High Dose Glucocorticoid-Induced Endothelial Dysfunction

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Abstract

Given their anti-proliferative and anti-inflammatory effects, glucocorticoids are frequently administered in high doses as therapy for a variety of ailments including cancer and autoimmune disorders. Unfortunately, the lifesaving benefits of glucocorticoid therapy are offset by grave side-effects that include the development of osteonecrosis of the femoral head (ONFH) in association with high-dose glucocorticoid administration. Studies into ONFH etiology implicate endothelial cell dysfunction leading to impaired haemostasis, vascular obstruction, and downstream ischemia in the observed tissue necrosis. Endothelial cells play a major role in orchestrating the various factors that regulate haemostasis, maintaining regular blood flow under normal physiological conditions and initiating clot formation only in response to vascular injury. Endothelial dysfunction results in a disruption of the balance of coagulatory (i-e, clotting) and fibrinolytic (i-e, clot lysing) factors produced by the endothelium, leading to an endothelial milieu that favours clot formation and stasis.

We have previously shown that high-dose glucocorticoid treatment alters endothelial haemostatic gene expression. In a subsequent report, we identified alpha-2-macroglobulin (A2M), a broad-spectrum protease inhibitor, as the highest differentially up-regulated gene in microarrays of diseased rat femoral heads. A2M plays an important role in haemostasis by binding and inhibiting a large number of the proteases involved in both coagulation and fibrinolysis. Imbalances in A2M levels have been linked to various coagulopathies. More recently, it has been shown that once A2M is activated by protease binding, it is rapidly taken up by its ubiquitously expressed receptor, the low-weight lipoprotein-receptor related protein 1 (LRP1), triggering a wide range of signaling events that can further modulate endothelial function. Another haemostatic mediator that has been heavily implicated in ONFH development is anti-fibrinolytic protein plasminogen activator inhibitor (PAI-1). Several studies have reported elevated PAI-1 levels in patients with ONFH.

Here, we explored the effect of high-dose glucocorticoid treatment on endothelial expression of haemostatic genes A2M and PAI-1, and investigated whether A2M signaling contributes to high-dose glucocorticoid induced endothelial dysfunction. Treatment of endothelial cells with 1mM dexamethasone, a synthetic glucocorticoid, up-regulated A2M gene expression. In an inflammatory setting, the dexamethasone-mediated increase in A2M expression was attenuated while an additive effect was noted for PAI-1 expression, which was elevated further upon stimulation of dexamethasone treated cells with the inflammatory mediator TNFα. This observed PAI-1 up-regulation translated into increased PAI-1 levels in the conditioned media. The dexamethasone and TNFα mediated increase in PAI-1 expression was sustained in spite of A2M silencing, and treatment with activated A2M had no effect of PAI-1 expression. However, the important role A2M plays in haemostasis implies that elevated endothelial A2M gene expression is an important facet of high-dose glucocorticoid-induced endothelial dysfunction that requires further investigation.

Résumé

En raison de leur effets anti-prolifératifs et anti-inflammatoires, les glucocorticoïdes sont fréquemment administrés à des doses élevées en tant que thérapie pour une variété de maladies telles que le cancer et les maladies auto-immunes. Malheureusement, leur utilisation est très souvent associée à des effets secondaires graves incluant le développement de l'ostéonécrose de la tête fémorale (ONTF). Les cellules endothéliales jouent un rôle primordial dans l'orchestration des différents facteurs qui régulent l'hémostase dans des conditions physiologiques ainsi que dans le déclenchement de la coagulation. Cette dernière se traduit par la formation de caillots sanguins en réponse à une lésion vasculaire. Toutefois, l'ONTF est caractérisée par un dysfonctionnement des cellules endothéliales conduisant à l'obstruction vasculaire et à l'ischémie en aval de la nécrose des tissus observés, résultant ainsi en la rupture de l'équilibre hémostatique, incluant la coagulation qui se définit comme étant la formation de caillots sanguins et la fibrinolyse qui n'est autre que la lyse du caillot sanguin.

nous avons précédemment montré que le traitement aux glucocorticoïdes à haute dose modifie l'expression des gènes impliqués dans l'hémostase. Des travaux réalisés dans notre laboratoire ont précédemment démontré une augmentation de l'expression de l'alpha-2-macroglobuline (A2M), un inhibiteur de protéase, au niveau des têtes fémorales de rats affectés. L'A2M joue un rôle important dans l'hémostase par la régulation d'un grand nombre de protéases impliquées dans la coagulation ainsi que la fibrinolyse. Les déséquilibres dans les niveaux A2M ont été liés à diverses coagulopathies. Plus récemment, il a été démontré qu'une fois que l'A2M est activée suite à sa liaison à une protéase, cette dernière se lie rapidement par son récepteur ubiquitaire. Cette liaison avec son récepteur (LRP1), déclenche une vaste gamme d'événements de signalisation qui peut en outre moduler la fonction cellulaire. Des études ultérieures ont démontré des niveaux accrus d'inhibiteur de l'activateur du plasminogène (PAI-1), un anti-fibrinolytique, chez des patients atteints d'une ONTF.

Dans le présent rapport, nous avons exploré l'effet du traitement de glucocorticoïdes à haute dose sur l'expression des gènes de l'endothélium A2M et PAI-1, et cherché à savoir si la signalisation de l'A2M est impliquée dans le mécanisme de réponse à des fortes doses de glucocorticoïdes. Le traitement des cellules endothéliales à la dexaméthasone, un glucocorticoïde synthétique, conduit à une augmentation de l'expression de l'A2M. Dans un contexte inflammatoire, l'augmentation de l'expression de l'A2M médiée par la dexamethasone était atténuée tandis qu'un effet additif a été noté pour l'expression de PAI-1. En outre, lors de la stimulation des cellules à la dexaméthasone traitées avec le médiateur inflammatoire TNFa, l'augmentation de l'expression de PAl-1 s'est traduite par une augmentation des niveaux de PAI-1 dans le milieu de culture. L'augmentation de l'expression de PAI-1 induite par la dexamethasone et TNFa est soutenue malgré la suppression de l'A2M. De plus, le traitement avec l'A2M activée n'exerce aucun effet sur PAI-1. En résumé l'induction de l'expression de l'A2M suite au traitement à des doses élevées de dexamethasone représente une facette importante du mécanisme de réponse à des doses élevées de dexamethasone, d'où la nécessité de faire des études plus poussées à ce sujet.

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My family, whose love is a shelter from every storm:

Abby, thank you for not just being my sister but also my friend, you get me through the rough times. Hussein, you inspire me to be more disciplined; thank you for motivating me to finally finish this document ☺ Hassan, our bright youngest, I look forward to all the great things you'll accomplish!

My parents, Dr. Thekra A. and Dr. Ramzi A.: I hope I make you proud.

To my family, as a token of my love

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List of Abbreviations

11b-HSD 11b-hydroxysteroid dehydrogenase

A2M Alpha-2-macroglobulin

BMP4 Bone morphogenetic protein-44

CAMs Cell adhesion molecules

EBM-2 Endothelial basal medium-2

EGM-2 Endothelial growth medium-2

ELISA Enzyme-linked immunosorbent assay

ETS E-twenty six

GR Glucocorticoid receptor

GRE Glucocorticoid response element

nGRE Negative glucocorticoid response element

GRP78 Glucose-regulated protein 78

HBSS Hank's balanced salt solution

HPA Hypothalamic-pituitary-adrenal

HPRT1 Hypoxanthine phosphoribosyl transferase-1

HSP90 Heat shock protein-90

HUVEC Human umbilical vein endothelial cells

ICAM-1 Inter-cellular adhesion molecule-1

IHH Indian hedgehog

IL-1 Interleukin-1

IL-6 Interleukin-6

LDL Low-density lipoprotein

LPS Lipopolysaccharide

LRP1 Low-density lipoprotein receptor-related protein-1

MMP9 Matrix metallo-proteinase-9

MRI Magnetic resonance imaging

MSC Mesenchymal stem cell

NF_KB Nuclear factor kappa beta

NGF-β Nerve growth factor beta

ONFH Osteonecrosis of the femoral head

PAI-1 Plasminogen activator inhibitor-1

PBS Phosphate buffered saline

PI3K Phosphatidylinositol-3-kinase

qRT-PCR Quantitative real time polymerase chain reaction

RMEC Rat microvascular endothelial cells

SiRNA Small interfering ribonucleic acid

SNP Single nucleotide polymorphism

STAT Signal transducer and activator of transcription

TF Tissue factor

TGF-β Transforming growth factor beta

TNFα Tumor necrosis factor alpha

TNS Trypsin neutralizing solution

TPA Tissue plasminogen activator

UPA Urokinase plasminogen activator

VCAM-1 Vascular cell adhesion molecule -1

VEGF Vascular endothelial growth factor

VWF Von willebrand factor

Y2H Yeast-2-hybrid assay

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Introduction

ONFH, High Dose Glucocorticoids, and the Endothelium

Often regarded as the heart disease of the hip, osteonecrosis of the femoral head (ONFH) is brought about by infarction of the intricate blood vessels feeding the head of the femoral bone, leading to tissue necrosis, femoral head collapse, and loss of the hip joint function [3]. 20,000 to 30,000 patients are diagnosed with ONFH annually in the US alone, with patients often being individuals in their prime productive years between the third and fifth decades of life [4, 5]. ONFH can be triggered by traumatic injury directly disrupting the femoral head blood supply such as femoral neck fracture, dislocation, or surgery-associated trauma [1, 6]. In the absence of traumatic disease-instigating events, ONFH development is most commonly associated with glucocorticoid treatment [7]. Due to their anti-inflammatory and anti-tumorigenic effects, glucocorticoids are routinely administered in high doses as therapy for a variety of conditions including cancer, organ transplantation, and autoimmune disorders, with treated patients incurring an increased risk for ONFH development. ONFH occurs at a rate of 10-30% in young children with acute lymphoblastic leukemia treated with glucocorticoids [8]. Immune disease and organ transplantation patients receiving glucocorticoid therapy also increased rates of developing ONFH [9-12]. With the absence of preventative measures and the lack of a durable treatment for the disease, glucocorticoid-induced ONFH severely impacts the quality of life of individuals who are already suffering from other debilitating conditions.

Although glucocorticoids act through multiple pathways to bring about the observed bone necrosis, it is thought that high dose glucocorticoids mediate ONFH mainly by disturbing the balance of haemostatic (i.e., blood clotting) factors produced by the endothelium of blood vessels, leading to pathologic blood clot formation and eventually vessel blockade and tissue necrosis (**Fig. 1)** [3, 13]. Of the various factors that govern blood clot formation (also referred to as coagulation) and lysis (also referred to as fibrinolysis), the anti-fibrinolytic factor plasminogen activator inhibitor 1 (PAI-1) has been among the most strongly associated with ONFH development [14]. More recently work by our group using a rat model of glucocorticoid-induced ONFH revealed an increase in the expression of the haemostatic regulator alpha-2-macroglobulin (A2M) in association with the disease [15].

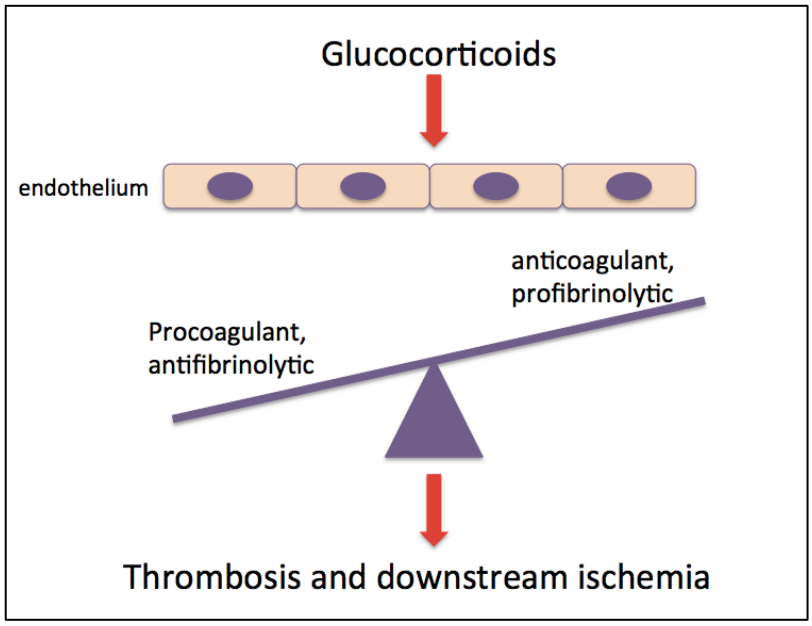

Figure 1. ONFH, high dose glucocorticoids, and the endothelium. It is thought that high dose glucocorticoids disturb the balance of coagulant and fibrinolytic factors produced by the endothelium, creating an endothelial environment with an increased likelihood of thrombosis and ONFH development.

A2M is a broad spectrum protease inhibitor that has been shown to inactivate many of the proteases involved in clot formation and lysis [16]. Once A2M binds a protease, it undergoes a conformational change that exposes its receptor-binding site. The protease-bound A2M is then said to be activated for binding to its ubiquitously expressed receptor, the low-density lipoprotein receptor-related protein 1 (LRP1) [2]. The binding of activated A2M to LRP1 can induce a wide range of signaling cascades that modulate cell function, such as the PI3K/Akt/NF_KB pathways [17, 18]. Elevations of A2M levels have been associated with the development of various coagulopathies [19]. Recently, various reports have highlighted A2M's signaling capacity as another important aspect of its multifaceted function that warrants further attention [20].

In this study, we attempt to explore the effect of high dose glucocorticoid treatment on the expression of endothelial PAI-1 and A2M and examine if A2M is involved in regulating endothelial PAI-1 gene expression in our glucocorticoid model. We also test the impact of A2M signaling on PAI-1 in an independent context to evaluate the potential contribution of A2M signaling to the endothelial dysfunction linked to ONFH development.

Chapter 1:

Literature Review

1.1 Osteonecrosis of the Femoral Head (ONFH): Heart Disease of the Hip

1.1.1 Anatomy, blood supply, and cells of the femoral head

An appreciation of the anatomical context, blood supply, and cellular composition of the femoral head is imperative to understanding the pathology of ONFH, as these factors interact to bring about a local disease manifestation of a systemic insult. While other anatomical sites of the body are also exposed to high dose glucocorticoids in the circulation, osteonecrosis develops preferentially in the femoral head, often with bi-lateral hip involvement [21]. It's thought that the high-pressure anatomical location of the femoral head, the intricate blood supply of the area, and the specific glucocorticoid response profile of the cells resident in the femoral head combine to cause osteonecrosis specifically at this site [3].

Anatomy & blood supply

The thighbone, clinically referred to as the femur, is the longest and strongest bone of the body. The proximal end of the femur consists of the greater and lesser trochanters, the femoral neck, and the femoral head- a nearly spherical bony structure that articulates with the acetabulum of the pelvis to form the hip joint [22] (**Fig. 2**). Due to its anatomical position, the femoral head is under the constant compressive forces of weight-bearing [23]. The profunda femoris artery feeds the deep tissue of the thigh. At the femoral neck, the profunda femoris branches into the lateral and medial circumflex arteries. From these vessels, the retinacular arteries branch out in an intricate, torturous pattern to feed the tissue of the femoral head.

