# Preeclampsia - A cardiovascular risk factor

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# Preeclampsia

## A cardiovascular risk factor

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**Title and subtitle: Preeclampsia - A cardiovascular risk factor**


**Abstract:**

Preeclampsia is a pregnancy complication affecting between 3 and 7% of all pregnancies. There is no definite treatment and the only cure is to deliver the placenta and the fetus. Recent studies have shown that Alpha-1-microglobulin (A1M), an endogenous heme and radical scavenger, may be an effective treatment against preeclampsia. Besides being associated with maternal and perinatal morbidity and mortality, preeclampsia has been shown to be a risk factor for cardiovascular disease (CVD). However, the pathophysiological mechanism connecting preeclampsia with long-term CVD is not completely undestood.

In the present thesis we seek to elucidate the maternal cardiac implications of severe late-onset preeclampsia using cardiac magnetic resonance imaging. In addition, this thesis intends to determine the therapeutic potential of A1M against preeclampsia and how exogenous administration of A1M affects the preeclampsia-associated changes in cardiac morhology and ultrastructure using a mouse model of preeclampsia. Part of the aim of this thesis is to understand the dynamics in changes of plasma levels of A1M in the women with risk-factors predisposing to preeclampsia who have an uncomplicated pregnancy (high-risk controls) and in the preeclamtic women.

Our results show that severe late-onset preeclampsia is associated with transient maternal cardiac hypertrophy; the cardiac changes dissapear at six months postpartum. In the mouse model of preeclampsia, we found cardiac hypertrophy, cardiac fibrosis, and changes in cardiac ultramorphology in terms of errupted mitochondria and irregularly organized myocardial fibers. The changes in ultramorphology, together with hypertension and proteinuria, were alleviated by exogenous administration of A1M. We found an increase in the maternal plasma levels of A1M in the high-risk controls between the first and second trimester of pregnancy. In contrast, the levels of A1M reduced in the preeclamptic women during the same period.

In conclusion, cardiac hypertrophy after severe late-onset PE is transient. Other pathophysiologic mechanisms, such as cardiac fibrosis, may be the culprit for increased long-term cardiovascular risk after preeclampsia. Exogenous administration of A1M is an effective treatment against preeclampsia and counteracts changes in cardiac ultramorhology associated with preeclampsia. Whether this effect can decrease the risk for future CVD is an important issue for future research. The increase in the A1M levels in the high-risk controls may reflect a protective response against systemic oxidative stress.



**Key words: Preeclampsia, Cardiovascular disease, Alpha-1-microglobulin, Oxidative stress**

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# List of Abbreviations

| | |
|---|---|
| ISSHP | International Society for the Study of Hypertension in Pregnancy |
| ACOG | American College of Obstetricians and Gynecologists |
| STB | Syncytiotrophoblast |
| HbF | Fetal hemoglobin |
| sFLT1 | Soluble FMS-like tyrosine kinase-1 |
| sENG | Soluble endoglin |
| PlGF | Placental growth factor |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| ROS | Reactive oxygen species |
| ER | Endoplasmic reticulum |
| HSP70 | 70 kilodalton heat shock proteins |
| HO-1 | Heme oxygenase-1 |
| IFN-γ | Interferon-gamma |
| NO | Nitric oxide |
| HPX | Hemopexin |
| A1M | Alpha-1-microglobulin |
| CVD | Cardiovascular disease |
| MRI | Magnetic resonance imaging |
| STOX1 | Storkhead box 1 |
| TEM | Transmission electron microscopy |
| PWV | Pulse wave velocity |
| ELISA | Enzyme-linked immunosorbent Assay |
| SGA | Small for gestational age |
| AGA | Appropriate for gestational age |

# Introduction

## Definition

Preeclampsia is a complex medical disorder associated with pregnancy, which affects 3 - 7% of all pregnancies according to global estimates [1]. According to the current definition by the International Society for the Study of Hypertension in Pregnancy (ISSHP) [2], preeclampsia is diagnosed by the presence of hypertension and one or more of the following conditions arising at or after 20 weeks of gestation:

- Proteinuria
- Uteroplacental dysfunction
- Maternal organ dysfunction, including:
    - Acute kidney injury (creatinine $\geq$ 1 mg/dL)
    - Liver involvement (alanine or aspartate aminotransferase > 40 IU/L)
    - Neurological complications
    - Hematological complications

In the current definition, a distinction is made between **preeclampsia de novo**, when hypertension arises at or after 20 weeks' gestation, and **preeclampsia superimposed on chronic hypertension**, when hypertension pre-dates pregnancy or is diagnosed in the first 20 weeks of pregnancy.

Regarding the distinction between **early-onset** and **late-onset** preeclampsia, there is weak consensus and no explicit definition. According to the results of a survey among expert members of the International Committee of ISSHP, preeclampsia occurring before 34 weeks of gestation is considered as **early-onset** preeclampsia [3]. However, according to the recently published guidelines by ISSHP, a distinction between early-onset and late-onset preeclampsia, though useful for research purposes, should not be used in clinical practice. The reason is that preeclampsia is associated with severe adverse outcomes, irrespective of gestational age at disease onset.

## Disease severity

According to the American College of Obstetricians and Gynecologists (ACOG) [4], **preeclampsia with severe features** is diagnosed when one or more of the following findings are present:

- Systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg, on two occasions, at least four hours apart
- Platelet count less than $10^5/\mu L$
- Progressive renal insufficiency, defined as serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease
- Impaired liver function, including:
    - Elevated liver enzymes
    - Severe persistent epigastric or right upper quadrant pain
- Pulmonary edema
- Persistent neurological symptoms such as severe headache or visual disturbances

There is a lack of population-based data on the incidence of preeclampsia with severe features according to the definition set by ACOG; the incidence has been found to be eight per 1000 deliveries, based on data collected from a tertiary care institution [5].

According to the guidelines set by ISSHP, classifying preeclampsia as mild or severe may be misleading and is not recommended in clinical practice. However, ISSHP recommends urgent treatment for women with ‘severe’ hypertension, defined as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110. Furthermore, ISSHP recommends the use of the PIERS (Preeclampsia Integrated Estimate of Risk) model in the assessment of women with preeclampsia as a predictive tool for ‘severe’ adverse maternal outcome [2]. In the PIERS model, platelet count, serum creatinine, aspartate aminotransferase, dyspnea, chest pain, oxygen saturation and gestational age are included. Taken together, both ISSHP and ACOG agree that there are severe features associated with a high risk of imminent, severe deterioration in maternal and fetal condition.

### Fetal growth

The relationship between preeclampsia and fetal growth differs by gestational age at preeclampsia onset. Early-onset preeclampsia is associated with the birth of small for gestational age (SGA) infants [6]. In late-onset preeclampsia, however, most infants are either of normal size or large for gestational age (LGA) [7].

It is necessary here to clarify that SGA, usually defined as a birthweight below the 10th centile [8], is a statistical deviation from the population standard and does not necessarily reflect impaired fetal growth. The term fetal growth restriction (FGR) describes more accurately whether the fetus reaches its growth potential. The definition of FGR, although there is no gold standard, is based on tests that evaluate abnormal placental function, namely raised umbilical and uterine artery Doppler impedance [8]. Interestingly, in a large cohort of pregnancies complicated by FGR before 32 gestational weeks, 60% were associated with some form of hypertensive disorder of pregnancy, clearly reflecting the strong association between early-onset preeclampsia and impaired fetal growth [9].

Taken together, there are differences in fetal growth between early-onset and late-onset preeclampsia that clearly reflect the underlying pathophysiological heterogeneity.

## Etiology

A key characteristic of preeclampsia is its unpredictability. The condition of the mother and the fetus can deteriorate rapidly and without warning. Classifying preeclampsia as mild or severe may therefore be misleading and is not recommended in clinical practice. For research purposes, however, it is important to make a distinction between early-onset and late-onset preeclampsia, based on differences in the clinical outcome for the mother and the fetus. In particular, early-onset preeclampsia is associated with higher maternal [10-13] and perinatal [10, 14] morbidity and mortality compared to late-onset preeclampsia, as well as with a higher rate of fetal growth restriction [7, 15-17].

In an attempt to find a unifying pathophysiological model explaining the clinical heterogeneity of preeclampsia, the revised two-stage model [18-20] has been proposed (**figure 1**). According to this model, the first stage is characterized by placental malperfusion, which leads to syncytiotrophoblast (STB) stress. The cause

of placental malperfusion may be either the shallow remodeling of the spiral arteries or the restricted intervillous perfusion due to a large placenta which outgrows the capacity of the uterus, thus leading to compression of the terminal villi. The second stage is characterized by a generalized maternal endothelial cell dysfunction, which affects the blood vessels of essentially all maternal organs and explains the various clinical manifestations of preeclampsia. The differences between early-onset and late-onset preeclampsia can be explained by the differences in the mechanisms causing placental malperfusion. Shallow spiral artery remodeling takes place early in pregnancy and results in poor placentation and poor fetal growth. In contrast, the compression of the terminal villi takes place in late pregnancy and is caused by a relatively large placenta which outgrows the capacity of the uterus, thus explaining why the fetus in late-onset preeclampsia may be large for the gestational age instead of growth-restricted.

The maternal endothelial cell dysfunction is the result of circulating toxic factors, which are released into the maternal circulation due to the STB stress. Multiple placenta-derived toxic factors have been identified in the plasma of women with preeclampsia, and include antiangiogenic factors, placenta-derived extracellular vesicles and cell-free fetal hemoglobin (HbF).

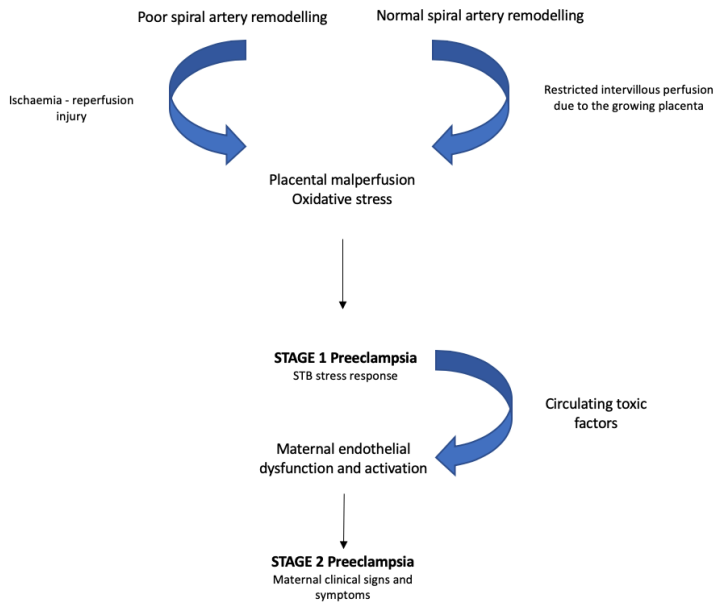


**Figure 1. The two-stage model of preeclampsia**

## Antiangiogenic factors

Soluble FMS-like tyrosine kinase-1 (sFLT1) and soluble endoglin (sENG) have been found to be elevated in the circulation of women with preeclampsia several weeks before the diagnosis and correlate with disease severity [21-25]. Soluble endoglin is an endogenous inhibitor of transforming growth factor β1 [26]. Soluble FMS-like tyrosine kinase 1 binds to and inhibits the activity of the proangiogenic factors placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) [27]. Vascular endothelial growth factor (VEGF) is important for the maintenance of endothelial cell function [28].

## Placenta-derived extracellular vesicles

Placentally-derived vesicles range from nanometer scale exosomes to microvesicles and are found in the maternal circulation both in normal pregnancies and in preeclampsia [29-33]. In preeclampsia, placentally derived vesicles are shed in increased amounts into the maternal circulation and have been shown to induce an inflammatory response by activating peripheral blood mononuclear cells to release proinflammatory cytokines [31, 34].

## Cell-free fetal hemoglobin

Recent evidence suggests that cell-free fetal hemoglobin plays a crucial role in the pathophysiology of preeclampsia [35]. The preeclamptic placenta is characterized by increased expression of fetal hemoglobin that is localized in the vascular lumen, the vascular endothelium, as well as in the villous stroma [36]. The harmful effects of cell-free hemoglobin on human placenta have been previously shown in an *ex vivo* study, where human placenta was perfused with cell-free hemoglobin in the fetal circulation, leading to breaches in the placental barrier and leakage into the maternal circulation [37]. In accordance with these findings, a series of clinical studies have shown that preeclampsia is characterized by increased levels of cell-free fetal hemoglobin in maternal circulation compared to normal pregnancy measurable from the first trimester [38-42]. Furthermore, a positive correlation has been found between maternal systolic blood pressure and the concentration of cell-free HbF in the maternal circulation [38].

# Oxidative stress in normal pregnancy and in preeclampsia

Reactive oxygen species (ROS) are produced from molecular oxygen both enzymatically or as by-products of aerobic metabolism [43]. The ROS are highly reactive and capable of damaging biological molecules, such as nucleic acids, lipids, and proteins, thus leading to cell and tissue injury. However, far from being only harmful by-products of aerobic metabolism, ROS have also been shown to participate in cellular signaling [44] and to be capable of modifying the activity of several transcription factors, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors [45, 46]. In order to control the undesired effects of ROS while permitting their physiological functions, a variety of endogenous antioxidants have been evolved to prevent, reduce, or repair the detrimental effects of ROS **(figure 2)**. Under physiological conditions, the production of ROS and their detoxification by antioxidants are carefully balanced. However, the redox homeostasis can be disturbed by either an increase in ROS production or inhibition/downregulation of the antioxidants. In these conditions, oxidative stress occurs as a result of excess ROS accumulation.

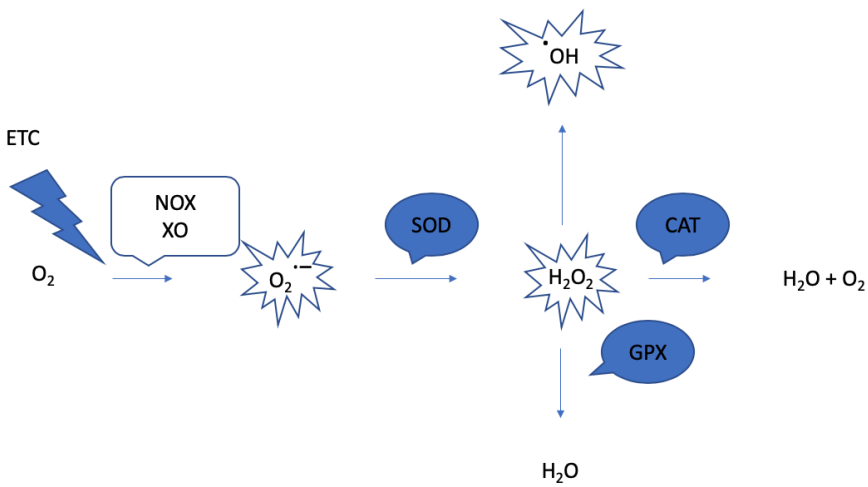


**Figure 2. Sources of reactive oxygen species and overview of the antioxidant mechanisms**
Superoxide anion ($O_2^{\cdot-}$) is the product of the one-electron reduction of oxygen. Electron leakage from the electron transport chain (ETC) leads to reduction of oxygen and generation of superoxide anion ($O_2^{\cdot-}$). The enzymes xanthine oxidase (XO) and NADPH oxidase (NOX) are also major sources of superoxide anion. Hydroxyl radical ($^{\cdot}OH$) is a powerful oxidant produced via the Fenton reaction. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to form hydrogen peroxide ($H_2O_2$). Catalase (CAT) catalyzes the decomposition of hydrogen peroxide to form water and molecular oxygen. Glutathione peroxidase (GPX) catalyzes the reduction of hydrogen peroxide to water.

It has been postulated that pregnancy *per se* is a condition characterized by increased production of ROS [47]. Despite this, few studies have investigated the longitudinal changes in ROS production during a normal pregnancy. According to a recent study by Mannaerts *et al.*, normal pregnancy is characterized by a stable concentration of superoxide anion radicals in the maternal circulation, albeit increased compared to the non-pregnant state [48]. In the placenta, however, excessive production of ROS has been shown to occur at certain windows during normal pregnancy, playing a central role in placental development. In particular, at the end of the first trimester, a steep rise in ROS production occurs in the normal placenta as a result of the onset of the utero-placental circulation [49]. The increase in ROS production is generally balanced by an increase in the expression of the antioxidant enzyme, copper-zinc superoxide dismutase, in the trophoblast cells, thus preserving the redox homeostasis. However, a shift in redox homeostasis has been found to occur in the periphery of the placenta, where the maternal blood flow begins at as early as eight weeks of pregnancy, exposing the trophoblast to a high local oxygen concentration, at a time point when the expression of the antioxidant enzyme copper-zinc superoxide dismutase in the trophoblast is absent. The ensuing trophoblastic oxidative damage leads to local villi regression in the periphery of the placenta, thus triggering the formation of chorion laeve [50].

Oxidative stress has been proved to play a pivotal role in the pathophysiology of preeclampsia. In the early preclinical stage of preeclampsia, placental malperfusion causes oxidative stress that affects the key part of the placenta, the multinucleated STB lining the placental villi. Excessive production of ROS may derive either from endoplasmic reticulum (ER) stress caused by ischemia-reperfusion injury [51, 52] or from mitochondrial stress [53]. In addition, the preeclamptic placenta is characterized by impaired antioxidant mechanisms, namely decreased expression of superoxide dismutase and glutathione peroxidase which further aggravates the oxidative damage [54]. As a consequence of the increased production of ROS and the impairment of the antioxidant mechanisms, oxidatively-stressed STB releases multiple toxic factors into the maternal circulation. These circulating placental-derived factors induce oxidative stress in maternal endothelial cells through multiple pro-oxidant intracellular pathways, thus leading to generalized maternal endothelial dysfunction and systemic inflammation [55]. Overall, oxidative stress appears to be both the primary placental problem in the early pre-clinical stage of preeclampsia and the point of convergence for maternal endothelial cell dysfunction in the later clinical stage of preeclampsia.

