Vascular and circulating biomarkers

in preeclampsia and uteroplacental acute atherosis

PhD thesis

by

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Dedicated to the memory of my grandfather, who sparked my curiosity,
and began challenging me intellectually long before I could read or write.

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Oslo, August 1st, 2022

Ingvild K. Fa

LIST OF PAPERS

Paper I: Fosheim IK, Alnaes-Katjavivi P, Redman C, Roald B, Staff AC, Størvold GL

Acute atherosis of decidua basalis; characterization of spiral arteries, endothelial status and activation

Placenta. 2019 Jul;82:10-16

Paper II: Fosheim IK, Johnsen GM, Alnaes-Katjavivi P, Turowski G, Sugulle M, Staff AC

Decidua basalis and acute atherosis: expression of atherosclerotic foam cell associated proteins

Placenta. 2021 Apr;107:1-7

Paper II corrigendum: Fosheim IK, Johnsen GM, Alnaes-Katjavivi P, Turowski G, Sugulle M, Staff AC

Corrigendum to "Decidua basalis and acute atherosis: expression of atherosclerotic foam cell associated proteins" [Placenta 107 (2021) 1-7]

Placenta. 2022 Jan;117:28

Paper III: Fosheim IK, Jacobsen DP, Sugulle M, Alnaes-Katjavivi P, Fjeldstad HES, Ueland T, Lekva T, Staff AC

Serum amyloid A1 and pregnancy zone protein in pregnancy complications and correlation with markers of placental dysfunction

Submitted

ABBREVIATIONS

AA	acute atherosis
ADRP	adipose differentiation-related protein
CD	cluster of differentiation
cFMC	cellular fetal microchimerism
CHT	chronic hypertension
CK7	cytokeratin 7
CVD	cardiovascular disease
DAB	diaminobenzidine
DM	diabetes mellitus
DoHAD	developmental origins of health and disease
DSM	decidual vacuum suction method
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EVT	extravillous trophoblast
FABP4	fatty acid binding protein 4
FFPE	formalin fixed, paraffin embedded
FGR	fetal growth restriction
GH	gestational hypertension
HDL	high-density lipoprotein
HDP	hypertensive disorders of pregnancy
HE	hematoxylin-eosin
HRP	horseradish peroxidase
hsCRP	high-sensitivity C-reactive protein
ICAM-1	intercellular adhesion molecule 1
ISSHP	International Society for the Study of Hypertension in Pregnancy
LDL	low-density lipoprotein
LOX-1	lectin-like oxidized LDL receptor 1
MSB	Martius Scarlet Blue
NHLBI	National Heart, Lung, and Blood Institute
OPB	Oslo Pregnancy Biobank
OUS	Oslo University Hospital (Norwegian: Oslo universitetssykehus)
PAS	periodic acid-Schiff
PE	preeclampsia
PECAM-1	platelet endothelial cell adhesion molecule 1 (also known as CD31)

PlGF	placental growth factor
PZP	pregnancy zone protein
REK	Regional Committees for Medical and Health Research Ethics (Norwegian: Regionale komiteer for medisinsk og helsefaglig forskningsetikk)
SAA1	serum amyloid A1
sFlt-1	soluble fms-like tyrosine kinase-1
STB	syncytiotrophoblast
TMB	tetramethylbenzidine
VCAM-1	vascular cell adhesion molecule 1
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor

SAMMENDRAG AV AVHANDLINGEN (NORWEGIAN SUMMARY)

Preeklampsi (svangerskapsforgiftning) er en svangerskapskomplikasjon definert ved nyoppstått hypertensjon og minst ett annet tegn på organdysfunksjon (f.eks. proteinuri, forhøyede levertransaminaser, koagulasjonsforstyrrelser eller eklampsi/svangerskapskramper), som definisjonsmessig oppstår etter 20. svangerskapsuke. Globalt forårsaker preeklampsi omfattende maternell sykelighet og dødelighet under svangerskapet. Syndromet kan også påvirke maternell helse senere i livet, ettersom overlevere av preeklampsi har økt risiko for å utvikle hjerte- og karsykdommer.

Akutt aterose er en svangerskapsspesifikk lesjon i uteroplacentære spiralarterier som forsyner morkaken med blod under svangerskapet. Denne arterielesjonen finnes hyppigst i decidua basalis, livmorslimhinnelaget som ligger direkte under morkaken. Akutt aterose er vanlig i svangerskap komplisert av preeklampsi eller diabetes mellitus, men kan også forekomme i ukompliserte svangerskap. Lesjonen likner på tidlige stadier av aterosklerose (ofte kalt «fatty streaks»), en lesjon som er viktig i utviklingen frem til kliniske manifestasjoner av hjerte- og karsykdom. Akutt aterose og «fatty streak»-lesjoner er begge karakterisert av lipidfylte (skum-)celler i karveggen, betennelse og oksidativt stress.

Vår forskningsgruppe har foreslått at akutt aterose, som ofte sees i svangerskap komplisert av preeklampsi, kan sees på som en mislykket stresstest av kvinnens hjerte- og karsystem under graviditeten. Preeklampsi er en kjønnsspesifikk risikofaktor for hjerte- og karsykdom, og vi har foreslått at akutt aterose kan representere en egen kjønnsspesifikk risikofaktor. Denne avhandlingen tar sikte på å avdekke morfologiske og molekylære vaskulære og sirkulerende biomarkøregenskaper ved akutt aterose og preeklampsi. Avhandlingen tar utgangspunkt i etablerte nøkkelkomponenter og biomarkører i

aterosklerosеprosessen og vurderer uttrykk av disse i decidua basalis-vev med og uten akutt aterose, samt i blodprøver fra kvinner med og uten akutt aterose og/eller preeklampsi. Avhandlingens hypotese er at vaskulære og sirkulerende biomarkører som er av betydning i aterosklerose likeens er assosiert med akutt aterose og preeklampsi.

Studiene i avhandlingen bruker decidua basalis-vev og maternelle blodprøver fra Oslo Pregnancy Biobank, fra kvinner med ukompliserte svangerskap eller svangerskap komplisert av hypertensive svangerskapskomplikasjoner (inkludert preeklampsi) og/eller diabetes mellitus. De fleste prøvene ble tatt i forkant av og under elektivt keisersnitt, mens noen av blodprøvene ble tatt ved andre tidspunkter før forløsning.

Decidua basalis-vev ble samlet ved hjelp av vår tidligere utviklede vakuumsugeteknikk av livmorveggen under morkaken. Serieparallelle vevssnitt fra decidua basalis ble farget med immunhistokjemiske og histokjemiske fargemetoder. Alle vevssnitt ble evaluert av PhD-kandidaten, med bistand fra erfarne placentapatologer og erfarne medlemmer av forskningsgruppen. Spiralarterier ble identifisert i henhold til forhåndsdefinerte kriterier. Akutt aterose ble definert som minst to tilstøtende skumceller (vakuolerte og CD68-positive), et evidensbasert kriterium som tidligere er utviklet og publisert av vår gruppe. Endotelceller ble identifisert ved bruk av immunhistokjemi med CD31 og von Willebrand-faktor (vWF). Endotelaktivering, som står sentralt i ateroskleroseutvikling, ble vurdert ved intercellular adhesion molecule 1 (ICAM-1), og definert som minst tre tilstøtende ICAM-1-positive endotelceller. Lipidhåndteringsproteinene fatty acid binding protein 4 (FABP4), perilipin-2 og lectin-like oxidized LDL receptor 1 (LOX-1), som alle er uttrykt i aterosklerose, ble vurdert i decidua basalis-spiralarterier med og uten akutt aterose.

Maternelle blodprøver (EDTA-plasma) ble vurdert for konsentrasjon av serum amyloid A1 (SAA1) og pregnancy zone protein (PZP), sirkulerende markører

for proteinmisfolding, ved bruk av enzymkoblet immunadsorberende analyse (ELISA). Proteinmisfolding er vist å fremskynde aterosklerose.

Artikkel I og II studerte morfologiske og molekylære trekk ved spiralarterier og akutt aterose i ukompliserte og preeklamptiske svangerskap. Vi fant fullt og delvis fysiologisk remodellerte decidua basalis-spiralarterier, samt akutt aterose, i prøver fra både preeklamptiske og ukompliserte svangerskap. Studiene valgte bevisst en overrepresentasjon av decidua basalis-prøver som hadde spiralarterier med akutt aterose, både fra ukompliserte og preeklamptiske svangerskap.

Akutt aterose var signifikant assosiert med en endret endotelfenotype (Artikkel I). «Unormale» endotelceller ble definert som svulmede eller uregelmessig formet, i motsetning til et normalt, flatt endotelcellelag. «Ødelagt» endotel ble definert som ikke-kontinuerlig og stedvis løsnet fra karveggen. Nesten alle akutt aterose-lesjoner hadde unormalt eller ødelagt endotelcellelag, og hadde lavere fargeintensitet av CD31 enn endotelceller i spiralarterier uten akutt aterose. Akutt aterose var ikke assosiert med endotelaktivering.

Nesten alle (93 %) decidua basalis akutt aterose-lesjoner hadde minst en perilipin-2 positiv skumcelle, og 55 % av alle observerte skumceller var positive for perilipin-2 (Artikkel II). FABP4-farging ble sjeldnere observert, og var kun til stede i 36 % av akutt aterose-lesjoner og i 13 % av alle skumceller. LOX-1 ble ikke observert i skumceller eller andre deler av akutt aterose-lesjonen, men ble signifikant oftere funnet i vevsområdet rundt spiralarterier med akutt aterose enn rundt spiralarterier uten akutt aterose.

Det var ingen signifikante forskjeller i karakteristika (endotelmorfologi og -aktivering, forekomst av perilipin-2- og FABP4-uttrykk) for akutt aterose mellom ukompliserte og preeklamptiske svangerskap (Artikkel I og II).

Artikkel III studerte sirkulerende biomarkører som er assosiert med proteinmisfolding (SAA1 og PZP) i blod fra ukompliserte svangerskap, samt fra

svangerskap komplisert av hypertensive lidelser (inkludert preeklampsi) og/eller diabetes mellitus. Forekomst av akutt aterose i livmorslimhinnen til de inkluderte kvinnene i Artikkel III ble også dokumentert.

Akutt aterose var til stede hos 39 % av kvinner med hypertensive svangerskapskomplikasjoner (inkludert preeklampsi), mot 9 % av ukompliserte svangerskap (Artikkel III), som er sammenlignbart med tidligere studier av decidua basalis. Det var ingen signifikant assosiasjon mellom akutt aterose og sirkulerende nivåer av SAA1 eller PZP.

Sirkulerende nivå av SAA1 var økt og PZP var redusert i gruppen med preeklampsi med svært tidlig forløsning (fødsel før 34. svangerskapsuke), sammenlignet med ukompliserte svangerskap (Artikkel III). Videre var SAA1 og PZP assosiert med markører for placentadysfunksjon, slik som føtal veksthemning og dysregulerte angiogeniske biomarkører.

Denne doktorgradsavhandlingen demonstrerer likheter så vel som forskjeller mellom akutt aterose-lesjoner i små decidua basalis spiralarterier og tidligere beskrevne ateroskleroseforandringer i større arterier. Avhandlingen fant fremtredende likheter mellom de to arterielesjonene: oksidativt stress omkring lesjonene og lignende proteinuttrykk i skumceller. Betydelige forskjeller ble også funnet: mangel på endotelaktivering, fravær av LOX-1-proteinuttrykk og manglende assosiasjon til SAA1 i akutt aterose er av spesiell interesse, siden disse egenskapene er sentrale i tidlige stadier av aterosklerosedannelse.

Resultatene i avhandlingen indikerer både felles og forskjellige veier for utvikling av akutt aterose og aterosklerose. Likhetene mellom de to arterielesjonene kan støtte vår hypotese om akutt aterose som en indikator på økt risiko for fremtidig hjerte- og karsykdom hos kvinner, men flere longitudinelle studier trengs for å bekrefte denne hypotesen.

SUMMARY OF THE THESIS

Preeclampsia is a pregnancy complication defined by new-onset hypertension and at least one other sign of organ dysfunction (e.g. proteinuria, elevated liver transaminases, coagulopathy, or eclampsia/seizures), by definition appearing in the second half of pregnancy. Preeclampsia causes significant maternal morbidity and mortality during pregnancy worldwide. Maternal long-term health may also be affected, as preeclampsia survivors have an increased risk of developing cardiovascular disease.

Acute atherosis is a pregnancy-specific lesion of the uteroplacental spiral arteries, which supply the placenta with blood during pregnancy. These arterial wall foam cell lesions are most commonly found in the decidua basalis, which lies directly beneath the placenta. Acute atherosis is common in pregnancies complicated by preeclampsia and/or diabetes mellitus, but also occurs in uncomplicated pregnancies. The lesions morphologically resemble early stages of atherosclerosis (“fatty streaks”), a lesion which is a leading cause of clinical cardiovascular disease. Acute atherosis and fatty streak lesions are both characterized by arterial wall lipid-filled (foam) cells, inflammation, and oxidative stress.

Our research group has proposed that acute atherosis, commonly seen in preeclampsia, could be viewed as a failed stress test of the female cardiovascular system during pregnancy. Preeclampsia is a sex-specific risk factor for cardiovascular disease, and we have hypothesized that acute atherosis may represent another sex-specific risk factor. This thesis aims to uncover morphological and molecular vascular and circulating biomarker features of acute atherosis and preeclampsia. The thesis compares acute atherosis and atherosclerosis by studying established key components and biomarkers of atherosclerosis in decidua basalis tissue with and without acute atherosis lesions. The hypothesis of the thesis is that vascular and circulating biomarkers of

known importance in atherosclerosis are associated with acute atherosis and preeclampsia as well.

The studies in this thesis use decidua basalis tissue and maternal blood samples from the Oslo Pregnancy Biobank, from women with uncomplicated pregnancies or pregnancies complicated by hypertensive disorders of pregnancy (including preeclampsia) and/or diabetes mellitus. Most biological samples were collected at the start of and during elective caesarean section, while some of the blood samples were collected at other times prior to delivery.

Decidua basalis tissue was collected using our previously developed vacuum suction technique. Decidua basalis serial tissue slides were stained with immunohistochemical and histochemical stains. All tissue sections were evaluated by the PhD candidate, with assistance from experienced placenta pathologists and experienced members of the research group. Spiral arteries were identified according to predefined criteria. Acute atherosis was defined as at least two adjacent foam cells (vacuolated and CD68-positive), an evidence-based criterion previously developed by our group. Endothelial cells were identified using immunohistochemical stains CD31 and von Willebrand factor (vWF). Endothelial activation, a central feature of early atherosclerosis, was assessed by intercellular adhesion molecule 1 (ICAM-1), and defined as at least three adjacent ICAM-1-positive endothelial cells. Foam cells were characterized by assessing the lipid-handling proteins fatty acid binding protein 4 (FABP4), perilipin-2, and lectin-like oxidized LDL receptor 1 (LOX-1), which are all present in atherosclerosis.

Maternal blood samples (EDTA plasma) were assessed for concentrations of serum amyloid A1 (SAA1) and pregnancy zone protein (PZP), circulating markers of protein misfolding, using enzyme-linked immunosorbent assay (ELISA). Protein misfolding has been shown to accelerate atherosclerosis.

Papers I-II studied spiral artery and acute atherosis morphology in uncomplicated and preeclamptic pregnancies. We observed fully and partially physiologically transformed decidua basalis spiral arteries, as well as acute atherosis, in samples from preeclamptic as well as uncomplicated pregnancies. The studies purposefully chose to over-represent acute atherosis decidua basalis samples from uncomplicated and preeclamptic pregnancies.

Decidua basalis acute atherosis was associated with an altered endothelial phenotype (Paper I). "Abnormal" endothelial cells were defined as swollen or irregularly shaped as opposed to a normal, flat endothelial cell layer. "Destroyed" endothelium was defined as disrupted and detached from the arterial wall. We found that almost all acute atherosis lesions had an abnormal or destroyed endothelial lining, and displayed lower staining intensity of CD31 than endothelial cells in spiral arteries without acute atherosis. Acute atherosis was not associated with endothelial activation.

Almost all (93%) decidua basalis acute atherosis lesions had at least one perilipin-2-positive foam cell, and 55% of all foam cells observed were positive for perilipin-2 (Paper II). FABP4 was less frequently observed, only present in 36% of acute atherosis lesions and in 13% of all foam cells. LOX-1 was not observed in foam cells or other parts of acute atherosis lesions, but was significantly more often found in the perivascular area of arteries with acute atherosis than around arteries without acute atherosis.

There were no significant differences in acute atherosis characteristics (endothelial morphology and activation, frequency of perilipin-2 and FABP4 expression) between uncomplicated and preeclamptic pregnancies (Papers I-II).

Paper III studied circulating biomarkers associated with protein misfolding (SAA1 and PZP) in blood samples from uncomplicated pregnancies and pregnancies complicated by hypertensive disorders (including preeclampsia)

and/or diabetes mellitus. Presence of acute atherosis in the pregnancies included to Paper III was also recorded.

Decidua basalis acute atherosis was present in 39% of the women with hypertensive disorders of pregnancy, compared to 9% of the uncomplicated pregnancies (Paper III), which is comparable to other decidua basalis studies. Acute atherosis was not associated with circulating levels of SAA1 or PZP.

Circulating levels of SAA1 were increased and PZP decreased, in pregnancies complicated by early-onset preeclampsia (delivery prior to 34 weeks' gestation) compared to uncomplicated pregnancies (Paper III). Further, SAA1 and PZP were associated with proxies for placental dysfunction, such as fetal growth restriction and dysregulated angiogenic biomarkers.

This PhD thesis demonstrates similarities as well as discrepancies between acute atherosis lesions of small decidua basalis spiral arteries and previously well described atherosclerosis lesions found in larger arteries. The thesis highlights some of the more prominent similarities between the two arterial lesions: local oxidative stress and similar foam cell protein expression. Significant discrepancies are also recognized. The lack of endothelial activation, LOX-1 protein expression, and circulating SAA1 in acute atherosis are particularly interesting differences, as these features are central in early stages of atherosclerosis.

In conclusion, the results of this thesis indicate both shared and differing pathways for development of acute atherosis and atherosclerosis. The similarities between the two lesions may support our hypothesis of acute atherosis as an indicator of increased future maternal cardiovascular risk, although additional longitudinal studies are needed to confirm this hypothesis.

1. INTRODUCTION

1.1 Spiral arteries: the end arteries of the uterine blood supply

The uterus receives its blood supply from the uterine arteries, which arise from the internal iliac arteries, and anastomose with the ovarian arteries (Figure 1.1). The uterine arteries give rise to the arcuate arteries, which run in the outer-middle part of the myometrium (the muscular layer of the uterine wall). The arcuate arteries give off branches called radial arteries, which penetrate the myometrium and end as the spiral arteries (named so because of their corkscrew shape), running through the inner myometrium through to the endometrium (the mucosal lining of the uterine cavity), supplying the functional layer of the endometrium. The radial arteries also give off branches called the basal arteries, which supply the basal layer of the endometrium (1-3).

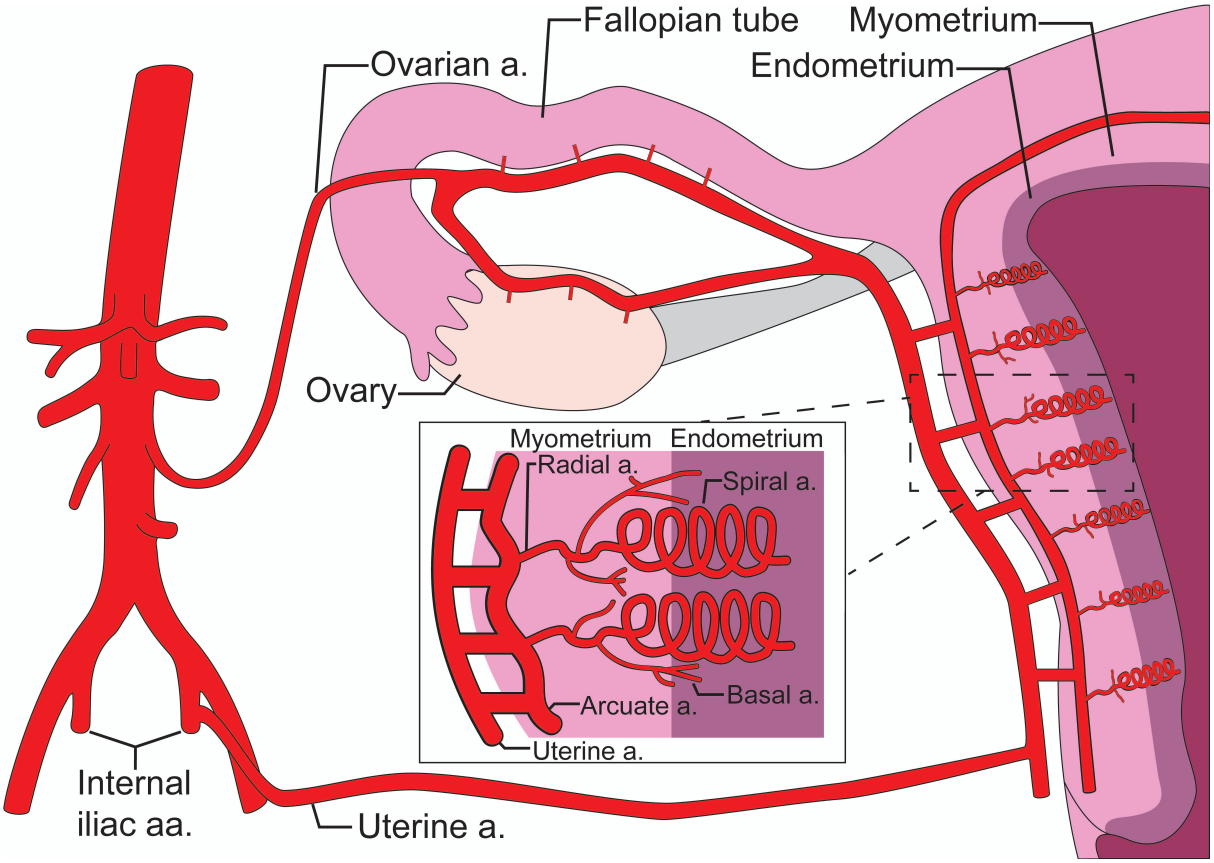

Figure 1.1. The uterine blood supply, nonpregnant uterus. Illustration by Ingrid K. Fosheim, in part based on images from Servier Medical Art.

1.2 Placentation and placental blood supply

The endometrium (termed “decidua” during pregnancy) and the spiral arteries undergo immense changes during pregnancy to allow the fetus and placenta to develop. Structural changes in the endometrium to facilitate pregnancy begin even before conception. The endometrium undergoes hormone-dependent changes, largely driven by progesterone, toward the end of each menstrual cycle, preparing it to receive the conceptus (4). Immediately following implantation of the blastocyst (early stages of the conceptus), the placenta begins to form. Uteroplacental anatomy is summarized in Figure 1.2.

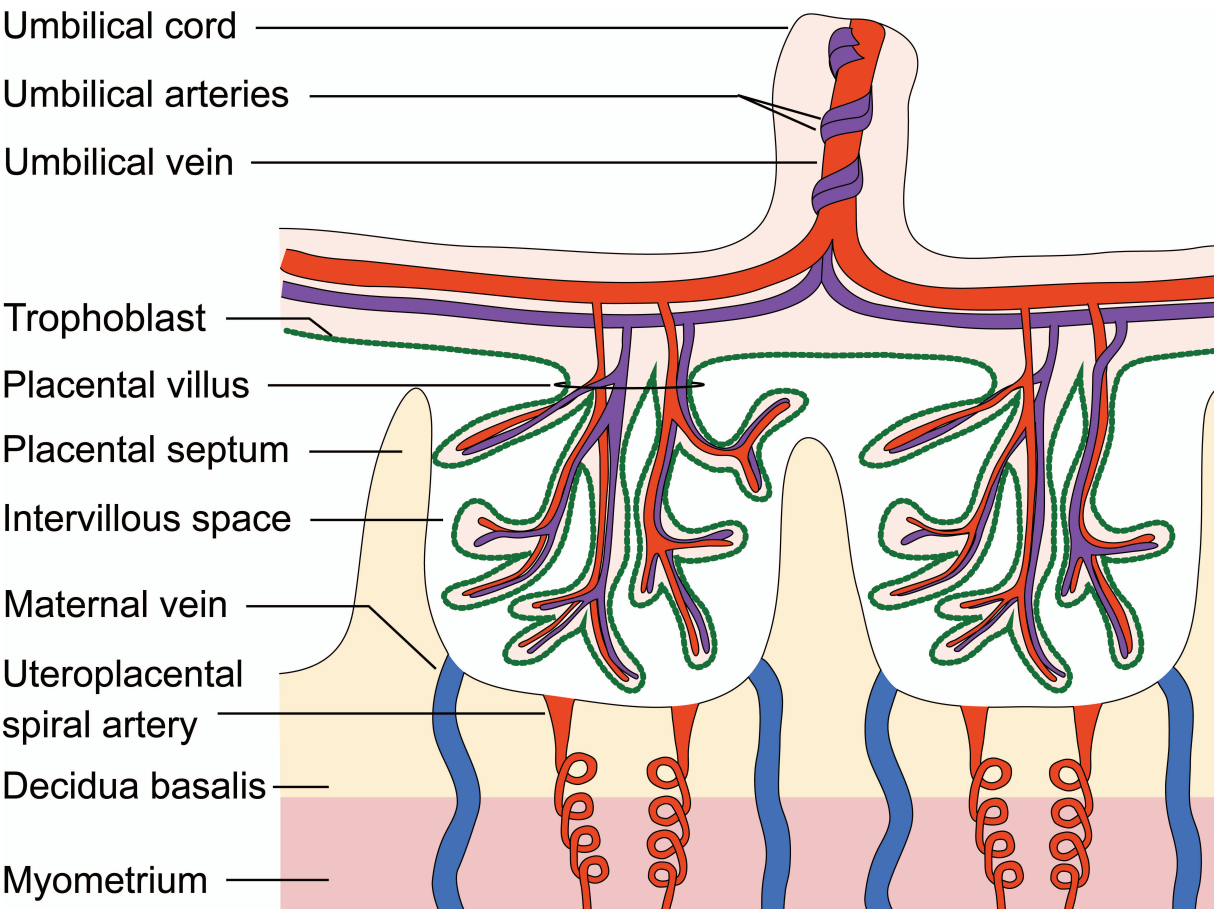

Figure 1.2. Uteroplacental anatomy and circulation. Illustration by Ingrid K. Fosheim.

Formation and development of the placenta is referred to as placentation. Normal placentation is vital for normal placental function throughout pregnancy (5). A healthy placenta is essential to a healthy pregnancy, as the placenta is a multi-talented organ responsible for several vital functions for the growing fetus, such as gas exchange, water and pH balance, numerous metabolic processes, endocrine functions, and more (6).

1.2.1 Early stages of placentation

After the blastocyst implants into the decidua, fetal cells (named trophoblasts) in the outer cell layer begin to invade the decidua (7).

The decidua is anatomically and functionally divided into two parts at term, illustrated in Figure 1.3. The decidua underlying the placenta is called "decidua basalis" (also named decidua placentalis), which is where spiral arteries that supply the placenta are located. "Decidua parietalis" (also named decidua vera) is the remaining decidua lining the uterine wall, where there is no placental tissue attached, and which has not been invaded by trophoblasts. "Decidua capsularis" refers to the part of decidua that grows over the embryo, but fuses with decidua parietalis by the fourth month of gestation (2). This thesis will focus on decidua basalis and the spiral arteries supplying the placenta (also referred to as uteroplacental spiral arteries).

There are two types of trophoblasts: the multinucleated syncytiotrophoblast and cytotrophoblasts (single nucleus). Cytotrophoblasts are further divided into extravillous trophoblasts (EVTs) and villous trophoblasts. Villous trophoblasts and the syncytiotrophoblast form the outer layers of the placental villi (Figure 1.2), while EVTs invade the decidua basalis and underlying myometrium (7).