Cells of the femoral head

A variety of cell types makeup the bone tissue of the femoral head. Osteoblasts are the major bone matrix forming cells, derived from mesenchymal stem cell progenitors. Once an osteoblast becomes surrounded by the bone matrix it had produced, it undergoes a terminal transition into an osteocyte, residing in a pit referred to as a lacuna within the matrix. Osteocytes comprise 90-95% of all bone resident cells. The activity of osteoblasts is countered by the action of osteoclasts, bone matrix resorbing cells derived from hematopoietic stem cell progenitors. A dynamic balance exists between the action of osteoblasts and osteoclasts to maintain bone tissue turnover and initiate repair responses when necessary [24]. The inner bone marrow compartment of the femoral bone contains mesenchymal stem cells, hematopoietic stem cells, and other stromal cells including adipose cells,

macrophages, and fibroblasts [25, 26] that also take part in regulating bone tissue biology. The endothelial cells that line the blood vessels feeding the femoral head play a major role in bone tissue hemostasis by regulating the tissue's blood supply.

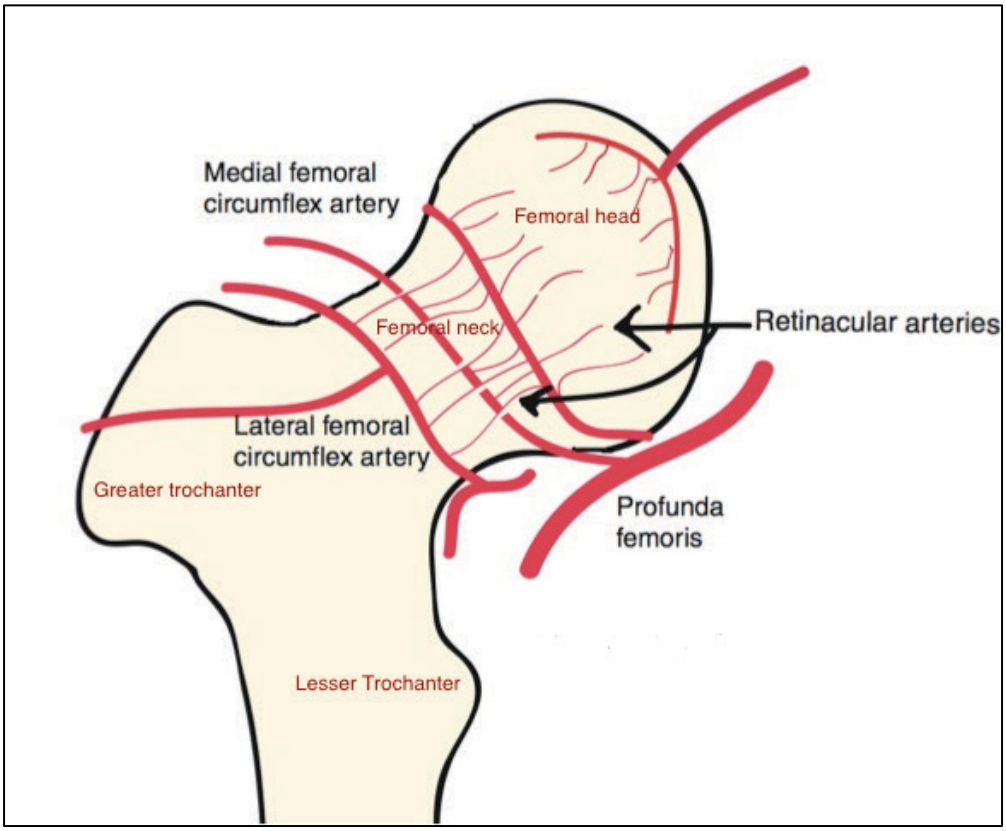

Figure. 2 Anatomy and blood supply of the femoral head. Together with the lesser trochanter, the greater trochanter, and the femoral neck, the femoral head marks the proximal end of the femur bone. The intricate retinacular arteries branch from the profunda femoris to provide the main blood supply of the femoral head. Adapted from Powell et al [7].

1.1.2 The Vascular hypothesis of ONFH etiology

Infarction of the retinacular arteries is the hallmark pathological feature of ONFH. Intricate with limited anastomoses (i.e., vessel reconnection points), the retinacular arteries are further predisposed to blockage due to the weight-bearing anatomical position of the femoral head [3, 7, 27, 28]. The concept of vascular obstruction as the main patho-mechanism underlying ONFH was put forth by Phemester et al in the early 1930's [29] and has been built upon by various investigators since. Upon performing vascular studies on early stage ONFH femoral heads, Atsumi et. al. detected impairments of blood flow through the retinacular arteries [27]. Starklint and colleagues performed a histological examination of 14 femoral heads with advanced ONFH and observed rampant vascular obstruction as well as elevated intra-femoral pressure associated with vascular blockade [30]. Intra-femoral pressure elevation in association with ONFH has also been reported by various other investigators and is thought to exacerbate ONFH pathology by further compressing the local vasculature and disrupting blood flow in the area [3, 31]. Disruption of blood flow through the retinacular arteries diminishes the nutrient supply of the femoral head, eventually leading to tissue ischemia and necrosis reflected by wide-spread bone cell death, inhibition of normal bone repair processes, eventually femoral head collapse and loss of joint function [3, 15].

1.1.3 Risk factors

While physical injury can directly cause femoral head vessel blockage, as is the case with trauma-induced ONFH, the pathology of non-traumatic ONFH is better understood through a multiple-hit model, whereby several risk factors converge to precipitate the disease [6]. Numerous risk factors associated with non-traumatic ONFH have been identified (**Table 1**), the strongest of which is glucocorticoid therapy. Other common risk factors include coagulation (i.e. blood clotting) disorders and alcoholism [1, 3, 7]. The potential pathological contributions of co-existing disease conditions such as hematologic malignancies or immune disorders confound the study of non-traumatic ONFH etiology, making it very difficult to tease out definitively causative factors. Nonetheless, it is agreed upon that though the various factors associated with ONFH are likely to act through separate mechanisms, their effects converge on a final pathway of vascular obstruction (**Fig. 3)**[32]**.**

Table 1. ONFH risk factors. Adapted from Choi et al. [1]

Trauma
Hip dislocation
Femoral neck fracture
Corticosteroid use
Solid organ transplantation
Bone marrow transplantation
Acute lymphoblastic leukemia
Alcohol consumption
Systemic lupus erythematosus
Coagulation disorders
Antithrombin III deficiency
Protein C deficiency
Protein S deficiency
Thrombocytosis
Disseminated intravascular coagulation
Human immunodeficiency virus (HIV) infection
Hemoglobinopathy
Sickle cell disease
Thalassemia
Polycythemia
Metabolic disease

Coagulation Disorders

The link between coagulation disorders and ONFH development is intuitive. To keep the blood clotting response in check, a fine balance must be maintained between the levels of coagulatory (i.e., clot-forming) and fibrinolytic (i.e., clot-resolving) factors. Acquired or genetic disturbances of this balance inevitably lead to a pathologic blood clotting response. Several studies have reported associations between ONFH development and heritable propensities towards clot formation and/or delayed clot resolution [14, 33-35]. Kim et. al. found significant associations between single nucleotide polymorphisms (SNPs) in the anti-fibrinolytic PAI-1 gene and the incidence of ONFH [14]. Glueck and his colleagues also reported a higher incidence of a PAI-1 polymorphism resulting in increased PAI-1 activity in ONFH patients vs. controls (41% vs. 20%, respectively) [34].

Glueck et al.'s genetic background analysis of a 244-patient ONFH group in a separate study uncovered a higher incidence of the pro-thrombotic factor V Leiden variant gene (9.3-9.6% depending on subgroup) in comparison to controls (1.9%) [36]. A mutation in the pro-thrombotic gene tissue plasminogen activator (tPA) was found at a higher frequency in ONFH patients than controls in a study by Gagala et al. [35]. Definitive conclusions linking ONFH incidence risk to certain genetic loci are difficult to make given the relatively low purported risk contribution of each loci and the sometimes conflicting findings of different studies. Nonetheless, the association between coagulopathies and ONFH is well established in the literature and is in line with the accepted vascular obstruction patho-mechanism model of ONFH.

Excessive alcohol intake

Excessive alcohol intake is thought to contribute towards ONFH development by deregulating lipid metabolism [7, 37]. In a rat-model of alcohol-induced ONFH, Wang et al. reported femoral head fat cell hypertrophy, a skewing of bone marrow mesenchymal stem cell differentiation towards the adipose (i.e., fat) lineage, and an increase in the level of blood triglycerides [37]. An increase in blood triglyceride levels is associated with an increased likelihood of fat emboli formation, akin to the patho-physiology of heart disease. Furthermore, fat cell hypertrophy within the femoral head causes elevated intra-osseous pressure, increasing the likelihood of pathologic clot formation [7].

Glucocorticoid treatment

Of the numerous risk factors contributing to ONFH development, high-dose glucocorticoid treatment remains the most strongly associated with non-traumatic ONFH development [6]. Studies of the patho-mechanism of glucocorticoid-induced ONFH indicate that glucocorticoids act through multiple pathways to trigger the disease. Several studies have shown that glucocorticoid administration influences the plasma levels of coagulation factors [7, 38, 39]. In a study by Van Giezen et. al , glucocorticoid administration increased rat plasma PAI-1 levels in a dose-dependent manner [40]. Van Zaane and colleagues performed a meta-analysis of 36 studies including clinical trials, cross-sectional studies, and drug interventions examining the effect of glucocorticoid administration on plasma haemostatic factor levels. Their analysis revealed a consistent inter-study up-regulation of plasma PAI-1 levels following glucocorticoid administration in an inflammatory setting [39].

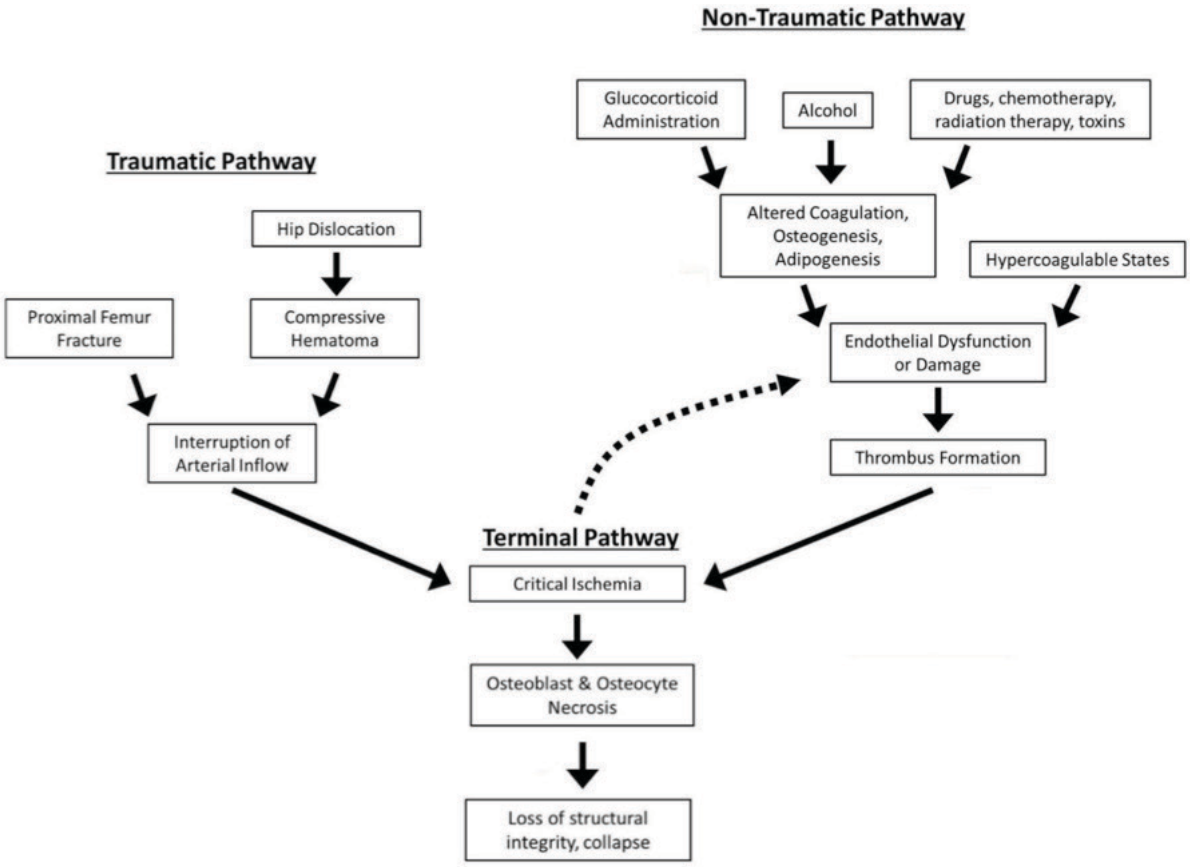

Figure 3. Hypothesis of ONFH etiology. It is thought that the different risk factors of ONFH act through multiple pathways to converge on a final pathway of endothelial dysfunction leading to thrombus formation, vessel obstruction, and eventually downstream tissue ischemia and femoral head collapse[32].

Using the Wistar Kyoto rat model of glucocorticoid-induced ONFH that our lab developed [41], our group demonstrated that glucocorticoid administration differentially increased the gene expression of coagulation regulator alpha-2-macroglobulin (A2M) in diseased rat hips. The microarray-detected increase in A2M was confirmed through quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry [15].

An *in vitro* study by our group demonstrated that high-dose glucocorticoids can directly modulate the expression of several endothelial haemostatic genes, up-regulating pro-coagulant genes tissue factor (TF) and von Willebrand factor (vWF) while down-regulating anti-coagulant thrombomodulin (TM) and pro-fibrinolytic urokinase plasminogen activator (uPA). High dose glucocorticoids also up-regulated cell adhesion molecules (CAMS), which translated functionally into increased neutrophil adhesion. Since neutrophil adhesion is a part of the inflammatory process, high-dose glucocorticoids appear to exacerbate inflammation at the endothelial surface [42].

Glucocorticoids have also been shown to contribute to ONFH development by deregulating fat metabolism through mechanisms similar to those observed in the molecular pathology of alcohol-induced ONFH. Wang and colleagues reported a 25% increase in femoral head fat cell numbers in steroid-treated rabbits [43]. Motomura and colleagues also used a rabbit model of ONFH to examine the patho-mechanism of glucocorticoid administration and found dose-dependent elevations in plasma triglyceride levels [44]. Li and colleagues reported a skewing of mesenchymal stromal cell differentiation away from the osteogenic and towards the adipose lineage [45]. Such changes would decrease the reservoir of potential osteoblasts available to participate in bone maintenance and instead result in an increase in fat cell numbers and thus femoral head pressure. Glucocorticoids also have numerous effects on the cells of the bone. Collectively, glucocorticoids suppress osteoblast, osteoclast, and osteocyte generation, prolong the lifespan of differentiated osteoclasts, and trigger osteoblast and osteocyte apoptosis [46, 47]. All in all, the multi-varied nature of glucocorticoid action renders it particularly challenging to attenuate the progression of glucocorticoid-induced ONFH.

