Exploring the role of Adenosine Deaminase 2 in Endothelial Homeostasis and in the Pathogenesis of Vascular Injury

A thesis submitted to Imperial College London for the degree of Doctor of Philosophy

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I, Andrew William Porter, confirm the work presented in this thesis is my own. I have indicated where work has been derived from other sources at the necessary places in this thesis.

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ABSTRACT

Deficiency of Adenosine Deaminase 2 (DADA2) is an autosomal recessive condition caused by biallelic variants in the Adenosine Deaminase 2 (ADA2) gene. Clinical manifestations are broad and include vasculitis, immunodeficiency and haematological involvement. The aim of this thesis was to explore the role of ADA2 in endothelial homeostasis and in the pathogenesis of vascular injury.

My work has confirmed that ADA2 is expressed by endothelial cells (EC) as a shortened transcript called ADA2 202 with undetectable protein and adenosine deaminase activity. We have demonstrated this using multiple transcriptomic technologies in both primary human endothelial cell lines and in patient derived endothelial colony forming cells (ECFC). This raises the question as to whether ADA2 202 RNA may function as a long non-coding RNA, or whether it is present as a protein at low undetectable levels in the cell, possibly in the insoluble fraction.

ADA2 202 was shown to have a role in endothelial homeostasis and its deficiency leads to inflammatory EC activation with a gene signature suggestive of both type I and type II interferon and Tumour Necrosis Factor α pathway activation together with induction of apoptotic pathways. ECFC from DADA2 patients were observed to have a stressed morphology, upregulation of interferon related genes, evidence of reduced proliferation and increased senescence. These data all suggest that EC deficient of ADA2 202 are more susceptible to activation, injury and subsequent vasculitis and therefore may play a role in DADA2 pathogenesis.

Despite the heterogeneity of DADA2, we did not find evidence of any patients with plasma ADA2 deficiency in a cohort of patients with large and small vessel vasculitis. Consistent with other inflammatory conditions, plasma ADA2 activity was elevated in vasculitis patients and correlated with immune cells and markers of inflammation.

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ABBREVIATIONS

ACTB – Actin – β

ADA – Adenosine deaminase

ADA1 – Adenosine deaminase 1

ADA2 – Adenosine deaminase 2

ADGFs - Adenosine deaminase growth factors

AMP – Adenosine monophosphate

ATP – Adenosine triphosphate

ANCA – Anti- neutrophil cytoplasmic antibody

BSA – Bovine serum albumin

Ctr – Control

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

DADA2 – Deficiency of adenosine deaminase 2

dAdo - Deoxyadenosine

Dino - Deoxyinosine

DAPI – 4′,6-diamidino-2-phenylindole

DBA - Diamond-Blackfan anaemia

ECFCs – Endothelial Colony Forming Cells

EGM2 – Endothelial Cell Growth Medium-2

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

ELISA – Enzyme-linked immunosorbent assay

FBS – Foetal bovine serum

FDR – False discovery rate

FKPM – Fragments Per Kilobase per Million

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GCA – Giant cell arteritis

GSEA – Gene set enrichment analysis

HC – Healthy control

HAEC – Human Aortic Endothelial Cells

HDVEC – Human Dermal Vascular Endothelial Cells

HSCT – Haematopoietic stem cell transplantation

HSVEC – Human Saphenous Vein Endothelial Cells

HSPCs – Haematopoietic stem and progenitor cells

HUVEC – Human Umbilical Vein Endothelial Cells

ICAM1 – Intercellular Adhesion Molecule 1

KD – Knockdown

KEGG – Kyoto Encyclopaedia of Genes and Genomes

LEC – Lymphatic Endothelial Cells

L2FC – Log 2 fold change

IFNγ – Interferon γ

IL6 – Interleukin 6

lncRNA – long non-coding RNA

LDGs – Low-density granulocytes

MS – Mass spectrometry

NES – Normalised enrichment score

NET – Neutrophil extracellular trap

NK – Natural Killer cell

PAN – Polyarteritis nodosa

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PMA – phorbol 12-myristate 13-acetate

PRCA – pure red cell aplasia

qPCR – quantitative reverse transcription polymerase chain reaction

rADA2 – Recombinant Adenosine deaminase 2

RFU – Relative fluorescence units

RIPA – Radio-Immunoprecipitation Assay

RLT – RNeasy Lysis Buffer

RLU – Relative light units

RNA-seq – RNA sequencing

RT – Room temperature

SA-β-Gal – senescence-associated expression of β-galactosidase

SE – Standard error

SPP – Signalling Pathways Project

TAK – Takayasu arteritis

TBS – Tris-buffered saline

TBS-T – Tris-buffered saline with 0.1% Tween-20

TNFα – Tumour necrosis factor α

Ub – Ubiquitin

VCAM – Vascular Cell Adhesion Molecule

WoS – Web of Science

CHAPTER 1: INTRODUCTION

1.1 Deficiency of adenosine deaminase 2 (DADA2)

1.1.1 Introduction

The study of rare monogenic diseases has enhanced understanding, while also revealing pathogenic pathways in polygenic diseases and influencing management [1]. In March 2014 two separate groups published descriptions of a monogenic disease, Deficiency of Adenosine Deaminase 2 (DADA2), of early-onset vasculopathy, haemorrhagic and ischaemic stroke and a polyarteritis nodosa (PAN) like vasculitis [2, 3]. This was linked to loss of function biallelic mutations in the Adenosine Deaminase 2 (ADA2) gene (previously CECR1; Cat Eye Syndrome Chromosome Region, Candidate gene 1) located on the q arm of chromosome 22 at position 11.1 [2, 3]. Over time it was recognised that a significant proportion of the patients also had immunodeficiency and haematological involvement including pure red cell aplasia and bone marrow failure [4, 5]

The ADA2 gene encodes the dimeric extracellular enzyme adenosine deaminase 2 (ADA2), which is a protein comprised of 4 domains and is involved in purinergic signalling [6]. Pathogenic mutations in this gene have been associated with very low or absent ADA2 plasma deaminase activity in in vitro assays [7]. Since 2014 more than 300 cases have been published in the literature [8-11]. These cases describe a highly variable phenotype with heterogeneity in presentation from being clinically asymptomatic to severe and sometime fatal symptoms including stroke, bone marrow failure and severe immunodeficiency [4]. The age of presentation ranges from birth to 59 years [2, 12-14].

1.1.2 Clinical features

1.1.2.1 Vasculitis/vasculopathy

Vascular manifestations are the commonest phenotype reported in the literature of large DADA2 case series [15]. There are many similarities with the presentation of polyarteritis nodosa, another form of vasculitis, with cutaneous and neurological involvement frequently seen [15]. These clinical manifestations are often accompanied by evidence of systemic inflammation and fever [2, 3, 12, 16].

A livedoid racemosa/reticularis is the commonest cutaneous manifestation, but subcutaneous nodules, Raynauds's phenomenon with extensive digital ulceration and gangrene are also described [3, 15]. Skin biopsies of the livedoid rash have a predominance of neutrophils and

macrophages, with perivascular T lymphocytes, however other skin biopsies have shown a necrotising vasculitis, nonspecific leukocytoclastic vasculitis, panniculitis or thrombosis of capillaries [2, 3, 17, 18].

Neurological involvement in the form of stroke occurs frequently and can be both ischaemic or less commonly haemorrhagic in nature [9, 15]. The ischaemic strokes are typically deep lacunar infarcts and haemorrhages usually parenchymal bleeds [12]. Cerebral angiography is typically normal and only occasionally demonstrates evidence of arterial stenoses or aneurysms [12, 19]. Other central nervous system manifestations have been reported and include seizures, headaches and spinal cord involvement [12, 19-21]. Peripheral nervous system involvement is much less common but can involve cranial nerve palsies, peripheral neuropathy, sensorineural hearing loss and ocular involvement [3, 9, 22, 23].

The vasculitis can also affect the bowel, liver, kidney and other organs. Gastrointestinal system involvement can result in inflammatory bowel disease, mesenteric aneurysms, ischaemic abdominal pain and rarely bowel perforation [2, 12, 21, 24, 25]. Liver disease with elevated transaminases, hepatosplenomegaly and portal hypertension is observed in <10% of patients [3, 21]. Renal involvement can result in renal artery stenosis, renal infarcts and systemic hypertension [12]. Testicular pain with a biopsy demonstrating vasculitis is also described [3, 12]. Pulmonary haemorrhage, pancreatitis, dilated cardiomyopathy and coronary aneurysms are also documented in case reports [3, 9, 13]. Muscle and joint involvement can also be present with arthritis and myositis [2, 26]

1.1.2.2 Immunodeficiency, Autoimmune and Lymphoproliferative

Hypogammaglobulinaemia and low serum IgA and IgM were reported in association with ADA2 mutations in the initial descriptions of the disease and subsequently feature commonly in case series [2, 3, 10]. Decreased number of B cells, a reduction in the terminal differentiation of B cells, low NK cells and low number of T cells has also been reported [24, 27, 28]. Increased susceptibility to both bacterial and viral infections can be seen in these groups of patients [24, 29, 30]. The autoimmune phenomena in DADA2 are less common but positive autoantibodies including anti-phospholipid, anti-nuclear antibodies (ANA) and anti-neutrophil cytoplasmic antibodies (ANCA) have been reported [3, 12, 31].

Generalised lymphadenopathy and hepatosplenomegaly are also well described features in DADA2 [13, 32, 33]. An autoimmune lymphoproliferative syndrome phenotype and a T cell large granular lymphocyte phenotype has been reported in patients with biallelic ADA2 mutations [28, 34].

1.1.2.3 Haematological

Haematological manifestations were largely absent from the original descriptions of the disease, but it is increasingly evident that there is a broad haematological phenotype ranging from mild immunodeficiency to bone marrow failure. These have been reported both with and without clinical evidence of inflammation or vasculitis. Lymphopenia is common and seen in many of the clinical phenotypes [12]. A severe refractory anaemia which resembles Diamond-Blackfan anaemia (DBA), and pure red cell aplasia (PRCA) is also described in approximately 10% of DADA2 patients [8, 35, 36]. Bone marrow failure (BMF) with neutropenia, thrombocytopenia or pancytopenia is also reported [5, 36, 37]. Rarer manifestations include myelofibrosis, acute myeloid leukaemia and macrophage activation syndrome [3, 13, 35, 38]. Given the heterogeneity of presentation, DADA2 should therefore be considered in the differential diagnosis of bone marrow failure and idiopathic aplastic anaemia even in the absence of an inflammatory or vasculitic phenotype.

1.1.2.4 Epidemiology

In a review of 316 DADA2 patients, 52% were male [15]. 79.7% of the patients were Caucasians with 13.6% reported to be South Asians and 6.6% other ethnicities [15]. The age of disease onset is variable and ranges from birth to 59 years [12-14]. The majority of patients do present before 18 years of age but adult onset disease is increasingly recognised [12]. Mortality is high with up to 8% of patients dying before the age of 30 [10]. The commonest cause of death includes complications from recurrent stroke or infections [10].

1.1.3 Genetics

DADA2 is caused by autosomal recessive mutations, both homozygous and more commonly compound heterozygous, in chromosome 22q11.1 in the ADA2 gene [2, 3]. This gene is made up of 10 exons and encodes the ADA2 protein of 511 amino acids [3]. More than 100 disease causing variants are described in the literature and the Infevers database, a genetic database for hereditary inflammatory conditions [15, 39]. These are predominantly missense substitutions, but deletions, insertions and duplications are reported [39]. DADA2 associated mutations are located over the entire coding region of ADA2 [40]. The most common disease variants described are p.Gly47Arg (p.G47R), p.Gly47Ala (p.G47A), p.Arg169Gln (p.R169Q), and p.Tyr453Cys (p.Y453C) [2, 3, 40]. Population based genetic studies have estimated the carrier frequency of deleterious ADA2 variants is approximately 1 in 236 individuals [41]. This

corresponds to an estimated DADA2 disease prevalence of 1 in 222,000 individuals and predicts 30,000 DADA2 cases amongst the worldwide population [41].

1.1.4 Diagnosis

A diagnosis of DADA2 can be made by measurement of plasma or serum ADA2 enzymatic activity or by ADA2 gene sequencing [42]. Spectrophotometric assays that couple the deamination of adenosine to the release of ammonia can be used to quantify ADA2 enzymatic activity [3, 43]. Near-absent ADA2 activity is diagnostic for DADA2 [42]. A genetic diagnosis of DADA2 can be made by identification of biallelic pathogenic or likely pathogenic ADA2 variants [42]. This can be done with targeted sequencing of the ADA2 gene or whole exome sequencing [42]. One challenge with the genetic diagnosis of DADA2 is determining the importance of variants of unknown significance. In these cases, measurement of plasma ADA2 activity alongside genetic testing can be helpful. Additionally, standard targeted sequencing techniques don't always identify variants in non-coding regions, splicing variants and gene structural variants so additional genetic evaluation can be considered if the clinical suspicion is high [44, 45].

Individuals who have monoallelic ADA2 variants are considered carriers. Typically, they have detectable plasma or serum ADA2 activity but lower than that of healthy controls and have no clinical features of DADA2 [8]. There are some reports in the literature of individuals with single pathogenic ADA2 variants and plasma ADA2 activity in the carrier range who have mild or late onset features of the disease [9]. There is much debate about whether these patients do have biallelic variants, one of which is yet to be identified, or there is a phenotype to being a carrier of a single ADA2 variant. Once a diagnosis of DADA2 is confirmed, screening of parents, siblings and family members who have symptoms of DADA2 should be carried out [42].

1.1.5 Genotype-phenotype correlation

Given the broad spectrum of clinical features of DADA2, attempts have been made to understand whether there are any genotype-phenotype correlations. Ozen et al assessed the genotype of 14 DADA2 patients with a vasculitis (polyarteritis nodosa – PAN) phenotype and 10 with haematological features (Diamond-Blackfan anaemia – DBA) [35]. They demonstrated that ADA2 activity was similar between the groups, although the patients with the vasculitis phenotype had mutations in the dimerisation domain of the protein, versus patients with the haematological phenotype who had mutations in the catalytic domain [35]. Further work

compared the genotype and phenotype of 152 DADA2 patients. They showed complete loss of ADA2 activity was associated with haematological manifestations and low level residual enzymatic activity, of at least 3%, was more associated with vasculitis [8]. Phenotypic variability has however been demonstrated in individuals with the same mutation, including within families [7]. There must therefore be additional influencing factors such as the environment or possibly epigenetic modifiers [15].

1.1.6 Treatment and outcomes

The treatment of DADA2 depends on the primary disease manifestation. Patients were traditionally treated with immunosuppressive medications used in the treatment of other forms of vasculitis, such as steroids, azathioprine, methotrexate, mycophenolate mofetil and cyclophosphamide [2, 3, 9, 12, 46]. The efficacy of these treatments has proved disappointing with temporary efficacy demonstrated at best, and relapses occurring on tapering steroids [9, 13]. They also do not ameliorate stroke risk [7]. Tumour necrosis factor (TNFα) inhibitors have been shown to be highly effective in treating the vasculitic component of the condition, with adalimumab and etanercept the most commonly used [7, 9, 47]. A retrospective analysis of 31 patients with genetically confirmed DADA2 demonstrated that anti-TNF treatment significantly reduced the incidence of ischaemic events including stroke and other DADA2 vasculitic manifestations [48]. Some patients require higher than usual doses and switches between different anti-TNF agents [12]. One of the challenges is that treatment with anti-TNF is likely to be required life-long and carries a risk of resistance due to immunogenicity over time [12]. Long term outcomes with anti-TNF treatment are not yet known. Other cytokine inhibitors including anti-interleukin-1 therapy with anakinra or canakinumab, anti-interleukin 6 therapy with tocilizumab have only shown limited or no efficacy [7, 49].

Although effective for the vasculitic component, anti-TNF was ineffective for those with haematological manifestations [48]. This has been confirmed in further studies which demonstrated patients with pure red cell aplasia and bone marrow failure were refractory to TNF inhibitors [8]. The use of the anti-B cell (CD20) monoclonal antibody, Rituximab, has shown some response in those with DADA2 related cytopenia [33].Other immunosuppressive agents have not been effective for those with haematological disease [8, 36].

Allogenic hematopoietic stem cell transplantation (HSCT) has demonstrated favourable results in treating DADA2 patients with 97% overall survival and 73% relapse-free survival in 2 years with resolution of haematological phenotypes [50, 51]. However, issues regarding HLA matched donor and HSCT associated morbidity may limit its use. Graft versus host rejection

is of great concern and to date no optimal graft and conditioning regime available to balance between the success and toxicity of HSCT in DADA2 patient [50, 52].

Autologous gene therapy has become a promising new potential treatment for DADA2. The concept of autologous gene corrected haematopoietic stem and progenitor cell (HSPC) transplantation in DADA2 was first described by Zoccolillo et al. in a preclinical study where lentiviral vector encoding ADA2 was transduced into HSPCs of DADA2 patients to correct the enzymatic defect [53]. Restoration of intracellular ADA2 expression in CD34+ enabled HSPCs to retain their potential to differentiate into erythroid and myeloid cells [53]. In addition, amelioration of macrophage inflammatory activation was seen following gene therapy [53]. This concept was further supported by recent ex-vivo study by Hong Y et.al where lentiviral vector mediated ADA2 gene transduction in 8 DADA2 patients HSPCs led to restoration of enzymatic activity, marrow function, correction of macrophage cytokine production and amelioration of macrophage-mediated endothelial activation [54]. The proven therapeutic efficacy and safety of gene therapy in preclinical studies along with the growing clinical need of alternative treatment in DADA2 strongly supports rapid clinical translation of a gene therapy approach to treat DADA2.

As ADA2 is present in plasma, infusions of fresh frozen plasma were trialled to try and normalise plasma ADA2 activity [55]. The short half-life of ADA2 and the large volumes of infusions mean this approach was not feasible [55]. Future studies will assess whether a pegylated form of ADA2 may have a longer half-life and therefore be more effective.

1.2 Adenosine deaminases

In humans, there are two adenosine deaminase proteins: Adenosine deaminase 1 (ADA1), deficiency of which is a cause of severe combined immunodeficiency (SCID) [56], and ADA2 which when deficient leads to the DADA2 phenotype as described. These proteins have partial structural homology with 27% amino acid identity between them. However, they differ in many aspects including function and cell-type expression pattern [57]. ADA1 is a predominantly intracellular, 41-kDa monomer which is ubiquitously expressed, with highest levels in lymphocytes [58]. ADA2 is found mainly in myeloid cells and is secreted by monocytes, macrophages, dendritic cells and B-cells [59, 60]. ADA2 is a 59-kDA protein made up of four domains; signal peptide, dimerisation domain, putative receptor-binding domain and catalytic domains [61] (Figure 1). The ADA2 protein forms homodimers and its signal peptide and glycosylation sites suggest it is secreted to the extracellular space [61]. ADA1 and ADA2 have

non-redundant roles in the immune system as they cannot compensate for each other when one is lacking [62].



Figure 1 – ADA2 protein domains

ADA2 is made up of 4 domains (including 2 catalytic domains). The amino acid number is annotated at the top of each domain. N terminus to C terminus.

The crystal structures of ADA1 and ADA2 reveal differences in the catalytic sites, which explains some of the differences in enzymatic activity and susceptibility to inhibitors [61]. Both isoforms play a role in the purinergic signalling pathway by converting adenosine to inosine and 2'-deoxyadenosine to 2'-deoxyinosine however ADA1 has 100 fold greater affinity for these substrates [6]. ADA2 is more stable at high temperatures and its optimum pH for activity is 6.5 [6]. This may suggest that the deaminase activity of ADA2 plays a more specialised role in regulating extracellular adenosine at site of hypoxia, inflammation, and oncogenesis [6]. In keeping with this, ADA2 activity has been shown to be elevated in a number of inflammatory conditions including infections, autoimmune diseases and cancer [43, 63-66]. Given the described enzymatic characteristics, it is probable that both ADA1 and ADA2 are required to control levels of extracellular adenosine. Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) binds and inhibits ADA1 but not ADA2 [67]. This purine analogue is often used in enzymatic assays to differentiate between ADA1 and ADA2 activity.

ADA1 and ADA2 also exhibit differential binding to cells. ADA2 preferentially binds CD39+ immune cells including neutrophils, CD16+ monocytes, B cells and Tregs [62]. The putative receptor binding domain of ADA2 sits at the top of the catalytic domain and gives a butterfly shape to the molecule [68]. It is thought to dock ADA2 to a receptor on the surface of immune cells [60, 61].

Although ADA2 can catalyse the deamination reaction as described, it is thought to have a number of non-enzymatic functions. It has growth factor-like properties, and when secreted by activated monocytes, can induce monocyte and T cell proliferation as well as macrophage differentiation [6, 60, 61]. This was shown to be mediated by direct binding of cellular receptors as separation with a pore membrane abolished this monocyte-induced T cell proliferation [60]. ADA2 also plays a role in the balance between so called pro-inflammatory (M1) and anti-inflammatory (M2) monocytes. $ADA2^{-/-}$ human monocyte/macrophages adopt an M1-like

phenotype, release high-levels of TNFα and IL-1 and may directly injure endothelial cells (EC) [3, 62, 69].

ADA2 also has a similar sequence to adenosine deaminase growth factors (ADGFs) which were first identified in insects [70]. The deaminase activity of ADGFs was demonstrated to be essential to the growth of flesh flies and Drosophila [71, 72]. Elimination of ADGF-A in a Drosophila model led to larval death and it was suggested that the ADGFs protected tissues again increased levels of adenosine and deoxyadenosine [73, 74]. Knockdown of ADGF/ADA2 homologues in frogs led to a reduction in body size and abnormalities in the body axis [75]. The absence of an identifiable murine ADA2 ortholog has hindered *in vivo* studies of ADA2 function. Transgenic expression of ADA2 in mice results in the abnormal development of the heart and kidneys [76]. This all suggests ADA2 may be involved in the regulation of cell proliferation and differentiation.

The mechanisms through which extracellular ADA2 exerts its effects remain unknown. It binds cell-surface heparan sulphate [6], CD39+ cells [62] and modulates adenosine receptor affinity, leading to speculation that it may also bind adenosine receptors [61, 75]. The ectonucleotidases CD39 and CD73 mediate stepwise phosphohydrolysis of ATP to extracellular adenosine and are expressed on the EC surface, where they regulate homeostasis and defence against injurious effects of inflammation and thrombosis [77, 78].

	ADA1	ADA2
Gene	ADA	ADA2
Chromosome	20q13.12	22q11.1
Number of amino acids	363	511
Protein	41-kDa monomer	59-kDA homodimer
Cells expressed	Ubiquitously expressed, highest in lymphocytes	Predominantly myeloid cells, monocytes, macrophages
Cellular location	Intracellular	Secreted into extracellular space
Adenosine deaminase activity	High	100 fold lower affinity for adenosine and 2'-deoxyadenosine
Optimum pH for deaminase function	7.5	6.5
Function	Key role in purine metabolism	?purine metabolism at sites of inflammation Growth factor
Inhibited by EHNA	Yes	No
Syndrome when deficient	SCID	DADA2
Animal model	Expressed in mice	No mouse orthologue

Table 1 – ADA1 and ADA2 characteristics

1.2.1 Adenosine signalling

Adenosine is a purine nucleoside that plays an important role as a mediator of the immune response. Under physiological conditions, intracellular adenosine is derived predominantly from adenosine monophosphate (AMP) and released into the extracellular space via nucleoside transporters [79]. Under conditions of inflammation, hypoxia, ischaemia, trauma or cancer, there can be significant increases in the extracellular levels of adenosine from low nano-molar to micro-molar concentrations [80]. This is due to increased extracellular dephosphorylation of adenosine triphosphate (ATP) [81-83]. Extracellular adenosine concentrations are regulated through re-uptake into the intracellular space through nucleoside transporters as well as conversion into inosine by either ADA2 extracellularly or ADA1 intracellularly [84]. Adenosine exerts its effect through G protein-coupled cell surface

adenosine receptors (AR) of which there are four; A1, A2A, A2B and A3 [82]. These receptors are widely distributed on immune cells but also on endothelial cells [85-87]. Acute rises in adenosine have immunoregulatory anti-inflammatory effects to protect tissues [86]. In contrast however, chronically elevated adenosine concentrations can promote tissue injury and fibrosis [86].

1.3 ADA2 expression in endothelial cells

The prominent vasculopathic phenotype, with stroke and small-medium vessel vasculitis, in DADA2 suggests that ADA2 has a critical role in the maintenance of vascular integrity. Early studies did not show ADA2 mRNA or protein expression in the endothelium [2, 3]. A zebrafish model in which embryos were injected with an inhibitory oligonucleotide sequence to CECR1b (the human ADA2 analogue), resulted in embryos with intracranial haemorrhage, endothelial disruption and neutropenia [3]. This was inhibited by coinjection with nonmutant human ADA2 mRNA [3]. The same study also showed that when siADA2-depleted U937 monocyte cells or monocytes from DADA2 patients were cocultured with monolayers of human primary dermal microvascular endothelial cells there was significant endothelial disruption [3].

Examination of brain and skin biopsies from DADA2 patients shows significant endothelial damage and activation, with positive E-selectin staining [3]. Furthermore, there was also increased staining for interleukin-1β, inducible nitric oxide synthase, and TNFα, indicating inflammation [3]. Therefore, the evidence from these experimental models and biopsy studies strongly suggest that DADA2 is associated with significant endothelial damage that is primarily being mediated by activated monocytes and other immune cells, with the endothelium being a target rather than primary participant in this process.

More recent work has detected ADA2 expression in the endothelium and implicated it in the pro-inflammatory phenotype seen in DADA2. ADA2 mRNA transcripts were demonstrated in multiple primary human endothelial lineage cells by quantitative reverse transcription polymerase chain reaction (qPCR), and ADA2 protein in cultured supernatants of human endothelial cells by enzyme-linked immunosorbent assay (ELISA) and Western blotting [88].

Through measurement of conversion of ^{15}N-labeled dAdo to dIno by mass-spectrometry, it was demonstrated that >90% of intracellular ADA activity measured in human umbilical vein endothelial cells (HUVEC) was from ADA1 and >75% of the extracellular ADA activity in supernatant was from ADA2 [88]. This is in keeping with results obtained in human plasma where the majority of the ADA activity is from ADA2 [89].

siRNA knockdown of ADA2 in HUVEC led to spontaneous induction of IFNβ mRNA, and interferon related genes including CCL5, CXCL10, RSAD2 and IFIT1 [88]. Addition of recombinant ADA2 or ADA1 inhibited this induction. These rescue effects were prevented on addition of pentostatin, a general inhibitor of ADA activity [88]. When co-culture was carried out with siADA2-treated HUVEC and untreated U937 cells there was IFNβ production in bystander U937 cells indicating a paracrine effect of ADA2 in HUVEC [88]. This indicates that loss of endothelial ADA2 results in release of proinflammatory cytokines which can induce inflammatory signalling in immune cells and thus has raised the concept that endothelial ADA2 may have a role is DADA2 pathogenesis.

1.4 Pathogenesis of DADA2

The exact physiological functions of ADA2 and how this relates to DADA2 pathophysiology is not fully understood. There are a number of theories including catalytic dysfunction, immune cell dysfunction, endothelial cell dysfunction and growth factor dysfunction. It is likely that these factors all interplay to result in the heterogenous and broad phenotype seen in DADA2.

1.4.1 Catalytic dysfunction

This hypothesis assumes that a key function of ADA2 is to degrade extracellular adenosine generated from ATP breakdown at sites of cellular stress, ischaemia or inflammation. Depending on the adenosine concentration in the microenvironment and the adenosine receptor profile of the cellular infiltrate, adenosine can be either pro- or anti-inflammatory [82, 90]. Although plasma levels of adenosine in DADA2 are variable, they can be significantly elevated and critically, local tissue adenosine concentrations are likely to be markedly increased [2, 3, 69, 90]. Recent data have shown that neutrophil extracellular traps (NET) are present in affected tissue and in the circulation of patients with DADA2 [90]. Adenosine incubation with healthy peripheral blood neutrophils led to significantly increased NET formation compared to untreated neutrophils. This was reduced in the presence of recombinant ADA2 [90]. In addition, when control neutrophils were incubated with supernatant from DADA2 macrophages there was increased NET formation, relative to healthy controls, which was reversed with the addition of recombinant ADA2 (rADA2) but not recombinant ADA1 (rADA1). This indicates that deficiency of ADA2 catalytic enzyme activity may contribute to adenosine mediated NET formation in DADA2. The fact that ADA2 activity is elevated in several inflammatory conditions again suggests that it may be involved in the degradation of excess extracellular adenosine at sites of inflammation where there is an acidic pH and elevated temperature, both conditions where it has higher deaminase activity. [43, 63]. Thus,

deficiency of ADA2 may lead to impaired adenosine catabolism and thus to chronic inflammation at the endothelium.

A counter argument to this hypothesis relates to the very low affinity ADA2 has for adenosine and 2'-deoxyadenosine relative to ADA1 [6]. Bidirectional nucleoside transporters on the cell surface, have a high affinity for adenosine so rapidly equilibrate intra- and extracellular adenosine concentrations [58]. The concentration of free intracellular adenosine is kept very low by the actions of intracellular ADA1. Any extracellular generated adenosine is rapidly taken up and intracellularly metabolised [58]. In patients with SCID, who have ADA1 deficiency but preserved ADA2 function, the adenosine levels are significantly higher (approaching or exceeding 10μM) than that seen in DADA2 patients (0.2-0.6μM) [90, 91]. ADA1 therefore has a more important role than ADA2 in controlling extracellular adenosine concentrations.

1.4.2 Immune cell dysfunction

ADA2 is expressed and secreted by a wide range of immune cells in both the innate and adaptive immune systems [92]. It is therefore not surprising that deficiency of ADA2 has a broad effect on many immune interactions.

1.4.2.1 Monocytes and Macrophages

Monocytes and macrophages have been identified as having a key role in the pathogenesis of DADA2 since the first reports of the disease demonstrated an increase in proinflammatory M1-like macrophages and a relative reduction in anti-inflammatory M2-like subtype in DADA2 patients [2, 3]. M1-like monocytes release proinflammatory cytokines including TNFα which leads to subsequent endothelial damage [93]. Correction of ADA2 activity in DADA2 patient macrophages with a lentiviral vector resolves this inflammatory phenotype [53, 54].

Immunophenotyping of DADA2 patients showed reduced proportions of classical monocytes with increased intermediate and non-classical monocytes [94]. This was further demonstrated through single cell RNA sequencing of DADA2 monocyte populations [95]. Non-classical monocytes are a prominent source of TNFα, a cytokine highly implicated in the disease pathogenesis and known to activate a wide range of immune cells including macrophages, dendritic cells, T-cells and B-cells [96, 97]. TNFα has been shown to be detectable in the blood of DADA2 patients and when inhibited with anti-TNF treatment significantly improves the vasculitic phenotype of the disease [3, 9, 48, 93, 95].

Dysregulation of both Type I and Type II Interferons (IFN) have recently been demonstrated to play an important role in the pathogenesis of DADA2. Transcriptomic analysis of DADA2 patient monocytes demonstrated upregulation of IFNα/β and IFNγ signalling as well as

elevated plasma IFNγ [95]. Similar results were demonstrated in studies which analysed the transcriptome and proteome in peripheral blood mononuclear cells (PBMCs) or whole blood from DADA2 patients [93, 98, 99]. It was postulated that IFNγ activates cell signalling resulting in differentiation of monocytes to M1 macrophages and the subsequent release of TNFα [95]. Interestingly, the IFN and NFκB signatures in whole blood of DADA2 patients normalised after anti-TNF treatment [93]. Many further studies have demonstrated elevated IFN scores and upregulated type I IFN signalling in DADA2 patients giving further evidence of its importance in disease pathogenesis [26, 31, 94, 100, 101].

1.4.2.2 Natural Killer and Dendritic cells

ADA2 has been shown to be able to bind to NK cells but their involvement in DADA2 pathogenesis is thought to be limited [62]. There are some reports of reduced NK cell numbers in DADA2 patients [62]. Other studies suggest that there are an increased proportion of NK regulatory cells which have high cytokine production including TNFα [94]. Dendritic cells (DC) express and secrete ADA2, however immune phenotyping of DCs in DADA2 patients shows no impact on their total numbers or proportions [60].

1.4.2.3 Neutrophils

Upregulation of neutrophil expressed gene transcripts has been demonstrated in two patients with DADA2 and their PBMCs were shown to have increased myeloperoxidase expression relative to controls [101]. It is therefore thought that ADA2 may regulate neutrophil activation and deficiency of ADA2 leads to neutrophil related endothelial damage [101].

Dysregulation of NETosis, triggered by excess extracellular adenosine is also thought to play a significant role in the disease. Patients with DADA2 have been shown to have higher circulating numbers of low-density granulocytes (LDGs) which have previously been implicated in NET formation in other forms of vasculitis [90, 102]. After these DADA2 patients were treated with anti-TNF medication there was a significant reduction in the numbers of circulating LDGs [90]. In addition, M1 macrophages incubated with NETs from DADA2 patients secreted greater amounts of TNFα than healthy controls [90]. This all points to neutrophils playing a significant role in the pathogenesis of DADA2.

1.4.2.4 T cells and B cells

More evidence has come to light in recent times of the involvement of the adaptive immune system in DADA2 patients with evidence of both T-cell and B-cell defects [92]. ADA2 is known bind to the surface of T cells and act as a growth factor to induce T-cell dependent differentiation of monocytes into macrophages [60]. It is not therefore surprising that when

there is deficiency of ADA2 there is a decrease in CD4 and CD8 memory T-cells and impairment in the function of follicular T-cells [27, 52, 94]. This results in a fall in the T-cell generation of IL-21 which is a key cytokine for B-cell function [27]. DADA2 patient T-cells have also been shown to express markers consistent with a senescent phenotype [94]. Consistent with the IFN signature seen in other immune cells, single-cell RNA sequencing in DADA2 patient T-cells demonstrated activation of IFNα and IFNγ pathways [103].

B cell deficits have also been identified in DADA2 patients including a reduction in the number of memory B cells, terminally differentiated B cells and plasma cells and higher rates of spontaneous B cell death [3, 27, 69]. This would be consistent with the clinically phenotype of hypogammaglobulinaemia and recurrent infections [24].

1.4.3 Endothelial cell dysfunction

Initial reports of DADA2 suggested that endothelial cells did not express ADA2 and were not directly involved in disease pathogenesis but were a target of an activated immune system deficient of ADA2 [2, 3]. There is more recent evidence to suggest that this is might not be the case. ADA2 has been shown to be expressed by EC and that ADA2 knock-down results in induction of IFNβ mRNA and interferon related genes [88]. In addition, siADA2 HUVEC can signal in a paracrine manner to lead to IFNβ production in U937 cells [88]. It is therefore possible to postulate that ECs in DADA2 play a role in generating the inflammatory immune response and may be more susceptible to activation, injury and subsequent vasculitis.

1.4.4 Growth factor dysfunction

Although there are many theories regarding the pathogenesis of the vasculitic component of DADA2, the cause of the haematological manifestations remains unclear. ADA2 is a growth factor as part of the ADGF family and has been shown to be important in growth and development [61, 74, 75]. The fact that plasma ADA2 activity is highest in children and decreases with age may also be in keeping with this role as a growth factor [43]. ADA2 is involved in the differentiation of monocytes, macrophage proliferation and when knocked-down in the zebrafish model results in neutropenia. This again shows its importance in haematopoiesis [3, 60]. Furthermore, bone marrow from DADA2 patients showed reduced numbers of haematopoietic stem cells and progenitors of multiple lineages [53]. Given complete loss of ADA2 activity was shown to be associated with haematological manifestations and low level residual enzymatic activity was more associated with vasculitis, it is possible to hypothesise that small amounts of ADA2 are sufficient for haematopoiesis [8].

1.4.5 Summary

Thus, DADA2 represents a ‘perfect storm’ in which a combination of; dysregulated pro-inflammatory macrophages and monocytes secreting excess inflammatory cytokines, activated neutrophils with NET formation and B and T-cell dysfunction alongside increased tissue adenosine levels and lack of extracellular ADA2 to counteract this predisposes to endothelial damage and vascular injury (Figure 2) [3, 90, 92]. The lack of ADA2 as a growth factor leads to ineffective haematopoiesis. One important question to be explored in this PhD thesis will be the role of endothelial ADA2 and whether intrinsic loss of endothelial ADA2 contributes to disease pathogenesis. Is the endothelial damage and activation secondary to extrinsic loss of ADA2 in immune cells or does intrinsic deficiency of endothelial ADA2 contribute to inflammatory signalling and result in ECs that are susceptible to injury and subsequent vasculitis?

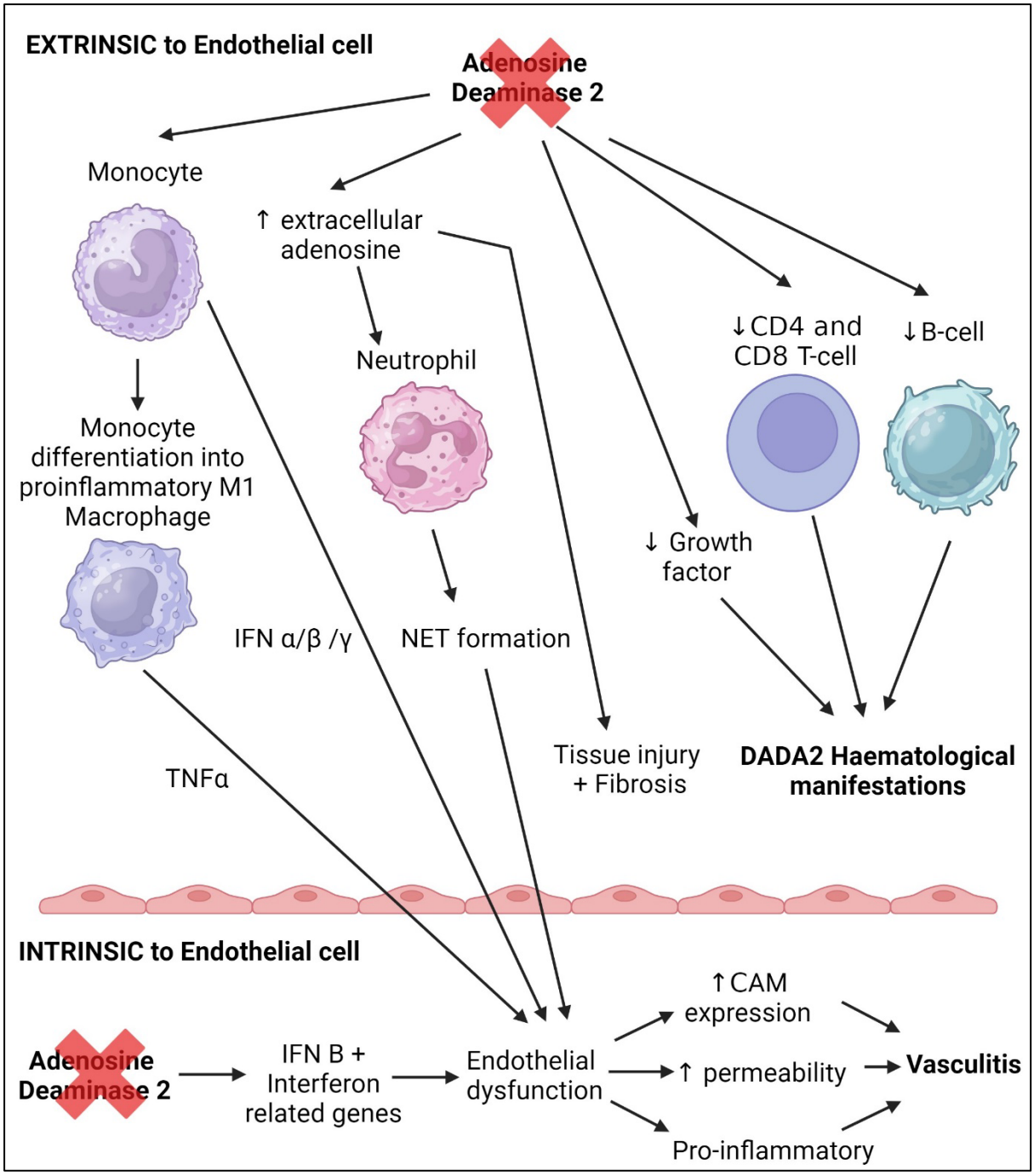


Figure 2 – Proposed pathogenesis of DADA2 considering mechanisms ADA2 deficiency intrinsic to EC and extrinsic to EC.

1.5 Endothelial cells in health and vasculitis

The lumen of all blood vessels and lymphatics is lined by a monolayer of EC and represents the interface between circulating blood and surrounding tissues. EC carry out a number of important functions to maintain vascular health including regulating the passage of nutrients and oxygen to underlying tissues, haemostasis, controlling vascular tone and permeability, angiogenesis, vascular repair and leucocyte adhesion and trafficking [104].

Persistent disturbance in any of these homeostatic mechanisms can lead to endothelial dysfunction [105]. This describes a pro-inflammatory, pro-coagulant, more permeable and less vasoactive endothelial state [105]. A number of factors and stimuli can lead to endothelial dysfunction including microbiological components, cytokines or damage-associated factors [105]. This pro-inflammatory state results in surface expression of leucocyte interacting proteins which leads to the recruitment and extravasation of leucocytes to the site of inflammation [106]. Leucocyte tethering, rolling and firm adhesion along the endothelium is modulated through a series of interactions and molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [106, 107]. This results in further release of inflammatory molecules, recruitment of yet more leucocytes and when these are activated and present chronically, leads to a number of the pathological features seen in vasculitis. The resulting damage to the endothelium also results in a pro-thrombotic state [108].

A persistent inflammatory response also induces angiogenesis, the formation of new blood vessels, to maintain leucocyte recruitment and the survival of activated immune cells at the site of inflammation [109]. Angiogenic molecules including vascular endothelial growth factor (VEGF) and TNFα are secreted by macrophages and other cell types which promotes neovascularisation [110, 111]. New blood vessel formation, driven by angiogenesis, is seen in patients with large vessel vasculitis (LVV) and is thought to be an important route of leucocyte trafficking into the arterial wall and therefore key in disease pathogenesis [112].

1.6 Endothelial colony forming cells

Endothelial colony forming cells (ECFC) are endothelial progenitor cells that can be isolated and cultured from peripheral blood [113]. They were first described in 2000 by Lin et al. who demonstrated the appearance of cells with endothelial characteristics after isolating and culturing human PBMCs on collagen-coated plates [114]. They had a cobblestone morphology, appeared after approximately 2-4 weeks of culture and were initially called blood

outgrowth endothelial cells (BOEC) [114]. They were shown to express endothelial markers including CD31, VE-cadherin, von Willebrand fractor, CD146, VEGFR2 and lacked the monocyte marker CD14 [113, 115-117].

ECFC have high proliferative potential and are reported to undergo similar population doublings as Human Umbilical Vein Endothelial Cells (HUVEC) [118-120]. *In vitro* they can form tubes and *in vivo* are involved in angiogenesis [121-123]. In animal models they have been shown to contribute to vascular repair through aiding neovascularisation and improving tissue perfusion in a number of conditions including hind limb ischaemia, myocardial infarction and stroke [124-126].

The precise origin of ECFC is not clear however both a bone marrow and tissue vascular niches have been suggested [114, 119]. RNA sequencing has demonstrated a similar endothelial transcriptional signature in ECFC when compared to HUVEC and human coronary artery EC (HCAEC) but also an overexpression of markers of all endothelial lineages including arterial, venous and lymphatics [127]. This may suggest they have not yet committed to a particular endothelial phenotype which may explain their multipotent proliferative potential [127].

ECFC have been used to assess endothelial molecular dysfunction in a number of diseases including diabetes, venous thromboembolism, pulmonary hypertension and ANCA vasculitis where they have been shown to have impaired proliferation and migration [128-133]. Although they cannot completely reflect the complex systemic inflammatory environment in DADA2, these patient derived cells represent a powerful tool to model disease-specific effects on endothelial function at a molecular and functional level.

1.7 Hypothesis and Aims

1.7.1 Hypothesis

ADA2 is expressed by EC and has an essential role in endothelial homeostasis. Thus, deficiency of endothelial ADA2 results in EC activation which contributes to the pathogenesis of DADA2.

1.7.2 Aims of study

1) Determine the role of ADA2 activity in other forms of vasculitis and establish rates of DADA2 in these cohorts
2) Determine ADA2 mRNA and protein expression and catalytic activity in EC
3) Establish the role of ADA2 in endothelial homeostasis
4) Characterise the role of endothelial ADA2 in the pathogenesis of DADA2

CHAPTER 2: Materials and Methods

2.1 ADA2 enzyme activity assay

2.1.1 Coupled spectrophotometric assay

An enzymatic coupled spectrophotometric assay for ADA2 activity was set up based on previously described methods [43, 134, 135]. The assay monitors the rate of ammonium release from adenosine by coupling to the glutamic dehydrogenase (GDH)-catalysed reaction of NH3 with α-ketoglutarate in the presence of B-Nicotinamide adenine dinucleotide (NADH) **(**Figure 3). ADA activity is estimated through the detection of NADH consumption, measured by kinetic tracking of absorbance at 340 nm for 2 hours at 37˚C. ADA2 activity is distinguished from total ADA by performing the assay with and without the addition of the purine analogue erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), a specific inhibitor of ADA1 but not ADA2 [134, 136]. Pentostatin, an inhibitor of both ADA1 and ADA2, was used to inhibit all ADA activity and confirm the background NADH consumption [137-139]

In each well of a 96 well plate, 20 µL of sample (plasma or serum) is combined with 40 µL of assay mix and 2 µL of either 10 mM EHNA or water (Table 3). The reaction is initiated by adding 140 µL of 25 mM adenosine (or water for the sample blank) and monitored for 2 hours on a plate reader during incubation at 37°C.

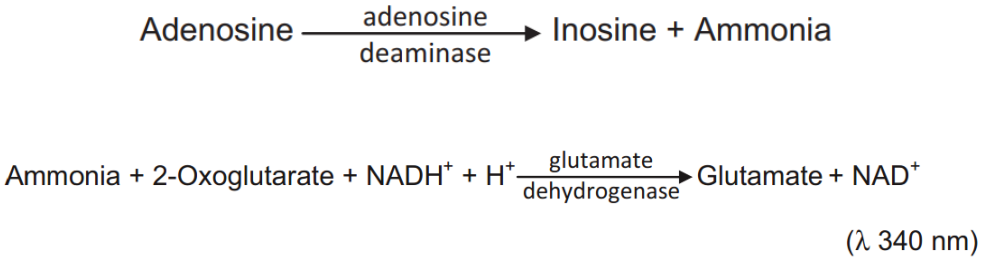


Figure 3 – ADA2 coupled spectrophotometric enzyme assay reaction

Reagent	Concentration	Volume to make 40 µL Assay mix (µL)	Supplier	Catalogue number
Tris Acetate Buffer	1M	29.5	Sigma	93295
Adenosine 5' – diphosphate sodium salt (ADP)	0.1M	2.1	Sigma	A2754
α Ketoglutaric Acid	0.2M	2.1	Sigma	75890
Glutamate dehydrogenase (GDH)	1.3U/ µL	2.1	Sigma	G2626
B-Nicotinamide adenine dinucleotide (NADH)	10mg/ml	4.2	Sigma	N8129
EHNA	10mM	N/A	Sigma	E114
Adenosine	25mM	N/A	Sigma	A9251

Table 2 – Reagents for the ADA2 activity assay

Well 1	Well 2	Well 3	Well 4
20µL sample	20µL sample	20µL sample	20µL sample
40µL Assay mix	40µL Assay mix	40µL Assay mix	40µL Assay mix
2µL Water	2µL Water	2µL EHNA	2µL EHNA
140µL water	140µL adenosine	140µL adenosine	140µL adenosine
Blank	**TOTAL ADA**	**ADA2**	**ADA2**

Table 3 – Summary of well contents for total ADA and ADA2 activity

For each sample, 4 wells are required to calculate: background activity, total ADA activity and 2 wells for ADA2 activity (Table 2). Based upon a conversion factor determined with purified rADA2 of known specific activity, multiplying the mean change in OD per minute, over 3 separate 30 minute time points, by the conversion factor 3600 gives the activity of the sample in U/L.

2.1.2 Filtration for use with supernatants and lysates

Optimisation experiments were conducted to measure ADA2 activity in cell supernatant. However, there were interactions between the cell media and the reagents in the activity assay that led to rapid consumption of exogenously supplied NADH and a significant fall in OD 340 even prior to the point at which the first reading could be made (Figure 4A+B). As the assay saturated very quickly, it was not possible to accurately measure the ADA2 activity. Amicon Ultra 3kDa centrifugal filters (Merck) were used to filter, concentrate and buffer exchange the supernatant with PBS (Phosphate Buffered Saline). This resulted in normalisation of the initial

OD and allowed accurate measurement of ADA2 activity in the sample (Figure 4A+B). Interactions between RIPA (Radio-Immunoprecipitation Assay) lysis buffer and the activity assay was also demonstrated therefore the same filtration process was carried out which led to acceptable ADA2 activity measurements (Figure 4C). Filtration of healthy control patient plasma and rADA2 in aqueous buffer established that approximately 15% of ADA2 activity was lost through the filtration process (Figure 4D).

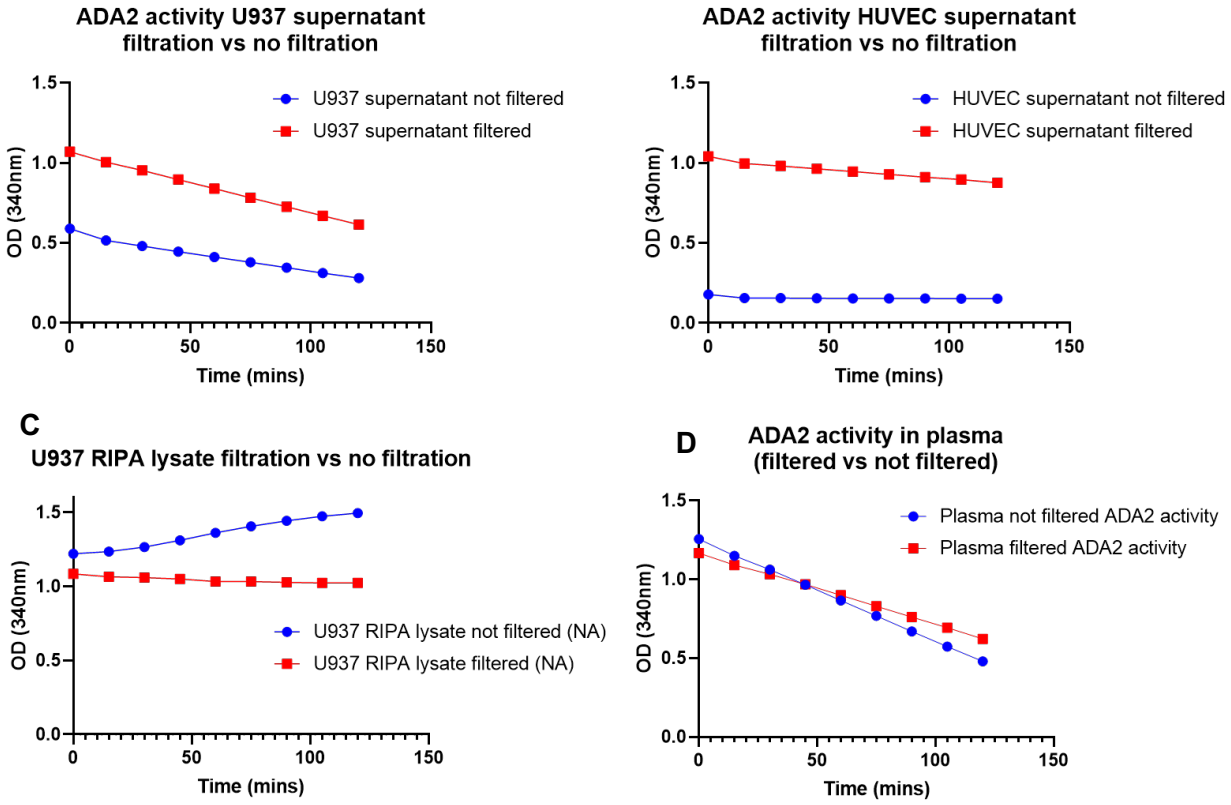


Figure 4 – ADA2 activity in filtered vs non-filtered cell supernatant, lysate and plasma

(A) U937 and (B) HUVEC cell supernatant and (C) U937 RIPA lysate was filtered, and buffer exchanged with PBS to prevent interactions between the cell media/ lysate and the activity assay reagents to allow accurate measurement of ADA2 activity. (D) Healthy control plasma was also filtered and buffer exchanged and a 15% loss of ADA2 activity was demonstrated relative to unfiltered plasma.

2.2 Cell culture

2.2.1 Cell propagation

Human Umbilical Vein Endothelial Cells (HUVEC) (PromoCell) and HAEC (PromoCell) were cultured in 75cm2 flasks (Sarstedt), pre-coated with 1% gelatin (Sigma), in Endothelial Cell Growth Medium-2 (EGM2) (PromoCell) supplemented with 10% foetal bovine serum (FBS) (PromoCell) and 100 U/ml penicillin/streptomycin (Sigma). EGM2 contains basal medium supplemented with 10 ng/ml basic fibroblast growth factor, 20 ng/ml insulin-like growth factor, 5 ng/ml human epidermal growth factor, 0.5 ng/ml vascular endothelial growth factor-165, 22.5 µg/ml heparin, 1 µg/ml ascorbic acid and 0.22 µg/ml hydrocortisone. HUVEC were seeded at 10,000 cells/cm2 in a tissue culture treated 75cm2 flask (Sarstedt). All HUVEC experiments were conducted at passage 3.

U937 cells (American Type Culture Collection) were cultured in RPMI 1640 medium (ThermoFisher) containing GlutaMaxTM-1, Phenol Red and 25 mM HEPES, supplemented with 10% foetal bovine serum and 100 U/ml penicillin/streptomycin. U937 were seeded in a 25cm^2 flask, no pre-coating, at a density of 40,000cell/cm^2. All cells were cultured at 37°C with 5% CO2.

HEK293T cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (Corning) containing 4.5g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin.

2.2.2 Cell freezing and thawing

For cryopreservation, HUVEC were washed with HyClone Dulbecco's Phosphate Buffered Saline (PBS), harvested with 0.25% trypsin/ 1 mM EDTA (Sigma) for 5 minutes at 37°C followed by centrifugation at 300g for 5 minutes. Cells were counted with a Cellometer Auto T4 Bright Field cell counter (Nexcelom Bioscience). Resuspension of cell pellets (0.75x10^6) was carried out in FBS with 10% DMSO (Sigma) and stored in a Mr Frosty (ThermoFisher) freezing container to allow a cooling rate of 1°C per minute at -80°C for 48 hours. Stocks of cells were transferred to liquid nitrogen for ongoing storage. Stocks of cells were rapidly thawed in a water bath a 37°C before seeding in 1% gelatin coated flasks in EGM2- 10% FBS. U937 cells are non-adherent so cells were counted as above, centrifuged at 300g for 5 minute before cell pellets were resuspended in FBS with 10% DMSO and stored as per HUVEC.

2.2.3 Cell treatments

2.2.3.1 Compound stimulation treatments

U937 cells were plated in six-well plates at $1x10^6$ cells per well prior to compound treatment. Three pools of HUVEC were seeded at $1x10^5$ cells per well 48 hours before treatment. 24 hour incubation with compounds was carried out prior to supernatant and RNA harvesting. Further time course experiments were conducted with U937 cells for 6, 24, 48 and 72 hours.

The treatments used are listed in Table 4. Concentrations of compounds were determined from literature searches, database analysis and preliminary experiments. Water, 0.1% dimethyl sulfoxide and 95% ethanol were used as vehicle controls depending on the vehicle of the compound.

Compound	Concentration	Purchased from	Catalogue number
dAdo	100 µM	Sigma	D8668
Ado	100 µM	Sigma	A9251
IL6	10 ng/ml	R&D	206-IL
TNFα	10 ng/ml	R&D	210-TA
Phorbol 12-myristate 13-acetate (PMA)	10 ng/ml	Sigma	P8139
Rapamycin	12 µM	Stratech	S1039-SEL
Rosiglitazone	1 µM	Sigma	R2408
Wortmannin	10 µM	Abcam	ab120148
2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1)	10 µM	Abcam	ab145522
Lipopolysaccharide (LPS)	100 ng/ml	Sigma	L2630
Interferon γ (IFNγ)	100 U/ml	Peprotech	300-02
Sodium butyrate	20 µM	Sigma	B5887
1,25 dihydroxyvitamin D3 (1,25D)	0.1 µM	Sigma	D1530

Table 4 – Compounds used in U937 and HUVEC treatment

2.2.4 siRNA transfection

HUVEC were seeded in a six-well plate at a density of $2.5x10^5$ cells per well and cultured in EGM2 with 10% FBS overnight. Cells were washed and fed with pre-warmed OptiMEM (Gibco). Transfection of HUVEC was carried out with specific siRNA and non-targeting control siRNAs (ON-TARGET plus Non-targeting Pool) at a concentration of 20 nM (Table 5). GeneFECTOR (Venn Nova) was used as the transfection reagent. The siRNA and GeneFECTOR in OptiMEM were incubated with HUVEC at 37°C, 5% CO_2, for 6 hours before being removed and replaced with EGM2 with 10% FBS. Cell supernatant, RNA or protein was harvested after 48 hours.

