obstructive pulmonary disease

CRP: C-reactive protein

CVD: Cardiovascular diseases

CXCL10: C-X-C Motif Chemokine Ligand 10

DADA2: Deficiency of adenosine deaminase 2

DAMPs: Danger-associated molecular patterns

DDAH: dimethylarginine dimethylaminohydrolase

DEA-NONOate: Diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate

DGEA: Differential gene expression analysis

DMEM: Dulbecco's Modified Eagle's Medium

EC: Endothelial cell

ECFCs: endothelial colony–forming cells

ECGM: Endothelial Cell Growth Media

ECM: Extracellular matrix

EDHF: Endothelium-derived hyperpolarization factor

EH: Essential hypertension

EHNA: Erythro-9-(2-hydroxy-3-nonyl) adenine

ELISA: Enzyme linked immunosorbent assay

eNOS, NOS3: Endothelial nitric oxide synthase

EPC: Endothelial progenitor cells

ET-1: Endothelin-1

FBS: Fetal bovine serum

GAG: glycosaminoglycan

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

gDNA: Genomic DNA

GEnC: Glomerular endothelial cells

GPC1: Glypican 1

GPCR: G protein-coupled receptors

GSEA: Gene set enrichment analysis

HHT: Hereditary haemorrhagic telangiectasia

HIV: Human Immunodeficiency Virus

HS: Heparan sulphate

HSCs: Hematopoietic stem cells

HSCT: Haematopoietic stem cell transplantation

HSPG2: Heparan Sulphate Proteoglycan 2

HUVEC: Human Umbilical Vein Endothelial cells

ICAM-1: Intercellular adhesion molecule 1

ICC: Immunocytochemistry

IEJ: Inter-endothelial junctions

IFN-β: Interferon beta

IFN-γ: Interferon gamma

IL: Interleukin

iNOS, NOS2: Inducible nitric oxide synthase

JAM: Junctional adhesion molecule

LDL-C: LDL cholesterol

MAS: Macrophage activation syndrome

MCP-1: Monocyte chemoattractant protein -1

MHC: Major histocompatibility class

NES: Normalised Enrichment Score

NETs: Neutrophil extracellular traps

NIH: National Institutes of Health

nNOS, NOS1: Neural nitric oxide synthase

NO: Nitric oxide

NOS: Nitric oxide synthase

PAH: Pulmonary Arterial Hypertension

PAMPs: Pathogen-associated molecular patterns

PAN: Polyarteritis nodosa

PBMCS: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PECAM: platelet endothelial cell adhesion molecule

PGI2: Prostacyclin

PKG protein kinase G

PMA: Phorbol-12-myristate-13-acetate

PNP: Purine nucleoside phosphorylase

POD: Peroxidases

PRB: Putative receptor binding

PRMTs: Protein arginine methyltransferases

qRT-PCR: Quantitative real time polymerase chain reaction

ROS: Reactive oxygen species

SCID: Severe combined immunodeficiency

SDC: Syndecan

SDC4: Syndecan 4

SEM: Standard error of the mean

sJIA: systemic juvenile idiopathic arthritis

SMC: smooth muscle cells

TA: Takayasu arteritis

TAE: Tris/Acetic acid/EDTA

TB: Tuberculosis

TMB: tetramethyl-benzidinthen

TNF: Tumour necrosis factor

VCAM-1: Vascular Cell Adhesion Molecule

VEGF: Vascular endothelial growth factor

VVOs: vesiculo-vacuolar organelles

vWF: von Willebrand factor

Table of Contents

List of Figures

List of Tables

Abstract

Autosomal recessive loss-of-function mutations in *Adenosine Deaminase 2 (ADA2)* cause deficiency of adenosine deaminase 2 (DADA2), a monogenetic systematic vasculitis, sometimes also associated with immune deficiency or marrow failure. Children with DADA2 exhibit: inflammation, endothelial cell (EC) damage and elevated plasma adenosine levels. ADA2 is typically expressed by leukocytes. Indirect evidence suggests that ADA2 is a growth factor for ECs. It was hypothesised that ADA2 might be expressed by, and functionally important to, EC biology. Recent developments in culture techniques to extract progenitive ECs from whole blood, known as blood outgrowth endothelial cells (BOEC) or endothelial colony-forming cells (ECFC), indicates that ECs can be isolated from a relatively small amount of donor's blood and represent an individual's epigenetic makeup. The aim of this thesis was to assess BOECs as an EC model, understand the ADA2 metabolite, adenosine's signalling mechanism within these cells, and then assess how ADA2 directly impacts EC structure, and function.

BOECs were isolated from healthy donors and compared to the endothelial model, HUVECs. Phenotype and functional assays showed that BOECs behaved typically of endothelial cells. Extensive glycocalyx expression and elevated quiescent *DDAH2,* (a gene involved in the regulation of nitric oxide production), levels in BOEC, suggest that BOECs might be a more relevant EC model, than HUVEC.

When investigating the influence of adenosine on BOECs, BOECs expressed more A_{2B} than A_{2A} adenosine receptor (AR) and HUVECs expressed more A_{2A} than A_{2B} AR. Extracellular adenosine inhibited BOEC glycocalyx expression and recovery. At high adenosine levels, adenosine downregulated BOEC *DDAH2* expression. Functional assays, proliferation and migration were increased with increasing concentrations of adenosine, and this was mediated by both ARs, but predominantly A_{2B}.

Finally, when investigating the ADA2 direct influence on BOECs, we demonstrated that although lower than monocytes, *ADA2* mRNA was expressed by BOECs, and ADA2 activity was found in BOEC-derived supernatants. Exogenously added ADA2 inhibited proliferation and migration of BOECs. ADA2 inhibition of BOEC migration was prevented by an A_{2B} AR antagonist and adenylate cyclase inhibitor, suggesting the mechanism is AR pathway-dependent. Exogenous ADA2 also promoted restoration of the glycocalyx and inhibited Tumour necrosis factor alpha (TNF-α) stimulated monocyte adhesion. ADA2 in the presence of 0.5μM adenosine, which is extreme physiological adenosine concentration (as found in mild hypoxia or DADA2 patients) improved *HSPG2* expression and glycocalyx restoration; upregulated *NOS3* expression; and promoted growth, restored cell migration to control and increased monocyte adhesion.

BOECs are ECs thought to repair the vascular endothelium. This work demonstrates an ADA2-dependent mechanism by which BOECs locally influence growth and repair, highlighting another means by which deficiency of ADA2 may lead to vascular dysfunction.

1 Introduction

1.1 Deficiency of Adenosine Deaminase 2

1.1.1 Earliest clinical description

In 2014, two independent groups simultaneously reported a group of children with vasculitis, who had mutations in the *Cat Eye Syndrome Critical Region Protein 1* (*CECR1)* gene (Navon Elkan et al., 2014; Zhou et al., 2014). These children had symptoms of early-onset strokes, livedo rashes and systematic vasculopathy, usually associated with polyarteritis nodosa (PAN), hepatosplenomegaly and hypogammaglobulinemia, which collectively did not correspond to any known familial autoinflammatory diseases. Disease onset was as young as 2 months old, and the severity of the clinical manifestations varied, with the most severe cases resulting in children dying before their first birthday (Navon Elkan et al., 2014). Due to the devastating nature of the symptoms and lack of a definitive diagnosis, an exploratory approach for a common genetic cause was employed: whole-exome sequencing. This technique has been used increasingly since its advent in 2009, to identify *de novo* genetic mutations for rare disorders (Ku et al., 2016).

Zhou et al., (2014) originally observed 3 unrelated patients with the same symptoms, and their asymptomatic parents, then expanded their search using candidate-gene sequencing to a further 3 patients with similar phenotypes, and finally studied their siblings; with a total of 9 patients across the US, UK and Turkey. The impetus for the work from Navon Elkan et al., (2014) differed. A study in Israel recognised the familial nature of paediatric PAN in patients with Jewish Georgian Caucasus ancestry and wanted to determine the genetic basis of the disease. Together, these studies recorded various loss-of-function mutations in the *CECR1* gene which is now described as the *ADA2* gene and this nomenclature will be used henceforth. The *ADA2* gene encodes for the enzyme: adenosine deaminase 2 (ADA2) and loss of function mutations result in a reduction in the quantity and enzymatic activity of ADA2 in the plasma, in comparison to healthy controls. Consequently, this disease is called deficiency of adenosine deaminase 2 (DADA2).

1.1.2 Prevalence

By July 2019, 206 patients around the world had been identified as having DADA2 and there was a mortality rate of 8.3% (Gibson et al., 2019). The two most frequent reasons for death were complications due to vasculopathy or immunodeficiency, collectively accounting for 82% of deaths (14/17). DADA2 disease prevalence across all populations, has been predicted using gnomAD v2, to be 1 in 320,000 (O'Donnell-Luria and Baxter, 2020). This estimate can be adjusted to 1 in 250,000 if analysis includes a particular variant: p.Phe355Leu. The p.Phe355leu variant has been reported in one DADA2 case and is found to have a high carrier rate in East Asia, but does not yet meet the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP), criteria for, 'Likely Pathogenic'. There are certain sub-populations which have a higher DADA2 prevalence. The Finnish sub-population has a prevalence of 1 in 124,000. This is mainly driven by the pathogenic variant p.Arg169Gln. The East Asian sub-population, has a prevalence of 1 in 43 million, though this rises markedly to 1 in 92,000, if the p.Phe355Leu variant is included. The carrier frequency across all populations was predicted at 1/283. This can be adjusted to 1/250 if the p.Phe355leu variant is included (O'Donnell-Luria and Baxter, 2020).

1.1.3 Signs and symptoms

Research stemming from pivotal publications in 2014, has broadened the clinical phenotype to include pathology across the vascular, haematological and immunological systems (see Figure 1.1).

Vasculopathy is still the hallmark of DADA2 with manifestations having been described in over 75% of cases and affecting many organs such as the skin, brain, and kidneys (Lee, 2018). One or more cutaneous manifestations are seen in 90% of patients (Lee, 2018). For 72% of patients this presents as livedo reticulitis and in 57% cutaneous vasculitis (Lee, 2018). This manifestation is similar to that of Sneddon syndrome, however, in DADA2, the livedo is more patchy (broken circles), named livedo racemose, and is localised to the lower extremities. In addition, low serum IgM differentiates DADA2 from Sneddon syndrome (Cowen, 2020; Santo et al., 2018). Another prominent feature of DADA2 is recurrent fevers, affecting 63% of patients (Lee, 2018). A skin biopsy of DADA2 pathology will illustrate transluminal neutrophilic and lymphocytic infiltration and fibrinoid necrosis through the vessel wall,

affecting medium vessels; capillaries are spared, but are often dilated (Chasset et al., 2020; Gonzalez Santiago et al., 2015; Navon Elkan et al., 2014; Zhou et al., 2014). Over half of the patients with PAN-like symptoms in one study, had proteinuria suggestive of endothelial dysfunction or chronic inflammation, as previous work has demonstrated that patients with proteinuria have EC dysfunction (Özen et al., 2020; Paisley et al., 2003). Neurological involvement most commonly manifests as ischemic stroke (38% of patients), though haemorrhagic stroke and aneurysms have also been reported (Dhanwani et al., 2020; Lee, 2018). Neuropathy occurs in 43% of DADA2 cases and is a distinguishing feature of DADA2 in comparison to childhood PAN (Lee, 2018). Other inflammatory manifestations often include an elevation of C-reactive protein (CRP), hepatosplenomegaly, portal hypertension and, similar to PAN, digital infarct and necrosis if inflammation occurs in the arteries supplying these areas.

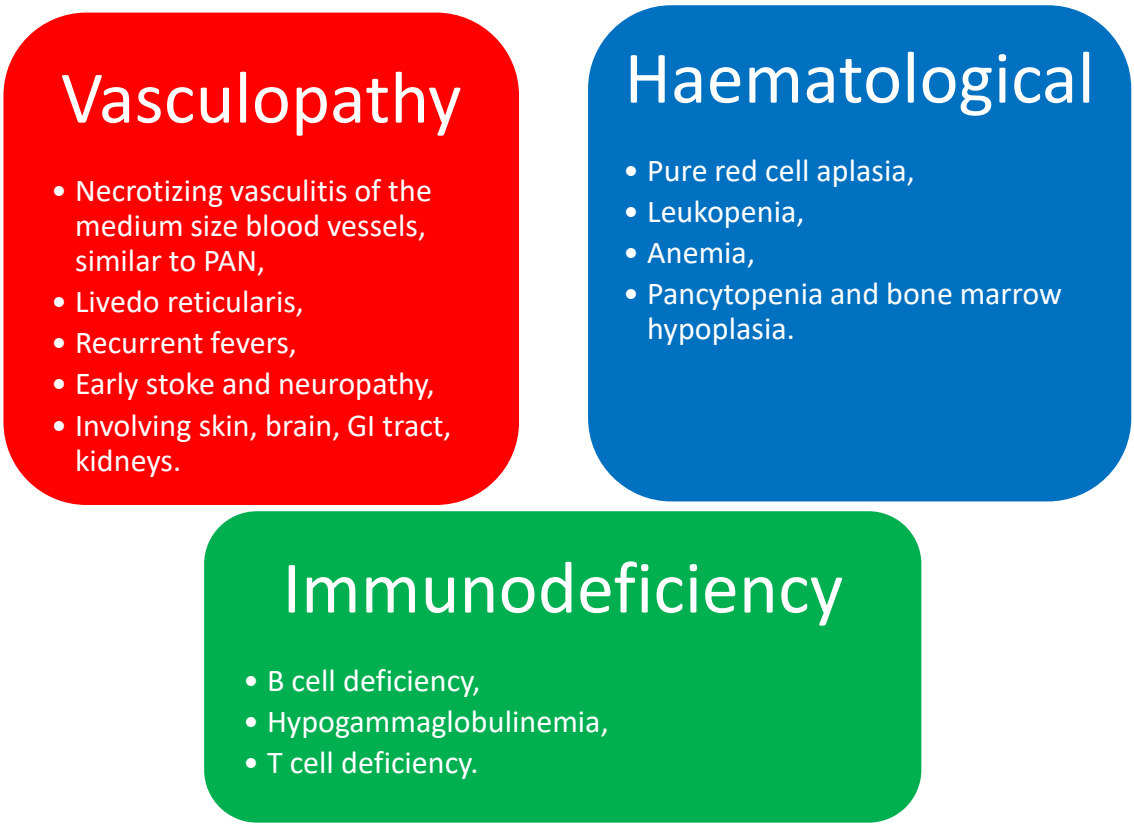


Figure 1.1 Diagram to show the three different DADA2 clusters of dysregulation and their corresponding phenotypes.

The vascular and haematological clusters are distinct whereas the immunological dysregulation often accompanies either cluster, to various degrees. Adapted from (Kendall and Springer, 2020; Lee et al., 2020a; Sag et al., 2020).

In the haematological cluster, anaemia is a common manifestation of DADA2 which is present in over 50% of patients. Some patients present with pure red cell aplasia which mimics Diamond-Blackfan Anaemia (Ben-Ami et al., 2016; Lee, 2018; Sasa et al., 2015). 60% of patients have leukopenia, variably affecting the myeloid and lymphoid cells, in contrast to thrombocytopenia, which is recorded in 32% of cases (Lee, 2018). Bone marrow biopsies have reported hypocellularity across the distribution of hematopoietic precursors, lymphocyte infiltration and reticular fibrosis (Ben-Ami et al., 2016; Ghurye et al., 2019; Michniacki et al., 2018; Trotta et al., 2018; Zhou et al., 2014).

The immunological cluster of symptoms, which overlaps with the haematological cluster, consist of leukopenia and hyper-activation of neutrophil NETosis function (Carmona-Rivera et al., 2019; Lee, 2018). Within the innate immune system, neutropenia occurs in 10-50% of DADA2 patients, the higher estimate was found within a marrow failure phenotype cohort (Lee et al., 2020a; Trotta et al., 2018). The literature has not reported any complement involvement, the only evidence for this is a case study of two siblings showing no complement related effects of DADA2, by C3 and C4 levels, as well as, normal CH50 function tests (Skrabl-Baumgartner et al., 2017). With regards to the adaptive immune system, pathogenic alternations are commonly found in DADA2 patients. Lymphopenia is seen in 10-50% of patients, with B cells more commonly affected than T cells (Schepp et al., 2017; Trotta et al., 2018). There is a decrease in CD27+ B memory cells in DADA2, and of this population, switched CD27+ is decreased in comparison to controls (Schena et al., 2020). Hypogammaglobulinemia is estimated to occur in at least 25% of patients (Meyts and Aksentijevich, 2018). Another study showed that of 160 patients 57% had low IgM, 52% low IgG and 35% combined low IgM and IgG (Lee, 2018). Affected patients often present with recurrent sino-pulmonary infections and immunoglobulin therapy helps to resolve this (Schepp et al., 2017). T cell lymphopenia varies among clusters of patients but can be up to 10% of DADA2 patients and sometimes collectively with B cell deficiency (Schena et al., 2020; Schepp et al., 2017). For those patients that do suffer from T cell lymphopenia, both CD4+ and CD8+ absolute number are reduced in DADA2 patients (Schena et al., 2020). This observation is unsurprising as ADA2 has been shown to be a T cell growth factor and functionally T cells are reported to respond normally to mitogens *in vitro* (Zavialov et al.,

2010b). On the other hand, Schene et al., (2020), suggests that there is a defect of function of T follicular helper cells, shown by decreased CD40L on *in vitro* stimulated T cells.

1.1.4 Clinical diagnosis

Clinical diagnosis of DADA2 is difficult due to the broad spectrum of symptoms and the lack of a commercially available ADA2 test, so obtaining a diagnosis can often only be achieved by measuring ADA2 activity in plasma/serum along with *ADA2* sequencing (Human and Pagnoux, 2019; Rama et al., 2018). When using ADA2 activity or level as a biomarker, it should be considered that the healthy paediatric population has a higher ADA2 activity in comparison to the healthy adult population, though the mechanism for the reduction in activity is not known (Bowers et al., 2020; Nanthapisal et al., 2015). Rama et al., (2018) proposed a decision tree for diagnosis of DADA2.

Accurate and prompt DADA2 diagnosis is imperative for two reasons. Firstly, to prevent potentially harmful treatments, such as aspirin (Human and Pagnoux, 2019). Secondly, to allow patients to start appropriate immunosuppressive medications which most, especially children do well with. This will be discussed further in the Treatment section of this thesis (1.1.6).

Ghurye and colleagues (2019), emphasised that clinicians should consider adult patients, who may fall under the DADA2 umbrella, as well as children. The range of both disease severity and symptoms, as well as recent identification of this disease, may mean some young adults or adults will not have been diagnosed, or may have been misdiagnosed. A tragic account of a 17-year-old who was diagnosed post-mortem with DADA2, regrettably proves the need for this clinical consideration and appropriate treatment (Ghurye et al., 2019).

Carriers, who are often siblings of the affected children, usually present as asymptomatic, but have also been shown to have reduced ADA2 activity. Thus, although there is no data yet, it has been proposed that this group may also be at risk in later life of strokes and vasculitis, due to their reduction in ADA2 activity, which requires further investigation.

1.1.4.1 Differentiating diagnostic methods

DADA2 is a disease with a vast phenotypic range and 'mimics' other diseases, for instance PAN. Özen et al., (2020) proposed that a routine platelet count could be helpful in discriminating DADA2 from PAN, preceding more expensive genetic sequencing. IgM levels are another method for DADA2 diagnosis as these have been shown to accurately differentiate between Sneddon's syndrome and DADA2 (Santo et al., 2018). Cytokine and chemokine signatures for DADA2 have been identified as type I IFN mediated, not type II (Insalaco et al., 2019; Rice et al., 2017; Skrabl-Baumgartner et al., 2017). This has been suggested as a biomarker for DADA2 and the effectiveness of treatment (Insalaco et al., 2019).

1.1.4.2 Potential non-invasive vascular/cardiac phenotype testing

In November 2020, an ongoing pilot study by the National Institutes of Health (NIH) is using a batch of non-invasive techniques to evaluate the vascular abnormalities present in DADA2 patients (Brofferio, 2020). The non-invasive techniques which have thus far shown significant differences in response between DADA2 and controls include digit brachial index, nailfold videocapillaroscopy, laser speckle contrast imaging, EndoPAT™ and near infrared spectroscopy. Those which have shown no detectable response in the DADA2 pilot study include electrocardiogram, echocardiogram, ankle brachial index and eye evaluation. Based on this pilot data of 9 patients, DADA2 patients seem to display an impaired vasodilator response, demonstrated using near infrared spectroscopy: the reperfusion time is doubled in DADA2 patients, showing reduced ability to vasodilate under hypoxia (Brofferio, 2020).

1.1.5 Genetic mutations

DADA2 is an autosomal recessive disease with either homozygous or compound heterozygous variants. Despite being a rare disease, by 2018 over 60 disease-associated mutations had been recorded, affecting all domains of the ADA2 protein; the catalytic, protein dimerization, the putative receptor binding (PRB) and the signal peptide domains (Lee et al., 2020a; Meyts and Aksentijevich, 2018). No known gain-of-function mutations have been identified for DADA2 to date.

Figure ***1.2*** illustrates the reported disease-causing mutations and their corresponding position across the gene and the domains of the ADA2 protein. For example, a mutation

associated with vasculitis is G47R, exon 2, affecting the dimerization domain (Meyts and Aksentijevich, 2018). Mutations preventing the dimerization of the ADA2 homodimer will affect ADA2's ability to bind to cell surface receptors (glycosaminoglycan-binding site), full enzymatic activity and secretion to the cell surface (Zavialov et al., 2010a). It is important to note, however, that it is not only mutations in the catalytic domain which have given rise to the disease phenotype. Discovering genotype-phenotype correlations has been a complex task. Investigation into mapping of the mutations, alongside the protein structure, has not clearly highlighted a correlation between the position of the mutation and disease severity. In fact, it has been reported that groups of patients with the same mutations, including siblings, have shown varying degrees of disease severity and age of onset (Batu et al., 2015; van Montfrans et al., 2014). This would suggest that epigenetic and environmental factors contribute to disease symptoms and severity.

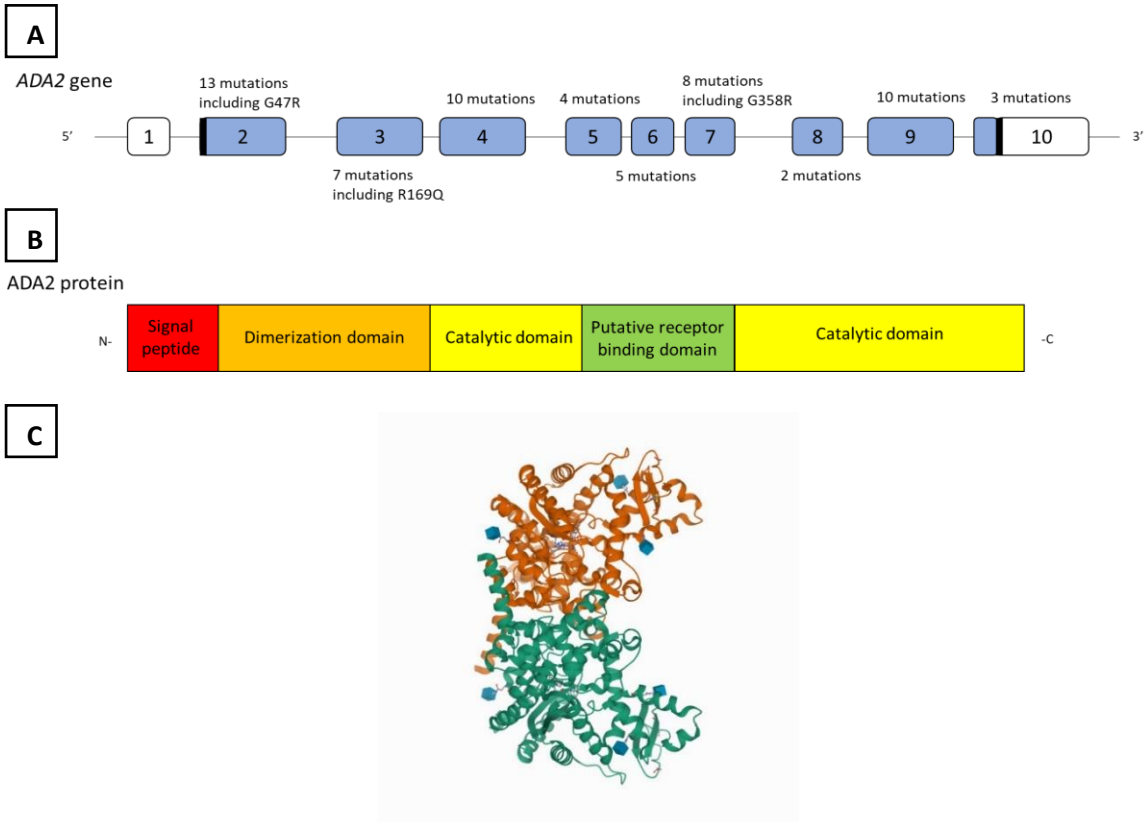


Figure 1.2 A schematic representation of the *ADA2* gene and protein, with the number of disease causing mutations identified.

A) The parts of the *ADA2* gene which are protein coding are presented in blue. 61 unique ADA2 mutations have been described across DADA2 patients. Mutations are scattered across a number of genes and the ADA2 protein. The G47R mutation is located in exon 2, R169Q is located in exon 3 and G358R mutation is located within exon 7. **B)** A schematic of the ADA2 protein. **C)** ADA2 homodimer 3D assembly PDB ID:3LGD. Adapted from (Lee et al., 2020a; Meyts and Aksentijevich, 2018; Zavialov et al., 2010a).

However, it is now becoming clear, through systematic review of all patients reported globally, that there are three main clusters of patients: those who have mainly vascular failure, haematological failure (pure red aplasia) or immunological (bone marrow) failure (Kendall and Springer, 2020; Lee et al., 2020a; Sag et al., 2020). When comparing patients with homozygous mutations, all mutations were missense for the vasculitis phenotype (63/63), but the proportion were a lot lower for the haematological (14/28) and immunological (12/19) cluster patients (Lee et al., 2020a). The most common biallelic mutations are G47R and R169Q (Gibson et al., 2019). The G47R variant is most common for patients with vasculitis and is frequently seen in the Georgian- Jewish population (Gibson et al., 2019; Meyts and Aksentijevich, 2018; Navon Elkan et al., 2014). R169Q has a more variable presentation across all three clusters (Gibson et al., 2019; Sahin et al., 2020). R169Q is the most common mutation for the immunological phenotype, the third most common for the haematological and second most common mutation for the vascular phenotype. G358R is the most common mutation for the haematological type, the second most common type for immunological phenotypes, and does not occur in the vascular cluster at all. Functional analysis of each mutation showed that the vascular mutations have a wide range of ADA2 activity and some residual activity. Whereas, for mutations reported with haematological or immunological clusters there is almost no residual activity. After modelling of patient genotypes with homozygous and heterogenous genotypes, rather than just each allele, a spectrum was proposed whereby, at one extreme, the vascular cluster was found with missense mutations and some residual ADA2 activity, and at the other extreme, haematological and immunological clusters with no residual activity and other mutation types (Lee et al., 2020a).

Overall this suggests that ADA2 might have both enzymatic activity-dependent functions which affect the bone marrow cells, and enzymatic activity-independent functions that influences the blood vessels (Schnappauf et al., 2020). As this is a recently described and a rare disease, it is expected that as more cases are diagnosed, this will help to map a path through the complexity of symptoms and mutations and in turn provide suitable treatment options for individual patients.

DADA2 research to date, has been focused on clinical signs and symptoms, and correlation of these to the genetic mutations, as well as treatment options. There is however, limited mechanistic knowledge for ADA2 and how it influences endothelial cells.

1.1.6 Treatment

It would seem most obvious to treat DADA2 by administering the ADA2 protein to replace the absent or non-functioning enzyme expressed as a result of various mutations. Ombrello et al., (2019) attempted to do so by giving patients fresh-frozen plasma administrations every day for 5 days. Whilst there were no safety concerns, the ADA2 was rapidly cleared from the body; donor plasma ADA2 concentrations averaged 9U/L, but <1U/L was detected in the plasma of DADA2 patients within 1 hour of administration and the median half-life was recorded as 6.4 hours.

First line medication used for PAN, corticosteroids, has been found to be only partially effective even at high doses for DADA2 and does not prevent the neurological involvement and number of strokes (Ombrello et al., 2019; Zhou et al., 2014). In terms of the two main clusters of DADA2 patients, anti-TNFα treatment has been more effective in treating the vascular phenotype, whereas, Haematopoietic stem cell transplantation (HSCT) is the only treatment option for those with the haematological phenotype.

1.1.6.1 Anti-TNF-α

Currently, the most effective treatment for patients suffering with DADA2, in particular those suffering from vasculitis, are anti-TNFα agents such as etanercept, adalimumab, infliximab or golimumab and this was reported in the earliest confirmed DADA2 patients (Navon Elkan et al., 2014; Ombrello et al., 2019). Ombrello et al., (2019) followed 15 patients for 733 patient months before and after starting anti-TNFα treatment and the number of strokes fell from 37 to 0. Caorsi et al., (2017) reported no severe infections or complications for the 10 patients in their study taking anti-TNF over a median time of 3.9 years. In a larger study of 31 DADA2 patients, 27/31 were given anti-TNF for median time of 32 months (Cooray et al., 2021). There was a reduction in the median event rate from 2.37 per 100 patient months to 0, post anti-TNF treatment. This covered both central nervous system (CNS) and non-CNS ischemic events. The paediatric vasculitis activity score (PVAS) was reduced over this time period from

a median of 20/63 to 2/63, post anti-TNF treatment, with livedo reticularis being the main persisting symptom. CRP and Erythrocyte Sedimentation Rate (ESR), inflammatory markers, were also significantly reduced after anti-TNF treatment (Cooray et al., 2021).

It is thought that anti-TNF-α treatment may be successful for DADA2 patients due to a disease mechanism where lack of ADA2 triggers adenosine-mediated neutrophil extracellular traps (NET) formation, in neutrophils. This in turn activates monocytes which release TNF-α (Carmona-Rivera et al., 2019). TNF-α is a pro-inflammatory cytokine which, in excess, can cause tissue damage as previously discussed in reviews (Tesser et al., 2021; Tracey et al., 2008). ADA2's functions will be discussed further in the function section of this thesis (1.2.2.3).

Thalidomide has also been suggested as a cheaper TNF blockade therapeutic with similar effectiveness to anti-TNFα treatment (Caorsi et al., 2017). However, this has side effects involving the peripheral nervous system and it was found that thalidomide treatment for 3 out of 6 DADA2 patients had to be discontinued due to neurologic toxicity (after 20 months to 5 years) (Caorsi et al., 2017).

Anti-TNF treatment has not been curative and life-long treatment is challenging (Özen et al., 2020). It has been reported that livedo rashes, neutropenia, red cell aplasia and peripheral vasculopathy were not fully resolved following anti-TNF treatment and some patients suffer from breakthrough inflammatory periods (Cooray et al., 2021; Ombrello, 2020; Ombrello et al., 2019; Özen et al., 2020). Anti-TNF agents can have variable effects on hepatomegaly, splenomegaly and immunodeficiency. ADA2 activity levels are not restored using anti-TNF agents, and this absence of ADA2 activity is most likely the reason that severe bone marrow deformities are not recovered. During the optimisation of the anti-TNF treatment for the patient, those who are on monoclonal antibodies, can start to produce anti-drug antibodies, and then experience breakthrough inflammatory periods, so therapeutics are needed to ward off this development such as, Methotrexate (Ombrello, 2020). Other agents in conjunction with anti-TNF have been reportedly used for DADA2 including; anti-IL-1 agents, directed T cell therapy for neutropenic patients and granulocyte colony stimulating factor (G-CSF) (Cooray et al., 2021; Ombrello, 2020). Lack of ADA2 and disease severity of DADA2 is correlated with

elevated IL-23 and serum IFN-γ (Ombrello, 2020). This information along with better understanding of the disease, may provide more therapeutic targets.

1.1.6.2 Hematopoietic stem cell transplantation

For those patients who do not respond to anti-TNF, in particular DADA2 patients with haematological and immunological predominant phenotypes, haematopoietic stem cell transplantation (HSCT) is the only effective treatment. A number of studies have shown HSCT restores enzyme activity rapidly; to normal levels within 14 days post-HCST (Barzaghi et al., 2019; Cooray et al., 2021; Dauwe et al., 2016, 2016; Hashem et al., 2017a, 2017b; Van Eyck et al., 2015). A study by Hashem et al., (2017b), where 14 DADA2 patients received an HSCT, showed successful elimination of haematological, immunogenic and vascular phenotypes associated with DADA2, in all 14 patients. But there were some complications which had to be treated. Moderate graft versus host disease was reported in 6/14 patients but was resolved under standard treatments. Other issues such as viral reactivation (10/14 patients) and post-HSCT haematological autoimmune phenomena (4/16) patients were also seen. Though HSCT is curative, this procedure is highly invasive and therefore only advised for patients unresponsive to anti-TNF (Hashem et al., 2017b).

1.1.6.3 Gene therapy

Gene therapy has been suggested as a prospective 'curative' treatment for DADA2. The rationale for this being that DADA2 is brought about by a recessive monogenic mutation and there are current protocols established based on adenosine deaminase deficient severe combined immunodeficiency (ADA1-SCID) that are successful in *ex-vivo* delivery to hematopoietic stem cells (HSCs) (Carbonaro et al., 2014; Eleftheriou, 2020; Kohn et al., 2021). Development for this treatment is in the pre-clinical stage, whereby an effective, *in vitro*, ADA2-deficient model has been developed for both THP-1 and U937 cell lines. When treated with a lentivirus vector for ADA2, the THP-1 and U937 knock outs have restored ADA2 protein expression and activity, as well as restored immunophenotype type and reduction in TNF-α expression (Eleftheriou, 2020; Mortellaro, 2020). *Ex vivo* studies have been performed on CD34+ HSCs utilising the lentivirus vector for ADA2 (Eleftheriou, 2020; Mortellaro, 2020). These studies have shown that HSC properties such as CFU are maintained. When performed using DADA2 patient CD34+ HSC, there is efficient lentiviral transfer, ADA2 restoration in patient macrophages and suppression of cytokine overproduction (Eleftheriou, 2020;

Mortellaro, 2020). There is still more work to be done pre-clinically, as well as in clinical trials including identifying any off-target effects, understand genotoxicity and biodistribution and be able to 'correct' the vast range of ADA2 mutations causing DADA2 (Eleftheriou, 2020). It is hoped that gene therapy will be a future treatment option, in particular for those where there is loss of efficacy of anti-TNF or severe immunodeficiency, and the success of the gene therapy for ADA-SCID maybe replicated (Eleftheriou, 2020).

1.1.7 Summary

As discussed, although DADA2 is a rare disease, recessive mutations can be prevalent in some populations and it is thought to be underreported. This is mainly due to the broad range of clinical phenotypes which are similar in nature to other diseases and diagnosis is reliant on clinical observation, in conjunction with genetic screening and activity testing. Treating DADA2 can be complex and, unfortunately, not always successful, and gene therapy is still a long way off being used routinely in the clinic. It is debated whether there is a clinical need to be proactively treating asymptomatic people with *ADA2* mutations, and there are concerns over continuous treatment with anti-TNFα. Gaining a complete understanding of the disease mechanisms and the recently characterised ADA2 enzyme, will hopefully lead to improved drug targets and better outcomes for patients. Therefore, the evidence surrounding the ADA2 functions will be explored in this thesis (section 1.2.2.3).

1.2 Adenosine deaminases

1.2.1 Adenosine deaminase family

The adenosine deaminase (ADA) enzyme is responsible for controlling the levels of adenosine by deaminating adenosine to inosine (see Figure 1.3). Currently there are three known ADAs; all of which are present in humans. ADA or ADA1 is coded for by the *ADA* gene (chromosome 20q13.12). Whereas, ADA2 the isoenzyme, is coded for by the *ADA2* gene, formally known as *CECR1* (chromosome 22q11.1). In the animal kingdom, rodents, for example, do not have ADA2, and flies do not have ADA1, which suggests, to some extent, that there is degeneracy. This also means that there is no mouse model for studying ADA2 deficiency, unlike ADA1. However, paralogs have been identified in Zebrafish, *cecr1a* and *cecr1b* (Zhou et al., 2014). ADA1 has been extensively studied, as deficiency of this enzyme causes a condition called severe combined immunodeficiency (SCID). Without the ADA1 enzyme, the adenosine and 2'-deoxyinosine builds up intracellularly in immune cells and provokes apoptosis, resulting in complete compromise of the immune system (Hershfield, 2005). However, much less is known about the role of the isoenzyme, ADA2 and even less about ADA3 which is yet to be characterised.

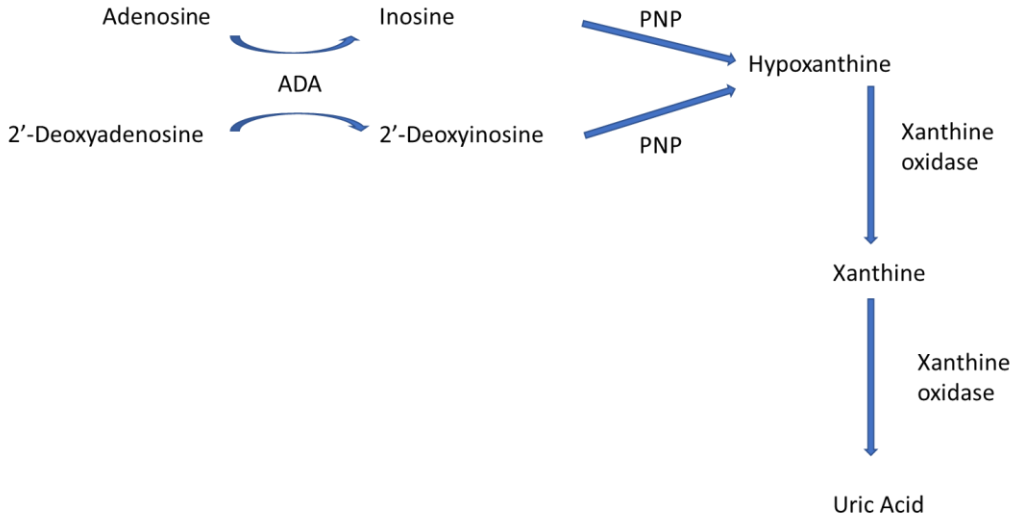


Figure 1.3 A schematic diagram of the adenosine deaminase pathway

ADA irreversibly metabolises the deamination of adenosine or 2-deoxyadenosine to inosine and 2'deoxyinosine respectively. These products are further processed with the formation of Uric acid. Uric acid as well as the other products of ADA metabolism can be measured by spectrophotometry assays. Adapted from (Sauer et al., 2012). **Abbreviation**- **ADA:** adenosine deaminase, **PNP:** purine nucleoside phosphorylase.

1.2.2 Adenosine deaminase 2 protein

1.2.2.1 Structure

Unlike ADA1, ADA2 can form dimers. This dimerization forms a glycosaminoglycan-binding site. *In vitro* binding experiments performed by Zavialov et al., (2010a), have shown that ADA2 binds to different types of cells, via both heparan sulphate and chondroitin sulphate proteoglycans. The dimerization is also thought to be essential for full enzymatic activity and secretion to the cell surface (Zavialov et al., 2010a).

1.2.2.2 Cellular expression

ADA2 is primarily found as an extracellular homodimer, unlike ADA1 (Zavialov et al., 2010a). It is believed that ADA2 is primarily expressed from monocytes/macrophages, but that dogma is changing, with various other cell types being identified as expressing ADA2 including dendritic cells, B cells and endothelial cells and it has been reported that antigen-presenting cells can also secrete ADA2 (Conlon and Law, 2004; Dhanwani et al., 2020; Schena et al., 2020; Zavialov et al., 2010b).

1.2.2.3 Function

As an adenosine deaminase, one role of ADA2 is the breaking down of adenosine into inosine. Though, in the extracellular environment, ADA2 has 100-fold lower enzymatic activity than ADA1 (Zavialov and Engström, 2005). It is thought that ADA2 is active at higher concentrations of adenosine, and when the extracellular pH is acidic, for example when there is inflammation, hypoxia or tumour growth (Zavialov and Engström, 2005). The higher concentrations of adenosine control the immune systems response to the site of injury. The mechanism for upregulation of ADA2 under hypoxic conditions is not yet known, but it is considered that this is in response to high adenosine levels and signalling through adenosine receptors (Wang et al., 2021; Zavialov et al., 2010a).

It has been proposed, however, that the enzymatic nature of ADA2 may not be its primary function. Kaljas et al., (2017) and Zavialov et al., (2010b) demonstrated that ADA2, secreted by monocytes binds to different immune cells: primarily to neutrophils but also monocytes, NK cells and B cells. This secretion is inhibited by IFN-γ *in vitro*. ADA2 binding is thought to occur primarily through the interaction between the glycocalyx, specifically the heparan and chondroitin sulphate proteoglycan chains on the surface of the cells, and the ADA2 glycocalyx binding site. ADA2 is also believed to bind to CD4 + T regulatory cells, expressing CD39 and

lacking CD26 (a receptor for ADA1), also, ADA2 preferentially binds CD16+ monocytes, which are pro-inflammatory, unlike ADA1 which has a preference for CD16- (Kaljas et al., 2017; Zavialov et al., 2010b). This presentation suggests that ADA2 has a role in inflammation and regulating the immune system.

In addition to ADA2's enzymatic activity, ADA2 is capable of being a mitogen for T cells. On T cells, along with the binding via the glycocalyx, there is an additional ADA2 specific receptor which is thought to be similar to the ADA-related growth factors (ADGFs) family and activation of this receptor causes CD4+, T cell proliferation (Zavialov et al., 2010a; Zavialov and Engström, 2005; Zavialov et al., 2010b). Interestingly, the ADA2 triggered proliferation of CD4+ T cells was dependent on physical interaction of these cells with monocytes; ADA2 alone was not enough to trigger this process (Zavialov et al., 2010b).

ADA2 also induces differentiation of monocytes to macrophages; that is a process dependent on CD4+ T cells (Zavialov et al., 2010b). Zavialov et al., (2010b) have demonstrated that ADA2 interacts with adenosine receptors (A_{2A} and A_{2B}) and modulates the receptor's affinity for its ligand. Therefore, it is suggested that rather than ADA2's primary purpose being catalytic, it may bind directly to adenosine receptors and form "molecular bridges" between interacting cells (Zavialov et al., 2010b). Caorsi et al., (2017), have shown that upon activation, monocytes from DADA2 patients produce more TNF, which suggests a disease mechanism.

Another alternative role for ADA2 is that of an intracellular nature. An intrinsic mechanism has been uncovered whereby ADA2 negatively regulates the type I interferon pathway in monocytes (Greiner-Tollersrud et al., 2020). This was indicated after an ADA2-deficient monocytic cell line, in comparison to wild-type cells, upregulated expression of many interferon- induced genes and this was also measured at protein level for CXCL10. ADA2, re-expression by lentiviral transduction restored the phenotype. Further to this in monocytes/macrophages, ADA2 is believed to be targeted to the lysosomes via a M6P-dependent pathway and may have alternative functions, aside from the adenosine deaminase function, as the lysosomal pH is highly acidic (Greiner-Tollersrud et al., 2020). Further evaluation of the active site in ADA2, in comparison to ADA1, as well as *in vitro* enzymatic assays, nuclease activity evaluation using FRET-based assays and modelling, uncovered a distinct ADA2 function: lysosomal DNase activity. This is reinforced by the clinical parallels

between DADA2 and DNase2 deficiency (Greiner-Tollersrud et al., 2020). Therefore, it has been proposed that in DADA2 there is attenuated degradation of dsDNA and that this is the stimulus for the type 1 interferonopathy recorded in DADA2 patients (Greiner-Tollersrud et al., 2020).

Carmona-Rivera and colleagues (2019) have demonstrated the role of ADA2 in neutrophil NET formation. The study demonstrated that DADA2 patients had elevated plasma levels of adenosine, and neutrophil NETs in the mesenteric arteries of the small bowel. *In vitro*, adenosine increased neutrophil NET formation, via A_1 and A_3 adenosine receptors, which was prevented by recombinant ADA2 (Carmona-Rivera et al., 2019).

Finally, *In vitro*, knock-out of ADA2 in endothelial cells stimulates IFN-β expression. This is thought to be a result of the increase in available extracellular deoxy-adenosine which is transported the cells and is catalysed by ADA1 in the cytosol (Dhanwani et al., 2020).

Aside from ADA2s function to breakdown extracellular adenosine, it is clear that ADA2 has effects independent of this. To date those roles include being a growth factor for T cells, differentiating monocytes to macrophages, preventing neutrophil NET formation and lysosomal DNase activity. ADA2 seemingly binds to many cell types but the influence of ADA2 on each cell type, the cell specific ADA2 receptors, and intracellular pathways, have not been fully elucidated.

1.2.3 Adenosine deaminase 2 in disease

ADA2 levels have been implicated as a prognostic and biomarker for other diseases aside from DADA2, especially those related to cardiovascular diseases. What is interesting is that both high levels, as well as deficiency (low or no active levels) of ADA2, are implicated in the pathogenesis of inflammatory diseases. This suggests a complex mechanism of regulation for this disease. Examples of adult rheumatic conditions where ADA2 is higher in disease, in comparison to healthy controls include rheumatoid arthritis, systemic lupus erythematosus and Crohn's disease (Maor et al., 2011; Saghiri et al., 2012; Sari et al., 2003; Valadbeigi et al., 2019). Furthermore, ADA2 is a biomarker for a life-threatening complication of systemic juvenile idiopathic arthritis (sJIA), macrophage activation syndrome (MAS) (Lee et al., 2020b). Other diseases where ADA2 levels have been studied include Tuberculosis (TB), Human Immunodeficiency Virus (HIV) and assessment of benign in comparison to malignant tumours.

For instance, ADA2 may be used as a biomarker for the diagnosis of tuberculous pleural effusion (Aghaei et al., 2005; Khodadadi et al., 2011; Li et al., 2014). ADA2's role in disease will be discussed further in chapter 5.

1.2.4 Summary

There are three adenosine deaminase proteins expressed in humans, of which two are characterised. Whilst there is some functional degeneracy, deficiency of either ADA1 or ADA2 gives rise to distinct pathophysiologies, signifying unique functions as well as their shared enzymatic activity. The mechanism for ADA1 deficiency has been well described, whilst the interactions and mechanisms of the ADA2 protein in terms of its inflammatory, immunological and haematological roles have not been fully elucidated. To date, ADA2 has been investigated primarily at a structural level, utilising knockdown in zebrafish, and knowledge of the expanding clinical phenotype of DADA2 patients. In addition to this, at a mechanistic level the interaction of ADA2 with neutrophils, T cells and monocytes has demonstrated niche functions. The observations particularly in knockdown zebrafish and co-cultures, have led researchers to suggest that ADA2 is a growth factor and vital for endothelial integrity (Zhou et al., 2014). Discovery of DADA2 has revealed a protein of unknown mechanism which must play a key role in homeostasis and the complex mechanisms of immunological and inflammatory response. However, empirical evidence to date is sparse and studies are needed to determine whether ADA2 can directly act upon endothelial cells; a key cell type mediating homeostasis in a vascular and immunological sense. It is unclear if the effect on endothelial cells is direct, or indirect, via other immune cells. This is turn raises the question about what the endothelial cell receptor for ADA2 might be and the contingent mechanistic pathways involved. The importance of the endothelium, the key cell type involved in vascular homeostasis and mediator of the body's immunological and inflammatory response, will now be discussed.

1.3 Vascular homeostasis

The vascular system is an intricate network of blood vessels; arteries, capillaries and veins, which are key to the delicate balance of cell homeostasis (Tennant and McGeachie, 1990).

1.3.1 Blood vessel structure

Most blood vessels are made up of three distinct main sections (Borysenko and Beringer, 1984). The structure, from outer-most layer in towards the lumen of the vessel, begins with the tunica adventitia. The tunica adventitia is primarily made of connective tissue. Nerve and lymphatic innervation are present, and in the larger blood vessels, the vaso vasorum provides the blood supply to the vessel. The subsequent layer, towards the lumen, is the tunica media, consisting predominantly of smooth muscle and elastic lamina. The external elastic lamina which provides structural support is frequently found in this layer. The inner most layer is the tunica intima made up of the basal lamina and the single layer of ECs, which are lumen facing (Borysenko and Beringer, 1984). Pericytes are embedded into the basement membrane in close proximity to the EC layer and have cytoplasmic processes which can surround EC cells (Sims, 1986). Pericytes cells can communicate with ECs by direct contact and paracrine signalling, as well as their structural function.

Across blood vessels, the thickness of the connective tissue and smooth muscle varies depending on the vessel type function and diameter (Shinaoka et al., 2013). However, the endothelial lining is always present, in fact capillaries and sinusoids have walls which are only endothelial cells and basal lamina, with dispersed pericytes (Borysenko and Beringer, 1984; Pugsley and Tabrizchi, 2000). Extending and remodelling of the network of blood vessels for tissues growth and repair is reliant on the activity of endothelial cells.

1.3.2 Endothelial structure

The vascular endothelium is the largest organ in the body, coating the inner side of all blood vessels, and acting as a barrier between the blood and the tissues. The number of EC in adult humans is thought to be 1-6x10^{13} cells, with a surface area of 1-7m^2 (Cines et al., 1998).

As described, endothelial cells are attached to the basal lamina, and the pair make up the vascular tunica intima. On a cellular level, EC are flat and slightly elongated. They are polarised cells with one side that is luminal- facing the blood constituents, and in opposition the basolateral surface, which is connected to a glycoprotein basement membrane, secreted by the endothelial cells themselves (Krüger-Genge et al., 2019). The luminal surface structure consists of the glycocalyx whose sugar branches protrude into the lumen (Luft, 1966; Reitsma et al., 2007). Expression of various cell membrane receptors are highly regulated including PECAM-1, Ang-1 and Ang-2 (Féraud et al., 2003; RayChaudhury et al., 2001).

1.3.2.1 Inter-endothelial junctions

Between cells there are inter-endothelial junctions (IEJ) which consist of tight junctions, gap junctions and adherens junctions as depicted in Figure 1.4. Tight junctions are made up of occludin, claudins and junctional adhesion molecules. On the other hand, gap junctions comprise of connexins, and VE-cadherin is necessary for creating adherens junctions. On the basolateral surface there are Integrins which ground the EC to the basal lamina or extracellular matrix (ECM) by interactions with matrix proteins vitronectin and fibronectin. The adhesion of IEJ and EC to the ECM produces a tight barrier and quiescent state (Bazzoni and Dejana, 2004).

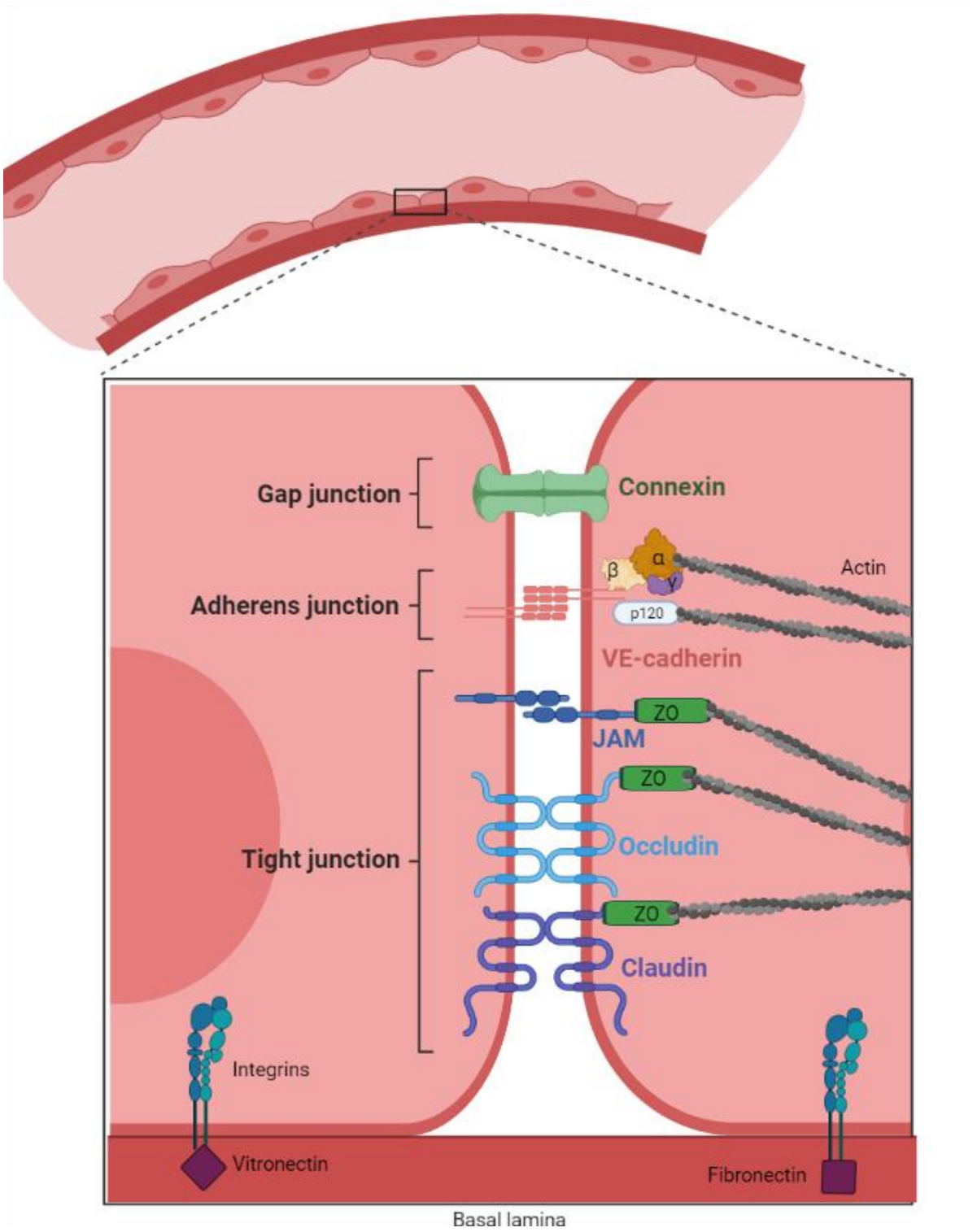


Figure 1.4 A schematic diagram of endothelial intercellular junctions

Inter-endothelial junctions are made up of tight, gap and adherens junctions which interact with the actin cytoskeleton. Adapted from (Sukriti et al., 2014) and 'Created with BioRender.com'. **Abbreviations: JAM-** junctional adhesion molecule, **ZO-** Zonula Occludens.

1.3.2.2 The glycocalyx

A prominent part of the luminal facing EC structure is the glycocalyx. Using transmission electron microscopy, the glycocalyx can be seen on the surface of the endothelium (Luft, 1966). The glycocalyx is an ever-remodelling, gel-like layer, which works like a sieve; made up of long chains of carbohydrates and soluble molecules, forming a mesh between the blood and endothelial surface (Reitsma et al., 2007). The glycocalyx, is made up of negatively charged proteoglycans, which are membrane bound core proteins, with unbranching chains of glycosaminoglycans (GAGs), and glycoproteins along with other soluble molecules, as illustrated in Figure 1.5 (Reitsma et al., 2007; Weinbaum et al., 2007). The thickness of this layer is dependent on vessel type and shear rate and the vascular bed (Jeansson and Haraldsson, 2006; van den Berg et al., 2006; van Haaren et al., 2003; Vink Hans and Duling Brian R., 1996).

Proteoglycan core protein groups include Syndecan, Glypican and Perlecan, each with many subtypes and linking different numbers of GAG chains (Rosenberg et al., 1997). There are five different GAGs; chondroitin sulphate, heparan sulphate (HS), hyaluronic acid, keratin and dermatan sulphates, with HS being the predominant type (Fullwood et al., 1996; Lee and Spicer, 2000; Oohira et al., 1983; Trowbridge and Gallo, 2002). These GAG chains covalently link to the proteoglycans, except hyaluronic acid which is attached via cell membrane receptors (CD44) (Lee and Spicer, 2000).

In contrast, the glycoproteins have short, branched carbohydrate side-chains and expression level varies widely across the endothelium depending on cell activation (Reitsma et al., 2007). There are three main classes of glycoproteins which are also EC adhesion molecules, the selectin and the integrin families as well as the immunoglobulin superfamily (Müller et al., 2002a; Sperandio, 2006; Xiong et al., 2003).

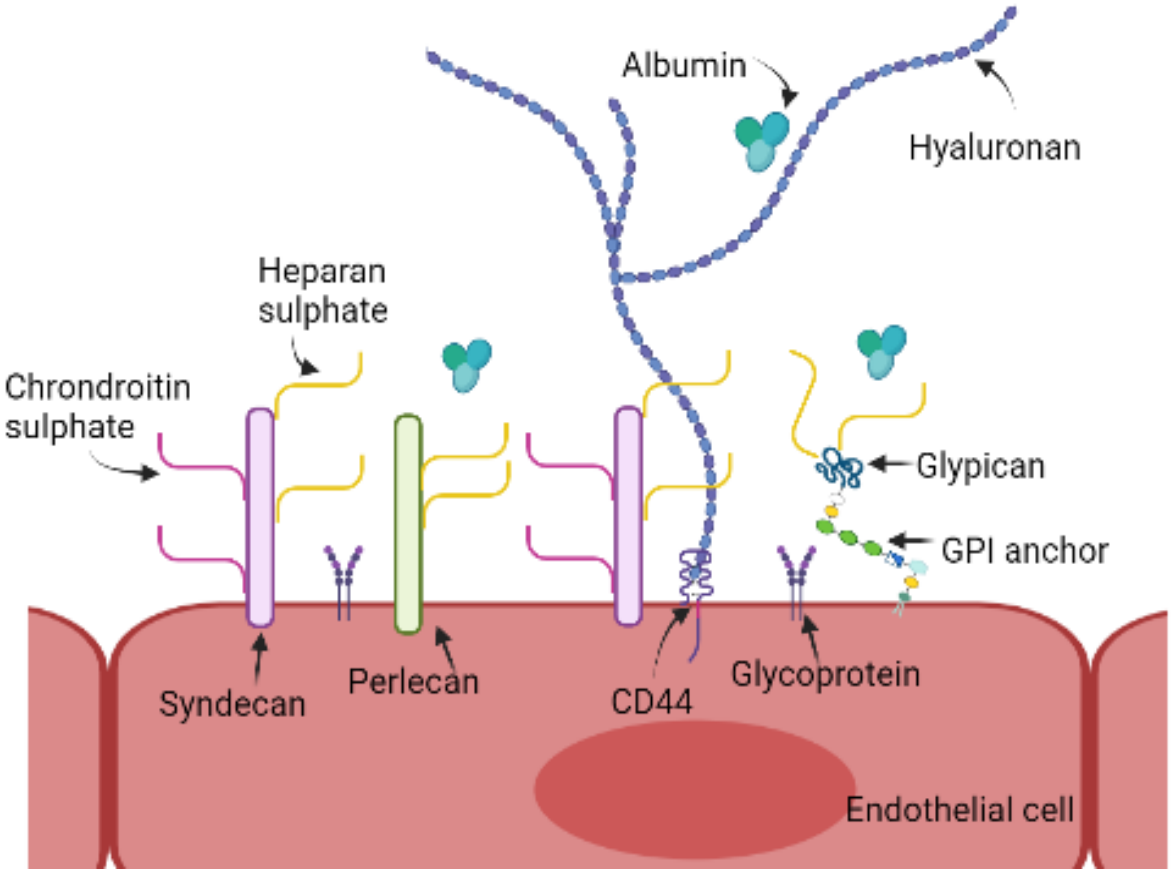


Figure 1.5 A schematic diagram of the endothelial glycocalyx

The structure of the endothelial glycocalyx. Adapted from (Yilmaz et al., 2019) and 'Created with BioRender.com'.

Research to date, shows that, *in vivo*, healthy vascular endothelial cells, must have an intact and functional glycocalyx. For example, knockout of SDC-1 in mice causes a proinflammatory endothelial phenotype (Voyvodic et al., 2014). The glycocalyx layer functions include;

- acting as a sensor and mechanotransducer for sheer stress,
- a modulator of permeability,
- forming a vascular barrier between the blood cells and endothelium,
- modulating adhesion of leukocyte and platelets,
- may also bind enzymes that metabolise oxygen radicals such as superoxide dismutase (Reitsma et al., 2007; Weinbaum et al., 2007; Yilmaz et al., 2019).

Overall, the presence of the glycocalyx aids the endothelium's homeostatic and anti-thrombogenic role.

Conversely, degradation or shedding of the glycocalyx is linked with endothelial cell dysfunction and pathologies such as diabetes, atherosclerosis and sepsis (Cancel et al., 2016; Lewis et al., 1982; Nelson et al., 2008; Nieuwdorp et al., 2006b, 2006a; van den Berg et al., 2006). Shear stress, oxidative stress in disease states, and/or substances such as TNF-α, high levels of adenosine, and enzymes such as heparanase (and its bacterial counterpart heparinase) and hyaluronidase, bring about shedding of the glycocalyx (Brands et al., 2013; Chappell et al., 2009a, 2008; Henry and Duling, 2000; Li and Wang, 2018; Nieuwdorp et al., 2007; Rubio-Gayosso et al., 2006). Overall studies have demonstrated that, deterioration of the glycocalyx will exacerbate inflammation, adhesion of leukocyte, cause oedema and loss of flow-dependent vasodilation (Bruegger et al., 2008; Jacob et al., 2007; Vink et al., 2000).

There has been limited investigation on substances, or approaches, to prevent shedding or restore the function of the glycocalyx. It is believed that *in vivo* glycocalyx recovery is 5-7 days whilst *in vitro* is 1-2 days, depending on flow conditions (Giantsos-Adams et al., 2013; Potter et al., 2009). However, finding methods to restore the glycocalyx may help treat chronic vascular and inflammatory diseases where glycocalyx shedding is correlated with endothelial cell dysfunction (Yilmaz et al., 2019).

An example of an agent which has been explored in terms of glycocalyx restoration, is Sulodexide. Sulodexide is a mixture of heparin sulphate and dermatan sulphate and has shown promise in animal models by improving glycocalyx recovery, decreasing vascular permeability and reducing damage and local inflammation (Li et al., 2017; Song et al., 2017). In human clinical trials there has been mixed success, where sulodexide failed to decrease urine albumin excretion in patients with Type 2 Diabetes and microalbuminuria (Lewis et al., 2011). However, another study has reported an partial restoration with sulodexide administration, of vascular permeability and glycocalyx layer associated with type 2 diabetes in humans (Broekhuizen et al., 2010). Aside from sulodexide, other approaches have been used, predominantly observed in animal models, albumin, fresh frozen plasma, glucocorticoids, TNF-α inhibitors, Nitric Oxide (NO), hyaluronan, heparin and adenosine agonists (Bruegger et al., 2008; Chappell et al., 2009a; Haywood-Watson et al., 2011; Jacob et al., 2009; Nieuwdorp et al., 2009; Platts et al., 2003; Rubio-Gayosso et al., 2006).

What is uncertain is whether the glycocalyx binds hormones, cytokines, chemokines and other signalling molecules, and if so, whether it enhances, or sequesters their activity (Li et al., 2014; Lortat-Jacob et al., 2002; Renné et al., 2000; Yu and Woessner, 2000). Moreover, what is yet to be studied is the pharmacological implications of the glycocalyx on drug action (Becker et al., 2015). Another interesting observation, which is yet to be fully described, is that there is very dense glycocalyx at the interface between maternal and foetal tissue in the placenta, on the villous surface. However, there is no glycocalyx in the fetal capillaries (Hofmann-Kiefer et al., 2013).

Current investigation of the glycocalyx has been more problematic *in vitro* and this has been discussed by (Haymet et al., 2021). This is because current endothelial cell models produce far less/no detectable glycocalyx, in comparison to what is found physiologically or *ex vivo* (Chappell et al., 2009b; Potter et al., 2009). Explanations for this include fixation methods leading to degradation of glycocalyx, flow conditions, the use of blood products or cell culture media and selection of EC (Chappell et al., 2009b; Dong et al., 2002; Fu and Tarbell, 2013; Urner et al., 2012). Due to the lack of knowledge about how the glycocalyx is involved in endothelial or immune cell signalling, or interaction with drugs, and how to accelerate restoration, finding an appropriate, and physiologically relevant, model for endothelial glycocalyx, is imperative. To date, popular *in vitro* methods have involved using rat fat pad and bovine aortic EC, or, as a human model, there has been mixed success with Human umbilical vein endothelial cells (HUVEC), where *in vitro* these cells are reported to express little glycocalyx in comparison to *ex vivo* tissues or no detectable levels (Giantsos-Adams et al., 2013; Haymet et al., 2021; Mensah et al., 2017; Potter et al., 2009; Thi et al., 2004; Yao et al., 2007; Zeng et al., 2012; Zeng and Tarbell, 2014).

1.3.2.3 Endothelial cell phenotype

Healthy endothelium has an anti-inflammatory, anti-angiogenic phenotype, expressing high levels of NO, prostacyclin and low levels of reactive oxygen species (ROS) and uric acid. In a quiescent state ECs express major histocompatibility class I (MHC I) (Lozanoska-Ochser and Peakman, 2009). *In vitro,* the culture of primary ECs will exhibit a cobblestone morphology for monolayers and can be identified by constitutively expressed cell surface markers. EC marker profile includes markers such as, platelet endothelial cell adhesion molecule (PECAM-1, CD31), VE-cadherin (CD144), angiotensin-converting enzyme (ACE, CD143) and Factor VIII-

related antigen (Breier et al., 1996; Müller et al., 2002b; Ryan et al., 1976). It is important to note that these are not exclusively expressed on endothelial cells, CD31 is also expressed on leukocytes, VE-cadherin has been identified on fetal hematopoietic stem cells, and CD143 is present on hematopoietic stem cells (Fleming, 2005; Jokubaitis et al., 2008; Stockinger et al., 1990). von Willebrand factor (vWF) can serve as a cytoplasmic endothelial cell marker and has been deemed a universal marker of EC, even if it is not expressed uniformly across EC (Jaffe et al., 1974, 1973). The lack of exclusivity of endothelial markers with other cell types illustrates the importance of using a panel of markers in order to reliably characterise endothelial cells (Fleming, 2005).

Inflammatory activation can switch the endothelial cell phenotype to a pro-inflammatory, coagulative and angiogenetic state. Under these conditions ECs can express MHC I and produce ROS. Inducible endothelial markers, are also expressed in response, for example, ICAM-1 (CD54), NCAM-1 (CD106), E-selectin (CD62E), P-selectin (CD62P), Flt-1 (VEGFR1), KDR (VEGF2) and Tie-2 (Müller et al., 2002b).

In addition to the markers already introduced there are markers which are specific to the endothelial cells within each organ, organ-associated antigens, and these will be discussed in the EC heterogeneity section (Ribatti et al., 2020).

1.3.3 Endothelial function

Until the 1970s this was described as an 'inert layer'. But it is now known that this layer has many functions aside from a simple barrier function; it is an endocrine organ and plays a pivotal role in homeostasis, responding to physical, chemical and environmental stimuli (Krüger-Genge et al., 2019).

1.3.3.1 Barrier function

The endothelium tightly controls the fluid, solute and cell exchange between the blood and the surrounding tissues and this movement is a process called extravasation (Wettschureck et al., 2019). This is a highly regulated barrier with different mediators working under either homeostatic or inflammatory conditions to control the barrier or increase the permeability. There is heterogeneity displayed between EC from different vascular beds and barrier structure and function, for example the blood brain barrier in comparison to the EC in the renal glomeruli which has fenestrae which facilitate transport (Aird, 2007).

There are two main forms of transport across the endothelium, transcellular vesicle transport by transcytosis, or, by paracellular movement and trans-endothelial cell migration (Wettschureck et al., 2019). Cells and solutes can pass through the barrier via vesicles in transcellular pathways (Komarova and Malik, 2010). Caveolae-mediated transcytosis is used mainly for albumin transport, whereas vascular endothelial growth factor (VEGF) and other inflammatory mediators, are thought to be transported via vesiculo-vacuolar organelles (Dvorak et al., 1996; Schnitzer and Oh, 1994). Although most cells move via the paracellular route, it has been reported that some cells move via a transcellular extravasation route but the molecular mechanisms are not clear (Feng et al., 1998). Paracellular mechanisms involve movement between EC, when there are alternations in cell-cell junctions and opening of IEJ (Szymborska and Gerhardt, 2018).

Under basal conditions the EC barrier has low permeability for fluid and solutes but under inflammatory conditions there is a localised permeability (Majno and Palade, 1961). Loss of barrier functionality is part of the pathology for various vascular disorders such as ischemic stroke, pulmonary oedema and, cancer and metastasis (Lee et al., 2007; Miyahara et al., 2007; Wang et al., 2007). To maintain the EC barrier integrity, there are several signalling processes which include; shear stress and notch, fibroblast growth factor, sphingosine-1-phosphate,

Angiopoietin-1/Tie2, prostacyclin (PGI_2) and prostaglandin E2 (Birukova et al., 2007; David et al., 2011; Hatanaka et al., 2012; Lee et al., 1999; Polacheck et al., 2017).

1.3.3.2 Vascular tone

The endothelium, along with the smooth muscle cells (SMC), control the amount of blood flow to the tissues and respond to changes in the tissue oxygen and metabolic needs. This is achieved by regulation of vasodilation and vasoconstriction. The main agents involved in vasodilation are NO and PGI_2 (Palmer et al., 1987). EC synthesise NO subsequent to stimulation by shear stress, and in response to molecules such as, acetylcholine and histamine (Chen et al., 1996; Lantoine et al., 1998; Rubanyi et al., 1986). EC also produce NO to maintain basal tone. NO diffuses into nearby SMCs which brings about vasodilation by stimulating the soluble guanylyl cyclase and cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) pathway (Furukawa et al., 1991; Sampson et al., 2001).

NO is produced by an enzyme, nitric oxide synthase (NOS), from L-arginine (see Figure 1.6). There are three isoforms of NOS, neural NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3). NOS1 is expressed in central and peripheral neurons, NOS2 can be induced in a number of cells types after stimulation and NOS3 is predominantly expressed by ECs. Asymmetric dimethylarginine (ADMA) inhibits NOS and the production of NO and elevated levels of ADMA are found in cardiovascular disease (Birukova et al., 2007; Böger et al., 2000; Goonasekera et al., 1997; Miyazaki et al., 1999; Vallance and Leiper, 2004). ADMA is produced by the action of protein arginine methyltransferases (PRMTs) (Clarke, 1993; McBride and Silver, 2001). On the other hand, ADMA is metabolised by dimethylarginine dimethylaminohydrolase (DDAH), of which there are two isoforms; 1 and 2 (Kimoto et al., 1995; Leiper et al., 1999; Ogawa et al., 1989). It is believed that DDAH2 is the predominant form in the vasculature and decreased DDAH2 is associated with cardiovascular disease (Lambden et al., 2015; Tran et al., 2000).

Another EC derived vasodilator is PGI_2. PGI_2 causes relaxation of SMC via an adenylate cyclase (AC) mechanism and increase of cyclic adenosine monophosphate (cAMP) (Dembinska-Kiec et al., 1979). PGI_2 is constitutively produced by EC and also considered to maintain basal tone but its release can also be stimulated by thrombin, histamine, ATP and bradykinin (McIntyre et al., 1985; Moncada et al., 1977; Weksler et al., 1978).

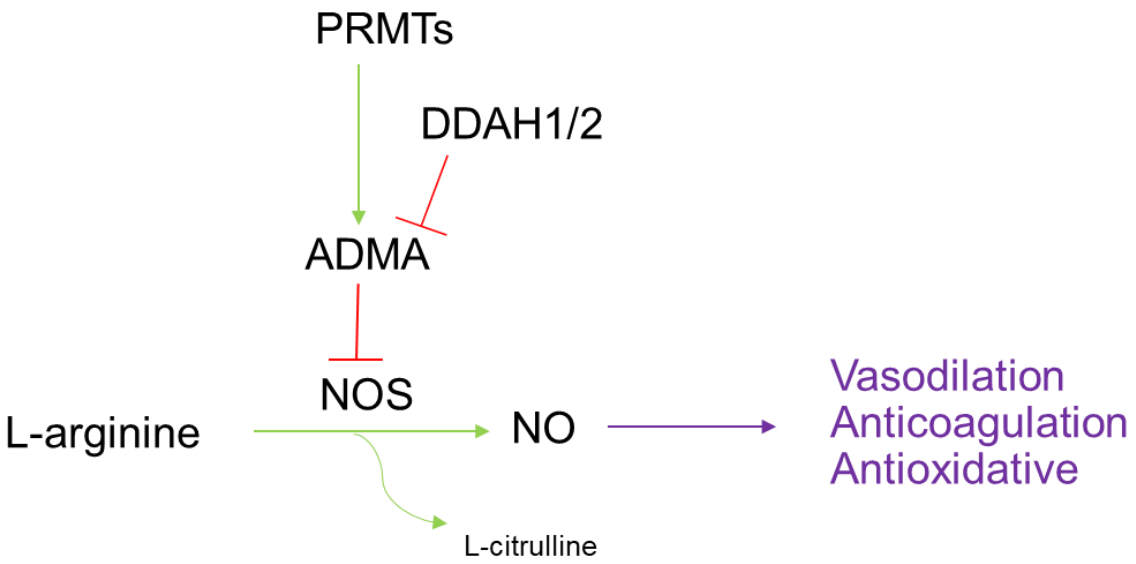


Figure 1.6 A schematic diagram of the NO/ADMA pathway

NO has vasoprotective functions and is produced by the action of NOS. NOS is regulated by the endogenous NOS inhibitor ADMA. This in turn is regulated by the enzyme DDAH, of which are two isoforms 1 and 2. DDAH metabolises ADMA. PRMTs catalyse arginine methylation and the formation of ADMA. **Abbreviations**- **ADMA:** asymmetric dimethylarginine, **DDAH:** dimethylarginine dimethylaminohydrolase, **NO:** nitric oxide **NOS:** nitric oxide synthase, **PRMTs:** protein arginine methyltransferases. Adapted from (Böger et al., 2005; Cooke, 2004).