In preeclamptic pregnancies, however, redox homeostasis is not affected solely by placental malperfusion; the presence of pregestational systemic diseases, such as diabetes mellitus, obesity and essential hypertension, may also contribute to excessive production of ROS. Essential hypertension has been found to be associated with high plasma hydrogen peroxide levels [56]. Likewise, diabetes mellitus and obesity have also been shown to be associated with oxidative stress [57, 58]. It can thus be postulated that women with obesity, diabetes mellitus and essential hypertension enter pregnancy in a state of higher oxidative stress, which predisposes them to the development of pregnancy complications by affecting the early stage of placental development. An example is shown in a study by Gauster *et al.*, in which diabetes mellitus type 1 was found to be associated with increased oxidative stress in the early placenta. It was indirectly shown by increased expression of 70 kilodalton heat shock proteins (HSP70) and heme oxygenase-1 (HO-1), which are normally upregulated in conditions of oxidative stress and inflammation. The authors suggested that the oxidative insult may alter trophoblastic function, thus leading to diabetes-associated pregnancy complications, such as preeclampsia and fetal growth restriction.

Although oxidative stress is considered a key factor in the pathogenesis of preeclampsia, clinical studies have failed to confirm any beneficial effect of early supplementation with the antioxidants vitamin C and E on prevention of preeclampsia in women either at low or high risk of developing preeclampsia [59-61]. When interpreting these results, the potential adverse effects of supplementation with dietary antioxidants should be considered. It has been postulated that supplementation with vitamin E may stimulate proinflammatory reactions at the fetal-maternal interface by acting as a potential interferon-gamma (IFN-γ) mimic [62]. Another possible explanation for why the exogenous administration of dietary antioxidants is ineffective may be the delayed initiation of the treatment, which occurs between 10 and 20 weeks of gestation, after trophoblast transformation of the spiral arteries.

There are alternative antioxidant strategies that may have a place in the prevention and treatment of preeclampsia. In animal models of preeclampsia, treatment with mitochondrial specific antioxidants, such as MitoTEMPO and MitoQ, has been found to decrease the mean arterial pressure [63]. In the list of effective antioxidant strategies, aspirin can be included. Evidence from randomized controlled trials shows that aspirin reduces the risk of preeclampsia [64], although the exact mechanism remains uncertain. Aspirin may mediate its beneficial effect through irreversible inactivation of cyclooxygenase activity in platelets, thus inhibiting the

synthesis thromboxane A2, which is a potent vasoconstrictor and stimulant of platelet aggregation [65]. In addition, aspirin may act by alleviating oxidative stress through directly scavenging hydroxyl radicals [66] or through the induction of HO-1 expression in endothelial cells [67].

In conclusion, antioxidants may have a role in preeclampsia treatment and prevention. However, oxidative stress is the result of multiple oxidative insults leading to increased ROS production through multiple converging pathways. However, for a rational antioxidant strategy to be proposed though, we need a complete understanding of the specific pathway, starting from the specific oxidative insult that activates the cascade that eventually leads to excessive ROS production. Furthermore, for a specific antioxidant substance to be proposed, we need evidence supporting the specific antioxidant's protective effect against oxidative damage.

## Alpha-1-microglobulin - a promising antioxidant strategy

Cell-free hemoglobin is a highly toxic molecule when released extracellularly during hemolysis [68]. Once released into the intravascular space, cell-free hemoglobin reacts with nitric oxide (NO) and physiologic oxidants, such as hydrogen peroxide and lipid peroxides [69]. When reacting with cell-free hemoglobin, NO is depleted and an acute hypertensive response is triggered. Furthermore, as a result of the reaction with the physiologic oxidants, cell-free hemoglobin is oxidized and heme is produced. Heme has multiple pathophysiologic effects; it is a source of redox-active iron and can cause oxidative damage through production of ROS [70]. Furthermore, heme is capable of binding to Toll-like receptor 4 [71], thus triggering inflammatory reactions. The kidneys are exposed to circulating heme's pro-oxidative toxicity and significant damage is inflicted through the non-apoptotic cell death pathway of ferroptosis [72].

Multiple defense mechanisms are involved in the protection against the adverse effects of cell-free hemoglobin and heme. The primary hemoglobin scavenger is haptoglobin [73, 74], which is an abundant plasma glycoprotein primarily secreted by the hepatocytes. When cell-free hemoglobin is released into the intravascular space, haptoglobin binds to hemoglobin and creates an irreversible complex that circulates in the plasma and binds to CD163, a transmembrane glycoprotein expressed in monocytes in the peripheral blood and in tissue-resident macrophages. After binding to CD163, hemoglobin is transferred intracellularly and undergoes

degradation; heme is released from globin and degraded into bilirubin and carbon monoxide by HO-1. Besides its hemoglobin scavenging activity, other functions of haptoglobin have been described, such as its anti-inflammatory effects [75]. The expression of haptoglobin in the hepatocytes is induced by proinflammatory cytokines, such as interleukin-1 and interleukin-6 [76, 77].

The role of hemopexin (HPX) [78] complements the role of haptoglobin. Like haptoglobin, hemopexin is primarily secreted by the hepatocytes. Hemopexin binds to heme with high affinity, when heme is released from cell-free hemoglobin as a result of oxidation. The complex which forms as a result of the interaction between hemopexin and heme binds to LRP/CD91 [79], a receptor expressed in macrophages, hepatocytes, neurons, syncytiotophoblast and other cell types, and is removed from the circulation. Like haptoglobin, hemopexin has pleiotropic effects other than its heme scavenging activity, such as anti-inflammatory effects [78]. However, the complex regulation of human hemopexin gene expression is not fully understood. Although the human hemopexin gene contains an interleukin-6 responsive element [80, 81], it has been postulated that hemopexin is not an acute phase protein in humans [82-84].

Alpha-1-microglobulin (A1M) is a small protein of the lipocalin family, which has been found to be evolutionarily conserved in all vertebrates [85]. Alpha-1-microglobulin has multiple anti-oxidant properties, such as acting as a reductase and scavenging free heme and free radicals [85]. Furthermore, A1M protects mitochondrial structure and function by binding to Complex I subunit [86]. Alpha-1-microglobulin is primarily synthesized in the liver and secreted into the blood, from where it rapidly reaches most tissues. After binding heme and free radicals, the complex reenters the circulation and is cleared by glomerular filtration, tubular reabsorption and kidney degradation. Due to its pleiotropic protective effects, the expression of A1M is regulated in multiple ways. In the liver, its expression is regulated by hepatocyte nuclear factors [87-89]. In addition, upregulated expression has been found under oxidative stress [90-92]. Although the specific transcriptional factors have not been identified, previous results have shown that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) may promote the upregulation of A1M under conditions of oxidative stress [93]. Nrf2 regulates the expression of many antioxidant factors and is activated under a variety of stress conditions, including oxidative stress.

## The endogenous hemoglobin scavenger system in preeclampsia

When cell-free fetal hemoglobin is released into the maternal intravascular space, the endogenous scavenger and detoxification system is activated in order to alleviate hemoglobin's harmful effects. How the hemoglobin scavenger system responds to cell-free fetal hemoglobin in the maternal circulation has been previously described in a series of studies. In parallel with increases in the levels of cell-free fetal hemoglobin in the maternal circulation, the levels of alpha-1-microglobulin have been found to be increased in preeclampsia compared to normal pregnancy as early as in the first trimester of pregnancy and close to delivery [38-41]. The increase in alpha-1-microglobulin in preeclampsia is accompanied by a decrease in both haptoglobin and hemopexin compared to normal pregnancy, presumably due to the prolonged presence of increased levels of cell-free hemoglobin and heme in the maternal circulation, leading to depletion of the hemoglobin scavenger proteins [38, 40-42]. A further look at the previous studies indicates that the level of alpha-1-microglobulin increases during a pregnancy complicated by preeclampsia, from 24.77 μg/ml in the first trimester to 33.5 μg/ml close to delivery. However, there is a lack of information on the dynamics of this increase and more research is needed to determine whether the increase in A1M levels takes place at an early preclinical stage of preeclampsia or after onset of the disease. Another unanswered question is how the levels of the hemoglobin scavengers are affected by the presence of pregestational risk factors, such as obesity, essential hypertension and diabetes mellitus, that predispose to inflammation and systemic oxidative stress. Women who enter pregnancy with the aforementioned risk factors are at considerably higher risk of developing preeclampsia compared to healthy women. It would therefore be interesting to compare the changes undergone by the hemoglobin scavenger in high-risk women who develop preeclampsia with high-risk women who eventually have an uncomplicated pregnancy.

Taken together, the previous findings provide important insights into the pathophysiology of preeclampsia and have paved the way for the development of novel therapeutic approaches. Considering the key role of cell-free hemoglobin's pro-oxidant properties in the pathophysiology of preeclampsia and the antioxidant properties of A1M, it is tempting to claim that A1M is a promising antioxidant strategy for the management of preeclampsia. In fact, the therapeutic potential of A1M against preeclampsia has previously been studied both *ex vivo* and *in vivo*. In an *ex vivo* placenta perfusion model, A1M has been found to inhibit oxidative damage induced by cell-free hemoglobin [37]. Furthermore, A1M has been shown to ameliorate placental and kidney damage in different preeclampsia animal models

[94, 95]. So far, however, there has been no study investigating whether exogenous administration of A1M confers any protection against preeclampsia-induced damage to the maternal heart.

## Cardiovascular effects of preeclampsia

Cardiovascular disease (CVD) is the leading cause of death among men and women globally, mainly due to uncontrolled risk factors in many adults [96]. Traditional cardiovascular risk factors, such as smoking and diabetes mellitus, affect both women and men, albeit with sex-specific differences as to their potency [97, 98]. However, there are cardiovascular risk factors which are unique to women, such as early menopause [99] and a history of certain pregnancy complications, including gestational diabetes [100], preterm delivery [101] and preeclampsia [102].

According to a recent report by the American College of Cardiology/American Heart Association's Task Force on clinical practice guidelines, a history of preeclampsia is considered to be an additional cardiovascular risk factor that can be used to revise the 10-year atherosclerotic cardiovascular disease (ASCVD) risk estimate [103]. The guidelines are based on epidemiologic studies which have revealed that a history of preeclampsia is associated with an increased risk of CVD later in life [104-107].

According to a recent meta-analysis by Wu *et al*. [104] which included 22 studies with over 258 000 preeclamptic women, a history of preeclampsia is associated with an increased risk of coronary heart disease, heart failure, stroke and death due to cardiovascular disease later in life. Interestingly, the risk persisted even after adjustment for traditional cardiovascular risk factors, such as age, body mass index and diabetes mellitus. However, preeclampsia is a heterogeneous disorder with a broad clinical spectrum and varying severity and the risk of long-term CVD varies depending on disease severity [12, 108, 109]. According to a meta-analysis by McDonald *et al.* [105], there is a positive correlation between the severity of preeclampsia and the risk of CVD later in life. Mild preeclampsia is associated with a twofold increased risk, while severe preeclampsia, defined as preeclampsia complicated by preterm delivery or fetal death, is associated with a fivefold increased risk for future cardiac disease.

Interestingly, preeclampsia and CVD share many risk factors, such as hypertension [110], obesity [111] and diabetes mellitus [112], which predispose to both

conditions. On the other hand, a woman who experienced preeclampsia in a previous pregnancy is more likely to develop hypertension [113, 114], diabetes mellitus [115] or dyslipidemia [116] later in life compared to a woman who had an uncomplicated pregnancy. Important questions that remain unanswered at present are: what is the link between preeclampsia and the occurrence of long-term CVD? Does preeclampsia per *se*, irrespective of the associated cardiovascular risk factors, have an impact on maternal cardiovascular health that persists after delivery? Can the association between CVD and preeclampsia solely be explained by the fact that common risk factors exist pregestationally and predispose to both conditions?

The questions have been the object of several epidemiologic studies with contradictory findings. Compared to women who had uncomplicated pregnancies, Romundstad *et al.* found increased body mass index and blood pressure, as well as an unfavorable lipid profile after delivery in women who had preeclampsia [117]. However, the difference was attenuated after adjustment for cardiovascular risk factors that were present before pregnancy, suggesting that the influence of pregestational cardiovascular risk on future cardiovascular disease risk is more important than preeclampsia *per se*. On the other hand, Berks *et al.* conducted a literature-based study and found that preeclampsia seems to be a true risk factor affecting the risk of maternal CVD later in life, independently of other co-existing cardiovascular risk factors [118]. However, the precise mechanism behind this association remains to be defined and a series of hypotheses have been proposed, such as the high rate of metabolic syndrome [119], the persistent abnormal endothelial function [120] and the persistent abnormal activity of the sympathetic nervous system in formerly preeclamptic women [121].

In recent years, there has been an increasing amount of literature on the structural and functional changes undergone by the maternal heart during a pregnancy complicated by preeclampsia. According to a recent meta-analysis by Castleman *et al*. [122], maternal left ventricular mass has been found to be significantly increased in preeclampsia compared to normal pregnancy. In addition, preeclampsia is associated with concentric remodeling of the left ventricle, in contrast to normal pregnancy which is characterized by eccentric left ventricular hypertrophy. Whether the aforementioned changes in maternal cardiac structure revert to normal or persist after delivery has been the subject of a series of echocardiographic studies. Gossein-Doha *et al.* evaluated maternal cardiac structure and function in formerly preeclamptic women nine months postpartum and found a significantly increased left ventricular mass index in women who subsequently developed hypertension compared to formerly preeclamptic women who remained normotensive [123].

Similarly, Melchiorre *et al*. evaluated the maternal cardiac structure and function in formerly preeclamptic women at one year postpartum. They found a persistent left ventricular altered geometric pattern in as many as 19% of women who previously developed term preeclampsia and in 41% of women who previously developed preterm preeclampsia [124]. In addition, they also showed that formerly preeclamptic women with persistent left ventricular structural and functional abnormalities were more likely to subsequently develop hypertension. Taken together, these results suggest that alterations in cardiac mass and morphology occurring during preeclamptic pregnancy may persist postpartum and may be one of the pathophysiologic mechanisms by which preeclampsia is associated with CVD later in life.

More recently, however, literature has emerged that offers contradictory findings about the long-term persistence of maternal cardiac structural changes after a pregnancy complicated by preeclampsia. Ersbøll *et al.* evaluated maternal cardiac structure and function in formerly preeclamptic women using cardiac magnetic resonance imaging (MRI), after a median time of 91 months from the delivery [125]. They could not detect any difference in the left ventricular mass between formerly severely preeclamptic women compared to women with uncomplicated pregnancies. These results suggest that the recovery of the maternal heart after a preeclamptic pregnancy may occur more often than has previously been shown.

It is obvious that there is abundant room for further progress in determining what the persistent maternal cardiovascular changes after a preeclamptic pregnancy are, how long they persist after delivery and in which group of preeclamptic women these changes are more prevalent. Among previously published studies, few have exclusively included women with preeclampsia with severe features as defined by the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. A recent study investigated the acute effects of severe preeclampsia on maternal cardiac structure and function [126]. This study included a high proportion of women with early-onset preeclampsia with severe features. They found a higher left ventricular septal and posterior wall thickness compared to controls.

To the best of our knowledge, there is a lack of studies focusing on the maternal cardiac changes associated with late-onset severe preeclampsia. This thesis intends to determine the extent to which maternal cardiac changes associated with late-onset severe preeclampsia persist after delivery. Considering that severe preeclampsia is a rare condition, we have used cardiac MRI for the evaluation of maternal cardiac

changes, a method which confers significant advantages over the echocardiographic approaches. Cardiac magnetic resonance imaging (MRI) allows for accurate quantification of cardiac volumes and function [127], blood flow [128] and pulse wave velocity [129], with the advantage of smaller sample size for similar power compared to echocardiography [130]. This thesis also intends to advance the understanding of the therapeutic effects of alpha-1-microglobulin against preeclampsia, including the possible cardio-protective effects. Considering that preeclampsia is associated with both short- and long-term adverse cardiovascular outcomes, our findings may have important implications for future practice.

# Aims

## Study I

The aim of the study was to evaluate the maternal plasma levels of A1M and HPX between 26 and 28 weeks of gestation in women affected by preeclampsia, in women without risk factors for preeclampsia who had an uncomplicated pregnancy (low-risk controls), and in women with risk factors for preeclampsia who did not develop preeclampsia during pregnancy (high-risk controls).

## Study II

The aim of this study was to investigate the therapeutic effects of A1M using the STOX1 mouse model of preeclampsia.

## Study III

The aim of the study was to investigate the structural and functional changes undergone by the maternal heart within six months postpartum in women with severe late-onset preeclampsia compared to women with uncomplicated pregnancies using cardiac MRI.

## Study IV

The aim of the study was to evaluate the longitudinal changes in maternal plasma levels of A1M and HPX in women with preeclampsia, high-risk controls, and low-risk controls. In each woman, the levels of A1M and HPX were measured at three different time points; at 12 to 14, 18 to 20, and 26 to 28 weeks of gestation.

# Material and Methods

## Studies I and IV

### Study population

The Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO) study is a prospective study conducted in Finland. Women with a singleton pregnancy who visited antenatal clinics in Finland for their first ultrasound screening were recruited between 2005 and 2009. Pregnant women with risk factors for preeclampsia and IUGR and pregnant women without risk factors were enrolled. The inclusion criteria are shown in **table 1**.

**Table 1. Inclusion and exclusion criteria for women with risk factors for preeclampsia and IUGR**

| **Inclusion criteria** |
|---|
| Obesity (body mass index over 30 kg/m2) |
| Chronic hypertension (BP140/90 mmHg or antihypertensive medication before 20 weeks of gestation) |
| Sjögren's syndrome |
| History of gestational diabetes |
| History of small for gestational age (birthweight < -2SD) |
| History of fetus mortus (fetal death after 22 weeks of gestation or >500 g weight in a previous pregnancy) |
| Systemic lupus erythematosus |
| Type I diabetes mellitus |
| |
| **Exclusion criteria** |
| Smoking (during this pregnancy) |
| Multiple pregnancy |
| History of asthma |
| History of peptic ulcer |
| Placental abruption |
| Inflammatory bowel diseases (Crohn's disease, ulcerative colitis) |
| Rheumatoid arthritis |
| Hemophilia or thrombophilia (previous venous or pulmonary thrombosis or coagulopathy) |

**Study I** and **IV** were conducted using randomly selected subjects who were enrolled in the PREDO study. In total, 142 women were included:

- 51 women without risk factors who had an uncomplicated pregnancy (**low-risk controls**)
- 49 women with risk factors who did not develop preeclampsia (**high-risk controls**)
- 42 women with risk factors who developed preeclampsia

## Blood samples

Fasting blood samples were collected from each patient in each trimester, at 12-14, 18-20 and 26-28 weeks of gestation. **Plasma** was separated from whole blood by centrifugation and stored at -80$^{o}$ C.