1.1.2 Diagnosis

Early stage ONFH is often asymptomatic. Late stage ONFH presents as pain in the groin region associated with limited hip mobility [28, 48]. Radiographs and magnetic resonance imaging (MRI) are the main diagnostic modalities used to detect ONFH, with MRI being the gold standard. Using MRI, a rimmed, band-like lesion with low signal intensity is indicative of the disease (**Fig. 4**) [1, 49]. Various staging systems have been developed to guide disease prognosis and treatment. One of the earliest of these systems was developed by Arlet and Ficat and relied on radiographic changes coupled with femoral head vascular studies and intra-femoral pressure measurements [50]. Following the advent of MRI, Steinberg and his colleagues at the University of Pennsylvania developed an improved system incorporating MRI use to allow for earlier detection and a more thorough evaluation of the size and location of the necrotic lesion, waiving the need for invasive vascular studies and intra-femoral pressure measurements. In the Steinberg staging system (alternatively known as the University of Pennsylvania staging system), hips are staged in progressively stages from 0 to VI, depending on the extent of the necrotic lesion [51]. A group of researchers from The Association Research Circulation Osseous (ARCO) developed yet another modified classification system that includes histological evaluations [5]. Currently, there is no consensus in the literature on the use of a given staging system [52], which hinders inter-study comparisons of findings and renders ONFH knowledge integration a difficult task.

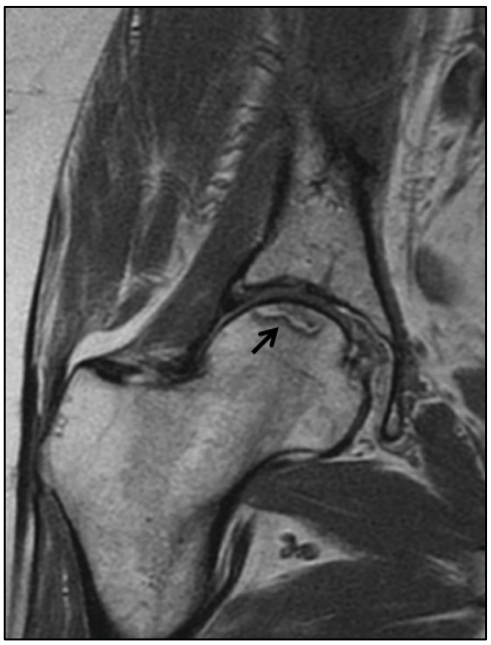

Figure 4. MRI of a femoral head with ONFH. A T2-weighted MRI image of a femoral head displaying the characteristic band-like lesion indicative of ONFH. Adapted from Choi et. al [1].

1.1.4 Treatment options

Current treatment options for ONFH are mostly palliative and do not address the underlying pathological disease processes. Non-surgical management involves lesion observation and limiting weight-bearing through crutches [48]. Bone decompression is the most common therapy for early stage ONFH [50]. It involves drilling into the diseased femoral head to alleviate the increased intra-femoral pressure and improve local blood flow. A meta-analysis of literature reports by Mont et al. revealed a 63.5% clinical femoral head improvements compared with 22.7% in patients [53]. Stulberg and colleagues further confirmed these findings with a randomized control study. Stulberg assigned 55 diseased hips to either a core decompression group or a non-operative group that used non-weight-bearing through crutches. Stulberg and colleagues then found that bone decompression lead to significant clinical improvements over non-operative treatment [54].

End-stage disease, defined by femoral head collapse, requires total hip replacement surgery. Clinically termed arthroplasty, the surgery involves removing the diseased tissue and installing artificial implants. The implants are not permanent and require multiple replacements. Furthermore, the surgery success rate is lower in ONFH patients versus other patient populations and it is hypothesized that inherent aspects of the disease pathophysiology hinder implant integration [31]. Since ONFH mostly afflicts children and relatively young individuals, multiple replacement surgeries are thus required over an individual's lifetime.

Therapies targeting early stage disease are currently emerging. Mesenchymal stem cell therapy has shown particular promise, having the potential to ameliorate multiple aspects of the underlying disease pathology by promoting new vessel formation and improving tissue repair. Its been reported that the femoral head mesenchymal stem cell pool is depressed in ONFH patients. As such, mesenchymal stem cell therapy also provides an additional source of cells with osteoblastic differentiation potential that would mitigate ONFH pathology [31, 55]. In spite of these advances in ONFH therapy, progress in preventing the disease is slow and understanding of the underlying disease pathology and the associated vascular changes would be of tremendous help in disease prevention efforts.

1.2 Glucocorticoid Therapy: A double-Edged Sword

Glucocorticoids are cholesterol-derived hormones secreted by the adrenal glands at levels regulated by the hypothalamic-pituitary-adrenal (HPA) axis in relation to circadian rhythms and stress levels. The major glucocorticoid secreted in man is cortisol, while rodents predominantly secrete corticosterone. The availability of endogenous glucocorticoids is tightly regulated through binding to corticosteroid-binding globin in the blood and by processing via 11b-hydroxysteroid dehydrogenase (11b-HSD) enzymes in tissues. Humans produce an average of 12.8mg of cortisol per day, resulting in a concentration of $7x10^{-7}$M at peak levels [56]. Glucocorticoids have wide-ranging effects on multiple physiological systems including the metabolic, nervous, reproductive, and immune systems. The anti-inflammatory, immune-suppressive, and anti-proliferative effects of glucocorticoids have rendered glucocorticoid treatment a main therapy for a variety of autoimmune disorders as well as an adjunct therapy for graft-transplantations and various hematologic malignancies [57]. Potent synthetic glucocorticoids such as dexamethasone, prednisone, and prednisolone are administered in doses as high as 40mg/day or even greater, sometimes for prolonged periods [21].

1.2.1 Glucocorticoid mechanisms of action

Glucocorticoids exert their effects through both genomic (i.e. gene expression modulating) and non-genomic mechanisms, resulting in a wide range of effects over multiple organ systems. The two modes of action differ in their speed and the downstream effectors they utilize (**Fig. 5**). Within the broader genomic and non-genomic mechanism categories, further variation exists in terms of effectors/signaling pathways, such that glucocorticoid treatment often causes numerous complex effects that are difficult to study in isolation.

1.2.1.1 Signaling through the glucocorticoid receptor

Signalling through the glucocorticoid receptor (GR) is considered the canonical mode of glucocorticoid action. The GR belongs to the super-family of ligand-inducible transcription factors. In the absence of glucocorticoid ligand, the GR resides in the cytoplasm bound to the heat shock protein-90 (HSP90) chaperone.

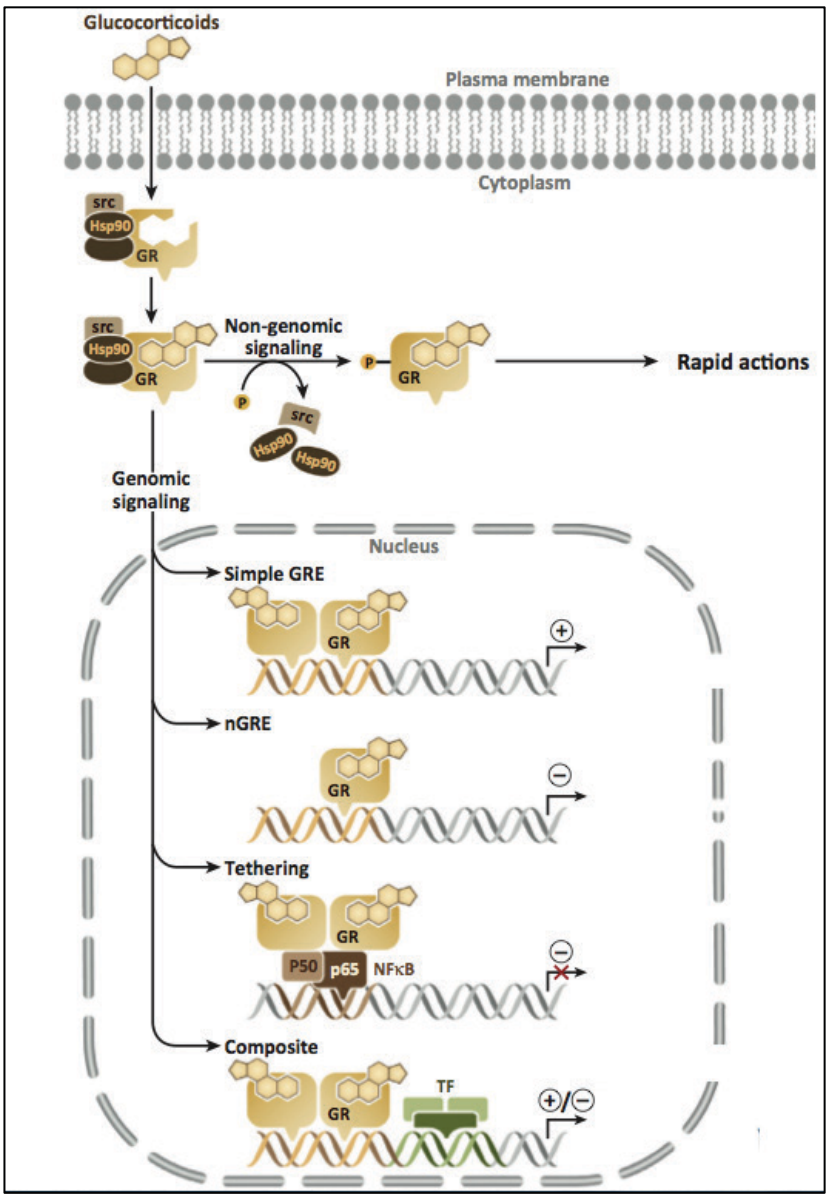

Figure 5. Glucocorticoid mechanisms of action. Glucocorticoids act through both genomic and non-genomic pathways. The genomic pathway involves signaling through the glucocorticoids receptor (GR). The GR translocates into the nucleus upon ligand binding. It homo-dimerizes and acts as transcription factor, binding glucocorticoid response elements (GREs) or negative glucocorticoid response elements (nGREs) to induce or suppress expression, respectively. Alternatively, glucocorticoids can also act through on-genomic pathways, activating or suppressing signaling through a wide range of signaling cascades, inducing rapid outcomes. Adapted from Kadmiel and Sidlowski [57].

Ligand binding induces a conformational change in the GR that allows it to dissociate from the HSP90 chaperone and translocate into the nucleus. There, it homo-dimerises with another glucocorticoid-bound GR to modulate the transcription of specific target genes. The GR recognizes consensus 6-base pair palindromic sequences. Sequences driving gene expression are referred to as glucocorticoid response elements (GRE), while those driving gene repression are referred to as negative glucocorticoid response elements (nGRE). GR bound to the consensus sequence then recruits co-activators/co-repressors to modulate the transcription of the target gene. The GR can also modulate the expression of target genes indirectly by interacting with transcription factors already bound to the DNA [57, 58] (**Fig. 5**).

1.2.1.2 Non-genomic glucocorticoid effects

In addition to regulating transcription, glucocorticoids can exert their effects in a more rapid manner by binding to as of yet unknown membrane receptors and modulating different signaling cascades. The signaling effects of glucocorticoids are independent of the action of the GR, although the two can occur in parallel. Glucocorticoids have been shown to activate signaling through the PI3K-Akt, ERK, and G protein-coupled receptor cascades. The rapid effects of glucocorticoids do not necessarily have to be in the same vein as the GR-mediated genomic effects of glucocorticoids. For instance, glucocorticoids have been shown to rapidly supress the stimulated release of insulin, which is in direct opposition to their transcriptionally mediated insulin increase [59].

1.2.2 Glucocorticoid excess in disease

Given their far-reaching effects, it's not surprising that disturbances in the levels of glucocorticoids have been associated with various disorders. Abnormally elevated levels of glucocorticoids have been associated with the development of Cushing's Syndrome, characterized by a suite of symptoms including central obesity, high blood pressure, thinned skin, glucose intolerance, depression, and sleep disturbances. In addition to the development of ONFH, exogenous glucocorticoid excess has been associated with hypertension, osteoporosis, muscle atrophy, and peripheral insulin resistance [60, 61].

1.3 Endothelium: A Dynamic Organ System

The Endothelium is the cellular lining of blood vessels. Due to its heterogeneity, vast spread across the body, and its ability to perform a wide array of functions, the endothelium has been viewed a bona fide organ system [62-64]. Developmentally, the endothelium arises from the mesoderm embryonic layer. Embryonic stem cells in the mesoderm differentiate into a common hematopoietic/endothelial precursor termed the hemangioblast, which then further differentiates into either a hematopoietic stem cell progenitor or an angioblast which has endothelial differentiation potential [65, 66]. The angioblast is then guided along the various differentiation stages of the endothelial lineage by the concerted action of a wide range of transcription factors, molecular effectors, and signal-transduction pathways. These include but are not limited to the molecular effectors fibroblast growth factor 2 (FGF-2), Indian hedgehog (IHH), bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor (VEGF); transcription factor family E-twenty six (ETS); and the Notch and Wnt singling pathways [67-72]. The aforementioned fate-determining transcription factors and molecular effectors are tightly regulated in space and time, and vary depending on the local environment of the progenitor cells, leading to the formation of heterogeneous endothelial populations. In spite of their marked heterogeneity, endothelial cells can be defined by their expression of a panel of markers most commonly comprised of the CD31, CD34, and vWF antigens [73, 74].

1.3.1 Endothelial Heterogeneity

The aforementioned endothelial heterogeneity encompasses gene expression, phenotype, and function. Many endothelial genes are vascular bed-specific, being expressed in response to stimuli specific to the local environment of the endothelial bed [68]. The alignment of endothelial cells varies in response to sheer stress in accordance to the local direction of blood flow. The continuity of the endothelial monolayer is also a variable phenotypic feature. The endothelium can be continuous, fenestrated with pores, or discontinuous with large gaps, depending on the type of junctions between adjacent cells [75, 76]. Three types of endothelial junctions have been described: tight junctions, adherens junctions, and gap junctions[77]. Adherens junctions are constitutively expressed in all endothelial beds and lend the endothelium its barrier function. Tight junctions impart an impermeable feature to the endothelium and are mainly expressed in specialized endothelial beds such as the blood-brain or the blood-retinal barriers. The more leaky gap junctions are found in the

vascular beds of the liver and kidney where extensive nutrient and cellular trafficking needs to occur [78]. Thus, as can be evident, endothelial heterogeneity arises to meet the unique functional requirements of the endothelium in a given part of the body [75, 76].

The unique properties of endothelial cells from different vascular beds render it difficult to draw uniform, overarching conclusions regarding endothelial cell behaviour. On the other hand, it is not always feasible to study endothelial cells from particular cell beds due to difficulty of isolation, as is the case with endothelial cells of the bone vasculature. Human umbilical vein endothelial cell (HUVEC) cultures have bridged this gap in the endothelial biology research due to their easy isolation, relatively high proliferation rate, and faithful recapitulation of characteristic endothelial features. HUVEC-based *in vitro* studies have provided the basis of many breakthrough findings into endothelial biology [79, 80].