When NO and PGI_2 production is therapeutically inhibited, vasodilation can still occur experimentally, and this is thought to be down to endothelium-derived hyperpolarization factor (EDHF), also of EC origin (Scotland et al., 2005). EDHF's effect is thought to be more prominent in smaller rather than larger vessels (Luksha et al., 2010).

In contrast, factors produced by ECs can also cause vasoconstriction, which is important in inflammation. This includes; endothelin-1 (ET-1), angiotensin II, prostaglandin H_2 (PGH_2) and reactive oxygen species (ROS) (de Gasparo et al., 2000; Martinez-Lemus et al., 2011; Tesfamariam, 1994; Yanagisawa et al., 1988).

1.3.3.3 Haemostasis and thrombosis

Haemostasis is a process to stop bleeding, and keep blood within a damage vessel, commencing the process of wound healing. Under homeostatic conditions EC release of NO and PGI_2 prevent platelet aggregation by increasing platelet intracellular cAMP (de Graaf et

al., 1992; Moncada et al., 1977). ATP and ADP both stimulate platelet aggregation. To prevent thrombosis in response to this, EC express ectonucleotides which hydrolase ATP and ADP to AMP and adenosine to decrease platelet adherence (Michal and Thorp, 1966). There are other anti-coagulant pathways that ECs support to prevent thrombosis such as the protein C/protein S pathway and the thrombomodulin (Esmon et al., 1982; Stern et al., 1986).

After vessel injury, and blood components coming into contact with collagen or vWF, they become activated and aggregate and trigger the formation of a blot clot, to prevent blood loss (Krüger-Genge et al., 2019). Platelets, once activated themselves, can activate ECs by releasing mediators including ADP and 5-HT (Cerrito et al., 1993). This process is highly regulated to prevent thrombosis (Mackman, 2012).

1.3.3.4 Angiogenesis

Angiogenesis is the process by which EC can form new blood vessels from existing ones in response to injury or hypoxic conditions (Folkman, 1971; Krupinski et al., 1994; Shweiki et al., 1995). There are two types of angiogenesis that occur in adults: sprouting angiogenesis and intussusceptive angiogenesis (Adair and Montani, 2010). Sprouting is stimulated by VEGF-A through the ECM and also δ-Notch signalling (Ruhrberg et al., 2002; Suchting et al., 2007). Intussusceptive angiogenesis where one vessel splits into two (Burri and Tarek, 1990). The angiogenesis process is highly controlled multi-step process with initial processes, degradation of the basal lamina, migration of ECs, chemotasis EC proliferation, lumen formation or tublerogenesis, and pericyte stabilisation (Duran et al., 2017). The angiogenic process can be both physiological – wound healing, and pathophysiological- cancer, inflammatory disorders, or diabetes mellitus (Barrientos et al., 2008; Kobayashi and Lin, 2009; Ribot et al., 2017).

1.3.3.5 Immune system interaction and function

ECs can actively participate in both innate and adaptive immune responses (Shao et al., 2020). Nonspecific immunity, innate immunity can be mediated by ECs, which is an immediate response, independent of antigens. In fact, EC perform 11 innate immune system roles that are also performed by macrophages including cytokine secretion, migration, pathogen-

associated molecular patterns (PAMPs) sensing and danger-associated molecular patterns (DAMPs) sensing; fuelling the debate as to whether ECs innate immune cells (Mai et al., 2013).

When inflammation or injury occurs, ECs attract immune cells locally. Inflammatory cytokines are produced by ECs, triggering the leukocyte migration and infiltration cascade (Ley et al., 2007). Monocyte chemoattractant protein -1 (MCP-1) is an example of an endothelial chemoattractant (Uguccioni et al., 1995). Leukocyte recruited will then undergo leukocyte rolling, adhesion, arrest and transmigration. This is facilitated by EC adhesion surface markers which are upregulated under inflammatory stimuli, such as vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) and CD62e, CD62p (Brown et al., 2001; Ley, 2001).

MHC II has also been found to be expressed on ECs, taking a role in antigen presentation and can also express co-stimulatory molecules for T cell activation such as CD80, highlighting the potential of ECs to contribute to the adaptive immune response (Kreisel et al., 2002; von Willebrand et al., 1985).

1.3.4 Heterogeneity

The endothelial structure and functions so far have demonstrated how important these cells are in homeostasis as well as pathogenesis. Though, it is important to put this in the context of EC heterogeneity shown within the body. Heterogeneity between ECs occurs at a morphological, functional, gene expression and antigen composition levels (Aird, 2012). As well as across organs and the vascular tree, heterogeneity can occur within the same organ and blood vessel type.

Structurally, an example of the heterogeneity is EC thickness, which varies from less than 0.1µm in the capillaries, to 1µm in the large arteries such as the aorta (Florey, 1966). Another difference is there is flow-dependent alignment of EC at many parts of the vascular tree, but not at branch points (Passerini et al., 2004). Furthermore, capillaries fall into three types, continuous, fenestrated or discontinuous (Aird, 2007). Discontinuous endothelium are only found in the liver; in parts of the sinusoidal bed. Fenestrated endothelium are similar to discontinuous, but have a larger diameter, they lack a diaphragm and can have a fragile

basement membrane. This type of endothelium is found in organs such as exocrine or endocrine glands and glomeruli, those organs which secret or filter (Florey, 1966).

Permeability of ECs also varies. The process of transcytosis is mediated by the caveolae and vesiculo-vacuolar organelles (VVOs). An example of heterogeneity for this EC function is that the caveolae are in far higher numbers per cell in the capillary endothelium, about 10,000 per cell, with the exception of the blood-brain barrier where caveolae are significantly reduced, than those found in all other types of vessel. The endothelium of the veins and venules are where VVOs are most conspicuous (Simionescu et al., 2002).

Another example of functional heterogeneity, is within the leukocyte trafficking steps. Firstly, this process is a site-specific, occurring in postcapillary venules (Aird, 2007). One of the adhesion molecules mediating the leukocyte rolling, is CD62E. This is a marker which is only expressed in activated endothelium and this activation is limited to postcapillary venules (Feuerhake et al., 1998).

1.3.5 Endothelial repair

Endothelial cells have the ability to respond to injury by stopping haemorrhaging and repairing, to prevent long-term dysfunction. This was discussed in the haemostasis and thrombosis, and angiogenesis sections. However, there is another means by which the endothelium repairs itself and that is via endothelial progenitor cells (EPCs). Until relatively recently it was thought that angiogenesis occurred only by the current EC dividing and migrating. However, since 1997, the discovery of a population of circulating CD34+ cells in the blood, termed EPCs, has challenged this (Asahara et al., 1997). In support of this, mathematical modelling of endothelium maintenance, and tumour angiogenesis, highlights the necessity of progenitive ECs in the maintenance of vascular health, during a human lifespan (Buijs et al., 2004; Stoll et al., 2003). Replacement of ECs by mere cell division, is not enough due to telomere shortening and senescence, and simply by angiogenesis or cell division alone, would lead to severe vascular wall damage (Buijs et al., 2004). Modelling predicts that these effects are overcome by ECs from elsewhere (Buijs et al., 2004).

In vitro, EPCs have been shown to be endothelial cell-like and stimulate vascular repair and angiogenesis in some experimental settings (Clarke et al., 2010). Since then, there has been

much progress made in defining EPC populations and correlating them with vessel repair and patient phenotypes. Isolated ECs from peripheral blood are increased in disease or mechanical damage of the vasculature, and ex vivo display abnormal EC function (Clarke et al., 2010; Fabbri-Arrigoni et al., 2012; Liu et al., 2007; Rigato et al., 2016; Smadja et al., 2014, 2013; Solovey et al., 1997). EPCs are being employed as a readily available source of ECs for research. ECs arising from peripheral blood are a far more readily available source of ECs, especially for isolation and study of human ECs. This is a particularly important consideration when studying the paediatric population.

1.3.5.1 Endothelial cell progenitor cells

Isolating EPCs from peripheral blood of patients, has circumvented the experimental limitation of retrieving ECs from patients. These ECs, can be isolated from peripheral blood mononuclear cells (PBMNCs) (Lin et al., 2000). Their subsequent culture in endothelial growth media supplements with endothelial growth factors, can be divided into two main types of cells, 'early EPCs' and 'Blood Outgrowth Endothelial cells' (BOECs) (see Figure 1.7) (Hebbel, 2017; Medina et al., 2017). The first forms within 7 days in culture. Whereas the BOECs, also known as 'late EPCs', 'progenies of endothelial forming colony cells' or 'endothelial colony–forming cells (ECFC)', usually form between 7-21 days. Studies have stained the different cells types in these cultures grown under these conditions. Early EPCs do express low levels of CD31 and vascular endothelial growth factor receptor 2 (VEGFR2), which are typical endothelial markers, however, they also express monocytic cells markers such as CD14. Isolation of EPCs from patients, shows a correlation between vasculature health and number of EPC colony forming units (Rigato et al., 2016). Continuing culture after 7 days, induces a secondary colony of cells with characteristic endothelial cobbled shape morphology. These BOECs, have typical mature EC surface marker expression including; CD31, CD144, vWF and lack of monocytic markers CD14, CD45 (Gulati et al., 2003; Ormiston et al., 2015).

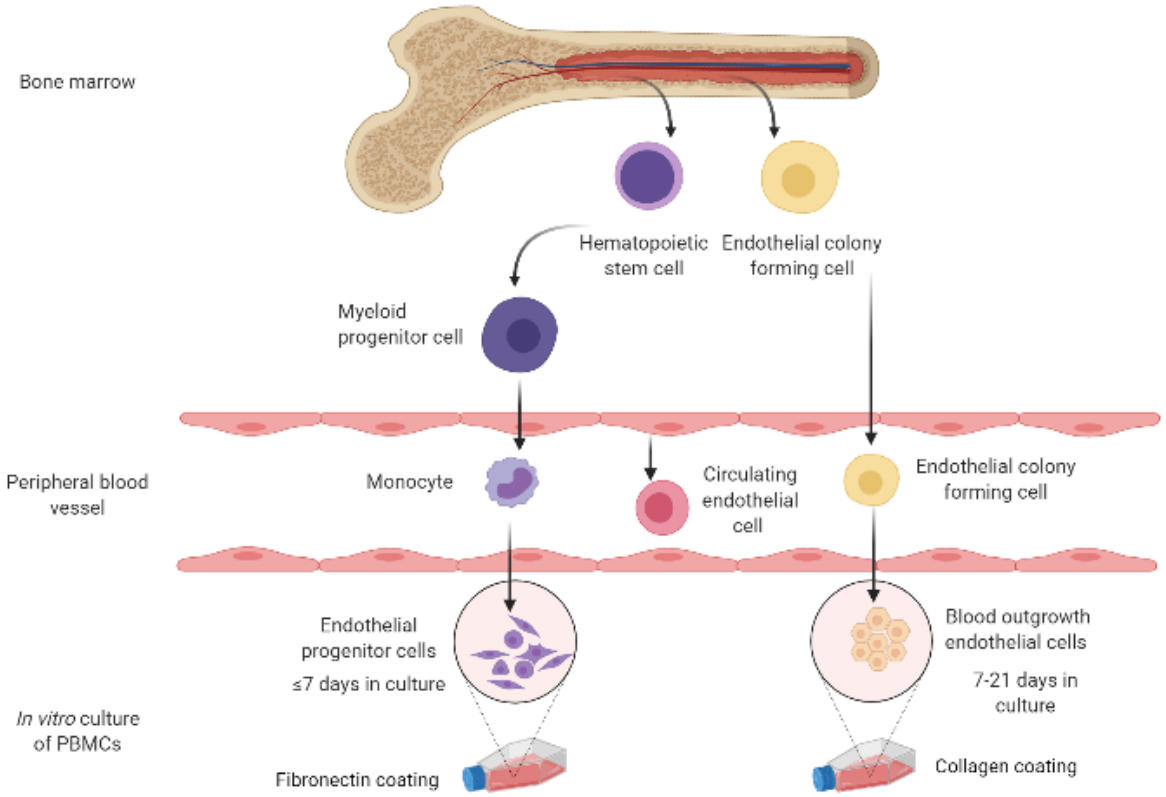


Figure 1.7 A diagram to show the origins of blood endothelial cell types

Blood outgrowth endothelial cells (BOECs), as mature endothelial cells, seem to be the progeny of endothelial colony forming cells (ECFC) which are found in both the circulation and vessel wall. In contrast endothelial progenitor cells (EPCs) arise from hematopoietic stem cells. Circulating endothelial cells (CEC) arise from the vessel wall as a consequence of injury or disease states. *In vitro* culture of peripheral blood mononuclear cells (PBMCs) causes formation of two different cell types. *In vitro* culture with collagen coating causes formation of BOEC colonies whereas, fibronectin coating causes EPC colony formation. Adapted from (Hebbel, 2017). Created with BioRender.com

1.3.6 Endothelial dysfunction

It is normal for endothelial cells to become activated under certain conditions, and it is in fact part of the endothelium's role to repair vascular tissue when damaged and to stop bleeding. However, there is a delicate balance that exists between an activated and quiescent EC state. When the balance is tipped too far in the direction of activation, under chronic exposure to risk factors, the endothelium may become dysfunctional and contribute to atherothrombosis, thrombosis (Schächinger et al., 2000; Verma and Anderson, 2002). As discussed previously, the endothelium is a key mediator in both the immune and cardiovascular systems, endothelial dysfunction is part of the aetiology of a range of diseases.

1.3.6.1 Inflammation

EC are essential cells in regulating and participating in the inflammatory process (Pober and Sessa, 2007). There are two main types of inflammatory activation (Pober and Cotran, 1990). Type 1 activation mediated by ECs, are quick, and concluded within 10-20 mins and receptor desensitisation concludes this activation (Pober and Cotran, 1990; Winsauer and Martin, 2007). This type of activation is independent of protein synthesis produces an increase in blood flow, adhesion of activation of neutrophils and extravastion into an inflammatory site, increase in permeability of the endothelium and movement of plasma proteins into the tissue (Lorant et al., 1991; Pober and Sessa, 2007; Teixeira et al., 1993). In contrast, Type II activation of ECs, requires protein synthesis, is slower onset, but can persist for hours or days (Pober and Cotran, 1990). This is mediated by pro-inflammatory cytokines, such as TNF or IL-1, ECs can go beyond to recruit monocytes and T cells, after neutrophils (Martin and Wesche, 2002). Type II activation also involves an increase in blood flow and leakage of the plasma proteins (Petrache et al., 2003; Pober et al., 1987; Zavoico et al., 1989). Type II activation persists until the resolution of the inflammatory stimulus (for example infection) and therefore the lack of production of inflammatory and activating cytokines (Winsauer and Martin, 2007).

If the type I and type II responses fail to resolve the inflammatory stimulus, then the inflammatory process will move from acute to chronic inflammation. EC may contribute to this process by presenting antigens to T cells and involvement in the adaptive immune response (Pober et al., 1986). IFNγ induced changes in the endothelium, such as producing chemokine CXCL10, and expression of adhesion molecules such as E-selectin and VCAM1,

recruiting T cells including T-helper cells (Austrup et al., 1997; Doukas and Pober, 1990; Fukuda et al., 1996; Luster et al., 1985).

If the adaptive immune system fails to resolve the inflammatory stimulus, eradicating the pathogen, then the next stages of chronic infection involve angiogenesis (discussed previously) and the development of tertiary lymphoid organs. These process may become prominent in inflammatory associated diseases such as rheumatoid arthritis (Paleolog, 2002).

1.3.6.2 Atherosclerosis

Any damage to the endothelium which can cause an imbalance between the vasodilation and vasoconstriction can trigger, or exacerbate, the events leading to atherosclerosis. In the presence of atherosclerosis, endothelial dependent vasodilation is hindered (Ludmer et al., 1986). Atherosclerosis is the deposition of fatty deposits in the intima layer of arteries (Libby et al., 2019). Over time this lipid material found in the intima can form an atherosclerotic plaque, or atheroma, which can become calcified and fibrous (Bentzon et al., 2014). The atheroma can stimulate thrombus formation, that can cause ischemia by obstruction of the lumen. Another way the lumen can be occluded is in advanced plaques which can encroach into the lumen of the blood vessel, decreasing blood flow and causing tissue ischemia. When atherosclerosis occurs in the circulation of the heart myocardial infarction or other chronic cardiovascular diseases (CVD) can be provoked. This atherosclerosis process is the cause of many ischemic strokes and other conditions such as the formation of aneurysms (Huang et al., 2001). In the peripheral arteries, atherosclerosis can be the causative agent of ulceration and gangrene (Libby et al., 2019). Despite preventative measures, CVD are the primary cause of death globally (WHO, 2021).

There are many stages in the atherosclerosis course. Atherosclerosis initiation is due to the cumulative exposure of excess LDL cholesterol (LDL-C) concentrations above physiological requirements, over time (Ference et al., 2017). Accumulation of LDL-C in the intima undergoes oxidation. Circulating classical monocytes with pro-inflammatory propensities, which are attracted to the vessel wall by chemoattractants, enter the intima, via adhesion with, and migration through, endothelial cells. Monocytes within the intima, can differentiate into macrophages and bind lipoprotein particles which via expression of scavenger receptors. Thus, become foam cells (Galis et al., 1995). In addition to this, T cells enter the intima and this accumulation of leukocyte in the region, and the release of a chemoattractant by

macrophages, can cause migration of SMCs from the tunica media into the intima (Bennett et al., 2016).

Progression of the atherosclerotic lesion is prompted by the SMCs and macrophages becoming entwined with the lipid. Other factors that are involved in the progression of atherogenesis include, smoking and hypertension, and inflammation (Chow et al., 2010; Gottesman et al., 2014). Inflammation itself, can alter the way that endothelial cells behave and promote angiogenesis disease progression. For example, angiotensin, part of the pathogenesis of hypertension, can trigger NF-κB beta inflammatory pathways (Kranzhöfer et al., 1999). Cytokines, released from sites of inflammation, can also promote atherosclerosis develop at other sites in the vascular system (Libby et al., 2016). Inflammation can change the production of nitric oxide and other vasodilators by endothelial cells (Ignarro and Napoli, 2005). Another way that endothelial cells can be activated is through a diet rich in cholesterol which stimulates the production of adhesion molecules (e.g. VCAM-1) and chemoattractants of EC origin (Cybulsky and Gimbrone, 1991; Li et al., 1993).

1.3.6.3 Vasculitis

Vasculitis is a term which encompasses systemic inflammation of the blood vessels, which culminates to organ dysfunction (Jennette et al., 2013). Vasculitis has traditionally been split into categories dependent on the size of the vessel involved; small, medium or large (Shavit et al., 2018). Examples of each of these categories include; ANCA-associated vasculitis under small vessels, PAN affecting the medium vessels and Takayasu arteritis (TA) large-vessel vasculitis (Shavit et al., 2018). In 2008, a paediatric vasculitis specific classification was proposed by The European League Against Rheumatism/Paediatric Rheumatology International Trials Organisation/ Paediatric Rheumatology European Society in Ankara (Arend et al., 1990; Leavitt et al., 1990; Lightfoot et al., 1990; Mills et al., 1990; Ozen et al., 2010). A revised nomenclature system was proposed in 2012 by the 'International Chapel Hill Consensus Conference Nomenclature of Vasculitides' (Jennette et al., 2013). However, as some of the classification criteria has never been validated in children, paediatricians have also collaborated to develop consensus using the 'Single Hub and Access point for paediatric Rheumatology in Europe' (SHARE) (de Graeff et al., 2019a, 2019b; Ozen et al., 2019).

Definitive causative agents for vasculitis remain unknown for most patients, however, it is thought that a combination of environmental triggers such as infections, and genetic susceptibility lead to the ensuring inflammation and damage to the endothelium observed (Brogan, 2007; Lidar et al., 2009). Most cases of vasculitis are facilitated by the accumulation of immune complexes within the cell wall, causing mast cell degranulation, neutrophil chemotaxis, which results in free oxygen radicals and proteolytic enzymes being release, and pro-inflammatory mediators resulting in injury to the vessel wall – including the endothelium (Schnabel and Hedrich, 2019; Yang et al., 2008).

Environmental triggers include infectious agents, as is speculated for Henoch-Schönlein purpura (HSP), also known as IgAV, and Kawasaki disease (KD), and non-infectious agents considered are silica, solvents and exposure to heavy metals, many classes of drugs and withdrawal of corticosteroids (Brogan, 2007; Eleftheriou et al., 2009; Kang et al., 2014; Stratta et al., 2001).

Genetic factors for vasculitis susceptibility are complex and many candidate genes have been identified. As paediatric vasculitis is rare, this confounds the genome exploration due to lower sample number power. For example, in childhood presentations polymorphisms of the

SNP7 subunit of the *C1GALT1* gene are associated with IgVA/HSP, polymorphisms of *ITPKC* is a susceptibility gene for KD, and most recently a monogenic form of vasculitis, DADA2, associated with *ADA2* gene mutations (He et al., 2012; Navon Elkan et al., 2014; Onouchi et al., 2008; Zhou et al., 2014).

There are numerous reported cases of patients who have been recently diagnosed with DADA2 who presented with clinical signs and symptoms which were misidentified for other AIDs, other vasculidities or autoimmune disorders, whom, without genetic sequencing, would have endured an incorrect diagnosis (Alaygut et al., 2019; Barzaghi et al., 2019; Ghurye et al., 2019; Sahin et al., 2018; Springer et al., 2018; van Well et al., 2019).

1.4 Summary

Whilst inflammation and vascular pathology have been linked to ADA2 deficiency due to the symptoms presented, including markers of endothelial activation, the pathogenic mechanism giving rise to endothelial dysfunction and vasculopathy, is not understood. It is important to understand ADA2's role and its interactions with adenosine, the endothelium and leukocyte. This in turn may also provide knowledge for the most suitable treatments for this group of patients. In addition, it may shed light on the best preventative treatment for the families of these ADA2 deficient children, who also have mutations in the *ADA2* gene, and a reduction in ADA2 activity, but are yet to show clinical signs. Whilst ADA2 deficiency is a rare condition, it can have fatal results for the children affected. Thus far, research into ADA2 has shown a wide range of clinical manifestations, a range of mutations in the *ADA2* gene, the need for asymptomatic patients to be closely monitored or treated, and the diagnostic potential of ADA2 for vasculitis (Nanthapisal et al., 2015; Zhou et al., 2014)

Nevertheless, key questions remain unanswered including: whether ADA2 is having a direct effect on the endothelium, which adenosine receptors and downstream pathways are involved, what is their contribution to the disease pathology seen in ADA2 deficiency, and what may be the most effective treatment for both, symptomatic and asymptomatic patients.

1.5 Overall hypothesis and specific aims

The direct impact of ADA2 on the endothelium will be investigated. The hypothesis is that ADA2 has a direct effect on the endothelium, regulating growth, integrity and angiogenesis in an anti-inflammatory manner. I conjecture that fluctuating levels of adenosine, as seen in response to cell injury and inflammation will impact ADA2's functionality and the endothelium's ability to respond to inflammatory stimuli. Therefore, at both ends of the spectrum, ADA2 deficient patients, in addition to inflammatory disease and over expression ADA2, will manifest in endothelial cell dysfunction. The overall aim is to understand the effect of ADA2 on the endothelium using *in vitro* and *ex vivo* techniques.

To meet these aims, the following objectives will be pursed:

Chapter 3: To isolate and grow Blood Outgrowth Endothelial Cells (BOECs) from healthy donors and examine this progenitor cell model comparing it to Human Umbilical Vein Endothelial cells (HUVEC).

Chapter 4: To identify any involvement of adenosine receptors, and downstream pathways on BOEC in comparison to HUVEC by means of expression profile, structure, and function.

Chapter 5: To elucidate any physiological or pathophysiological effects of ADA2, in the presence of adenosine, on the endothelial cell expression, structure, and function.

2 General Methods

2.1 Introduction

This chapter contains materials and methods used to generate results in all proceeding results chapters. Where the methodologies have been adapted for specific experiments, these are discussed within each chapter, in further detail. The methodological process involved examination of endothelial cell types at the mRNA level using transcriptomics and quantitative real time polymerase chain reaction (qRT-PCR). Isolation and culture of different sources of endothelial cells was achieved alongside cellular assessment at the protein level using flow cytometry and immunofluorescence. The analysis of soluble signalling mediator production was quantified using colorimetric assays. Further to this, endothelial cell function was analysed using a number of assays including: colorimetric enzyme activity, colorimetric cell proliferation enzyme linked immunosorbent assay (ELISA) (BrdU), migration and cell adhesion assays.

2.2 Materials

The suppliers of the chemicals and reagents are listed in Appendix I. Recipes for the buffers and solutions are outlined in Appendix II.

2.3 Bioinformatics

A database for cross-study endothelial cell transcriptomics was published called, 'EndoDB' (Khan et al., 2019). The purpose of EndoDB is to facilitate the re-use of publicly available data; at the time of publication 360 datasets were curated and pre-analysed. The aim was to compare two endothelial models before starting work *in vitro*, exercising differential gene expression and gene set enrichment analysis available on EndoDB.

Studies were specifically selected involving blood outgrowth endothelial cells (BOECs) and human umbilical vein endothelial cells (HUVEC). I used this database to carry out meta-analysis comparing changes of pathway and gene expression across studies, for endothelial cells arising from different origins (varying experimental conditions and disease states).

The normalised data was then downloaded, manually sorted and examined using GraphPad Prism9 software.

2.4 Cell culture

2.4.1 Culture and isolation of human blood outgrowth endothelial cells

Ethical approval granted for the isolation of BOECs from healthy donor blood, Ref 1617/024, and the donor consent form, can be found in Appendix III. The protocol is adapted from protocols kindly given by Prof J. Mitchell's group at Imperial College London, with small amendments made using other published methods (Ingram et al., 2004; Martin-Ramirez et al., 2012a; Ormiston et al., 2015; Reed et al., 2015).

Donor recruitment criteria included participants who were over 18 years old, non-smokers and self-reported as healthy. Participants were aged between 20 to 58 years old and 16 out of 18 participants recruited for this study, were successful during venepuncture. The rate of success of BOEC colony isolation from whole blood was 81%, 13 out of 16. Of the 13 successful BOEC isolations the cells were isolated from 7 female and 6 male participants.

Up to 35 mL of blood was collected in sodium citrate vacutainers (BD) and processed under sterile conditions. The blood was diluted 1:1 with sterile PBS and layered on the top of Ficoll-plaque™ PLUS (GE healthcare). Within 20 mins of collection the layered Ficoll was centrifuged for 35 mins (400 x g) with no brake. The buffy coat and plasma layer were then removed and washed three times with PBS (10% FBS) by centrifugation at 520 x g for 10 mins (acceleration 9, deceleration 3). After the third wash the cells were re-suspended in EBM2 media (LONZA) with 10% FBS (Hyclone FBS, Thermos Scientific). The cells were counted using a FastRead counting slide (Immune systems Ltd.) and plated at a seeding density of 3.0 x 10^7 cells/4ml, onto a rat tail collagen (50µg/mL) pre-coated 6 well plate (Gibco, Life Technologies). The isolated cells were then incubated at 37°C, 5% CO2 in an incubator. For the first three days, the cells were very carefully washed with media and the media was changed. The cells were then incubated for 2 days until day 6 onwards, where the media was carefully changed three times a week until the endothelial cell colonies started to form, which took between 7-21 days. There was an 81% success rate of colony formation after blood processing. If colonies failed to appear by 21 days, the cells were disposed of. The endothelial colonies were

identified by their attachment to the bottom of the well and the characteristic cobble-shaped morphology. Once the colony stopped expanding, the cells were trypsinised using TrypLE™ Select Enzyme (Gibco, Life Technologies) and transferred to a T25 (coated with rat tail collagen). Once the flask was confluent, the BOECs were transferred to a T75 flask. When the BOECs were established and expanded to 3 T75 flasks (passage 3), the BOECs were cryopreserved using Freezing Medium Cryo-SFM (PromoCell) at a density of $0.5x10^6$ cells/mL and stored in -80˚C for 24 hours and then moved for long term storage in liquid nitrogen. The BOECs were thereafter cultured and grown as an endothelial cell line, when needed, as described in part 2.4.2.

2.4.2 Cell lines

Human umbilical vein endothelial cells (HUVEC), pooled (Sigma-Aldrich) and BOECs were cultured in Endothelial Cell Growth Media (ECGM) (Cell Applications Inc.), with 10% heat inactivated Foetal Bovine Serum (FBS) (Sigma-Aldrich). ECGM with 10% FBS, will be referred to as complete media henceforth. Passages 2-5 were used for all experiments.

THP-1 cells (a kind gift from the Institute of Child Health, UCL) and NB4 cells (a kind gift from Mrs Sappal, Kingston University), were grown in RPMI 1640 (Sigma-Aldrich) with L-glutamine and sodium bicarbonate (Sigma-Aldrich) and supplemented with 100U/mL Penicillin (Sigma-Aldrich) and 100µg/mL Streptomycin (Sigma-Aldrich), and 10% FBS. The media was stored at 4˚C and every two weeks the L-glutamine was replenished with 2mM L-glutamine (Sigma-Aldrich), as L-glutamine is not very stable in aqueous form. In some experiments THP-1s were differentiated into macrophages by addition of 10ng/mL phorbol-12-myristate-13-acetate (PMA) and incubated for up to 72 hours. At this time the differentiation was verified by changes in the cell morphology and adherence to the flask or plate.

HEK 293T cells (a kind gift from Dr Natasha Hill's group, Kingston University London) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate (Sigma-Aldrich), supplemented with 10% FBS. Passages 13-20 were used for experiments.

Hep G2 and Caco-2 cells (a kind gift from Dr Stolinski's group, Kingston University London), were grown in DMEM media supplemented with 10% FBS and 2mM L-glutamine. Passages 6-8 were used for experiments.

All cell lines were cultured under a humidified atmosphere of 5% CO_2 at 37°C and cell viability was assessed using trypan blue dye. The adherent primary cells or cell lines (BOEC, HUVEC, HEK 293T, and Hep G2), were trypsinised when 80% confluent, every 2-3 days. This process involved removal of the used media and washing of the cells with PBS before the addition of 1x Trypsin-EDTA solution (Sigma-Aldrich). After incubation with the trypsin for 2-5mins and the detachment of the cells, the trypsin was neutralised by the addition of complete media and transferred to a 50ml falcon tube and centrifuged for 5mins at 1000rpm. The cell pellet was resuspended in 1ml of fresh complete media. The cells were then counted and cell viability assessed using trypan blue. The suspension cell line, THP-1, had the media renewed, every 2-3 days, when the cell concentration reached ~$0.8x10^6$ cells/mL.

2.5 RNA extraction, First strand cDNA synthesis

2.5.1 Cell culture and lysis of samples

All plastic wear and tips were sterilised and RNAse-free for RNA extraction. The surfaces were wiped down with RNaseZap decontamination solution to remove RNase contamination from surfaces and pipettes. Cells were seeded in 6 well plates for 2-3 days until confluent. The media was removed and 0.3ml of TRIzol™ reagent (Invitrogen) was added directly into the culture plate to lyse the cells, following the manufacturer's protocol. The lysate was pipetted up and down several times to homogenise. The lysates were then incubated for 5 mins. Samples were then stored at -20°C, for up to one month, until the rest of the procedure was carried out.

2.5.2 Precipitation of the RNA

66µl of chloroform was added to the cell lysate in TRIzol™ reagent and shaken vigorously for 15 secs and incubated for 2-3 mins. The samples were then centrifuged for 15 mins at 12,000 x g at 4°C. The mixture then separated into the colourless upper aqueous phase, the interphase and the lower phenol-chloroform phase. The aqueous phase containing the RNA was transferred into a new tube.

2.5.3 Wash of the RNA

5µg of RNase-free glycogen per sample was added as a carrier to each aqueous phase. 150µl of isopropanol was added to the aqueous phase and incubated for 10 mins. This was then centrifuged at 12,000 x g for 19 mins at 4°C. Without disrupting the RNA cell pellet, the supernatant was discarded.

2.5.4 Solubilisation of RNA

The RNA pellet was resuspended in 0.3ml of 5% ethanol. The samples were vortexed and centrifuged at 7500 x g for 5 mins at 4°C. The supernatant was removed, and the RNA pellet was air dried for 5 mins. The pellet was resuspended in 20µl of RNase-free water and incubated at 55-60˚C for 15mins.

2.5.5 Determination of RNA yield

A BioDrop µLITE (BioDrop Ltd) determined RNA yield and purity, by the ratio of absorbance values at 260nm and 280nm, as well as the ratio between the absorbance at 260nm and A230nm.

2.5.6 cDNA synthesis

The volume of RNA needed to synthesise 1µg of cDNA was calculated from the RNA concentration derived in section 2.5.5. Genomic DNA (gDNA) was removed using DNase I (ThermoFisher Scientific), following the manufacturer's instructions. 1µl of 10X reaction buffer with $MgCl_2$ and DNase I was added to 1µg of RNA. The preparation was made up to 10µL using DEPC-treated water. This was incubated at 37˚C for 30 mins in a thermal cycler (Veriti™ 96-Well Thermal Cycler, Applied Biosystems™). 1µl of 50mM EDTA was added and the resulting mixture was incubated at 65˚C for 10mins.

After gDNA removal, first strand cDNA synthesis was carried out following the manufacturer's protocol (RevertAid First Strand cDNA synthesis Kit, ThermoFisher Scientific), volumes were added in order, as detailed in Table 2.1. The samples were then incubated at 42˚C for 60mins and the reaction was terminated by heating to 70˚C for 5mins. The resulting cDNA solution was then diluted to a concentration of 5ng/µL (1 in 10) with nuclease-free water for qRT-PCR.

Table 2.1 cDNA synthesis components
Table showing the cDNA synthesis components used in this study and the required volumes per sample.

Components	Volume (µl)
Template total RNA (1µg)	11
Oligo $(dT)_{18}$ primer	1
5X Reaction Buffer	4
RiboLock RNase Inhibitor (20U/µl)	1
10mM dNTP Mix	2
RevertAid M-MuLV RT (200U/µl)	1
Total	20

2.6 Quantitative reverse transcription polymerase chain reaction

2.6.1 Primer design

NCBI gene and nucleotide databases were used to find the reference sequences for each of the genes of interest. Using these template sequences, Primer Blast (Ye et al., 2012) was employed to design the primers for the qRT-PCR experiments. Parameters were set during the design process which included: at least one primer within the primer pair should be exon-exon spanning, the product size should be between 70-200 base pairs (bp) in length and the T_m should be around 63˚C (i.e an optimum annealing temperature of 60˚C). Primers were manufactured and supplied at 100µM, by Sigma-Aldrich (UK).

2.6.2 Primer validation

Primers were titrated to find the optimum working concentration of primer for the qRT-PCR reactions of 300nM. Primer pairs were further validated by 10 fold cDNA serial dilutions (1:10 – 1:100000) to calculate the efficiency. Primers were only used for experiments if they fell between 90-110% efficiency and the internal control gene and the target gene, were always within 10%, as outlined in guidelines by (Dauwe et al., 2016). Post thermal cycling, melt curves were performed to verify one peak indicating one product. This was confirmed by the PCR products being run on an agarose gel (see section 2.7) to confirm one product, at the

predicted size. An example of the primer validation can be found in Appendix IV. Further details of specific primer sequences and efficiencies are detailed in the methods section of the relevant results chapters, however, the house keeping genes are listed in Table 2.2. Three housekeeping genes were used to normalise the target gene qRT-PCR data, by using an average ct across all three housekeeping genes.

Table 2.2 Housekeeping gene primer sequences

Table showing the house keeping genes and their primer sequences used in this study. The expected product length and the efficiency calculated after cDNA serial dilutions is also listed.

Gene	Sequence		Product length (bp)	Efficiency (%)
	Forward Primer	Reverse Primer		
ACTB	GCCGCCAGCTCACCA	ATCCTTCTGACCCATGCCCA	167	100.54
GAPDH	CCTCCTGTTCGACAGTCAGC	ACGACCAAATCCGTTGACTCC	105	101.33

2.6.3 Sample preparation and thermal cycling parameters

A reaction master mix was made with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). Primers were diluted 1: 10, from stock with ddH_2O. cDNA had been diluted 1: 10 after synthesis with dH_2O. Each sample was made up to a final volume of 10µl, as shown in Table 2.3.

Samples were then loaded into a PROPLATE48 (Cole Parmer) and the PCR thermal cycling programme was executed using a Prime Pro 48 Real-time qPCR machine; the programme used is displayed in Figure 2.1. Samples were analysed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Table 2.3 qRT-PCR components

Table showing the components, volume and concentrations used in all qRT-PCR reactions.

	Volume per well (µl)	Working concentration
2x PowerUp™ SYBR™ Green Master Mix	5.00	1x
10µM Forward primer	0.30	300nM
10µM Reverse primer	0.30	300nM
cDNA	4.40	2.2ng/µl

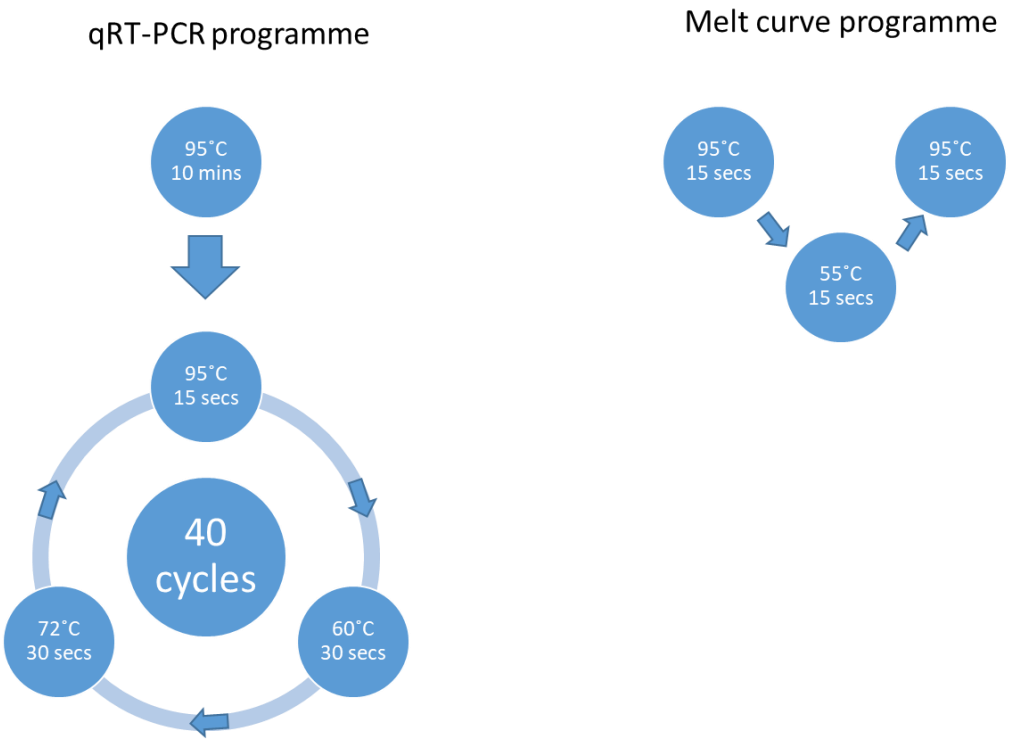


Figure 2.1 The qRT-PCR and melt curve programmes used

A schematic diagram to illustrate qRT-PCR and melt programmes used on the Prime Pro 48 Real-time qPCR machine for this study.

2.7 Gel electrophoresis

Products of qRT-PCR were analysed on 2% w/v agarose gel in 1x Tris/Acetic acid/EDTA (TAE) buffer, see Appendix II. 1x GelRed® (Biotium) fluorescent nucleic acid stain was added to the molten gel and the gels were cast in BIO-RAD gel casting trays. 6X TriTrack DNA Loading Dye (ThermoFisher Scientific) was added to each sample in a 1:5 ratio. 6µl of sample/loading dye mix was added to each well of the agarose gel. A GeneRuler Low Range DNA Ladder (ThermoFisher Scientific), was used as a molecular weight marker. The 2% agarose gels were run for 45-60 mins at 100V (BIO-RAD Power-Pac 300), using 1x TAE as a running buffer. Gels were imaged using Molecular Imager Gel Doc™ XR+ and ImageLab 5.2.1 software (BIO-RAD).

2.8 Flow cytometry characterisation of isolated cells

Flow cytometry was used to characterise and assess purity of isolated BOECs, as well as the cell line, HUVECs.

2.8.1 Preparation of cells

Isolated BOECs (see sections 2.4.1 and 2.4.2) and HUVEC were washed and trypsinised, once confluent, and resuspended in FACs buffer (2% FBS in PBS) at a density of $1x10^5$ cells/200µl. Isolated neutrophils were also resuspended at a density of $1x10^5$ cells/200µl in FACs buffer. The cells were centrifuged at 1400rpm for 4 mins.

2.8.2 Antibody incubations

The cell pellets were then resuspended in the appropriate diluted antibodies (see table of antibodies outlined within each results chapter) at 4˚C for 30mins in the dark. The cells were then washed twice with FACS buffer by centrifugation at 1400rpm for 4 mins and resuspended in 1x CellFIX (BD).

2.8.3 Acquisition and analysis

Stained cells were examined using BD™ LSR II flow cytometer, within the Flow Cytometry Core Facility, University College London, Great Ormond Street Hospital, London. Instrument settings used linear FSC/SSC scale and samples were acquired on a low flow rate with 10,000 events acquired per sample. Analysis was carried out using FlowJo™ Software (BD, USA). The specific gating strategy is described in the methods section of Chapter 3 (section 3.3.3.2).

2.9 Immunofluorescence microscopy

2.9.1 Preparation of cells

BOECs and HUVECs were plated at a seeding density of $1x10^4$ cells/well, on a chamber slide pre-coated with 2% gelatin, for 2-3 days until confluent.

For glycocalyx recovery experiments, the cells were then treated for 2 hours with glycocalyx degrading enzymes, Heparinise III 25 U/L or Chrondroitinase abc, and washed and incubated with pharmacological treatments for 18 hours. The cells were then washed 3x with PBS.

2.9.2 Preparation of slides

The cells were fixed at room temperature with 4% paraformaldehyde for 10 mins. The slides were then washed with PBS 3x. For permeabilization, when necessary, the slides were

incubated with 0.05% Tween 20 in 1xTBS (see Appendix II) for 3 mins and washed with PBS. The slides were blocked with 3% BSA for 30 mins at room temperature.

2.9.3 Antibody incubations

The primary antibody was then diluted in 1% BSA. The slides were incubated with the primary antibody overnight at 4˚C (see antibody dilutions in

Table 2.4 Primary antibodies used for immunofluorescence experiments in this study

Table 2.4). The antibody solution was removed and the cells were washed three times with PBS. A fluorescently conjugated secondary antibody was diluted 1in 200, in 2% normal goat serum and the slides were incubated in this antibody solution for 1 hour. For mouse primary antibodies, Anti-Mouse IgG+IgM H&L (FITC) (Abcam) was used, whereas, with rabbit primary antibodies, anti-rabbit IgG Alexa Fluor 488 was used. Secondary antibody only wells were used as negative controls. The slide was washed three times with PBS and then stained with Hoechst 33342 (ThermoFisher Scientific) (1in 2000) for 3 mins. This was washed 3x, mounted and imaged.

For staining of F-actin, following fixation, cells were stained with 1x Rhodamine Phalloidin Reagent (Abcam) for 60 mins at RT. The cells were then washed in PBS and the process continued as per above from staining of Hoechst 3342.

Further details of microscope settings and image analysis can be found in the relevant results chapters.

Table 2.4 Primary antibodies used for immunofluorescence experiments in this study

Table displaying the primary antibodies used in this study including their isotype, clone, host species, dilution, source and experiment usage. Use in this thesis G= glycocalyx coverage, P= protein expression.

Target Protein	Isotype	Clone	Host	Dilution	Source	Usage
ADA2	IgG	NBP1-89238	Rabbit	1:100	Bio-techne	P
Heparan Sulphate	IgMκ	F58-10E4	Mouse	1:100	AMS biotechnology	G

2.9.4 Glycocalyx image analysis

Image analysis to determine Heparan sulphate coverage was carried was carried out utilising FIJI software and applying a macro across images; one for HS (green channel), for Hoechst 33342 (blue channel) as shown in Figure 2.2. The output of the each macro gave an area of HS coverage and number of cells, respectively.

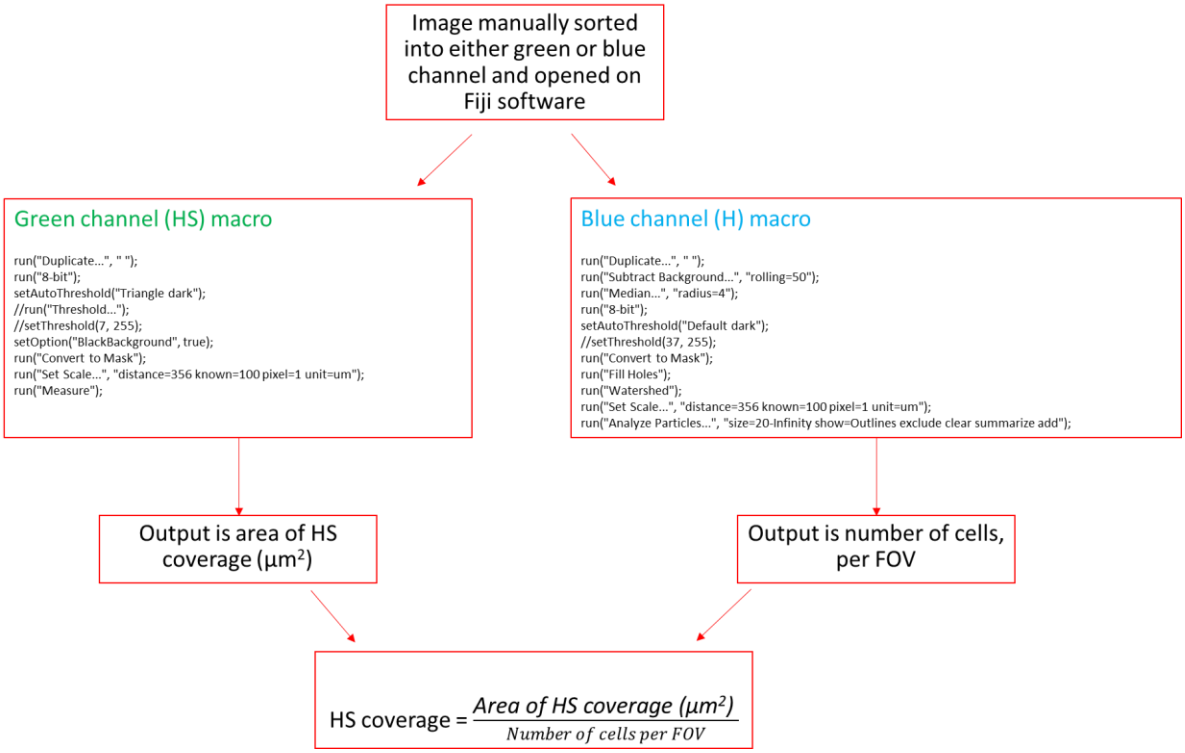


Figure 2.2 Workflow to evaluate heparan sulphate coverage

Workflow for colour channels to evaluate the heparan sulphate coverage. Images were processed using Fiji software (Schindelin et al., 2012) by a macro either for green or blue channels. The output of the macro gave area of HS for the green channel and number of cells for the blue channel.

2.10 Soluble mediators

Biological messengers produced by endothelial cells such as nitric oxide and ADA2 were assessed.

2.10.1 Nitrite/nitrate assay

Nitric oxide expression was assessed using a colorimetric Nitrite/nitrate Assay Kit (Sigma-Aldrich). After 24 hours of treatment, cell supernatants were centrifuged at 1000 x g, at 4^{o}C for 15 mins, and stored at -80 ^{o}C. Samples were assessed within a month from collection for nitric oxide metabolite production (nitrate and nitrite) following the manufacturer's guidelines, using an Epoch 2 Microplate Spectrophotometer (BioTek®) and measuring the absorbance at 540nm.

2.10.2 ADA activity assay

Activity of the cell mediator, ADA2, was measured using a colorimetric Adenosine Deaminase Assay Kit (Diazyme) in accordance with the manufacturer's protocol, but an amendment was made using 100nM Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), to specifically inhibit ADA1 activity and record ADA2 activity only (Nanthapisal et al., 2015). Absorbance was measured at 540nm at 5 min intervals over 30 mins, at 37˚C, using Fluostar Optima spectrophotometer (BMG Labtech).

2.11 Colorimetric BrdU ELISA

Growth of endothelial cells was assessed by a Cell Proliferation ELISA, BrdU (colorimetric) (Roche) assay. Cells were plated onto a 96 well plate and were serum starved (0.5% FBS) for 24 hours. The media was then changed containing 2% FBS with the addition of the treatments to the cells for 24 hours. The cultured cells were then incubated with BrdU for 3 hours, following the manufacturer's protocol. The cells were then fixed, and the DNA denatured. The fixed cells were incubated with anti-BrdU antibody conjugated with peroxidases (POD), for 90mins. A POD substrate, tetramethyl-benzidinthen (TMB), was added to develop the colour. The absorbance was measured using an Epoch 2 Microplate Spectrophotometer after 15 mins, at 370 nm (reference wavelength 492nm).

2.12 Wound healing assay

HUVEC and BOEC migratory capacity was assessed by observing cell migration and ability to close a 'wound' made in a confluent monolayer. HUVEC and BOEC cells were plated at a density of 7.5 x10^3 cells/well in a 96 well plate overnight, in complete media. Homogenous scratch wounds were made in each well, across the confluent monolayer, using a sterile WoundMaker™ tool (Essenbioscience). The wells were gently rinsed with PBS. All treatments were added in 2% FBS, ECGM. Plates were incubated inside an incubator with an Incucyte® system, whereby images were taken every 3 hours, over a 24 hour period. Image analysis was automatically generated using Incucyte® ZOOM software, measuring relative wound density over 24 hours (see Figure 2.3).

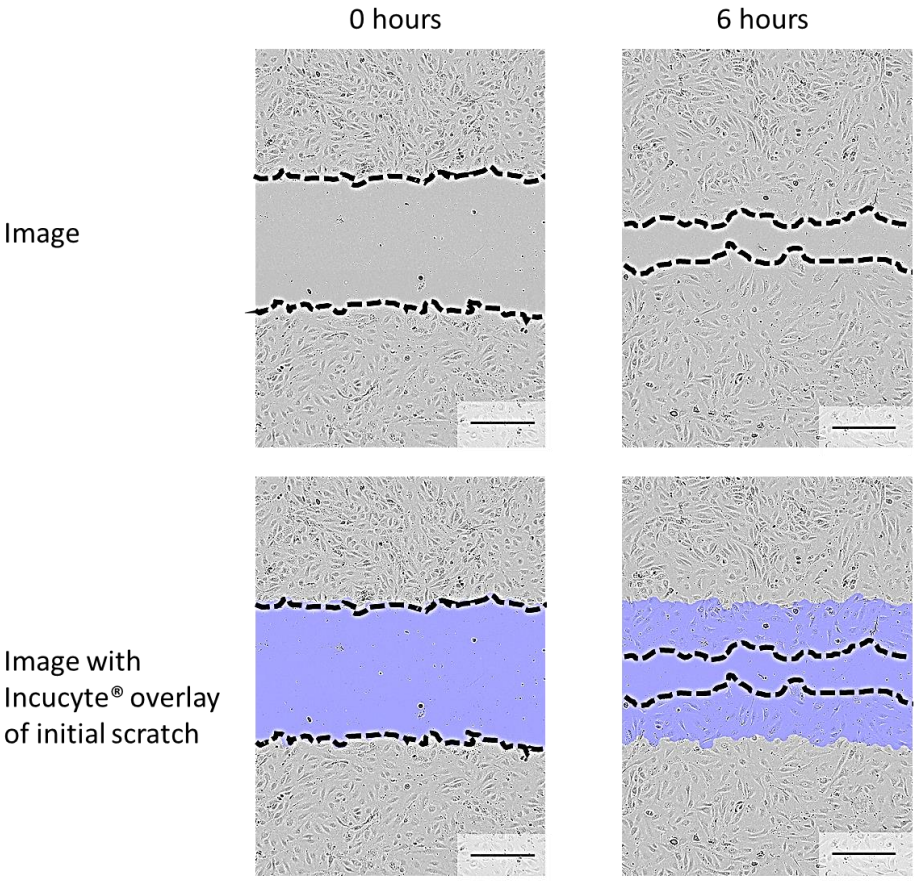


Figure 2.3 Representative images showing wound healing assay analysis

Analysis of endothelial cell migration over time to was achieved by Incucyte® ZOOM software. The software detects the initial scratch area (purple) and this overlay is used to work out the relative confluency of the cells within this area, at each time point. The dashed, black line indicates a manual trace of the wound front. Scale bar represents 300µm.

2.13 Static leukocyte adhesion assay

5x10^3 BOEC and HUVECs cells were seeded onto a 96 well plate and incubated at 37˚C for 2 days, until confluent. The cells were then activated with 10ng/mL TNF-α, for 4 hours. THP-1s monocytes were diluted to 1x10^6 cells/mL in PBS and labelled with 0.1µM Calcein AM (ThermoFisher Scientific), for 30mins in the dark, at 37˚C. Calcein AM is a cell permanent dye which is converted to green-fluorescent calcein by live cells. The fluorescently labelled cells were then washed three times with PBS and resuspended in basal RPMI media. Calcein AM-labelled monocytes were then added to the endothelial monolayer at concentrations of 0.5x10^6 cells/mL and incubated at 37˚C for 1 hour. Non-adherent monocytes were then washed off with PBS, three times. Three random fields of the fluorescently labelled adherent cells were taken using a Leica DM IL LED (Leica Microsystems, UK), inverted microscope. THP-1 monocyte adherence was quantified using Fiji software and the workflow for this is illustrated in Figure 2.4.

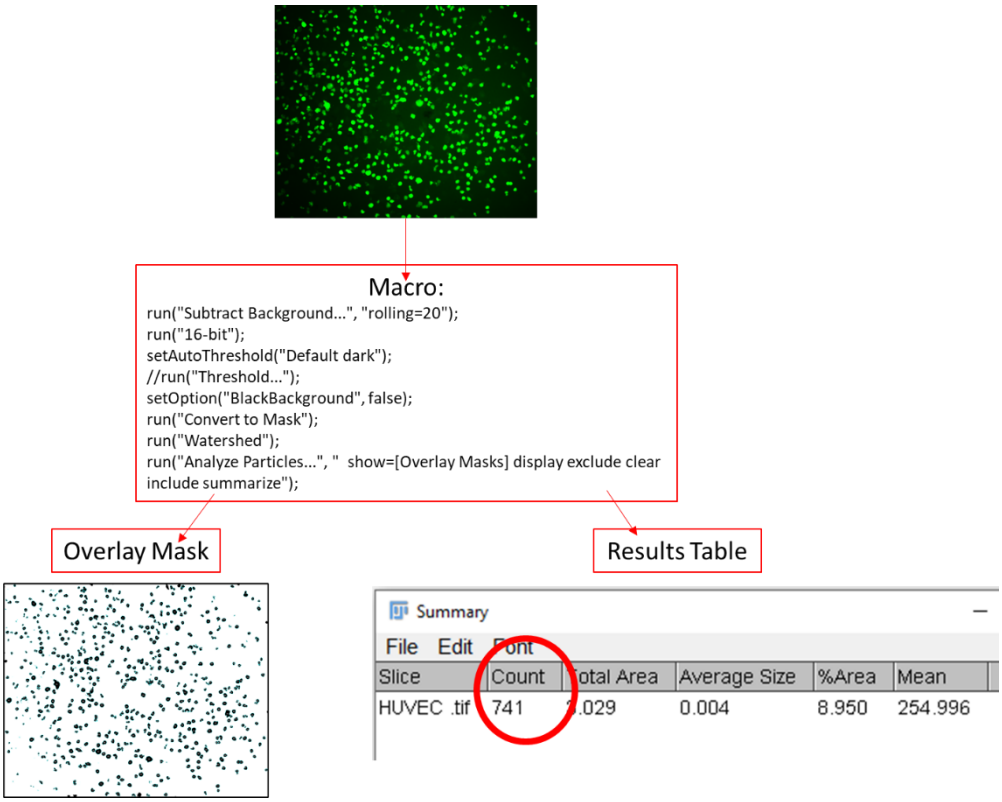


Figure 2.4 Workflow for processing images for monocyte adhesion to pre-stimulated endothelial cells after 1 hour

Adhered THP-1s are distinguished from endothelial cells by green, fluorescent stain (Calcein AM). THP-1 cell counts were calculated using a macro created on Fiji software (Schindelin et al., 2012) using the 'Analyse Particles' function, producing an overlay mask and results table.

2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism9 software. All statistical tests are stated in the relevant chapter and cited in the figure legends. For *in vitro* based experiments one-way or two-way ANOVA statistical tests were deployed to allow comparison of one or two independent variables (e.g. cell types and treatment conditions). The relevant posthoc test was chosen for multiple comparisons depending on whether the mean was compared to every other mean (Tukey's multiple comparisons test), or if every mean was compared to the control mean (Dunnett Multiple comparisons test). All graphs show the mean $\pm$ standard error of the mean (SEM), unless otherwise stated. A p value of ≤ 0.05 was considered a significant difference.

3. Results 1- Endothelial nature of BOECs

3.1. Introduction

This chapter, will investigate the suitability of adopting BOECs as an endothelial model preferentially to HUVECs. Many *in vitro* models study endothelial cell responses as endothelial dysfunction is a key feature of cancer, aging, cardiovascular, and inflammatory disease pathology, which includes the pathophysiological role of DADA2 (Rajendran et al., 2013; Zhou et al., 2014).

The method of harvesting ECs that are isolated and cultured from individuals' peripheral blood, has been recently developed (Ingram et al., 2004; Lin et al., 2000; Ormiston et al., 2015; Yoder et al., 2007). It is thought that these ECs arise from circulating endothelial progenitor cells (EPCs), which develop into bona fide endothelial cells known as endothelial colony–forming cells (ECFC) or blood outgrowth endothelial cells (BOECs) (Hebbel, 2017; Medina et al., 2017). Whilst BOECs can be isolated from cord blood and peripheral blood; this study will be discussing BOECs from peripheral blood only.

To date, BOECs have been isolated from a relatively small amount of patient's blood, including patients with various vascular and inflammatory disorders (see Table 3. 1). The isolated BOECs from these donors reflect the donor's vascular health and, specific disease phenotypes are exhibited by differences in endothelial cell function (Hebbel, 2017). For example, one study compared BOECs from children with sickle cell anaemia with and without vasculopathy, which leads to childhood stroke. Between the BOECs isolated from the two groups of children, there were no significant differences in gene expression profiling. However, using predetermined gene sets for 9 biological systems, the Biological Systems Scores did show changes in inflammatory signalling between children with and without vasculopathy, in isolated BOECs. Subsequent stimulation *in vitro* by IL-1β and TNF-α, of BOECs isolated from children with sickle cell anaemia who were at risk of ischemic stroke, revealed they were more susceptible to the inflammatory cytokines, demonstrated by an amplified ReIA (also known as p65, part of the NF-κB family) activation, than those without vasculopathy (Milbauer et al., 2008). This is an example of where BOECs, isolated from a patient group with clinical subsets, reflected both genetic and functional differences, which may make BOECs a more suitable *in vitro* EC model.

Table 3. 1 Studies which have isolated peripheral BOECs from patient groups

Human patient group	Author(s) and year of publication
Acute myocardial infarction	(Massa et al., 2009)
	(Meneveau et al., 2011)
Abdominal aortic aneurysm	(Sung et al., 2013)
Atherosclerotic-renal-artery-stenosis	(Chen et al., 2014)
Bicuspid aortic valve	(van de Pol et al., 2019)
Burns	(Rignault-Clerc et al., 2013)
Cerebral cavernous malformations	(Spiegler et al., 2019)
Chronic myeloproliferative disorders	(Otten et al., 2008)
	(Piaggio et al., 2009)
	(Teofili et al., 2011)
Chronic Obstructive Pulmonary Disease	(Paschalaki et al., 2013)
	(Paschalaki et al., 2018)
Coronary artery disease	(Güven et al., 2006)
	(Fernandez et al., 2014)
	(Martin-Ramirez et al., 2014)
	(Matveeva et al., 2018)
	(Stroncek et al., 2009)
	(Stroncek et al., 2011)
	(Stroncek et al., 2012)
Premature coronary artery disease	(Brittan et al., 2015)
Early coronary atherosclerosis	(Hebbel et al., 2020)
Early infantile epileptic encephalopathy type 4	(van Breevoort et al., 2014)
Essential hypertension (EH)	(Kim et al., 2018)
	(Chen et al., 2014)
Hemophilia A	(Bittorf et al., 2020)
Hereditary haemorrhagic telangiectasia (HHT)	(Fernandez-L et al., 2005)
Hermansky-Pudlak syndrome type 2	(Karampini et al., 2019)
Idiopathic systemic capillary leak syndrome	(Sek et al., 2015)

Idiopathic pulmonary fibrosis	(Smadja et al., 2013)
	(Smadja et al., 2014)
	(Bacha et al., 2018)
Ischemic cardiomyopathy	(Dauwe et al., 2016)
Neovascular age-related macular degeneration	(Grierson et al., 2013)
	(Thill et al., 2011)
Obesity	(Tobler et al., 2010)
	(Richards et al., 2014)
Peripheral artery disease	(Essaadi et al., 2018)
Preterm-born adults	(Bertagnolli et al., 2018)
Pulmonary arterial hypertension	(Toshner et al., 2009)
	(Geti et al., 2012)
	(Dunmore et al., 2013)
	(Ormiston et al., 2013)
	(Lavoie et al., 2014)
	(George et al., 2014)
	(Wojciak-Stothard et al., 2014)
	(Long et al., 2015)
	(Caruso et al., 2017)
	(Ferrer et al., 2018)
	(Smits et al., 2018)
	(Long et al., 2020)
Rheumatoid arthritis	(de Villeroché et al., 2010)
Sickle cell anaemia	(Milbauer et al., 2008)
	(Sakamoto et al., 2013)
Smokers	(Paschalaki et al., 2013)
Transradial Cardiac Catheterization	(Mitchell et al., 2017)
Type 1 diabetes mellitus	(Coppens et al., 2013)
	(Mathur et al., 2019)
Type 2 diabetes	(Leicht et al., 2011)
	(Richards et al., 2014)

Variant microvillus inclusion disease	(Schillemans et al., 2018)
Venous thromboembolic disease	(Alvarado-Moreno et al., 2016)
	(Hernandez-Lopez et al., 2017)
von Willebrand disease	(Wang et al., 2013)
	(Starke et al., 2013)
	(Groeneveld et al., 2015)
	(Noone et al., 2016)
	(Yadegari et al., 2016)
	(Selvam et al., 2017)
	(Khursigara et al., 2020)

The ways in which BOECs are a good model to cover the clinical phenotypic heterogeneity has also been demonstrate in other vascular diseases. In patients with Pulmonary Arterial Hypertension (PAH), where the common cause is mutation of *BMPR-II*, BOECs isolated from these patients demonstrated a hyperproliferative phenotype and impaired ability to form vascular networks (Smits et al., 2018). In contrast, BOECs harvested from individuals with subclinical atherosclerosis, had impaired proliferation compared to healthy controls, that was restored following statin therapy (Martin-Ramirez et al., 2014).

Use of BOECs *in vitro* is only one of the suggested applications for these cells. Many studies are looking at how BOECs can be used as autologous cell therapy for vascular repair, as a delivery system for gene therapy and for tissue bioengineering (Bertelsen et al., 2014; Fuchs et al., 2010; Glynn and Hinds, 2014; Lin et al., 2002; Paschalaki and Randi, 2018).

Despite the apparent advantages of using BOECs, the misuse of the term "endothelial progenitor cell (EPC)" for populations of cells that do not mature into endothelial cells has caused confusion whereby the nomenclature does not always align with the latest scientific evidence. Along with naming inconsistencies and biological complexities, this has fuelled the debate surrounding the existence of EPCs from blood. Attempts to distinguish between cell types and promote consistent nomenclature has been made by both Hebbel (2017) and Medina et al., (2017).

Therefore, to study human vascular diseases such as; atherosclerosis, inflammation, diabetes and tumour angiogenesis some researchers have remained with more traditional models. Primary cells at a macrovascular level include Human Coronary Artery Endothelial Cells, Human Aortic Endothelial Cells, Human Pulmonary Artery Endothelial Cells and Human Umbilical Vein Endothelial Cells (HUVEC). At a microvascular level (organ-specific endothelial cells) there are Human Dermal Microvascular Endothelial Cells, Human Pulmonary Microvascular Endothelial Cells and Human Brain Microvascular Endothelial Cells (Hauser et al., 2017). Examples of commonly used and well characterised endothelial cell lines include Ea.Hy926 and HMEC-1, which originate from primary endothelial cells that have undergone fusion or transfection to make them immortalised (Ades et al., 1992; Edgell et al., 1983). Primary ECs are more difficult to handle, have a shorter life span and greater variability due to assorted donors but, are regarded as preferential to EC lines, as they are more physiologically relevant and the cell lines can have significant differences in response to inflammatory cytokines (Lidington et al., 1999).

The most commonly used *in vitro* model, HUVECs, were first isolated and described in 1973 (Jaffe et al., 1973). These have been the predominant model of ECs as they are primary human cells, and before BOECs were discovered, were more readily available than other sources of primary ECs. This is down to other primary ECs only being isolated from organs after the time of death or during transplantation. Consequently, investigation into endothelial dysfunction is limited due to the availability of primary ECs, in a non-invasive manner, from patients with vascular disorders (Hauser et al., 2017). In this respect, HUVECs were an alternative source, in spite of their fetal nature.

HUVECs have a limited life span and are typically used experimentally between passages 2-5 (Medina-Leyte et al., 2020). The proliferation rate and the morphology of the cells differs over their life span (Gimbrone et al., 1974). This can cause issues around reproducibility and makes data arising from the use of HUVECs rather difficult compared to data arising from studying other cell types. To avoid this problem, HUVECs can be pooled from many donors, but this then removes donor specific traits (Glee et al., 2001; Hunt et al., 2010). For diseases at a specific organ level, HUVECs may not be as relevant, as it is known that ECs have a high degree of heterogeneity, adapting to local needs (McCarthy et al., 1991).

Therefore, I wanted to investigate BOECs as a preferred human EC model *in vitro,* and validate the method of isolating endothelial cells from patients and healthy donors, which would be reflective of an individual's vascular health. BOECs from various diseases with matched healthy controls has been explored by many groups – a "reverse approach" to disease modelling (refer to Table 3. 1), though not from DADA2 patients (Medina et al., 2012). A number of disease states have been modelled using BOECs *in vitro*, including; co-culture with early EPCs, astrocytes and pericytes, osteoblasts, and fibroblasts; oxygen-glucose deprivation; hypoxia; high glucose exposure; shear stress response; cytokine storm responses; factor VIII secretion; generation of tissue engineered blood vessels; anti-Platelet Profiling; miR-150 transfection; response to infected cells and toxins; and CRISPR/Cas9- von Willebrand factor–deficient engineered (Abdulkadir et al., 2019; Ahmann et al., 2011; Au et al., 2008; Chantzichristos et al., 2018, 2018; Du et al., 2020; Ecklu-Mensah et al., 2018; Feitz et al., 2020; Hendrickx et al., 2010; Herzog et al., 2014; Medina et al., 2012; Reed et al., 2015; Schillemans et al., 2019; Tasev et al., 2018; Torres et al., 2014; van Beem et al., 2009; Wang and Cooper, 2013). However, BOECs response to pharmacological agents, especially those that mimic inflammatory conditions, along with the EC structural component, the glycocalyx, have not been examined and compared to that of HUVECs.

In this chapter, BOEC and HUVECs have been compared by transcriptomics, morphology, cell surface marker expression, protein and secondary messenger expression, structure, function, and response to inflammatory signals.

3.2. Specific Aims

This chapter assesses the suitability of using BOECs to study endothelial dysfunction in inflammatory disease, by comparison with HUVECs at mRNA, protein and functional levels. The null hypothesis is that there is no difference in the way that BOEC and HUVEC respond to inflammatory stimuli by mRNA, protein expression and EC function.

The following aims will be addressed:

1. Use the EndoDB to investigate transcriptomic data from BOEC vs HUVEC in health and disease.
2. Compare and contrast BOEC and HUVEC in the following ways;
    a. To characterise the phenotype with respect to morphology and cell surface marker expression by flow cytometry.
    b. Observe the structure of the glycocalyx that covers the luminal surface of the different cell types.
    c. Quantify expression of the soluble signalling mediator nitric oxide and regulators of this pathway.
    d. Assess EC functional responses to inflammatory stimuli by proliferation, migration, and adhesion assays.

3.3. Methodology

The methodologies used in this section are generally as outlined in Chapter 2. However, where there are specific adaptations applied to the general methods, relevant to the results presented in this chapter, these are also described.

3.3.1. Transcriptomics

The publicly available EndoDB database consists of endothelial transcriptomics from 309 studies, 4741 samples and 5847 single cell transcriptomes (Khan et al., 2019). Specifically, there were 146 HUVEC studies and 4 BOEC studies. 4 additional studies were labelled as BOEC, but these were excluded, as an in-depth review of the data source revealed that these cells were not BOEC in identity. The normalised data was then downloaded, manually sorted and examined using GraphPad Prism9 software. The results discussed from EndoDB, are accurate as of the last accessed date, July 2020. There were two steps to the bioinformatics analysis.

Step one: Gene set enrichment analysis (GSEA) was compared across two studies to ascertain if BOEC and HUVEC cell types from inflammatory associated diseases, relative to healthy controls, had similar regulation of gene sets. GSEA or pathway analysis methodology involves comparing pre-grouped genes on the basis of shared biological function. EndoDB used the KEGG gene set and corresponding list of genes. This is a popular method when working with a large number of genes and their expression data, to decipher biologically relevant results, in a non-biased manner. Unsurprisingly, the database only contained one study involving HUVECs isolated from affected mothers (Accession ID: E-GEOD-49524). The other 145 HUVEC studies concerned *in vitro* manipulation of cells isolated from healthy donors. This is a limitation of working with HUVECs, as a model of disease, as the nature of these cells means it is difficult to isolate them from various affected patient groups. The BOEC study (Accession ID: E-GEOD- 9877), was chosen from the four BOEC studies as a representative study, due to the cells being from individual's afflicted with vascular diseases (sickle cell anaemia with predicted stroke risk), and this study contained the largest number of samples of the BOEC studies.

Step two: To further examine the *in vitro* response of BOECs and HUVECs to pharmacological treatment the EndoDB was searched for any matching treatments in both BOEC and HUVEC studies. One HUVEC study was found (Accession ID: E-GEOD-27631) that also treated their

isolated cells with BMP-6 and BMP-9, as was the case in the BOEC study (Accession ID: E-GEOD-54416

). The sample numbers were limited for the HUVEC study, so GSEA could not be performed. Therefore, relative gene expression of all genes involved in the four chosen gene sets, were acquired from EndoDB and control was compared with BMP-6 and BMP-9 treatment conditions. The data was manually sorted and heatmaps were produced to examine how BOECs and HUVECs respond to matching treatments in vitro.

3.3.2. BOEC isolation

In total 18 participants were recruited for this study. Two donations were unsuccessful during venepuncture, leaving 16 blood donations. 3/16 BOEC isolations were unsuccessful by day 21 and therefore, ultimately BOECs were isolated from 13 donors; the rate of success was thus 81%, 13/16. The BOECs were isolated and cultured as described in section 2.4.1. Donors were recruited if they were over 18, non-smokers and self-reported as healthy. Participants were aged between 20 to 58 years old, and of those with successful donation and BOEC isolation, 7 were female and 6 were male. For experiments, donor cells were not pooled and were used individually as biological replicates, with two technical replicates per donor, unless otherwise stated in the relevant methodology section.

3.3.3. Flow cytometry

3.3.3.1. Characterisation of endothelial cells

Isolated BOECs were examined in terms of their endothelial phenotype- cell surface marker expression, in comparison to HUVECs. The methodology in section 2.8 was followed, using the antibody panels laid out in Table 3. 2.