*Hemopexin*

Plasma levels of hemopexin were measured using the Human Hemopexin ELISA kit from Genway Biotech Inc. In this assay the hemopexin reacts with the anti-hemopexin antibodies which have been pre-coated onto a well plate. After the removal of unbound proteins by washing, anti-hemopexin antibodies conjugated with horseradish peroxidase (HRP) are added and form complexes with the previously bound hemopexin. An enzymatic reaction catalyzed by HRP occurs after adding the chromogenic substrate tetramethylbenzidine (TMB). The density of coloration is read by absorbance at 450 nm.

*Alpha-1-microglobulin*

Plasma levels of A1M are measured with an in-house ELISA. In this assay the A1M reacts with the mouse monoclonal anti-A1M antibodies which have been pre-coated onto a well plate. The mouse monoclonal anti-A1M antibodies are prepared against human urinary A1M by Agrisera AB. After the removal of unbound proteins by washing, anti-A1M mouse monoclonal antibodies conjugated with HRP are added and form complexes with the previously bound A1M. After adding the chromogenic substrate TMB, an enzymatic reaction catalyzed by HRP occurs and the density of coloration is read by absorbance at 450 nm.

### Statistical analysis

In **Study I**, we evaluated the maternal plasma levels of A1M and hemopexin at 26-28 weeks of gestation. Kruskal-Wallis and Mann-Whitney tests were used for group comparisons. Bonferroni corrections were used in post hoc comparisons. In all analyses, a p-value $< 0.05$ was considered statistically significant.

I **Study IV**, we evaluated the longitudinal changes in maternal plasma levels of A1M and hemopexin. Repeated measures analysis of variance was used for group comparisons. The statistical tests were performed on logarithmically transformed data due to the positively skewed distribution. In all analyses, a p-value $< 0.05$ was considered statistically significant.

### Ethical considerations

Ethical approval was given by the regional ethical review board. All participants provided written informed consent before participating.

## Study II

### Storkhead box 1 (STOX1) mouse model of preeclampsia

In **Study II**, the STOX1 transgenic mouse model of preeclampsia was used. By mating wildtype females with STOX1 transgenic males, the pregnant females overexpress the STOX1 transcription factor in the fetoplacental unit and develop symptoms similar to severe, early-onset preeclampsia in humans, such as hypertension, proteinuria, decreased litter size and increased plasma levels of sFLT-1 and sENG. Furthermore, it has been previously shown that preeclamptic mice overexpressing STOX1 in the fetoplacental unit develop cardiac hypertrophy.

In Study II, four groups of mice were included:

- wildtype females mated to wildtype males and injected with buffer
- wildtype females mated to wildtype males and injected with human recombinant A1M
- wildtype females mated to STOX1 transgenic males and injected with buffer

- wildtype females mated to STOX1 transgenic males and injected with human recombinant A1M

The experiment lasted from the time of mating until termination at 17.5 days post coitum (dpc). The females were given six intraperitoneal injections of either buffer or human recombinant A1M every second day starting at 6.5 dpc. The experiments were conducted at two different locations; Paris (France) and Lund (Sweden). The experimental design is shown in **figure 3**.

## Human recombinant A1M

Human recombinant A1M was donated by A1M Pharma AB (Lund, Sweden).

## Measurement of blood pressure

Blood pressure was measured using a non-invasive tail-cuff device. The mice were trained for one week to accustom them to the manipulation. During the blood pressure measurement, the female was non-anesthetized and placed on a warming platform and in an animal restrainer of appropriate size.

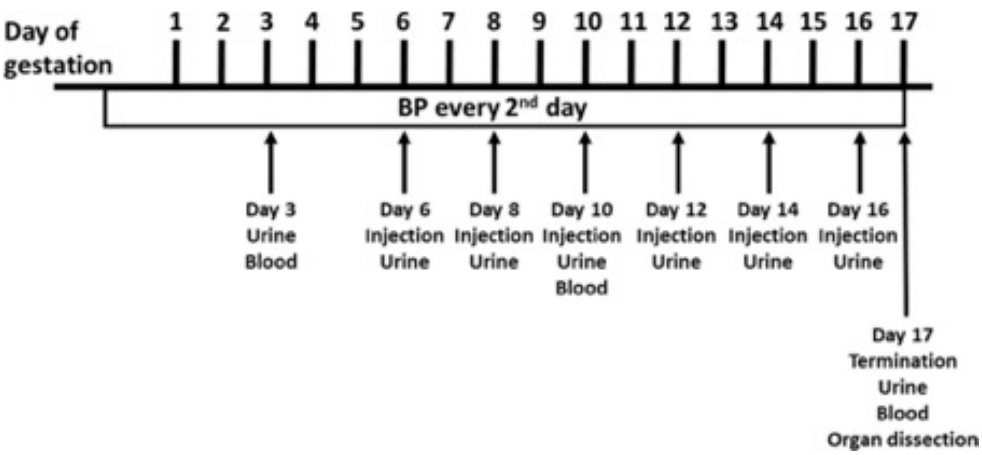


**Figure 3. Illustration of the experimental design in Study II [131]**

## Blood and urine analysis

A urine sample was obtained non-invasively by placing the mice on a cold metal surface to induce urination. The concentration of albumin was measured using the mouse albumin ELISA kit Albuwell M from Exocell. The concentration of creatinine was measured using the assay The Creatinine Companion from Exocell. The albumin-to-creatinine ratio is calculated by dividing the albumin concentration in micrograms by the creatinine concentration in milligrams.

A blood sample was obtained from the saphenous vein and from the posterior vena cava. Plasma was separated from whole blood by centrifugation and was stored at -80º C. Plasma levels of sFlt1 are measured with the Mouse VEGFR1/Flt-1 Quantikine ELISA Kit from R&D Systems. Plasma levels of sEng were measured with the Mouse Endoglin/CD105 Quantikine ELISA Kit from R&D Systems.

## Assessment of placental hypoxia and placental oxidative stress

Immunohistochemical analysis using pimonidazole hydrochloride was used for the assessment of placental hypoxia. Pimonidazole hydrochloride (Hydroxyprobe$^{TM}$) is injected intraperitoneally two hours before termination. Pimonidazole is reductively activated in hypoxic cells and forms adducts with thiol groups in proteins, peptides, and amino acids. A monoclonal antibody binds to the stable adducts and is detected by immunoperoxidase staining after appropriate tissue collection, formalin fixation, and paraffin embedding. The amount of pimonidazole that is detected is directly proportional to the level of hypoxia.

In conditions of oxidative stress, nitric oxide reacts with superoxide to form peroxynitrite, which nitrates tyrosine residues in proteins. Nitrotyrosine therefore serves as a marker of oxidative stress and is detected in protein extract from placenta by using the OxiSelect Nitrotyrosine ELISA kit from Cell Biolabs Inc.

## Cardiac MRI

MRI examination was performed at 17.5 dpc. A 9.4 Tesla MRI scanner was used. The pregnant mouse was sedated and positioned on a warming pad to maintain a temperature of 37ºC. A fast low angle shot (FLASH) sequence with respiratory and electrocardiogram (ECG) triggering was used to acquire short-axis cine images covering the ventricles. Manual segmentation of the endocardial and epicardial borders of the left ventricle was performed at end-systole and end-diastole, allowing

for accurate measurement of end-diastolic and end-systolic ventricular volume, ejection fraction, cardiac output and left ventricular mass.

### Analysis of gene expression in the kidney

We evaluated the expression of genes involved in the protection against oxidative stress in the kidney. Total RNA was extracted from frozen kidney biopsies. The RNA is first converted into cDNA using reverse transcription. The cDNA was then used as a template for exponential amplification using real-time PCR for the genes heme oxygenase-1 (HO-1), catalase (CAT) and superoxide dismutase 2 (SOD2). The gene expression levels were normalized to the hypoxanthine phosphoribosyltransferase (HPRT) levels.

### Microscopy

Histologic analysis of placental, renal and cardiac tissue was performed using hematoxylin and eosin staining or Massons's trichrome staining. Transmission electron microscopy (TEM) was performed on placental, renal and cardiac biopsies.

### Ethical considerations

The study was approved by the local ethics committees for animal studies at Lund University (Lund, Sweden) and Institut National de Recherche Agronomique (Jouy-en-Josas, France).

## Study III

### Study population

Women with severe late-onset preeclampsia, occurring after 34 gestational weeks, and women with uncomplicated pregnancy were prospectively recruited at Skåne University Hospital in Malmö and in Lund between 2014 and 2017. The severity of preeclampsia was defined according to the criteria set by the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Six women with preeclampsia and eight women with uncomplicated pregnancy were

enrolled in the study and undergone cardiac MRI at one to three days postpartum, one week postpartum and six months postpartum.

## Cardiac MRI

Study participants underwent cardiac magnetic resonance imaging using a 1.5 Tesla MRI scanner. A balanced steady state free precession (bSSFP) sequence was used to acquire cine images in the standard projections. Manual segmentation of the endocardial and epicardial borders of the left ventricle and right ventricle was performed at end-systole and end-diastole, allowing for accurate measurement of end-diastolic and end-systolic ventricular volume, stroke volume and ejection fraction. Left ventricular mass was calculated as the left ventricular wall volume multiplied by the left ventricular myocardial density (1.05 g/ml).

For the assessment of pulse wave velocity and cardiac output, phase-contrast quantitative flow data were acquired. Flow curves were obtained from the ascending aorta at the level of the pulmonary trunk and from the descending aorta at the level of the diaphragm. Pulse wave velocity was calculated as the distance between the flow planes divided by the transit time of the flow curve from the descending aorta to the descending aorta. Cardiac output was obtained by multiplying the ascending aortic flow by the heart rate.

## Ethical considerations

Ethical approval was given by the regional ethical review board. All participants provided written informed consent before participating.

# Summary of results

## Study I

The aim of the study was to evaluate the maternal plasma levels of A1M and HPX between 26 and 28 weeks of gestation in women affected by preeclampsia, in women without risk factors for preeclampsia who had an uncomplicated pregnancy (**low-risk controls**), and in women with risk factors for preeclampsia who did not develop preeclampsia during pregnancy (**high-risk controls**).

### Maternal plasma levels of A1M

The high-risk controls had higher levels of A1M compared to the low-risk controls. We found no difference in plasma levels of A1M between the women with preeclampsia and the high-risk controls, and between the women with preeclampsia and the low-risk controls. Interestingly, the median plasma concentration of A1M was higher in the high-risk controls compared to the women with preeclampsia, although the difference was not statistically significant.

The women with severe preeclampsia had higher plasma A1M levels compared to the women with non-severe preeclampsia.

### Maternal plasma levels of HPX

The women with preeclampsia had higher plasma levels of HPX compared to the low-risk controls. There was no difference in plasma levels of HPX between the high-risk controls and the low-risk controls, and between the high-risk controls and the women with preeclampsia.

# Study II

The aim of this study was to investigate the therapeutic effects of A1M using the STOX1 mouse model of preeclampsia.

We found an increase in systolic blood pressure in the preeclamptic females at midgestation and at late gestation compared to the controls. The intraperitoneal administration of human recombinant A1M led to a significant decrease in systolic blood pressure at midgestation in preeclamptic females.

We found a decreased placental weight and a trend toward increased levels of placental hypoxia and placental nitrative stress in preeclamptic females compared to controls. Treatment with human recombinant A1M resulted in decreased levels of hypoxia and protein nitration in the placentas of the preeclamptic females. The histological analysis revealed areas with necrosis and edema in the placentas of the untreated preeclamptic females. In contrast, the placentas of the treated preeclamptic females were indistinguishable from the controls.

The preeclamptic females had increased heart weight compared to the controls. Treatment with human recombinant A1M did not reduce the heart weight of the preeclamptic females. However, the cardioprotective effects of A1M were visible in electron microscopy. We found structural changes in the heart of the preeclamptic females, namely swollen and erupted mitochondria and irregularly organized muscle fibers. When treated with A1M, the preeclamptic females showed more organized structure and less mitochondrial damage. Cardiac MRI showed increased left ventricular mass in untreated preeclamptic females compared to the controls. We found no difference in the left ventricular ejection fraction between untreated preeclamptic females and controls.

We found proteinuria in the untreated preeclamptic females at late gestation, which was not present in the preeclamptic females receiving treatment with human recombinant A1M. The expressions of genes involved in protection against oxidative stress was reduced in the kidneys of the preeclamptic females treated with A1M compared to the untreated preeclamptic females, suggesting that exogenous administration of A1M alleviates renal oxidative stress. Transmission electron microscopy revealed glomerular changes in the kidneys of the untreated preeclamptic females, namely effacement of podocyte foot processes, swollen and irregular glomerular basement membrane, and structurally aberrant endothelial fenestration. In contrast, treatment of the preeclamptic females with human recombinant A1M resulted in normal glomerular morphology.

# Study III

The aim of the study was to investigate the structural and functional changes undergone by the maternal heart within six months postpartum in women with severe late-onset preeclampsia compared to women with uncomplicated pregnancies using cardiac MRI.

## Left ventricular mass

We found increased left ventricular mass index in the women with severe late- onset preeclampsia compared to the women with uncomplicated pregnancies at one to three days after delivery (57 g/m$^2$ versus 48 g/m$^2$). However, there was no difference between the two groups at six months after delivery. In the preeclamptic women, the left ventricular mass index was decreased by 19% between one to three days after delivery and six months after delivery (**figure 4**).

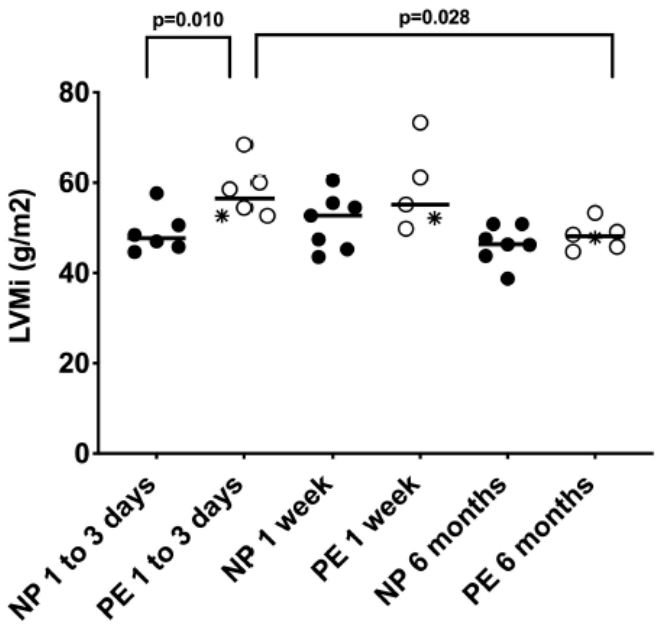


**Figure 4. Left ventricular mass index (LVMi) in normal pregnancy (NP) and in severe late-onset preeclampsia (PE)**

### Pulse wave velocity

Pulse wave velocity (PWV) was increased in the preeclamptic women compared to the women with uncomplicated pregnancies, although the difference was not statistically significant (6.1 m/s versus 5.6 m/s). In the preeclamptic women, PWV decreased from 6.1 m/s at one to three days after delivery to 5 m/s at six months after delivery.

## Study IV

The aim of the study was to evaluate the longitudinal changes in maternal plasma levels of A1M and HPX in women with preeclampsia, high-risk controls and low-risk controls. In each woman, the levels of A1M and HPX were measured at three different time points; at 12 to 14, 18 to 20, and 26 to 28 weeks of gestation.

### Maternal plasma levels of A1M

- At **12 to 14** weeks of gestation, the maternal plasma levels of A1M were higher in women with preeclampsia compared to the high-risk and low-risk controls. The plasma levels of A1M were higher in the high-risk controls compared to the low-risk controls.
- At **18 to 20** and **26 to 28** weeks of gestation, plasma levels of A1M were higher in the high-risk controls compared to the preeclamptic women and the low-risk controls. The plasma levels of A1M were higher in the preeclamptic women compared to the low-risk controls at **18 to 20** weeks of gestation.
- At **18 to 20** and **26 to 28** weeks of gestation, the preeclamptic women with a small for gestational age (SGA) fetus had higher plasma levels of A1M compared to the preeclamptic women with an appropriate for gestational age (AGA) fetus.
- When comparing the preeclamptic women to the high-risk controls, there were differences in the changes in A1M between the first and the second time points; the concentration of A1M increased in the high-risk controls between the first and the second time points. In contrast, the concentration of A1M was decreased in the preeclamptic women during the same period.

## Maternal plasma levels of HPX

- At **12 to 14** weeks of gestation, the maternal plasma levels of HPX were lower in the high-risk controls compared to the preeclamptic women and low-risk controls.
- At **18 to 20** and **26 to 28** weeks of gestation, the maternal plasma levels of HPX were higher in the preeclamptic women compared to the high-risk and low-risk controls.
- There were differences in the changes in maternal plasma levels of HPX between the low-risk and high-risk controls; we found a decrease in the mean plasma concentration of HPX in the low-risk controls between the second (18 to 20 weeks of gestation) and the third time points (26 to 28 weeks of gestation). In contrast, the levels of HPX in the high-risk controls slightly increased during the same period.

# Discussion

## Cardiac implications of preeclampsia (Studies II and III)

The key question of the **third study** was whether late-onset preeclampsia with severe features is associated with structural and functional cardiac changes that persist postpartum and contribute to the increased risk of long-term CVD. The reason why we chose to study women with severe features is that in these women we expected to find cardiac changes which were more prominent during pregnancy and persist longer after delivery compared to preeclamptic women without severe features. As expected, we found increased left ventricular mass index in the women with preeclampsia compared to the women with uncomplicated pregnancy at one to three days after delivery. However, the difference was rapidly attenuated and disappeared at six months after delivery in parallel with the normalization of blood pressure.

A possible explanation for our results may be that the increased left ventricular mass index in late-onset severe preeclampsia is the result of increased cardiac afterload due to severe hypertension. As blood pressure normalizes after delivery, the cardiac changes attenuate. In contrast, in early-onset preeclampsia, where structural and functional cardiac changes have previously been shown to persist for several months after delivery, other pathophysiologic mechanisms may be involved in the persistent cardiac hypertrophy [124]. In a recent study by Benschop *et al.* [132], low mid-pregnancy levels of PlGF were found to be associated with high left ventricular mass six years after pregnancy. A low level of PlGF throughout pregnancy is considered to be an indicator of abnormal placentation and identifies the group of preeclamptic women with an early disease onset [133]. It can thus be suggested that low levels of PlGF may account for the persistent cardiac changes after early-onset preeclampsia.