1.3.2 Endothelial Functions

Blood to tissue trafficking

Long thought of as a merely passive vascular coating, emerging reports paint a picture of the endothelium as an active participant in the health and disease processes of the vasculature and the organs it feeds. As the interface between the blood and the underlying tissues, the endothelium acts as a semi-permeable membrane, controlling the trafficking of cells, nutrients, and other effectors from the blood [77, 81, 82]. Passage across the endothelium can occur either through cells, a process termed transcytosis, or between cells, a process termed paracytosis [75]. While fluids and small solutes can generally diffuse passively through the endothelium, the passage of cells and macromolecules through the endothelium is an active process that requires energy expenditure and the engagement of various receptors. Movement across the endothelium is determined in part by the types of junctions between the cells of the endothelial monolayer. As described above, the specific type of junctions in an endothelium varies depending on the functional requirements of a given vascular bed. Another determinant of cross-endothelial movement is the stimulus-dependent expression of cell adhesion receptors. This property is particularly important for leukocyte and immune cell honing to sites of injury. Endothelial cells at injury sites produce various cytokines and chemokines to attract leukocytes, immune effectors, and platelets, initiating various inflammatory

and immune responses to injury. These cytokines also act in a paracrine fashion, inducing the expression of adhesion molecules on neighbouring endothelial cells. Endothelial cell adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) are expressed at low levels under normal physiological conditions but undergo rapid up-regulation upon endothelial stimulation. The interactions between these receptors and their corresponding ligands on target leukocytes and immune effectors mediate cell extravasation through the endothelium into the injured tissue [75].

Regulation of vasomotor tone

The endothelium also acts as a sensor, detecting changes in oxygen levels, metabolite amounts, and shear stress, which act as a proxy for the metabolic state of the tissue. The endothelium then produces potent mediators that act on the underlying vasculature to regulate flow in response to local tissue needs. These endothelium-derived mediators can have a dilatory effect on the underlying blood vessels, as is the case with nitric oxide and prostacyclin, or they can have a constrictive effect as is the case with the mediators thromboxane and endothelin-1 [83].

Regulation of blood clotting

The endothelium plays a key role in stemming blood loss from sites of vascular injury through blood clotting. Vascular injury induces the formation of blood clots which act as plugs that stem blood flow and allow healing to begin [84]. However, the body also initiates a complementary clot lysis response that ensures blood clots are dissolved once they have served their function [85]. For the response to remain adaptive, a very delicate balance must be maintained between the action of clotting and fibrinolytic factors, a process termed haemostasis. If the aforementioned balance is disturbed, various pathologies arise including haemophilia, atherosclerosis, and the focus of our current study: ONFH [3, 85, 86].

1.3.2.1 Haemostasis

Haemostasis is regulated by the concerted action of endothelial cells, platelets, and humoral coagulation and fibrinolysis enzymes [84]. Vascular injury exposes circulating platelets to sub-endothelial structures, leading to platelet activation and adhesion to sites of injury. Activated platelets release a variety of effectors that promote further platelet aggregation. Vascular injury also induces the activation of the coagulation cascade, which produces fibrin to reinforce the platelet plug. Fibrin is produced by the liver and travels in the blood as the inactive zymogen fibrinogen. Cleavage of fibrinogen by the enzyme thrombin leads to the formation of rapidly aggregating fibrin polymers; the final event of the coagulation cascade [84]. The coagulation cascade describes a series of coupled reactions by which a **serine** protease is activated by cleavage and is then free to activate the next enzyme in the cascade. Serine proteases are referred to as such due to the presence of a serine residue in the active site of the protease [87]. The stepwise nature of the coagulation cascade enables the body to fine-tune and amplify the response to injury.

The classical paradigm of coagulation posits that activation can occur through two different avenues; the intrinsic and the extrinsic pathways. In the **intrinsic** pathway, contact of the blood with sub-endothelial collagen fibers leads to activation of factor **XII**. Factor XII in turn activates factor **XI**, which itself activates factor **IX**, and the latter then activates factor **X**. By contrast, the **extrinsic** pathway of blood coagulation is initiated when factor **VII** interacts with **TF** expressed in the sub-endothelial space to activate factor **IX,** which in turn activates factor **X** with the help of cofactor **VIII** produced by the endothelium. As might be evident, factor X represents the point of convergence of the two pathways. It activates factor **II**, the serine protease otherwise known as thrombin, with the help of endothelium-derived cofactor **V**. The activated thrombin then proceeds to cleave fibrinogen into fibrin polymers as mentioned above [84, 86, 88, 89] (**Fig. 6**). Although the early enzymes involved differ between the two, in both pathways of coagulation a breach of endothelial integrity is what provides the initial stimulus for coagulation.

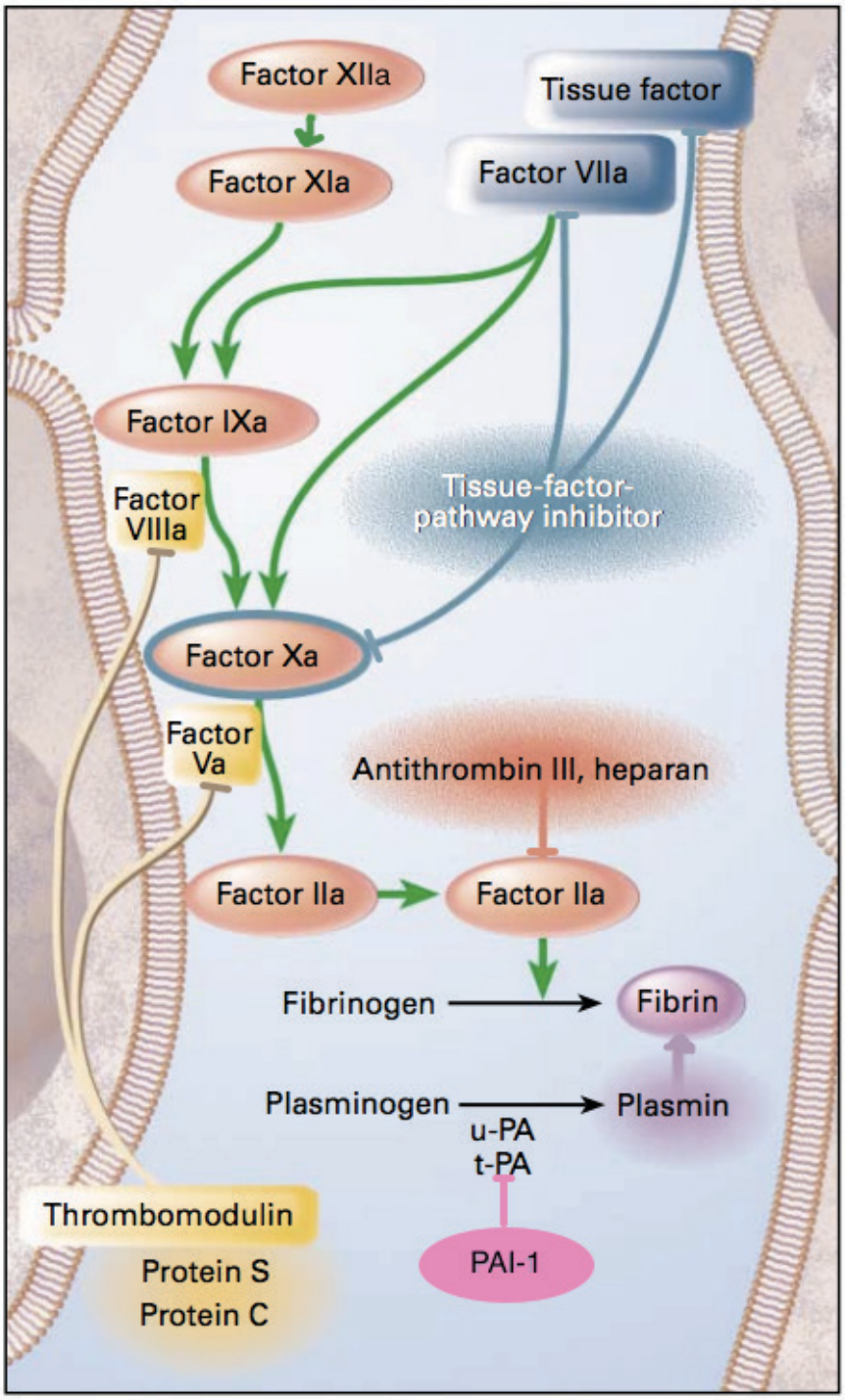

Figure 6. Haemostasis. Haemostasis is a balance between the two arms of coagulation and fibrinolysis. The processes of coagulation and fibrinolysis involve multiple steps that allow the fine-tuning of the haemostatic response. A balance must exist between the two processes in order for haemostasis to remain beneficial. Adapted from Rosenberg and Aird [88].

Natural inhibitors exist at each step of the coagulation cascade to fine-tune and regulate the response. Anti-thrombin III and heparan sulphate act in concert to inhibit thrombin. The tissue-factor-pathway inhibitor forms a complex with TF, factor VII, and factor X, inhibiting their function. Furthermore, endothelial cells express TM, a cell surface protein capable of binding thrombin and modifying its function so that it assumes an anti-clotting role. TM-bound thrombin activates protein C, a serine protease which inhibits cofactors V and VIII necessary for coagulation [88].

In addition to the action of the aforementioned coagulation inhibitors, the process of fibrinolysis acts in direct opposition of coagulation, targeting its final product, the fibrin clot. Fibrinolysis (i.e., fibrin lysis) occurs mainly through the action of plasmin, a serine protease that degrades fibrin into small fragments. Plasmin is activated from its plasminogen precursor by serine proteases uPA and endothelium-derived tissue-type plasminogen activator (tPA). tPA is thought to be more relevant to vascular fibrinolysis[90]. Both tPA and uPA are inhibited by PAI-1, a serine protease inhibitor that is produced by the endothelium, liver, and adipose tissue[87] [85, 91, 92].

1.3.3 Endothelial activation and dysfunction

In response to inflammatory stimuli, endothelial cells modify their expression profile towards a leaky, pro-adhesive, and pro-coagulant phenotype. This 'activation' response occurs over a continuum, rather than being an 'all-or-none' phenomenon [93]. Endothelial responses to a given stimuli can vary depending on the activation state of the cells. Common endothelial activators include lipopolysaccharide (LPS), TNFα, and interleukin -1 (IL-1).

Endothelial activation does not necessarily result in endothelial dysfunction. Endothelial activation is an important component of the adaptive physiological responses of wound healing, angiogenesis, and host defense against infections. It is only when activation is injurious to the host (e.g. in cases of maladaptive chronic activation) that the term dysfunction applies; as is the case when the response is chronic or mismatched in magnitude, timing, or location. Taking the aforementioned points into account, endothelial dysfunction can be thought of as a maladaptive change in the expression profile of endothelial cells [62, 93]. The endothelium is the first layer exposed to systemic stimuli. As such, it's the most susceptible to dysfunction and it play a pivotal role in the pathophysiology of numerous diseases such as atherosclerosis, cancer, and most relevant to this study, ONFH.

1.4 Haemostatic Regulator Alpha-2-Macroglobulin (A2M)

Alpha-2-Macroglobulin (A2M) is a 720 kilodalton homo-tetrameric glycoprotein produced predominantly by the liver, but also by astrocytes, macrophages, monocytes, and endothelial cells [18, 94-96]. A2M acts as a broad-spectrum protease inhibitor that is unique in its 'cage-like' mechanism of inhibition. It is capable of inhibiting all four major types of proteases: metallo, serine, aspartic, and thiol proteases [2, 97]. The production of A2M differs between the human and the rat; an animal model frequently used in A2M studies. Both rat and human A2M become elevated in response to interleukin-6 (IL-6)-mediated inflammation. But while human A2M baseline production is very robust, with A2M plasma concentration hovering around 2μM [98], rat A2M is classified as an acute phase reactant whose baseline plasma levels are low, hovering around 40nM [99]. In humans, A2M levels are highest during childhood but decline steadily to adult baseline levels [100]. The importance of maintaining A2M levels within normal physiological ranges is underscored by the variety of diseases associated with imbalances in A2M. Abnormal A2M production has been associated with Alzheimer's disease, rheumatoid arthritis, glaucoma, and thrombotic disorders [19, 101-103]. The diversity of the disorders A2M is implicated in is a testament to its broad inhibitory activity. Intriguingly, however, A2M knockout mice models appear phenotypically normal but are more prone to diet-induced acute pancreatitis [104].

1.4.1 Activation and function

Evolutionarily, A2M developed as a part of the innate immune defense repertoire, providing broad-spectrum defense by neutralizing various proteases released by invading pathogens [2, 105]. Each A2M subunit has a 'bait region'; an amino acid sequence that is highly susceptible to cleavage by proteases. Cleavage of this region induces a conformational change that entraps the protease, sterically preventing it from accessing its substrates (**Fig. 7**). The conformational change also unveils A2M's receptor binding site, 'activating' A2M for receptor binding. Although A2M has four bait regions, it is functionally capable of binding two small weight proteases or one large protease. In addition to protease cleavage, A2M can also be activated by reaction with primary amines such as methylamine, which interact directly with the bait region to induce the activated A2M conformation.

Figure 7. A2M activation. Protease binding to A2M induces a conformational change that reveals A2M receptor-binding sites and activates it for receptor binding. Adapted from Rehman et al. [2]

Methylamine-activated A2M triggers equivalent signaling events as protease-activated A2M [106]. A2M also has a distinct cytokine/growth factor-binding site that remains accessible in all of its conformations. This site allows A2M to act as a cytokine/growth factor carrier [2]. A2M binds a wide range of cytokines and growth factors including transforming growth factor beta (TGF-β), nurotrophin-4, and nerve growth factor beta (NGF-β). A2M's carrier effects vary and can be stabilizing or destabilizing depending on the cytokine and A2M's conformation [20].

1.4.2 A2M Signaling

Activated A2M-protease complexes are cleared up within a few minutes by A2M's endocytic receptors [2]. The complexes are taken up into lysosomes and subsequently degraded [107, 108]. Two A2M receptors have been identified to date; the low-density lipoprotein receptor-related protein 1 (LRP1), and cell-surface-associated glucose-regulated protein-78 (GRP78). GRP78 normally functions as an endoplasmic-reticulum protein chaperone but has been detected on the cell surface as a receptor in a variety of cancer cells and in activated macrophages [106]. By contrast, LRP1 is a constitutive cell surface receptor expressed broadly across multiple tissues [109]. LRP1 is a 600 kilodalton protein comprised of two polypeptide chains associated through non-covalent interactions. It belongs to the low-density lipoprotein (LDL) receptor family and is a promiscuous receptor with more than 30 ligands identified to date [107].