Table 3. 2 Fluorochrome conjugated antibodies for flow cytometry characterisation of isolated cells

Panel	Target	Isotype	Conjugate	Clone	Dilution	Source
1	CD14	IgG2a, κ	PB	M5E2	1:50	BioLegend
1	CD16	IgG1, κ	PCP-Cy5.5	3G8	1:50	BD
1	CD31	IgG1, κ	PE-Cy7	WM59	1:50	BioLegend
1	CD144	IgG1	APC	16B1	1:100	Invitrogen
1	HLA-DR	IgG2a, κ	FITC	G46-6	1:50	BD
2	CD34	IgG2a, κ	PE	561	1:50	BioLegend
2	CD45	IgG1, κ	FITC	HI30	1:50	BioLegend
2	CD54	IgG1, κ	PB	HA58	1:50	BioLegend
2	CD105	IgG1, κ	APC	266	1:50	BD
2	CD106	IgG1, κ	PCP-Cy5.5	51-10C9	1:50	BD
2	CD144	IgG1	PE-Cy7	16B1	1:100	Invitrogen
3	CD62e	IgG1, κ	PE	68-5H11	1:50	BD
3	CD144	IgG1	APC	16B1	1:100	Invitrogen
4	CD62p	IgG1, κ	PE	AK4	1:50	BioLegend
4	CD144	IgG1	APC	16B1	1:100	Invitrogen

3.3.3.2. Flow cytometry gating strategy

Samples were acquired as described in section 2.8.3. After flow cytometry acquisition, the data was analysed using Flow-Jo software (BD, USA). The gating strategy for the characterisation of BOEC and HUVECs is demonstrated with representative plots Figure 3. 1.

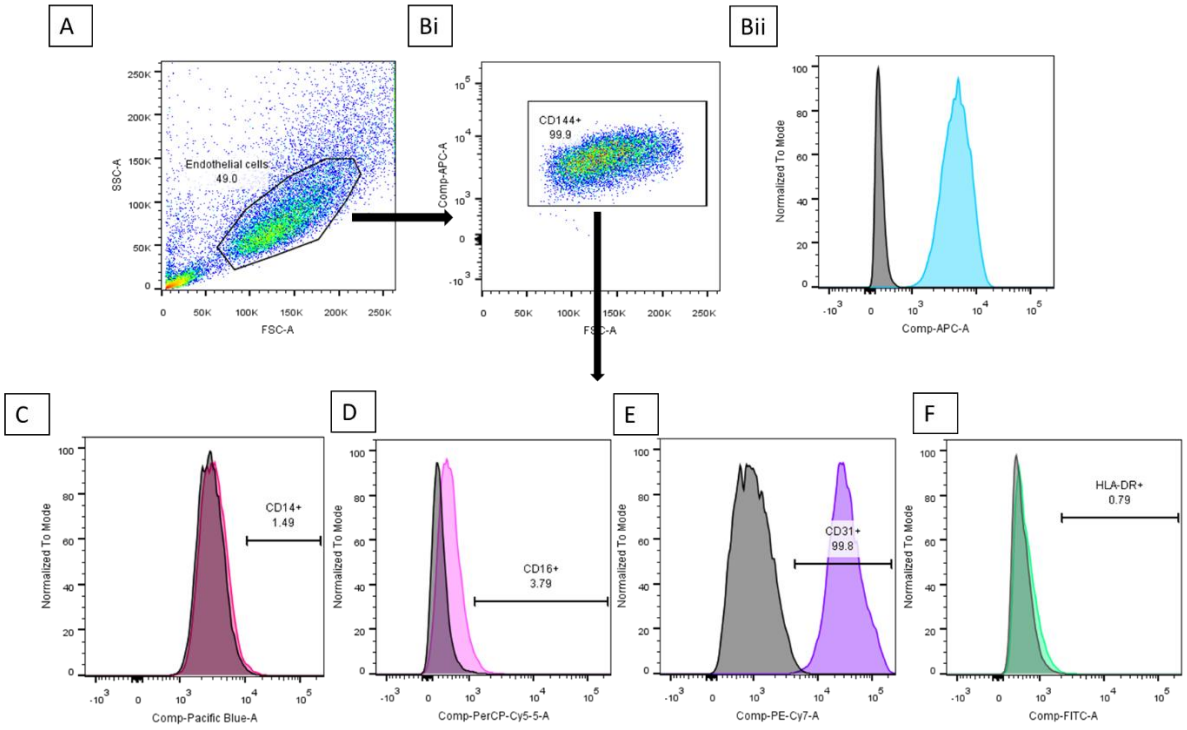


Figure 3. 1 Flow cytometry dot plots and histograms demonstrating how the cell population was selected, endothelial cells gated, and specific marker expression was based upon percentage expression.

Representative dot plots and histograms for panel 1. **A)** From the forward scatter, side scatter plot, the cell population was identified and gated upon. **Bi)** To determine the number of endothelial cells within this cell population, the CD144+ population was selected. **Bii)** Histogram plot to show CD144+ in blue and unstained in grey. Within the CD144+ population, further cell surface marker expression was measured by percentage expression; **C)** CD14+, **D)** CD16+, **E)** CD31+, **F)** HLA-DR+.

3.3.4. Immunocytochemistry

Immunocytochemistry (ICC) was a method utilised was used to compare the glycosaminoglycan (GAG) composition protruding out of the cell membrane for the two EC types. The antibodies and method, in section 2.9, were adhered to for this purpose. Briefly, the GAG antibody which targeted extracellular polysaccharide chains of Heparan Sulphate (HS), along with the fluorescently labelled secondary antibody, were validated to check for non-specific binding, in addition to using a secondary antibody only control. This meant that before fixation of the cells, another control well was established by incubation with GAG degrading enzymes, heparinase III (25 U/L), for 2 hours. The cells were then washed, fixed and stained as described previously (refer to section 2.9). Images were captured using a ZEISS LSM 900 confocal microscope.

3.3.5. qRT-PCR

qRT-PCR was used to look at mRNA expression for genes involved in the composition of the glycocalyx and the nitric oxide pathway under control and inflammatory conditions, after treatment with TNF-α (10ng/ml) for 24 hours. Primers for glycocalyx components such as the genes for proteoglycan core proteins; *Glypican 1 (GPC1), Heparan Sulphate Proteoglycan 2 (HSPG2)* and *Syndecan 4 (SDC4)*, were designed and validated as outlined in the general methods. The list of the primer sequences, used for the qRT-PCR assessment of the BOEC and HUVEC glycocalyx components, can be found in Table 3. 3 and the positive control used for the primers were HEK 293T or Hep G2 cells. The changes in mRNA level of the enzymes and key regulators involved in the production of the soluble mediator nitric oxide, were measured, using the primers listed in Table 3. 4 and the positive cDNA control for *DDAH1* and *DDAH2 was* HEK 293T; *NOS2*, CACO-2, and *NOS3* was HUVEC.

Table 3. 3 Primers designed to detect components of the glycocalyx, at mRNA level

Table listing the primer sequences for *GPC1*, *HSPG2* and *SDC4* genes, as well as the expected product length and the calculated efficiency from primer validation.

Gene	Sequence		Product length (bp)	Efficiency (%)
	Forward Primer 5'-3'	Reverse Primer 5'-3'		
GPC1	CAGCTGTCCTGAACCGACTGA	TGGCACTGGCAGGGTTATTATG	178	96.72
HSPG2	TACACACGCCACCTGATCTC	GCTGCCAGTAGAAGGACTCA	181	103.07
SDC4	GAGCCCTACCAGACGATGAG	TCTAGAGGCACCAAGGGATGG	139	109.87

Table 3. 4 Primers used for the examination of the nitric oxide pathway

Table listing the primer sequences *DDAH1, DDAH2, NOS2* and *NOS3*, as well as the expected product length and the calculated efficiency from primer validation. The *NOS2* primer sequence was taken from (Lambden, 2016).

Gene	Sequence		Product length (bp)	Efficiency (%)
	Forward Primer 5'-3'	Reverse Primer 5'-3'		
DDAH1	TATGCAGTCTCCACAGTGCC	TCATCTGTTGCATGATCTTAAGGG	133	96.05
DDAH2	CTCCATCGACCTCTGGCCTA	CAAAGCTCAAAGGGAGCACG	191	98.93
NOS2	TGGCCAGATGTTCCTCTATT	CCAAAGGGATTTTAACTTG	182	97.25
NOS3	GGAGAATGGAGAGAGCTTTGCAG	TCTGAGCAGGAGATGCTGTTG	120	101.00

3.3.6. Nitrite/nitrate assay

Nitrite and nitrate production was quantified in cell culture supernatants by colorimetric assay after 24 hours of stimulation with TNF-α. The quantification was performed as detailed in section 2.10.1, following the manufacturer's instructions.

3.3.7. Colorimetric BrdU ELISA

A cell proliferation ELISA, BrdU (colorimetric) kit was purchased from Sigma-Aldrich (Roche) and was employed to assess BOEC and HUVEC proliferation under control and activated conditions (TNF-α treatment over 24 hours). A more detailed description of the procedure can be found in section 2.11.

3.3.8. Wound healing assay

To assess cell migration a wound was rendered into a confluent monolayer of endothelial cells using a sterile WoundMaker™ tool (Essenbioscience), as communicated in section 2.12. The cells were then subjected to inflammatory cytokine activation (TNF-α treatment) or control conditions. Images were taken every 3 hours over a 24-hour period, using an Incucyte® incubation system. The movement of the cells to close the wound was measured by relative cell density of the wound, over time, using Incucyte® ZOOM software.

3.3.9. Static leukocyte adhesion assay

To observe BOEC and HUVECs' ability to recruit leukocyte after inflammatory stimulation a static adhesion assay was carried out. A confluent monolayer of endothelial cells was stimulated with TNF-α, for 0,4 or 24 hours. THP-1 monocytes were stained with a fluorescent label calcein AM (100nM) and co-cultured with the pre-stimulated endothelial cells for one hour. Images were taken using a Leica DM IL LED microscope. The number of adherent monocytes was quantified using Fiji software (Schindelin et al., 2012).

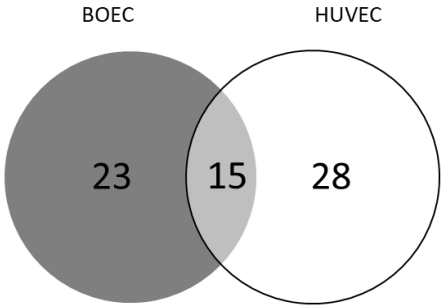
3.4. Results

3.4.1. Meta-analysis of endothelial transcriptomics

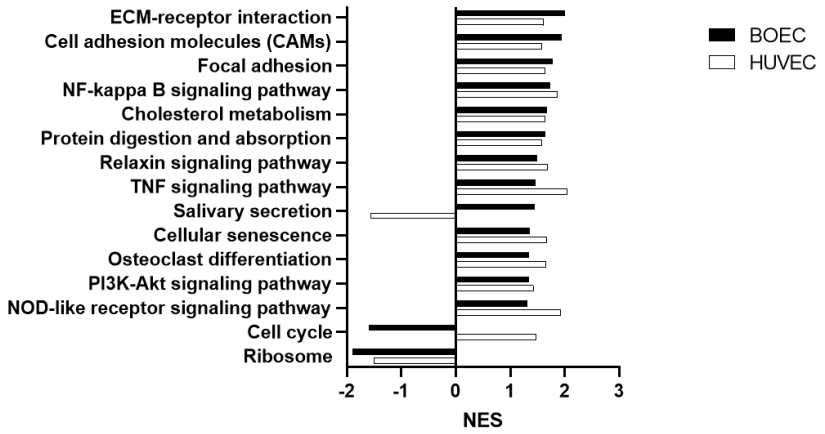
Initially meta-analysis was carried out using a public database of endothelial cell transcriptomics, named 'EndoDB' to investigate the importance of BOECS in the literature and to test their mature endothelial nature, rather than haemopoietic stem cell, monocyte, macrophage or other cell phenotype (Khan et al., 2019). The purpose was to establish two points. Firstly, whether BOEC and HUVECs, from healthy in comparison to inflammatory associated diseases, behave in a similar manner. Secondly, to understand if BOECs respond to pharmacological treatment *in vitro,* by reflecting appropriate transcriptomic behaviour.

The GSEA, (Figure 3. 2) highlights 15 shared gene sets across BOECs and HUVECs. Within this group 13/15 show a parallel trend of expression of these genes sets. Of interest to this study and modelling an inflammatory disease, there was an overlapping response to inflammatory disease for both cell types whereby there was an upregulation of the ECM-receptor interaction, cell adhesion molecules (CAMs), focal adhesion and TNF signalling gene sets. BOEC and HUVECs isolated from donors with inflammatory associated diseases, behave in a similar manner in comparison to controls.

A **Total number of gene set shared and differentially expressed in inflammatory associated disease**



B **15 Shared gene sets differentially expressed in inflammatory associated disease**



C **Gene sets differentially expressed for each cell type**

BOEC; 23 gene sets

Gene set	Direction	NES	P value
Lysosome	Up	1.9560	0.0036
Drug metabolism - cytochrome P450	Up	1.8715	0.0027
Mineral absorption	Up	1.7941	0.0027
Collecting duct acid secretion	Up	1.7701	0.0208
Metabolism of xenobiotics by cytochrome P450	Up	1.7455	0.0056
Glycerolipid metabolism	Up	1.6643	0.0028
Synaptic vesicle cycle	Up	1.6463	0.0113
Glycosaminoglycan degradation	Up	1.6179	0.0297
Leukocyte transendothelial migration	Up	1.6162	0.0070
Tyrosine metabolism	Up	1.6090	0.0455
Gastric acid secretion	Up	1.4768	0.0316
Phagosome	Up	1.4297	0.0260
Natural killer cell mediated cytotoxicity	Up	1.4296	0.0344
Adherens junction	Down	-1.4311	0.0423
mRNA surveillance pathway	Down	-1.4752	0.0378
Protein processing in endoplasmic reticulum	Down	-1.5027	0.0092
Homologous recombination	Down	-1.5351	0.0309
Ribosome biogenesis in eukaryotes	Down	-1.5464	0.0205
Fanconi anemia pathway	Down	-1.5870	0.0201
Oocyte meiosis	Down	-1.6375	0.0028
Cysteine and methionine metabolism	Down	-1.7154	0.0079
RNA transport	Down	-1.8790	0.0013
Spliceosome	Down	-2.0340	0.0014

HUVEC; 28 gene sets

Gene set	Direction	NES	P value
Hippo signaling pathway	Up	1.8765	0.0015
TGF-beta signaling pathway	Up	1.8382	0.0016
IL-17 signaling pathway	Up	1.7639	0.0016
Steroid hormone biosynthesis	Up	1.7592	0.0017
Cytokine-cytokine receptor interaction	Up	1.6131	0.0014
Arginine and proline metabolism	Up	1.6088	0.0152
Folate biosynthesis	Up	1.6050	0.0226
Wnt signaling pathway	Up	1.5977	0.0091
Platelet activation	Up	1.5898	0.0078
p53 signaling pathway	Up	1.5813	0.0211
FoxO signaling pathway	Up	1.5786	0.0105
Signaling pathways regulating pluripotency of stem cells	Up	1.5777	0.0121
C-type lectin receptor signaling pathway	Up	1.5204	0.0189
Jak-STAT signaling pathway	Up	1.5201	0.0121
Hedgehog signaling pathway	Up	1.5167	0.0341
Ovarian steroidogenesis	Up	1.4936	0.0387
Chemokine signaling pathway	Up	1.4901	0.0105
Necroptosis	Up	1.3913	0.0422
Regulation of actin cytoskeleton	Up	1.3706	0.0310
MAPK signaling pathway	Up	1.3600	0.0177
Axon guidance	Up	1.3527	0.0298
Purine metabolism	Down	-1.3410	0.0150
Pyrimidine metabolism	Down	-1.3489	0.0390
Cardiac muscle contraction	Down	-1.3582	0.0413
Renin-angiotensin system	Down	-1.5936	0.0323
Oxidative phosphorylation	Down	-1.6207	0.0059
Proteasome	Down	-1.6408	0.0120
Proximal tubule bicarbonate reclamation	Down	-1.7545	0.0113

Figure 3. 2 Cross-study GSEA: differential expression in inflammatory associated disease, compared to healthy controls, for BOEC and HUVEC

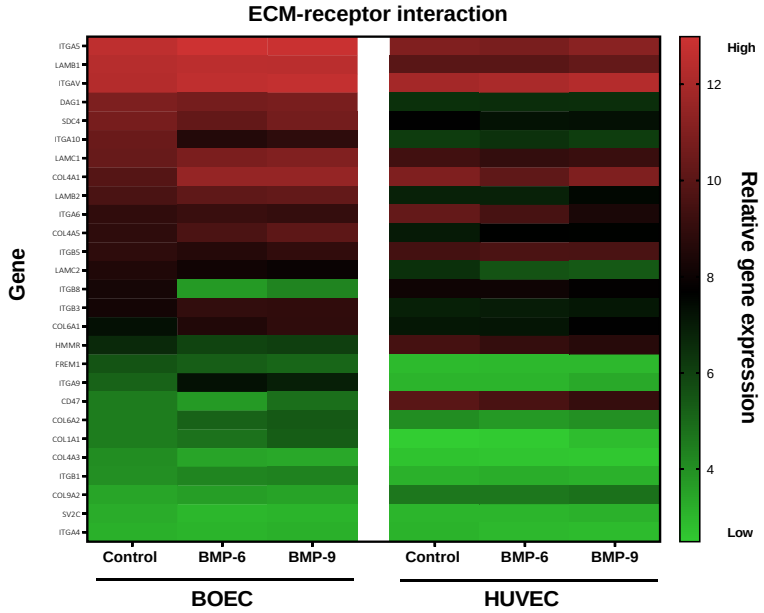
Gene set enrichment analysis was performed using data curated by EndoDB (Khan et al., 2019) across two studies with accession IDs: E-GEOD-9877 (BOEC) and E-GEOD-49524 (HUVEC). For each study, the reference conditions were samples from healthy donors and the experimental conditions were samples from patients with an inflammatory associated disease (sickle cell at risk of ischemic stroke- BOEC: E-GEOD-9877, and gestational diabetics- HUVEC: E-GEOD-49524). Gene sets were selected as differentially expressed if the normalised enrichment score (NES) had a significant p-value. A positive NES indicates an increase in gene set expression whereas a negative NES, indicates the reverse. **A** Venn-diagram to display the total number of gene sets that were shared and differentially expressed in disease compared to healthy samples. 15 gene sets were shared between BOEC and HUVEC samples. BOEC and HUVEC differentially expressed 23 and 28 other gene sets, respectively. **B** Interleaved plot illustrating the 15 shared gene sets. The normalised enrichment score reflects the relative expression of each gene set in disease compared to healthy samples. **C** Tables listing the non-shared gene sets which were differentially expressed for BOEC or HUVEC. **Abbreviation**- **NES**: Normalised Enrichment Score, **ECM:** extracellular matrix.

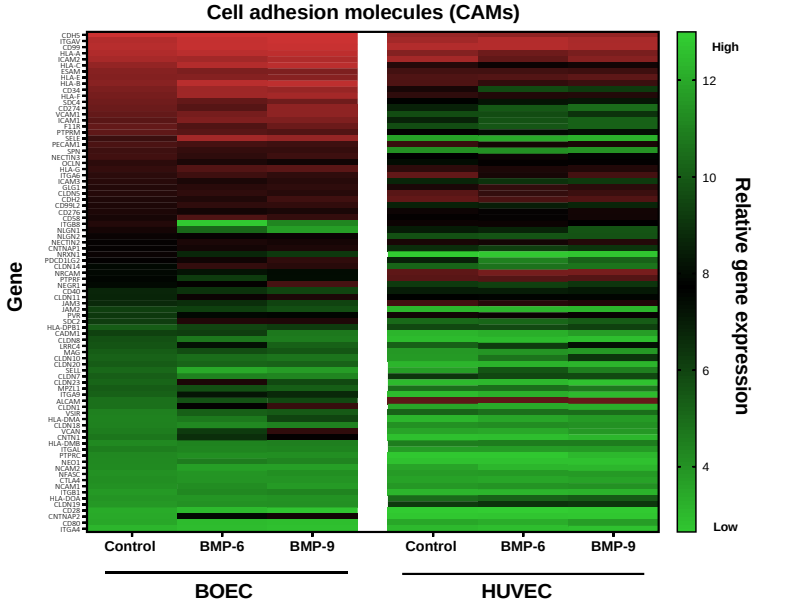
The four gene shared genes sets which were identified from Figure 3. 2 (ECM-receptor interaction, cell adhesion molecules (CAMs), focal adhesion and TNF signalling) were then examined after *in vitro* pharmacological treatment (step two of the transcriptomic analysis). The results from can be visualised as cross-study heatmaps for the four gene sets (Figure 3. 3 and Figure 3. 4). The heatmaps for the four individual gene sets (Figure 3. 3), show relative gene expression for a number of genes with a colour coded scale from low expression, green, to high expression, red. When comparing control samples of BOEC and HUVECs, the relative gene expression is largely parallel, in the ECM-receptor interaction, cell adhesion molecule and focal adhesion gene sets. The gene set which showed the great disparity between BOECs and HUVECs was the TNF-signalling pathway gene set, whereby there is a greater proportion of genes expressed highly (in red) in the BOEC samples, whereas HUVEC control samples express the TNF-signalling pathway set at lower relative expression, illustrated by more green bars. An example of one gene with differential expression in BOECs and HUVECs, across the TNF-signalling pathway and CAMs gene sets, is *CD54* (*ICAM-1*), where there is high expression (in red) for BOECs across control, BMP-6 and BMP-9 treatments whereas, in HUVECs there is low expression (green) in all three conditions.

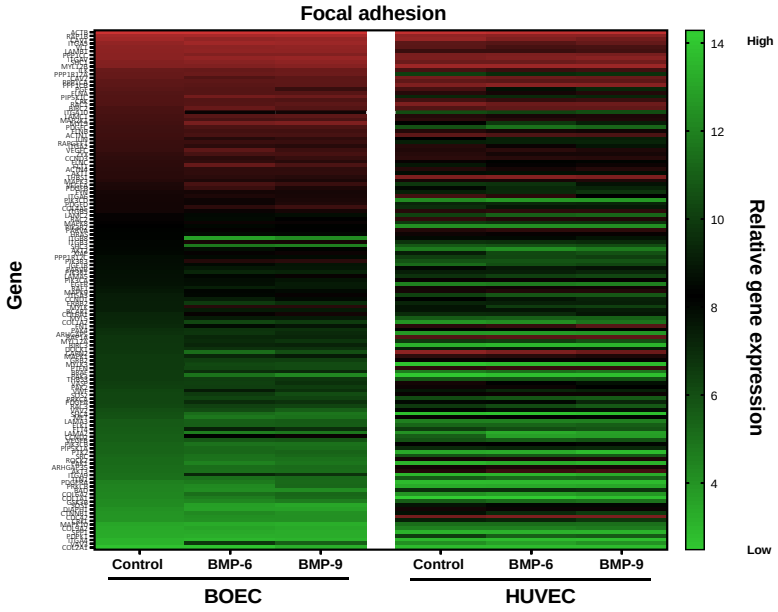
Across all four gene sets, BMP-6 and BMP-9 treatment does not markedly shift relative gene expression from control, for either BOEC or HUVECs. HUVEC gene expression stays constant with BMP treatment across all four gene sets, however, there are particular genes within each gene set which are differentially expressed with pharmacological treatment for BOECs. For example, within the ECM-receptor gene set, incubation with either BMP-6 or 9 increases *ITGA10* and *ITGA9* coding for integrin alpha subunits and decreases *ITBG8* (Integrin Subunit Beta 8) in BOECs, but this is not seen in HUVECs. With the TNF-signalling pathway, there are a number of genes (*VEGFC, JAG1, CX3CL1, PTGS2, MAPK13* and *CXCL6*) which BMP-6 and 9 upregulate in BOECs. This is in contrast to the absence of any HUVEC response, again.

Figure 3. 4 brings together all 4 gene sets into one heatmap and this highlights the similarity of control expression levels in the gene sets, ECM-receptor interaction, cell adhesion molecules and focal adhesions, but how the levels of expression of the TNF signalling pathway for HUVECs, are lower overall. HUVEC lacked a transcriptomic response to stimulation with BMP-6 and 9, whereas particular genes are differentially expressed in response to stimulation for BOECs.

Overall, the meta-analysis of endothelial transcriptomics has demonstrated that BOECs respond comparably to HUVECs in terms of healthy versus disease, and in a superior manner in response to pharmacological agents, when handled *in vitro*. With this information, BOECs from healthy donors were isolated and used in subsequent experimentation.


ECM-receptor interaction
Gene
ITGA5
LAMB1
ITGAV
DAG1
SDC4
ITGA10
LAMC1
COL4A1
LAMB2
ITGA6
COL4A5
ITGB5
LAMC2
ITGB8
ITGB3
COL6A1
HMMR
FREM1
ITGA9
CD47
COL6A2
COL1A1
COL4A3
ITGB1
COL9A2
SV2C
ITGA4
High
12
10
8
6
4
Low
Relative gene expression
Control
BMP-6
BMP-9
Control
BMP-6
BMP-9
BOEC
HUVEC


Cell adhesion molecules (CAMs)
Gene
High
12
10
8
6
4
Low
Relative gene expression
Control
BMP-6
BMP-9
Control
BMP-6
BMP-9
BOEC
HUVEC


Focal adhesion
Gene
High
14
12
10
8
6
4
Low
Relative gene expression
Control
BMP-6
BMP-9
Control
BMP-6
BMP-9
BOEC
HUVEC

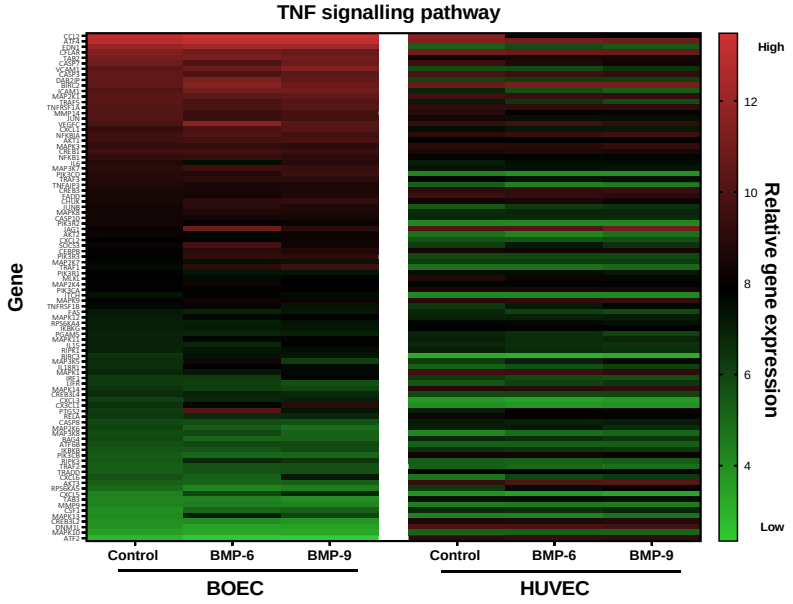

TNF signalling pathway
Gene
High
12
10
8
6
4
Low
Relative gene expression
Control
BMP-6
BMP-9
Control
BMP-6
BMP-9
BOEC
HUVEC

Figure 3. 3 Cross-study heatmaps, displaying relative gene expression after *in vitro* pharmacological treatment with BMP-6 and BMP-9 of BOEC and HUVEC samples

Four heatmaps plotting different gene sets and the relative expression of the genes under control or treatment with BMP-6 or BMP-9. The data was normalised and sourced from EndoDB (Khan et al., 2019) with accession IDs E-MTAB-2495 (BOEC) and E-GEOD-27631 (HUVEC). The key shows a gradient from low to high expression using shades of green (low) to red (high).

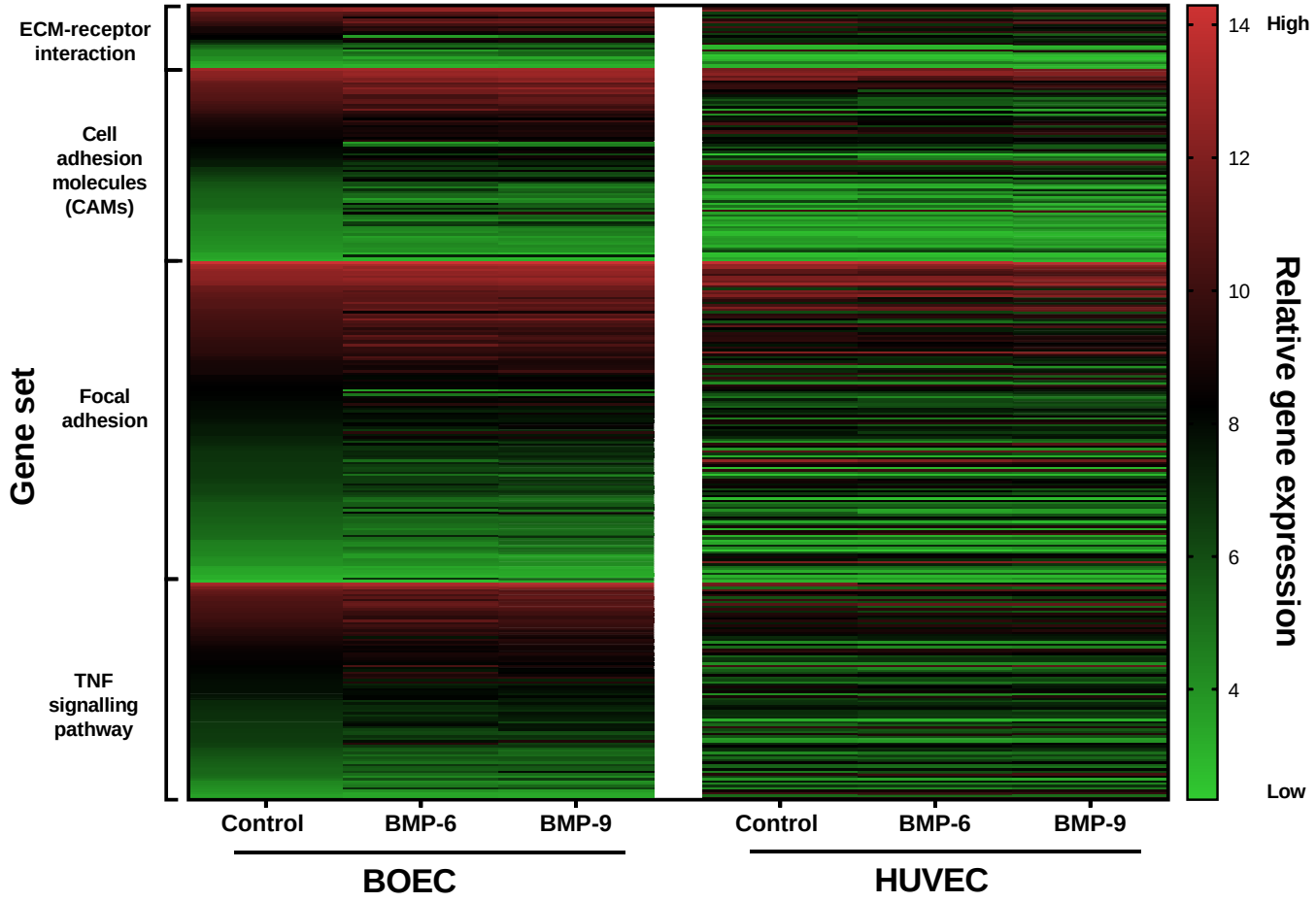


Figure 3. 4 Overall heatmap displaying cross-study comparison of relative gene expression, after *in vitro* treatment
Heat map plotting the relative expression of the genes within four sets of genes, under control or treatment with BMP-6 or BMP-9. The data was normalised and sourced from EndoDB (Khan et al., 2019) with accession IDs E-MTAB-2495 (BOEC) and E-GEOD-27631 (HUVEC). The key shows a gradient from low to high expression using shades of green (low) to red (high).

3.4.2. Cell structure

Between days 7-21 after donation and start of culture, colonies of cells with cobbled shaped morphology emerged.

Figure 3. 5 shows the culture of PBMCs from one donor at days 1, 7, 14 until a BOEC colony appearing, captured at day 17. BOECs were then grown and expanded within T75 culture flasks. Representative images of confluent BOEC and HUVEC T75 flasks are shown.

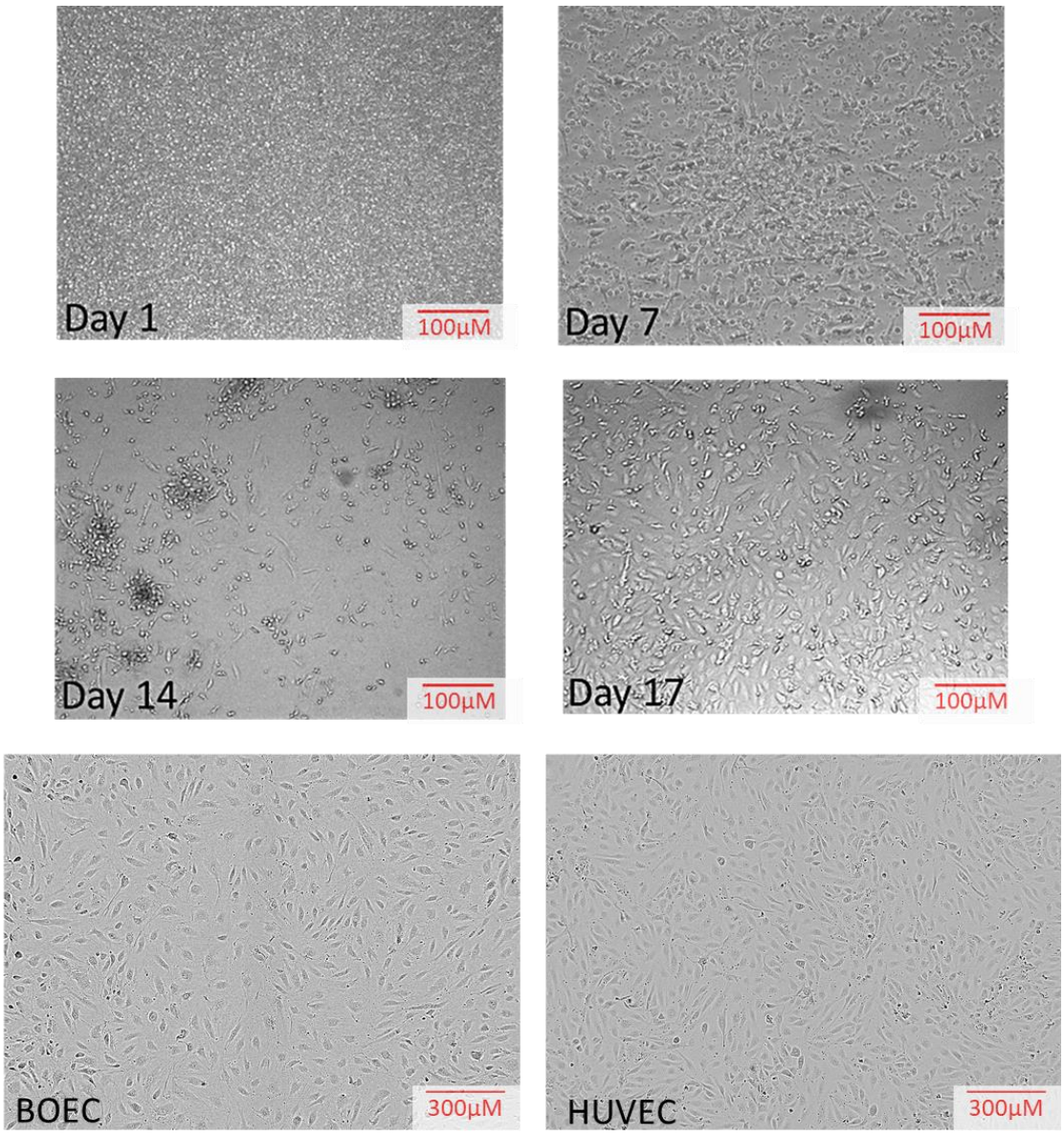


Figure 3. 5 Isolated BOECs have cobbled shaped morphology akin to HUVECs

Representative images of BOEC colony formation from one donor days 1, day 7, day 14 and day 17 where a BOEC colony is observed. The BOEC colony was expanded to a T75 flask. Representative images of BOEC and HUVEC confluent T75 flasks are shown. Scale bars represent either 100 or 300 µm, as indicated.

As the morphology of the isolated BOECs was consistent with HUVECs and distinctive of ECs, other structural components were examined. A key structural component and mediator of endothelial signalling *in vivo* is the glycocalyx. The components and structure of the BOEC and HUVEC glycocalyx *in vitro* were studied at mRNA and protein levels. Shedding of GAGs and proteoglycan core proteins is considered a cause of endothelial dysfunction (Becker et al., 2015). Both are shed from ECs, under conditions such as TNF-α stimulation (Ramnath et al., 2014; Wiesinger et al., 2013). Proteoglycan core proteins which are able to covalently bind at least one GAG chain, are more difficult to detect at protein level, as enzymatic removal of the GAG chains is typically required before targeting with antibodies. Therefore, proteoglycan expression levels were not measured in terms of proteins, but at mRNA level. Quantification of mRNA for the proteoglycan core proteins *Glypican 1* (*GPC1), Heparan Sulphate Proteoglycan 2 (HSPG2), Syndecan 4 (SDC4)* was determined by qRT-PCR (Figure 3. 6).

In a quiescent state, BOECs *HSPG2* was expressed significantly more than *GPC1* (p=0.0005) or *SDC4* (p=0.0192), but the difference between *SDC4* and *GPC1* was not significantly different. The same trend was seen in HUVECs, where *HSPG2* was expressed significantly more than *GPC1* (p=0.0038) or *SDC4* (p=0.0097), but the difference between *SDC4* and *GPC1* was not significantly different.

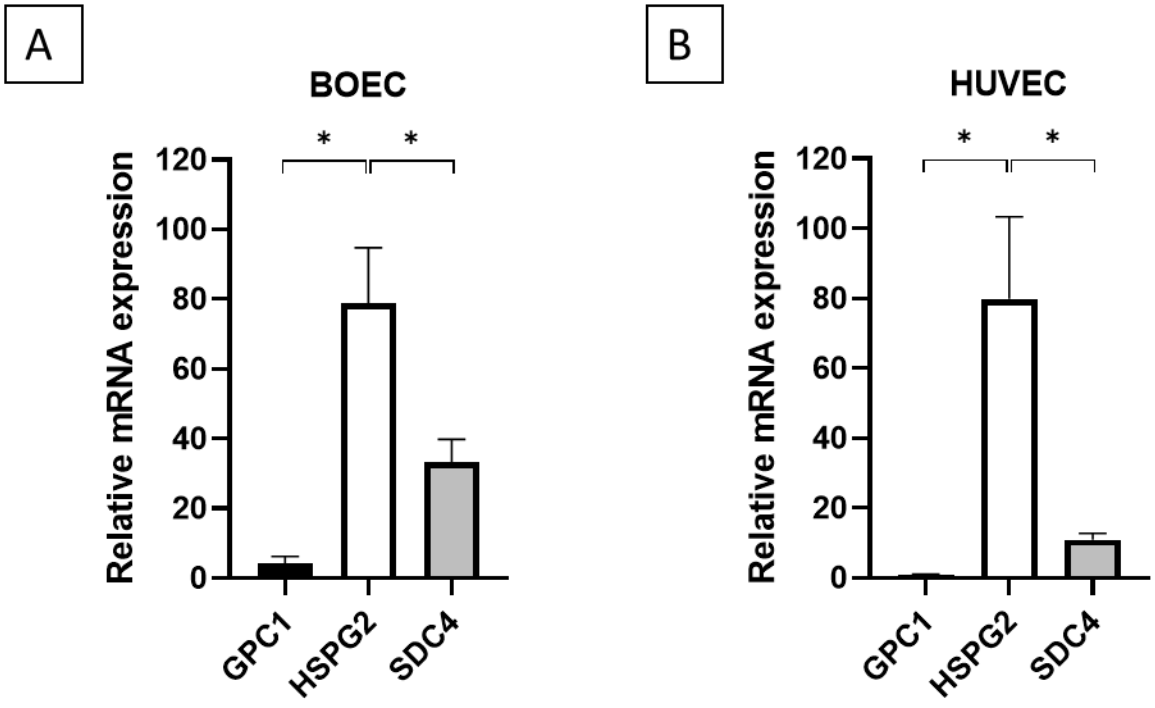


Figure 3. 6 BOECs and HUVECs express more *HSPG2* than *SDC4* and *GPC1*

Comparison of endothelial cell *GPC1, HSPG2* and *SDC4* mRNA by qRT-PCR using $2^{-\Delta\Delta ct}$ method, of **A)** BOECs and **B)** HUVECs. The data was obtained by 5 independent biological replicates (n=5), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. All columns represent mean ± SEM. Statistical analysis (one-way ANOVA with Tukey's multiple comparisons test) showed significant differences, $p<0.05$, denoted by (*).

The proteoglycan mRNA expression was then examined under quiescent and inflammatory conditions; TNF-α (10ng/ml) (Figure 3. 7). *GPC1* expression was 3.8x higher in BOECs than HUVEC (p=0.0191). *HSPG2* mRNA expression was equivalent in BOECs and HUVECs and there was significantly more BOEC, then HUVEC, *SDC4* expression under quiescent conditions (6.8x, p=0.0359)

Inflammatory stimulation caused a downregulation of *GPC1* expression for BOECs only (p=0.0356). *HSPG2* did not significantly change under inflammatory stimulation for either EC type. *SDC4* expression was upregulated under inflammatory stimulation in BOECs (p=0.0325).

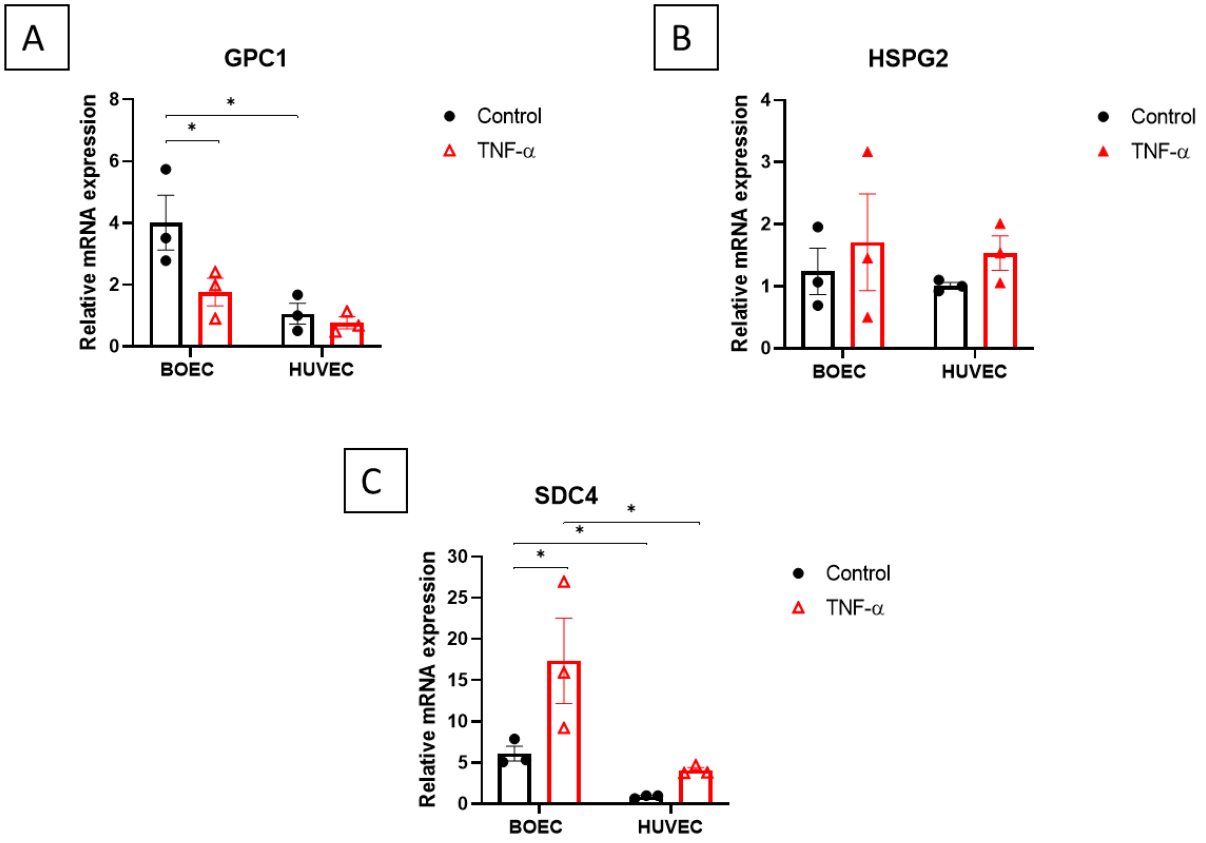


Figure 3. 7 BOECs respond to inflammatory stimulation by regulation of proteoglycan core protein mRNA expression

Determination of endothelial cell **A)** *GPC1,* **B)** *HSPG2* and **C)** *SDC4,* mRNA by qRT-PCR using $2^{-\Delta\Delta ct}$ method. Endothelial cells were incubated with media only (control) or TNF-α (10ng/ml), fo 24 hours. The data was obtained by 3 independent biological replicates (n=5), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. All columns represent mean ± SEM. Statistical analysis (2way ANOVA with Tukey's multiple comparisons test) showed significant differences, p<0.05, denoted by (*).

The endothelial glycocalyx components were further assessed using confocal microscopy. The coverage of heparan sulphate (HS), the most prevalent glycosaminoglycan (GAGs) *in vivo,* were measured *in vitro*, for BOEC in comparison to HUVEC. Due to concerns in the literature that trypsinisation of cells would remove the endothelial glycocalyx and limit detection, it was decided that immunocytochemistry was the most appropriate method to measure GAG coverage, over flow cytometry (Potter et al., 2009). Incubation with the Heparinase III enzyme for 2 hours, successfully removed HS coverage across the cells (Figure 3.8).

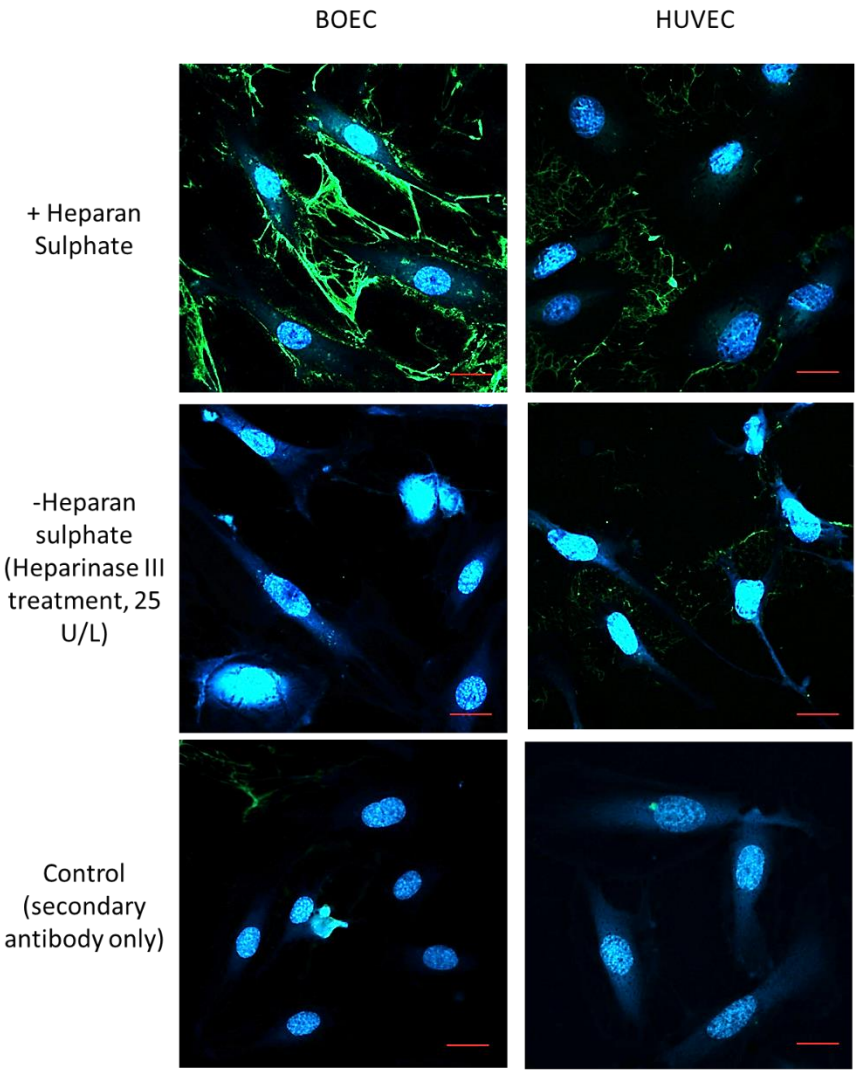


Figure 3. 8 Representative confocal images of Heparan sulphate

Representative images from experiments carried out n=3 of heparan sulphate (green) expression in BOEC cells and HUVEC cells. Nuclei were counterstained with Hoechst 33342 (blue). There is greater coverage of HS across BOEC than HUVEC. HS is removed after treatment with Heparinase III treatment (25U/L), for 2 hours. Magnification x400, scale bar represents 20µm.

3.4.3. Immune phenotype

Once the BOECs were identified by light microscopy as a colony of cells with cobbled-shaped morphology, they were expanded to T75 flasks, up to passage 3. Preliminary confocal images for 3 BOEC donors, showed that BOECs expressed another characteristic endothelial marker, vWF, intracellularly (Appendix V). Multi-colour flow cytometry was used to assess the cell surface marker expression, in comparison to HUVECs (Figure 3. 9). Flow cytometry was chosen to look at cell surface marker expression as, unlike western blots, tertiary protein structure is maintained. The methodology described in section 2.8 was followed, using 4 panels of antibodies. BOECs have classical endothelial marker expression (CD144, CD31), akin to HUVECs. There was also low expression of monocytic (CD14, CD16, CD45), smooth muscle cell (PDGF) and haematopoietic progenitor surface antigens (CD34). Activated monocytic marker (HLA-DR), and activated endothelial cell markers (CD106, CD105, CD62e, CD62p) were expressed in low amounts. Only CD54 (also known as ICAM-1) was expressed significantly more in BOECs than HUVECs, from 2.64 to 7.93% (p=0.0174).

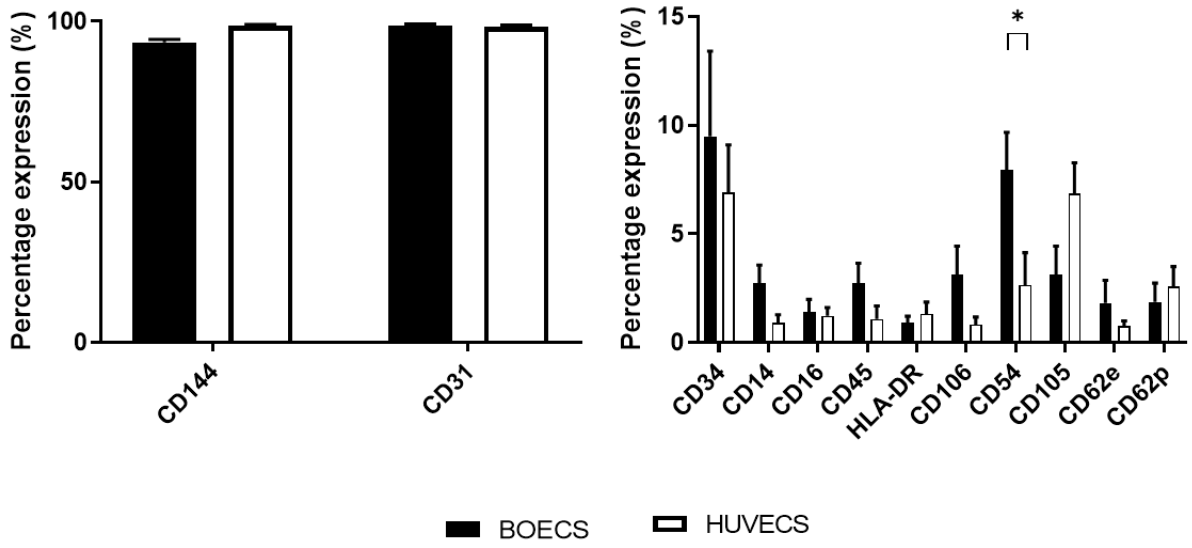


Figure 3. 9 Resting BOECs present similar phenotype to HUVECs

Cell surface marker expression as measured by flow cytometry and presented as a percentage of all cells. BOEC n=5, HUVEC n=5, error bars show mean ± SEM. Statistical analysis (two-way ANOVA and Sidak's multiple comparison test) show CD54 is significantly more expressed on BOECs (*p=0.0174).

3.4.4. Nitric oxide pathway

The ability of ECs to produce the signalling molecule, NO, via NOS3 (eNOS), is critical to maintaining vascular homeostasis, by inhibiting platelet aggregation, causing smooth muscle relaxation and endothelial migration, amongst other effects. Decreased NO production is a prominent part of endothelial cell dysfunction. Therefore, expression of the regulators of the NO pathway (*NOS3, DDAH1* and *DDAH2*) and the production of NO under inflammatory conditions, was evaluated using qRT-PCR and a colorimetric nitrite/nitrate assay. The results (Figure 3. 10), demonstrate that mRNA expression of *NOS3* was equivalent in BOECs and HUVECs, and under inflammatory stimulation (TNF-α, 10ng/ml), both EC types responded by downregulating *NOS3* (BOEC, $p<0.0001$; HUVEC, $p=0.0002$). No *NOS2* expression was identified under these conditions for BOEC or HUVECs.

NOS expression is highly regulated and so, it was essential to look at the expression of the regulators of NOS. NOS is inhibited by ADMA, which in turn is regulated by the DDAH enzymes. *DDAH1* mRNA expression was equivalent in BOECs and HUVECs (Figure 3. 11), however, *DDAH2* expression was much higher in BOECs than HUVECs ($p<0.0001$). *DDAH2* was expressed 12.3x more than *DDAH1* in BOEC ($p<0.0001$), whereas HUVECs only expressed 4.7x more *DDAH2* than *DDAH1*. Under inflammatory conditions, *DDAH1* expression stayed constant for both BOECs and HUVECs. For *DDAH2*, under inflammatory conditions, the mRNA expression did not significantly change in BOEC, however, in HUVECs *DDAH2* expression was upregulated ($p=0.0254$).

Quantification of nitrates and nitrates, reflecting NO production, revealed that both BOECs and HUVEC produce little basal NO (Figure 3. 12), which did not change under inflammatory conditions. The NO donor, DEA-NONOate, significantly increased nitrite and nitrate production in the cell supernatants (BOEC, $p<0.0001$; HUVEC, $p<0.0001$), with higher amounts of nitrite and nitrates produced in BOEC supernatants than HUVEC ($p=0.0106$).

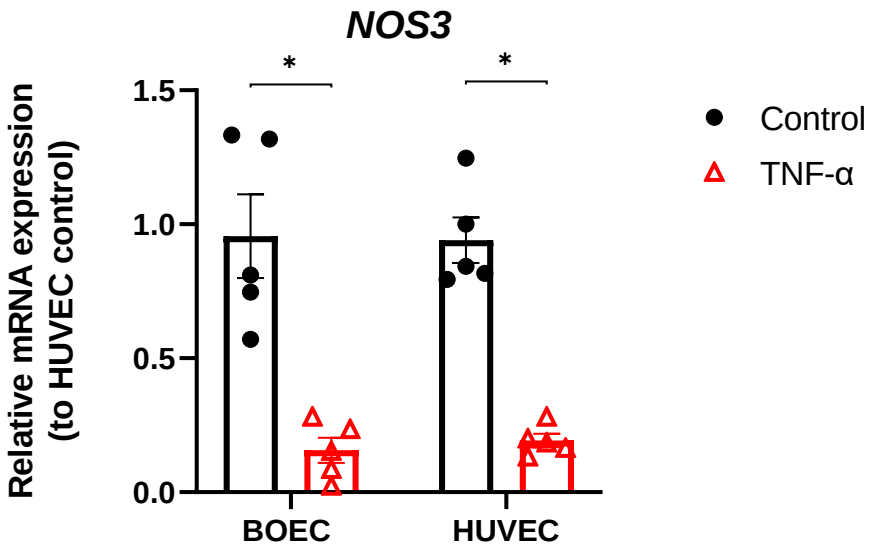


Figure 3. 10 TNF-α downregulates *NOS3* in BOECs and HUVECs

Relative *NOS3* mRNA expression of BOEC and HUVEC after TNF-α treatment for 24 hours. The expression was normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. Error bars show the mean ± SEM. Experiments were carried in duplicate, five biological replicates. Statistical analysis, 2way ANOVA with Šídák's multiple comparisons test confirmed significant differences, $p<0.05$, shown by (*).

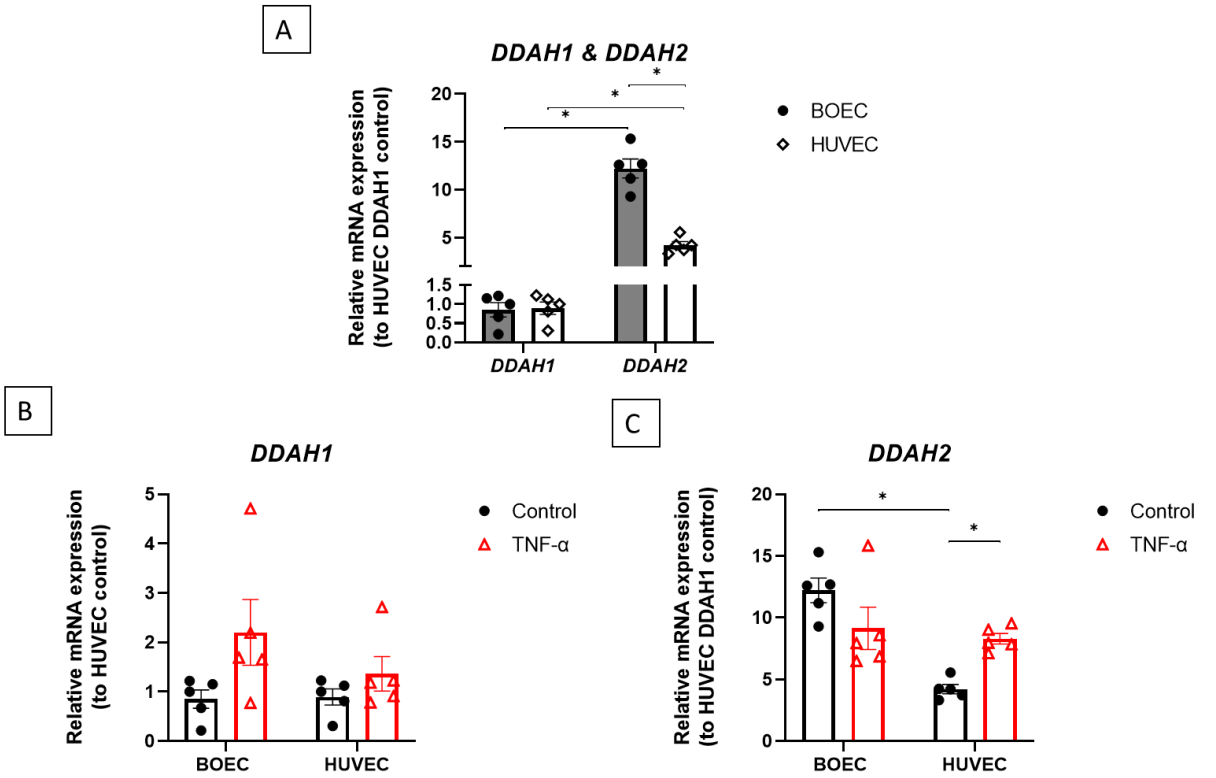


Figure 3. 11 BOECs express more *DDAH2* than HUVECs

Relative *DDAH1* and *DDAH2* mRNA expression in BOECs and HUVECs, determined using the $2^{-\Delta\Delta ct}$ method. **A)** *DDAH1* was similar in BOECs an HUVECs. Both EC types expressed more *DDAH2* than *DDAH1*. BOECs expressed significantly more *DDAH2* than HUVECs. **B)** *DDAH1* expression under inflammatory (TNF-α, 10ng/ml) did not significantly change from control conditions for both EC types. **C)** *DDAH2* expression under inflammatory conditions did not change for BOECs, but for HUVECs DDAH2 was upregulated. Error bars show the mean ± SEM. Experiments were carried in duplicate, five biological replicates. Statistical analysis (2way ANOVA with Šídák's multiple comparisons test) demonstrated significant differences, $p<0.05$, denoted by (*).

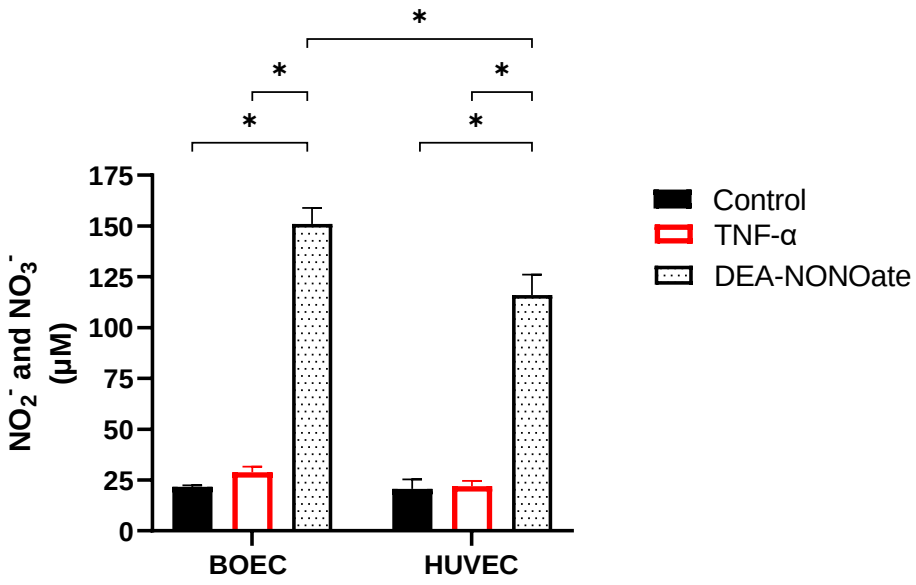


Figure 3. 12 NO donor increases nitrite and nitrate production to a greater extent in BOECs than HUVECs

Cell supernatants were collected from culturing BOECs or HUVECs in presence of media alone, TNF-α (10ng/ml) or DEA-NONOate (100µM). The supernatants were analyzed for the production of nitric oxide by determining nitrite and nitrate levels using a nitrite/nitrate assay colorimetric kit. Error bars show the mean ± SEM. Experiments were carried in duplicate, three biological replicates. Statistical analysis (2way ANOVA with Tukey's multiple comparisons test) demonstrated that there was a significant increase in production of nitrites and nitrates from control or TNF-α in comparison with DEA-NONOate treatment for BOEC ($p<0.0001$) and HUVEC ($p<0.0001$), but the increase observed in BOEC supernatants was significantly higher than in HUVECs ($p=0.0106$). **Abbreviation**- DEA-NONOate: 2-(N,N-Diethylamino)-diazenolate 2-oxide sodium salt hydrate.

3.4.5. Endothelial function in BOEC vs HUVEC

The analysis of BOECs within this study thus far has focused on transcriptomics, morphology, immune phenotype and structure. To fully access BOECs as a model, key endothelial functions were explored such as growth, migration, and adhesion of leukocyte.

3.4.5.1. Proliferation

Proliferation and migration and are stages in the progression towards angiogenesis. This study looked at how BOECs responded to inflammatory cytokines through these key functions. Cell proliferation was assessed using a BrdU ELISA. Figure 3. 13 confirms that the BOECs and HUVECs behave in a similar manner under TNF-α stimulation, which reduces growth in BOECs by 46.0% (p=0.0062) and by 64.4% in HUVECs (p=0.0009).

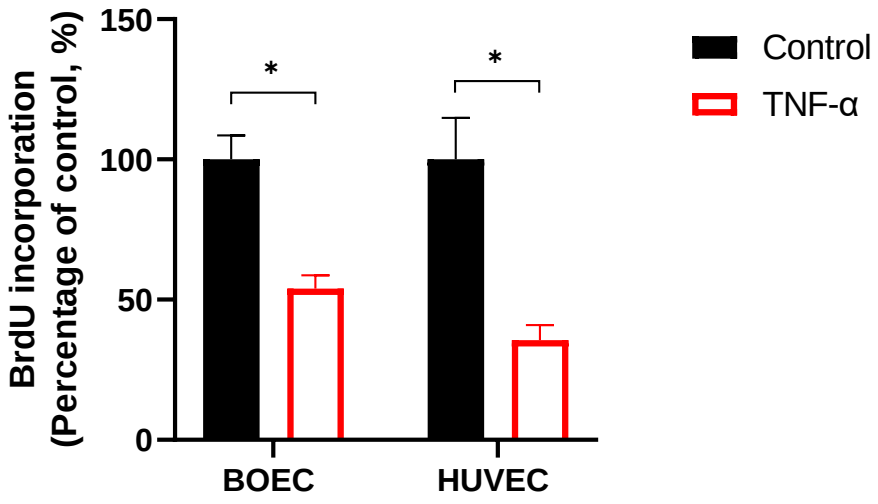


Figure 3. 13 Both BOEC and HUVEC cell proliferation is attenuated after stimulation with TNF-α

Endothelial cells were plated and left overnight, before undergoing inflammatory stimulation with TNF-α, or treated with media only as a control. Cell proliferation was assessed using cell proliferation BrdU ELISA. Error bars show the mean ± SEM. Experiments were carried in duplicate, 6 biological replicates. Statistical analysis (2way ANOVA with Šídák's multiple comparisons test) showed significant differenced from control indicated by (*), where $p<0.05$.

3.4.5.2. Migration

One of the steps in the angiogenic process is cell migration. The two endothelial cell types were assessed in terms of their ability to migrate and close a 'wound' made into a confluent monolayer of cells. HUVECs were able to migrate and close the wound by 24 hours, with a relative wound density of 84.1 ± 5.0%, whereas by 24 hours the mean wound density for BOECs was 54.5 ± 4.6%. Overall HUVECs had a significantly higher relative wound density (p=0.0360), representing faster migration than BOEC (Figure 3. 14).

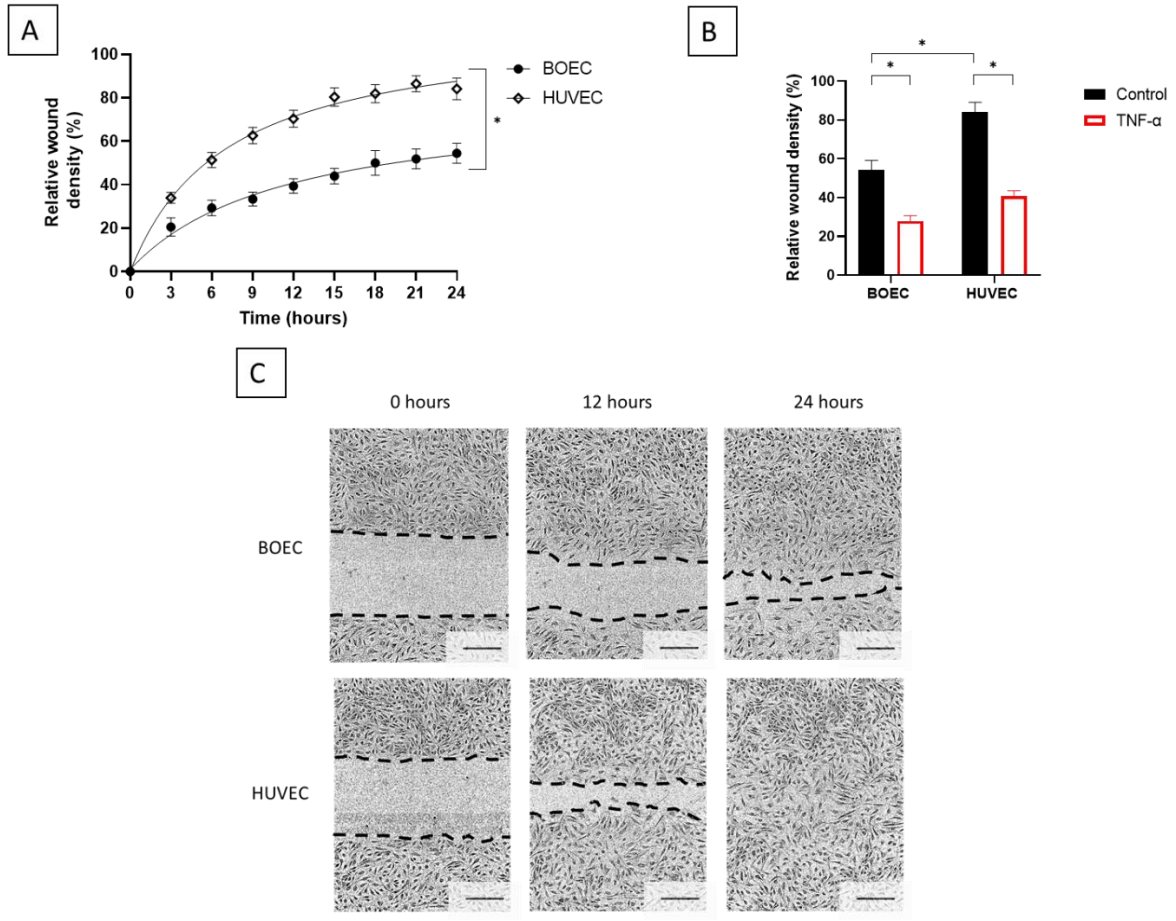


Figure 3. 14 TNF-α inhibits endothelial cell migration and HUVEC migrate faster than BOEC

A 'wound' was made into a confluent monolayer of endothelial cells and the cells were allowed to recover for 24 hours. Images were taken every 3 hours and analysed by an Incucyte® ZOOM system. Cell migration was quantified by measuring the cell density of the wound. **A)** HUVECs migrate faster than BOECs over 24 hours (p=0.0002, Paired T test). **B)** At 24 hours TNF-α (10ng/ml) stimulation inhibits cell migration in both BOEC (p=0.0378) and HUVEC (p=0.0018) determined by statistical analysis, 2way ANOVA with Tukey's multiple comparisons test. **C)** Representative images of wound healing assay at 0, 12 and 24 hours after scratch. The black dashed line indicates a manual trace of the wound front. Scale bar represents 300µm. Experiments were carried out in duplicate, with 9 biological replicates (n=9).

3.4.5.3. Monocyte adhesion

As part of the stages of the inflammatory process, endothelial cells respond by recruiting and adhering leukocyte before trans- endothelial migration. To model this, confluent ECs were stimulated with TNF-α (10 ng/ml) for 0, 4 or 24 hours and then co-cultured with THP-1 monocytes for 1 hour. Prior to this the THP-1s had been fluorescently labelled with calcein AM.

The results show (Figure 3. 15) that after 4 hours of TNF-α stimulation both BOECs ($p<0.0001$) and HUVECs ($p<0.0001$) significantly enhanced the adherence of THP-1 monocytes. 24 hours of TNF-α stimulation did not improve the adhesion of THP-1s further than the levels seen at 4 hours, for either BOECs or HUVECs.