Target gene	siRNA	Sequence	Conc	Company
Control	ON-TARGET plus Non-targeting Pool	1. UGGUUUACAUGUCGACUAA 2. UGGUUUACAUGUUGUGUGA 3. UGGUUUACAUGUUUUCUGA 4. UGGUUUACAUGUUUUCCUA	5 nM	Dharmacon
ADA1	ON-TARGET plus Human ADA siRNA – SMARTpool	1. GGGCUCGGCCGAAGUAGUA 2. CAAGUUUGACUACUACAUG 3. GGACAUGGGCUUUACUGAA 4. CCAAAGAGGGCGUGGUGUA	5 nM	Dharmacon
ADA2	ON-TARGET plus Human ADA2 siRNA - SMARTpool	1. UCGCAGAAUCCAUCCGAAU 2. GCCCAAAGCUAGUUAGUAC 3. CUUCCACGCCGGAGAAACA 4. GUGCCAAAGGCUUGUCCUA	5 nM	Dharmacon
ADA2 202	Custom ADA2 202 siRNA x3	1. GGAACUGGGCUCUGGUGUAUU 2. UGGCGAUCCCAGAGAGCAUUU 3. CUGGAAUGGAACUGGGCUCUU	20 nM	Dharmacon

Table 5 – siRNA sequences used

2.2.4.1 TNFα treatment of siRNA KD HUVEC

HUVEC were seeded in a 25cm^2 flask in EGM2 with 10% FBS overnight at a density of 20,000 cells/cm^2. siRNA transfection was carried out as per the above method. The following day cells were harvested with trypsin, centrifuged and counted before being seeded into six-well plates at a density of 2.5x10^5 cells per well in EGM2 with 10% FBS. The following day TNFα (R&D) was added at a concentration of 0 ng/ml, 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml or 10 ngm/ml. RNA and supernatant was harvested after 24 hours of treatment.

2.2.5 Plasmid expansion and purification

Two pcDNA 3.1 plasmids (ThermoFisher), one containing the coding sequence (CDS) of ADA2 201 and the other ADA2 202 were generated by our collaborator Dr Pui Lee (Boston Children's Hospital). These plasmids also contain a coding sequence for both a HIS and MYC tag to enable purification.

Heat shock transformation of competent E. coli was carried out. 1ul of plasmid was added to 50ul of E. coli on ice and incubated for 30 minutes. This was then transferred to a water bath at 42°C for 30 seconds before being transferred back on ice and incubated for 2 minutes. 250ul of SOC media (ThermoFisher) was added and the tube placed in a shaker at 37°C for 1 hour. This was streaked onto a LB-agar plate containing ampicillin and cultured at 37°C overnight.

3 colonies from ADA2 201 and 3 from ADA2 plates were labelled, picked and placed in 2ml of LB-broth. This was cultured overnight on a shaker at 37°C. A QIAprep Spin Miniprep kit (Qiagen) was used as per the manufacturer's protocol to purify the DNA plasmid. The purified plasmids were sent to Azenta Life Sciences for sanger sequencing using T7 and BGHR primers to confirm the plasmid sequences (Supplementary figure 1). Once matching of the plasmid sequences to the appropriate CDS was confirmed (Clustal Omega, EBI), a HiSpeed® Plasmid Maxi Kit (Qiagen) was used, as per protocol, to purify a large quantity of the pcDNA plasmids from the same bacterial colonies.

2.2.6 Plasmid transfection

HEK293T cells were seeded in a six- well plate at a density of 2x10^5 per well in DMEM + FBS 10% overnight. Cells were washed and fed with pre-warmed OptiMEM. FuGENE HD

transfection reagent and the DNA plasmid was added in a 2:1 ratio respectively according to the manufacturer's protocol (Fugene 6ul:Plasmid 3ug). Supernatant, protein lysate and RNA was harvested at the desired time point (48-72hrs).

2.2.7 Proliferation assay

Proliferation of HUVEC was determined using the Click-iT EdU proliferation assay (ThermoFisher) for microplates which measures the incorporation of Edu (5-ethynyl-2´-deoxyuridine) into DNA during active DNA synthesis. This was carried out as per the manufacturer's protocol. siRNA KD of HUVEC was performed as above. 24 hours later, cells were re-seed on a 96 well plate at a density of 2,500 cells per well. To partially synchronise cell cycles, the cells were serum starved for 24 hours prior to being restored in media with the desired FBS concentration +/- TNFα.

EdU was added to the cells and incubated for 4 hours. The media was removed, and the cells fixed and then washed. The Click-iT reaction cocktail, containing a horseradish peroxidase with an azide group which binds to the alkylne group on EdU , was added and incubated for 30 minutes followed by blocking, washing and then addition of Amplex UltraRed reaction. This reaction was stopped through adding Amplex UltraRed stop reagent and then fluorescence measured on a plate reader.

2.2.8 Apoptosis and necrosis assay

The levels of apoptosis and necrosis in HUVEC were measured using the RealTime-Glo Annexin V Apoptosis and Necrosis assay (Promega) as per the manufacturer's protocol. siRNA KD of HUVEC was performed and cells re-seeded on a 96 well white plate at a density of 10,000 cells per well. Cells were incubated in media for 24 hours prior to the experiment being conducted. The detection reagent containing Annexin NanoBIT substrate, Calcium chloride, Necrosis detection reagent, Annexin V-smBiT and Annexin V-LgBiT were added and luminescence and fluorescence were measured on a plate reader.

2.2.9 Matrigel tubule formation assay

Matrigel tubule formation assay was used to measure the angiogenic potential of HUVEC. siRNA KD was carried out as per manufacturers protocol. Growth factor reduced Matrigel

(Corning) was placed into a 48- well plate and left for 30 minutes to solidify. siCtr and siADA2 HUVEC were seeded at a density of 50,000 cells per well. The plate was incubated at 37°C, 5% CO^2 and microscope images taken at 6 and 20 hours (x4 magnification). Images were analysed using the Image J angiogenesis plugin [140]. The measurements analysed are recorded in Table 6.

Measurement	Description
Nodes	Number of nodes in the analysed area
Junctions	Number of junctions in the analysed area
Segments	Number of segments in the analysed area
Mesh	Number of meshes in the analysed area
Mesh area	Sum of mesh areas detected in analysed area
Total length	Sum of the length of the segments, isolated elements and branches in the analysed area

Table 6 – Table of the recorded measurements from the Matrigel tubule formation assay

2.2.10 Senescence assay

The senescence of siCtr and siADA2 HUVEC was measured using a Senescence Detection Kit (BioVision) which histochemically detects senescence-associated β-galactosidase (SA-β-Gal). HUVEC siCtr or siADA2 were seeded in a 12-well plate and cultured in EGM2 10% FBS for 48 hours at 37°C, 5% CO^2. Media was removed and cells washed x1 with PBS. Cells were fixed with fixative solution and then washed twice with PBS before the staining solution mix was added and incubated overnight. Fixate solution was removed and cells overlayed with glycerol prior to microscope imaging. Image J was used to count the number of SA-β-Gal positive cells in the sample.

2.2.11 Immunofluorescence

Sterile glass coverslips were placed in a 12-well plate and coated with 1% gelatin solution for 30 minutes at 37°C. HUVEC were seeded on top of the coverslips at a density of $1x10^5$ per well in media with 10% FBS and cultured for 48 hours. Once cells were appropriately

confluent, media was aspirated, and cells washed 3x with PBS and then fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes. Following further washes with PBS the cells were permeabilised with 0.2% Triton X-100 in PBS and then washed. Primary antibodies were diluted in 3% BSA/0.2% Triton X-100 solution and 100ul added and incubated overnight at 4°C on a rocking platform.

Coverslips were washed 3x with 0.2% Triton X-100 in PBS and secondary antibodies diluted in 3% BSA/0.2% Triton X-100 solution and incubated for 1 hour. Coverslips were washed x3 with PBS and DAPI stain carried out to stain the nucleus of individual cells. Coverslips were transferred to glass slides and imaged on the Zeiss 780 confocal microscope. The antibodies used are shown in Table 7.

Antibody	Primary/ secondary	Company	Concentration
Adenosine deaminase 2	Primary	Sigma (HPA007888)	1 in 100
Phalloidin TRITC	Primary	Bio-techne (5783)	1 in 100
Anti-rabbit Alexa Fluor 488	Secondary	Invitrogen (A21206)	1 in 500
DAPI	N/A	BioLegend (422801)	1 in 2000

Table 7 – Primary and secondary antibodies used for immunofluorescence

2.2.12 Endothelial colony forming cells (ECFC)

2.2.12.1 Patient recruitment

Patients with genotype confirmed DADA2 were recruited from Hammersmith Hospital (Imperial College London) and the Royal Free Hospital with the assistance of Dr Richard Stratton. Blood samples were collected for plasma, PBMC and ECFC isolation. Written informed consent was obtained for all subjects prior to their inclusion. Patient samples were collected with ethical approval from Imperial College Healthcare Trust Tissuebank (Subcollection NHL_JM_18_033). ANCA plasma samples were kindly provided by Dr Steven McAdoo.

2.2.12.2 PBMC isolation

PBMCs were isolated from blood as per previous documented protocols [141]. Blood samples were collected in sodium heparin tubes (Greiner) and spun at 400g for 10 minutes. The plasma was removed, transferred to a new tube and spun at 3,000g to deplete platelets before being aliquoted and stored at -80°C. The remaining blood components in the sodium heparin tubes were diluted 1:1 in PBS (HyClone) + 2% FBS. This was then layered onto a SepMate tube (StemCell) containing Ficoll paque (GE Life Sciences) and centrifuged at 1200g for 10 minutes. The PBMC fraction from the SepMate was poured into new tubes and diluted with PBS + FBS 2% topped up to 50ml. This was centrifuged at 350g for 10 minutes with the brake on half. The supernatant was aspirated and the PBMC pellet resuspended in PBS + FBS 2% before further centrifugation at 350g for 10 minutes with half brake. The pellet was resuspended in PBS + FBS 2% and PBMCs counted using a haemocytometer. Flasks were pre-coated with rat-tail collagen (50ug/ml) (Corning) before PBMCs were plated in EGM-2 supplemented with ultra-low endotoxin FBS 20% (HyClone), EGM-2 supplement pack (Promocell) and 100 U/ml penicillin/streptomycin.

Additional PBMCs were resuspended in FBS with 10% DMSO (Sigma) and stored in a Mr Frosty (ThermoFisher) freezing container to allow a cooling rate of 1°C per minute at -80°C for 48 hours. Stocks were transferred to liquid nitrogen for longer term storage.

2.2.12.3 ECFC colony generation

Media in the flasks containing the seeded PBMCs was changed every 48-72 hours and screened for ECFC colonies every 48 hours after 1 week of culture. As per ISTH guidelines, ECFC colonies were identified as “well-circumscribed colonies with a cobblestone appearance

and with more than 50 adherent cells of endothelial morphology" [142]. The number of days to first passage and number of colonies generated prior to the first passage was recorded for each ECFC culture.

2.2.12.4 ECFC propagation

ECFC colonies were expanded to passage 3 and then cryopreserved. Functional experiments were carried out at passage 4.

2.3 RNA

2.3.1 RNA isolation

Cells were washed twice with ice cold PBS and lysed with 350µL RNeasy Lysis Buffer (RLT; Qiagen) supplemented with β- mercaptoethanol at a concentration of 10µL/mL. RNA was then harvested and isolated using the RNeasy Mini Kit (Qiagen) as per the manufacturer's protocol. RNA quantification and assessment of purity was carried out using on a Nanodrop 2000 Spectrophometer (ThermoFisher).

2.3.2 cDNA synthesis

RNA (1 µg) was diluted with RNAse-free water to a volume of 16µL and then mixed with 4µL of Quanta qScript cDNA mix (Quanta Biosciences) to make a total volume of 20µL. cDNA was generated on the Thermocycler using the following protocol; 5 minutes at 22°C, 30 minutes at 42°C and 5 minutes at 85°C. cDNA was stored at -20°C. Priming of Quanta reverse transcription reaction is achieved using both oligo dT and random hexamer primers.

2.3.3 Quantitative real-time polymerase chain reaction (qPCR)

A master mix of 5µL of iQTM SYBR® Green Supermix (BioRad), 0.12µL forward primer (600nM), 0.12µL reverse primer (600nM) and 0.26µL RNase free water was prepared and 5.5µL added to each well of a 384- well plate. cDNA was diluted to 2.2ng/ul and 4.5ul added to the master mix in the well (10ng total per reaction). Assays were subsequently run on QuantStudio 6 Flex Real-Time PCR System (ThermoFisher). The PCR protocol comprised a denaturation step of 95°C for 3min and 40 cycles of 95°C for 10sec and 58°C for 45sec. Target gene expression was normalised to a housekeeper gene; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or Actin – β (ACTB). Results were analysed with QuantStudio™ Real-Time PCR Software (ThermoFisher) using relative threshold methods (2^-CT). Primer pair sequences were designed using Primer-BLAST (NCBI) and purchased from Invitrogen. Oligonucleotide sequences of the primers used are listed in Table 8.

Primer	Forward sequence	Reverse sequence
ACTB	TGAAGTGTGACGTGGACATC	GGAGGAGCAATGATCTTGAT
GAPDH	CAACAGCCTCAAGATCAT	GAGTCCTTCCACGATACC
ADA1	GGGCTGCTGAACGTCATTG	AGGCATGTAGTAGTCAAACTTGG
ADA2 (pan)	ACCATGACGAAGAGTGGTCAG	CCATTCGGATGGATTCTGCG
ADA2 – 201	AGGATTATCGGAAGCGGGTG	GAAATTCCTCAGCAAGCTGTCA
ADA2 - 202	TTCCGGAGGGGAGATGGATT	CCACTGAGCTCATACACCAGA
ICAM1	ATGCCCAGACATCTGTGTCC	GGGGTCTCTATGCCCAACAA
VCAM	TTTGACAGGCTGGAGATAGACT	TCAATGTGTAATTTAGCTCGGCA
ESEL	CAGCAAAGGTACACACACCTG	CAGACCCACACATTGTTGACTT
IFNα	GTGAGGAAATACTTCCAAAGAATCAC	TCTCATGATTTCTGCTCTGACAA
IFNβ	GCTTGGATTCCTACAAAGAAGCA	ATAGATGGTCAATGCGGCGTC
IFIT1	TTGATGACGATGAAATGCCTGA	CAGGTCACCAGACTCCTCAC
RSAD2	ATGTGGGTGCTTACACCTGC	GAGAGCTCAGAGGTTGCCTG
CXCL10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
IFIT3	AAAAGCCCAACAACCCAGAAT	CGTATTGGTTATCAGGACTCAGC
MX1	GGTGGTCCCCAGTAATGTGG	CGTCAAGATTCCGATGGTCCT
OASL	CTGATGCAGGAACTGTATAGCAC	CACAGCGTCTAGCACCTCTT
IFIT2	GACACGGTTAAAGTGTGGAGG	TCCAGACGGTAGCTTGCTATT
OAS1	TGTCCAAGGTGGTAAAGGGTG	CCGGCGATTTAACTGATCCTG
IFI44L	TCTGCCATTTATGTTGTGTGACA	CAGGTGTAATTGGTTTACGGGAA
IRF7	GCTGGACGTGACCATCATGTA	GGGCCGTATAGGAACGTGC
CCL5	CTCTHHHTTGGCACACACTT	CCAGCAGTCGTCTTTGTCAC

Table 8 - Oligonucleotide sequences used for qPCR analysis

2.3.4 RNA sequencing

RNA was sent to a commercial service provider (Genewiz, Germany) for poly-A, strand-specific sequencing targeting 30 million paired-end reads per sample (150 base pairs each). Prior to library synthesis, the RNA integrity number was evaluated by capillary electrophoresis and shown to be >9. Subsequent processing steps were carried out by Dr Robert Maughan (Imperial) according to an established pipeline (Figure 5). Read quality scores, per base sequence quality and presence of adapter sequences were assessed using FastQC

(version.0.14.2) and it was determined that read trimming or other exclusion of data was required. Transcript quantification was performed using Salmon (version 1.10.2) [143] and the Human reference transcriptome (GRCh38.p13, Gencode, release 35) [144]. Transcript count data was imported into the R software environment and summarised at the gene level using tximport [145] and then normalised using the standard EdgeR methodology [146]. Genes displaying expression levels below 5 counts per million (CPM) in at least 3 individual libraries were filtered out. Differential expression (DE) analysis was performed between si-control and si-ADA2 pairs as well as between si-control and si-ADA1 pairs using quasilikelihood F-tests in EdgeR accounting for HUVEC donor ID (pairwise comparison). P values were adjusted for multiple comparisons using Benjamini-Hochberg and differentially expressed genes were defined as having adjusted P $\leq$ 0.05 (5% false discovery rate) and Log2 fold change $\geq$/$\leq$ 0.6 (equivalent to a 1.5 fold increase). Various data visualisation tools were then used to enable the interpretation of the data. Over-representation and gene-set enrichment analysis was carried out and analysed again the hallmark geneset [147].

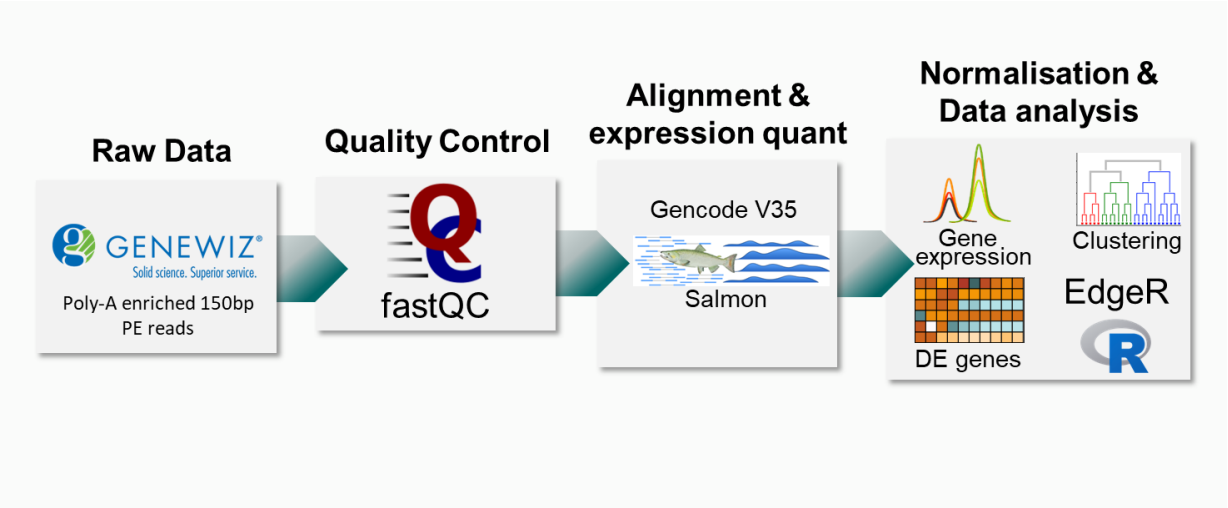


Figure 5 – Overview of RNA sequencing analysis pipeline

2.4 Protein

2.4.1 Protein collection and determination of concentration

Cells were washed twice with ice cold PBS followed by the addition of RIPA lysis buffer (100µL per 6 well, ThermoFisher) supplemented with complete protease and phosphatase inhibitors (Roche). This was left on ice for 30 minutes before the contents of each well scraped and pipetted into pre-cooled microcentrifuge tubes and centrifuged at 4°C for 20 minutes at 16,000 g to remove insoluble matter. The supernatant from this was collected and transferred into a fresh tube. The pellet that remained was further lysed in an 8M urea lysis buffer containing 8M urea, glycerol, 20% sodium dodecyl sulfate (SDS), 1.5M Tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT) (Sigma) and phenylmethylsulfonyl fluoride (PMSF).

Protein concentrations were measured with the Pierce BCA Protein Assay Kit (ThermoFisher) using the manufacturer's protocol.

2.4.2 Immunoblot analysis

Samples were prepared by mixing 20µg of protein lysate with a 4x sample buffer containing lithium dodecyl sulfate, phenol red, DTT and tracking dyes (ThermoFisher) and made up to 20µL with water followed by boiling on a hot plate for 5 minutes at 99°C. Gel electrophoresis was carried out using Bolt 4-12% Bis-Tris gels (Invitrogen) and MOPS SDS running buffer (Invitrogen) for 1.5 hours at 150V. iBlot transfer stacks (Invitrogen) were used to transfer onto a membrane using the iBlot 2 Gel transfer device for 10.5 minutes at 20V. 3% milk in Tris-buffered saline (TBS) was used to block the membrane for 1 hour at room temperature (RT). This was followed by incubation with primary antibodies (Table 9) diluted in 5% BSA TBS-T (Bovine serum albumin in Tris-buffered saline with 0.1% Tween-20) overnight at 4^{o}C. The membranes were washed three times with TBS-T for 5 minutes each time and then incubated with the secondary fluorescent antibody in 3% milk TBS-T for 1 hour at RT in the dark. After three further washes with TBS-T for 5 minutes each the membranes were imaged using the Odyssey CLX infrared imaging system (LI-COR Biosciences).

Antibody	Species	Company (reference)	Concentration
ADA2 polyclonal	Rabbit	Sigma (HPA007888)	1:1000
ADA2 monoclonal	Mouse	Sigma (SAB1403267)	1:1000
GAPDH	Mouse	Cell signalling (97166)	1:2000
Alfa tubulin	Mouse	Cell signalling (3873)	1:2000
Anti-Rabbit IgG Alexa Fluor 680	Goat	Invitrogen (A32734)	1:5000
Anti-Mouse IgG Alexa Fluor 680	Goat	Invitrogen (A32729)	1:5000
Anti-Rabbit IgG DryLight 800	Goat	Invitrogen (SA5-35571)	1:5000
Anti-Mouse IgG DryLight 800	Goat	Invitrogen (SA5-10176)	1:5000

Table 9 – Antibodies used for Immunoblot

2.4.2.1 Optimisation of ADA2 antibody use in Immunoblot

To determine the most appropriate ADA2 antibody and concentration to use, pilot experiments were conducted with 2 commercially available ADA2 antibodies (Table 9). Immunoblot was carried out with cell lysate from HUVEC, U937 and THP1 as well as recombinant ADA2 (rADA2). Primary antibody concentrations of 1:1000 and 1:2000 were used. Immunoblot showed that the ADA2 polyclonal antibody (HPA007888) demonstrated lower levels of non-specific binding and detection of rADA2 relative to the ADA2 monoclonal antibody (SAB1403267) where no rADA2 binding was demonstrated (Figure 6A+B). An antibody concentration of 1:1000 led to an appropriate balance between signal detection and non-specific binding with the ADA2 polyclonal antibody. Our collaborator (Dr Pui Lee) demonstrated that there was loss of the ADA2 band in U937 with specific CRISPR knockout of the ADA2 gene using the Sigma polyclonal antibody.

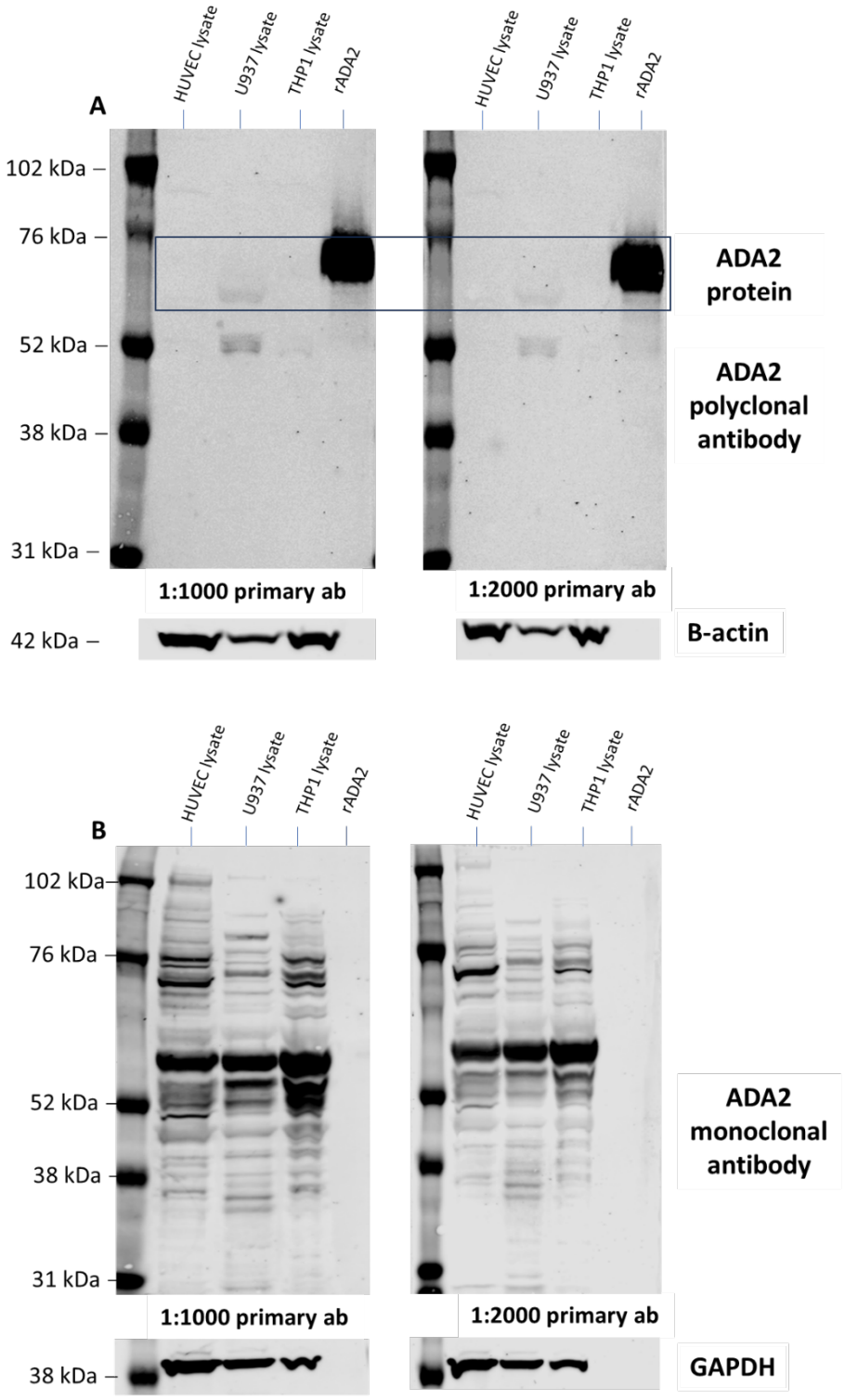


Figure 6 – Immunoblot of optimisation of ADA2 antibodies and concentrations. Immunoblot analysis of HUVEC, U937, THP1 lysate and rADA2 as a positive control. Whole cell protein lysates were separated by SDS-PAGE. Proteins were detected with (A) an ADA2 polyclonal antibody or (B) an ADA2 monoclonal antibody at a concentration of either 1:1000 or 1:2000. The loading controls used were (A) B-actin or (B) GAPDH.

2.4.3 ELISA

A commercially available sandwich ELISA (R&D DuoSet) was used to measure protein concentrations of ICAM1, VCAM and CXCL10 in both cell lysate and supernatant. Samples were diluted to ensure that the values were within the range of the standard curve. The protocol was followed as per the manufacturer's instructions.

2.4.4 HIS tag purification with Nickel columns

A HisPur Ni-NTA purification kit was used to purify His tag recombinant proteins generated through plasmid transfection in HEK293T cells. The columns contain a nickel-charged nitrilotriacetic acid (NTA) chelate which bind Hi-tagged proteins. Differing concentrations of Imidazole were used for the equilibration, wash and elution buffers (10mM, 23mM and 250mM respectively) as per the manufacturer's instructions. The protein lysate samples were mixed with equilibration buffer and added to the column and centrifuged at 700g for 2 minutes. The flow through was collected and stored. The resin was washed three times with wash buffer before the elution buffer added and the column centrifuged at 700g for 2 minutes. 2 further additions of elution buffer were conducted and collected. Samples were analysed by immunoblot and Coomassie blue staining.

2.4.5 Mass spectrometry

Cell lysate protein samples were prepared with RIPA buffer as per previous methods. Further experimental work and analysis of these samples was carried out by Dr Harry Whitwell, lecturer in proteomic and integrative data analysis, Imperial College London. PreOmics iST kits (Preomics) were used as per manufacturer's instructions to remove the detergents in the RIPA buffer.

Protein digestion of samples was performed using the departmental standardised protocol. Protein reduction, alkylation and digestion was performed in a single-pot reaction. A digestion buffer was generated containing ammonium bicarbonate (6µL of 1M stock per sample), chloracetamide (6µL of 400mM stock per sample), TCEP (1.2µM of 500mM stock per sample) and trypsin (2ul of a 0.1ug/ul stock per sample) in a 2mL LoBind Eppendorf Tube.

The digested peptides are then added and drawn through the plate with a vacuum. The plate is then washed 3 times. A sample 96 well Max Quant 700ul (Waters) plate is then put under

the HLB µElution plate for elution. Peptides are eluted by the addition of 50ul 60% acetonitrile which is drawn through the plate under vacuum for 30 seconds. A second elution is then performed with 100uµL 60% acetonitrile with manual control of the vacuum. The samples are then dried in a Speedyvac (Eppendorf) at 60◦C until completely dry (typically this is between 90-120 minutes).

Samples were thawed at room temperature for 30 minutes before being dissolved in 0.1% formic acid (FA) to 0.1µg/µL by sonicating for 10 minutes in a water bath and centrifuging for 1 minute at 1000x g. Samples were loaded into a UPLC system (M-Class Acquity, Waters) and 4µL was separated across a Kinetex™ 150x0.3mm 2.6µm XB-C18 column (Phenomenex) interfaced with a high-performance Q-TOF mass spectrometer (MS) (ZenoTOF 7600, Sciex). Chromatography was performed at 10µL/minute with 3% organic phase (acetonitrile with 0.1% FA) increasing to 30% between 1 and 21 minutes. The column was then washed with 80% organic for 3 minutes before equilibrating for 5 minutes prior to the next injection. The MS was run in positive mode with a zenoSWATH method comprising 85 variable windows with ionisation scheduling between 2 and 27 minutes. MS data was acquired over 400-1500mz with an accumulation time of 0.1s. MSMS was acquired over 140-1800mz with an accumulation time of 0.013 and dynamic collision energy enabled. For DDA experiments, samples were acquired using the gradient described above. In each acquisition cycle, the top 45 most intense ions were selected for fragmentation with ramped collision energy and a 6s exclusion window.

2.4.6 Proteomics analysis

MS files (.wiff) were imported into DIA-NN version 1.8.1 [148] and searched against a predicted library of human proteins from the Swissprot database (Uniprot.org) and Yeast enolase, a reference standard frequently used on the MS. The library was generated in DIA-NN using default settings. Peptide specific MS2 fragment ion peaks were selected using a 1% false discovery rate with the maximum number of variable modifications set to 2, methionine variably oxidised and all cysteines assumed carbamidomethylated. Match between runs was enabled and no normalisation was specified. Peptide intensity was then derived from peak area and protein abundance was quantified using the sum of the top 3 corresponding peptide intensity values.

The subsequent processing steps were carried out by Dr Robert Maughan (Imperial). Protein abundance data was next imported into the R software environment and assessed for protein detectability, uniformity of abundance values and outliers using a combination of boxplots, signal intensity plots and principal component analysis. A single si-control sample was

removed due to its substantially lower level of protein detection and significant difference in protein abundance levels compared to other samples. The remaining data was Log2 transformed and then filtered for proteins expressed in all samples, final protein n=2,311. After quantile normalisation, the differential expression of proteins between si-control and si-ADA2 donor pairs was assessed using the standard LIMMA empirical bayes moderated linear model methodology [149]. P values were adjusted for multiple comparisons using the Benjamini-Hochberg method and differentially expressed proteins were defined as having adjusted $P \leq 0.05$ (5% false discovery rate).

Data dependent acquisition data was searched in Mascot (Matrix Science) with modifications as specified above with a 0.6Da peptide and fragment mass tolerance, matches were filtered to a significance threshold of 0.05.

2.5 Identification of compounds for ADA2 stimulation experiments

2.5.1 Literature review

A literature search was conducted using PubMed and Web of Science (WoS). For both searches, keywords including “ADA2” and “CECR1” were combined with “induction”, “upregulation” and “increase” or “role” or “production” by Boolean logical operators (“AND” used between target protein name and other terms, “OR” used between alternative terms). “NOT” was used to exclude unrelated results involving biomolecules with similar abbreviations as ADA2 (e.g., Ada2a and Ada2b genes encoding for the transcriptional adapter 2 protein in the histone acetylation complex). Truncations were applied by using asterisk (*) at the end of each word.

2.5.2 Public database analysis

Two publicly available databases were used for analysis; a multi-omics database for cellular signalling pathways, The Signaling Pathways Project (SPP) *https://www.signalingpathways.org/index.jsf* [150], and a chemical perturbation database of molecular and cellular signatures SigCom LINCS *https://maayanlab.cloud/sigcom-lincs/#/* [151]. SPP allows queries of transcriptomic data of specific genes in cell lines from different human system categories. ADA2 transcriptomic data in cell lines from the ‘human immune system’ category upon compound treatment was extracted from the ‘Ominer’ database. SigCom LINCS provides transcriptomic information of different genes upon chemical administrations in different cell lines. Metadata search of the CECR1/ADA2 gene was performed in U937, THP-1 and HUVEC cell lines, and data with CECR1 upregulation signatures were downloaded.

2.6 Ethics

Patient samples were collected with ethical approval from Imperial College Healthcare Trust Tissuebank (Subcollection NHL_JM_18_033). The patient information sheet and example consent form has been included in the appendix.

2.7 Data analysis and statistics

Data was initially analysed by the normality (Shapiro-Wilk) and lognormality test on GraphPad to examine if results were normally distributed. If normality criteria was met, unpaired t-tests were performed otherwise Mann-Whitney tests were performed for non-normally distributed groups. For comparisons of multiple datasets, one-way ANOVA was performed followed by Dunnett's multiple comparisons test when comparing against a control group.

All HUVEC experiments were performed in parallel using at least 3 donor pools (each pool had 3 donors). Pairwise comparisons for HUVEC were performed using a two-tailed Student's t-test. All data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Statistical significance was defined as follows: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 and **** p-value < 0.0001.

CHAPTER 3: Plasma ADA2 activity and prevalence of deficiency in a cohort of patients with vasculitis

3.1 Introduction

Deficiency of ADA2 leads to a broad phenotype that causes vasculitis in some patients but in others it can result in haematological manifestations such as bone marrow failure and immunodeficiency [2, 3, 8, 35]. In initial reports, DADA2 associated vasculitis was described as being similar to polyarteritis nodosa but subsequent reports have demonstrated that small, medium and large sized arteries can be affected [2, 19]. Involvement of the skin, with a livedoid rash, and the neurological system, with ischaemic or haemorrhage stroke, is common however the vasculitis can also involve the gut, liver, kidney and heart [7, 10, 13, 21, 152]. Given the heterogeneity in presentation of DADA2 vasculitis there can be overlapping features with many other vasculitic diseases, including Takayasu arteritis and giant cell arteritis, and vasculitis that predominantly affects small vessels, including ANCA vasculitis [153]. The estimated mortality of DADA2 is approximately 8% before the age of 30 so early diagnosis and treatment are key to improving patient outcomes and reducing severe manifestations of DADA2 vasculitis [154]. Misdiagnosis with a different form of vasculitis or autoinflammatory condition is common and can have catastrophic but avoidable outcomes since anti-TNF treatment is highly effective in DADA2 vasculitis but is not used widely in other vasculitides [48].

A diagnosis of DADA2 can be determined through measurement of serum or plasma ADA2 enzymatic activity or by sequencing the ADA2 gene [42]. Near absent ADA2 activity in plasma or serum or identification of biallelic pathogenic ADA2 variants in ADA2 gene sequencing is considered diagnostic [42]. The limited availability of these diagnostic tools has been identified as one of the barriers to DADA2 diagnosis [42]. The development and validation of an ADA2 activity assay was therefore one of the key tools required for this project, not only to be able to determine ADA2 activity in patient samples but also to measure activity in cell supernatant and lysate samples.

Deficiency of ADA2 is well documented to lead to vasculitis but low ADA2 activity has also been demonstrated in patients with chronic heart failure [155]. Conversely, ADA2 activity has been shown to be elevated in a number of conditions. These include infections such as HIV [156, 157], hepatitis B and C [158], active tuberculosis (both in the serum and pleural effusions) [159, 160], SARS-CoV 2 infection [161], autoimmune conditions including juvenile rheumatoid arthritis and systemic lupus erythematosus [63, 162], systemic juvenile idiopathic arthritis with macrophage activation syndrome [43], malignancies including breast cancer [66],

adult T-cell leukaemia [156], and ovarian tumours [163] and in non-alcoholic fatty liver disease [164]. Therefore, it has been proposed that elevated ADA2 activity is a potential marker of immune system activation and perturbation [165].

Given the predicted DADA2 disease prevalence is 1 in 222,000 individuals, and DADA2 vasculitic features are highly heterogenous, one of the aims of this chapter was to establish whether any patients in a cohort of patients diagnosed with a large or small vessel vasculitis had ADA2 deficiency [41]. Previous work has looked at identifying disease-causing variants in the ADA2 gene in patients with polyarteritis nodosum (PAN) and ANCA vasculitis [166]. They demonstrated that 3.4% patients with PAN had biallelic pathogenic variants in the ADA2 gene with 3 patients identified to have deficient serum ADA2 activity [166]. Given the elevated ADA2 enzymatic activity in other inflammatory conditions, additional aims will be to determine ADA2 activity in this cohort and establish whether ADA2 has a role in other forms of vasculitis. Assessment of whether blood markers and/ or disease activity scores correlate with ADA2 activity will also be determined.

3.2 Hypothesis of the chapter

Given the heterogeneity of DADA2 presentation, we expect to identify ADA2 deficiency in the plasma of some patients in a cohort who have been previously diagnosed with another form of vasculitis. In the vasculitis patients who do not have DADA2, ADA2 activity will be elevated and will correlate with vasculitis and immune cell activity markers in the blood and disease activity scores.

3.3 Aims of the chapter

1) Develop and validate an ADA2 enzymatic activity assay.
2) Establish rates of ADA2 deficiency in a cohort of patient with large vessel and ANCA vasculitis.
3) Determine ADA2 activity in patients with vasculitis
4) Evaluate whether any vasculitis disease activity markers or clinically measured blood parameters correlate with ADA2 activity.

3.4 Development and optimisation of an ADA enzymatic activity assay

A coupled spectrophotometric assay for ADA enzyme activity was established based on previously described methods [43, 134, 135]. As described in methods, the assay couples the catalysis of adenosine to inosine and ammonia by ADA/ADA2 to the consumption of ammonia and NADH by glutamate dehydrogenase as quantified continuously at 340 nm for 2 hours during incubation at 37˚C. ADA/ADA2 activity can therefore be estimated using the measured consumption rate of NADH. The dynamics of the assay were determined using a calibrator of rADA1 of known ADA enzyme activity (Figure 7A). There is natural degradation of NADH over time, even in the absence of adenosine (Figure 7B), so calculations of activity for each sample are corrected for this.

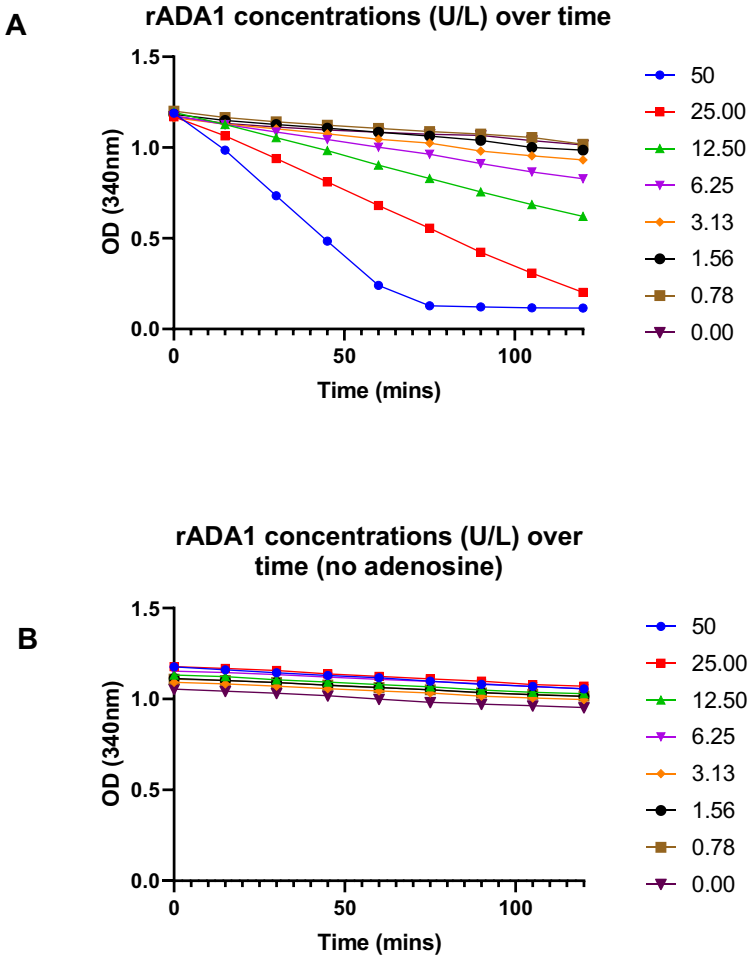


Figure 7 – Change in optical density over time with serial dilutions of rADA1.

(A) Serial dilations of rADA1 of known ADA enzymatic activity were loaded onto a 96 well plate and ADA activity measured using the described enzymatic assay. The OD (340nm) was measured over 2 hours to understand the dynamics of the assay. (B) A no adenosine (NA)

control for each sample was also loaded onto the 96 well plate and OD (340nm) was measured over 2 hours to understand the background degradation of NADH over time which is independent of ADA activity.

Addition of EHNA (Erythro-9-(2-hydroxy-3-nonyl)-adenine) to rADA1 led to complete loss of enzyme activity whereas rADA2 activity was unaffected (Figure 8A+B). Pentostatin, an inhibitor of both ADA1 and ADA2, led to loss of activity of both rADA1 and rADA2 (Figure 8A+B). The rADA1 was a calibrator of a known ADA activity whereas the rADA2 enzymatic activity was given as a range by the company (R&D) and had to be estimated. Hence the apparent difference in activity between rADA1 and rADA2 25U/L in the absence of inhibitor.

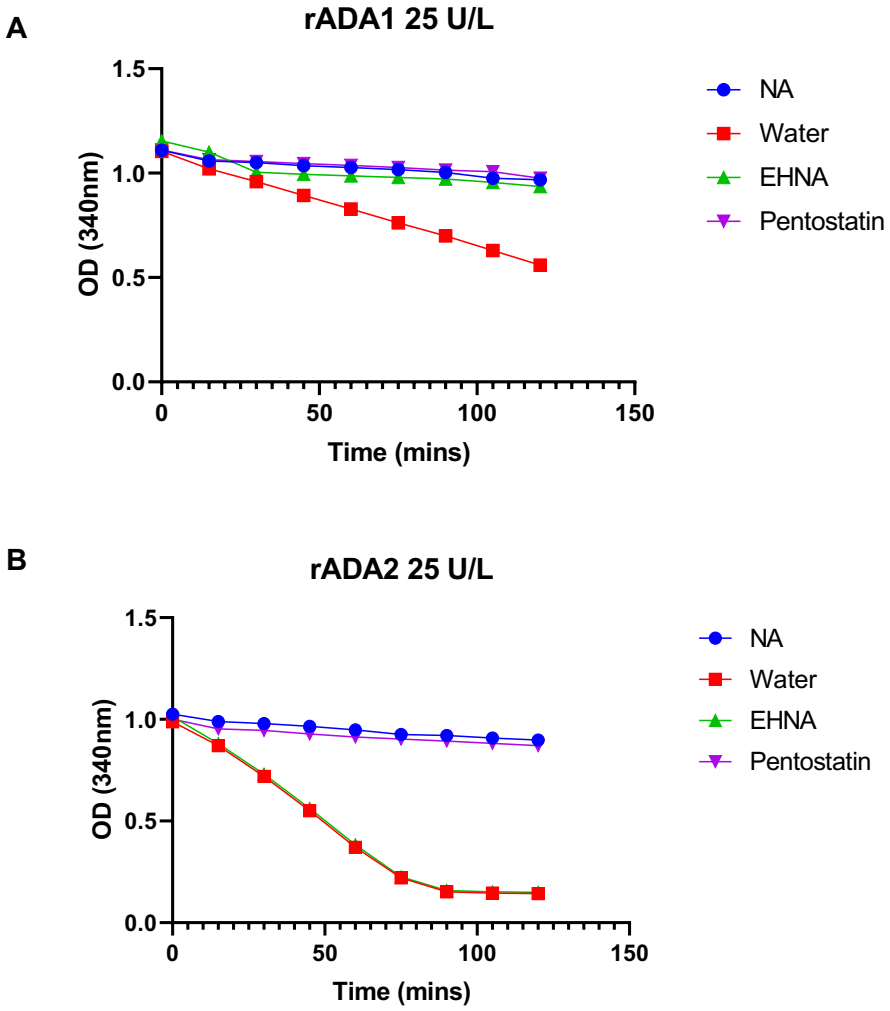


Figure 8 – rADA1 activity is inhibited with EHNA but rADA2 activity is unaffected. Pentostatin leads to complete loss of rADA2 and rADA2 activity.

(A) rADA1 and (B) rADA2 with 25U/L ADA activity was loaded in duplicate on a 96 well plate in the presence of water, EHNA and pentostatin. ADA activity was also measured with no adenosine (NA) to correct for background degradation of NADH over time.

ADA activity is calculated by the mean change in OD over 3 separate 30 minute time points, corrected for the natural degradation of NADH in the no adenosine sample. This value is then multiplied by the conversion factor 3600 to determine ADA activity in U/L (Figure 9).

$$\text{Mean}\ \frac{\text{OD 30} - \text{OD 60}}{30}\quad\frac{\text{OD 45 - OD 75}}{30}\quad\frac{\text{OD 60} - \text{OD 90}}{30}$$

X3600 (conversion factor)

Figure 9 – Calculation of ADA2 activity

ADA activity is calculated from the mean change in OD of 3x 30 minute time intervals. This is then multiplied by 3600 (conversion factor) to determine ADA activity in U/L

This conversion factor was initially determined by our collaborator Dr Pui Lee (Harvard, USA) using a standard curve prepared from dilutions of purified rADA2 of known specific activity, who assisted in setting up this assay. To ensure that this conversion factor was applicable to our assay, serial dilutions of the calibrator of known ADA activity were run in the assay and ADA activity was calculated using the formula and conversion factor. The results demonstrate that the calculated activity using the conversion factor gives the correct activity when compared to the known activity (Figure 10).

Calibrator of known ADA activity (U/L)	Measured activity using x3600 conversion factor (U/L)
50	49.49
25	27.44
12.5	14.74
6.25	7.28
3.13	3.8
1.56	2.12
0.78	0.72
0.39	0.5

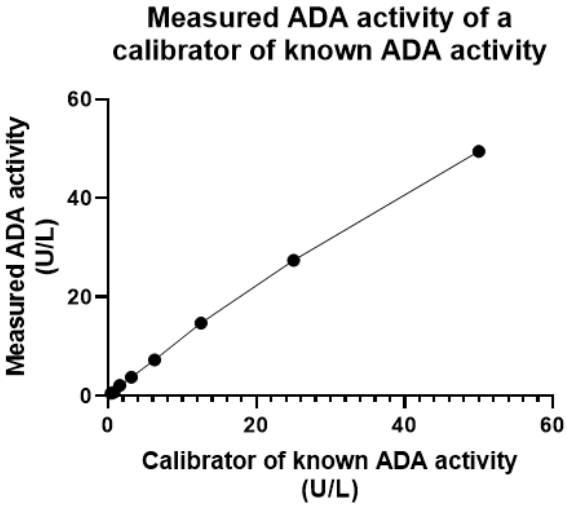


Figure 10 – Measured ADA activity of a calibrator of known ADA activity.

To confirm the conversion factor (x3600), serial dilutions of the calibrator of known ADA activity were loaded onto a 96 well plate and ADA activity measured using the described assay. ADA activity was calculated using the conversion factor and compared to the known ADA activity.

Patient plasma samples and serial dilutions of rADA1 and rADA2 run on the same plate and across multiple plates demonstrated very good reproducibility with an inter and intra-assay variation of 7% and the lower limit of detection being 1.56 U/L while the upper limit of detection was 50 U/L (Figure 10).

3.4.1 Use of Plasma samples from DADA2 patients and heterozygous carriers to determine specificity of assay to detect deficient enzymatic activity

To test the assay's capacity to detect of ADA2 enzyme deficiency, plasma from 11 patients with genotype confirmed DADA2 and 10 patients heterozygous for pathogenic variants in ADA2 were run on the assay. All the DADA2 patients had very low or deficient activity (0 – 0.90 U/L, 9 patients had 0 U/L activity), while the heterozygous carriers had intermediate activity levels (2.02 – 10.62 U/L) versus healthy controls (6.48 – 30.3 U/L) (Figure 11).

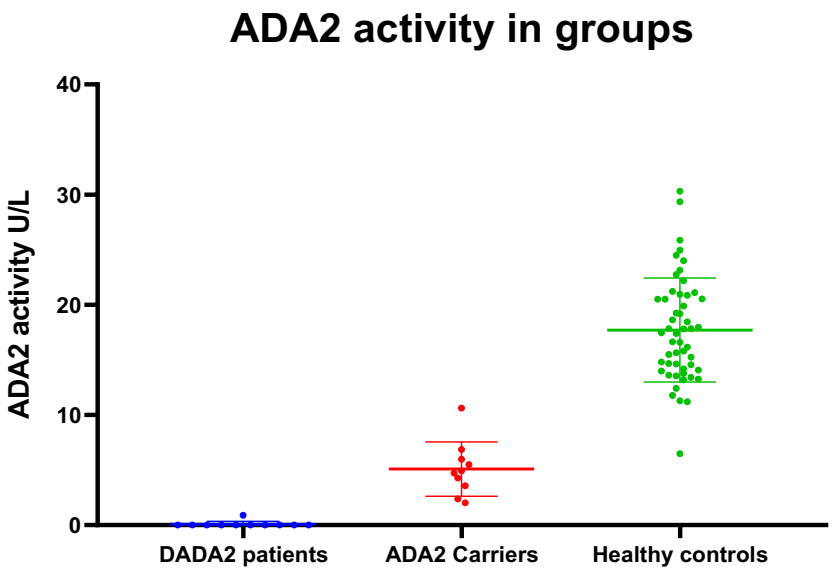


Figure 11 – ADA2 activity in DADA2 patients, heterozygous carriers of variants in ADA2 gene and Healthy controls.

ADA2 activity was measured, using the described ADA activity assay, in the plasma of 11 patients with genotyped DADA2, 10 patients heterozygous for DADA2 variants and 52 healthy controls.

The change in OD (340nm) over time from one of the DADA2 patients is shown in Figure 12A. There is no fall in OD 340nm in addition to the background degradation (no adenosine control) when EHNA is present indicating no ADA2 activity. Figure 12B shows that in the DADA2 patient a small amount total ADA activity remains when there is no EHNA present which represents residual ADA1 activity.

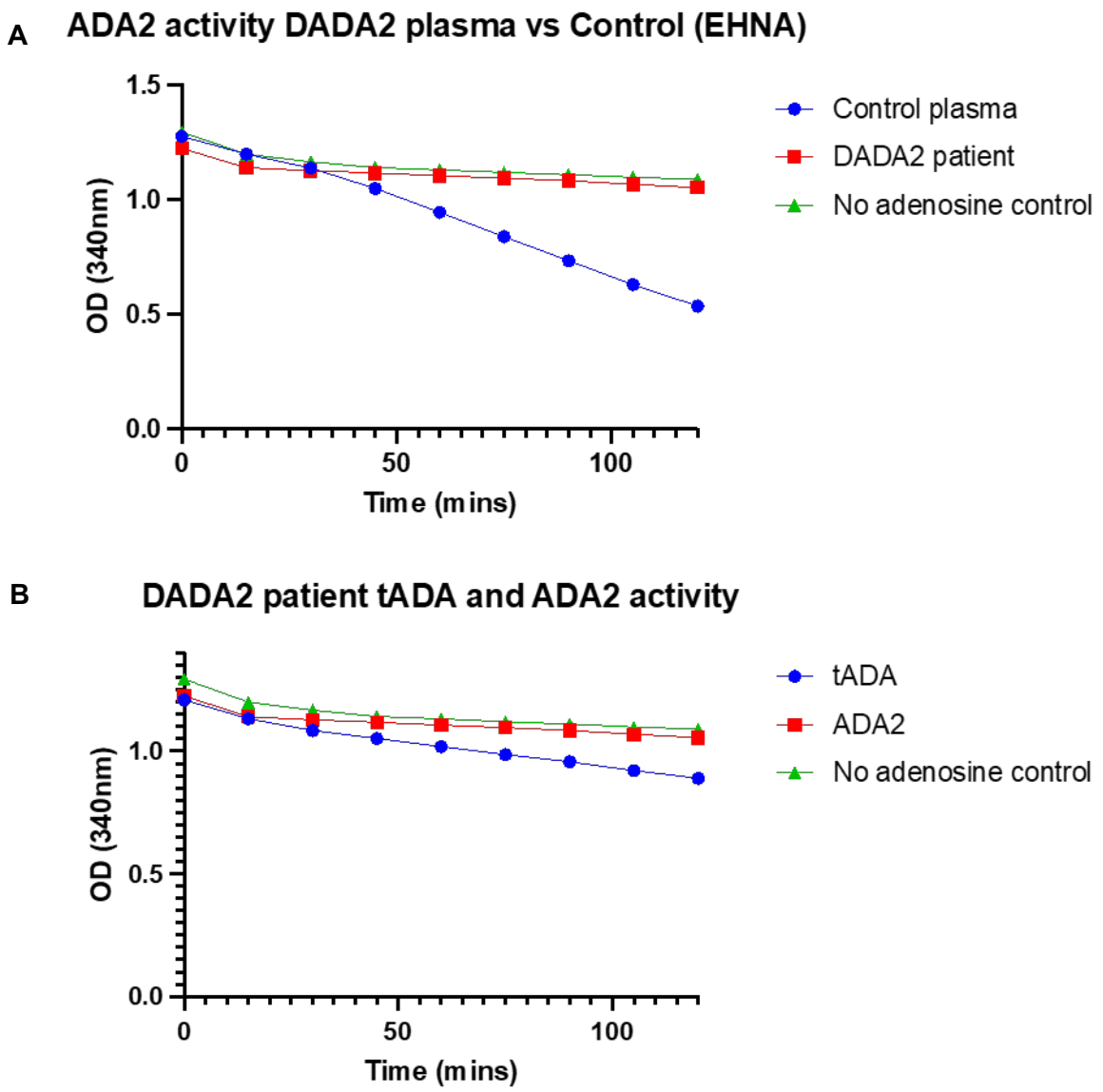


Figure 12 – ADA activity in a patient with DADA2

(A) ADA2 activity was measured in the plasma of a patient with DADA2 and a healthy control. The addition of EHNA inhibits ADA1 activity. In the DADA2 patient there is no fall in OD 340nm in addition to the background decline seen in the no adenosine control. This indicates there is negligible ADA2 activity in the sample. There is a fall in the healthy control sample in keeping with ADA2 activity. (B) Total ADA activity and ADA2 activity (with EHNA) were measure in the DADA2 patient sample. The fall in OD 340nm in the tADA sample is secondary to ADA1 activity, this is inhibited with the addition of EHNA.