Previously, the degradation of A2M-protease complexes was thought to be the only outcome of activated A2M uptake. However, emerging reports have shown that the binding of activated A2M to its receptors can induce a wide array of signaling events through the PI3K-Akt, ERK 1/2, and MAPK pathways, resulting in significant modulation of cellular responses [17, 18, 20, 110]. Misra and colleagues investigated the effect of activated A2M signaling in prostate cancer cells. They report that treatment of cells from the 1-LN prostate cancer cell line with 50pM of activated A2M resulted in an increase in the activation of the Akt, ERK1/2, and p38 MAPK kinases as well as the NF_KB transcription factor activity. GRP78 gene silencing attenuated the observed Akt and NK_KB activation. Signaling through the Akt, ERK 1/2, and p38 MAPK kinases has a proliferative effect, while NF_KB signaling is known to be anti-apoptotic. Thus, A2M signaling collectively has a pro-proliferative, anti-apoptotic effect on tumor cells [111].

A2M signaling has also been shown to regulate cellular responses under normal physiological conditions. Caceres et. al. showed that in the J774 and Raw264.7 macrophage cell lines, A2M signaling through LRP1 induced the production of matrix metalloproteinase-9 (MMP9). Further experiments revealed that the observed induction of MMP9 was mediated by signaling through the MAPK-ERK1/2 and NF_KB pathways [18]. It is worth noting that A2M is also capable of enzymatically inhibiting MMP9 [112], illustrating an important point about A2M's different functionalities; namely that they can be antagonistic. This concept is elegantly illustrated by the work of Mantuano et. al. To study the signaling and growth carrier functions of A2M in isolation,

Mantuano and her team expressed the A2M growth factor carrier site and the LRP1 recognition domain as separate fusion proteins. The team used PC12 cells, a model system for neuronal differentiation, to examine the activity of the fusion proteins on neuronal differentiation, as measured by neurite outgrowth. Nerve growth factor-β, a known neuronal differentiation-promoting factor, was used as a positive control. Mantuano and her team found that the fusion protein containing the LRP1 binding domain activated the Akt and ERK/MAP Kinases, as did NGF-β. The LRP1 binding domain fusion protein also promoted neurite outgrowth similarly to NGF-β. Interestingly, however, although the fusion protein containing the growth carrier site had no effect on its own, it abolished the effects of NGF-β when they were added together [20]. This seminal study made it clear that A2M signaling is an important facet of the action of A2M and a holistic consideration of all of A2M's functionalities is needed in order to appreciate it's overall effect on cellular responses.

1.4.3 A2M in Haemostasis

Most of what is known about A2M's role in haemostasis relates to its enzymatic inhibitory action. As a broad-spectrum protease inhibitor, A2M inhibits several proteases belonging to both the coagulation and fibrinolysis pathways, including thrombin, the tPA/uPA plasminogen activators, plasmin, and APC (**Fig. 8**) [19, 113]. As such, A2M's haemostatic profile possesses both anti-coagulant and anti-fibrinolytic features and the net effect of A2M on haemostasis depends on the physiological context and the amount of A2M present.

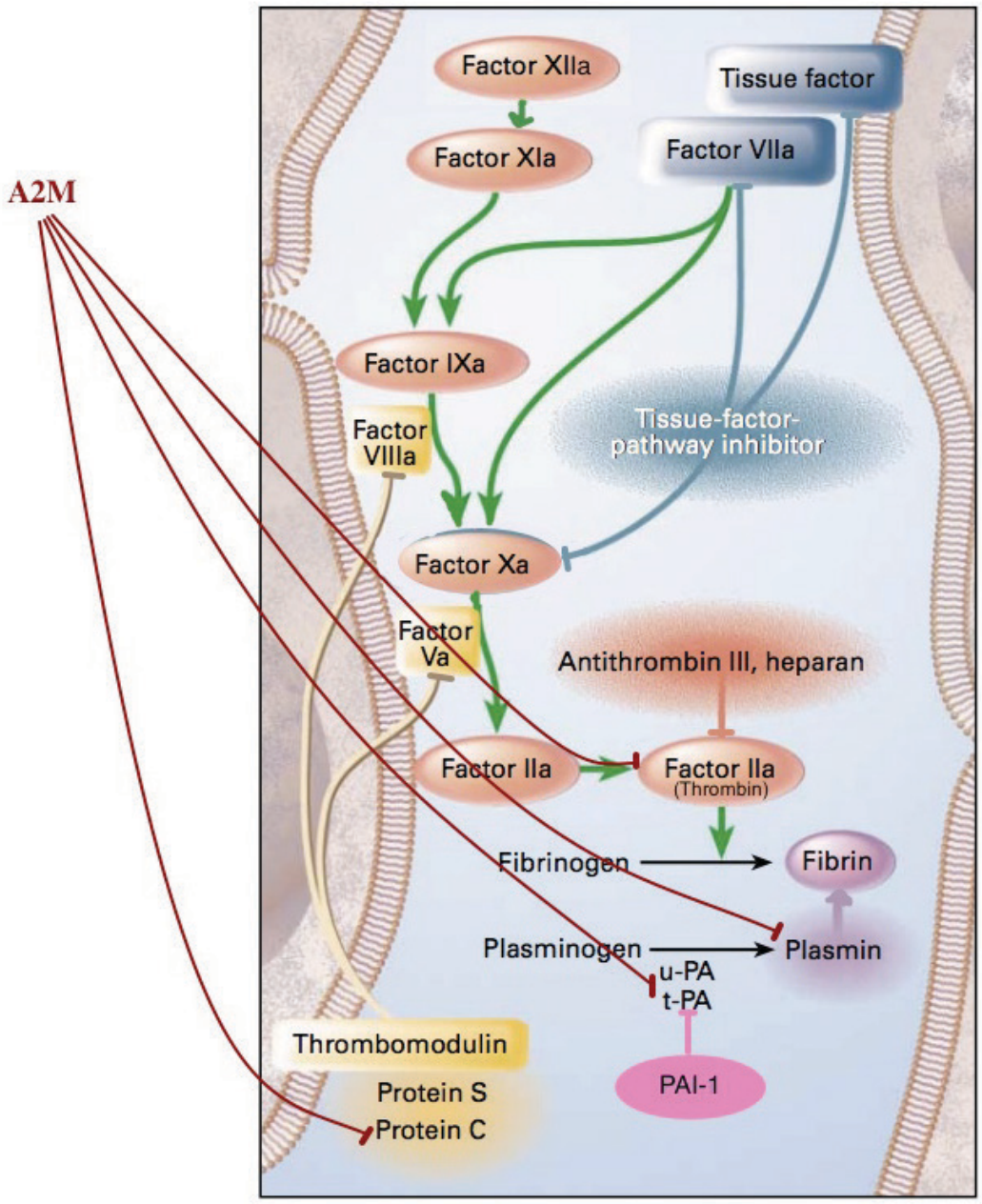

Figure 9. A2M in haemostasis. A2M inhibits proteases from both the coagulation and fibrinolysis arms of haemostasis. A2M exerts anti-coagulatory effects by inhibiting thrombin. However, A2M also inhibits APC, exerting a pro-coagulatory effect. It inhibits fibrinolysis both directly through inhibition of plasmin, and indirectly, through inhibition of plasminogen activators tPA and uPA. As such, an evaluation of A2M's net impact must take into consideration the physiological context and the levels of A2M. Figure adapted from Rosenberg and Aird [114].

The complexity of A2M's role in haemostasis can be appreciated further by pondering the various intriguing reports on anti-thrombin III deficiency in children. Anti-thrombin III is the main inhibitor of thrombin, accounting for 80% of anti-thrombin activity in the blood [115]. Interestingly, children with congenital anti-thrombin III rarely experience coagulation problems, with such issues usually commencing in adulthood. As mentioned previously, A2M levels are elevated in children compared to adults, and it has been suggested that the increased levels of A2M compensate for the anti-thrombin III deficiency through increased inhibition of thrombin. This theory is supported by the work of Mitchell et al. [100], who examined the thrombin inhibitory capacity of plasma from anti-thrombin III deficient adults and children compared with age-matched controls. As expected, they found that the plasma of adults with the deficiency inhibited significantly less thrombin than both controls and children with the deficiency. Furthermore, they reported that the plasma of children with anti-thrombin III deficiency inhibited normal amounts of thrombin relative to controls. They also reported that within anti-thrombin III deficient subjects, both children and adults, the levels of A2M highly correlated with the amount of inhibited thrombin. Based on these findings Mitchell and colleagues concluded that in anti-thrombin III deficient subjects, A2M plays a protective, anti-coagulant role.

It is important, however, to note that the anti-coagulant action of A2M is context-specific. This is highlighted by Cvirn et al.'s work, which showed that increasing the amount of A2M in umbilical cord and adult plasma containing normal levels of anti-thrombin III had no effect on thrombin inhibition [116]. Furthermore, in subsequent work Cvirn et al showed that increasing the concentrations of enzymatically active A2M in normal umbilical cord and adult human plasma resulted in a dose-dependent decrease of APC anti-coagulant activity [98]. Cvirn's work indicates that under normal physiological conditions, elevated levels of A2M have a pro-coagulant effect through inhibition of APC. This conclusion is also supported by the work of Beheiri and colleagues, who reported that children with deep vein thromboembolism have significantly higher levels of A2M than in age-matched controls [19].

Collectively, the aforementioned research reports imply that beyond normal physiological levels, A2M acquires pro-coagulant effects. This implication is in line with the evolutionary role of A2M in innate defense. In addition to increased neutralization of pathogen proteases, elevated levels of A2M are thought to increase pathogen capture by blood clots, thereby preventing systemic dissemination of pathogens. Chairkeeratisak and his colleagues studied the role of A2M in the response of *Penaeus monodon* shrimp to *Vibrio harveyi* bacterial infection and found that silencing the expression of shrimp A2M resulted in a significantly reduced ability of the clotting system to capture bacteria. They also reported 3.3 fold higher numbers of bacteria that systemically disseminated after infection [117]. Asokan and colleagues showed that A2M binds to clots in the *Limulus polyphemus* horseshoe crab and they posited that A2M might play a role in protecting the clot from lysis.

Overall, currently available data provides a compelling case for A2M's involvement in haemostasis as a protease inhibitor, but the impact of A2M signaling on haemostasis remains unexplored. Given the prominent role of A2M in haemostasis and the wide array of signaling cascades its can potentially activate, an investigation of the impact of A2M signaling in haemostasis is much required.

Chapter 2:
Study Rationale and Objectives

Glucocorticoid-induced ONFH is thought to be caused by impaired endothelial regulation of haemostasis. Our lab has previously shown *in vitro* that high dose glucocorticoid treatment induces an abnormal haemostatic expression profile favoring clot formation [42]. These findings illustrated that glucocorticoids can directly cause the endothelial dysfunction thought to underlie ONFH pathology. Subsequently, a microarray examination of gene expression changes in our rat model of glucocorticoid-induced ONFH revealed that the most significant change of expression in the hips of diseased rats was a 3.5 fold increase in the mRNA levels of the haemostatic regulator A2M [41]. Several reports have linked A2M to various coagulopathies. Another haemostatic regulator that has also been heavily implicated in the development of ONFH is fibrinolysis inhibitor PAI-1; work by several investigators has shown elevated PAI-1 levels in association with the disease [14]. Based on these collective findings, we sought to further explore the high dose glucocorticoid-induced endothelial dysfunction response, homing in on A2M and PAI-1. We also sought to investigate the potential role A2M plays in regulating PAI-1 gene expression as a facet of its haemostatic regulatory function. Given its unexplored potential, we focused on investigating the signaling action of A2M in isolation of its proteinase-neutralizing enzymatic function.

In summary, our objectives were to explore:

1. The effect of high dose glucocorticoids on the expression of endothelial A2M and PAI-1

2. If A2M plays a role in modulating PAI-1 gene expression in our glucocorticoid treatment model
3. If A2M signaling can directly impact endothelial PAI-1 gene expression in isolation of glucocorticoid treatment

Because endothelial responses can diverge between stimulated vs. un-stimulated settings, we tested both conditions.

Chapter 3:

Materials and Methods

3.1 HUVEC cell culture

HUVEC were purchased from Lonza (Allendale, NJ, USA) and expanded in endothelial growth medium (EGM-2; Lonza) at 37°C and 5% CO_2 conditions. The media was changed every other day. To passage cells, 90% confluent HUVEC were washed with HBSS (Lonza), detached through a 5-minute incubation with 0.25% Trypsin/EDTA solution (Lonza), and re-plated in T-75 flasks in 15ml of EGM-2 following trypsin neutralization by the addition of 2 volumes of trypsin neutralizing medium (TNS; Lonza). Cells were used in experiments at passage 5, and their growth and appearance monitored regularly using a light microscope.

3.2 RMEC cell culture

RMEC were purchased from VEC Technologies (Rensselaer, NY, USA) and expanded in MCDB131 complete medium (VEC Technologies) at 37°C, 5% CO_2. Culture vessels were coated with 10μg/mL fibronectin in HBSS at 4°C overnight which was aspirated prior to cell seeding. To passage cells, 90% confluent RMEC were washed with PBS (Lonza), detached through a 5- minute incubation with 0.25% Trypsin/EDTA, and the trypsin was then neutralized through the addition of 2 volumes of TNS. The growth and appearance of the cells was monitored regularly and the cells were used in experiments at passage 5.

3.3 Glucocorticoid treatment

Cells were seeded at a density of $2x10^5$ cells per well in 6-well plates (Costar, USA). 90% confluent cells were then treated with 1mM water-soluble dexamethasone (Sigma, Burlington, ON) for 48 hours, in serum starved media (Endothelial basal medium -2 (EBM-2) for HUVEC and basal MCDB131 for RMEC) with 0.5% serum. As an additional treatment condition, cells were also stimulated with 10ng/mL TNFα (Sigma) for the final 4 hours of the experiment to check for possible divergences between basal and stimulated endothelial responses to high-dose dexamethasone treatment.

3.4 Cell viability determination

To measure cell viability, cells were detached as described above and suspended in 2ml of appropriate media. 100µL of the mixture were incubated with an equal volume of 0.4% trypan blue (Sigma). Inclusion of trypan blue into the cell is a sign of compromised membrane integrity, indicative of cell death. Total live cells were counted using a hemocytometer and a cell counter (Coulter, Hialeah, FL, USA).

3.5 A2M siRNA transfection

Pre-designed *Silencer*® Select siRNAs were purchased from ThermoFisher Scientific (USA) and dissolved in RNase-free water to prepare 2µM working solutions. Lipid-based forward transfection was initially attempted with lipofectamine RNAiMAX reagent (ThermoFisher Scientific). However, the high associated cytotoxicity observed rendered the use of this reagent unfeasible, and sensitive primary-cell compatible HiPerFect reagent (Giagen, USA) was utilized instead. Cells were seeded in 6-well plates at a density of $1.5x10^5$ cells per well. After 24 hours, the media was changed to 2.3 ml fresh EGM-2 media without antibiotic. 12µl per well of 2µM A2M siRNA (Cat. # 4390824) non-targeting negative control siRNA (Cat. # 4390843), or GAPDH positive control siRNA (Cat. # 4390849) were diluted in 76µl per well of EBM-2 in 1.5 mL microfuge tubes. 12µl per well of HiPerFect reagent (Qiagen) were added and the mixture was gently vortexed then incubated for 10 minutes at room temperature before being added drop-wise to cells, for a final respective siRNA concentration of 10nM. The media was changed to fresh EGM-2 media after 4 hours. Cells were treated with dexamethasone 24 hours following transfection.