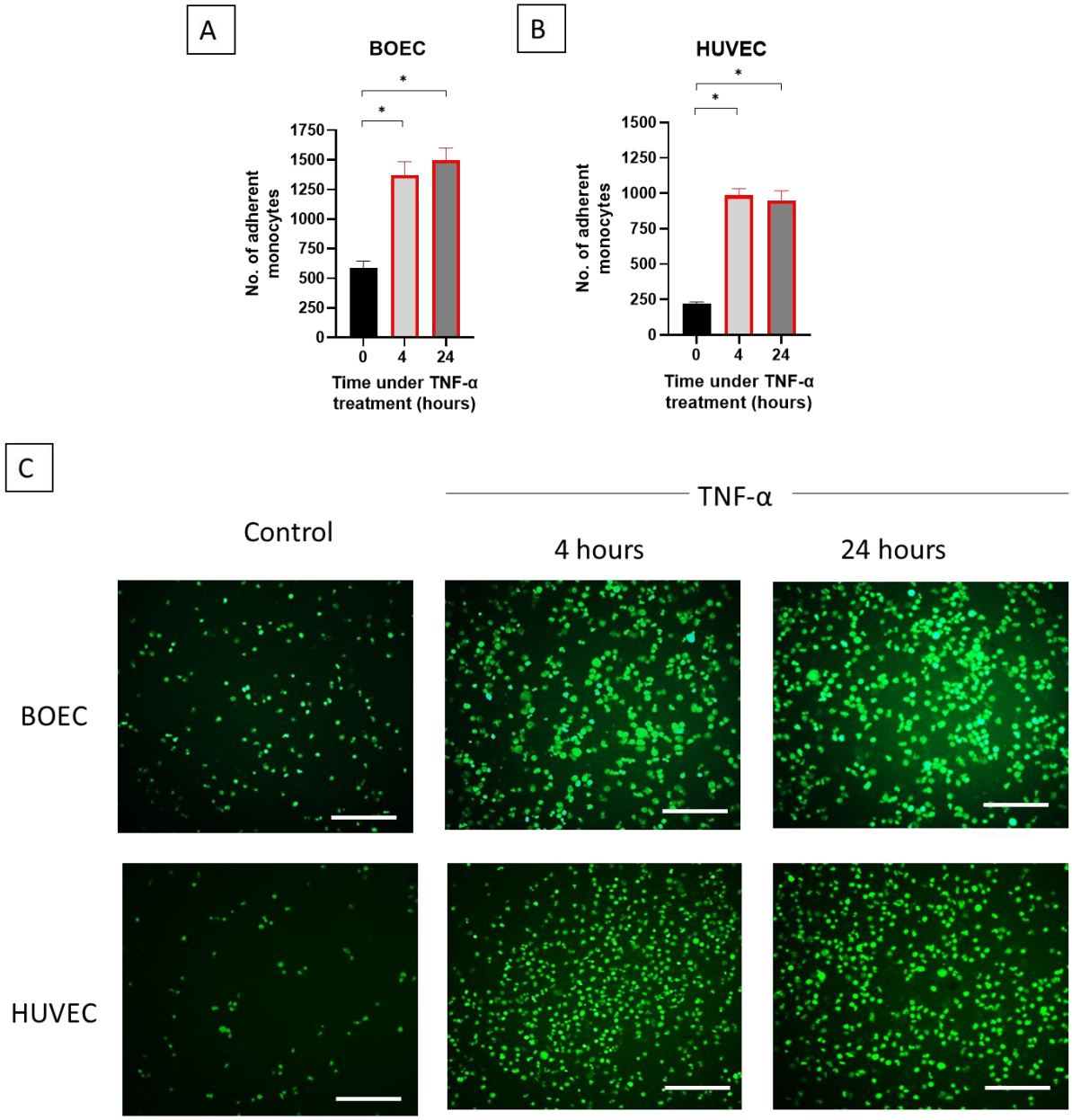


Figure 3. 15 Stimulated BOECs and HUVECs adhere monocytes

Confluent BOEC and HUVECs were treated for 0, 4 and 24 hours with TNF-alpha before being co-cultured with calecin AM labelled-THP-1s. 3 random fields of vision were taken after one-hour of co-culture. Adherent cells were quantified using Fiji software (Schindelin et al., 2012). Experiments were carried out n=3. **A)** BOECs, and, **B)** HUVECs after 4 and 24 hours of TNF-α stimulation adhered significantly more THP-1s than control, but increasing the activation time from 4 to 24 hours did not significantly improve monocyte adhesion. **C)** Representative images of THP-1 monocytes (green), adhered to a confluent monolayer of endothelial cells, with no TNF-α (control), or after 4, or, 24 hours of TNF-α stimulation (10ng/ml). Statical analysis used one-way ANOVA and showed significant differences denoted using (*), $p \leq 0.0001$. Scale bar represents 100µM.

3.5. Discussion

The specific aims listed in section 3.2 have been met as follows:

I. The transcriptomics from cells isolated from disease vs healthy, and those pharmacologically treated *in vitro* have been considered and BOECs follow the same trend as HUVECs,

II. The endothelial characterisation of BOECs has been compared to HUVECs by the morphology and cell surface marker expression, which has also demonstrated BOECs are highly similar to HUVECs, apart from CD54 expression, which remains low but is still higher in BOECs than HUVECs,

III. The expression of proteoglycan core proteins was determined as well as coverage of the glycosaminoglycan, heparan sulphate, *in vitro*, revealing a greater coverage in BOEC than HUVEC for *GPC1, SDC4* and Heparan sulphate.

IV. Production of nitric oxide and expression of the nitric oxide pathway mediators has revealed some unique features of BOEC; elevated *DDAH2* for quiescent BOEC and higher nitrite and nitrate production with the NO donor treatment. Though, BOEC follow similar trends, as those set by HUVEC, under inflammatory stimulation such as downregulation of *NOS3*.

V. Proliferation, migration and adhesion assays have been performed to assess typical endothelial cell function and response to inflammatory stimuli. BOECs performed at a slower rate in migration assays, but otherwise BOECs responded to inflammatory stimuli, consistent with HUVECs.

The transcriptomics results showed HUVECs and BOECs reflect the patients they come from, and has shown that *in vitro*, BOECs respond to pharmacological stimulation more so than HUVECs. This might be ascribed to HUVECs being fetal in nature. Whilst BOECs, can be expanded from all ages of patient groups.

In support of BOECs being endothelial in nature, a number of typical EC characteristics were examined. Both BOECs and HUVECs have the same cobbled-shaped morphology, which was confirmed with BOECs from our donors, in line with previously published work (Lin et al., 2000; Ormiston et al., 2015). This is in contrast to early EPCs which do not have mature endothelial cell morphology (Hebbel, 2017). Immune phenotype was compared between

HUVECs and BOECs and the quiescent BOECs isolated in this study showed classical endothelial cell marker expression, including high CD144, CD31 and lack of haematopoietic progenitor and monocytic markers CD34, CD14 and CD16. The only difference observed was CD54 expression or ICAM-1, which was higher in BOECs, than HUVEC, suggesting a more activated phenotype (Li et al., 2013). The transcriptomics cross-study data, also showed a difference in *CD54* expression where BOECs expressed higher levels of *CD54* than HUVECs. Therefore, the CD54 expression difference between BOECs and HUVECs was consistent at mRNA and protein expression levels. Although, the CD54 protein expression level was still relatively low, <8%, and other markers of endothelial cell activation such as CD62e and CD62p were low and not different between the two EC types.

BOECs *in vitro*, express more glycocalyx shown at mRNA (*GPC1* and *SDC4* not *HSPG2*) and protein levels (HS). As far as I am aware this is the first time that BOEC glycocalyx expression has been reported *in vitro*. For HUVECs the literature is mixed; some groups have reported the glycocalyx at protein level in HUVECs, others have not (Chappell et al., 2009b; Delgadillo et al., 2021; Klein et al., 1992; Potter and Damiano, 2008). Our study involved pooled donor HUVECs so, perhaps the donor variance is why this study reported very little expression at protein level, despite proteoglycan core protein mRNA expression. Other proposals for the lack of glycocalyx *in vitro* is the flow conditions, the use of blood products, such as FBS, or cell culture media (Chappell et al., 2009b; Dong et al., 2002; Fu and Tarbell, 2013; Urner et al., 2012).

In response to inflammatory stimuli at mRNA level, the amount of *GPC1* is downregulated by BOECs, but stays constant in HUVECs. *HSPG2* stays constant in both BOECs and HUVECs, but, BOEC *SDC4* expression increases with TNF-α treatment. Consistent to the results shown in this chapter, it has been reported previously that *in vitro* TNF-α increased *SDC4* mRNA expression in glomerular endothelial cells (GEnC) (Ramnath et al., 2014). Despite the upregulation at mRNA level, TNF-α caused shedding of SDC4 and HS into the culture media and animal models have shown TNF-α induced shedding of the endothelial glycocalyx over 10 minute infusions (Chappell et al., 2009a; Ramnath et al., 2014). The divergence in response of BOECs in terms of *SDC4* and *GCP-1* to inflammatory stimuli may be down to a compensatory mechanism, which has been previously implied before when loss of SDC-1 increases GPC-1 (Voyvodic et al., 2014).

Examination of the regulators of NO production revealed that *NOS3* expression is equivalent in BOECs and HUVECs. In response to inflammatory stimuli both EC types downregulated *NOS3*. This response has been observed by others for HUVECs (Caravà et al., 2021; Choi et al., 2017; Sala et al., 2002; Yan et al., 2008).

Both BOECs and HUVECs express more *DDAH2* than *DDAH1*. This result was expected as DDAH2 is the predominant form found highly vascularised tissues (Tran et al., 2000). *DDAH2* expression was greater in BOECs in comparison to HUVECs, however, under inflammatory stimulation *DDAH2* expression remains constant in BOECs but upregulated in HUVECs.

DDAH1 expression does not change in either cell type. Literature has shown that HUVEC *DDAH1* mRNA has been downregulated after TNF-α stimulation (Sundar et al., 2019; Yang et al., 2018). This is in contrast to the results presented in this study, however, the concentrations of TNF-α that these publications used, 30 and 50ng/ml over 24 hours, which are very high and causes cell death (Sundar et al., 2019; Yang et al., 2018; Zhou et al., 2017). A lower concentration of 10ng/ml was used and this may account for the observed difference. *DDAH2* expression following 24 hours of TNF-α stimulation (10ng/ml), has been previously reported to be decreased in HUVECs and whilst a similar observation was found in BOECs, it was not seen in HUVECs, and could be attributed to the source of the HUVECs (Pullamsetti et al., 2011).

Nitrite and nitrate levels reflecting NO production, were low in HUVEC and BOEC under quiescent conditions and this did not change under inflammatory conditions. Following DEA-NONOate treatment it is unclear why nitrite or nitrate levels were higher for BOEC, than HUVEC supernatants. This may be due to a substance in the BOEC supernatant specifically that reacts with the NO donor, or the NO donor creating an upregulation of NO production in BOECs.

At a functional level TNF-α stimulation inhibited BOEC and HUVEC proliferation and migration, in this study. This phenomenon has been reported previously for HUVECs, though I believe that this is the first time this has been reported in BOECs (Jiang et al., 2016; Kim et al., 2017). In response to inflammatory stimuli, adhesion of monocytes was increased, which has been consistently reported prior to this for HUVECs (Kwon et al., 2007; Wang et al., 2016). At a basal level however, adhesion of leukocytes was higher in BOEC than HUVECs and this may

be due to the higher CD54 level seen in BOECs than HUVECs. The representative images seen in Figure 3.15 of calecin AM labelled-THP-1s, adhered to the monolayer of endothelial cells, show a different spread of the THP-1 cells dependent on the EC type adhered to. The appearance of the THP-1s adhered to BOECs seemed more 'clumped' whereas the images of the THP-1s adhered to HUVECs, appear more like single cells. The experiments on each of the EC types were performed simultaneously with the same batch of calecin AM labelled THP-1s. Hence, the difference observed is likely to be due to a difference in quantity and distribution of EC surface adherence markers expression (e.g. CD54) in BOECs in comparison to HUVECs.

This chapter has demonstrated the suitability of BOECs as an endothelial model in terms of structure, function and inflammatory response at mRNA and protein levels. In many ways BOECs in control conditions and inflammatory stimuli, respond in a similar fashion to HUVEC with respect to transcriptomics, morphology, cell surface marker expression, *NOS3* expression, proliferation and adhesion of leukocyte. However, BOECs express some unique features such as elevated *DDAH2* levels and extensive *in vitro* glycocalyx expression, unlike the pooled HUVECs. These characteristics make BOECs a preferential model to study the endothelium as they appear to replicate those found *in vivo*. BOECs are thought to show epigenetic individual differences and evidence has been presented previously, where isolated BOECs from smokers and COPD patients, in comparison to healthy controls showed epigenetic regulation of DNA damage and senescence (Paschalaki et al., 2013). Accompanied by the fact that BOECs reflect a patient's vascular health and can be isolated from all ages of patient groups, this chapter has established that BOEC are a better model than HUVEC to study endothelial health. In Chapter 4 elements of the adenosine pathway in the BOECs will be studied, consolidating knowledge of the adenosine pathway in ECs.

4. Results 2- Adenosine pathway of BOECs

4.1. Introduction

In order to understand the impact of ADA2 on BOECs, the role of its metabolite, adenosine, must be understood. Adenosine is a purine nucleoside which has many important roles in cellular metabolism. These include binding to its transmembrane receptors for downstream signalling and forming part of the composition of the universal energy currency ATP and ADP, and of the secondary signalling molecule, cyclic adenosine monophosphate (cAMP). Both intracellular and extracellular adenosine levels are regulated by the hydrolysis of ATP to adenosine by a series of transmembrane enzymes found on endothelial cells and immune cells (CD39, Alkaline Phosphatase and CD73) (Kaczmarek et al., 1996; Millán, 2006; Yegutkin, 2008; Zimmermann, 1992). ATP, which is maintained at low intracellular concentrations physiologically (30-1000nM), is released from ECs as well as other immune cells under hypoxic or inflammatory conditions (Bergfeld and Forrester, 1992; Bodin and Burnstock, 1995; Gorman et al., 2007; Schenk et al., 2008). ATP is generally believed to be a pro-inflammatory agent, but can also act as anti-inflammatory agent depending on its concentration (Haskó et al., 2000b). The prolonged ATP pro-inflammatory stimulation of cells, is prevented by the hydrolysis of ATP to adenosine (Yegutkin, 2008).

As previously discussed adenosine is considered anti-inflammatory in its actions and is usually maintained at low concentrations; however, the adenosine concentration can increase when there is cell damage, inflammation or hypoxia (Marquardt et al., 1984; Ontyd and Schrader, 1984; Rudolphi et al., 1992; Van Belle et al., 1987; Winn et al., 1981). Locally, adenosine levels are controlled by adenosine deaminases, which decrease the concentration of adenosine by deamination of adenosine and 2'-deoxyadenosine, to inosine and deoxyinosine, respectively, or alternatively, by nucleoside transporters transporting adenosine intracellularly, expressed on ECs and some immune cells (Haskó et al., 2008; Wei et al., 2004). Maintaining a delicate balance between ATP and adenosine concentrations controls the stages of inflammation between the activation and enhancement of inflammation and to the inhibition and resolution of inflammation (Faas et al., 2017). Disruption of this balance can occur under extreme physiological conditions such as heavy exercise or hypoxia but can also occur with

pathologies, such as atherosclerosis or preeclampsia, or on the other hand cancer and chronic viral infection (Faas et al., 2017).

Extracellular adenosine binds to cells via one of the four P1 purinergic receptors, also known as the adenosine receptors (AR), which are outlined in Table 4. 1.

Table 4. 1 Summary of the four purinergic or adenosine receptors

A table describing the receptor, gene name as well as signalling properties of each adenosine receptor. Adapted from (Chen et al., 2013; Effendi et al., 2020; Faas et al., 2017).

Adenosine receptor	Gene	Coupled G protein	Intracellular signalling	Affinity for adenosine
A_1	*ADORA1*	$G_{i/o}$;	↓cAMP; ↑Ca^{2+}	High
A_{2A}	*ADORA2A*	$G_{s/olf}$	↑cAMP	High
A_{2B}	*ADORA2B*	G_s; $G_{q/11}$; $G_{12/13}$	↑cAMP; ↑Ca^{2+}	Low
A_3	*ADORA3*	$G_{i/o}$;$G_{q/11}$	↓cAMP; ↑Ca^{2+}	Low

AR are all examples of G protein-coupled receptors (GPCR) that have different affinities for adenosine and either inhibit or activate adenylate cyclase and influence cAMP production (Fredholm et al., 2001a). Although, there are some G protein independent effects of these receptors (Cronstein, 1994).

Agonist-receptor binding to Gs linked receptors, such as A_{2A} and A_{2B}, increases production of cAMP by activating adenylyl cyclase to catalyse ATP conversion to cAMP. cAMP is an important secondary messenger as it binds to effector molecules such as PKA or EPAC. In contrast, agonist receptor binding to Gi linked receptors, such as A_1 and A_3 receptors, inhibits adenylate cyclase and prevents the catalysis of ATP to cAMP (Calker et al., 1979; Fredholm et al., 1994).

In addition to the range of linked-G proteins to each AR, the affinity of adenosine for each of the ARs is concentration dependent. It is thought that A_1, A_{2A} and A_3 receptors, if there are abundant A_3 receptors present, are activated at low, physiological concentrations of

adenosine (Fredholm et al., 2001b). Whereas, under extreme physiological conditions, such as heavy exercise, and pathophysiological conditions such as ischemia and apoptosis or necrotic cell death, adenosine concentrations will increase and A_{2B} receptors will also be affected (Fredholm et al., 2001b; Fredholm, 2007).

A_1 receptors are mostly found in the central nervous system, kidneys and testis; A_{2A} ARs are predominantly distributed across the liver, heart, lungs and cells and organs within the immune system and the CNS; A_{2B} ARs, the CNS, some immune cell types, lungs, bladder and bowel; A_3 AR in the testis with high expression and to a lesser extent in the lungs, kidneys, heart and some parts of the CNS (Dixon et al., 1996; Fredholm et al., 2000; Zhou et al., 1992). ECs of different origins are thought to express predominantly A_{2A} and/or A_{2B} receptors (Feoktistov et al., 2002; Nguyen et al., 2003).

Adenosine is known to be a potent vasodilator, primarily acting via the A_{2A} receptor, so much so, that adenosine is used in clinical cardiovascular stress tests, to look at blood flow to the heart (Berne, 1963; Drury and Szent-Györgyi, 1929). Here adenosine is given via an intravenous pump, and images of the heart are taken before and after adenosine administration to imitate the effect of stress on the heart (similar to that which is experienced under exercise). Adenosine's other function is considered generally to be anti-inflammatory and plays a part in the resolution of inflammation (Faas et al., 2017). Evidence to substantiate this is diminished neutrophil recruitment, inhibition of pro-inflammatory classical macrophage activation and cytokine release (Cronstein et al., 1990; Csóka et al., 2012; Haskó et al., 2000a). It is believed to be mediated via A_{2A} ARs, which most commonly have anti-inflammatory effects. A_{2A} AR agonists are in development for the treatment of asthma and COPD, arthritis, inflammatory bowel disease and wound healing (Flögel et al., 2012; Golzar and Doukky, 2014; Montesinos et al., 1997; Odashima et al., 2005). Only Regadenoson 21, an A_{2A} AR agonists is approved for human use (Jacobson et al., 2019; Palani and Ananthasubramaniam, 2013).

However, adenosine's homeostatic and anti-inflammatory categorisation in the literature maybe oversimplified, especially as the other ARs have roles as both pro-inflammatory and anti-inflammatory mediators, depending on duration and concentration. For example, A_1 AR agonists reduce the inflammatory response, preventing septic peritonitis, in mouse models,

though conversely will also induce secretion of VEGF and promote monocyte differentiation into osteoclasts (Clark et al., 2007; Gallos et al., 2005; Kara et al., 2010). A_{2B} ARs mediate most of their anti-inflammatory effects by coupling with G_s and, pro-inflammatory effects by coupling with G_q (Linden, 2006). This is demonstrated where activation of A_{2B} AR stimulates IL-6 in ECs but in macrophages, TNF-α production is inhibited (Figler et al., 2011; Németh et al., 2005). A_3 AR activation also has conflicting roles within the inflammatory process. For example, these receptors promote inflammatory cell influx in the lung of eosinophils and macrophages, though A_3 AR agonists suppress production of TNF-α, can be used to treat rheumatoid arthritis (Silverman et al., 2008; Spruntulis and Broadley, 2001).

Moreover, there are conditions where chronic overproduction of adenosine is pathological such as; asthma, pulmonary fibrosis, cancer, Parkinson's disease, and more recently DADA2 (Allard et al., 2014; Carmona-Rivera et al., 2019; Cieślak et al., 2008; Karmouty-Quintana et al., 2013; Luo et al., 2016). High adenosine concentrations have been also reported in fetal plasma and from patients with preeclampsia. Primary HUVECs isolated from these patients have displayed elevated A_{2B} AR expression in comparison to healthy controls (Acurio et al., 2014; Yoneyama et al., 1996).

Adenosine, and signalling via its four receptors, has been shown to exhibit both anti-inflammatory and pro-inflammatory mechanisms, leading to the development and use of pharmacological interventions (Effendi et al., 2020). These opposing roles seem to depend on the abundance of the receptor and/or adenosine, and the duration of stimulation, where adenosine's protective signalling functions (e.g. A_{2A} AR anti-inflammatory functions) are unable to protect against chronic insults, and can even exacerbate disease states (Haskó et al., 2008). This makes clinically successful pharmacological targeting of the ARs problematic, as discussed by a number of reviews (Borea et al., 2018; Chen et al., 2013; Effendi et al., 2020; Haskó et al., 2008; Peleli et al., 2017).

To add to the understanding of how ECs respond to the changing extracellular environment and if this response is consistent across EC models, *in vitro*, this chapter will explore EC AR profile for BOECs in comparison to HUVECs and aim to understand how physiological (~0.02-0.5µM), extreme physiological, such as found with strenuous exercise (~0.5-5µM), and

pathophysiological extracellular adenosine concentrations, such as found during ischemia (≥5μM), influences the structure and function of these cells.

4.2. Specific aims

1. To examine adenosine receptor expression profile in BOECs and HUVECs by transcriptomic and qRT-PCR analysis.
2. *In vitro* quantification at mRNA level of adenosine receptor profile of BOECs and HUVECs, subsequent to manipulation of extracellular adenosine.
3. To understand how extracellular adenosine influences BOECs in the following ways:
    a. The EC glycosaminoglycan profile.
    b. The expression of soluble signalling mediators such as nitric oxide and its regulators.
    c. The function of BOECs by looking at proliferation, migration and leukocyte adhesion.

4.3. Methodology

4.3.1. Transcriptomics

EndoDB was used to identify adenosine receptor mRNA expression across multiple previous studies (Khan et al., 2019). An advanced search by gene was performed for each of the four receptors *ADORA1, ADORA2A, ADORA2B* and *ADORA3* to observe normalised log fold change in expression across samples, within each study. Search constraints 'Homo sapiens', and cell types, 'Blood outgrowth endothelial cells' and 'Umbilical vein endothelial cells' were selected. The results for each of the genes were downloaded and manually sorted. Samples were excluded if the reference cell type was not either HUVEC or BOEC in nature. There were 4 BOEC studies and 146 HUVEC studies in total. To compare healthy versus inflammatory-associated disease the studies utilised for BOEC were accession ID E-GEOD- 9877 (sickle cell at risk of ischemic stroke) and for HUVEC accession ID E-GEOD-49524 (gestational diabetes). Comparisons of BOEC and HUVEC *in vitro* treatment with BM-6 and BMP-9 was not carried out as the sample size was too small, n=1, for HUVEC (BOEC accession IDs E-MTAB-2495, and HUVEC E-GEOD-27631).

4.3.2. qRT-PCR

To examine how adenosine influences the expression of particular genes in BOECs and HUVECs, qRT-PCR was used. Cells were treated with increasing concentrations of adenosine (0.05, 0.5 and 5µM) spanning physiological, extreme physiological (such as strenuous exercise or high altitude), and pathophysiological adenosine concentrations respectively. The RNA was extracted and cDNA synthesised as described in the general methods. As well as the primers validated in Chapter 2 and Chapter 3, additional primers for each adenosine receptor were validated from sequences previously published for the four adenosine receptors (Table 4.2) (Tajadini et al., 2014). qRT-PCR was carried out as described in the general methods. Cq values were analysed to observe relative gene expression employing the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Table 4. 2 Primers to target the genes of the four adenosine receptors

Table listing the primer sequences (Tajadini et al., 2014) for *ADORA1, ADORA2A, ADORA2B, ADORA3,* as well as the expected product length and the calculated efficiency from primer validation.

Gene	Sequence		Product length (bp)	Efficiency (%)
	Forward Primer 5'-3'	Reverse Primer 5'-3'		
ADORA1	TCGCCATCCTCATCAACA	ACCATCTTGTACCGGAGAG	161	98.8
ADORA2A	CTCCATCTTCAGTCTCCTGG	AAGCCATTGTACCGGAGC	75	96.7
ADORA2B	CTCCATCTTCAGCCTTCT	ACCAAACTTTTATACCTGAGC	78	96.0
ADORA3	TTGCCTACTGCTTATCTT	TCTTGTATCTGACGGTAA	98	98.8

4.3.3. Immunocytochemistry

BOEC cells were plated onto gelatine coated chamber slides and grown until fully confluent, as detailed in the general methods. HUVECs were not used in these experiments as results in chapter 3 highlighted the lack of heparan sulphate expressed, by HUVECs *in vitro*.

To observe the effect that extracellular adenosine had on the recovery of the glycocalyx, the confluent cells were incubated with the degrading enzyme, Heparinase III (25 U/L) for 2 hours. Another two wells with no enzyme treatment were utilised for the secondary antibody control and the positive control. The cells were then gently washed with pre-warmed PBS and incubated with adenosine (0.5µM) or media only. The glycocalyx was then allowed to recover for 18 hours. The cells were then washed 3x with PBS and fixed in 4% PFA and blocked with 3% BSA for 30 mins. The slides were then incubated overnight at 4˚C with the primary antibody, targeting heparan sulphate at a dilution of 1 in 100 in 1% BSA. Following this, the primary antibody solution was removed, the cells were washed and then incubated with a fluorescently labelled FITC secondary antibody (at a dilution of 1 in 200). Further staining with Hoechst 33342, (1 in 2000) targeted the nucleus of each cell.

4.3.4. Nitrite/nitrate assay

Cell supernatants were collected from cells treated for 24 hours with increasing concentrations of adenosine (0, 0.05, 0.5 and 5μM) in DMEM complete media. Addition of the NO donor, DEA-NONOate (100μM), was used as a positive control. DMEM media was used as this does not contain either nitrites or nitrates, which would influence the readings. The manufacturer's instructions for the Nitrite/nitrate kit were followed as described in Chapter 2.

4.3.5. Colorimetric BrdU ELISA

ECs were treated with increasing adenosine concentrations from 10nM (10^{-8} M) to 0.1mM (10^{-4}M) spanning physiological, extreme physiological and pathophysiological concentrations, to form log dose response curves. In conjunction with this cells were treated with a combination of the following:

- adenylate cyclase inhibitor, SQ22536, 100μM,
- adenosine receptor antagonists,
    - the A_{2A} receptor antagonist SCH58261 (Sigma-Aldrich), 1μM, or,
    - the A_{2B} receptor antagonist Alloxazine (Sigma-Aldrich), 10μM.

4.3.6. Wound healing assay

A log dose response curve of adenosine (0, 0.05, 0.5 and 5μM) in the presence and absence of the AR antagonists was performed. Uniform scratches were made across confluent monolayers of ECs using a 'wound maker'. The cells were then washed once with PBS and the pharmacological treatments were applied for 24 hours. Images were taken every 2-3 hours by an Incucyte® ZOOM system. Cell migration to close the wound and relative wound density was measured using Incucyte® ZOOM software as explained in the general methods.

4.3.7. Static leukocyte adhesion assay

To understand if adenosine affects BOEC and HUVEC adhesion to monocytes, ECs were grown to confluence for 2-3 days in 96 well micro plates. ECs were then pre-treated for 24 hours with increasing concentrations of adenosine (0, 0.05, 0.5 and 5μM) in EC defined medium with 2% FBS. The media was removed and replaced with TNF-α (10ng/ml) for 4 hours to

stimulate the cells. THP-1 monocytes were counted and $1x10^6$/mL cells were resuspended in PBS containing, calcein AM (0.1µM) cell permanent dye for 30mins, at 37°C. Conversion of calcein AM to green-fluorescent calein occurred in live cells and therefore fluorescently labelled the THP-1 monocytes. The cells were washed 3x with PBS and resuspended in RPMI without FBS, at a seeding density of $5x10^5$ cells/mL. After stimulation with TNF-α, the ECs were then co-incubated with 100µL of the calcein AM labelled THP-1s at a seeding density of $5x10^5$ cells/mL, for 1 hour at 37°C. The co-cultures were then washed with PBS to remove any unbound monocytes. The EC with monocytes bound were then fixed with 4% PFA for 10mins and washed with PBS. 3 random fields of each well were captured on a Leica DM IL LED microscope and image analysis to count the number of adherent cells was achieved using Fiji software, outlined in Chapter 2 (Schindelin et al., 2012).

4.4. Results

4.4.1. Adenosine receptor expression in BOECs and HUVECs

The mRNA expression of the four adenosine receptors was observed across a variety of studies using the EndoDB. Table 4. 3 highlights the inconsistencies in reported expression across studies, for the same EC type. For instance, although there are 4 BOEC studies in total, only 3 reported any *ADORA1* expression in any of the samples, whereas there was no reported *ADOR2A* in any of the studies, and *ADORA2B* and *ADORA3* expression were reported in all of them. For HUVEC, with a total of 146 studies in the EndoDB, only 103 reported *ADORA1*, 42 studies reported *ADORA2A* and *ADORA2B*, and only 54 studies reported *ADORA3*.

Across the studies which reported gene expression in any of the four ARs, comparisons were carried out between cells isolated from healthy and inflammatory associated disease patients, with the aim to identify any changes in the expression with disease state. Figure 4.1 shows that across the studies, no significant change in any of the four receptors expression in various disease states. There was no reported expression of *ADORA2A* within BOEC samples.

Table 4. 3 The number of studies which report expression of the four adenosine receptors across the EndoDB and the BOEC or HUVEC samples

Table listing the number of studies which report each of the four adenosine receptors *ADORA1, ADORA2A, ADORA2B, ADORA3* across any of the BOEC and HUVEC samples, in the EndoDB (Khan et al., 2019).

	Total number of studies	***ADORA1***	***ADORA2A***	***ADORA2B***	***ADORA3***
BOEC	**4**	3	0	4	4
HUVEC	**146**	103	42	42	54

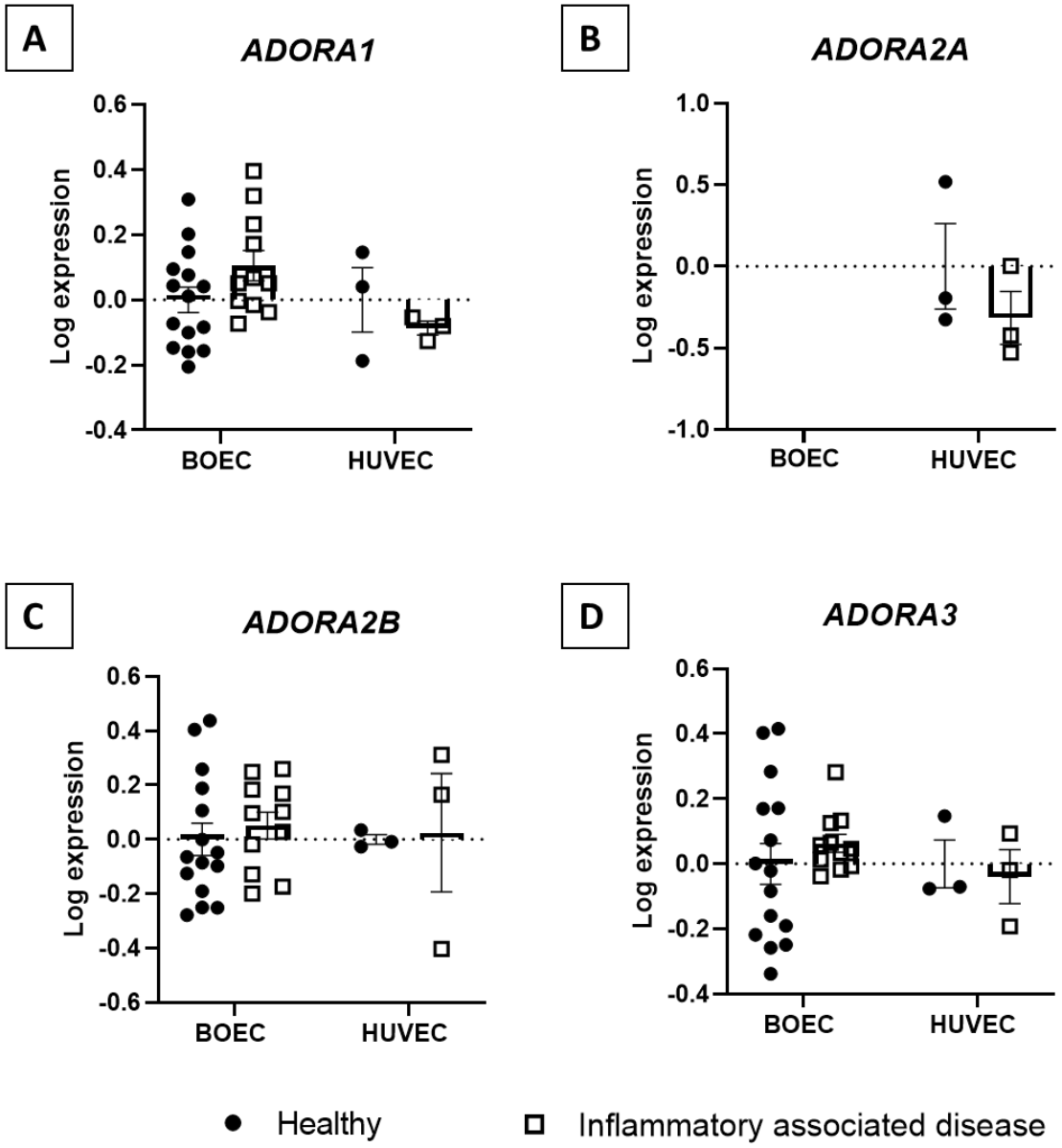


Figure 4. 1 Transcriptomic meta-analysis of BOEC and HUVEC shows no change in adenosine receptor expression in healthy vs inflammatory associated disease.

Interleaved scatter with bars plot, for the four adenosine receptor gene sets displaying the log expression of BOEC and HUVEC samples from healthy and disease cohorts. The data source was EndoDB (Khan et al., 2019). Study accession ID BOEC: E-GEOD- 9877 (sickle cell at risk of ischemic stroke), HUVEC: E-GEOD-49524 (gestational diabetes). Each dot represents a gene expression level in a sample, relative to control. BOEC healthy n=5, BOEC inflammatory associated disease n=11; HUVEC healthy n=3, HUVEC inflammatory associated disease, n=3. The error bars show the mean ± SEM. No expression of *ADORA2A* in BOECs was reported in controls or inflammatory associated disease. Statistical analysis (2way ANOVA Šídák's multiple comparisons test) shows no significant change in regulation of the adenosine receptor genes, *ADORA1, ADORA2A, ADORA2B, ADORA3, with* disease.

The results produced from the EndoDB, show that AR expression in both EC types does not seem to change with inflammatory associated disease, however, there is inconsistent reporting of ARs across studies, highlighting the need to investigate the samples isolated in within this study.

4.4.2. Extracellular adenosine and adenosine receptor expression

Within the samples isolated for this study, exploration of AR mRNA expression has shown a difference in *ADORA2A* vs *ADORA2B* proportions in BOEC and HUVEC EC types (Figure 4.2). HUVECs express 8.6x more *ADORA2A* mRNA than BOECs (from 0.125 ±0.026, to 1.079 ±0.077 $p<0.001$, n=5). Whereas BOECs produce 3.9x more *ADORA2B* mRNA than HUVECs (from 0.078 ±0.028 to, 0.308 ±0.038 $p=0.0065$, n=5).

After inflammatory stimulation with TNF-α, *ADORA2A* expression significantly increased in BOECs 5.3-fold (from 0.125 ±0.026 to, 0.659 ±0.211, $p=0.0461$, n=5) but remained constant in HUVECs. *ADORA2B* expression did not significantly change in either EC cell type after TNF-α stimulation. Hep G2 cells were used as a positive control for *ADORA2A* and *ADORA2B* expression.

No expression was observed by *ADORA1* or *ADORA3* in BOEC or HUVEC under control or after stimulation with TNF-α. The positive controls for *ADORA1* and *ADORA3* were HEK 293T and NB-4 cells, respectively.

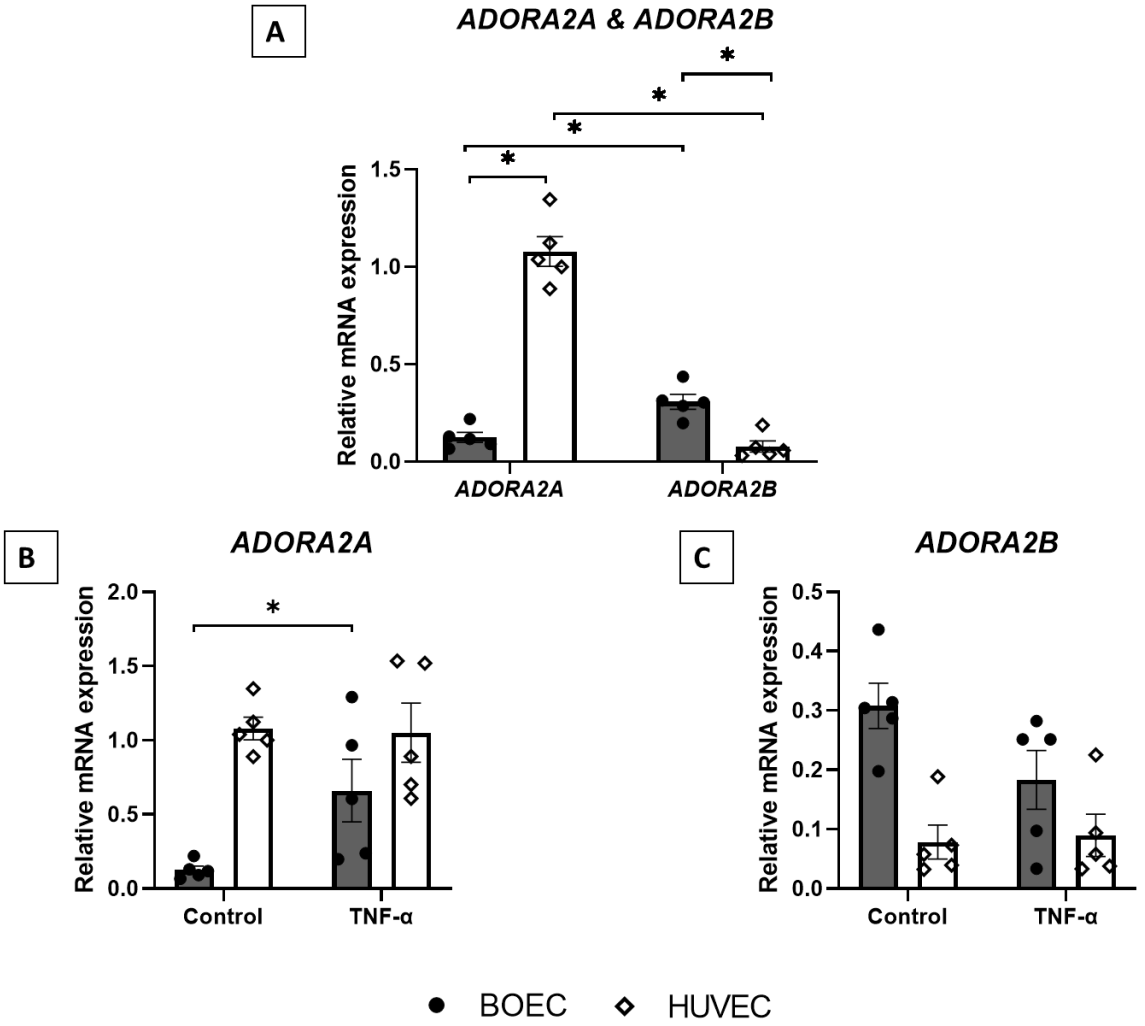


Figure 4. 2 *In vitro* HUVECs constitutively express more *ADORA2A* mRNA than BOECs, but BOECs express more *ADORA2B* than HUVECs

Determination of the relative mRNA expression of *ADORA2A* and *ADORA2B* in ECs *in vitro*. **A)** *ADORA2A* & *ADORA2B* distribution in BOEC in comparison to HUVEC. HUVECs significantly express more *ADORA2A* mRNA than BOECs (8.6x, *p<0.001, n=5). BOECs produce more *ADORA2B* mRNA than HUVECs (3.9x, *p=0.0065, n=5). **B)** Stimulation with TNF-α increases BOEC *ADORA2A* expression (5.3x, *p=0.0461, n=5) but remained constant in HUVECs. **C)** *ADORA2B* expression did not significantly change in either EC cell type after TNF-α stimulation. The error bars show the mean ± SEM. Experiments were carried in duplicate with three biological replicates. Statistical analysis was carried out employing 2way ANOVA with Tukey's multiple comparisons test.

In vitro, the ECs were treated with increasing concentrations of extracellular adenosine (Figure 4.3). HUVECs responded to this stimulation from extracellular adenosine, by a diminished *ADORA2A* mRNA expression, at adenosine concentrations of 0.05µM (p=0.002, n=3) and 5µM (p=0.0023, n=3), however, this effect was not significant at 0.5µM. BOEC *ADORA2A* mRNA expression remained unchanged from control. ADORA2B mRNA remained level with control expression levels for both BOEC and HUVEC across extracellular adenosine concentrations ranging from physiological to pathophysiological levels. When treated with increasing concentrations of extracellular adenosine in HUVECs *ADORA1 and ADORA3* expression was not detected for either EC type.

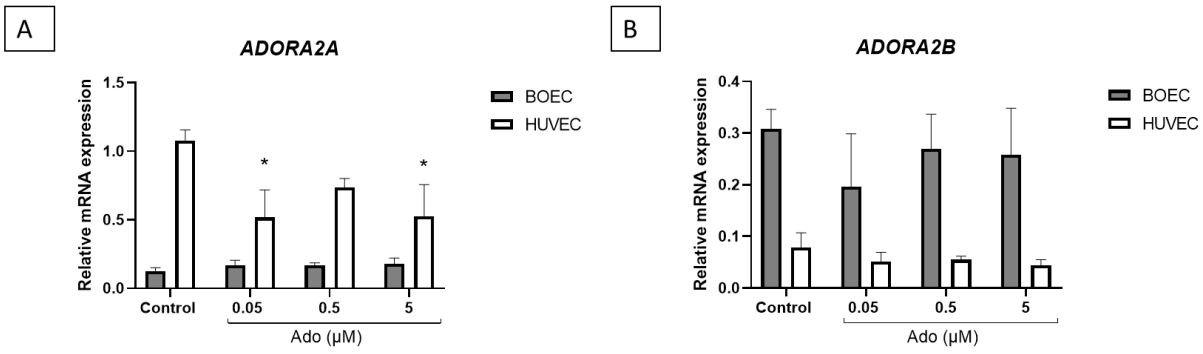


Figure 4. 3 Extracellular adenosine downregulates *ADORA2A* expression in HUVECs only

In vitro exploration of the influence of extracellular adenosine on the relative mRNA expression of *ADORA2A* and *ADORA2B* in ECs. **A)** *ADORA2A* HUVEC expression is reduced at 0.05µM (*p= 0.002, n=3) and 5µM ado (*p=0.0023, n=3). There is no significant change in BOEC expression. **B)** Both BOEC and HUVEC *ADORA2B* expression does not significantly change after extracellular adenosine stimulation. The error bars show the mean ± SEM. Experiments were carried in duplicate with three biological replicates. Statistical analysis was carried out using 2way ANOVA with Dunnett's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

4.4.3. The influence of adenosine on cell glycocalyx structure

The influence of extracellular adenosine on the expression of EC glycosaminoglycan core proteins glycpican-1 (*GPC1*), heparan sulphate proteoglycan 2 (*HSPG2*) and syndecan 4 (*SDC4*) was evaluated by qRT-PCR (Figure 4.4). In HUVEC, expression of all three core proteins remained low under the influence of increasing concentrations of extracellular adenosine. In BOECs high, pathophysiological concentrations of adenosine (5μM) significantly diminished *GPC1* expression (p=0.0316, n=3). In contrast at physiological concentrations of adenosine, *HSPG2* and *SDC4* expression was significantly elevated (0.05μM, *HSPG2* p=0.0113, *SDC4* p=0.0027, n=3) and fell to back control levels at extreme physiological concentrations.

In vivo Glycocalyx removal precedes endothelial cell damage. The ability of the glycocalyx to repair following enzymatic degradation was observed, using immunocytochemistry, for the glycosaminoglycan, heparan sulphate (HS) (Figure 4.5). BOECs were treated with Heparinase III for 2 hours and then allowed to recover with media only or adenosine. As a positive control a treatment without enzymes was used. 18 hours post enzymatic degradation, control cells, treated with media only, had recovered to 52 ±3.6% (p=0.0055, n=3) of their initial coverage. It took a further 18 hours before control ECs recovered their full glycocalyx expression. HS recovery was inhibited by exogenously applied adenosine. At 18 hours, ECs treated with extracellular adenosine had only recovered to 10.16 ±1.2% (p= 0.0002, n=3) of their initial coverage, which was 42±9.4% less than media only controls (p=0.0098, n=3). No HUVEC were used for these experiments as previous experiments, illustrated in chapter 3, had shown a lack of HS coverage *in vitro* for pooled HUVECs.

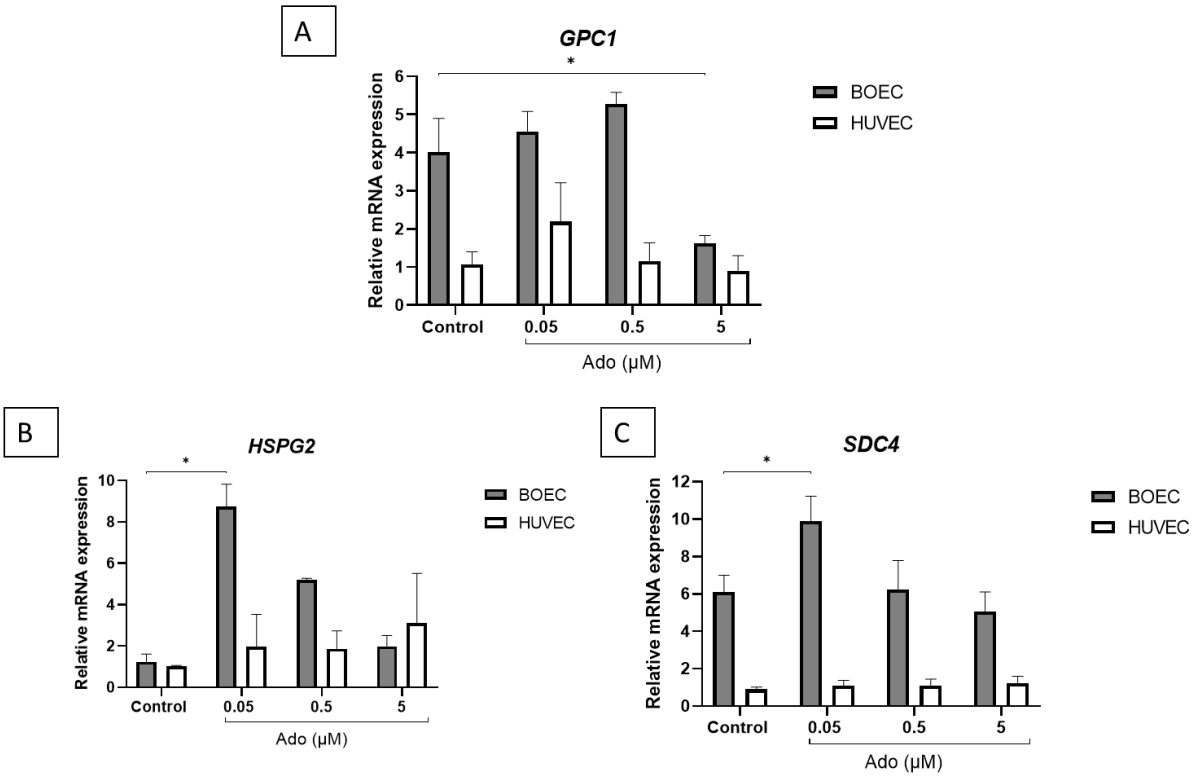


Figure 4. 4 Adenosine dose dependently influences mRNA expression of GAG core proteins in BOECs

In vitro glycosaminoglycan core proteins *GCP1, HSPG2, SDC4* mRNA *expression* under the influence of increasing concentrations of extracellular adenosine. **A)** *GPC1* expression is only significantly reduced at pathophysiological concentration of adenosine (5μM, *p=0.0316, n=3) for BOECs, no change is seen in HUVECs. **B)** *HSPG2* expression is significantly enhanced in BOECs, at low, physiological concentrations of adenosine (0.05 μM, *p=0.0113, n=3). **C)** *SDC4* expression is significantly enhanced in BOECs, at low, physiological concentrations of adenosine (0.05 μM, *p=0.0027, n=3). The error bars show the mean ± SEM. Experiments were carried in duplicate with three biological replicates. Statistical analysis was carried out using 2-way ANOVA with Dunnett's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

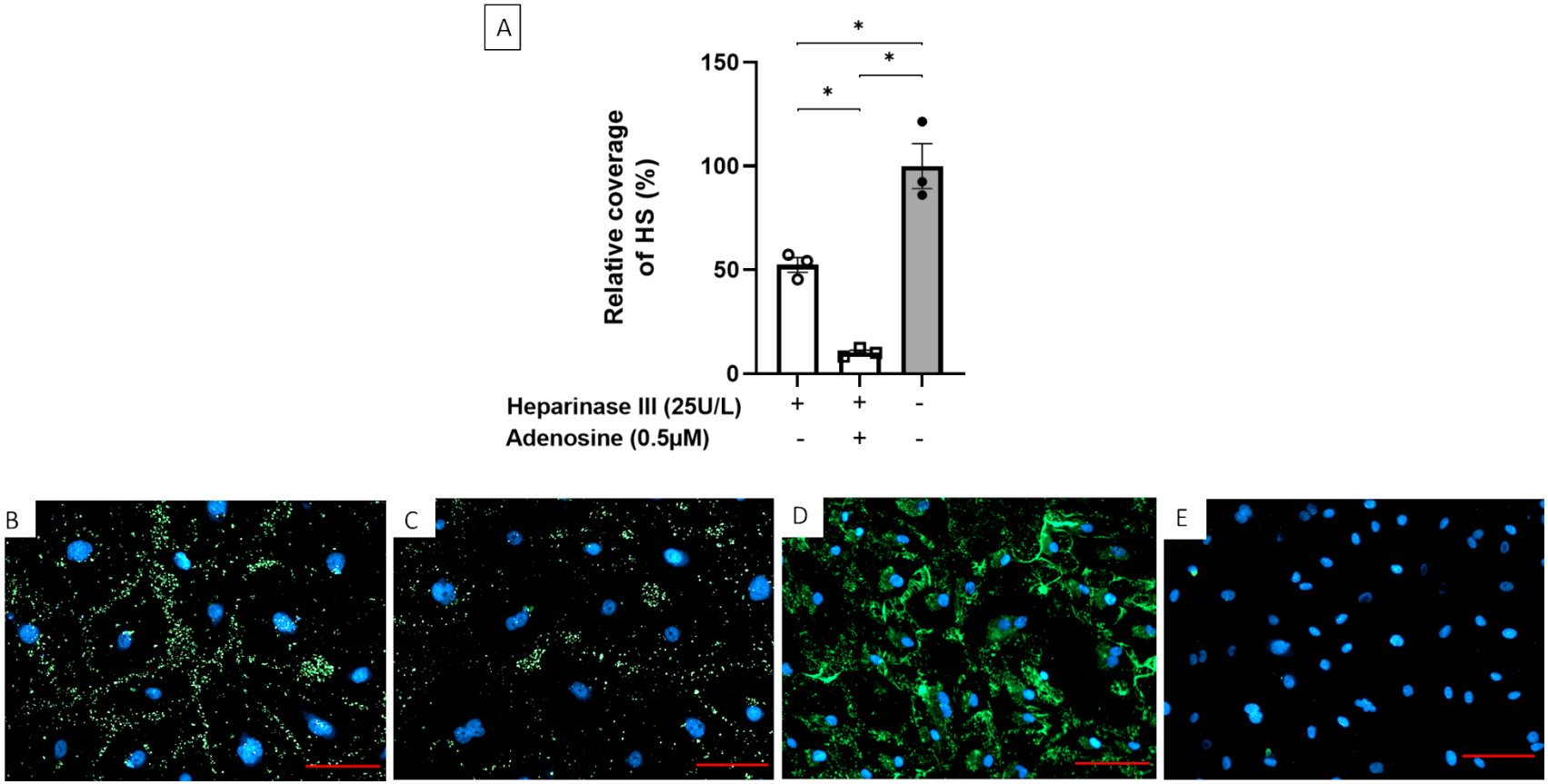


Figure 4. 5 Extracellular adenosine decreases the recovery of heparan sulphate in BOECs.

Ability of BOECs to recover Heparan sulphate glycosaminoglycan coverage, *in vitro.* **A)** BOECs were treated with Heparinase III for 2 hours to remove HS. Then treated with media alone or extracellular adenosine. At 18 hours media only control had recovered 52 ±3.6% (*p=0.0055, n=3) and adenosine 10.16 ±1.2% (*p= 0.0002, n=3), in comparison to no enzymatic degradation of HS. Extracellular adenosine significantly inhibited HS recovery from media only control (*p=0.0098, n=3). The error bars show the mean ± SEM. Scale bar =100µM. Statistical analysis was carried out using One-way ANOVA with multiple comparisons. Representative images **B)** media alone control, **C)** adenosine (0.5µM), **D)** no heparinise III treatment (positive control) **E)** secondary antibody only. Scale bar =100µM. Abbreviation- **HS:** Heparan sulphate.

4.4.4. The influence of adenosine on the NO pathway

Expression of NO is a marker of EC function. The impact of extracellular adenosine on the expression of NO and the proteins involved in regulating NO production was scrutinised at the mRNA level looking at the genes for endothelial nitric oxide synthase (*NOS3*), dimethylarginine dimethylaminohydrolase 1 (*DDAH1*), and dimethylarginine dimethylaminohydrolase 2 (*DDAH2*), as well as, NO concentration in supernatants.

Figure 4.6 demonstrated that *NOS3* was higher in BOECs than HUVECs at physiological concentrations of adenosine (0.05µM, p=0.0387). *DDAH2* expression was significantly elevated in BOECs in comparison to HUVECs at control (p<0.0001), physiological (0.05µM, p=0.0204) and extreme physiological (0.5µM, p=0.0012) concentrations of adenosine.

Increasing the concentration of exogenously applied adenosine did not significantly affect *NOS3* or *DDAH1* levels from control conditions, in either BOECs, or HUVECs. However, adenosine decreased BOEC *DDAH2* levels at pathophysiological concentrations, from a relative expression of 12.2±0.9 to 7.7±1.3 (p=0.0307, n=3).

Typically, at basal conditions BOECs and HUVECs produced low levels of basal NO *in vitro*, however, at 0.5µM adenosine where there was a significant increase in NO metabolites in BOEC supernatants from 1.0±0.6, to, 9.8±1.9% (p=0.0170, n=3) (Figure 4.7). At this concentration of extracellular adenosine BOECs produced significantly more NO than HUVECs (p=0.0081, n=3). Exogenously applied adenosine did not alter NO production in HUVECs at any concentration.

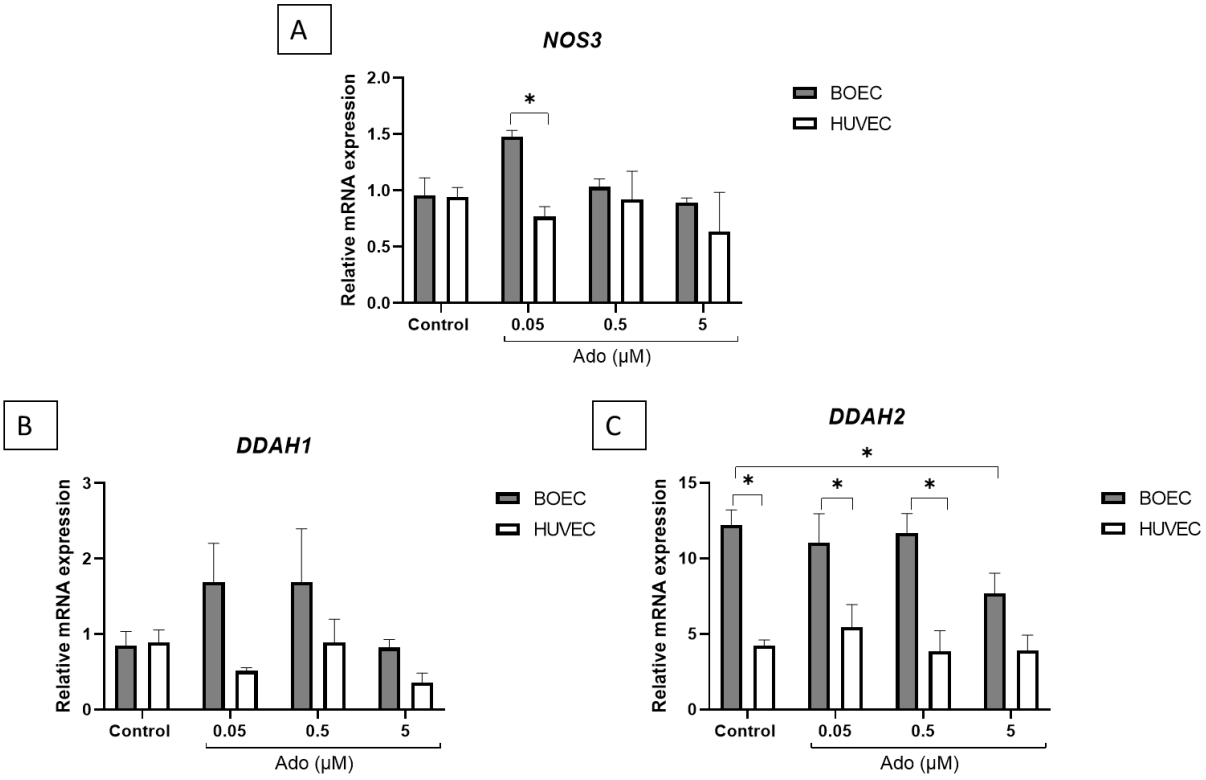


Figure 4. 6 Extracellular adenosine decreased DDAH2 expression at pathophysiological concentrations of adenosine in BOECs only, though no significant effect was observed in the mRNA expression of NOS3 and DDAH1 in ECs

Determination of the influence of extracellular adenosine on the relative mRNA expression of *NOS3, DDAH1 and DDAH2* in ECs. **A)** At 0.05μM adenosine BOECs express more *NOS3* than HUVECs (*p=0.0387, n=3) though, *NOS3* expression does not significantly change from control for BOECs or HUVECs. **B)** Both BOEC and HUVEC *DDAH1* expression does not significantly change after extracellular adenosine stimulation. **C)** At 0, 0.05, and 0.5μM adenosine BOECs express more *DDAH2* than HUVECs (*p<0.0001, *p=0.0204, *p=0.0012, respectively). *DDAH2* expression is decreased at 5μM adenosine for BOECs (*p=0.0307, n=3). No significant change in HUVEC *DDAH2* expression. Error bars show the mean ± SEM. Statistical analysis was carried out using 2way ANOVA with Šídák's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

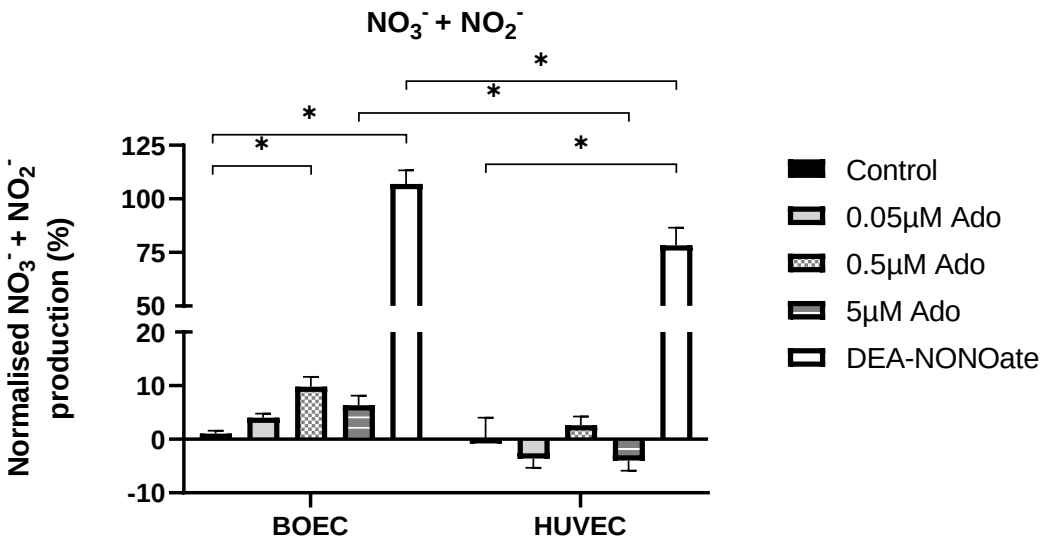


Figure 4. 7 Extracellular adenosine stimulates nitric oxide production in BOECs at extreme physiological concentrations

Normalised percentage of total nitrite and nitrate production in EC supernatants from cells exposed to DEA-NONOate, or after stimulation with extracellular adenosine. 0.5µM adenosine significantly increases NO production in BOECs (*p=0.0170, n=3), but not at any other adenosine concentration. 0.5µM adenosine was the only concentration whereby there was a significant difference between BOEC and HUVEC total nitrite/nitrate production (*p=0.0081, n=3). No adenosine concentration significantly changed the NO production from control in for HUVEC cells. The positive control, DEA-NONOate (100µM) significantly increased the NO production for both BOECs (*p<0.0001, n=3) and HUVECs (*p<0.0001, n=3) in comparison to control levels, and the increase was significantly greater in BOECs than HUVECs (*p=0.0001, n=3). Error bars show the mean ± SEM. Statistical analysis was carried out using 2way ANOVA with Tukey's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

4.4.5. The influence of adenosine on BOEC functionality

4.4.5.1. Proliferation

An important part of EC function is the ability to repair the endothelial layer and vessels via the process of angiogenesis and cell proliferation is one of the steps contributing to the process. The effect of extracellular adenosine and AR receptors on cell proliferation was explored (Figure 4. 8). Extracellular adenosine promoted BOEC cell proliferation in a dose dependent manner; at 0.5 and 5µM cell proliferation increased by 60.4% (p=0.0198) and 67.6% (p=0.0082) from control, respectively. At 0.5µM, this effect was mediated by A_{2A} receptor, illustrated by the presence of the A_{2A} receptor antagonist, SCH58261, which decreased proliferation from 0.5µM adenosine alone (-63.4%, p=0.0114). At 5µM adenosine the rise in proliferation was mediated, in part by A_{2A} and A_{2B} receptors; demonstrated following the addition of either SCH58261, or the A_{2B} receptor antagonist, Alloxazine, and each significantly reduced BOEC proliferation (SCH58261, - 52.9%, p=0.0381 and Alloxazine, -59.7%, p=0.0178).

A similar trend was seen in HUVEC, whereby adenosine increased cell proliferation at 0.5µM and 5µM by 103% (p=0.0009) and 119.5% (p=0.0001) respectively from control. Inhibition of the A_{2A} receptor by SCH58261, significantly diminished the adenosine induced HUVEC proliferation, at all concentrations of adenosine, HUVEC proliferation was reduced by 107.5%, (0.05µM p=0.0009), 160.3% (0.5µM p<0.0001) and by 165.8 % (5µM p<0.001). Alloxazine treatment did not influence the adenosine induced proliferation at any adenosine concentration.

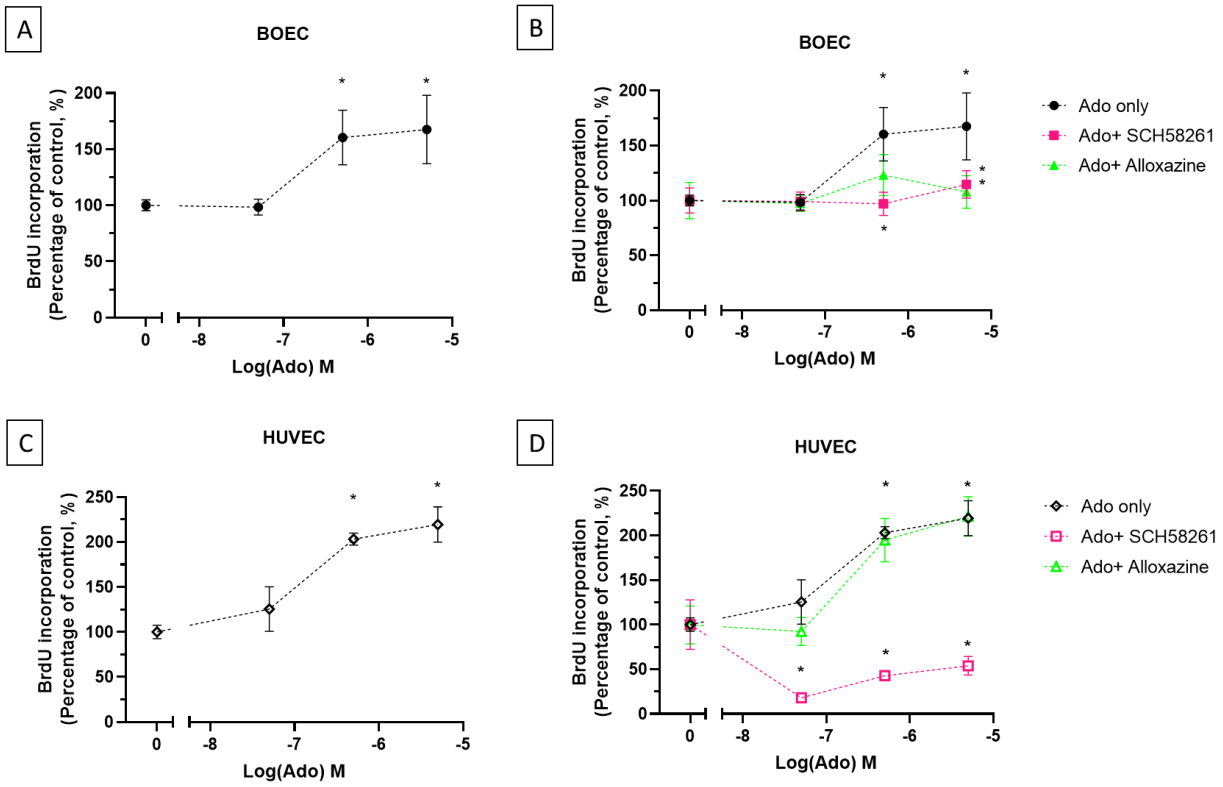


Figure 4. 8 Adenosine has a dose dependent effect on EC cell proliferation and which is mediated by both A_{2A} and A_{2B} receptors in BOEC, but only A_{2A} in HUVEC

Investigation of EC proliferation by examining the log dose response of adenosine vs. BrdU incorporation relative to control. **A)** Adenosine dose dependently increases BOEC proliferation at 0.5µM (*p= 0.0198, n=3) and 5µM (*p=0.0082, n=3). **B)** BOEC proliferation is removed at 0.5µM by the A2A receptor antagonist SCH58261 (*p=0.0114, n=3). At 5µM ado BOEC proliferation is reduced in the presence of either SCH58261 (*p=0.0381, n=3) or Alloxazine (*p=0.0178, n=3). **C)** HUVEC proliferation is increased at 0.5µM (*p=0.0009, n=3) and 5µM (*p=0.0001, n=3) adenosine. **D)** HUVEC enhanced proliferation is removed by A2AR antagonists (SCH58261) at 0.05µM (*p=0.0009, n=3), 0.5µM (*p<0.0001, n=3) and 5µM (*p<0.0001, n=3) adenosine. There is no significant change with the A2BR antagonist, Alloxazine, at any adenosine concentration. Error bars show the mean ± SEM. Experiments were carried in duplicate with three biological replicates. Statistical analysis was carried out using 2way ANOVA with Dunnett's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

4.4.5.2. Migration

Another step in the angiogenesis process is migration of cells. Adenosine dose-dependently increased EC migration in both cell types, mediated by the A_{2A} and A_{2B} adenosine receptors (Figure 4.9). For BOECs the increase in cell migration was only seen at extreme physiological adenosine concentrations (0.5µM), mediated by both A_{2A} and A_{2B} adenosine receptors, as in the presence of the A_{2A} receptor antagonist, SCH58261, and to a greater extent A_{2B} receptor antagonist, Alloxazine, removed the increase in migration seen at 0.5µM adenosine. The migration seen at 5µM adenosine was also mediated by the A_{2B} receptor and not the A_{2A} receptor, illustrated by the greatly reduced cell migration at the pathophysiological concentration, in the presence of Alloxazine. HUVEC cell migration was also dose-dependently increased in the presence of adenosine and is significantly increased at 0.5 and 5µM adenosine. The HUVEC migration is mediated by A_{2A} receptors at physiological and pathophysiological concentrations of adenosine, shown by the reduction in the presence of SCH58261 antagonist at 0.05 and 0.5µM. At 5µM, HUVEC cell migration is mediated by A_{2B} receptors alone, where there was a reduction in the increased cell migration, in the presence of Alloxazine, at 5µM.

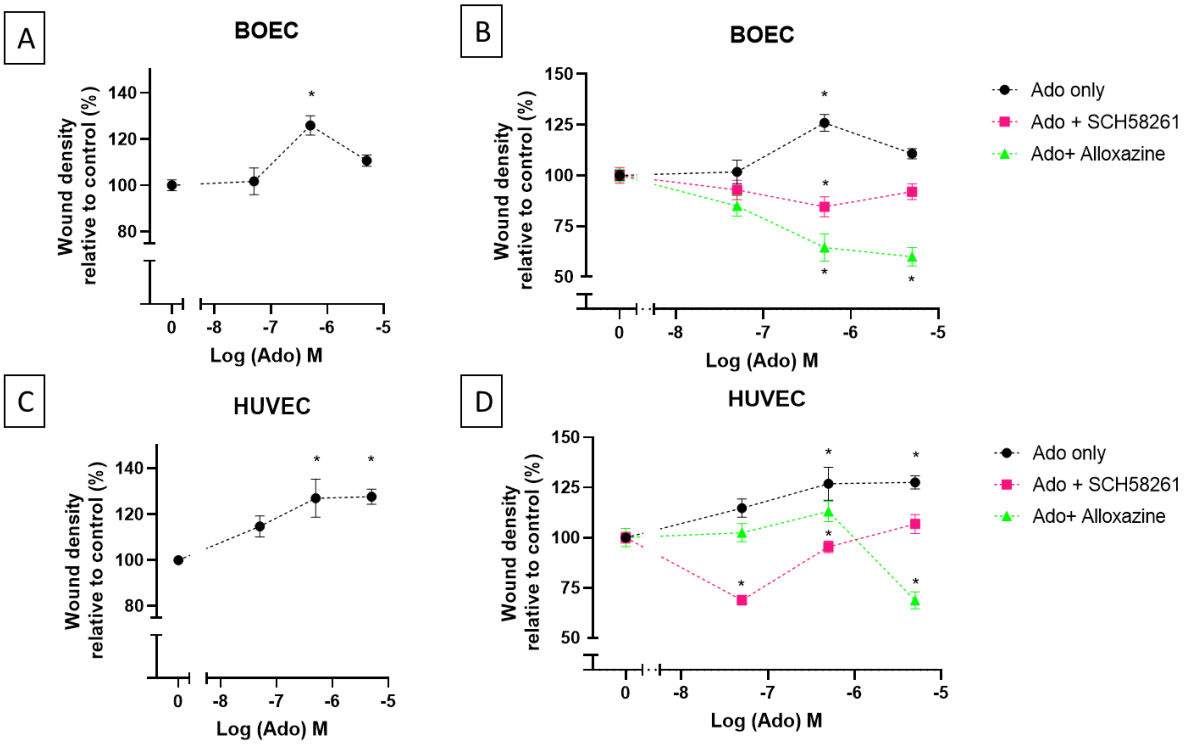


Figure 4. 9 Adenosine dose dependently improves EC migration, and this is mediated via both A_{2A} and A_{2B} receptors, in each EC type

Investigation of EC migration by log dose response for adenosine vs. density of the wound, relative to control. Confluent monolayers of EC were scratched, introducing a 'wound' to form and the cells were allowed to heal by cell migration for 12 hours, under different pharmacological treatments. **A)** BOEC migration is improved at 0.5µM ado (*p=0.008, n=3). **B)** The adenosine improvement in migration at 0.5µM is removed by the A2A receptor antagonist, SCH58261 (*p<0.0001, n=3), and to a greater extent A2B receptor antagonist, Alloxazine (*p<0.0001, n=3). Alloxazine also significantly reduced BOEC migration at 5µM ado (*p<0.0001, n=3). **C)** HUVEC migration is significantly improved at 0.5µM (*p=0.0042, n=3) and 5µM (*p=0.003, n=3) ado. **D)** This improvement in HUVEC migration is removed in the presence of SCH58261 at 0.05µM (*p<0.0001, n=3) and 0.5µM (p<0.0004, n=3) and Alloxazine at 5µM (*p<0.0001). Error bars show the mean ± SEM. Experiments were carried in duplicate with three biological replicates. Statistical analysis was carried out using 2way ANOVA with Tukey's multiple comparisons test. Abbreviation- **Ado**: Adenosine.