3.5 Investigation of ADA2 activity in a cohort of patients with vasculitis

3.5.1 Design of study

To evaluate the potential role of ADA2 in other vasculitides, ADA2 activity was measured in adults with clinician confirmed, Takayasu arteritis (TA), ANCA vasculitis, large vessel giant cell arteritis (LV-GCA), intracranial vasculitis (ICV) and healthy control (HC) subjects using a coupled spectrophotometric assay as described. Biobanked plasma samples were used and the patient's clinical phenotype, clinical blood results and vasculitis disease activity scores at the time of sampling were recorded. Disease groups were balanced across the 96-well plates and samples were run in duplicate. The same control plasma sample was run on every plate to ensure that there was no significant inter-assay variation. rADA1 was also included on each plate with and without EHNA to confirm ADA1 inhibition.

Plasma from 11 patients with genotyped DADA2 and 10 patients heterozygous for pathogenic variants in ADA2 were used to define thresholds for deficiency and carrier status respectively (DADA2 activity 0 - 0.90 U/L, ADA2 carrier activity 2.02 - 10.62 U/L). Activity levels above the upper limit of normal (ULN) were defined as >99th percentile of the HC activity (30.3 U/L). To assess potential associations with TA disease status, ADA2 was measured prospectively in matched active and remission samples.

TA disease activity was determined as active, grumbling (those with suspected low level activity) or stable using clinical and radiological features, physician impression, C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR) and Indian Takayasu clinical Activity Score (ITAS). ANCA vasculitis disease activity was recorded using the Birmingham Vasculitis Activity Score (BVAS) (BVAS=0 defined as remission, BVAS >0 defined as active).

Median values are presented with differences between groups analysed using non-parametric tests. $P<0.05$ was considered significant.

3.5.2 Patient demographics

ADA2 activity was measured in 302 vasculitis patients and 52 HC subjects. Demographic details of the subjects are provided in Table 10. As per the known female predominance in TA and LV-GCA there were significantly more females in these groups (91.1 and 81.2% respectively) [112]. There was a range of ethnicities of subjects as reflective of the local demographics. A range of patients with very active vasculitis, reflected by high disease activity

scores, and non-active disease were included (TA group; active = 41 (30.4%), grumbling = 23 (17%), stable = 71 (52.6%), ANCA group; active = 61 (52.6%), remission = 55 (47.4%)).

	Takayasu arteritis	ANCA vasculitis	LV-GCA	Intra-cranial vasculitis	Healthy control
N	135	116	44	7	52
Female	123 (91.1)	55 (47.4)	36 (81.2)	4 (57.1)	42 (81)
Age	43.2 [17.1 – 73.8]	62.7 [23.8 – 86.0]	67.3 [56.8 – 84.1]	50.0 [33.4 – 77.9]	42.6 [22.7 – 70.0]
Ethnicity					
Caucasian	71 (52.6)	63 (54.3)	35 (79.5)	5 (71.4)	40 (77)
Asian	48 (35.5)	24 (20.7)	5 (11.4)	2 (28.6)	7 (13.4)
Mixed	3 (2.2)	3 (2.6)	1 (2.3)	0	0
Afro-Caribbean	3 (2.2)	5 (4.3)	1 (2.3)	0	3 (5.8)
Middle East	5 (3.7)	0	0	0	2 (3.8)
African	3 (2.2)	0	0	0	0
Other	2 (2.2)	21 (18.1)	2 (4.5)	0	0
ITAS 2010	0 [0 -22]	N/A	N/A	N/A	N/A
ITAS 2010 ESR	2 [0 – 22]	N/A	N/A	N/A	N/A
ITAS 2010 CRP	1 [0 -22]	N/A	N/A	N/A	N/A
BVAS	N/A	3 [0 - 26]	N/A	N/A	N/A

Table 10 – Patient demographic data

3.5.3 There were no patients with ADA2 deficiency in this cohort

In this cohort of 302 vasculitis patients and 52 HCs, there were no cases of deficient ADA2 activity (Figure 13). There were 9 subjects that had activity within the carrier range (1 HC (1.9%), 2 ANCA (1.7%), 5 TA (3.7%) and 1 LV-GCA (2.2%)) (Figure 13). Fishers exact test showed no statistically significant difference in the rate of carriers in the HC vs vasculitis cohort. Genetic testing was not possible in these patients due to lack of appropriate samples for DNA isolation, but these levels may indicate the heterozygous presence of pathogenic ADA2 variants.

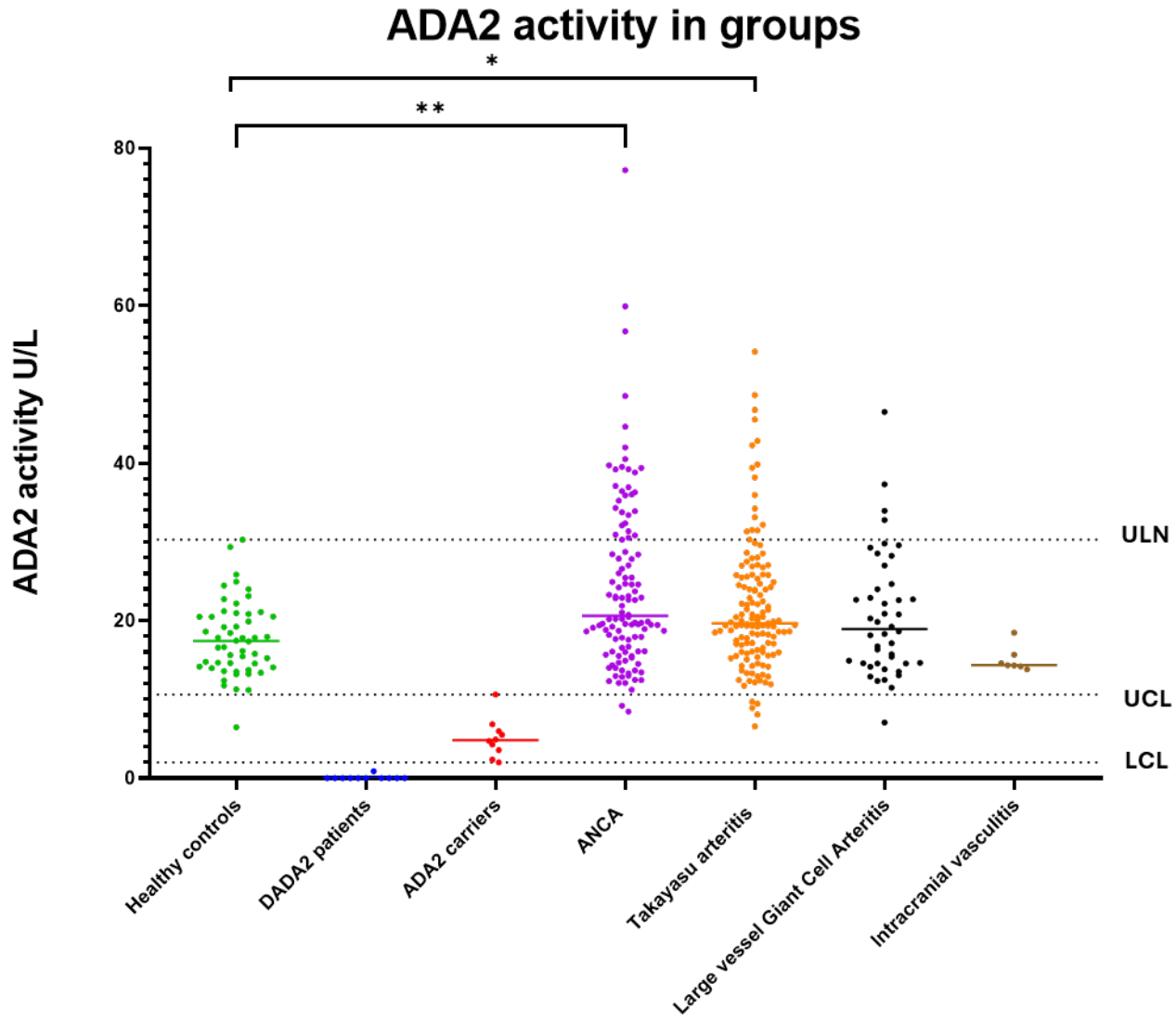


Figure 13 – ADA2 activity in vasculitis patients and healthy control subjects

*ADA2 activity was measured in the plasma of vasculitis patients and healthy control subjects using the previously described ADA2 activity assay. Upper limit of normal (ULN) were defined as >99th percentile of the HC activity (30.3 U/L), Upper (UCL) and lower carrier level (LCL) were defined by the range of the 10 patients who were heterozygous for ADA2 variants (*2.02 - 10.62 U/L). One-way ANOVA with Dunnett's post-hoc tests were performed (* $p<0.05$, ** $p<0.001$).

3.5.4 ADA2 activity was elevated in patients with Takayasu arteritis and ANCA vasculitis

Compared to HC levels (median value 17.43 U/L [14.11 – 20.78]), activity in the TA and ANCA groups was significantly increased (TA 19.68 U/L [6.6 – 54.16], p=0.0113; ANCA 20.62 U/L [8.5 – 77.2], p=0.0002), while LV-GCA and ICV were not statistically different (Figure 13). In 50 cases, activity exceeded the upper limit of normal; 16 TA (11.9%, p=0.0068; 4 active, 2 grumbling, 10 stable), 30 ANCA (25.9%, p<0.0001, 14 active, 16 remission) and 4 LV-GCA (9.1%, p=0.0409) (Figure 13).

Subgroup analysis within the TA group revealed that ADA2 activity was most prominently elevated in the TA stable group relative to HCs (TA stable 20.54 U/L [8.08 - 48.66], median +17.8%, p= 0.0006), with no significant difference in active or grumbling TA (Figure 14A). In ANCA, activity was elevated in both the active and remission groups (active 21.04 U/L [8.48 – 77.22], p= 0.0008, remission 19.88 U/L [12.12 – 47.82], p= 0.0021) (Figure 14B).

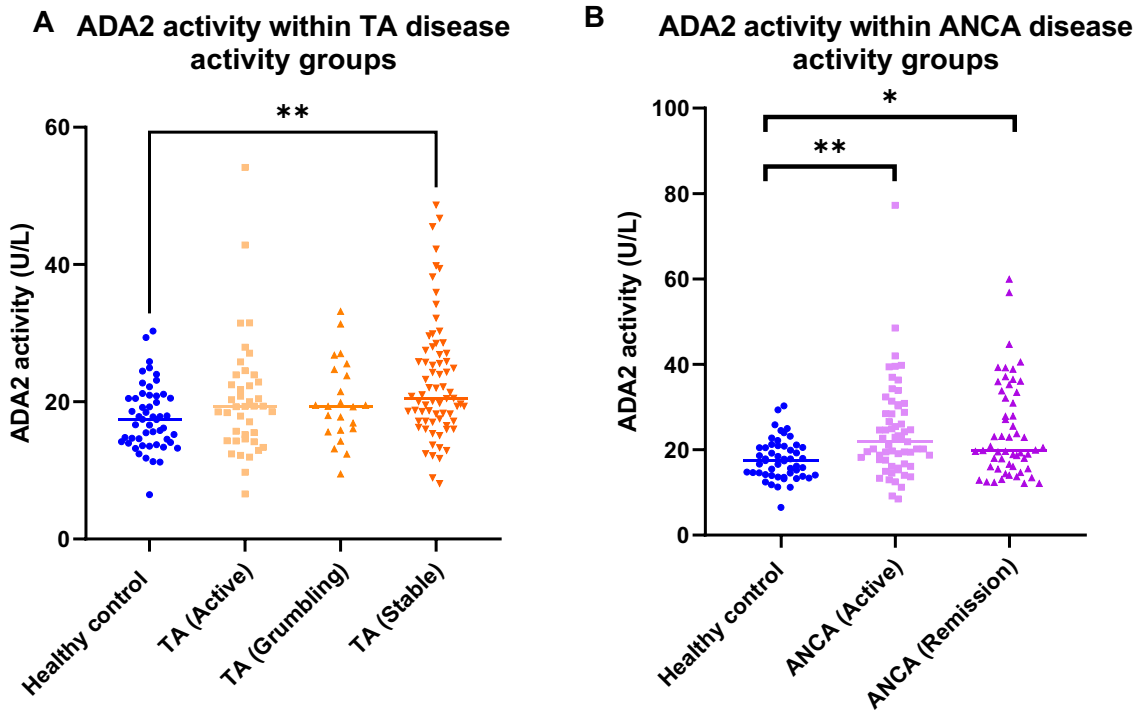


Figure 14 – ADA2 activity within TA and ANCA activity groups

ADA2 activity was compared between healthy controls and (A) TA patients with active, grumbling or stable disease or (B) ANCA vasculitis with active disease or those in remission. One-way ANOVA with Dunnett's post-hoc tests were performed (* p<0.05, ** p<0.001)

3.5.5 Prospective analysis of ADA2 activity in active and remission disease activity samples in TAK

Consistent with the ADA2 activity being most prominently elevated in the TA stable group, prospective analysis demonstrated a significant increase in ADA2 activity in remission vs active disease (p=0.024), with activity increasing in 18 of 29 (62%) patients achieving remission (Figure 15).

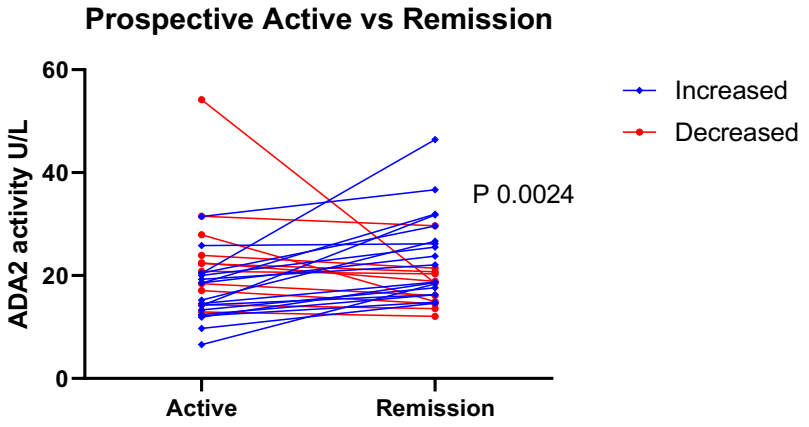


Figure 15 – There was a significant increase in ADA2 activity in prospective analysis of TA samples in remission vs active disease

ADA2 activity was measured prospectively in the plasma of TA patients with active disease and then in remission. Wilcoxon paired rank test statistics were performed.

3.5.6 ADA2 activity was associated with age, immune subset counts and ESR

The ADA2 activity in the plasma of the vasculitis patients (n=135) was correlated with a number of clinically measured blood markers which had been recorded at the time of sampling. These were collected from the electronic patient records at Imperial College NHS Healthcare Trust. All patients provided written consent. In the TA group there was a significant positive correlation between ADA2 activity and monocyte count (Spearman r = 0.297, p=0.0009), eosinophil count (r = 0.448, p<0.0001), ESR (r = 0.2857, p= 0.001) and age (r = 0.204, p=0.0175). There was a significant negative correlation between ADA2 activity and neutrophil count (Spearman r = -0.254, p=0.005). There was no significant correlation between ADA2 activity and TA disease activity score (ITAS 2010). A correlation matrix demonstrating these data is shown in Figure 16A.

In the LV-GCA group (n=44) there was a significant positive correlation between ADA2 activity and lymphocyte count (r = 0.305, p=0.049), monocyte count (r = 0.305, p=0.049), eosinophil count (r = 0.315, p=0.042), CRP (r = 0.330, p=0.029), ESR (r = 0.407, p=0.008) and age (r = 0.317, p=0.036) (Figure 16B). The only significant correlation with ADA2 activity in the ANCA (n=116) group was with age (r = 0.270, p=0.003) (Figure 16C). Figure 16D shows ADA2 activity vs age in all TA, LV-GCA and ANCA patients, the only significant correlation in all groups (r = 0.213, p=0.0002). Analysis of ADA2 activity in our HC cohort showed no correlation with age albeit this group had narrow age range (median 38 years (22.7-68.9)).

To assess the independence and relative importance of these associations, a linear regression analysis was performed in the TA group with all variables that were significantly correlated with ADA2. The linear regression model showed that there were significant and independent association between ADA2 activity and eosinophil count in the TA group (estimate 12.07, standard error [SE] = 5.63, p=0.034), neutrophil count (estimate -0.51, SE = 0.23, p=0.032), ESR (estimate 0.09, SE = 0.03, p=0.002) and age (estimate 0.10, SE = 0.05, p=0.045).

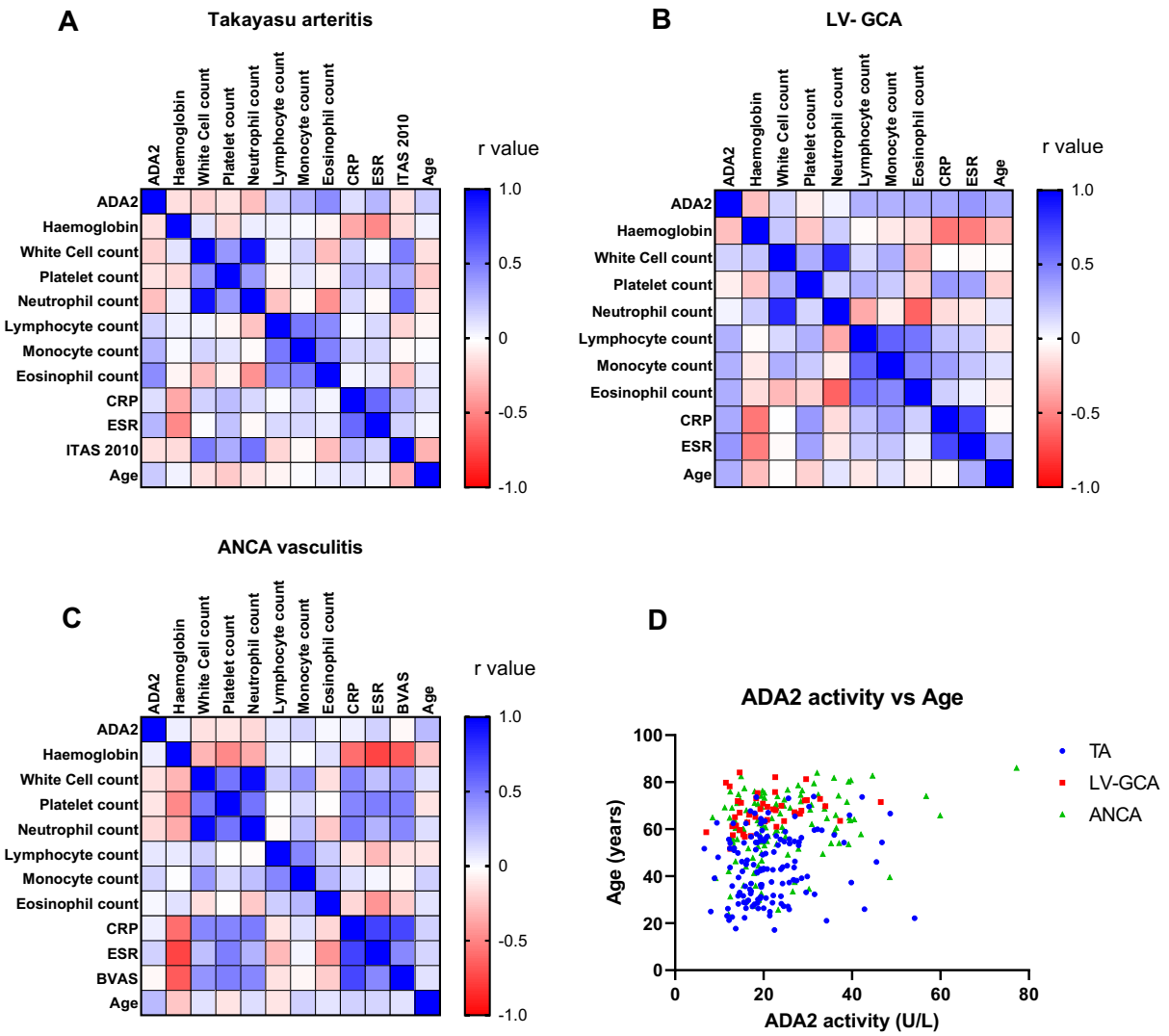


Figure 16 – Assessment of correlation between plasma ADA2 activity and blood markers, age and disease activity scores.

Plasma ADA2 activity was correlated with blood parameters, age and disease activity scores in patients with (A) Takayasu arteritis, (B) LV-GCA and (C) ANCA vasculitis. Correlation matrices were generated and Spearman r value for each data pair calculated. (D) ADA2 activity was plotted against age for patients with TA, LV-GCA and ANCA vasculitis. Spearman rank correlation was performed.

3.6 Discussion

In this chapter an ADA2 enzymatic assay was developed and validated in patient plasma. The assay appropriately identified completely deficient ADA2 activity in the plasma of patients with genotyped DADA2 and partial loss of activity in patients heterozygous for DADA2 variants. ADA2 activity was also measured in the plasma of 302 vasculitis patients, with both large and small vessel involvement. No patients with deficient activity were detected but 8 had plasma ADA2 activity within the carrier range. Consistent with other inflammatory conditions, ADA2 activity was significantly increased in the plasma of patients with TA and ANCA vasculitis. Although ADA2 activity did not correlate with vasculitis disease activity scores, there were correlations demonstrated between ADA2 activity and clinically measured blood markers including monocyte, lymphocyte and eosinophil count as well as markers of inflammation including CRP and ESR.

The development and optimisation of the ADA2 enzymatic activity assay is a vital tool for the primary aim of this thesis in determining the role of ADA2 in the endothelium. ADA2 protein detection has been well documented to be challenging to accurately quantify and has often required the use of a HIS/MYC tag to be added [12, 167]. While this assay has not been clinically accredited for use in our laboratory, the assay may be used to indicate the possibility of deficient ADA2 plasma activity in local cases of suspected DADA2. In many cases, the interpretation of ADA2 genotyping results can be challenging since the pathogenic consequences of certain variants is not always clear. An ADA2 enzymatic activity assay allows a functional quantification of whether ADA2 enzymatic activity is present and therefore permits rapid, cost-effective screening to allow early diagnosis and commencement of effective treatment in those who have DADA2.

Given the heterogeneity in presentation of DADA2, with many overlapping features with other forms of vasculitis, one of the aims of this chapter was to establish whether any patients in a large cohort of vasculitis patients had deficient ADA2 activity in the plasma. Despite measuring ADA2 activity in 302 patients, there were no cases of deficient ADA2 activity identified. The lack of ADA2 deficient cases found in this relatively large cohort, demonstrates that undiagnosed DADA2 is not prevalent in patients with large and small vessel vasculitis. This would be consistent with the predicted DADA2 disease prevalence of 1 in 222,000 individuals [41]. The recent international consensus statement on DADA2 suggests patient groups that should be screened [42]. These include individuals with polyarteritis nodosa, livedo racemosa, unexplained autoinflammatory syndromes, ischaemic or haemorrhagic stroke during childhood or early adulthood and certain haematological conditions [42]. Our work supports

that more widespread ADA2 activity testing in vasculitis patients is not indicated and should be confined to the above groups or those who are refractory to conventional treatment.

Of interest, 9 individuals (1 HC, 2 ANCA, 5 TA and 1 LV-GCA) had ADA2 activity within a predicted range for a carrier of an ADA2 variant. Population genetics studies estimate the carrier frequency of deleterious ADA2 variants is 1 in 236 individuals [41]. In a cohort of 302 vasculitis patients the predicted number of patients with an ADA2 variant would be 1.3 patients. The 8 identified patients is therefore, although not significant on fishers exact test, more than would be expected. This raises interesting questions as to whether being a carrier of a deleterious ADA2 variant may leave you at higher risk of developing another form of vasculitis. The long-term clinical consequences of carrying heterozygous ADA2 variants are poorly defined. There are some reports which suggest that individuals with heterozygous genotypes that also have interim levels of plasma ADA2 activity, have certain clinical manifestations suggestive of DADA2 [9]. Many however would disagree and suggest that these individuals have an alternative disease which results in overlapping clinical features to those seen in DADA2. Further work needs to be carried out to understand whether the presence of monoallelic DADA2-associated variants predisposes to polygenic vasculitis and other vascular diseases such as cardiovascular disease and stroke. As of yet, genome-wide association studies have not linked common variants in the ADA2 gene to these vascular disorders [168, 169].

Consistent with other inflammatory conditions, plasma ADA2 activity was elevated in both TA and ANCA patients. A number of hypotheses have been proposed as to why ADA2 activity is elevated in certain infections, autoimmune conditions and malignancies. One hypothesis is that this represents a marker of immune activation to fight infection or cancerous cells [157, 158, 165]. More specifically it is thought to represent activation of monocytes and macrophages as is consistent with its high level of expression in these cells [43, 159, 160, 170]. The elevated levels in malignant versus benign breast and ovarian cancers is thought to be caused by macrophage infiltration into malignant tissues and subsequent ADA2 production as well as increased ADA2 production as a compensatory mechanism against toxic accumulation of substrates such as adenosine [66, 163]. Monocyte and macrophages are well known to play a key role in the pathogenesis of both large and small vessel vasculitis [171, 172]. It would therefore be possible to hypothesise that the elevated ADA2 activity seen in TA and ANCA patients is likely to represent monocyte and macrophage activation and subsequent ADA2 production. It remains unclear as to whether the secreted ADA2 has a role to deaminate excess adenosine at sites of inflammation or it is a by-product of activated monocytes.

The hypothesis of immune activation is further supported from the correlation analysis. In TA and LV-GCA groups there was a significant correlation between higher ADA2 activity and monocyte and eosinophil counts. There was also a significant correlation with lymphocyte count and ADA2 activity in the LV-GCA group. Although myeloid cells have the highest level of expression of ADA2, other immune cells such as eosinophils and lymphocytes also express it [60, 62]. Given the complex multi-cellular interactions in the pathogenesis of vasculitis it is likely that the correlation between these immune cells and ADA2 activity represents immune activation as part of the pathogenesis of the disease [173]. The significant positive correlation between blood markers of inflammation; ESR and ADA2 activity in TA group and CRP and ESR with ADA2 activity in LV-GCA group, gives further evidence that this is related to inflammation likely as a consequence of immune cell activation. Interestingly however despite significant higher ADA2 activity in ANCA patients vs HC, the immune cell and inflammatory marker correlations were not demonstrated in the ANCA group.

Although previous studies have demonstrated correlation between disease activity and ADA2 activity in rheumatoid arthritis and systemic lupus erythematosus, this was not evident in our vasculitis patient data [63, 162]. ITAS score in TA patients and BVAS score in ANCA patients showed no correlation with ADA2 activity. This is slightly surprising in the TA group as there was correlation demonstrated between immune cell count and ESR so one may have hypothesised that there would have been a correlation seen with disease activity. This may represent some of well-known challenges and inaccuracies of disease activity scoring systems. It must also be noted that for a number of the TA patients there was retrospective ITAS scoring from the date of sampling which is well documented to have a degree of inaccuracy.

Subgroup analysis demonstrated that ADA2 activity was most prominently elevated in the TA stable group relative to HCs and prospective analysis demonstrated a significant increase in ADA2 activity in remission vs active disease in TA patients. This is interesting and would not be what is expected given the previous hypothesis and data to support that the elevated ADA2 activity is related to immune cell activation and inflammation. One possible explanation for this could be differences in steroid doses between the groups which could affect the number of immune cells and their activation. This was also not demonstrated in the ANCA group where activity was elevated in both the active and remission groups. It is therefore important to take this subgroup analysis, which has small numbers, into context of the results as a whole.

Interestingly there was a significant positive correlation between ADA2 activity and age in the TA, LV-GCA and ANCA groups. Previous studies however have demonstrated that in healthy control adults and children there is a negative correlation with age and ADA2 activity [43]. Our

group is of course a cohort of patients with vasculitis whom, in the TA and ANCA groups, had significantly elevated ADA2 activity compared to HCs which may explain the opposite correlation previously described with age. Analysis of ADA2 activity in our HC cohort showed no correlation with age albeit this group had narrow age range (median 38 years (22.7-68.9).

Despite some very interesting and novel results in this chapter, there are some limitations. We have not controlled for the potential impact of treatments that the patients were receiving. In addition, the predicted ADA2 carrier activity range in this study was determined from the measured plasma activity of 10 patients heterozygous for DADA2 variants. Carrying out genetic testing on the 8 individuals with carrier ADA2 plasma activity to confirm ADA2 variants would have been confirmatory, but this was not possible as samples appropriate for DNA isolation were unavailable and patient recall on this basis was not covered by our ethical approval. Another important consideration is that we are using a functional assay and not measuring ADA2 protein concentration. Attempts were made to optimise an ADA2 ELISA to assess whether protein concentration correlated with enzymatic activity. These ELISA results were not found to be reproducible, so this analysis was not performed. It would also have been interesting to measure plasma adenosine concentrations to help determine whether elevated ADA2 activity in TA and ANCA patients was as a consequence of elevated adenosine concentrations which may be explained by inflammation.

In summary, we have developed and optimised an ADA2 activity assay which was an invaluable tool for this PhD in determining the role of ADA2 in the endothelium. The results confirm that deficient ADA2 activity in a large cohort of vasculitis patients is not prevalent however there may be enrichment of heterozygous carriers in this group. Consistent with other inflammatory conditions, ADA2 activity was elevated in patients with TA and ANCA vasculitis and correlated with immune cells and markers of inflammation. This would therefore be consistent with being a marker of immune activation. Questions remain to understand whether this secreted ADA2 has a role in the pathogenesis of vasculitis or is merely a by-product of an activated immune system. Determining ADA2 expression in the endothelium and understanding its role will be essential to answering this question and determining its role in the pathogenesis of DADA2.

CHAPTER 4: Adenosine Deaminase 2 isoform expression in endothelial cells

4.1 Introduction

The prominent vasculopathic phenotype in DADA2 suggests that ADA2 could have a critical role in the maintenance of vascular integrity. The initial studies on the DADA2 phenotype did not show ADA2 expression in the endothelium and it was thought that the endothelial damage seen in knockdown zebrafish embryos and in brain and skin biopsies from DADA2 patients was secondary to dysfunctional immune cells [2, 3].

More recently, Dhanwani et al reported demonstration of ADA2 mRNA and protein detection in ECs with qPCR, ELISA and immunoblotting [88]. They detected ADA2 enzymatic activity in the supernatant of HUVEC and showed that depletion of ADA2 in ECs resulted in IFNβ stimulation [88]. This suggested a potential role of ADA2 in endothelial homeostasis with loss of EC ADA2 expression being involved in the pathogenesis of DADA2.

The aim of this chapter was to confirm ADA2 RNA and protein expression in the endothelium. The catalytic enzymatic activity of endothelial ADA2 was explored as well as its stimulation and regulation.

4.2 Hypothesis of the chapter

ADA2 is expressed by EC and has a role in endothelial homeostasis.

4.3 Aims of the chapter

1) Determine ADA2 mRNA and protein expression in EC
2) Investigate the catalytic activity and function of ADA2 protein in EC
3) Evaluate the response to stimulation and transcriptional regulation of ADA2

4.4 ADA2 isoforms and their expression

Classically ADA2 has been described as a protein consisting of 511 amino acids which is transcribed from a gene consisting of 3925 base pairs. Review of public resources including Ensembl [174] showed that multiple of isoforms of ADA2 exist (Table 11). A number of these are potentially protein coding and have differing amino acid sequences to the classically

described ADA2 protein of 511 amino acids. An exon map of the 3 predominant isoforms expressed across multiple human tissue types in the GTEx Portal, ADA2 201, 202 and 203 are shown in Figure 17A [175].

Transcript ID	Name	bp	Protein	Biotype
ENST00000262607.3	ADA2-201	3925	511aa	Protein coding
ENST00000330232.9	ADA2-202	3199	270aa	Protein coding
ENST00000399837.8	ADA2-203	4355	511aa	Protein coding
ENST00000399839.5	ADA2-204	3986	511aa	Protein coding
ENST00000449907.8	ADA2-205	4010	469aa	Protein coding
ENST00000469063.2	ADA2-206	3606	No protein	Retained intron
ENST00000480276.1	ADA2-207	570	No protein	Retained intron
ENST00000543038.2	ADA2-208	3810	511aa	Protein coding
ENST00000610390.5	ADA2-209	3987	391aa	Protein coding
ENST00000648061.2	ADA2-210	2937	69aa	Nonsense mediated decay
ENST00000648343.1	ADA2-211	470	No protein	Protein coding CDS not defined
ENST00000648668.1	ADA2-212	3140	No protein	Protein coding CDS not defined
ENST00000649310.2	ADA2-213	4343	511aa	Protein coding
ENST00000649540.1	ADA2-214	1932	469aa	Protein coding
ENST00000649746.2	ADA2-215	5333	511aa	Protein coding
ENST00000649915.2	ADA2-216	3600	187aa	Nonsense mediated decay
ENST00000650635.1	ADA2-217	969	No protein	Retained intron
ENST00000696189.1	ADA2-218	496	No protein	Retained intron
ENST00000696196.1	ADA2-219	4383	511aa	Protein coding
ENST00000696197.1	ADA2-220	5353	511aa	Protein coding
ENST00000696198.1	ADA2-221	576	No protein	Retained intron
ENST00000696218.1	ADA2-222	1816	No protein	Retained intron
ENST00000696220.1	ADA2-223	3455	253aa	Protein coding
ENST00000696221.1	ADA2-224	2476	261aa	Protein coding
ENST00000696222.1	ADA2-225	1750	359aa	Protein coding
ENST00000696223.1	ADA2-226	1268	274aa	Protein coding
ENST00000696224.1	ADA2-227	3828	309aa	Protein coding
ENST00000696225.1	ADA2-228	4931	511aa	Protein coding
ENST00000696226.1	ADA2-229	1717	267aa	Protein coding
ENST00000696227.1	ADA2-230	1354	187aa	Protein coding

Table 11 – Isoforms of ADA2 listed on the ENSEMBL genome browser

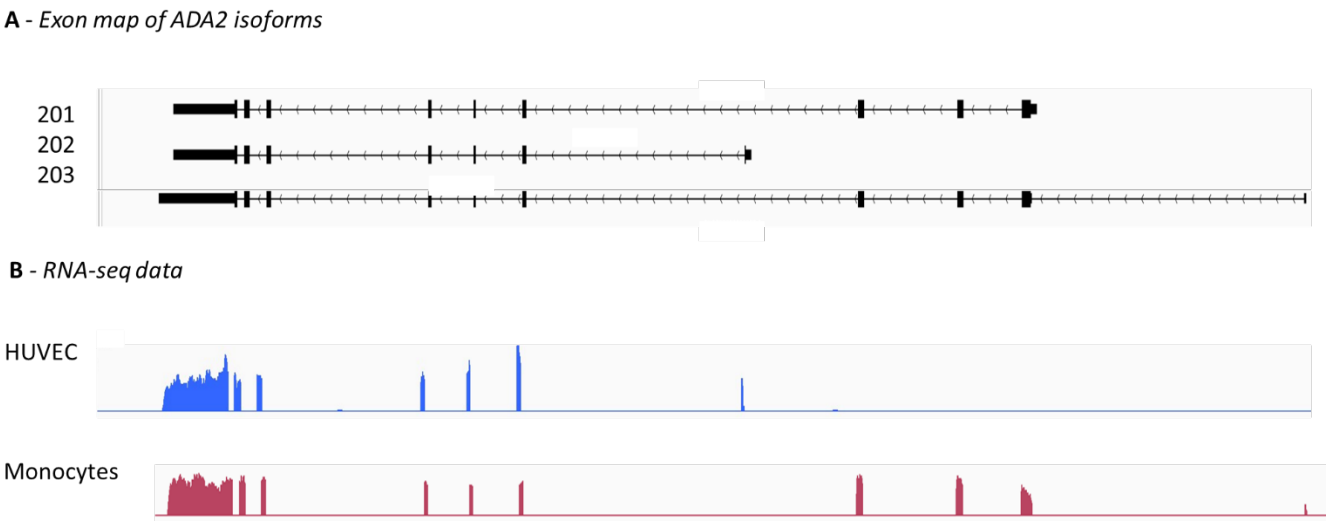


Figure 17 – HUVEC express the ADA2 202 isoform whereas monocytes express ADA2 201.

(A) Exon map from GTEX database demonstrating the predominantly expressed isoforms of ADA2. (B) Signal plot of RNA-seq from the ENCODE project showing HUVEC only express exons present in ADA2 202 with no expression of any exons that are upstream of this. Monocytes only express exons in ADA2 201/203 and not in exon exclusive to ADA2 202.

Review of transcriptomic databases demonstrate that various cell and tissue types express different isoforms of ADA2. As previously described, myeloid cells (particularly monocytes and macrophages) predominantly expressed ADA2 201 and 203 which is the classically described 511 amino acid protein (Figure 18). These are also the predominant isoforms in B and T cells as well as NK cells, eosinophils, and basophils (Figure 18).

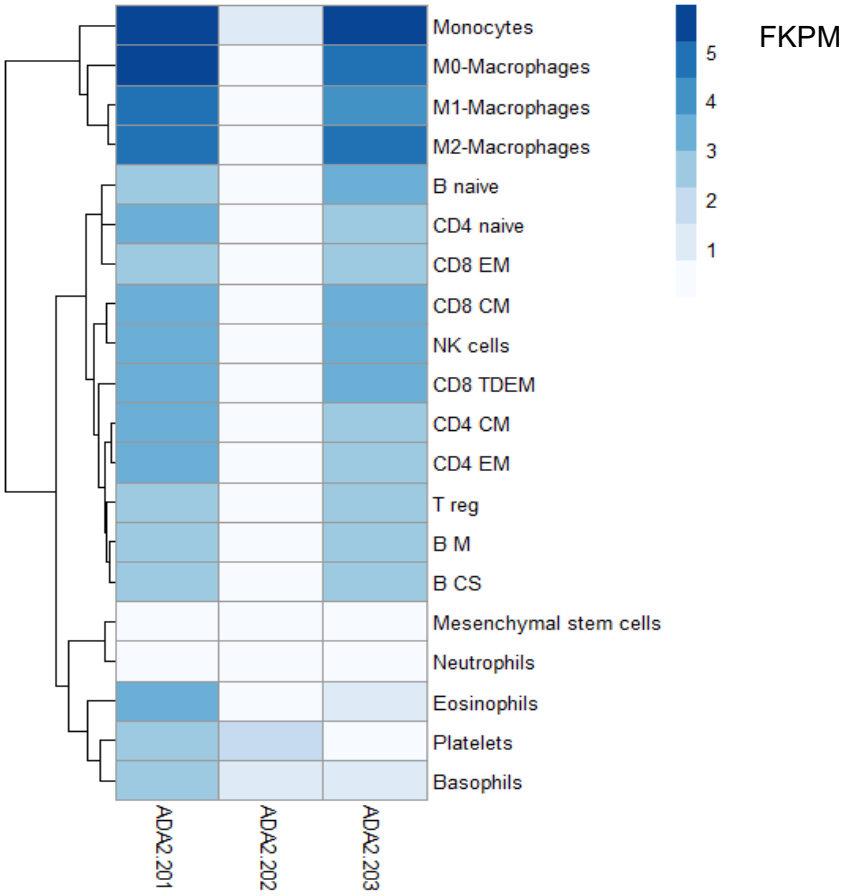


Figure 18 – ADA2 201 and 203 are the predominantly expressed isoforms in myeloid and other immune cells.

Transcriptomic data was analysed from the BLUEPRINT blood database to assess ADA2 isoform RNA expression in blood derived cells.

In tissue extracts the predominant expressed isoform is ADA2 202 which encodes a protein made up of 270 amino acids (Figure 19). The highest levels of ADA2 202 expression were observed in the spleen as well as the pituitary, lung and brain.

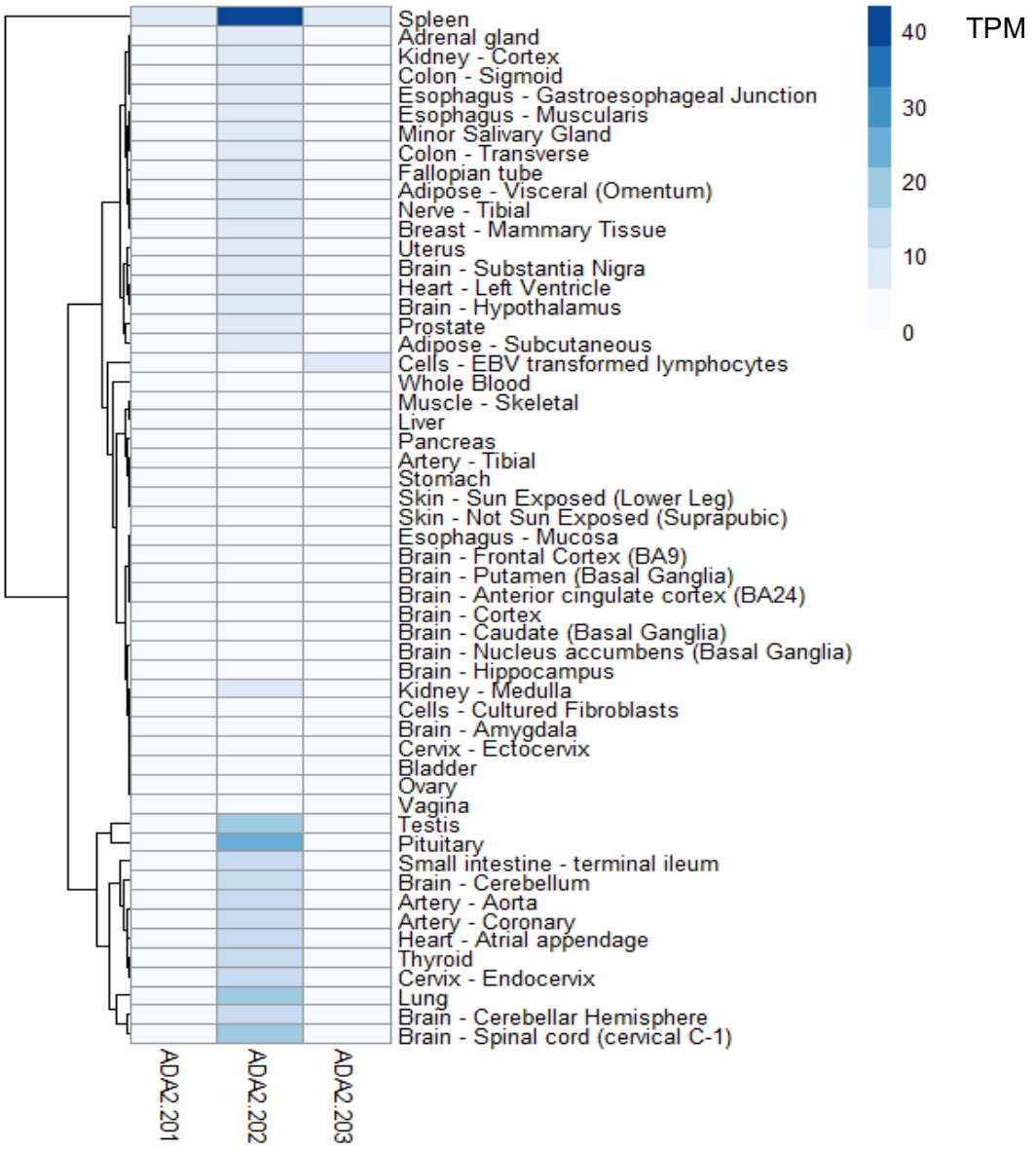


Figure 19 – ADA2 202 is the predominantly expressed isoform expressed in tissue extracts.

Transcriptomic analysis from the GTEX database was carried out on ADA2 isoform RNA expression in tissues.

4.5 Endothelial cells express the ADA2 202 isoform

Analysis of the ENCODE database demonstrated that ADA2 202 is the predominant isoform expressed in HUVEC (Figure 20) [176]. This is confirmed in the signal plot from this RNA analysis in HUVEC which demonstrates there is no evidence of any expression of other exons upstream of ADA2 202 (Figure 17B). This contrasts with monocyte RNA-sequencing (RNA-seq) data which show evidence of expression of all exons in ADA2 201 (Figure 17B).

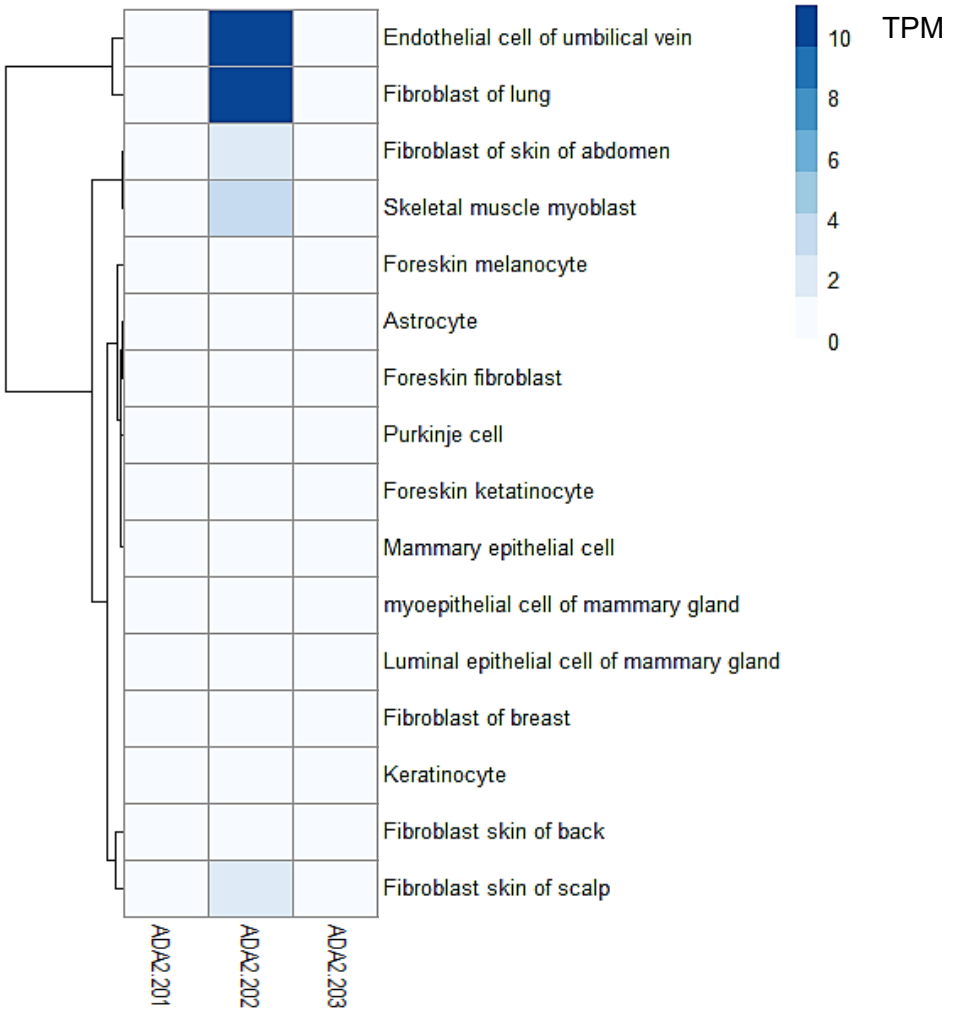


Figure 20 –ADA2 202 is the predominant isoform expressed in HUVEC.
Transcriptomic analysis from the ENCODE libraries was carried out on ADA2 isoform RNA expression in primary cells.

To rule out that there may be multiple shorter isoforms present at these genetic loci, we also examined previously published HUVEC long-read RNA-sequencing data [177]. In this data, there is only one transcript present, and it matches ADA202 with 99.87% identity and 84.8% coverage. The full 813 nucleotide protein coding region of ADA2 202 is captured in this long read sequence with the missing portions primarily being 24bp untranslated region at the 5' end and 518bp at the 3' end.

To further characterise the ADA 202 genomic region, the presence of H3Kme1, H3Kme3 and H3K27ac histone marks in HUVEC and CD14+ monocytes were compared in publicly available ChIP-seq datasets [144] analysed alongside RNA-seq data. H3Kme1 and H3K27ac are associated with active enhancer sites while H3Kme3 is found enriched at gene promoter sites [178]. This analysis clearly identifies an active promoter at the transcriptional start site of the ADA 202 transcript in HUVEC (Figure 21, highlighted in black box). This promoter was not found in monocytes where the histone marks were enriched proximal to the ADA 201 and 203 start sites (Figure 21, highlighted in red box).

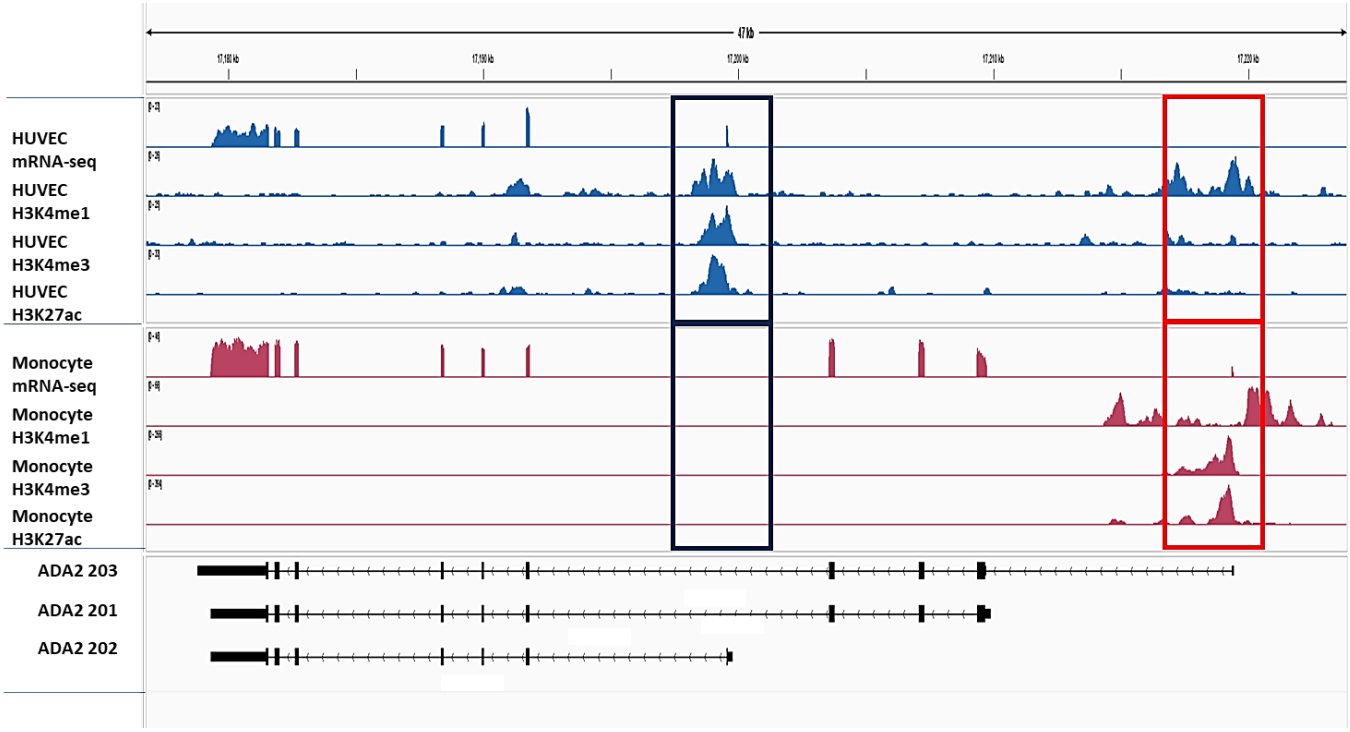


Figure 21 – Histone markers suggest that ADA2 202 is actively transcribed and regulated.

Histone marker data from the ENCODE project was analysed. Histone markers that are enhancers (H3Kme1), mark the transcription start sites of active genes (H3Kme3) and are at active sites of genetic regulation (H3K27ac) are located at the first exon of ADA2 202

(highlighted in black box). Monocytes express these histone markers at the first exon for the ADA2 203 isoform (highlighted in red box)

To validate the exclusive expression of the ADA2 202 transcript in HUVEC, primers were designed to target sequences only present of ADA2 201/203 and sequences unique to ADA2 202. qPCR was used to quantify ADA2 mRNA transcripts in endothelial cells; HUVEC, HAEC, HDVEC, HSVEC, LEC, as well as monocyte cell lines: U937 and THP1 and PBMCs from healthy volunteers. Consistent with the transcriptomic analysis, ADA2 202 was the predominantly expressed isoform in endothelial cells and ADA2 201 + 203 in monocytic cells lines and PBMCs (Figure 22).

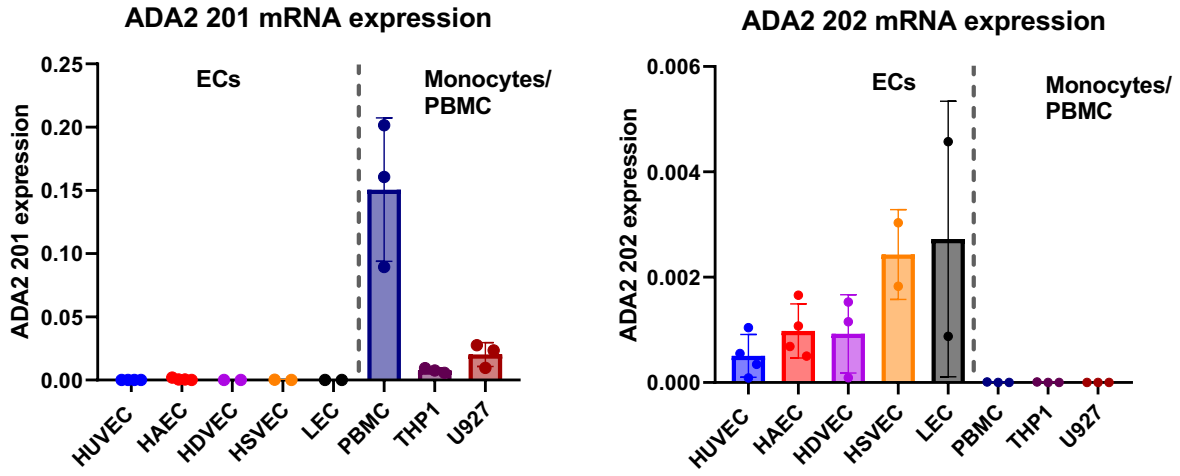


Figure 22 – qPCR analysis confirmed ADA2 201 is predominantly expressed in monocytes/PBMCs whereas ECs express ADA2 202.

HUVEC – Human Umbilical Vein Endothelial Cell, HAEC – Human Aortic Endothelial Cell, HDVEC – Human Dermal Vascular Endothelial Cell, HSVEC – Human Saphenous Vein Endothelial Cell, LEC – Lymphatic Endothelial Cell, PBMC, THP1 and U937 cells were isolated, and RNA extracted (n=2 or 3). cDNA was generated and primers specific to ADA2 201 and ADA2 202 were used to measure gene expression which was normalised to GAPDH.

4.6 ADA2 202 isoform expression in other species

To gain some evolutionary context regarding the ADA2 202 transcript, the ADA2 201 and 202 amino acid sequences were analysed in both BLASTP AND BLASTN (NCBI) to assess other species that have these proteins. The percentage identity and coverage compared to the human ADA2 201 and 202 amino acid sequences was recorded. Any results where there was >80% amino acid identity were identified. Only primates were identified as having ADA2 201 using these criteria, with many other species have a percentage identity <80% (Table 12). Interestingly only 6 primates were identified who have ADA2 202 all of which have genomes which are closely related to humans [179-182]. A dendrogram in Figure 23 demonstrates the similarity of ADA2 201 and 202 in other species.

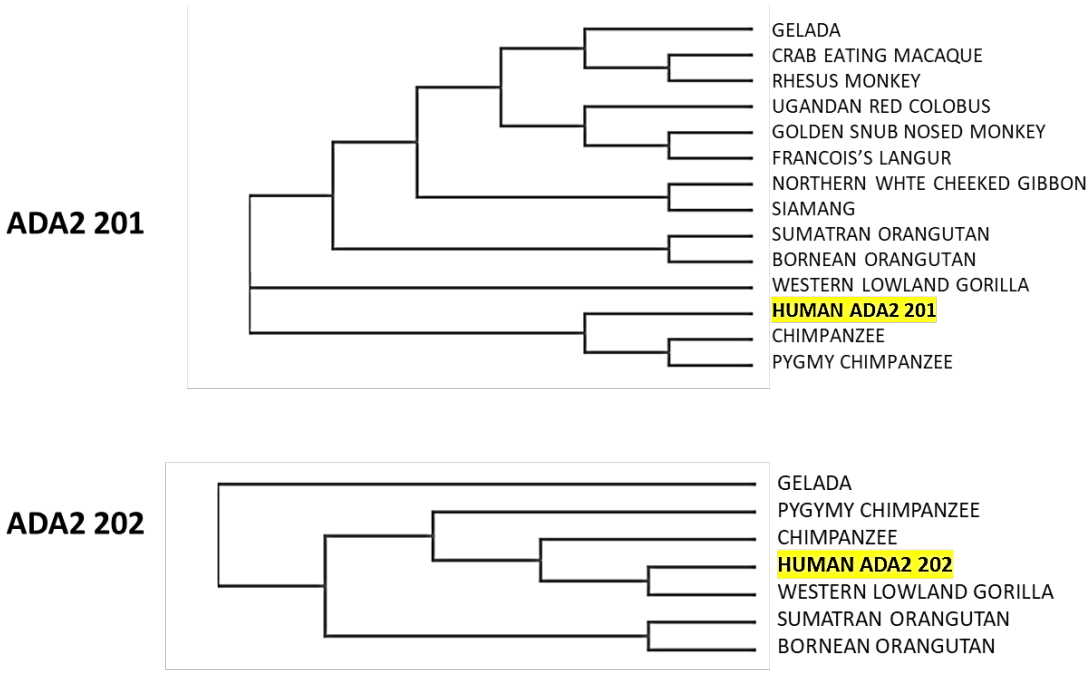


Figure 23 – A dendrogram demonstrating similarity of ADA2 201 and 202 in other species.