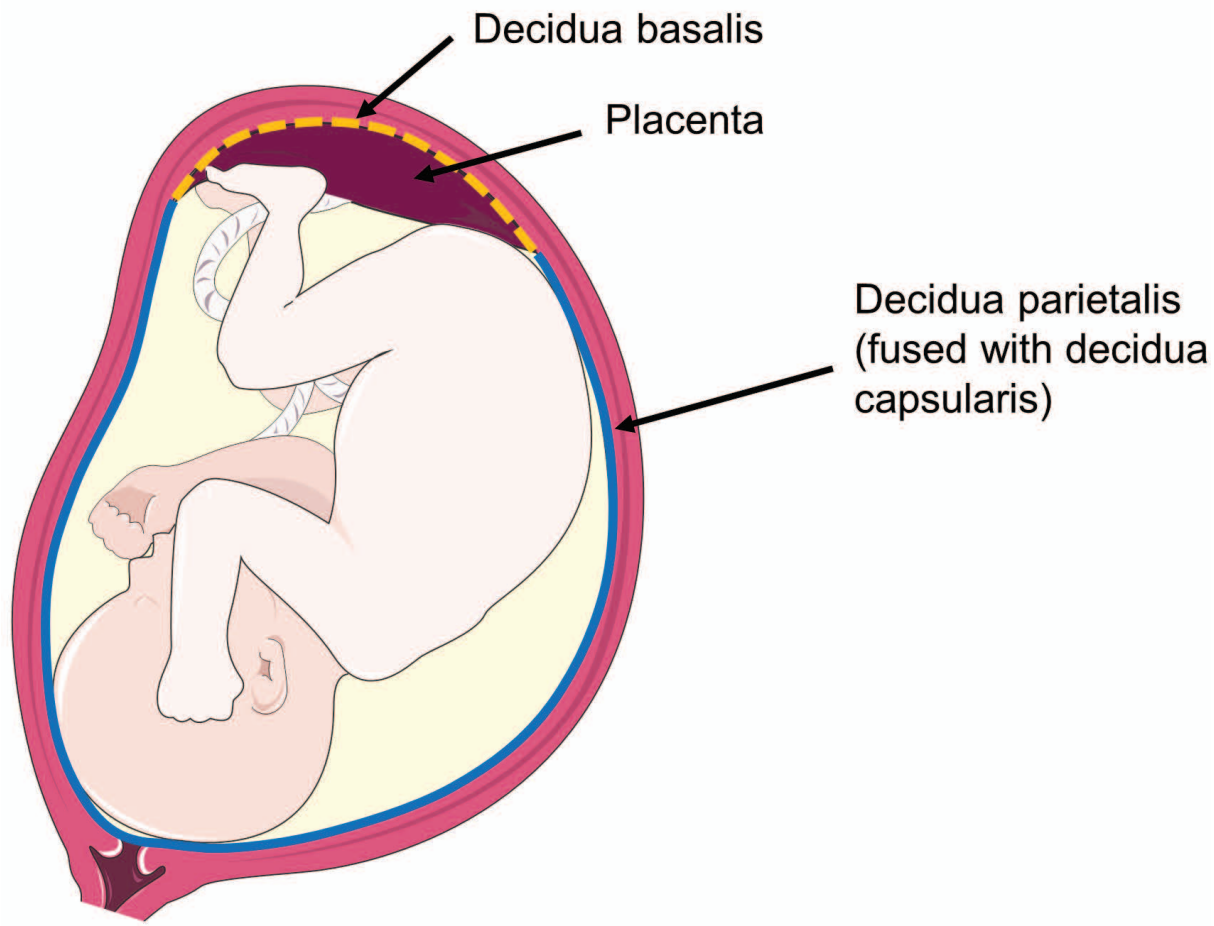

Figure 1.3. Anatomy of the decidua at term. Decidua basalis (orange dashed line) underlying the placenta. Decidua parietalis fused with decidua capsularis (blue line). Created using Servier Medical Art.

EVTs are divided into interstitial and endovascular trophoblasts (8). Endovascular EVTs "plug" the uteroplacental spiral arteries during the first 8-12 weeks of pregnancy (9). The developing placenta requires a physiological hypoxic environment to thrive, and these EVT plugs hinder oxygen-rich blood from reaching the immature trophoblasts too early during placentation (10). Inadequate EVT plugging and premature uteroplacental circulation is associated with oxidative stress, damage to the developing placenta, and miscarriage (11).

While the spiral arteries are plugged early in pregnancy (prior to week 9 of pregnancy), and there is no uteroplacental circulation, the endometrial glands provide the placenta and fetus with nutrients, termed histiotrophic nutrition (12).

As the trophoblast plugs begin to dissolve around week 9 of pregnancy (9), the spiral arteries begin to perfuse the intervillous space of the placenta with maternal blood (Figure 1.2).

1.2.2 Physiological transformation of uteroplacental spiral arteries

The EVTs invade the decidua basalis and the spiral arteries at the blastocyst insertion site (i.e. where the placenta is developing). Consequently, trophoblast invasion of uteroplacental spiral arteries is typically more extensive in the spiral arteries directly underlying the placental center (i.e. cord insertion site), less in the placental margins, and not at all in the spiral arteries not involved in placental blood supply (i.e. decidua parietalis) (13-15).

Trophoblast invasion of the decidua basalis and spiral arteries is an important feature of spiral artery transformation, also referred to as remodeling or conversion. Spiral artery transformation is a physiological process involving changes in the spiral artery walls and is central for normal placentation and placental function (8, 16). Transformation involves both trophoblast independent and trophoblast dependent processes, and has been proposed to occur through five stages (8). The first stage begins before spiral arteries are in contact with trophoblasts (17), and includes beginning artery dilation and endothelial vacuolization. The following four stages (2^{nd}: disorganization of vascular smooth muscle by interstitial EVTs, 3^{rd}: endovascular EVT migration, 4^{th}: EVTs embedded within intramural fibrinoid, 5^{th}: re-endothelialization) all involve EVTs (8). Spiral arteries are transformed from both the luminal and adventitial sides in order to adequately meet the blood supply demands of the placenta. Interstitial EVTs invade the decidual tissue first, followed by endovascular EVTs after dissolution of the EVT plugs. EVTs (thought to be predominantly endovascular) migrate into the spiral artery wall (8). Endovascular and interstitial EVTs work together with maternal immune cells (18, 19), through

complex cross-talk mechanisms (20), to break down the vascular smooth muscle layer, resulting in replacement by fibrinoid (8, 21). In the central part of the decidua basalis, spiral artery transformation with intramural trophoblasts and fibrinoid replacement of the artery wall is present even from week 10 of pregnancy, while the spiral arteries in the placental margin take longer to become transformed (13). Removing the smooth muscle layer from the uteroplacental spiral arteries exempts the arteries from blood pressure regulation exerted by vascular smooth muscle cells and ensures a steady, low-velocity blood flow to the placenta (22).

In normal pregnancy, EVT invasion and spiral artery transformation extends into the inner third of the myometrium (14, 23). Here, the EVTs fuse and become multinucleated "giant cells", marking the end point of normal trophoblast invasion depth (13).

1.3 Placental dysfunction: cause of main obstetrical syndromes

Failure of physiological transformation is called insufficient (also termed incomplete, partial, or failed) physiological transformation, and contributes to poor placentation. Shallow trophoblast invasion is assumed to mediate insufficient physiological transformation of the spiral arteries, and in particular in the myometrial segments (24). The spiral arteries thereby retain some or all of their vascular smooth muscle cells, resulting in a pulsatile blood flow and ischemia-reperfusion injuries to the placenta (22), including oxidative and endoplasmic reticulum stress (25, 26).

Poor placentation with insufficient transformation of uteroplacental spiral arteries may lead to placental dysfunction and subsequent "placental syndromes" with adverse outcome for mother and newborn. Placental syndromes include preeclampsia, fetal growth restriction, preterm delivery,

preterm prelabor rupture of membranes, placental abruption, and intrauterine fetal death (27, 28). Among placental syndromes, preeclampsia, fetal growth restriction, and spontaneous preterm deliveries comprise the largest groups. In Norway, preeclampsia affects 3%, fetal growth restriction 10-15%, and preterm delivery (defined as delivery prior to gestational week 37) 6% of all deliveries, respectively (not excluding possible overlap) (29-31).

Preeclampsia is a hypertensive disorder of pregnancy (HDP). HDPs also include gestational hypertension, chronic hypertension, and chronic hypertension with superimposed preeclampsia, all defined in Table 3.1 in Chapter 3 (Materials and methods). The 2018 revised preeclampsia definition by ISSHP (International Society for the Study of Hypertension in Pregnancy) acknowledges the many clinical aspects of the syndrome, with proteinuria no longer being a mandatory criterion in addition to new-onset hypertension (Table 3.1) (32).

Preeclampsia has gained much research attention due to its large impact on maternal and fetal mortality and morbidity worldwide (33). As recently reviewed by us (3), placental malperfusion and injury caused by insufficient spiral artery transformation triggers release of pro-inflammatory placental molecules (e.g. antiangiogenic factors). This leads to excessive systemic maternal inflammation, ultimately resulting in maternal hypertension and preeclampsia-associated organ dysfunction, the clinical manifestation of preeclampsia.

Two distinct subtypes of preeclampsia are recognized: early- and late-onset preeclampsia (defined in Table 3.1). Redman and Staff have proposed that both early- and late-onset preeclampsia result from placental malperfusion and syncytiotrophoblast stress, but that the causes and timing of the placental dysfunction differ (28, 34-36), illustrated in Figure 1.4.

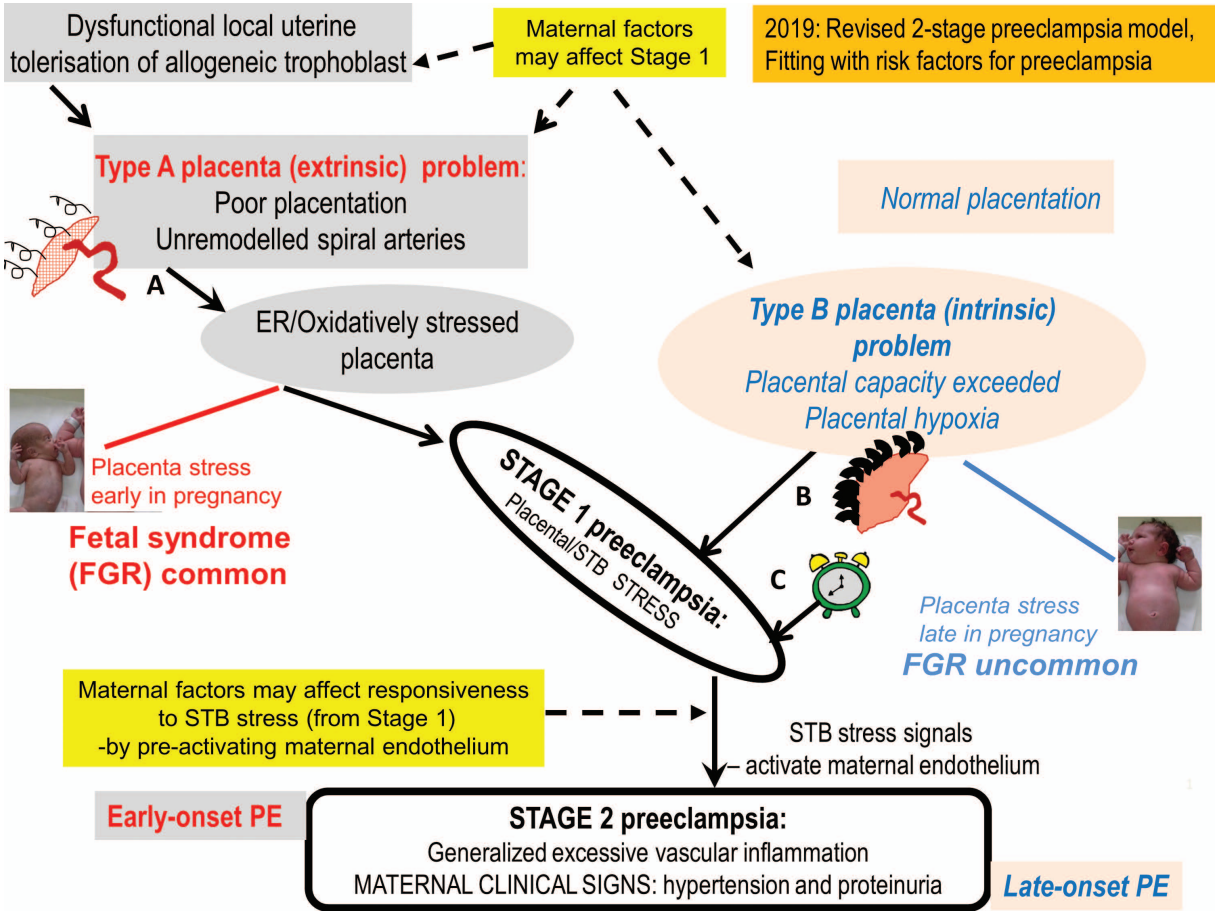

Figure 1.4. Reprinted from (28) via Open Access. The 2019 revised two-stage model of preeclampsia (PE): two placental pathways to clinical preeclampsia. Early-onset preeclampsia involves poor placentation with insufficient physiological transformation of uteroplacental spiral arteries, while late-onset preeclampsia is associated with placental hypoxia due to villous overcrowding towards term. ER: endoplasmic reticulum. FGR: fetal growth restriction. STB: syncytiotrophoblast.

Early-onset preeclampsia is characterized by shallow EVT invasion and insufficient spiral artery transformation. It has been a common misunderstanding that both interstitial and endovascular invasion routes are affected in early-onset preeclampsia. As discussed by Pijnenborg et al. (8), interstitial EVT invasion occurs prior to endovascular EVT invasion, but it is only the depth of the latter that is reduced in preeclampsia while the interstitial trophoblast invasion depth remains normal, as confirmed by Lyall et al. (37).

Late-onset preeclampsia is most often not affected by poor placentation and insufficient spiral artery transformation (5, 28, 35) (Figure 1.4). Per the two-stage model of preeclampsia, dysfunctional placentation, occurring in early pregnancy, constitutes the first stage in early-onset preeclampsia. In late-onset preeclampsia, the first stage may be caused by villous overcrowding and placental malperfusion in a large placenta towards term. The common second stage for both early- and late-onset preeclampsia is syncytiotrophoblast stress (36). This revised two-stage model of preeclampsia fits better with the clinical heterogeneity of preeclampsia as well as gestational hypertension, and with the role of the placenta-associated circulating angiogenic biomarkers soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) (28, 34-36).

1.4 Acute atherosis

Acute atherosis is a lesion affecting spiral arteries (Figure 1.5). It is most frequently found at the tips of decidua basalis spiral arteries, but is also found in spiral arteries in the decidua parietalis and myometrium (16, 38-40). Hertig was among the first to describe pathology of the decidual vessels, and in 1945 he described “an acute degenerative arteriolitis” with subintimal foamy leukocytes, fibrinoid degeneration of the tunica media and stenosis (41). This lesion was then termed “acute atherosis” by Zeek and Assali in 1950 (38).

Acute atherosis is typically found downstream of spiral arteries with insufficient or incomplete transformation in the myometrial segment of the artery (16). As reviewed by us (3), the frequency of acute atherosis varies greatly between different studies and is affected by sampling method (40), but is more prevalent in preeclampsia (10-52% of pregnancies) compared to normotensive pregnancies (0.4-11%) (42-44). Another possible explanation for the large variation in rates lies in the nature of acute atherosis lesions, as they do not necessarily affect the entire circumference or length of a spiral artery, nor all

spiral arteries (8, 44). The highest acute atherosis rates are found in pregnancies with profound placentation problems and insufficient physiological transformation of spiral arteries, such as in autoimmune maternal disease like systemic lupus erythematosus (45), where the lesions have been found as early as gestational week 12 (46).

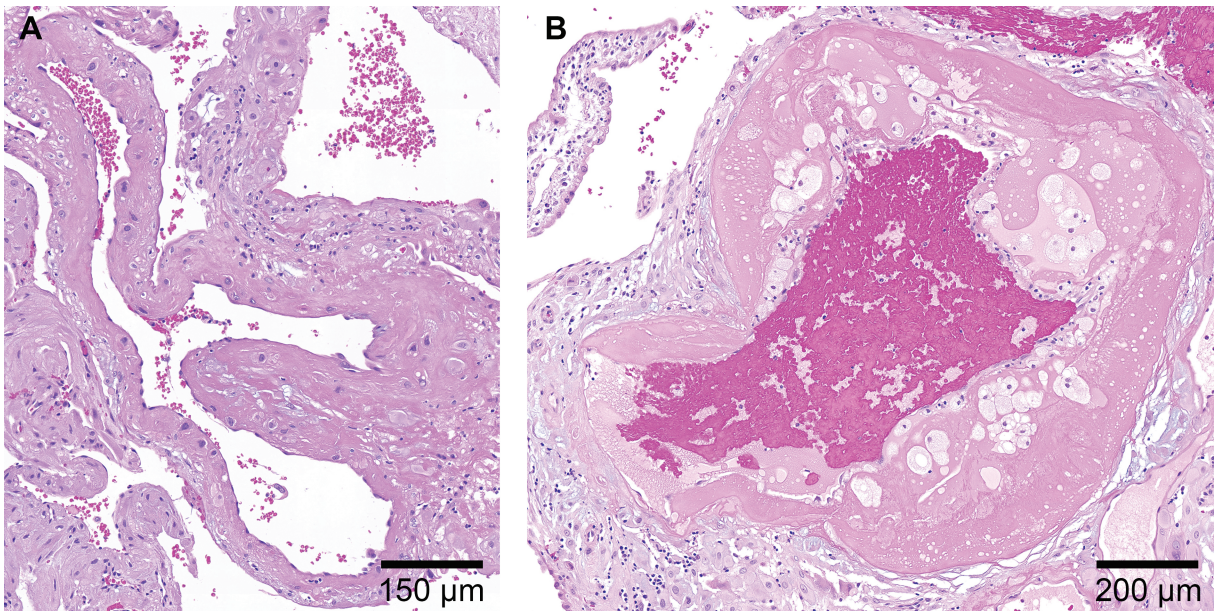

Figure 1.5. Decidua basalis spiral arteries. A, spiral artery without acute atherosis. B, spiral artery with acute atherosis. Photos by Ingrid K. Fosheim.

1.4.1 Defining acute atherosis: new evidence-based criteria by our group

Zeek and Assali expanded on Hertig's definition and described acute atherosis as containing intimal "fatty material" in "large mononuclear cells", "fibrinoid necrosis", and "inflammatory exudation" (38) – giving rise to the "classical" definition of acute atherosis consisting of subintimal foam cells, medial fibrinoid necrosis, and perivascular mononuclear inflammation.

As argued in several publications by our group (3, 44, 47), a clear, reproducible definition of what constitutes each of the three acute atherosis criteria has been lacking, making comparisons between studies on acute atherosis more difficult.

Our group has therefore suggested an evidence-based research definition of acute atherosis as two or more adjacent subintimal, vacuolated, CD68-positive cells ("foam cells"), omitting fibrinoid necrosis and perivascular inflammation as necessary for a diagnosis of acute atherosis (44).

Other researchers have studied spiral artery lesions in the decidua parietalis and/or basalis that are similar to acute atherosis. These lesions, termed "decidual vasculopathy" have artery wall fibrinoid necrosis (with or without foam cells) or smooth muscle hypertrophy as the main diagnostic features (48-50).

1.4.2 Potential mechanisms for acute atherosis development

The causes and development mechanisms of acute atherosis are not fully understood, though researchers for decades have worked towards a greater understanding of the lesion. As mentioned, acute atherosis typically occurs downstream of incomplete transformation of spiral arteries (16), which causes pulsatile flow through the spiral artery (22). This aberrance from normal pregnancy hemodynamics may result in disturbed shear stress and endothelial damage. Indeed, signs of endothelial damage are observed in acute atherosis, with resulting loss of endothelial barrier integrity and leakage of fibrin material into the artery wall (50-52). Endothelial damage and dysfunction in acute atherosis is further discussed in section 1.7 ("Endothelial cells").

There is substantial evidence in favor of an excessive inflammatory component to acute atherosis, and Labarrere proposed already in 1988 that acute atherosis represented a "hallmark of immune aggression" based on his and others' findings of immune involvement in the lesion (53). Several studies have documented immunoglobulin deposits, complement involvement, and increased levels of leukocytes (immunohistochemically identified as primarily consisting of macrophages and T-lymphocytes) in the artery wall and perivascular area of acute atherosis lesions (44, 48, 53, 54). Further, maternal immune tolerization is

also involved, as certain genetic combinations of maternal and fetal protein expression thought to promote inflammation are associated with acute atherosis in preeclampsia (55).

We have recently reviewed (3) our concept of multiple pathways (56-58) leading to decidual inflammation and the formation of acute atherosis in decidua basalis. As illustrated in Figure 1.6, incomplete spiral artery transformation is not a mandatory prerequisite for downstream acute atherosis formation in our model. Other pathways to excessive decidual inflammation are also likely; thereby multiple pathways to acute atherosis are possible. This is in line with acute atherosis also being observed in late-onset preeclampsia (44), where spiral artery transformation most often is normal.

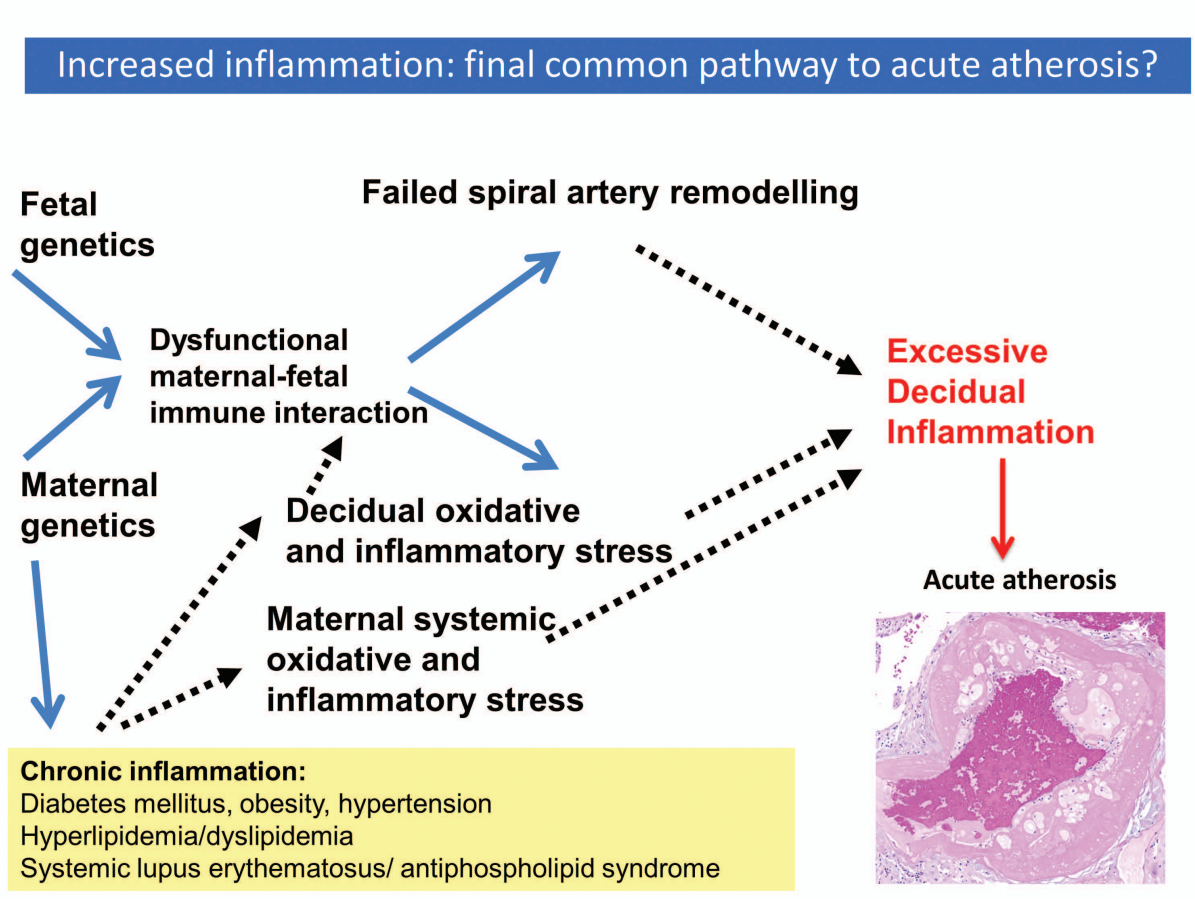

Figure 1.6. Reprinted from (3) via Open Access. Multiple possible pathways to excessive decidual inflammation and resulting acute atherosis.

Our two recent reviews (3, 47) summarize our concepts regarding possible mechanisms behind acute atherosis lesion initiation and development specifically for early- and late-onset preeclampsia, illustrated in Figure 1.7. This model highlights the likely shared pathways between acute atherosis and preeclampsia as well as the crosstalk between the two clinical entities. For early-onset preeclampsia, we propose that poor placentation caused by shallow endovascular EVT invasion with ensuing insufficient spiral artery transformation and placental malperfusion leads to excessive decidual inflammation and acute atherosis. Late-onset preeclampsia may also lead to acute atherosis through excessive decidual inflammation, but caused by a senescent ("ageing") placenta or placental malperfusion caused by overcrowding of placental villi towards the end of pregnancy. This theory fits with the proposed two-stage preeclampsia model (28) and converges at syncytiotrophoblast stress, known to cause inflammation (36), proposed by us to result in acute atherosis (3, 58).

1.4.3 Clinical consequences of acute atherosis and effect on pregnancy outcome

As illustrated in Figure 1.7, acute atherosis may in itself contribute to placental dysfunction and ensuing clinical syndromes such as preeclampsia and fetal growth restriction. Acute atherosis constricts the spiral artery lumen, which likely worsens the already dysfunctional uteroplacental flow (22, 47). Further, decidual vasculopathy (often resembling acute atherosis) is associated with increased uterine artery pulsatility index, indicating disturbed uteroplacental flow (59). Thrombosis is frequently observed in spiral arteries affected by acute atherosis (44, 60) and is associated with downstream placental infarcts (38, 61). Preeclamptic pregnancies with acute atherosis are associated with more severe clinical outcomes such as preterm delivery and fetal growth restriction (40, 48), and in some studies fetal death and perinatal infant mortality (43, 49).

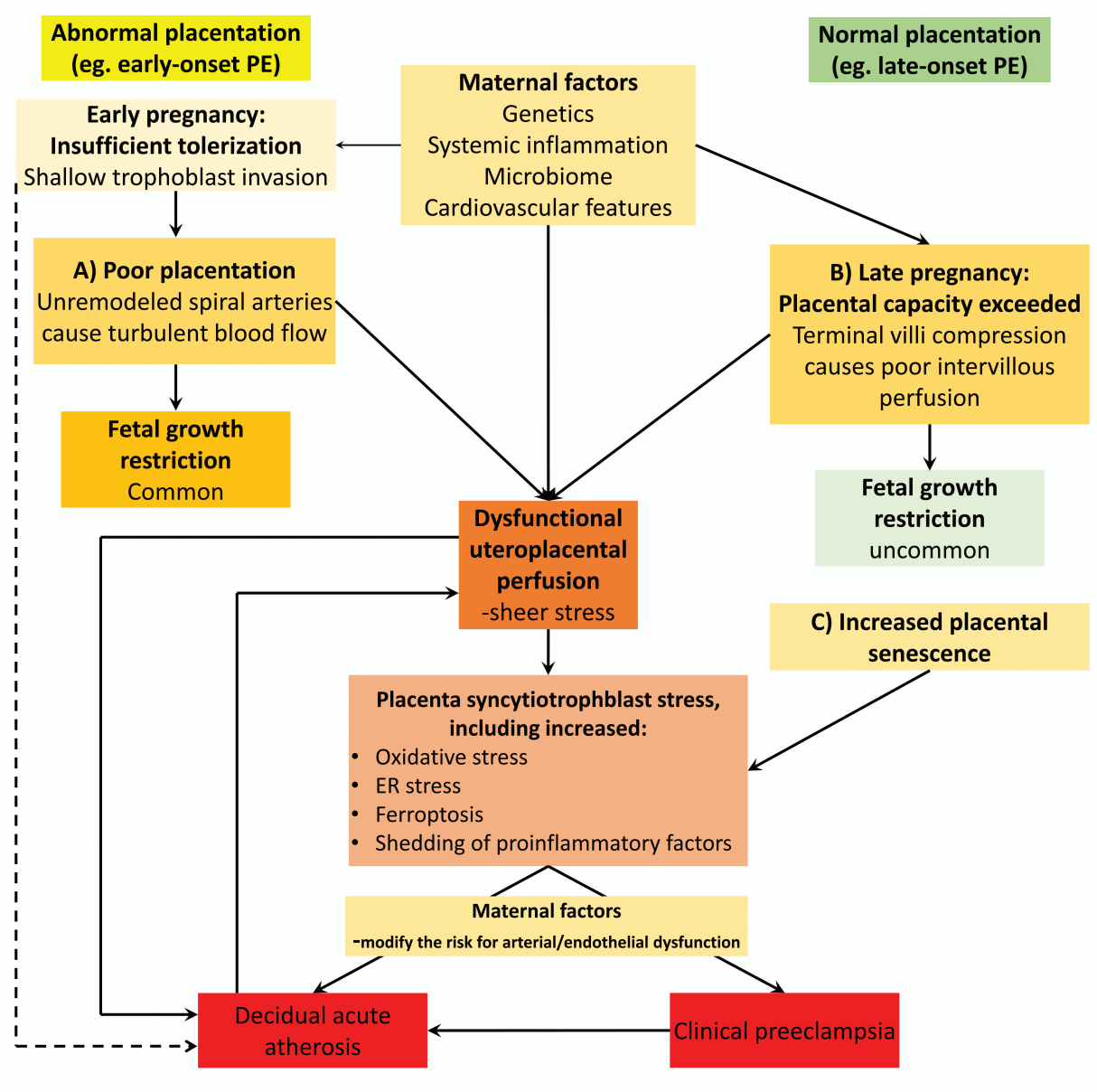

Figure 1.7. Reprinted from (47) via Open Access. Acute atherosis in early- and late-onset placental dysfunction. The figure illustrates possible routes to acute atherosis formation in abnormal placentation caused by insufficient spiral artery transformation (as illustrated here for early-onset preeclampsia (PE)). Other routes than abnormal placentation may also lead to acute atherosis (as seen in late-onset preeclampsia). The figure also illustrates how acute atherosis may lead to or aggravate preexisting preeclampsia through dysfunctional uteroplacental perfusion. ER: endoplasmic reticulum.