4.4.5.3. Monocyte adhesion

Another feature of the inflammatory process, leukocyte adhesion to EC, was investigated. Treatment with different concentrations of adenosine preceding stimulation with TNF-α, dose-dependently reduced the number of adherent leukocyte for both EC types (Figure 4.10). For BOECs, treatment with adenosine at 0.5µM ($p=0.0283$) and 5µM ($p=0.0014$) significantly reduced monocyte adhesion compared to TNF-α only enhancement, and the levels were not significantly different from control (media only). For HUVECs, adenosine reduced leukocyte adhesion from TNF-α at all adenosine concentrations (0.05µM $p=0.0246$, 0.5µM $p=0.0051$, 5µM $p<0.0001$). However, monocyte adhesion was only diminished to control levels at pathophysiological concentrations of adenosine, 5µM. Representative images are shown in Figure 4. 11.

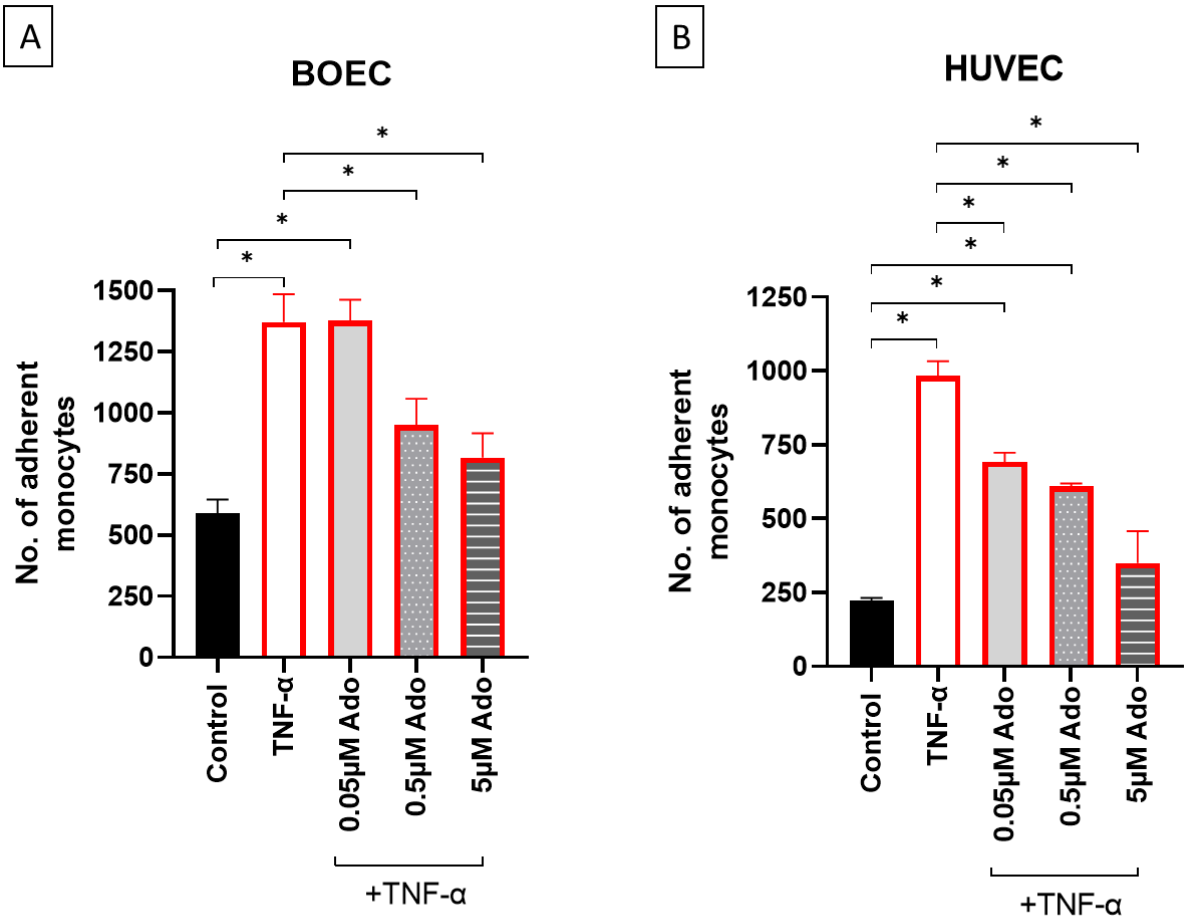


Figure 4. 10 Adenosine dose dependently diminishes monocyte adhesion and this is enhanced by TNF-α stimulation

Confluent ECs, BOECs or HUVECs, were pre-treated with adenosine (0, 0.05, 0.5, or, 5μM) for 24 hours and then stimulated with TNF-α for 4 hours, before co-culture with calcein AM labelled THP-1 monocytes, for 1 hour. The adhered monocytes were washed once with PBS and fixed with paraformaldehyde. Images were captured of 3 random fields and the number of adherent monocytes were determined using Fiji software. **A)** For BOECs co-culture, TNF-α enhanced THP-1 adhesion (*p<0.0001). There was no significant difference after pre-stimulation with 0.05μM ado from TNF-α stimulated and this was significantly different from control (*p<0.0001). Both 0.5μM (*p=0.0283) and 5μM (*p=0.0014) diminished the TNF-α stimulated adhesion and reduced the number of adherent cells back to control. **B)** For HUVEC co -cultures, TNF-α enhanced monocyte adhesion (*p<0.0001). Pre-treatment with all concentrations of ado, reduced TNF enhanced monocyte adhesion (0.05μM *p=0.0246, 0.5μM *p=0.0051, 5μM *p<0.0001). THP-1 adhesion was still significantly higher than control at 0.05μM (*p=0.0009) and 0.5μM (*p=0.0038) ado, but was returned to control levels with 5μM ado pre-treatment. Error bars show the mean ± SEM, n=3. Statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison test. Abbreviation- **Ado**: Adenosine.

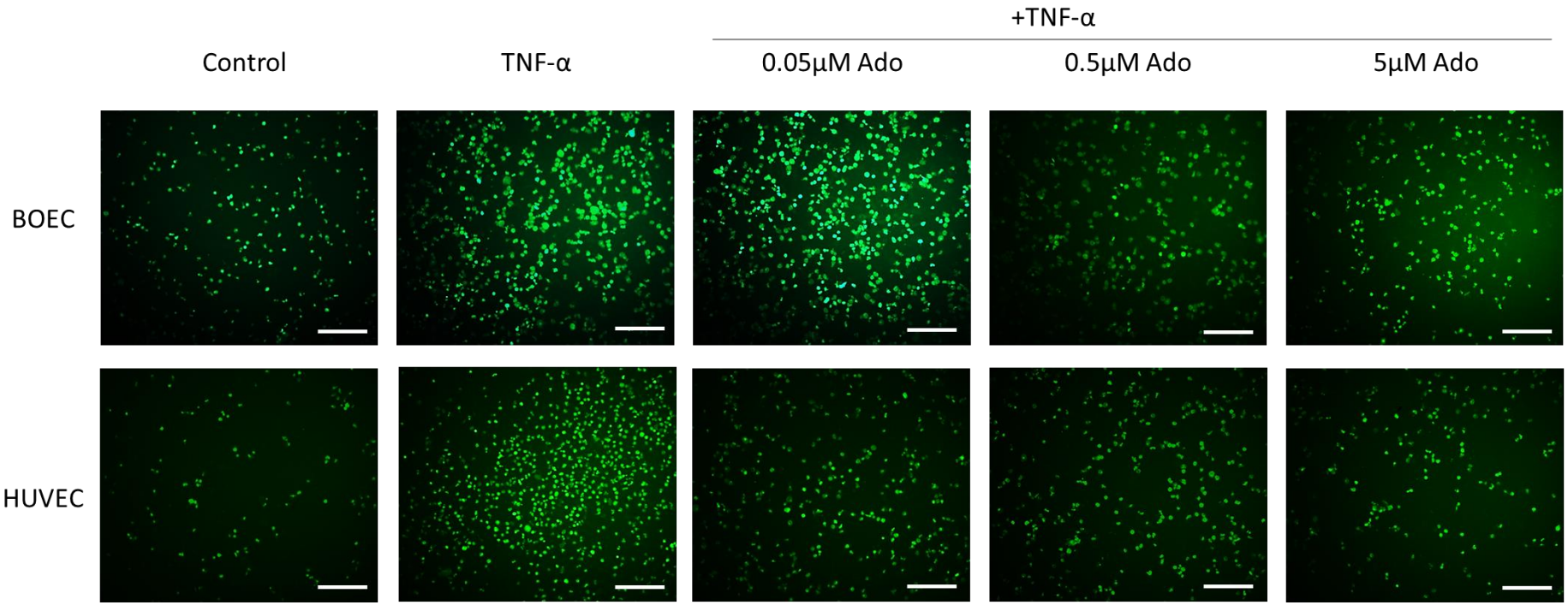


Figure 4. 11 Calcein AM labelled THP-1 cells adhering to ECs, pre-treated with adenosine and stimulated by TNF-α.

Representative images of calcein AM labelled THP-1s adhered to confluent monolayers of BOEC and HUVECs, following EC pre-treatment with adenosine (0, 0.05, 0.5, or, 5µM) for 24 hours and then stimulated with TNF-α for 4 hours. Images taken at 100x magnification, scale bar 100µM. Abbreviation- **Ado**: Adenosine.

4.5. Discussion

The objectives listed for this chapter have been met as follows:

1. The AR expression profile in BOECs and HUVECs was evaluated using cross-study transcriptomic assessment and quantitative PCR analysis of this study's donor cells and pooled HUVEC stocks. This showed a difference in AR distribution in the EC types, HUVECs A_{2A}>A_{2B}, BOECs A_{2B}>A_{2A}.
2. Quantification of adenosine receptors at the mRNA level successfully showed that extracellular adenosine dose dependently decreases the adenosine receptor profile of *ADORA2A* from HUVECs, though *ADORA2B* remains constant in HUVECs and BOECs.
3. Extracellular adenosine's influence on EC structure, production of soluble mediators and function were observed in a number of ways:
    a. *In vitro* extracellular adenosine influenced the glycocalyx structure and recovery shown at both mRNA and protein levels, particularly in BOECs.
    b. At physiological concentrations of adenosine, *NOS3* expression was higher in BOECs than HUVECs. BOEC expression was also higher than HUVEC for the NO associated gene, *DDAH2*, at basal, physiological and pathophysiological concentrations of adenosine. Extracellular adenosine dose-dependently influenced BOEC *DDAH2* mRNA, by reduction at pathophysiological adenosine concentrations, but other NO-related genes remained constant in BOECs. NO levels in EC supernatants increased at 0.5µM adenosine for BOECs, but no change from low basal levels was seen in HUVECs.
    c. EC function was influenced by extracellular adenosine whereby proliferation, migration was increased, mediated by A_{2A} and A_{2B} receptors, in contrast to leukocyte adhesion which was inhibited by adenosine dose dependently.

Adenosine receptors are responsible for many downstream pathways and behaviours in ECs and immune cells. It has been reported that different endothelial cell models have differing expression levels of adenosine receptors expression. Work by Feoktistov and colleagues (2002) has suggested that EC derived from larger vessels express predominantly A_{2A} receptors and A_{2B} in predominantly small vessels or capillaries, and vessels of microvascular origin. In particular, it has been reported that HUVEC preferentially express A_{2A}, over A_{2B} whereas,

HMEC-1, express A_{2B} (Feoktistov et al., 2002). The *in vitro* evaluation of the samples generated in this study, showed that BOECs and HUVECs only express *ADORA2A* and *2B*. BOECs predominantly express *ADORA2B* and to a greater extent than the levels reported for HUVEC. HUVECs predominantly express *ADORA2A,* a result which is consistent with previous studies (Feoktistov et al., 2002; Hassanian et al., 2014). *ADORA2A* expression in HUVEC was much greater than BOECs, in which low levels were detected. *ADORA2A* expression in both EC types was an expected result for an EC, but this was in contrast to the cross study transcriptomic analysis, where no BOEC *ADORA2A* expression was reported. This difference is might be a donor specific difference from our study, to the studies in the EndoDB, or, the analytical sensitivity for the microarray data in the EndoDB, may be inferior to the qRT-PCR data for a low expression gene, as has been reported before (Morey et al., 2006; Raymond et al., 2009).

Under inflammatory conditions, the BOECs upregulating *ADORA2A* expression, to levels similar to those expressed by HUVECs. This suggest that the BOECs could respond to the inflammatory stimuli by producing more anti-inflammatory receptors (*ADORA2A*).

A_1 and A_3 receptors have not been reported in HUVECs or have been identified at very low levels *in vitro* (Feoktistov et al., 2002; Hassanian et al., 2014; Wyatt et al., 2002). In a similar fashion, our study found no expression of *ADORA1* or *ADORA3* for either EC type. Any small variability in A_1 and A_3 receptors reported by *in vitro* studies might be attributed to donors from which the cells are derived and whether the ECs were pooled, as well as the conditions of culture (in particular the presence of AR agonists). The ambiguity in A_1 and A_3 expression was reflected in the cross-study transcriptomic analysis, where, for HUVECs, a differing number of studies reported each of the ADORA genes. As far as I am aware, BOEC adenosine receptor profile has not been reported in the literature previously, however, scrutiny of the publicly available EndoDB showed that 3 of the ADORA genes, aside from *ADORA2A,* were reported across the 4 BOEC studies. Our qRT-PCR data, which illustrated *ADORA2A* expression in contrast to this, maybe be a donor specific expression. To add to this, there are only 4 BOEC studies in the database, and as seen with the HUVEC studies, there are a large proportion of studies which do not report some or any of the ADORA genes in their samples at all, when this would be expected for an EC profile. For example, the *ADORA1* gene was most frequently reported with 103/146 studies, but *in vitro* studies have reported *ADORA1*

gene expression at low levels, or not all (Feoktistov et al., 2002; Hassanian et al., 2014; Wyatt et al., 2002). This suggests that there are some inconsistencies in Microarray and qPCR data, a phenomenon which has been reported and discussed previously, but the qPCR data presented within this chapter is consistent with both the broad expectation that ECs physiologically express AR, and previous *in vitro* HUVEC data (Morey et al., 2006).

Extracellular adenosine administration only influenced *ADORA2A* expression in HUVECs, unlike the expression in BOEC where *ADORA2A* levels remained constant. The application of extracellular adenosine onto cells did not influence *ADORA2B* expression in either EC type. Many G-protein coupled receptors are believed to be negatively regulated by stimulation with their agonist, which may explain why higher concentrations of adenosine downregulate the HUVEC *ADORA2A* expression (Tsao et al., 2001). BOEC *ADORA2A* expression was independent of the adenosine concentration and this may be due to the far lower levels seen in comparison to HUVEC. However, neither the BOEC nor HUVEC *ADORA2B* expression was affected by adenosine concentration, unlike *ADORA2A* and this might be explained by the fact that *ADORA2B* is a low affinity receptor if only the high adenosine concentrations and is therefore not negatively regulated to the same extent as the other G protein coupled receptor *ADORA2A*.

In this study, assessment of adenosine receptors was limited to mRNA analysis due to the lack of specific antibodies for A_{2B} AR and this limitation has been discussed by others previously (Carmona-Rivera et al., 2019). Once suitable antibodies for the ARs become available, it would be important to observe whether the results presented in this chapter at mRNA level are also reflected at protein level.

Adenosine has been implicated in the maintenance of the EC glycocalyx, in opposing, concentration dependent ways. High adenosine levels are believed to cause EC shedding, and reduced exclusion of circulating blood, as a pro-inflammatory mechanism, along with other inflammatory mediators such as TNF-α (Brands et al., 2013; Henry and Duling, 2000). *In vivo* A_3 AR activation causes modification of the glycocalyx in hamster and mouse capillaries, whereby there is failure to exclude dextran-labelled plasma, supplementing the A_3 AR-dependent pro-inflammatory effects already reported (Platts and Duling, 2004). However, *in vivo* it has been demonstrated that glycocalyx modification by ischemia-reperfusion is

attenuated by adenosine A_{2A} AR activation (Platts et al., 2003). Therefore, adenosine has both concentration-dependent effects and AR profile-dependent effects producing diverse actions of adenosine on the glycocalyx. Our results show that at low levels of adenosine there is an upregulation of the glycosaminoglycan core proteins *HSPG2* and *SDC4* but this falls back to controls levels at higher concentrations, in BOEC only, and *GPC1* is reduced below control at pathophysiological concentrations of adenosine, in BOEC. This may reflect a switch from an anti-angiogenic profile at low adenosine concentrations to pro-angiogenic profile at higher adenosine concentrations. Once the EC glycocalyx has been removed by enzymatic degradation (mimicking inflammation and endothelial cell injury), the results demonstrated that extreme physiological concentrations of adenosine inhibited glycocalyx recovery (Arfian et al., 2019). This result suggests that in BOECs, adenosine-mediated prevention of glycocalyx recovery, may contribute to increased permeability and mechanotransduction, but that a chronic lack of EC glycocalyx recovery can lead to endothelial cell dysfunction (Nieuwdorp et al., 2006a).

Extracellular adenosine can also increase NO production, in a dose-dependent manner and this has been demonstrated previously, using porcine carotid artery endothelial cells (PCAEC) (Li et al., 1995). The same study, did not find this in the human model, human saphenous vein endothelial cells, and have suggested that there may be heterogeneity between EC from artery and veins (Li et al., 1995). Using rat aortic endothelium, *in vitro* assays have shown that adenosine stimulated NO release was via A_1 and A_{2A} receptors and that activation of the A_{2A} receptor releases NO in two ways. The first by being coupled to K_{Ca} channels to facilitate Ca^{2+} influx, so activating eNOS, and secondly, adenylate cyclase activation, causing an increase in cAMP downstream of this and subsequent phosphorylation of eNOS by PKA (Ray and Marshall, 2006). In HUVECs isolated from healthy pregnancies in comparison to those isolated from preeclamptic pregnancies and late-onset preeclampsia (LOPE) pregnancies, there is a high level of adenosine extracellularly, and upregulation of A_{2B} receptors and endothelial dysfunction. The HUVECs isolated from LOPE pregnancies have an altered A_{2B} AR dependent L-arginine/NO signalling pathway. This is thought to be caused by A_{2B} AR signalling increasing P-Thr^{495} of eNOS which inactivates this enzyme and results in a reduction of NO (Acurio et al., 2014; Salsoso et al., 2021). These examples provide evidence that adenosine and its receptors are influencing NO and EC function.

In this chapter the results reflect that extracellular adenosine can influence NO production and the expression of NO related genes, in BOECs. BOEC's expression of NO related genes is higher than HUVEC for *NOS3* mRNA at physiological concentrations of adenosine, and for *DDAH2* at physiological and extreme physiological adenosine concentrations. DDAH is known to be downregulated in disease pathology resulting in an increase in ADMA and depletion of NO leading to endothelial dysfunction (Fliser et al., 2005; Lambden et al., 2015; Sibal et al., 2010; Tran et al., 2000). This is manifested in the results whereby *DDAH2*, which is more highly expressed in BOECs, in comparison to HUVECs, is significantly downregulated under pathophysiological concentrations of adenosine.

NOS3 and *DDAH1* expression remain constant despite stimulation with adenosine. However, the quantification of NO metabolites in cell supernatants revealed that adenosine upregulates NO production by BOECs under extreme physiological adenosine conditions, in line with previous literature (Li et al., 1995). However, under pathophysiological conditions, the NO levels returned to low basal levels, which occurred concurrently with a downregulation of *DDAH2*. This suggests a mechanism whereby extreme physiological adenosine concentrations (0.5-5µM) increase NO, as a vasoprotective function, but pathophysiological concentrations of adenosine (>5µM) may cause a decrease in DDAH2, which in turn increases ADMA and the inhibition of NOS3, reducing the ability of BOECs to produce NO.

Angiogenesis is another AR dependent function in ECs. Functionally extracellular adenosine has pro-angiogenic effects on the ECs. In relation to cell proliferation, an *in vitro* study of HUVECs has shown extracellular adenosine to be a mitogen with effects mediated by the A_{2A} AR (Sexl et al., 1995). Adenosine is also believed to influence EC growth by inducing macrophages to produce VEGF, via the A_{2A} AR (Hashimoto et al., 1994; Leibovich et al., 2002). The results in this chapter show how adenosine dose dependently increases EC growth. In HUVECs this was mediated via the A_{2A} AR from low to high concentrations of extracellular adenosine and not the A_{2B} AR. BOEC cell proliferation was mediated by both the A_{2A} and A_{2B} ARs at high concentrations of adenosine (extreme and patho-physiological). This could be attributed to the fact that *ADORA2B* is a low affinity receptor, that is expressed at low levels in HUVEC but not BOEC.

Another stage in the angiogenic pathway is cell migration and extracellular adenosine has influenced this pathway as well. In animal models, topical A_{2A} AR agonist application, significantly accelerated wound healing related angiogenesis, thought to be via a cAMP-dependent pathway (Montesinos et al., 1997). It has also been reported that HUVEC A_{2A} AR overexpression by gene transfer blocks E-selectin mediated monocyte adhesion to TNF-α activated ECs (Sands et al., 2004). In this study it was found that HUVEC migration was dose-dependently increased by adenosine, and this was via the A_{2A} AR, at physiological and extreme physiological adenosine concentrations, and, via the A_{2B} AR at pathophysiological concentrations. In BOECs, cell migration was only significantly increased at extreme physiological concentrations of adenosine and was diminished to control levels at high adenosine. The enhancement of cell migration was mediated via an A_{2A} and A_{2B} AR mechanism.

Another important component of the balance between endothelial damage from inflammation and repair, is leukocyte adhesion. Adenosine is reported to inhibit leukocyte adhesion. EC also recruit leukocyte as a part of a wider response to inflammation, whilst inhibiting leukocyte adhesion to maintain homeostasis. *In vitro*, adenosine dose dependently inhibits monocyte adhesion for stimulated HUVECs via an A_{2A} mediated mechanism (Hassanian et al., 2014). This occurs due to down-regulation of the cell surface markers VCAM-1, ICAM-1 and E-selectin and inhibition of IL-6 and MCP-1 (Hassanian et al., 2014). Our results also show dose-dependent effects, whereby increasing adenosine concentrations inhibits TNF-α-enhanced monocyte adhesion. For BOECs it took extreme physiological and patho-physiological concentrations of adenosine to reduce monocyte adhesion back to levels seen with control conditions. For HUVECs, the cells responded at lower adenosine concentrations to significantly reduce stimulated monocyte adhesion. However, in a significant fashion, it was only at patho-physiological concentrations of adenosine that brought the levels back to control. The images in Figure 4.11 show a difference in the distribution of calcein-AM labelled THP-1s adhered to each of the EC types. This is thought to be due to an increase in overall CD54 percentage expression per cell for BOECs in comparison to HUVECs, as discussed in Chapter 3.

In summary, BOECs respond in similar ways to adenosine stimulation as HUVEC. There were three concentrations at which adenosine was observed to create a variety of opposing

responses. These responses were mediated via diverse receptors in each cell type leading to different responses from these EC types. Adenosine-mediated actions in BOEC seemed to be predominantly via the A_{2B} AR, whereas, HUVEC was predominantly mediated via the A_{2A}, mirroring the difference in AR profile.

At low adenosine concentrations, in BOEC, anti-inflammatory and anti-angiogenic effects, were promoted, where GAG related genes are upregulated (BOEC only), NO-related genes were unchanged from control, and migration and proliferation remain unchanged. Adhesion of monocytes to stimulated endothelial cells remained constant in BOECs.

At extreme physiological concentrations of adenosine, increased BOEC proliferation, migration and NO production, whilst decreasing the ability of ECs to restore the glycocalyx and in the presence of TNF-α, inhibiting leukocyte recruitment. At pathophysiological concentrations of adenosine, NO levels were diminished to control levels and *DDAH2* expression attenuated. While proliferation and migration were maintained alongside the decrease in adhesion of leukocyte.

In DADA2 patients, elevated adenosine plasma levels, occur in the ranges between 0.2-0.6µM, which this study has labelled as extreme physiological concentrations, which is similar to levels reported in plasma in other inflammatory diseases (Carmona-Rivera et al., 2019). What is interesting when looking at the results from this chapter, is that one concentration of adenosine, 0.5µM ($5x10^{-7}$ M), which falls within the elevated range seen in DADA2 patients, seems to have a positive pivotal effect on the glycocalyx recovery, NO production and *DDAH2* expression, growth, migration, and adhesion of leukocyte for BOECs.

This chapter has observed the effect of extracellular adenosine via its receptors on BOECs and compared to typically studied HUVECs. The results from this chapter will contextualize and inform the next chapter which will explore the ADA2 pathway in BOECs (using HUVECs as a comparator), in the presence of its substrate adenosine, which is elevated in the DADA2 disease.

5. Results 3- Adenosine deaminase 2 pathway of BOECs

5.1. Introduction

ADA2 is an extracellular enzyme which deaminates adenosine into inosine under conditions induced by hypoxia and low pH. ADA2 is predominantly expressed by monocytes/macrophages and has been reported in B cells and endothelial cells (Dhanwani et al., 2020; Schena et al., 2020; Zavialov et al., 2010b; Zavialov and Engström, 2005). In humans, ADA2 activity decreases with age from childhood onwards, though no established ranges for the healthy population are widely accepted; highlighting the concern for the possible lack of appropriate age-matched controls when measuring ADA2 (Bowers et al., 2020; Nanthapisal et al., 2016; Zhou et al., 2014). A causative mechanism for the reduction of ADA2 activity in adults, with age, remains unclear, but is not reflected in total ADA2 concentration, and the reason for this lack in correlation remains unknown (Bowers et al., 2020).

Altered ADA2 levels have been implicated in disease, including; breast cancer, viral infections such as HIV, inflammatory-associated diseases such as rheumatic diseases and COPD (Aghaei et al., 2005; Goodarzi et al., 2010; Khodadadi et al., 2011; Sari et al., 2003). Most recently lack of either ADA2 production, or, if present, low activity, has been implicated in a paediatric monogenic form of vasculitis, DADA2 (Navon Elkan et al., 2014; Zhou et al., 2014). In contrast, abnormally high levels of ADA2 are associated with negative clinical outcomes in disease, such as with triple negative breast cancer where high plasma ADA2 is found at the most advanced stages of cancer development (Kutryb-Zajac et al., 2021). As ADA2 activity changes with age, and either very high or very low ADA2 levels are associated with disease states, it seems that ADA2 levels and activity must be tightly controlled to fall within a physiological useful range.

ADA2 binds to diverse populations of immune cell types, predominantly neutrophils, but to a lesser extent monocytes, natural killer cells, B cells and T cells (Kaljas et al., 2017). Research to date suggests that ADA2 has a short range of action performing paracrine or autocrine signalling with a short half-life in serum of about 6.4 hours (Ombrello et al., 2015). Many studies have focused on the relationship of ADA2 with monocytes and macrophages. Human ADA2 is shown to induce T cell-dependent differentiation of monocytes to macrophages (Zavialov et al., 2010b). ADA2 acts as a mitogen for macrophages and ADA2 deficiency *in vitro*, polarises monocytes to differentiate into M1 pro-inflammatory macrophages (Zavialov

et al., 2010b; Zhou et al., 2014). It has been observed that for both macrophages and monocytes, ADA2 is located lysosomally. Under these acidic conditions, ADA2 functions as a DNase enzyme (Greiner-Tollersrud et al., 2020). It is believed that this drives the DADA2 interferonopathy reported (Greiner-Tollersrud et al., 2020).

In other immune cells, such as T helper cells, ADA2 behaves as a growth factor (Zavialov and Engström, 2005; Zavialov et al., 2010b). In contrast, for neutrophils, ADA2 inhibits adenosine-mediated NETosis formation (Carmona-Rivera et al., 2019).

Less is known about the function of ADA2 on ECs, or whether there is an endothelial receptor for ADA2. Indirect evidence has suggested that ADA2 might be a growth factor for endothelial cells (Zhou et al., 2014). The reasoning behind this includes the vasculopathy displayed by DADA2 patients, the loss of EC integrity seen *in vitro* when EC are co-cultured with DADA2 patient monocytes, and the intracranial bleeding observed with knockdown of a zebrafish ADA2 homologue (Zhou et al., 2014). A role for ADA2 involvement in innate immunity has been elucidated, where loss of ADA2 in HUVECs, stimulates IFN-β and this is thought to occur by the uptake of extracellular deoxy-adenosine (Dhanwani et al., 2020).

Overall, ADA2 and its deficiency is implicated in a number of diseases and seems to have some adenosine-dependent and adenosine-independent effects, across multiple cell types, as well as both intracellular and extracellular functions. Moreover, the impact of elevated adenosine levels as a consequence of its deficiency, makes ADA2's functions unclear, and consequently, poses a threat to finding more therapeutics to treat the vast array of symptoms DADA2 displays (Carmona-Rivera et al., 2019). This chapter aims to reveal any direct influence of ADA2 on endothelial cells.

5.2. Specific aims

This chapter builds on the results in the previous chapters utilising BOEC as an endothelial model and understanding of the influence of adenosine on BOECs. In doing so, it aims to expose the influence that extracellular ADA2 has on endothelial cells.

Specifically, this chapter will;

1. Elucidate whether BOECs express ADA2:
    a. At mRNA level, using transcriptomics and qRT PCR.
    b. At protein level using immunofluorescence.
    c. At functional level using the ADA activity assay.
2. Assess the influence of ADA2 on:
    a. Cellular glycocalyx structure.
    b. Adenosine receptor expression.
    c. *ADA2* mRNA expression.
    d. Nitric oxide regulation and expression.
    e. EC function in terms of growth, migration, and adhesion.
3. Evaluate the influence of ADA2 in the presence of its substrate adenosine on:
    a. Adenosine receptor expression.
    b. Cellular glycocalyx structure.
    c. Nitric oxide and inflammatory cytokine expression.
    d. EC function in terms of growth, migration, and adhesion.

5.3. Methods

5.3.1. Cross-study *ADA2* transcript analysis

A gene of interest search on the EndoDB database was carried out on the *ADA2* gene, constrained to species: 'Homo sapiens' and cell type: 'Umbilical vein endothelial cells' and 'Blood outgrowth endothelial cells' (Khan et al., 2019). There was not a large enough data set (HUVEC samples; n=1, accession ID: E-GEOD-49524), to carry out pre-calculated pair-wise differential gene expression analysis (DGEA) between experimental conditions, so comparisons between BOEC and HUVEC in disease and healthy were not made.

5.3.2. qRT-PCR

BOECs were grown until confluent and then treated with ADA2 (0, 5, 10 U/L), with or without ADA2's substrate, adenosine (0.5µM). 0.5µM Adenosine was chosen as this falls within the elevated range reported in DADA2 plasma levels (Carmona-Rivera et al., 2019). RNA was isolated, cDNA synthesised and qRT-PCR was carried out described in chapter 2. In addition to the primers validated in the previous chapters; *ADORA2A, ADORA2B, DDAH1, DDAH2, GCP1, HSPG2, NOS3* and *SDC4*, a primer was used to target the *ADA2* mRNA (QT01667862; Qiagen).

5.3.3. Immunofluorescence

5.3.3.1. ADA2 protein

To observe intracellular ADA2 in BOECs and THP-1s, cells were plated on chamber slides and incubated until confluent. The cells were then fixed, permeabilised, and blocked as outlined in the general methods. The primary anti-ADA2 antibody was diluted in 1% BSA, 1in 50, overnight at 4˚C. The slides were washed three times with PBS and incubated with anti-rabbit IgG Alexa Fluor 488, in 2% goat serum, for 1 hour (1:200). Secondary antibody only was used as a negative control for each cell type. The slide was washed three times with PBS and then stained with Hoechst 33342 (ThermoFisher Scientific) (1:2000, for 3 mins). This was washed 3x, mounted and imaged. 3 random fields of vision were taken, for three biological replicates (n=3), using a Leica DM IL LED inverted microscope.

5.3.3.2. Heparan sulphate glycosaminoglycan recovery

Heparan sulphate glycosaminoglycan coverage was analysed by fluorescent microscopy. Samples were prepared as described in Chapter 2. 5 random fields of vision were captured using an EVOS M5000 Imaging System, the negative controls were used to set exposure time for acquisition of all images.

5.3.4. ADA activity assay

There are a number of methods and kits whereby ADA2 activity can be measured and activity can be presented as U/L or ng/ml. For the purposes of this study, ADA2 activity was measured using a colorimetric ADA activity assay (Diazyme), with 100nM Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), to inhibit the ADA1, following the protocol as previously described (Nanthapisal et al., 2016). This method was chosen primarily because this publication compared ADA2 levels in 4 different patient groups: DADA2 patients, carriers, healthy controls and paediatric controls, and measuring our cell culture supernatants and recombinant ADA2 levels in the same way, enabled direct comparisons to physiological levels. The activity assay was carried out as per the manufacturer's instructions with the addition of EHNA (100nM) to each of the samples. An internal ADA2, positive control, of known ADA2 concentrations was used as a standard, as well as an ADA1 standard.

5.3.5. Comparison of recombinant ADA2 activity

Throughout this study (2017-2021), two commercially available ADA2 products were recombinantly made, by 2 different companies. Using the same concentration of ADA2 (738ng/ml), the two products produced different activity levels. The R&D systems ADA2 was used for the standards for patient samples as previously described (Nanthapisal et al., 2016). This was far more active than the Biorbyt enzyme (Figure 5.1) (8.5x more active, $p<0.0001$). The majority of the R&D systems enzyme activity could be inhibited by either adding a neutralising antibody ($p<0.0001$) or the pan-ADA inhibitor, pentostatin ($p<0.0001$) and therefore the R&D systems ADA2 was used in future assays in this thesis.

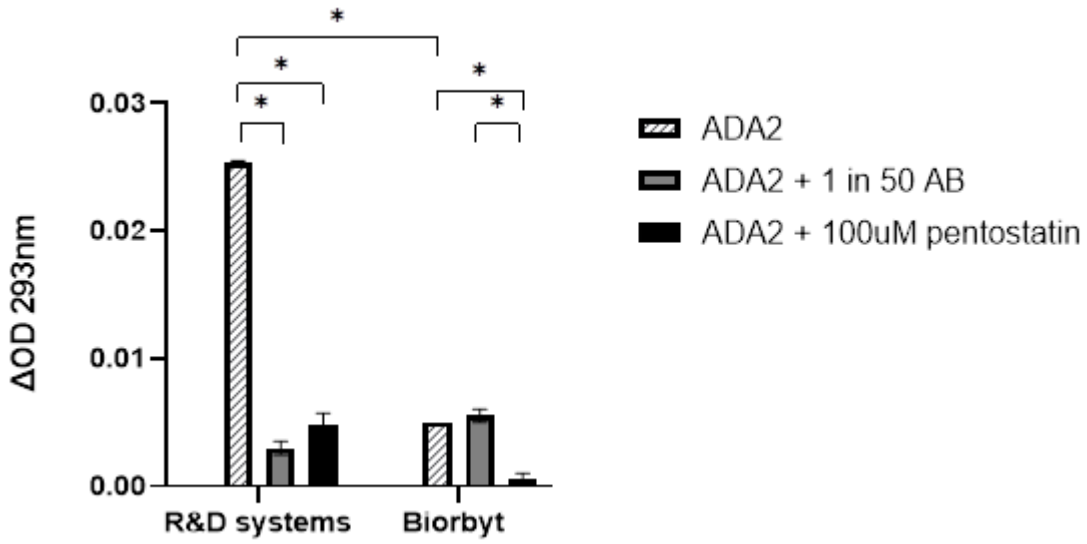


Figure 5. 1 Comparison of ADA2 activity from commercially available ADA2

Recombinant ADA2 was available from two commercial companies. These two differently sourced proteins were compared in terms of ADA2 activity using the same concentration of ADA2 (738ng/ml) and inhibition by an ADA2 antibody, and a pan-adenosine deaminase 2 inhibitor, pentostatin. Statistical analysis used 2way ANOVA with Tukey's multiple comparisons test and showed significant difference donated by (*), $p<0.05$.

5.3.6. Nitrite/nitrate assay

BOECs were grown until confluent and then treated for 24 hours with ADA2 alone (0, 5 and 10U/L) and in the presence of adenosine (0.5µM). The cell supernatants were collected after 24 hours, centrifuged at 1000 x g for 5 mins and stored at -80˚C, for up to one month, before the Nitrite/nitrate assay was carried out. The manufacturer's guidelines were followed as detailed in the general methods.

5.3.7. Colorimetric BrdU ELISA

To assess cell proliferation a colorimetric BrdU ELISA (Roche) assay was utilised. At a seeding density of $2x10^4$ cell/ml, EC were plated onto a 96 well plate and incubated overnight for cells to adhere. The spent media was removed carefully, and ECGM 2% FBS was added containing pharmacological agents in duplicate. These included; recombinant ADA2 (0,5,10U/L), with and without adenosine (0, 0.05, 0.5 & 5µM), the A2A receptor antagonist SCH58261 (1µM), or, the A2B receptor antagonist Alloxazine (10µM).

5.3.8. Wound healing assay

The ability of ECs to migrate following pharmacological intervention was measured over 24 hours. ECs were plated onto 96 well plates and allowed to reach confluence over 2-3 days. A sterile WoundMaker™ tool (Essenbioscience) created homogenous scratch wounds in each well, across the confluent monolayer. The ECs were washed with PBS and treatments were added in duplicate, including: recombinant ADA2 (0, 2.5, 5, 10, 20 U/L), adenosine (0, 0.05, 0.5, 5μM), the A2A receptor antagonist SCH58261 (1μM), the A2B receptor antagonist Alloxazine (10μM), or the adenylate cyclase inhibitor, SQ22536 (100μM).

5.3.9. Static leukocyte adhesion assay

In order to assess the influence of ADA2 on BOECs and their ability to adhere to monocytes (THP-1 cells), an adhesion assay was carried out. A confluent monolayer of BOECs were pre-treated for 24 hours with ADA2 (0, 5, 10 U/L) in the presence or absence of adenosine (0.5μM). Then the cells were stimulated for 4 hours with TNF-alpha (10ng/ml) and a TNF-α only condition was included as a positive control. After this time the BOEC were washed once with PBS and co-cultured with $5x10^5$ THP-1 cells/mL, at 37˚C for 1 hour. Prior to co-culture with the EC monolayer, the monocytes were fluorescently labelled with the cell-permeant dye, calcein AM (0.1μM). To achieve this, $1x10^6$ cells/mL in PBS were labelled with 0.1μM calcein AM (ThermoFisher Scientific), for 30mins in the dark, at 37˚C. The fluorescently labelled cells were then washed three times with PBS and resuspended in basal RPMI media. After 1 hour of co-culture non-adherent monocytes were then washed off with PBS three times. Three random fields of the fluorescently labelled adherent monocytes were taken using a Leica DM IL LED (Leica Microsystems, UK), inverted microscope. Monocyte adherence was quantified using Fiji software (Schindelin et al., 2012), as outlined in the general methods, Chapter 2.

5.4. Results

5.4.1. ADA2 expression in EC- comparing BOEC with HUVEC

5.4.1.1. Measuring *ADA2* expression in quiescent and activated ECs across the EndoDB transcriptomics database

The EndoDB database was used to observe whether the *ADA2* gene was expressed across studies for BOECs and HUVECs (Khan et al., 2019). 4/4 BOEC studies but only 60/146 HUVEC studies reported *ADA2* expression in the study's samples.

5.4.1.2. Measuring *ADA2* expression by qRT-PCR in quiescent and activated ECs

Within this study, ADA2 expression in the BOECs isolated were compared to the pooled HUVECs, in addition to the positive control, the monocytic cell line, THP-1s. ADA2 mRNA expression was assessed using qRT-PCR (Figure 5. 2). Both BOECs and HUVECs expressed ADA2 and there was significantly more ADA2 expressed in BOEC than HUVEC ($p=0.0039$, $n=5$; BOEC 2.177±0.17, HUVEC 1.043±0.16). However, when compared to THP-1 cells, expression of ADA2 in both EC types was low (BOEC vs THP-1 $p<0.0001$, HUVEC vs THP-1 $p<0.0001$; THP-1 12.16±1.37, $n=5$).

5.4.1.3. ADA2 expression observed by immunofluorescence

Immunofluorescence microscopy detected ADA2 in monocyte cells (THP-1s) however, ADA2 was undetectable in either EC type (Figure 5. 3).

5.4.1.4. Determination of ADA2 activity

Figure 5. 4, shows the results for the assessment of ADA2 activity in cell supernatants and revealed the presence of enzymatic activity in the BOEC (1.657 ± 0.6 U/L) and THP-1 (5.456 ± 0.4 U/L) supernatants, whilst HUVEC supernatants contained negligible amounts of active ADA2 enzyme (0.03397 ± 0.03 U/L). Comparing ADA2 activity in BOEC and HUVEC, BOEC cell supernatants produced significantly more than HUVEC ($p=0.0002$), but significantly less than THP-1 supernatants ($p<0.0001$).

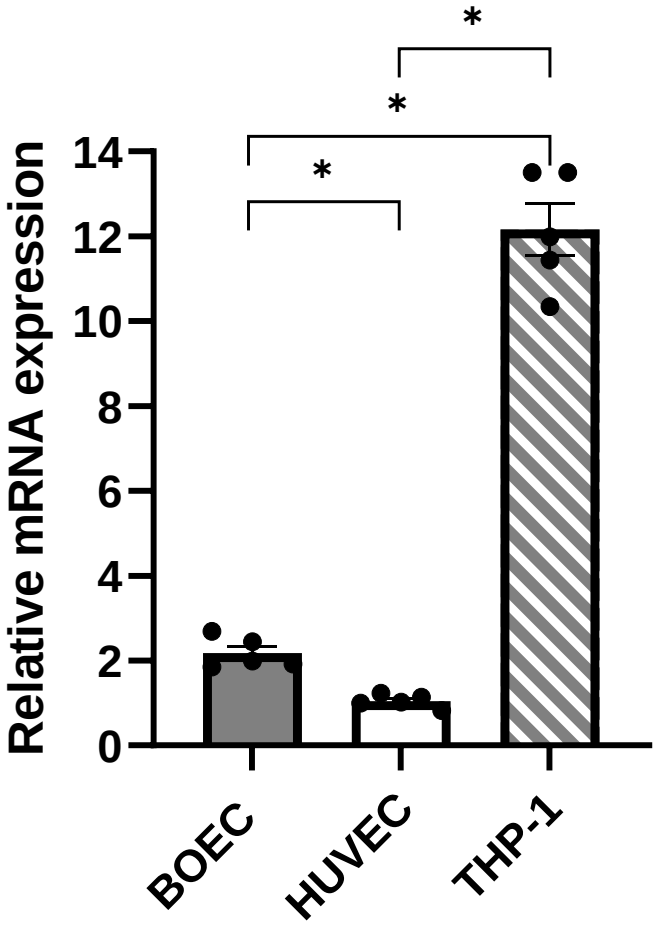


Figure 5. 2 BOECs express more *ADA2* mRNA than HUVEC

Determination of *ADA2* mRNA expression by qRT-PCR. The data was obtained by 5 independent biological replicates, with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. The error bars show the mean ± SEM. BOEC 2.177±0.17, HUVEC 1.043±0.16 and THP-1 12.16±1.37. Statistical analysis (Brown-Forsythe and Welch ANOVA with Dunnett's multiple comparisons test) shows that THP-1s expressed significantly more ADA2 than both BOEC (*p<0.0001) and HUVEC (*p<0.0001). BOECs expressed significantly more *ADA2* than HUVEC (2.1x, *p=0.0039).

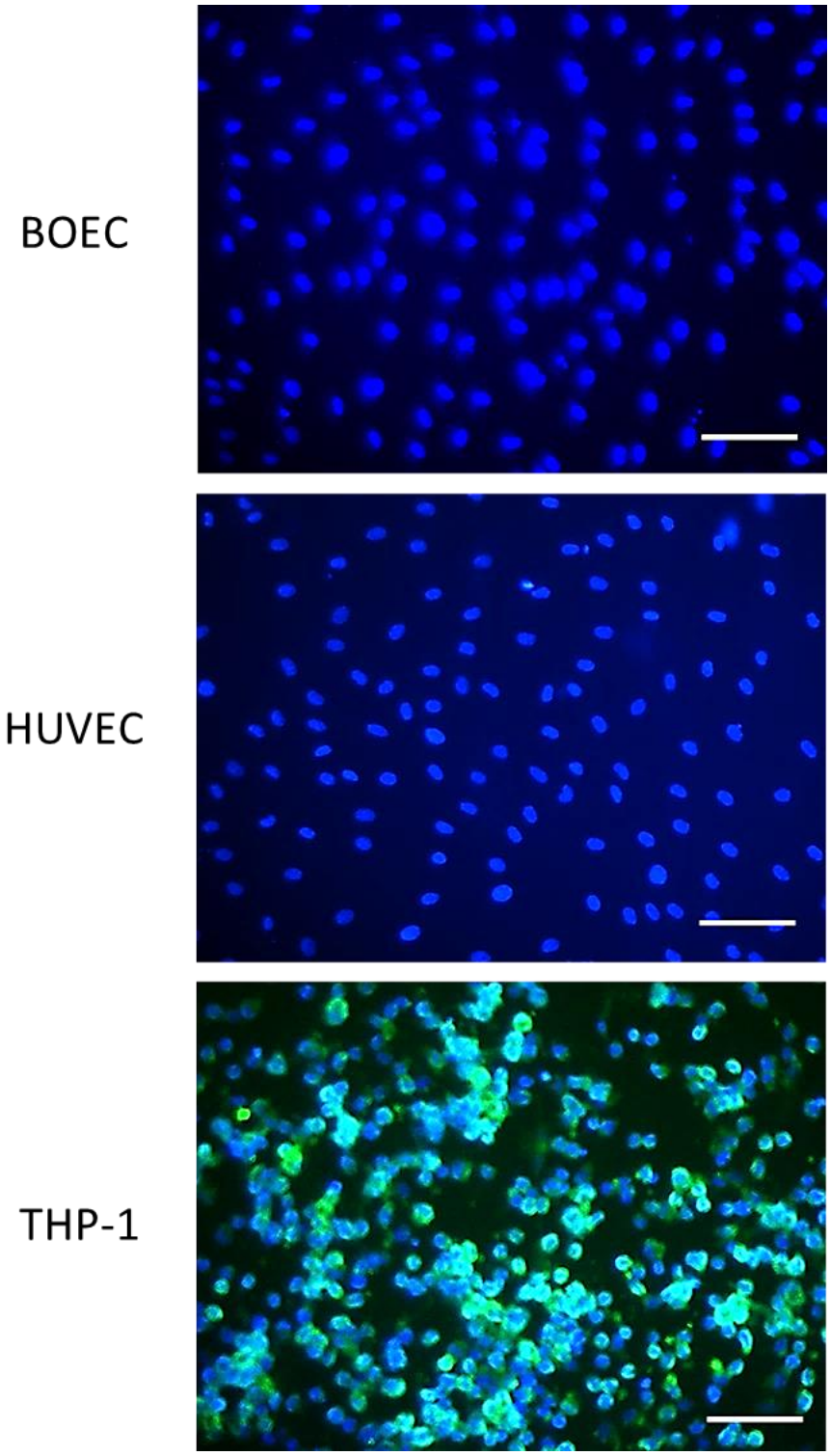


Figure 5. 3 ADA2 is detected intracellularly in THP-1 cells but not BOEC

Representative immunofluorescence images of THP-1, BOEC and HUVEC cells showing intracellular ADA2 protein expression in THP-1s, but absent in HUVECs and BOECs. Cells were fixed and permeabilised with 0.5% Triton X-100 in PBS. ADA2 protein was targeted by incubation with a primary antibody, anti-ADA2, and fluorescent secondary antibody, anti-rabbit 4 (green) and the cell nucleus was stained with Hoechst 33342 (Blue). 3 random fields were taken, n=3. Scale bar represents 50µm.

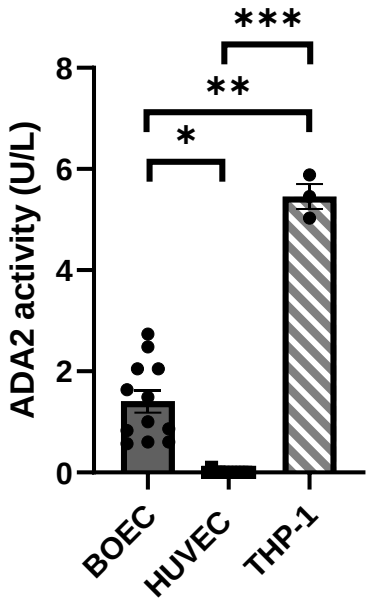


Figure 5. 4 BOECs produce active ADA2 which is detected in cell supernatants

ADA2 activity measured by ADA activity assay with the addition of EHNA (Erythro-9-(2-hydroxy-3-nonyl) adenine, ADA1 specific inhibitor). BOEC express active ADA2 enzyme extracellularly (1.657 $\pm$ 0.6 U/L, n=12), but little to no enzyme activity is shown from HUVEC supernatants (0.03397 $\pm$ 0.03 U/L, n=12) (BOEC vs HUVEC, *p=0.0002). THP-1s express 5.456 $\pm$ 0.4 U/L (n=3) extracellularly and this is significantly more than both BOECs (**p<0.0001) and HUVECs (***p=0.0043). Statistical analysis utilised the Brown-Forsythe and Welch ANOVA test with Dunnett's T3 multiple comparisons test.

5.4.2. Influence of recombinant ADA2 on BOECs

ADA2 expression in BOEC is approximately 3.3x less than THP-1 monocytes. To examine potential effects of exogenous ADA2 on EC structure and function, BOEC were cultured for up to 24 hours with physiologically relevant concentrations of exogenous ADA2 (0-10U/L). Following this, EC structure (the glycocalyx), protein expression (ADA2, ARs), signalling molecule production (nitric oxide and its pathway), and function (proliferation, migration and adhesion, were studied. The following subsections examine how exogenous ADA2 influences each of these in turn.

5.4.2.1. *ADA2* mRNA expression

To confirm that basal *ADA2* expression was not altered by this variable in BOEC, exogenous ADA2 was incubated with confluent ECs and mRNA expression was measured (HUVEC was used as a reference). EC *ADA2* expression did not significantly alter in the presence of recombinant ADA2 (Figure 5. 5).

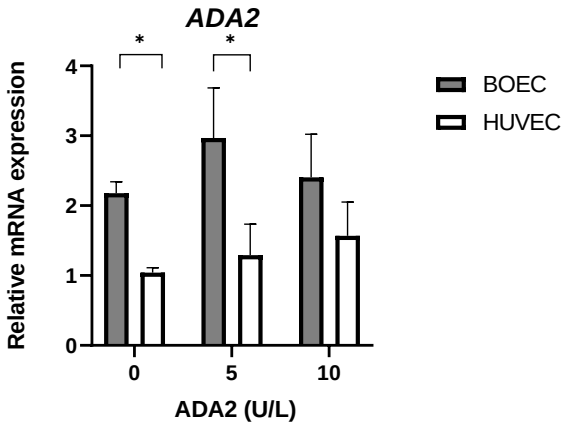


Figure 5. 5 Exogenous recombinant ADA2 does not influence *ADA2* mRNA expression

Assessment of EC *ADA2* mRNA expression by qRT-PCR, after pharmacological treatment with recombinant ADA2 (0, 5, 10 U/L). There was no change in ADA2 expression at any concentration and there was significantly more ADA2 expressed by BOECs than HUVECs, at 0 (*p=0.0039), and 5 U/L (*p=0.0374, 2way ANOVA with Šídák's multiple comparisons test). Although the difference between BOEC and HUVEC expression became non-significant at 10U/L. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. The error bars show the mean ± SEM.

5.4.2.2. The glycocalyx structure

Results reported in Chapter 4 demonstrated the impact of diverse pharmacological treatments on the structure of the EC glycocalyx. This section examined the impact of ADA2 on the glycocalyx, and its components such as, the proteoglycan core protein and the glycosaminoglycans (Figure 5. 6).

Proteoglycan core protein mRNA expression was significantly improved, from control, in BOECs, for *GPC1*, at 10U/L ($p<0.0001$), HSPG2 at both 5 ($p=0.0003$), and 10 U/L ($p=0.0003$), and SDC4 at 10U/L ($p=0.0009$). As a reference expression in HUVEC cells was examined but there was no significant change in the proteoglycan core protein mRNA expression for HUVECs, and I continued to use BOECs as the principal model for the glycocalyx. Likewise, glycosaminoglycan, HS, in response to prior heparinase action, was more abundant in the presence of exogenous ADA2, in comparison to media only controls (an increase of 72.01±13.74%, $p=0.0027$). Over 18 hours ADA2 restored the HS coverage to a level equivalent to the no heparinase III treatment control (representative images are illustrated in Figure 5.15).

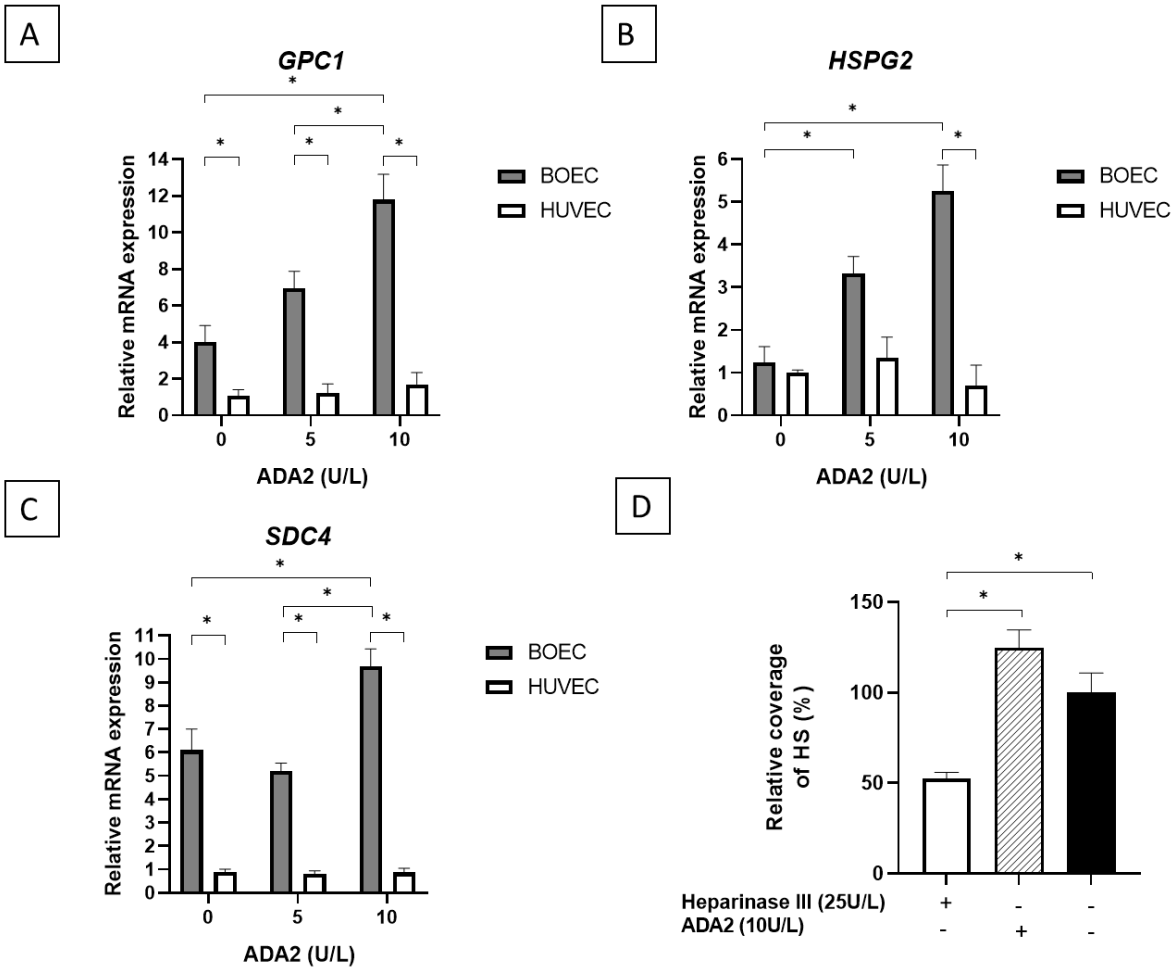


Figure 5. 6 ADA2 increases BOEC proteoglycan core protein and GAG expression

The EC glycocalyx was evaluated by observing changes in proteoglycan core protein mRNA expression utilising qRT-PCR, and immunocytochemistry targeting HS, after recombinant ADA2, *in vitro* treatment (0, 5 and 10 U/L). **A)** *GCP1* was upregulated at 10U/L ADA2 for BOECs alone. BOEC expression was significantly higher than HUVEC at all concentrations. **B)** *HSPG2* expression was increased at both 5 and 10 U/L ADA2 for BOECs alone. 10U/L ADA2 created a significant difference in BOEC and HUVEC *HSPG2* expression. **C)** BOEC *SDC4* was increased at 10U/L ADA2. All concentrations of ADA2 yielded a significantly higher BOEC, than HUVEC SDC4 expression. **D)** 18 hours after enzymatic HS removal, ADA2 improves HS recovery, above control (p= p=0.0027), and back to coverage in accordance with no heparinase II treatment. HS coverage was achieved by capturing images of 3 random fields of vision across, three biological replicates. The error bars show the mean $\pm$ SEM. The qRT-PCR data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample and normalised to an average of two housekeeping genes, GAPDH and ACTB. Statistical analysis employed either 2way ANOVA with Tukey's multiple comparison test, for qRT-PCR data, or, one-way ANOVA with Tukey's multiple comparison test for HS coverage and revealed significant differences shown by (*), where $p<0.05$. Abbreviation- **HS**: Heparan sulphate.

5.4.2.3. AR mRNA expression

As previously described in Chapter 4, BOEC predominantly expressed *ADORA2B*, unlike HUVEC, under quiescent conditions. The application of exogenous ADA2 (Figure 5. 7) did not alter this relationship and *ADORA2A* expression remained lower in BOEC than HUVEC mRNA expression at 5 (p=0.0187), and 10 U/L (p<0.0001). *ADORA2B* remained higher in comparison to HUVEC at 10 U/L (p=0.0106), but not at 5U/L (p=0.1696).

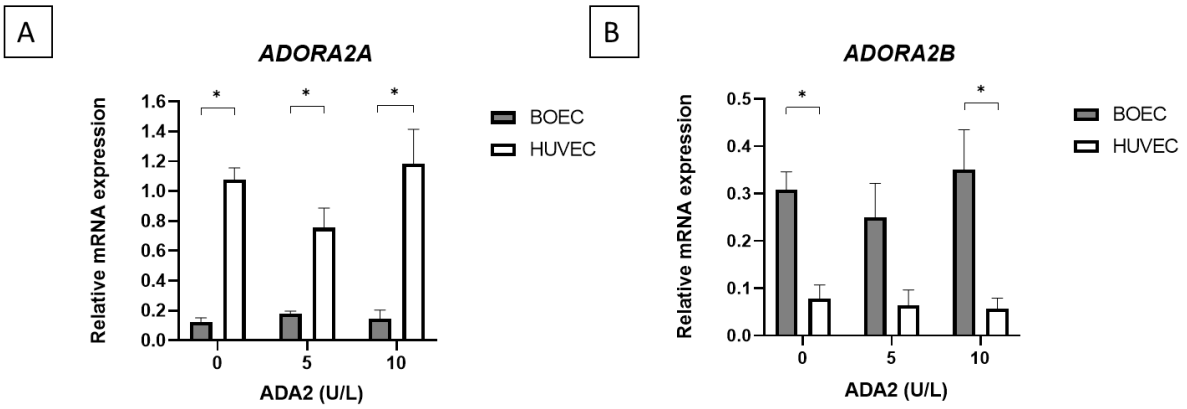


Figure 5. 7 BOEC *ADORA2A* and *ADORA2B* receptor expression is not influenced by recombinant ADA2

ADORA2 and *ADORA2B* mRNA expression determined by qRT-PCR, after recombinant ADA2 *in vitro* treatment (5 and 10 U/L). **A)** No significant change in *ADORA2A* for either BOECs or HUVECs was seen and HUVEC levels remained superior to BOEC at all ADA2 concentrations (0U/L *p<0.0001, 5U/L *p=0.0187, and, 10U/L *p<0.0001). **B)** ADA2 did not significantly alter the expression of *ADORA2B* for either EC type. BOEC *ADORA2B* amount was higher in comparison to HUVEC at 0 and 10U/L (*p=0.0098, and *p=0.0106 respectively). The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. The error bars show the mean $\pm$ SEM. Statistical analysis used 2way ANOVA with Tukey's multiple comparisons test.

5.4.2.4. mRNA expression in the nitric oxide pathway

NO production is a key secondary signalling molecule produced by ECs. Disruption to the NO generating pathway leads to endothelial cell dysfunction. Therefore, ADA2's impact on the mediators of NO production, and NO production in cell supernatants was assessed (Figure 5. 8).

NOS3 expression did not significantly increase from controls with increasing concentrations of ADA2, in either BOEC or HUVEC. However, the trend was for ADA2 at 10U/L to decrease *NOS3* in BOEC and increase in HUVEC, such that *NOS3* expression was significantly different between each EC type ($p=0.0125$). *DDAH1* expression was upregulated from control in BOECs, at both 5 ($p=0.0109$) and 10U/L ($p=0.0005$) ADA2, and was significantly enhanced in comparison to HUVEC at 5U/L ($p=0.0421$), and 10U/L ($p=0.0039$). Although, *DDAH2* expression remained relatively high in BOECs in comparison to HUVECs at control ($p<0.0001$) and 5U/L ($p=0.0083$). *DDAH2* was not significantly different from control at any ADA2 concentration.

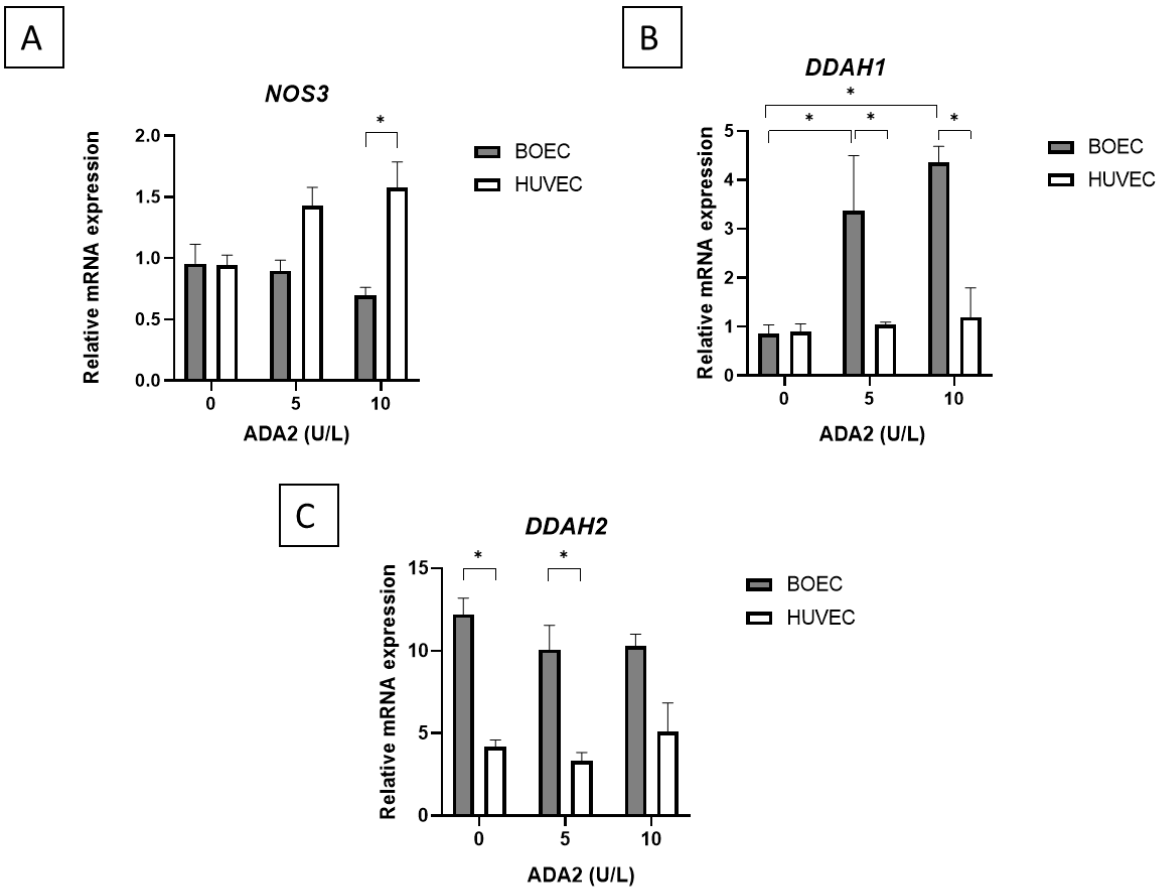


Figure 5. 8 ADA2 upregulates *DDAH1* expression significantly in BOECs

The nitric oxide pathway was evaluated using qRT-PCR observing changes in expression of *NOS3, DDAH1* and *DDAH2* and determination of NO production in cell supernatants using a nitrite/nitrate kit, after ADA2 *in vitro* treatment (0, 5 and 10 U/L). **A)** *NOS3* expression does not significantly change for BOECs or HUVECs across the ADA2 concentrations but the difference between *NOS3* BOEC and HUVEC at 10U/L, becomes significantly more in HUVECs. **B)** *DDAH1* mRNA expression is upregulated in BOECs at 5 and 10 U/L and is significantly higher in BOECs than HUVEC at these concentrations. **C)** *DDAH2* expression remains consistent for BOECs and HUVECs despite ADA2 treatment. There is significantly more DDAH2 expressed by BOECs than HUVECs, although the difference because non-significant at 10U/L ADA2. he data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. qRT-PCR data was normalised to an average of two housekeeping genes, *GAPDH* and *ACTB using the* $2^{-\Delta\Delta ct}$ method. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences shown by (*), where $p<0.05$.

5.4.2.5. Nitrite and nitrate production

NO metabolites, nitrite and nitrates, in the cell supernatants, were low in BOEC and HUVEC and did not significantly change under ADA2 pharmacological treatment *in vitro,* although an upward trend was seen with increasing concentrations of ADA2 on BOEC (Figure 5. 9).

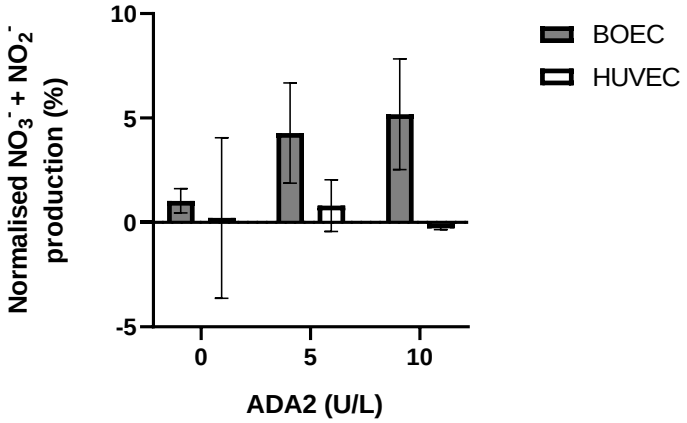


Figure 5. 9 ADA2 influence on nitrite and nitrate production

Determination of NO production in cell supernatants using a nitrite/nitrate kit, after ADA2 *in vitro* treatment (0, 5 and 10 U/L). ADA2 treatment does not affect the low basal nitrite/nitrate production seen in BOEC and HUVEC cell supernatants. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed no significant differences where $p<0.05$.

5.4.2.6. EC function

The aim of the following experiments was to investigate the impact of ADA2 on BOEC function using HUVEC as a reference.

5.4.2.6.1.Proliferation

The BrdU assay was used to assess the effect of ADA2 on EC proliferation. The results shown in Figure 5. 10 show that ADA2 reduces EC proliferation. For BOECs this effect was significant at 10 U/L ($p=0.0132$) where there was a 44.09% reduction in cell proliferation. In HUVECs the inhibition of cell proliferation was seen at both 5U/L ($p=0.0012$), and at 10U/L, where there was a 67.93% decrease in cell proliferation ($p=0.0002$).

To assess if ADA2 inhibition in EC proliferation was mediated by ARs, BOECs and HUVECs were cultured in the presence of ADA2, with or without either the A_{2A} receptor antagonist SCH58261, or, the A_{2B} receptor antagonist, Alloxazine. There was no significant change in cell proliferation in BOEC from 55.91±2.7% for ADA2 treated cells, when cultured in the presence of either AR antagonist, or in HUVECs.

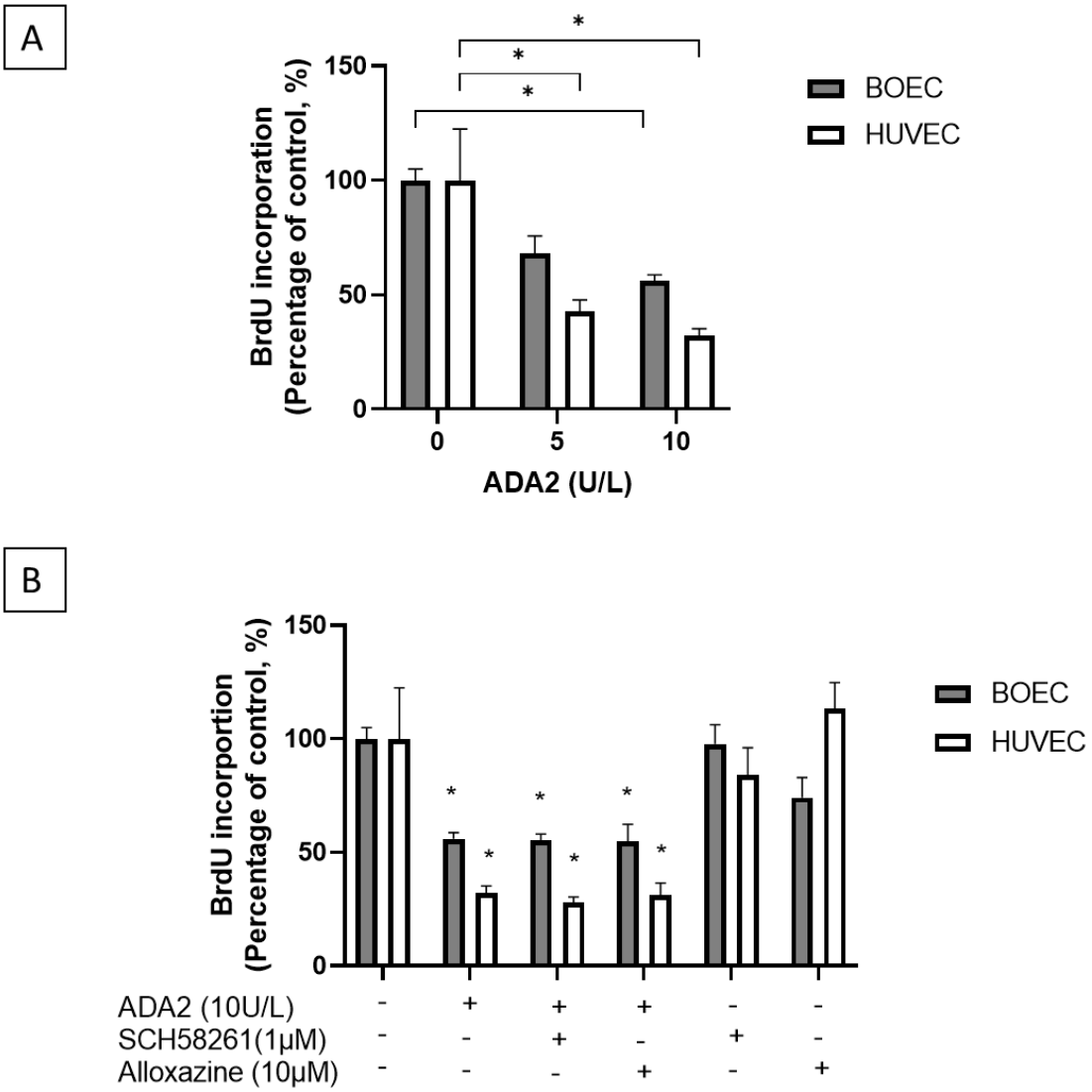


Figure 5. 10 ADA2 dose dependently diminishes EC proliferation and this is not AR mediated

Extracellular ADA2's *in vitro* influence on BOEC and HUVEC proliferation was measured by Cell Proliferation ELISA, BrdU, after 24 hours of treatment. **A)** ADA2 reduces cell proliferation for BOECs at 10U/L and for HUVECs significantly at 5 and 10U/L. **B)** For both EC types, incubation with either SCH58261 or, Alloxazine, in the presence of ADA2, did not significantly change the proliferation from ADA2 treatment alone. SCH58261 and alloxazine treatment alone, did not change the cell proliferation significantly from control. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences from control, indicated by (*), where $p<0.05$.

5.4.2.6.2.Migration

EC cell migration was mimicked *in vitro* by a 'wound healing' assay. Scratches were made into confluent monolayers of EC and then cells were allowed to migrate to close the wound in the presence of ADA2, with or without AR antgonists for 24 hours, as previouly described.