		ADA2 201			ADA2 202		
Family	**Organism**	**Present**	**% Identity**	**% Coverage**	**Present**	**% Identity**	**% Coverage**
Primate	Chimpanzee	Y	99.2	100	Y	98.5	100
Primate	Western lowland gorilla	Y	98.8	100	Y	99.3	100
Primate	Pygmy chimpanzee	Y	98.4	100	Y	97.8	100
Primate	Northern white-cheeked gibbon	Y	96.9	100	N		
Primate	Siamang	Y	96.3	100	N		
Primate	Sumatran orangutan	Y	97.3	100	Y	97.4	100
Primate	Bornean orangutan	Y	96.9	100	Y	96.7	100
Primate	Ugandan red colobus	Y	94.5	100	N		
Primate	Golden snub-nosed monkey	Y	93.9	100	N		
Primate	Francois's langur	Y	93.9	100	N		
Primate	Crab eating macaque	Y	93.9	100	N		
Primate	Gelada	Y	93.4	100	Y	93.7	100
Primate	Rhesus monkey	Y	93.5	100	N		
Primate	Olive baboon	Y	89.3	100	N		
Primate	Bolivian squirrel monkey	Y	86.3	99	N		
Primate	White-tuffed ear marmoset	Y	87.7	99	N		
Primate	Drill	Y	87.4	100	N		
Primate	Black snub-nosed monkey	Y	85.4	100	N		
Primate	Cocquerel's sifaka	Y	80.7	100	N		
Primate	Gray mouse lemur	Y	80.1	100	N		
Primate	Angolan colobus	Y	82.8	100	N		

Table 12 – Organisms that have ADA2 201 and ADA2 202

4.7 Predicted functional implications of ADA2 202 amino acid sequence

Review of the Human Protein Atlas database shows that ADA2 202 is made up of 270 amino acids vs 511 in ADA2 201 (predicted protein 31kDa vs 59 kDa respectively). Comparing these 2 isoforms, the first 241 amino acids are missing in ADA2 202 compared to ADA2 201 and amino acids 242-251 have an alternative sequence. From 252-511 the amino acid sequence is the same (Figure 24). This has significant implications with regards to the possible function of ADA2 202. It does not have a signal peptide which could impair its ability to be secreted (Figure 24). It is also missing the whole of the first adenosine deaminase catalytic domain and a portion of the second as well as the dimerisation and receptor binding domains (Figure 24). This will have implications in terms of its ability to deaminate adenosine to inosine and raises important questions as to what it's function may be.

The predicted ADA2 201 and 202 protein structures were generated using AlphaFold (Google Deepmind) and are shown in Figure 25. The figure shows human ADA2 201 and Pan Troglodytes (Chimpanzee) ADA2 202 as human ADA2 202 was not available. Pan Troglodytes ADA2 202 is made up of 270 AA and has 98.52% AA identity to the human form.

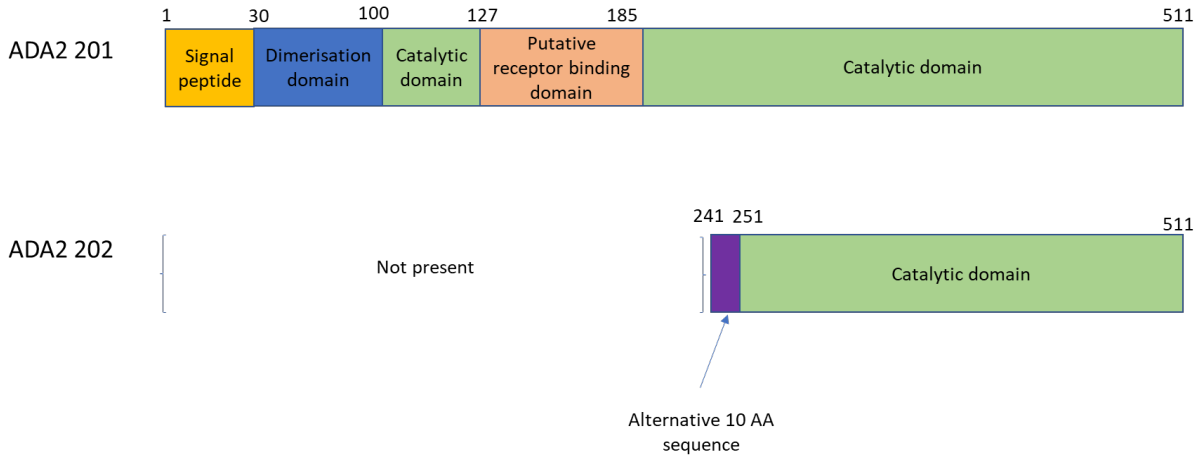


Figure 24 – ADA2 202 is lacking the amino acids for signal peptide, dimerisation domain, putative receptor binding domain and a significant proportion of its catalytic domain.

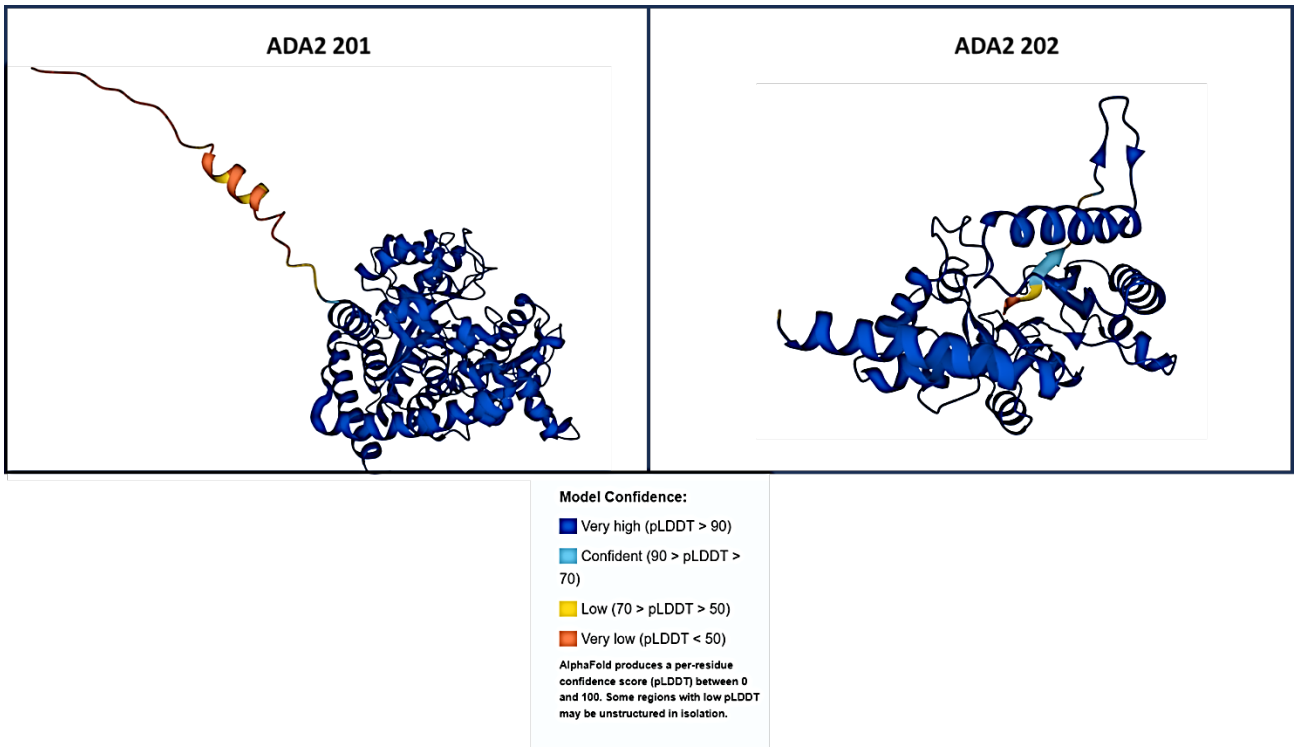


Figure 25 – ADA2 201 and 202 predicted protein structure.

AlphaFold (Google Deepmind) was used to generated predicted protein structures for ADA2 201 and 202 using the amino acid sequences.

4.8 ADA2 202 protein was not detected in endothelial cells

To assess ADA2 protein expression in EC, cell lysates and supernatant were collected from 2 pools of HUVEC and HAEC. U937 lysate and supernatant and recombinant ADA2 201 protein was used as a positive control. Immunoblot was carried out using a previously validated ADA2 antibody. This was confirmed to bind ADA2 202 protein (Figure 30). No bands consistent with ADA2 201 or 202 (59kDa or 31kDa) were identified in the HUVEC or HAEC lysate or supernatant (Figure 26). A band at 59kDa was present in the U937 lysate and supernatant consistent with ADA2 201 (Figure 26). The rADA2 used (R&D) has an additional C-terminal 6-His tag which may explain why the molecular weight appears higher on the immunoblot than ADA2 in U937 cells. The rADA2 product data sheet states that under reducing conditions the molecular weight will be 58-66 kDa.

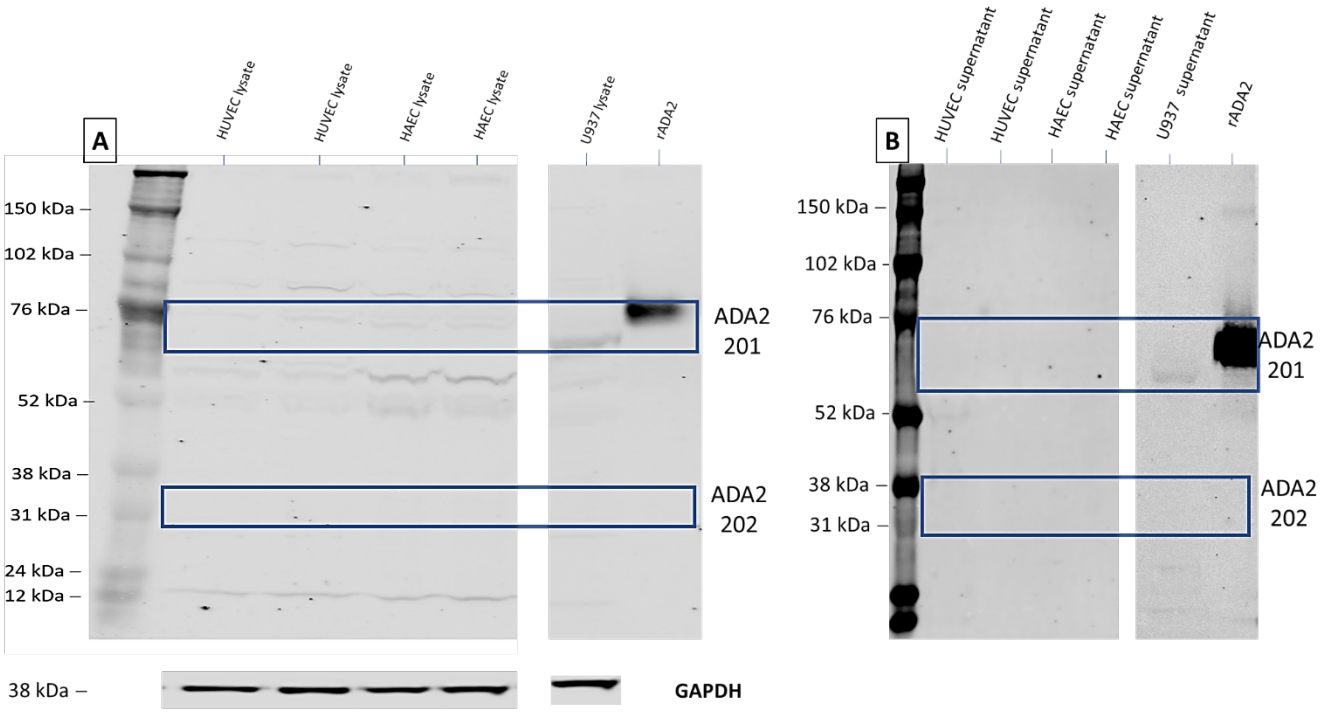


Figure 26 – ADA2 202 or 201 protein was not detected in EC lysate (A) or supernatant (B) but ADA2 201 was detected in U937 monocyte lysate (A) and supernatant (B).
Immunoblot analysis of HUVEC (n=2), HAEC (n=2) and U937 (n=1) lysate (using RIPA lysis buffer) and supernatant as well as recombinant ADA2 as a positive control. Whole cell protein lysates were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (can detect 201 and 202 isoforms) and GAPDH.

To assess whether ADA2 202 protein was present in the insoluble fraction of the cell lysate, urea extraction of pelleted remnants of RIPA lysate was carried out on 3 HUVEC pools. An immunoblot with ADA2 antibody showed no evidence of ADA2 202 protein in the urea fraction of HUVEC (Figure 27).

Mass spectrometry was carried out on lysates from 4 HUVEC pools and recombinant ADA2 201 protein. A manual search was carried out for peptides that were enriched in the recombinant sample and there was no evidence of ADA2 201 or 202 peptides in HUVEC.

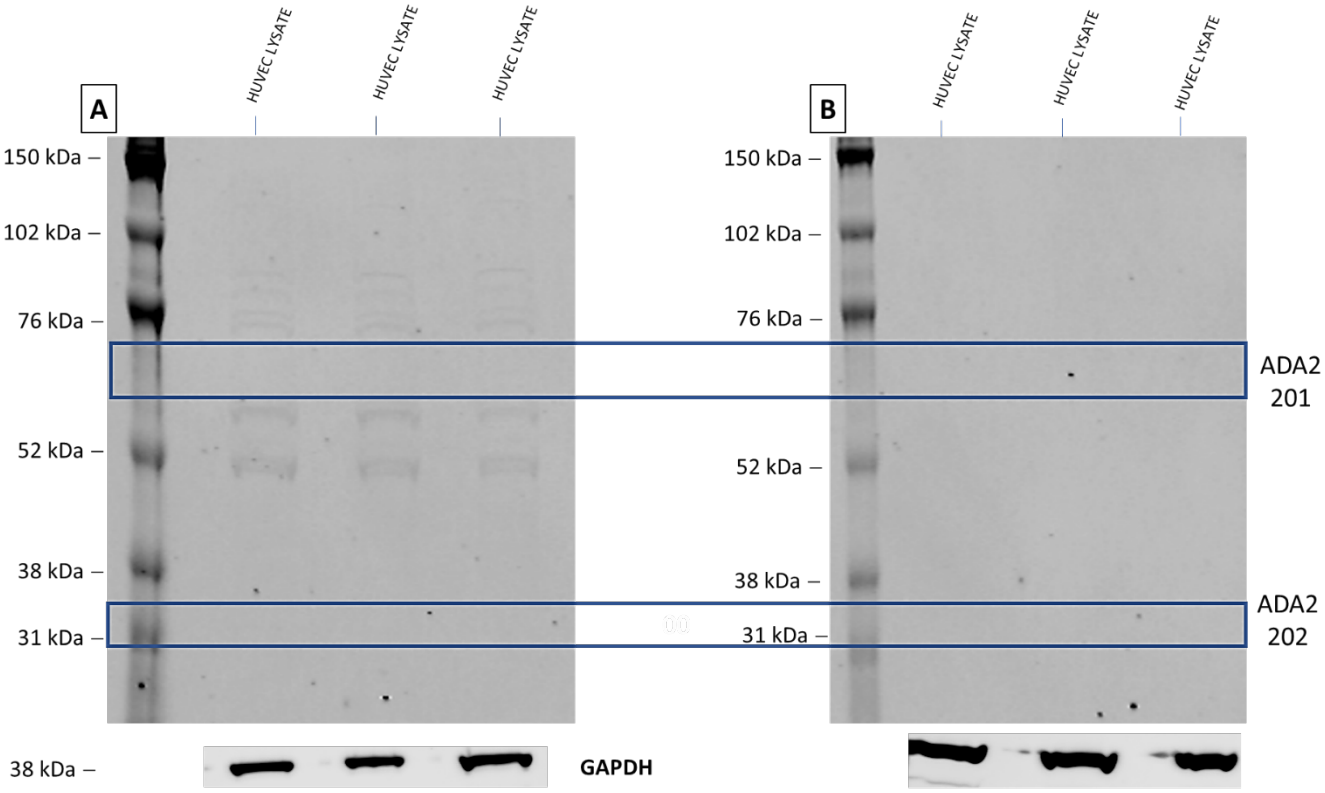


Figure 27 – ADA2 201 or 202 protein was not detected in HUVEC RIPA lysate (A) or in HUVEC Urea lysate (B).

Immunoblot analysis for HUVEC (n=3) lysate that was generated using RIPA lysis buffer (A) or with 8M Urea lysis buffer on the insoluble pellet remaining after RIPA lysate. These lysates were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (both 201 and 202 isoforms) and GAPDH.

4.9 ADA2 protein was not detected by immunofluorescence staining of endothelial cells

Immunofluorescent staining for ADA2 protein was carried out on HUVEC and PBMCs. Cellular F-actin and nuclei were also stained using phalloidin and DAPI. ADA2 staining was positive in PBMC but was not detectable in HUVEC (Figure 28).

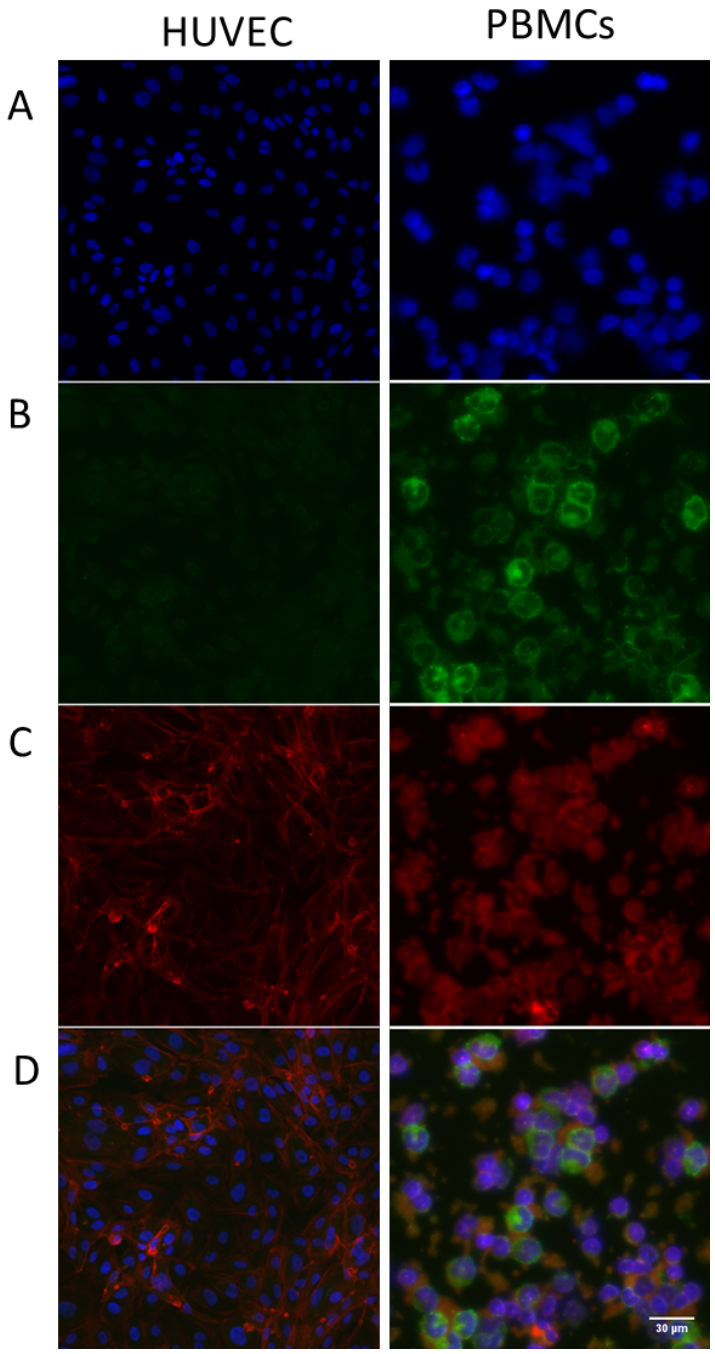


Figure 28 – ADA2 protein was not detectable in HUVEC but was in PBMCs with immunofluorescence (A-D).

HUVEC and Healthy control PBMCs were fixed and stained with antibodies for ADA2 (green) (Alexa Flur 488) (B), Phalloidin-TRITC (red) (C) and mounted with VectaShield Antifade Mounting Medium with DAPI (blue) (A). Imaging was carried out at 20x using an inverted Zeiss LSM-780.

4.10 ADA2 activity was not detected in lysate or supernatant of endothelial cells

The previously described ADA2 activity assay was optimised for use with cell lysate and supernatants. Due to interaction of RIPA in the lysate buffer and cell media in the supernatant with the assay, samples were filtered, and buffer exchanged as described in methods. EHNA was used to inhibit ADA1 activity and pentostatin used to inhibit all ADA activity.

ADA2 activity was detectable in U937 cell lysate and cell supernatant but not in HUVEC lysate or supernatant even at maximal loading concentration of 100ug of cell lysate and 10x concentration of HUVEC supernatant via filtration (Figure 29). As expected, given it is a predominantly an intracellular enzyme, ADA1 activity was higher in lysate samples in comparison to cell supernatants in both U937 and HUVEC. The use of pentostatin inhibited all ADA activity (Table 13).

Sample	Total ADA activity (U/L	ADA2 activity (U/L)	ADA1 activity U/L)	ADA activity with pentostatin (U/L)
U937 50ug lysate	53.92	22.8	31.12	0
U937 25ug lysate	57.72	12	45.72	0
U937 12.5ug lysate	37.12	3.35	33.77	0
HUVEC 100ug lysate	24.52	0	24.52	0
HUVEC 50ug lysate	10.92	0	10.92	0
HUVEC 25ug lysate	2.04	0	2.04	0
U937 supernatant (x10 concentrated)	36.8	10.62	26.18	0
HUVEC supernatant (x10 concentrated)	1.32	0	1.32	0

Table 13 – ADA activity in U937 and HUVEC lysate and supernatant

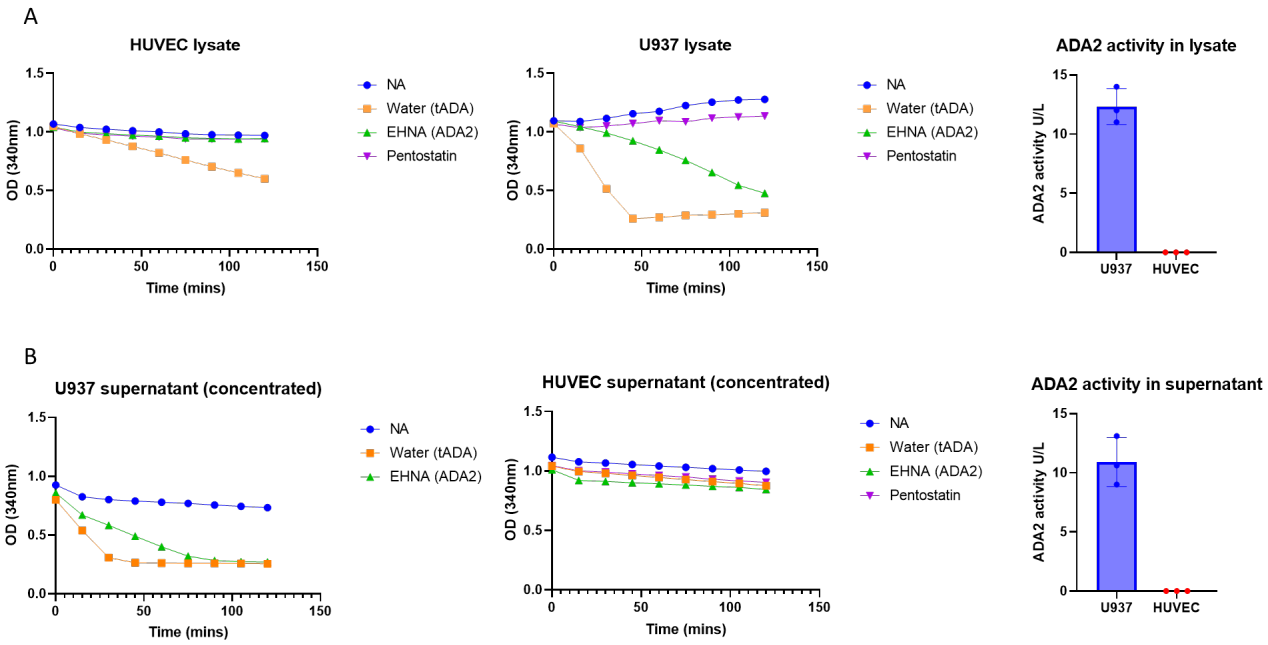


Figure 29 – There was no detectable ADA2 activity in HUVEC lysate or supernatant.
HUVEC and U937 cell supernatant and whole cell lysate was collected. These samples were buffer exchanged and concentrated prior to analysis on the described ADA activity assay. Total ADA activity (tADA), ADA2 activity (ADA2) through the addition of EHNA were measured. Pentostatin (ADA inhibitor) was used to confirm the activity measured was due to ADAs. tADA activity (corresponding to ADA1) was detected in all samples. ADA2 activity was detected in U937 lysate (A) and supernatant (B) but not in HUVEC lysate (A) or supernatant (B).

4.11 Use of a pcDNA plasmid to synthesise ADA2 201 and 202 protein

4.11.1 Transfection of HEK293T cells with ADA2 201 and 202 pcDNA plasmids

Given ADA2 202 protein was not detected in EC, further work was carried out to explore the function and structure of this protein. A pcDNA plasmid containing the ADA2 202 coding sequence and another plasmid with the ADA2 201 coding sequence was generated by our collaborator Dr Pui Lee (Boston Children's Hospital). These plasmids were transformed and expanded in E.coli cells and then plasmid DNA was purified and sequenced to confirm matching to the coding sequences (Supplementary figure 1). After confirmation of the coding sequences, HEK293T cells were transfected with these plasmids. Supernatant and cell lysate were harvested at 72 hours.

After transfection a qPCR was carried out to confirm ADA2 RNA expression in both the 201 and 202 transfected cells (Figure 30A). There were in higher levels of ADA2 RNA noted in the 202 compared to the 201 transfected cells.

Immunoblot with ADA2 antibody of lysate and supernatant from ADA2 201 transfected HEK293T cells show a new detectable band at ~60kDa consistent with ADA2 201 protein (Figure 30B). A much fainter new band is present in the ADA2 202 transfected lysate at ~30kDa which is not present in the non-transfected cells (Figure 30B). Densitometry quantification shows that ADA2 201 protein quantity is 32-fold higher than ADA2 202 despite the same quantity of plasmid being transfected, and lysate loaded onto the gel.

Immunoblot from the supernatant of these transfected cells shows that ADA2 201 is detectable in the supernatant of 201 transfected cells however ADA2 202 is not (Figure 30B). This would be in keeping with the lack of identifiable signal peptide in the ADA 202 protein sequence thereby affecting its secretion (Figure 24).

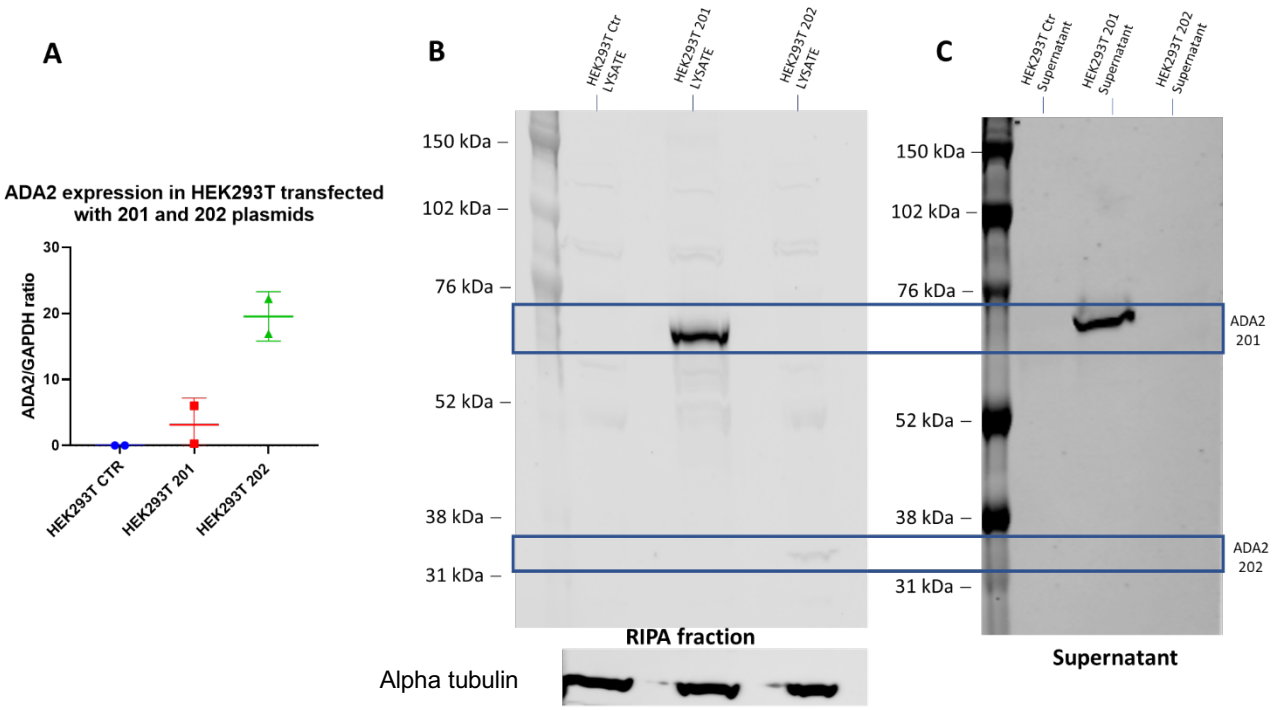


Figure 30 – ADA2 201 and 202 RNA was detected in transfected HEK293T cells (A) however much lower levels of ADA2 202 relative to ADA2 201 were detected in transfected lysate (B). ADA2 201 protein was detected in transfected supernatant but not ADA2 202 (C).

HEK293T cells were transfected with an ADA2 201, ADA2 202 or no plasmid (Ctr). RNA from the transfected cells was extracted and ADA2 201 and 202 gene expression analysed (n=2) (A). Cell lysates (B) and supernatant (C) from the transfected cells was collected and immunoblot carried out. Samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa) and Alpha tubulin (50kDa).

4.11.2 Nickel column purification of translated ADA2 201 and 202 proteins

Both recombinant proteins were designed to contain a His tag for efficient purification. Nickel column purification was used as per the methods section to purify the translated HIS tagged ADA2 protein from these samples. Both lysate and supernatant from plasmid transfected HEK293Ts was purified. This resulted in a flow through, 2x wash samples and 3x elution fractions for each sample.

Figure 31 shows an immunoblot for ADA2, of the products from the nickel column lysate purification. In the 201 transfected lysate a band consistent with ADA2 201 protein is present at ~60kDa. Some of this protein is detectable in the nickel column flow through and wash samples, however a significant concentrated quantity is detectable in all 3 elution fractions. In the 202 transfected lysate, as previously, there is a band present at ~30kDa but in a much lower quantity than in the 201 transfected lysate. This band is detectable in low quantities in the nickel column flow through, wash and elution fractions without any significant concentration of protein.

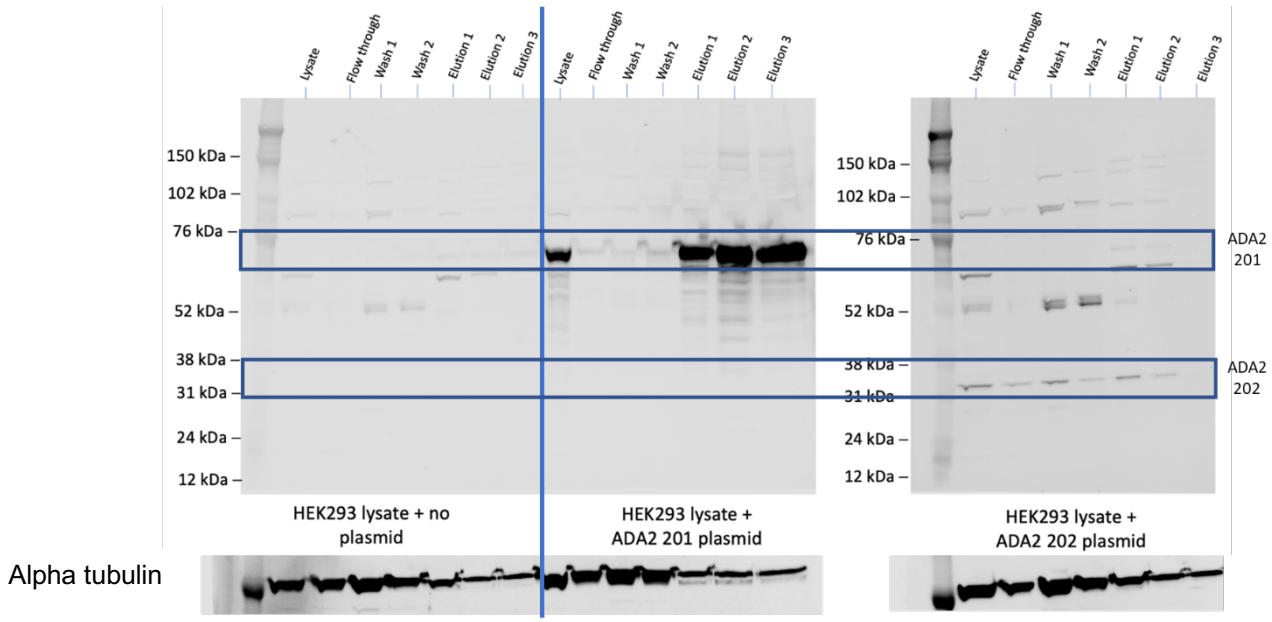


Figure 31 – Nickel column purification of ADA2 201 and 202 from transfected HEK293T lysate.

HEK293T cells were transfected with an ADA2 201, ADA2 202 or no plasmid (Ctr). Whole cell protein lysate was collected and run through a nickel purification column to bind any HIS tagged proteins. The initial flow through, 2 washes and 3 elution fractions were collected. Samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa) and Alpha tubulin (50kDa).

Figure 32 shows the immunoblot of the products from the nickel column supernatant purification from control, ADA2 201 and ADA2 202 transfected HEK293T cells. No ADA2 protein is detectable in the control or ADA2 202 supernatant. A significant quantity of ADA2 201 protein is present in the supernatant of the ADA2 201 transfected cells. Some of this protein is detectable in the flow through and wash samples but a much greater quantity is present in the elution samples.

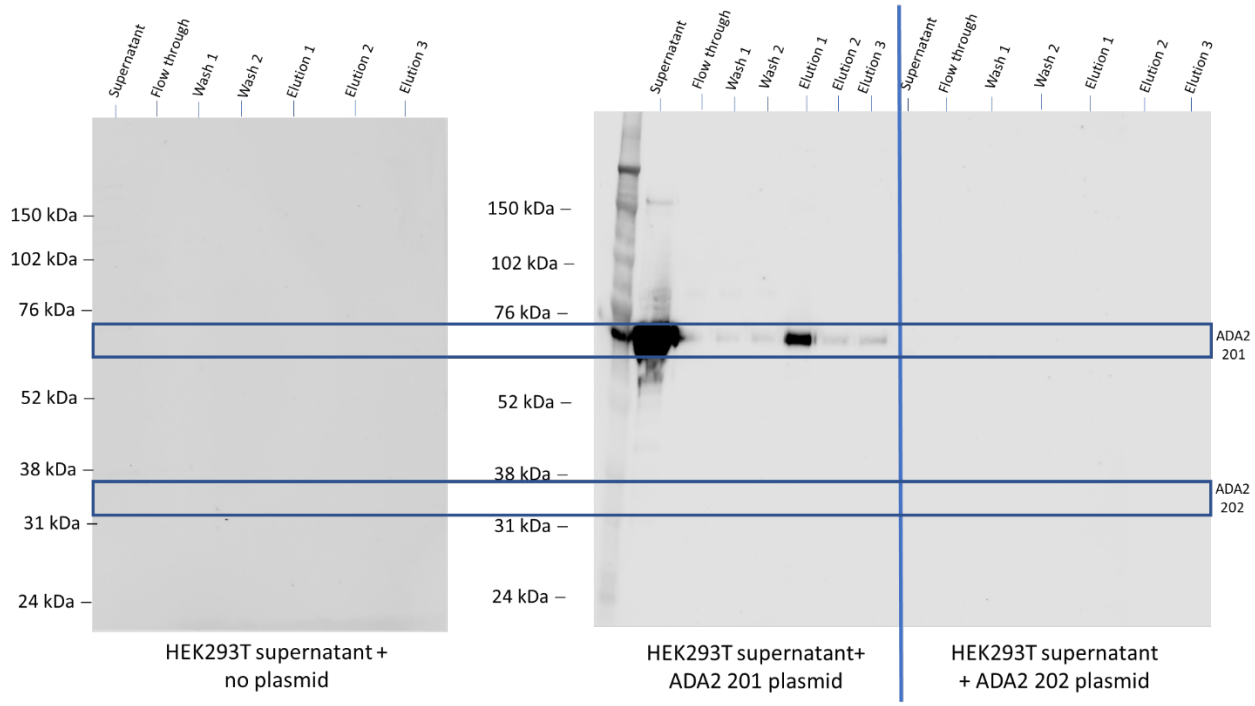


Figure 32 – Nickel column purification of ADA2 201 and 202 from transfected HEK293T supernatant.

HEK293T cells were transfected with an ADA2 201, ADA2 202 or no plasmid (Ctr). Cell supernatant was collected and run through a nickel purification column to bind any HIS tagged proteins. The initial flow through, 2 washes and 3 elution fractions were collected. Samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa).

4.11.3 ADA2 202 protein is predominantly found in the insoluble fraction of transfected HEK293T cells

The fact that the ADA2 202 protein was in a significantly lower quantity relative to ADA2 201 protein in cell lysate and that purification and concentration was not achieved raises questions whether a functional 202 protein was translated or whether it was being degraded or targeted to another cellular compartment that is less accessible with RIPA buffer lysis. To assess this further, an 8M urea buffer was used lyse the insoluble pellet that is left after RIPA lysis and centrifugation. An immunoblot carried out on both the RIPA fraction and Urea fraction demonstrated that although there were similar quantities, according to densitometry, of ADA2 201 in the urea vs RIPA fraction ((1.05 (43800/41800), there was 47 times more ADA2 202 in the urea fraction vs RIPA fraction (55900/1190) (Figure 33).

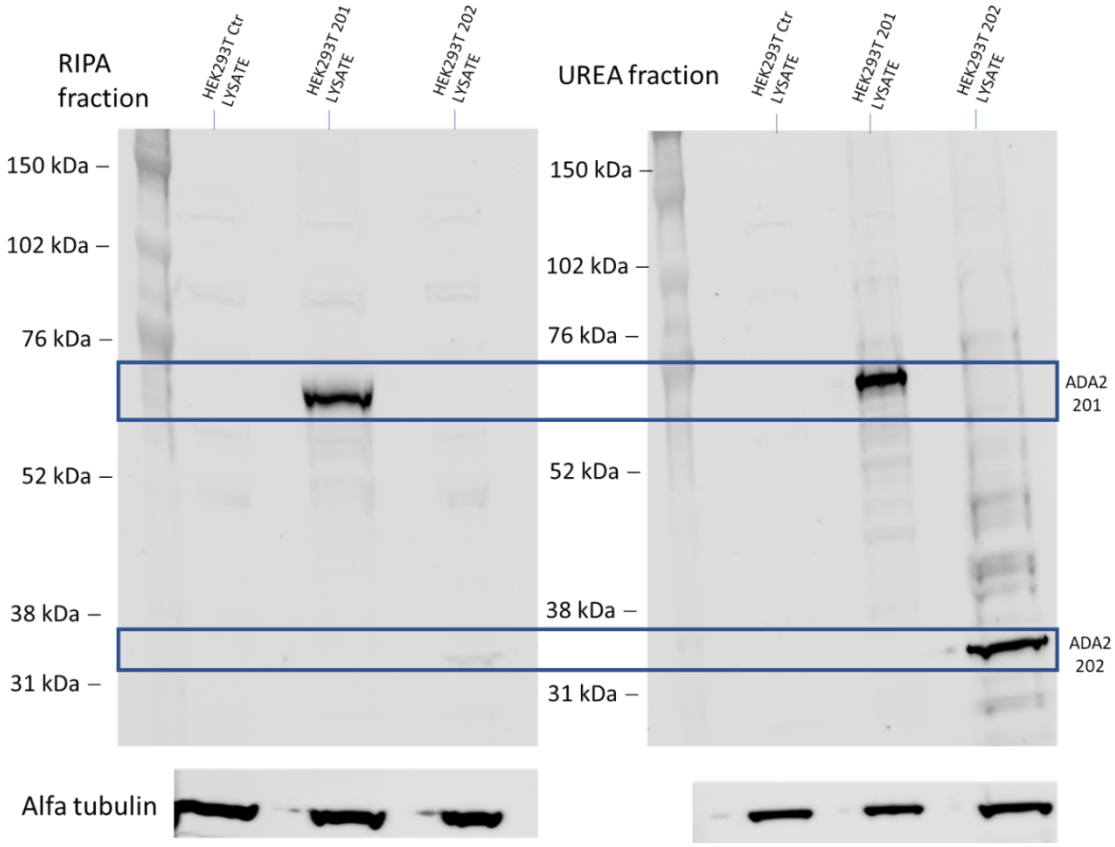


Figure 33 –ADA2 202 protein was detected in the insoluble fraction (Urea lysate fraction) of transfected HEK293T lysate

HEK293T cells were transfected with an ADA2 201, ADA2 202 or no plasmid (Ctr). RIPA lysis buffer was used to achieve whole cell lysates. These samples were spun at 16,000G and the pellet of the insoluble cellular components collected and removed. This pellet was then solubllised in 8M urea lysis buffer. Samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa) and Alfa tubulin (50kDa).

Nickel column His tag purification was carried out on the urea lysates from the 201 and 202 transfected HEK293Ts. Immunoblot for ADA2 in the products of the purification steps are shown in Figure 34. For 201, a significant quantity of the 201 protein was lost in the flow through and wash with only a small amount present in the elution fraction. For 202, significant quantities were lost in the flow through and wash steps with no 202-protein present in the elution fraction. Given that the urea lysis denatures the protein structure, this likely impacts His tag binding efficiency to the column and explains the significant loss of recombinant 201 and 202 protein in the flow through and wash steps.

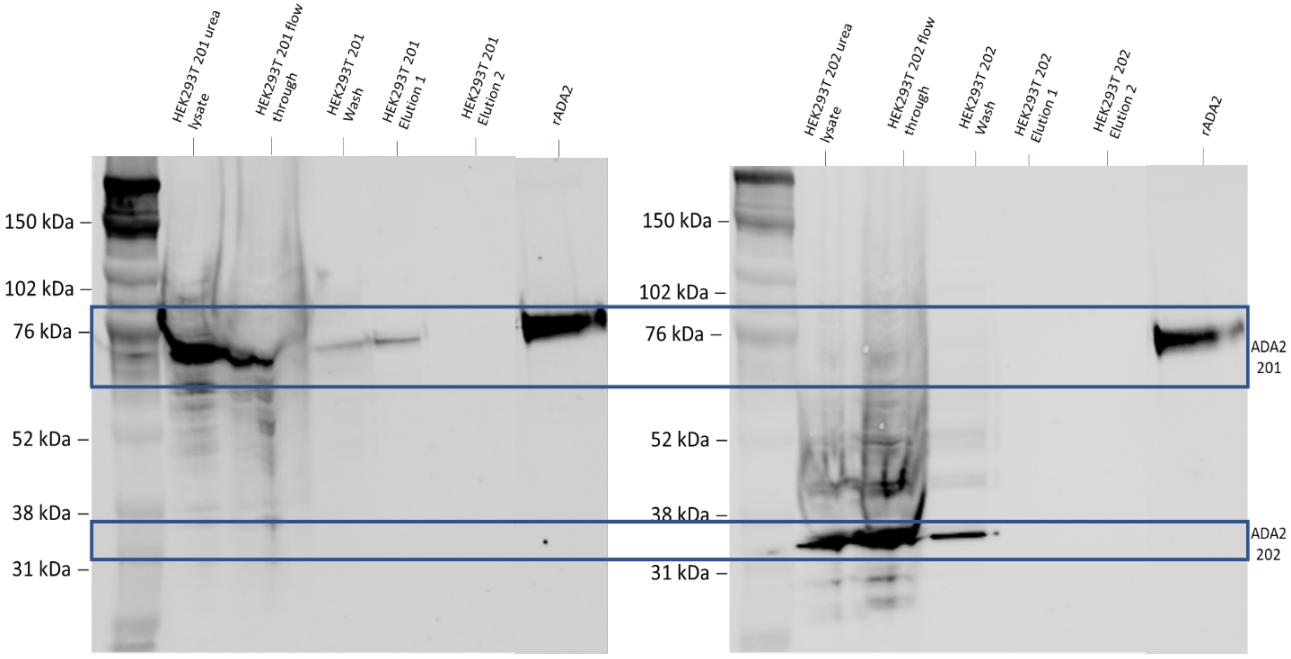


Figure 34 – ADA2 immunoblot of the products of nickel column purification of ADA2 201 and 202 in urea lysate

HEK293T cells were transfected with an ADA2 201, ADA2 202 or no plasmid (Ctr). RIPA lysis buffer was used to achieve whole cell lysates. These samples were spun at 16,000G and the pellet of the insoluble cellular components collected and removed. This pellet was then solubllised in 8M urea lysis buffer. HIS tag purification was carried out on the urea lysis samples to collect a flow through, a wash and 2 elution fractions. These samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa). A significant quantity of 201 and 202 protein was lost in flow and wash samples. This is likely due to the urea lysis buffer causing denatured protein structure and reduced HIS tag binding to nickel column

Given that the most ADA2 202 protein was present in the urea lysate flow through and wash samples, attempts were made to purify this using larger nickel gravity flow columns. A greater quantity of lysate was added to the column and Amicon 3kDa filters were used to x10 concentrate the 10ml of flow through and wash samples.

Immunoblot quantification of these samples using a known concentration of rADA2 demonstrated 116ng and 121ng of ADA2 202 in each 20µg of flow through and wash sample respectively (Figure 35). This would indicate a purity of 0.58% and 0.61% of ADA2 202 relative to other proteins in the flow through and wash samples respectively. Consistent with this, Coomassie blue staining of these samples demonstrated additional proteins were present, although it was the purest sample obtained in this project.

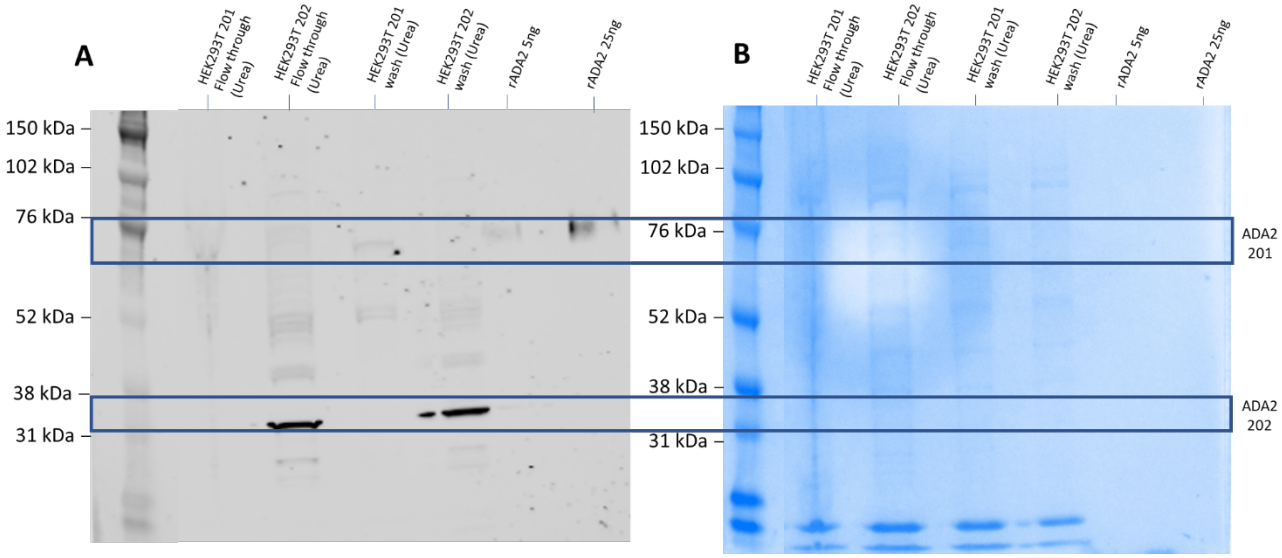


Figure 35 – ADA2 immunoblot (A) and Coomassie blue staining (B) of nickel gravity column purification and concentration of urea lysate flow through and wash samples
Larger nickel columns were used to purify greater quantities of urea lysate from ADA2 201, 202 and Ctr transfected HEK239Ts. The generated flow through and wash samples were x10 concentrated with Amicon 3kDa filters. Samples were separated by SDS-PAGE. Proteins were detected with antibodies against ADA2 (201 – 59kDa, 202 – 30kDa) (A). Purity of these samples was assessed with Coomassie blue staining (B).

This purified sample was sent for mass spectrometry (MS) analysis which confirmed the presence of peptides derived from ADA2. Analysis demonstrated that only peptides after amino acid 242 in the 511 ADA2 201 sequence were detected i.e. only those in ADA2 202 and 201. No amino acids that are only present in 201 were detected. This is therefore in keeping with the urea wash sample containing ADA2 202 protein. A specific search was carried out for the 10 amino acid sequence that is unique to ADA2 202 but this was not identified (Figure 36). MS analysis of rADA2 demonstrated detection of peptides throughout the 511 sequence (Figure 36)

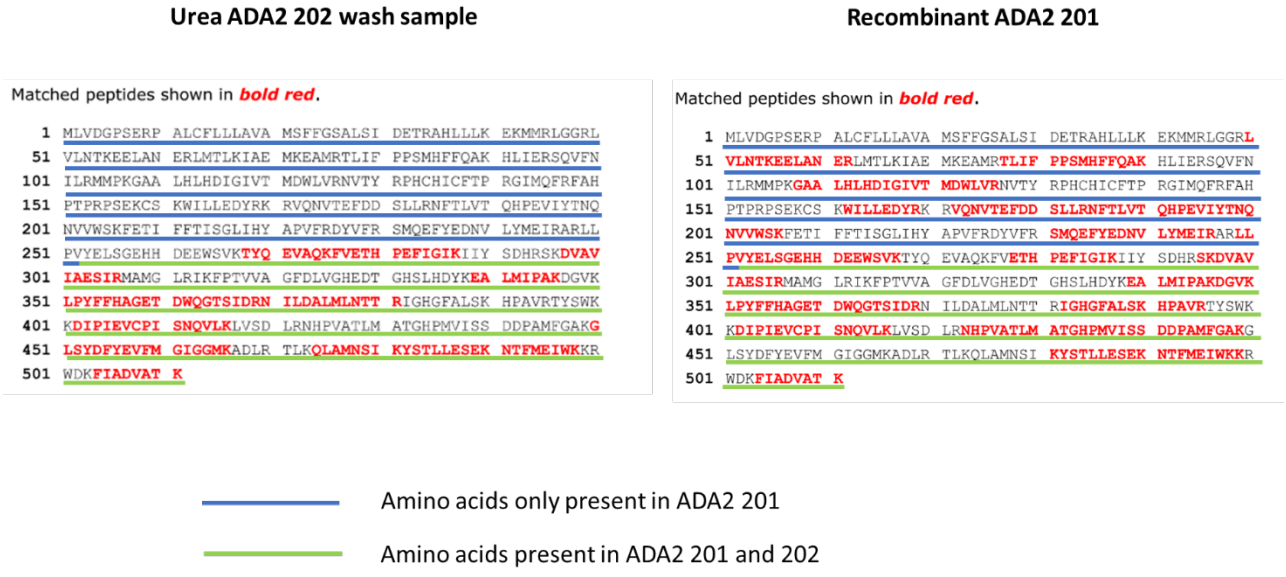


Figure 36 – The urea lysate nickel column wash sample from ADA2 202 transfected HEK293Ts confirmed peptides in ADA2 202 were detected but no peptides unique to ADA2 201.

The urea lysate nickel column wash samples were sent for mass spectrometry along with recombinant ADA2 201. Matched peptides to the ADA2 201 and 202 protein were identified.

4.11.4 ADA2 enzymatic activity of ADA2 201 and 202 protein

To evaluate the enzymatic activity of generated recombinant ADA2 201 and 202 proteins, RIPA lysate and supernatant from the transfected cells were assessed in the ADA2 activity assay. In both the lysate and supernatant from 201 transfected cell, ADA2 activity was detected (24 U/L and 31 U/L respectively) (Figure 37A). No ADA2 activity was detected in the RIPA lysate or supernatant from the control or 202 transfected cells. However, this analysis is limited by the low concentration of ADA2 202 in the RIPA fraction of the cell lysate and so the possibility remains that there is low level activity that is below the assay's lower limit of detection.

There was no ADA2 activity in the urea lysate for 201 or 202 transfected HEK293Ts (Figure 37A). This is despite an immunoblot showing similar concentrations of ADA2 201 protein in the urea and RIPA lysate (Figure 33) and ADA2 activity was detected in the 201 RIPA lysate (Figure 37A). This likely relates to the fact that the urea lysis buffer denatures the protein meaning it is not folded appropriately to have enzymatic function. There was also no ADA2 activity in the nickel column flow-through from urea lysates from either 201 or 202 transfected cells (Figure 37B). This is despite >100ng of ADA2 202 protein being present in 20ug of 202 flow through (Figure 35). Again, this may be due to denaturation caused by the urea lysis buffer.

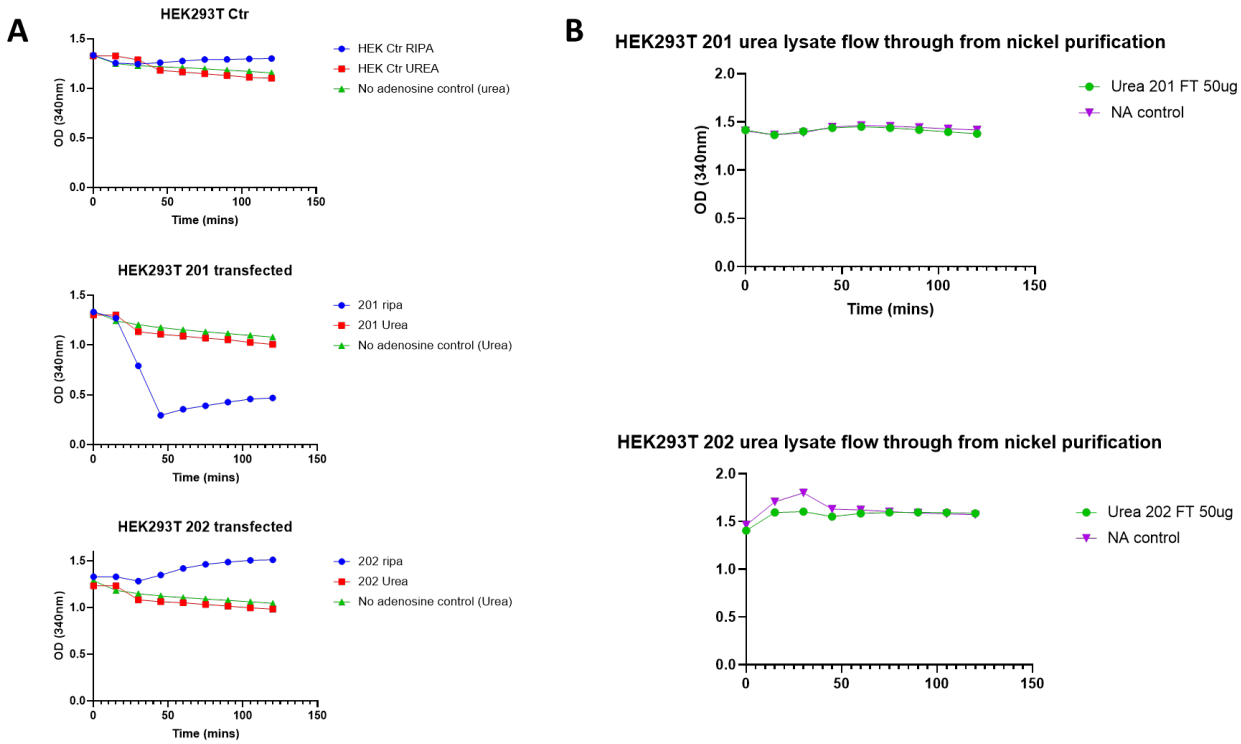


Figure 37 – (A) ADA2 activity was detected in the RIPA lysate from 201 but not 202 transfected HEK293T. No ADA2 activity was detected in the 201 or 202 urea lysates. (B) no ADA2 activity was detected in the purified ADA2 201 or 202 samples from the urea lysate.