3.6 Activated A2M treatment effect

To examine the effect of A2M signaling on endothelial function, HUVEC were treated with activated A2M (BioMac, Germany). A2M activation was performed via the reaction of human plasma-purified A2M with methylamine, with a 100% supplier-guaranteed activation rate as measured by monoclonal antibody-based immunosorbent assays (BioMac). Since activation of A2M induces a conformational change that precludes substrate binding and exposes the receptor binding site, treatment with activated A2M allows for the specific examination of the signaling functions of A2M in isolation of its other protease-inhibitor function. In activated A2M treatment experiments,

90% confluent HUVEC in EGM-2 medium were treated for 24 hours with 100nM activated A2M reconstituted with PBS. For time-response experiments, cells were harvested following 3, 6, 12, or 24 hours of activated A2M treatment. For dose-response experiments, 90% confluent HUVEC were treated for 24 hours with 10, 20, 40, 60, 80, 100, 140, or 180nM activated A2M reconstituted with PBS.

3.7 RNA extraction and cDNA synthesis

Total RNA was isolated from cells using PureLink® RNA Mini Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Total RNA concentration and purity were measured using a Tecan Infinite M200 PRO instrument (Tecan, Männedorf, Switzerland) and analyzed with the i-control 1.9 software (Tecan). The Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used to reverse-transcribe 1µg of total RNA into complementary DNA (cDNA) in a Biometra thermal cycler (Biometra, Göttingen, Germany) according to kit instructions.

3.8 Semi-quantitative PCR

To assay prepared cDNA quality for downstream quantitative real-time polymerase chain reaction (qRT-PCR) applications, semi-quantitative PCR was performed for the GAPDH housekeeping gene using *Taq* DNA Polymerase with the associated buffers from ThermoFisher Scientific as well as the following primers:

Human:

Forward primer : GGAGCGAGATCCCTCCAAAAT

Reverse primer : GGCTGTTGTCATACTTCTCATGG

Rat:

Forward primer : ATCACTGCCACTCAGAAG

Reverse primer : AAGTCACAGGAGACAACC

The PCR reaction was run on a Biometra thermal cycler according to kit instructions. The resulting PCR products were run on a 1% agarose gel with SYBR® Safe DNA gel stain (ThermoFisher Scientific) at 100V for 40 minutes. The DNA bands were visualized by a UV trans-illuminator on an ImageQuant LAS4000 machine (GE Healthcare Life Sciences, Mississauga, Canada).

3.9 QRT-PCR evaluation of gene expression

QRT-PCR was performed on an Applied Biosystems StepOnePlus machine (Applied Biosystems, Carlsbad, CA, USA) in a 96-well, singleplex format.100ng cDNA per well (prepared as described above) were used with the TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific) and the appropriate TaqMan® Gene Expression Assay (Table 2). Relative gene expression was determined using the comparative threshold cycle (ΔΔCT) method, normalized against the hypoxanthine phosphoribosyltransferase 1 (HPRT1) housekeeping gene levels.

Table 2. TaqMan® Gene Expression Assays used in the study

Gene	Gene Assay Catalogue #
HPRT1	Hs02800695_m1
A2M	Hs00929971_m1
PAI-1	Hs01126606_m1

3.10 ELISA analysis of conditioned media protein levels

At the end of the glucocorticoid 48-hour treatment period, the conditioned media was collected and centrifuged at 12000x g for 5 minutes at 4° to pellet cell debris. The supernatant was stored at -80° until analysis. The levels of A2M in the conditioned media were evaluated using a Kamiya human A2M ELISA kit (Kamiya Biomedical Company, USA) as per the manufacturer's instructions. PAI-1 levels were assayed using a human PAI-1 platinum ELISA kit (eBioscience, USA) as per the manufacturer's instructions. Colorimetric absorbance was measured on a Tecan Infinite M200 PRO machine and analyzed with the i-control 1.9 software (Tecan).

3.11 Statistical analysis

Experiments were performed in triplicate and the results presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and *post hoc* Tukey tests were performed to examine the significance of any differences between treatment groups. $P<0.05$ values were considered significant for this study. All statistical analyses were performed using the Graphpad Prism 6 software (La Jolla, CA, USA).

Chapter 4:

Results

4.1 High Dose Glucocorticoid Regulation of A2M and PAI-1 Gene Expression in Basal and Stimulated Endothelial Settings

In this study, HUVEC were utilized to model endothelial responses due to their ease of handling, widespread use, and applicability to human disease. To examine the effect of high dose glucocorticoid treatment on the endothelial expression of haemostatic genes A2M and PAI-1, nearly confluent HUVEC were treated with 1mM of the synthetic glucocorticoid dexamethasone for 48 hours to simulate the chronic, high glucocorticoid exposure frequently encountered in a clinical setting. The dexamethasone dose was selected based on previous literature reports examining the *in vitro* effects of high dose glucocorticoid treatment [118, 119]. Since endothelial responses can be modulated by the cells' activation state, dexamethasone treatment effects were examined under both basal and TNFα-stimulated conditions, wherein 10ng/mL of TNFα were used to stimulate the cells during the final 4 hours of dexamethasone treatment.

Dexamethasone treatment caused a significant 3.5 fold up-regulation of A2M mRNA levels relative to untreated control HUVEC, as determined through qRT-PCR. 10ng/mL TNFα alone caused a down-regulation of A2M mRNA levels to 0.5 fold relative to control, but this effect did not reach statistical significance. The dexamethasone-induced A2M up-regulation was attenuated to 2.6 fold when cells were also stimulated with TNFα (**Fig. 9A**). TNFα stimulation alone caused a significant 3.5 fold up-regulation of PAI-1 gene expression. Dexamethasone treatment also up-regulated PAI-1 expression by 2.8 fold, but this effect only reached statistical significance upon activation of endothelial cells with TNFα, whereby the additive effect of both treatments reached a significant 4.7 fold increase in PAI-1 mRNA levels in comparison to control (**Fig. 9B**).

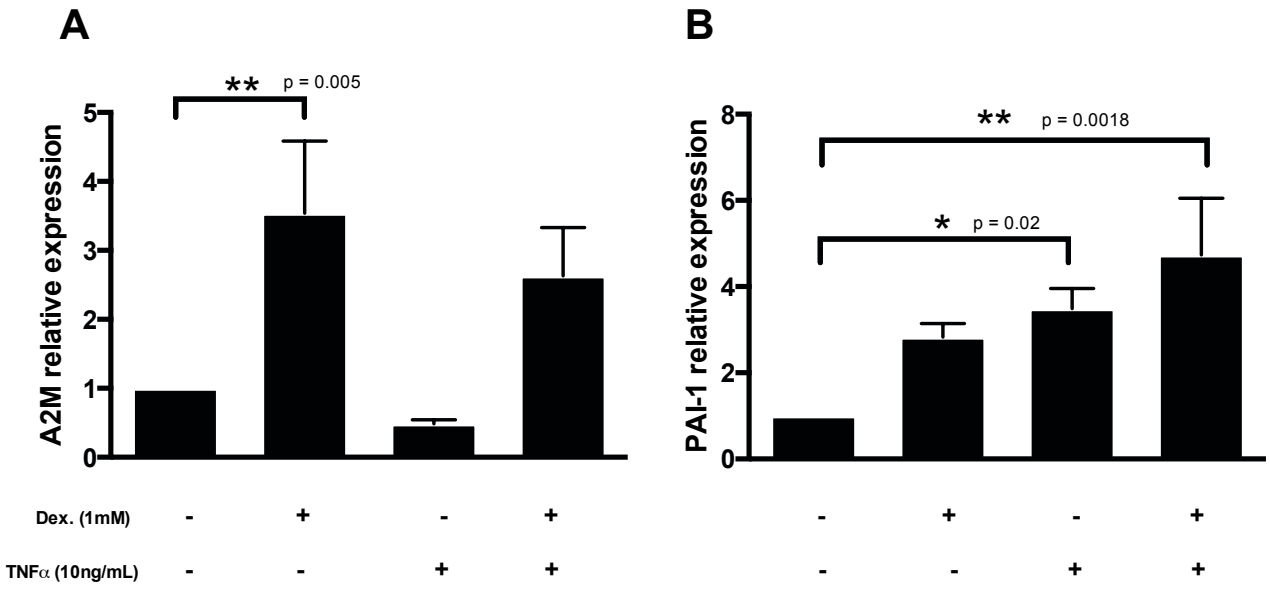

Figure 9. High dose glucocorticoid regulation of A2M and PAI-1 gene expression in HUVEC. Nearly confluent HUVEC were treated with 1mM of the synthetic glucocorticoid dexamethasone for 48 hours and stimulated with 10ng/mL TNFα for the final 4 hours of treatment. Dexamethasone treatment significantly increased A2M gene expression by 3.5 fold compared to untreated control HUVEC. TNFα stimulation attenuated the dexamethasone-induced A2M up-regulation to a non-significant 2.6 fold (A). TNFα treatment resulted in a statistically significant 3.5 fold increase in PAI-1 levels, which increased further to 4.7 fold in cells treated with dexamethasone and then stimulated with TNFα (B). Data represented as the mean of three experiments ± SEM. Statistical significance was determined with one-way ANOVA, with $P<0.05$ considered statistically significant. * denotes $p<0.05$; ** denotes $p<0.01$; 'Dex.' denotes dexamethasone.

As previously mentioned, the regulation of A2M production differs between humans and rats. While inflammation and infection increase A2M levels in both species, A2M baseline levels are robust in human plasma but very low in rat plasma, rendering rat A2M an acute phase reactant. Since our microarray-based findings of A2M gene expression up-regulation were observed in a rat model of ONFH, we also examined the response of rat endothelial cells to high dose glucocorticoids (**Fig. 10**).

Nearly confluent RMEC were treated with dexamethasone and stimulated with TNFα as described above. Similar to our findings in HUVEC, high dose glucocorticoid treatment led to an increase in A2M and PAI-1 gene expression in RMEC. Treatment of RMEC with dexamethasone led to a significant 3 fold up-regulation of A2M mRNA levels in comparison to control. Stimulation of RMEC with TNFα alone or in combination with dexamethasone treatment did not affect A2M levels. (**Fig. 10A**).

Dexamethasone treatment caused a 2.1 fold up-regulation of PAI-1 levels that did not reach statistical significance. TNFα stimulation alone induced a statistically significant 2.4 fold up-regulation of PAI-1 mRNA levels relative to untreated RMEC control levels. Furthermore, stimulation of dexamethasone-treated RMEC with TNFα resulted in a synergistic, significant 2.8 fold up-regulation of PAI-1 levels compared to control (**Fig. 10B**).

After confirming the effect of high dose dexamethasone in RMEC-endothelial cells of rat origin- all other experiments were carried out on HUVEC and subsequent results are all obtained form our HUVEC model of high dose glucocorticoid treatment.

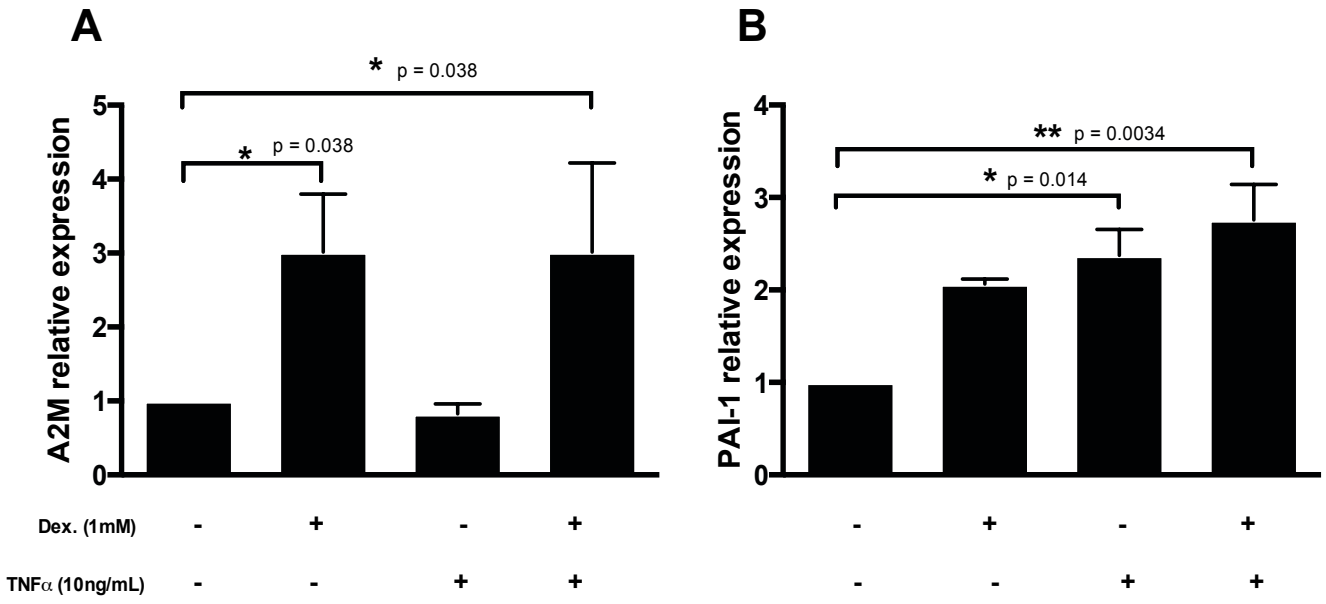

Figure 10. High dose glucocorticoid regulation of A2M and PAI-1 gene expression in RMEC. Nearly confluent RMEC were treated with 1mM dexamethasone for 48 hours and stimulated with 10ng/mL TNFα for the final 4 hours of treatment. Dexamethasone treatment significantly increased A2M gene expression by 3 fold compared to untreated RMEC. TNFα stimulation had no effect alone or in combination with dexamethasone treatment (A). TNFα treatment resulted in a statistically significant 2.4 fold increase in PAI-1 levels that was elevated further to 2.8 fold with combined dexamethasone treatment (B). Data represented as the mean of three experiments ± SEM. Statistical significance was determined with one-way ANOVA, with $P<0.05$ considered statistically significant. * denotes $P<0.05$; ** denotes $p<0.01$. 'Dex.' denotes dexamethasone.

4.2 High Dose Glucocorticoid Regulation of A2M and PAI-1 Conditioned Media Levels in Basal and Stimulated Endothelial Settings

As mentioned previously, both A2M and PAI-1 are soluble proteins that are released from cells to carry out their functions in the extracellular milieu. PAI-1 released into the media inactivates both tPA and uPA, forming complexes that are subsequently cleared up [90]. A2M is released in its native form to the extracellular space or media where it can inhibit various proteinases leading to its activation and rapid clearance [2]. In order to evaluate the impact of high-dose glucocorticoid treatment on endothelial cells at the functional protein level, we utilized ELISA analyses to assay the total levels of A2M and PAI-1 protein in the conditioned media of HUVEC treated with glucocorticoids as per our treatment model.