ADA2 dose-dependently, reduced BOEC and HUVEC migration Figure 5. 11. The IC50 for ADA2 was lower in BOECs (IC50 8.81 U/L) than that observed for HUVECs (IC50 9.98 U/L). ADA2 reduction in BOEC migration (to 31.6±6.7% by 10U/L ADA2, $p<0.0001$) did not significanlty change in the presence of the A_{2A} receptor antagonist, SCH58261 and was also significantly reduced in comparison to control conditions ($p=0.0031$). Alloxazine, the A_{2B} receptor antagonist, restored BOEC migration to control levels in the samples incubated with ADA2. 10U/L ADA2 reduced HUVEC migration to 42.0±6.4% of control ($p<0.0001$) and in the presence of SCH58261 there was a return to migration levels observed under control conditions. However, for HUVECs, ADA2 with alloxazine, did not alter the reduction in wound density and this was still significantly diminished from control ($p<0.0001$).

As the ADA2 mediated reduction in BOEC migration was mediated by A_{2B} ARs, it was speculated that this might be cAMP-dependent. To test this, BOECs were incubated with ADA2 in combination with the adenylate cyclase inhibitor, SQ22536 (100µM). When the adenylate cyclase inhibitor was added in combination with ADA2, BOEC migration was restored to control, removing the ADA2 effect (Figure 5. 12).

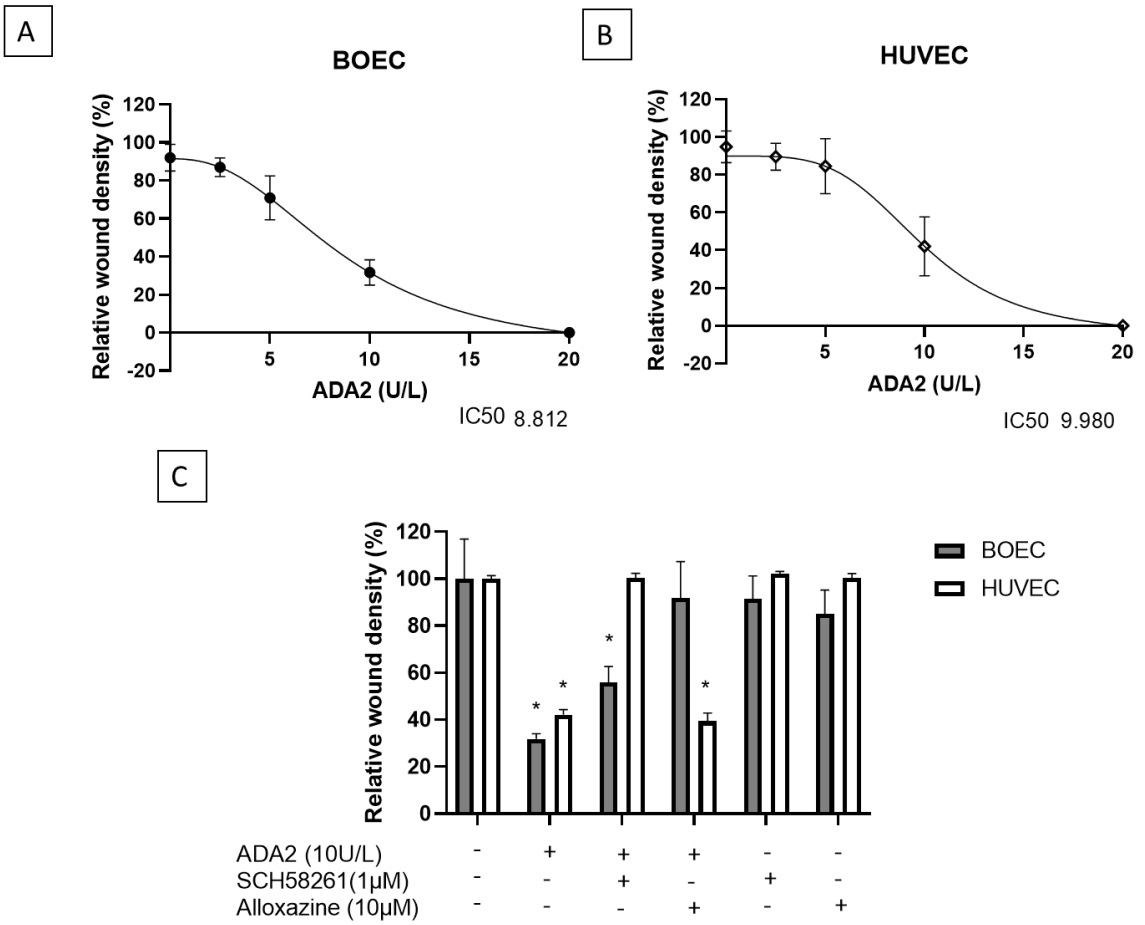


Figure 5. 11 Extracellular ADA2 dose-dependently reduces EC migration and in BOECs this effect is mediated by the A_{2B} adenosine receptor, whereas in HUVECs this effect is mediated by the A_{2A} receptor.

BOEC and HUVECs were treated with extracellular ADA2 in the presence or absence of adenosine receptor antagonists, for 24 hours of treatment after a 'wound' was created in a confluent monolayer of cells. Cell migration was estimated by measuring the density of cells migrated within the wound, using Incucyte® ZOOM software. **A)** ADA2 dose-dependently reduces wound density/cell migration for BOECs with an IC50 of 8.812. **B)** ADA2 dose-dependently reduces wound density/cell migration for HUVECs with an IC50 of 9.980. **C)** For both cell types, ADA2 significantly reduced cell migration at 10U/L. For BOECs this was restored back to control condition levels when co-incubated with Alloxazine, the A_{2B} receptor antagonist. For HUVECs cell migration was restored when treated with both ADA2 and SCH58261, the A_{2A} receptor antagonist. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences from control, indicated by (*), where $p<0.05$.

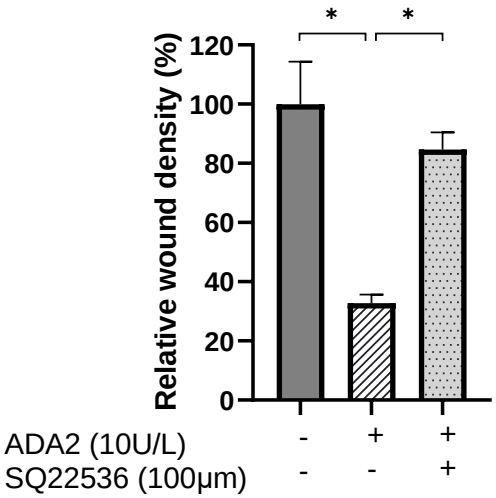


Figure 5. 12 Extracellular ADA2 reduction in BOEC migration is mediated by adenylate cyclase

BOECs were treated with extracellular ADA2 in the presence or absence of the adenylate cyclase inhibitor SQ22536, for 24 hours of treatment after a 'wound' was created in a confluent monolayer of cells. Cell migration was estimated by measuring the density of cells migrated within the wound, using Incucyte® ZOOM software. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean ± SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences from control, indicated by (*), where $p<0.05$.

5.4.2.6.3. Monocyte adhesion

EC adhesion was simulated *in vitro* by co-culture of TNF-α stimulated confluent ECs with monocytes (THP-1s). In both BOECs and HUVECs TNF-α stimulated cells encouraged monocyte adhesion (Figure 5. 13). ADA2 dose-dependently decreased cell adhesion but this was not significant, until 10U/L (BOEC p=0.0401, HUVEC p=0.0019), but this was still significantly greater than control (BOEC p=0.0386, HUVEC p=0.0105).

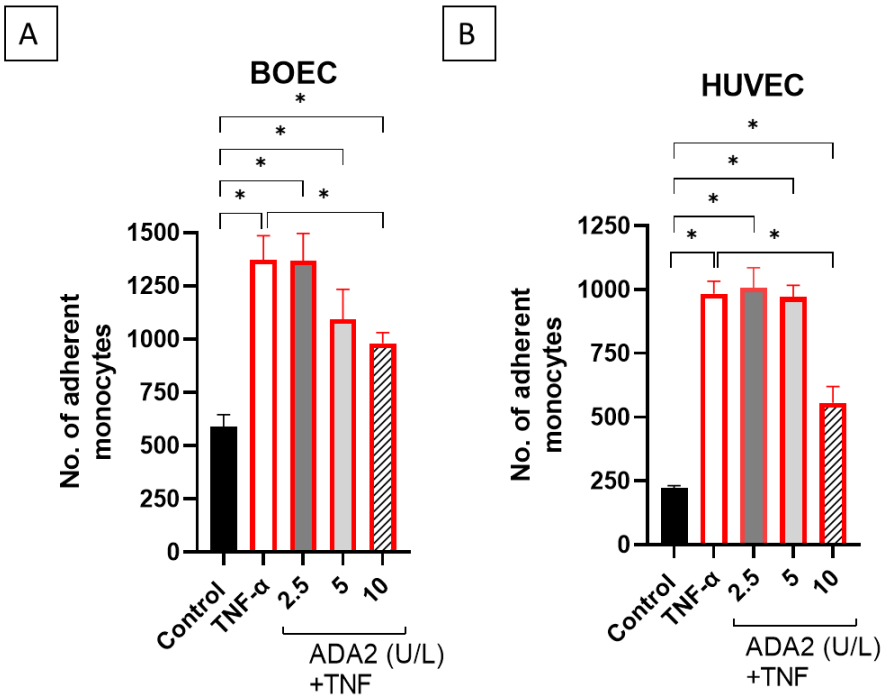


Figure 5. 13 ADA2 decreased TNF-α stimulated monocyte adherence to endothelial cells

Confluent ECs, BOECs or HUVECs, were pre-treated with ADA2 (0, 5, 10 U/L) for 24 hours and then stimulated with TNF-α for 4 hours, before co-culture with calcein AM labelled THP-1 monocytes, for 1 hour. Images were captured of 3 random fields and the number of adherent monocytes were determined using Fiji software. **A)** For BOECs co-culture, TNF-α enhanced THP-1 adhesion. There was no significant difference with pre-stimulation with 2.5 or 5U/L ADA2 from TNF-α stimulated and these were significantly different from control. 10 U/L diminished the TNF-α stimulated adhesion but this was still significantly enhanced from control conditions. **B)** For HUVEC co -cultures, TNF-α enhanced monocyte adhesion. Pre-treatment only with 10U/L significantly reduced TNF-α enhanced monocyte adhesion but this was still above adhesion seen under control conditions. 2.5 and 5U/L failed to reduced monocyte adhesion from TNF-α stimulated HUVECs. Error bars show the mean ± SEM, n=3. Statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparison test.

5.4.3. Influence of ADA2 in the presence of adenosine on BOECs

ADA2's substrate is adenosine, and the extracellular adenosine concentration depends on the cellular local environment (as previously discussed in Chapter 4). It is reported in a number of diseases, including DADA2, that plasma adenosine levels are elevated, to between 0.2-0.6µM, and is elevated in comparison to healthy controls (Dhanwani et al., 2020). Therefore, it was important to describe how ADA2, in the presence of adenosine, would influence EC structure (the glycocalyx), protein expression (ADA2, ARs), signalling molecule production (nitric oxide and its pathway), and function (proliferation, migration and adhesion). The following subsections examine how exogenous ADA2, with adenosine, influence each of these in turn. Investigating ADA2 in combination with adenosine is directed towards gaining insight as to what may occur in patients deficient in ADA2. A concentration of 0.5µM of adenosine was used based on the work in Chapter 4 which demonstrated that this concentration had a positive effect on BOEC: glycocalyx recovery, NO production, growth, migration, and adhesion of leukocyte, as well as, this concentration falling within the range of plasma adenosine levels seen in DADA2.

5.4.3.1. The glycocalyx structure

The results reported previously (Figure 5. 6, page 145) show that for BOECs, ADA2 alone increased proteoglycan core protein mRNA expression, and ADA2 improves HS GAG recovery above control. Proteoglycan core protein expression was examined by qRT-PCR after ADA2 in combination with adenosine (0.5µM), was incubated with the cells. The results in Figure 5. 14 illustrate that for *GPC1* and *SCD4* adenosine negated the positive impact of ADA2. This was not observed however, for *HSPG2*, where adenosine had a negligible effect on enhanced proteoglycan core protein expression induced by ADA2 (*HSPG2*, ADA2 and adenosine is significantly more than control, p=0.0073).

Analysis of immunofluorescence images shows that following enzymatic degradation (Figure 5. 15), the combination of both ADA2 and adenosine, improved the adenosine-mediated inhibition of the HS restoration, in line with control. The combination of both ADA2 and adenosine was significantly less than ADA2 alone (p=0.0320), though significantly more than adenosine alone (p=0.0056).

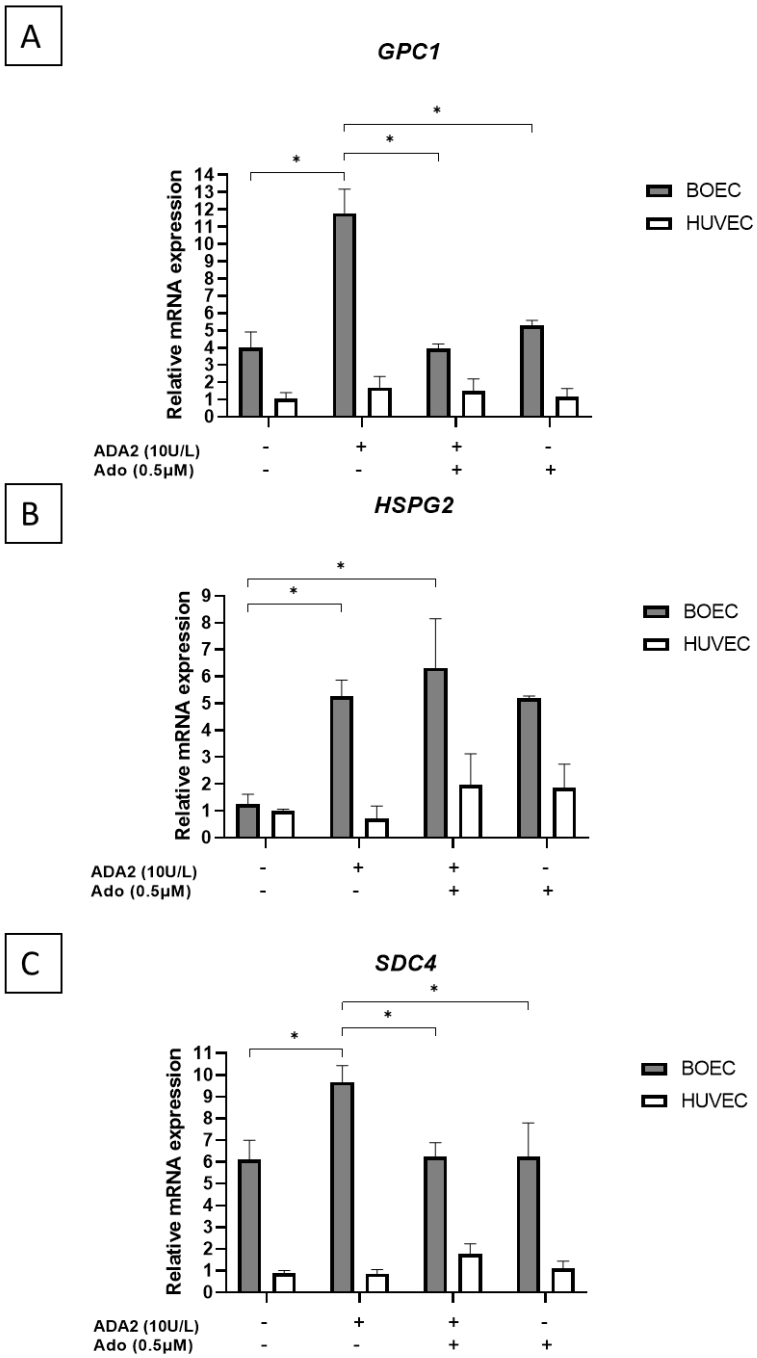


Figure 5. 14 In the presence of extracellular adenosine ADA2 enhancement of *GPC1* and *SDC4* mRNA expression is removed, but *HSPG2* remains high

A) *GCP1,* ***B)*** *HSPG2* and ***C)*** *SDC4* BOEC and HUVEC mRNA expression by qRT-PCR, after *in-vitro* treatment with ADA2 (10 U/L) in the presence or absence of adenosine (0.5µM). The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. The error bars show the mean ± SEM. Statistical analysis used One-way ANOVA with Tukey's multiple comparisons. (*) represents a significant difference, $p<0.05$. Abbreviation- **Ado**: Adenosine.

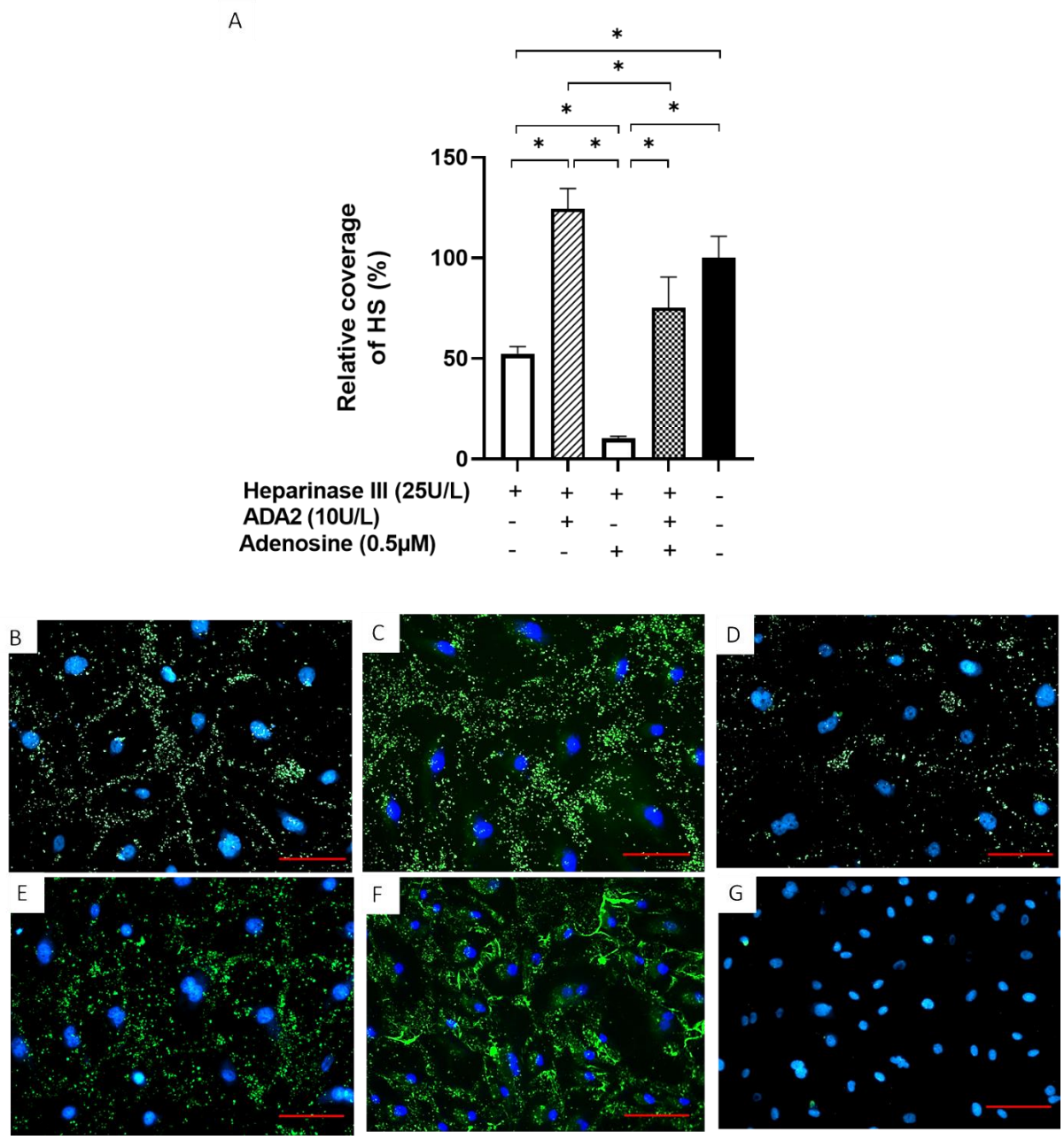


Figure 5. 15 ADA2 improves HS recovery and restores adenosine mediated inhibition of HS recovery

A) Recovery of HS following enzymatic treatment with Heparinase III. Data were plotted as mean values of 5 random fields ±SEM, n=3. (*) represents a significant difference, $p<0.05$. BOECs were treated with heparinase III and then allowed to recover with **B)** media alone, **C)** ADA2 (10U/L), **D)** adenosine (0.5µM), and **E)** ADA2 in the presence of adenosine, for 18 hours. **F)** No heparinase III treatment (positive control), **G)** No heparinase III treatment and secondary only antibody (negative control). Scale bar represents 100µM.

5.4.3.2. AR mRNA expression

As described in section 5.4.2.3, page 146, ADA2 did not impact the expression of *ADORA2A* (predominantly expressed by HUVEC) or *ADORA2B* (predominantly expressed by BOEC). When treating ECs with ADA2, in the presence of adenosine, (Figure 5. 16), *ADORA2A* mRNA expression was not affected in BOECs. There was no change seen in EC *ADORA2B* mRNA expression, either.

It should be noted that, as a reference, HUVEC *ADORA2A* expression was significantly reduced by the combination of both ADA2 and adenosine, from control ($p=0.0018$) and ADA2 ($p=0.0011$). Neither ADA2, nor, adenosine (0.5µM) alone significantly changed the ADORA2A expression from control.

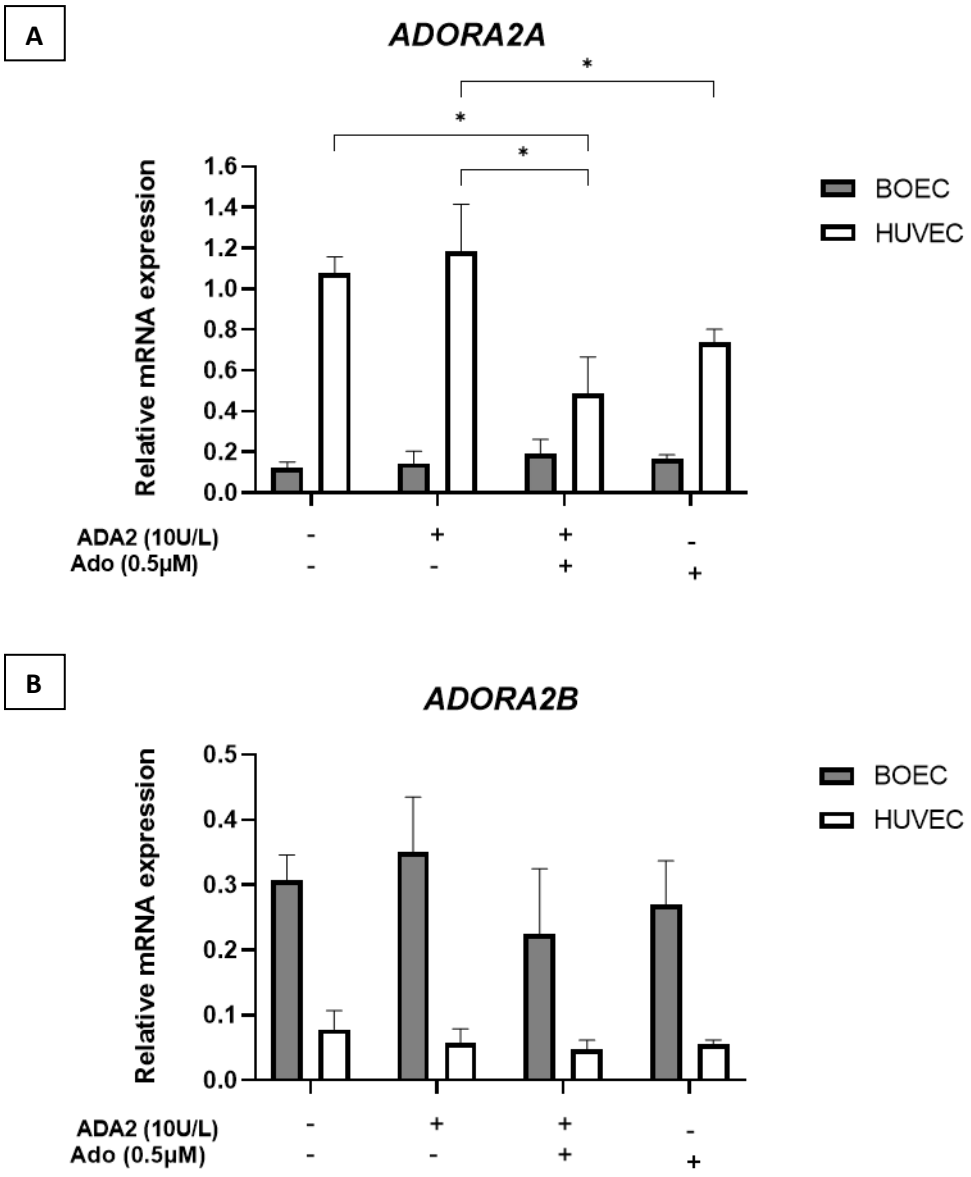


Figure 5. 16 ADA2 in the presence of adenosine had no effect on BOEC AR mRNA expression

ADORA2A and *ADORA2B* mRNA expression was assessed by qRT-PCR, after recombinant ADA2 (10U/L) in the presence or absence of adenosine (0.5µM). **A)** No significant change in *ADORA2A* expression for BOECs. For HUVECs, ADA2 with adenosine downregulates *ADOAR2A* to be significantly less than control and ADA2 alone. ADA2 or adenosine alone were not significantly different from control. **B)** *ADORA2B* for either EC type was not statistically significantly altered under the pharmacological treatments for either BOECs or HUVECs. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample and normalised to an average of two housekeeping genes, *GAPDH* and *ACTB*. The error bars show the mean $\pm$ SEM. Statistical analysis (2way ANOVA with Tukey's multiple comparisons test) demonstrated significant differences, $p<0.05$, denoted by (*). Abbreviation- **Ado**: Adenosine.

5.4.3.3. mRNA expression in the nitric oxide pathway

Examining the mRNA expression of the regulators of the NO pathway showed that *NOS3* expression was upregulated from control when treated with both ADA2 and adenosine together (BOEC, p=0.0205) (Figure 5. 17). This combination of both ADA2 and adenosine significantly expressed more *NOS3* than ADA2 alone (p=0.0200), but not adenosine only. This was also reflected in HUVEC.

In BOECs, the ADA2-induced increase in *DDAH1* expression, was removed in the presence of adenosine (p=0.0027). There was no alternation in *DDAH2* expression in either BOECs or HUVECs.

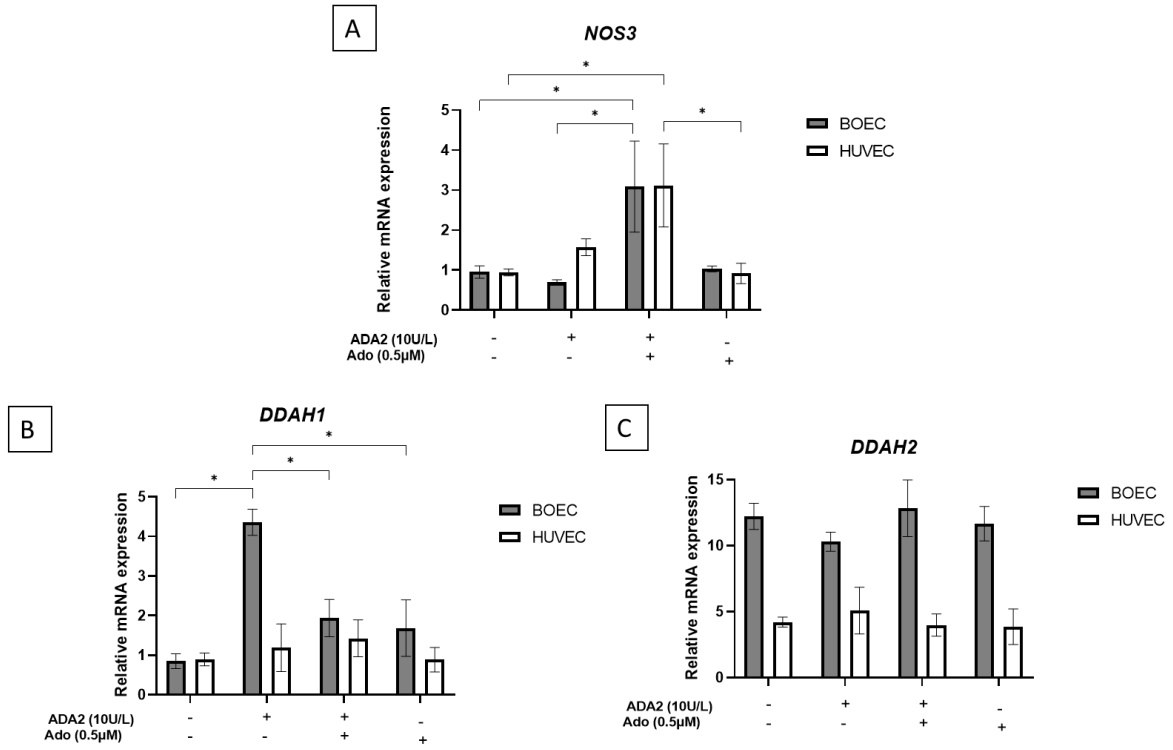


Figure 5. 17 BOEC upregulate *NOS3* expression in the presence of both ADA2 and adenosine

Regulators of the nitric oxide pathway expression were assessed using qRT-PCR observing changes in expression of **A)** *NOS3, **B)** DDAH1* and **C)** *DDAH2*. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The data was normalised to an average of two housekeeping genes, *GAPDH* and *ACTB using the* $2^{-\Delta\Delta ct}$ method. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences shown by (*), where p<0.05. Abbreviation- **Ado**: Adenosine.

5.4.3.4. Nitrite and nitrate production

In cell supernatants, adenosine by itself mediated an increase in nitrite and nitrate production (Figure 4. 7, page 116). This was no longer present in the presence of ADA2, and the levels were not significantly different from control, for BOECs (Figure 5. 18).

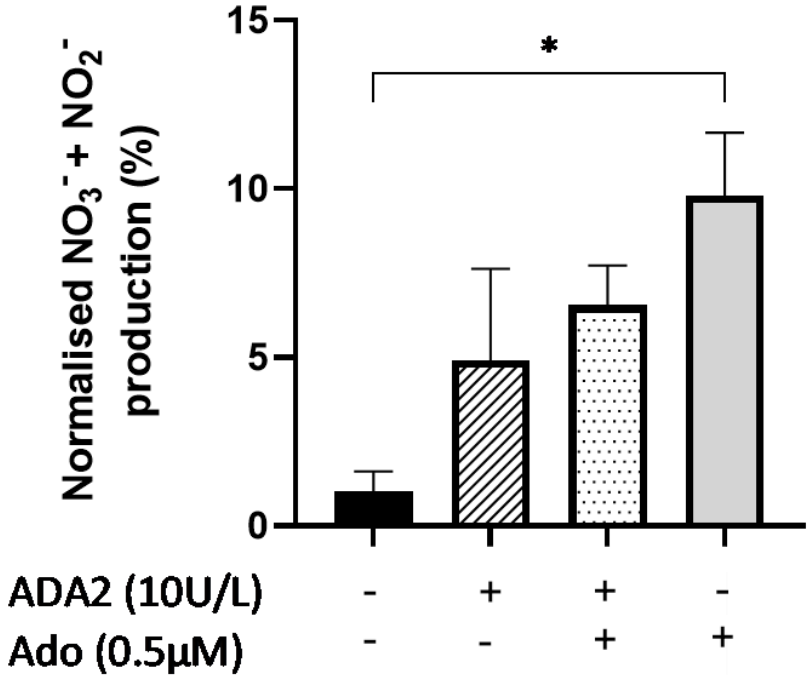


Figure 5. 18 The adenosine increase in nitrites and nitrates is removed in the presence of ADA2, for BOECs

Determination of NO production in cell supernatants was carried out using a nitrite/nitrate kit, after ADA2 (10U/L) with or without adenosine (0.5µM), *in vitro* treatment. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean ± SEM. Statistical analysis used a 2way ANOVA with Tukey's multiple comparison test and revealed significant differences shown by (*), where $p<0.05$. Abbreviation- **Ado**: Adenosine.

5.4.3.5. Endothelial cell function

5.4.3.5.1.Proliferation

Figure 5. 19 illustrates how ADA2 in the presence of adenosine influences EC growth, utilising the BrdU cell proliferation assay. The null hypothesis that the EC50 ratio is 1.0 (no shift), was tested within GraphPad Prism version 9.2.0. ADA2 in the presence of adenosine causes a left shift in BOEC proliferation from adenosine alone; the LogEC50 of ADA2 with adenosine is -7.3M, in comparison to adenosine alone, LogEC50 -6.8M ($p<0.001$). An opposite trend is seen in HUVECs. There is a right shift in HUVEC proliferation, from adenosine only logEC50 -6.9M, to, ADA2 in the presence of adenosine logEC50 -6.2M ($p<0.0001$).

ADA2 alone, as reported in section 5.4.2.6.1 page, 150, decreases proliferation in BOECs. However, in the presence of both ADA2 and adenosine cell proliferation increased by 59.9% from control ($p=0.0103$), and by 104% from ADA2 alone ($p<0.0001$), to be equivalent to cell proliferation seen with adenosine only. HUVEC cell proliferation in the presence of both ADA2 and adenosine was reduced by 54.9% from control ($p=0.0224$), to be equivalent to the reduction seen in ADA2 only.

The observed trends reported when incubating BOECs with recombinant ADA2, in combination with adenosine, were also observed when treating BOECs with total ADA (ADA1 and ADA2), extracted from intestine (Sigma-Aldrich), in combination with the ADA1 specific inhibitor (EHNA) and adenosine (Appendix VI).

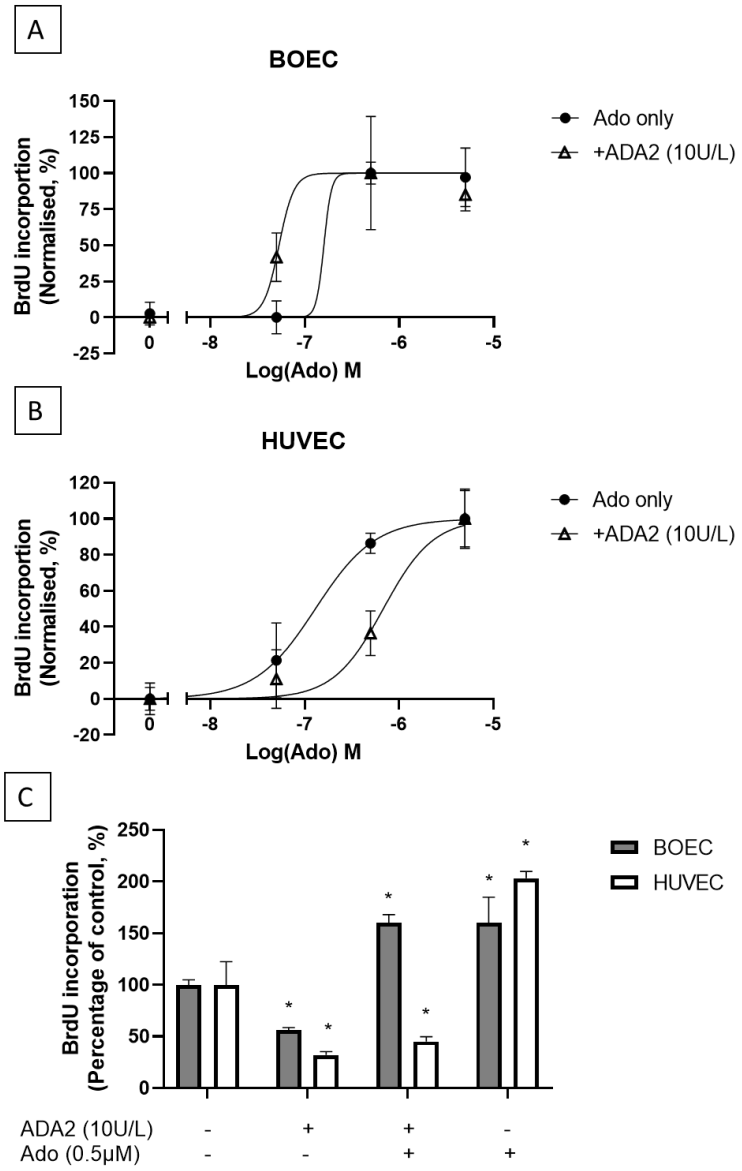


Figure 5. 19 ADA2 in the presence of adenosine promotes cell proliferation in BOECs but reduces cell proliferation in HUVECs

Endothelial cell proliferation was quantified using BrdU cell proliferation assay. **A)** BOEC log dose adenosine with or without ADA2, shows a left shift in the presence of ADA2. **B)** HUVEC log dose shows a right shift with adenosine in the presence of ADA2 **C)** The treatment of ADA2 (10U/L) in the presence or absence of adenosine (0.5µM) was observed. For BOECs, the ADA2 inhibition of proliferation was removed, and enhanced, to adenosine-only augmented levels. Whereas, for HUVECs, in the presence of both ADA2 and adenosine proliferation remained low, significantly reduced from both control and adenosine only. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean $\pm$ SEM. Statistical analysis used a 2way ANOVA with Šídák's multiple comparisons test and revealed significant differences from control, shown by (*), where $p<0.05$. Abbreviation- **Ado**: Adenosine.

5.4.3.5.2.Migration

EC cell migration was determined by a wound healing assay and the quantification of wound cell density (Figure 5. 20). For BOECs, there was no difference in the dose response curve to either adenosine or adenosine with ADA2, as the same logEC50 of -6.7M was observed.

As a reference, for HUVEC migration there is a right shift for ADA2 with adenosine, LogEC50 -6.5M, in comparison to adenosine only, LogEC50 -7.4M ($p<0.0001$).

Focusing on one adenosine concentration (0.5µM), adenosine restored the decrease in wound healing created by ADA2, to control levels. This effect was not seen in HUVECs where ADA2 with adenosine, continues to significantly reduce cell migration, from control (-43.3% $p<0.0001$) and adenosine alone ($p<0.0001$).

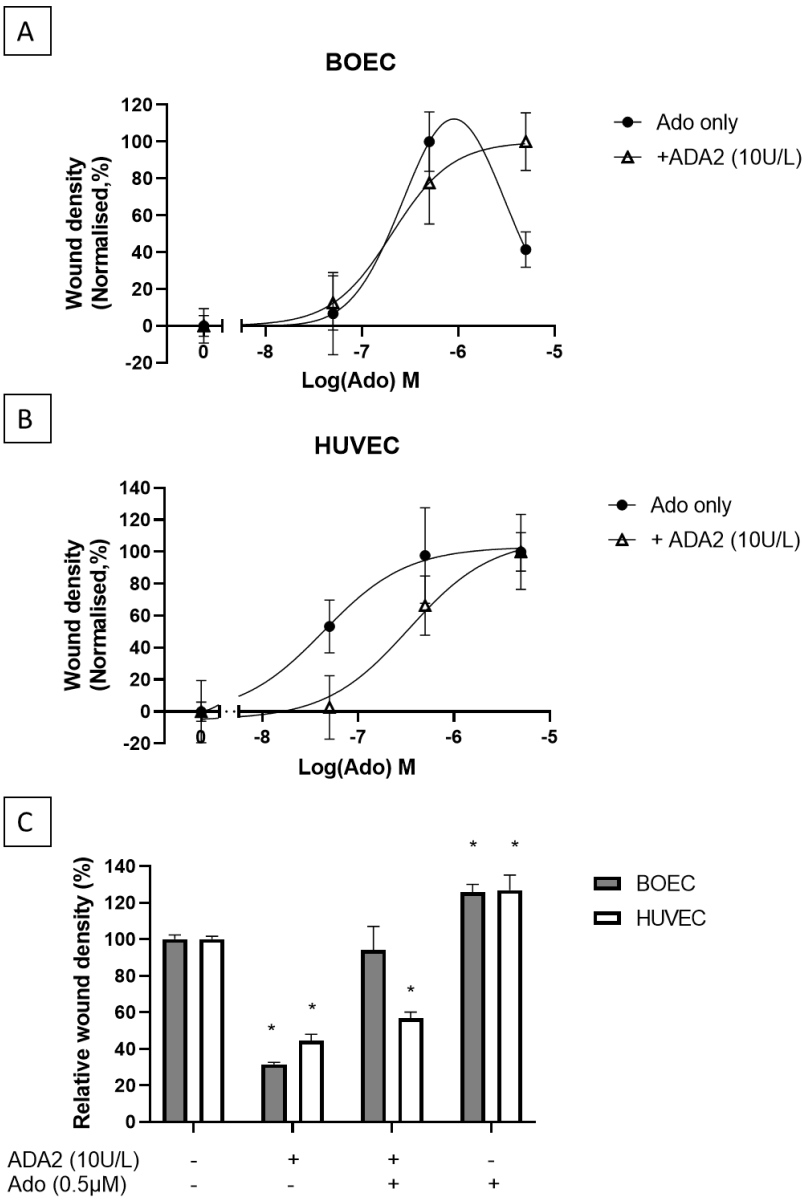


Figure 5. 20 ADA2 in the presence of adenosine restores the ADA2 induced reduction in BOEC migration

Endothelial cell migration was quantified using Incucyte® Zoom software analysis of wound density. **A)** For BOECs the log dose curve for adenosine with or without ADA2, shows no significant shift in the presence of ADA2. **B)** For HUVECs the log dose curve shows a right shift with adenosine in the presence of ADA2 **C)** The treatment of ADA2 (10U/L) and/or adenosine (0.5µM) was observed. For BOECs, the ADA2 inhibition of migration was removed to control levels. Whereas, for HUVECs, the ADA2 reduction in cell migration was not improved in the presence of adenosine. The data was obtained by 3 independent biological replicates (n=3), with two technical replicates per sample. The error bars show the mean ± SEM. Statistical analysis used a 2way ANOVA with Šídák's multiple comparisons test and revealed significant differences from control, shown by (*), where $p<0.05$. Abbreviation- **Ado**: Adenosine.

5.4.3.5.3. Monocyte adhesion

EC were pre-treated with ADA2 in the presence or absence of adenosine, and then stimulated with TNF-α for 4 hours. The stimulated ECs were co-cultured with calcein AM labelled-THP-1s. The number of adherent monocytes after various combination of treatments, was quantified and displayed in Figure 5. 21.

When BOECs were pre-treated with both ADA2 and adenosine, the monocyte adhesion returned to control levels, compared to the reduction seen with ADA2 ($p=0.0043$), or adenosine alone ($p=0.0017$). ADA2 in combination with adenosine was also, significantly different from control ($p<0.0001$) and equivalent to TNF-α alone in BOECs.

Pre-treatment with ADA2 and adenosine, restores HUVEC monocyte adhesion significantly from control ($p<0.0015$). Monocyte adhesion to HUVEC was inhibited by either ADA2, or adenosine alone, and this was not significantly increased when treated with ADA2 in combination with adenosine. However, the increase in adhesion of monocytes to HUVECs with ADA2 in combination with adenosine, was increased to levels to become non-significant from TNF-α alone.

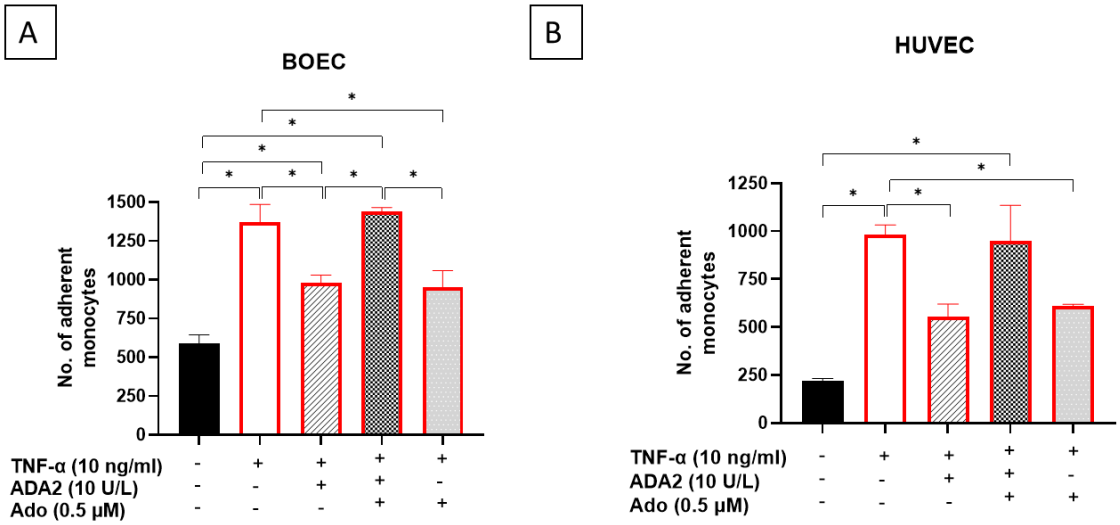


Figure 5. 21 ADA2 in the presence of adenosine enhances monocyte adhesion to stimulated BOECs and HUVECs.

Monocyte adhesion to TNF-α stimulated BOECs or HUVECs, was evaluated after pre-treatment with ADA2 (10 U/L), in the presence, or absence of adenosine (0.5µM). Images were captured of 3 random fields and quantification of adherent monocytes was achieved using Fiji software (Schindelin et al., 2012). **A)** BOEC reduction in monocyte adhesion by ADA2, or adenosine, is reversed after pre-treatment with both ADA2 and adenosine together. **B)** HUVEC monocyte adhesion reduction by ADA2 or adenosine is restored by ADA2 in the presence of adenosine. The error bars show the mean $\pm$ SEM. Statistical analysis used a one-way ANOVA with Tukey's multiple comparisons test and revealed significant differences, shown by (*), where $p<0.05$. Abbreviation- **Ado**: Adenosine.

5.5. Discussion

The objectives listed in chapter have been met as follows:

1. ADA2 is expressed by both BOECs and HUVECs, but to a greater extent in BOECs, demonstrated by cross-study transcriptomics, qRT-PCR, and activity assay. Extracellular adenosine or ADA2 does not influence this.
2. ADA2 influenced EC structure by dose-dependently upregulating proteoglycan core protein genes, *GCP1, HSPG2* and *SDC4,* in BOECs, and improving heparan sulphate recovery after its removal. BOEC *ADORA2A* and *ADORA2B* mRNA expression was not influenced by ADA2. Within the NO pathway, *DDAH1* was upregulated in BOECs after ADA2 treatment, though NO production remains low. EC functional assays have demonstrated how ECs were influenced dose-dependently by ADA2, for both BOECs and HUVECs, as proliferation, migration and adhesion are inhibited. ADA2 dependent inhibition of cell migration, was mediated via A_{2B} AR for BOECs, and A_{2A} AR for HUVECs.
3. In the presence of its substrate adenosine, ADA2 affected both EC structure and function. The observed ADA2-induced increase in proteoglycan core protein mRNA (*GPC1* and *SDC4)*, was removed in the presence of adenosine. In contrast, adenosine did not influence the effects observed with ADA2 for BOEC *HSPG2* mRNA expression, and this was reflected in the ICC images where HS coverage was restored to control levels by ADA2, following adenosine mediated inhibition. In BOEC mRNA expression of either *ADORA2A* or *ADORA2B* was not affected by adenosine in the presence of ADA2. NO pathway; *NOS3* expression was upregulated in BOEC with ADA2 and adenosine. EC functional assays showed the two EC types behaving differently in response to ADA2 in the presence of adenosine. In BOECs ADA2 in the presence of adenosine promoted cell proliferation and migration, whereas in HUVECs, this combination reduced proliferation and migration. In terms of monocyte adhesion, when both EC types were pre-treated with ADA2 in combination with adenosine, there was an increase in monocyte adhesion from adenosine or ADA2 treatment alone.

5.5.1. ADA2 expression in EC- comparing BOEC with HUVEC

When DADA2 was first identified in 2014, it was originally thought that ECs (HUVECs and HCACE) did not express ADA2 (Zhou et al., 2014). However, scrutinization of protein levels in cell supernatants has shown that various endothelial cell types, including HUVEC, express ADA2 and secrete ADA2 protein extracellularly, unlike monocytes which express large amounts of ADA2, but this is predominantly active intracellularly (Dhanwani et al., 2020). The data presented in this chapter supports the view that ECs express *ADA2* and secrete active ADA2 extracellularly, as well as reporting BOEC ADA2 expression for the first time.

However, examination of intracellular ADA2 protein by ICC failed to observe any ADA2 protein expression in BOECs, when compared to THP-1s as the positive control, despite the *ADA2* mRNA and activity in BOECs. Previously published data has also failed to detect ADA2 protein in EC lysates (intracellular protein) (Zhou et al., 2014). Preliminary titrations of the anti-ADA2 antibody at 1:50 was the most appropriate dilution and within manufacturer's guidelines. As THP-1 *ADA2* mRNA levels were 5.6x more, and enzyme activity 3.3x higher, than BOEC, ADA2 protein might actually be present at such concentrations to require amplification of the antibody signals.

However, there might be a biological reason for this result. The ICC reported in this chapter examined ADA2 intracellularly by permeabilising the cells. Dhanwani and colleagues (2020) detected ADA2 in western blots of concentrated EC supernatants (extracellular protein). ADA2 is an ectoenzyme with a classical signal peptide and perhaps ECs are rapidly exporting ADA2, which is why this study, along with previously published work, failed to detect ADA2 protein intracellularly, despite the presence of mRNA and supernatant activity (Dhanwani et al., 2020; Zavialov et al., 2010a; Zhou et al., 2014). In future, it would be beneficial to explore whether non-permeabilization ICC technique or flow cytometry could identify any membrane bound ADA2. In addition to this, following a similar methodology to Dhanwani et al., (2020), western blot analysis of the concentrated cell supernatants could be used to look for ADA2 using the available anti-ADA2 antibody. The methodology reported to detect ADA2 in the concentrated cell supernatants of ECs involved serum-starvation for 48 hours and this fell outside the initial scope of this study to establish whether ADA2 was expressed under basal conditions. Although, for future work it would be beneficial to establish if the results seen in other ECs are reproducible in BOECs (Dhanwani et al., 2020).

This chapter also highlights that BOECs produce more ADA2 than HUVECs, in terms of mRNA and activity assay. This reflects the data published by Dhanwani and colleagues (2020) where, across four EC lines (HKVEC, HBVEC, HDVEC and HUVEC), HUVEC expressed the least *ADA2* at mRNA level and the lowest secreted ADA2 activity. Although ADA2 activity is approximately 3.3x less in BOECs than THP-1 monocytes, the ADA2 that is produced by ECs may have an autocrine effect on ECs and add to the exogenously available ADA2. BOECs are thought to arise from circulating ECFC and may repair the endothelium. The intrinsic ADA2 expression level that BOECs express, may aid their proposed role in the repair of the endothelium.

5.5.2. The influence of recombinant ADA2 on BOECs

5.5.2.1. AR mRNA expression

Once it was established that BOECs and HUVECs were also contributing to extracellular ADA2 pools, albeit to a lesser extent than monocytes, the influence of extracellular adenosine on EC structure and function was studied. *ADORA2A* and *ADORA2B* mRNA expression was not influenced by extracellular ADA2 for either BOECs or HUVECs. Although, the mRNA expression was not affected, previous work has shown, in a different cell phenotype to ECs (Chinese hamster ovary cells); ADA2 increases the binding of adenosine receptor agonists (for A_{2A} and A_{2B} receptors), and modulates adenosine receptor affinities towards their ligands, similar to ADA1 (Zavialov et al., 2010b).

5.5.2.2. The glycocalyx structure

ADA2 also influenced BOEC proteoglycan core protein mRNA expression of *GPC1*, *HSPG2* and *SDC4*, in a dose-dependent manner. ADA2 also significantly improved heparan sulphate recovery. The trend was not emulated in HUVECs, which might be due to the overall significantly lower proteoglycan core protein mRNA expression and heparan sulphate protein expression for HUVECs. It is known that dimerization of ADA2 forms a glycosaminoglycan-binding site, ADA2 binds to both HS and CS, and the dimerization is required for full enzymatic activation (Zavialov et al., 2010a). It is feasible that the ADA2 effects seen in BOEC and not in HUVEC, may be driven by the EC glycocalyx produced *in vitro*, by each cell type.

5.5.2.3. mRNA expression in the nitric oxide pathway, and nitrite/nitrate production

Another difference reported in this chapter, between BOECs and HUVECs, is the expression of *DDAH1*, which is an upstream regulator of NOS production. This is dose-dependently upregulated by ADA2 in BOECs, but not in HUVECs. Although *NOS3* and *DDAH2* expression remained constant in both EC types, upregulation of *DDAH1* in BOECs, may promote more NOS3 activity and NO production, by the metabolism of ADMA. However, nitrite and nitrate levels for BOECs and HUVECs remained low and similar to basal levels.

5.5.2.4. EC function

Furthermore, EC function was affected by extracellular ADA2 and this is the first time that this has been directly studied in either BOECs or HUVECs. BOEC and HUVEC proliferation, migration and leukocyte adhesion were dose-dependently inhibited by ADA2. This effect was not adenosine receptor-mediated for EC proliferation but was for EC migration. The ADA2 reduction in migration was mediated by A_{2B} receptors in BOEC and the A_{2A} adenosine receptor in HUVEC. The difference in AR dependency may be attributed to the fact that BOECs express A_{2B}>A_{2A} and HUVECs A_{2A}>A_{2B}. When the adenylate cyclase inhibitor (SQ22536) was added in the presence of ADA2, ADA2's effects were removed, providing further evidence that this ADA2 effect was cAMP-dependent. The receptor mediating the cell proliferation effect was neither A_{2A} nor A_{2B} ARs. However, it has been demonstrated that in T cells ADA2 can also bind via a GAG binding site and another unknown receptor (Zavialov et al., 2010b). Therefore, the ADA2-mediated decrease in cell proliferation could signal by binding to the glycosaminoglycans, or another unknown receptor.

In summary, extracellular ADA2 on its own has homeostatic effects; inhibiting proliferation, migration, upregulating *DDAH1* and promoting coverage of the glycocalyx and decreasing leukocyte adhesion. These effects were mediated via either adenosine receptors, or other receptors independent of ARs. However, this is probably an oversimplified initial model. Under inflammatory conditions, and in DADA2 disease, the substrate for ADA2, adenosine, is produced at elevated levels, which may alter the effects observed by ADA2 alone.

5.5.3. Influence of ADA2 in the presence of adenosine on BOECs

5.5.3.1. AR mRNA expression

In the presence of ADA2, with adenosine, no change was seen in BOEC *ADORA2A* expression or BOEC and HUVEC *ADORA2B* expression. It was important to establish that ADA2 in the presence of adenosine, was not influencing the AR expression for BOECs, as this may have impacted the subsequent experimental work. Although, HUVECs downregulate ADORA2A mRNA expression compared to control and ADA2 alone.

5.5.3.2. The glycocalyx structure

Proteoglycan core proteins, *GPC1* and *SDC4,* mRNA expression enhancement mediated by ADA2, was removed when ADA2 was in the presence of extreme physiological concentrations of adenosine. However, for the proteoglycan core protein, *HSPG2*, mRNA expression was unaffected. It was previously reported (section 4.4.3) that under conditions where adenosine is low, there was an increase in *HSPG2* expression but when adenosine levels were elevated there was no change in *HSPG2* expression from control. The combination of the *HSPG2* expression results from Chapter 4 and Chapter 5 may suggest that ADA2 is working independently of the adenosine deaminase function to promote EC glycocalyx and this result is particularly important as this is the proteoglycan core protein which is expressed significantly more than the other two proteins.

5.5.3.3. mRNA expression of the nitric oxide pathway and nitrite/nitrate production

The nitric oxide pathway is also influenced by exogenous ADA2 in the presence of extreme physiological concentrations of adenosine. *NOS3* expression was upregulated in ECs when treated with ADA2 in the presence of adenosine. This was enhanced from ADA2 or adenosine only effects, which may suggest an effect independent of the enzymatic function of ADA2. The observed increase in *DDAH1* expression by ADA2, was not present with adenosine. This may be reflective of ADA2 metabolising adenosine or ADA2 and adenosine competing for the same receptors. The nitrite and nitrate levels which are increased by BOECs in the presence of adenosine, were removed with the addition of ADA2, which implies an effect of metabolising adenosine.

5.5.3.4. EC function

When incubated with ADA2 in the presence of adenosine, the EC functional responses became divergent for BOECs and HUVECs. BOEC proliferation was enhanced with both ADA2 and adenosine, causing a left shift in the dose response curves. This result may suggest a deaminase-independent effect where ADA2 acts as a growth factor under these conditions. Whereas, for HUVECs the adenosine effect caused a right shift, suggesting ADA2 was working by metabolising extracellular adenosine (a deaminase-dependent effect).

Adenosine improved BOEC migration mediated via A_{2A} and A_{2B} ARs (reported in Chapter 4), whereas ADA2 reduced migration below control, via A_{2B} ARs. BOEC migration was restored to control levels by adenosine, when ADA2 and adenosine were in combination. These results combined suggest that adenosine and ADA2 might be via working two different signalling mechanisms, where ADA2 metabolises adenosine reducing migration, in addition to reducing migration below control by another mechanism. In contrast, for HUVECs ADA2 in the presence of adenosine reduces the adenosine-mediated effect, causing a right shift in the dose response curves, suggesting an effect of metabolising adenosine. This could be potentially due to a number of differences in BOEC and HUVEC cell types, and their individual responses to ADA2, which will need to be investigated further. However, proposed explanations may involve, the fact that BOECs produce more glycosaminoglycans *in vitro* than HUVECs, and so more ADA2 can bind to BOECs via the glycosaminoglycan binding site or, the fact that ADA2 in the presence of adenosine downregulates *ADORA2A* in HUVECs.

Preceding stimulation with TNF-α, adenosine in the presence of ADA2, increased monocyte adhesion in BOECs and HUVECs, restoring effects seen either with adenosine or ADA2 alone. This might be attributed to ADA2 and adenosine binding. This flip from anti-inflammatory with either ADA2 alone, or adenosine alone, to pro-inflammatory with ADA2 and adenosine together, suggests a repair mechanism which is evoked in response to inflammatory stimuli.

In summary, ADA2 in the presence of adenosine, has beneficial effects of repair and renewal on the ECs, which are more evident in BOECs than HUVECs. For example, increasing glycosaminoglycan recovery, upregulation of *NOS3*, enhancement of proliferation, restoring migration to control and increasing monocyte adhesion in response to inflammatory stimulation.

5.5.4. Summary

The results in this chapter, have demonstrated that ADA2 has a direct effect on endothelial cells, rather than simply just metabolising extracellular adenosine or effects mediated via other inflammatory cells. Extracellular ADA2 in unstimulated conditions promotes homeostasis whereas, ADA2 in the presence of extracellular adenosine, which is elevated in extreme physiological (strenuous exercise) and pathological conditions, promotes repair and pro-inflammatory mechanisms. Both the lack of ADA2 and the increase in adenosine can both have deleterious effects, and, therefore, in ADA2-deficient, DADA2 patients, this may contribute to the endothelial damage causing the inflammation and vasculitis that is observed in these patients. In addition, in conditions where ADA2 is over-expressed, this may be damaging as there are chronic repair and pro-inflammatory mechanisms which do not resolve the inflammation. Nevertheless, this work does not directly identify the ADA2 receptor for ECs, and this would be of great interest moving forward in ADA2 and EC studies.

6. Conclusions

DADA2 is a rare monogenic disease with fatal outcomes for some young patients (Navon Elkan et al., 2014; Zhou et al., 2014). This disease has an expansive phenotype (Lee et al., 2020a). Whilst treatment options such as TNF-α inhibitors seem to work well, especially for patients within the vascular cluster, HSCT is the only alternative to effectively treat the symptoms for patients within the haematological, or immunodeficiency clusters (Ombrello et al., 2019). As a highly invasive treatment, this is not routine. In addition, there are several family members of affected children, who have less severe symptoms (or asymptomatic) DADA2, as well as carriers. This raises the question as to whether these patients need to be pro-actively treated before onset of symptoms. Therefore, there is an urgent need to understand the disease mechanism and identify appropriate therapeutic targets.

The overall aim of the research presented throughout this thesis, was to improve upon the understanding of ADA2's action on the endothelium. Exploring the endothelial pathways and functions which might be directly affected by ADA2 was addressed, in the hope of driving towards consensus on ADA2's physiological functions. This in turn would gain insight into the understanding of the pathogenesis of deficiency of adenosine deaminase 2, and elucidating novel therapeutic targets. In the following subsections, each aim from section 1.5 is stated, and will be discussed.

6.1. To isolate and grow BOECs from healthy donors and examine this progenitor cell model comparing it to HUVEC

BOECs were successfully isolated, and confirmation of endothelial cell characteristics were confirmed in Chapter 3. Morphology and endothelial cell surface marker phenotype were confirmed in this study and this in in agreement with previous work (Martin-Ramirez et al., 2012b; Ormiston et al., 2015). Overall, BOECs and HUVECs behaved in a similar fashion. However, BOECs showed potential to be a more relevant endothelial cell model *in vitro,* due to enhanced glycocalyx expression and elevated *DDAH2* expression, in comparison to HUVECs, and I believe that this is the first time that this has been shown.

The ability to isolate BOECs is also important in terms of personalised medicine. BOECs have the potential to be used for autologous therapies (Paschalaki and Randi, 2018). Of particular

interest to monogenetic diseases akin to DADA2, BOECs have been shown to be useful as a carrier cell for gene therapy (Lin et al., 2002). An example of a recent study were BOECs were used as the vector for gene delivery is in a rat model of pulmonary hypertension (Somani et al., 2019). Rat BOEC cultures were established and genetically altered to over-express human eNOS. The infusion of the genetically modified BOECs showed encouraging and significant efficacy for both prevention and intervention in rats with pulmonary hypertension (prevented worsening of right ventricular hypertrophy and partially reversed arteriolar muscularisation) (Somani et al., 2019). Therefore, exploring the use of BOECs in the development of DADA2 gene therapies would be beneficial.

6.2. To identify any involvement of adenosine receptors, and downstream pathways on BOEC in comparison to HUVEC by means of expression profile, structure, and function.

Elucidating the effect of the ADA2 metabolite, adenosine, on BOECs, was important before moving onto understanding ADA2 itself. Adenosine works via ARs. *In vitro* EC models have been reported as having predominantly *ADORA2A* and *ADORA2B* expression (Feoktistov et al., 2002). The results from chapter 4 were in agreement with this and revealed the BOEC AR profile to be $A_{2B} > A_{2A}$, in contrast to HUVECs that displayed $A_{2A} > A_{2B}$.

Adenosine is generally regarded as anti-inflammatory in its actions, though this is concentration dependent (Faas et al., 2017). The results concurred with this as the effect of increasing adenosine concentrations on BOECs, increased cell growth and migration, mediated by A_{2A} and A_{2B} ARs, whilst reducing TNF-α stimulated monocyte-endothelial adhesion. I also found that BOECs upregulate *ADORA2A* under inflammatory conditions, which may represent the BOECs trying to compensate by upregulating the adenosine receptor known to have anti-inflammatory actions downstream.

Adenosine levels can rise under conditions such as strenuous exercise (extreme physiology) or ischemia (pathophysiology). At these high concentrations there is some evidence of a switch to thrombotic and pro-inflammatory functions in an attempt by the ECs to repair (GAG inhibited recovery as well as increased proliferation, migration). Though, potential endothelial dysfunction was observed by downregulation of *DDAH2.* It is known that a

decrease in DDAH2 is associated with cardiovascular disease (Lambden et al., 2015). Chapter 4 showed that *DDAH2* was downregulated at pathophysiological concentrations of adenosine by BOECs. This suggests that in disease states such as DADA2, where adenosine plasma levels are elevated, nitric oxide production may also be reduced, leading to the endothelial dysfunction that is observed.

6.3. To elucidate any physiological, or pathophysiological, effects of ADA2 in the presence of adenosine, on the endothelial cell expression, structure and function

The earliest reports of DADA2 disease suggested that ADA2 was not expressed by endothelial cells (Zhou et al., 2014). However, recently, work by Dhanwani and colleagues (2020) have demonstrated ADA2, at mRNA and protein levels, in four EC types. Specifically measuring ADA2 in endothelial cell culture supernatants rather than cell lysates, identified ADA2 protein and activity. As ADA2 is an ectoenzyme with a signalling peptide, these observations appear to be in line with predicted function. The results in Chapter 5 agree with this and contribute BOECs as another EC type which express ADA2, to the other four types identified.

The transcriptomic analysis of ADA2 expression in Chapter 5 used EndoDB, however, other datasets are available. EndoDB was chosen as a specialist endothelial database, which was important due to the ambiguity surrounding the definition of endothelial progenitor cells and BOECs, despite EndoDB being a smaller dataset. Geo Datasets were checked for additional BOEC data sets and ADA2 expression data. A Geo profiles search using both ADA2 as the Gene Symbol, and, blood outgrowth endothelial cells, returned no items. Manually inspecting any BOEC study data and using the 'Analyze with GEO2R' function provided no additional datasets for ADA2 expression in BOECs and therefore transcriptomic analysis was limited to the data already scrutinised and discussed from the EndoDB in Chapter 5.

Data presented in Chapter 5 identifies direct influences of ADA2 on the endothelium. Increased proteoglycan core protein structure, alongside accelerated glycocalyx recovery and upregulation of *DDAH1* is indicative of vaso-protective actions.

Indirect evidence in the literature, to date, has suggested that ADA2 might be a growth factor for ECs (Zavialov and Engström, 2005; Zhou et al., 2014). In contrast, the results have

demonstrated that ADA2 dose-dependently decreases proliferation, migration (A_{2B} AR mediated) and monocyte adhesion, reflecting anti-angiogenic and antithrombotic actions. However, the action of ADA2 changes in the presence of its substrate adenosine. This is important to determine as adenosine levels fluctuate, responding to the extracellular environment (strenuous exercise or hypoxia), and plasma adenosine is elevated in DADA2 patients.

For BOECs, ADA2 in the presence of adenosine promotes cell proliferation, overcoming the ADA2 mediated reduction, and increases proliferation above the adenosine only effect. This result suggests that ADA2's effect is not just as an adenosine deaminase, but perhaps a receptor mediated effect. Migration is also promoted in the presence of ADA2 and adenosine. In accordance with this result, ADA2 in the presence of adenosine, reverses the reduction in monocyte adhesion caused by either adenosine, or ADA2, alone (after TNF-α stimulation). Finally, ADA2 with adenosine upregulates *NOS3.* The positive effect on proliferation, migration and *NOS3* expression, ADA2 had in the presence of adenosine, appears to be an adenosine deaminase-independent effect. ADA2 in the presence of adenosine, switches to a pro-angiogenic action supporting growth and repair.

In contrast to the adenosine metabolism-independent effects, for BOECs, ADA2 in the presence of adenosine, also has an adenosine deaminase-dependent effect. This is where ADA2 with adenosine restores glycocalyx recovery to control levels, from the adenosine inhibition.

The results presented therefore suggest that, in the absence of ADA2, and with increased adenosine levels, as found in DADA2 patients, there is a decrease in structural integrity of the glycocalyx, alongside the proliferation and migration, working directly and indirectly through NOS3 and DDAH2.

The aims of this thesis have been met as discussed above. The results explored in this thesis, contribute to the current understanding of the endothelial cell model, BOECs, and provide evidence for direct ADA2 effects on ECs. This work highlights the need for appropriate endothelial *in vitro* models, and the complex nature of ADA2 in the presence of adenosine signalling pathways.

6.4. Limitations of this study

Measurement of NO is known to be technically difficult (Csonka et al., 2015; Möller et al., 2019). NO has a short half-life, 0.1-10 seconds, and so indirect methods are often deployed measuring the derivatives of NO. However, there are many advantages and disadvantages of these methods which have been discussed widely in the literature (Coneski and Schoenfisch, 2012; Hetrick and Schoenfisch, 2009). In this study, a well established technique was used, quantification of nitrite/nitrate production, as metabolites of NO, to indicate NO production in the cell supernatants. Despite the increases in expression of *DDAH2* mRNA in BOECs in comparison to HUVECs, the results showed no significant increase nitrite/nitrate production and the levels remained low in both cell types under basal and inflammatory conditions. The reason for this might be due to this assay being unable to detect low levels of NO production via nitrites/nitrates and it is thought that human cells in culture produce less NO than animal cells (Hetrick and Schoenfisch, 2009). Electrochemical NO sensors are another alternative that could be used but these also have disadvantages such as the signal reduction by oxygen, carbon monoxide and other interferents (Hetrick and Schoenfisch, 2009). Therefore, the data at mRNA level for genes involved with the NO pathway (*NOS3, DDAH1* and *DDAH2*), provided more useful insight into the NO pathway than the nitrite/nitrate production data.

As candidate receptors for ADA2, and the receptor for ADA2's metabolite, ARs were studied. This analysis was limited to observing changes of mRNA expression and was not assessed further at protein level. This is due to the lack of specific A_{2B} AR antibodies, as discussed within Chapter 4 (Carmona-Rivera et al., 2019). Once these antibodies become available in the future, it would be prudent to look at the influence both adenosine and ADA2 has on the cell surface expression of the ARs using methods such as immunofluorescence, flow cytometry or western blot.

The isoenzyme of ADA2, ADA1, is also expressed in BOECs. Transcriptomic analysis using the EndoDB showed expression of *ADA1* mRNA across all 4 BOEC data sets (data not shown). The expression of another adenosine deaminase by this cell type, raises the question as to whether this could influence the results reported in Chapters 4 & 5 by altering the concentrations of adenosine. In this study we chose not to inhibit ADA1 produced by the cells during *in vitro* experiments for a number of reasons. Firstly, although ADA1 & ADA2 are both adenosine deaminases, they differ in optimal conditions for enzymatic activity. For instance,

by location: ADA1 is primarily located intracellularly, unlike ADA2 which is found extracellularly, and there are great differences in the optimal pH and environment such as normoxia versus hypoxia. In addition to this, inhibiting ADA1 and influencing adenosine levels intracellularly will influence cell signalling, introducing another variable that would not be related to the extracellular ADA2 effect that this study intended to be measure.

6.5. Future work

In terms of developing the understanding of both the BOEC model, and DADA2 pathogenesis, it would be interesting to see how BOECs isolated from DADA2 patients might behave in vitro and whether any BOEC function/dysfunction could be restored with therapeutic agents.

This study has showed adenosine deaminase-independent effects of ADA2 which might be attributed to ADA2 binding to an endothelial receptor, potentially an AR, and altering downstream signalling pathways. Identification of the ADA2 endothelial receptor, is pivotal and would advance the understanding of the mechanism of action of this protein and drug targets and potential therapy for patients. In other cell types, such as monocytes and T cells, this has been carried out by fluorescently labelling ADA2 and allowing it to bind to the cells, in combination with pharmacological agents, such a GAG degrading enzymes, and observing any changes in ADA2 binding, by flow cytometry (Zavialov et al., 2010b). For a number of immune cells lack of recombinant ADA2 binding was seen after enzymatic removal of the glycocalyx. However, for T cells the binding of the fluorescently labelled ADA2 was only partially prevented when the glycocalyx was removed and this was suggestive of another ADA2 receptor on T cells. Similar experiments should be carried out with endothelial cells, such as BOECs, to identify the ADA2 receptor on these cells. In addition to this, techniques such as immunoprecipitation would be useful in identifying the ADA2 receptor.

Appendix I- Materials and suppliers

Supplier	Materials
Abcam	Goat Anti-Mouse IgG+IgM H&L (FITC) preadsorbed (ab47830)
Amsbio	Heparinase III (Heparitinase I) Flavobacterium heparinum (EC 4.2.2.8), 0.1 IU
	Ab Heparan Sulfate, purified (clone F58-10E4), 50ug pack
Biorbyt	Human CECR1 protein
Cole Parmer	PROPLATE48
	PROSEAL48
Lonza	EGM-2 MV BulletKit
PromoCell	Freezing Medium Cryo-SFM
Qiagen	QuantiTect Primer Assays, *ADA2* primer (QT01667862)
R&D systems	Recombinant Human Adenosine Deaminase 2/CECR1 Protein, CF
	Adenosine Deaminase 2/CECR1 Antibody (NBP1-89238),
	Pentostatin- pan-adenosine deaminase inhibitor
	EHNA hydrochloride – selective ADA1 inhibitor
SIGMA-aldrich	Adenosine
	Alloxazine – A2B receptor antagonist
	Bovine Serum Albumin
	Cell proliferation kit ELISA BrdU
	Custom oligo primers (various)
	Diethylamine NONOate diethylammonium salt
	Dulbecco's Phosphate Buffered Saline
	Dulbecco's Modified Eagle's Medium
	Ethylenediaminetetraacetic acid solution
	Endothelial Cell Basal Medium without Phenol Red (500 ml)
	Endothelial Cell Growth Supplement without FBS (5ml-2 parts)
	Glacial Acetic Acid
	RNaseZAP™, Cleaning agent for removing RNase
	Nitrite/Nitrate Assay Kit, colorimetric
	RPMI-1640 Medium
	SCH 58261 – A2A receptor antagonist
	SQ22536 - adenylate cyclase inhibitor
	Tris base
	Trypsin Solution 10X
ThermoFisher Scientific	6X TriTrack DNA Loading Dye
	8-well Chamber Slide w/ removable wells
	Collagen I Rat Protein, Tail
	DNase I, RNase-free (1 U/µL)
	Fetal Bovine Serum, qualified, heat inactivated, US origin 100ml (batch 1856052)
	Ficoll-Paque PLUS
	GeneRuler Low Range DNA Ladder, ready-to-use

	Glycogen, RNA grade
	Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488
	Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg
	Maxima SYBR Green/Fluorescein qPCR Master Mix (2X),
	RevertAid First Strand cDNA Synthesis Kit
	Sodium citrate vacutainers
	TRIzol™ Reagent
	TrypLE™ Select Enzyme (1X), no phenol red (100ml),
	Water, nuclease-free
VWR	GelRed® nucleic acid gel stain, 10,000x in water

Appendix II- Buffers and solution

TAE buffer

10x stock of TAE buffer was prepared using 12.1g Tris, 2.85ml of glacial acetic acid and 5ml of 0.5mM EDTA. dH_2O was used to make the solution up to 250ml.