(A) RIPA and urea lysates were prepared from ADA2 201 and 202 transfected HEK293T cells. Lysates were buffer exchanged with PBS using amicon filters. These samples were then run on the ADA2 activity assay to measure enzymatic activity. (B) Urea lysate from ADA2 201 and 202 transfected HEK293Ts was placed through a nickel column purification column. Previous work had shown that much of the generated ADA2 201 and 202 protein was present in the flow through so these samples were buffer exchanged and ADA2 activity measured using the activity assay.

4.12 Stimulation and transcriptional regulation of ADA2 in endothelial cells and monocytes

Given ADA2 202 mRNA was detectable in EC but protein was not, experiments were carried out to assess whether 202 mRNA could be upregulated in certain conditions or with specific compounds.

4.12.1 Identification of ADA2 upregulating agents from literatures

To define a panel of compounds that could induce ADA2 upregulation, a systematic literature search was performed in PubMed and WoS. For PubMed, 3 keyword combinations returned 166, 129 and 67 results, respectively (Supplementary figure 2). For WoS, 3 keyword searches returned 132, 149 and 28 results, respectively. After review of the full texts, 9 compounds which could induce ADA2 gene and/or activity were identified. 4 compounds were selected for U937 and HUVEC treatments; butyrate, a short chain fatty acid produced from bacterial anaerobic fermentation of dietary fibres [183, 184], the inflammatory cytokines IFNγ and TNFα [185], and the glycolipid molecule in bacterial outer membranes, LPS [183] (Table 14).

4.12.2 Selection of potential ADA2 inducing agents from public databases

We next reviewed the Signaling Pathways Project and SigCom LINCS databases which provide predictions of compounds that might regulate target genes based on large compendiums of transcriptomic and genomic regulation data [150, 151]. Two compounds were identified from the SPP analysis; rosiglitazone (agonist of peroxisome proliferator-activated receptor gamma) [186] and 1,25D (active form of vitamin D).

SigCom LINCS database search of ADA2 gene upregulation data in U937, THP-1 and HUVEC cell lines demonstrated 24, 253 and 148 distinct compounds respectively, that could induce ADA2 expression. Compounds that appeared in more than one search were selected for experimental testing. This included 3 compounds: TCPA-1, the IκB kinase-2 (IKK2) inhibitor [187], wortmannin (inhibitor of phosphoinositide 3-kinase, PI3K) [188] and rapamycin (inhibitor of molecular target of rapamycin, mTOR) [189].

Four further compounds of interest were included as per Table 14; Phorbol 12-myristate 13-acetate (PMA) as it is known to be a proinflammatory molecule that induces inflammation [190, 191], IL-6 as it has been proposed to be involved in DADA2 pathogenesis, deoxy-adenosine as it has been shown to induce IFNβ in monocytes [88] and adenosine as it has been shown to be elevated in plasma from DADA2 patients and induces neutrophil extracellular trap (NET) formation in neutrophils [90].

Compound	Concentration	Cell line	Treatment duration	Source
Literature search				
TNFα	N/A†	Peripheral blood mononuclear cells	5 days	[185]
IFNγ	1000 U/ml	Peripheral blood mononuclear cells	5 days	[185]
Butyrate	0, 5, 10, 20 μM	THP-1 cells	12, 24, 36, 48 hours	[183]
LPS	N/A	THP-1 cells	N/A	[183]
SPP database analysis				
Rosiglitazone	1 μM	CD14+ monocytes cultured with 500 U/ml interleukin 4 and 800 U/ml Granulocyte macrophage colony-stimulating factor	24 hours	[186, 192]
	2.5 μM		5 days	
1,25D	0.1 μM	Terminally differentiated THP-1 cells	24 hours	[193]
SigCom LINCs database analysis				
TCPA-1	0.12 μM	HUVEC	24 hours	
	10 μM	U937	6 hours	
	10 μM	THP-1	24 hours	
Wortmannin	0.37 μM	U937	6 hours	
	10 μM			
	3.33 μM		24 hours	
	0.04 μM	THP1-1	6 hours	
Rapamycin	12 μM	U937	6 hours	
	0.04 μM	THP-1	6 hours	
	0.25 μM		24 hours	
Additional compounds				
PMA	10 ng/ml	U937 cells	24 hours	
IL6	N/A	N/A	N/A	
dAdo	100 μM	U937 cells	24 hours	
Ado	100 μM	U937 cells	24 hours	

Table 14 – Summary of compounds selected for cell treatments from Literature search, SPP database analysis, SigCom LINCs database analysis

4.12.3 Increased ADA2 gene expression and activity level in compound-treated monocytes

4.12.3.1 Upregulation of ADA2 mRNA expression in compound treated monocytes

U937 cells were incubated with each compound for 24 hours before ADA2 mRNA was measured using qPCR. Relative to vehicle controls, TNFα, wortmannin and TPCA-1 demonstrated significant upregulation in ADA2 gene expression with 2.12 ± 0.4 fold ($p<0.05$), 4.5 ± 0.4 fold ($p<0.01$) and 3.2 ± 0.9 fold ($p<0.001$) increases, respectively (Figure 38A+B). All other compound stimulations did not significantly alter ADA2 gene expression (Figure 38C+D).

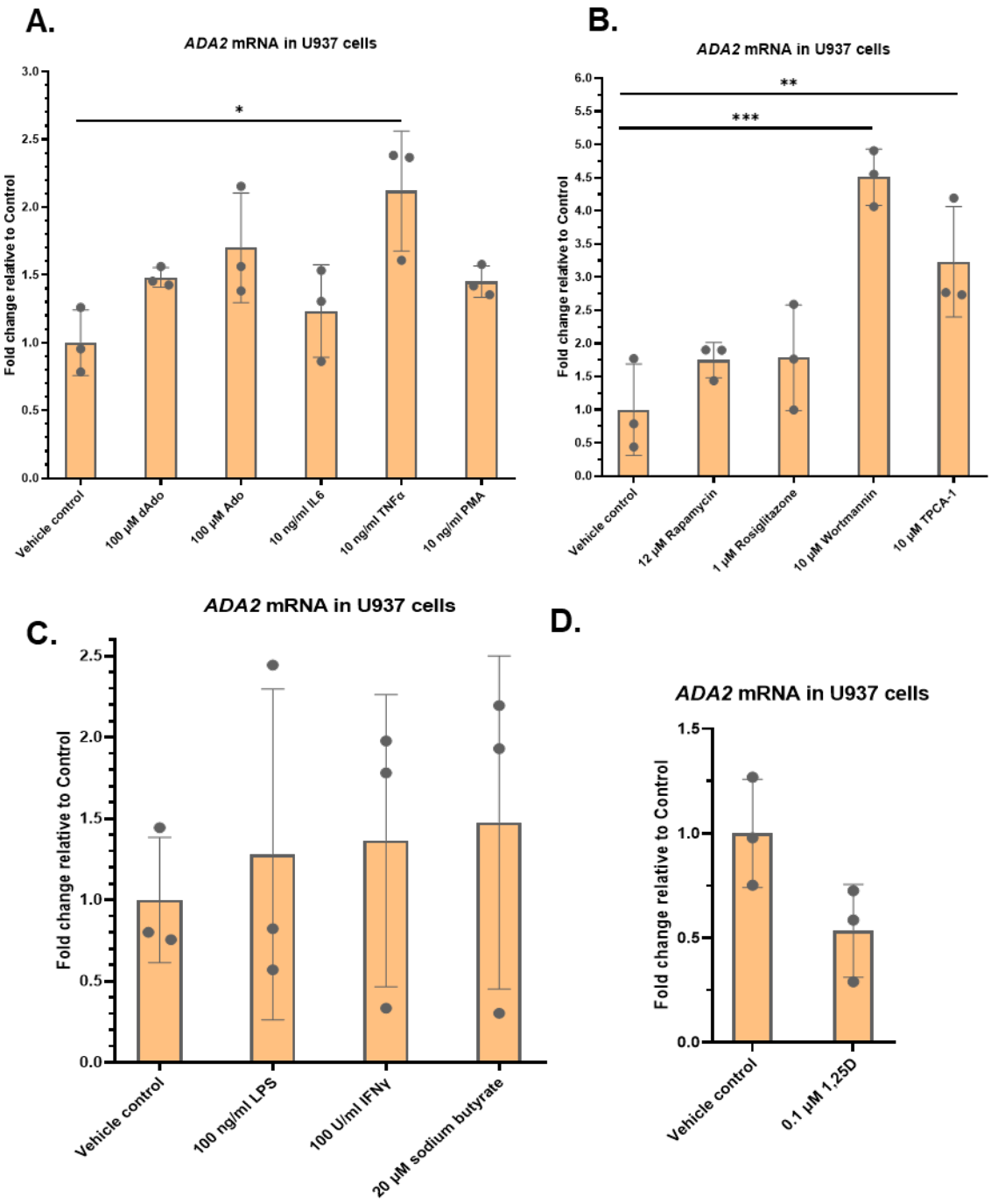


Figure 38 – There was significant upregulation of ADA2 mRNA expression in TNFα, wortmannin and TPCA-1 treated U937 cells (A-D).

U937 cells were treated with the above compounds for 24 hours prior to RNA being harvested and extracted (n=3). ADA2 gene expression ratio was measured using qRT-PCR. This was normalised to GAPDH and represented as fold change relative to the mean of vehicle controls. (A-C) *One-way ANOVA with Dunnett's post-hoc tests and (D) Student's t-test were performed. *indicates p<0.05, **p<0.01 and ***p<0.001, otherwise data have p>0.05.*

4.12.3.2 ADA2 activity remains unchanged in compound-treated monocytes

ADA2 enzymatic supernatant was measured in the supernatant of U937 cells treated with TNFα, wortmannin and TPCA-1. Despite the increases in ADA2 mRNA level, no corresponding increase in ADA2 enzymatic activity was observed after 24 hours (Figure 39).

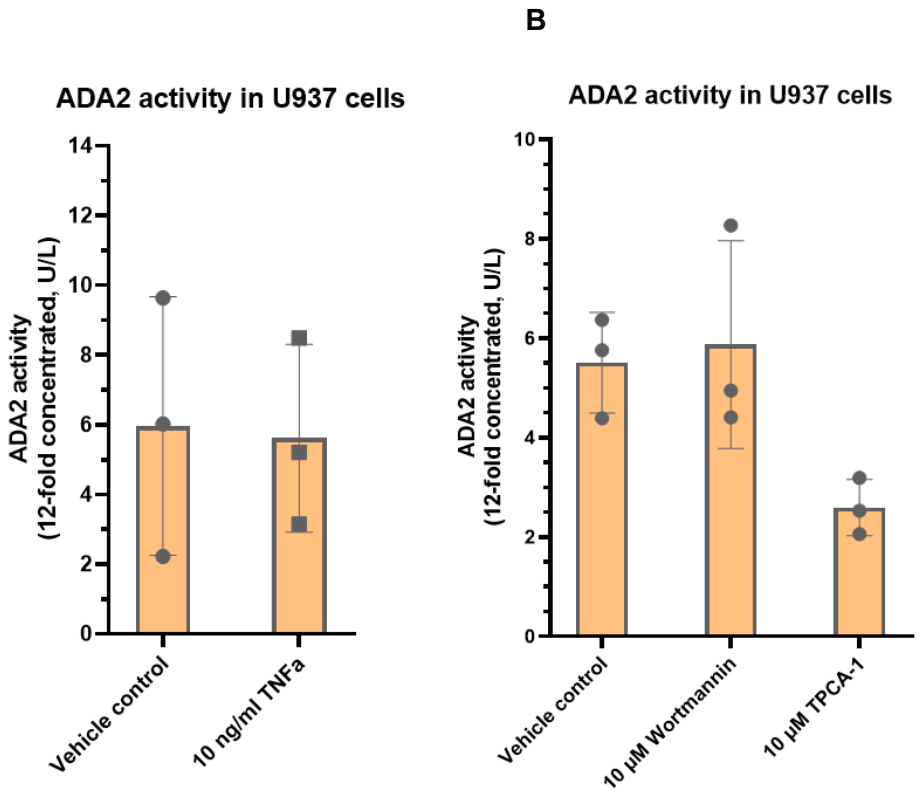


Figure 39 – There was no increase in ADA2 activity in the supernatant of TNFα, wortmannin and TPCA-1 treated U937 cells.

U937 cells were treated with the above compounds for 24 hours before the cell supernatant was harvested. The supernatant was buffer exchanged, and 12-fold concentrated. ADA2 activity was measured using the ADA2 activity assay. All data are presented as mean ± SD, n=3. (A) Student's t-test and (B) One-way ANOVA with Dunnett's post-hoc test were performed. All data have p>0.05.

4.12.3.3 ADA2 enzymatic activity was elevated in monocytes treated with wortmannin and TNFα for 72 hours

To ascertain whether activity may increase at a timepoint later than 24 hours, U937 cells were treated with wortmannin and TNFα over an extended time course of 24, 48 and 72 hours. Supernatant ADA2 activity was measured.

ADA2 activity increased over time in both control and treatment conditions however there was significant elevation in activity at 72 hours in the wortmannin treated cell supernatant with a 1.4 ± 0.1-fold induction relative to controls ($p<0.05$, Figure 40A). Compared to vehicle controls, 72-hour U937 supernatants treated with TNFα demonstrated a 1.25 ± 0.1 fold elevation of ADA2 activity ($p<0.01$,Figure 40B).

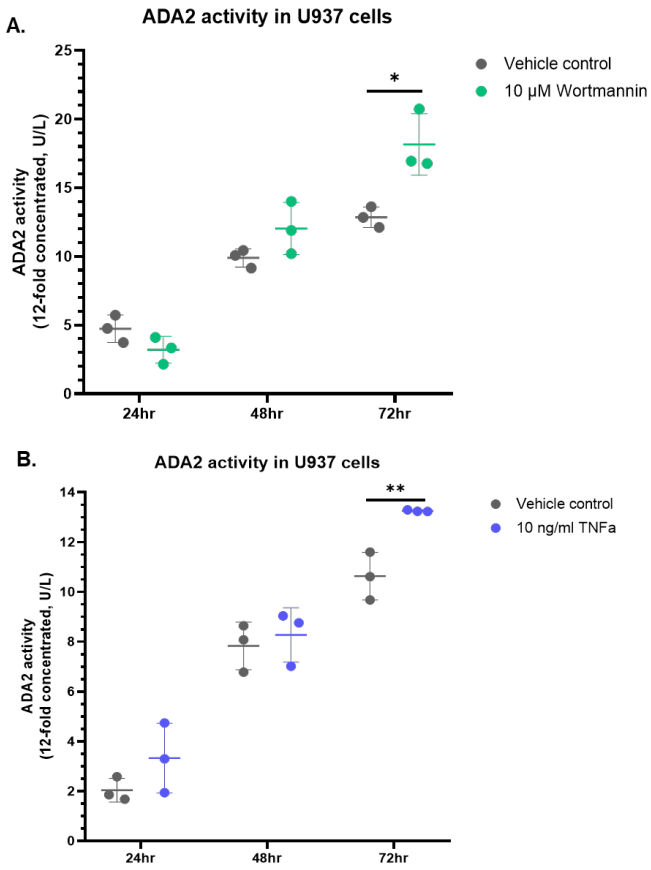


Figure 40 – ADA2 enzymatic activity was elevated in the supernatant U937 cells treated with wortmannin and TNFα for 72 hours.

*U937 cells were treated with wortmannin or TNFα for 24, 48 or 72 hours prior to supernatant harvesting (n=3). Supernatant ADA2 activity was measured using the ADA2 activity assay. All data are presented as mean ± SD, n=3. Student's t-test were performed within each time point. * indicates $p<0.05$ and **$p<0.01$, otherwise data have $p>0.05$.*

4.12.4 ADA2 mRNA expression level was unaltered in compound-stimulated ECs

To assess whether ADA2 202 could be induced in EC, 3 separate HUVEC pools were treated with the same compounds for 24 hours. ADA2 gene expression was measured using primers specific to ADA2 202. No significant elevation of ADA2 mRNA expression was observed in any compound treated HUVECs (Figure 41). There were however trends for increased ADA2 gene expression with sodium butyrate in all 3 HUVEC pools. Additionally, despite the inductive effect in U937 cells (Figure 38), wortmannin tended to decrease ADA2 expression in HUVEC albeit without statistical significance ($p>0.05$, Figure 41).

Analysis of changes in ADA2 activity was not performed since protein and enzymatic activity was previously not identified in HUVEC lysate or supernatant (Figure 26,Figure 29).

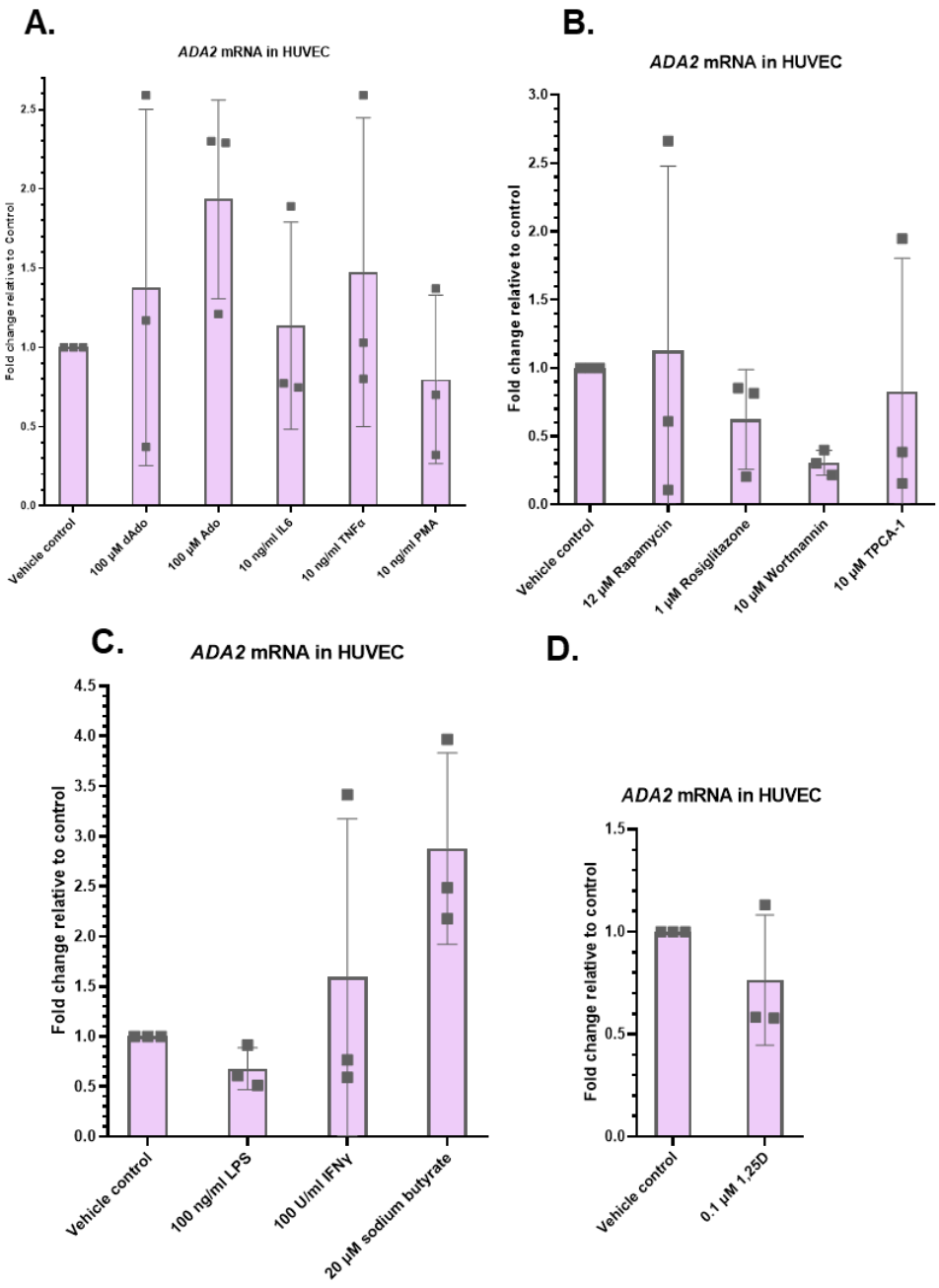


Figure 41 – There was no significant change in ADA2 202 mRNA expression levels in compound-stimulated ECs.

HUVEC were treated with the above compounds for 24 hours prior to RNA being harvested and extracted (n=3). ADA2 gene expression ratio was measured using qRT-PCR. This was normalised to GAPDH and represented as fold change relative to the mean of vehicle controls. All data are presented as mean ± SD, n=3. (A-C) One-way ANOVA with Dunnett's posthoc tests and (D) Student's t-test were performed. All data have p>0.05.

4.13 ADA2 202 expression in the endothelium under flow

Assessment of ADA2 expression in endothelial cells under flow was carried out through assessing published RNAseq data sets [194, 195]. This group performed shear stress experiments with HUVEC and HAEC under pulsative shear flow (12 ± 4 dyn/cm^2) or oscillatory shear flow (1 ± 4 dyn/cm^2). In HUVEC, RNA was harvested at 10 time points between 1 and 24 hours. In HAEC, RNA was harvested at 1, 4 and 24 hours. ADA2 202 RNA data was collated at each time points. In HUVEC there was upregulation of ADA2 202 expression with pulsatile stress (1.8-fold increase time 1 hour to 24 hours) but not with oscillatory stress (Figure 42). In HAEC there was upregulation of ADA2 202 with both pulsatile and oscillatory stress (3.1 and 2-fold increase 1 hour to 24 hours respectively) (Figure 42). This indicates that ADA2 202 RNA can be upregulated thus suggesting that it may have a role in endothelial homeostasis despite the lack of detectable protein.

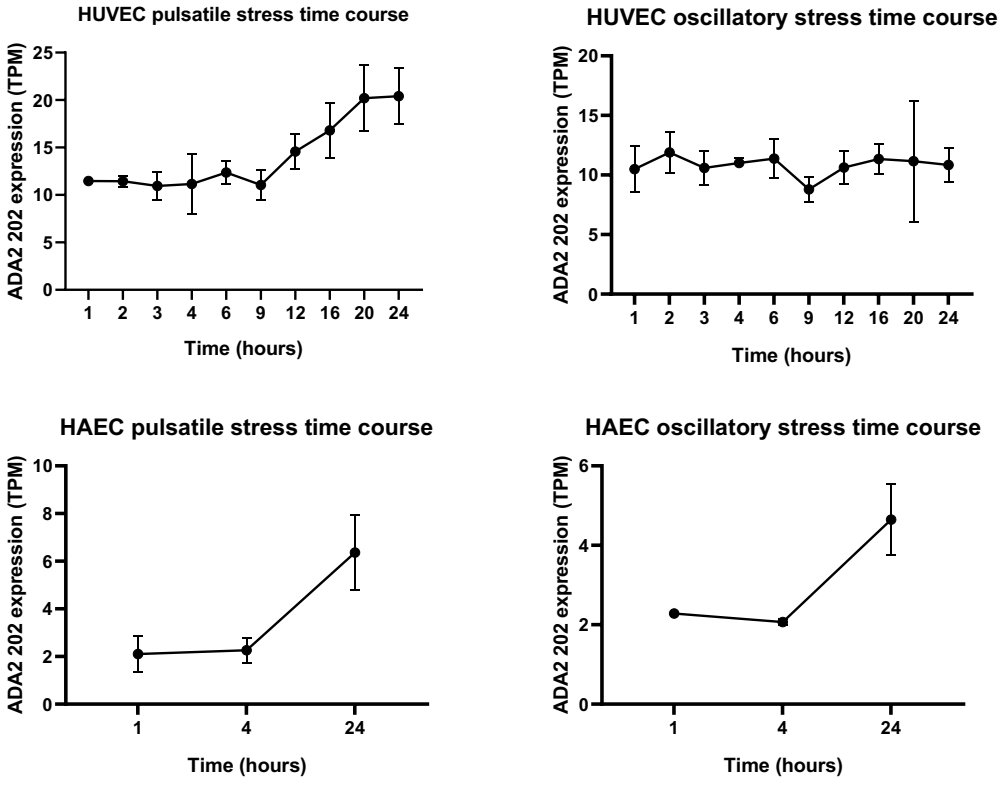


Figure 42 – ADA2 202 expression can be upregulated in HUVEC through pulsatile stress and in HAEC with both pulsatile and oscillatory stress

HUVEC and HAEC were put under pulsative shear flow or oscillatory shear flow. RNA was harvested at different time points and sent for RNAseq. Data for ADA2 202 expression was analysed from this publicly available database [194, 195]

4.14 Discussion

In this chapter, I confirmed that EC express ADA2 but exclusively as a shortened transcript isoform and the encoded protein appears to lack deaminase activity and other significant functional domains. Tissue databases suggest that there is widespread expression of this isoform. Attempts to induce ADA2 expression demonstrated that 202 and 201/203 are under divergent systems of transcriptional regulation which is in keeping with the alternative promoter regions identified in this chapter.

The ADA2 202 transcript encodes a truncated ADA2 202 protein of 270AA in contrast to the classically described 511AA ADA2 201 (Table 11). Exploratory analysis of tissue-wide transcriptomic datasets revealed that ADA2 202 was widely expressed in tissues including the spleen, pituitary, lung , and brain as well as ECs (Figure 19, Figure 20). Myeloid cells, particularly monocytes and macrophages express high levels of ADA2 201 and little or no 202 (Figure 18). These findings were validated by qPCR using our own ADA2 isoform specific primers (Figure 22). Given the widespread expression, it would suggest that ADA2 202 may have a functional role in these tissues and ECs. The epigenetic analysis highlights 2 different promoters for 201 and 202 and differing expression suggests that they are under independent regulation and serve different functional roles (Figure 21).

Investigation of its expression in other species demonstrates that ADA2 202 is only present when ADA2 201 is also present (Table 12). Interestingly, only primates with genomes closely related to humans seem to have the capacity to express ADA2 202. This suggests that ADA2 202 emerged relatively recently in evolutionary history and that it may serve an important function in Hominidae type primates.

The truncated predicted amino acid sequence of ADA2 202 compared to 201 has important implications for its potential function as a protein (Figure 24). It lacks a classic signal peptide which would impair its ability to be secreted and function as an extracellular enzyme or growth factor. This is in keeping with our results where no ADA2 202 protein was detected in the supernatant of ECs or in the supernatant of our HEK293T overexpression model (Figure 26, Figure 30). It also lacks a significant proportion of its catalytic domains which would undoubtedly affect any potential deaminase activity. This is consistent with the results of this chapter, whereby no enzymatic activity could be detected in the lysate of ECs nor the lysate of HEK293Ts overexpressing ADA2 202 (Figure 37). The missing disulphide bond and dimerisation domains again are important as they are known to be key for ADA2 201 structure and function. Overexpression of ADA2 202 in HEK293Ts was found enriched in the insoluble

fraction which might imply disordered protein folding and certainly rules out typical behaviour and localisation of an extracellular protein such as full length ADA2.

Our experimental work has in fact failed to detect any ADA2 protein expression or enzymatic activity in EC lysate or supernatant (Figure 26, Figure 29). The use of a pcDNA plasmid containing the ADA2 202 coding sequence showed that when 202 protein was artificially overexpressed it accumulated the insoluble protein fraction. These proteins are more difficult to detect and quantify using standard techniques such as immunoblot and mass spectrometry. It is estimated that 20-30% of the proteins encoded by the human genome are insoluble, a large proportion of which are membrane proteins [196]. The insoluble protein fraction contains proteins that have been targeted for degradation [197, 198]. The two main pathways involved in protein disposal are the proteasome or lysosome [199, 200]. The proteasome is responsible for degradation, by the ubiquitin-proteasome system, of short-lived proteins and those that are damaged, unfolded and useless [198, 201]. The 76-residue ubiquitin protein is attached to proteins via a lysine bond as a post-translational modification through a Ub activating enzyme, a Ub conjugating enzyme and a Ub ligase [202, 203]. Proteins marked with Ub chains are then targeted to the proteasome for degradation. Lysosomes are involved in the degradation of long-lived proteins and insoluble protein aggregates via endocytosis, phagocytosis or autophagy pathways [204]. After endocytosis some cell membrane proteins are recycled to the plasma membrane or to other organelles, whereas some are ubiquitinated and sent to degradation pathways [205]. The fact that ADA2 202 can only be detected in an artificially overexpression model and not in EC may suggest that it is misfolded and non-functional so not present in basal conditions. When a model overexpresses the protein, it is translated but may be misfolded or non-functional so is rapidly targeted for degradation and in therefore detected in the insoluble fraction.

Another hypothesis would be that the ADA2 202 protein is only present at low levels (below the lower limit of detection of our assays) in the insoluble fraction of ECs. It may well have a function in this cellular component that we have not yet identified. The fact that we can detect the protein in the forced expression model, but no corresponding activity is consistent with our functional hypothesis that this protein does not have enzymatic activity. Further work would include assessing whether in the overexpression model, ADA2 202 has post-translational modifications with Ub chains indicating it is targeted for degradation. This could be carried out through purification of the protein and assessment of ubiquitination with an immunoblot.

The initial descriptions of DADA2 did not demonstrate ADA2 mRNA or protein expression [2, 3]. Subsequently, Dhanwani *et al.* demonstrated ADA2 mRNA transcripts were present in multiple primary human endothelial cells consistent with full length ADA2 (HKVEC, HBVEC,

HDVEC, HUVEC) [88]. The Taqman ADA2 primer pair sequences that were used for those qPCR experiments would detect both ADA2 201 and 202 RNA and would not differentiate between the 2 (Supplementary figure 3). Given our RNA sequencing findings of ADA2 202 expression in ECs, we designed and used 202 specific primers to validate and confirm solely ADA2 202 expression in multiple types of EC (Figure 22).

Dhanwani et al, detected ADA2 201 protein in cultured supernatants of HUVEC by ELISA and western blotting as well as ADA2 activity. [88]. This contrasts to our results where no ADA2 201 transcript is detected in HUVEC, no ADA2 protein is present in HUVEC lysate, supernatant nor in the supernatant of the ADA2 202 forced overexpression model. My results are consistent with the predicted implications of a shorter ADA2 protein that lacks a signal domain and lack of secretory capacity of ADA2 202. This significant difference in results is likely explained by the presence of non-specific protein banding that is consistently visible in all immunoblots undertaken with the antibody used in my work and in the work of Dhanwani et al. Extensive optimisation and comparison of 3 different ADA2 antibodies demonstrated this multiple banding pattern and confirmation of the ADA2 protein was only possible with collaborative work with Dr Pui Lee who demonstrated that one of these bands disappeared in ADA2 U937 CRISPR knockout. Therefore, it may be that the ADA2 201 described in Dhanwani et al was in fact one of these non-specific bands that is present at approximately 60 kDa.

Dhanwani et al, detected ADA2 enzymatic activity in HUVEC supernatant through measuring de novo conversion of ^{15}N-labeled dAdo to dIno by mass spectrometry [88]. This is likely to be a more sensitive assay than our spectrophotometric coupled assay and could detect lower levels of ADA2 activity however the ADA2 202 protein that ECs express lacks a significant proportion of its catalytic domain meaning that enzymatic activity is unlikely.

To try and understand whether ADA2 202 was stimulated and regulated in the same way as ADA2 201, compounds were picked to treat U937 monocytes (201 expressing) and HUVEC (202 expressing). In monocytes, TNFα, wortmannin and TPCA-1 were shown to upregulate ADA2 201 mRNA expression at 24 hours. ADA2 activity was increased in supernatant from monocytes treated with TNFα and wortmannin for 72 hours. In contrast, no significant increase was seen in ADA2 202 gene expression in compound stimulated HUVEC. This suggests differential regulation of the different ADA2 transcripts. Interestingly the enzyme substrates, adenosine and dAdo were not shown to promote ADA2 production in monocytes or ECs. The evidence of increased ADA2 202 expression in HUVEC under pulsatile stress and HAEC under both pulsatile and oscillatory stress suggests that this transcript is regulated, can be stimulated, and it may be functional.

Limitations with this chapter include whether the immunoblot and ADA2 activity assays are sensitive enough to detect low levels of protein or activity that may be present in ECs, particularly if it is present in the insoluble compartment of the cells. In the overexpression model, the 202 protein is at a much lower concentration in the RIPA soluble fraction than the 201 protein so may be below the lower limit of detection of the activity assay.

The use of a urea lysis buffer did yield significantly more ADA2 202 protein from the insoluble fraction, but urea is thought to bind to proteins and compete with native interactions therefore leading to unfolding [206]. This meant that enzymatic activity was lost in the 201 urea sample, compared to the 201 RIPA sample. The fact that activity was not detected in the 202 urea sample could therefore be due to unfolding of the native 202 protein. Further work to obtain a greater concentration of folded ADA2 202 protein would be include attempting to refold the denatured 202 protein from the urea purification to confirm that it was enzymatically inactive.

These novel findings have implications in the understanding of the pathogenesis of DADA2. The location of the patient's genetic variant will determine whether ADA2 201 alone is affected (AA 1-251) or both ADA2 201 and 202 are affected (AA 252-511) (Figure 43). Given the widespread tissue and EC expression, the preservation of ADA2 202 in the context of an ADA2 201-only variant may contribute to the heterogeneity seen in the clinical phenotypes of DADA2. Crucial to understanding this will be determining the function of ADA2 202 in endothelial cells.

In summary, endothelial ADA2 is expressed exclusively as the shortened 202 transcript but its protein form is undetectable. Artificially overexpressed 202 accumulated in the insoluble protein fraction, lacked deaminase activity and thus appeared non-functional. This raises questions as to whether ADA2 202 has a functional role in the endothelium.

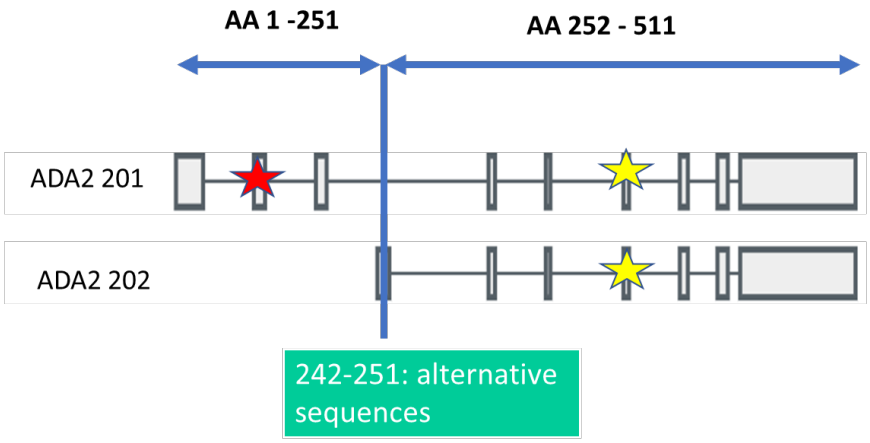


Figure 43 – The location of DADA2 variants determine the ADA2 isoforms affected.

CHAPTER 5: A model of ADA2 deficiency in endothelial cells

5.1 Introduction

The confirmation of ADA2 expression in endothelial cells naturally leads to the question of what functional role might ADA2 have in endothelial cells and whether perturbations of such functions have a role in the manifestations of DADA2. Previous work has demonstrated that siADA2 in HUVEC results in induction of an IFNβ stimulated gene signature as well as differentially expressed genes encoding proinflammatory cytokines, chemokines and innate immune response proteins [88]. This was reversed with the addition of rADA2 or rADA1 and further inhibited with the addition of pentostatin, an inhibitor of ADA enzymatic activity [88]. It was also demonstrated that dAdo is required for IFNβ production in siADA2 HUVEC as the use of dipyridamole, an inhibitor of equlibrative nucleoside transporters (ENT) reduced this signature [88]. Finally, co-culture of siADA2 HUVEC with untreated U937 cells resulted in IFNβ production in the bystander U937s suggesting a paracrine effect of ADA2 in HUVEC [88].

Despite the lack of detectable ADA2 protein or enzymatic activity, ADA2 202 expression in EC may yet have a functional relevance as a long non-coding RNA (lncRNA) [207]. An alternative hypothesis is that ADA2 202 protein may be present at low levels, below the lower limits of detection of our assays, as an insoluble protein [196, 208]. Furthermore, our inability to detect protein may have been due to technical limitations such as lower detection limits. In many cases proteins localised to specific compartments can be difficult to detect using standard buffers. Nevertheless, understanding the function of the ADA2 202 isoform in EC, if any, is critical for deciphering whether DADA2 mutations may play an “intrinsic” (primary effect within EC) in DADA2.

This chapter aims to understand the function of ADA2 202 in endothelial cells using a multiomic approach. We will assess the impact of siRNA based ADA2 KD on HUVEC phenotype through transcriptomic and proteomic profiling in addition to assessment of key EC functions.

5.2 Hypothesis of chapter

The ADA2 202 transcript has a role in endothelial homeostasis.

5.3 Aims of the chapter

1) Carry out siRNA knock-down of ADA2 in HUVEC
2) Perform transcriptomic and proteomic assessment of siADA2 knock-down in HUVEC
3) Investigate the functional effects of siADA2 knock-down in HUVEC

5.4 Optimisation of siRNA knockdown of ADA2 in HUVEC

siRNA KD of ADA2 was carried out in HUVEC. A commercially available pool of 4 siRNAs to target all isoforms of ADA2 was used (siADA2) and a pool of 3 siRNAs was designed to target the exon that is specific to ADA2 202 (si202) (Figure 44). A time course experiment was carried out to determine the optimal point for RNA harvesting after 6 hours of siRNA treatment. These results showed that 94% ADA2 KD was achieved at 24 hours, 96% at 48 hours and 84% at 72 hours (Figure 45A). The 48 hour time point was therefore chosen for optimal KD efficiency.

Experiments were then carried out to compare the effectiveness of siADA2 vs si202 KD with 4 separate pools of HUVEC. One further condition, with a commercially available siRNA to ADA1, was also used for comparison of the effect of loss of ADA1 and ADA2 in ECs. Figure 45B demonstrates that both siADA2 and si202 achieved KD of ADA2 202 in HUVEC. There was however a greater efficiency of KD with siADA2 vs si202 (86.6% vs 75.5%) and so this siRNA was used for further experimental work (Table 15). ADA2 expression remained stable in the siADA1 treated HUVEC (Figure 45B). 86% KD of ADA1 was achieved with siADA1 (Figure 45C). There was no effect of siADA2 or si202 on ADA1 expression.

	Pool 1 (% ADA2 KD)	Pool 2 (% ADA2 KD)	Pool 3 (% ADA2 KD)	Pool 4 (% ADA2 KD)	Mean (% ADA2 KD)
siCtr	0	0	0	0	0
siADA2	93.7	86.8	89.7	76.4	86.7
si202	79.6	84.5	82.7	55.2	75.5

Table 15 - Percentage knockdown of ADA2 achieved in each HUVEC pool with each siRNA

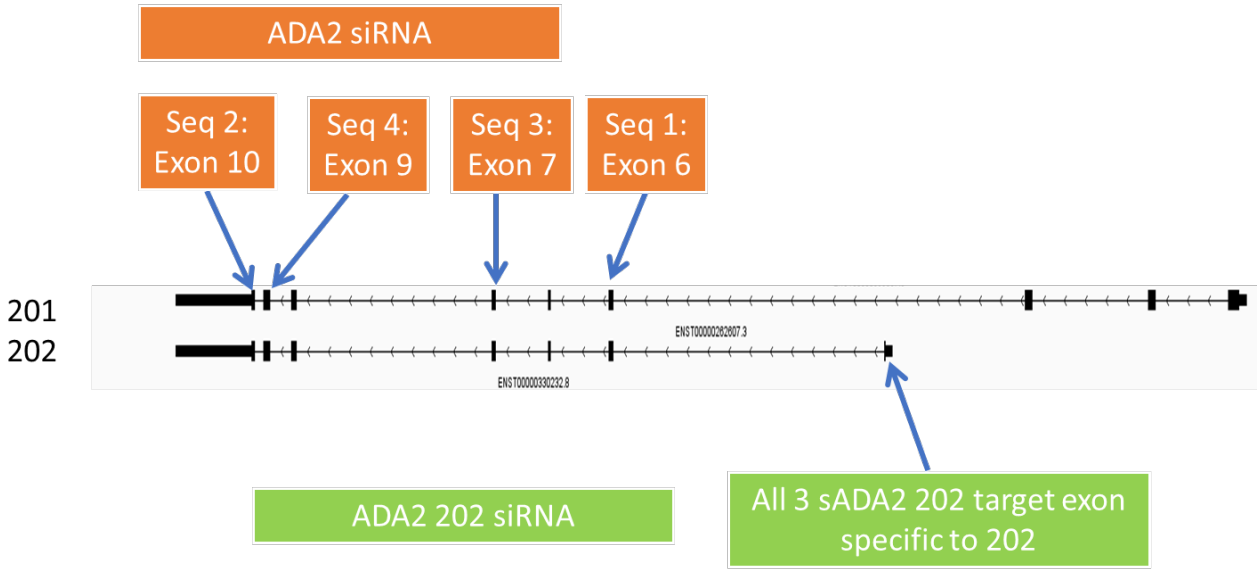


Figure 44 – Exon map of ADA2 201 and 202 demonstrating the regions targeted by specific ADA2 siRNAs.

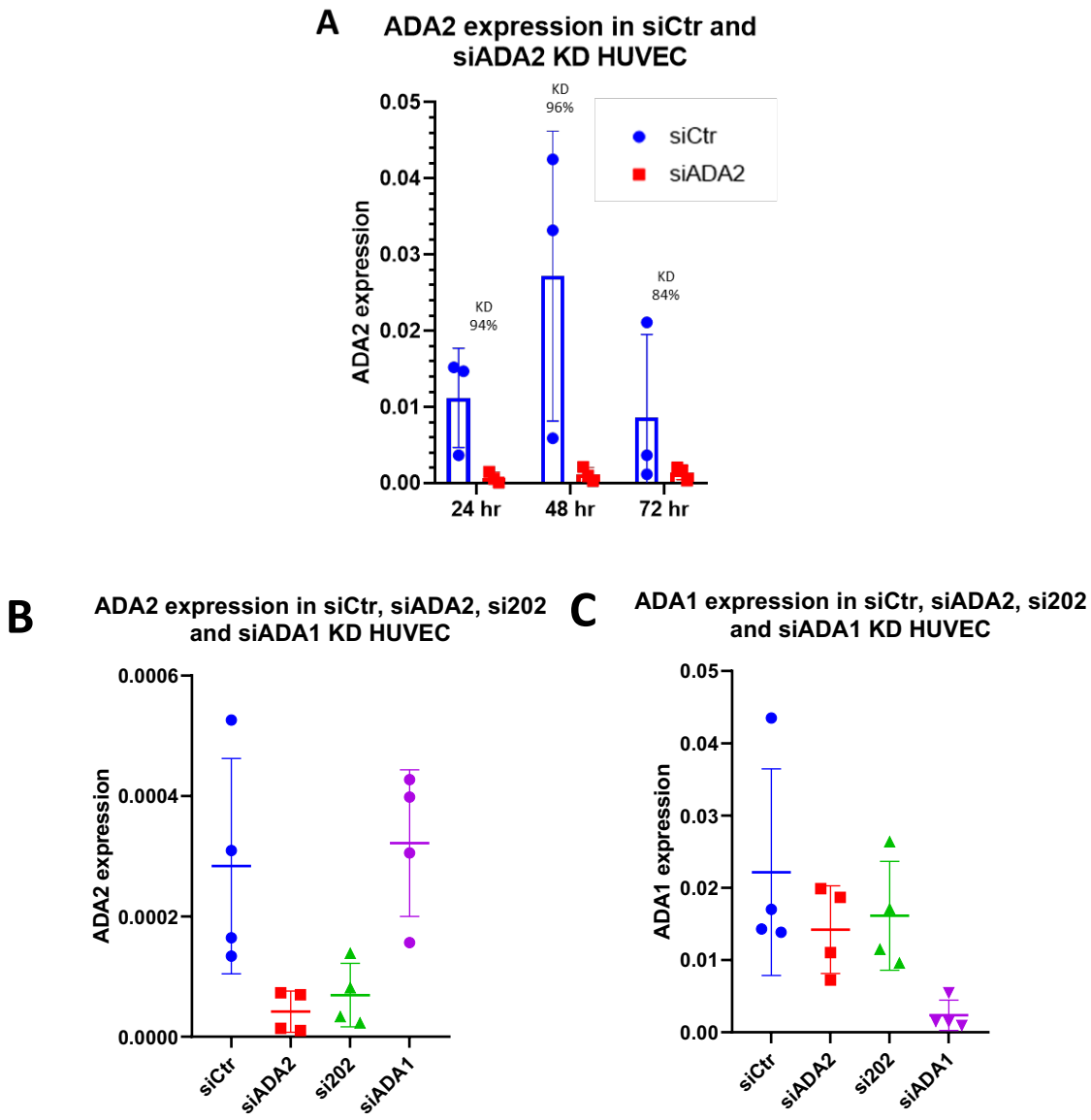


Figure 45 – specific siRNA knockdown of ADA2 was achieved without off target effect on ADA1.

(A) HUVEC (n=3) were treated with siCtr or siADA2 siRNAs for 6 hours with transfection reagent. Cells were harvested at 24, 48 and 72hrs and RNA purified. ADA2 gene expression was measured using qRT-PCR. (B) HUVEC (n=4) were treated with siCtr, siADA2, si202 or siADA2 siRNAs for 6 hours with transfection reagent. Cells were harvested at 48hrs, RNA purified and ADA2 gene expression measured using qRT-PCR. (C) ADA1 gene expression was also measured.

Immunoblot of protein lysates from siCtr and siADA2 HUVEC demonstrated that there was no band consistent with ADA2 202 protein and no loss of any bands with siADA2 (Figure 46). rADA2 202 was included as a positive control.

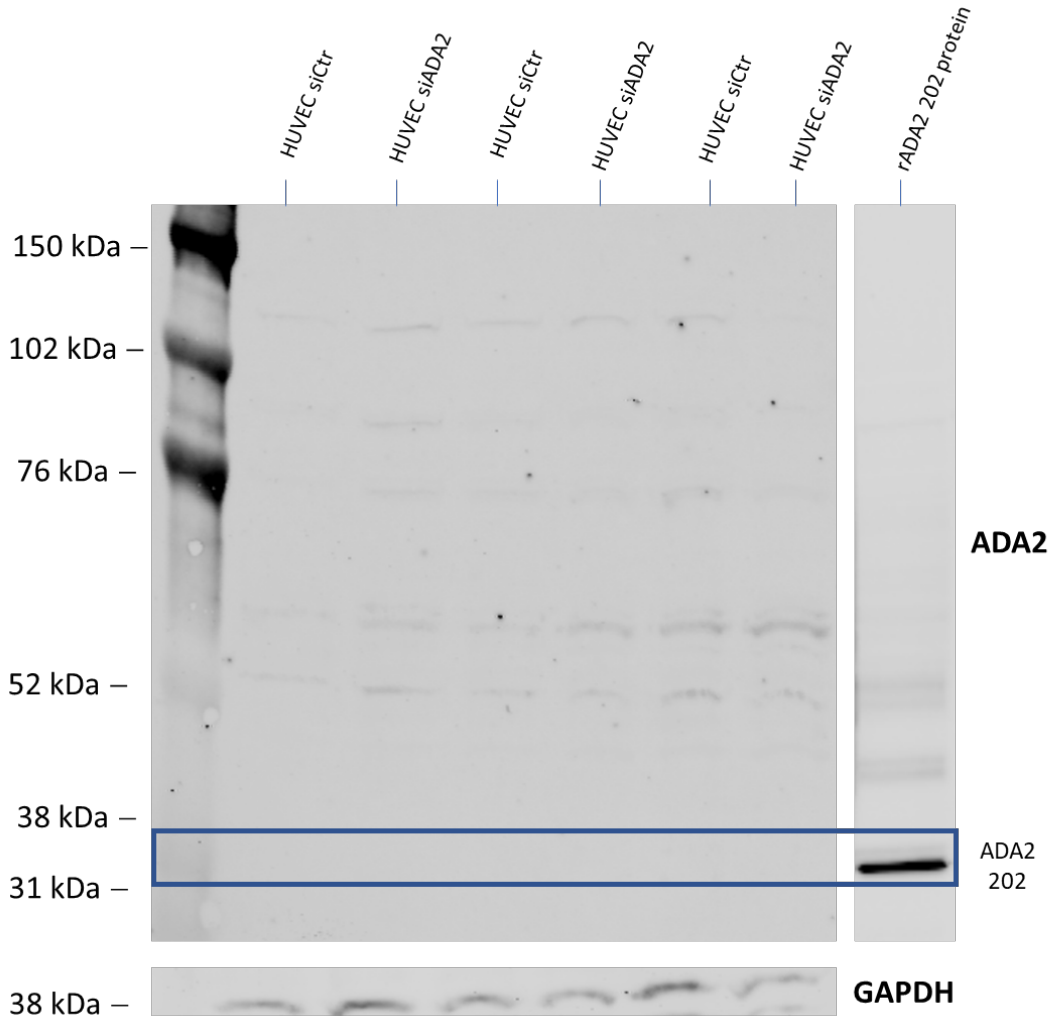


Figure 46 – No ADA2 protein was detected in HUVEC siCtr or siADA2 lysates

HUVEC were treated with siCtr or siADA2 siRNAs for 6 hours with transfection reagent. Protein was harvested after 48 hours using RIPA lysis buffer. These lysates were separated by SDS-PAGE and immunoblot analysis carried out. Proteins were detected with an ADA2 antibody (both 201 and 202 isoforms) and GAPDH.

5.5 RNA sequencing of siADA2 knockdown HUVEC

5.5.1 Design of experiment

To gain an unbiased assessment of the function of the ADA 202 transcript in endothelial cell, RNA sequencing of siADA2 KD HUVEC was carried. 4 pools of HUVEC were treated with siCtr and 3 with siADA2. One further condition with a commercially available siRNA to ADA1 was also used for comparison of the effect of loss of ADA1 and ADA2 in ECs. Imaging of the HUVEC demonstrated no significant difference between treatments at 48 hours when RNA was harvested (Figure 47).

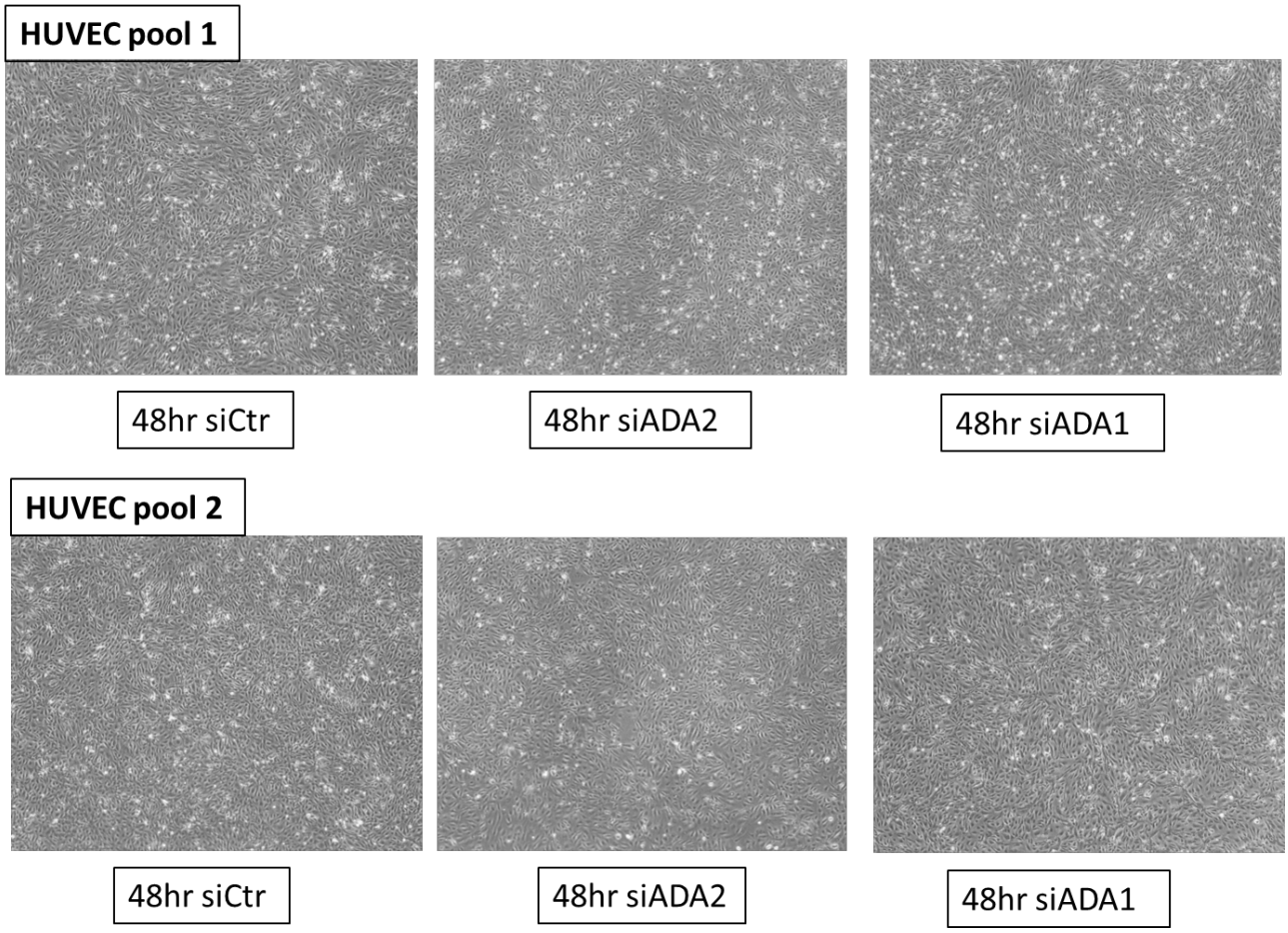


Figure 47 – 4x microscope images of 2 pools of HUVEC treated with siCtr, siADA2 and siADA1.

HUVEC were treated with siCtr, siADA2 or siADA1 siRNAs for 6 hours with transfection reagent. 4X microscope images were taken at 48 hours.

5.5.2 RNAseq analysis

RNA was harvested at 48 hours, isolated and confirmed to have good RNA integrity as indicated by RNA integrity number >9 for all samples. RNA samples were sent to Genewiz for poly-A, strand-specific paired-end sequencing targeting 30 million paired-end reads per sample as per ENCODE guidelines (Figure 5) [176]. FASTQC analysis demonstrated that the RNA-sequencing data quality was excellent as shown by Phred quality scores >30 in 96% of reads for all samples and data did not require trimming before mapping. Read mapping rates per sample were satisfactory and >97% of mapped reads corresponded to protein coding genes in each sample as expected with poly-A RNA-seq data (Table 16). Prior to differential expression analysis, gene-level read counts were confirmed to have similar distributions between samples. Mapping stats and normalisation of the data is shown in (Figure 48). Differential gene expression was defined as an false discovery rate<0.05 and Log 2 fold change > 0.6.