1.5 Pregnancies with placental dysfunction: adverse long-term health outcomes for offspring and mother

Offspring that survive pregnancies complicated by placental dysfunction, such as preterm birth, fetal growth restriction, and preeclampsia, often have long-term health problems due to their prematurity at birth (62). Placental dysfunction may also lead to long-term maternal health problems, as discussed in the 5th Edition of Chesley's Hypertensive Disorders of Pregnancy (62). Over the last several decades, developmental origins of health and disease (DOHaD) has received much research attention, whereas less interest has been paid to the mother's long-term health, until the last two decades.

Chesley remarked almost 50 years ago that women with previous preeclampsia-eclampsia seemed to have increased risk of premature cardiovascular death (63). Population-based studies in Norway from over 20 years ago uncovered that women with a history of placental dysfunction, such as preeclampsia or preterm delivery, have increased long-term mortality compared to women with uncomplicated pregnancies (64). Furthermore, women with preeclampsia and preterm delivery have an 8-fold increased risk of death from cardiovascular disease (CVD) (64). Preeclampsia remains a risk factor for CVD after adjusting for established CVD risk factors (65).

CVD is the leading cause of death for females and males in most countries worldwide (66). Several different vasculopathies cause CVD, with atherosclerosis being the main cause (67). Atherosclerosis is the name of the pathological process ending in fatty ("athero-") lesions of the arterial wall, causing the arteries to harden ("-sclerosis"), obstructing the artery lumen as the lesion grows, with the final stage of the disease being rupture of the fatty and fibrous plaque causing thrombosis and occlusion of the affected artery (67). The understanding of atherosclerosis pathophysiology has evolved over time: from the view of atherosclerosis being a lipid storage disorder to the introduction of

smooth muscle cells as key players in the disease, to the current understanding of atherosclerosis as primarily being an inflammatory artery wall disease, with a vast number of risk factors and pathways (68).

1.6 Acute atherosis: a risk factor for premature cardiovascular disease?

Preeclampsia is related to vascular disease both during and after pregnancy, through the associations to acute atherosis and atherosclerotic CVD. Further, acute atherosis and atherosclerosis (particularly early stages of the lesion, often called "fatty streaks") share several morphological features, as reviewed by Staff and Redman (69). Thus, our group has hypothesized that acute atherosis itself could present a sex-specific risk factor for CVD in addition to preeclampsia (3, 56-58, 69).

As mentioned in section 1.4.3 ("Clinical consequences of acute atherosis"), acute atherosis has been associated with more severe clinical outcomes in patients with preeclampsia, in particular preterm delivery and fetal growth restriction. Such adverse outcomes further increase the woman's risk of premature CVD (64), again possibly linking acute atherosis to long-term CVD.

One of the most prominent similarities between acute atherosis and atherosclerosis is the presence of subintimal CD68-immunopositive foam cells (44, 70, 71). Both lesions are also characterized by inflammation (for acute atherosis further detailed in section 1.4.2 "Potential mechanisms for acute atherosis development"), with involvement from both the innate and adaptive immune systems (53, 54, 72), and dyslipidemia (73-75). We have argued (3) that there may be a role for oxidative stress in acute atherosis, which is also implicated in atherosclerosis (76), as increased oxidative stress is observed in decidua basalis from preeclamptic women (77), who have high rates of acute atherosis. Oxidative stress has been demonstrated in the related decidual

vasculopathy lesion (50). Sites of arterial branching or bifurcation are more prone to atherosclerosis development due to disturbed shear stress with ensuing endothelial dysfunction and inflammation (78). Acute atherosis is generally found in decidua basalis segments (i.e. downstream) of spiral arteries with insufficient physiological transformation of the myometrial segments, which generates a pulsatile flow through the decidua basalis (16), and implicates shear stress in acute atherosis.

There are important differences between the lesions as well. The size of the affected arteries greatly differs between acute atherosis and atherosclerosis. While spiral arteries at term are up to 2-3 mm in diameter where they open into the intervillous space (22), atherosclerosis affects larger arteries up to several centimeters in diameter (79). The time course of the two lesions also represents a major difference. Acute atherosis is only present during pregnancy (38), thus developing over the course of weeks or months, while atherosclerosis is a chronic vasculopathy that develops over decades before reaching symptomatic stages. The final stages of atherosclerosis development involve plaque formation with calcification and plaque rupture (67). Plaque stability in atherosclerosis relies on several factors, one being cholesterol crystal structure, which is also important for clinical endpoints (80). While plaque formation and calcification are not known to occur in acute atherosis, a recent study identified presence of cholesterol crystals in decidua basalis tissue and near decidual vessels in preeclampsia, with *in vitro* findings of cholesterol crystals activating an inflammasome pathway similar to what is seen in atherosclerosis (81). Though the study did not report acute atherosis presence, this finding supports our concept of decidual inflammation at the fetal-maternal border as similar to inflammation in atherosclerosis (58). Whether acute atherosis lesions have the capacity to form fibrous plaques with calcification if pregnancy and lesion duration were longer, is unknown.

1.7 Endothelial cells: key players in vascular health and disease

1.7.1 Normal endothelial function

Vascular endothelial cells line all blood vessels of the human body. The endothelium is involved in processes such as thrombosis, inflammation, and blood pressure regulation (82). A healthy endothelium is essential for normal regulation of these processes and a healthy and well-functioning cardiovascular system. Consequently, endothelial dysfunction is central to several cardiovascular diseases (83).

1.7.2 Endothelial dysfunction in atherosclerosis and preeclampsia

Atherosclerotic lesions typically occur in places of bifurcation or other areas of the arterial tree where the normal laminar blood flow is altered, leading to disturbed shear stress (78, 84). Endothelial shear stress also promotes atherosclerosis lesion development and affects plaque stability (85).

The endothelium also plays a role in atherogenesis through endothelial activation, a process in which the endothelial cells express adhesion molecules to promote attachment and recruitment of circulating leukocytes, inducing proatherogenic vascular inflammation (82). Endothelial activation is considered an important stage during early atherosclerosis lesion development (72). Adhesion molecules shown to be expressed in atherosclerosis include intercellular adhesion molecule 1 (ICAM-1), E-selectin, and vascular cell adhesion molecule 1 (VCAM-1) (86).

The endothelium is also important in preeclampsia pathogenesis. As described in section 1.3 (“Placental dysfunction”), placental malperfusion and dysfunction following insufficient spiral artery transformation (as in early-onset preeclampsia) or senescence and villous overcrowding (as in late-onset preeclampsia) leads to systemic inflammation (3). Systemic endothelial

dysfunction (87, 88) and activation (89) have been identified as important features of this systemic maternal response, leading to the clinical features of preeclampsia (34).

1.7.3 Endothelial cells in uteroplacental spiral arteries: some controversies

Over the years, there has been considerable controversy regarding whether uteroplacental spiral arteries are lined by invading EVTs or maternal endothelial cells (90), as recently reviewed by us (3). One proposed scenario is that EVTs for a short period of time replace the endothelial cells, and that the spiral arteries later are re-endothelialized (8).

Endothelial cells have been studied in acute atherosis and the related decidual vasculopathy lesion, with several authors reporting signs of endothelial damage in relation to the lesion (50-52), describing the endothelium as being "locally discontinuous" (51) and "disrupted" (52). Further, our group has demonstrated that plasma concentration of thrombomodulin, a marker of endothelial dysfunction, was increased in women with preeclampsia who also had decidua basalis acute atherosis, compared to women with preeclampsia without acute atherosis (91).

Little work has been performed to explore whether endothelial activation and expression of adhesion molecules may play a role in acute atherosis. One study found endothelial activation (demonstrated by presence of ICAM-1) in spiral arteries in placentae where acute atherosis was detected in neighboring arteries, but they did not report endothelial activation in the spiral arteries that had acute atherosis (92).

1.8 Lipid-handling proteins in atherosclerotic lesions

As described in section 1.6 (“Acute atherosis”), one of the more striking similarities between acute atherosis and fatty streak lesions of atherosclerosis is the presence of numerous foam cells. Histologically, foam cells of acute atherosis and atherosclerosis are similar: they are vacuolated, rather large, with a single small nucleus, have little cytoplasm, and label positive for CD68 (44, 71, 93).

Foam cells have been studied in detail in atherosclerosis, with particular focus on cellular origin of the foam cells, different development mechanisms, and intracellular lipid metabolism, as reviewed by several authors (94, 95).

The vacuoles of the foam cells represent cytoplasmic lipid droplets in both acute atherosis and atherosclerosis – as confirmed by Oil Red O and Sudan Black B staining (96-98). In atherosclerosis, foam cells contain triglycerides, phospholipids, and cholesterol esters, and accumulate neutral lipids in lipid droplets (96). Several different proteins are located on the surface of lipid droplets. Perilipin-2, also called adipophilin or adipose differentiation-related protein (ADRP), is the most abundant of these surface proteins (95). Increased expression of perilipin-2 in macrophages leads to intracellular lipid accumulation (99) and foam cell formation (98), and a mouse model showed that perilipin-2 deficiency protected mice from atherosclerosis (100).

Fatty acid binding protein 4 (FABP4) is involved in intracellular lipid transport and is located to macrophages and endothelial cells in atherosclerotic lesions (71, 101). Lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX-1) promotes lipid accumulation by binding and internalizing oxidized LDL (102). LOX-1 is expressed by endothelial cells in early stages of atherosclerosis (103) and is suggested to promote foam cell formation from smooth muscle cells and macrophages (76, 103).

FABP4, perilipin-2, and LOX-1 are not only of importance in atherosclerosis. Studies of preeclamptic placentae (using villous tissue) have shown increased protein expression of FABP4 and LOX-1 and increased gene expression of perilipin-2 compared to normotensive pregnancies (104-106). FABP4, perilipin-2, and LOX-1 have not been studied in uteroplacental acute atherosis, and their potential role in the lesion is unknown.

1.9 Protein misfolding and hypercoagulability

1.9.1 Protein folding and misfolding

Following protein synthesis, the polypeptide chain (also termed the "primary" protein structure) folds (or coils) into the secondary protein structure, such as the α-helix or β-sheet. Protein folding is an important process to ensure normal function of the synthesized protein (107).

Protein folding may go wrong and form misfolded proteins, or the protein may lose its ability to maintain its functional fold (108). Misfolded proteins either lose their normal function or gain potentially undesirable functions (108). They may form fibrous aggregates called amyloid (from Greek; "starch-like"), consisting of numerous fibrils with a cross-β-sheet structure (109). This makes the amyloid fibrils stable, with a slow spontaneous dissociation. Because of their stability as well as large size, amyloid fibrils have been linked to several chronic diseases, as they accumulate in various tissues, disturb cellular and tissue structure, and cause organ dysfunction (109, 110).

1.9.2 Evidence of protein misfolding in atherosclerosis and preeclampsia

Protein misfolding and amyloid accumulation have long been associated with several chronic, progressive, and inflammatory diseases including

Alzheimer's disease, arthritis, and atherosclerosis (109, 110). Protein misfolding has been identified in preeclampsia as well over the last decade, with amyloid accumulating in the urine, serum, and placenta of women with preeclampsia (111, 112). Kell and Pretorius have proposed that a common mechanism for these inflammatory diseases may lie in stress-induced iron dysregulation which awakens dormant microbes in the host (113, 114), resulting in release of microbe-associated inflammatory molecules such as lipopolysaccharide, in turn promoting thrombosis, cell death, and further inflammation.

1.9.3 Serum amyloid A1 and pregnancy zone protein: knowledge gaps in placental dysfunction

Several different proteins may form amyloid (109). One such protein is serum amyloid A1 (SAA1), an acute phase protein that is highly upregulated by the liver following inflammatory stimuli (110). SAA1 is elevated in patients with risk of CVD like myocardial infarction (115) and cerebral thrombosis (116), and has been proposed to promote early stages of atherosclerosis (117). SAA1 forms amyloid when SAA1 levels are elevated over time (110), but may also promote amyloid formation by displacing apolipoproteins from LDL or high-density lipoprotein (HDL) (118). SAA1 has been shown to affect platelets to become more prothrombotic *in vitro* (119), linking SAA1 and hypercoagulability.

Apart from being involved in amyloidosis and atherosclerosis, SAA1 may play a role in initiating parturition (120, 121). A possible relationship between SAA1 and placental dysfunction is unclear, as few studies have examined SAA1 in relation to preeclampsia (122-127) and other syndromes of placental dysfunction (128), and existing studies are small and with conflicting findings.

Protein folding is aided by chaperones, which may prevent protein misfolding and amyloid formation (107). An example of one such protein chaperone is pregnancy zone protein (PZP). PZP binds misfolded proteins and prevents

aggregation into amyloid fibrils. PZP is highly upregulated during normal pregnancy, and dysregulation of PZP has been suggested to play a role in placentation and risk of preeclampsia (129), though this theory has not been tested.

The roles of SAA1 and PZP are unclear in preeclampsia. Studies from other syndromes of placental dysfunction, like fetal growth restriction, are largely lacking. Likewise, data of SAA1 or PZP in relation to uteroplacental acute atherosis or any form of decidual vasculopathy have not been published.

2. AIMS OF THE THESIS

Uteroplacental spiral artery acute atherosis is a poorly understood and understudied pregnancy-specific lesion. The prevalence of this arterial wall foam cell lesion is high in pregnancies with a dysfunctional placenta, such as in preeclampsia. The lesion bears resemblance to early stages of atherosclerosis. Women who survive preeclampsia have an increased risk of premature atherosclerotic cardiovascular disease, but the mechanisms remain largely unknown. Rates of severe cardiovascular disease such as acute myocardial infarction are rising in young women (130), underscoring the importance of identifying female-specific risk factors and disease mechanisms. We have previously hypothesized that women with acute atherosis (affecting small-caliber spiral arteries) are at higher risk for premature atherosclerotic cardiovascular disease (affecting larger arteries).

The main aim of the thesis was therefore to study vascular and circulating biomarkers in pregnancies affected by preeclampsia and/or uteroplacental acute atherosis in order to uncover shared and discrepant molecular features of preeclampsia, acute atherosis, and atherosclerosis.

Specifically, the thesis aims to answer the following questions:

1. Fetal trophoblasts have been proposed to replace maternal endothelial cells in spiral arteries during pregnancy (90). Are third trimester decidua basalis spiral arteries, with or without acute atherosis lesions, lined by maternal endothelial cells or fetal trophoblasts?

2. If decidua basalis spiral arteries are lined by endothelial cells; is the cellular morphology of the endothelial layer in acute atherosis similar or discrepant to what is known from atherosclerotic lesions, including evidence of endothelial cell activation?

3. Do arterial wall foam cells of decidua basalis acute atherosis lesions express lipid-handling proteins similar or discrepant to what is known of foam cell formation in larger artery atherosclerosis?

4. Preeclampsia is associated with oxidative stress in decidua basalis (77). Does decidua basalis tissue surrounding acute atherosis artery lesions (interstitial tissue) express increased levels of lipid-handling proteins and signs of oxidative stress?

5. Are there differences in molecular properties of decidua basalis acute atherosis when present in preeclamptic pregnancies versus uncomplicated normotensive pregnancies regarding endothelial morphology and activation, as well as lipid-handling protein expression?

6. Is decidua basalis acute atherosis associated with circulating signs of protein misfolding, as in early stages of atherosclerosis?

7. Are pregnancies complicated by preeclampsia or signs of placental dysfunction (e.g. fetal growth restriction or dysregulated angiogenic biomarkers) associated with circulating signs of protein misfolding, as seen in atherosclerosis and cardiovascular disease?

3. MATERIALS AND METHODS

3.1 Study population

This PhD study utilizes clinical data and biological samples from women recruited to the Oslo Pregnancy Biobank (OPB) during pregnancy or prior to elective caesarean delivery.

The OPB is an ongoing, prospective biobank study established in 2001 by Professor Annetine Staff, and is located at the Division of Obstetrics and Gynaecology, Oslo University Hospital, location Ullevål. OPB has since its conception recruited pregnant women with clinically uncomplicated pregnancies as well as pregnancies complicated by hypertensive disorders of pregnancy (HDP), fetal growth restriction, diabetes mellitus (DM; pregestational or gestational), and more (e.g. postterm pregnancies). The overarching goal of the OPB study is, as communicated to study participants, to improve the understanding of pregnancy complications and their short- and long-term effect on maternal and offspring health.

The OPB collects biological samples from pregnancy, including prior to elective caesarean delivery, and at postpartum follow-up. Samples collected during pregnancy include maternal blood and urine. At caesarean delivery, comprehensive biological samples are harvested: maternal blood and urine, subcutaneous fat, amniotic fluid, placental biopsies, decidua basalis biopsies, and umbilical cord biopsies, as well as cord arterial and venous blood.

The Biobank coordinator, PhD candidates, and Medical Student Research Program students recruit study participants, gather comprehensive clinical data, and collect biological samples. Clinical data and biological samples are collected, processed, and stored in accordance with strict predefined OPB protocols, which are in line with international state-of-the-art procedures (131). The collection, processing, and storage of biological samples used in this PhD

study are further detailed in sections 3.3 “Decidua basalis tissue collection and processing”, 3.4 “Tissue sectioning and staining”, 3.6 “Maternal blood sampling”, and 3.7 “Blood sample analysis”.

3.1.1 Formal study approval

The OPB study is approved by the Regional Committee for Medical and Health Research Ethics (REK) in South-Eastern Norway, as are all specific research projects utilizing data and samples from OPB. All OPB study participants provide informed written consent prior to collection of clinical and biological data. The work in the present thesis is part of the REK approved OPB sub-studies HAPPY/PATH (ref no. 2013/2092) and Compass (ref no. 8949), which include acute atherosis studies and circulating biomarker studies.

3.1.2 OPB study participants selected for this PhD thesis

This PhD study comprises biomarker studies of decidua basalis tissue and maternal blood. In women delivered by caesarean section, the indication for caesarean delivery was always decided by a clinician (following informed and shared decision making with the woman), independent of the OPB studies. Participation in the OPB study does not affect choice of delivery method.

OPB study participants selected for the papers of this thesis were recruited to the biobank between 2001 and 2017. Among those recruited during 2016-17, several were recruited by the PhD candidate during her time in the Medical Student Research Program at the University of Oslo. In total, 567 pregnant women have contributed with comprehensive health data and biological samples to this thesis. Figure 3.1 provides an overview of OPB study participants selected for this PhD thesis.

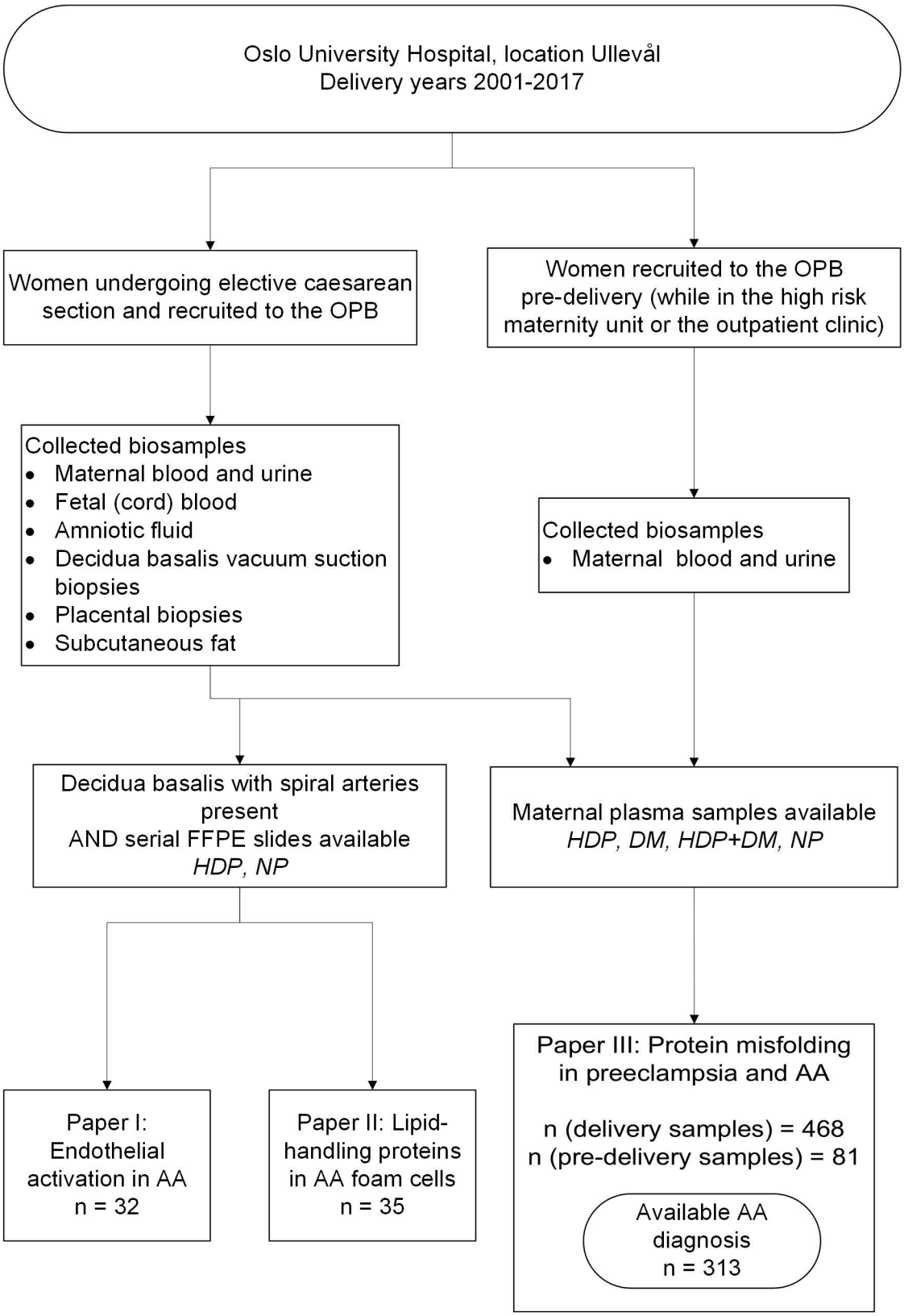

Figure 3.1. Overview of study participant selection to Papers I-III. OPB: Oslo Pregnancy Biobank, FFPE: formalin fixed, paraffin embedded tissue, HDP: hypertensive disorders of pregnancy, DM: diabetes mellitus (pregestational or gestational), NP: normal (normotensive and euglycemic) pregnancy, AA: acute atherosis (in decidua basalis tissue).

All biological samples used in this thesis are collected from women with intact fetal membranes, without regular laboring contractions or signs of infection. For samples collected at caesarean delivery, all (except 16 women with HDP included to Paper III, of which 2 were also included to Paper II) were fasting (defined by us as no food or fluid intake in the last 6 hours). The majority of samples collected prior to delivery (only applies to Paper III; 70 of 81) were from non-fasting women.

Study participants in this thesis contributing with decidual tissue and/or blood collected at caesarean delivery were diagnosed according to final obstetric outcome at delivery (Papers I-III). Participants contributing with blood samples from pregnancy (n = 81 participants in Paper III) were diagnosed according to their obstetric diagnosis at blood sampling. The thesis includes participants with clinically uncomplicated (euglycemic and normotensive) pregnancies, HDP (preeclampsia, gestational hypertension, chronic hypertension, superimposed preeclampsia), DM (pregestational (both type I and II) or gestational), and HDP+DM (participants with an HDP and a DM diagnosis).

For Papers I and II, preeclampsia was defined using "classical" criteria (132): new-onset hypertension (blood pressure $\geq$ 140 mmHg systolic and/or $\geq$ 90 mmHg diastolic) and new-onset proteinuria (repeated $\geq$ 1+ protein on dipstick in the absence of urinary tract infection or $\geq$ 30 mg/mmol total protein/creatinine ratio (29)) at $\geq$ 20 weeks' gestation. For Paper III, preeclampsia and other HDP were defined according to the 2018 International Society for the Study of Hypertension in Pregnancy (ISSHP) classification (32), permitting new-onset signs of organ dysfunction other than proteinuria as sufficient for diagnosing preeclampsia in the presence of new-onset hypertension. The diagnostic criteria for types of HDP included in this thesis are further detailed in Table 3.1. DM (pregestational or gestational) was diagnosed in the clinic by endocrinologists, according to current guidelines (133, 134).

Table 3.1. Definitions of hypertensive disorders of pregnancy (HDP) categories, and those used in the papers of this thesis

HDP category	Definition	Ref.	Paper
CHT	Chronic hypertension: hypertension (≥ 140 mmHg systolic and/or ≥ 90 mmHg diastolic on ≥ 2 occasions ≥ 6 hours apart) present prior to pregnancy or discovered during the first half of pregnancy	(29, 32, 132, 135, 136)	II, III
GH (prior to 2018)	Gestational hypertension: new-onset hypertension without proteinuria, developing after 20 weeks' gestation	(132, 135, 136)	Not used
GH (from 2018)	Gestational hypertension: new-onset hypertension without other preeclampsia-related features, developing after 20 weeks' gestation	(29, 32)	III
PE (prior to 2018)	Preeclampsia: new-onset hypertension and proteinuria (≥ 1+ protein on dipstick more than once or ≥ 30 mg/mmol total protein/ creatinine ratio), developing after 20 weeks'	(132, 135, 136)	I, II
PE (from 2018)	Preeclampsia: new-onset hypertension and ≥ 1 preeclampsia-related feature(s), such as proteinuria, acute kidney injury, elevated liver transaminases, neurological symptoms (e.g. eclampsia), hematological complications, and fetal growth restriction, developing after 20 weeks' gestation	(29, 32)	III
Early-onset PE	PE and delivery < 34 weeks' gestation	(28, 137)	I-III
Late-onset PE	PE and delivery ≥ 34 weeks' gestation	(28, 137)	I-III
Superimposed PE (prior to 2018)	A woman with CHT develops de novo proteinuria after 20 weeks' gestation	(132, 136)	II
Superimposed PE (from 2018)	A woman with CHT develops ≥ 1 preeclampsia-related feature(s) after 20 weeks' gestation	(29, 32)	III

Gestational age at delivery was defined by routine ultrasound at 17-20 weeks' gestation, offered to all pregnant women in Norway as part of the public health antenatal care program. Where ultrasound was unavailable (n = 32 participants in Paper III), gestational age was defined by embryo transfer date in the case of in vitro fertilization (n = 25), or first day of the last menstrual period before pregnancy (assuming pregnancy length of 282 days, n = 7). Sex-specific newborn weight percentages were calculated according to Norwegian fetal ultrasound-based growth curves of uncomplicated pregnancies (138). Small for gestational age (used in Papers I-II) was defined as birth weight $\leq 10^{th}$ percentile. Fetal growth restriction was defined in Paper I as birth weight $\leq 3^{rd}$ percentile, with or without pathological antenatal fetal Dopplers (absent or reversed umbilical artery flow, umbilical artery pulsatility index $> 95^{th}$ percentile, cerebral artery pulsatility index $< 5^{th}$ percentile, and/or cerebroplacental ratio ≤ 1.08) (139, 140), and in Paper III as birth weight $\leq 3^{rd}$ percentile.