The STOX1 model of preeclampsia provides a useful tool to advance our knowledge on the underlying mechanisms linking preeclampsia to long-term CVD [134-137]. Previous studies using the STOX1 model have reported increased heart weight and cardiac fibrosis in the preeclamptic mice. Interestingly, the cardiac changes

persisted at eight months after the pregnancy, even though there was no significant difference in blood pressure between the preeclamptic mice and controls. Furthermore, gene expression analysis showed a strong upregulation of interleukin-6 (IL-6) in the endothelial cells of the preeclamptic mice at eight months after the pregnancy [136]. Interleucin-6 is a pleiotropic cytokine with a series of adverse effects, such as endothelial cell dysfunction [138] and myocardial remodeling characterized by myocardial fibrosis, concentric hypertrophy and diastolic dysfunction [139]. One question that needs to be asked, however, is what factor triggers the sustained alterations in IL-6 expression in endothelium several months after a complicated pregnancy. As shown in a recent study by Cronqvist *et al.* [140], the sustained alterations in gene expression in endothelial cells may be the result of microRNA deposition, transferred from the preeclamptic placenta through circulating syncytiotrophoblast-derived extracellular vesicles.

In the **second study**, we sought to elucidate the protective effects conferred by A1M using the STOX1 model of preeclampsia. We found that exogenous administration of human recombinant A1M, besides reducing blood pressure during mid and late gestation in the STOX1 model of preeclampsia, reduces the level of oxidative stress and the tissue damage seen in the preeclamptic placenta and kidneys. The most interesting finding, however, was that A1M has a cardio-protective effect. Transmission electron microscopy revealed that preeclamptic mice treated with A1M showed more regular myocardial fiber organization and less mitochondrial damage compared to untreated preeclamptic mice. Interestingly, we found increased heart weight in the preeclamptic mice compared to the controls, which was the result of both myocyte hypertrophy and increased collagen deposition in the myocardium. The heart weight was not reduced by treatment with A1M, even though the treatment led to a significant reduction in blood pressure. The results may be explained by the fact that a reduction in blood pressure reduces the cardiac afterload but does not affect cardiac fibrosis. It would be difficult to study at which extent cardiac fibrosis occurs after preeclamptic pregnancy in humans, since the current gold standard of diagnosing cardiac fibrosis is endomyocardial biopsy [141]. However, it is tempting to hypothesize that cardiac fibrosis occurs in the subgroup of women who develop long-lasting alterations in cardiac structure and function and may contribute to the long-term cardiovascular risk. Another implication of our findings is the possibility that A1M could be effective in reducing the long-term risk of CVD by counteracting changes in cardiac ultramorphology or possibly even cardiac hypertrophy and fibrosis in higher doses. This is an important issue for future research.

# Dynamics of scavenger proteins (Studies I and IV)

Treatment of preeclampsia with A1M is promising but many uncertainties remain. The timing and the duration of exposure to exogenously administered A1M are uncertain. In addition, the concentration to which A1M should be raised in the maternal circulation has not previously been studied. However, these uncertainties cannot be clarified without a previous understanding of the dynamics of changes in the levels of A1M in the maternal circulation.

The aim of the **first study** was to evaluate the maternal plasma levels of A1M at the late second trimester, between 26 and 28 weeks of pregnancy. We included patients with preeclampsia, healthy pregnant women (low-risk controls) and pregnant women who entered the pregnancy with risk factors predisposing them to preeclampsia (high-risk controls) but who had an uncomplicated pregnancy. The median plasma concentration of A1M was found to be significantly higher in the high-risk controls compared to the low-risk controls. These results may be explained by the fact that the high-risk controls entered pregnancy with risk factors associated with systemic inflammation and oxidative stress, such as diabetes mellitus, essential hypertension and obesity. The higher level of oxidative stress may have triggered a rise in the levels of A1M.

Although the median value of A1M was higher in the women with preeclampsia compared to the low-risk controls, the difference was not statistically significant, in contrast to previous studies where a statistically significant difference was found between the two groups [41]. However, in the present study, non-parametric tests were used for the comparison of the median values between the groups and subtle differences may have gone undetected. Another explanation may be that the difference in the levels of A1M between the preeclamptic women and the low-risk controls becomes more prominent at a later stage of the pregnancy, close to gestational age at disease onset. This remains to be elucidated in a future study, where it will be necessary to include blood sampling at a later stage of pregnancy.

Surprisingly, we found a trend toward higher plasma concentration of A1M in the high-risk controls compared to the women with preeclampsia between 26 and 28 weeks of pregnancy, although the difference was not statistically significant. We therefore performed the **fourth study** in order to elucidate the longitudinal changes in maternal plasma levels of A1M during pregnancy. The concentration of A1M in maternal plasma was measured on three occasions during pregnancy, at a median gestational age of 13 weeks, 19.4 weeks and 27.1 weeks of gestation. We found

differences in the dynamics of changes in maternal plasma levels of A1M between the women with preeclampsia and the high-risk controls. In particular, the levels of A1M in the preeclamptic women, although initially higher compared to the high-risk controls, decreased between the first and the second measurements. In contrast, the levels of A1M increased in the high-risk controls and surpassed the levels of A1M in the preeclamptic women during the same period. The importance of the difference in the dynamics of changes in the levels of A1M is not clear. It is tempting to hypothesize that the increase in maternal plasma levels of A1M in the high-risk controls reflects their high endogenous capacity to counteract the pro-oxidant factors released from the dysfunctional placenta, such as cell-free HbF, thus protecting them from the development of clinically overt preeclampsia later in pregnancy. In contrast, the decrease in plasma levels of A1M in the women who developed preeclampsia indicates that the defense mechanism against cell-free HbF is inadequate; A1M is depleted, thus resulting in clinically overt preeclampsia.

Interestingly, we found higher levels of A1M in the preeclamptic women with SGA fetuses compared to the preeclamptic women with AGA fetuses. A possible explanation may be the increased leakage of cell-free HbF from the circulation of the SGA fetuses into the maternal circulation. According to a recent study by Brook *et al.* [142], the levels of cell-free HbF are higher in pregnancies complicated by fetal growth restriction compared to normal pregnancies. Hence, it could be hypothesized that the increased levels of HbF in the circulation of SGA fetuses can damage the placental barrier and leak into the maternal circulation, upregulating the expression of A1M. Furthermore, cell-free HbF in the maternal circulation may further aggravate maternal cardiac function; it has previously been shown that free heme induces contractile dysfunction in human cardiomyocytes [143, 144]. Taken together, we hypothesize that cell-free HbF could be the mechanistic link connecting the dysfunctional placenta with the dysfunctional myocardium; cell-free HbF in the circulation of the growth restricted fetus leaks into the maternal circulation after damaging the placental barrier. In the maternal circulation, cell-free HbF affects the myocardial contractility and reduces the cardiac output. The reduced cardiac output, in turn, could further compromise fetal growth, thus resulting in a positive feedback loop which exacerbates both placental dysfunction and myocardial dysfunction. The proposed pathophysiologic mechanism may explain why preeclampsia with fetal growth restriction is characterized by low maternal cardiac output [145, 146].

An arguable weakness of the studies is that we have not measured the concentration of cell-free HbF in the maternal circulation or other markers of oxidative stress and systemic inflammation. Further work needs to be done to establish how A1M levels

are affected by the leakage of cell-free HbF into the maternal circulation in high-risk controls and by the excessive ROS production and systemic inflammation due to pregestational conditions such as essential hypertension and diabetes mellitus.

# Conclusions

The high-risk controls have increased plasma concentration of A1M compared to the low-risk controls. The increased plasma levels of A1M may be associated with increased systemic oxidative stress. More research needs to be undertaken before the association can be more clearly understood. It is suggested that the association of plasma levels of A1M with markers of systemic oxidative stress should be investigated in future studies.

Alpha-1-microglobulin has been found to be effective in alleviating hypertension and proteinuria as well as cellular and subcellular myocardial changes associated with preeclampsia. By counteracting myocardial damage, A1M can possibly confer a long-term cardio-protective effect against cardiovascular implications of preeclampsia. This is an important issue for future research.

Severe late-onset preeclampsia is associated with increased left ventricular mass compared to normal pregnancy. However, the cardiac changes attenuate postpartum and disappear at six months after delivery. This indicates that left ventricular hypertrophy after severe late-onset preeclampsia may be a secondary physiological response to increased peripheral resistance in preeclampsia and not the primary link connecting preeclampsia with CVD later in life.

# Populärvetenskaplig sammanfattning

Havandeskapsförgiftning är en graviditetskomplikation som drabbar 3–7 procent av alla gravida kvinnor i Sverige och leder till signifikant sjuklighet hos mor och barn. Havandeskapsförgiftning kan debutera sent eller tidigt i graviditeten och kan manifesteras med allt från lätta till allvarliga symtom. Idag finns ingen annan behandling för preeklampsi än förlossning. Tidigare studier har visat att kvinnor som utvecklar preeklampsi under graviditeten drabbas ofta av hjärt-kärlsjukdomar senare i livet. Mekanismerna är dock okända och kunskapen om hur havandeskapsförgiftning påverkar hjärtat hos såväl foster som mamma är väldigt begränsad.

Avhandlingens syfte är att studera hur moderns hjärta påverkas vid havandeskapsförgiftning. I studie III undersöker vi med magnetkamera hur moderns hjärta påverkas vid allvarlig havandeskapsförgiftning som debuterar sent i graviditeten. Studie II avser att undersöka, i en djurmodell, hur moderns hjärta påverkas vid allvarlig havandeskapsförgiftning som debuterar tidigt i graviditeten. I samma studie undersöker vi huruvida hjärtats celler återfår sin normala form efter behandling med Alpha-1-Microglobulin (A1M) som är människokroppens eget renhållningsprotein. Studier I och IV avser att öka kunskapen om hur nivåerna av A1M i blodet ändras under graviditeten.

Våra resultat visar att allvarlig preeklampsi som debuterar sent i graviditeten leder till en temporär förstoring av hjärtmuskeln som försvinner inom sex månader efter förlossningen. Allvarlig preeklampsi som debuterar tidigt i graviditeten leder däremot till fibros i hjärtat som eventuellt persisterar efter förlossningen och leder till hjärtsjukdomar senare i livet. Behandling med A1M ger symtomlindring och leder till återställning av hjärtcellernas form. Det finns skillnader i hur nivåerna av A1M ändras under gravidteten mellan kvinnorna som utvecklar havandeskapsförgiftning och kvinnor som har en okomplicerad graviditet.

Sammanfattningsvis, visar resultaten att hjärtpåverkan vid havandeskapsförgiftning varierar. Hos vissa kvinnor kan persisterande hjärtpåverkan uppstå, nämligen fibros i hjärtat, som eventuellt leder till

hjärtsjukdomar senare i livet. Alpha-1-Microglobulin kan vara en effektiv behandling mot havandeskapsförgiftning.

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# Plasma Heme Scavengers Alpha-1-Microglobulin and Hemopexin as Biomarkers in High-Risk Pregnancies

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Women with established preeclampsia (PE) have increased plasma concentration of free fetal hemoglobin. We measured two hemoglobin scavenger system proteins, hemopexin (Hpx) and alpha-1-microglobulin (A1M) in maternal plasma using enzyme-linked immunosorbent assay during the late second trimester of pregnancy in women with high and low risk of developing PE. In total 142 women were included in nested case-control study: 42 women diagnosed with PE and 100 controls (49 randomly selected high-risk and 51 low-risk controls). The concentration of plasma A1M in high-risk controls was higher compared to low-risk controls. Women with severe PE had higher plasma A1M levels compared to women with non-severe PE. In conclusion, the concentration of plasma A1M is increased in the late second trimester in high-risk controls, suggesting activation of endogenous protective system against oxidative stress.

**Keywords: preeclampsia, heme, hemopexin, alpha-1 microglobulin, aspirin**

## INTRODUCTION

Preeclampsia (PE) is a relatively common hypertensive disorder in pregnancy, affecting 4,6% of pregnancies worldwide (Abalos et al., 2013). The specific etiology of PE is, however, still not completely described. According to the most well-accepted model, PE is a two-stage disorder (Redman, 1991; Redman et al., 2014). The abnormal modification of the spiral arteries during

placental development is thought to be the initial stage leading to reduced utero-placental perfusion and increased oxidative stress that in turn causes placental damage. Circulating toxic factors derived from the placenta cross the blood-placenta barrier and leak into the maternal circulation where they in turn trigger an inflammatory response and general endothelial damage. As a consequence of that, general organ damage develops, which leads to the typical manifestations of PE after 20th week of gestation, including hypertension, edema and proteinuria. Circulating syncytiotrophoblast microvesicles (Smarason et al., 1996), free fetal DNA (Zhong et al., 2002), cytokines (Hamai et al., 1997) and antiangiogenic factors (Powe et al., 2011) have been postulated as endothelial toxic factors derived from the fetus and the placenta. However, we still lack a full explanation on how placental damage leads to distinct maternal and fetal manifestations that occur either during early pregnancy or late pregnancy, so called early onset PE and late-onset PE. Early onset PE is linked to poor placentation while the late-onset is more determined by maternal risk factors such as obesity, diabetes mellitus and chronic hypertension, which are associated with a higher pre-pregnancy level of vascular inflammation (Roberts and Redman, 1993; Ness and Roberts, 1996).

Extracellular fetal hemoglobin (HbF) has been introduced in a series of earlier studies and suggested to have a crucial role in the etiology of PE (Hansson et al., 2013). Increased synthesis of HbF in the placenta was indicated by an up-regulation of the HbF genes and there was an accumulation of extracellular HbF in the vascular lumen of PE placenta (Centlow et al., 2008). Extracellular HbF induces oxidative stress by formation of reactive oxygen species resulting in damage to the blood-placenta barrier and leakage of extracellular HbF into the maternal circulation (May et al., 2011). As a consequence, plasma concentration of extracellular HbF has been shown to be increased in maternal plasma as early as the first trimester in women who later develop PE (Anderson et al., 2011). Increased plasma levels in the late third trimester has been shown to correlate to the maternal blood pressure (Anderson et al., 2018).

There are several defense mechanisms which protect against the harmful effects of extracellular hemoglobin (Hb). Haptoglobin (Hp) is the most important protective scavenger protein that binds extracellular Hb in plasma resulting in a complex that in turn is cleared via CD163 receptors on macrophages (Kristiansen et al., 2001; Schaer et al., 2006). Hemopexin (Hpx) has a complementary role to bind extracellular heme that is released as a metabolite when Hb is degraded by the rate-limiting enzyme heme-oxygenase (HO-1) (Nielsen et al., 2010; Tolosano et al., 2010). The resulting complex is cleared from the circulation by liver parenchymal cells via receptor-mediated endocytosis involving CD91/LRP1. Alpha-1-microglobulin (A1M) is another component of the heme scavenger system (Akerstrom and Gram, 2014). It is a lipocalin with heme-binding properties as well as being an antioxidant due to radical-scavenging and reductase properties. In a series of studies (Gram et al., 2015; Anderson et al., 2018), it has been shown that the plasma levels of Hp and Hpx are reduced, suggesting that in cases where the maternal endogenous protection system against extracellular HbF is overwhelmed, PE becomes clinically manifest. Cellular A1M expression of A1M is upregulated by increased oxidative stress and Hb/heme exposure (Olsson et al., 2007) and previous investigations have shown increased circulating plasma levels of A1M in women pregnant with PE (Olsson et al., 2010; Anderson et al., 2011) consistent with high circulating levels of Hb, heme and oxidants in this disease.

In the present study, we analyzed the plasma levels of A1M and Hpx in order to further understand the dynamics of these components of the Hb/heme scavenger system in the second trimester in women with high and low risk of developing PE. The cohort of patients were stratified according to known maternal risk factors, intervention with acetylsalicylic acid (ASA) as well as neonatal outcome.

# MATERIALS AND METHODS

## Study Population

The present nested case-control study is a part of the multidisciplinary "Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction" (PREDO) project. Women with known risk factors for PE were prospectively recruited between September 2005 and June 2009 at ten participating maternity clinics in Finland. The ethics Committee at the Helsinki and Uusimaa Hospital District approved the study and written informed consent was obtained from all participants.

In total 142 women were included in this study: 42 women diagnosed with PE and 100 controls (49 randomly selected high-risk and 51 low-risk controls). Seven women with PE participated in the ASA trial (part of the PREDO project), as well, and were treated with low dose acetylsalicylic acid (mini-ASA 100mg/d) starting before 14th week of gestation. Three women who were taking mini-ASA and did not develop PE, were included as controls for this sub-group. The inclusion and exclusion criteria are described in **Supplementary Table S1**.

Preeclampsia was defined as a systolic blood pressure $\geq$140 mmHg and/or a diastolic blood pressure $\geq$90 mmHg occurring after 20th weeks of gestation combined with a urinary 24-h protein excretion of $\geq$0.3 g or the dipstick equivalent in two consecutive measurements. Severe PE was defined as systolic blood pressure $\geq$160 mmHg and/or diastolic blood pressure $\geq$110 mmHg and/or proteinuria $\geq$5 g/24 h. Small for gestational age (SGA) was defined as a birthweight $\leq$minus 2 SDs.

All participants had their first visit at $12^{+0}$–$14^{+0}$ weeks of gestation. Uterine artery blood flow was measured with Doppler ultrasound examination. Gestational age was confirmed by crown-rump length measurement. The first trimester mean arterial pressure (MAP) was calculated with the equation: MAP = diastolic blood pressure + (systolic blood pressure – diastolic blood pressure)/3.

Fasting blood samples were collected in all three trimesters. Plasma was separated within an hour by centrifugation and stored in $-80^{\circ}$C until analysis. In the present study we determined serum A1M and Hpx concentrations from samples drawn at $26^{+0}$ to $28^{+0}$ weeks of gestation.

## Hemopexin ELISA

The Hpx concentrations were measured with a Human Hemopexin ELISA Kit from Genway Biotech Inc. The analysis was performed according to manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter.