	Total Reads	Mapped Reads	% Mapped	P.Coding % of mapped reads
Pool 1 – siCtr	3.94E+07	3.26E+07	82.56	98.11
Pool 1 – siADA2	5.05E+07	4.20E+07	83.22	97.99
Pool 1 – siADA1	6.09E+07	5.07E+07	83.13	98.06
Pool 2 – siCtr	4.76E+07	4.05E+07	85.02	98.33
Pool 2 – siADA2	5.01E+07	4.24E+07	84.65	98.28
Pool 2 – siADA1	4.52E+07	3.80E+07	84.05	98.21
Pool 3 – siCtr	4.54E+07	3.80E+07	83.66	98.17
Pool 3 – siADA1	4.46E+07	3.69E+07	82.88	98.23
Pool 4 – siCtr	6.54E+07	5.41E+07	82.62	98.04
Pool 4 – siADA2	5.03E+07	4.13E+07	82.04	97.63
Pool 4 – siADA1	4.11E+07	3.46E+07	84.03	97.99
Min	3.94E+07	3.26E+07	82.04	97.63
Max	6.54E+07	5.41E+07	85.02	98.33
Median	4.76E+07	4.05E+07	83.22	98.11

Table 16 – Read and mapping data from the RNAseq analysis

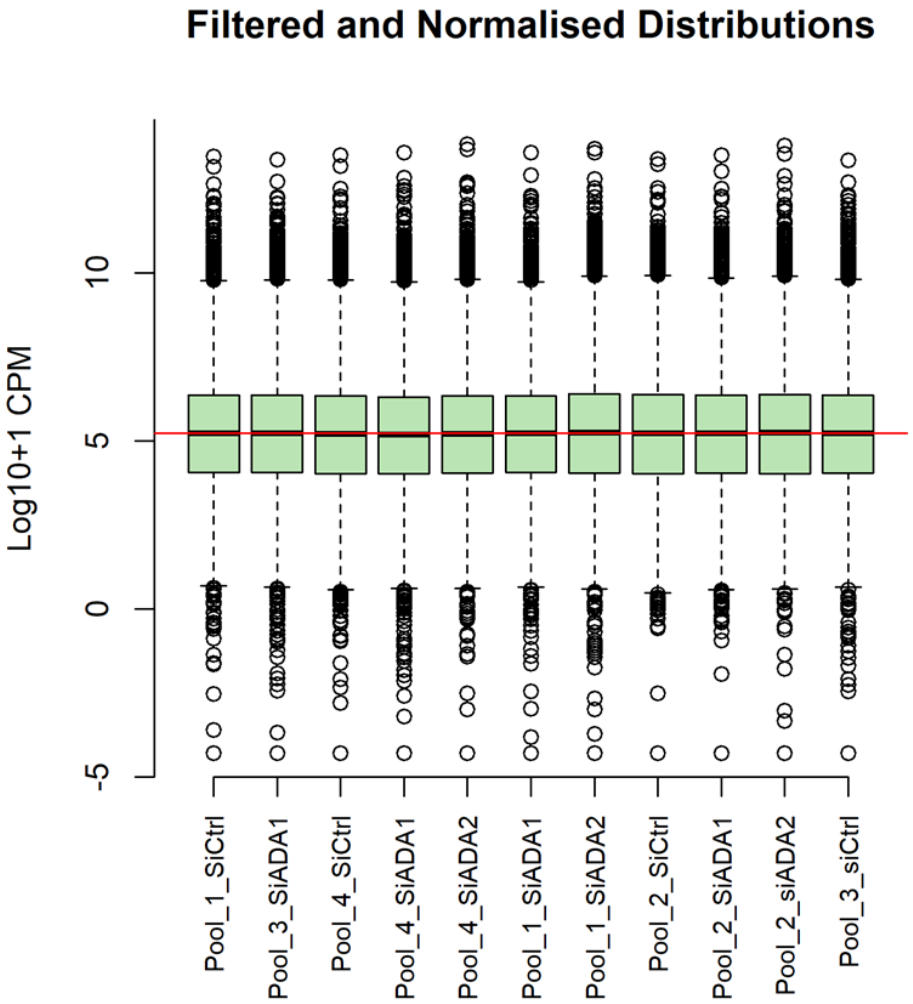


Figure 48 – RNAseq read count distribution after normalisation and filtering

5.5.3 Specific knockdown of ADA2 and ADA1 were achieved.

Analysis of ADA2 isoform expression in the HUVEC siCtr samples confirmed that ADA2 202 is the sole isoform expressed. The signal plot in Figure 49 demonstrates effective KD with loss of ADA2 202 exon signals in the siADA2 HUVEC.

88% KD of ADA2 was achieved in siADA2 vs siCtr HUVEC with no change in ADA1 expression. 81% KD of ADA1 was achieved in siADA1 vs siCtr HUVEC with no change in ADA2 expression.

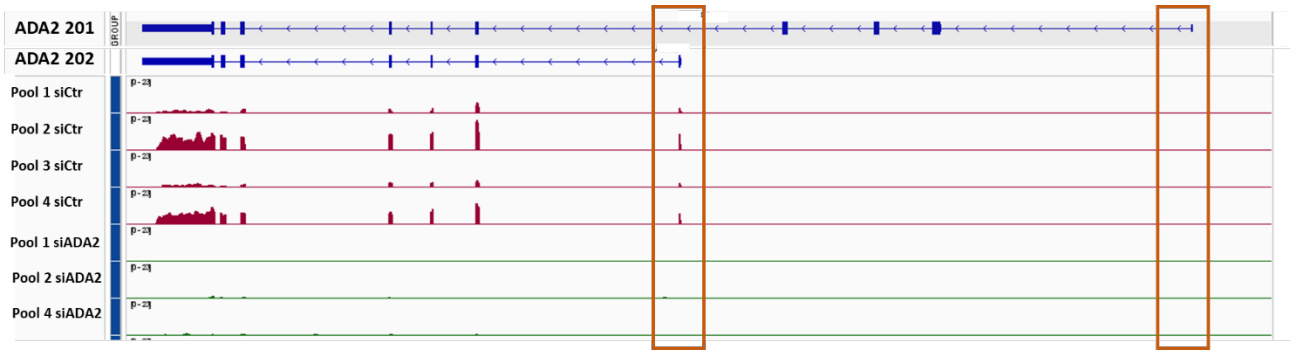


Figure 49 – Treatment with siADA2 led to loss of signal of the exons of ADA2 202 in HUVEC.

HUVEC were treated with siCtr or siADA2 siRNAs for 6 hours with transfection reagent. RNA was harvested at 48 hours sent to Genewiz for poly-A, strand-specific paired-end sequencing. A signal plot was generated.

5.5.4 siADA2 knockdown in HUVEC led to differential regulation of a number of genes.

In siADA2 vs siCtr HUVEC there were 352 differentially expressed (DE) genes, 204 up and 148 down (FDR<0.05 and L2FC>0.6) (Table 17, Figure 50). The number of genes affected by ADA2 KD suggests that ADA2 202 is functional despite the lack of detectable protein levels in HUVEC.

In siADA1 v siCtr HUVEC there were 53 differentially expressed genes, 10 up and 43 down (Table 17). There were 11 genes that were differentially expressed in both siADA2 and siADA1; 2 that were upregulated in both, 2 that were downregulated in both and 7 that were discordant (Table 17). The 2 upregulated genes were NLRC3 – a cytosolic regulator of innate immunity, and MN1 – a transcriptional activator [209]. The 2 downregulated genes were KCNAB1 – encodes voltage-gated potassium channel subunit and MAP1A – a microtubule-associated protein [209].

Number of genes with FDR <0.05 & L2FC > 0.6			
	siADA2	**siADA1**	**Overlap**
Total	352	53	11
Up	204	10	2
Down	148	43	2

Table 17 – Numbers of differentially expressed genes in siADA2 and siADA1 KD HUVEC.

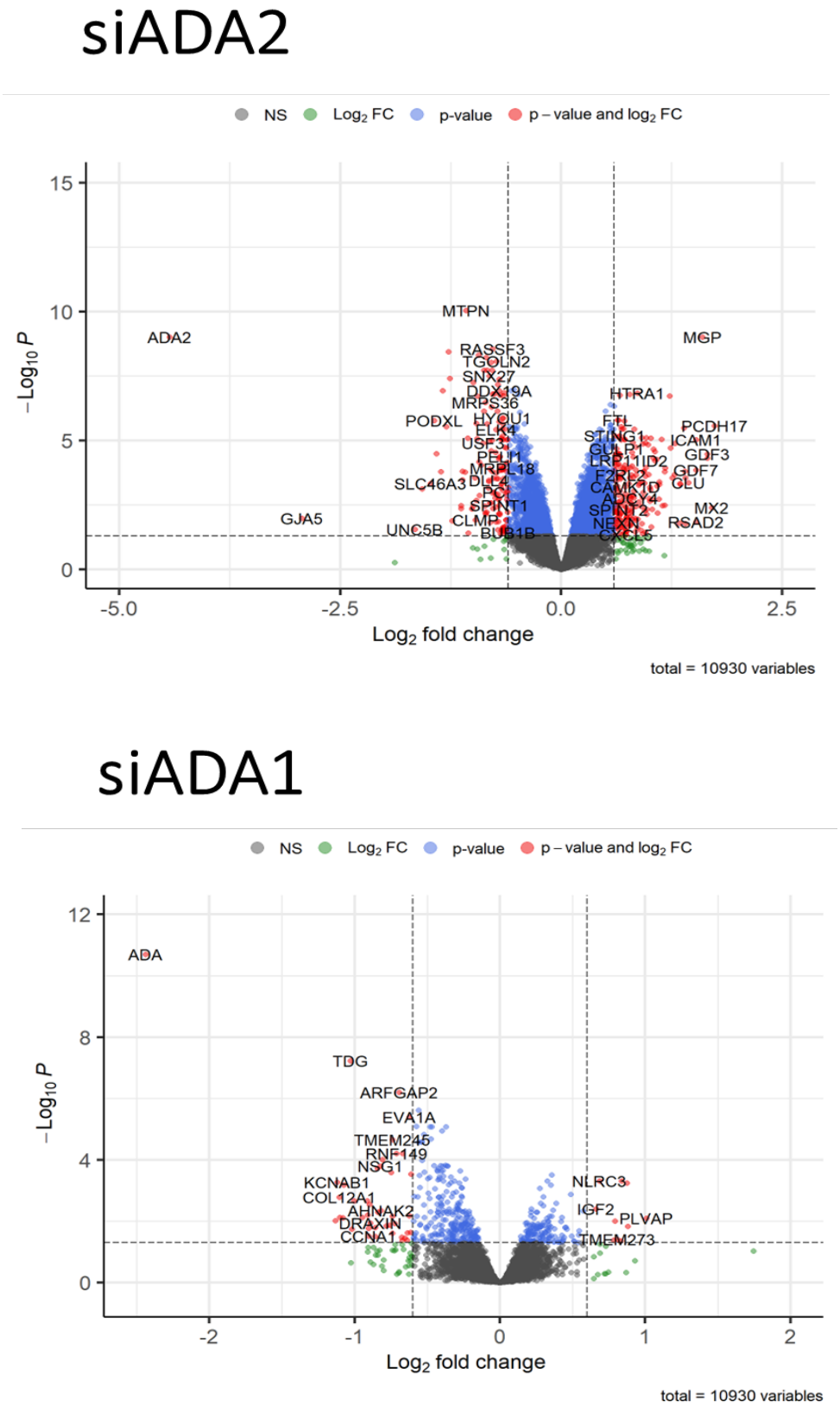


Figure 50 – Volcano plot of differentially expressed genes in siADA2 and siADA1.

A volcano plot was generated from the RNAseq data of differentially expressed genes with an FDR<0.05 and L2FC>0.6.

5.5.5 Pathway analysis of differentially expressed genes.

Gene set enrichment analysis of siADA2 related changes identifies signatures relevant to vasculitis and EC dysfunction (Table 18). Given the clinical phenotype of DADA2 is with vasculitis the pathways associated with the inflammatory cytokines; IFNα and γ pathways as well as TNFα signalling via NFκB and inflammatory response are very important. Anti-TNF is known to be a very effective treatment for the vasculitic component of the disease [48]. Work has also shown evidence of an IFN signature in the RNA of patient blood samples and patient PBMCs [100, 207]. In addition, there was enrichment of apoptotic pathways. There were no meaningful enrichment results with the 10 increased differentially expressed genes in siADA1 HUVEC.

Pathway	NES	FDR
Interferon alfa response	1.85	0.000
Interferon gamma response	1.67	0.005
Coagulation	1.58	0.022
TNFα signalling via NFκB	1.57	0.018
Epithelial Mesenchymal transition	1.56	0.017
Apoptosis	1.52	0.026
Inflammatory response	1.47	0.031

Table 18 – Table demonstrating gene set enrichment pathways of differentially expressed genes in siADA2 HUVEC.

NES – Normalised Enrichment Score. FDR – False Discovery Rate

5.5.6 Gene ontology analysis of differentially expressed genes

Gene ontology analysis of the differentially expressed genes was carried out using the online g:Profiler tool [210]. Ontology analysis of biological processes and cellular component was carried out and negative Log10 adjusted p-values recorded (Figure 51A-B). Biological processes involved in blood vessel morphogenesis (Up 73.3%), vasculature development (Up 74.3%), blood vessel development (Up 74.3%) and angiogenesis (73.3%) were identified which is of interest given the hypothesis that ADA2 has a role a growth factor in the endothelium (Figure 51A). Ontology analysis of the cellular component showed the most significant results were in the cell periphery and extracellular matrix (Figure 51B).

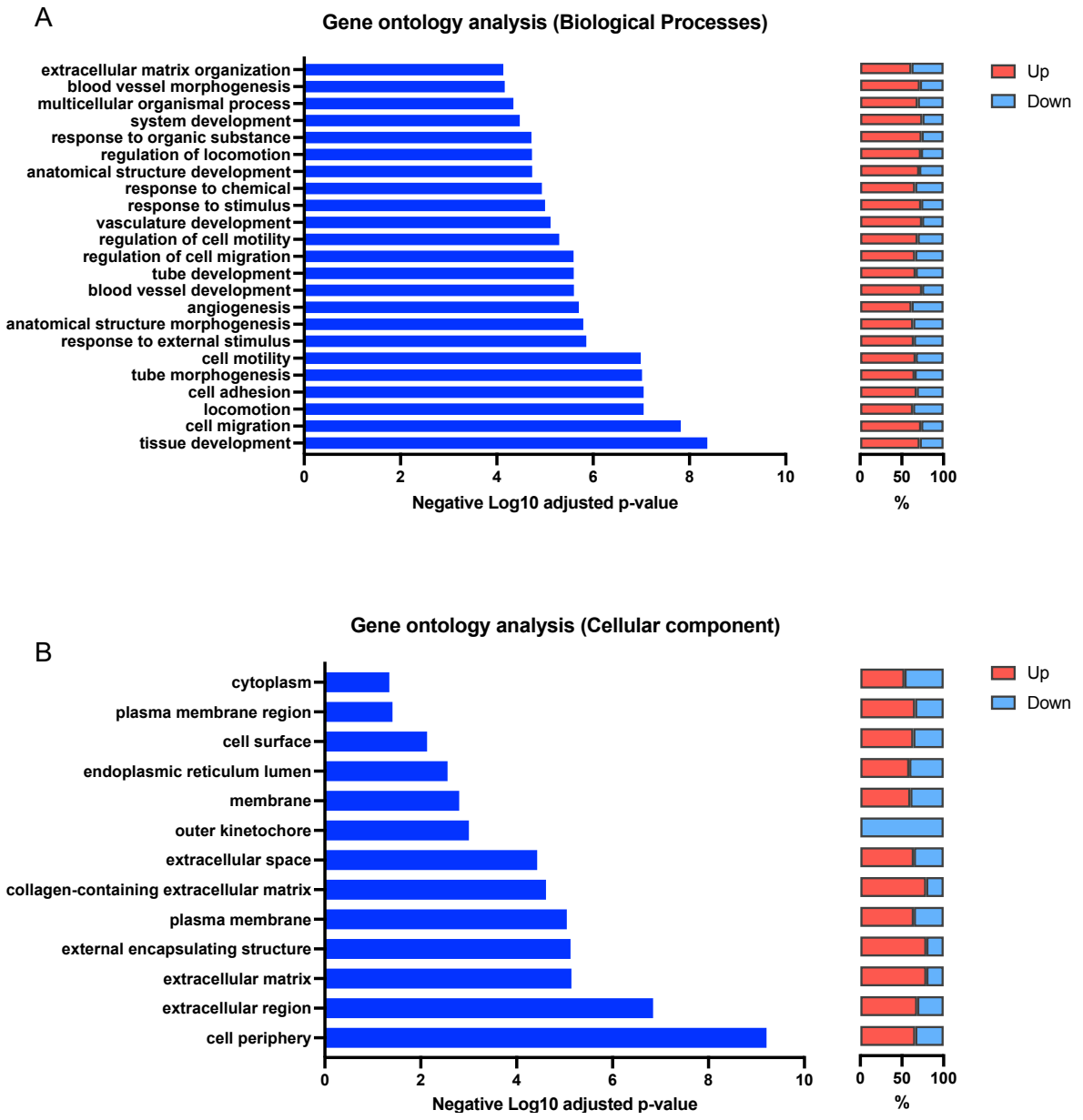


Figure 51 – Gene ontology of differentially expressed genes in siADA2 HUVEC.

Gene ontology analysis was carried out on differentially expressed genes (350, FDR >0.05, L2FC <0.6) using the g:Profiler online tool. Negative Log10 adjusted p-value was generated for (A) Biological Processes and (B) Cellular Component analysis (LHS). The percentage of the genes Up-regulated or Down-regulated were also recorded (RHS).

5.5.7 Comparison of RNAseq results with previously published data

Dhanwani et al have previously carried out siRNA KD of ADA2 in a single HUVEC pool replicated 4 times [88]. In my analysis, ADA2 KD was performed in 3 separate pools i.e. 3 independent biological replicates. Re-analysis of the Dhanwani data (GEO accession GSE150540) identified 1,447 differentially expressed genes (FDR >0.05, L2FC>0.6) with 942 upregulated and 505 downregulated.

Reasonable agreement was observed between the differentially expressed genes identified in both datasets with 38.7% of the upregulated and 17.6% of the downregulated genes found in my dataset represented in the Dhanwani analysis. Comparing the Log2 fold change parameter between both datasets revealed even better agreement with a Pearson R of 0.49. 73.1% of the Dhanwani differentially expressed genes had a concordant direction of change in my analysis (Figure 52). Notably Dhanwani et al also demonstrated an IFN-associated gene expression signature, however in IFNβ rather than IFNα and γ as we have [88].

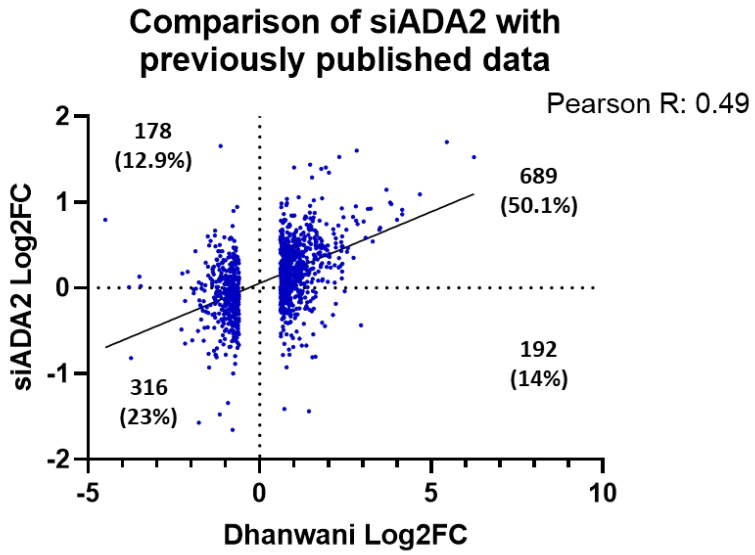


Figure 52 – Comparison to previously published siADA2 RNAseq data in HUVEC.
Comparison of Log2 fold change parameter between our RNA seq data and previously published data was carried out.

5.6 Gene expression and protein validation of RNAseq results in siADA2 HUVEC

5.6.1 siADA2 KD HUVEC have increased expression of interferon related genes and trends to elevated CXCL10 protein relative to siCtr

To validate the IFN signature that had been demonstrated in the RNAseq analysis of siADA2 KD in HUVEC, a further siADA2 KD experiment was carried out, RNA harvested, and qPCR done with IFN related targets (IFIT1, RSAD2 and CXCL10). There was a significant increase in IFIT1 expression in siADA2 KD vs siCtr (p=0.035) and non-significant trends to increase in RSAD2 and CXCL10 expression (Figure 53).

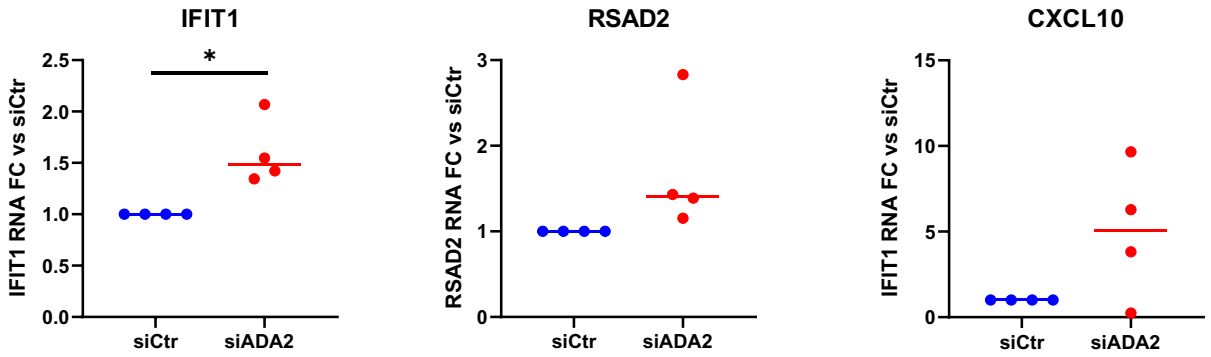


Figure 53 – Interferon related genes expression (IFIT1, RSAD2 and CXCL10) were elevated in siADA2 KD HUVEC.

HUVEC (n=4) were treated with siCtr or siADA2 siRNAs for 6 hours with transfection reagent. Cells were harvested at 48hrs, RNA purified and IFIT1, RSAD2 and CXCL10 gene expression measured using qRT-PCR

Assessment of CXCL10 supernatant protein expression through ELISA was carried out in both siCtr and siADA2 KD HUVEC. Increasing concentrations of TNFα (0, 0.1, 1 and 10ng/ml) were used to stimulate CXCL10 expression so it was detectable above the lower limit of detection of the ELISA. Consistent with the gene expression, there were trends to increase in CXCL10 protein in siADA2 HUVEC vs siCtr with TNFα 10ng/ml (p=0.07). (Figure 54).

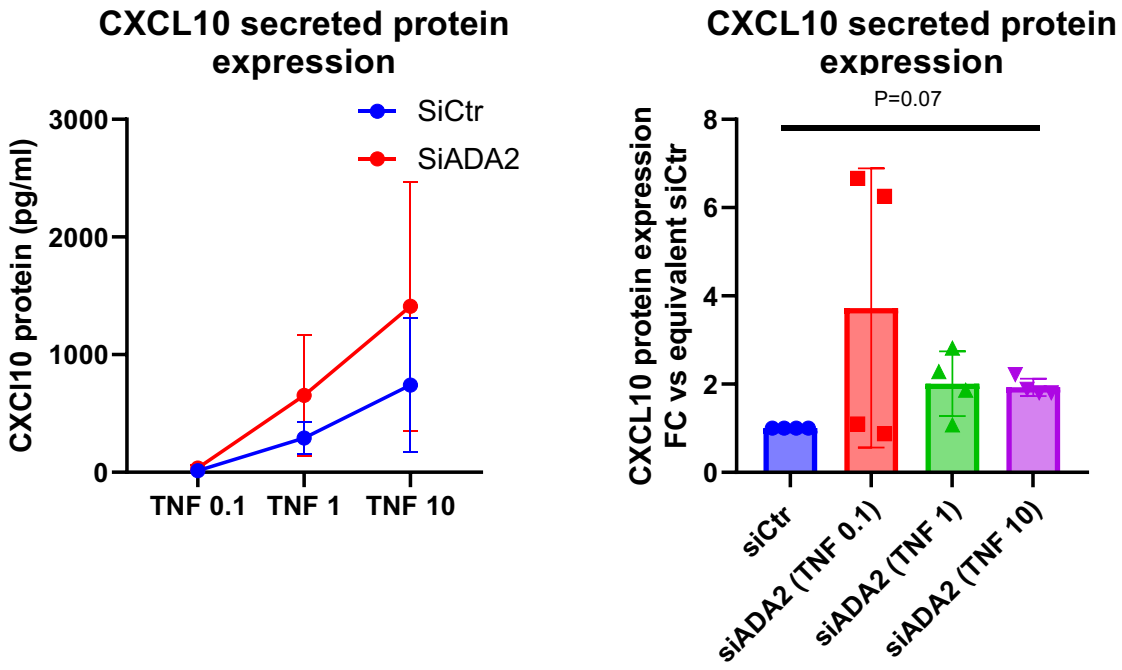


Figure 54 – There were trends to increased CXCL10 protein expression in siADA2 HUVEC treated with TNFα 10ng/ml.

siADA2 and siCtr HUVEC (n=4) were treated with increasing concentrations of TNFα (0, 0.1, 1 and 10 ng/ml). Supernatant was harvested after 24 hrs and supernatant CXCL10 protein concentration measured using an ELISA. One-way ANOVA with Dunnett's post-hoc test were performed.

5.6.2 siADA2 KD HUVEC express higher levels of cell adhesion molecules and are more sensitive to TNFα.

5.6.2.1 ICAM1

Given the vasculitic phenotype seen in DADA2 and that GSEA analysis identified upregulation in TNFα signalling and in cell adhesion molecule expression, ICAM expression in untreated and TNFα treated siCtr and siADA2 KD HUVEC was measured. siCtr and siADA2 HUVEC were treated with 24 hours with TNFα 0, 0.1 and 1ng/ml.

There was significant upregulation of ICAM1 mRNA in siADA2 KD in basal conditions with no TNFα ($p=0.048$) (Figure 55B). TNFα exposure resulted in a dose dependent increase in ICAM1 RNA expression in both siCtr and siADA2 KD HUVEC (Figure 55A + B). There were non-significant trends to elevated ICAM1 RNA levels in the siADA2 vs siCtr cells at each TNFα concentration (Figure 55A + B).

When ICAM1 protein expression was assessed using an ELISA, no significant differences were seen in siADA2 vs siCtr with TNFα 0 and 0.1ng/ml (Figure 55C + D). With TNFα 1ng/ml there was saturation of the ICAM1 protein signal detected in the ELISA (Figure 55C).

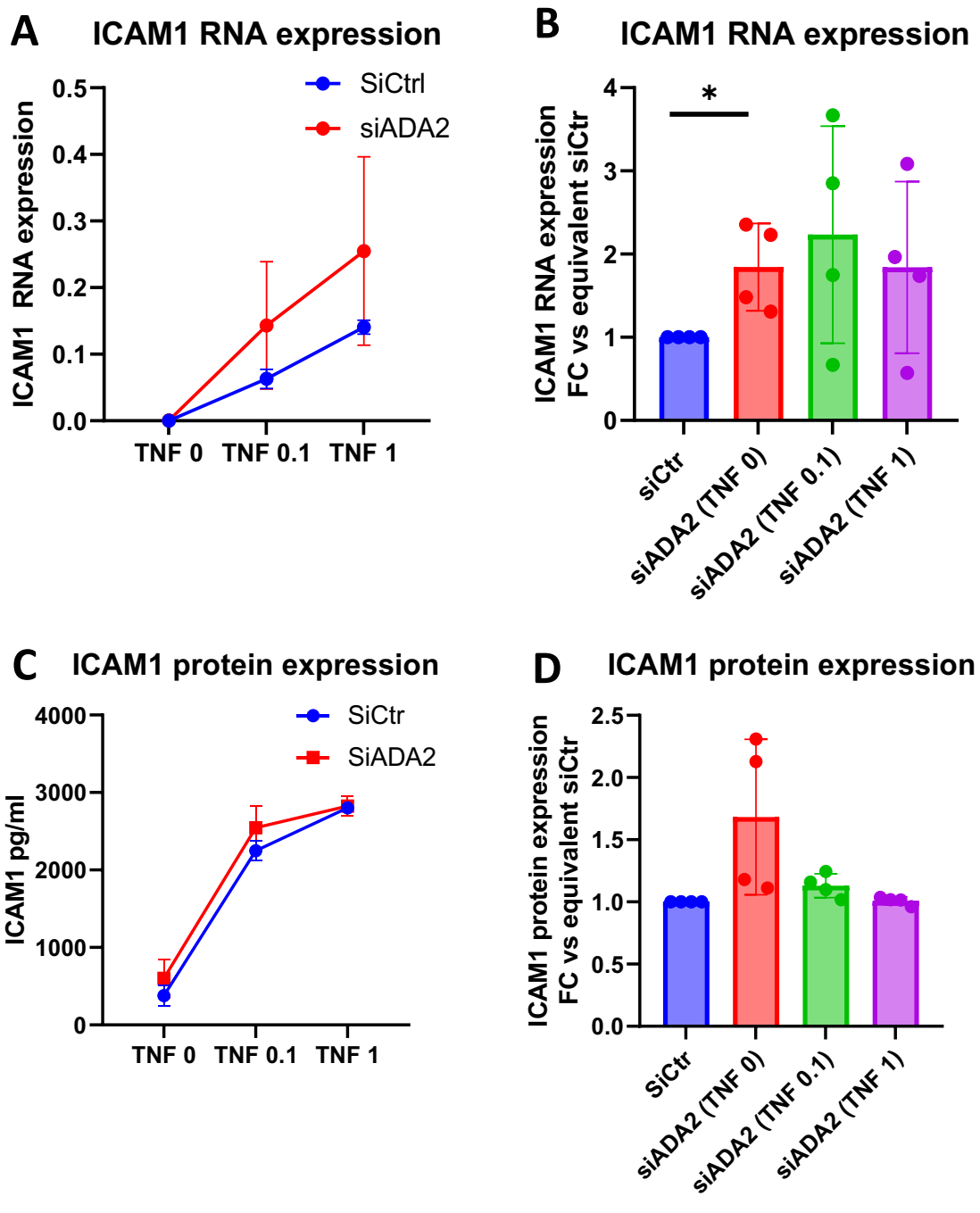


Figure 55 – ICAM1 RNA and protein expression in siADA2 HUVEC under basal conditions and with TNFα stimulation.

siCtr and siADA2 HUVEC were treated with TNFα (0, 0.1 and 1 ng/ml) for 24 hours. RNA and cell lysate for protein were harvested. (A) ICAM1 RNA expression (relative to GAPDH) was measured using qRT-PCR. (B) ICAM1 RNA expression fold change relative to the equivalent siCtr TNFα concentration was calculated. (C) ICAM1 protein concentration in cell lysates was measured using an ELISA. (D) ICAM1 protein expression fold change relative to the equivalent

siCtr TNFα concentration was calculated. One-way ANOVA with Dunnett's post-hoc test were performed.

5.6.2.2 VCAM

There was no significant difference in VCAM RNA expression between siCtr and siADA2 HUVEC with no TNFα treatment. There were however again non-significant trends for increased VCAM expression in siADA2 KD at TNFα concentrations of 0.1ng/ml and 1 ng/ml (Figure 56A + B). Assessment of VCAM protein expression with an ELISA demonstrated no significant differences between siCtr and siADA2 cells at any TNFα concentration (Figure 56C + D).

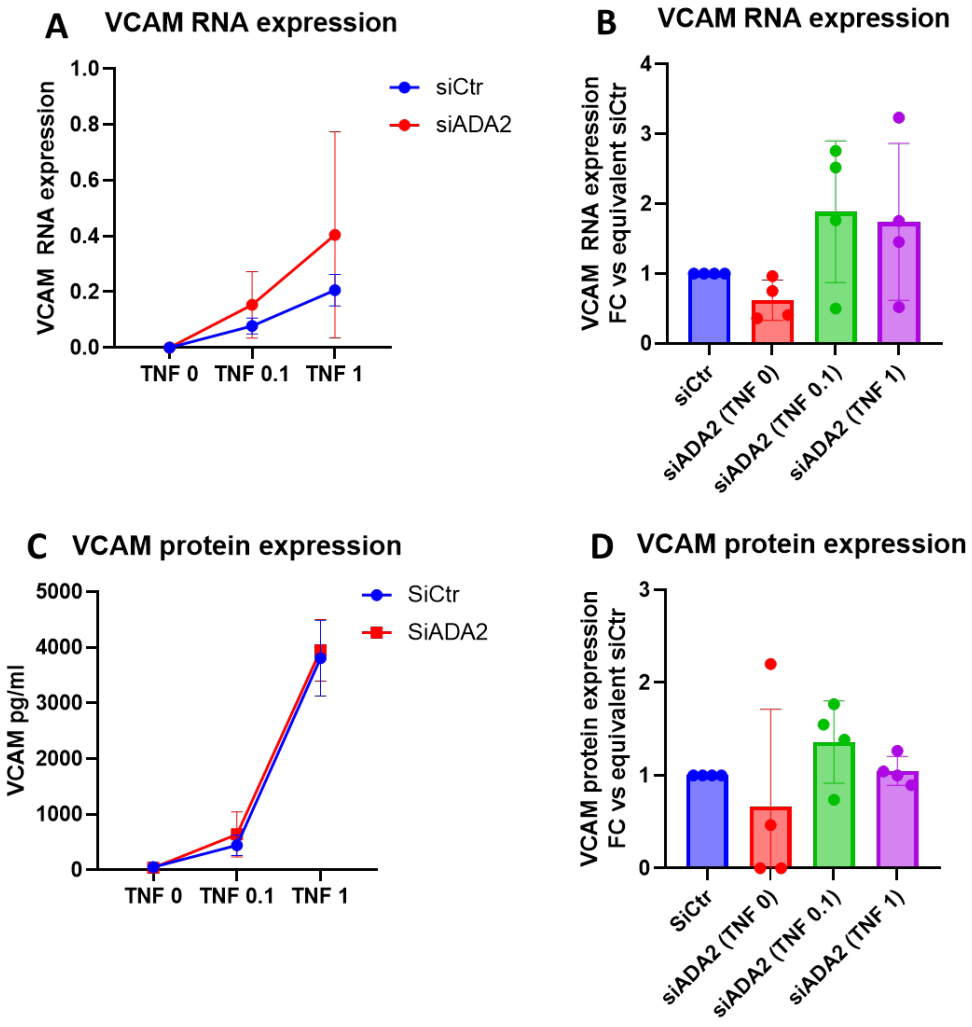


Figure 56 - VCAM RNA and protein expression in siADA2 HUVEC under basal conditions and with TNFα stimulation

siCtr and siADA2 HUVEC were treated with TNFα (0, 0.1 and 1 ng/ml) for 24 hours. RNA and cell lysate for protein were harvested. (A) VCAM RNA expression (relative to GAPDH) was measured using qRT-PCR. (B) VCAM RNA expression fold change relative to the equivalent siCtr TNFα concentration was calculated. (C) VCAM cell lysate protein expression was measured using an ELISA. (D) VCAM protein expression fold change relative to the equivalent siCtr TNFα concentration was calculated.

5.7 Proteomics in siADA2 HUVEC

Protein lysates from siCtr and siADA2 cells were sent for mass spectrometry analysis. Proteomic analysis was carried out as per methods section. 125 differentially expressed (DE) proteins between the 2 groups were identified (47 upregulated in siADA2 and 78 downregulated). Ontology analysis using g:Profiler demonstrated biological process of the differentially expressed proteins were involved in translation (Down 61.9%), peptide metabolic processing (Down 59.0%) and non-coding RNA processing (Down 83.3%) (Figure 57A). Cellular component ontology analysis identified the cytoplasm and cytosol as prominent categories amongst DE proteins (Figure 57B).

There were 20 proteins that were undetectable in all 4 the siCtr samples but became detectable in all 3 of the siADA2 samples. Ontology analysis of cellular component in g:Profiler demonstrated that 11 of these 20 proteins were involved in vesicles (TBC1D4, VTN, MGP, CDH13, PIP4K2C, KIF21B, FLOT1, TSPAN14, SCAMP4, GNB4, HBS1L).

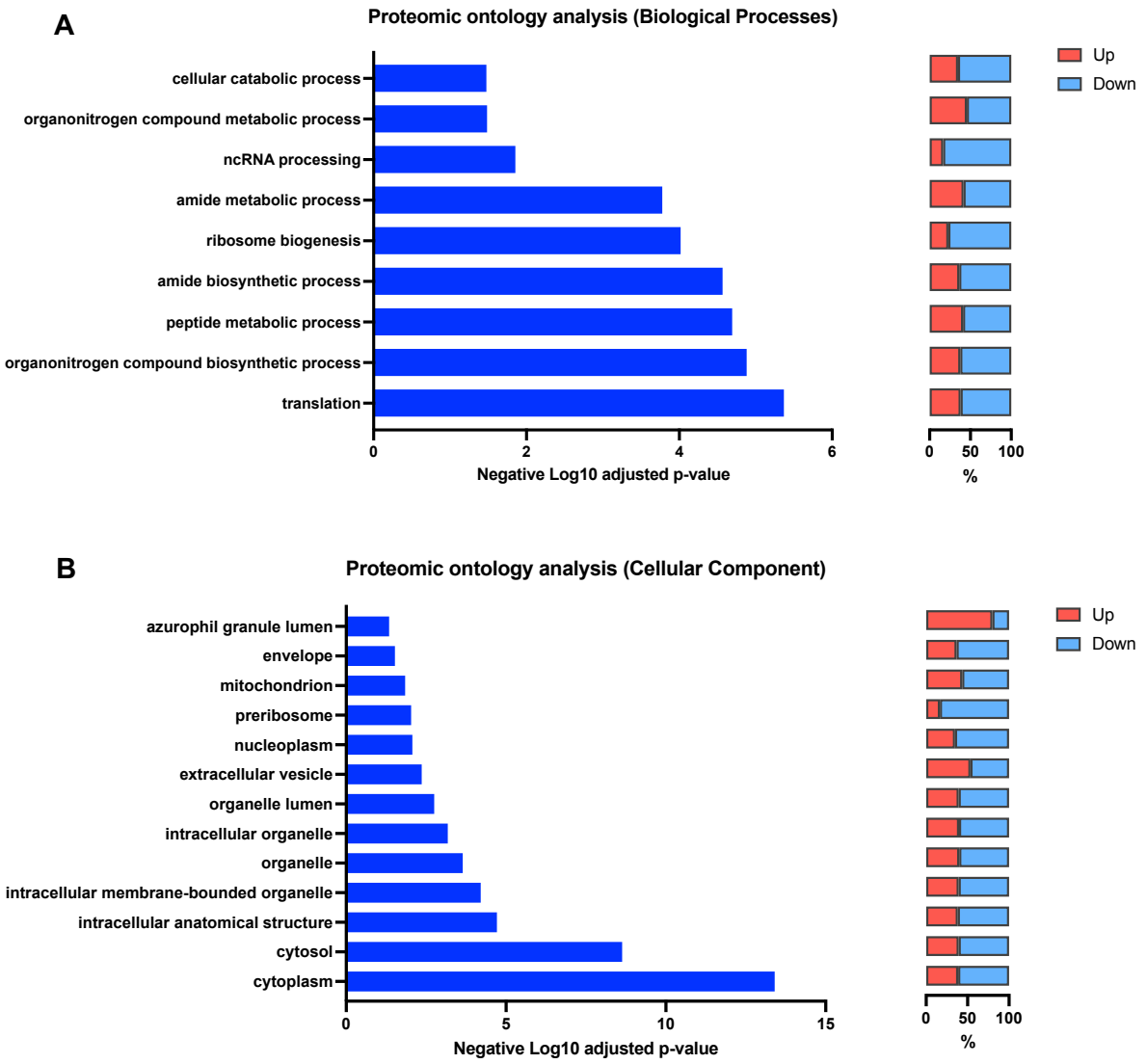


Figure 57 – Proteomic analysis in siADA2 HUVEC

Protein lysates from siCtr and siADA2 cells were sent for mass spectrometry analysis. Differentially expressed proteins between the groups were identified and proteomic ontology analysis was carried out looking at (A) Biological processes and (B) the Cellular component.

5.8 Comparison of differentially expressed genes from RNAseq and differentially expressed proteins from proteomics in siADA2 HUVEC

Comparison of the 352 differentially expressed genes from the RNAseq results and 126 differentially expressed proteins from the proteomics identified an overlap of 5 (Table 19). All 5 were concordant between the RNAseq and proteomic analysis. The NCBI gene summary for each is included in the table. Of interest some of these targets are involved in the ubiquitination pathway (UBA6), RNA binding (RPL22L1) and protein binding (DCTN4). In addition, RPL22L1 is associated with Diamond Blackfan anaemia, a congenital cause of pure red cell aplasia which has been shown to be linked to biallelic mutations in the ADA2 gene and deficiency of ADA2 activity in plasma [211].

	RNAseq	Proteomic	NCBI Gene summary
ITGA2	Down	Down	This gene encodes the alpha subunit of a transmembrane receptor for collagens and related proteins. The encoded protein forms a heterodimer with a beta subunit and mediates the adhesion of platelets and other cell types to the extracellular matrix. Loss of the encoded protein is associated with bleeding disorder platelet-type 9. Antibodies against this protein are found in several immune disorders, including neonatal alloimmune thrombocytopenia
UBA6	Down	Down	Modification of proteins with ubiquitin (UBB; MIM 191339) or ubiquitin-like proteins controls many signaling networks and requires a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3). UBE1L2 is an E1 enzyme that initiates the activation and conjugation of ubiquitin-like proteins
RPL22L1	Down	Down	Predicted to enable RNA binding activity. Predicted to be a structural constituent of ribosome. Predicted to be involved in cytoplasmic translation. Predicted to be located in ribosome
DCTN4	Down	Down	Enables protein N-terminus binding activity. Located in centrosome

CTSA	Up	Up	This gene encodes a member of the peptidase S10 family of serine carboxypeptidases. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed to generate two chains that comprise the heterodimeric active enzyme. This enzyme possesses deamidase, esterase and carboxypeptidase activities and acts as a scaffold in the lysosomal multienzyme complex. Mutations in this gene are associated with galactosialidosis

Table 19 – Comparison of the differentially expressed genes from the RNAseq results and differentially expressed proteins from proteomics

5.9 Functional phenotype of siADA2 HUVEC

5.9.1 Apoptosis and necrosis

Given the identification of apoptosis in RNA-seq gene-set enrichment analysis as well as the dysregulation of proteins involved in key homeostatic processes such as translation, I next evaluated whether silencing of ADA2 might affect HUVEC cell death. The levels of apoptosis and necrosis after siCtrl and siADA2 treatment were assessed using commercially available probes that enable real-time detection without cell lysis (see methods for full details). The experiment was conducted in 3 conditions: i) normal endothelial growth media #2 (EGM2) + 10% FBS, ii) EGM2 + 10% FBS + TNFα 10ng/ml and iii) EGM2 + 0% FBS. This was done to assess cell death in basal conditions (EGM2 + 10%) as well as under cell stress i.e. TNF and serum starvation. Prior to this experiment, serum starvation for 24 hours, 0% FBS, was shown to significantly increase apoptosis in HUVEC (p=0.003).

There was no significant difference in the level of apoptosis or necrosis between siCtr and siADA2 under any of the conditions (Figure 58A + B).

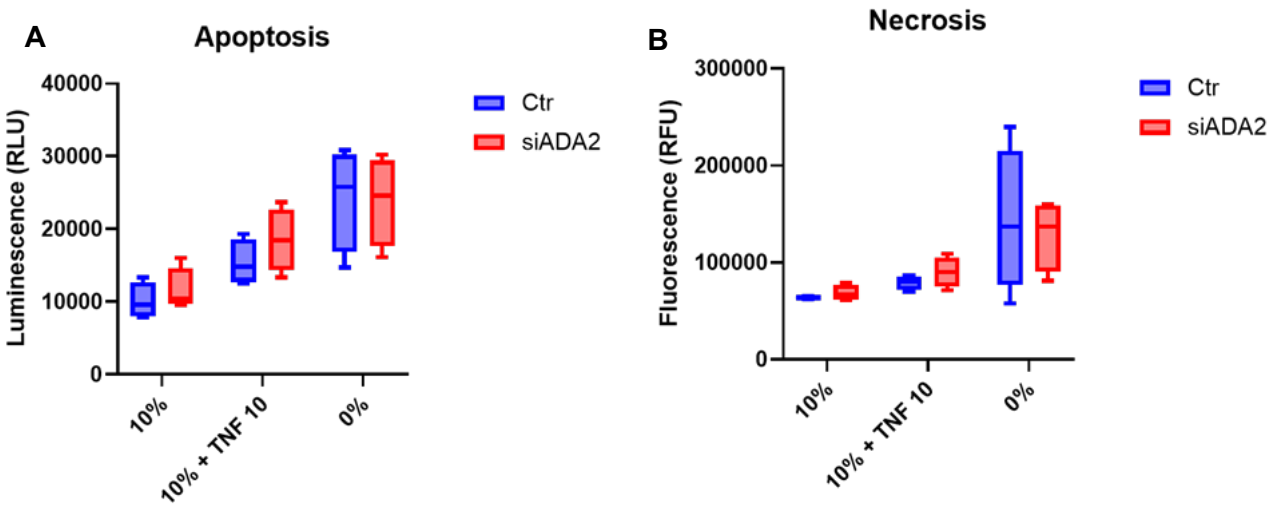


Figure 58 – Apoptosis and necrosis in siCtr and siADA2 HUVEC

(A) Apoptosis and (B) necrosis in SiCtr and siADA2 HUVEC were measured using the RealTime-Glo Annexin V Apoptosis and Necrosis assay (Promega). Prior to the assay, cells were exposed to 24 hours of; EGM2+ FBS 10%, EGM2 + FBS 10% + TNFα 10ng/ml or EGM2 + no FBS.

5.9.2 Proliferation

To assess whether siRNA KD of ADA2 in HUVEC affected the ability of the cells to proliferate, HUVEC were transfected with siCtr or siADA2 in a 25cm2 flask and after 24 hours re-seeded on 96 well plates. After 4 hours in media with FBS, cells were serum starved with 0% FBS for 24 hours to facilitate synchronisation of cell cycles. The cells were then restored with EGM2 + 0, 0.5, 2 or 10% FBS and grown for an additional 24 hours. Cell proliferation was measured by EdU incorporation in a microplate assay. There was increased proliferation with increasing serum concentrations in both siCtr and siADA2 HUVEC (Figure 59A). There were no significant differences in the proliferation between siCtr and siADA2 HUVEC at any serum concentration (Figure 59A).

The effect of TNFα on proliferation in siADA2 HUVEC was assessed using the protocol above but with inclusion of TNF 10ng/ml in restoration media that included 0% and 10% FBS. The addition of TNFα led to a fall in proliferation at both 10% and 0% FBS concentrations (Figure 59). There were no significant differences in the degree of proliferation between siCtr and siADA2 with any of the conditions (Figure 59B).

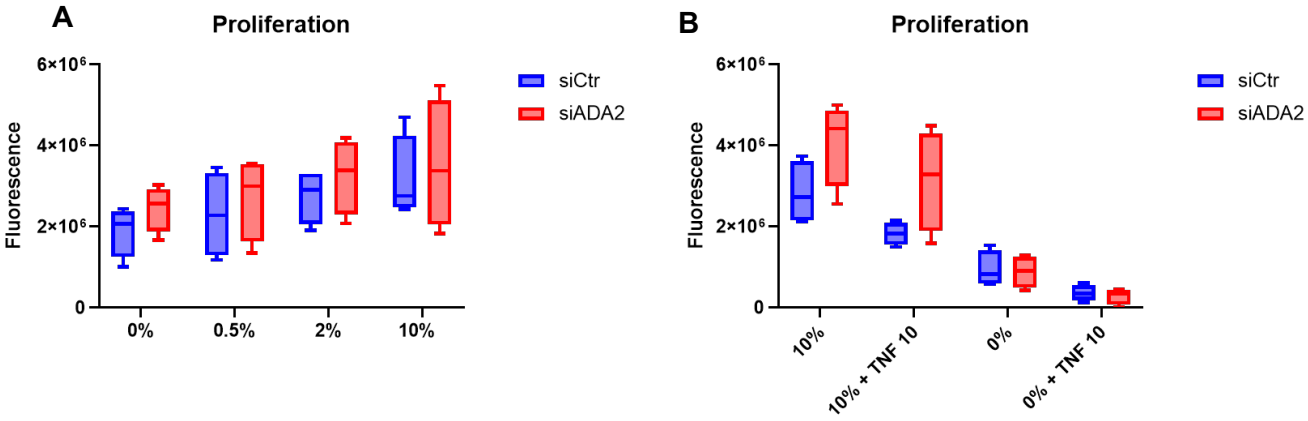


Figure 59 – Proliferation in siCtr and siADA2 HUVEC with different serum concentrations and TNFα treatment

Proliferation in SiCtr and siADA2 HUVEC was measured using the Click-iT EdU proliferation assay (ThermoFisher). Cells were serum starved with 0% FBS for 24 hours and then exposed to (A) EGM2 + 0, 0.5, 2 or 10% FBS and grown for an additional 24 hours prior to the assay or (B) EGM2 + 10% FBS, 10% FBS + TNFα 10ng/ml, 0% FBS, 0% FBS + TNFα 10ng/ml.

5.9.3 Senescence

Senescence was measured in siCtr and siADA2 cells using the described senescence assay. There was a non-significant trend to increased senescence in siADA2 compared to siCtr HUVEC (Figure 60A). Comparison of each HUVEC pool individually demonstrated increased senescence with siADA2 treatment vs siCtr. Figure 60B demonstrates increased SA-B-GAL staining with siADA2 in 2 of these pools.

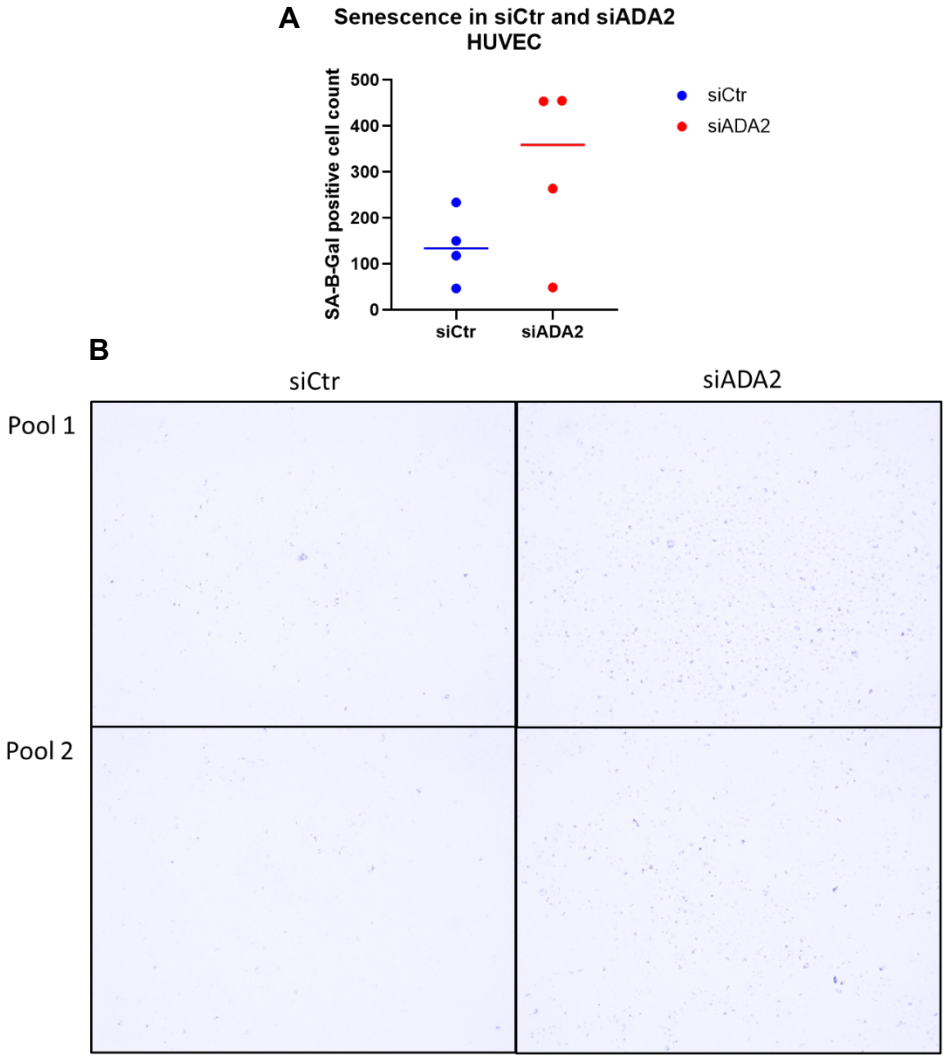


Figure 60 – Senescence in siADA2 HUVEC

Senescence of siCtr and siADA2 HUVEC was measured using a Senescence Detection Kit (BioVision). (A) SA-β-Gal positive cell count was measured by counting the number of blue stained cells. (B) 4X Microscope images of 2 pools of SA-β-Gal stained (Blue) siCtr and siADA2 HUVEC.

5.9.4 Angiogenesis

Angiogenesis was measured with the Matrigel tubule formation assay. siCtr and siADA2 HUVEC were seeded in either 0% or 2% media. Microscope images were taken at 6 and 20 hours and analysed with the image J angiogenesis plugin (Figure 61). The measurements analysed are described in Table 6. There were significantly more nodes formed at 24 hours in siADA2 vs siCtr cells (p=0.033). There were no significant differences between the KD and control cells in any of the other measured parameters (Figure 62).

There were significant increases in the number of nodes, junctions, segments, meshes and mesh area with 2% media vs 0% media in the siCtr cells (p= 0.021, 0.016, 0.003, 0.011, 0.028 respectively). This was not seen with the siADA2 cells.