3.2 Study design

3.2.1 Study design of Papers I -II

Papers I-II are case control studies using histochemistry and immunohistochemistry methodology to evaluate detailed features of decidua basalis acute atherosis lesions. OPB study participants whose decidua basalis tissue sample had previously been assessed and where presence of spiral arteries (with or without acute atherosis) was confirmed, *and* who had additional decidua basalis tissue sections available in the OPB, were selected for these studies (Figure 3.1). Cases were participants with confirmed decidua basalis acute atherosis. Controls were participants without decidua basalis acute atherosis (but with present decidua basalis spiral arteries in the tissue section).

We included participants with preeclampsia as well as participants with normotensive pregnancies in both the case (acute atherosis present) and control (acute atherosis absent) groups.

Papers I-II only include OPB study participants with normotensive or preeclamptic pregnancies (all included were euglycemic throughout pregnancy). In Paper I, participants with normotensive pregnancies were termed "normotensive controls". From a study design perspective, these participants could be part of both the control (acute atherosis absent) and case (acute atherosis present) groups. In Paper II, two of the participants in the preeclampsia group had preexisting chronic hypertension, with new-onset proteinuria after 20 weeks' gestation (= superimposed preeclampsia). All other participants in the preeclampsia group in Papers I-II had new-onset hypertension and proteinuria after 20 weeks' gestation. Hypertension and proteinuria are defined in Table 3.1.

3.2.2 Study design of Paper III

Paper III is a cross-sectional study where OPB study participants (pregnant women recruited pre-delivery and/or at caesarean delivery) with sufficient volume of available maternal plasma in the biobank were included. A subset of the participants included at caesarean delivery had been assessed for decidua basalis acute atherosis. The study used enzyme-linked immunosorbent assay (ELISA) to describe circulating biomarkers in preeclampsia and acute atherosis.

Paper III includes OPB study participants with euglycemic and normotensive pregnancies (termed "controls"), as well as HDP, DM, and HDP+DM pregnancies.

3.3 Decidua basalis tissue collection and processing

Decidua basalis tissue was collected using the decidual vacuum suction method previously developed and evaluated by our group (40, 77, 141, 142). Following delivery of the fetus and placenta at elective caesarean delivery, the placental bed is gently suctioned with a vacuum curette (as routinely used during surgical evacuation of the uterus following retained pregnancy tissues of early abortions) by the obstetrician performing the caesarean delivery. The collected tissue is immediately flushed with 500 mL sterile 0.9% saline solution at room temperature to remove blood. A representative part of the decidua basalis tissue is then covered with 4% formalin for fixation and subsequently embedded in paraffin.

3.4 Tissue sectioning and staining

3.4.1 Decidua basalis tissue sectioning and preparation

Decidua basalis formalin fixed, paraffin embedded (FFPE) blocks were cut to 3 µm thick sections using a Micron HM 355 microtome (Thermo Fisher Scientific Inc., USA). Sections were mounted on SuperFrost slides (Menzel Gläser, Germany).

Prior to staining, FFPE sections were deparaffinized and rehydrated (detailed in Appendix 1). A complete list of immunohistochemical stains, including antibodies, dilutions, clones, manufacturers, and positive control tissues used in Papers I-II is shown in Table 3.2.

Table 3.2. Overview of immunohistochemical stains used in Papers I-II

Stain	Target antigen	Clone / Producer / Dilution	Positive control tissue
Monoclonal mouse anti-human Cytokeratin 7	Cytokeratin (CK) 7	OV-TL 12/30 / Dako, Denmark / 1:300	Placenta
Monoclonal mouse anti-human CD68	CD68 scavenger receptor	KP1 / Dako, Denmark / 1:1500	Appendix (appendicitis), tonsil
Monoclonal mouse anti-human CD31	CD31	JC70A / Dako, Denmark / 1:400	Appendix (appendicitis), kidney
Monoclonal mouse anti-human ICAM-1	Intercellular adhesion molecule 1	23G12 / Thermo Fisher Scientific, USA / 1:10 + amplification	Appendix (appendicitis), kidney
Polyclonal rabbit anti-human vWF	von Willebrand factor	polyclonal / Dako, Denmark / 1:120000	Kidney, placenta
Polyclonal rabbit anti-human FABP4	Fatty acid binding protein 4	polyclonal / Sigma-Aldrich, USA / 1:1000	Placenta
Monoclonal mouse anti-human PLIN2	Perilipin-2	AP125 / Progen, Germany / 1:300	Liver
Monoclonal mouse anti-human LOX-1	Lectin-like oxidized LDL receptor 1	9E12.1 / Millipore, USA / 1:750	Placenta

3.4.2 Histochemical and immunohistochemical staining for Paper I

Serial 3 µm sections of decidua basalis tissue were stained with CD31 (also known as platelet endothelial cell adhesion molecule 1 (PECAM-1)), intercellular adhesion molecule 1 (ICAM-1), von Willebrand factor (vWF) +

periodic acid-Schiff (PAS), and Martius Scarlet Blue (MSB). Sections were stained with CD31, ICAM-1, and vWF using a Ventana BenchMark XT automated immunostainer (Roche, USA), a system based on the avidin-biotin-peroxidase complex method. Positive control tissues used are listed in Table 3.2. Appropriate negative reagent controls (mouse or rabbit, depending on antibody) were used. Staining with MSB and co-staining with PAS (for vWF slides) was performed manually according to routine (detailed in Appendix 1).

In addition, serial sections stained with hematoxylin-eosin (HE; in Paper I, the term H+E was used), desmin+PAS, CK7+PAS, and CD68+PAS were already available for the cohort selected for Paper I, as these sections had been used in a previously published study by our group (44).

3.4.3 Histochemical and immunohistochemical staining for Paper II

All sections used for Paper II were manually stained. Serial 3 µm sections of decidua basalis tissue were stained with HE, CK7+PAS, CD68+PAS, fatty acid binding protein 4 (FABP4), perilipin-2, and lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1). Antibody clones listed in the original publication (Paper II) were incorrect; correct clones are provided in the Corrigendum to Paper II and in Table 3.2.

Following deparaffinization and rehydration, sections were stained with HE according to standard routines or immunostained: by heat-induced antigen retrieval, background peroxidase blocking, target detection with primary antibody (Table 3.2) and horseradish peroxidase (HRP)-labelled secondary antibody (anti-mouse or anti-rabbit depending on primary antibody), visualization with chromagen diaminobenzidine, and counterstaining with hematoxylin. Staining protocols are detailed in Appendix 1.

Positive control tissues used are listed in Table 3.2. Non-immune mouse or rabbit ascites was used as primary antibody for negative reagent control.

Co-staining with PAS (for sections immunostained with CK7 and CD68) was performed as for Paper I, detailed in Appendix 1.

One human aorta sample with early atherosclerotic lesions ("fatty streaks") and one sample with advanced atherosclerotic lesions were stained with FABP4, perilipin-2, and LOX-1. The same antibodies in the same dilutions as for decidua basalis sections were used for methodological comparison of subcellular location of immunostaining.

3.5 Decidua basalis tissue evaluation

All decidua basalis tissue sections used in Papers I-II were evaluated by the PhD candidate after training with an experienced placenta pathologist as well as a PhD candidate with extensive experience in the field of decidua basalis microscopy. For Paper I, decidua basalis serial sections were assessed using a Motic BA410E light microscope, using x4, x10, and x40 objectives. Pictures used for publication were obtained using an Axio Scan slide scanner (Zeiss, Germany) and Zen Blue 2.6 (Zeiss, Germany). For Paper II, all sections were scanned using an Axio Scan slide scanner (Zeiss, Germany). Zen Blue 2.6 (Zeiss, Germany) was used for tissue evaluation and for obtaining images for publication. Table 3.3 gives a summary of definitions and terminology regarding decidua basalis tissue evaluation.

Table 3.3. Morphological classifications and definitions used in the evaluation of decidua basalis tissue in Papers I-II

Entity	Definition	Ref.	Paper
Trophoblast (extravillous)	Large CK7-positive cell, found in spiral artery walls (intramural) or scattered in the decidual interstitium	(93)	I-II
Decidual stromal cell	Identified using HE slides as large cells with a round-oval shape and a central, rather large, single nucleus, located in the decidual interstitium	(143)	I-II
Decidua basalis tissue	Decidua basalis tissue origin confirmed by presence of extravillous (interstitial) trophoblasts and decidual stromal cells	(93)	I-II
Decidua basalis spiral artery	Arteries in decidua basalis with a diameter ≥ 140 µm, measured from outer to outer artery wall border	(44, 93)	I-II
Vacular smooth muscle cell	Desmin-positive cell, typically with a spindle shape, located in the spiral artery wall	(93)	I
Foam cell	Vacuolated (in HE slides), mononuclear, CD68-positive cell	(44, 93)	I-II
Physiological fibrinoid (of spiral artery wall)	Bright purple or magenta PAS stain, appearing with a grainy or heterogeneous texture, typically with embedded intramural trophoblasts	(93)	I-II
Fibrinoid necrosis (of spiral artery wall)	Pale pink PAS stain, sometimes with a grey or blue shade, appearing glassy or homogeneous, often with embedded foam cells	(93)	I-II
Complete physiological transformation (of spiral artery)	Spiral artery with intramural trophoblasts, physiological fibrinoid, and absence of mural vascular smooth muscle cells. Also referred to as “completely transformed spiral artery” and (by other authors) “fully remodeled/converted”		I

Partial physiological transformation	Spiral artery with a combination of intramural trophoblasts and physiological fibrinoid, and area(s) with remaining mural vascular smooth muscle cells (lacking fibrinoid in these areas). Also referred to as "partially transformed spiral artery" and (by other authors) "partially/ incompletely remodeled/converted"		I
Non-transformed spiral artery	Spiral artery with smooth muscle cells in entire artery wall circumference, absence of physiological fibrinoid and trophoblasts. By other authors also referred to as "unremodeled spiral artery" or "failure of physiological remodeling"		I
Acute atherosis	≥ 2 adjacent intramural foam cells in a spiral artery wall	(44)	I-II
non-AA artery	Spiral artery without acute atherosis. Includes completely transformed and partially transformed spiral arteries		I-II
AA artery	Spiral artery with acute atherosis lesion		I-II
non-AA sample	Decidua basalis tissue sample with only *non-AA arteries*		I-II
AA sample	Decidua basalis tissue sample with ≥ 1 *AA artery*		I-II
Endothelial cell	CD31- and vWF-positive cell lining the vascular wall (CK7-negative)		I
Normal endothelium	Flat, continuous endothelial cells		I
Abnormal endothelium	Swollen or irregularly shaped endothelial cells. If a spiral artery has both normal and abnormal endothelium, the endothelium is classified as abnormal if > 25% of the endothelium has this phenotype		I
Destroyed endothelium	Disrupted endothelial cells, often detached from the artery wall		I
Endothelial activation	≥ 3 adjacent ICAM-1-positive endothelial cells	(144)	I

Perivascular area	The interstitial decidua basalis area surrounding spiral arteries, stretching up to 100 µm from the outer artery wall margin, avoiding other vascular structures	(54)	II
Scattered perivascular staining pattern	Single immunopositive cells		II
Focal perivascular staining pattern	≥ 5 immunopositive clustered cells		II
Generalized perivascular staining pattern	Larger portions of the perivascular cells are immunopositive		II

3.5.1 Spiral artery identification

Decidua basalis serial tissue sections were evaluated systematically, using a predefined chart (Appendix 2), based on Appendix 1 of a previous PhD thesis by a candidate from our group (145). First, the HE section was evaluated in order to identify and count spiral arteries. Only arteries with a diameter ≥ 140 µm, measured from outer to outer artery wall border, were evaluated, in order to exclude basal arteries (44, 93).

Next, desmin+PAS (Paper I) and CK7+PAS (Papers I-II) slides were assessed. Desmin is an intermediate filament (cytoskeleton component) in smooth muscle cells, and identifies vascular and myometrial smooth muscle cells (93, 146). Desmin positivity in spiral artery tunica media was noted, in particular the location in the artery wall, using a modified categorization after Lyall et al. (37). The degree of disruption of the media was categorized as “no desmin positivity”, “separate desmin positive cells”, “disrupted/discontinuous desmin positive cells”, or “continuous/intact desmin positivity” (Appendix 2).

CK7, also a cytoskeleton component, is frequently used as a marker of glandular epithelium, and identifies decidual glandular epithelium and trophoblasts (93, 147). Presence of interstitial trophoblasts was noted and used as confirmation of decidua basalis tissue origin. Presence or absence of intramural trophoblasts was logged for each spiral artery (Appendix 2).

In Paper I, degree of physiological spiral artery transformation was noted. We defined complete physiological transformation as spiral arteries with intramural trophoblasts, physiological fibrinoid, and absence of mural vascular smooth muscle cells. Partial physiological transformation was defined as spiral arteries with a combination of intramural trophoblasts and physiological fibrinoid, and area(s) with remaining mural smooth muscle cells. Non-transformed (the term untransformed was used in Paper I) spiral arteries were defined as containing smooth muscle cells in the entire artery wall circumference, being entirely absent of physiological fibrinoid and trophoblasts (Table 3.3).

3.5.2 Acute atherosis diagnosis

Spiral artery acute atherosis was diagnosed using an evidence-based research definition previously published by our group (44). This definition identifies acute atherosis as presence of ≥ 2 adjacent foam cells, identified as subintimal, vacuolated, and CD68-positive cells. CD68 is a scavenger receptor, and is used to identify monocytes and macrophages. Subintimal, vacuolated cells were identified on HE sections, then assessed for CD68 positivity. As listed in Table 3.3, spiral arteries with acute atherosis were termed *AA arteries* (term used in Papers I-II and throughout the thesis). Conversely, spiral arteries lacking acute atherosis were termed *non-AA arteries* (term used in Paper II and throughout the thesis; in Paper I, the term *nonAA arteries* was used). Decidua basalis tissue samples with ≥ 1 *AA artery* were termed *AA samples* (term used in Papers I-II and throughout the thesis)*;* tissue samples containing only

non-AA arteries were termed *non-AA samples* (term used in Paper II and throughout the thesis; in Paper I, the term *nonAA samples* was used).

3.5.3 Fibrinoid characterization

Spiral artery mural fibrinoid was assessed and characterized using HE, PAS, and MSB. Fibrinoid was identified on HE and PAS, and presence was graded by visually estimating the proportion of fibrinoid in the artery wall circumference ranging from no fibrinoid, < 25%, 25-49%, 50-74%, to ≥ 75%, as in our group's previous publication on acute atherosis (44). Mural fibrinoid was categorized using PAS as normal/physiological (bright purple or magenta stain, appearing grainy or with a heterogeneous texture, typically with embedded trophoblasts) or fibrinoid necrosis (pale pink, sometimes with a grey or blue shade, appearing glassy or homogeneous) (93). In Paper I, MSB was used to assess which color of the trichrome stain (blue, red, or yellow) the mural fibrinoid adopted.

3.5.4 Assessment of spiral artery endothelium (Paper I)

In Paper I, spiral artery endothelium was described according to morphology and activation status. Endothelial cells were identified using CD31 and vWF (148). Endothelial cell morphology was categorized qualitatively as "normal" (flattened endothelial cells lining the artery wall), "abnormal" (endothelial cells that were swollen or more irregular in shape compared to normal, flat endothelium), or "destroyed" (disrupted endothelial cell layer, often detached from the artery wall, or artery walls partly lacking endothelial lining). If a spiral artery showed signs of both normal and abnormal endothelial cell morphology, the endothelium was classified as abnormal if > 25% of the endothelium was abnormal. Veins, capillaries, and arteries measuring < 140 µm were used as internal positive controls for CD31 and vWF.

Additionally, the intensity of CD31 positivity in endothelial cells was measured. CD31 slides were scanned as described in section 3.5 ("Decidua basalis tissue evaluation"). The endothelial cell layer was outlined and representative sections of "low" and "high" CD31 intensity were indicated manually in Zen Blue 2.6, after which the total CD31 positive (endothelial) area as well as areas of "high" and "low" CD31 staining intensity were calculated by the software.

Endothelial activation was defined according to a definition used in a previous publication on endothelium in atherosclerotic plaques as ≥ 3 adjacent ICAM-1-positive endothelial cells (144).

We initially planned on using vascular cell adhesion molecule 1 (VCAM-1) and E-selectin to identify endothelial activation in addition to ICAM-1 as all three adhesion molecules had previously been used to identify endothelial activation in atherosclerotic endothelium (86). Unfortunately, we were unsuccessful in achieving a satisfying staining quality using the VCAM-1 (clone 1.4C3, Thermo Fisher Scientific) and E-selectin (clone BBIGE4, R&D Solutions) stains we acquired, and we only went forward with ICAM-1.

3.5.5 Assessment of lipid-handling proteins in foam cells and decidua basalis (Paper II)

Foam cells, identified on HE and CD68 slides, were manually marked and counted in Zen Blue 2.6. We counted the number of foam cells per lesion by counting foam cell nuclei. Co-localization of FABP4, perilipin-2, and LOX-1 was assessed for each identified foam cell.

Expression of FABP4, perilipin-2, and LOX-1 surrounding the spiral arteries (i.e. perivascularly) was also assessed. We defined "perivascularly" as a perimeter extending 100 µm from the outer artery wall (Figure 3.2) (avoiding other vascular structures or decidual glands), as previously described by our

group (54). Perivascular expression of FABP4, perilipin-2, and LOX-1 was categorized as "no stain" (no immunopositive cells), "scattered" (single immunopositive cells, but seemingly without relation to one another), "focal" (clusters of ≥ 5 immunopositive cells), and "generalized" (large areas of immunopositive cells) (Table 3.3).

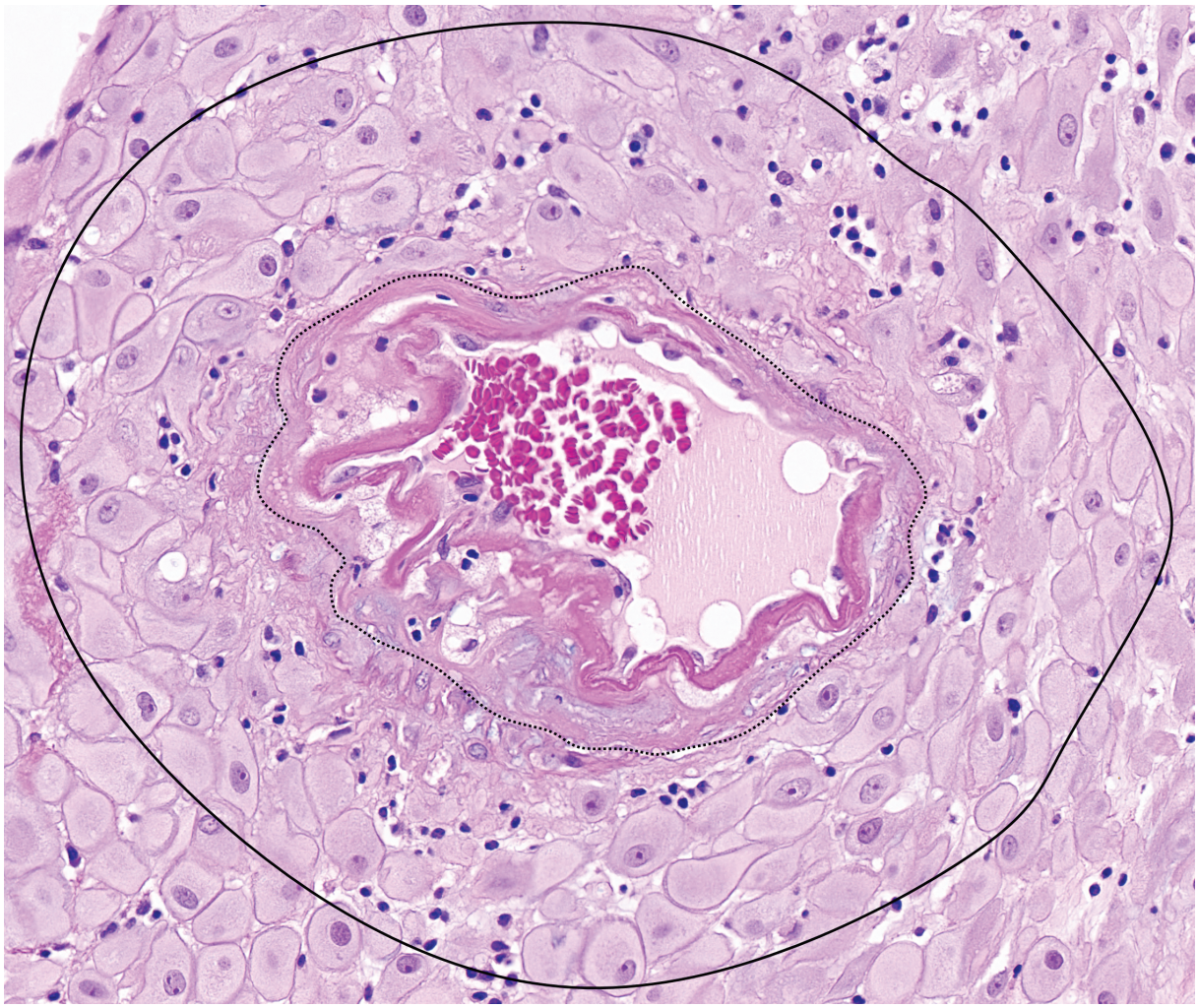

Figure 3.2. Perivascular area surrounding an *AA artery*. The perivascular area was predefined as the area extending up to 100 µm (solid line) from the outer artery wall (dotted line) (54). Photo by Ingrid K. Fosheim.

3.6 Maternal blood sampling

For OPB participants contributing with blood samples during pregnancy (Paper III), blood was sampled from an antecubital vein into ethylenediaminetetraacetic acid (EDTA) and serum gel tubes. For participants contributing with blood sampled immediately prior to caesarean section, blood was obtained from an antecubital vein or an intravenous cannula (prior to flushing or administration of fluids through the cannula) into EDTA and serum gel tubes. For 20 participants, blood was collected from an arterial catheter. EDTA blood samples were immediately placed on ice, and centrifuged at 1800*g* for 10 min at 4 °C within two hours after sampling. EDTA plasma was then isolated and aliquoted into 0.5 mL Matrix storage vials (Thermo Scientific, USA) before storage at -80 °C until thawing and immunoassay. Serum gel tubes were kept at room temperature for 30-60 min after sampling and then centrifuged at 1800*g* for 10 min at room temperature. Serum was aliquoted and stored as described for plasma samples.

3.7 Blood sample analyses

EDTA plasma samples were analyzed for concentrations of serum amyloid A1 (SAA1) and pregnancy zone protein (PZP) in duplicates using indirect sandwich enzyme-linked immunosorbent assay (ELISA). Reagents were obtained from R&D Systems, USA; catalogue number for SAA1: DY3019-05, for PZP: DY8280-05. All assays were performed according to the manufacturer's instructions (detailed in Appendix 3); 96-well microtiter plates were coated and incubated with a capture antibody and blocked as plate preparation. Plates were then incubated with samples or standards and washed. Plates were incubated with a biotinylated detection antibody and washed, incubated with streptavidin-HRP and washed, then incubated with a chromophore substrate (TMB, tetramethylbenzidine), before the enzymatic reaction was stopped with sulfuric

acid. Optical density was determined at 450 nm and corrected at 540 nm. Coefficients of variation were 6.4% for SAA1 and 2.4% for PZP.

Serum concentration of soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) were available for 547 of the included 549 pregnancies, and had been measured as previously described (149); using Elecsys® immunoassays (Roche Diagnostics, Rotkreuz, Switzerland) utilizing Cobas E analyzer, a fully automated electrochemiluminescence immunoassay platform (Roche Diagnostics). Different modules were used for analyses; 306 samples were analyzed using module E170, 186 using module 601, and 55 using module 801. sFlt-1 and PlGF results were comparable across the different modules used (data not published).

Serum levels of high-sensitivity C-reactive protein (hsCRP) were available for 380 pregnancies, and had been measured in either fresh (n = 219) or thawed (n = 161) samples in accordance with clinical routine at the Department of Clinical Biochemistry, Oslo University Hospital, using a particle-enhanced turbidimetric method (Cobas 8000 c702, Roche Diagnostics), as described previously (74).

3.8 Statistical analyses

Statistical analyses in all three papers were performed using SPSS Statistics (IBM, USA). For Papers I-II: version 25, for Paper III: version 26.

For Papers I-II, Fisher's exact test was used for comparison of categorical data, expected cell count < 5. For Paper III, Chi-square test was used for categorical data; Fisher's exact test was used for analyses with expected cell count < 5. For all three papers, non-parametric Mann-Whitney U test was used for continuous data. For Paper III, Spearman's rank correlation test (r_S) was used to analyze correlations between continuous variables. A probability (p value) < 0.05 was

considered statistically significant. The statistical analyses for Paper I were performed by postdoc/researcher Gro L. Størvold. The analyses for Papers II-III were performed by the candidate.

The main focus of Papers I-II was the acute atherosis lesion, its morphology, and its molecular characteristics. For this reason, spiral arteries were primarily analyzed artery by artery (comparing *non-AA arteries* to *AA arteries*) instead of tissue sample by tissue sample in Paper I. Foam cell expression of lipid-handling proteins in Paper II were analyzed both *AA artery* by *AA artery* and cell by cell.

4. SUMMARY OF RESULTS

Figure 4.1 presents a graphical overview of the results in Papers I-III.

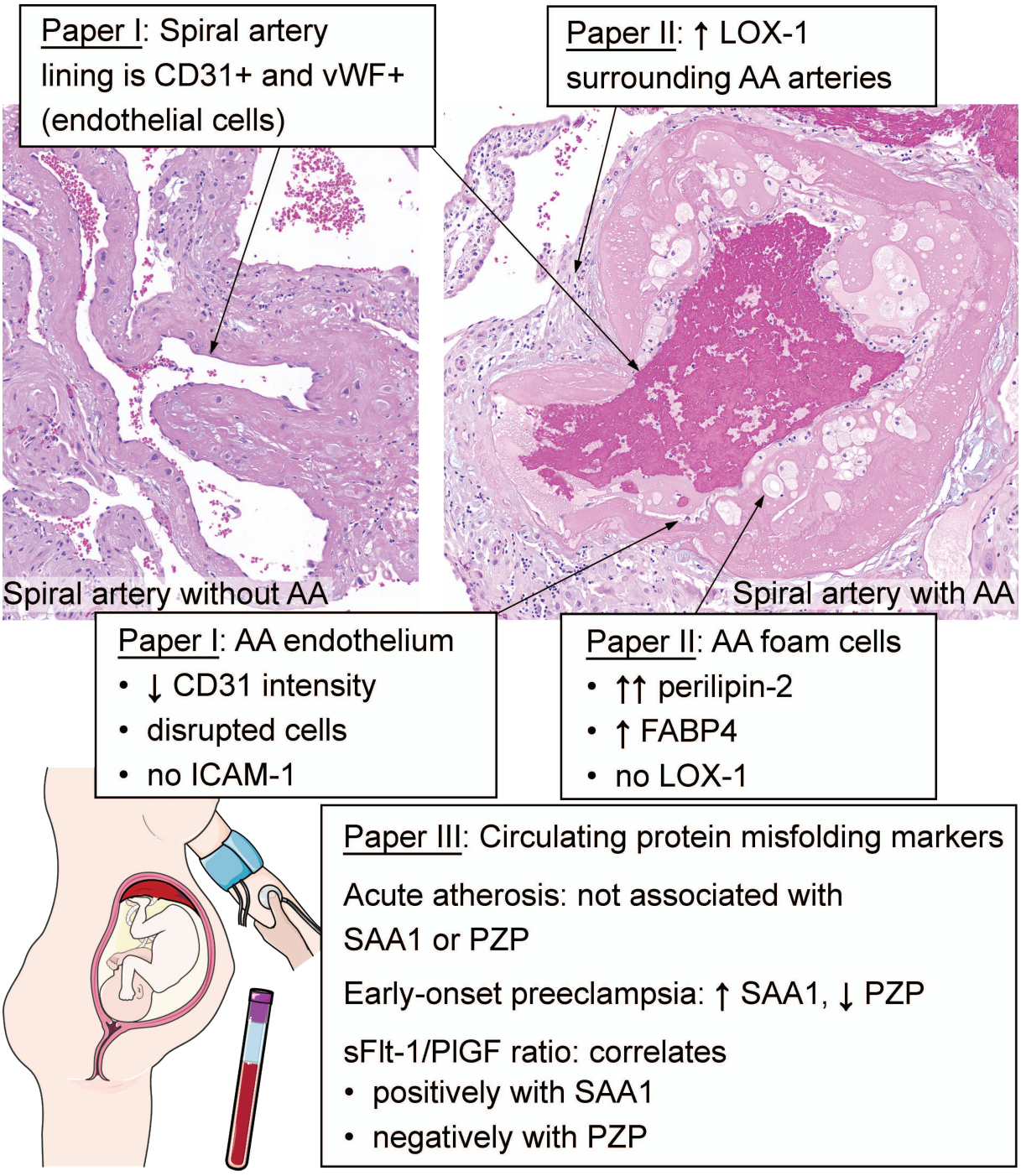

Figure 4.1. Summary of the main results of Papers I-III. AA: acute atherosis. vWF: von Willebrand factor. ICAM-1: intercellular adhesion molecule 1. FABP4: fatty acid binding protein 4. LOX-1: lectin-like oxidized LDL receptor 1. SAA1: serum amyloid A1. PZP: pregnancy zone protein. Spiral artery photos by Ingrid K. Fosheim. Created in part using Servier Medical Art.