## A1M ELISA

The A1M concentrations were measured with an in-house A1M ELISA. Flat-bottom ninety six-well microtiter plates were coated with mouse monoclonal anti-A1M antibodies (clone 35.14) by incubation overnight at +4°C under sealing film with 100 μl/well of a 5 μg/ml-solution in PBS. After washing three times with PBS + 0.05% tween-20, 100 μl of human urinary A1M reference standard samples (1.56 – 100 ng/ml in PBS + 0.05% tween-20) or unknown plasma samples (diluted 1000× with PBS + 0.05% tween-20) were added to the wells and incubated under sealing film for 1 h at room temperature, darkness and rotational shaking 250–500 rpm. After washing three times with PBS + 0.05% tween-20, 100 μl/well of the detection antibody solution was added (horse radish peroxidase-coupled mouse monoclonal anti-A1M antibody clone 57.10; 5 ng/ml in PBS + 0.05% tween-20) and incubated under sealing film for 1 h at room temperature, darkness and rotational shaking 250–300 rpm. After washing three times with PBS + 0.05% tween-20, 100 μl/well of TMB substrate (SureBlue$^{TM}$ TMB Microwell Peroxidase Substrate, KPL cat. no. 50-00-04) was added, sealed, and incubated 20 min without shaking, and the reaction was stopped by adding 100 μl/well of 1 M sulfuric acid. Absorbance at 450 nm was read in a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences). The monoclonal anti-A1M antibodies were prepared against human urinary A1M by Agrisera AB (Vännäs, Sweden). Human urinary A1M was prepared in our lab as described (Akerstrom et al., 1995).

## Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 statistic software package. Normally distributed data were analyzed using one-way ANOVA followed by Tukey's *post hoc* tests. Kruskal-Wallis and Mann-Whitney test were used in case the data were not normally distributed and Bonferroni corrections were used in *post hoc* comparisons. Statistical significance was defined as $p < 0.05$.

# RESULTS

Patient demographics and clinical characteristics are shown in **Table 1**, **2**. Women affected by PE had higher body mass index (29,3 kg/m$^2$) compared to controls (23,7 kg/m$^2$). There were less primiparas among women affected by PE compared to controls. There were less women with a previous pregnancy complicated by PE among controls compared to women affected by PE.

The distributions of plasma Hpx and A1M across the groups are shown in **Figures 1A,B**. Pairwise comparisons using significance values adjusted by the Bonferroni correction for multiple tests revealed that the concentration of Hpx in women with PE was higher compared to low-risk normotensive women (median concentration 1.21 mg/ml versus 1.04 mg/ml, $p = 0.014$). There was no significant difference in Hpx concentration between women with PE and high-risk normotensive women (median concentration 1.11 mg/ml) or between high-risk and low-risk normotensive women. The concentration of A1M in high-risk controls was higher compared to low-risk controls (median concentration 16.08 μg/ml vs. 13.09 μg/ml, $p = 0.002$). There was no significant difference in A1M concentration between women with PE and high-risk controls or between women with PE and low-risk controls.

The concentrations of plasma Hpx and A1M in women with PE are shown in **Table 3**. We found no difference in Hpx or A1M concentrations between women with PE who gave birth to SGA infant and women with PE who gave birth to non-SGA infant. The concentration of A1M in women with severe PE was higher compared to women with non-severe PE. The distributions of Hpx and A1M across the subgroups of PE, high- and low-risk controls are shown in **Figures 1C,D**.

**TABLE 1** | Demographics of patients and controls.

| | Women not affected by PE (n = 100) | Women affected by PE (n = 42) | *P*-value | OR[a] | 95% CI | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| Age, years (SD)[b] | 31.3 (4.4) | 31.6 (5.2) | 0.74 | 1.01 | 0.94 | 1.10 |
| BMI, pre-pregnancy, kg/m$^2$ (IQR)[c] | 23.7 (7.3) | 29.3 (10.6) | <0.01 | 1.09 | 1.03 | 1.15 |
| Primiparous, n (%) | 42 (42.0) | 10 (23.8) | 0.04 | 1.53 | 1.01 | 2.30 |
| Infertility treatment, n (%) | 11 (11.0) | 5 (11.9) | 0.77 | 1.19 | 0.38 | 3.70 |
| Chronic disease, n (%) | 37 (37.0) | 22 (52.4) | 0.67 | 0.18 | 0.80 | 3.26 |
| Education, n (%) | | | | | | |
| Elementary or less[d] | 0 (0.0) | 3 (7.9) | 0.02 | | | |
| High school or vocational school | 22 (22.0) | 8 (19.0) | 0.64 | 0.90 | 0.36 | 2.26 |
| Intermediate | 32 (32.0) | 19 (45.2) | 0.83 | 0.90 | 0.36 | 2.26 |
| University | 40 (40.0) | 7 (17.0) | 0.01 | 0.32 | 0.13 | 0.79 |
| Prior preeclampsia | 14 (14.0) | 21 (50.0) | <0.01 | 0.16 | 0.07 | 0.37 |
| SGA in previous pregnancy | 9 (9.0) | 4 (9.5) | 0.92 | 0.94 | 0.27 | 3.24 |
| Chronic hypertension | 14 (14.0) | 10 (23.8) | 0.16 | 0.52 | 0.21 | 1.29 |
| Prior GDM | 4 (4.0) | 4 (9.5) | 0.32 | 0.50 | 0.13 | 1.96 |
| BMI ≥ 30 kg/m$^2$ | 22 (22.0) | 16 (38.1) | 0.05 | 0.46 | 0.21 | 1.00 |
| Prior fetus mortuus[d] | 2 (2.0) | 1 (2.4) | 1.00 | | | |

*[a]binary logistic regression, [b]mean, [c]median, [d]fisher's exact test; PE = preeclampsia, OR = odds ratio, CI = confidence interval, SD = standard deviation, BMI = body mass index. There was one type I diabetes mellitus and one Sjögren's syndrome in women who did not develop preeclampsia. There was not systemic lupus erythematosus in either group.*

**TABLE 2 |** Clinical characteristics of patients and controls.

| | Women not affected by PE *n* = 100 | Women affected by PE *n* = 42 | *P*-value | OR | 95% CI Upper | 95% CI Lower |
|---|---|---|---|---|---|---|
| Weight change during pregnancy, kg/m$^2$ | 14.5 (7.8) | 12.0 (6.6) | 0.18 | 0.95 | 0.88 | 1.02 |
| Gestational diabetes, n (%) | 17 (17.0) | 12 (28.6) | 0.12 | 0.51 | 0.22 | 1.20 |
| I trimester mean arterial pressure, mmHg | 89.7 (15.7) | 98.8 (13.8) | <0.01 | 1.07 | 1.04 | 1.11 |
| I trimester mean uterine artery PI | 0.99 (0.35) | 1.25 (0.47) | <0.01 | 14.64 | 3.45 | 62.04 |
| Highest mean arterial pressure, mmHg | 100.0 (13.5) | 128.5 (15.2) | <0.01 | 1.16 | 1.11 | 1.22 |
| Gestational weeks at birth | 40.3 (1.9) | 38.4 (3.2) | <0.01 | 0.53 | 0.41 | 0.70 |
| Apgar score at 5 min | 9 (1) | 9 (2) | 0.32 | 0.80 | 0.51 | 1.25 |
| Umbilical artery pH | 7.25 (0.13) | 7.25 (0.13) | 0.24 | 0.08 | <0.01 | 5.57 |
| Newborn birthweight, g | 3590 (651) | 3109 (1259) | <0.01 | 0.999 | 0.998 | 0.999 |
| Placental weight, g | 605 (175) | 540 (173) | <0.01 | 0.995 | 0.992 | 0.998 |

*Continuous variables are presented as median values with interquartile range in parenthesis. n=number of cases, PE=preeclampsia.*

Seven PE women received mini-ASA during the pregnancy and one of them gave birth to SGA infant. Thirty-five PE women did not receive mini-ASA and eight of them gave birth to SGA infant. Women who received ASA had higher Hpx concentration compared to women who did not receive ASA (median concentration 1,28 mg/ml versus 1,09 mg/ml, $p = 0{,}025$).

## DISCUSSION

To the best of our knowledge, this is the first study evaluating the levels of maternal plasma Hpx and A1M in the late second trimester in PE women as well as in high- and low-risk controls without PE. The strength of the study is a carefully characterized cohort, where both women with predetermined risk factors for PE and a low-risk reference group were prospectively recruited (Girchenko et al., 2017).

Our analysis shows a significantly higher plasma A1M concentration in high-risk controls compared to low-risk controls, while there was no significant difference in concentration of A1M between PE women and controls. In previous studies (Olsson et al., 2010; Anderson et al., 2011, 2016; Gram et al., 2015), the concentration of A1M in PE women was increased compared to controls when it was analyzed during the first trimester and third trimester, 24 h prior to delivery. In the aforementioned studies, normotensive pregnant women had been studied as a single undivided group, irrespective of risk factors for PE. However, it is reasonable to hypothesize that high-risk women are a distinct group characterized by increased oxidative stress compared to low-risk women.

In the present cohort, pre-pregnancy obesity was among the criteria conferring high-risk status to normotensive pregnant women. Obesity is associated with systemic oxidative stress (Furukawa et al., 2004). It can therefore be assumed that in high-risk controls, the endogenous protection system against oxidative stress is activated. The housekeeping protein A1M is an extravascular scavenger and tissue repair protein which has an important role in cleaning oxidative radicals and heme. It is upregulated during oxidative stress in general and by hemolysis specifically (Akerstrom and Gram, 2014). Thus, our results may suggest that increased oxidative stress is present in high-risk normotensive pregnant women compared to low-risk controls in late second trimester. A serious weakness with this argument, however, is that we have not included evaluation of oxidative stress markers. Evaluation of oxidative stress markers and reactive oxidative species measurements need to be undertaken before the association between A1M and oxidative stress in high-risk normotensive women is more clearly understood. Although A1M has not been shown to be an acute-phase protein, increased levels of A1M in high-risk normotensive women as a result of chronic inflammation related to obesity might be another explanation and needs to be clarified in further studies (Akerstrom and Gram, 2014).

Interestingly, the median plasma A1M concentration in high-risk normotensive women was increased compared to PE women, although the difference was not statistically significant. Higher concentration of A1M may confer its protective effect ameliorating the clinical impact of oxidative stress and preventing the development of PE in high-risk controls who remain asymptomatic. In line with this assumption, intravenous administration of a recombinant version of A1M in PE animal models has been successful in eliminating or at least significantly reducing the manifestations of preeclampsia (Sverrisson et al., 2014; Nääv et al., 2015; Gunnarsson et al., 2017). One can therefore speculate that the administration of exogenous A1M might have a similar effect in humans and PE women also.

Recent evidence suggests that extracellular HbF is elevated in the fetal circulation of pregnancies complicated by fetal growth restriction (Brook et al., 2018). We expect that among SGA infants in our cohort there are both constitutionally small infants and infants that have not reached their growth potential because of placental dysfunction. In the latter case, we expect that extracellular HbF in the fetal circulation is elevated causing, as we have previously shown (Centlow et al., 2008; May et al., 2011), damage to blood-placenta barrier and consequently leaking into maternal circulation, depleting Hpx. We hypothesized thus that women with PE and SGA infant would have lower levels of Hpx compared to women with PE and non-SGA infant, because Hpx binds and detoxifies heme and HbF that is released from the feto-placental unit. Although the levels of plasma Hpx was lower in women with PE who gave birth to SGA infant compared to women with PE and non-SGA infant, the difference was not significant. Type II error is possible due to a small sample size. One woman in the group of women with PE who gave birth to SGA infant had exceptionally high plasma Hpx and A1M values and this increased variability substantially. This woman differed from the other in that group by having a medical history of

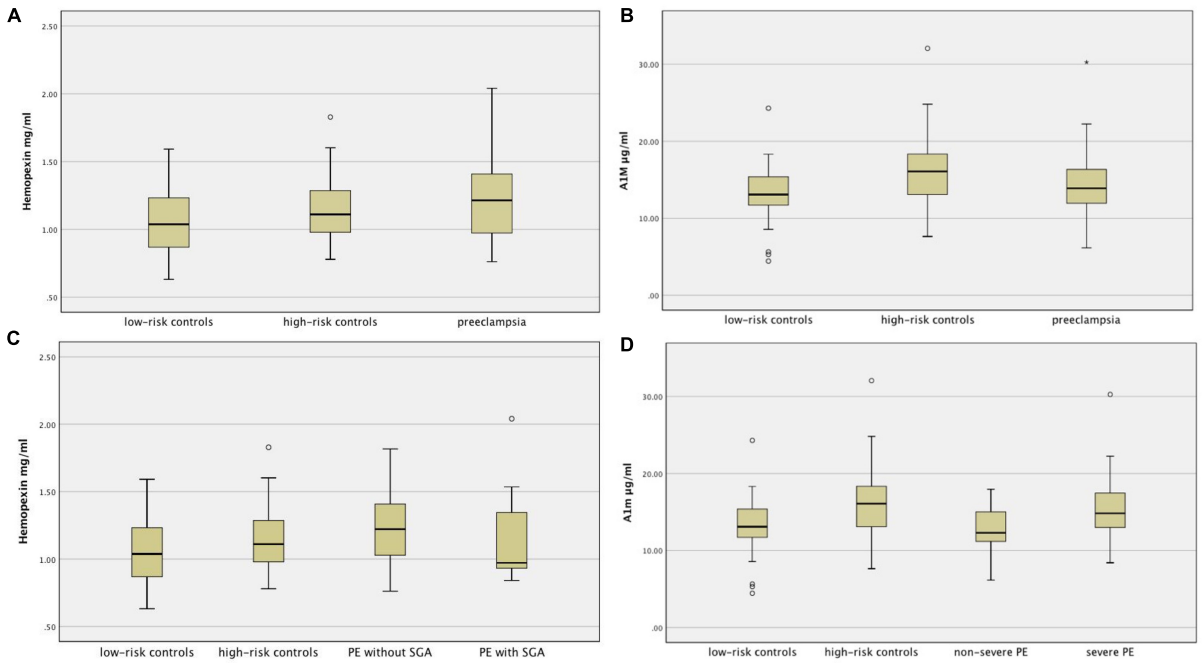


**FIGURE 1 | (A)**: Distribution of hemopexin across groups. **(B)**: Distribution of A1M across groups. **(C)**: Distribution of hemopexin across subgroups of PE, high- and low-risk controls. **(D):** Distribution of A1M across subgroups of PE, high- and low-risk controls.

**TABLE 3 |** Values of A1M and hemopexin across subgroups of women with PE.

| | | Hemopexin (mg/ml) | A1M (µg/ml) |
|---|---|---|---|
| PE onset | late onset ($n$ = 31) versus early onset ($n$ = 11) | 1.22 (0.32) versus 1.16 (0.64), $p$ = 0.66 | 13.34 (3.26) versus 16.66 (3.96), $p$ = 0.07 |
| SGA | no ($n$ = 34) versus yes ($n$ = 8) | 1.22 (0.41) versus 0.97 (0.52), $p$ = 0.183 | 13.20 (3.84) versus 16.85 (5.83), $p$ = 0.11 |
| PE severity | mild ($n$ = 21) versus severe ($n$ = 21) | 1.22 (0.56) versus 1.21 (0.44), $p$ = 0.84 | 12.30 (4.46) versus 14.83 (5.23), $p$ = 0.04 |
| ASA | no ($n$ = 35) versus yes ($n$ = 7) | 1.20 (0.43) versus 1.31 (0.51), $p$ = 0.16 | 14.24 (4.42) versus 12.13 (3.10), $p$ = 0.30 |

*Continuous variables are presented as median values with interquartile range in parenthesis. n=number of cases.*

chronic hypertension and thus superimposed PE and she was the only woman who received mini-ASA in the group of women with PE who gave birth to SGA infant.

Recent studies (Ferrazzi et al., 2018; Tay et al., 2018) suggest that women with PE and SGA or growth restricted fetus have a distinct cardiovascular phenotype characterized by lower cardiac output and higher peripheral vascular resistance. It is tempting to speculate that there is a connection between the impaired cardiovascular function and the decreased levels of heme scavenger Hpx. A possible explanation may be leakage of extracellular HbF of fetoplacental origin in maternal circulation. As a consequence, heme is released from metabolized extracellular HbF and depleting Hpx as recently shown (Anderson et al., 2018). Imbalance in the scavenging capacity causes vasoconstriction by reducing nitric oxide availability and impaired cardiac function. In fact, heme has been shown to induce contractile dysfunction in human cardiomyocytes *in vitro* (Alvarado et al., 2015). Furthermore, Hpx has been shown to have cardio-protective effect and it preserves systolic function by limiting heme-driven oxidative stress in mice (Ingoglia et al., 2017). Further studies, where cardiac function is evaluated simultaneously with the concentration of heme in maternal circulation, are needed to further clarify whether this suggested mechanism has clinical relevance.

In contrast to previous studies, we found increased plasma concentration of Hpx in PE women compared to low-risk controls. After excluding controls and PE women treated with mini-ASA, there was no statistically significant difference in the concentrations of Hpx between PE women and controls. In previous studies, where women treated with mini-ASA were not included, the concentration of Hpx was decreased, albeit marginally, in early pregnancy in women who later developed PE (Anderson et al., 2016). The concentration of Hpx in PE women has been shown to be even more decreased compared to controls just before delivery (Anderson et al., 2018). Thus, when focusing on women not treated with mini-ASA, our results suggest that Hpx depletion, due to heme-Hpx binding, is a continuous process, which starts slowly in early pregnancy and intensifies in third trimester when PE is manifested clinically. Further studies with a significantly larger patient group are needed if we try to detect the very marginal differences of the early stages of this process in order to clarify the dynamics of this process.

As regards to mini-ASA prophylaxis, 3 high-risk controls and 7 PE women who had participated in ASA trial were included in this study. Overall, these 10 women had significantly higher plasma Hpx concentration compared to controls and PE women who did not receive mini-ASA prophylaxis. Mini-ASA could possibly have an impact on Hpx concentration by improving placental perfusion. We have previously hypothesized that the up-regulation of HbF gene expression in the preeclamptic placenta may be induced by hypoxia caused by insufficient placental perfusion (Gram et al., 2015). Mini-ASA may improve the placental perfusion through inhibition of the potent vasoconstrictor Thromboxane A2 (TXA2) or by preventing placental thrombosis formation improving the blood flow in the PE placenta. Improved placental perfusion could then possibly prevent up-regulation of the HbF gene expression thereby reducing the leakage of HbF and heme into the maternal circulation. As previously shown, small amount of heme administered intravenously in rhesus monkeys increases the Hpx levels by increasing the rate of Hpx synthesis (Foidart et al., 1982). In contrast, large amount of heme has been shown to reduces Hpx levels by increased Hpx catabolism. Further studies are needed to understand the role of mini-ASA on Hpx dynamics.

## CONCLUSION

This study shows increased concentration of plasma A1M in high-risk normotensive pregnant women in late second trimester.