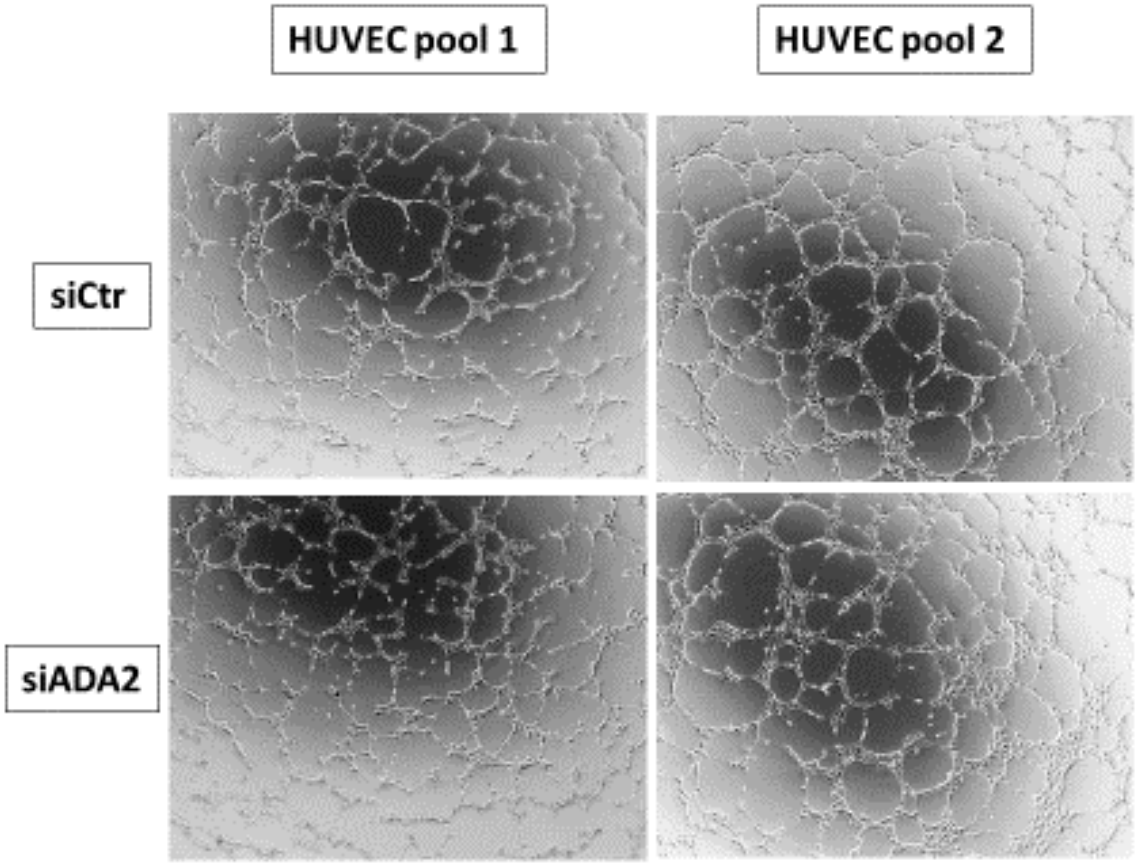


Figure 61 – 4x microscope images of siCtr and siADA2 HUVEC seeded in Matrigel

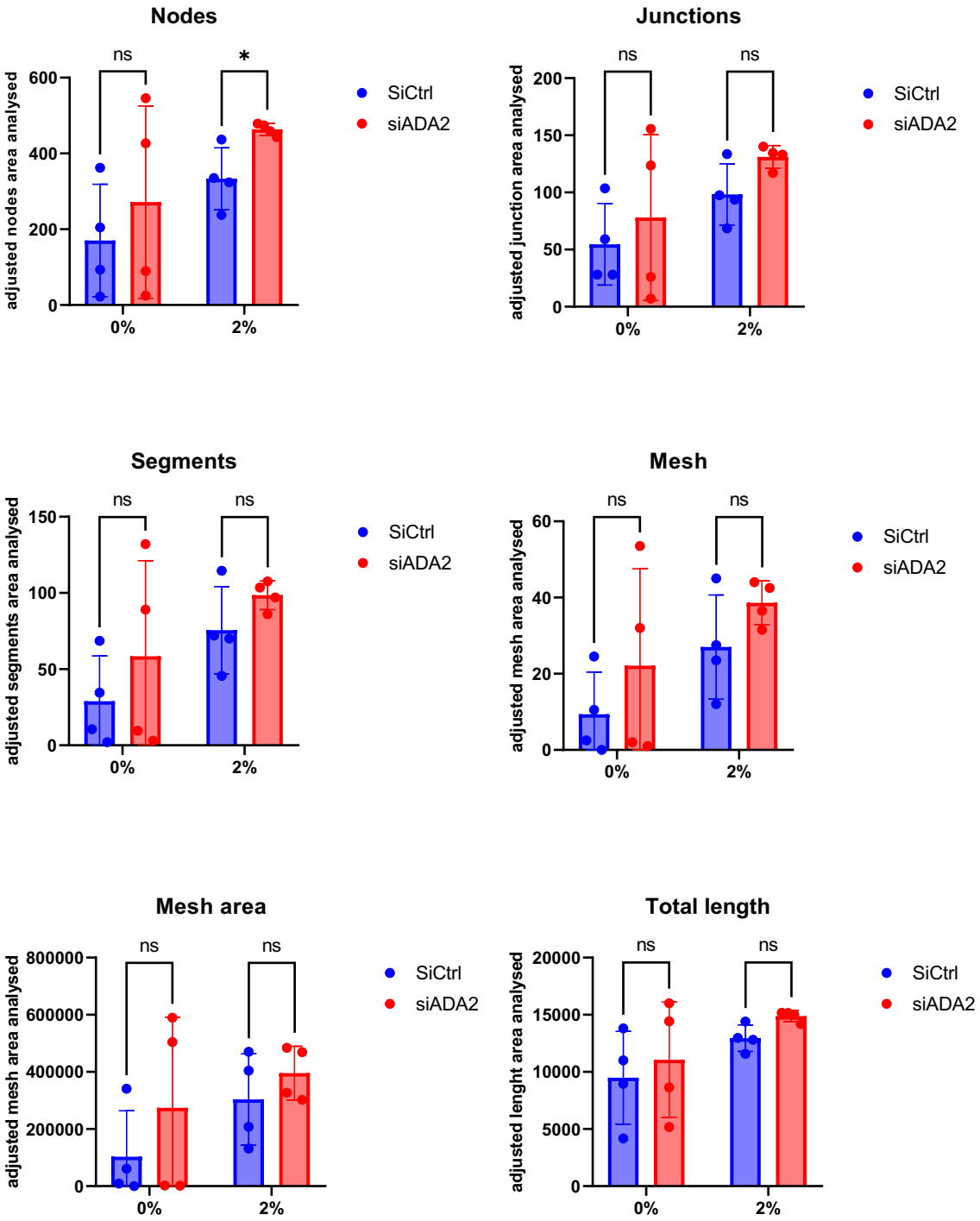


Figure 62 – Angiogenesis of siCtr and siADA2 HUVEC

Senescence of siCtr and siADA2 HUVEC was measured using the Matrigel tubule formation assay. Cells were seeded in EGM2 + 0% or 2% FBS. 4x microscope images were taken at 24 hrs and analysed using image J angiogenesis plugin to measure Nodes, Junctions, Segments, Mesh, Mesh area and Total length. Student's t-test were performed.

5.10 Discussion

In this chapter, transcriptomic and proteomic analyses of ADA2 KD in ECs revealed the dysregulation of 352 genes and 125 proteins. IFNα, IFNγ, TNFα, and apoptotic gene pathways were enriched in the transcriptomic profile whereas the proteomic signature reflected dysregulation of protein translation, peptide processing and non-coding RNA processing. This demonstrates that ADA2 202 has a functional role in EC. There was an overlap of 5 genes/proteins between RNAseq and proteomic analysis with these targets involved in the ubiquitination pathway, RNA binding and protein binding. KD HUVEC were shown to have increased expression of interferon related genes and trends to elevated CXCL10 protein relative to siCtr cells. ADA2 KD in HUVEC also led to the expression of higher levels of the cell adhesion molecule ICAM1 and a trend towards enhanced sensitivity to TNFα stimulation. Surprisingly, despite prominent changes in gene and protein expression, we did not find measurable differences in the levels of apoptosis, necrosis, proliferation or senescence between siADA2 KD and siCtr cells. Assessment of angiogenesis showed that increasing serum concentrations resulted in elevation in the markers of angiogenesis in the siCtr cells but not siADA2. All of these observed differences between siADA2 and siCtr cells indicates that deficiency of ADA2 202 in ECs leads to an inflammatory phenotype. This is in keeping with our hypothesis that ADA2 202 has a role in endothelial homeostasis.

Interestingly, comparison of our HUVEC KD work to transcriptomic profiling of PBMCs from DADA2 patients demonstrates an almost identical gene set enrichment analysis [99]. In both, there was upregulation in genes involved in TNFα signalling via NFκB, IFNα response, IFNγ response and inflammatory response [99]. Chen et al demonstrated that this signature was seen in all of the DADA2 PBMCs independent of the different patient clinical phenotypes; DADA2 vasculitis, DADA2 PRCA, DADA2 asymptomatic [99]. This is of particular interest as despite the PBMCs expressing the full length ADA2 201/203 isoform, its deficiency leads to the same transcriptomic signature as KD of ADA2 202 in HUVEC.

Enrichment of both a type I (IFNα) and type II (IFNγ) interferon response have been demonstrated in our gene analysis of the siADA2 HUVEC. Studies have identified dysregulation in the IFN type I and II axis as possibly having a significant role in the pathogenesis of DADA2. $CD14^+$ monocytes from DADA2 patients analysed using single-cell RNA sequencing showed upregulated immune response pathways, including IFNα, β and γ [95]. Consistent with this they also showed elevated patient plasma levels of IFNγ and TNFα [95]. Similar results were demonstrated in studies which analysed the transcriptome and proteome in whole blood or PBMCs from DADA2 patients [93, 98]. STAT1, a key molecule of

the Janus kinase/signal transducer involved in IFN signalling has also been shown to be activated in DADA2 PBMCs and T-cells [98, 103].

Further studies have shown elevated IFN scores and upregulated type I IFN signalling in DADA2 patients [26, 31, 94, 100, 101, 212]. The authors suggested that elevated IFNγ activates cell signalling and leads to differentiation of monocytes into M1 macrophages resulting in release of TNFα [95]. This IFN signalling was originally thought to be entirely derived from immune cells however our results suggest that IFN signalling may also be present in ECs in DADA2 and therefore could contribute to the immune cells activation and pathogenesis of the disease. This all has important clinical implications, and a greater understanding of the role of IFN in DADA2 may lead to the use of novel therapeutic targets including Janus kinase (JAK) inhibitors that inhibit downstream signalling of IFNs and anti-IFN antibodies e.g. Anifrilomab which binds to the type I IFN receptor [213, 214].

TNFα has also been shown to be an important cytokine in the pathogenesis of DADA2 with patients found to have elevated plasma concentrations relative to healthy controls [62, 99]. Brain and skin biopsies from DADA2 patients have also shown increased staining for TNFα [3]. Consistent with this, our siADA2 KD HUVEC model demonstrated enrichment in genes in the TNFα signalling via NFκB pathway (Table 18). There were also trends to increased CXCL10 protein secretion in siADA2 HUVEC treated with TNFα 10ng/ml vs siCtr HUVEC demonstrating enhanced TNFα sensitivity of these cells (Figure 54). Although the majority of the increased plasma TNFα in DADA2 will be from macrophages, in ADA2 deficient ECs there may be some additional production plus enhanced sensitivity contributing to the pathogenesis. All of these factors may explain why anti-TNFα medication has been shown to be highly effective in treating the vasculitic component of DADA2 [48].

We have demonstrated significant upregulation in the gene expression of the CAM, ICAM1 in siADA2 HUVEC in the absence of any external signalling from immune cell activation (Figure 55B). CAMs such as ICAM1 play a vital role in leucocyte-endothelial cell interactions in vasculitis [215-217]. EC activation results in CAM expression, leucocyte binding and activation followed by transendothelial migration [218, 219]. A number of different factors are known to lead to EC activation and CAM expression including bacterial toxins, cytokines e.g TNFα and oxidants [220-222]. Our work showing that siADA2 KD HUVEC leads to significantly increased ICAM1 gene expression and trends to increased sensitivity to TNFα suggests that a lack of endothelial ADA2 results in EC activation. This suggests that in DADA2, the endothelium may not be simply a target of an activated immune system but that ECs deficient of ADA2 are more susceptible to activation, injury and subsequent vasculitis.

To determine whether the demonstrated gene and protein differences in siADA2 HUVEC led to changes in EC phenotype, a series of functional assays were carried out. There were no significant differences in the level of apoptosis, necrosis, or proliferation between the siCtr and siADA2 HUVEC. There were trends towards increased levels of senescence in siADA2 HUVEC however this was not statistically significant. When assessing angiogenesis, there were no significant differences between siCtr and siADA2 HUVEC at both 0% and 2% serum concentrations. Interestingly, increasing the serum concentration from 0% to 2% led to significant increases in the markers of angiogenesis in the siCtr cells but not in siADA2. Gene ontology analysis of the DEGs had previously suggested a signal in angiogenesis in siADA2 with identification of upregulation in blood vessel morphogenesis, vasculature development and angiogenesis (Figure 51).

The lack of demonstrated phenotypic difference in siADA2 is one limitation of this work. I would postulate that a number of trends were identified however statistical significance was not achieved. One of the main factors for this was variability amongst HUVEC donors. We limited ourselves to n=4 in this project to allow more parameters to be tested. However, we saw that functional phenotypes were variably affected amongst donors. If more time were available, more HUVEC pools might be procured to add biological replicates to areas where trends were observed e.g. Proliferation & Angiogenesis. It's also possible that siRNA based gene silencing is sufficient to generate a temporary transcriptional and proteomic phenotype but that this does not last long enough to manifest as phenotypic change. Future work might consider the use of permanent gene silencing methods such as CRISPR or transfection of a silencing plasmid.

The fact that knockdown of ADA2 in ECs leads to differential expression of a significant number of genes and proteins indicates that ADA2 202 does have a functional role in the endothelium and it is not a redundant transcript. Given we have no evidence of ADA2 enzymatic activity or protein in HUVEC there are two hypotheses regarding its function. Firstly, it acts as a long non-coding RNA or secondly it is present at low levels, below the lower limits of detection of our assays, as an insoluble protein.

LncRNAs are defined as RNAs longer than 200 nucleotides that are not translated into functional proteins [223]. Human GENCODE suggests that there are more than 16,000 lncRNAs in the human genome, however other estimates exceed 100,000 lncRNAs [224-226]. Although some lncRNAs are likely transcription by-products, it is well known that an increasing number of lncRNAs have important cellular functions [223]. There is evidence that they can control gene transcription, are involved in mRNA splicing, turnover, translation and can interact with DNA, proteins and signalling pathways [223, 227, 228]. These diverse functions can affect cell differentiation, growth and can activate or supress the expression of genes encoding

inflammatory molecules [207]. ADA2 202 may act in this way and lead to either the activation or suppression on genes encoding inflammatory molecules. Of note, ontology proteomic analysis of the biological process of the differentially expressed proteins in siADA2 HUVEC demonstrated involvement in non-coding RNA processing. 83.3% of these identified proteins in this pathway were downregulated suggesting that KD of ADA2 202 leads to downregulation of proteins involves in non-coding RNA processing.

The alternative hypothesis is that ADA2 202 is an insoluble protein that we are unable to detect. Approximately 20-30% of human genome proteins are insoluble, most of which are integral membrane proteins but also include those in the nuclear fraction [196, 208]. Interestingly 11 of the 20 proteins that became detectable in the siADA2 proteomic samples are involved in vesicles. In addition of the 5 overlapping results from the RNA-seq and proteomic analysis; 1 of these targets is involved in the ubiquitination pathway (UBA6) and another in protein binding (RPL22L1). It may be possible to speculate therefore that ADA2 202 could well be present in vesicles as an insoluble protein where it is involved in protein binding, trafficking and possibly the ubiquitination pathway. The lack of a significant proportion of its catalytic domain would suggest it has a non-enzymatic function. Further work to understand how ADA2 202 functions would be to carry out an RNA pulldown of ADA2 202 and perform RNAseq to assess what other RNAs it is bound to and mass spectrometry to review the proteins bound.

The identification of significant RPL22L1 downregulation in both the RNAseq and protein analysis is particularly intriguing since this gene is associated with Diamond-Blackfan anaemia, a congenital cause of PRCA, linked to ADA2 deficiency in plasma [211]. It is challenging to understand how loss of the ADA2 202 EC transcript could lead to downregulation of a gene and protein known to be closely linked to one of the haematological manifestations of DADA2. We know that there is widespread ADA2 202 transcript tissue expression so it may have a role here that we have yet to identify.

As previously discussed in Chapter 4, the location of the DADA2 patient's genetic variant will determine whether ADA2 201 alone is affected (AA 1-251) or both ADA2 201 and 202 are affected (AA 252 – 511) (Figure 43). Review of published genotypes demonstrates no significant phenotypic difference between patients who have variants affecting ADA2 201 alone, and hence have preserved ADA2 202, or those that affect both ADA2 201 and 202 [8]. Patients can therefore develop vasculitis whether or not they have dysfunction in ADA2 202. A stronger predictor of phenotype was whether there was complete loss of ADA2 activity, which was associated with haematological manifestations, or low level residual activity which was more associated with vasculitis [8]. Ozen et al assessed the genotype of 14 DADA2

patients with a vasculitis (polyarteritis nodosa – PAN) phenotype and 10 with haematological features (Diamond-Blackfan anaemia – DBA) [35]. They demonstrated that ADA2 activity was similar between the groups however the patients with the vasculitis phenotype had mutations in the dimerisation domain, thus affecting ADA2 201 only, versus the patients with the haematological phenotype had mutations in the catalytic domain which would affect ADA2 201 and 202 [35]. All of this would suggest that dysfunctional ECs due to deficiency of ADA2 202 is not the sole pathological driver in DADA2 but may well be contributory. Further work to assess whether severity of vasculitis presentation or response to treatment in those with and without variants affecting ADA2 202 would provide further understanding.

In this chapter some surprising results were that in the RNAseq there were fewer differentially expressed genes in siADA1 relative to siADA2 (53 vs 352 respectively) despite the fact that ADA1 is known to be a key metabolic enzyme [138]. This may relate to the fact that ADA1 is very efficacious; it has a >100 fold higher affinity for adenosine and 2'-deoxyadenosine than ADA2 [6]. Severe combined immune deficiency (SCID), the clinical phenotype of ADA1 deficiency, develops when there is <1% ADA1 activity [229]. This may mean that a KD efficiency of 81% is not enough to produce a phenotype and the residual ADA1 that remains despite KD is sufficient to maintain its function.

In addition, differences between the individual HUVEC pools were noted, with siADA2 KD in some pools leading to a much greater inflammatory gene signature and sensitivity to TNFα. 3 separate pools of HUVEC were used for the RNAseq experimental work to try and account for this. Comparison of our siADA2 HUVEC RNAseq work to previously published data demonstrated reasonable agreement; the Log2 fold change parameter between both datasets showed a Pearson R of 0.49 [88]. Some of the observed differences between the 2 datasets may be explained by the fact that only a single HUVEC pool replicated 4 times was used by Dhanwani et al in comparison to 3 pools used in our analyses [88].

In conclusion, these findings suggest that ADA2 202 has a role in endothelial homeostasis and its deficiency leads to EC activation and an inflammatory signature. There are still unanswered questions regarding whether it acts as a lncRNA or an insoluble protein with non-enzymatic functions and further work is needed to address this. It is currently not clear regarding the exact contribution of ADA2 202 to the pathogenesis of DADA2. The use of endothelial samples from DADA2 patients in the form of ECFCs may help to understand this further.

CHAPTER 6: Phenotype of Endothelial Colony forming cells from DADA2 patients

6.1 Introduction

Due to the lack of expression of ADA2 by mice and other rodents, which have no orthologue of the human gene, there are limited animal models of DADA2 [230]. The zebrafish model of DADA2 did demonstrate a similar phenotype with intracranial haemorrhage and endothelial disruption however the pathogenesis of how this occurs remains elusive [3]. A significant body of work has been carried out to assess leukocyte biology from DADA2 patients including monocytes, macrophages and neutrophils [3, 90, 101]. There has however been no experimental work on cultured endothelial cells from DADA2 patients and their role in disease pathogenesis.

Endothelial colony forming cells (ECFC) derived from DADA2 patients may be able to provide new insights with this. These are cells with a true endothelial phenotype that can be isolated and cultured from peripheral blood mononuclear cells (PBMC) using endothelial growth media [113]. They express endothelial and not myeloid cell surface markers, have high proliferative capacity and promote vascular formation *in vitro* and *in vivo* [113, 115-117]. The precise origin of these cells is not clear however both a bone marrow and tissue vascular niches have been suggested [114, 119]. ECFC have been used to assess endothelial molecular dysfunction in several diseases including diabetes, venous thromboembolism and pulmonary hypertension [128-130]. In addition, dysfunction in ECFC has been shown in patients with ANCA vasculitis where they have impaired ability to differentiate, proliferate and migrate [131, 132]. Although they cannot completely model the complex systemic inflammatory environment present in a DADA2, ECFC can provide insight into phenotypic conditioning due to disease states and enable the investigation of the impact of specific genotypes on endothelial phenotypes.

Work in the previous chapters of this thesis was directed towards determining ADA2 expression in EC and understanding the role of ADA2 202 in endothelial homeostasis. As ECFC can be isolated from patients, these cells allow a novel and patient derived model to validate our previous results (Chapter 5). This will include whether these ECs from DADA2 patients express ADA2 202, both protein and mRNA, and assess whether they have any evidence of ADA2 activity. It will allow us to determine whether they have the transcriptomic signature see in siADA2 HUVEC with enrichment in IFNα, IFNγ, TNFα and inflammatory pathways as well as their functional phenotype. These cells will of course have experienced the inflammatory environment of ADA2 deficient monocytes, macrophages and other immune cells which may have led to some level of imprinting and alteration in gene expression and

function. Therefore, there may be an extrinsic effect on ECs of ADA2 201 deficient myeloid cells as well as, depending on the patient's genotype, an intrinsic effect of loss of endothelial ADA2. This chapter will therefore aim to understand the phenotype of patient derived ECs and their potential role in the pathogenesis of DADA2.

6.2 Hypothesis of chapter

Patient derived ECFC will express ADA2 202 RNA as the predominant isoform. Consistent with previous results there will be no detectable ADA2 202 protein or enzymatic activity. ECFCs from DADA2 patients will have a pro-inflammatory profile comparable to that seen in siADA2 HUVEC

6.3 Aims of the chapter

1) Validate expression of ADA2 202 RNA protein and enzymatic activity in patient derived ECFC
2) Assess whether ADA2 202 is the predominant expressed isoform in ECFC
3) Compare gene signature and protein expression in DADA2 ECFC to siADA2 HUVEC
4) Assess the functional phenotype of DADA2 ECFCs

6.4 DADA2 patient demographics

Blood samples were collected from 4 patients with genotype confirmed DADA2 for plasma, PBMC and ECFC isolation as per methods. Patient demographics are summarised in Table 20. Median age was 27.5 years (24-39) with 2 males and 2 females. 3 of the 4 patients were currently receiving treatment with Adalimumab (anti-TNF biologic). All 4 patients had undetectable ADA2 enzymatic activity in the plasma. 1 of the 4 patients had a genotype that would affect the ADA2 202 isoform in addition to the 201 isoform. The other 3 patients had variants affecting only ADA2 201 (Table 20). Four age and sex matched healthy control ECFCs donors were used for comparative analysis.

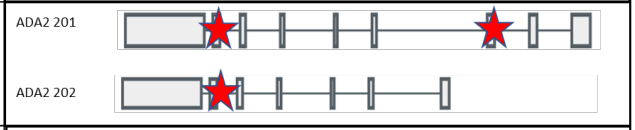
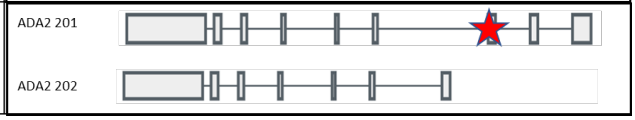
Patient	Age	Gender	Current treatment	ADA2 activity in plasma	Genotype	202 affecting variant	Exon map demonstrating location of variant
1	24	Male	Adalimumab	0	ADA2 homozygous p.P251L	No	
2	39	Female	Adalimumab	0	c.634_636del TTC (p.Phe212del) and c.1225C>T (p.Pro409Ser)	Yes	
3	26	Female	IVIG + Prednisolone	0	ADA2 homozygous p.G47W	No	
4	29	Male	Adalimumab	0	ADA2 homozygous p.P251L	No	

Table 20 - DADA2 patient demographics, genotype and treatment received

6.5 DADA2 patient ECFCs

ECFC were cultured and propagated from all 4 DADA2 patients. Colony generation data was recorded in the 4 DADA2 ECFC samples along with the 42 HC ECFC samples that have been generated by the group (Figure 63 A-D). DADA2 ECFC generation parameters were highly comparable to the HC group. The median number of colonies generated prior to the first passage was 3.5 colonies in both DADA2 and HC ECFC (range 2 - 6 vs 0 – 37 colonies) (Figure 63A). When normalising this to the number of PBMC that were seeded this was 0.4 vs 0.6 Colonies / 10^7 PBMC in DADA2 vs HC ECFC (range 0.2670 – 1.79 vs 0 – 4.85) (Figure 63B). The median number of days to first split was 32.5 vs 29 in the DADA2 vs HC ECFC

(range 31 – 33 vs 16 – 43) (Figure 63C). The median day of first colony appearance was 14.5 in DADA2 vs 10 in the HC ECFC (range 10 – 20 vs 7 – 24) (Figure 63D).

Microscope images show that despite the same number of cells being seeded, DADA2 ECFC at passage 3 are less numerous relative to HCs at day 5 (Figure 64). They also appear more elongated consistent with a more stressed phenotype.

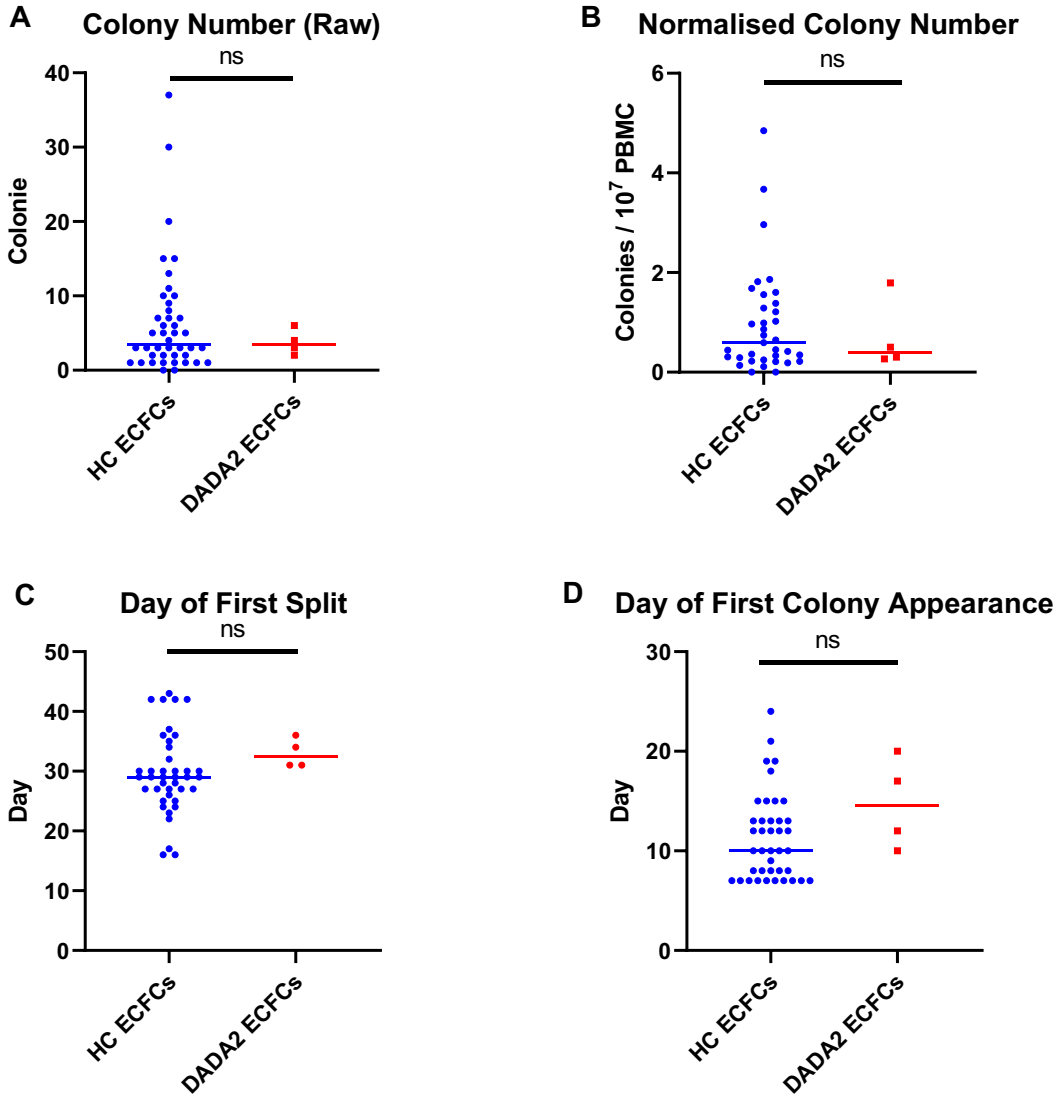


Figure 63 – There was no significant differences in markers of colony generation in DADA2 and HC ECFCs

DADA2 ECFCs were cultured and propagated. The number of colonies, normalised colony number (to total PBMCs seeded), day of first split and day of first colony appearance were recorded and compared with 42 HC ECFC samples that had been generated by the group. Median value shown. Mann-Whitney tests were performed.

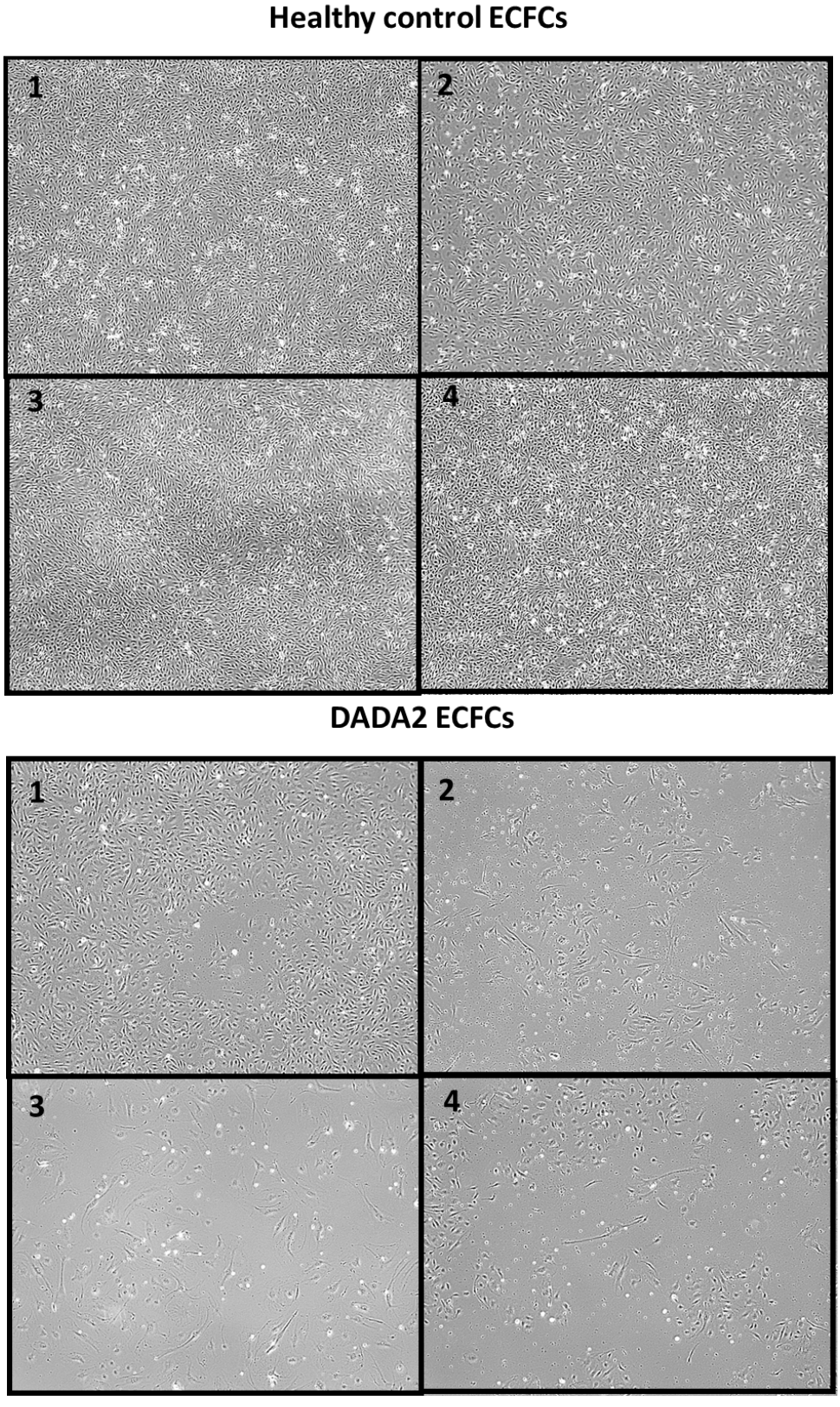


Figure 64 – 4x microscope images of healthy control and DADA2 ECFC

0.25x10^6 HC and DADA2 ECFC were seeded in a 25cm^2 flask as per methods. X4 microscope images Day 5 post seeding were taken.

6.6 ADA2 expression in DADA2 and healthy control ECFC

6.6.1 ADA2 isoform expression in DADA2 ECFC

RNA was extracted and purified from 4 HC and 4 DADA2 ECFC at passage 3. qPCR analysis of all 8 ECFC RNA samples was performed to quantify the expression of ADA2 201 and ADA2 202 isoforms. Figure 65A demonstrates that ADA2 202 was the predominant isoform expressed in ECFC, in both DADA2 and HCs. Every sample had a greater expression level of ADA2 202 vs 201, including those with low ADA2 202 expression and on average there was 30.5x more ADA2 202 vs 201 (range 3.9 – 78.6) (Figure 65A). On assessment of the 2 groups separately, there was significantly higher expression of ADA2 202 (p=0.022) in DADA2 ECFCs vs HCs but no difference in ADA2 201 (Figure 65B).

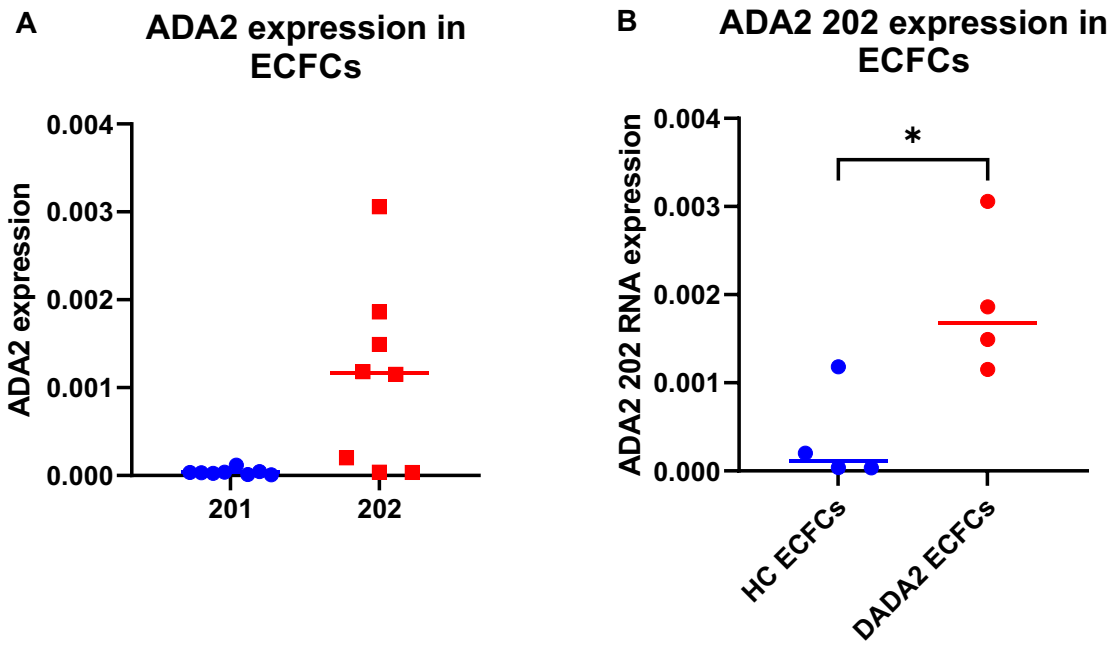


Figure 65 – ADA2 202 was the predominant isoform expressed in ECFC

RNA was harvested and purified from HC and DADA2 ECFC. ADA2 201 and 202 expression was measured using qRT-PCR. (A) ADA2 202 was the predominant isoform expression in both DADA2 and HC ECFC. (B) There was significantly elevated ADA2 202 RNA expression in DADA2 ECFC vs HC (p=0.022). Mann-Whitney tests were performed.

Immunoblot of both cell lysate and supernatant from HC and DADA2 ECFC showed no evidence of ADA2 protein (Figure 66A). In addition, there was no detectable ADA2 enzymatic activity in cell lysate or concentrated supernatant from HC or DADA2 ECFC (Figure 66B). ADA1 activity was present in these samples (Figure 66B)

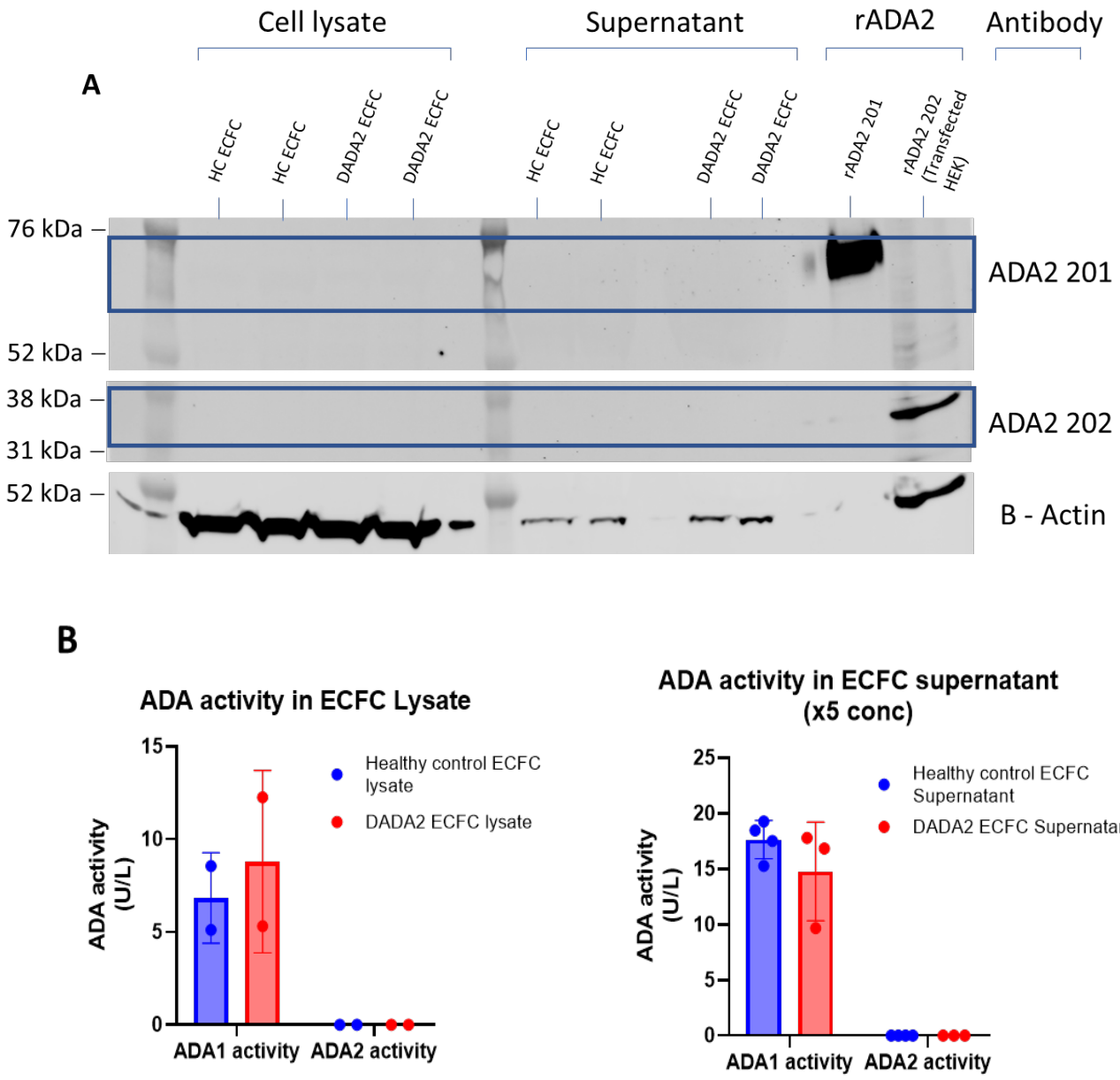
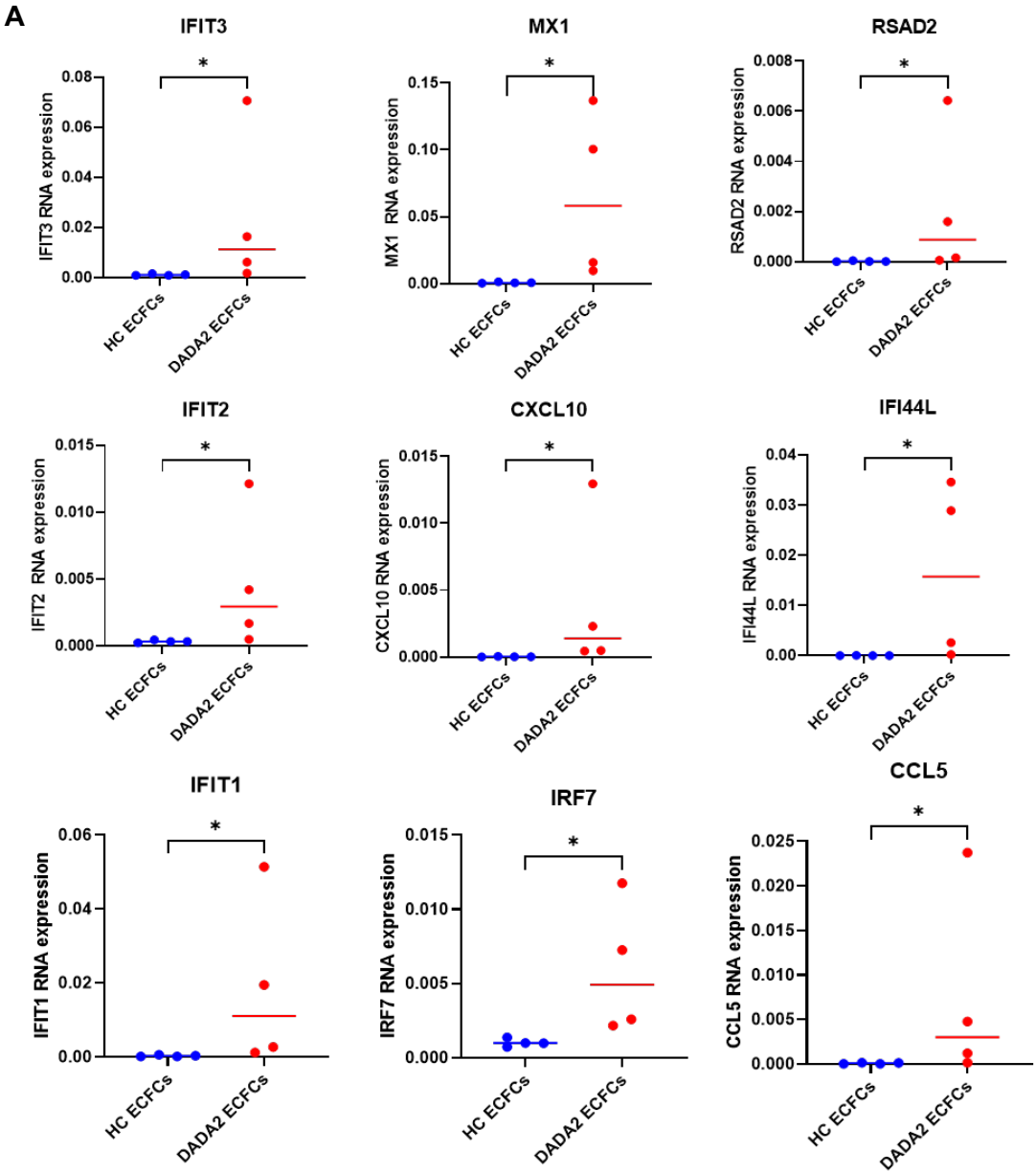


Figure 66 – There was no evidence of ADA2 protein or enzymatic activity in HC or DADA2 ECFC lysate or supernatant.

HC and DADA2 ECFC (passage 3) were cultured until confluent in a 6 well dish. Protein was harvested after using RIPA lysis buffer and supernatant was collected. (A) These lysates and supernatant were separated by SDS-PAGE and immunoblot analysis carried out. Proteins were detected with antibodies against ADA2 (both 201 and 202 isoforms) and B-Actin. (B) ADA1 and ADA2 activity were measured in these lysates and x5 concentrated supernatants.

6.7 Interferon expression in DADA2 and HC ECFCs

To assess whether DADA2 ECFC had the same IFN signature that was demonstrated in the siADA2 KD HUVEC, qPCR of RNA from ECFC was performed on a number of IFN related genes (Figure 67A). These genes were chosen based on IFN related genes from ADA2 KD in HUVEC (both our own data and previously published from Dhanwani et al), genes selectively regulated by IFN in endothelial cells and IFN genes used to calculate interferon scores in other autoimmune diseases [88, 231, 232].There was significantly elevated IFIT3, MX1, RSAD2, IFIT2, CXCL10, IFI44L, IFIT1, IRF7, CCL5 in DADA2 vs HC ECFCs (p=0.029) (Figure 67A). Figure 67B shows these results as Log2fold change vs HC results and again demonstrated elevated IFN related gene expression in the DADA2 ECFCs.



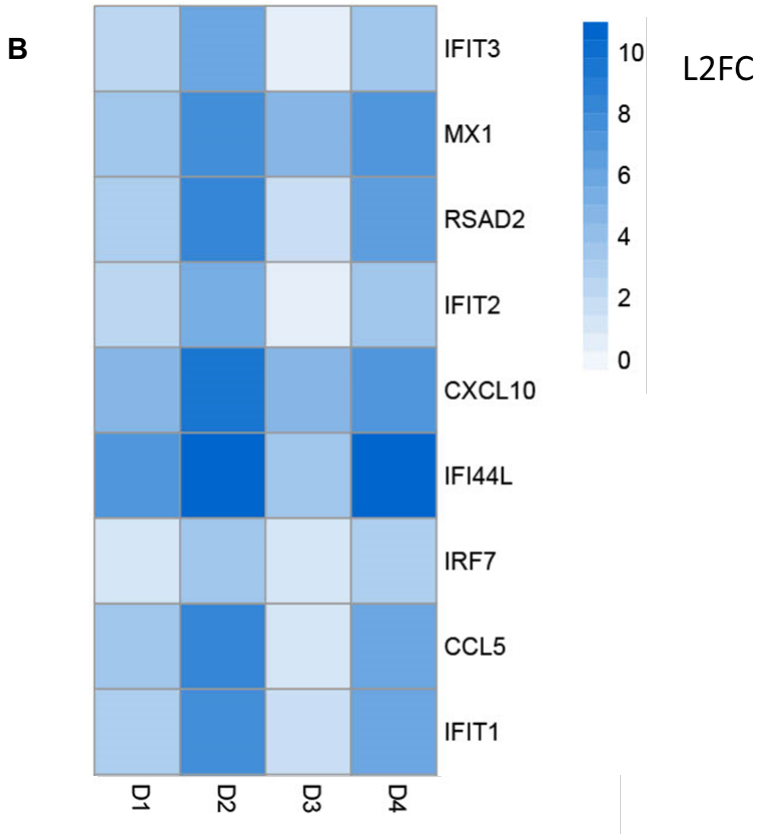


Figure 67 – Interferon related gene expression is elevated in DADA2 vs HC ECFC

RNA was harvested and purified from HC and DADA2 ECFC. (A) IFN related gene expression was measured using qRT-PCR. (B) Log2fold change of DADA2 ECFC IFN related gene expression vs HC was calculated. Mann-Whitney tests were performed.

6.7.1 Secreted CXCL10 protein in IFNγ treated HC and DADA2 ECFCs

Given the IFN signature detected in the siADA2 KD model in the previous chapter, HC and DADA2 ECFC were seeded at a concentration of $5x10^4$ in a 12 well plate and treated for 24 hours with IFNγ 1 U/L or 10 U/L. Supernatant was collected, and an ELISA used to measure the concentration of secreted CXCL10. In 2 of the DADA2 samples there was significant levels of cell death and no CXCL10 signal was detected in the supernatant. There were trends to increased levels of CXCL10 protein in the supernatant of DADA2 vs HC ECFCs however no statistical tests were possible due to the small number of samples (Figure 68).

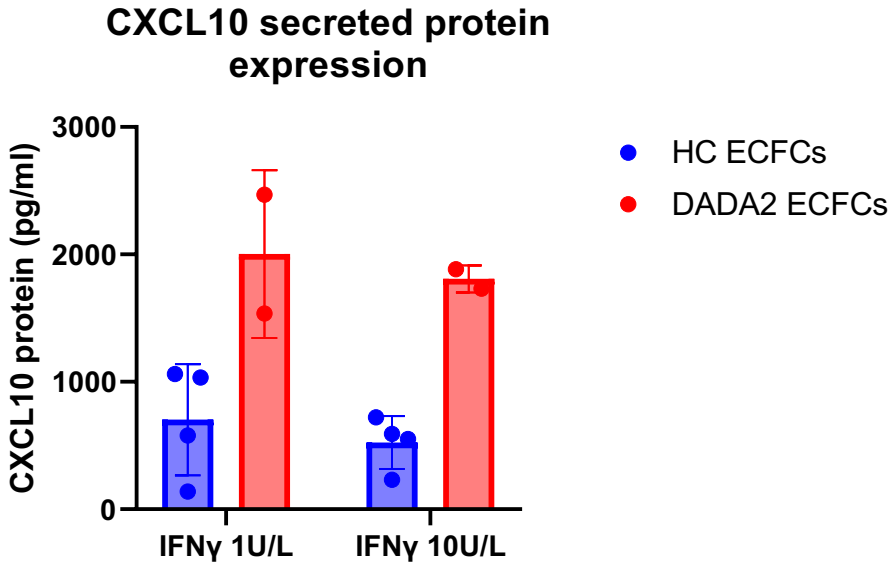


Figure 68 – Secreted CXCL10 protein expression in IFNγ treated HC and DADA2 ECFC
HC and DADA2 ECFC were treated with IFNγ at a concentration of 1 U/L or 10 U/L for 24 hours. Supernatant was collected and an ELISA used to measure concentrations of CXCL10 protein.

6.8 Cell adhesion molecule expression in DADA2 and HC ECFCs

Given the elevated CAM response seen in HUVEC siADA2, qRT-PCR was used to quantify ICAM1, VCAM and E-Selectin RNA expression in HC and DADA2 ECFC. Significantly higher expression levels of all 3 measured CAMs were demonstrated in the DADA2 vs the HC ECFC (p=0.029) (Figure 69).

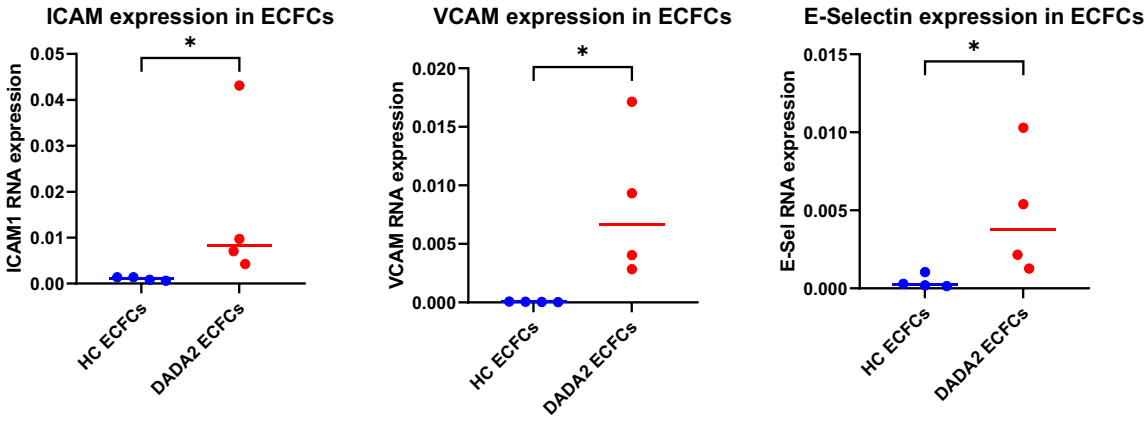


Figure 69 – Significantly higher expression levels of ICAM1, VCAM and E-Selectin were demonstrated in DADA2 vs HC ECFC.

RNA was harvested and purified from HC and DADA2 ECFC. ICAM1, VCAM and E-Selectin gene expression was measured using qRT-PCR. Mann-Whitney tests were performed.

6.9 Functional phenotype of DADA2 ECFCs

6.9.1 Apoptosis and necrosis in DADA2 ECFCs

Levels of apoptosis and necrosis in ECFC were determined using the previous described assay. Cells were treated with 4 conditions; EGM2 + FBS 10%, EGM2 + FBS 10% + TNFα 10ng/ml, EGM2 + no FBS or EGM2 + no FBS + TNFα 10ng/ml. This was to assess whether inducing stress with either serum starvation or TNFα led to a greater degree of apoptosis or necrosis in the DADA2 ECFC. There were no significant differences in the degree of apoptosis or necrosis between DADA2 and HC ECFC in any of the conditions (Figure 70).

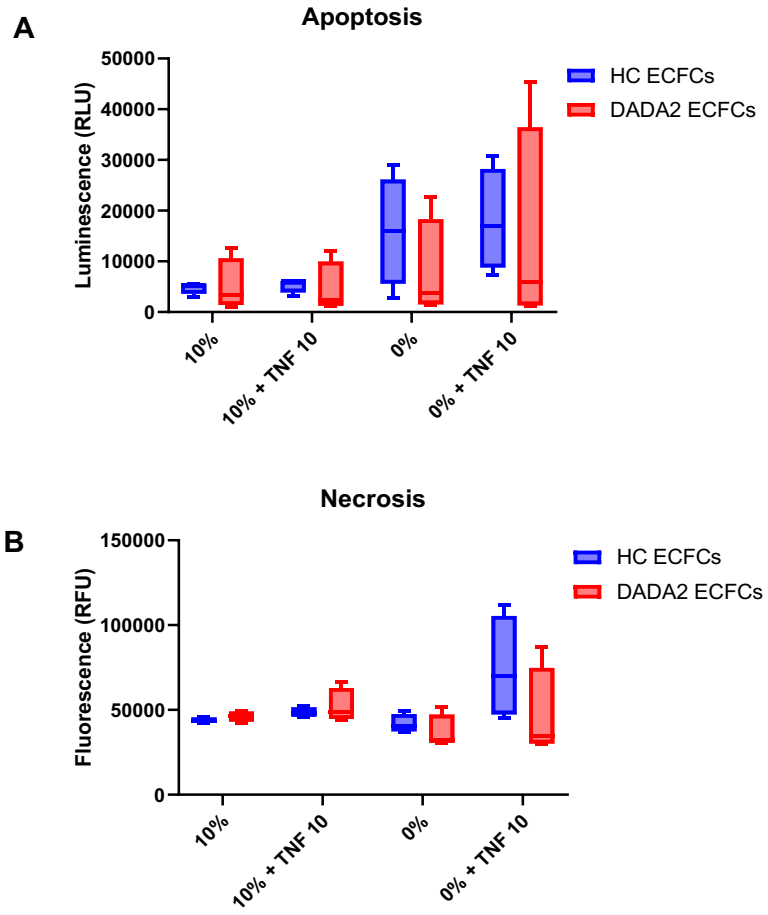


Figure 70 – There was no significant difference in apoptosis or necrosis in HC vs DADA2 ECFC

(A) Apoptosis and (B) necrosis in HC and DADA2 ECFC were measured using the RealTime-Glo Annexin V Apoptosis and Necrosis assay (Promega). Prior to the assay, cells were exposed to 24 hours of; EGM2+ FBS 10%, EGM2 + FBS 10% + TNFα 10ng/ml, EGM2 + no FBS or EGM2 + no FBS + TNFα 10ng/ml.

6.9.2 Proliferation in DADA2 ECFCs

Proliferation was measured in ECFC using the previously described EDU incorporation assay. Given the fact that anti-TNF treatment is very effective in the vasculitic component of DADA2 plus the TNFα gene signature that was demonstrated in siADA2 KD HUVEC, the effect TNFα 10ng/ml on proliferation of these ECFC was also assessed. There were trends to lower proliferation in the DADA2 ECFC vs HC with a serum concentration of 10% (Figure 71A). There was however a significantly lower degree of proliferation in the DADA2 vs HC ECFC with the addition of TNFα (p=0.045). Serum starvation led to a significant fall in proliferation with no difference between the groups.

Further assessment of proliferation was carried out through seeding $0.25x10^6$ stocks of ECFC in a $25cm^2$ flask as per methods. Cells were harvested after 4 days of culture prior to seeding for experimental analysis and counted. There was a significantly lower total cell count at harvesting in the DADA2 ECFC relative to HCs (median cell count; DADA2 $0.3x10^6$ vs HC $2.3x10^6$, p= 0.029) (Figure 71B).