TBS buffer

10x stock of TBS buffer was prepared by 24g Tris base and 88g NaCl dissolved in 900ml of dH_2O and adjusted to pH 7.6. dH_2O was added to make a final volume of 1L.

Appendix III- Consent form

1617/024 V2 CONSENT FORM

WRITTEN CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Statement by participant

- I confirm that I have read and understood the information sheet/letter of invitation for this study. I have been informed of the purpose, risks, and benefits of taking part.

Use of blood and blood derived cells to study vascular inflammation.

- I understand what my involvement will entail and any questions have been answered to my satisfaction.
- I understand that my participation is entirely voluntary, and that I can withdraw at any time without prejudice.
- I understand that all information obtained will be confidential.
- I agree that research data gathered for the study may be published provided that I cannot be identified as a subject.
- Contact information has been provided should I (a) wish to seek further information from the investigator at any time for purposes of clarification (b) wish to make a complaint.

Participant's Signature----------

Date ----------

Statement by investigator

- I have explained this project and the implications of participation in it to this participant without bias and I believe that the consent is informed and that he/she understands the implications of participation.

Name of investigator ----------

Signature of investigator ----------

Date ----------

Appendix IV- qRT-PCR primer validation

Each of the primers in this study was validated by performing a cDNA serial dilution as described in the general methods (Figure A.1). Primer effeciency was calculated using the equation of the line (Cq values plotted against the log of the quantity of cDNA used). To confirm that a single pcr product is formed and no off-target amplification, a melt cruve was performed at the end of each qRT-PCR run (confired by a single peak), and then the pcr products were run on a 2% agarose gel (confirmed by a single band at the expected product sizeand no amplifiaction in the no template control).

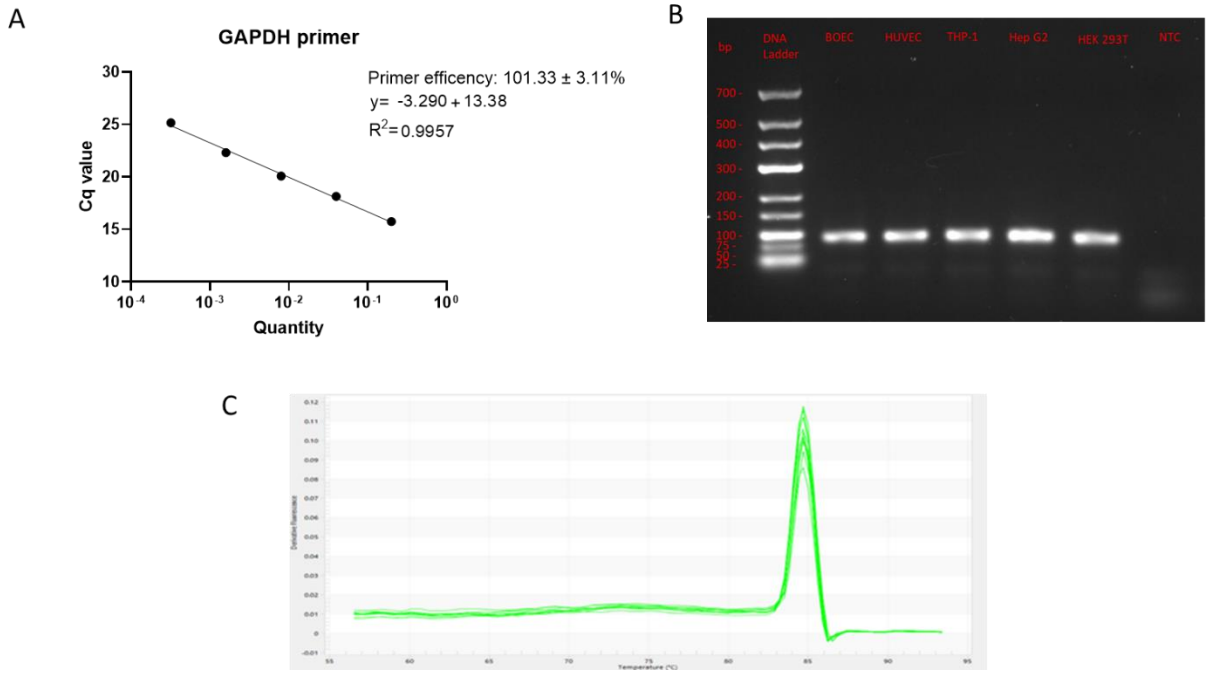


Figure A. 1 Example of primer validation

Primer efficiency was validated for all primers using cDNA serial dilutions. This figure shows GAPDH primer validation. **A)** The equation of the line was used to calculate the efficiency and an R^2 value. **B)** The PCR products for each of the cell lines and a no template control (NTC) were run on a 2% w/v agarose gel, stained with 1x GelRed® (Biotium) fluorescent nucleic acid stain. Visualization shows 1 product at the expected product size of 105bp. **C)** The derivative melt curve was used to screen for unintended products. Only one peak was seen for the GAPDH serial dilution, suggestive of one pcr product.

Appendix V- Preliminary observation of intracellular BOEC vWF expression

Another characteristic marker of ECs is von Willebrand Factor (vWF). Expression of vWF was compared using ICC and confocal microscopy, as this method allowed visualisation of the distribution of vWF intracellularly. BOECs express vWF as shown in the representative images from 3 BOEC donors (Figure A. 2).

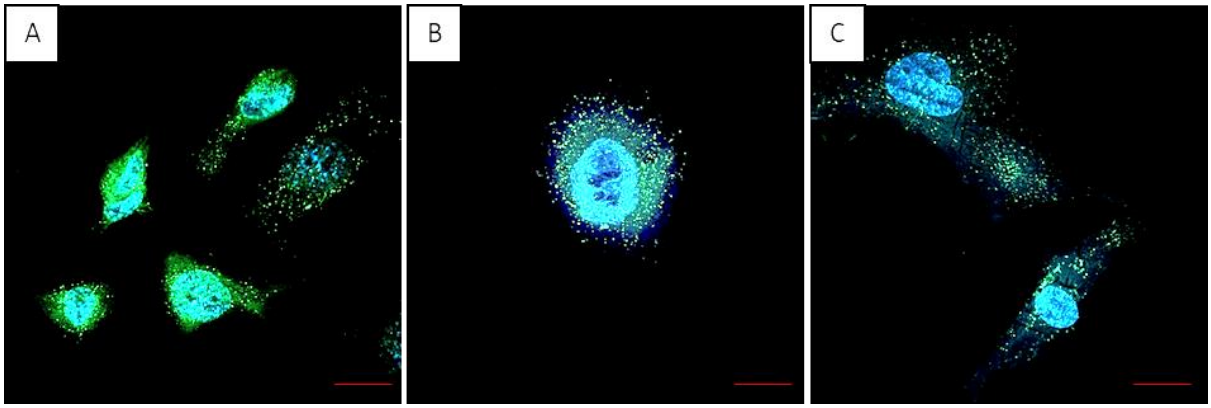


Figure A. 2 Representative confocal images show vWF intracellularly in BOECs

Images taken of BOECs isolated from 3 different donors (A, B and C), using confocal imaging showing, vWF (green) and the nuclear stain, Hoechst 33342 (blue). Magnification x400, scale bar represents 20µM.

Appendix VI- Effect of total ADA on BOEC proliferation

BOEC cells were treated with increasing concentrations of adenosine, total ADA (isolated from calf intestine, Sigma-Aldrich), in the absence or presence of the ADA1 specific inhibitor, EHNA, for 24 hours. BOEC proliferation was measured using a BrdU ELISA as outlined in the general methods. Total ADA (both ADA1 and ADA2) did not influence BOEC proliferation, however, in the presence of the ADA1 specific inhibitor, EHNA, proliferation was reduced; an ADA2 mediated effect (Figure A. 3). Adenosine affects BOEC proliferation in a dose dependent manner and in the presence of total ADA, proliferation is increased further at every concentration of adenosine. In the presence of increasing concentrations of adenosine, total ADA and the ADA1 specific inhibitor (EHNA), a significant spike in growth is seen at 0.5µM; an ADA2 influence on BOEC proliferation at extreme physiological concentrations of adenosine (+65 +/- 17.4% n=5, p <0.01).

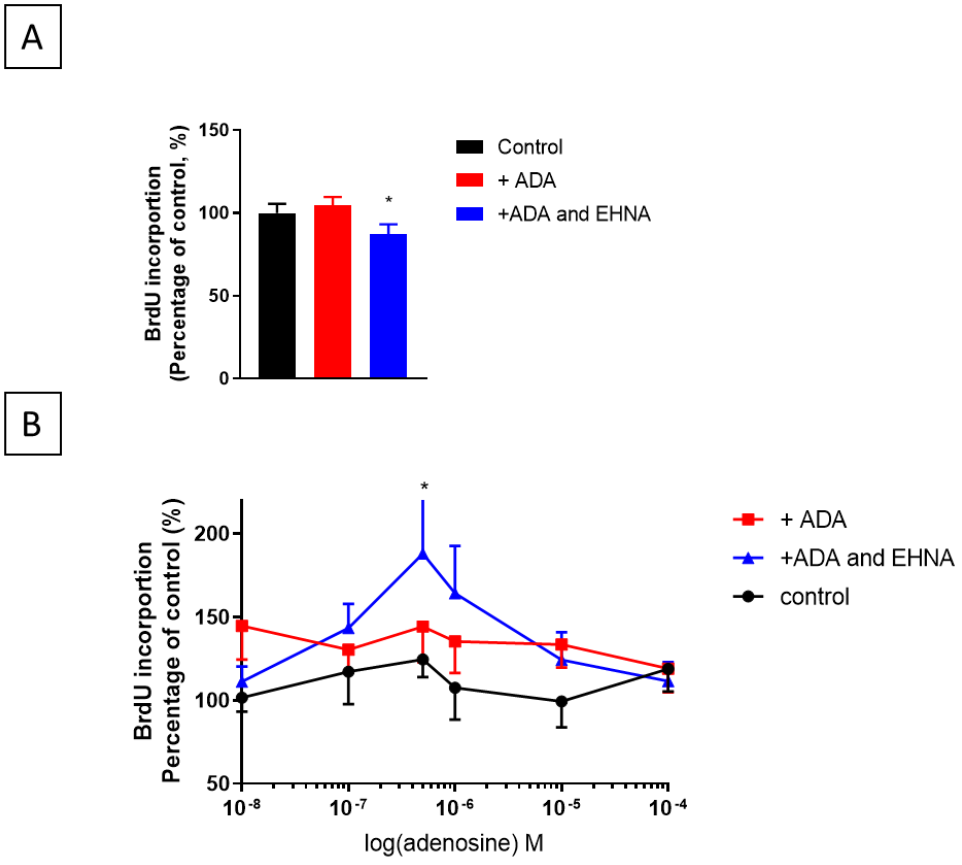


Figure A. 3 At 0.5µM adenosine, ADA2 influences BOEC proliferation

BOEC proliferation was measured by colorimetric BrdU assay. **A)** BOECs were incubated with total ADA, in the presence or absence of EHNA (the ADA1 specific inhibitor). Total ADA with EHNA significantly decreased BOEC proliferation (*p=0.0221, unpaired t test). **B)** ADA2 only influences BOEC proliferation in the presence of adenosine, at 0.5µM, creating a significant spike in growth (p=0.0150, 2way ANOVA). The error bars show the mean ± SEM, n=5.

References

Abdulkadir, R.R., Alwjwaj, M., Othman, O.A., Rakkar, K., Bayraktutan, U., 2019. Outgrowth endothelial cells form a functional cerebral barrier and restore its integrity after damage. Neural Regen Res 15, 1071–1078.

Acurio, J., Troncoso, F., Bertoglia, P., Salomon, C., Aguayo, C., Sobrevia, L., Escudero, C., 2014. Potential Role of A2B Adenosine Receptors on Proliferation/Migration of Fetal Endothelium Derived from Preeclamptic Pregnancies. BioMed Research International 2014, e274507.

Adair, T.H., Montani, J.-P., 2010. Overview of Angiogenesis, Angiogenesis. San Rafael (CA): Morgan & Claypool Life Sciences.

Aghaei, M., Karami-Tehrani, F., Salami, S., Atri, M., 2005. Adenosine deaminase activity in the serum and malignant tumors of breast cancer: the assessment of isoenzyme ADA1 and ADA2 activities. Clin Biochem 38, 887–891.

Ahmann, K.A., Johnson, S.L., Hebbel, R.P., Tranquillo, R.T., 2011. Shear stress responses of adult blood outgrowth endothelial cells seeded on bioartificial tissue. Tissue Eng Part A 17, 2511–2521.

Aird, W.C., 2012. Endothelial Cell Heterogeneity. Cold Spring Harb Perspect Med 2.

Aird, W.C., 2007. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res 100, 158–173.

Alaygut, D., Alparslan, C., Öncel, E.P., Mutlubaş, F., Özdemir, T., Yavaşcan, Ö., Kasap Demir, B., 2019. A Child Diagnosed With Treatment-Resistant Polyarteritis Nodosa: Can the Clinical Diagnosis Be Different? Arch Rheumatol 34, 338–342.

Allard, B., Turcotte, M., Stagg, J., 2014. Targeting CD73 and downstream adenosine receptor signaling in triple-negative breast cancer. Expert Opinion on Therapeutic Targets 18, 863–881.

Alvarado-Moreno, J.A., Hernandez-Lopez, R., Chavez-Gonzalez, A., Yoder, M.C., Rangel-Corona, R., Isordia-Salas, I., Hernandez-Juarez, J., Cerbulo-Vazquez, A., Gonzalez-Jimenez, M.A., Majluf-Cruz, A., 2016. Endothelial colony-forming cells: Biological and functional abnormalities in patients with recurrent, unprovoked venous thromboembolic disease. Thrombosis Research 137, 157–168.

Arfian, N., Setyaningsih, W.A.W., Romi, M.M., Sari, D.C.R., 2019. Heparanase upregulation from adipocyte associates with inflammation and endothelial injury in diabetic condition. BMC Proc 13, 17.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., Zee, R. van der, Li, T., Witzenbichler, B., Schatteman, G., Isner, J.M., 1997. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. Science 275, 964–966.

Au, P., Daheron, L.M., Duda, D.G., Cohen, K.S., Tyrrell, J.A., Lanning, R.M., Fukumura, D., Scadden, D.T., Jain, R.K., 2008. Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. Blood 111, 1302–1305.

Austrup, F., Vestweber, D., Borges, E., Löhning, M., Bräuer, R., Herz, U., Renz, H., Hallmann, R., Scheffold, A., Radbruch, A., Hamann, A., 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature 385, 81–83.

Bacha, N.C., Blandinieres, A., Rossi, E., Gendron, N., Nevo, N., Lecourt, S., Guerin, C.L., Renard, J.M., Gaussem, P., Angles-Cano, E., Boulanger, C.M., Israel-Biet, D., Smadja, D.M.,

2018. Endothelial Microparticles are Associated to Pathogenesis of Idiopathic Pulmonary Fibrosis. Stem Cell Rev and Rep 14, 223–235.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., Tomic-Canic, M., 2008. Perspective article: Growth factors and cytokines in wound healing. Wound Repair and Regeneration 16, 585–601.

Barzaghi, F., Minniti, F., Mauro, M., Bortoli, M.D., Balter, R., Bonetti, E., Zaccaron, A., Vitale, V., Omrani, M., Zoccolillo, M., Brigida, I., Cicalese, M.P., Degano, M., Hershfield, M.S., Aiuti, A., Bondarenko, A.V., Chinello, M., Cesaro, S., 2019. ALPS-Like Phenotype Caused by ADA2 Deficiency Rescued by Allogeneic Hematopoietic Stem Cell Transplantation. Frontiers in Immunology 9, 2767.

Batu, E.D., Karadag, O., Taskiran, E.Z., Kalyoncu, U., Aksentijevich, I., Alikasifoglu, M., Özen, S., 2015. A Case Series of Adenosine Deaminase 2-deficient Patients Emphasizing Treatment and Genotype-phenotype Correlations. J Rheumatol 42, 1532–1534.

Bazzoni, G., Dejana, E., 2004. Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis. Physiological Reviews 84, 869–901.

Becker, B.F., Jacob, M., Leipert, S., Salmon, A.H.J., Chappell, D., 2015. Degradation of the endothelial glycocalyx in clinical settings: searching for the sheddases. British Journal of Clinical Pharmacology 80, 389–402.

Ben-Ami, T., Revel-Vilk, S., Brooks, R., Shaag, A., Hershfield, M.S., Kelly, S.J., Ganson, N.J., Kfir-Erenfeld, S., Weintraub, M., Elpeleg, O., Berkun, Y., Stepensky, P., 2016. Extending the Clinical Phenotype of Adenosine Deaminase 2 Deficiency. The Journal of Pediatrics 177, 316–320.

Bennett, M.R., Sinha, S., Owens, G.K., 2016. Vascular smooth muscle cells in atherosclerosis. Circ Res 118, 692–702.

Bentzon, J.F., Otsuka, F., Virmani, R., Falk, E., 2014. Mechanisms of Plaque Formation and Rupture. Circulation Research 114, 1852–1866.

Bergfeld, G.R., Forrester, T., 1992. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovascular Research 26, 40–47.

Berne, R.M., 1963. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. American Journal of Physiology-Legacy Content 204, 317–322.

Bertagnolli, M., Xie, L.F., Paquette, K., He, Y., Cloutier, A., Fernandes, R.O., Béland, C., Sutherland, M.R., Delfrate, J., Curnier, D., Bigras, J., Rivard, A., Thébaud, B., Luu, T.M., Nuyt, A.M., 2018. Endothelial Colony-Forming Cells in Young Adults Born Preterm: A Novel Link Between Neonatal Complications and Adult Risks for Cardiovascular Disease. J Am Heart Assoc 7.

Bertelsen, L.B., Bohn, A.B., Smith, M., Mølgaard, B., Møller, B., Stødkilde-Jørgensen, H., Kristensen, P., 2014. Are endothelial outgrowth cells a potential source for future re-vascularization therapy? Experimental Gerontology 58, 132–138.

Birukova, A.A., Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J.R., Birukov, K.G., 2007. Prostaglandins PGE2 and PGI2 promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. Experimental Cell Research 313, 2504–2520.

Bittorf, P., Bergmann, T., Merlin, S., Olgasi, C., Pullig, O., Sanzenbacher, R., Zierau, M., Walles, H., Follenzi, A., Braspenning, J., 2020. Regulatory-Compliant Validation of a Highly Sensitive qPCR for Biodistribution Assessment of Hemophilia A Patient Cells. Mol Ther Methods Clin Dev 18, 176–188.

Bodin, P., Burnstock, G., 1995. Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells. Experientia 51, 256–259.

Böger, R.H., Bode-Böger, S.M., Sydow, K., Heistad, D.D., Lentz, S.R., 2000. Plasma Concentration of Asymmetric Dimethylarginine, an Endogenous Inhibitor of Nitric Oxide Synthase, Is Elevated in Monkeys With Hyperhomocyst(e)inemia or Hypercholesterolemia. Arteriosclerosis, Thrombosis, and Vascular Biology.

Borea, P.A., Gessi, S., Merighi, S., Vincenzi, F., Varani, K., 2018. Pharmacology of Adenosine Receptors: The State of the Art. Physiological Reviews 98, 1591–1625.

Borysenko, M., Beringer, T., 1984. Functional histology. Little, Brown and Company.

Bowers, S.M., Gibson, K.M., Cabral, D.A., Brown, K.L., 2020. Adenosine deaminase 2 activity negatively correlates with age during childhood. Pediatric Rheumatology 18, 54.

Brands, J., van Haare, J., Vink, H., VanTeeffelen, J.W.G.E., 2013. Whole-body recruitment of glycocalyx volume during intravenous adenosine infusion. Physiol Rep 1, e00102.

Breier, G., Breviario, F., Caveda, L., Berthier, R., Schnürch, H., Gotsch, U., Vestweber, D., Risau, W., Dejana, E., 1996. Molecular Cloning and Expression of Murine Vascular Endothelial-Cadherin in Early Stage Development of Cardiovascular System. Blood 87, 630–641.

Brittan, M., Hunter, A., Boulberdaa, M., Fujisawa, T., Skinner, E.M., Shah, A.S., Baker, A.H., Mills, N.L., 2015. Impaired vascular function and repair in patients with premature coronary artery disease. European Journal of Preventive Cardiology 22(12):1557-66.

Broekhuizen, L.N., Lemkes, B.A., Mooij, H.L., Meuwese, M.C., Verberne, H., Holleman, F., Schlingemann, R.O., Nieuwdorp, M., Stroes, E.S.G., Vink, H., 2010. Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus. Diabetologia 53, 2646–2655.

Brofferio, A., 2020. Non-Invasive Vascular Testing and Cardiac Phenotype in DADA2. The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Brogan, P.A., 2007. What's new in the aetiopathogenesis of vasculitis? Pediatr Nephrol 22, 1083–1094.

Brown, M.D., Wick, T.M., Eckman, J.R., 2001. Activation of vascular endothelial cell adhesion molecule expression by sickle blood cells. Pediatr Pathol Mol Med 20, 47–72.

Bruegger, D., Rehm, M., Jacob, M., Chappell, D., Stoeckelhuber, M., Welsch, U., Conzen, P., Becker, B.F., 2008. Exogenous nitric oxide requires an endothelial glycocalyx to prevent postischemic coronary vascular leak in guinea pig hearts. Crit Care 12, R73.

Buijs, J.O. den, Musters, M., Verrips, T., Post, J.A., Braam, B., van Riel, N., 2004. Mathematical modeling of vascular endothelial layer maintenance: the role of endothelial cell division, progenitor cell homing, and telomere shortening. American Journal of Physiology-Heart and Circulatory Physiology 287, H2651–H2658.

Burri, P.H., Tarek, M.R., 1990. A novel mechanism of capillary growth in the rat pulmonary microcirculation. The Anatomical Record 228, 35–45.

Calker, D. van, Müller, M., Hamprecht, B., 1979. Adenosine Regulates Via Two Different Types of Receptors, the Accumulation of Cyclic Amp in Cultured Brain Cells. Journal of Neurochemistry 33, 999–1005.

Cancel, L.M., Ebong, E.E., Mensah, S., Hirshberg, C., Tarbell, J.M., 2016. Endothelial glycocalyx, apoptosis and inflammation in an atherosclerotic mouse model. Atherosclerosis 252, 136–146.

Caorsi, R., Penco, F., Grossi, A., Insalaco, A., Omenetti, A., Alessio, M., Conti, G., Marchetti, F., Picco, P., Tommasini, A., Martino, S., Malattia, C., Gallizi, R., Podda, R.A., Salis, A.,

Falcini, F., Schena, F., Garbarino, F., Morreale, A., Pardeo, M., Ventrici, C., Passarelli, C., Zhou, Q., Severino, M., Gandolfo, C., Damonte, G., Martini, A., Ravelli, A., Aksentijevich, I., Ceccherini, I., Gattorno, M., 2017. ADA2 deficiency (DADA2) as an unrecognised cause of early onset polyarteritis nodosa and stroke: a multicentre national study. Annals of the Rheumatic Diseases 76, 1648–1656.

Caravà, E., Moretto, P., Caon, I., Parnigoni, A., Passi, A., Karousou, E., Vigetti, D., Canino, J., Canobbio, I., Viola, M., 2021. HA and HS Changes in Endothelial Inflammatory Activation. Biomolecules 11, 809.

Carbonaro, D.A., Zhang, L., Jin, X., Montiel-Equihua, C., Geiger, S., Carmo, M., Cooper, A., Fairbanks, L., Kaufman, M.L., Sebire, N.J., Hollis, R.P., Blundell, M.P., Senadheera, S., Fu, P.-Y., Sahaghian, A., Chan, R.Y., Wang, X., Cornetta, K., Thrasher, A.J., Kohn, D.B., Gaspar, H.B., 2014. Preclinical Demonstration of Lentiviral Vector-mediated Correction of Immunological and Metabolic Abnormalities in Models of Adenosine Deaminase Deficiency. Mol Ther 22, 607–622.

Carmona-Rivera, C., Khaznadar, S.S., Shwin, K.W., Irizarry-Caro, J.A., O'Neil, L.J., Liu, Y., Jacobson, K.A., Ombrello, A.K., Stone, D.L., Tsai, W.L., Kastner, D.L., Aksentijevich, I., Kaplan, M.J., Grayson, P.C., 2019. Deficiency of adenosine deaminase 2 triggers adenosine-mediated NETosis and TNF production in patients with DADA2. Blood 134(4):395-406.

Caruso, P., Dunmore, B.J., Schlosser, K., Schoors, S., Santos, C.D., Perez-Iratxeta, C., Lavoie, J.R., Zhang, H., Long, L., Flockton, A.R., Frid, M.G., Upton, P.D., D'Alessandro, A., Hadinnapola, C., Kiskin, F.N., Taha, M., Hurst, L.A., Ormiston, M.L., Hata, A., Stenmark, K.R., Carmeliet, P., Stewart, D.J., Morrell, N.W., 2017. Identification of miR-124 as a major regulator of enhanced endothelial cell glycolysis in pulmonary arterial hypertension via PTBP1 and PKM2. Circulation 136, 2451–2467.

Cerrito, E., Lazzaro, M.P., Gaudio, E., Arminio, P., Aloisi, G., 1993. 5HT2-receptors and serotonin release: Their role in human platelet aggregation. Life Sciences 53, 209–215.

Chantzichristos, V.G., Gkrozou, F., Stellos, K., Paschopoulos, M.E., Tselepis, A.D., 2018. Comparative Anti-Platelet Profiling Reveals a Potent Anti-Aggregatory Effect of CD34+ Progenitor Cell-Derived Late-Outgrowth Endothelial Cells in vitro. JVR 55, 13–25.

Chappell, D., Hofmann-Kiefer, K., Jacob, M., Rehm, M., Briegel, J., Welsch, U., Conzen, P., Becker, B.F., 2009a. TNF-alpha induced shedding of the endothelial glycocalyx is prevented by hydrocortisone and antithrombin. Basic Res Cardiol 104, 78–89.

Chappell, D., Jacob, M., Paul, O., Rehm, M., Welsch, U., Stoeckelhuber, M., Conzen, P., Becker, B.F., 2009b. The glycocalyx of the human umbilical vein endothelial cell: an impressive structure ex vivo but not in culture. Circ. Res. 104, 1313–1317.

Chappell, D., Jacob, M., Rehm, M., Stoeckelhuber, M., Welsch, U., Conzen, P., Becker, B.F., 2008. Heparinase selectively sheds heparan sulphate from the endothelial glycocalyx. Biol Chem 389, 79–82.

Chasset, F., Fayand, A., Moguelet, P., Kouby, F., Bonhomme, A., Franck, N., Goldman-lévy, G., Fraitag, S., Barbaud, A., Queyrel, V., Touitou, I., Boursier, G., Sarrabay, G., Ducharme-Benard, S., Dupin, N., Francès, C., Bessis, D., Georgin-Lavialle, S., 2020. Clinical and pathological dermatological features of Deficiency of Adenosine Deaminase 2: Multicenter retrospective observational study. Journal of the American Academy of Dermatology 83(6):1794-1798.

Chen, H., Chiang, I.-P., Jen, C.J., 1996. Exercise training increases acetylcholine-stimulated endothelium-derived nitric oxide release in spontaneously hypertensive rats. J Biomed Sci 3, 454–460.

Chen, J.-F., Eltzschig, H.K., Fredholm, B.B., 2013. Adenosine receptors as drug targets — what are the challenges? Nat Rev Drug Discov 12, 265–286.

Chen, Z., Herrmann, S.M.S., Zhu, X., Jordan, K.L., Gloviczki, M.L., Lerman, A., Textor, S.C., Lerman, L.O., 2014. Preserved function of late outgrowth endothelial cells in medically-treated hypertensive patients under well-controlled conditions. Hypertension 64, 808–814.

Choi, S., Kim, J., Kim, J.-H., Lee, D.-K., Park, W., Park, M., Kim, S., Hwang, J.Y., Won, M.-H., Choi, Y.K., Ryoo, S., Ha, K.-S., Kwon, Y.-G., Kim, Y.-M., 2017. Carbon monoxide prevents TNF-α-induced eNOS downregulation by inhibiting NF-κB-responsive miR-155-5p biogenesis. Exp Mol Med 49, e403–e403.

Chow, C.K., Jolly, S., Rao-Melacini, P., Fox, K.A.A., Anand, S.S., Yusuf, S., 2010. Association of Diet, Exercise, and Smoking Modification With Risk of Early Cardiovascular Events After Acute Coronary Syndromes. Circulation 121, 750–758.

Cieślak, M., Komoszyński, M., Wojtczak, A., 2008. Adenosine A2A receptors in Parkinson's disease treatment. Purinergic Signal 4, 305–312.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, E.S., McCrae, K.R., Hug, B.A., Schmidt, A.-M., Stern, D.M., 1998. Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders. Blood 91, 3527–3561.

Clark, A.N., Youkey, R., Liu, X., Jia, L., Blatt, R., Day, Y.-J., Sullivan, G.W., Linden, J., Tucker, A.L., 2007. A1 Adenosine Receptor Activation Promotes Angiogenesis and Release of VEGF From Monocytes. Circulation Research 101, 1130–1138.

Clarke, L.A., Hong, Y., Eleftheriou, D., Shah, V., Arrigoni, F., Klein, N.J., Brogan, P.A., 2010. Endothelial injury and repair in systemic vasculitis of the young. Arthritis & Rheumatism 62, 1770–1780.

Clarke, S., 1993. Protein methylation. Current Opinion in Cell Biology 5, 977–983.

Coneski, P.N., and Schoenfisch, M.H. (2012). Nitric Oxide Release Part III. Measurement and Reporting. Chem Soc Rev *41*, 3753–3758.

Conlon, B.A., Law, W.R., 2004. Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses. Clinical and Experimental Immunology 138, 14–20.

Cooray, S., Omyinmi, E., Hong, Y., Papadopoulou, C., Harper, L., Al-Abadi, E., Goel, R., Dubey, S., Wood, M., Jolles, S., Berg, S., Ekelund, M., Armon, K., Eleftheriou, D., Brogan, P.A., 2021. Anti-tumour necrosis factor treatment for the prevention of ischaemic events in patients with deficiency of adenosine deaminase 2 (DADA2). Rheumatology.

Coppens, V., Heremans, Y., Leuckx, G., Suenens, K., Jacobs-Tulleneers-Thevissen, D., Verdonck, K., Lahoutte, T., Luttun, A., Heimberg, H., De Leu, N., 2013. Human blood outgrowth endothelial cells improve islet survival and function when co-transplanted in a mouse model of diabetes. Diabetologia 56, 382–390.

Cowen, E., 2020. Dermatological phenotype of DADA2. The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Cronstein, B.N., 1994. Adenosine, an endogenous anti-inflammatory agent. Journal of Applied Physiology 76, 5–13.

Cronstein, B.N., Daguma, L., Nichols, D., Hutchison, A.J., Williams, M., 1990. The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O2 generation, respectively. J Clin Invest 85, 1150–1157.

Csóka, B., Selmeczy, Z., Koscsó, B., Németh, Z.H., Pacher, P., Murray, P.J., Kepka-Lenhart, D., Morris, S.M., Gause, W.C., Leibovich, S.J., Haskó, G., 2012. Adenosine promotes alternative macrophage activation via A2A and A2B receptors. FASEB J 26, 376–386.

Csonka, C., Páli, T., Bencsik, P., Görbe, A., Ferdinandy, P., and Csont, T. (2015). Measurement of NO in biological samples. Br J Pharmacol *172*, 1620–1632.

Cybulsky, M.I., Gimbrone, M.A., 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251, 788–791.

Dauwe, D., Pelacho, B., Wibowo, A., Walravens, A., Verdonck, K., Gillijns, H., Caluwe, E., Pokreisz, P., van Gastel, N., Carmeliet, G., Depypere, M., Maes, F., Vanden Driessche, N., Droogne, W., Van Cleemput, J., Vanhaecke, J., Prosper, F., Verfaillie, C., Luttun, A., Janssens, S., 2016. Neovascularization Potential of Blood Outgrowth Endothelial Cells From Patients With Stable Ischemic Heart Failure Is Preserved. J Am Heart Assoc 5 (4):e002288.

David, S., Park, J.-K., Meurs, M. van, Zijlstra, J.G., Koenecke, C., Schrimpf, C., Shushakova, N., Gueler, F., Haller, H., Kümpers, P., 2011. Acute administration of recombinant Angiopoietin-1 ameliorates multiple-organ dysfunction syndrome and improves survival in murine sepsis. Cytokine 55, 251–259.

de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W., Unger, T., 2000. International Union of Pharmacology. XXIII. The Angiotensin II Receptors. Pharmacol Rev 52, 415–472.

de Graaf, J.C., Banga, J.D., Moncada, S., Palmer, R.M., de Groot, P.G., Sixma, J.J., 1992. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation 85, 2284–2290.

Delgadillo, Luis.F., Lomakina, Elena.B., Kuebel, J., Waugh, Richard.E., 2021. Changes in endothelial glycocalyx layer protective ability after inflammatory stimulus. American Journal of Physiology-Cell Physiology 320, C216–C224.

Dembinska-Kiec, A., Rücker, W., Schönhöfer, P.S., 1979. PGI2 enhanced cAMP content in bovine coronary arteries in the presence of isobutylmethylxanthine. Naunyn-Schmiedeberg's Arch. Pharmacol. 308, 107–110.

Dhanwani, R., Takahashi, M., Mathews, I.T., Lenzi, C., Romanov, A., Watrous, J.D., Pieters, B., Hedrick, C.C., Benedict, C.A., Linden, J., Nilsson, R., Jain, M., Sharma, S., 2020. Cellular sensing of extracellular purine nucleosides triggers an innate IFN-β response. Sci Adv 6.

Dixon, A.K., Gubitz, A.K., Sirinathsinghji, D.J., Richardson, P.J., Freeman, T.C., 1996. Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol 118, 1461–1468.

Dong, J., Moake, J.L., Nolasco, L., Bernardo, A., Arceneaux, W., Shrimpton, C.N., Schade, A.J., McIntire, L.V., Fujikawa, K., López, J.A., 2002. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood 100, 4033–4039.

Doukas, J., Pober, J.S., 1990. IFN-gamma enhances endothelial activation induced by tumor necrosis factor but not IL-1. The Journal of Immunology 145, 1727–1733.

Drury, A.N., Szent-Györgyi, A., 1929. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J Physiol 68, 213–237.

Du, X., Hu, N., Yu, H., Hong, L., Ran, F., Huang, D., Zhou, M., Li, C., Li, X., 2020. miR-150 regulates endothelial progenitor cell differentiation via Akt and promotes thrombus resolution. Stem Cell Res Ther 11.

Dunmore, B.J., Drake, K.M., Upton, P.D., Toshner, M.R., Aldred, M.A., Morrell, N.W., 2013. The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations. Hum Mol Genet 22, 3667–3679.

Duran, C.L., Howell, D.W., Dave, J.M., Smith, R.L., Torrie, M.E., Essner, J.J., Bayless, K.J., 2017. Molecular Regulation of Sprouting Angiogenesis. Compr Physiol 8, 153–235.

Dvorak, A.M., Kohn, S., Morgan, E.S., Fox, P., Nagy, J.A., Dvorak, H.F., 1996. The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. J. Leukoc. Biol. 59, 100–115.

Ecklu-Mensah, G., Olsen, R.W., Bengtsson, A., Ofori, M.F., Hviid, L., Jensen, A.T.R., Adams, Y., 2018. Blood outgrowth endothelial cells (BOECs) as a novel tool for studying adhesion of Plasmodium falciparum-infected erythrocytes. PLoS One 13.

Effendi, W.I., Nagano, T., Kobayashi, K., Nishimura, Y., 2020. Focusing on Adenosine Receptors as a Potential Targeted Therapy in Human Diseases. Cells 9, 785.

Eleftheriou, D., 2020. Can DADA2 be “cured” by gene therapy? The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Eleftheriou, D., Melo, M., Marks, S.D., Tullus, K., Sills, J., Cleary, G., Dolezalova, P., Ozen, S., Pilkington, C., Woo, P., Klein, N., Dillon, M.J., Brogan, P.A., 2009. Biologic therapy in primary systemic vasculitis of the young. Rheumatology 48, 978–986.

Esmon, N.L., Owen, W.G., Esmon, C.T., 1982. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J Biol Chem 257, 859–864.

Essaadi, A., Nollet, M., Moyon, A., Stalin, J., Simoncini, S., Balasse, L., Bertaud, A., Bachelier, R., Leroyer, A.S., Sarlon, G., Guillet, B., Dignat-George, F., Bardin, N., Blot-Chabaud, M., 2018. Stem cell properties of peripheral blood endothelial progenitors are stimulated by soluble CD146 via miR-21: potential use in autologous cell therapy. Sci Rep 8.

Faas, M.M., Sáez, T., de Vos, P., 2017. Extracellular ATP and adenosine: The Yin and Yang in immune responses? Molecular Aspects of Medicine, Adenosine - from molecular actions to pathophysiology 55, 9–19.

Fabbri-Arrigoni, F.I., Clarke, L., Wang, G., Charakida, M., Ellins, E., Halliday, N., Brogan, P.A., Deanfield, J.E., Halcox, J.P., Klein, N., 2012. Levels of circulating endothelial cells and colony forming units are influenced by age and dyslipidaemia. Pediatr Res 72, 299–304.

Feitz, W.J.C., van de Kar, N.C.A.J., Cheong, I., van der Velden, T.J.A.M., Ortiz-Sandoval, C.G., Orth-Höller, D., van den Heuvel, L.P.J.W., Licht, C., 2020. Primary Human Derived Blood Outgrowth Endothelial Cells: An Appropriate In Vitro Model to Study Shiga Toxin Mediated Damage of Endothelial Cells. Toxins 12, 483.

Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F., Dvorak, A.M., 1998. Neutrophils Emigrate from Venules by a Transendothelial Cell Pathway in Response to FMLP. J Exp Med 187, 903–915.

Feoktistov, I., Goldstein, A.E., Ryzhov, S., Zeng, D., Belardinelli, L., Voyno-Yasenetskaya, T., Biaggioni, I., 2002. Differential Expression of Adenosine Receptors in Human Endothelial Cells: Role of A_{2B} Receptors in Angiogenic Factor Regulation. Circulation Research 90, 531–538.

Féraud, O., Mallet, C., Vilgrain, I., 2003. Expressional Regulation of the Angiopoietin-1 and -2 and the Endothelial-Specific Receptor Tyrosine Kinase Tie2 in Adrenal Atrophy: A Study of Adrenocorticotropin-Induced Repair. Endocrinology 144, 4607–4615.

Ference, B.A., Ginsberg, H.N., Graham, I., Ray, K.K., Packard, C.J., Bruckert, E., Hegele, R.A., Krauss, R.M., Raal, F.J., Schunkert, H., Watts, G.F., Borén, J., Fazio, S., Horton, J.D., Masana, L., Nicholls, S.J., Nordestgaard, B.G., van de Sluis, B., Taskinen, M.-R., Tokgözoğlu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J.K., Chapman, M.J., Catapano, A.L., 2017. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J 38, 2459–2472.

Fernandez, C.E., Obi-onuoha, I.C., Wallace, C.S., Satterwhite, L.L., Truskey, G.A., Reichert, W.M., 2014. Late-outgrowth endothelial progenitors from patients with coronary artery disease: Endothelialization of confluent stromal cell layers. Acta Biomaterialia 10, 893–900.

Fernandez-L, A., Sanz-Rodriguez, F., Zarrabeitia, R., Pérez-Molino, A., Hebbel, R.P., Nguyen, J., Bernabéu, C., Botella, L.-M., 2005. Blood outgrowth endothelial cells from Hereditary Haemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc Res 68, 235–248.

Ferrer, E., Dunmore, B.J., Hassan, D., Ormiston, M.L., Moore, S., Deighton, J., Long, L., Yang, X.D., Stewart, D.J., Morrell, N.W., 2018. A Potential Role for Exosomal Translationally Controlled Tumor Protein Export in Vascular Remodeling in Pulmonary Arterial Hypertension. Am J Respir Cell Mol Biol 59, 467–478.

Feuerhake, F., Füchsl, G., Bals, R., Welsch, U., 1998. Expression of inducible cell adhesion molecules in the normal human lung: immunohistochemical study of their distribution in pulmonary blood vessels. Histochem Cell Biol 110, 387–394.

Figler, R.A., Wang, G., Srinivasan, S., Jung, D.Y., Zhang, Z., Pankow, J.S., Ravid, K., Fredholm, B., Hedrick, C.C., Rich, S.S., Kim, J.K., LaNoue, K.F., Linden, J., 2011. Links Between Insulin Resistance, Adenosine A2B Receptors, and Inflammatory Markers in Mice and Humans. Diabetes 60, 669–679.

Fleming, W.H., 2005. Endothelial cell–specific markers: going... going... gone. Blood 106, 769–769.

Fliser, D., Kronenberg, F., Kielstein, J.T., Morath, C., Bode-Böger, S.M., Haller, H., Ritz, E., 2005. Asymmetric Dimethylarginine and Progression of Chronic Kidney Disease: The Mild to Moderate Kidney Disease Study. JASN 16, 2456–2461.

Florey, 1966. The endothelial cell. Br Med J 2, 487–490.

Folkman, J., 1971. Tumor Angiogenesis: Therapeutic Implications. N Engl J Med 285, 1182–1186.

Fredholm, B.B., 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ 14, 1315–1323.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, K.T., Jacobson, K.A., Leff, P., Williams, M., 1994. VI. Nomenclature and Classification of Purinoceptors. Pharmacol Rev 46, 143–156.

Fredholm, B.B., Arslan, G., Halldner, L., Kull, B., Schulte, G., Wasserman, W., 2000. Structure and function of adenosine receptors and their genes. Naunyn-Schmied Arch Pharmacol 362, 364–374.

Fredholm, Bertil B., IJzerman, A.P., Jacobson, K.A., Klotz, K.-N., Linden, J., 2001a. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. Pharmacol Rev 53, 527–552.

Fredholm, Bertil B, Irenius, E., Kull, B., Schulte, G., 2001b. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary. Biochemical Pharmacology 61, 443–448.

Fu, B.M., Tarbell, J.M., 2013. Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. Wiley Interdiscip Rev Syst Biol Med 5, 381–390.

Fuchs, S., Dohle, E., Kolbe, M., Kirkpatrick, C.J., 2010. Outgrowth Endothelial Cells: Sources, Characteristics and Potential Applications in Tissue Engineering and Regenerative Medicine, in: Kasper, C., van Griensven, M., Pörtner, R. (Eds.), Bioreactor Systems for Tissue Engineering II: Strategies for the Expansion and Directed Differentiation of Stem Cells, Advances in Biochemical Engineering / Biotechnology. Springer, Berlin, Heidelberg, pp. 201–217.

Fukuda, T., Fukushima, Y., Numao, T., Ando, N., Arima, M., Nakajima, H., Sagara, H., Adachi, T., Motojima, S., Makino, S., 1996. Role of interleukin-4 and vascular cell adhesion molecule-1 in selective eosinophil migration into the airways in allergic asthma. Am J Respir Cell Mol Biol 14, 84–94.

Fullwood, N.J., Davies, Y., Nieduszynski, I.A., Marcyniuk, B., Ridgway, A.E., Quantock, A.J., 1996. Cell surface-associated keratan sulfate on normal and migrating corneal endothelium. Invest. Ophthalmol. Vis. Sci. 37, 1256–1270.

Furukawa, K., Ohshima, N., Tawada-Iwata, Y., Shigekawa, M., 1991. Cyclic GMP stimulates Na+/Ca2+ exchange in vascular smooth muscle cells in primary culture. J Biol Chem 266, 12337–12341.

Galis, Z.S., Sukhova, G.K., Kranzhöfer, R., Clark, S., Libby, P., 1995. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. Proc Natl Acad Sci U S A 92, 402–406.

Gallos, G., Ruyle, T.D., Emala, C.W., Lee, H.T., 2005. A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. American Journal of Physiology-Renal Physiology 289, F369–F376.

George, P., M., Oliver Eduardo, Dorfmuller Peter, Dubois Olivier D., Reed Daniel M., Kirkby Nicholas S., Mohamed Nura A., Perros Frederic, Antigny Fabrice, Fadel Elie, Schreiber Benjamin E., Holmes Alan M., Southwood Mark, Hagan Guy, Wort Stephen J., Bartlett Nathan, Morrell Nicholas W., Coghlan John G., Humbert Marc, Zhao Lan, Mitchell Jane A., 2014. Evidence for the Involvement of Type I Interferon in Pulmonary Arterial Hypertension. Circulation Research 114, 677–688.

Geti, I., Ormiston, M.L., Rouhani, F., Toshner, M., Movassagh, M., Nichols, J., Mansfield, W., Southwood, M., Bradley, A., Rana, A.A., Vallier, L., Morrell, N.W., 2012. A Practical and Efficient Cellular Substrate for the Generation of Induced Pluripotent Stem Cells from Adults: Blood-Derived Endothelial Progenitor Cells. Stem Cells Transl Med 1, 855–865.

Ghurye, R.R., Sundaram, K., Smith, F., Clark, B., Simpson, M.A., Fairbanks, L., Adhya, Z., Mufti, G.J., Marsh, J.C.W., Ibrahim, M.A.A., 2019. Novel ADA2 mutation presenting with neutropenia, lymphopenia and bone marrow failure in patients with deficiency in adenosine deaminase 2 (DADA2). British Journal of Haematology 186(3):e60-e64.

Giantsos-Adams, K.M., Koo, A.J.-A., Song, S., Sakai, J., Sankaran, J., Shin, J.H., Garcia-Cardena, G., Dewey, C.F., 2013. Heparan Sulfate Regrowth Profiles Under Laminar Shear Flow Following Enzymatic Degradation. Cel. Mol. Bioeng. 6, 160–174.

Gibson, K.M., Morishita, K.A., Dancey, P., Moorehead, P., Drögemöller, B., Han, X., Graham, J., Hancock, R.E., Foell, D., Benseler, S., Luqmani, R., Yeung, R.S.M., Shenoi, S., Bohm, M., Rosenberg, A.M., Ross, C.J., Cabral, D.A., Brown, K.L., 2019. Identification of novel Adenosine Deaminase 2 gene variants and varied clinical phenotype in pediatric vasculitis. Arthritis & Rheumatology 71(10):1747-1755.

Gimbrone, M.A., Cotran, R.S., Folkman, J., 1974. Human vascular endothelial cells in culture. J Cell Biol 60, 673–684.

Glee, P.M., Cutler, J.E., Benson, E.E., Bargatze, R.F., Hazen, K.C., 2001. Inhibition of Hydrophobic Protein-Mediated Candida albicans Attachment to Endothelial Cells during Physiologic Shear Flow. Infect Immun 69, 2815–2820.

Glynn, J.J., Hinds, M.T., 2014. Endothelial Outgrowth Cells: Function and Performance in Vascular Grafts. Tissue Eng Part B Rev 20, 294–303.

Gonzalez Santiago, T.M., Zavialov, A., Saarela, J., Seppanen, M., Reed, A.M., Abraham, R.S., Gibson, L.E., 2015. Dermatologic Features of ADA2 Deficiency in Cutaneous Polyarteritis Nodosa. JAMA Dermatology 151, 1230–1234.

Goodarzi, M.T., Abdi, M., Tavilani, H., Nadi, E., Rashidi, M., 2010. Adenosine Deaminase Activity in COPD Patients and Healthy Subjects. Iranian Journal of Allergy, Asthma and Immunology 7–12.

Goonasekera, C.D.A., Rees, D.D., Woolard, P., Frend, A., Shah, V., Dillon, M.J., 1997. Nitric oxide synthase inhibitors and hypertension in children and adolescents. Journal of Hypertension 15, 901–909.

Gorman, M.W., Feigl, E.O., Buffington, C.W., 2007. Human Plasma ATP Concentration. Clinical Chemistry 53, 318–325.

Gottesman, R.F., Schneider, A.L.C., Albert, M., Alonso, A., Bandeen-Roche, K., Coker, L., Coresh, J., Knopman, D., Power, M.C., Rawlings, A., Sharrett, A.R., Wruck, L.M., Mosley, T.H., 2014. Midlife hypertension and 20-year cognitive change: The Atherosclerosis Risk in Communities Neurocognitive Study. JAMA Neurol 71, 1218–1227.

Greiner-Tollersrud, O.K., Boehler, V., Bartok, E., Krausz, M., Polyzou, A., Schepp, J., Seidl, M., Olsen, J.O., Smulski, C.R., Raieli, S., Hübscher, K., Trompouki, E., Link, R., Ebersbach, H., Srinivas, H., Marchant, M., Staab, D., Guerini, D., Baasch, S., Henneke, P., Kochs, G., Hartmann, G., Geiger, R., Grimbacher, B., Warncke, M., Proietti, M., 2020. ADA2 is a lysosomal DNase regulating the type-I interferon response. bioRxiv 2020.06.21.162990.

Grierson, R., Meyer-Rüsenberg, B., Kunst, F., Berna, M.J., Richard, G., Thill, M., 2013. Endothelial Progenitor Cells and Plasma Vascular Endothelial Growth Factor and Stromal Cell-Derived Factor-1 During Ranibizumab Treatment for Neovascular Age-Related Macular Degeneration. Journal of Ocular Pharmacology and Therapeutics 29, 530–538.

Groeneveld, D.J., Bekkum, T. van, Dirven, R.J., Wang, J.-W., Voorberg, J., Reitsma, P.H., Eikenboom, J., 2015. Angiogenic characteristics of blood outgrowth endothelial cells from patients with von Willebrand disease. Journal of Thrombosis and Haemostasis 13, 1854–1866.

Gulati Rajiv, Jevremovic Dragan, Peterson Timothy E., Chatterjee Suvro, Shah Vijay, Vile Richard G., Simari Robert D., 2003. Diverse Origin and Function of Cells With Endothelial Phenotype Obtained From Adult Human Blood. Circulation Research 93, 1023–1025.

Güven, H., Shepherd, R.M., Bach, R.G., Capoccia, B.J., Link, D.C., 2006. The Number of Endothelial Progenitor Cell Colonies in the Blood Is Increased in Patients With Angiographically Significant Coronary Artery Disease. Journal of the American College of Cardiology 48, 1579–1587.

Hashem, H., Kelly, S.J., Ganson, N.J., Hershfield, M.S., 2017a. Deficiency of Adenosine Deaminase 2 (DADA2), an Inherited Cause of Polyarteritis Nodosa and a Mimic of Other Systemic Rheumatologic Disorders. Curr Rheumatol Rep 19, 70.

Hashem, H., Kumar, A.R., Müller, I., Babor, F., Bredius, R., Dalal, J., Hsu, A.P., Holland, S.M., Hickstein, D.D., Jolles, S., Krance, R., Sasa, G., Taskinen, M., Koskenvuo, M., Saarela, J., van Montfrans, J., Wilson, K., Bosch, B., Moens, L., Hershfield, M., Meyts, I., 2017b. Hematopoietic stem cell transplantation rescues the hematological, immunological, and vascular phenotype in DADA2. Blood 130, 2682–2688.

Hashimoto, E., Kage, K., Ogita, T., Nakaoka, T., Matsuoka, R., Kira, Y., 1994. Adenosine as an Endogenous Mediator of Hypoxia for Induction of Vascular Endothelial Growth Factor mRNA in U-937 Cells. Biochemical and Biophysical Research Communications 204, 318–324.

Haskó, G., Kuhel, D.G., Chen, J.-F., Schwarzschild, M.A., Deitch, E.A., Mabley, J.G., Marton, A., Szabó, C., 2000a. Adenosine inhibits IL-12 and TNF-α production via adenosine A2a receptor-dependent and independent mechanisms. The FASEB Journal 14, 2065–2074.

Haskó, G., Kuhel, D.G., Salzman, A.L., Szabó, C., 2000b. ATP suppression of interleukin-12 and tumour necrosis factor-α release from macrophages. Br J Pharmacol 129, 909–914.

Haskó, G., Linden, J., Cronstein, B., Pacher, P., 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov 7, 759–770.

Hassanian, S.M., Dinarvand, P., Rezaie, A.R., 2014. Adenosine regulates the proinflammatory signaling function of thrombin in endothelial cells. J Cell Physiol 229, 1292–1300.

Hatanaka, K., Lanahan, A.A., Murakami, M., Simons, M., 2012. Fibroblast Growth Factor Signaling Potentiates VE-Cadherin Stability at Adherens Junctions by Regulating SHP2. PLOS ONE 7, e37600.

Haymet, A.B., Bartnikowski, N., Wood, E.S., Vallely, M.P., McBride, A., Yacoub, S., Biering, S.B., Harris, E., Suen, J.Y., Fraser, J.F., 2021. Studying the Endothelial Glycocalyx in vitro: What Is Missing? Front. Cardiovasc. Med. 8.

Haywood-Watson, R.J., Holcomb, J.B., Gonzalez, E.A., Peng, Z., Pati, S., Park, P.W., Wang, W., Zaske, A.M., Menge, T., Kozar, R.A., 2011. Modulation of syndecan-1 shedding after hemorrhagic shock and resuscitation. PLoS One 6, e23530.

He, X., Zhao, P., Kang, S., Ding, Y., Luan, J., Liu, Z., Wu, Y., Yin, W., 2012. C1GALT1 polymorphisms are associated with Henoch–Schönlein purpura nephritis. Pediatr Nephrol 27, 1505–1509.

Hebbel, R., Wei, Peng, Milbauer, Liming, Corban, Michel, Solovey, Anna, Kiley, James, Pattee, Jack, Lerman, Lilach O., Pan, Wei, Lerman, Amir, 2020. Abnormal Endothelial Gene Expression Associated With Early Coronary Atherosclerosis. Journal of the American Heart Association 9, e016134.

Hebbel, R.P., 2017. Blood endothelial cells: utility from ambiguity. J Clin Invest 127, 1613–1615.

Hendrickx, B., Verdonck, K., Berge, S.V. den, Dickens, S., Eriksson, E., Vranckx, J.J., Luttun, A., 2010. Integration of Blood Outgrowth Endothelial Cells in Dermal Fibroblast Sheets Promotes Full Thickness Wound Healing. STEM CELLS 28, 1165–1177.

Henry, C.B.S., Duling, B.R., 2000. TNF-α increases entry of macromolecules into luminal endothelial cell glycocalyx. American Journal of Physiology-Heart and Circulatory Physiology 279, H2815–H2823.

Hernandez-Lopez, R., Chavez-Gonzalez, A., Torres-Barrera, P., Moreno-Lorenzana, D., Lopez-DiazGuerrero, N., Santiago-German, D., Isordia-Salas, I., Smadja, D., C. Yoder, M., Majluf-Cruz, A., Alvarado-Moreno, J.A., 2017. Reduced proliferation of endothelial colony-forming cells in unprovoked venous thromboembolic disease as a consequence of endothelial dysfunction. PLoS One 12.

Hershfield, M.S., 2005. New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. European Journal of Immunology 35, 25–30.

Herzog, D.P.E., Dohle, E., Bischoff, I., Kirkpatrick, C.J., 2014. Cell Communication in a Coculture System Consisting of Outgrowth Endothelial Cells and Primary Osteoblasts. Biomed Res Int 2014.

Hetrick, E.M., and Schoenfisch, M.H. (2009). Analytical Chemistry of Nitric Oxide. Annu Rev Anal Chem (Palo Alto Calif) *2*, 409–433.

Hofmann-Kiefer, K.F., Chappell, D., Knabl, J., Frank, H.G., Martinoff, N., Conzen, P., Becker, B.F., Rehm, M., 2013. Placental Syncytiotrophoblast Maintains a Specific Type of Glycocalyx at the Fetomaternal Border: The Glycocalyx at the Fetomaternal Interface in Healthy Women and Patients With HELLP Syndrome. Reprod Sci 20, 1237–1245.

Huang, H., Virmani, R., Younis, H., Burke, A.P., Kamm, R.D., Lee, R.T., 2001. The Impact of Calcification on the Biomechanical Stability of Atherosclerotic Plaques. Circulation 103, 1051–1056.

Human, A., Pagnoux, C., 2019. Diagnosis and management of ADA2 deficient polyarteritis nodosa. International Journal of Rheumatic Diseases 22, 69–77.

Hunt, M.A., Currie, M.J., Robinson, B.A., Dachs, G.U., 2010. Optimizing Transfection of Primary Human Umbilical Vein Endothelial Cells Using Commercially Available Chemical Transfection Reagents. J Biomol Tech 21, 66–72.

Ignarro, L.J., Napoli, C., 2005. Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis. Curr Diab Rep 5, 17–23.

Ingram, David A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D., Yoder, M.C., 2004. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 104, 2752–2760.

Insalaco, A., Moneta, G.M., Pardeo, M., Caiello, I., Messia, V., Bracaglia, C., Passarelli, C., De Benedetti, F., 2019. Variable Clinical Phenotypes and Relation of Interferon Signature with Disease Activity in ADA2 Deficiency. The Journal of Rheumatology jrheum.180045.

Jacob, M., Paul, O., Mehringer, L., Chappell, D., Rehm, M., Welsch, U., Kaczmarek, I., Conzen, P., Becker, B.F., 2009. Albumin Augmentation Improves Condition of Guinea Pig Hearts After 4 hr of Cold Ischemia. Transplantation 87, 956–965.

Jacob, M., Rehm, M., Loetsch, M., Paul, J.O., Bruegger, D., Welsch, U., Conzen, P., Becker, B.F., 2007. The Endothelial Glycocalyx Prefers Albumin for Evoking Shear Stress-Induced, Nitric Oxide-Mediated Coronary Dilatation. JVR 44, 435–443.

Jacobson, K.A., Tosh, D.K., Jain, S., Gao, Z.-G., 2019. Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. Front. Cell. Neurosci. 13:124.

Jaffe, E.A., Hoyer, L.W., Nachman, R.L., 1974. Synthesis of von Willebrand Factor by Cultured Human Endothelial Cells. Proc Natl Acad Sci U S A 71, 1906–1909.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of Human Endothelial Cells Derived from Umbilical Veins. IDENTIFICATION BY MORPHOLOGIC AND IMMUNOLOGIC CRITERIA. J Clin Invest 52, 2745–2756.

Jeansson, M., Haraldsson, B., 2006. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. American Journal of Physiology-Renal Physiology 290, F111–F116.

Jennette, J.C., Falk, R.J., Bacon, P.A., Basu, N., Cid, M.C., Ferrario, F., Flores-Suarez, L.F., Gross, W.L., Guillevin, L., Hagen, E.C., Hoffman, G.S., Jayne, D.R., Kallenberg, C.G.M., Lamprecht, P., Langford, C.A., Luqmani, R.A., Mahr, A.D., Matteson, E.L., Merkel, P.A., Ozen, S., Pusey, C.D., Rasmussen, N., Rees, A.J., Scott, D.G.I., Specks, U., Stone, J.H., Takahashi, K., Watts, R.A., 2013. 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. Arthritis & Rheumatism 65, 1–11.

Jiang, C., Fang, X., Jiang, Y., Shen, F., Hu, Z., Li, X., Huang, X., 2016. TNF-α induces vascular endothelial cells apoptosis through overexpressing pregnancy induced noncoding RNA in Kawasaki disease model. The International Journal of Biochemistry & Cell Biology 72, 118–124.

Jodon de Villeroché, V., Avouac, J., Ponceau, A., Ruiz, B., Kahan, A., Boileau, C., Uzan, G., Allanore, Y., 2010. Enhanced late-outgrowth circulating endothelial progenitor cell levels in rheumatoid arthritis and correlation with disease activity. Arthritis Res Ther 12, R27.

Jokubaitis, V.J., Sinka, L., Driessen, R., Whitty, G., Haylock, D.N., Bertoncello, I., Smith, I., Péault, B., Tavian, M., Simmons, P.J., 2008. Angiotensin-converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal, and adult hematopoietic tissues. Blood 111, 4055–4063.

Kaczmarek, E., Koziak, K., Sévigny, J., Siegel, J.B., Anrather, J., Beaudoin, A.R., Bach, F.H., Robson, S.C., 1996. Identification and Characterization of CD39/Vascular ATP Diphosphohydrolase *. Journal of Biological Chemistry 271, 33116–33122.

Kaljas, Y., Liu, C., Skaldin, M., Wu, C., Zhou, Q., Lu, Y., Aksentijevich, I., Zavialov, A.V., 2017. Human adenosine deaminases ADA1 and ADA2 bind to different subsets of immune cells. Cell. Mol. Life Sci. 74, 555–570.

Kang, Y., Park, J., Ha, Y.-J., Kang, M., Park, H.-J., Lee, S.-W., Lee, S.-K., Park, Y.-B., 2014. Differences in Clinical Manifestations and Outcomes between Adult and Child Patients with Henoch-Schönlein Purpura. J Korean Med Sci 29, 198–203.

Kara, F.M., Chitu, V., Sloane, J., Axelrod, M., Fredholm, B.B., Stanley, E.R., Cronstein, B.N., 2010. Adenosine A1 receptors (A1Rs) play a critical role in osteoclast formation and function. The FASEB Journal 24, 2325–2333.

Karampini, E., Schillemans, M., Hofman, M., van Alphen, F., de Boer, M., Kuijpers, T.W., van den Biggelaar, M., Voorberg, J., Bierings, R., 2019. Defective AP-3-dependent VAMP8

trafficking impairs Weibel-Palade body exocytosis in Hermansky-Pudlak Syndrome type 2 blood outgrowth endothelial cells. Haematologica 104, 2091–2099.

Karmouty-Quintana, H., Weng, T., Garcia-Morales, L.J., Chen, N.-Y., Pedroza, M., Zhong, H., Molina, J.G., Bunge, R., Bruckner, B.A., Xia, Y., Johnston, R.A., Loebe, M., Zeng, D., Seethamraju, H., Belardinelli, L., Blackburn, M.R., 2013. Adenosine A2B Receptor and Hyaluronan Modulate Pulmonary Hypertension Associated with Chronic Obstructive Pulmonary Disease. Am J Respir Cell Mol Biol 49, 1038–1047.

Kendall, J.L., Springer, J.M., 2020. The Many Faces of a Monogenic Autoinflammatory Disease: Adenosine Deaminase 2 Deficiency. Curr Rheumatol Rep 22.

Khan, S., Taverna, F., Rohlenova, K., Treps, L., Geldhof, V., de Rooij, L., Sokol, L., Pircher, A., Conradi, L.-C., Kalucka, J., Schoonjans, L., Eelen, G., Dewerchin, M., Karakach, T., Li, X., Goveia, J., Carmeliet, P., 2019. EndoDB: a database of endothelial cell transcriptomics data. Nucleic Acids Res 47, D736–D744.

Khodadadi, I., Abdi, M., Ahmadi, A., Wahedi, M.S., Menbari, S., Lahoorpour, F., Rahbari, R., 2011. Analysis of serum adenosine deaminase (ADA) and ADA1 and ADA2 isoenzyme activities in HIV positive and HIV–HBV co-infected patients. Clinical Biochemistry 44, 980–983.

Khursigara, M.R., Schlam, D., Noone, D.G., Bruno, V., Ortiz-Sandoval, C.G., Pluthero, F.G., Kahr, W.H.A., Bowman, M.L., James, P., Grinstein, S., Licht, C., 2020. Vascular endothelial cells evade complement-mediated membrane injury via Weibel-Palade body mobilization. Journal of Thrombosis and Haemostasis 18, 1484–1494.

Kim, J., Lee, K.-S., Kim, J.-H., Lee, D.-K., Park, M., Choi, S., Park, W., Kim, S., Choi, Y.K., Hwang, J.Y., Choe, J., Won, M.-H., Jeoung, D., Lee, H., Ryoo, S., Ha, K.-S., Kwon, Y.-G., Kim, Y.-M., 2017. Aspirin prevents TNF-α-induced endothelial cell dysfunction by regulating the NF-κB-dependent miR-155/eNOS pathway: Role of a miR-155/eNOS axis in preeclampsia. Free Radical Biology and Medicine 104, 185–198.

Kim, S.R., Eirin, A., Herrmann, S.M.S., Saad, A., Juncos, L.A., Lerman, A., Textor, S.C., Lerman, L.O., 2018. Preserved endothelial progenitor cell angiogenic activity in African American essential hypertensive patients. Nephrol Dial Transplant 33, 392–401.

Kimoto, M., Whitley, G.S.J., Tsuji, H., Ogawa, T., 1995. Detection of NG, NG-Dimethylarginine Dimethylaminohydrolase in Human Tissues Using a Monoclonal Antibody. The Journal of Biochemistry 117, 237–238.

Klein, N.J., Shennan, G.I., Heyderman, R.S., Levin, M., 1992. Alteration in glycosaminoglycan metabolism and surface charge on human umbilical vein endothelial cells induced by cytokines, endotoxin and neutrophils. J Cell Sci 102 (Pt 4), 821–832.

Kobayashi, H., Lin, P.C., 2009. Angiogenesis Links Chronic Inflammation with Cancer, in: Kozlov, S.V. (Ed.), Inflammation and Cancer: Methods and Protocols: Volume 1: Experimental Models and Practical Approaches, Methods in Molecular Biology™. Humana Press, Totowa, NJ, pp. 185–191.

Kohn, D.B., Booth, C., Shaw, K.L., Xu-Bayford, J., Garabedian, E., Trevisan, V., Carbonaro-Sarracino, D.A., Soni, K., Terrazas, D., Snell, K., Ikeda, A., Leon-Rico, D., Moore, T.B., Buckland, K.F., Shah, A.J., Gilmour, K.C., De Oliveira, S., Rivat, C., Crooks, G.M., Izotova, N., Tse, J., Adams, S., Shupien, S., Ricketts, H., Davila, A., Uzowuru, C., Icreverzi, A., Barman, P., Campo Fernandez, B., Hollis, R.P., Coronel, M., Yu, A., Chun, K.M., Casas, C.E., Zhang, R., Arduini, S., Lynn, F., Kudari, M., Spezzi, A., Zahn, M., Heimke, R., Labik, I., Parrott, R., Buckley, R.H., Reeves, L., Cornetta, K., Sokolic, R., Hershfield, M., Schmidt, M., Candotti, F., Malech, H.L., Thrasher, A.J., Gaspar, H.B., 2021. Autologous

Ex Vivo Lentiviral Gene Therapy for Adenosine Deaminase Deficiency. New England Journal of Medicine 384(21):2002-2013.

Komarova, Y., Malik, A.B., 2010. Regulation of Endothelial Permeability via Paracellular and Transcellular Transport Pathways. Annual Review of Physiology 72, 463–493.

Kranzhöfer, R., Browatzki, M., Schmidt, J., Kübler, W., 1999. Angiotensin II Activates the Proinflammatory Transcription Factor Nuclear Factor-κB in Human Monocytes. Biochemical and Biophysical Research Communications 257, 826–828.

Kreisel, D., Krupnick, A.S., Balsara, K.R., Riha, M., Gelman, A.E., Popma, S.H., Szeto, W.Y., Turka, L.A., Rosengard, B.R., 2002. Mouse Vascular Endothelium Activates CD8+ T Lymphocytes in a B7-Dependent Fashion. The Journal of Immunology 169, 6154–6161.

Krüger-Genge, A., Blocki, A., Franke, R.-P., Jung, F., 2019. Vascular Endothelial Cell Biology: An Update. Int J Mol Sci 20.

Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., Wang, J.M., 1994. Role of angiogenesis in patients with cerebral ischemic stroke. Stroke 25, 1794–1798.

Ku, C.-S., Cooper, D.N., Patrinos, G.P., 2016. The Rise and Rise of Exome Sequencing. PHG 19, 315–324.

Kutryb-Zajac, B., Harasim, G., Jedrzejewska, A., Krol, O., Braczko, A., Jablonska, P., Mierzejewska, P., Zielinski, J., Slominska, E.M., Smolenski, Ryszard.T., 2021. Macrophage-Derived Adenosine Deaminase 2 Correlates with M2 Macrophage Phenotype in Triple Negative Breast Cancer. Int J Mol Sci 22, 3764.

Kwon, H.-M., Choi, Y.-J., Choi, J.-S., Kang, S.-W., Bae, J.-Y., Kang, I.-J., Jun, J.-G., Lee, S.-S., Lim, S.S., Kang, Y.-H., 2007. Blockade of cytokine-induced endothelial cell adhesion molecule expression by licorice isoliquiritigenin through NF-kappaB signal disruption. Exp Biol Med (Maywood) 232, 235–245.

Lambden, S., Kelly, P., Ahmetaj-Shala, B., Wang, Z., Lee, B., Nandi, M., Torondel, B., Delahaye, M., Dowsett, L., Piper, S., Tomlinson, J., Caplin, B., Colman, L., Boruc, O., Slaviero, A., Zhao, L., Oliver, E., Khadayate, S., Singer, M., Arrigoni, F., Leiper, J., 2015. Dimethylarginine Dimethylaminohydrolase 2 Regulates Nitric Oxide Synthesis and Hemodynamics and Determines Outcome in Polymicrobial Sepsis. Arteriosclerosis, Thrombosis, and Vascular Biology 35, 1382–1392.

Lantoine, F., Iouzalen, L., Devynck, M.A., Millanvoye-Van Brussel, E., David-Dufilho, M., 1998. Nitric oxide production in human endothelial cells stimulated by histamine requires Ca2+ influx. Biochem J 330, 695–699.

Lavoie, J.R., Ormiston Mark L., Perez-Iratxeta Carol, Courtman David W., Jiang Baohua, Ferrer Elisabet, Caruso Paola, Southwood Mark, Foster William S., Morrell Nicholas W., Stewart Duncan J., 2014. Proteomic Analysis Implicates Translationally Controlled Tumor Protein as a Novel Mediator of Occlusive Vascular Remodeling in Pulmonary Arterial Hypertension. Circulation 129, 2125–2135.

Lee, J.-M., Zhai, G., Liu, Q., Gonzales, E.R., Yin, K., Yan, P., Hsu, C.Y., Vo, K.D., Lin, W., 2007. Vascular Permeability Precedes Spontaneous Intracerebral Hemorrhage in Stroke-Prone Spontaneously Hypertensive Rats. Stroke 38, 3289–3291.

Lee, J.Y., Spicer, A.P., 2000. Hyaluronan: a multifunctional, megaDalton, stealth molecule. Current Opinion in Cell Biology 12, 581–586.

Lee, M.-J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., Hla, T., 1999. Vascular Endothelial Cell Adherens Junction Assembly and Morphogenesis Induced by Sphingosine-1-Phosphate. Cell 99, 301–312.

Lee, P.Y., 2018. Vasculopathy, Immunodeficiency, and Bone Marrow Failure: The Intriguing Syndrome Caused by Deficiency of Adenosine Deaminase 2. Front Pediatr 6.

Lee, P.Y., Kellner, E.S., Huang, Y., Furutani, E., Huang, Z., Bainter, W., Alosaimi, M.F., Stafstrom, K., Platt, C.D., Stauber, T., Raz, S., Tirosh, I., Weiss, A., Jordan, M.B., Krupski, C., Eleftheriou, D., Brogan, P., Sobh, A., Baz, Z., Lefranc, G., Irani, C., Kilic, S.S., El-Owaidy, R., Lokeshwar, M.R., Pimpale, P., Khubchandani, R., Chambers, E.P., Chou, J., Geha, R.S., Nigrovic, P.A., Zhou, Q., 2020a. Genotype and functional correlates of disease phenotype in deficiency of adenosine deaminase 2 (DADA2). Journal of Allergy and Clinical Immunology 145(6):1664-1672.e10.

Lee, P.Y., Schulert, G.S., Canna, S.W., Huang, Y., Sundel, J., Li, Y., Hoyt, K.J., Blaustein, R.B., Wactor, A., Do, T., Halyabar, O., Chang, M.H., Dedeoglu, F., Case, S.M., Meidan, E., Lo, M.S., Sundel, R.P., Richardson, E.T., Newburger, J.W., Hershfield, M.S., Son, M.B., Henderson, L.A., Nigrovic, P.A., 2020b. Adenosine deaminase 2 as a biomarker of macrophage activation syndrome in systemic juvenile idiopathic arthritis. Annals of the Rheumatic Diseases 79, 225–231.