## AUTHOR CONTRIBUTIONS

SH, BÅ, and HL conceived and designed the analysis. GK and KM performed the analysis and wrote the manuscript. KM, PV, KR, EH, EK, and HL collected the data and contributed the data.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00300/full#supplementary-material

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**Conflict of Interest Statement:** SH and BÅ holds patent related to diagnosis and treatment of preeclampsia and are co-founders of A1M Pharma and Preelumina Diagnostics (www.a1m.se). The pre-existing intellectual properties involve 4 patents owned by A1M Pharma;

1. HBF and A1M as early stage markers for preeclampsia-1550535
2. Medical use of A1M-2638915
3. Diagnosis and treatment of preeclampsia-201500335
4. Biomarkers for preeclampsia-PA 2015 70146

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SCIENTIFIC REPORTS

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# Alpha-1 microglobulin as a potential therapeutic candidate for treatment of hypertension and oxidative stress in the STOX1 preeclampsia mouse model

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**Preeclampsia is a human placental disorder affecting 2–8% of pregnancies worldwide annually, with hypertension and proteinuria appearing after 20 weeks of gestation. The underlying cause is believed to be incomplete trophoblast invasion of the maternal spiral arteries during placentation in the first trimester, resulting in oxidative and nitrative stress as well as maternal inflammation and organ alterations. In the Storkhead box 1 (STOX1) preeclampsia mouse model, pregnant females develop severe and early onset manifestations as seen in human preeclampsia e.g. gestational hypertension, proteinuria, and organ alterations. Here we aimed to evaluate the therapeutic potential of human recombinant alpha-1 microglobulin (rA1M) to alleviate the manifestations observed. Human rA1M significantly reduced the hypertension during gestation and significantly reduced the level of hypoxia and nitrative stress in the placenta. In addition, rA1M treatment reduced cellular damage in both placenta and kidneys, thereby protecting the tissue and improving their function. This study confirms that rA1M has the potential as a therapeutic drug in preeclampsia, and likely also in other pathological conditions associated with oxidative stress, by preserving normal organ function.**

Preeclampsia is a human placental disorder that clinically presents after 20 weeks of gestation with maternal manifestations including hypertension and proteinuria[1,2]. Around 2–8% of pregnancies worldwide are affected annually[3]. The placenta plays a key role in the development of the disease, as its removal results in resolution of the clinical signs but also contributes to the high rate of premature births. Even if the maternal manifestations appear in the third trimester, the underlying cause is believed to be incomplete trophoblast differentiation/invasion of the maternal spiral arteries during placentation in the first trimester. Defective remodeling of the maternal spiral arteries is believed to result in high pressure flow entering the intervillous space, causing physical disruption of the placental villous architecture and fluctuations in oxygen delivery with relative hypoxia in the placenta[4]. This leads to increased inflammation[5,6], oxidative stress[7,8], and nitrative stress[9,10]. In addition, oxidative stress is closely linked to endoplasmic reticulum (ER) stress and redox homeostasis[11,12]. Normal pregnancy is in a state of oxidative stress compared to the non-pregnant state, generating reactive oxygen species (ROS)[13,14], which is further elevated in complicated pregnancies such as preeclampsia[8,15]. The maternal manifestations occurring after 20 weeks of gestation can be viewed as the maternal response towards placenta-derived circulating factors released through a disrupted placental barrier, resulting in systemic endothelial activation and dysfunction[16]. Women suffering from severe preeclampsia can develop eclampsia and seizures. Damage to the heart can cause peripartum cardiomyopathy[17], postpartum cardiovascular impairment[18], and severe cases also have increased

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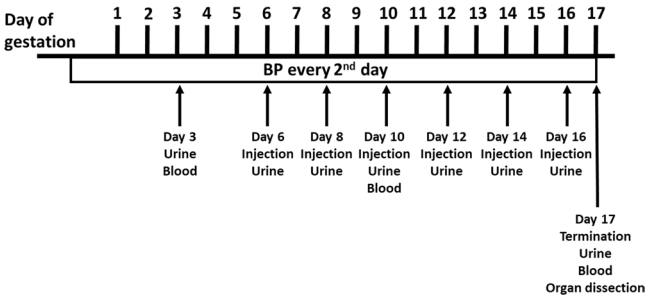


**Figure 1.** Experimental design. Illustration of the experimental design, indicating time points for collection of urine and blood, blood pressure measurements, injections and terminations.

risk of cardiovascular disease and stroke later in life[19]. Pre-symptomatic low-dose aspirin treatment (before 16 weeks of pregnancy) is efficient to prevent the onset of preeclampsia[20], but there is a clear lack of pharmaceutical therapeutic solutions able to alleviate the symptoms once they occur, allowing to safely prolong the pregnancy. Therefore such therapeutic approaches are much needed.

In a transgenic mouse model overexpressing the transcription factor Storkhead box 1 (STOX1) gene, pregnant females develop typical human preeclampsia manifestations such as gestational hypertension, proteinuria, kidney and placental tissue alterations, reduced litter size, and increased plasma levels of the anti-angiogenic factors soluble fms-like tyrosine kinase 1 (s-Flt1) and soluble endoglin (sEng)[21]. By mating wildtype (wt) females with STOX1 transgenic males, the transgene expression is restricted to the fetoplacental unit, making this one of the few animal models representing a severe and early onset form of preeclampsia. Furthermore, these pregnant females display cardiac hypertrophy and endothelial cell deregulation in gene networks linked to oxidative stress, cell cycle, and hypertrophy[22]. In addition, the placentas show alterations in mitochondria-related pathways and a disrupted nitroso-redox balance[23]. The STOX1 overexpression also results in hyperactive mitochondria, which leads to increased free radical production. In addition, nitric oxide (NO) production pathways are activated, generating peroxynitrite as a result of NO reacting with superoxide. Peroxynitrite is highly unstable and reacts with tyrosine residues on proteins, producing nitrotyrosine (protein nitration)[24]. Protein nitration has been shown to occur in a number of pathological conditions associated with inflammation, including preeclampsia[25,26]. Elevated levels of ROS that reacts with NO would result in decreased levels of NO, which is an important vasodilator in the vascular system, thereby potentially contributing to the hypertension described in this mouse model.

The endogenous protein alpha-1-microglobulin (A1M) is a ~30 kDa protein with haem- and radical-binding capacity as well as reductase activity. It has been shown to be protective against oxidative stress, as well as an inducer of natural tissue repair mechanisms[27]. Mainly synthesized by the liver, A1M is present both in the circulation as well as in the extravascular compartments. In the circulation, it forms complexes with other proteins in particular prothrombin. It is re-cycled in the kidneys and if present in urine, it is a major marker of renal tubular function[28]. It has also been shown to prevent intracellular oxidation, protect mitochondria, prevent mitochondrial swelling, inhibit cell lysis, and repair lesions caused by oxidative stress (reviewed in[27]). Exogenously administered recombinant A1M (rA1M) has previously been shown to have a therapeutic effect against organ damage induced by cell-free haemoglobin in organs such as placenta and kidneys, both *ex vivo*[29] and *in vivo* in other less specific preeclampsia animal models[30,31].

Here we used the STOX1 mouse model of severe preeclampsia to explore in detail the therapeutic possibilities of rA1M treatment in preeclampsia. The STOX1 transgene mouse model provides a useful model for analysing in depth and in an organ-targeted way the pathophysiological consequences of preeclampsia. It also offers the opportunity to investigate ways of reducing oxidative stress in preeclampsia, as well as testing new therapeutic avenues.

## Results

**Human rA1M significantly alleviates hypertension during mid- and late gestation.** The experimental set-up is described in Fig. 1. Females were given six i.p. injections of either buffer or rA1M every second day starting at 6.5 dpc. Human rA1M was detected in plasma at timepoint 10.5 dpc from rA1M-treated females, confirming that i.p. injected rA1M reached the circulation (Supplementary Fig. S1). Blood pressure (BP) was measured throughout gestation and the preeclamptic females (PE-buff) showed a significant increase in systolic BP during both mid- ($p = 5 \times 10^{-9}$) and late-gestation ($p = 5 \times 10^{-4}$) when compared to control groups (Fig. 2a and Supplementary Fig. S2). This increase was significantly alleviated by rA1M treatment during mid-gestation compared to PE-buff group ($p = 0.007$) and to some extent also during late-gestation. There was no increase in BP during gestation in the control groups.

**Human rA1M improves placental weight.** Preeclamptic females showed a tendency towards reduced litter size at 17.5 days post coitum (dpc) compared to controls (Table 1), which was not affected by rA1M treatment. Also, preeclamptic females demonstrated significantly reduced placental weight compared to controls ($p = 0.0001$), which was alleviated in the PE-A1M group (Table 2). There was no significant difference in foetal weight at day 17.5 dpc between any of the groups.

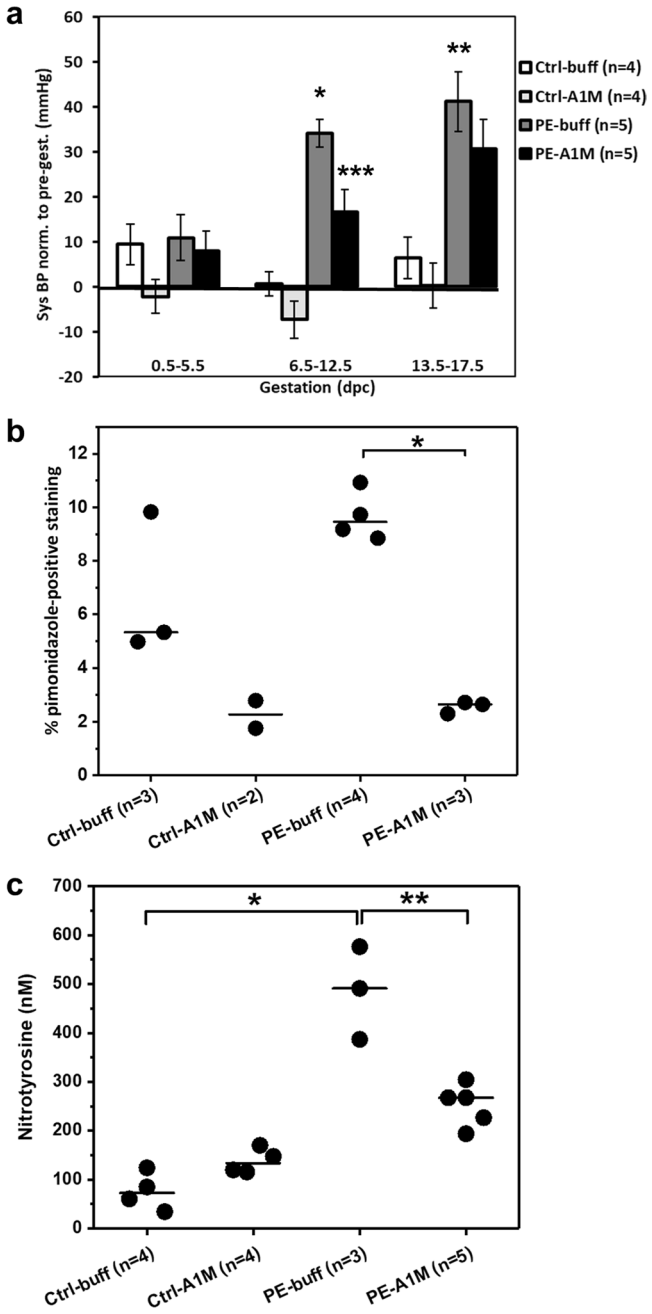


**Figure 2.** Human rA1M significantly reduces hypertension and placental hypoxia/nitrative stress levels in preeclamptic females. (**a**) Systolic BP measurements during early-, mid- and late pregnancy, normalised to pre-gestation pressure (mmHg). The PE-buff group displayed significantly elevated BP at mid and late gestation, compared to Ctrl-buff (*$p = 5 \times 10^{-9}$, **$p = 5 \times 10^{-4}$). Human rA1M significantly reduced BP mid-gestation, compared to PE-buff group (***$p = 0.007$). Shown is mean ± SEM, with 10–18 BP measurements for each gestation period and group. (**b**) Hypoxyprobe immunohistochemistry demonstrated a trend of higher levels of hypoxia in the junctional zone of preeclamptic placentas at 17.5 dpc, compared to controls. This was significantly reduced by rA1M treatment (PE-A1M vs PE-buff, *$p < 0.0001$). The line represent the median, and n = number of females analysed with three-four placentas/female. (**c**) Significantly elevated levels of protein nitration in the preeclamptic placentas at 17.5 dpc compared to controls (*$p = 0.05$), which was significantly reduced by rA1M treatment (**$p = 0.04$). The line represents median, and n = number of females analysed with one placenta/female.

| Groups | Ctrl-buff (n = 4) | Ctrl-A1M (n = 4) | PE-buff (n = 5) | PE-A1M (n = 5) |
|---|---|---|---|---|
| pups/litter | 8 (4–9) | 7 (6–8) | 5 (1–8) | 5 (2–11) |

**Table 1.** Litter size. Shown is median (range) at time of termination (17.5 dpc). N = number of females.

| Groups | Placental weight (mg) | Foetal weight (mg) |
|---|---|---|
| Ctrl-buff (n = 4) | 69 (55–114) | 841 (679–955) |
| Ctrl-A1M (n = 2) | 66 (56–88) | 845 (736–1188) |
| PE-buff (n = 6) | 61 (46–79)* | 856 (619–1117) |
| PE-A1M (n = 3) | 66 (40–96) | 883 (605–1074) |

**Table 2.** Placental and foetal weight at 17.5 dpc. Shown is median (range) at time of termination (17.5 dpc). Mann-Whitney U-test: PE-buff vs Ctrl-buff *$p = 0.0001$. N = number of females.

**Human rA1M reduces the level of hypoxia and nitrative stress in the preeclamptic placenta.** We used Hypoxyprobe™ immunohistochemistry to identify hypoxic regions in the mouse placenta at gestational age 17.5 dpc (Supplementary Fig. S3). The majority of Hypoxyprobe staining was in the junctional zone of the placenta, and there was a trend of higher levels of hypoxia in the preeclamptic placentas compared to controls ($p = 0.06$), which was significantly reduced by rA1M treatment ($p = 1 \times 10^{-6}$) (Fig. 2b). Furthermore, the preeclamptic mouse placentas showed significantly elevated levels of protein nitration when compared to controls ($p = 0.05$) (Fig. 2c). Human rA1M treatment resulted in significantly lower levels in the rA1M-treated preeclampsia group compared to PE-buff ($p = 0.04$).

**Human rA1M protects the placental tissue.** Histological analysis of placentas using hematoxylin & eosin (H&E) staining revealed an overall appearance of fuzzy and disrupted structures in the labyrinth zone of the placentas from the PE-buff group, with unclear boundaries between vessels and cells as well as areas of necrotic appearance (Fig. 3c and Supplementary Fig. S4). In this zone, there were also areas with both swollen cells and structures. Human rA1M treatment resulted in structures similar to the control placentas, with reduced swelling and no signs of necrosis (Fig. 3d). The control placentas displayed clear and well-defined structures over the whole labyrinth zone with no signs of necrosis or swollen structures (Fig. 3a,b). The structural changes observed in the preeclamptic placentas were more clearly visible by transmission electron microscopy (TEM) analysis, where morphological changes were demonstrated on the cellular level (Fig. 3g and Supplementary Fig. S5). The tissue showed signs of severe tissue disruption with cells displaying loss of plasma membrane integrity and organelle breakdown, as well as extensive amounts of apoptotic bodies. Swollen and distorted mitochondria and dilated ER were regularly observed. Empty extracellular space containing cell debris from dead cells indicated breakdown of structural components such as collagen fibers. Human rA1M treatment protected tissue structure, membrane integrity, and organelle morphology in preeclampsia (Fig. 3h). Also, no disrupted mitochondria or blebbing was seen, and apoptotic cells were rare. The placenta from both control groups displayed normal tissue morphology (Fig. 3e,f).

**Human rA1M alleviates the glomeruli damage observed in the kidneys.** The preeclamptic females developed a tendency to proteinuria at late gestation, which was not present in subjects receiving rA1M treatment (Fig. 4a). Histology analysis of kidneys from preeclamptic females revealed a tendency of glomerular tuft swelling (in 1–2 glomeruli per 10 examined), resulting in reduction of the Bowman's space, indicative of glomerular endotheliosis as seen in human preeclampsia (Fig. 5c). Treatment with rA1M alleviated these glomerular changes (Fig. 5d) to the level of the control groups (Fig. 5a,b). The TEM analysis revealed tissue damage at the cellular level in the glomeruli of the preeclamptic females (Fig. 5g), present in all glomeruli analysed. The pathological changes included podocytes with swollen and disrupted mitochondria and ER, and with an abundance of intracellular vesicular bodies. There was effacement of podocyte foot processes seen as fused and irregular shapes. The glomerular basal membranes were swollen and irregular in thickness. The endothelial fenestration lining the basal membrane was irregular and structurally aberrant. The vascular lumen contained extracellular vesicles and lumen occlusion was evident. Treatment with rA1M protected the tissue, resulting in podocytes with normal cell morphology, podocyte foot processes that were not fused and regular in shape, as well as thinner basal membranes with smooth texture (Fig. 5h). The vascular lumen contained fewer vesicles and the fenestration showed normal frequency. The control groups displayed normal glomeruli morphology (Fig. 5e,f). The expression of genes involved in the protection against oxidative stress or apoptosis was analysed in kidney at day 17.5 dpc of preeclamptic females and rA1M-treated preeclamptic females (Fig. 4b). Human rA1M treatment resulted in significant reduction of both heme oxygenase-1 (*HO-1*) ($p = 0.04$) and catalase (*CAT*) ($p = 0.04$) levels, and a trend towards reduced superoxide dismutase 2 (*SOD2*) ($p = 0.07$) in kidneys from the PE-A1M group compared to the PE-buff group.