4.1 Spiral artery morphology (Papers I-II)

Papers I-II describe decidua basalis spiral artery morphology and presence or absence of spiral artery acute atherosis.

In Paper I, 32 decidua basalis samples were evaluated; 12 with acute atherosis present (*AA samples*; 7 from preeclamptic pregnancies and 5 from uncomplicated, normotensive pregnancies), and 20 without acute atherosis (*non-AA samples*; 9 from preeclamptic pregnancies and 11 from uncomplicated pregnancies). In Paper II, a total of 35 decidua basalis samples were evaluated; 20 *AA samples* (16 preeclampsia, 4 uncomplicated, normotensive pregnancies) and 15 *non-AA samples* (7 preeclampsia, 8 uncomplicated, normotensive pregnancies).

In Paper I, the median number of spiral artery sections per decidua basalis tissue sample was 3 (range 1-10), similar to the median number of spiral artery sections in Paper II (4, range 1-16). There were no significant differences in number of observed spiral artery sections per tissue sample between the normotensive and preeclampsia groups in Paper I or Paper II. Similarly, there were no differences in number of spiral arteries when comparing *non-AA samples* to *AA samples*, neither in Paper I nor in Paper II (Table 1 in Paper I and Supplemental Table 1 in Paper II). All observed spiral artery sections had artery wall periodic acid-Schiff (PAS)-positive fibrinoid. Fibrinoid was further classified into "physiological fibrinoid" (mostly found in arteries without acute atherosis; *non-AA arteries*) or "fibrinoid necrosis" (mostly found in arteries with acute atherosis; *AA arteries*), further detailed in section 4.1.2 ("Acute atherosis morphology"), defined in Table 3.3.

Upon Martius Scarlet Blue (MSB) staining, physiological fibrinoid stained blue, while fibrinoid necrosis stained red (Figure 1 in Paper I).

4.1.1 *AA samples* have fewer completely transformed spiral arteries (Paper I)

Complete and partial physiological transformation (types of *non-AA arteries*) of decidua basalis spiral arteries are defined in Table 3.3. Spiral arteries with complete physiological transformation (intramural trophoblasts, physiological fibrinoid, absence of mural smooth muscle cells) were observed in tissue samples from preeclamptic as well as normotensive pregnancies, and there was no significant difference in number of spiral arteries with complete physiological transformation between the groups (Table 1 in Paper I). Spiral arteries with complete physiological transformation were observed in both *non-AA samples* and *AA samples*, and the fraction of spiral arteries with complete physiological transformation was significantly lower in *AA samples* compared to *non-AA samples* within the normotensive and preeclampsia group (33% vs. 100%, $p < 0.05$ both for the normotensive and the preeclampsia group; Table 1 in Paper I). We also observed some partially transformed spiral arteries, although less frequently and with no significant differences between the study groups. Non-transformed decidua basalis spiral arteries, defined as arteries with a diameter ≥ 140 µm having an intact desmin-positive vascular smooth muscle layer and lacking PAS-positive mural fibrinoid (Table 3.3), were not observed. For Paper II, we did not stain decidua basalis tissue sections with desmin; hence degree of physiological spiral artery transformation was not assessed.

4.1.2 Acute atherosis morphology (Papers I-II)

All *AA arteries* contained, as predefined by us, ≥ 2 adjacent vacuolated and CD68-positive (foam) cells. All *AA arteries* assessed in Paper I had intramural fibrinoid necrosis (homogeneous, pale pink PAS stain, Table 3.3) in part of or in the entire artery wall circumference. In Paper II, we observed six *AA arteries* (10.9%) that did not have fibrinoid necrosis, but instead had varying amounts of normal/physiological fibrinoid (purple or magenta PAS stain, Table 3.3, data not

published). Almost all *AA arteries* were absent of CK7-positive intramural trophoblasts. To our interest, three *AA samples* in Paper I had one *AA artery* each that displayed a combination of foam cells (thereby defining the spiral artery section as containing acute atherosis), fibrinoid necrosis in part of the artery wall, and physiological fibrinoid with embedded CK7-positive trophoblasts. The physiological fibrinoid was found as an outer layer covering the foam cell lesion and fibrinoid necrosis (representative artery shown in Figure 1d in Paper I), or found in another part of the artery wall (without foam cells and fibrinoid necrosis). We also observed this combination of features in eight *AA arteries* distributed across five *AA samples* in Paper II (data not published).

4.2 Endothelial cells in third trimester decidua basalis spiral arteries (Paper I)

Paper I also describes endothelial cell morphology and presence of endothelial activation in decidua basalis spiral arteries.

4.2.1 Spiral arteries at term are lined by endothelial cells, not by CK7-positive trophoblasts

We observed endothelial cells (positive for CD31 and von Willebrand factor (vWF)) lining all decidua basalis spiral arteries. In some samples, we observed CK7-positive trophoblasts within the spiral artery lumen (data not published), but we never observed trophoblasts lining the spiral artery wall.

4.2.2 "Normal" endothelium is significantly more common in *non-AA arteries*

The endothelium of each spiral artery section was categorized as appearing "normal", "abnormal", or "destroyed", as defined in Table 3.3. Representative examples are shown in Figure 2 in Paper I. There were no significant differences in distribution between the endothelial phenotypes when comparing fully and partially transformed spiral arteries. For this reason, fully and partially transformed arteries were merged into the "*non-AA arteries*" category (the term *nonAA arteries* was used in the publication) for subsequent analyses (Supplementary Table 2 in Paper I). Nor did we observe any such differences when comparing the normotensive and preeclampsia groups (Supplementary Table 2 in Paper I). There were no differences in the frequencies of the "normal", "abnormal", and "destroyed" endothelial phenotypes in *non-AA arteries* when comparing *AA samples* to *non-AA samples* (Figure 3a in Paper I). "Normal" endothelium was significantly more common in *non-AA arteries*, both in *non-AA arteries* from *non-AA samples* (45%) and *non-AA arteries* from *AA samples* (61%), compared to *AA arteries* (17%, $p \leq 0.02$ for both comparisons, Figure 3a in Paper I).

4.2.3 *AA arteries* are associated with an altered endothelial phenotype

AA arteries significantly more often displayed a "destroyed" endothelium compared to *non-AA arteries* in *non-AA samples* (33% vs. 7%, $p = 0.004$) and *non-AA arteries* in *AA samples* (33% vs. 3%, $p = 0.007$, Figure 3a in Paper I). Further, the endothelium of *AA arteries* showed significantly lower intensity of CD31 expression than *non-AA arteries* in *non-AA samples* ($p < 0.001$) and *non-AA arteries* in *AA samples* ($p = 0.03$) within the "abnormal" endothelial phenotype (Figure 3b in Paper I).

4.2.4 Acute atherosis is not associated with endothelial activation

Nearly all decidua basalis spiral artery sections had single, scattered endothelial cells positive for intercellular adhesion molecule 1 (ICAM-1). This was not considered "endothelial activation" by us, as we for this categorization used the definition of ≥ 3 adjacent ICAM-1-positive endothelial cells (Table 3.3) (144). We chose not to assess spiral arteries with a "destroyed" endothelial phenotype for endothelial activation, as the endothelial cells in these arteries were disintegrated. Thus, only spiral arteries with a "normal" or "abnormal" endothelial phenotype were assessed for endothelial activation. Within the endothelial phenotypes, there were no significant differences in frequency of endothelial activation when comparing *non-AA arteries* to *AA arteries* (Figure 3c in Paper I). There was no difference in frequency of endothelial activation when comparing *non-AA arteries* to *AA arteries*, regardless of endothelial phenotype (23% vs. 44%, p = 0.12, Supplementary Table 3 in Paper I).

When comparing samples from preeclamptic pregnancies to uncomplicated, normotensive pregnancies, there were no differences in how frequently neither *non-AA arteries* nor *AA arteries* showed endothelial activation (Supplementary Table 3 in Paper I).

4.3 Lipid-handling proteins in and around acute atherosis lesions (Paper II)

Paper II describes foam cells of acute atherosis lesions and their expression of atherosclerosis-related lipid-handling proteins, as well as expression of these proteins in decidua basalis tissue surrounding spiral arteries.

We found that the median number of foam cells per *AA artery* wall was 4 foam cells in the normotensive group and 16 foam cells in the preeclampsia group, but this difference was not statistically significant (p = 0.6).

4.3.1 Perilipin-2 is abundantly expressed in acute atherosis lesions

Perilipin-2, associated with foam cell formation in atherosclerosis, was expressed in foam cells in nearly all *AA arteries* (93%), and in 55% of all observed foam cells. Fatty acid binding protein 4 (FABP4) was found in foam cells in roughly one third of *AA arteries* (36%), but in only 13% of all foam cells (Figure 2 in Paper II). Perilipin-2 was significantly more frequently detected in *AA arteries* than was FABP4 ($p < 0.001$). We did not observe lectin-like oxidized LDL receptor 1 (LOX-1) expression in acute atherosis foam cells, nor in other cell types in the *AA artery* wall. We observed LOX-1 stain in the cell membrane of decidua basalis stromal cells (Figure 1 a6, b6, and c6 in Paper II). This was in line with the manufacturer's report of decidual cells in placental tissue as being positive control for LOX-1 (membrane stain expected), and with the observed positive membrane stain of decidual cells in our positive control tissue (placenta). LOX-1 was occasionally observed in intramural trophoblasts and endothelial cells in *non-AA arteries*.

We did not find any significant differences between the normotensive and preeclampsia groups when comparing the frequency of perilipin-2 ($p = 0.76$) or FABP4 ($p = 0.37$) expression in foam cells for each *AA artery*. Perilipin-2 and FABP4 were rarely expressed by cell types other than foam cells in *AA arteries*.

As detailed in section 3.4.3 ("Histochemical and immunohistochemical staining for Paper II"), we stained aorta atherosclerosis samples (one early and one advanced lesion) for methodological comparison of subcellular location of FABP4, perilipin-2, and LOX-1. In CD68-positive foam cells, FABP4 was located to cytoplasmic granules, while perilipin-2 was located to intracellular lipid droplets. Foam cells did not label for LOX-1 (Methods section 2.3 in Paper II).

4.3.2 LOX-1 and perilipin-2 are frequently expressed surrounding *AA arteries*

The three assessed lipid-handling proteins in Paper II were also assessed in the perivascular area (up to 100 µm from the outer artery wall, Figure 3.2) of both *AA arteries* and *non-AA arteries*. Expression of these three proteins was observed in three distinct patterns: "scattered", "focal", and "generalized", as defined in Table 3.3. Representative examples are shown in Figure 1 in Paper II. LOX-1 was often observed in the "generalized" pattern surrounding *AA arteries*, and significantly more frequently than around *non-AA arteries* (38% vs. 15%, $p < 0.001$). Perilipin-2 was also significantly more often expressed in the perivascular area of *AA arteries* compared to *non-AA arteries* (59% vs. 32%, $p < 0.001$). In contrast, FABP4 was more frequently expressed surrounding *non-AA arteries* compared to *AA arteries* (70% vs. 53%, $p = 0.03$).

Perilipin-2 and LOX-1 were often expressed around the same *AA arteries*, though not necessarily by the same perivascular cells. Both proteins were most commonly found in decidual stromal cells: perilipin-2 in intracellular lipid droplets (number of positive droplets was not quantified, but varied between the stromal cells), and LOX-1 in the cell membrane. LOX-1 was occasionally found in interstitial CK7-positive trophoblasts.

There were no significant differences in how often *AA arteries* and *non-AA arteries* displayed the different staining patterns ("scattered", "focal", and "generalized") for each of FABP4, perilipin-2, and LOX-1, in the perivascular area (Supplemental Figure 1 in Paper II). Similarly, there were no differences in perivascular staining patterns for FABP4, perilipin-2, or LOX-1 when comparing the normotensive and preeclampsia groups (data not published).

4.4 Protein misfolding markers in acute atherosis and preeclampsia (Paper III)

Paper III investigated proteins associated with atherosclerosis, protein misfolding, and hypercoagulability and their relation to acute atherosis, preeclampsia, and markers of placental dysfunction.

4.4.1 SAA1 correlates positively with some proxies for placental dysfunction, but not acute atherosis

Circulating levels of serum amyloid A1 (SAA1), which is associated with protein misfolding and cardiovascular disease outside pregnancy, was increased in women with hypertensive disorders of pregnancy (HDP, defined in Table 3.1) when compared to normotensive and euglycemic controls (9.9 vs. 5.1 µg/mL, $p < 0.001$; Table 1 in Paper III). In contrast, median SAA1 levels did not differ between women with diabetes mellitus (DM) and controls, neither for DM alone nor for DM in combination with HDP (HDP+DM) (Table 1 in Paper III).

The highest median concentration of SAA1 was found in women with early-onset preeclampsia (17.1 µg/mL, Supplemental Table 1 in Paper III). This was significantly increased compared to controls (5.1 µg/mL, $p < 0.001$, Supplemental Table 1 in Paper III), and when compared to all remaining subgroups of HDP (Figure 1a in Paper III). We found no differences in SAA1 levels when comparing the control group to late-onset preeclampsia, gestational hypertension, or chronic hypertension (Figure 1a and Supplemental Table 1 in Paper III).

We then assessed whether circulating SAA1 levels correlated with different proxies for placental dysfunction. We did not find any association between SAA1 levels and acute atherosis presence within any of the pregnancy groups (Supplemental Table 3 in Paper III). Circulating SAA1 was, however, increased in pregnancies complicated by fetal growth restriction (13.3 vs. 5.1 µg/mL,

$p < 0.001$). Further, SAA1 levels correlated positively with the antiangiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) to placental growth factor (PlGF) ratio in the total cohort ($r_S = 0.24$, $p < 0.001$) as well as in the HDP group ($r_S = 0.40$, $p < 0.001$). This was also the case for the early-onset preeclampsia group ($r_S = 0.24$, $p = 0.04$), but not for the other HDP subgroups (data not published).

SAA1 showed a positive correlation with the inflammation marker high-sensitivity C-reactive protein (hsCRP) in the total cohort ($r_S = 0.46$, $p < 0.001$) as well as in all study groups (controls: $r_S = 0.35$; HDP: $r_S = 0.58$; DM: $r_S = 0.35$; HDP+DM: $r_S = 0.62$, all $p \leq 0.04$). SAA1 was also significantly increased in pregnancies with hsCRP above the upper reference range (4 mg/L); 9.4 vs. 4.3 µg/mL ($p < 0.001$). Similarly, hsCRP was significantly increased in the HDP group compared to controls (4.4 vs. 3.1 mg/L, $p = 0.002$, Table 1 in Paper III). Of the HDP subgroups, hsCRP was increased in early-onset preeclampsia compared to controls (6.8 vs. 3.1 mg/L, $p < 0.001$), but not in any other HDP subgroup compared to controls (Supplemental Table 1 in Paper III).

SAA1 and pregnancy zone protein (PZP) did not correlate in the total cohort or in any of the study groups, except for HDP+DM ($r_S = -0.37$, $p = 0.01$). Neither levels of SAA1 nor levels of PZP were affected by fetal sex (Supplemental Results in Paper III).

4.4.2 PZP is decreased in early-onset preeclampsia and correlates negatively with sFlt-1/PlGF

Circulating levels of PZP, which may act as a chaperone and prevent protein misfolding, did not differ significantly between the HDP group and controls (Table 1 in Paper III). However, PZP levels were lower both in the DM group and the HDP+DM group compared to controls (DM: 683 µg/mL,

HDP+DM: 548 µg/mL, controls: 892 µg/mL, both comparisons p = 0.01, Table 1 in Paper III).

Although the HDP group as a whole did not display any difference in circulating PZP levels when compared to controls, PZP levels were significantly decreased in early-onset preeclampsia when compared to the control group, (590 vs. 892 µg/mL, p = 0.002, Supplemental Table 1 in Paper III), and when compared to women with chronic hypertension (590 vs. 1302 µg/mL, p = 0.04, Figure 1b in Paper III). The other HDP subgroups did not differ in PZP levels compared to controls (Supplemental Table 1 in Paper III).

Further, PZP was lower in pregnancies with pregestational DM compared to controls (controls: 892 µg/mL; DM type 1: 683 µg/mL, p = 0.02; DM type 2: 580 µg/mL, p = 0.003, Supplemental Table 2 in Paper III). In contrast, PZP in pregnancies complicated by gestational DM did not differ from the control group.

PZP correlated negatively with antiangiogenic sFlt-1/PlGF ratio in the total cohort (r_S = -0.13, p = 0.002) as well as in the HDP group (r_S = -0.19, p = 0.02) and the HDP+DM group (r_S = -0.30, p = 0.049). PZP did not correlate with sFlt-1/PlGF within any of the HDP subgroups (data not published). PZP did, however, correlate positively with PlGF in early-onset preeclampsia (r_S = 0.24, p = 0.04), but not in any other HDP subgroup (data not published). Of the DM subgroups, PZP correlated with sFlt-1/PlGF only in DM type 1 (r_S = -0.29, p = 0.02), not in DM type 2 or gestational DM (data not published). PZP levels were not different when comparing pregnancies with and without acute atherosis within each pregnancy group (Supplemental Table 3 in Paper III). PZP did not correlate with hsCRP (data not published), in contrast to SAA1 (as presented above).

5. DISCUSSION

5.1 Methodological discussion

5.1.1 Study population

Women scheduled to deliver at Oslo University Hospital (OUS), location Ullevål, are invited to participate in the Oslo Pregnancy Biobank (OPB) study. OUS has two delivery locations and is the local obstetric hospital for most women living in Oslo, Norway. OUS is also a secondary and tertiary center for many women in South-Eastern Norway with fetal or maternal complications during pregnancy requiring intensified obstetric care. Thus, both healthy women with clinically uncomplicated pregnancies as well as women with underlying diseases and/or pregnancy complications are delivered at the hospital, and available for invitation to participate in the OPB.

Only women who can communicate in Norwegian and/or English are eligible for recruitment to the OPB, as a requirement for recruitment is ability to understand written and oral information from study personnel. OPB has not produced study information material in more languages and has no funding for interpreters, disabling recruitment of women who do not understand Norwegian or English, often applying to newly immigrated women. As a consequence, pregnant women with non-white ethnicities are under-represented in the OPB compared to the general Oslo population (unpublished data). Preeclampsia is more prevalent in non-whites (33), introducing a selection bias to our biobank. The same bias may also apply to decidual acute atherosis, as this lesion is more prevalent in preeclampsia than in uncomplicated pregnancies (42, 44), though few studies have assessed acute atherosis in relation to ethnicity (49).

While there is potential selection bias in the recruitment process, sampling of biological material and clinical data is standardized through predefined

protocols. This ensures that samples harvested by different study personnel or in different years are still comparable.

Due to restricted resources, the recruited women also represent a convenience population, as personnel (mostly PhD candidates or medical students in a Medical Student Research Program) were not available for recruitment every day. Recruitment was also often limited outside daytime Monday-Friday due to the same resource restriction. We do however believe that this convenience recruitment did not induce a systematic population bias within the clinical study groups.

5.1.2 Study design of Papers I-III

The case control design in Papers I-II, detailed in section 3.2 ("Study design"), allowed for us to study molecular features of decidua basalis spiral arteries with acute atherosis compared to decidua basalis spiral arteries without acute atherosis. As there were pregnancies with and without preeclampsia in both the case (acute atherosis present) and control (acute atherosis absent) groups, we were able to further discern whether any of the features studied and observed were primarily associated with preeclampsia or with acute atherosis. We are, through these studies, unable to determine whether the reported molecular characteristics associated with acute atherosis lead to or are a consequence of acute atherosis. Longitudinal data are required to accurately determine cause and effect. Acute atherosis is currently impossible to study longitudinally, as all available methods of harvesting enough tissue of decidua basalis require delivery of the placenta (40, 93), making repeated decidual biopsies within the same pregnancy impossible.

The cross-sectional design of Paper III, detailed in section 3.2 ("Study design"), allowed for identification of possible relationships between acute atherosis, preeclampsia, placental dysfunction, and the protein misfolding markers serum

amyloid A1 (SAA1) and pregnancy zone protein (PZP). We had available longitudinal blood samples from only 4 pregnancies complicated by hypertensive disorders of pregnancy (HDP) included in Paper III. We chose not to include the longitudinal results in Paper III or this thesis as the data were limited in number (n = 4), diagnosis (only HDP pregnancies), and short time from first to second sample (1-11 days). Similar to Papers I-II, the study design of Paper III is not suitable for evaluation of causality between circulating protein misfolding markers and preeclampsia or acute atherosis.

5.1.3 Clinical diagnosis criteria

Types of HDP and diagnostic criteria used in this thesis are summarized in Table 3.1. Papers I-II include pregnancies with preeclampsia and uncomplicated normotensive pregnancies. All pregnancies included in Papers I-II were euglycemic throughout pregnancy. Paper III includes pregnancies with any type of HDP, diabetes mellitus (DM, pregestational or gestational), and pregnancies with both HDP and DM (HDP+DM), as well as uncomplicated normotensive and euglycemic pregnancies.

Although Papers I-II were published after the 2018 International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (32) were published, we chose to use the "classic" preeclampsia diagnostic criteria (new-onset hypertension and proteinuria after 20 weeks' gestation), as listed by the 2003 National Heart, Lung, and Blood Institute (NHLBI) (132), as these were the criteria used worldwide at the time of patient recruitment to our study. The clinical diagnosis (e.g. preeclampsia) of each participant included to OPB is recorded by the OPB recruiter at time of recruitment and reevaluated after delivery. A clinical HDP diagnosis prior to the recruitment is based on the working diagnosis set in the clinic, and as such, it is not always a final diagnosis. To reduce misdiagnosis, the laboratory and medical chart information collected

on all study participants later undergo comprehensive review by the OPB Diagnosis Revisory Group, consisting of experienced obstetricians and translational researchers, ensuring that the final diagnosis used in our analyses and publications is in line with the chosen guideline. Our publication diagnoses are therefore based on comprehensive revision of all singular and documented elements that feed into the final HDP diagnosis.

Papers I-II did not include any OPB study participants with gestational hypertension. Two participants in Paper II had chronic hypertension and new-onset proteinuria after 20 weeks' gestation, thus classifying as superimposed preeclampsia. Because Papers I-II did not include pregnancies complicated by gestational hypertension or chronic hypertension (without superimposed preeclampsia), we avoided the risk of "missing" a preeclampsia diagnosis per the 2018 ISSHP criteria. All participants in Papers I-II classified as having preeclampsia or superimposed preeclampsia per the 2003 NHLBI criteria would therefore have been diagnosed similarly had we applied the 2018 ISSHP criteria.

Between the publication of Paper II and Paper III, the OPB undertook an extensive review of the medical charts of each study participant, and gathered even more detailed unique data to facilitate diagnosing HDP according to the 2018 ISSHP criteria, which are summarized in Table 3.1. The HDP diagnosis was then determined using a syntax-based model in SPSS. In a few cases where the syntax was unable to generate a diagnosis, such as conflicting data input or insufficient data, the clinical data were reviewed and a diagnosis was set by two PhD candidates and the principal investigator (Professor Staff). Thus, the criteria for HDP differ slightly between Papers I-II and Paper III. The consequence of utilizing different criteria is likely minimal, although some participants with preeclampsia in Paper III would have been classified as having gestational hypertension if 2003 NHLBI criteria were used, as they lacked documented

presence of new-onset proteinuria, but had other new-onset preeclampsia-associated features, such as elevated liver transaminases, fetal growth restriction, acute kidney injury, hematological complications, and so on (Table 3.1).

5.1.4 Collection and preparation of decidua basalis tissue samples

Decidua basalis tissue was harvested by vacuum suction of the placental bed following delivery of the fetus and placenta at caesarean delivery. The technique, termed the Decidual Suction Method (DSM), was developed by Staff et al. (77, 141) in 1997 (published in 1999), and its clinical safety as well as efficacy in providing biopsies with decidua basalis spiral arteries was later assessed by Harsem et al. (142). The DSM technique has a higher success rate of sampling decidua basalis spiral arteries and in detecting acute atherosis compared to other frequently used biopsy techniques, including placenta basal plate biopsies, membrane roll biopsies, and placental bed biopsies (40, 142).

Following vacuum suction and flushing of the harvested tissue, the OPB recruiter places the tissue sampling box on ice, and assesses whether the decidua basalis tissue appears to be contaminated by other tissues, such as membranes, placental villous tissue, or myometrial tissue. Decidua basalis tissue macroscopically is near transparent compared to the brick red villous placental tissue. The harvested decidua basalis is portioned by the OPB recruiter and approximately equal portions are either fixated in formalin or snap-frozen in liquid nitrogen. This tissue aliquotation is done at random, and poses the possibility of loss of acute atherosis detection, as we generally use FFPE samples and not frozen samples to diagnose acute atherosis. A few of our frozen samples have been used for immunohistochemistry (including acute atherosis diagnosis), but the majority of these samples have been used for decidua basalis gene expression analyses. Acute atherosis is a focal lesion, and in theory it is possible that in some biopsies all spiral arteries with acute atherosis lesions were

portioned to snap freezing and not FFPE, or vice versa. Such an instance could affect the outcome of acute atherosis frequency reported in Paper III, however it likely did not, as the acute atherosis frequency we found (39% for HDP pregnancies, 9% for uncomplicated normotensive pregnancies) is comparable to previous decidua basalis studies (42, 44). Over- or under-representation of acute atherosis frequency due to tissue aliquotation would not have affected the outcome of Papers I-II, as OPB participant selection for these papers was based in part on existing acute atherosis diagnosis from previous studies (44), allowing us to purposefully over-represent acute atherosis in both the normotensive and preeclampsia groups to study the molecular biology of the lesion and not its frequency.

While the DSM technique provides high success rates of sampling spiral arteries, as discussed above, tissue orientation is lost during vacuum suction. We are generally able to follow the (assumed) same spiral artery across several serial DSM tissue sections, but due to lack of orientation, we cannot make statements regarding which end of the spiral artery is closer to the placenta or myometrium. Consequently, we cannot assess what is upstream or downstream from any spiral artery pathology we may observe (acute atherosis, thrombi, etc.) within the same artery. For the same reason, we are unable to comment on whether any given part of the biopsy is from the central or peripheral part of the placental bed.

By definition, traditional placental bed biopsies should contain myometrial tissue (93) and tissue orientation is kept, making these biopsies superior to DSM biopsies for studying deeper placentation problems and insufficient spiral artery transformation in the myometrium. However, the highest rates of acute atherosis are found in the decidual portion of the uteroplacental spiral arteries (39). Thus, DSM sampling of decidua basalis tissue is superior in decidual spiral artery evaluation and the major tissue location of acute atherosis lesions. In addition,

the placental bed biopsy procedure is more invasive and the success rate of sampling spiral arteries is lower than for the DSM technique, due to less tissue volume being collected (142).

The DSM technique, like the placental bed biopsy technique, can only be performed at caesarean section. This of course limits the number of women available for obtaining DSM biopsies, as the majority of women (with or without pregnancies complicated by HDP) in Norway deliver vaginally (150), but it does not affect the outcome of this thesis, as study participants for the papers in this thesis were selected from the OPB based on availability of previously harvested biological samples. For vaginally delivered placentae, placental basal plate biopsies or membrane roll biopsies may be collected, but offer a lower success rate in sampling spiral arteries and detecting acute atherosis, repeatedly demonstrated by our group (40, 142).

5.1.5 Staining and evaluation of decidua basalis tissue samples

All tissue samples used in this thesis are FFPE, a method well suited for providing long-lasting tissue blocks. However, FFPE preparation limits usable staining methods. During deparaffinization, all lipids, intra- and extracellular, are removed from the tissue. Thus, it is impossible to study lipids directly (e.g. stain with Oil Red O or Sudan Black B) in FFPE tissue. Frozen tissue samples are better suited for such studies. This limits us from identifying foam cells by their lipid content in our sampled decidual tissue; we have instead chosen immunohistochemical staining of the scavenger receptor CD68 to detect foam cells. CD68 also labels (non-vacuolated) macrophages, monocytes, and fibroblasts (151), which is why we only consider CD68-positive cells that are also vacuolated to be foam cells (Table 3.3). Lipid-loss through deparaffinization also affected the selection of stains for Paper II. As FFPE

tissue does not permit direct assessment of the lipids associated with foam cells and acute atherosis, we instead studied lipid-associated proteins.