Contrary to our gene expression findings, ELISA analysis of total A2M levels in the media of HUVEC following the 48-hour experimental period revealed consistent A2M levels that were not altered by any treatment (**Fig. 11A**). Dexamethasone treatment and TNFα stimulation caused modest increases in PAI-1 conditioned media protein levels that did not reach statistical significance. TNFα stimulation had a synergistic effect with dexamethasone treatment and the combination of both resulted in a significant increase of PAI-1 protein levels to 844ng/mL from the 552ng/mL detected in untreated control HUVEC (**Fig. 11B**).

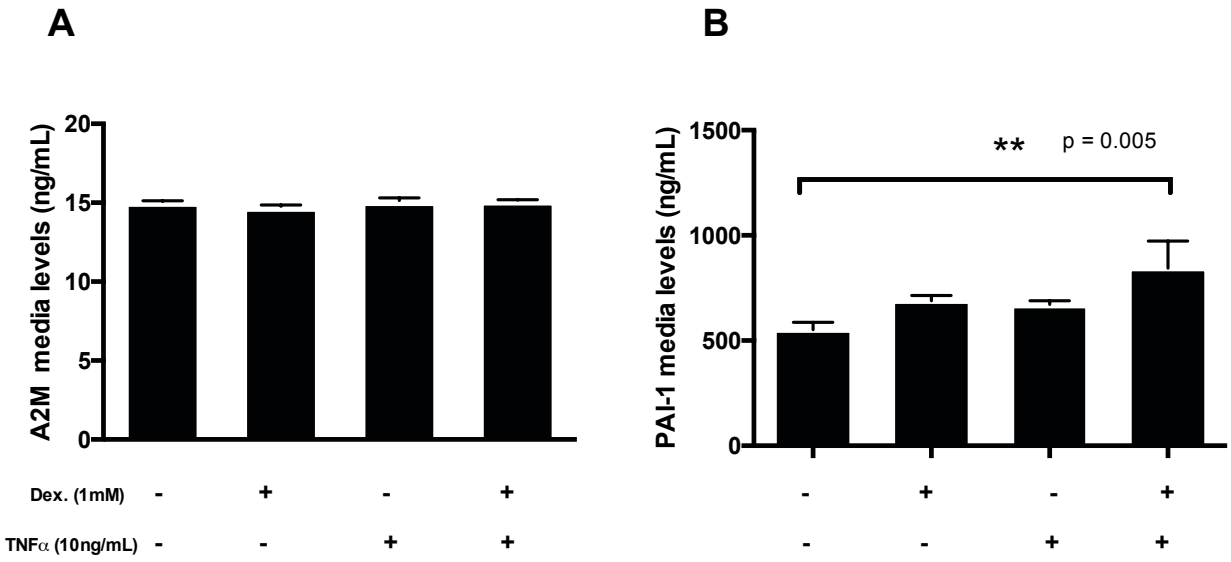

Figure 11. High dose glucocorticoid regulation of A2M and PAI-1 media protein levels. ELISA analyses were performed to determine the A2M and PAI-1 protein content of the conditioned media following 48 hours of 1mM dexamethasone treatment and 4 hours of 10ng/mL TNFα stimulation. ELISA analysis of A2M protein levels in HUVEC conditioned media at the end of the experimental period did not uncover any differences across the various treatments (A). Dexamethasone treatment combined with TNFα stimulation resulted in a statistically significant increase in the levels of PAI-1 protein in the media (B). Data represented as the mean of three experiments ± SEM. Statistical significance was determined with one-way ANOVA, with $P<0.05$ considered statistically significant. * denotes $P<0.05$; ** denotes $p<0.01$. 'Dex.' denotes dexamethasone.

4.3 Effect of A2M Silencing on PAI-1 Gene Expression in the Glucocorticoid Model

A2M's regulatory effects on haemostatic effectors are multi-faceted and its impact remains largely unexplored. We hypothesized that a glucocorticoid-mediated increase in A2M expression would lead to an increase in activated A2M levels and therefore cause increased signaling through the A2M receptors on the endothelium, potentially impacting PAI-1 levels and adding another layer of modulation of PAI-1 expression. To examine whether the observed dexamethasone-induced A2M gene expression up-regulation contributes to the modulation of PAI-1 gene expression in our glucocorticoid treatment model, we transiently silenced A2M gene expression. 24 hours after treating HUVEC with complexes of A2M siRNA and lipid-based HiPerFect transfection reagent, dexamethasone treatment was initiated as per the established glucocorticoid treatment protocol.

When gene expression was assayed at the end of the treatment period via qRT-PCR, we found that treatment with A2M siRNA led to a significant reduction of A2M expression to 0.4 fold or lower of the expression levels of control cells transfected with non-targeting siRNA. As expected, A2M silencing abolished the previously observed dexamethasone-induced up-regulation of A2M. However, A2M silencing did not impact PAI-1 gene expression in any of the tested conditions. As per previous findings, PAI-1 gene expression increased to 2 fold with dexamethasone treatment and further to a statistically significant 4.4 fold following TNFα stimulation (**Fig. 12**).

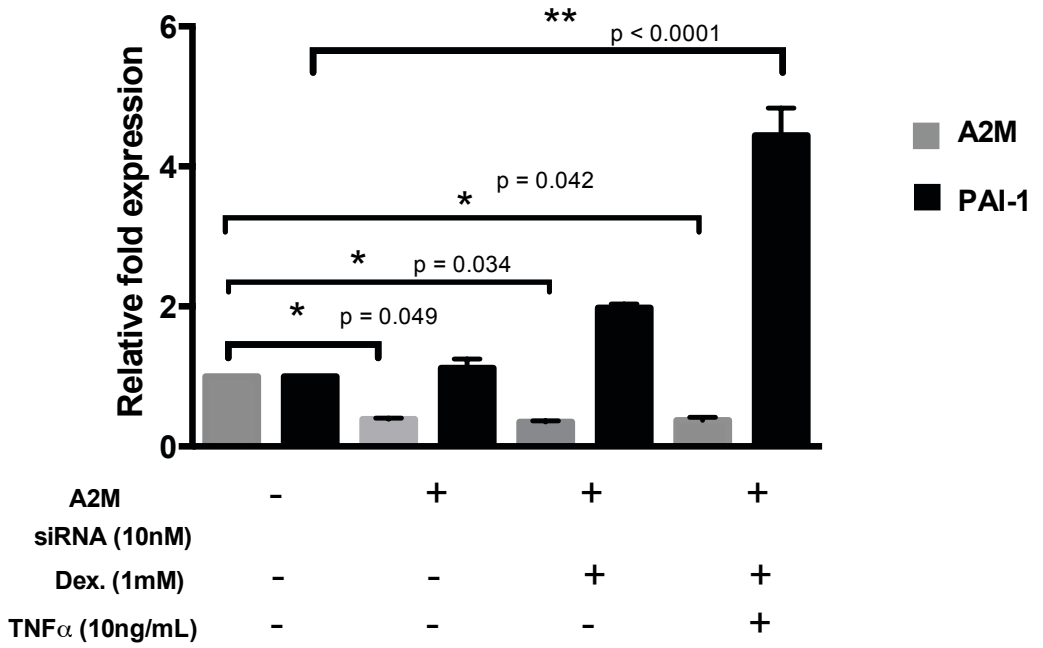

Figure 12. PAI-1 gene expression following A2M gene silencing. HUVEC were treated with 10nM of either A2M siRNA or non-targeting control siRNA. 24 hours following transfection, cells were treated with 1mM dexamethasone for 48 hours, and stimulated with10ng/mL TNFα for the final4 hours of experiments. Treatment of HUVEC with A2M siRNA led to statistically significant reduction of A2M expression to at least 0.4 fold of the levels of expression in cells treated with non-targeting siRNA. A2M silencing had no impact on PAI-1 gene expression, which, as per previous findings, reached 2 fold with dexamethasone treatment and was further elevated to a statistically significant 4.4 fold when cells were stimulated with 10ng/mL TNFα. Data represented as the mean of three experiments ± SEM. Statistical significance was determined with one-way ANOVA, with $P<0.05$ considered statistically significant. * denotes $P<0.05$; ** denotes $p<0.01$. Dex. denotes dexamethasone.

4.4 Effect of Activated A2M Treatment on PAI-1 Gene Expression

The lack of an observable A2M-silencing effect on PAI-1 gene expression could be due to the fact that glucocorticoids induce numerous molecular events that might mask A2M's action in our glucocorticoid model. As such, we set out to investigate the effect of A2M on endothelial PAI-1 gene expression in isolation of glucocorticoid treatment. Given that A2M signaling through its ubiquitous endocytic receptors activates various signaling pathways that impact cell function, we focused on A2M's signaling effects through treatment with activated A2M.

4.4.1 Dose-and time-dependent evaluations of activated A2M response

Since activated A2M is incapable of neutralizing substrates and has the receptor-binding site exposed, treatment with activated A2M allows for a specific examination of A2M signaling effects independent of A2M's protease neutralizing functions. Treatment of nearly confluent HUVEC with varying doses of activated A2M (0 to 180nM) for 24 hours had no effect on HUVEC PAI-1 gene expression as determined by qRT-PCR (**Fig. 13A**). We sought to uncover any transient expression-modulating effects of activated A2M treatment that might occur for a limited amount of time within the 24-hour treatment period. To this end, we performed a time-based evaluation of HUVEC PAI-1 gene expression after 3, 6, 12, and 24 hours of treatment with 100nM activated A2M. The specific dose of activated A2M was chosen based on previous literature reports [17, 18, 20, 110]. QRT-PCR evaluation of gene expression at the aforementioned time points did not uncover any limited duration- activated A2M effects (**Fig. 13B**).

4.4.2 Stimulated endothelial responses to activated A2M

We also sought to characterize the activated A2M response in a stimulated endothelial context. Nearly confluent HUVEC were treated with 100nM of activated A2M for 24 hours and stimulated with 10ng/mL TNFα for the final 4 hours of treatment. Stimulation with TNFα yielded the expected, statistically significant 3.5 fold up-regulation of PAI-1 and this up-regulation was not affected by treatment of cells with activated A2M prior to stimulation (**Fig. 13C**).

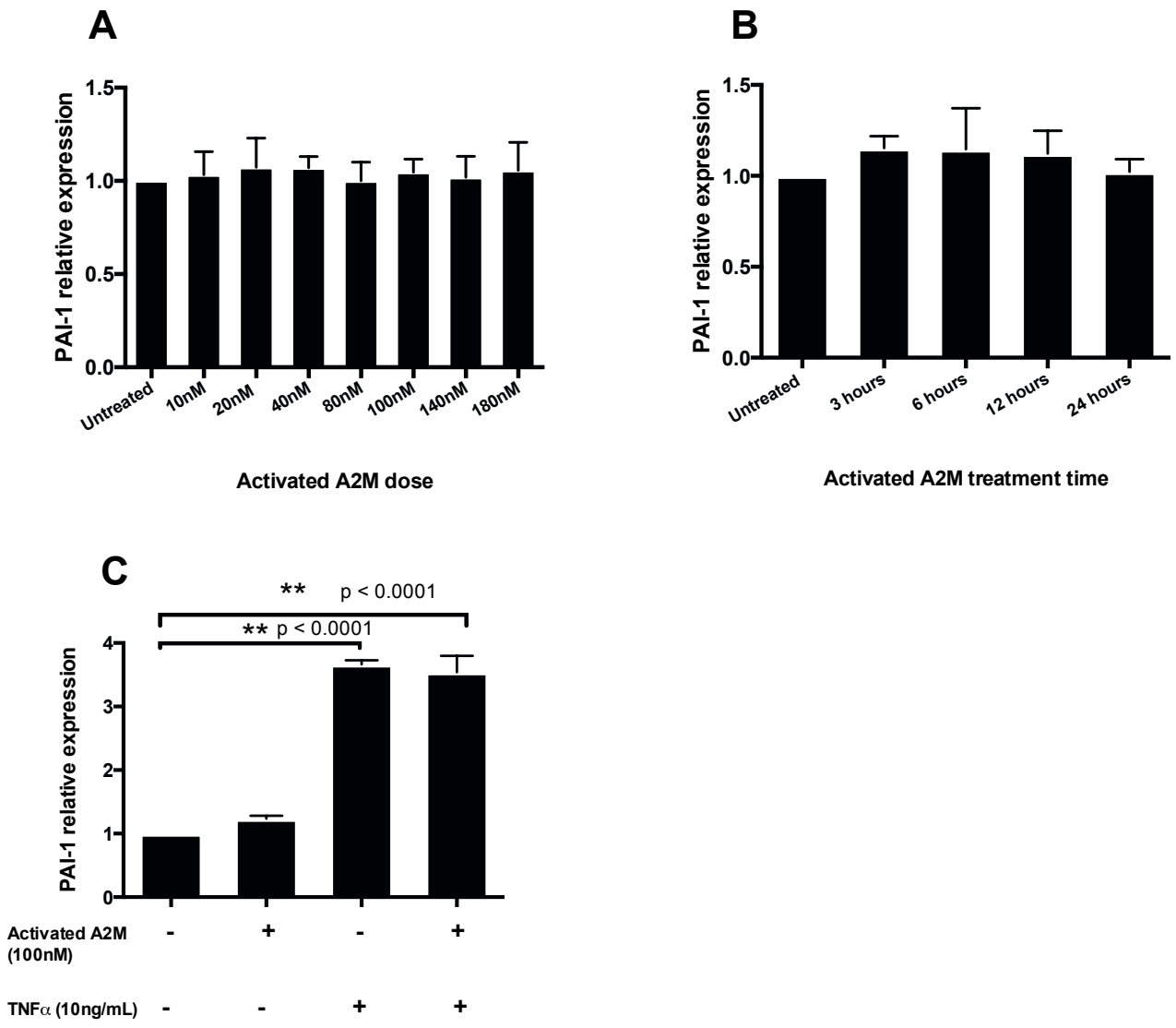

Figure 13. PAI-1 gene expression following activated A2M treatment**.** The effect of A2M signaling on endothelial PAI-1 expression was examined via treatment of nearly confluent HUVEC with activated A2M. Treatment with varying doses of activated A2M for 24-hours had no effect on HUVEC PAI-1 expression (A). A time-dependent evaluation of the effect of 100nM activated A2M treatment did not uncover any effects on PAI-1 expression either (B). Stimulation of HUVEC with 10ng/mL TNFα alone resulted in an expected 3.5 fold up-regulation of PAI-1 but no additional effect was detected when cells were treated with 100nM of activated A2M (C). Data represented as the mean of three experiments ± SEM. Statistical significance was determined with one-way ANOVA, with $P<0.05$ considered statistically significant. * denotes $P<0.05$; ** denotes $P<0.001$.

Chapter 4:

Discussion

5.1 High Dose Glucocorticoid Regulation of A2M/PAI-1 Gene Expression in Relation to ONFH

The majority of studies examining the effects of glucocorticoid treatment employ relatively low glucocorticoid doses that do not approximate the high levels associated with the development of ONFH. In this study, we utilized an *in vitro* endothelial model of high dose glucocorticoid treatment to explore the effect of high dose glucocorticoids on the endothelial production of A2M and PAI-1; two important haemostatic effectors that have been associated with ONFH development.