**Human rA1M alleviate cardiac tissue damage seen in the STOX1 preeclampsia model.** The heart weight of the preeclamptic females was significantly increased at time of termination (17.5 dpc) compared to controls ($p = 0.002$) (Fig. 4c), which was not alleviated by rA1M treatment (PE-A1M vs Ctrl-buff; $p = 0.008$). At 17.5 dpc, both the control groups showed similar heart weight as non-pregnant females. Histology analysis using H&E staining of heart biopsies revealed that preeclampsia caused structural changes to the heart muscle, with increased extracellular space and swollen cells (Fig. 6c). This was also observed in the rA1M treated preeclampsia group, however with increased extracellular space to a lesser extent (Fig. 6d). Both control groups displayed slender muscle fibers that were densely packed together (Fig. 6a,b). Masson's Trichrome staining revealed an intense overall blue staining for collagen being present throughout the whole biopsy from the preeclamptic heart (Fig. 6g and Supplementary Fig. S6), which was not alleviated by rA1M treatment (Fig. 6h). The TEM analysis confirmed

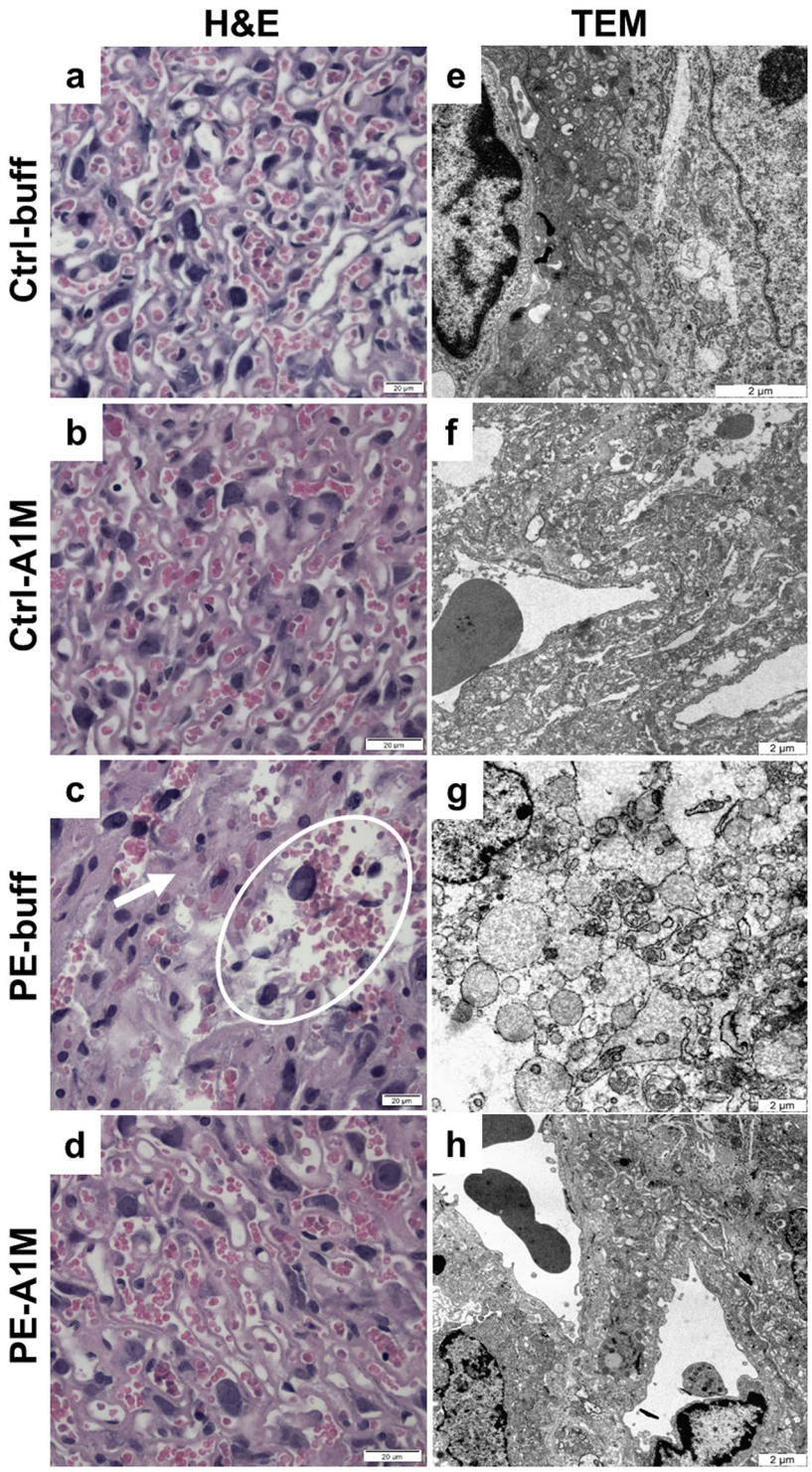


**Figure 3.** Histological and TEM analyses of placenta tissue structure. Morphological analyses of placenta biopsies at 17.5 dpc using H&E staining (**a**-**d**, scale bar = 20 µm) and TEM analysis (**e**–**h**, scale bar = 2 µm), showing representative images. (**a,b**) Control groups displayed normal tissue morphology. (**c**) The labyrinth zone of preeclamptic placentas displayed areas of necrosis (circle) and swollen tissue (arrow). (**d**) Placentas from rA1M-treated preeclamptic females showed no swelling and no necrosis, and were indistinguishable from control groups. (**e,f**) Control groups displayed normal tissue and cell morphology. (**g**) The preeclamptic placenta showed severe tissue damage and necrotic cells with loss of plasma membrane integrity and organelle breakdown, as well as extensive blebbing. Distorted mitochondria and dilated ER along with extensive cell debri was seen. (**h**) Human rA1M treatment protected tissue structure, membrane integrity and organelle morphology.

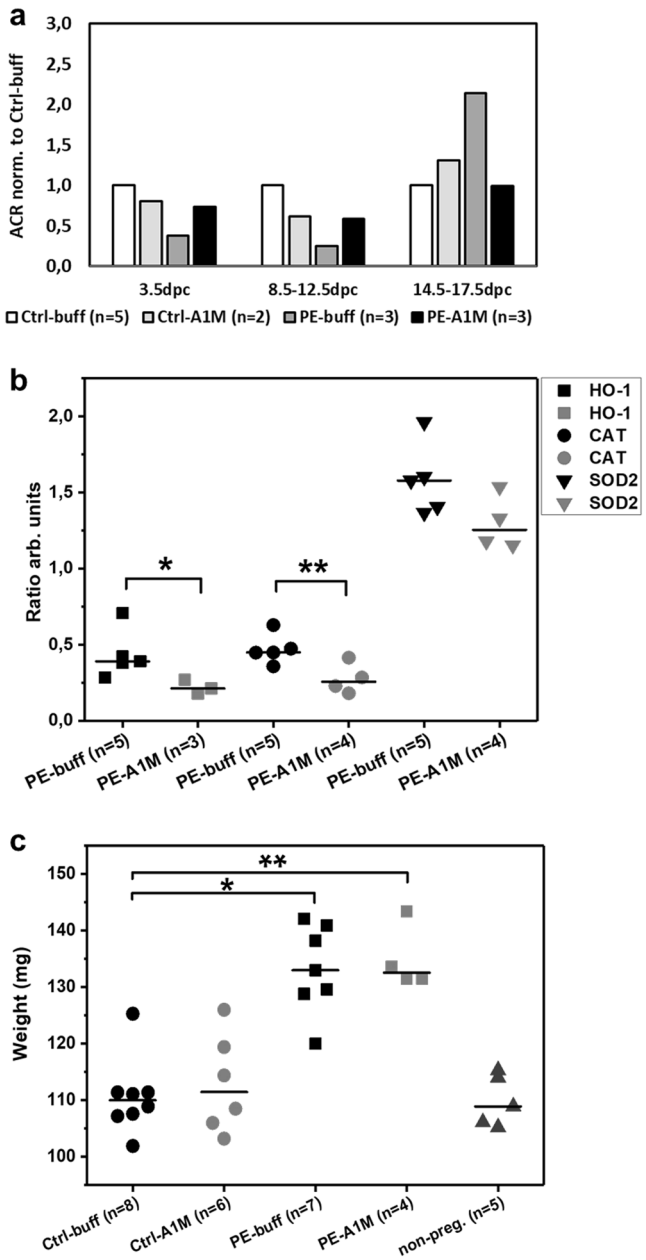


**Figure 4.** Human rA1M protects kidney function but no effect on cardiac hypertrophy. (**a**) Albumin/Creatinine ratio (ACR) analysis of urine from pregnant females showed an increase of ACR at late-gestation in the preeclamptic females, which was absent in the rA1M-treated group. Shown is the mean ACR normalised to Ctrl-buff values for each gestation period, and n = number of females analysed. (**b**) Gene expression levels for *HO-1*, *CAT* and *SOD2*, normalised to the *HPRT*-gene levels in kidney from PE-buff and PE-A1M females, demonstrating significant reduction of *HO-1* and *CAT* expression (*p = 0.04; **p = 0.04) after rA1M-treatment. The line represents the median and n = number of females analysed. (**c**) Preeclamptic females showed increased heart weight compared to Ctrl-buff at 17.5 dpc (*p = 0.002), which could not be alleviated by rA1M treatment (PE-A1M vs Ctrl-buff; **p = 0.008). Control groups showed similar heart weight as non-pregnant females. The line represents the median and n = number of females analysed.

that the preeclamptic heart showed structural and cellular damages, with the typical striated appearance missing to a large extent. Swollen and erupted mitochondria, along with irregular organization of muscle fibers and mitochondria were commonly seen, indicating disrupted tissue integrity (Fig. 6k and Supplementary Fig. S7). Human rA1M treatment could to some extent protect the cardiac macrostructure and cellular structures (Fig. 6l), with more organized striated muscle fiber structures and less mitochondrial damage. The control groups displayed an organized striated muscle fiber structure and densely packed mitochondria (Fig. 6i,j). Magnetic resonance imaging (MRI) analysis on a separate set of non-treated pregnant controls and preeclamptic females (not part of the treatment groups) revealed no significant difference in left or right ventricular end-diastolic or end-systolic

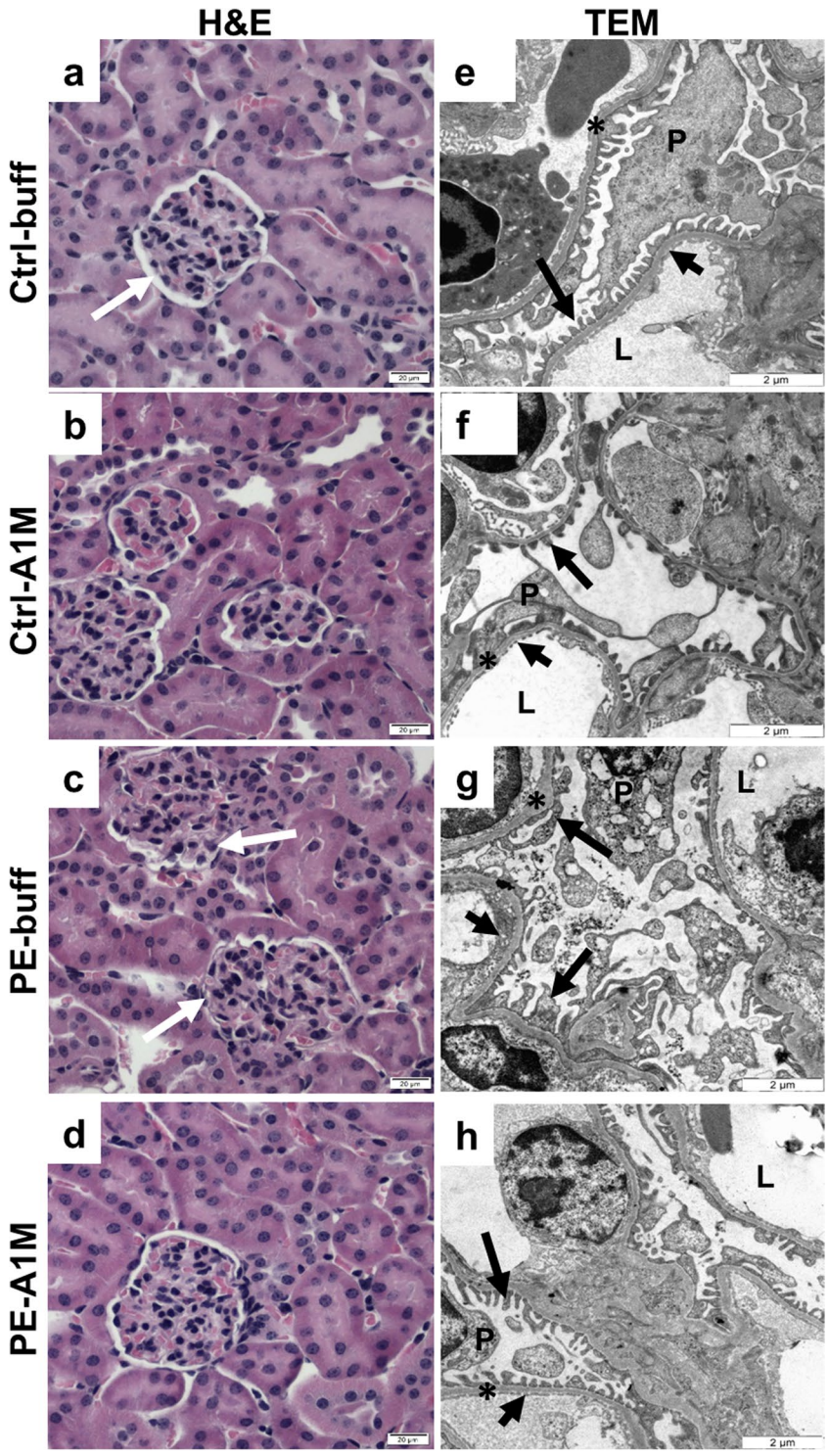


**Figure 5.** Histological and TEM analyses of kidney tissue structure. Morphological analysis of kidney biopsies at 17.5 dpc using H&E staining (**a**–**d**, scale bar = 20 µm) and TEM analysis (**e**–**h**, scale bar = 2 µm), showing representative images. (**a**–**b**) Control groups displayed normal tissue morphology. (**c**) Kidneys from preeclamptic females displayed glomerular tuft swelling resulting in reduced Bowman's space. (**d**) Treatment with rA1M alleviated these glomerular changes to a level similar to the control groups. (**e,f**) Control groups displayed normal tissue and cell morphology. (**g**) Kidneys from preeclamptic females showed pathological changes including podocytes with intracellular vesicular bodies and disrupted mitochondria and ER, swollen and irregular glomerular basal membrane, effacement of podocyte foot processes and irregular and structurally aberrant endothelial fenestration. (**h**) Human rA1M-treatment protected the structure of the tissue, showing normal cell morphology and tissue organisation. White arrow = Bowman's space, P = podocyte, L = lumen, *basal membrane, long black arrow = podocyte foot processes, short black arrow = endothelial fenestration.

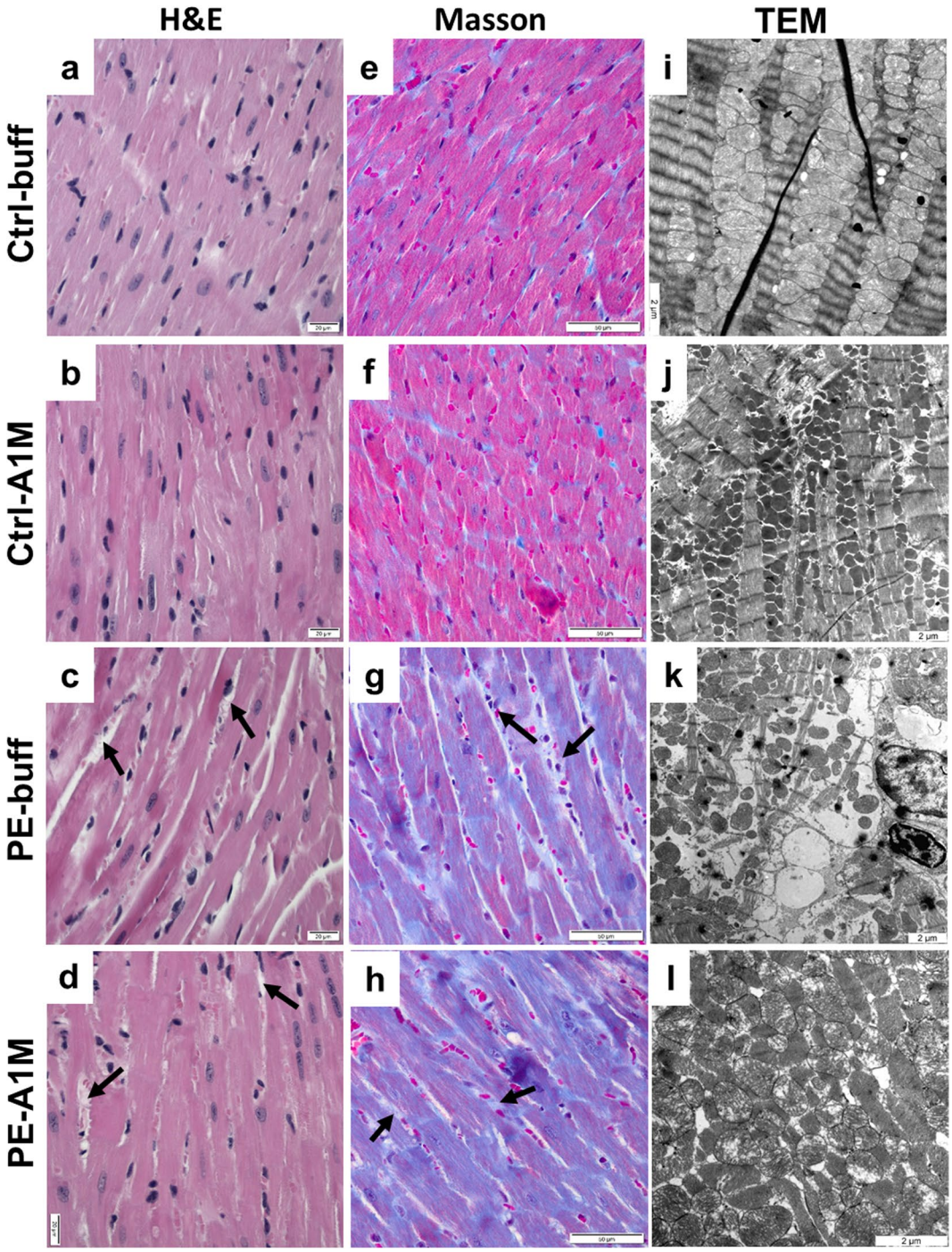


**Figure 6.** Histological and TEM analyses of heart tissue structure. Morphological analysis of heart biopsies at 17.5 dpc using H&E staining (**a**–**d**, scale bar = 20 µm), Masson trichrome staining (**e**–**h**, scale bar = 50 µm) and TEM analysis (**i**–**l**, scale bar = 2 µm), showing representative images. (**a**,**b**,**e**,**f**) Control groups displayed slender muscle fibers that were densely packed and stained pink. (**c**) Hearts from preeclamptic females displayed structural changes with increased extra-cellular space (arrows), cellular swelling and (**g**) an intense overall blue staining, indicating presence of collagen. (**d**,**h**) The same structural changes and intense Masson trichrome blue staining was seen in the rA1M-treated females. (**i**,**j**) The control groups displayed an organised structure between muscle fibers and healthy mitochondria. (**k**) The preeclamptic hearts had structural and cellular damages with swollen and erupted mitochondria, along with irregular organization of muscle fibers and mitochondria. Increased extra-cellular space could be observed in many places, indicating disrupted tissue. (**l**) Human rA1M treatment resulted in to some extent a more organized structure, with less mitochondrial damage.

volumes, left ventricular mass or ejection fraction between groups (Supplementary Table S1), although there was a trend towards increased left ventricular mass in mice with preeclampsia. Left ventricular cardiac output was increased in the preeclampsia group ($p = 0.029$) combined with a trend towards an increased heart rate in these females.