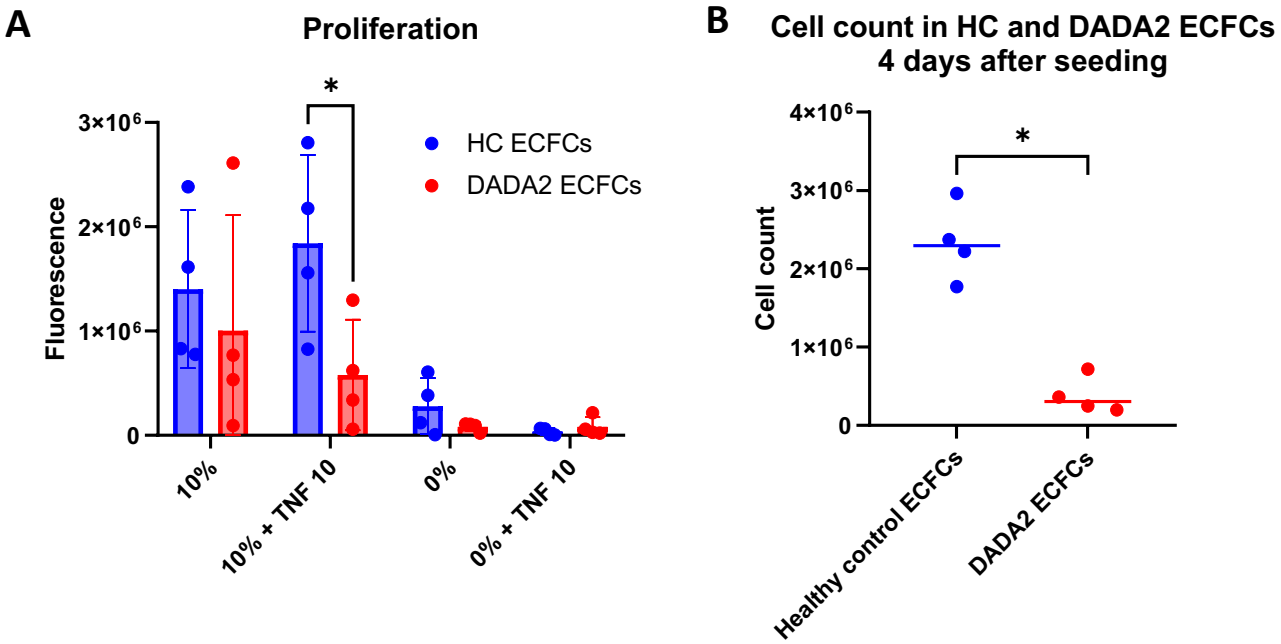


Figure 71 – Proliferation in DADA2 ECFC

(A) Proliferation in HC and DADA2 ECFC was measured using the Click-iT EdU proliferation assay. Cells were serum starved with 0% FBS for 24 hours and then exposed to 24 hours of; EGM2+ FBS 10%, EGM2 + FBS 10% + TNFα 10ng/ml, EGM2 + no FBS or EGM2 + no FBS + TNFα 10ng/ml. (B) $0.25x10^6$ stocks of ECFCs were seeded in a $25cm^2$ flask. Cells were harvested after 4 days and counted. Mann-Whitney tests were performed.

6.9.3 Senescence in DADA2 ECFC

Senescence was measured in HC and DADA2 ECFCs using the described senescence assay. There was a non-significant trend to increased senescence in the DADA2 compared to HC ECFC, with higher SA-β-GAL cell counts in 3 of the 4 DADA2 patients vs HC (Figure 72A). Figure 72B demonstrates SA-B-GAL staining in 2 HC and 2 DADA2 ECFC samples.

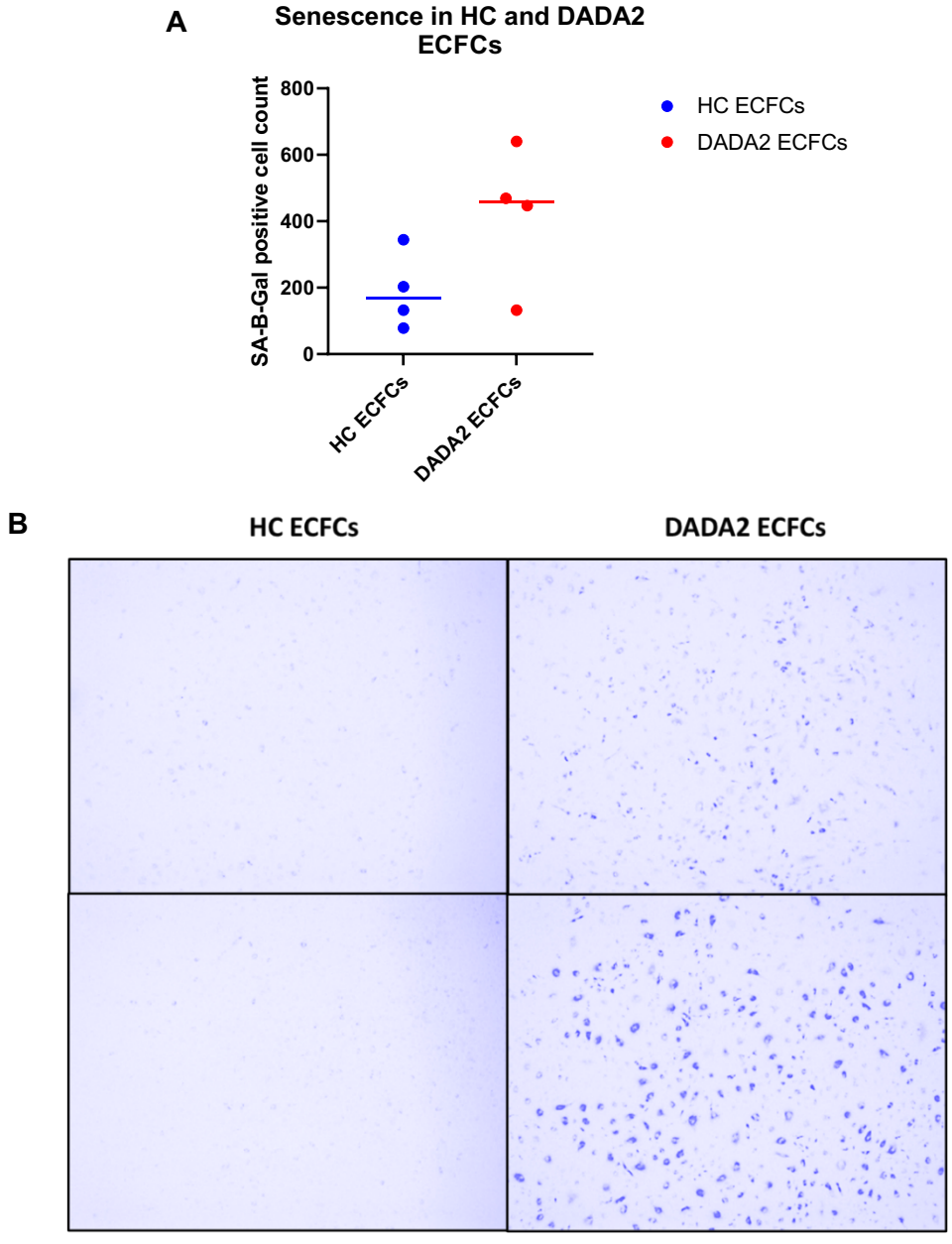


Figure 72 – Senescence in DADA2 ECFC

Senescence of HC and DADA2 ECFC was measured using a Senescence Detection Kit (BioVision). (A) SA-β-Gal positive cell count was measured by counting the number of blue stained cells. (B) 4X Microscope images of SA-β-Gal staining (Blue) in 2 HC and 2 DADA2 ECFC. Mann-Whitney tests were performed.

6.10 Discussion

The goal of this chapter was to determine whether patient derived ECFCs express ADA2 and assess whether DADA2 patient ECFC have a similar inflammatory gene signature, protein expression and functional phenotype to siADA2 HUVEC. It must be noted however that the DADA2 ECFC represent a different disease model compared to the siADA2 HUVEC. Only 1 DADA2 patient in this study has a genotype that would be predicted to affect ADA2 202, the rest would have preserved EC ADA2 202. This therefore mainly represents a model of the effect of extrinsic ADA2 immune cell deficiency on EC versus the effect of loss of intrinsic ADA2 202 loss in the siADA2 HUVEC.

The data presented in this chapter confirm that ADA2 202 RNA is the predominant ADA2 isoform expressed in ECFC. As seen in other EC cell types, no evidence of ADA2 202 protein or ADA2 enzymatic activity was detectable in either the lysate of supernatant of these cells. Interestingly, ADA2 202 RNA expression was higher in the DADA2 compared to the HC ECFC. DADA2 ECFC were consistently observed to have an elongated “stressed” morphology in all 4 donors. A similar interferon gene signature and elevated CAM RNA expression was seen in DADA2 ECFC that was demonstrated in siADA2 HUVEC. DADA2 ECFC also had evidence of reduced proliferation and increased senescence relative to HC. This work provides novel insights as no previous studies have assessed cultured endothelial cells from DADA2 patients. It answers the important question regarding whether ADA2 is expressed by the endothelium, as well as suggesting that DADA2 ECFC have evidence of endothelial dysfunction with upregulation of IFN related genes and cell adhesion molecules.

A common question arising in the DADA2 literature is whether ADA2 is expressed by the endothelium with some groups demonstrating expression and others not [2, 3, 88]. In Chapter 4 we confirmed ADA2 RNA expression in HUVEC in the form of the ADA2 202 isoform. The results from this chapter confirm the expression of ADA2 202 RNA isoform in patient derived ECFC but again without detectable protein or enzymatic activity. There was some detectable ADA2 201 RNA in the samples, albeit 30.5x less than ADA2 202 expression. This most likely represents some ADA2 201 RNA from residual PBMCs that remained in the sample during the ECFC culture and propagation process. There was no detectable ADA2 201 protein or enzymatic activity which would be expected if ADA2 201 RNA was present in sufficient quantities.

ADA2 202 RNA expression was detected in both HC and DADA2 ECFC. This is interesting as depending on the genotype one would have hypothesised that there would be deficiency of the ADA2 RNA. On detailed assessment of the 4 DADA2 patient genotypes (Table 20), 3

patients had a variant that would only affect the ADA2 201 isoform (p.G47W, p.P251L – 2 patients). These are both missense mutations that are characterised in the literature to lead to the vasculitis phenotype and deficient ADA2 activity when plasmids with these sequences are transfected into HEK 293T cells [7, 8, 233]. p.G47W affects the dimerisation domain of the protein and p.P251L the catalytic domain [8]. The fourth patient had novel compound heterozygous variants, one of which is a deletion affecting catalytic domain of ADA2 201 and the other affecting the catalytic domain of both ADA2 201 and 202 (c.634_636delTTC (p.Phe212del) and c.1225C>T (p.Pro409Ser)) [234]. The patient had a clinical phenotype of vasculitis and Diamond-Blackfan anaemia and had deficient ADA2 activity in her plasma. These genotypes are important as only one of the patients would have a theoretical intrinsic deficiency of ADA2 202 in EC as well as extrinsic effect of ADA2 201 deficiency in myeloid cells (Figure 24). The other 3 patients would theoretically have preserved intrinsic EC ADA2 202 but loss of extrinsic ADA2 201 in myeloid cells.

The fact that ADA2 202 RNA expression was significantly higher in the DADA2 vs HC ECFC is very interesting. One might speculate that given the previous work demonstrating that silencing of ADA2 results in upregulation of inflammatory genes, this increased generation of ADA2 202 RNA in the DADA2 ECFC could be a compensatory mechanism to reduce inflammation in the activated ECFC. The lack of detected ADA2 202 protein or enzymatic activity is consistent with our results from other EC types. This again raises the question as to whether ADA2 202 RNA may function as a lncRNA, or whether it is present as a protein at low undetectable levels in the cell, possibly in the insoluble fraction.

DADA2 ECFC demonstrated significantly upregulated IFN related genes relative to HCs. This is consistent with the enrichment of both a type I (IFNα) and type II (IFNγ) interferon response demonstrated in our siADA2 HUVEC work and the previously discussed studies that show upregulation in IFN in DADA2 monocytes, PBMCs, whole blood and plasma [26, 88, 93-95, 98, 100, 101]. To my knowledge, this is the first time that the IFN signature has been demonstrated in endothelial cells from DADA2 patients and although this a different model of disease, these results are also consistent with the IFN signature demonstrated in siADA2 HUVEC (Chapter 5). As previously discussed, only 1 of the DADA2 patients in this study had a genotype that would be predicted to affect ADA2 202. It is likely therefore that exposure to ADA2 201 deficient immune cells in the body has led to ECFC dysfunction and contributed to the IFN response seen. Of interest, the DADA2 patient with the ADA2 202 affecting genotype consistently had the highest IFN response out of all the patients. This of course is only a single sample however it would be plausible to speculate that the upregulation of IFN may be multifactorial and involve both EC and immune cells. Further work to collect additional patient ECFC samples to assess any transcriptomic or phenotypic difference between patients with

variants affecting solely ADA2 201 vs those with ADA2 201 and 202 affecting variants would shed further light on this. This is of key importance in further understanding the pathogenesis of the disease and for the development of IFN targeted therapeutics for patients.

Consistent with the previous results demonstrating upregulation in the gene expression of ICAM1 in siADA2 HUVEC, we have shown a similar CAM response in DADA2 ECFC with significant upregulation in the expression of ICAM1, VCAM and E-selectin RNA relative to HC ECFC. This is a novel finding in cultured DADA2 patient endothelial cells and is consistent with evidence of E-selectin staining in both brain and skin biopsy specimens from patients with DADA2 [3]. As previously stated, CAMs play a vital role in leucocyte-endothelial cell interactions in vasculitis [215-217]. It is therefore possible to postulate that deficiency of intrinsic functional ADA2 202 in DADA2 EC leads to increased CAM expression and therefore susceptibility to leucocyte activation, injury and vasculitis. As previously discussed regarding the different genotypes, this is likely to be multifactorial and involve both intrinsic ADA2 202 EC loss and extrinsic loss of ADA2 201 in myeloid cells. Understanding of this further would again be improved with a comparison of CAM response in patients with ADA2 201 vs ADA2 201 and 202 affecting variants.

Another important question addressed in this chapter was whether there were any functional differences between HC and DADA2 ECFC. Assays were used to measure apoptosis, necrosis, proliferation and senescence. As in the siADA2 HUVEC model there were no significant differences in apoptosis or necrosis between the groups. Although there were no differences in the degree of proliferation, measured by EdU incorporation, between the groups with standard media, there was a significant reduction in the degree of proliferation in DADA2 ECFC with the addition of TNFα. This may suggest that DADA2 ECFC are more sensitive to TNFα, a cytokine well characterised in the pathogenesis of the DADA2 which when inhibited with anti-TNF medication is an effective treatment for the condition [48, 62, 99]. There was no difference in ECFC colony number, day to first split and day of first colony appearance between the groups however there was evidence of impaired proliferation in that there was significantly reduced cell counts in the DADA2 ECFC after seeding equivalent numbers of cells. This is comparable with ECFC cultured from patients with ANCA vasculitis which have been shown to have impaired proliferation and migration relative to HCs [131-133]. Given one of the roles of ECFC is promotion of vascular formation and repair, impaired ability to proliferate and carry out this role in vasculitis where there is endothelial and vascular injury could contribute to the disease pathogenesis [113, 115, 116].

Of particular interest was the demonstration that DADA2 ECFC had trends to increased levels of senescence relative to HCs. Senescence is a state of cell cycle arrest which occurs due to

a number of different activators and aims to prevent uncontrolled proliferation of damaged cells [235]. Senescent endothelial cells are refractory to changes in response to shear stress, secrete inflammatory molecules and contribute to vascular dysfunction [236, 237]. Increased senescence has been demonstrated in several other vascular diseases including atherosclerosis and heart failure [238, 239]. A link has also previously been shown between the upregulation of IFN signalling and endothelial senescence [240]. It is possible to suggest that increased senescence in DADA2 ECFC through the above mechanisms may contribute to the vasculitis disease pathogenesis. Consistent with these results, unpublished data presented at the DADA2 conference also demonstrated increased senescence in mesenchymal stomal cells in DADA2 patients.

All 4 of the DADA2 patients who were sampled for ECFC in this work were in clinical remission on immunosuppression and had no clinical evidence of active vasculitis or inflammation. The work in this chapter however has demonstrated that despite this there is evidence of ongoing endothelial dysfunction. This is of particular importance as this may lead to increased long term cardiovascular risk in this cohort of young patients. Increased cardiovascular risk is also seen in other autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis where dysfunction in ECFC is also detectable [241, 242]. Given this disease was only first described 10 years ago, long term follow-up for these patients including cardiovascular outcomes is not yet known [2, 3]. This work raises important considerations in terms of whether more aggressive control of conventional cardiovascular risk factors, including glycaemic, blood pressure and lipid control, should be considered alongside immunosuppressive treatment.

There are some limitations to this work. Firstly, this is a small sample of DADA2 patients (n=4). Given this is a rare condition, identifying patients for blood sampling for ECFC isolation is extremely challenging. As previously discussed, these patients also have different genotypes meaning only one these patients has a genotype that would theoretically affect the ADA2 202 isoform. There is therefore a clear imprinted effect upon patient ECFC caused by exposure to ADA2 201 deficient immune cells *in vivo*. This work provides a greater understanding of the extrinsic basis of EC dysfunction in DADA2 rather than intrinsic. There were also differences in the treatments that these patients are receiving; 3 are currently using anti-TNF in the form of adalimumab whereas one patient is refractory to anti-TNF so is receiving IVIG and prednisolone. These different treatments may affect the phenotype of the ECFC samples. These factors may explain some of the heterogeneity in the DADA2 ECFC cultures and mean that achieving statistical significance in some of the experimental work is challenging with this number of biological replicates. Further work to validate these results would include sampling

from more DADA2 patients, including a range of patients with ADA2 202 affecting variants and if possible, sampling from treatment naïve patients.

The results in this chapter help to validate one of the central questions in this thesis; is ADAD2 expressed in the endothelium. ADA2 202 RNA expression has now been confirmed in endothelial cell lines as well as patient derived endothelial cells. This work provides novel insights into the effect of ADA2 deficiency on ECFC and gives more evidence that dysregulation in the IFN type I and II axis has a role in the pathogenesis of DADA2. Further consideration should therefore be given to novel targeted IFN treatments to improve patient outcomes. The evidence of ECFC dysfunction despite clinical remission raises important questions regarding the long term cardiovascular disease risk for these patients.

CHAPTER 7: Concluding remarks

7.1 Discussion and Future Work

7.1.1 ADA2 is expressed by EC but as the 202 transcript with undetectable protein and deaminase activity

The goal of this thesis was to confirm ADA2 expression in EC and determine its role in endothelial homeostasis and in the pathogenesis of DADA2. Despite the original studies reporting that endothelial cells lack ADA2, more recent work has demonstrated the presence of both ADA2 mRNA and protein expression in multiple EC types [2, 3, 88]. Our work has confirmed that ADA2 is indeed expressed by ECs but contrary to what was previously understood, we show that it is expressed as a shortened ADA2 202 transcript with undetectable protein and adenosine deaminase activity. We have demonstrated this using multiple transcriptomic technologies in both primary human endothelial cell lines and in patient derived ECFC. Analysis of publicly available transcriptomic data showed that this 202 transcript is not just found in EC but has widespread expression in multiple cell-types and tissues. Moreover, the identification of the ADA2 202-specific coding regions in primates suggests that it may be evolutionarily conserved and therefore functional.

The observation that ADA2 202 protein was not detectable in EC supernatant was not surprising given the truncated 270AA protein would lack its signal peptide. However, one would have still expected it to be present intracellularly but we were similarly unable to detect it in such samples. This was despite extensive efforts including multiple western blots inputting large quantities of protein and searching for ADA2 202 specific peptides with mass spectrometry. Forced expression of ADA2 202 in HEK293T cells demonstrated that this protein accumulated in the insoluble protein fraction. The question remains as to whether this is because the protein is misfolded/non-functional and is therefore targeted for degradation or whether it might be functional, but present at very low levels in the insoluble fraction, perhaps as a membrane bound protein. Of course, it is also possible that the ADA2 202 transcript functions as a lncRNA with no associated protein. Further work is required to fully elucidate this and would include RNA pull down of the 202 transcript to understand what other RNAs or proteins it is bound to.

The fact that we can detect and purify ADA2 202 protein in the forced expression model but cannot detect any adenosine deaminase activity is consistent with our functional hypothesis that this protein does not have enzymatic activity due to a lack of a significant proportion of

the catalytic domain. This raises the question as to what the function of this protein is. There is lots of evidence to suggest that ADA2 201 has many non-enzymatic functions including as a growth factor for immune cells and the endothelium [60, 61]. If ADA2 202 is functional as a protein, and not as a lncRNA, our work has demonstrated that it is not functioning as an enzyme with deaminase activity.

7.1.2 ADA2 has a role in EC homeostasis

The fact that a strong transcriptomic and proteomic signature was demonstrated with KD of ADA2 in HUVEC demonstrates that ADA2 202 has a role in EC homeostasis, and it is not a redundant transcript. KD of ADA2 in EC leads to upregulation of genes involved in inflammatory cytokines (IFNα, IFNγ and TNFα), inflammatory apoptotic gene pathways and CAMs suggesting that ADA2 has a role that when deficient results in endothelial dysfunction. KD of ADA2 in HUVEC previously has been associated with intracellular accumulation of deoxyinosine, interfering with the cellular methionine cycle and driving epigenomic hypomethylation resulting in IFNβ production [88]. Contrary to this study, our results showing a lack of adenosine deaminase activity by ADA2 202 would suggest that a non-enzymatic function has been lost in these KD experiments.

Consistent with previously published work, we have demonstrated enrichment of both a type I (IFNα) and type II (IFNγ) interferon response in siADA2 HUVEC [88]. Of particular note was that comparison of our HUVEC KD to transcriptomic profiling of DADA2 patient PBMC, demonstrated an almost identical gene set enrichment analysis with upregulation in genes involved in TNFα signalling via NFκB, IFNα response, IFNγ response and inflammatory response [99]. This is interesting that despite PBMC expressing the ADA2 201/203 isoform, its deficiency leads to the same transcriptomic signature as KD of ADA2 202 in HUVEC and possibly suggests that ADA2 201 and 202 have some shared functions in preventing cellular dysfunction.

The siADA2 HUVEC proteomic results may suggest preliminary hypotheses regarding the function of ADA2 202. KD resulted in changes, predominantly downregulation, in the expression of proteins involved in translation, peptide processing and non-coding RNA processing. It is possible to hypothesise therefore that ADA2 202 acts to suppress genes encoding inflammatory molecules and when deficient there is a reduction in these inhibitory pathways and subsequent upregulation of inflammatory genes. In addition, there was upregulation in a number of proteins involved in vesicle function in keeping with previous hypotheses that ADA2 may be lysosomal [243]. These results of course do not tell the full picture and much more work is required to fully understand ADA2 202 function.

Despite the transcriptomic and proteomic profiles, we have yet to show a functional change in the phenotype of siADA2 HUVEC in the assays that were performed. This is certainly one limitation of the work. One of the challenges was the variation in the responses seen among the four HUVEC pools that were used. Future work would focus on generating a CRISPR (clustered regularly interspaced short palindromic repeats) ADA2 knockout in HUVEC to allow a more stable cell line for functional phenotyping.

ADA2 202 therefore does have a role in EC homeostasis but whether this is as a lncRNA or an insoluble protein without adenosine deaminase activity remains to be seen. It appears to act to protect against endothelial dysfunction and when deficient results in upregulation of inflammatory and apoptotic gene pathways and is therefore likely to be involved in DADA2 pathogenesis.

7.1.3 Deficiency of intrinsic ADA2 in EC contributes to DADA2 pathogenesis

Classically the pathogenesis of DADA2 vasculitis was described as activation of the immune system and upregulation of inflammatory pathways as a consequence of lack of ADA2 with resulting endothelial damage by activated immune cells. The work data generated in this project suggests that intrinsic deficiency of ADA2 in EC may play a much greater role than previously thought. We have shown that intrinsic deficiency of the ADA2 202 isoform in EC resulted in induction of IFNα and IFNγ, TNFα, inflammatory apoptotic gene pathways, increased CAM expression and some trends towards enhanced sensitivity to TNFα.

Dysregulation of both type I and type II interferons has been implicated in DADA2 pathogenesis and has been demonstrated in a number of different DADA2 immune cells including PBMC and $CD14^+$ monocytes. Elevated levels of IFNγ have also been shown in DADA2 patient plasma [95, 98]. It was hypothesised that elevated IFNγ activated cell signalling which leads to differentiation of monocytes into M1 macrophages and release of TNFα [95]. Given our results demonstrating upregulation of both type I and type II IFNs in siADA2 KD HUVEC, it may be possible to postulate that some of this IFN signalling is from EC which could lead to further production from monocytes. This hypothesis would be consistent with previous work showing siADA2 HUVEC can induce IFNβ production from bystander U937 monocytes [88]. This has important clinical implications, and improved understanding of the role of both type I and type II IFN in DADA2 pathogenesis may yield novel targeted therapeutic agents including JAK inhibitors, which inhibit downstream signalling of IFNs, and anti-IFN antibodies which binds to the type I IFN receptor. Given the IFN signature in EC that we have shown, these therapeutics would be targeting both the dysfunctional immune system and EC which may have additional clinical benefits.

TNFα is well characterised as being an important cytokine in DADA2 with elevated TNFα concentrations in patient plasma and increased staining for TNFα shown in patient brain and skin biopsies [3, 62, 99]. The enrichment of TNFα genes in siADA2 HUVEC could suggest that, although the majority of the increased plasma TNFα in DADA2 will be from macrophages, the intrinsic loss of ADA2 in EC contributes to the generation of, and enhanced EC sensitivity to TNFα. This may explain why anti-TNF medication has been shown to be highly effective in treating the vasculitic component of DADA2 [48]

Further evidence of the involvement of EC ADA2 in the pathogenesis of DADA2 comes from our data demonstrating siADA2 202 KD in HUVEC resulted in significantly increased ICAM1 expression and trends towards these EC being more sensitive to TNFα. This may suggest that the endothelium is involved in disease pathogenesis, and that EC deficient of ADA2 202 have increased expression of cell adhesion molecules, are more susceptible to activation, injury and subsequent vasculitis.

Significantly increased cell adhesion molecule expression and upregulation of IFN related genes were also demonstrated in the ECFC of DADA2 patients. It must be stressed however that this represents a different EC disease model. The siADA2 202 KD in HUVEC represents solely intrinsic deficiency of ADA2 202 with reduced/silenced expression of ADA2 202 and would therefore only represent patients with an ADA2 variant affecting an amino acid >252 in the sequence. Intrinsic ADA2 202 expression was still shown to be present in the DADA2 ECFCs even in the patient who would in theory have a ADA2 202 affecting variant. The differences in DADA2 ECFCs vs HC seen therefore likely represent an imprinting effect on the ECFC from extrinsic deficiency of ADA2 in the patient's immune cells. It is interesting however that despite this being a different model there were a number of overlapping transcriptomic features which may give further evidence to the importance of these pathways in the disease pathogenesis. Further work to assess and compare ECFC from patients with variants affecting ADA2 201 only or both 201 and 202 to assess whether there are differences in gene expression of functioning would certainly help to improve understanding of the role of ADA2 202 in ECFC and disease pathogenesis.

The evidence of reduced proliferation in the DADA2 ECFCs may suggest that in the context of vasculitis where there is endothelial injury, the DADA2 ECFCs are unable to carry out one of their important roles in vascular repair [113, 115, 116]. This lack of reparative involvement could therefore contribute to ongoing vascular injury and thus be contributing to disease pathogenesis. Given the senescent, hypoproliferative phenotype seen in the DADA2 ECFCs if may be possible to hypothesise that there is also senescence of other progenitor cells which may contribute to the haematological manifestations in DADA2. This would be consistent with

unpublished data demonstrating increased senescence in DADA2 mesenchymal stromal cells.

The improved understanding of DADA2 pathogenesis from this thesis has clinical implications and may help explain some of the heterogeneity in disease presentations. It would be helpful to carry out more detailed phenotyping of patients who have a variant affecting ADA2 201 only versus those in whom 201 and 202 are affected. In particular, to assess their specific disease manifestations and response to treatment. A demonstration of a clear difference between these patients would give further evidence for the role of ADA2 202.

It is clear that the primary driver in the pathogenesis of the vasculitis in DADA2 is loss of immune cell ADA2 201 and resultant activation and consequential endothelial damage. This is demonstrated by the fact that the clinical features can develop in patients with preserved ADA2 202. This chapter has shown however, that EC ADA2 202 plays a much greater role than was previously understood.

7.1.4 ADA2 plasma activity is elevated in patients with other forms of vasculitis consistent with immune cell activation

In keeping with other inflammatory conditions including certain infections, autoimmune disorders and malignancies, plasma ADA2 activity was elevated in TA and ANCA vasculitis patients. Our results showing a significant positive correlation between ADA2 activity and monocyte and eosinophil count is consistent with the hypothesis that the elevated ADA2 activity results from immune cell activation. The significant positive correlation between blood markers of inflammation; ESR and ADA2 activity in TA group and CRP and ESR with ADA2 activity in LV-GCA group, gives further evidence that the elevated activity is likely related to inflammation.

Despite the heterogeneity of DADA2, with many clinical features that overlap with other forms of vasculitis, we did not find evidence of any patients in a large vasculitis cohort who had plasma ADA2 deficiency. There was however a suggestion that there was some enrichment in the frequency of patients who had ADA2 activity within a predicted range for a carrier of an ADA2 variant.

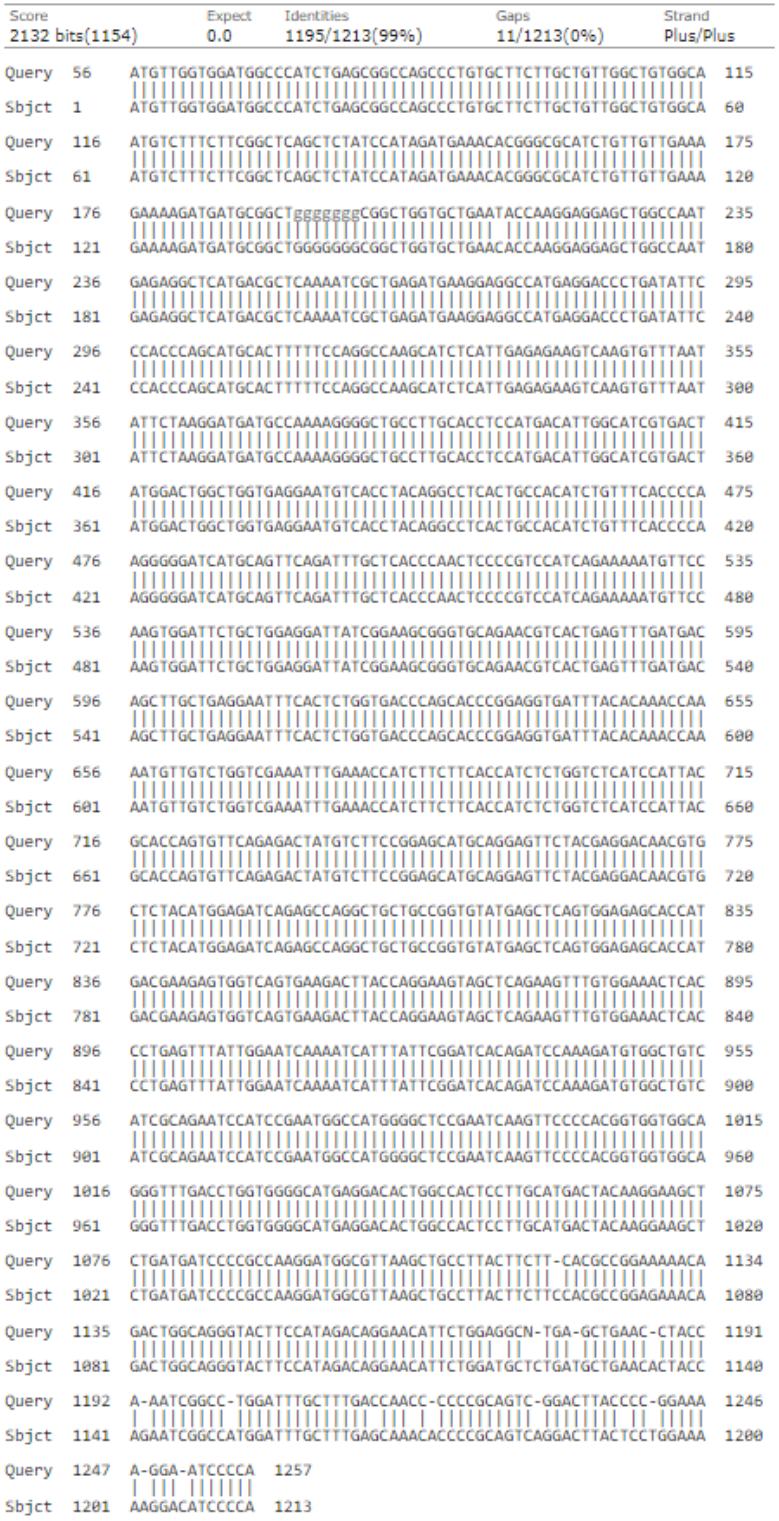
7.2 Conclusion

This thesis has answered the important question of whether ADA2 is expressed by EC. The data has confirmed that this is as the ADA2 202 transcript with undetectable protein and deaminase activity. We have shown that ADA2 202 has an important role in endothelial homeostasis and when deficient results in upregulation of inflammatory and apoptotic gene pathways. EC therefore likely directly contribute to DADA2 disease pathogenesis. The data has also demonstrated that ADA2 plasma activity is elevated in TA and ANCA vasculitis patients consistent with immune cell activation.

CHAPTER 8: Supplementary figures

Supplementary figure 1 – Forward coding sequence of ADA2 201 and ADA2 202 plasmids

A – ADA2 201 plasmid forward sequence vs ADA2 201 CDS sequence



Score	Expect	Identities	Gaps	Strand
2132 bits(1154)	0.0	1195/1213(99%)	11/1213(0%)	Plus/Plus

```
Query  56    ATGTTGGTGGATGGCCCATCTGAGCGGCCAGCCCTGTGCTTCTTGCTGTTGGCTGTGGCA  115
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGTTGGTGGATGGCCCATCTGAGCGGCCAGCCCTGTGCTTCTTGCTGTTGGCTGTGGCA  60

Query  116   ATGTCTTTCTTCGGCTCAGCTCTATCCATAGATGAAACACGGGCGCATCTGTTGTTGAAA  175
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    ATGTCTTTCTTCGGCTCAGCTCTATCCATAGATGAAACACGGGCGCATCTGTTGTTGAAA  120

Query  176   GAAAAGATGATGCGGCTgggggggCGGCTGGTGCTGAATACCAAGGAGGAGCTGGCCAAT  235
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GAAAAGATGATGCGGCTGGGGGGGCGGCTGGTGCTGAACACCAAGGAGGAGCTGGCCAAT  180

Query  236   GAGAGGCTCATGACGCTCAAAATCGCTGAGATGAAGGAGGCCATGAGGACCCTGATATTC  295
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   GAGAGGCTCATGACGCTCAAAATCGCTGAGATGAAGGAGGCCATGAGGACCCTGATATTC  240

Query  296   CCACCCAGCATGCACTTTTTCCAGGCCAAGCATCTCATTGAGAGAAGTCAAGTGTTTAAT  355
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   CCACCCAGCATGCACTTTTTCCAGGCCAAGCATCTCATTGAGAGAAGTCAAGTGTTTAAT  300

Query  356   ATTCTAAGGATGATGCCAAAAGGGGCTGCCTTGCACCTCCATGACATTGGCATCGTGACT  415
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   ATTCTAAGGATGATGCCAAAAGGGGCTGCCTTGCACCTCCATGACATTGGCATCGTGACT  360

Query  416   ATGGACTGGCTGGTGAGGAATGTCACCTACAGGCCTCACTGCCACATCTGTTTCACCCCA  475
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ATGGACTGGCTGGTGAGGAATGTCACCTACAGGCCTCACTGCCACATCTGTTTCACCCCA  420

Query  476   AGGGGGATCATGCAGTTCAGATTTGCTCACCCAACTCCCCGTCCATCAGAAAAATGTTCC  535
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   AGGGGGATCATGCAGTTCAGATTTGCTCACCCAACTCCCCGTCCATCAGAAAAATGTTCC  480

Query  536   AAGTGGATTCTGCTGGAGGATTATCGGAAGCGGGTGCAGAACGTCACTGAGTTTGATGAC  595
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   AAGTGGATTCTGCTGGAGGATTATCGGAAGCGGGTGCAGAACGTCACTGAGTTTGATGAC  540

Query  596   AGCTTGCTGAGGAATTTCACTCTGGTGACCCAGCACCCGGAGGTGATTTACACAAACCAA  655
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   AGCTTGCTGAGGAATTTCACTCTGGTGACCCAGCACCCGGAGGTGATTTACACAAACCAA  600

Query  656   AATGTTGTCTGGTCGAAAATTTGAAACCATCTTCTTCACCATCTCTGGTCTCATCCATTAC  715
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Query  716   GCACCAGTGTTCAGAGACTATGTCTTCCGGAGCATGCAGGAGTTCTACGAGGACAACGTG  775
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Sbjct  661   GCACCAGTGTTCAGAGACTATGTCTTCCGGAGCATGCAGGAGTTCTACGAGGACAACGTG  720

Query  776   CTCTACATGGAGATCAGAGCCAGGCTGCTGCCGGTGTATGAGCTCAGTGGAGAGCACCAT  835
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Sbjct  721   CTCTACATGGAGATCAGAGCCAGGCTGCTGCCGGTGTATGAGCTCAGTGGAGAGCACCAT  780

Query  836   GACGAAGAGTGGTCAGTGAAGACTTACCAGGAAGTAGCTCAGAAGTTTGTGGAAACTCAC  895
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Sbjct  781   GACGAAGAGTGGTCAGTGAAGACTTACCAGGAAGTAGCTCAGAAGTTTGTGGAAACTCAC  840

Query  896   CCTGAGTTTATTGGAATCAAAATCATTTATTCGGATCACAGATCCAAAGATGTGGCTGTC  955
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CCTGAGTTTATTGGAATCAAAATCATTTATTCGGATCACAGATCCAAAGATGTGGCTGTC  900

Query  956   ATCGCAGAATCCATCCGAATGGCCATGGGGCTCCGAATCAAGTTCCCCACGGTGGTGGCA  1015
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   ATCGCAGAATCCATCCGAATGGCCATGGGGCTCCGAATCAAGTTCCCCACGGTGGTGGCA  960

Query  1016  GGGTTTGACCTGGTGGGGCATGAGGACACTGGCCACTCCTTGCATGACTACAAGGAAGCT  1075
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   GGGTTTGACCTGGTGGGGCATGAGGACACTGGCCACTCCTTGCATGACTACAAGGAAGCT  1020

Query  1076  CTGATGATCCCCGCCAAGGATGGCGTTAAGCTGCCTTACTTCTT-CACGCCGGAAAAACA  1134
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||| |||||
Sbjct  1021  CTGATGATCCCCGCCAAGGATGGCGTTAAGCTGCCTTACTTCTTCCACGCCGGAGAAACA  1080

Query  1135  GACTGGCAGGGTACTTCCATAGACAGGAACATTCTGGAGGCN-TGA-GCTGAAC-CTACC  1191
             |||||||||||||||||||||||||||||||||||||||||| ||  ||| ||||||| |||||
Sbjct  1081  GACTGGCAGGGTACTTCCATAGACAGGAACATTCTGGATGCTCTGATGCTGAACACTACC  1140

Query  1192  A-AATCGGCC-TGGATTTGCTTTGACCAACC-CCCCGCAGTC-GGACTTACCCC-GGAAA  1246
             | |||||||| |||||||||||||| ||| | |||||||||| |||||||| || |||||
Sbjct  1141  AGAATCGGCCATGGATTTGCTTTGAGCAAACACCCCGCAGTCAGGACTTACTCCTGGAAA  1200

Query  1247  A-GGA-ATCCCCA  1257
             | ||| |||||||
Sbjct  1201  AAGGACATCCCCA  1213
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B – ADA2 202 plasmid forward sequence vs ADA2 201 CDS sequence

Score	Expect	Identities	Gaps	Strand
1496 bits(810)	0.0	810/810(100%)	0/810(0%)	Plus/Plus

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Query  32   ATGGATTCCCTGGAATGGAACTGGGCTCTGGTGTATGAGCTCAGTGGAGAGCACCATGAC  91
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Sbjct  1    ATGGATTCCCTGGAATGGAACTGGGCTCTGGTGTATGAGCTCAGTGGAGAGCACCATGAC  60

Query  92   GAAGAGTGGTCAGTGAAGACTTACCAGGAAGTAGCTCAGAAGTTTGTGGAAACTCACCCT  151
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAAGAGTGGTCAGTGAAGACTTACCAGGAAGTAGCTCAGAAGTTTGTGGAAACTCACCCT  120

Query  152  GAGTTTATTGGAATCAAAATCATTTATTCGGATCACAGATCCAAAGATGTGGCTGTCATC  211
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GAGTTTATTGGAATCAAAATCATTTATTCGGATCACAGATCCAAAGATGTGGCTGTCATC  180

Query  212  GCAGAATCCATCCGAATGGCCATGGGGCTCCGAATCAAGTTCCCCACGGTGGTGGCAGGG  271
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCAGAATCCATCCGAATGGCCATGGGGCTCCGAATCAAGTTCCCCACGGTGGTGGCAGGG  240

Query  272  TTTGACCTGGTGGGGCATGAGGACACTGGCCACTCCTTGCATGACTACAAGGAAGCTCTG  331
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TTTGACCTGGTGGGGCATGAGGACACTGGCCACTCCTTGCATGACTACAAGGAAGCTCTG  300

Query  332  ATGATCCCCGCCAAGGATGGCGTTAAGCTGCCTTACTTCTTCCACGCCGGAGAAACAGAC  391
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATGATCCCCGCCAAGGATGGCGTTAAGCTGCCTTACTTCTTCCACGCCGGAGAAACAGAC  360

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Sbjct  361  TGGCAGGGTACTTCCATAGACAGGAACATTCTGGATGCTCTGATGCTGAACACTACCAGA  420

Query  452  ATCGGCCATGGATTTGCTTTGAGCAAACACCCCGCAGTCAGGACTTACTCCTGGAAAAAG  511
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ATCGGCCATGGATTTGCTTTGAGCAAACACCCCGCAGTCAGGACTTACTCCTGGAAAAAG  480

Query  512  GACATCCCCATAGAAGTCTGTCCCATCTCTAACCAGGTGCTGAAACTGGTGTCTGACTTG  571
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Sbjct  481  GACATCCCCATAGAAGTCTGTCCCATCTCTAACCAGGTGCTGAAACTGGTGTCTGACTTG  540

Query  572  AGGAACCACCCTGTAGCCACTCTGATGGCCACTGGGCACCCCATGGTGATCAGCTCTGAT  631
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AGGAACCACCCTGTAGCCACTCTGATGGCCACTGGGCACCCCATGGTGATCAGCTCTGAT  600

Query  632  GACCCAGCTATGTTTGGTGCCAAAGGCTTGTCCTATGATTTCTATGAGGTCTTCATGGGC  691
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Sbjct  601  GACCCAGCTATGTTTGGTGCCAAAGGCTTGTCCTATGATTTCTATGAGGTCTTCATGGGC  660

Query  692  ATTGGGGGGATGAAGGCTGACCTGAGGACCCTCAAACAGCTGGCCATGAACTCTATCAAG  751
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  ATTGGGGGGATGAAGGCTGACCTGAGGACCCTCAAACAGCTGGCCATGAACTCTATCAAG  720

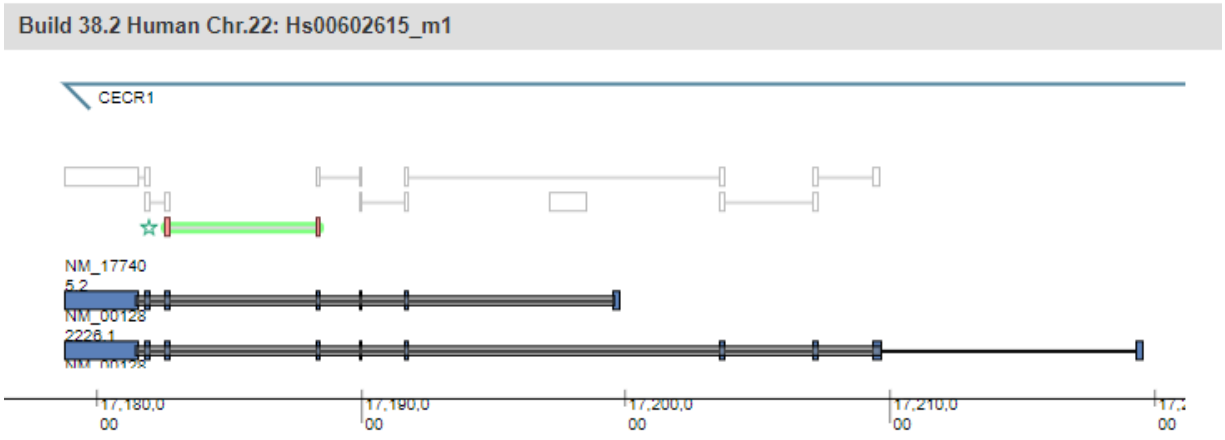
Query  752  TACAGTACCCTGTTGGAGAGTGAGAAAAATACTTTCATGGAAATCTGGAAGAAGAGATGG  811
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TACAGTACCCTGTTGGAGAGTGAGAAAAATACTTTCATGGAAATCTGGAAGAAGAGATGG  780

Query  812  GATAAGTTCATAGCAGATGTGGCTACAAAG  841
            |||||||||||||||||||||||||||||
Sbjct  781  GATAAGTTCATAGCAGATGTGGCTACAAAG  810
```

Supplementary figure 2 – Search keywords for literature search for ADA2 upregulating agents

Keywords		Compounds identified	Source
Keyword combination 1	(ADA2* OR CECR1) AND (induction* OR increase* OR upregulation) NOT (Ada2a* OR Ada2b)	IFNγ	[185]
		TNFα	
		Interleukin 12	
		Interleukin 18	
		Early secretory antigenic target protein 6	[170]
		Culture filtrate protein 10	
Keyword combination 2	(ADA2* OR CECR1) AND (role) NOT (Ada2a* OR Ada2b)	Butyrate	[183]
		LPS	
Keyword combination 3	(ADA2* OR CECR1) AND (production) NOT (Ada2a* OR Ada2b)	Amadori-glycated albumin	[244]

Supplementary figure 3 – ADA2 Taqman probe ID HS00602615_m1 would target ADA2 201 and 202 isoforms



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CHAPTER 10: Appendix

10.1 Tissue bank ethics patient information sheet

Imperial College Healthcare NHS Trust

Tissue Bank

Information for patients, relatives and carers version 6 18/4/18

Introduction

This leaflet provides information about the Imperial College Healthcare Tissue Bank (ICHTB) run by Imperial College Healthcare NHS Trust. We hope it will help you to understand:

- the aims and purposes of ICHTB
- what research is likely to be performed
- what your participation (if you agree to it) will involve

Please read the following information carefully and feel free to ask us if anything is unclear or if you would like more information: telephone 020 3311 7173 or email us via tissuebank@imperial.ac.uk

What is ICHTB?

ICHTB is a project that will help the Trust and its partner, Imperial College London, carry out high quality healthcare-related research to better understand diseases and improve future treatments.

ICHTB aims to provide researchers with access to a very large resource of human tissue samples that are vital in helping us to:

- better understand your health conditions
- reach our goal of developing new medicines and other treatments
- improve ways of detecting diseases earlier

By taking part, you will be helping researchers investigate and understand why some individuals have or develop a particular disease, and to understand the biology of the disease so we can develop better treatments.

In addition, healthcare professionals may need to study tissue or fluids as part of their training. It is hoped that this knowledge will help other patients in the future.

We also want to give you the option to take part in further research studies related to your condition or that are relevant to you. To do this we need to be able to contact you to provide further details of these studies so you can decide if you want to take part. NHS clinical staff will contact you to explain the study and ask for your

permission to share your contact details with the researcher running it. You have the right to say no.

Do I have to join ICHTB?

No. It is completely up to you whether or not you wish to join. If you decide not to it will not affect the standard of care that you receive in any way. If you do decide to join, you will be free to withdraw at any time and without having to give a reason.

Allowing us to keep your biological samples for research

When you attend hospital your care team will often need to perform tests and procedures to diagnose and treat your condition. These procedures may involve the removal of fluids, pieces of tissue or sometimes a whole organ (known as biological samples). These biological samples are sent to a laboratory to be tested by doctors or scientists. This can help them diagnose your condition.

Not all of the biological samples may be needed for diagnosis. If you consent to join ICHTB, any leftover biological samples after diagnosis may be kept for research (under normal circumstances these would be thrown away).

If you are having a biopsy procedure, two or three extra samples may be taken for research which may mean the procedure would take a few extra minutes. If you have already had your operation you can still help us by consenting - we can then ask the pathologist for a small amount of tissue already used for your diagnosis.

After treatment, your doctor may invite you back for further visits and this may involve taking other biological samples. If, at a later stage, you have blood or tissues taken as part of your future care, we may wish to access these samples too. This will allow researchers to make potentially helpful comparisons with your original samples. If you do not wish to provide further samples, you do not have to.

Allowing us to take additional blood samples

As part of your regular hospital treatment, a doctor may need to take blood samples from you. If you consent to ICHTB, when you provide these blood samples, we may take up to an additional 45 millilitres (ml) - three tablespoons - of blood to be stored for research. You will not need to attend any extra appointments - we will only take this extra blood when you are required to provide blood samples as part of your regular clinical care. If you do not wish to provide further samples, you do not have to.

How will I benefit from joining ICHTB?

You may not benefit personally from any research carried out using your samples and data. However, the results of the research may benefit patients with your type of illness in the future. You will not receive a financial reward now or in the future for providing samples. The medical team involved in your treatment and care will receive no payment because of your donation.

Your sample will not be sold for profit to researchers, but the use of your samples may lead to the development of new drugs, treatments or tests by both academic and commercial organisations.

Will the samples be tested for (genetic) inherited disorders?

We want to know how genes influence disease. Genes are made out of DNA. We may, now or in the future, isolate, analyse and store a sample of your DNA from your donated biological samples. Using current advanced laboratory techniques or those developed in the future, we may determine your genetic make-up. This could include determining the sequence of all or part of your DNA code.

ICHTB will not disclose information about your genetic make-up in any way that could harm you or your family. Your personal identifiable information will never be shared without your permission.

Who will use the stored samples?

Your sample may be used by researchers based in academic institutions, the NHS or commercial companies worldwide. All research will be thoroughly reviewed by a tissue management committee before being allowed access to your samples and data. This committee will ensure that the research is scientifically valid, in the interests of human health and has appropriate security measures to protect your information. All of your information will be anonymised which means it will not contain details that could identify you. Your identity will never be shared without your permission.

What will happen to the results of the study?

Research studies usually take several years to complete. The results from these studies will be used to improve treatment and care of patients in the future. Results will be published as appropriate in scientific papers and magazines and regular updates of research in progress, research results and other relevant information will be published on the Imperial College London website at www.imperial.ac.uk/tissuebank.

You will not be identified in any publication or through any information on the website.

What if you find something new about my health?

Results for individual patients from particular research studies will not normally be given to you or your doctor. In very rare situations, some research projects could identify changes to your diagnosis or treatment or may indicate an inherited disease that could affect you or your family members. Your hospital doctor will be notified of this information.

When and how will I be contacted about research studies that I may wish to join?

Approved researchers will be able to search your anonymised health data, data obtained from your biological samples, and other data approved for use in research to find people who may be suitable to take part in research studies. For example, a search might be carried out to find people who are over the age of 40 and have diabetes. If your data matches the requirements for a research study that may be

beneficial to you, we will allow an NHS clinician at Imperial College Healthcare NHS Trust to contact you and provide you with more information. If you are interested in finding out more, the NHS clinician will pass your contact details on to the researcher who will get in touch with you to discuss it further.

You decide if you want to take part. It is your choice and you do not have to take part in any studies if you do not wish to. If you decide not to take part it will not affect the standard of care that you receive in any way.

What will happen to my data if I join ICHTB?

Your information is protected under the General Data Protection Regulations 2016. We have a legal duty to keep your information confidential, secure and hold the information only as long as necessary. The Trust collects and stores information about you in order to provide safe and effective healthcare. Your health records are held on paper and on secure computer systems. Your full identifiable medical record will never be seen by researchers.

In order to conduct high quality research and to improve NHS services, Imperial College London may need to link your health information to other data held about you, this may include other information about your health or other data that may be relevant to ethically approved research studies.

All data linkage will be approved in full by Imperial College Healthcare NHS Trust and any other organisation that holds data about you. Linkage will be completed by NHS staff and they will only see the minimum data required to link your records – when using identifiable data they will never see other information about you, for example your medical record. After linking your information, the details that could identify you are removed and will not be used in research without your separate explicit consent or we are legally obliged to provide them. Your electronic information is linked, stored and transferred using secure, encrypted mechanisms.

We will use your contact details to tell you about research studies that may be beneficial to you. You do not have to take part in any studies if you do not wish to.

You have a right to a copy of the information that we hold about you. To request this information please write to the Subject access requests team via the details listed at the end of this leaflet.

I would like to join ICHTB – what should I do next?

If you have been approached by one of trained consenters in the clinic, just let them know that you wish to consent and they will help you enrol via our secure IT system. If you are discussing consent with an Imperial College Healthcare NHS Trust clinician at the hospital they will show you how to join ICHTB.

I no longer want to be a member of ICHTB – what do I need to do?

You can leave ICHTB at any time without giving a reason and without your medical care or legal rights being affected. We will remove your details from the database

and destroy any biological samples that we still have in storage from you for research and teaching. If you withdraw sometime after joining, some of your samples may have already been used in research.

If you want to leave ICHTB please contact the team directly on 020 3311 7173 or via email at tissuebank@imperial.ac.uk

Who has approved/reviewed the collection of human tissue samples as part of ICHTB?

A Research Ethics Committee based in Wales (Wales REC3) has approved and will regularly review this project.

Contact details for the data protection officer

Post: Data protection officer, Information Governance, ICT Directorate, Charing Cross Hospital, London, W6 8RF
Email: Imperial.dpo@nhs.net
Telephone: 020 3311 7344

Enquiries relating to Subject Access Requests (DPA) should be sent to - imperial.accesstohealthrecords@nhs.net

Imperial College Healthcare NHS Trust is a registered data controller under the Information Commissioner's Office. Further information can be found at:
Information Commissioners Office, Wycliffe House, Water Lane, Wilmslow, Cheshire, SK9 5AF
Website: www.ico.org.uk/concerns
Phone: 0303 123 1113

If you are not satisfied with our response or believe we are processing your personal data not in accordance with the law you can complain to the Information Commissioner's Office

Contact details for Imperial College Healthcare's patient advice and liaison service (PALS)

If you have any questions or comments about your care, please contact PALS on **020 3313 0088** (Charing Cross, Hammersmith and Queen Charlotte's & Chelsea hospitals), or **020 3312 7777** (St Mary's and Western Eye hospitals). You can also email PALS at pals@imperial.nhs.uk The PALS team will listen to your concerns, suggestions or queries and is often able to help solve problems on your behalf.

Alternative formats

This leaflet can be provided on request in large print, as a sound recording, in Braille, or in alternative languages. Please contact the communications team on **020 3312 5592**.

10.2 Patient consent form

Imperial College Healthcare NHS
NHS Trust

Patient Agreement to Provision of Extra Samples for Research

Patient Details (or Printed Label)

Hospital No _____

Patient's First Names _____

Patient's Surname/Family Name_____

Date of Birth _____ ☐ Male ☐ Female

Name of Consultant _____

Job Title _____

Patient's Special Requirements
(e.g. other language/other communication method)
_____

Name of proposed procedure or course of treatment (include brief explanation if medical term not clear)

.....

.....

.....

Statement of health professional (to be filled in by health professional with appropriate knowledge of proposed procedure, as specified in consent policy)

I have explained the procedure to the patient. In particular, I have explained that the following extra samples will be taken:

_____

_____

_____

_____

Use of Extra Tissues and Fluid Samples for Research

Statement of health professional
(to be filled in by health professional with appropriate knowledge of the use of tissue and fluid samples for research)

☐ I have explained that this procedure may involve the collection and use of tissue, blood or fluid samples for research purposes

☐ The patient has received the following leaflet/tape PIS v6 18.04.18 .....

Signed ..... Name (PRINT) ..... Date .....

Statement of interpreter (where appropriate)
I have interpreted the information above to the best of my ability and in a way that s/he can understand.
Signed: Name: Date

Statement of patient

I have confirmed that I have read and understood the patient information leaflet "Tissue Bank Information for patients, relatives and carers".

I understand that my participation is voluntary and will not affect my treatment in any way.

I understand that sections of my medical notes may be accessed by healthcare professionals from the Tissue Bank. I give permission for these individuals to access my medical notes.

I understand that parts of my medical information may be passed to other organisations involved in the research on the understanding that my personal patient confidentiality will be maintained.

I understand, and agree to, data relating to my donated samples being stored electronically.

☐ I **AGREE** to the collection and use of my tissue blood or fluid samples for approved research

☐ I **DISAGREE** to the collection and use of my tissue, blood or fluid samples for approved research

Signed ..... Date .....

Name (PRINT) _____

A witness should sign below if the patient is unable to sign but has indicated his or her consent. Young people/children may also like a parent to sign here

Signed ..... Date .....

Name (PRINT) _____

Confirmation of consent (to be completed by the health professional following signature by the patient)

Signed ..... Date .....

Name (PRINT) _____ Job Title _____

Please note:
Top copy (gold) in health records, white copy to patient, green copy to research staff with specimen

Copy accepted by patient? Yes/No (please ring)