5.1.5.1 Defining the degree of spiral artery transformation (Paper I)

Spiral arteries with insufficient physiological transformation retain some or all of their vascular smooth muscle cell layer (93), so we chose to define non-transformed and partially transformed spiral arteries accordingly (Table 3.3). For accurate identification of retained vascular smooth muscle cells in the spiral artery walls, we used desmin immunohistochemistry staining instead of relying on identification of smooth muscle cells by morphology alone (e.g. by using hematoxylin-eosin (HE) stained slides). Thus, we were only able to comment on degree of spiral artery transformation in Paper I, as we did not stain decidua basalis tissue sections with desmin for Paper II.

5.1.5.2 Identifying acute atherosis lesions and fibrinoid necrosis (Papers I-II)

Papers I-III use our evidence-based research definition of acute atherosis (Table 3.3) as this has proven a reliable definition with low inter-observer variability (44). The definition does not require fibrinoid necrosis or perivascular inflammatory infiltrates for a diagnosis of acute atherosis, although fibrinoid necrosis was frequently, though not always, observed in spiral arteries with acute atherosis in Papers I-II. Fibrinoid necrosis may be detected using HE, periodic acid-Schiff (PAS), or Martius Scarlet Blue (MSB, or other trichrome) stains. However, these are all histochemical stains and lack the specificity of immunostains. There are, at present, no uniform, specific criteria to diagnose fibrinoid necrosis, or consensus regarding the quantity of fibrinoid necrosis sufficient for acute atherosis (or decidual vasculopathy) diagnosis. For these reasons, we chose to not include fibrinoid necrosis in our acute atherosis diagnosis, but rather describe the phenomenon in Papers I-II.

5.1.5.3 Endothelial markers and endothelial activation (Paper I)

CD31, von Willebrand factor (vWF), and intercellular adhesion molecule 1 (ICAM-1) had all been tested on decidual tissue prior to our studies in Paper I (52, 92). One weakness of our investigation of endothelial activation in acute atherosis of decidua basalis spiral arteries lies in that it only utilizes one adhesion molecule (ICAM-1) to assess endothelial activation.

The method applied for measuring intensity of CD31 stain is vulnerable to bias, as several steps were carried out manually (outlining the endothelial cell layer, indicating examples of "high" and "low" intensity CD31 stain) before the Zen blue 2.6 software calculated the areas of "high" vs. "low" CD31 staining intensity. "High" intensity was checked against an internal positive control (vein or capillary), whereas "low" was subjectively determined. We did not measure staining intensity for vWF, as we did not observe differences in intensity for vWF.

Our definition of endothelial activation (≥ 3 adjacent ICAM-1-positive endothelial cells, Table 3.3), based on a previous atherosclerosis publication (144), had not previously been applied to or validated in uteroplacental spiral arteries. Paper I does not attempt at validating this definition for use in spiral arteries. Applying a specific definition attempts to reduce bias. However, we may only speculate whether our chosen definition accurately detects endothelial activation of biological or clinical significance.

5.1.5.4 Markers of lipid-handling proteins (Paper II)

In Paper II, foam cells of acute atherosis lesions were marked and counted if they were CD68-positive, vacuolated, and had a nucleus present. Each counted foam cell was then followed in the next serial slides to assess expression of fatty acid binding protein 4 (FABP4), perilipin-2, and lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX-1). Due to the nature of serial slides, not all

foam cells could be rediscovered on subsequent slides (3 µm farther into the tissue). The total number of foam cells identified is higher than the total number of FABP4- and perilipin-2-positive foam cells, respectively (no foam cells expressed LOX-1). We did not exclude foam cells that were not located on all serial slides from analysis, thus the reported fractions of FABP4- and perilipin-2-positive foam cells (13% and 55%, respectively) are likely underestimating the true prevalence.

Similarly, FABP4 and perilipin-2 (never LOX-1) were sometimes observed in cells appearing to be foam cells. These cells were not present on the HE and CD68 slides and could not be verified to be foam cells per our definition (Table 3.3), and were excluded from further analysis, possibly also contributing to underestimation of the true prevalence of FABP4- and perilipin-2-positive foam cells. We did not perform co-staining of different immunostains on our FFPE slides. It is possible that triple immunostaining with CD68, FABP4, and perilipin-2 on the same slides could have corrected the potential underestimation.

5.1.6 Collection, preparation, and storage of maternal blood samples (Paper III)

Maternal EDTA blood samples were collected and prepared in a uniform way (section 3.6 “Maternal blood sampling”), reducing variation in pre-analytical factors. However, some variation in collection method occurred, as detailed below.

A few of the blood samples were collected from arterial catheters (n = 20, of which early-onset preeclampsia: 11, late-onset preeclampsia: 7, gestational hypertension: 1, gestational DM: 1), as opposed to venous cannula or catheter, from which the majority of blood samples were collected. We have not found any reports suggesting that levels of serum amyloid A1 (SAA1) or pregnancy zone protein (PZP) would be different in arterial vs. venous blood. Levels of

SAA1 or PZP were not significantly different between arterial and venous samples in our cohort, except in the late-onset preeclampsia group, where SAA1 was increased in arterial samples (57.0 vs. 5.4 μg/mL, p = 0.02). As we did not see any significant difference in SAA1 levels when comparing arterial to venous samples in other HDP subgroups or in DM, we hypothesize that the observed difference in the late-onset preeclampsia group may be explained by a worse placental dysfunction and more severe clinical phenotype in participants with late-onset preeclampsia with arterial catheters, as this intervention is generally reserved for unstable patients requiring intensified clinical monitoring.

Not all participants were fasting at sampling time. While fasting status affects many biochemical analyses, we have been unable to find reports addressing the potential impact of non-fasting on plasma levels of SAA1 or PZP in pregnant women. Levels of SAA1 or PZP were not significantly different between fasting and non-fasting samples in our cohort (data not published).

Aliquots of plasma samples were kept at -80 °C until assay, and had been frozen for 4-20 years before assay. One study found PZP to be stable when PZP purified from plasma samples was stored at -20 or -70 °C for 2 years (152). We have not found reports assessing PZP assays from thawed vs. fresh plasma samples, or any reports assessing whether freezing affects SAA1 levels. Our reported levels of SAA1 and PZP are largely comparable to other publications from pregnancy (124, 126, 129) where samples were also frozen prior to enzyme-linked immunosorbent assay (ELISA). However, freezing time was not specified in any of these publications.

5.1.7 Analysis of maternal plasma samples (Paper III)

ELISA was performed in one single laboratory, under similar conditions, and by the same personnel. Assay coefficients of variation were 6.4% for SAA1 and 2.4% for PZP, indicating that the data are accurate and reliable.

SAA1 has been suggested to play a role in initiating parturition (120), and elevated levels may be a sign of imminent parturition. None of the participants included in this thesis had signs of labor (intact fetal membranes, no uterine contractions). We did not document whether the included participants had had a cervical examination, though we would not have any reason to suspect they might show cervical ripening, as none included had any other signs of labor. We cannot exclude that some of the included participants would have gone into spontaneous labor soon after sampling, had they not been delivered by caesarean section first. If that were the case, we assume this would be equally distributed between the study groups, as they appeared similar in terms of absent signs of labor.

Glucocorticoids are frequently used to enhance lung maturity in cases of iatrogenic or spontaneous early preterm labor (prior to 34 weeks' gestation), and while glucocorticoids generally reduce the expression of acute-phase proteins such as SAA1, direct glucocorticoid stimulation of epithelial cells has been shown to increase SAA1 expression (153), though it is not clear whether this increased tissue expression results in increased circulating SAA1 levels. Some of the participants with HDP (n = 72) received glucocorticoids (Celestone, administered intramuscularly) prior to OPB recruitment and blood sampling. SAA1 levels were increased in this group compared to participants with HDP who did not receive glucocorticoids (15.8 vs. 5.5 µg/mL, $p < 0.001$). However, we believe this difference is primarily explained by the fact that the majority of participants who received glucocorticoids (67 of 86) had early-onset preeclampsia, which was the group with the highest median SAA1 values. There was no statistically significant difference in SAA1 levels within the early-onset preeclampsia group when comparing participants who received glucocorticoids (median: 17.1 µg/mL) to those who did not (median: 27.4 µg/mL, $p = 0.92$). We

therefore believe that intramuscular glucocorticoid treatment likely did not significantly alter circulating SAA1 levels in our study.

5.1.8 Statistical analyses

We did not perform sample size estimation for Papers I-III as previous studies were mostly lacking. Papers I-III therefore represent pilot studies and are intended to explore molecular characteristics of acute atherosis and preeclampsia, not epidemiological data on acute atherosis or preeclampsia prevalence.

Sample sizes in Papers I-II were small ($n = 32$ and 35, respectively), and continuous variables did not follow normal distribution, as assessed by histograms and the Shapiro-Wilk test (data not published). For Paper III, the Shapiro-Wilk test revealed that maternal age was normally distributed, while all other continuous variables (including SAA1 and PZP levels) did not follow normal distribution (data not published). For these reasons, Mann-Whitney U test was used for continuous data in Papers I-III.

For Paper III, we did not correct for gestational age at sampling. Gestational age at sampling correlated significantly with SAA1 and PZP only in the early-onset preeclampsia group ($r_S = -0.25$ and 0.30, respectively, both $p \leq 0.04$, Supplemental Results in Paper III), believed by us to reflect a more severe clinical phenotype requiring earlier delivery. As gestational age did not correlate with SAA1 or PZP in any other groups, we do not believe gestational age to significantly affect the levels of SAA1 or PZP in our samples from the second half of pregnancy. Similarly, body mass index, early pregnancy blood pressures, or fetal sex did not correlate with SAA1 or PZP levels, except in a few instances (listed in Supplemental Results in Paper III). For this reason, we did not correct for these exposures in our analyses of SAA1 and PZP.

In Paper III, circulating biomarker concentrations (SAA1, PZP, high-sensitivity C-reactive protein (hsCRP), soluble fms-like tyrosine kinase-1 (sFlt-1), and placental growth factor (PlGF)) were not significantly different in blood samples collected during pregnancy up to 31 days prior to delivery (n = 81; for hsCRP: n = 28) compared to blood samples collected at delivery (n = 468; for hsCRP: n = 352) (data not published), and were analyzed together. Levels of hsCRP were not significantly different in fresh vs. thawed samples, except in the control group (2.8 vs. 4.7 mg/L, p = 0.003), and were analyzed together.

5.2 Results discussion

5.2.1 Are acute atherosis lesions only found in non-transformed spiral arteries?

Acute atherosis has been suggested to only occur in spiral arteries that have not undergone physiological transformation (non-transformed spiral arteries) (43, 92), while others claim acute atherosis mainly occurs downstream (in decidua basalis segments) of spiral arteries whose myometrial segments are non-transformed (16, 154). In a few decidua basalis tissue samples explored in Papers I-II, we observed acute atherosis lesions in combination with intramural trophoblasts and "physiological" artery wall fibrinoid (Table 3.3, representative spiral artery shown in Figure 1d in Paper I). This finding demonstrates that acute atherosis indeed can occur in segments of spiral arteries that have undergone physiological transformation, in contrast to some previous suggestions. We did not have access to samples from the myometrial parts of the placental bed and therefore cannot state whether the spiral arteries with decidua basalis acute atherosis had undergone physiological transformation or not in their myometrial segments.

Despite the few spiral arteries showing a combination of physiological transformation and acute atherosis, the majority of decidua basalis spiral arteries

with acute atherosis were absent of intramural CK7-positive trophoblasts and desmin-positive vascular smooth muscle cells (VSMCs) (Papers I-II). However, all acute atherosis lesions we report occurred in spiral arteries measuring ≥ 140 µm. Basal arteries and non-transformed spiral arteries are reported to measure < 100 µm (93). Thus, it is likely that all decidua basalis spiral arteries in which we observe acute atherosis lesions have undergone some degree of physiological transformation in their decidual parts despite lacking intramural trophoblasts, as reported by our group and others previously (44, 154).

Almost all decidua basalis samples with acute atherosis spiral arteries also contained spiral arteries without acute atherosis (Papers I-II). In Paper I, spiral arteries without acute atherosis were further classified as showing complete or partial physiological transformation. Spiral arteries with complete physiological transformation were far more common than arteries with partial transformation, also in samples with concomitant acute atherosis arteries. This finding suggests that decidua basalis acute atherosis is not an indicator of generalized defects in physiological transformation, at least not in the decidua basalis segment of spiral arteries.

5.2.2 Third trimester spiral arteries: lined by maternal endothelium or fetal trophoblasts?

Some authors have claimed that maternal endothelial cells are replaced by fetal trophoblasts during pregnancy (90), while others argue that this replacement is only temporary (8). This controversy prompted us to investigate whether spiral arteries in our third trimester decidua basalis samples were lined by fetal trophoblasts or maternal endothelial cells. In our samples, all uteroplacental spiral arteries (with or without acute atherosis) were lined by cells positive for CD31 and vWF and negative for CK7, inferring that these cells are endothelial cells and not trophoblasts (Paper I). Decidua basalis spiral arteries are lined by

endothelial cells in the third trimester, in line with the five-stage model proposed by Pijnenborg et al. for physiological transformation of uteroplacental spiral arteries (8).

5.2.3 Endothelial damage in acute atherosis: what is the significance?

Paper I found that spiral arteries with acute atherosis were associated with an altered endothelial phenotype, such as "destroyed" or "abnormal" endothelium (defined in Table 3.3), and lower intensity of CD31 stain in the endothelium. An altered endothelial phenotype in acute atherosis may be the result of preceding endothelial damage, potentially caused by locally disturbed shear stress, upstream thrombosis, or local vascular inflammation, all of which are features associated with acute atherosis (22, 54, 61). Whether such an altered endothelial phenotype is the cause or consequence of acute atherosis is not known, as longitudinal studies of acute atherosis are not feasible, for methodological and ethical reasons (further discussed in section 5.1.2 "Study design of Papers I-III"). However, it is tempting to speculate that the "destroyed" phenotype represents the extreme on a scale of endothelial damage, with the "abnormal" phenotype either representing a preceding step toward "destroyed" endothelium or a response to less severe endothelial damage than that causing the "destroyed" endothelium.

Previous reports have also shown endothelial damage in acute atherosis (50-52), and de Wolf et al. proposed that endothelial damage may cause fibrin leakage into the vessel wall (51). We found fibrinoid necrosis (identified by MSB and PAS stain) of the vessel wall in nearly all acute atherosis arteries (Papers I-II), in line with the hypothesis put forward by de Wolf.

When assessing spiral arteries without acute atherosis (*non-AA arteries*) in *non-AA samples* and *non-AA arteries* in *AA samples*, we consistently found that the endothelium in these arteries displayed similar endothelial morphology, such

as a “normal” type endothelium and strong intensity of CD31 endothelial stain (Paper I). Thus, the presence of neighboring spiral arteries with acute atherosis did not affect the endothelial phenotype of *non-AA arteries* in *AA samples*, reflecting the focal nature of acute atherosis lesions.

5.2.4 Is the endothelium of acute atherosis spiral arteries activated?

One previous study using basal plate biopsies found endothelial activation (assessed by ICAM-1 expression) to associate with spiral arteries in biopsies where acute atherosis was also identified (92). Whether ICAM-1 was found in the endothelium of spiral arteries with acute atherosis was not specified in the study. We were unable to find associations between acute atherosis and endothelial activation, assessed by ICAM-1 endothelial expression (Paper I). Endothelial activation did not associate with decidua basalis spiral arteries with or without acute atherosis, in contrast to previous reports (92).

Our results do not entirely exclude the possibility that endothelial activation plays a role in the pathogenesis of acute atherosis. ICAM-1 is only one of several markers of endothelial activation (86). We may only speculate whether vascular endothelial adhesion molecule 1 (VCAM-1) and E-selectin (section 3.5.4 “Assessment of spiral artery endothelium”) would have demonstrated results similar to those of ICAM-1 had we succeeded in optimizing the antibodies for staining on decidua basalis tissue. Endothelial activation has been implicated in first trimester samples of decidua basalis (17), and may play a role in attracting endovascular trophoblasts into the spiral artery wall during physiological transformation. However, should this process become dysregulated and result in excessive endothelial activation, one may speculate that such excessive endothelial activation could represent an early event in acute atherosis development, which would be in line with endothelial activation in early atherosclerosis lesions (144). If this were true, it may also explain the

absence of endothelial activation in our third trimester decidua basalis samples with acute atherosis.

5.2.5 What are the potential sources of foam cells in acute atherosis?

Foam cells in acute atherosis lesions are CD68-positive and likely from a monocyte-macrophage origin (44, 97). Circulating monocytes may be attracted to the spiral artery walls through endothelial activation, and have been suggested to phagocytose VSMCs (97). Alternatively, decidual interstitial macrophages may be recruited to the artery wall. However, other cell types may also be sources of acute atherosis foam cells; electron microscopy studies have indicated VSMCs as a source of foam cells in acute atherosis (51).

Our group has previously found that oxidative stress induces lipid uptake and LOX-1 expression in a trophoblastic cell line (155) (further discussed in section 5.2.7 "Outside foam cell lesions"). Though LOX-1 was mostly expressed by decidual stromal cells in our samples, we also observed LOX-1 expression by intramural trophoblasts in spiral arteries without acute atherosis. We therefore propose that trophoblasts may also be a source of foam cells in spiral arteries, particularly in an oxidative environment, such as in preeclampsia (77) and acute atherosis (50). In line with this hypothesis, Zhang and Baergen have previously reported "foamy cells" of proposed trophoblast origin (CD56-positive, CD68-negative) in spiral arteries from basal plate biopsies, and termed this lesion "atherosis of trophoblast type" (156). However, they did not stain with CK7, our chosen trophoblast marker. Acute atherosis foam cells in our samples were negative for CK7, a cytoskeleton protein. We hypothesize that if trophoblasts accumulate lipid droplets (as sometimes observed by us as perilipin-2-positive droplets in CK7-positive trophoblasts) and develop into foam cells, their intracellular structure may become altered by the accumulating lipid droplets to the extent that they no longer stain positive for CK7. A similar

trend is seen in foam cells of VSMC origin: VSMCs represent a foam cell source in both acute atherosis and atherosclerosis (51, 94). Atherosclerotic foam cells, despite sometimes being of VSMC origin, do not stain positive for frequently used VSMC markers such as desmin or α-actin (71), indicating that the cytoskeleton/contractile filaments may disappear or become significantly altered and no longer label for desmin or α-actin.

LOX-1 is frequently expressed by endothelial cells during early atherogenesis, and promotes foam cell formation (103). LOX-1 protein expression was never found in endothelial cells (or foam cells) in acute atherosis in our third trimester decidua basalis samples (Paper II). Lack of LOX-1 protein expression in acute atherosis endothelium in the third trimester could indicate differing development mechanisms for atherosclerosis and acute atherosis. As discussed for ICAM-1, it is possible that the endothelium of acute atherosis arteries has expressed LOX-1 at earlier gestation. This theory is strengthened by our observation of LOX-1 sporadically being positive in endothelial cells in spiral arteries without acute atherosis (Paper II), perhaps indicating that LOX-1 is predominantly present in the endothelium prior to foam cell formation, though longitudinal studies (infeasible in decidua basalis studies) would be needed to test such a hypothesis.

As recently reviewed by our group (47, 157), fetal cells may be transferred to the mother during pregnancy, termed cellular fetal microchimerism (cFMC). cFMC is more frequent in preeclampsia, and may have beneficial as well as detrimental effects on maternal health both short and long term (157). cFMC has also been linked to cardiovascular disease (158). To our knowledge, cFMC has not been studied in acute atherosis. We speculate that trophoblasts may, as discussed above, represent a source of foam cells in acute atherosis (Paper II), and through cFMC (47) also be involved in future maternal cardiovascular disease.

5.2.6 What is the significance of atherosclerosis-related lipid-handling proteins in acute atherosis foam cells?

Both FABP4 and perilipin-2 have been identified in atherosclerotic foam cells previously, and participate in foam cell formation (71, 98).

To our knowledge, comparisons of frequency of FABP4 and perilipin-2 expression in atherosclerotic foam cells are lacking. Despite the possible underestimation of FABP4 and perilipin-2 expression in acute atherosis foam cells (section 5.1.5.4 "Markers of lipid-handling proteins"), we believe the observed difference in frequency between FABP4 (positive in 36% of acute atherosis lesions) and perilipin-2 (positive in 93% of acute atherosis lesions) reflects that perilipin-2 truly is more frequently expressed in acute atherosis lesions than FABP4 (Paper II). High levels of perilipin-2 is linked to lipid loading and foam cell generation (98, 99), indicating that third trimester acute atherosis foam cells are still scavenging lipids, and not fully developed. Increased FABP4 is, in atherosclerosis, an indicator of advanced lesions and plaque instability (71, 101). Acute atherosis lesions do not form calcified plaques as in atherosclerosis, though it is possible that acute atherosis lesions with higher fraction of FABP4-positive foam cells represent more advanced acute atherosis lesions.

5.2.7 Outside foam cell lesions: what is the expression of lipid-handling proteins in the decidua basalis interstitium?

Both perilipin-2 and LOX-1 were significantly more frequently expressed surrounding spiral arteries with acute atherosis (Paper II), suggesting increased lipid metabolism and lipid contents in decidua basalis tissue immediately surrounding spiral arteries with acute atherosis.

Perilipin-2 coats lipid droplets consisting of neutral lipids (95), pointing to generally increased lipid contents surrounding spiral arteries with acute atherosis. Increased levels of phospholipids, cholesterol, and neutral lipids (demonstrated by Oil Red O staining) in decidua basalis tissue in preeclampsia has been shown by our group (141) and others (159) previously, though these studies did not state whether increased decidua basalis lipid contents also associated with acute atherosis presence.

Increased LOX-1 indicates increased levels of oxidized LDL locally surrounding spiral arteries with acute atherosis, implying a role for oxidative stress in the pathophysiology of acute atherosis. Indeed, signs of oxidative stress have been identified in decidua parietalis (the part of the decidua not underlying the placenta, Figure 1.3) samples with acute atherosis (50). Our group has previously described signs of oxidative stress in decidua basalis tissue from women with preeclampsia (77), and found that oxidative stress induces lipid uptake and LOX-1 expression in a trophoblastic cell line (155). Though we predominantly found LOX-1 expression in decidual stromal cells (Paper II), we also observed positive LOX-1 staining of interstitial trophoblasts. These results suggest a role for oxidative stress in interstitial trophoblasts and stromal cells of decidua basalis in the pathophysiology of acute atherosis, likely leading to increased local inflammation and acute atherosis formation, in line with our hypothesis (3, 57, 58). This suggestion is strengthened by the recent finding of cholesterol crystals in decidua basalis tissue in preeclampsia and their *in vitro* ability to induce inflammation (81).

Interestingly, FABP4 was significantly more often expressed surrounding spiral arteries without acute atherosis (Paper II), suggesting that different lipid-related proteins and thus, different types of lipids, may have differing roles in normal and pathological pregnancies.

5.2.8 Circulating proteins associated with amyloid in acute atherosis and preeclampsia: what is the relevance to atherosclerosis development?

In atherosclerosis, aggregates of misfolded protein (amyloid depositions) have been discovered within lesions (160). Increased gene expression of SAA1, a circulating precursor of amyloid, has been found in atherosclerotic endothelium (144), and increased circulating levels induce atherosclerosis lesion progression in mice (117). Amyloid also accumulates in the urine, serum, and placenta of women with preeclampsia (111, 112), though the range of different amyloidogenic proteins involved in preeclampsia is unknown. Protein misfolding and amyloid has until now (Paper III) not been explored in acute atherosis.

Presence of decidua basalis acute atherosis was not associated with circulating levels of amyloidogenic SAA1 or anti-amyloidogenic PZP (Paper III). However, levels of SAA1 were increased, and PZP decreased, in early-onset preeclampsia (Paper III), one of the pregnancy complications most often affected by acute atherosis (43, 44) and with high risk of future cardiovascular disease (64), indicating that SAA1 and PZP may play a role in connecting pregnancy complications and future maternal health. Further, SAA1 and PZP correlated with a dysregulated angiogenic biomarker profile (i.e. increased sFlt-1/PlGF ratio) and fetal growth restriction, proxies for placental dysfunction. Although fetal sex may impact placental function (161), and SAA1 and PZP correlated with placental dysfunction, we did not find an association between SAA1 or PZP and fetal sex (Paper III).

Increased SAA1 and decreased PZP may indicate increased amyloid deposition, as increased SAA1 may lead to amyloid-A deposition (the amyloid type associated with SAA1) (110). PZP has been found to prevent aggregation of amyloidogenic proteins other than SAA1 (129). Whether PZP may also inhibit amyloid-A aggregation from SAA1 is unknown. We speculate that PZP may

have a similar role in SAA1 amyloidogenesis, thus decreased PZP together with increased SAA1 in preeclampsia and placental dysfunction may further indicate increased amyloid-A deposition in these pregnancies. Mechanistic studies are needed to confirm this hypothesis.

Existing studies of SAA1 in pregnancy and preeclampsia vary in size and conclusions differ considerably (122-127). Of HDPs, we found that only early-onset preeclampsia was associated with increased SAA1 (Paper III). No previous study of SAA1 has made a similar distinction between early- and late-onset preeclampsia (delivery < vs. ≥ 34 weeks' gestation). We consider this, in addition to the relatively large population size (549 pregnancies), to be some of the main strengths of Paper III, which establishes that circulating SAA1 is increased in early-onset preeclampsia and placental dysfunction.

Circulating PZP levels in placental dysfunction have not been well described previously. Two small studies using immunodiffusion gel methodology demonstrated low PZP levels in preeclampsia (162, 163). Interestingly, we found immunoassay levels of PZP to be decreased both in early-onset preeclampsia and in pregestational diabetes mellitus. As low circulating PZP levels have been linked to pregnancy loss (164), we suggest that low PZP in women with pregestational diabetes and early-onset preeclampsia may reflect the profound placentation issues typically associated with these conditions. We speculate that low PZP may identify women with inadequate placentation, who are at risk of early-onset placental dysfunction.

Additional studies are required to elucidate the mechanistic role of SAA1 and PZP in preeclampsia and acute atherosis. Dysregulation of circulating SAA1 and PZP in early-onset preeclampsia may be an indicator of amyloid-A deposition. Tissue studies, such as immunohistochemistry of placenta or decidua basalis tissue, could be used to test our hypothesis of amyloid deposition correlating

with circulating SAA1. We have suggested this as a potential future study in continuation of Paper III (Chapter 7, Future studies).

SAA1 is an acute-phase protein synthesized by the liver in response to inflammatory stimuli (110). Another acute-phase protein, hsCRP, is often used to monitor inflammatory diseases, and hsCRP measurements are typically more accessible in the routine clinical setting. SAA1 and hsCRP correlate in several clinical populations (115, 124), including ours (Paper III). Further, hsCRP is elevated in atherosclerosis (115). In our cohort, hsCRP was increased in preeclampsia (Paper III), reaffirming that preeclampsia is characterized by excessive inflammation, and linking pregnancy to future cardiovascular disease. These results are also a further indication of possible amyloid-A depositions in preeclampsia, as chronic low-grade inflammation is the typical mechanism for SAA1 aggregation into amyloid-A (110). Though hsCRP measurements are more readily available, measuring hsCRP alone would not necessarily give indication of amyloid deposition in preeclampsia, as hsCRP does not form amyloid, in contrast to SAA1 (109).

In a mouse model, elevated circulating SAA1 was shown to associate with increased size of atherosclerotic lesions, even in the case of short-lasting (14 days) SAA1 elevation (117). We speculate that elevated SAA1 in preeclampsia may promote atherogenesis, both through short-term elevation (by increasing existing atherosclerosis lesions during pregnancy) and through long-term elevation (leading to amyloid-A deposition), and thereby cardiovascular disease. Longitudinal and mechanistic studies are needed to test our hypothesis that amyloid and acute-phase proteins in women with a history of preeclampsia and placental dysfunction are contributing factors in the development of atherosclerotic cardiovascular disease.

5.2.9 Characteristics of acute atherosis lesions: affected by pregnancy outcome?

Several studies show that acute atherosis not only affects preeclamptic pregnancies, but also a low rate of uncomplicated, normotensive pregnancies (42-44). The question as to whether acute atherosis characteristics differ or are similar in preeclampsia and normotensive pregnancies remains to be discussed. Previous studies including acute atherosis in both preeclamptic and normotensive pregnancies show that the lesion is characterized by foam cells, fibrinoid necrosis, and a varying degree of inflammatory infiltrates (42-44). Although attention to acute atherosis mainly has been directed at preeclamptic pregnancies, our group has previously identified that lymphocyte types in and around acute atherosis lesions in normotensive and preeclamptic pregnancies do not differ significantly (54).