**Blood analysis.** Human rA1M treatment had no alleviating effect on the plasma levels of sFlt1, or sEng in the preeclamptic females (Supplementary Fig. S8).

## Discussion

In the present study, we analysed the structural, ultrastructural, and functional alterations induced by preeclampsia on the maternal organs and the placenta, and the therapeutic effect of rA1M on these changes.

Our results demonstrate the potential therapeutic capacity of rA1M to alleviate typical manifestations of preeclampsia, such as elevated BP, proteinuria, and organ damage. Similar protective effects have previously been demonstrated in other animal models of preeclampsia[30,31], with reduced proteinuria, and reverted kidney and placenta damage. However, in this study we use a preeclampsia animal model to demonstrate a significant effect by rA1M on lowering an elevated BP, as well as proteinuria. The strongest therapeutic effect on elevated BP was seen mid-gestation, but also to some extent at late-gestation. We used the same dose of A1M throughout the experiments, and a higher dose might have been needed at late-gestation to have the same strong effect. Endogenous A1M has been shown to be up-regulated in women with preeclampsia[32], suggesting that it plays a role in the endogenous defense against increased oxidative and nitrative stress. By supplementing with rA1M, the suggested therapy mimics nature's own way of responding to the problem. Today, aspirin is used as a prophylactic treatment in high-risk pregnancies to prevent preeclampsia but is only effective when it is administered in early pregnancy before the symptoms appear, as a prophylaxis[20]. Clearly, a therapeutic molecule, able to reverse the symptoms once they appear is missing. Currently, to cure preeclampsia, delivery remains the only option. New research approaches, such as apheresis to extract excess sFlt1 from the maternal blood have been proposed[33]. However, such a method is expensive and likely not suitable for a broad clinical implementation, particularly not in low- and middle-income countries, where preeclampsia is a large problem. Human rA1M treatment could be a promising alternative, since it is an endogenous protein, naturally produced by the liver that could be supplemented to patients without fearing severe side effects.

The protective effects of rA1M treatment regarding both elevated BP and oxidative and nitrative stress supports its role as an endogenous radical-scavenging and tissue repair protein[27]. It has been shown that A1M has a protective role against oxidative stress and binds to mitochondria to help maintain morphological structure and ATP production[34,35]. The reduction in kidney expression levels of *HO-1*, *CAT* and *SOD2* in the rA1M-treated females supports this protective role, since these are genes involved in responses towards oxidative stress or apoptosis. HO-1 is a stress response protein and is upregulated in response to its substrate haem, but also to oxidative stress-inducing factors such as ROS[36]. CAT is involved in the protection of cells from oxidative damage by hydrogen peroxidase[37], while SOD2 plays an anti-apoptotic role against oxidative stress by clearing mitochondrial ROS and protecting against cell death[38]. In this study, A1M may stabilize the hyperactive mitochondria, reduce their increased free radical production, and reduce activation of NO production pathways, which in turn would lead to reduced levels of peroxynitrite and nitrotyrosine. Experiments have demonstrated a number of nitrated proteins being present in the normal placenta[39], where it is thought to occur as a posttranslational modification of proteins[24]. For preeclampsia, heightened levels of oxidative and nitrative stress has been reported[7,10], elevated levels of peroxynitrite[25], and an abundance of nitrotyrosine residues are present in the placenta[10,40,41]. Elevated levels of peroxynitrite can result in significantly altered function of proteins and mitochondria, as well as inflicting damage to nucleic acids. Nitrated proteins have also been reported to be able to elicit immune responses and to be involved in autoimmune diseases[42,43].

In this study, we could not detect foetal intra uterine growth restriction at day 17.5 dpc, as has previously been reported for days 17.5 and E18.5 dpc, but not for 16.5[44]. This observation could be due to breeding conditions, leading to difference in growth kinetics. However, in the present study, we could detect a significant decrease of placental weight as a result of preeclampsia. The placental weight was normalized by rA1M treatment, probably by protection of the placenta against oxidative/nitrative stress, and thereby reducing their damaging effects on the tissue integrity. This would result in a more normal placental function. Since before, A1M has been shown to both protect and repair tissue from oxidative lesions[27]. Furthermore, we used cardiac MRI to evaluate the effect of preeclampsia on functional properties of the left and right ventricle, but found no decrease in left ventricular ejection fraction in the preeclamptic females as previously reported[44]. An increase in left ventricular cardiac output was shown, which is in line with prior clinical studies describing a subgroup of preeclamptic women with increases in cardiac output in preeclampsia[45]. Further, a slight increase in left ventricular mass was found in preeclamptic females, which consisted of a general hypertrophy including the papillary muscles and trabeculation. To have a more definitive answer regarding the effect of preeclampsia on myocardial hypertrophy and cardiac function, rigorous adjustments taking into account individual cardiac and body weights, and other known confounding factors, as well as an increase in the number of animals analysed would be required. Nevertheless, we find the observation consistent with increase of heart mass and anomalies of its structure.

Human rA1M protected tissue integrity in the kidneys, resulting in normal glomeruli morphology and reduced proteinuria, in line with previous *in vivo* studies[30,31]. The heart of the preeclamptic females showed hypertrophy similar to what has been reported for preeclamptic women[18], and consistent with our previous observations in the STOX1 model[22]. This was seen as altered tissue morphology, with swollen mitochondria and disrupted muscle fibers with collagen, demonstrating that oxidative stress in the placenta can cause alterations in the heart. Human rA1M protected the mitochondria and reduced the extent of disrupted cardiac tissue. However, the weight of the heart was not reduced by rA1M treatment, probably due to the persisting collagen. This is in

line with reports for a subgroup of preeclamptic women that develop persistent alterations in cardiac function as a consequence of the disease[46]. The reason for this is poorly understood. As previously shown in a mouse model, overexpression of sFlt-1 has no long-term effect on BP and vascular function in the postpartum mothers[47]. Thus, collagen deposits and mitochondrial damage may provide a better explanation for long-term cardiac effects of preeclampsia. Overall, compared to kidneys and placentas, the heart was protected to a lesser extent by rA1M treatment. The exact mechanisms behind the development of these heart alterations needs to be further studied, in combination with rA1M treatment at earlier time points. The considerable protection of the kidney is of importance, as this was both structural and functional. It is established that preeclampsia induces long-term deleterious cardiovascular and renal consequences for the mothers[48–50], and protecting the organs already during pregnancy is likely crucial. We have shown in a rabbit preeclampsia model and in women with preeclampsia that urinary shedding of extracellular vesicles from podocytes is increased and correlates with the renal injury and proteinuria[51]. Furthermore, human rA1M-treatment of the pregnant rabbits had a protective role and reduced the levels of both Annexin-V$^+$ and Podocin$^+$ vesicles in urine (unpublished data). We therefore hypothesis that rA1M could be efficient for preserving normal organ function in general, and kidney function specifically, improving the long-term function.

This study confirms that rA1M has a protective role against both organ damage and secondary effects as elevated BP in preeclampsia. Recombinant A1M thus has potential as a therapeutic drug in preeclampsia and likely also in other pathological conditions associated with oxidative stress.

## Methods

**Ethics committee approval.** This study used the STOX1 transgenic mouse model on FVB/N strain background[21], and was approved by the local ethics committee for animal studies at Lund University, Lund, Sweden (permit no: M29-14) and Institut National de Recherche Agronomique (INRA), Jouy-en-Josas, France (permit no: 12/035 (06.29.2012)). Mice were kept at the Bio Medical Center (BMC) animal facility, Lund University, Sweden or at the animal facility of INRA, Jouy en Josas (outside Paris), France in a controlled environment (light/dark cycle, temperature, free access to food and water). Animal experiments were carried out in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioural Research with Animals (Directive 86/609EC). All efforts were made to minimize suffering.

**Human recombinant A1M.** Human rA1M was donated by A1M Pharma AB (Lund, Sweden). The full polypeptide of human plasma A1M preceded by an N-terminal His8-tag was prepared as previously described[52], and tested for activity[53]. The rA1M solution was dissolved in 10 mM Tris-HCl pH 8.0, 0.125 M NaCl at a concentration of 2.25 mg/ml (0.08 EU/mg) and sterile filtered. The solution was kept at −80 °C until use. Mice were injected i.p. with 0.27 mg rA1M. The control mice were injected with an equal volume of buffer (10 mM Tris-HCl pH 8.0, 0.125 M NaCl). Human rA1M was detected in mouse plasma after the i.p. injections by using an in-house human A1M-specific radioimmunoassay (RIA) described previously[32].

**Experimental set-up.** Figure 1 illustrates the experimental design. The four experimental groups were two control groups (wt females mated to wt males and injected with buffer during pregnancy (Ctrl-buff) and wt females mated to wt males and injected with rA1M during pregnancy (Ctrl -A1M)), and two preeclampsia groups (wt females mated to transgenic males and injected with buffer during pregnancy (PE-buff) and wt females mated to transgenic males and injected with rA1M during pregnancy (PE-A1M)). The females used were 10–20 weeks old. The experiment lasted from time of mating until termination at gestation day 17.5 dpc. The females were given six i.p. injections of either buffer or rA1M every second day starting at 6.5 dpc. BP (systolic and diastolic) was measured every second day, starting before mating to get a baseline and throughout the experiment (for ~35 consecutive days per mouse), by a non-invasive tail-cuff device (CODA8 with four channels, EMKA Technologies). Non-anesthetized mice, previously trained for 1 week to the manipulation, were placed in animal restrainers of appropriate size and placed on a warming platform. The system uses volume pressure recording sensors and an occlusion tail-cuff to repeatedly determine changes in the tail volume, corresponding to systolic and diastolic pressure, with at least 3 satisfactory measurements per day. Systolic and diastolic BP always displayed similar curve profiles in all groups analysed, therefore only systolic BP is shown. Urine was collected during the experiment non-invasively by putting female mice on a cold metal surface to induce urination, and stored at −80 °C until use. However, urine samples were difficult to collect from all females at every time point, and therefore results were pooled within each group for each gestational period (early, mid and late). Whole blood was collected from the Saphena vein at early- and mid-gestation, and from the Cava vein at time of termination in Li-Heparin tubes. Plasma was separated from whole blood and then stored at −80 °C until use. Females were sacrificed at day 17.5 dpc and organs collected. The organs were dissected and biopsies were fresh-frozen on dry ice, paraffin-embedded, or fixed for TEM. Pups were euthanized, counted, and weighed. The placentas and maternal hearts were weighed. Differences in number of animals per group are related to different experimental locations; Paris, France and Lund, Sweden (Supplementary Table S2). The following data analysis were performed on mouse experiments executed in Paris: BP measurements, Nitrotyrosine analysis, hA1M-RIA analysis, qPCR analysis, and plasma sEng/sFlt1 analysis. The following data analysis were performed on mouse experiments executed in Lund: Hypoxyprobe analysis, HE/Masson/TEM microscopy analysis, ACR analysis and MRI analysis. Heart weight was included from both locations, explaining the higher n-numbers.

**Gene expression analysis in kidney.** Total RNA was purified from frozen kidney biopsies using TRIzol (Life Technologies) followed by an E.Z.N.A. Total RNA kit (Omega Bio-Tek, VWR). Reverse transcription with random hexamers was done using TaqMan Reverse Transcription kit (Applied Biosystems, Roche). Real-time PCR was performed for *HO-1*, *CAT*, *SOD2*, with hypoxanthine phosphoribosyltransferase (*HPRT)* as

endogenous control, using TaqMan Gene Expression Assays specific for mouse (*HO-1* – Mm00516005_m1, *CAT* – Mm00437992_m1, *SOD2* – Mm01313000_m1, *HPRT* – Mm01545399_m1, Life Technologies). The analyses were done using the relative standard curve method, where a 4-fold dilution series of a complementary DNA (cDNA) from mouse placenta was used as an arbitrary standard. This was used to give an arbitrary unit for each sample as defined by a standard curve for each gene tested. The arbitrary unit for the gene of interest was normalized against the value for *HPRT* to give an expression ratio that could be compared between samples. All samples were run as duplicates.

**Blood and urine analysis.** The plasma was analysed for sFlt1 using a Quantikine Mouse VEGF R1/Flt-1 Immunoassay (R&D Systems), and for sEng using a Quantikine Mouse Endoglin/CD105 Immunoassay (R&D Systems). The level of proteinuria in urine was measured as the albumin/creatinine ratio (ACR) at early, mid and late gestation, by combining a murine urinary albumin ELISA kit (Albuwell M, Exocell) and a creatinine chemical assay (the Creatinine Companion, Exocell). For all analysis, samples were run in duplicates.

**Hypoxyprobe.** For detection of tissue hypoxia, we used the hypoxia marker pimonidazole hydrochloride, also known as Hypoxyprobe-1 (Hypoxyprobe Inc.). Pimonidazole is reductively activated in hypoxic cells and form stable adducts with thiol groups in proteins in both normal and malignant tissue. A monoclonal antibody binds specifically to formed adducts, allowing their detection by immunoperoxidase analysis of formalin-fixed paraffin-embedded sections. Two hours before termination on day 17.5 dpc, Hypoxyprobe-1 (at 116 mg/ml in 0.9% saline solution) was injected i.p. in pregnant females at 60 mg/kg body weight. Placentas were cut through the mid-section, formalin-fixed and paraffin-embedded for immunohistochemistry using peroxidase detection on sections according to manufacturer's protocol. On average, four placentas per female were analysed and stained sections were scanned as 20x magnification high resolution images using a Hamamatsu Nanozoomer S60 scanner (Hamamatsu Photonics). To quantify the peroxidase staining in the placenta, an algorithm was developed using the ImageJ software platform, where positive peroxidase stained area was calculated as percent of the whole area of the specimen (any holes excluded).

**Protein nitration assay.** To detect nitrotyrosine recidues on nitrated proteins in placenta, we used the OxiSelect Nitrotyrosine ELISA kit (Cell Biolabs Inc.) according to manufacturer's protocol. Protein extractions from placenta were prepared by adding homogenizing buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.5% NP-40, one Complete mini protease inhibitor cocktail tablet/10 ml buffer (Roche)) to biopsies and sonicated for 10 s. The tissue was further shredded by pipetting and thereafter centrifuged at 10000 × g for 30 min. The supernatant was collected and protein concentration measured by Pierce BCA Protein assay kit (Thermo scientific).

**Histology.** For histology analysis of tissue morphology, we used H&E staining according to standard protocols, or Masson's Trichrome staining (a three-color staining, including Aniline Blue that is specific for collagen) according to manufacturer's protocol (Reactifs RAL) on formalin-fixed paraffin-embedded biopsies. Biopsies were sectioned at four µm thickness using standard protocols and morphology was evaluated by light microscopy using an Olympus BX60 microscope with cellSens Entry micro imaging software. Microscopy was performed on sections from four placentas, one kidney and the heart per female and with two or three females per experimental group.

**Transmission electron microscopy.** Biopsies from placenta, kidney, and heart (3 × 3 × 3 mm) were fixed for two hours at room temperature in fixative (1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M Sörensen buffer pH 7.2), washed and stored overnight at 4 °C in Sörensen buffer. The fixed samples were prepared for ultrathin sectioning and subjected to TEM as reviewed in[54]. Microscopy was performed on duplicate sections from one biopsy per organ and female, with two-three females per experimental group. For kidney sections, 3–5 glomeruli were present per section.

**Magnetic resonance imaging analysis.** MRI experiments were performed on pregnant control and preeclamptic females (not included in the treatment groups) at gestational day 17.5 dpc using a 9.4 T horizontal bore MR scanner (Bruker, Germany) with the sedated pregnant mouse positioned on a warming pad to maintain constant body temperature at 36–37 °C. A prospectively electrocardiogram and respiration-triggered fast low angle shot (FLASH) sequence was used to acquire cine MR images in a short-axis stack covering the ventricles and two- and four-chamber views. To ensure full coverage of the cardiac cycle, 24 frames with a temporal resolution of 6 ms were acquired. Imaging parameters were field of view 25 × 25 mm, matrix 192 × 192, slice thickness 1 mm, no gap, echo time 2.1 ms, repetition time 6 ms, flip angle 15°, and number of averages 1. Manual segmentation of left and right ventricular endocardial borders and left ventricular epicardial borders at end-diastole and end-systole were performed in Segment v2.0 (Medviso AB, Sweden)[55] for end-diastolic and end-systolic volumes, ejection fraction, cardiac output and left ventricular mass. For left ventricular mass the papillary muscles and trabeculation were included as myocardium to quantify potential hypertrophy related to preeclampsia.

**Statistical analysis.** All data were analysed by Origin 8 software (Microcal Northampton, USA). Data are presented as mean ± SEM or as median (range), where appropriate. Differences between groups were evaluated using the 2-sample Student's *t*-test with Welch corrections (Fig. 2A) and Mann-Whitney U-test (all other figures and tables). P-values of $p = 0.05$ were considered statistically significant. No statistical test was performed for ACR-values in urine (Fig. 4) due to pooled and normalised results.

## Data Availability

Materials, data and associated protocols are available upon request.

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## Author Contributions

Study design – L.E., J.L.V., D.V. and S.R.H. Data collection – L.E., A.D., J.C., I.Z. and G.K. Analysis and interpretation of data – L.E., I.Z., G.K. and E.H. Writing of the manuscript – L.E., D.V. and S.R.H. Review and/or revision of the manuscript – All authors.

## Additional Information

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**Competing Interests:** Stefan R. Hansson holds patents related to diagnosis and treatment of preeclampsia and is co-founder of A1M Pharma AB and Preelumina Diagnostics (www.a1m.se), as well as a shareholder. Lena Erlandsson and Erik Hedström are minor shareholders of A1M Pharma AB. All the remaining authors (A.D., J.C., I.Z., G.K., J.L.V. and D.V.) declare no competing interests.

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