Our analysis of gene expression in human (HUVEC) and rat (RMEC) endothelial cells following treatment with 1mM of the synthetic glucocorticoid dexamethasone revealed a significant increase in A2M gene expression in both cell models. PAI-1 gene expression was also elevated with dexamethasone treatment, although this effect only reached significance when the cells where stimulated with the synergistically-acting TNFα. Given the small number of experiments conducted in this exploratory report, it is possible that the observed trend would reach significance with more data, providing solid evidence that PAI-1 gene expression regulation is an important facet of high dose glucocorticoid action. As it is, the synergistic action of dexamethasone and TNFα-resulting in a statistically significant up-regulation of PAI-1 gene expression in our study- bears a strong relevance to ONFH pathology. TNFα-mediated inflammatory events can occur as part of many physiological processes. However, in patients receiving glucocorticoids, the effect of these TNFα-mediated events on the endothelium is exacerbated through the concurrent action of glucocorticoids, resulting in strong, pathological changes in PAI-1 levels.

Previous literature reports corroborate our findings. Yamamoto and his team performed a study with a premise similar to our present examination but utilizing a much lower dose of glucocorticoids. The team used 1μM dexamethasone to treat HUVEC for a period of 48 hours. In line with our findings, Yamamoto et al also observed an up-regulation of PAI-1, which they measured at 2-fold [120]. Kimura et al studied the effect of glucocorticoid treatment on the production of PAI-1 in human proximal renal tubular epithelial cells by treating the cells with varying, relatively low doses of dexamethasone (0-5μM) for 24 hours. The team found that glucocorticoid-induced up-regulation of PAI-1 peaked at 3.4 fold following treatment with 1μM dexamethasone, and that the aforementioned

induction was attenuated to 2.2 fold when the dosage was increased to 5μM. These findings demonstrate that the dosage-response relationship for glucocorticoids in a given system is not always linear, illustrating the need for careful, dose-conscious examinations of glucocorticoid effects [121]. Literature reports also support our glucocorticoid-mediated A2M up-regulation findings. Several studies have shown that glucocorticoid treatment induces A2M expression in the liver, the major site of A2M production [99, 122, 123]. An early report by Kurokawa and colleagues demonstrated that treatment of hepatocyte cultures with 1μM dexamethasone alone caused an approximately 3-fold increase in the levels of media-secreted A2M [99]. Since both the A2M and PAI-1 genes have glucocorticoid response elements in their respective promoter regions, the up-regulation of these genes following glucocorticoid treatment is mechanistically plausible, likely through direct GR action [121, 123]. However, given the complexity of the regulatory networks governing A2M and PAI-1 gene expression, further experimentation is needed to tease out the specific glucocorticoid regulatory mechanisms involved.

The A2M/ PAI-1 regulation trends observed in our study are in line with the proposed ONFH pathology. Numerous reports have associated PAI-1 abnormalities with ONFH development [14, 34, 124, 125]. As an inhibitor of the activation of the fibrinolytic plasmin, an increase in PAI-1 levels reduces clot resolution. This translates to blood clots that remain longer than the body requires, creating a higher likelihood of vessel obstruction [126]. Likewise, elevations of A2M levels have been associated with coagulopathies that involve vessel obstruction [16, 19]. Increases in the level of A2M can affect haemostasis in two ways. First, as an inhibitor of a number of proteases involved in coagulation and fibrinolysis, pathological increases in A2M can tip the balance in favor clot formation and stasis, for example by increased affinity for and inhibition of anti-coagulant factors. In fact, several reports indicate that increases in the level of A2M inhibitory activity preferentially inhibit the anti-coagulant protein APC [16, 98, 127]. Another route through which A2M can tip the balance in favor of clot formation is by signaling through its receptor LRP1; an unexplored aspect of A2M function that we focused on in this study. In this scenario, A2M produced by endothelial cells (or nearby stromal cells) and activated by protease binding can bind to LRP1 expressed on neighboring endothelial cells to activate a variety of signaling pathways, including the PI3K/Akt/NFkB pathways, which can increase the production of anti-fibrinolytic effectors such as PAI-1. Using a Yeast-2-hybrid (Y2H) assay, Chaikeeratisak's study of A2M's anti-fibrinolytic

function in shrimp revealed that the receptor binding domain of A2M associates with important shrimp clotting enzymes, co-localizing with blood clots and protecting them from fibrinolysis [117]. Based on the above, our observations on A2M and PAI-1 glucocorticoid-mediated gene regulation are in line with the proposed ONFH pathology.

The regulation of A2M and PAI-1 gene expression differed slightly between basal and stimulated endothelial contexts. While TNFα stimulation had an additive effect to dexamethasone on PAI-1 expression, the same stimulation attenuated the dexamethasone-induced A2M up-regulation in the HUVEC but not RMEC model (0.5 fold; **Fig. 9**). TNFα's enhancing effect on PAI-1 expression is well documented in the literature [120, 121, 128]. TNFα modulates gene expression in part through the NF_KB transcription factor. Work by Swiatkowska and her team demonstrated that the TNFα-induced up-regulation of PAI-1 occurs through a TNFα response element in the PAI-1 promoter region and is mediated by the transcriptional action of NF_KB [129].NF_KB also plays a role in regulating A2M gene expression. IL-6, the main inducer of the acute phase response, acts through the Signal Transducer and Activator of Transcription (STAT) family of transcription factors to induce A2M gene expression [130]. Uskokovic and her team used a rat liver model to show that NF-kB competes with STAT3 for promoter binding to overlapping STAT3/NF-kB binding sites on the A2M promoter, with increasing concentrations of NF-kB antagonizing A2M gene transcription . Interestingly, however, they also found that the presence of NF-kB in the promoter region is essential for A2M transcription to take place [131]. Thus, it appears that there is a requirement for the presence of a minimum amount of NF-kB for transcription to occur, after which NF-kB assumes an antagonizing role on A2M transcription. These mechanistic findings help explain a TNFα-mediated decrease of A2M expression in the HUVEC model but it remains unclear why TNFα had no effect on A2M expression in the RMEC model. It is possible that the observed divergence in responses of HUVEC and RMEC is a demonstration of the previously discussed phenomenon of endothelial heterogeneity, highlighting the limitations of attempting to model endothelial behavior in general.

Overall, the aforementioned results highlight some interesting points of consideration regarding our model. When comparing our results to the literature, our treatment yielded similar A2M and PAI-1 gene expression changes compared to results obtained using much lower doses of glucocorticoids. The upper range of glucocorticoid dose patients are exposed to is equivalent to 100μM. Given that

the dose-response to glucocorticoids might not be always linear, it is possible that basing our glucocorticoid treatment model on the relatively lower 100μM dose might yield a greater up-regulation of PAI-1. However, it is still prudent to maintain a relatively high dose of glucocorticoids to remain faithful to the clinical situation. Gene expression assays, such as the one utilized for PAI-1 in our present study and others mentioned herein, provide a powerful tool for examining the effects of a given treatment. However, they do not allow for a complete examination of all the genetic responses to high vs. low dose glucocorticoid treatment and it is entirely possible that great differences would be observed across other genes important in ONFH pathology if a high throughput gene expression assay technique like microarray technology were used.

Because our work sought to examine the effect of glucocorticoids on the endothelium in relation to human ONFH pathology, we focused on HUVEC responses to high dose glucocorticoids. However, since we originally discovered the association of A2M with ONFH in a rat model, we also characterized the glucocorticoid response in rat endothelial cells. Interestingly, we found that the glucocorticoid treatment yielded very similar results on A2M expression in both HUVEC and RMEC, in spite of their different sources and endothelial subtypes (large umbilical vein vs. cardiac microvascular), but the responses were not identical. Overall HUVEC showed slightly greater responses; the same dose of dexamethasone caused a 3.5 fold up-regulation of A2M in HUVEC vs. 3 fold in RMEC; and combined TNFα and dexamethasone treatment caused a 4.5 fold up-regulation of PAI-1 gene expression in HUVEC compared to 2.8 in RMEC. These results illustrate how the responses of different endothelial subsets can mostly follow the same trends but differ slightly in the magnitude of the response, illustrating the concept of endothelial heterogeneity in action. As such, although HUVEC are an ideal model for preliminary explorations, more advanced studies should aim to utilize the specific endothelial cell population involved in the pathology. In the case of ONFH, although bone endothelial cells are known to be difficult to isolate and propagate [132], it is an endeavor worth the effort if one were to obtain a more faithful analysis of the pathology.

5.2 Regulation of PAI-1/A2M Released Protein Levels

We found that dexamethasone treatment slightly up-regulated the levels of PAI-1 protein in the conditioned media of HUVEC, and that stimulation with TNFα had a similar effect; though neither reached statistical significance in isolation. However, the combination of dexamethasone treatment and TNFα stimulation had a synergistic effect and resulted in an additive, significant increase of 1.7 fold. These findings are in line with the findings of Soeda, who stimulated HUVEC with a 200U/ml dose of TNFα for 3 hours and found a 2.8 fold increase in the release of PAI-1 into the media. Soeda's investigation also revealed that the TNFα-mediated release of PAI-1 involves a lysosomal signaling event [133]. It's worthwhile to note that the observed TNFα-mediated changes in PAI-1 media levels are related to increased release rather than synthesis of PAI-1. Since we stimulated HUVEC with TNFα for a relatively short period of a few hours, its observed effects on PAI-1 gene expression and subsequent protein synthesis are not accounted for in this analysis and a larger change in PAI-1 media levels would likely be observed if ELISA analysis is conducted at a further point in time. As it is, however, since PAI-1 exerts its inhibitory effects on tPA/uPA in the extracellular space/blood; the observed increase in conditioned media levels of PAI-1 following glucocorticoid treatment is in line with the proposed disease etiology.

Contrary to our expectations, we could not detect any differences in the level of A2M in the conditioned media of different treatments. In our previous study, the A2M gene expression up-regulation detected with microarray technology was confirmed at the protein level with immuno-histochemistry (**Fig. 14**). A possible explanation for the absence of the expected up-regulation of A2M conditioned media protein levels is that given the fast half-life of activated A2M, estimated at a few minutes, the A2M released in response to glucocorticoids might have already rapidly reacted with the endothelial-derived proteases in the conditioned media and cleared up by LRP1 by the end of the 48-hour treatment period when the ELISA assay was performed. Thus, it might be more appropriate to measure A2M release at earlier intervals following glucocorticoid treatment. Alternatively, it is also possible that the observed endothelial gene expression up-regulation doesn't carry through to the protein level. In this case, the A2M protein up-regulation observed in our previous study might have been derived from macrophages and other cells in the femoral head that are also capable of producing A2M.

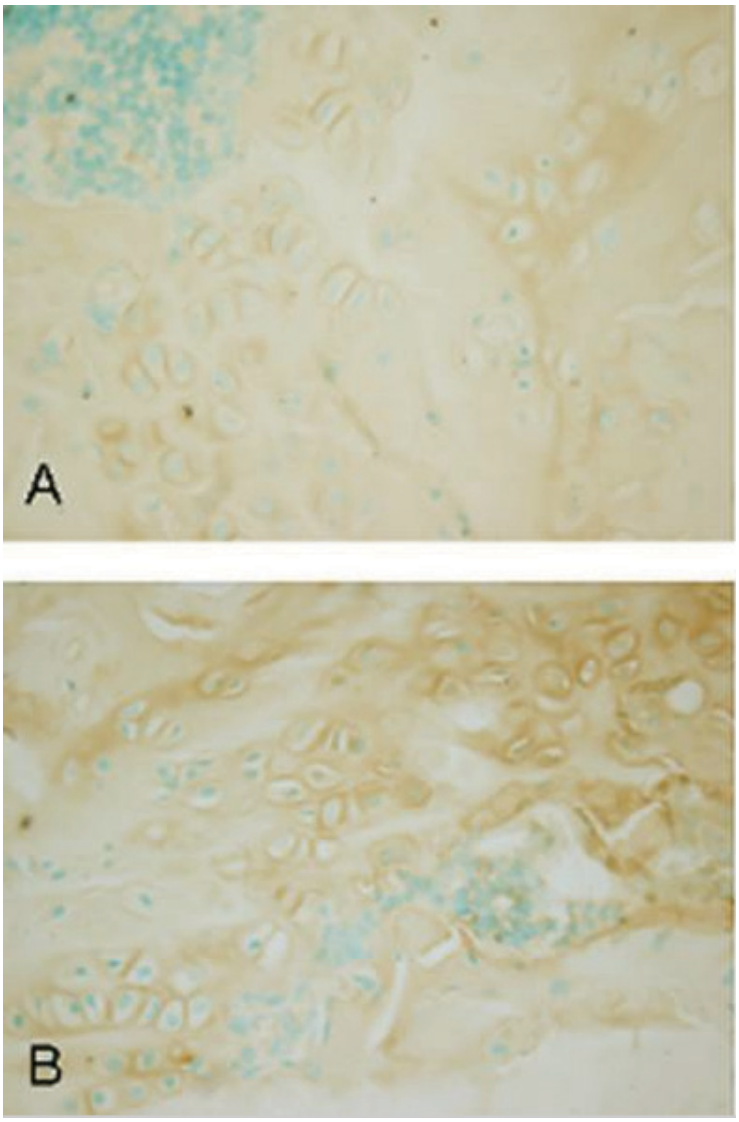

Figure 14. Immuno- histochemical staining for A2M in control and diseased rat hips. Staining for the A2M protein (shown in brown) in control (A) and ONFH (B) rat hips revealed an up-regulation of the protein in diseased hip tissue. Adapted from Kerachian et al [15].

5.3 A2M Signaling In Haemostatic Gene Expression Regulation

We found that abolishing the glucocorticoid-induced increase in A2M gene expression through gene silencing had no impact on PAI-1 expression. Furthermore, examining the effect of activated A2M treatment on PAI-1 expression revealed that A2M singaling does not play a role in PAI-1 regulation, since dose and time response examinations did not uncover any changes in PAI-1 levels after activated A2M treatment. It is still possible, however, that A2M signaling might influence the expression of other genes related to endothelial function and haemostasis, and this possibility is worth investigating given A2M's link to various coagulopathies.

In this study, we were unable to detect A2M in the conditioned media of endothelial cells. It is possible that other sources of A2M, including macrophages and other femoral head-resident cells, are more biologically relevant to the study of A2M's role in glucocorticoid-induced ONFH. In that vein, it would also be worthwhile to investigate the effect of A2M on other types of effectors that are biologically relevant to disease progression. For example, it has been reported that A2M is capable of inhibiting Bone Morphogenetic Protein 1 (BMP1), a metalloproteinase important in cartilage and bone formation [134, 135]. Thus, A2M's broad range of action leaves many avenues still open for exploration.

Given A2M's role in haemostasis, its strong association with various coagulopathies, and our previously reported association between A2M and ONFH, further investigation into the different modes of A2M's functions is definitely warranted. However, it seems prudent from our current investigation and previous reports to shift the focus of future investigations to the enzymatic inhibitory role of A2M on haemostasis and endothelial function.

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