Leibovich, S.J., Chen, J.-F., Pinhal-Enfield, G., Belem, P.C., Elson, G., Rosania, A., Ramanathan, M., Montesinos, C., Jacobson, M., Schwarzschild, M.A., Fink, J.S., Cronstein, B., 2002. Synergistic Up-Regulation of Vascular Endothelial Growth Factor Expression in Murine Macrophages by Adenosine A2A Receptor Agonists and Endotoxin. The American Journal of Pathology 160, 2231–2244.

Leicht, S.F., Schwarz, T.M., Hermann, P.C., Seissler, J., Aicher, A., Heeschen, C., 2011. Adiponectin Pretreatment Counteracts the Detrimental Effect of a Diabetic Environment on Endothelial Progenitors. Diabetes 60, 652–661.

Leiper, J.M., Maria, J.S., Chubb, A., Macallister, R.J., Charles, I.G., Whitley, G.St.J., Vallance, P., 1999. Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases. Biochemical Journal 343, 209–214.

Lewis, E.J., Lewis, J.B., Greene, T., Hunsicker, L.G., Berl, T., Pohl, M.A., Zeeuw, D. de, Heerspink, H.L., Rohde, R.D., Atkins, R.C., Reutens, A.T., Packham, D.K., Raz, I., 2011. Sulodexide for Kidney Protection in Type 2 Diabetes Patients With Microalbuminuria: A Randomized Controlled Trial. American Journal of Kidney Diseases 58, 729–736.

Lewis, J.C., Taylor, R.G., Jones, N.D., St Clair, R.W., Cornhill, J.F., 1982. Endothelial surface characteristics in pigeon coronary artery atherosclerosis. I. Cellular alterations during the initial stages of dietary cholesterol challenge. Lab Invest 46, 123–138.

Ley, K., 2001. Functions of Selectins, in: Crocker, P.R. (Ed.), Mammalian Carbohydrate Recognition Systems, Results and Problems in Cell Differentiation. Springer, Berlin, Heidelberg, pp. 177–200.

Ley, K., Laudanna, C., Cybulsky, M.I., Nourshargh, S., 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7, 678–689.

Li, H., Cybulsky, M.I., Gimbrone, M.A., Libby, P., 1993. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb 13, 197–204.

Li, J.M., Fenton, R.A., Cutler, B.S., Dobson, J.G., 1995. Adenosine enhances nitric oxide production by vascular endothelial cells. American Journal of Physiology-Cell Physiology 269, C519–C523.

Li, M., Wang, H., Wang, X., Huang, J., Wang, J., Xi, X., 2014. Diagnostic accuracy of tumor necrosis factor-alpha, interferon-gamma, interlukine-10 and adenosine deaminase 2

in differential diagnosis between tuberculous pleural effusion and malignant pleural effusion. Journal of Cardiothoracic Surgery 9, 118.

Li, Q., Syrovets, T., Simmet, T., Ding, J., Xu, J., Chen, W., Zhu, D., Gao, P., 2013. Plasmin induces intercellular adhesion molecule 1 expression in human endothelial cells via nuclear factor-κB/mitogen-activated protein kinases-dependent pathways. Exp Biol Med (Maywood) 238, 176–186.

Li, S., Pettersson, U.S., Hoorelbeke, B., Kolaczkowska, E., Schelfhout, K., Martens, E., Kubes, P., Van Damme, J., Phillipson, M., Opdenakker, G., 2014. Interference with Glycosaminoglycan-Chemokine Interactions with a Probe to Alter Leukocyte Recruitment and Inflammation In Vivo. PLoS One 9, e104107.

Li, T., Liu, X., Zhao, Z., Ni, L., Liu, C., 2017. Sulodexide recovers endothelial function through reconstructing glycocalyx in the balloon-injury rat carotid artery model. Oncotarget 8, 91350–91361.

Li, W., Wang, W., 2018. Structural alteration of the endothelial glycocalyx: contribution of the actin cytoskeleton. Biomech Model Mechanobiol 17, 147–158.

Libby, P., Buring, J.E., Badimon, L., Hansson, G.K., Deanfield, J., Bittencourt, M.S., Tokgözoğlu, L., Lewis, E.F., 2019. Atherosclerosis. Nature Reviews Disease Primers 5, 1–18.

Libby, P., Nahrendorf, M., Swirski, F.K., 2016. Leukocytes Link Local and Systemic Inflammation in Ischemic Cardiovascular Disease: An Expanded "Cardiovascular Continuum." J Am Coll Cardiol 67, 1091–1103.

Lidar, M., Lipschitz, N., Langevitz, P., Shoenfeld, Y., 2009. The infectious etiology of vasculitis. Autoimmunity 42, 432–438.

Lin, Y., Chang, L., Solovey, A., Healey, J.F., Lollar, P., Hebbel, R.P., 2002. Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. Blood 99, 457–462.

Lin, Y., Weisdorf, D.J., Solovey, A., Hebbel, R.P., 2000. Origins of circulating endothelial cells and endothelial outgrowth from blood. J. Clin. Invest. 105, 71–77.

Linden, J., 2006. New insights into the regulation of inflammation by adenosine. J Clin Invest 116, 1835–1837.

Liu, L., Liu, Hui, Jiao, J., Liu, Huiquan, Bergeron, A., Dong, J.-F., Zhang, J., 2007. Changes in Circulating Human Endothelial Progenitor Cells after Brain Injury. J Neurotrauma. 24(6):936-43.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408.

Long, L., Ormiston, M.L., Yang, X., Southwood, M., Gräf, S., Machado, R.D., Mueller, M., Kinzel, B., Yung, L.M., Wilkinson, J.M., Moore, S.D., Drake, K.M., Aldred, M.A., Yu, P., Upton, P.D., Morrell, N.W., 2015. Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. Nat Med 21, 777–785.

Long, L., Yang, X., Southwood, M., Moore, S., Crosby, A., Upton, P.D., Dunmore, B.J., Morrell, N.W., 2020. Targeting translational read-through of premature termination mutations in BMPR2 with PTC124 for pulmonary arterial hypertension. Pulm Circ 10.

Lorant, D.E., Patel, K.D., McIntyre, T.M., McEver, R.P., Prescott, S.M., Zimmerman, G.A., 1991. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. Journal of Cell Biology 115, 223–234.

Lortat-Jacob, H., Grosdidier, A., Imberty, A., 2002. Structural diversity of heparan sulfate binding domains in chemokines. Proc Natl Acad Sci U S A 99, 1229–1234.

Lozanoska-Ochser, B., Peakman, M., 2009. Level of major histocompatibility complex class I expression on endothelium in non-obese diabetic mice influences CD8 T cell adhesion and migration. Clin Exp Immunol 157, 119–127.

Ludmer, P.L., Selwyn, A.P., Shook, T.L., Wayne, R.R., Mudge, G.H., Alexander, R.W., Ganz, P., 1986. Paradoxical Vasoconstriction Induced by Acetylcholine in Atherosclerotic Coronary Arteries. N Engl J Med 315, 1046–1051.

Luft, J.H., 1966. Fine structures of capillary and endocapillary layer as revealed by ruthenium red. Fed Proc 25, 1773–1783.

Luksha, L., Luksha, N., Kublickas, M., Nisell, H., Kublickiene, K., 2010. Diverse Mechanisms of Endothelium-Derived Hyperpolarizing Factor-Mediated Dilatation in Small Myometrial Arteries in Normal Human Pregnancy and Preeclampsia1. Biology of Reproduction 83, 728–735.

Luo, F., Le, N.-B., Mills, T., Chen, N.-Y., Karmouty-Quintana, H., Molina, J.G., Davies, J., Philip, K., Volcik, K.A., Liu, H., Xia, Y., Eltzschig, H.K., Blackburn, M.R., 2016. Extracellular adenosine levels are associated with the progression and exacerbation of pulmonary fibrosis. FASEB J 30, 874–883.

Luster, A.D., Unkeless, J.C., Ravetch, J.V., 1985. γ -Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 315, 672–676.

Mackman, N., 2012. New insights into the mechanisms of venous thrombosis. J Clin Invest 122, 2331–2336.

Mai, J., Virtue, A., Shen, J., Wang, H., Yang, X.-F., 2013. An evolving new paradigm: endothelial cells – conditional innate immune cells. J Hematol Oncol 6, 61.

Majno, G., Palade, G.E., 1961. STUDIES ON INFLAMMATION : I. The Effect of Histamine and Serotonin on Vascular Permeability: An Electron Microscopic Study. The Journal of Biophysical and Biochemical Cytology 11, 571–605.

Maor, I., Rainis, T., Lanir, A., Lavy, A., 2011. Adenosine deaminase activity in patients with Crohn's disease: distinction between active and nonactive disease. European Journal of Gastroenterology & Hepatology 23, 598–602.

Marquardt, D.L., Gruber, H.E., Wasserman, S.I., 1984. Adenosine release from stimulated mast cells. PNAS 81, 6192–6196.

Martin, M.U., Wesche, H., 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, The Molecular Biology of Cytokines 1592, 265–280.

Martinez-Lemus, L.A., Zhao, G., Galiñanes, E.L., Boone, M., 2011. Inward remodeling of resistance arteries requires reactive oxygen species-dependent activation of matrix metalloproteinases. Am J Physiol Heart Circ Physiol 300, H2005–H2015.

Martin-Ramirez, J., Hofman, M., van den Biggelaar, M., Hebbel, R.P., Voorberg, J., 2012a. Establishment of outgrowth endothelial cells from peripheral blood. Nature Protocols 7, 1709–1715.

Martin-Ramirez, J., Hofman, M., van den Biggelaar, M., Hebbel, R.P., Voorberg, J., 2012b. Establishment of outgrowth endothelial cells from peripheral blood. Nature Protocols 7, 1709–1715.

Martin-Ramirez, J., Kok, M.G.M., Hofman, M., Bierings, R., Creemers, E.E., Meijers, J.C.M., Voorberg, J., Pinto-Sietsma, S.-J., 2014. Individual with Subclinical Atherosclerosis Have Impaired Proliferation of Blood Outgrowth Endothelial Cells, Which Can Be Restored by Statin Therapy. PLoS One 9.

Massa, M., Campanelli, R., Bonetti, E., Ferrario, M., Marinoni, B., Rosti, V., 2009. Rapid and large increase of the frequency of circulating endothelial colony-forming cells (ECFCs) generating late outgrowth endothelial cells in patients with acute myocardial infarction. Experimental Hematology 37, 8–9.

Mathur, T., Singh, K.A., Pandian, N.K.R., Tsai, S.-H., Hein, T.W., Gaharwar, A.K., Flanagan, J.M., Jain, A., 2019. Organ-on-Chips made of Blood: Endothelial Progenitor Cells from Blood reconstitute Vascular Thromboinflammation in Vessel-Chips. Lab Chip 19, 2500–2511.

Matveeva, V., Khanova, M., Sardin, E., Antonova, L., Barbarash, O., 2018. Endovascular Interventions Permit Isolation of Endothelial Colony-Forming Cells from Peripheral Blood. Int J Mol Sci 19.

McBride, A.E., Silver, P.A., 2001. State of the Arg: Protein Methylation at Arginine Comes of Age. Cell 106, 5–8.

McCarthy, S.A., Kuzu, I., Gatter, K.C., Bicknell, R., 1991. Heterogeneity of the endothelial cell and its role in organ preference of tumour metastasis. Trends Pharmacol. Sci. 12, 462–467.

McIntyre, T.M., Zimmerman, G.A., Satoh, K., Prescott, S.M., 1985. Cultured endothelial cells synthesize both platelet-activating factor and prostacyclin in response to histamine, bradykinin, and adenosine triphosphate. J Clin Invest 76, 271–280.

Medina, R.J., Barber, C.L., Sabatier, F., Dignat-George, F., Melero-Martin, J.M., Khosrotehrani, K., Ohneda, O., Randi, A.M., Chan, J.K.Y., Yamaguchi, T., Van Hinsbergh, V.W.M., Yoder, M.C., Stitt, A.W., 2017. Endothelial Progenitors: A Consensus Statement on Nomenclature. Stem Cells Transl Med 6, 1316–1320.

Medina, R.J., O'Neill, C.L., O'Doherty, T.M., Wilson, S.E.J., Stitt, A.W., 2012. Endothelial Progenitors as Tools to Study Vascular Disease. Stem Cells Int 2012.

Medina-Leyte, D.J., Domínguez-Pérez, M., Mercado, I., Villarreal-Molina, M.T., Jacobo-Albavera, L., 2020. Use of Human Umbilical Vein Endothelial Cells (HUVEC) as a Model to Study Cardiovascular Disease: A Review. Applied Sciences 10, 938.

Meneveau, N., Deschaseaux, F., Séronde, M.-F., Chopard, R., Schiele, F., Jehl, J., Tiberghien, P., Bassand, J.-P., Kantelip, J.-P., Davani, S., 2011. Presence of endothelial colony-forming cells is associated with reduced microvascular obstruction limiting infarct size and left ventricular remodelling in patients with acute myocardial infarction. Basic Res Cardiol 106, 1397–1410.

Mensah, S.A., Cheng, M.J., Homayoni, H., Plouffe, B.D., Coury, A.J., Ebong, E.E., 2017. Regeneration of glycocalyx by heparan sulfate and sphingosine 1-phosphate restores inter-endothelial communication. PLOS ONE 12, e0186116.

Meyts, I., Aksentijevich, I., 2018. Deficiency of Adenosine Deaminase 2 (DADA2): Updates on the Phenotype, Genetics, Pathogenesis, and Treatment. J Clin Immunol 38, 569–578.

Michal, F., Thorp, R.H., 1966. Enhanced Adenosine Inhibition of Platelet Aggregation in the Presence of Cardiac Glycosides. Nature 209, 208–209.

Michniacki, T.F., Hannibal, M., Ross, C.W., Frame, D.G., DuVall, A.S., Khoriaty, R., Vander Lugt, M.T., Walkovich, K.J., 2018. Hematologic Manifestations of Deficiency of Adenosine Deaminase 2 (DADA2) and Response to Tumor Necrosis Factor Inhibition in DADA2-Associated Bone Marrow Failure. J Clin Immunol 38, 166–173.

Milbauer, L., C., Wei, P., Enenstein, J., Jiang, A., Hillery, C.A., Scott, J.P., Nelson, S.C., Bodempudi, V., Topper, J.N., Yang, R.-B., Hirsch, B., Pan, W., Hebbel, R.P., 2008. Genetic endothelial systems biology of sickle stroke risk. Blood 111, 3872–3879.

Millán, J.L., 2006. Alkaline Phosphatases. Purinergic Signalling 2, 335.

Mitchell, A., Fujisawa, T., Mills, N.L., Brittan, M., Newby, D.E., Cruden, N.L.M., 2017. Endothelial Progenitor Cell Biology and Vascular Recovery Following Transradial Cardiac Catheterization. J Am Heart Assoc 6.

Miyahara, T., Hamanaka, K., Weber, D.S., Drake, D.A., Anghelescu, M., Parker, J.C., 2007. Phosphoinositide 3-kinase, Src, and Akt modulate acute ventilation-induced vascular permeability increases in mouse lungs. American Journal of Physiology-Lung Cellular and Molecular Physiology 293, L11–L21.

Miyazaki, H., Matsuoka, H., Cooke, J.P., Usui, M., Ueda, S., Okuda, S., Imaizumi, T., 1999. Endogenous Nitric Oxide Synthase Inhibitor. Circulation 99, 1141–1146.

Möller, M.N., Rios, N., Trujillo, M., Radi, R., Denicola, A., and Alvarez, B. (2019). Detection and quantification of nitric oxide–derived oxidants in biological systems. Journal of Biological Chemistry *294*, 14776–14802.

Moncada, S., Higgs, E.A., Vane, J.R., 1977. Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. Lancet 1, 18–20.

Montesinos, M.C., Gadangi, P., Longaker, M., Sung, J., Levine, J., Nilsen, D., Reibman, J., Li, M., Jiang, C.-K., Hirschhorn, R., Recht, P.A., Ostad, E., Levin, R.I., Cronstein, B.N., 1997. Wound Healing Is Accelerated by Agonists of Adenosine A2 (Gα s-linked) Receptors. J Exp Med 186, 1615–1620.

Morey, J.S., Ryan, J.C., Van Dolah, F.M., 2006. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol Proced Online 8, 175–193.

Mortellaro, A., 2020. Efficacy of lentiviral-mediated gene therapy for the treatment of DADA2. The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Müller, A.M., Hermanns, M.I., Cronen, C., Kirkpatrick, C.J., 2002a. Comparative Study of Adhesion Molecule Expression in Cultured Human Macro- and Microvascular Endothelial Cells. Experimental and Molecular Pathology 73, 171–180.

Müller, A.M., Hermanns, M.I., Skrzynski, C., Nesslinger, M., Müller, K.-M., Kirkpatrick, C.J., 2002b. Expression of the Endothelial Markers PECAM-1, vWf, and CD34 in Vivo and in Vitro. Experimental and Molecular Pathology 72, 221–229.

Nanthapisal, S., Murphy, C., Omoyinmi, E., Hong, Y., Standing, A., Berg, S., Ekelund, M., Jolles, S., Harper, L., Youngstein, T., Gilmour, K., Klein, N.J., Eleftheriou, D., Brogan, P.A., 2016. Deficiency of Adenosine Deaminase Type 2: A Description of Phenotype and Genotype in Fifteen Cases. Arthritis & Rheumatology 68, 2314–2322.

Nanthapisal, S., Murphy, C., Omoyinmi, E., Standing, A., Hong, Y., Gomes, S., Klein, N., Eleftheriou, D., Brogan, P., 2015. Monogenic polyarteritis nodosa caused by ADA2 Deficiency: the GOSH experience. Pediatr Rheumatol 13, P8, 1546-0096-13-S1-P8.

Navon Elkan, P., Pierce, S.B., Segel, R., Walsh, T., Barash, J., Padeh, S., Zlotogorski, A., Berkun, Y., Press, J.J., Mukamel, M., Voth, I., Hashkes, P.J., Harel, L., Hoffer, V., Ling, E., Yalcinkaya, F., Kasapcopur, O., Lee, M.K., Klevit, R.E., Renbaum, P., Weinberg-Shukron, A., Sener, E.F., Schormair, B., Zeligson, S., Marek-Yagel, D., Strom, T.M., Shohat, M., Singer, A., Rubinow, A., Pras, E., Winkelmann, J., Tekin, M., Anikster, Y., King, M.-C., Levy-Lahad, E., 2014. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. N. Engl. J. Med. 370, 921–931.

Nelson, A., Berkestedt, I., Schmidtchen, A., Ljunggren, L., Bodelsson, M., 2008. Increased levels of glycosaminoglycans during septic shock: relation to mortality and the antibacterial actions of plasma. Shock 30, 623–627.

Németh, Z.H., Lutz, C.S., Csóka, B., Deitch, E.A., Leibovich, S.J., Gause, W.C., Tone, M., Pacher, P., Vizi, E.S., Haskó, G., 2005. Adenosine Augments IL-10 Production by Macrophages through an A2B Receptor-Mediated Posttranscriptional Mechanism. J Immunol 175, 8260–8270.

Nguyen, D.K., Montesinos, M.C., Williams, A.J., Kelly, M., Cronstein, B.N., 2003. Th1 cytokines regulate adenosine receptors and their downstream signaling elements in human microvascular endothelial cells. J Immunol 171, 3991–3998.

Nieuwdorp, M., Haeften, T.W. van, Gouverneur, M.C.L.G., Mooij, H.L., Lieshout, M.H.P. van, Levi, M., Meijers, J.C.M., Holleman, F., Hoekstra, J.B.L., Vink, H., Kastelein, J.J.P., Stroes, E.S.G., 2006a. Loss of Endothelial Glycocalyx During Acute Hyperglycemia Coincides With Endothelial Dysfunction and Coagulation Activation In Vivo. Diabetes 55, 480–486.

Nieuwdorp, M., Holleman, F., de Groot, E., Vink, H., Gort, J., Kontush, A., Chapman, M.J., Hutten, B.A., Brouwer, C.B., Hoekstra, J.B.L., Kastelein, J.J.P., Stroes, E.S.G., 2007. Perturbation of hyaluronan metabolism predisposes patients with type 1 diabetes mellitus to atherosclerosis. Diabetologia 50, 1288–1293.

Nieuwdorp, M., Meuwese, M.C., Mooij, H.L., van Lieshout, M.H.P., Hayden, A., Levi, M., Meijers, J.C.M., Ince, C., Kastelein, J.J.P., Vink, H., Stroes, E.S.G., 2009. Tumor necrosis factor-alpha inhibition protects against endotoxin-induced endothelial glycocalyx perturbation. Atherosclerosis 202, 296–303.

Nieuwdorp, M., Mooij, H.L., Kroon, J., Atasever, B., Spaan, J.A.E., Ince, C., Holleman, F., Diamant, M., Heine, R.J., Hoekstra, J.B.L., Kastelein, J.J.P., Stroes, E.S.G., Vink, H., 2006b. Endothelial Glycocalyx Damage Coincides With Microalbuminuria in Type 1 Diabetes. Diabetes 55, 1127–1132.

Noone, D.G., Riedl, M., Pluthero, F.G., Bowman, M.L., Liszewski, M.K., Lu, L., Quan, Y., Balgobin, S., Schneppenheim, R., Schneppenheim, S., Budde, U., James, P., Atkinson, J.P., Palaniyar, N., Kahr, W.H.A., Licht, C., 2016. Von Willebrand factor regulates complement on endothelial cells. Kidney Int 90, 123–134.

O'Donnell-Luria, A., Baxter, S., 2020. Can DADA2 Prevalence be Estimated? The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Ogawa, T., Kimoto, M., Sasaoka, K., 1989. Purification and properties of a new enzyme, NG, NG-dimethylarginine dimethylaminohydrolase, from rat kidney. Journal of Biological Chemistry 264, 10205–10209.

Ombrello, A., 2020. Efficacy of TNf inhibitors in treating DADA2. The 3rd International Conference on Deficiency of Adenosine Deaminase 2 (Virtual, online).

Ombrello, A.K., Qin, J., Hoffmann, P.M., Kumar, P., Stone, D., Jones, A., Romeo, T., Barham, B., Pinto-Patarroyo, G., Toro, C., Soldatos, A., Zhou, Q., Deuitch, N., Aksentijevich, I., Sheldon, S.L., Kelly, S., Man, A., Barron, K., Hershfield, M., Flegel, W.A., Kastner, D.L., 2019. Treatment Strategies for Deficiency of Adenosine Deaminase 2. New England Journal of Medicine 380, 1582–1584.

Onouchi, Y., Gunji, T., Burns, J.C., Shimizu, C., Newburger, J.W., Yashiro, M., Nakamura, Yoshikazu, Yanagawa, H., Wakui, K., Fukushima, Y., Kishi, F., Hamamoto, K., Terai, M., Sato, Y., Ouchi, K., Saji, T., Nariai, A., Kaburagi, Y., Yoshikawa, T., Suzuki, K., Tanaka, T., Nagai, T., Cho, H., Fujino, A., Sekine, A., Nakamichi, R., Tsunoda, T., Kawasaki, T.,

Nakamura, Yusuke, Hata, A., 2008. ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms. Nat Genet 40, 35–42.

Ontyd, J., Schrader, J., 1984. Measurement of adenosine, inosine, and hypoxanthine in human plasma. J Chromatogr 307, 404–409.

Oohira, A., Wight, T.N., Bornstein, P., 1983. Sulfated proteoglycans synthesized by vascular endothelial cells in culture. J Biol Chem 258, 2014–2021.

Ormiston, M.L., Southgate, L., Treacy, C., Pepke-Zaba, J., Trembath, R.C., Machado, R.D., Morrell, N.W., 2013. Assessment of a Pulmonary Origin for Blood Outgrowth Endothelial Cells by Examination of Identical Twins Harboring a BMPR2 Mutation. Am J Respir Crit Care Med 188, 258–260.

Ormiston, M.L., Toshner, M.R., Kiskin, F.N., Huang, C.J.Z., Groves, E., Morrell, N.W., Rana, A.A., 2015. Generation and Culture of Blood Outgrowth Endothelial Cells from Human Peripheral Blood. J Vis Exp. 106:e53384.

Otten, J., Schultze, A., Schafhausen, P., Otterstetter, S., Dierlamm, J., Bokemeyer, C., Brummendorf, T.H., Fiedler, W., Loges, S., 2008. Blood outgrowth endothelial cells from chronic myeloid leukaemia patients are BCR/ABL1 negative. Br. J. Haematol. 142, 115–118.

Özen, S., Batu, E.D., Taşkıran, E.Z., Özkara, H.A., Ünal, Ş., Güleray, N., Erden, A., Karadağ, Ö., Gümrük, F., Çetin, M., Sönmez, H.E., Bilginer, Y., Ayvaz, D.Ç., Tezcan, I., 2020. A Monogenic Disease with a Variety of Phenotypes: Deficiency of Adenosine Deaminase 2. The Journal of Rheumatology 47, 117–125.

Paisley, K.E., Beaman, M., Tooke, J.E., Mohamed-Ali, V., Lowe, G.D.O., Shore, A.C., 2003. Endothelial dysfunction and inflammation in asymptomatic proteinuria. Kidney International 63, 624–633.

Palani, G., Ananthasubramaniam, K., 2013. Regadenoson: Review of Its Established Role in Myocardial Perfusion Imaging and Emerging Applications. Cardiology in Review 21, 42–48.

Paleolog, E.M., 2002. Angiogenesis in rheumatoid arthritis. Arthritis Research & Therapy 4, S81.

Palmer, R.M.J., Ferrige, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Paschalaki, K.E., Randi, A.M., 2018. Recent Advances in Endothelial Colony Forming Cells Toward Their Use in Clinical Translation. Front Med (Lausanne) 5.

Paschalaki, K.E., Starke, R.D., HU, Y., Mercado, N., Margariti, A., Gorgoulis, V.G., Randi, A.M., Barnes, P.J., 2013. Dysfunction of Endothelial Progenitor Cells from Smokers and Chronic Obstructive Pulmonary Disease Patients Due to Increased DNA Damage and Senescence. Stem Cells 31, 2813–2826.

Paschalaki, K.E., Zampetaki, A., Baker, J.R., Birrell, M.A., Starke, R.D., Belvisi, M.G., Donnelly, L.E., Mayr, M., Randi, A.M., Barnes, P.J., 2018. Downregulation of MicroRNA-126 Augments DNA Damage Response in Cigarette Smokers and Patients with Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 197, 665–668.

Passerini, A.G., Polacek, D.C., Shi, C., Francesco, N.M., Manduchi, E., Grant, G.R., Pritchard, W.F., Powell, S., Chang, G.Y., Stoeckert, C.J., Davies, P.F., 2004. Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta. Proc Natl Acad Sci U S A 101, 2482–2487.

Peleli, M., Fredholm, B.B., Sobrevia, L., Carlström, M., 2017. Pharmacological targeting of adenosine receptor signaling. Molecular Aspects of Medicine, Adenosine - from molecular actions to pathophysiology 55, 4–8.

Petrache, I., Birukova, A., Ramirez, S.I., Garcia, J.G.N., Verin, A.D., 2003. The Role of the Microtubules in Tumor Necrosis Factor-α–Induced Endothelial Cell Permeability. Am J Respir Cell Mol Biol 28, 574–581.

Piaggio, G., Rosti, V., Corselli, M., Bertolotti, F., Bergamaschi, G., Pozzi, S., Imperiale, D., Chiavarina, B., Bonetti, E., Novara, F., Sessarego, M., Villani, L., Garuti, A., Massa, M., Ghio, R., Campanelli, R., Bacigalupo, A., Pecci, A., Viarengo, G., Zuffardi, O., Frassoni, F., Barosi, G., 2009. Endothelial colony-forming cells from patients with chronic myeloproliferative disorders lack the disease-specific molecular clonality marker. Blood 114, 3127–3130.

Platts, S.H., Duling, B.R., 2004. Adenosine A3 Receptor Activation Modulates the Capillary Endothelial Glycocalyx. Circulation Research 94, 77–82.

Platts, S.H., Linden, J., Duling, B.R., 2003. Rapid modification of the glycocalyx caused by ischemia-reperfusion is inhibited by adenosine A2A receptor activation. American Journal of Physiology-Heart and Circulatory Physiology 284, H2360–H2367.

Pober, J.S., Collins, T., Gimbrone, M.A., Libby, P., Reiss, C.S., 1986. Inducible expression of class II major histocompatibility complex antigens and the immunogenicity of vascular endothelium. Transplantation 41, 141–146.

Pober, J.S., Cotran, R.S., 1990. THE ROLE OF ENDOTHELIAL CELLS IN INFLAMMATION. Transplantation 50, 537–544.

Pober, J.S., Lapierre, L.A., Stolpen, A.H., Brock, T.A., Springer, T.A., Fiers, W., Bevilacqua, M.P., Mendrick, D.L., Gimbrone, M.A., 1987. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. The Journal of Immunology 138, 3319–3324.

Pober, J.S., Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. Nature Reviews Immunology 7, 803–815.

Polacheck, W.J., Kutys, M.L., Yang, J., Eyckmans, J., Wu, Y., Vasavada, H., Hirschi, K.K., Chen, C.S., 2017. A non-canonical Notch complex regulates adherens junctions and vascular barrier function. Nature 552, 258–262.

Potter, D.R., Damiano, E.R., 2008. The Hydrodynamically Relevant Endothelial Cell Glycocalyx Observed In Vivo Is Absent In Vitro. Circulation Research 102, 770–776.

Potter, D.R., Jiang, J., Damiano, E.R., 2009. The Recovery Time Course of the Endothelial Cell Glycocalyx In Vivo and Its Implications In Vitro. Circulation Research 104, 1318–1325.

Pugsley, M.K., Tabrizchi, R., 2000. The vascular system: An overview of structure and function. Journal of Pharmacological and Toxicological Methods, Methods in Vascular Pharmacology 44, 333–340.

Pullamsetti, S.S., Savai, R., Schaefer, M.B., Wilhelm, J., Ghofrani, H.A., Weissmann, N., Schudt, C., Fleming, I., Mayer, K., Leiper, J., Seeger, W., Grimminger, F., Schermuly, R.T., 2011. cAMP Phosphodiesterase Inhibitors Increases Nitric Oxide Production by Modulating Dimethylarginine Dimethylaminohydrolases. Circulation 123, 1194–1204.

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., Nishigaki, I., 2013. The Vascular Endothelium and Human Diseases. Int J Biol Sci 9, 1057–1069.

Rama, M., Duflos, C., Melki, I., Bessis, D., Bonhomme, A., Martin, H., Doummar, D., Valence, S., Rodriguez, D., Carme, E., Genevieve, D., Heimdal, K., Insalaco, A., Franck, N.,

Queyrel-Moranne, V., Tieulie, N., London, J., Uettwiller, F., Georgin-Lavialle, S., Belot, A., Koné-Paut, I., Hentgen, V., Boursier, G., Touitou, I., Sarrabay, G., 2018. A decision tree for the genetic diagnosis of deficiency of adenosine deaminase 2 (DADA2): a French reference centres experience. Eur J Hum Genet 26, 960–971.

Ramnath, R., Foster, R.R., Qiu, Y., Cope, G., Butler, M.J., Salmon, A.H., Mathieson, P.W., Coward, R.J., Welsh, G.I., Satchell, S.C., 2014. Matrix metalloproteinase 9-mediated shedding of syndecan 4 in response to tumor necrosis factor α: a contributor to endothelial cell glycocalyx dysfunction. The FASEB Journal 28, 4686–4699.

Ray, C.J., Marshall, J.M., 2006. The cellular mechanisms by which adenosine evokes release of nitric oxide from rat aortic endothelium. J Physiol 570, 85–96.

RayChaudhury, A., Elkins, M., Kozien, D., Nakada, M.T., 2001. Regulation of PECAM-1 in Endothelial Cells during Cell Growth and Migration. Exp Biol Med (Maywood) 226, 686–691.

Raymond, F., Carbonneau, J., Boucher, N., Robitaille, L., Boisvert, S., Wu, W.-K., Serres, G.D., Boivin, G., Corbeil, J., 2009. Comparison of Automated Microarray Detection with Real-Time PCR Assays for Detection of Respiratory Viruses in Specimens Obtained from Children. Journal of Clinical Microbiology 47, 743–750.

Reed, D.M., Paschalaki, K.E., Starke, R.D., Mohamed, N.A., Sharp, G., Fox, B., Eastwood, D., Bristow, A., Ball, C., Vessillier, S., Hansel, T.T., Thorpe, S.J., Randi, A.M., Stebbings, R., Mitchell, J.A., 2015. An autologous endothelial cell: Peripheral blood mononuclear cell assay that detects cytokine storm responses to biologicsReed, D. M. et al. (2015) 'An autologous endothelial cell: Peripheral blood mononuclear cell assay that detects cytokine storm respon. FASEB Journal 29, 2595–2602.

Reitsma, S., Slaaf, D.W., Vink, H., van Zandvoort, M.A.M.J., oude Egbrink, M.G.A., 2007. The endothelial glycocalyx: composition, functions, and visualization. Pflugers Arch 454, 345–359.

Renné, T., Dedio, J., David, G., Müller-Esterl, W., 2000. High Molecular Weight Kininogen Utilizes Heparan Sulfate Proteoglycans for Accumulation on Endothelial Cells *. Journal of Biological Chemistry 275, 33688–33696.

Ribatti, D., Tamma, R., Ruggieri, S., Annese, T., Crivellato, E., 2020. Surface markers: An identity card of endothelial cells. Microcirculation 27, e12587.

Ribot, J., Caliaperoumal, G., Paquet, J., Boisson-vidal, C., Petite, H., Anagnostou, F., 2017. Type 2 diabetes alters mesenchymal stem cell secretome composition and angiogenic properties. Journal of Cellular and Molecular Medicine 21, 349.

Rice, G.I., Melki, I., Frémond, M.-L., Briggs, T.A., Rodero, M.P., Kitabayashi, N., Oojageer, A., Bader-Meunier, B., Belot, A., Bodemer, C., Quartier, P., Crow, Y.J., 2017. Assessment of Type I Interferon Signaling in Pediatric Inflammatory Disease. J Clin Immunol 37, 123–132.

Richards, W.O., Prutzman, K.B., O'Hea, M.F., Audia, J.P., Alvarez, D.F., 2014. Bariatric surgery improves the circulating numbers and biological activity of late outgrowth endothelial progenitor cells. Surgery for Obesity and Related Diseases 10, 906–913.

Rigato, M., Avogaro, A., Fadini, G.P., 2016. Levels of Circulating Progenitor Cells, Cardiovascular Outcomes and Death: A Meta-Analysis of Prospective Observational Studies. Circ Res 118, 1930–1939.

Rignault-Clerc, S., Bielmann, C., Delodder, F., Raffoul, W., Waeber, B., Liaudet, L., Berger, M.M., Feihl, F., Rosenblatt-Velin, N., 2013. Functional late outgrowth endothelial progenitors isolated from peripheral blood of burned patients. Burns 39, 694–704.

Rosenberg, R.D., Shworak, N.W., Liu, J., Schwartz, J.J., Zhang, L., 1997. Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? J Clin Invest 99, 2062–2070.

Rubanyi, G.M., Romero, J.C., Vanhoutte, P.M., 1986. Flow-induced release of endothelium-derived relaxing factor. American Journal of Physiology-Heart and Circulatory Physiology 250, H1145–H1149.

Rubio-Gayosso, I., Platts, S.H., Duling, B.R., 2006. Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury. American Journal of Physiology-Heart and Circulatory Physiology 290, H2247–H2256.

Rudolphi, K.A., Schubert, P., Parkinson, F.E., Fredholm, B.B., 1992. Neuroprotective role of adenosine in cerebral ischaemia. Trends in Pharmacological Sciences 13, 439–445.

Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C., Shima, D.T., 2002. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16, 2684–2698.

Ryan, U.S., Ryan, J.W., Whitaker, C., Chiu, A., 1976. Localization of angiotensin converting enzyme (kininase II). II. Immunocytochemistry and immunofluorescence. Tissue and Cell 8, 125–145.

Sag, E., Demir, S., Ozen, S., 2020. Clusters in Pediatric Rheumatic Diseases. Curr Rheumatol Rep 22, 28.

Saghiri, R., Ghashghai, N., Movaseghi, S., Poursharifi, P., Jalilfar, S., Bidhendi, M.A., Ghazizadeh, L., Ebrahimi-Rad, M., 2012. Serum adenosine deaminase activity in patients with systemic lupus erythematosus: a study based on ADA1 and ADA2 isoenzymes pattern. Rheumatol Int 32, 1633–1638.

Sahin, S., Adrovic, A., Barut, K., Ugurlu, S., Turanli, E.T., Ozdogan, H., Kasapcopur, O., 2018. Clinical, imaging and genotypical features of three deceased and five surviving cases with ADA2 deficiency. Rheumatol Int 38, 129–136.

Sahin, S., Adrovic, A., Kasapcopur, O., 2020. A monogenic autoinflammatory disease with fatal vasculitis: deficiency of adenosine deaminase 2. Current Opinion in Rheumatology 32, 3–14.

Sakamoto, T.M., Lanaro, C., Ozelo, M.C., Garrido, V.T., Olalla-Saad, S.T., Conran, N., Costa, F.F., 2013. Increased adhesive and inflammatory properties in blood outgrowth endothelial cells from sickle cell anemia patients. Microvascular Research 90, 173–179.

Sala, R., Rotoli, B.M., Colla, E., Visigalli, R., Parolari, A., Bussolati, O., Gazzola, G.C., Dall'Asta, V., 2002. Two-way arginine transport in human endothelial cells: TNF-α stimulation is restricted to system y+. American Journal of Physiology-Cell Physiology 282, C134–C143.

Salsoso, R., Mate, A., Toledo, F., Vázquez, C.M., Sobrevia, L., 2021. Insulin requires A2B adenosine receptors to modulate the L-arginine/nitric oxide signalling in the human fetoplacental vascular endothelium from late-onset preeclampsia. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1867, 165993.

Sampson, L.J., Plane, F., Garland, C.J., 2001. Involvement of cyclic GMP and potassium channels in relaxation evoked by the nitric oxide donor, diethylamine NONOate, in the rat small isolated mesenteric artery. Naunyn-Schmied Arch Pharmacol 364, 220–225.

Sands, W.A., Martin, A.F., Strong, E.W., Palmer, T.M., 2004. Specific inhibition of nuclear factor-kappaB-dependent inflammatory responses by cell type-specific mechanisms upon A2A adenosine receptor gene transfer. Mol Pharmacol 66, 1147–1159.

Santo, G.C., Baldeiras, I., Guerreiro, R., Ribeiro, J.A., Cunha, R., Youngstein, T., Nanthapisal, S., Leitão, J., Fernandes, C., Caramelo, F., Almeida, M. do R., Brás, J., Santana, I., Hospitalar e Universitário de Coimbra, C., 2018. Adenosine Deaminase Two and Immunoglobulin M Accurately Differentiate Adult Sneddon's Syndrome of Unknown Cause. CED 46, 257–264.

Sari, R.A., Taysi, S., Yilmaz, O., Bakan, N., 2003. Correlation of serum levels of adenosine deaminase activity and its isoenzymes with disease activity in rheumatoid arthritis. Clin Exp Rheumatol 21, 87–90.

Sasa, G.S., Elghetany, M.T., Bergstrom, K., Nicholas, S., Himes, R., Krance, R.A., Hershfield, M., van Montfrans, J., Bertuch, A., 2015. Adenosine Deaminase 2 Deficiency As a Cause of Pure Red Cell Aplasia Mimicking Diamond Blackfan Anemia. Blood 126, 3615–3615.

Sauer, A.V., Brigida, I., Carriglio, N., Aiuti, A., 2012. Autoimmune Dysregulation and Purine Metabolism in Adenosine Deaminase Deficiency. Front Immunol 3, 265.

Schächinger, V., Britten, M.B., Zeiher, A.M., 2000. Prognostic Impact of Coronary Vasodilator Dysfunction on Adverse Long-Term Outcome of Coronary Heart Disease. Circulation 101, 1899–1906.

Schena, F., Penco, F., Volpi, S., Pastorino, C., Caorsi, R., Kalli, F., Fenoglio, D., Salis, A., Bertoni, A., Prigione, I., Bocca, P., Insalaco, A., De Benedetti, F., Antonini, F., Grossi, A., Signa, S., Damonte, G., Ceccherini, I., Filaci, G., Traggiai, E., Gattorno, M., 2020. Dysregulation in B-cell responses and T follicular helper cell function in ADA2 deficiency patients. European Journal of Immunology 51:206-219.

Schenk, U., Westendorf, A.M., Radaelli, E., Casati, A., Ferro, M., Fumagalli, M., Verderio, C., Buer, J., Scanziani, E., Grassi, F., 2008. Purinergic Control of T Cell Activation by ATP Released Through Pannexin-1 Hemichannels. Sci. Signal. 1, ra6.

Schepp, J., Proietti, M., Frede, N., Buchta, M., Hübscher, K., Restrepo, J.R., Goldacker, S., Warnatz, K., Schmid, J.P., Duppenthaler, A., Lougaris, V., Uriarte, I., Kelly, S., Hershfield, M., Grimbacher, B., 2017. Screening of 181 Patients With Antibody Deficiency for Deficiency of Adenosine Deaminase 2 Sheds New Light on the Disease in Adulthood. Arthritis & Rheumatology 69, 1689–1700.

Schillemans, M., Karampini, E., van den Eshof, B.L., Gangaev, A., Hofman, M., van Breevoort, D., Meems, H., Janssen, H., Mulder, A.A., Jost, C.R., Escher, J.C., Adam, R., Carter, T., Koster, A.J., van den Biggelaar, M., Voorberg, J., Bierings, R., 2018. Weibel-Palade Body Localized Syntaxin-3 Modulates Von Willebrand Factor Secretion From Endothelial Cells. Arterioscler Thromb Vasc Biol 38, 1549–1561.

Schillemans, M., Kat, M., Westeneng, J., Gangaev, A., Hofman, M., Nota, B., van Alphen, F.P.J., de Boer, M., van den Biggelaar, M., Margadant, C., Voorberg, J., Bierings, R., 2019. Alternative trafficking of Weibel-Palade body proteins in CRISPR/Cas9-engineered von Willebrand factor–deficient blood outgrowth endothelial cells. Res Pract Thromb Haemost 3, 718–732.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682.

Schnabel, A., Hedrich, C.M., 2019. Childhood Vasculitis. Front Pediatr 6, 421.

Schnappauf, O., Ombrello, A.K., Kastner, D.L., 2020. Deficiency of adenosine deaminase 2: Is it an elephant after all? Journal of Allergy and Clinical Immunology S0091674920305728.

Schnitzer, J.E., Oh, P., 1994. Albondin-mediated capillary permeability to albumin. Differential role of receptors in endothelial transcytosis and endocytosis of native and modified albumins. J Biol Chem 269, 6072–6082.

Scotland, R.S., Madhani, M., Chauhan, S., Moncada, S., Andresen, J., Nilsson, H., Hobbs, A.J., Ahluwalia, A., 2005. Investigation of Vascular Responses in Endothelial Nitric Oxide Synthase/Cyclooxygenase-1 Double-Knockout Mice. Circulation 111, 796–803.

Sek, A.C., Xie, Z., Terai, K., Long, L.M., Nelson, C., Dudek, A.Z., Druey, K.M., 2015. Endothelial Expression of Endothelin Receptor A in the Systemic Capillary Leak Syndrome. PLoS One 10.

Selvam, S.N., Casey, L.J., Bowman, M.L., Hawke, L.G., Longmore, A.J., Mewburn, J., Ormiston, M., Archer, S.L., Maurice, D.H., James, P.D., 2017. Abnormal angiogenesis in blood outgrowth endothelial cells derived from von Willebrand disease patients. Blood Coagul Fibrinolysis 28, 521–533.

Sexl, V., Mancusi, G., Baumgartner-Parzer, S., Schütz, W., Freissmuth, M., 1995. Stimulation of human umbilical vein endothelial cell proliferation by A2-adenosine and beta 2-adrenoceptors. Br J Pharmacol 114, 1577–1586.

Shao Ying, Saredy Jason, Yang William Y., Sun Yu, Lu Yifan, Saaoud Fatma, Drummer Charles, Johnson Candice, Xu Keman, Jiang Xiaohua, Wang Hong, Yang Xiaofeng, 2020. Vascular Endothelial Cells and Innate Immunity. Arteriosclerosis, Thrombosis, and Vascular Biology 40, e138–e152.

Shavit, E., Alavi, A., Sibbald, R.G., 2018. Vasculitis—What Do We Have to Know? A Review of Literature. The International Journal of Lower Extremity Wounds 17, 218–226.

Shinaoka, A., Momota, R., Shiratsuchi, E., Kosaka, M., Kumagishi, K., Nakahara, R., Naito, I., Ohtsuka, A., 2013. Architecture of the Subendothelial Elastic Fibers of Small Blood Vessels and Variations in Vascular Type and Size. Microscopy and Microanalysis 19, 406–414.

Shweiki, D., Neeman, M., Itin, A., Keshet, E., 1995. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proc Natl Acad Sci U S A 92, 768–772.

Sibal, L., Agarwal, S.C., Home, P.D., Boger, R.H., 2010. The Role of Asymmetric Dimethylarginine (ADMA) in Endothelial Dysfunction and Cardiovascular Disease. Curr Cardiol Rev 6, 82–90.

Silverman, M.H., Strand, V., Markovits, D., Nahir, M., Reitblat, T., Molad, Y., Rosner, I., Rozenbaum, M., Mader, R., Adawi, M., Caspi, D., Tishler, M., Langevitz, P., Rubinow, A., Friedman, J., Green, L., Tanay, A., Ochaion, A., Cohen, S., Kerns, W.D., Cohn, I., Fishman-Furman, S., Farbstein, M., Yehuda, S.B., Fishman, P., 2008. Clinical evidence for utilization of the A3 adenosine receptor as a target to treat rheumatoid arthritis: data from a phase II clinical trial. The Journal of Rheumatology 35, 41–48.

Simionescu, M., Gafencu, A., Antohe, F., 2002. Transcytosis of plasma macromolecules in endothelial cells: A cell biological survey. Microscopy Research and Technique 57, 269–288.

Sims, D.E., 1986. The pericyte—A review. Tissue and Cell 18, 153–174.

Skrabl-Baumgartner, A., Plecko, B., Schmidt, W.M., König, N., Hershfield, M., Gruber-Sedlmayr, U., Lee-Kirsch, M.A., 2017. Autoimmune phenotype with type I interferon signature in two brothers with ADA2 deficiency carrying a novel CECR1 mutation. Pediatr Rheumatol Online J 15.

Smadja, D.M., Dorfmüller, P., Guerin, C.L., Bieche, I., Badoual, C., Boscolo, E., Kambouchner, M., Cazes, A., Mercier, O., Humbert, M., Gaussem, P., Bischoff, J., Israël-Biet, D., 2014. Cooperation between human fibrocytes and endothelial colony-forming cells increases angiogenesis via the CXCR4 pathway. Thromb Haemost 112, 1002–1013.

Smadja, D.M., Mauge, L., Nunes, H., d'Audigier, C., Juvin, K., Borie, R., Carton, Z., Bertil, S., Blanchard, A., Crestani, B., Valeyre, D., Gaussem, P., Israel-Biet, D., 2013. Imbalance of circulating endothelial cells and progenitors in idiopathic pulmonary fibrosis. Angiogenesis 16, 147–157.

Smits, J., Tasev, D., Andersen, S., Szulcek, R., Botros, L., Ringgaard, S., Andersen, A., Vonk-Noordegraaf, A., Koolwijk, P., Bogaard, H.J., 2018. Blood Outgrowth and Proliferation of Endothelial Colony Forming Cells are Related to Markers of Disease Severity in Patients with Pulmonary Arterial Hypertension. Int J Mol Sci 19.

Solovey, A., Lin, Y., Browne, P., Choong, S., Wayner, E., Hebbel, R.P., 1997. Circulating Activated Endothelial Cells in Sickle Cell Anemia. N Engl J Med 337:1584-1590.

Somani, A., Nair, S.L., Milbauer, L.C., Zhu, G., Sajja, S., Solovey, A., Chen, Y., and Hebbel, R.P. (2019). Blood Outgrowth Endothelial Cells overexpressing eNOS mitigate pulmonary hypertension in rats: A unique carrier cell enabling autologous cell-based gene therapy. Transl Res *210*, 1–7. https://doi.org/10.1016/j.trsl.2019.04.005.

Song, J.W., Zullo, J.A., Liveris, D., Dragovich, M., Zhang, X.F., Goligorsky, M.S., 2017. Therapeutic Restoration of Endothelial Glycocalyx in Sepsis. J Pharmacol Exp Ther 361, 115–121.

Sperandio, M., 2006. Selectins and glycosyltransferases in leukocyte rolling in vivo. The FEBS Journal 273, 4377–4389.

Spiegler, S., Rath, M., Much, C.D., Sendtner, B.S., Felbor, U., 2019. Precise CCM1 gene correction and inactivation in patient-derived endothelial cells: Modeling Knudson's two-hit hypothesis in vitro. Mol Genet Genomic Med 7.

Springer, J.M., Gierer, S.A., Jiang, H., Kleiner, D., Deuitch, N., Ombrello, A.K., Grayson, P.C., Aksentijevich, I., 2018. Deficiency of Adenosine Deaminase 2 in Adult Siblings: Many Years of a Misdiagnosed Disease With Severe Consequences. Front Immunol 9.

Spruntulis, L.M., Broadley, K.J., 2001. A3 receptors mediate rapid inflammatory cell influx into the lungs of sensitized guinea-pigs. Clinical & Experimental Allergy 31, 943–951.

Starke, R.D., Paschalaki, K.E., Dyer, C.E.F., Harrison-Lavoie, K.J., Cutler, J.A., McKinnon, T.A.J., Millar, C.M., Cutler, D.F., Laffan, M.A., Randi, A.M., 2013. Cellular and molecular basis of von Willebrand disease: studies on blood outgrowth endothelial cells. Blood 121, 2773–2784.

Stern, D.M., Nawroth, P.P., Harris, K., Esmon, C.T., 1986. Cultured bovine aortic endothelial cells promote activated protein C-protein S-mediated inactivation of factor Va. J Biol Chem 261, 713–718.

Stockinger, H., Gadd, S.J., Eher, R., Majdic, O., Schreiber, W., Kasinrerk, W., Strass, B., Schnabl, E., Knapp, W., 1990. Molecular characterization and functional analysis of the leukocyte surface protein CD31. The Journal of Immunology 145, 3889–3897.

Stoll, B.R., Migliorini, C., Kadambi, A., Munn, L.L., Jain, R.K., 2003. A mathematical model of the contribution of endothelial progenitor cells to angiogenesis in tumors: implications for antiangiogenic therapy. Blood 102, 2555–2561.

Stratta, P., Messuerotti, A., Canavese, C., Coen, M., Luccoli, L., Bussolati, B., Giorda, L., Malavenda, P., Cacciabue, M., Bugiani, M., Bo, M., Ventura, M., Camussi, G., Fubini, B., 2001. The role of metals in autoimmune vasculitis: epidemiological and pathogenic

study. Science of The Total Environment, Environment, Human Health and Immune System (selected papers from International Symposium on Occupational and Environmental Allergy and Immune Diseases,Chieti(Italy),April 27-30,1999 270, 179–190.

Stroncek, J.D., Grant, B.S., Brown, M.A., Povsic, T.J., Truskey, G.A., Reichert, W.M., 2009. Comparison of Endothelial Cell Phenotypic Markers of Late-Outgrowth Endothelial Progenitor Cells Isolated from Patients with Coronary Artery Disease and Healthy Volunteers. Tissue Eng Part A 15, 3473–3486.

Stroncek, J.D., Ren, L.C., Klitzman, B., Reichert, W.M., 2012. Patient-derived endothelial progenitor cells improve vascular graft patency in a rodent model. Acta Biomaterialia 8, 201–208.

Stroncek, J.D., Xue, Y., Haque, N., Lawson, J.H., Reichert, W.M., 2011. In Vitro Functional Testing of Endothelial Progenitor Cells That Overexpress Thrombomodulin. Tissue Eng Part A 17, 2091–2100.

Suchting, S., Freitas, C., Noble, F. le, Benedito, R., Bréant, C., Duarte, A., Eichmann, A., 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. PNAS 104, 3225–3230.

Sundar, U.M., Ugusman, A., Chua, H.K., Latip, J., Aminuddin, A., 2019. Piper sarmentosum Promotes Endothelial Nitric Oxide Production by Reducing Asymmetric Dimethylarginine in Tumor Necrosis Factor-α-Induced Human Umbilical Vein Endothelial Cells. Front Pharmacol 10, 1033.

Sung, S.-H., Wu, T.-C., Chen, J.-S., Chen, Y.-H., Huang, P.-H., Lin, S.-J., Shih, C.-C., Chen, J.-W., 2013. Reduced number and impaired function of circulating endothelial progenitor cells in patients with abdominal aortic aneurysm. International Journal of Cardiology 168, 1070–1077.

Szymborska, A., Gerhardt, H., 2018. Hold Me, but Not Too Tight-Endothelial Cell-Cell Junctions in Angiogenesis. Cold Spring Harb Perspect Biol 10, a029223.

Tajadini, M., Panjehpour, M., Javanmard, S.H., 2014. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. Adv Biomed Res 3.

Tasev, D., Dekker-Vroling, L., van Wijhe, M., Broxterman, H.J., Koolwijk, P., van Hinsbergh, V.W.M., 2018. Hypoxia Impairs Initial Outgrowth of Endothelial Colony Forming Cells and Reduces Their Proliferative and Sprouting Potential. Front Med (Lausanne) 5.

Teixeira, M.M., Williams, T.J., Hellewell, P.G., 1993. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin. British Journal of Pharmacology 110, 1515–1521.

Tennant, M., McGeachie, J.K., 1990. Blood Vessel Structure and Function: A Brief Update on Recent Advances. Australian and New Zealand Journal of Surgery 60, 747–753.

Teofili, L., Martini, M., Iachininoto, M.G., Capodimonti, S., Nuzzolo, E.R., Torti, L., Cenci, T., Larocca, L.M., Leone, G., 2011. Endothelial progenitor cells are clonal and exhibit the JAK2V617F mutation in a subset of thrombotic patients with Ph-negative myeloproliferative neoplasms. Blood 117, 2700–2707.

Tesfamariam, B., 1994. Selective impairment of endothelium-dependent relaxations by prostaglandin endoperoxide. J Hypertens 12, 41–47.

Tesser, A., Pin, A., Mencaroni, E., Gulino, V., Tommasini, A., 2021. Vasculitis, Autoimmunity, and Cytokines: How the Immune System Can Harm the Brain. Int J Environ Res Public Health 18, 5585.

Thi, M.M., Tarbell, J.M., Weinbaum, S., Spray, D.C., 2004. The role of the glycocalyx in reorganization of the actin cytoskeleton under fluid shear stress: A “bumper-car” model. Proc Natl Acad Sci U S A 101, 16483–16488.

Thill, M., Berna, M.J., Kunst, F., Wege, H., Strunnikova, N.V., Gordiyenko, N., Grierson, R., Richard, G., Csaky, K.G., 2011. SU5416 induces premature senescence in endothelial progenitor cells from patients with age-related macular degeneration. Mol Vis 17, 85–98.

Torres, E.N., Yang, B., Brahmbhatt, A., Mukhopadhyay, D., Misra, S., 2014. Blood outgrowth endothelial cells reduce hypoxia-mediated fibroblast to myofibroblast conversion by decreasing pro-angiogenic cytokines. J Vasc Res 51, 458–467.

Toshner, M., Voswinckel, R., Southwood, M., Al-Lamki, R., Howard, L.S.G., Marchesan, D., Yang, J., Suntharalingam, J., Soon, E., Exley, A., Stewart, S., Hecker, M., Zhu, Z., Gehling, U., Seeger, W., Pepke-Zaba, J., Morrell, N.W., 2009. Evidence of Dysfunction of Endothelial Progenitors in Pulmonary Arterial Hypertension. Am J Respir Crit Care Med 180, 780–787.

Tracey, D., Klareskog, L., Sasso, E.H., Salfeld, J.G., Tak, P.P., 2008. Tumor necrosis factor antagonist mechanisms of action: A comprehensive review. Pharmacology & Therapeutics 117, 244–279.

Tran, C.T.L., Fox, M.F., Vallance, P., Leiper, J.M., 2000. Chromosomal Localization, Gene Structure, and Expression Pattern of DDAH1: Comparison with DDAH2 and Implications for Evolutionary Origins. Genomics 68, 101–105.

Trotta, L., Martelius, T., Siitonen, T., Hautala, T., Hämäläinen, S., Juntti, H., Taskinen, M., Ilander, M., Andersson, E.I., Zavialov, A., Kaustio, M., Keski-Filppula, R., Hershfield, M., Mustjoki, S., Tapiainen, T., Seppänen, M., Saarela, J., 2018. ADA2 deficiency: Clonal lymphoproliferation in a subset of patients. Journal of Allergy and Clinical Immunology 141, 1534-1537.e8.

Trowbridge, J.M., Gallo, R.L., 2002. Dermatan sulfate: new functions from an old glycosaminoglycan. Glycobiology 12, 117R-125R.

Tsao, P., Cao, T., Zastrow, M. von, 2001. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends in Pharmacological Sciences 22, 91–96.

Uguccioni, M., D’Apuzzo, M., Loetscher, M., Dewald, B., Baggiolini, M., 1995. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1α and MIP-1β on human monocytes. European Journal of Immunology 25, 64–68.

Urner, M., Herrmann, I.K., Buddeberg, F., Schuppli, C., Z’graggen, B.R., Hasler, M., Schanz, U., Mehr, M., Spahn, D.R., Schimmer, B.B., 2012. Effects of Blood Products on Inflammatory Response in Endothelial Cells In Vitro. PLOS ONE 7, e33403.

Valadbeigi, S., Saghiri, R., Ebrahimi-Rad, M., Khatami, S., Akhbari, H., 2019. Adenosine Deaminase Activity and HLA-DRB as Diagnostic Markers for Rheumatoid Arthritis. Curr Rheumatol Rev 15, 44–49.

Vallance, P., Leiper, J., 2004. Cardiovascular Biology of the Asymmetric Dimethylarginine:Dimethylarginine Dimethylaminohydrolase Pathway. Arteriosclerosis, Thrombosis, and Vascular Biology 24, 1023–1030.

van Beem, R.T., Verloop, R.E., Kleijer, M., Noort, W.A., Loof, N., Koolwijk, P., van der Schoot, C.E., van Hinsbergh, V.W.M., Zwaginga, J.J., 2009. Blood outgrowth endothelial cells from cord blood and peripheral blood: angiogenesis-related characteristics in vitro. J. Thromb. Haemost. 7, 217–226.

Van Belle, H., Goossens, F., Wynants, J., 1987. Formation and release of purine catabolites during hypoperfusion, anoxia, and ischemia. American Journal of Physiology-Heart and Circulatory Physiology 252, H886–H893.

van Breevoort, D., Snijders, A.P., Hellen, N., Weckhuysen, S., van Hooren, K.W.E.M., Eikenboom, J., Valentijn, K., Fernandez-Borja, M., Ceulemans, B., De Jonghe, P., Voorberg, J., Hannah, M., Carter, T., Bierings, R., 2014. STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. Blood 123, 3185–3194.

van de Pol, V., Bons, L.R., Lodder, K., Kurakula, K.B., Sanchez-Duffhues, G., Siebelink, H.-M.J., Roos-Hesselink, J.W., DeRuiter, M.C., Goumans, M.-J., 2019. Endothelial Colony Forming Cells as an Autologous Model to Study Endothelial Dysfunction in Patients with a Bicuspid Aortic Valve. Int J Mol Sci 20.

van den Berg, B.M., Spaan, J.A.E., Rolf, T.M., Vink, H., 2006. Atherogenic region and diet diminish glycocalyx dimension and increase intima-to-media ratios at murine carotid artery bifurcation. American Journal of Physiology-Heart and Circulatory Physiology 290, H915–H920.

Van Eyck, L., Hershfield, M.S., Pombal, D., Kelly, S.J., Ganson, N.J., Moens, L., Frans, G., Schaballie, H., De Hertogh, G., Dooley, J., Bossuyt, X., Wouters, C., Liston, A., Meyts, I., 2015. Hematopoietic stem cell transplantation rescues the immunologic phenotype and prevents vasculopathy in patients with adenosine deaminase 2 deficiency. J Allergy Clin Immunol 135, 283-287.e5.

van Haaren, P.M.A., VanBavel, E., Vink, H., Spaan, J.A.E., 2003. Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy. American Journal of Physiology-Heart and Circulatory Physiology 285, H2848–H2856.

van Montfrans, J., Zavialov, A., Zhou, Q., 2014. Mutant ADA2 in Vasculopathies. N Engl J Med 371, 478–481.

van Well, G.T.J., Kant, B., van Nistelrooij, A., Sirma Ekmekci, S., Henriet, S.V., Hoppenreijs, E., van Deuren, M., van Montfrans, J., Nierkens, S., Gül, A., van Gijn, M.E., 2019. Phenotypic variability including Behçet's disease-like manifestations in DADA2 patients due to a homozygous c.973-2A>G splice site mutation. Clin. Exp. Rheumatol. 37 Suppl 121, 142–146.

Verma, S., Anderson, T.J., 2002. Fundamentals of Endothelial Function for the Clinical Cardiologist. Circulation 105, 546–549.

Vink, H., Constantinescu, A.A., Spaan, J.A.E., 2000. Oxidized Lipoproteins Degrade the Endothelial Surface Layer. Circulation 101, 1500–1502.

Vink Hans, Duling Brian R., 1996. Identification of Distinct Luminal Domains for Macromolecules, Erythrocytes, and Leukocytes Within Mammalian Capillaries. Circulation Research 79, 581–589.

von Willebrand, E., Lautenschlager, I., Inkinen, K., Lehto, V.-P., Virtanen, I., Häyry, P., 1985. Distribution of the major histocompatibility complex antigens in human and rat kidney. Kidney International 27, 616–621.

Voyvodic, P.L., Min, D., Liu, R., Williams, E., Chitalia, V., Dunn, A.K., Baker, A.B., 2014. Loss of Syndecan-1 Induces a Pro-inflammatory Phenotype in Endothelial Cells with a Dysregulated Response to Atheroprotective Flow. J Biol Chem 289, 9547–9559.

Wang, L., Londono, L.M., Cowell, J., Saatci, O., Aras, M., Ersan, P.G., Serra, S., Pei, H., Clift, R., Zhao, Q., et al. (2021). Targeting Adenosine with Adenosine Deaminase 2 to Inhibit Growth of Solid Tumors. Cancer Research *81*, 3319–3332.

Wang, H., Song, S., Kou, G., Li, B., Zhang, D., Hou, S., Qian, W., Dai, J., Tian, L., Zhao, J., Guo, Y., 2007. Treatment of hepatocellular carcinoma in a mouse xenograft model with an immunotoxin which is engineered to eliminate vascular leak syndrome. Cancer Immunol Immunother 56, 1775–1783.

Wang, J.-W., Bouwens, E.A.M., Pintao, M.C., Voorberg, J., Safdar, H., Valentijn, K.M., de Boer, H.C., Mertens, K., Reitsma, P.H., Eikenboom, J., 2013. Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. Blood 121, 2762–2772.

Wang, X., Cooper, S., 2013. Adhesion of Endothelial Cells and Endothelial Progenitor Cells on Peptide-Linked Polymers in Shear Flow. Tissue Eng Part A 19, 1113–1121.

Wang, Y., Cao, J., Fan, Y., Xie, Y., Xu, Z., Yin, Z., Gao, L., Wang, C., 2016. Artemisinin inhibits monocyte adhesion to HUVECs through the NF-κB and MAPK pathways in vitro. International Journal of Molecular Medicine 37, 1567–1575.

Wei, K., Karen, E., Joanne, W., 2004. (Section A: Molecular, Structural, and Cellular Biology of Drug Transporters) Mammalian Nucleoside Transporters. Current Drug Metabolism 5, 63–84.

Weinbaum, S., Tarbell, J.M., Damiano, E.R., 2007. The Structure and Function of the Endothelial Glycocalyx Layer. Annual Review of Biomedical Engineering 9, 121–167.

Weksler, B.B., Ley, C.W., Jaffe, E.A., 1978. Stimulation of Endothelial Cell Prostacyclin Production by Thrombin, Trypsin, and the Ionophore A 23187. J Clin Invest 62, 923–930.

Wettschureck, N., Strilic, B., Offermanns, S., 2019. Passing the Vascular Barrier: Endothelial Signaling Processes Controlling Extravasation. Physiological Reviews 99, 1467–1525.

WHO, 2021. Cardiovascular diseases (CVDs). World Health Organization. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (Accessed: 7.26.21).

Wiesinger, A., Peters, W., Chappell, D., Kentrup, D., Reuter, S., Pavenstädt, H., Oberleithner, H., Kümpers, P., 2013. Nanomechanics of the Endothelial Glycocalyx in Experimental Sepsis. PLOS ONE 8, e80905.

Winn, H.R., Rubio, R., Berne, R.M., 1981. Brain adenosine concentration during hypoxia in rats. American Journal of Physiology-Heart and Circulatory Physiology 241, H235–H242.

Winsauer, G., Martin, R. de, 2007. Resolution of inflammation: Intracellular feedback loops in the endothelium. Thromb Haemost 97, 364–369.

Wojciak-Stothard, B., Abdul-Salam Vahitha B., Lao Ka Hou, Tsang Hilda, Irwin David C., Lisk Christina, Loomis Zoe, Stenmark Kurt R., Edwards John C., Yuspa Stuart H., Howard Luke S., Edwards Robert J., Rhodes Christopher J., Gibbs J. Simon R., Wharton John, Zhao Lan, Wilkins Martin R., 2014. Aberrant Chloride Intracellular Channel 4 Expression Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension. Circulation 129, 1770–1780.

Wyatt, A.W., Steinert, J.R., Wheeler-Jones, C.P.D., Morgan, A.J., Sugden, D., Pearson, J.D., Sobrevia, L., Mann, G.E., 2002. Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. The FASEB Journal 16, 1584–1594.

Xiong, J.-P., Stehle, T., Goodman, S.L., Arnaout, M.A., 2003. Integrins, cations and ligands: making the connection. Journal of Thrombosis and Haemostasis 1, 1642–1654.

Yadegari, H., Biswas, A., Akhter, M.S., Driesen, J., Ivaskevicius, V., Marquardt, N., Oldenburg, J., 2016. Intron retention resulting from a silent mutation in the VWF gene that structurally influences the 5' splice site. Blood 128, 2144–2152.

Yan, G., You, B., Chen, S.-P., Liao, J.K., Sun, J., 2008. TNF-alpha Downregulates Endothelial Nitric Oxide Synthase mRNA Stability via Translation Elongation Factor 1-alpha 1. Circ Res 103, 591–597.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yang, D., Tan, S., Yang, Z., Jiang, P., Qin, C., Yuan, Q., Dang, R., Yao, X., Qu, J., Lu, Q., Xu, P., Zhang, B., Xiang, D., Chen, L., 2018. Dihydromyricetin Attenuates TNF-α-Induced Endothelial Dysfunction through miR-21-Mediated DDAH1/ADMA/NO Signal Pathway. BioMed Research International 2018, e1047810.

Yang, Y.-H., Chuang, Y.-H., Wang, L.-C., Huang, H.-Y., Gershwin, M.E., Chiang, B.-L., 2008. The immunobiology of Henoch–Schönlein purpura. Autoimmunity Reviews, Special Issue on Mosaic of Autoimmunity 7, 179–184.

Yao, Y., Rabodzey, A., Dewey, C.F., 2007. Glycocalyx modulates the motility and proliferative response of vascular endothelium to fluid shear stress. American Journal of Physiology-Heart and Circulatory Physiology 293, H1023–H1030.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13, 134.

Yegutkin, G.G., 2008. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1783, 673–694.

Yilmaz, O., Afsar, B., Ortiz, A., Kanbay, M., 2019. The role of endothelial glycocalyx in health and disease. Clinical Kidney Journal.

Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T., Ingram, D.A., 2007. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109, 1801–1809.

Yoneyama, Y., Sawa, R., Suzuki, S., Shin, S., Power, G.G., Araki, T., 1996. The relationship between uterine artery Doppler velocimetry and umbilical venous adenosine levels in pregnancies complicated by preeclampsia. American Journal of Obstetrics and Gynecology 174, 267–271.

Yu, W.-H., Woessner, J.F., 2000. Heparan Sulfate Proteoglycans as Extracellular Docking Molecules for Matrilysin (Matrix Metalloproteinase 7) *. Journal of Biological Chemistry 275, 4183–4191.

Zavialov, Anton V., Yu, X., Spillmann, D., Lauvau, G., Zavialov, Andrey V., 2010a. Structural Basis for the Growth Factor Activity of Human Adenosine Deaminase ADA2. J Biol Chem 285, 12367–12377.

Zavialov, A.V., Engström, Å., 2005. Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity. Biochem J 391, 51–57.

Zavialov, A. V., Gracia, E., Glaichenhaus, N., Franco, R., Zavialov, A. V., Lauvau, G., 2010b. Human adenosine deaminase 2 induces differentiation of monocytes into macrophages and stimulates proliferation of T helper cells and macrophages. Journal of Leukocyte Biology 88, 279–290.

Zavoico, G.B., Ewenstein, B.M., Schafer, A.I., Pober, J.S., 1989. IL-1 and related cytokines enhance thrombin-stimulated PGI2 production in cultured endothelial cells without affecting thrombin-stimulated von Willebrand factor secretion or platelet-activating factor biosynthesis. The Journal of Immunology 142, 3993–3999.

Zeng, Y., Ebong, E.E., Fu, B.M., Tarbell, J.M., 2012. The Structural Stability of the Endothelial Glycocalyx after Enzymatic Removal of Glycosaminoglycans. PLOS ONE 7, e43168.

Zeng, Y., Tarbell, J.M., 2014. The Adaptive Remodeling of Endothelial Glycocalyx in Response to Fluid Shear Stress. PLOS ONE 9, e86249.

Zhou, P., Lu, S., Luo, Y., Wang, S., Yang, K., Zhai, Y., Sun, G., Sun, X., 2017. Attenuation of TNF-α-Induced Inflammatory Injury in Endothelial Cells by Ginsenoside Rb1 via Inhibiting NF-κB, JNK and p38 Signaling Pathways. Front. Pharmacol. 0.

Zhou, Q., Yang, D., Ombrello, A.K., Zavialov, A.V.A. V., Toro, C., Zavialov, A.V.A. V., Stone, D.L., Chae, J.J., Rosenzweig, S.D., Bishop, K., Barron, K.S., Kuehn, H.S., Hoffmann, P., Negro, A., Tsai, W.L., Cowen, E.W., Pei, W., Milner, J.D., Silvin, C., Heller, T., Chin, D.T., Patronas, N.J., Barber, J.S., Lee, C.-C.R., Wood, G.M., Ling, A., Kelly, S.J., Kleiner, D.E., Mullikin, J.C., Ganson, N.J., Kong, H.H., Hambleton, S., Candotti, F., Quezado, M.M., Calvo, K.R., Alao, H., Barham, B.K., Jones, A., Meschia, J.F., Worrall, B.B., Kasner, S.E., Rich, S.S., Goldbach-Mansky, R., Abinun, M., Chalom, E., Gotte, A.C., Punaro, M., Pascual, V., Verbsky, J.W., Torgerson, T.R., Singer, N.G., Gershon, T.R., Ozen, S., Karadag, O., Fleisher, T.A., Remmers, E.F., Burgess, S.M., Moir, S.L., Gadina, M., Sood, R., Hershfield, M.S., Boehm, M., Kastner, D.L., Aksentijevich, I., Heller, T., Chin, D.T., Patronas, N.J., Barber, J.S., Lee, C.-C.R., Wood, G.M., Ling, A., Kelly, S.J., Kleiner, D.E., Mullikin, J.C., Ganson, N.J., Kong, H.H., Hambleton, S., Candotti, F., Quezado, M.M., Calvo, K.R., Alao, H., Barham, B.K., Jones, A., Meschia, J.F., Worrall, B.B., Kasner, S.E., Rich, S.S., Goldbach-Mansky, R., Abinun, M., Chalom, E., Gotte, A.C., Punaro, M., Pascual, V., Verbsky, J.W., Torgerson, T.R., Singer, N.G., Gershon, T.R., Ozen, S., Karadag, O., Fleisher, T.A., Remmers, E.F., Burgess, S.M., Moir, S.L., Gadina, M., Sood, R., Hershfield, M.S., Boehm, M., Kastner, D.L., Aksentijevich, I., 2014. Early-Onset Stroke and Vasculopathy Associated with Mutations in ADA2. New England Journal of Medicine 370, 911–920.

Zhou, Q.Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L., Civelli, O., 1992. Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. Proc Natl Acad Sci U S A 89, 7432–7436.

Zimmermann, H., 1992. 5'-Nucleotidase: molecular structure and functional aspects. Biochem J 285, 345–365.