Acute atherosis is, as previously reported by our group and others before us (42-44, 60), more common in pregnancies complicated by preeclampsia (Paper III), but lesion characteristics are similar across different pregnancy outcomes. For pregnancies with acute atherosis, we found no differences between the normotensive and preeclampsia groups in terms of local endothelial activation (Paper I) or expression of lipid-handling proteins (Paper II). Morphologically, acute atherosis lesions displayed similar characteristics of endothelial damage, number of foam cells per lesion, fibrinoid necrosis, and absence of trophoblasts and vascular smooth muscle cells across the different pregnancy outcome groups (Papers I-II). Circulating levels of SAA1 and PZP were not affected by acute atherosis presence in any pregnancy group (Paper III).

The results from Papers I-III imply that acute atherosis presents morphologically and molecularly similar regardless of clinical diagnosis or pregnancy outcome, fitting our hypothesis of acute atherosis potentially being an independent identifier of women with an increased cardiovascular risk (3, 57, 58).

5.2.10 Clinical implications: does acute atherosis represent an accelerated form of atherosclerosis, fueled by the excessive inflammatory processes in pathologic pregnancies?

Staff and Redman (69) have previously discussed some of the similarities and most important differences between uteroplacental acute atherosis and early stages of atherosclerosis ("fatty streak" lesions) of larger arteries. The morphological and molecular results from Papers I-III expand on the similarities and differences between the two arterial lesions, as summarized in Table 5.1.

Among the most prominent similarities uncovered in this thesis is the finding that acute atherosis and atherosclerotic foam cells express similar lipid-handling proteins. It was known from previous work that the two lesions, per definition, have CD68-positive foam cells in common; however, the finding that foam cells from both lesions express perilipin-2 and FABP4 is novel (Paper II). This result further strengthens the notion that acute atherosis resembles early atherosclerosis lesions (69), as the perilipin-2 abundance indicates foam cell generation and formation (98, 99), similar to early stages of atherogenesis.

One significant difference between acute atherosis and atherosclerosis is the lack of ICAM-1- and LOX-1-positive endothelial cells in acute atherosis (Papers I-II). We interpret these results as a lack of endothelial activation (lack of ICAM-1) and that endothelial cells do not assist in oxidized LDL-uptake (lack of LOX-1) in third trimester acute atherosis, though both processes have been identified as important in early atherogenesis (103, 144). The lack of LOX-1-positive endothelium may also imply that endothelial cells do not play a role in foam cell formation in acute atherosis, differing from atherosclerosis (103).

Table 5.1. Major similarities and discrepancies between uteroplacental acute atherosis and atherosclerosis. Expanded on from Staff and Redman (69). Roman numerals (I-III) refer to findings presented in Papers I-III included in this thesis

	Atherosclerosis	**Acute atherosis**
Artery diameter	Typically > 0.5 mm (165), up to several cm (79)	Typically from 120-140 µm up to 2-3 mm (22, 93)
Time course	Long (years)	Short (pregnancy: months)
CD68+ foam cells	Yes (71)	Yes (I-II) (44, 97)
Foam cells: perilipin-2, FABP4 positivity	Yes (71, 99, 101)	Yes (II)
Foam cells: origin		
1. Monocyte-macrophages	1. Yes (71, 94)	1. Yes (I-II) (44, 97)
2. VSMCs	2. Yes (94)	2. Likely (51, 97)
3. Endothelial cells	3. Yes (94)	3. Possible (II)
4. Trophoblasts	4. Possible, through fetal microchimerism (157)	4. Possible (II)
Vascular inflammation	Yes (72)	Yes (54)
Fibrinoid necrosis	No (not reported)	Yes (I-II)
Fibrosis	Yes, in late stages (67)	No (I-II)
Plaque formation	Yes, in late stages (67)	No (I-II)
Thrombosis	Yes, in late stages (67)	Yes, variably (44, 61)
Complement deposition	Yes, previously reviewed by the candidate (166)	Yes, previously reviewed by the candidate (166)
Endothelial damage/ disruption	No (85)	Yes (I) (50-52)
Endothelial activation	Yes (86, 144)	No (I)
Oxidative stress	Yes (103)	Yes (II) (50)
Protein misfolding, circulating	Yes (115, 117)	No (III)
Protein misfolding, locally	Yes (160)	Unknown (to be explored by us)

Rather than endothelial *activation*, we found *damaged* endothelium in acute atherosis (Paper I). This is in contrast to atherosclerosis, where the endothelium is reported as being intact at all times, at least according to one report (85). This implies a different role for endothelium in acute atherosis versus atherosclerosis, and the results may be explained by the unique hemodynamic conditions in uteroplacental spiral arteries, both with and without acute atherosis present (22). The role of protein misfolding in atherosclerosis and cardiovascular disease is gaining increasing awareness (110, 160). The full extent of different amyloid precursors involved in atherosclerosis remains to be uncovered. SAA1 represents one of several amyloid-associated proteins that play a role in atherosclerotic lesions on a local (144) and systemic (117) level. The lack of an association between decidua basalis acute atherosis and circulating SAA1 (Paper III) sets this arterial lesion apart from atherosclerosis. Whether SAA1 may play a role in acute atherosis pathogenesis locally remains to be investigated. However, the distinct association between circulating SAA1 and early-onset preeclampsia as well as with proxies for placental dysfunction (Paper III) supports a link between placental dysfunction in pregnancy and future cardiovascular disease.

While atherosclerosis is one of the leading causes of cardiovascular disease for both sexes (66, 67), women are more likely to be affected by coronary microvascular dysfunction than men (167). Microvascular dysfunction, like acute atherosis, affects smaller (< 0.5 mm diameter) vessels (165), and is associated with inflammation (168, 169), endothelial dysfunction (170), and oxidative stress (increased LOX-1) (171). While SAA1 has not been studied in coronary microvascular dysfunction, CRP is elevated in the disease (169). In contrast to acute atherosis, microvascular dysfunction lacks foam cell lesions, and is associated with endothelial activation (172).

The results from Papers I-III reflect that acute atherosis and atherosclerosis likely have shared as well as unique development mechanisms. The shared features and the potentially shared development mechanisms of acute atherosis and atherosclerosis may be indicative of two opposing concepts. One being that the shared features may be general, non-specific features of any arterial foam cell lesion (another example of foam cell lesions being graft vasculopathy). The other possibility is that acute atherosis represents an accelerated atherosclerotic process, and that the short time aspect of acute atherosis gives rise to some of the observed differences between the two lesions, exemplified by the lack of plaque formation in acute atherosis. Alternatively, that the observed differences are pregnancy-specific and influenced by the unique hemodynamic and pro-inflammatory conditions of pregnancy.

Further studies are needed to explore potential similarities between acute atherosis and atherosclerosis, and to fully uncover the pathophysiological mechanisms of pregnancy-specific spiral artery acute atherosis, further discussed in Chapter 7 (Future studies). Increasing knowledge of the potential links between acute atherosis and atherosclerosis may aid in understanding whether uteroplacental acute atherosis poses a sex-specific risk factor for premature cardiovascular disease in women, although longitudinal studies are needed to confirm this hypothesis.

6. CONCLUSIONS

The studies and results presented in this PhD thesis add to existing knowledge of vascular and circulating biomarkers associated with preeclampsia and acute atherosis of the uteroplacental spiral arteries in decidua basalis tissue. Understanding the links between preeclampsia, preeclampsia-associated acute atherosis, and cardiovascular disease is essential to improving female cardiovascular health outcomes. The thesis explores whether preeclampsia and acute atherosis may share local and circulating molecular features with chronic, larger artery atherosclerosis.

The main conclusions from the thesis are illustrated in Figure 6.1.

1. Uteroplacental spiral arteries (both with and without acute atherosis) in third trimester decidua basalis samples are lined by maternal endothelial cells, not fetal extravillous trophoblasts (Paper I).

2. Endothelial cells in spiral arteries with acute atherosis show signs of endothelial damage, but not endothelial activation. These findings differ from endothelial activation seen in early stages of atherosclerosis (Paper I).

3. Arterial wall foam cells of acute atherosis lesions express lipid-handling proteins (perilipin-2 and fatty acid binding protein 4) similar to what is found in atherosclerosis. At the same time, endothelial cells lining acute atherosis artery walls do not express the lipid-scavenging receptor lectin-like oxidized LDL receptor 1 (LOX-1), as opposed to endothelial cells in early atherosclerotic lesions (Paper II).

4. Interstitial decidua basalis tissue (stromal cells) surrounding spiral arteries with acute atherosis was frequently positive for the lipid-scavenging LOX-1 receptor, indicating increased oxidative stress surrounding acute atherosis lesions (Paper II).

5. Acute atherosis of decidua basalis spiral arteries displays similar morphological and molecular properties across pregnancy outcomes, with no major differences in local lesion characteristics between pregnancies complicated by preeclampsia and uncomplicated, normotensive pregnancies (Papers I-II).

6. Decidua basalis acute atherosis is not associated with circulating serum amyloid A1 (SAA1) or pregnancy zone protein (PZP), potential markers of protein misfolding and amyloid aggregation, in contrast to increased circulating SAA1 in atherosclerosis (Paper III).

7. Amyloidogenic SAA1 is increased in early-onset preeclampsia, while anti-amyloidogenic PZP is decreased. Similarly, SAA1 and PZP correlate with an antiangiogenic biomarker profile and fetal growth restriction. This suggests increased amyloid-A deposition in early-onset preeclampsia and placental dysfunction, providing a possible link between placental dysfunction and the observed epidemiological increased risk of future cardiovascular disease (Paper III).

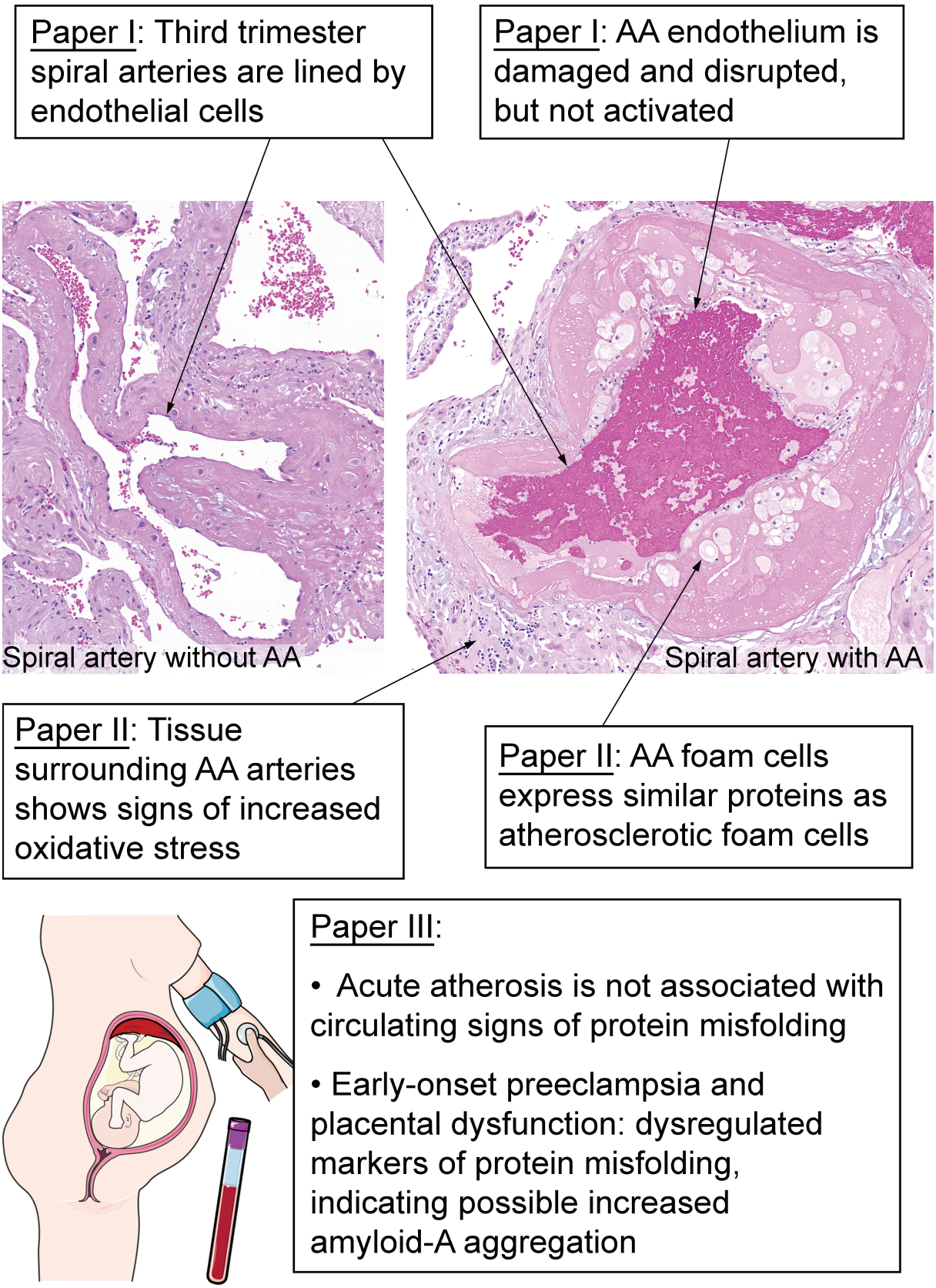

Figure 6.1. Main conclusions of the thesis. AA: acute atherosis. Spiral artery photos by Ingrid K. Fosheim. Created in part using Servier Medical Art.

7. FUTURE STUDIES

This PhD thesis has identified some hitherto unknown vascular and circulating features of uteroplacental spiral artery acute atherosis of decidua basalis. Still, more studies are needed to completely understand the development of acute atherosis, as well as the potential effects on long-term maternal cardiovascular health. Some future study ideas include:

1. In continuation of Paper III, our group has plans of studying local amyloid deposition in decidua basalis acute atherosis, similar to what has been identified in atherosclerosis (160). To identify whether amyloid depositions may play a role in acute atherosis pathogenesis, we will stain decidua basalis sections with Congo red or thioflavin T. These stains are specific for amyloid, but do not indicate which amyloidogenic protein the deposition consists of. Serum amyloid A1 (SAA1) is associated with amyloid-A depositions and atherosclerosis. Hence, we will stain decidua basalis tissue for SAA1 and amyloid-A as well.
2. Cholesterol crystals, which are part of the atherosclerotic lesion, have recently been identified in decidua basalis tissue (81). We hope to explore whether cholesterol crystals may be located to decidua basalis acute atherosis lesions, further linking acute atherosis to atherosclerosis.
3. The onset of acute atherosis is unknown, though lesions have been identified in first trimester abortion samples from women with systemic lupus erythematosus (45). High uterine artery pulsatility index near delivery has been associated with acute atherosis (59, 173). Longitudinal measurements of uterine artery pulsatility index during such high-risk pregnancies as well as other risk pregnancies (e.g. previous early-onset preeclampsia with fetal growth restriction) may therefore be explored to improve identification of the onset of acute atherosis formation. Development of other imaging methods, including tissue strain/elasticity and deep learning algorithms that have been used in

vascular research (174), may also represent new ways to explore vascular tissue changes during pregnancy in the decidua basalis (175).

4. Ultimately, if a tool for detecting *in vivo* development of acute atherosis were available, medical therapy may be tested during pregnancy and effect on spiral artery acute atherosis could be evaluated. Statins are anti-atherogenic and anti-inflammatory (176), and may be introduced after the first trimester fetal organogenesis period. Our group has suggested that use of statins may improve uteroplacental perfusion and thereby pregnancy outcome in pregnancies with acute atherosis (3, 58). In support of this, small studies of statin use in women with antiphospholipid syndrome have shown promising results with improved uteroplacental perfusion (177), but randomized trials are lacking.

8. OTHER RELEVANT PUBLICATIONS DURING THE PHD PERIOD

Staff AC, Fjeldstad HE, **Fosheim IK**, Moe K, Turowski G, Johnsen GM, Alnaes-Katjavivi P, Sugulle M

Failure of physiological transformation and spiral artery atherosis: their roles in preeclampsia

Am J Obstet Gynecol. 2022 Feb;226(2S):S895-S906.

doi: 10.1016/j.ajog.2020.09.026. Epub 2020 Sep 21

Jacobsen DP, Fjeldstad HE, Johnsen GM, **Fosheim IK**, Moe K, Alnæs-Katjavivi P, Dechend R, Sugulle M, Staff AC

Acute Atherosis Lesions at the Fetal-Maternal Border: Current Knowledge and Implications for Maternal Cardiovascular Health

Front Immunol. 2021 Dec 14;12:791606. doi: 10.3389/fimmu.2021.791606

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10. APPENDICES

Appendix 1: Decidua basalis tissue staining protocol

Deparaffinization and rehydration

Prior to staining, formalin fixed, paraffin embedded (FFPE) sections used in Paper I were deparaffinized and rehydrated according to routines at the Department of Pathology, Oslo University Hospital, Ullevål.

Sections used in Paper II were heated to 60 °C for 45-60 min using a hybridization oven, cooled to room temperature, immersed in Histo-Clear (VWR, USA) for 3 min in three successive baths (deparaffinization), then immersed in absolute ethanol for 3 min in three successive baths and finally in tap water for 5 min (rehydration).

Hematoxylin-eosin staining

Slides were immersed in filtered hematoxylin (Dako, Denmark) and incubated for 5 min. Slides were then washed using several changes of tap water, destained/differentiated using 4% acetic acid for 20 sec, washed in several changes of tap water, incubated in Scott's bluing water for 2 min, then soaked in tap water for 1 min. Slides were stained with eosin for 1 min and dipped/rinsed in two successive tap water baths before dehydration: 30 sec in each of 70% - 95% - 95% - 100% ethanol baths followed by 5 min in Xylene.

Immunohistochemical staining

Immunohistochemical staining for Paper I was performed using the fully automated Ventana BenchMark XT immunostainer (Roche, USA). Immunohistochemical staining for Paper II was performed as follows:

1) Antigen retrieval: slides were placed in antigen retrieval solution (buffer depending on the antibody) and heated until boiling point using a microwave oven (2 min, 900 W), placed in a pre-heated vegetable steamer for 20 min, then cooled at room temperature until temperature was below 20 °C.

2) Peroxidase blocking: slides were soaked in TBS-T (0.5% Tween in tris-buffered saline) for 5 min, then transferred to TBS-BSA (0.1% bovine serum albumin in TBS) for 5 min, then drained. A hydrophobic (PAP) pen was used to draw a circle around the tissue. Dako (Denmark) EnVision+ Peroxidase Block was dripped onto the tissue, covering the whole sample. Slides were placed in a wet chamber and incubated for 30 min, then rinsed with TBS-T.

3) Primary antibody incubation: slides were drained. Primary antibody was diluted in TBS-BSA to optimal dilution (Table 3.2), and applied to the slide. Slides were placed in a wet chamber and incubated overnight at 4 °C. Negative reagent controls were antibody dependent (mouse or rabbit) and used in the same dilution as primary antibody.

4) Secondary antibody incubation: slides were rinsed in TBS-T for 5 min in two successive baths, drained, and placed in a wet chamber. Dako (Denmark) EnVision+ Labelled Polymer-HRP (horseradish peroxidase) anti-mouse or anti-rabbit (depending on primary antibody, Table 3.2) was dripped onto the tissue. Slides were incubated at room temperature for 60 min, then rinsed in TBS-T for 5 min followed by TBS-BSA for 5 min.

5) Chromagen-DAB (diaminobenzidine): Dako (Denmark) DAB+ Substrate Buffer and DAB+ Chromagen was used in the ratio 1 ml to 1 drop, using

96 µl substrate buffer per slide and incubated for 5 min. Slides were then placed in tap water for 5 min.

6) Counterstaining: slides were immersed in filtered hematoxylin (Dako, Denmark) and incubated for 5 min. Slides were then washed using several changes of tap water, destained using 4% acetic acid for 20 sec, washed in several changes of tap water, incubated in Scott's bluing water for 2 min, then soaked in tap water for 1 min.

Periodic acid-Schiff co-staining

FFPE sections stained with von Willebrand factor (vWF, Paper I), CK7 and CD68 (Paper II) were co-stained with periodic acid-Schiff (PAS) following immunostaining, but prior to counterstaining with hematoxylin. Sections were incubated with 1% periodic acid for 10 min, rinsed in tap water for 10 min, incubated in Schiff's reagent for 10 min, and rinsed again in tap water for 10 min.

Martius Scarlet Blue staining

FFPE sections for Martius Scarlet Blue (MSB) trichrome staining were incubated in Weigert's iron hematoxylin for 15 min, rinsed in tap water for 5 min followed by absolute ethanol, stained with Martius Yellow for 2 min, rinsed in distilled water, stained with Brilliant Crystal Scarlet 6R for 10 min, rinsed in distilled water, treated with phosphotungistic acid for 2 min, rinsed in distilled water, counterstained with Methyl blue for 4 min, and rinsed in 1% acetic acid.

Appendix 2: Decidua basalis evaluation protocol for Papers I-II

Patient #:	Date:	Decidua basalis tissue y/n? (interstitial CK7+ trophoblasts):	Total # spiral arteries:	# acute atherosis arteries:		
Used in paper	Stain	Histological finding	Vessel 1 (V1)	V2	V3	…
I, II	HE	Vessel size (< vs. ≥ 140 µm)				
		Perivascular decidua y/n				
		Mural vacuolated cells y/n, quadrant(s)				
I, II	PAS	Mural fibrinoid impression ("normal purple", "glossy pale pink")				
		Fibrinoid position: quadrant(s)				
I, II	CK7	Intramural cells y/n, quadrant(s)				
		Perivascular cells y/n				
I, II	CD68	Intramural cells y/n				
		Position: quadrant(s)				
		Positive vacuolated cells y/n				
		Positive vacuolated cells: quadrant(s)				
		≥ 2 adjacent pos. vacuolated (AA): y/n				
		Perivascular cells y/n				
I	Desmin	Mural cells y/n (intact, disrupted, separate)				
		Position: quadrant(s)				
		Perivascular myometrium y/n				

I	MSB	Fibrinoid color (red, blue)																			
		Fibrinoid position: quadrant(s)																			
I	CD31	Cells lining vessel lumen y/n (normal, abnormal, destroyed)																			
		Position: quadrant(s)																			
		Staining intensity vs. internal pos. ctrl. (weaker, stronger)																			
I	vWF	Cells lining vessel lumen y/n																			
		Position: quadrant(s)																			
I	ICAM-1	Positive endothelial cells y/n																			
		≥ 3 adjacent pos. endothelial cells y/n																			
		Position: quadrant(s)																			
II	Perilipin-2	Positive vacuolated mural cells y/n																			
		# vacuolated CD68+ perilipin-2+ cells																			
		Positive mural cells (not CD68+) y/n																			
		Positive perivascular cells y/n (scattered, focal, generalized)																			
II	FABP4	Positive vacuolated mural cells y/n																			
		# of vacuolated CD68+ FABP4+ cells																			
		Positive mural cells (not CD68+) y/n																			
		Positive perivascular cells y/n																			
II	LOX-1	Positive vacuolated mural cells y/n																			
		# of vacuolated CD68+ LOX-1+ cells																			
		Positive mural cells (not CD68+) y/n																			
		Positive perivascular cells y/n																			

Based on Appendix 1 of Alnaes-Katjavivi P. Decidual acute atherosis: immunohistochemical definition, immune cell involvement, and tissue heterogeneity [Doctoral thesis]. Oslo: University of Oslo; 2020.

Appendix 3: Enzyme-linked immunosorbent assay (ELISA) protocol

For all steps, a combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, USA) dispenser/washer was used.

Plate preparation: The capture antibody was diluted to the working concentration in phosphate-buffered saline (PBS, Thermo Fisher, USA), and microplates immediately coated with 20 µL per well of the diluted capture antibody. Microplates were sealed and incubated overnight at 4 °C. Each well was aspirated and washed with 90 µL wash buffer (0.05% Tween in PBS) for a total of three washes. After the last wash, remaining wash buffer was aspirated. 90 µL blocking buffer (1% bovine serum albumin (BSA) in PBS) was added to each well to block plates. Plates were incubated at room temperature for 1 hour. Aspiration/wash was performed as described above.

Assay procedure: 20 µL of sample or standards in sample buffer (reagent diluent (0.5% BSA, 0.05% Tween in PBS) + 20% fetal calf serum (FCS)) was added to each well. Plates were covered with an adhesive strip and incubated overnight at 4 °C. Aspiration/wash was performed as described above. 20 µL of the detection antibody, diluted in reagent diluent, was added to each well. Plates were covered with an adhesive strip and incubated for 2 hours at room temperature. Aspiration/wash was performed as described above. 20 µL of the working dilution of streptavidin-horseradish peroxidase (HRP) was added to each well. Plates were covered with an adhesive strip and incubated for 20 min at room temperature, avoiding direct light. Aspiration/wash was performed as described above. 20 µL of substrate solution (3, 3', 5, 5'-tetramethylbenzidine (TMB), Thermo Fisher, USA) was added to each well. Plates were incubated for 20 min at room temperature, avoiding direct light. 50 µL of 2M H_2SO_4 was added to each well. Plates were gently tapped to ensure thorough mixing. Optical density was determined immediately using an ELISA microplate reader (BioTek, USA) set to 450 nm. Wavelength correction was performed at 540 nm.

11. ERRATA

In the original publication of Paper II (Fosheim IK et al. Decidua basalis and acute atherosis: expression of atherosclerotic foam cell associated proteins. Placenta. 2021 Apr;107:1-7), in the Materials and methods section, the listed clones for antibodies used for immunohistochemistry were incorrect. Correct clones are provided in: Fosheim IK et al. Corrigendum to "Decidua basalis and acute atherosis: expression of atherosclerotic foam cell associated proteins" [Placenta 107 (2021) 1-7]. Placenta. 2022 Jan;117:28. Clones listed in the thesis (Table 3.2) are correct.

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Acute atherosis of decidua basalis; characterization of spiral arteries, endothelial status and activation

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ABSTRACT

Introduction: Acute atherosis (AA) is a lesion affecting uteroplacental spiral arteries during pregnancy, most frequently in preeclampsia but occasionally in normal pregnancy. It is commonly observed in untransformed spiral arteries (intact smooth muscle cell layer, lacking intramural trophoblasts). The mechanism causing lesion development is unknown. AA shares some morphological similarities with atherosclerosis, in which endothelial activation occurs early. Here we histologically characterize decidua basalis spiral arteries with and without AA, focusing on endothelial status and activation.

Methods: Formalin-fixed and paraffin-embedded decidua basalis tissue sections from 32 patients (16 normotensive, 5 with AA, 16 preeclampsia, 7 with AA) were stained with H + E, PAS, MSB (Martius Scarlet Blue), desmin, CK7, CD68, CD31, vWF and ICAM-1. We logged remodeling status, presence of AA, endothelial morphology, endothelial CD31 intensity and activation (ICAM-1-positive cells).

Results: We observed fully or partially transformed spiral arteries in most decidua basalis samples, and no untransformed arteries. AA arteries were also observed, characterized by intramural CD68-positive vacuolated cells and fibrinoid necrosis. They lacked a smooth muscle cell layer and intramural trophoblasts. The fibrinoid necrosis in AA lesions stained red with MSB. AA arteries were associated with lower CD31 staining intensity of endothelial cells. More arteries had an abnormal or destroyed endothelium relative to arteries without AA. Endothelial activation was not observed in the majority of AA arteries.

Discussion: Our results indicate an altered endothelial phenotype as important in the development of AA, supporting previous observations. The histology of AA differs from that of atherosclerosis.

1. Introduction

Acute atherosis (AA) affects the uteroplacental spiral arteries, which perfuse the placental intervillous space during pregnancy. AA occurs in 10–40% of preeclamptic pregnancies [1,2], but also in a lower proportion of normotensive pregnancies [2,3]. It has been described in spiral arteries in the myometrium, decidua basalis, placental basal plate, and decidua parietalis [2,4–6]. The lesion is commonly associated with incomplete spiral artery remodeling (intact smooth muscle cell layer, lacking intramural trophoblasts) [7,8], also described as incomplete physiological transformation.

AA is characterized by intramural CD68-positive vacuolated cells ("foam cells"), thus appearing morphologically similar to early atherosclerosis lesions [9,10]. Further, it is associated with fibrinoid necrosis and occasionally a mononuclear perivascular infiltrate [1,4].

The mechanisms of AA are not understood. It is not clear whether the lesions in the myometrium, decidua basalis and parietalis represent the same pathological processes, even though they share some similar features. Based on the similarity to atherosclerosis, which is established as an inflammatory vascular lesion, we have proposed that vascular inflammation may also be associated with the development of AA [9]. Initial stages of atherosclerosis include endothelial activation, as demonstrated by an increased expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) [11]. Expression of these

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molecules promotes attachment and recruitment of circulating monocytes into the vessel wall [12].

This study gives a detailed and systematic histological characterization of decidua basalis spiral arteries with and without AA, to determine whether endothelial status and activation is consistent with our hypothesis.

2. Methods

2.1. Patient recruitment and tissue collection

Pregnant women were recruited as described previously [1], and all gave informed written consent. Preeclampsia was defined as new onset of hypertension (blood pressure ≥140/90 mmHg) and proteinuria (≥1 + on dipstick or ≥30 total protein/creatinine ratio) at ≥20 weeks of gestation [13]. None of the women had regular uterine contractions or signs of infection, and membranes were intact at time of delivery. None had known chronic diseases. Decidua basalis tissue was obtained by vacuum suction of the placental bed following elective caesarian section, as described previously [14]. The collected tissue was fixed in buffered formalin and embedded in paraffin (FFPE).

2.2. Decidua basalis tissue evaluation

For all patients, FFPE tissue sections (3 μm) of the decidua basalis stained for desmin, CK7, and CD68, counterstained with Periodic Acid-Schiff (PAS), were available [1]. Decidua basalis origin was confirmed by the presence of CK7-positive interstitial trophoblasts and decidual cells. Spiral arteries with complete physiological transformation (fully remodeled) were identified by the presence of CK7-positive trophoblasts in the vessel wall, of intramural fibrinoid (bright purple upon PAS staining) and of complete absence of mural smooth muscle cells (no desmin stain) [15]. Partially transformed spiral arteries (partially remodeled) had both intramural fibrinoid and trophoblasts, and scattered areas with remaining mural smooth muscle cells (desmin-positive). Acute atherosis (AA) arteries were defined as arteries with ≥2 intramural adjacent vacuolated CD68-positive cells in the vessel wall [1]. Vessels with an intact desmin-positive smooth cell muscle layer and absence of intramural trophoblasts were defined as untransformed spiral arteries (unremodeled) or basal arteries (typically < 100 μm in diameter) [15]. Only arteries with a diameter of at least 140 μm were included in order to exclude basal arteries from the analysis. Arteries with AA lesions were categorized as *AA arteries*. Arteries lacking AA lesions were categorized as *nonAA arteries*. Decidua basalis samples lacking AA arteries were categorized as *nonAA samples*. Decidua basalis samples harboring ≥1 artery with AA were categorized as *AA samples*. AA is a focal lesion, seldom affecting all arteries in a sample, so *nonAA arteries* in samples also harboring *AA arteries* were defined as *nonAA arteries in AA sample*. In total 32 patients were included in the study, 16 normotensive controls (NC) (5 with AA, which is a purposeful overrepresentation of the 10% AA rate we have previously found in controls [1]) and 16 with preeclampsia (PE) (7 with AA). The clinical characteristics of the patient groups are presented in Supplementary Table 1.

2.3. Immunohistochemical and histological staining

Additional 3 μm serial slides were stained with CD31 (PECAM-1) (Dako, mouse, clone JC70A, 1:400), von Willebrand Factor (vWF, Dako, rabbit polyclonal 1:120000) counterstained with PAS, and intercellular adhesion molecule-1 (ICAM-1, mouse, clone 23G12, Thermo Fisher Scientific, 1:10 + amplification) using a Ventana XT autostainer (Roche) following the manufacturer's protocol. All reagents were obtained from Roche. Pictures were obtained using an Axio Scan slide scanner and Zen Blue software (Zeiss). Specificity of antibody staining was verified using appropriate positive and negative controls. An example of positive and negative controls for ICAM-1 is shown in Supplementary Fig. 1. Serial slides were stained with Martius Scarlet Blue (MSB) [16] and Hematoxylin + Eosin (H + E) using standard histological techniques.

2.4. Evaluation of endothelial status and activation

Endothelial cells in spiral arteries were identified by positive CD31 and vWF staining. The histological appearance could be divided into three main categories; “Normal”; flattened CD31-positive endothelial cells lining the vessel wall, “Abnormal”; CD31-positive endothelial cells, but more irregular in shape than normal flat endothelium, and “Destroyed”; the endothelial cell layer is disrupted and detached from the vessel wall or partly lacking. Arteries with mixed endothelial cell appearance (abnormal + normal) were classified as abnormal if > 25% of the endothelium had this phenotype.

For each vessel, we also evaluated intensity of CD31 staining across the endothelium. The Image Analysis module of the Zen Blue software (Zeiss) was used to identify and quantify the areas of high and low CD31 intensity for the endothelium lining each vessel. Thresholds for high and low intensity of stain were set manually in Image Analysis in Zen Blue, after ensuring similar intensity level between each scanned slide. NonAA vessels were used to define high intensity level. An example of the image analysis is shown in Supplementary Fig. 2.

Endothelial activation was defined as ≥3 consecutive ICAM-1-positive endothelial cells, as used previously in a study of endothelial activation in atherosclerosis [17]. Samples were evaluated without knowledge of the patients’ clinical data.

2.5. Statistics

The data were analyzed using SPSS Statistics 25.0 (IBM). For continuous variables a non-parametric Mann-Whitney *U* test was used. Fisher's exact test was used for categorical variables, expected cell count < 5. A p-value < 0.05 was regarded as significant.

3. Results

3.1. Decidual basalis spiral artery identification and characterization

We identified a total of 124 spiral arteries across all evaluated samples. The data were primarily analyzed by artery not by case, as the focus of this study was the histology of the AA lesion. The arteries were categorized according to presence of AA (*AA artery*) or not (*nonAA artery*) as described in the methods section (Table 1).

The median number of spiral arteries observed per decidua basalis tissue section was higher in normotensive controls (NC) than preeclampsia (PE) (Table 1), however the difference was not significant. There were no significant differences in the median number of spiral arteries observed in *nonAA samples* vs *AA samples* for either NC or PE groups. Spiral arteries with complete physiological transformation (Fig. 1a) were observed both in NC and PE samples (Table 1), with no significant difference in the fraction of arteries with complete physiological transformation per sample between PE and NC. In *AA samples*, the fraction of *AA arteries* per total arteries observed was similar between NC and PE (Table 1). In *AA samples*, we also frequently observed completely transformed arteries (60% of NC and 71% of PE cases). The fractions of completely transformed arteries per total arteries in *AA samples* were similar between NC and PE, and it was significantly lower than for *nonAA samples* for both NC and PE. Partially transformed spiral arteries (Fig. 1b) were less frequently observed, with no difference in frequency between study groups (Table 1). Arteries with an intact desmin-positive smooth muscle cell layer were rarely observed, and excluded as basal arteries because of their size (< 140 μm). None of these had AA lesions. We did not observe any non-remodeled arteries with a diameter > 140 μm.

Table 1
Observed distribution of spiral arteries and categories across the study groups. The total number of spiral arteries observed within each study group, as well as the number of nonAA and AA arteries. The median number (and range) of spiral artery sections observed per evaluated tissue section for each study group. NC = Normotensive controls. PE = preeclampsia. *nonAAsample*; sample lacking artery with acute atherosis (AA), *AA sample*; sample containing ≥1 artery with acute atherosis. The distribution (in percentage) of completely transformed, partially transformed and AA arteries of the total number of spiral arteries observed per tissue section is presented as median (and range) across the samples in each category. *NonAA arteries* = arteries with complete or partial physiological transformation without AA.

	NC n = 16, $n_{arteries}$ = 66		*PE n = 16, $n_{arteries}$ = 58*	
	nonAAsample n = 11	*AA sample n = 5*	*nonAAsample n = 9*	*AAsample n = 7*
Total number of arteries observed per study group, n =	42	24	27	31
nonAA arteries, n =	42	16	27	15
AA arteries, n =	–	8	–	16
Median number of spiral arteries observed per tissue section	4 (1–7)	6 (1–9)	2 (1–8)	3 (1–10)
% arteries with complete physiological transformation	100% (0–100)	33% (0–50)[a)]	100% (50–100)	33% (0–67)[b)]
% arteries with partial physiological transformation	0% (0–100)	17% (0-44)	0% (0-50)	0% (0-33)
% AA arteries	–	50% (11–100)	–	50% (17–100)

Statistical differences were calculated for the total group of normotensive controls (NC) against preeclampsia (PE), and within the NC and PE group for samples without acute atherosis (*nonAA sample*) against women with acute atherosis (*AA sample*). We compared medians of continuous variables by non-parametric Mann-Whitney *U* test. Differences were considered to be statistically significant when $p < 0.05$. [a)] Significantly different when comparing *nonAA sample* vs *AA sample* for NC group. [b)] Significantly different when comparing *nonAA sample* vs *AA sample* for PE group.

AA arteries were always associated with fibrinoid necrosis; visualized as areas of grey-pink PAS stain in the vessel wall, which were also bright red with MSB staining. These arteries lacked the bright purple fibrinoid PAS-stain of completely transformed spiral arteries (Fig. 1c). In contrast, red MSB stain was not observed in completely or partially transformed arteries (*nonAA arteries*), except for a few cases where some traces were observed. For most *AA arteries* we observed no intramural CK7-positive trophoblasts, and only remnants or complete

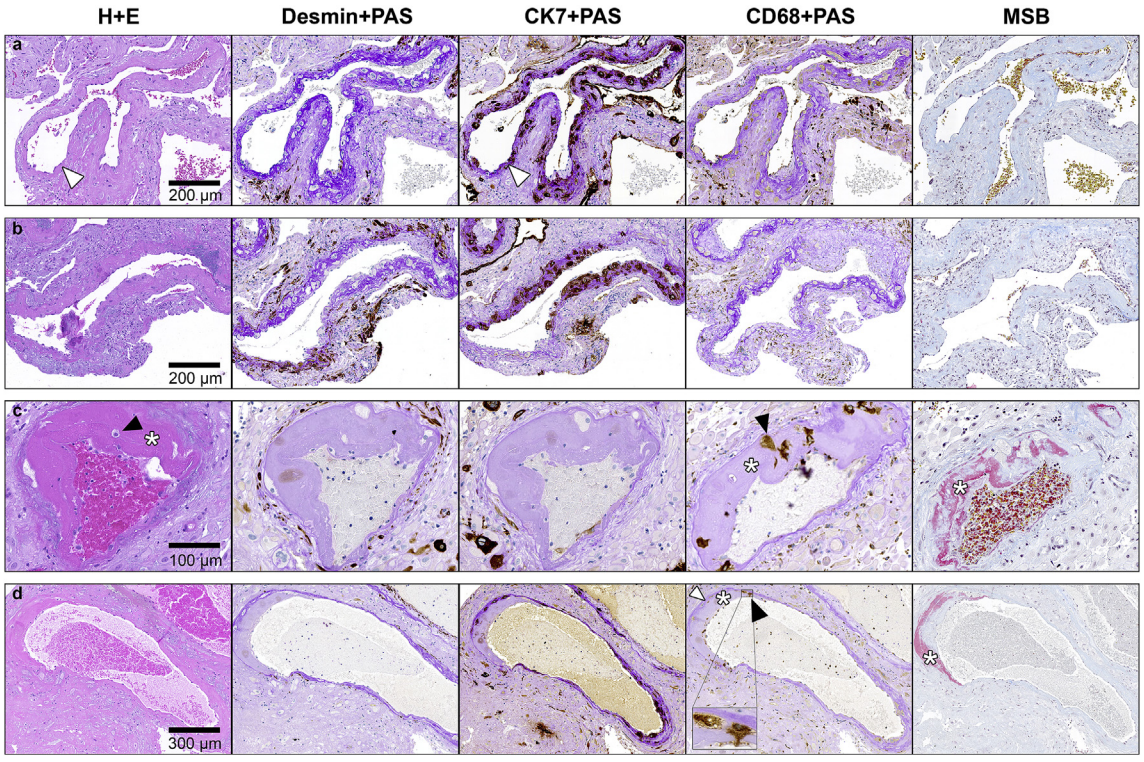

Fig. 1. Staining of serial FFPE sections of decidua basalis tissue to identify spiral arteries. Slides are stained with (from left to right) Hematoxylin + Eosin (H + E), desmin + PAS, CK7 + PAS, CD68 + PAS and Martius Scarlet Blue (MSB). Representative images of **a)** a spiral artery from a normotensive control with complete physiological transformation, characterized by presence of CK7-positive trophoblasts and intramural fibrinoid (bright purple upon PAS staining, white arrowhead) in the vessel wall, and complete absence of intramural smooth muscle cells (no desmin stain). **b)** Spiral artery from a PE patient with partial physiological transformation (both intramural fibrinoid and trophoblasts (CK7-positive) as well as areas with traces of mural smooth muscle cells (desmin-positive)). **c)** Spiral artery with acute atherosis from same sample as in b), lacking bright purple fibrinoid and CK7-positive trophoblasts in the vessel wall. Traces of intramural smooth muscle cells (desmin positive) are seen. Fibrinoid necrosis is visible as a grey-pink material in the vessel wall (asterisk), which stains red upon MSB staining (asterisk). Erythrocytes in the lumen of the AA artery stains red-brown upon MSB staining. Intramural CD68-positive foam cells are present (black arrowhead). **d)** Spiral artery from a PE patient with almost complete physiological transformation (lack of desmin-positive smooth muscle cells, presence of CK7-positive trophoblasts), yet acute atherosis lesion present (asterisk; fibrinoid necrosis, black arrowhead; foam cells, white arrowhead; purple physiological fibrinoid). Inset; higher power inset of foam cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

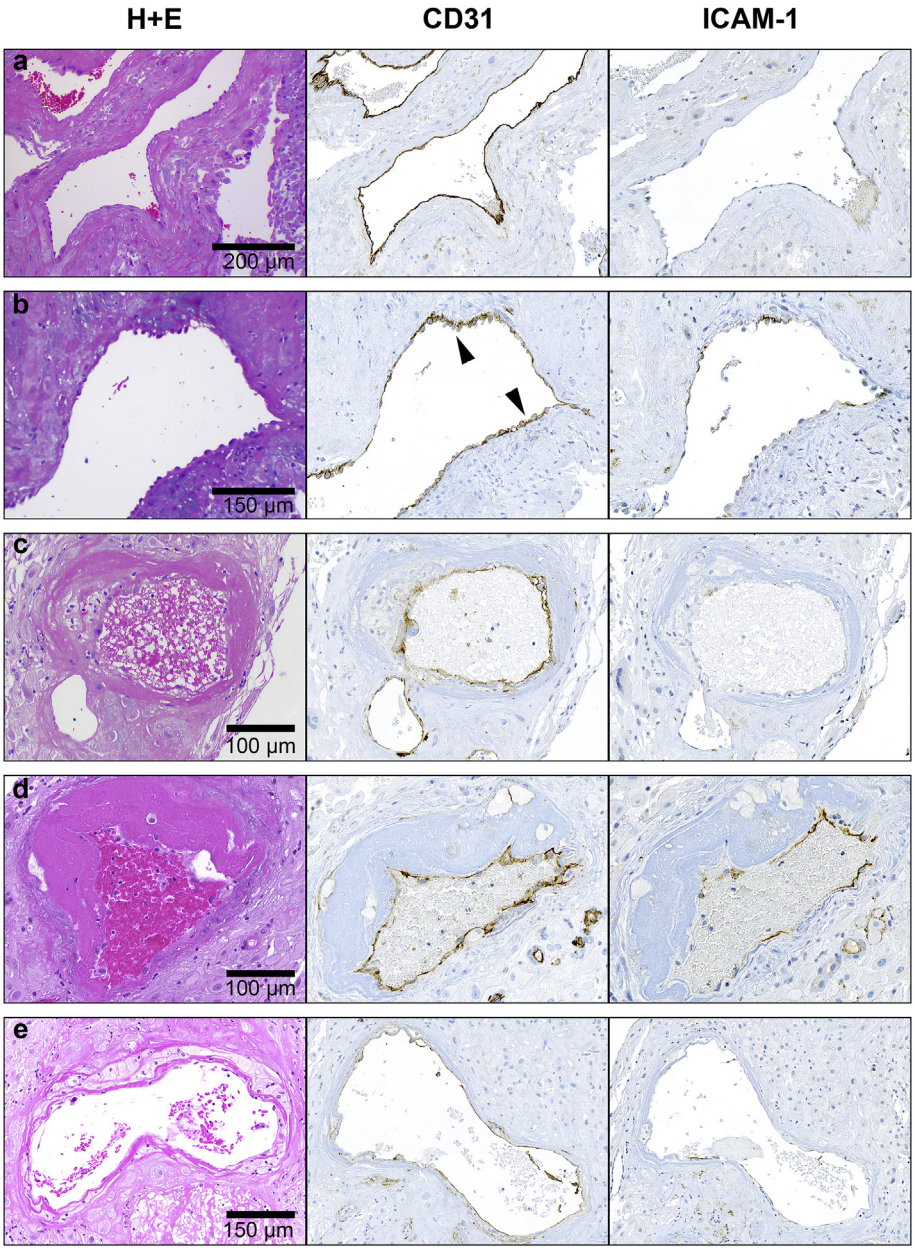

Fig. 2. Staining of serial FFPE sections of decidua basalis tissue to evaluate endothelial status and activation. Slides are stained with (from left to right) Hematoxylin + Eosin (H + E), CD31, and ICAM-1. a) and b) are vessels from the same PE patient sample. The full staining for vessel d) is shown in Fig. 1c **a)** NonAA-artery: normal endothelium with high intensity CD31 stain. Single ICAM-1-positive cells, but no endothelial activation. **b)** (PE) NonAA-artery: abnormal endothelium (arrowhead) in combination with normal endothelium. **c)** Example of AA-artery from a PE patient without endothelial activation: intact, but abnormal endothelium with low intensity CD31 stain. No ICAM-1 positive stain. **d)** Example of AA-artery with endothelial activation: intact, but abnormal endothelium with low intensity CD31 stain. Endothelial activation (≥3 consecutive ICAM-1-positive cells). **e)** (PE) AA-artery: destroyed endothelium, no endothelial activation.

absence of desmin-positive smooth muscle cells (Fig. 1c), as reported previously [1]. However, for three *AA arteries* across three different patients (2 PE, 1 NC) we observed foam cells and fibrinoid necrosis in combination with trophoblasts and normal PAS-positive purple fibrinoid in the same artery (Fig. 1d).

For spiral arteries with complete or partial physiological transformation, individual study variables were distributed similarly regardless of remodeling status (Supplementary Table 2), and these arteries were merged into one *nonAA artery* category. The subdivision of the *nonAA artery* category into *nonAA artery in nonAA sample* and *nonAA artery in AA sample* (was done in order to detect potential differences in the same vessel category (*nonAA artery*) between samples with and without AA arteries. Arteries from NC and PE were merged for plotting and statistical analysis, as they displayed a similar distribution across the individual study variables (Supplementary Tables 2 and 3).

3.2. AA is associated with an altered endothelial phenotype

Spiral artery endothelium was identified by positive CD31 and vWF staining. The histological appearance was classified as normal (Fig. 2a), abnormal, but not destroyed (Fig. 2b, c, d), or destroyed (Fig. 2e), as defined in the methods section. The distribution of the three spiral artery categories across the endothelial subtypes is shown in Fig. 3a.

NonAA arteries, both in *nonAA samples* and *AA samples,* were equally distributed between a normal and abnormal endothelial cell pattern and few had a destroyed phenotype (Fig. 3a and Supplementary Table 2). In contrast, significantly less of the arteries with AA (*AA arteries in AA sample*) had a normal endothelial phenotype, and most of the *AA arteries* (50%) were categorized as abnormal. For the remaining 30% of the *AA arteries* a destroyed phenotype was observed, which was significantly higher relative to both *nonAA artery* categories (Fig. 3a).

3.3. AA is associated with weaker CD31 staining of abnormal endothelium

There were differences in CD31 staining intensity between arteries (Fig. 2), and CD31 staining intensity of areas of intact endothelium was categorized as either high or low (Supplementary Fig. 2), as outlined in the methods section. The percentage of vessel endothelium with a low

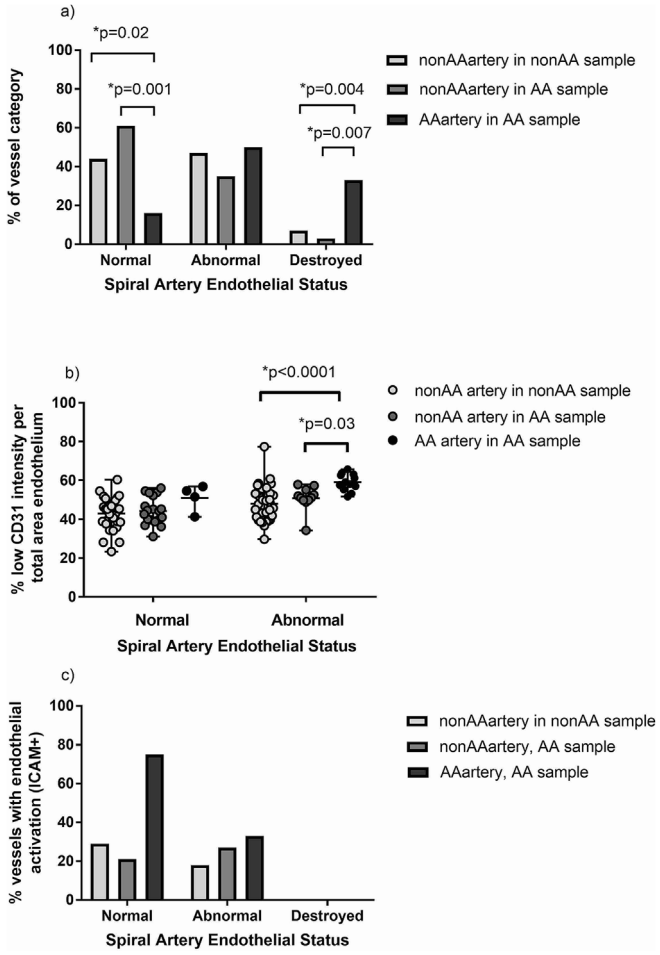

Fig. 3. Spiral artery and endothelial cell characterization across vessel categories. **a)** Distribution of spiral artery categories across the endothelial cell phenotypes (Normal, abnormal and destroyed). Bars show the percentage of arteries in each artery category associated with each endothelial subtype. *NonAA-arteries* are shown as light grey bars, *nonAA-arteries in AA samp*les are shown as darker grey bars and *AA-arteries (in AA samples)* are shown as black bars. **b)** Percentage of area of low CD31 intensity relative to total area of endothelium per vessel for each artery subtype across the normal and abnormal endothelial cell categories. **c)** Percentage of vessels with positive ICAM-1 stain (≥3 consecutive cells) for each artery subtype across endothelial cell categories.

CD31 intensity relative to total area of endothelium for each vessel across different spiral artery categories and endothelial subtypes is shown in Fig. 3b. Quantification of CD31 intensity for the destroyed phenotype was not performed, as the endothelial cells were destroyed and disintegrated. For the normal endothelial phenotype, no significant differences in CD31 intensity were observed between any of the spiral artery categories (Fig. 3b). For the abnormal endothelial phenotype, *AA arteries* had a higher percentage of areas with low CD31 intensity relative to total endothelium per vessel. This was significantly more than for both *nonAA artery in nonAA sample* and *nonAA artery in AA sample* categories (Fig. 3b), however the endothelial lining was still intact and present for all vessel categories.

The vWF staining of endothelial cells appeared in general weaker than for CD31, possibly because of the PAS counterstain (Supplementary Fig. 3). We did not observe a clear difference in staining intensity for vWF between *nonAA* and *AA arteries*, unlike our observations regarding CD31.

3.4. Endothelial activation is not detectable in a majority of AA vessels

Almost all decidua basalis spiral arteries had some scattered ICAM-1-positive endothelial cells, so endothelial activation was defined as presence of ≥3 adjacent ICAM-1-positive endothelial cells (Fig. 2d), as used previously in a study on atherosclerosis [17]. None of the vessels with destroyed endothelium could be evaluated for ICAM-1, as the endothelium was destroyed and disintegrated. Within the normal endothelial phenotype there was a tendency towards higher endothelial activation in the *AA arteries* (constituting 16.7% of all AA arteries), however the numbers were very low (only 4 observations, of which 3 were positive), and the difference not statistically significant.

There were no significant differences in endothelial activation between the spiral artery categories within the abnormal endothelial phenotype (Fig. 3c), only 4/12 (33%) of the AA vessels with abnormal phenotype had endothelial activation.

4. Discussion

The characteristics of AA at different sites in the uteroplacental unit have been investigated previously [18–21]. However, only a few studies [19,20] have histologically explored the morphology of AA by thoroughly characterizing spiral arteries in the decidua basalis. Our study of samples collected by vacuum suction after removal of the placenta during elective cesarean section is unique.

Our first observation is that AA arteries are associated with an altered endothelial phenotype. A majority of the *AA arteries* (50%) were associated with abnormal endothelium with weaker CD31 staining relative to arteries without AA, or a damaged endothelium (30% of AA arteries). Endothelial damage associated with AA has previously been described both in the myometrium, decidua basalis [21,22], as well as in the decidua parietalis, as observed by Hecht et al. [20]. Hecht et al. also used endothelial markers; however they did not report alterations in staining intensity [20]. Positive red staining with MSB in the AA artery wall, as observed by us and others [7,19], indicates deposition of fibrin (or fibrin-like material) most likely caused by a leakage of factors from the maternal circulation into the vessel wall possibly because of an altered endothelial phenotype [22].

The causes of reduced CD31 staining in AA arteries require further investigation. The reduced staining could indicate that the CD31-positive cells are in a state of disintegration, which could also affect ICAM-1 staining. However, in some cases we observed both weak CD31 stain as well as positive ICAM-1 stain. Weaker CD31 staining in AA arterial endothelium could indicate an altered endothelium as a first response to damaging or inflammatory stimuli. Cellular stress responses could affect global protein translation [23] causing lower CD31 staining. This would not affect ICAM-1 translation as this protein is selectively translated during stress conditions via the ATF4 transcription factor. As we do not observe clear reduction in vWF staining, it could indicate that the reduction in staining is not caused by general endothelial cell degeneration.

We were in general not able to find an association between endothelial activation (ICAM-1-positive stain) and presence of AA in decidua basalis arteries. For arteries with abnormal endothelium (constituting 50% of the AA arteries) endothelial activation was only observed in 25% of the arteries. In 30% of the AA arteries (destroyed phenotype) we could not assess ICAM-1 staining because the endothelial cell lining was completely disintegrated or partly lacking). For AA arteries with a normal endothelial phenotype (constituting only 16.7% of AA arteries), a trend towards higher ICAM-1 staining was observed; 3/4 (75%) were ICAM-1-positive, however because of the low numbers of observations the trend was not significant. Even though we did observe endothelial activation in some AA vessels, especially for the normal phenotype, it rarely affected the whole circumference of the vessel, and it was not clearly associated with the location of the foam cells embedded in the vessel wall. The overall histological appearance

of AA arteries differs from atherosclerotic lesions where an intact endothelial cell layer is observed with clear ICAM-1-positive staining, and without deposition of fibrin, even in the advanced stages of the lesion [11,17,24,25]. In summary, our data suggest that the decidual basalis AA lesion is associated with an altered endothelium; however it differs histologically from the observations found in atherosclerosis. This is in line with the conclusions of Katabuchi et al., in their characterization of the AA lesion [19]. Even though AA in our material is histologically dissimilar from atherosclerosis, we cannot exclude from our findings that ICAM-1-positive staining is present at an earlier stage of the AA lesion formation.

A recent study of endothelial activation in spiral arteries from the placental basal plate [5] showed that for arteries in preeclamptic samples, with complete failure of remodeling, both the arterial endothelium and the surrounding trophoblasts labelled for ICAM-1. AA was only observed in placentas harboring ICAM-1-positive vessels; however the investigators did not report whether ICAM-1-positive endothelial cells and AA localized in the same artery, nor the endothelial status. In contrast, we observed no ICAM-1-positive trophoblasts in the decidua basalis, and very few arteries with complete failure of physiological transformation (intact smooth muscle cell layer and absence of intramural trophoblasts), none with a diameter > 140 μm. We did not assess ICAM-1 status for excluded vessels. A further difference from this and other reports [15,26], is that we find that spiral arteries in decidual samples from PE patients in our cohort were predominantly fully remodeled. It is possible that the absence of unremodeled arteries in PE samples in our study could be because the PE cases included are less severe, as we have a mix of early and late PE with and without fetal growth restriction, with no preexisting hypertension. The observation of completely unremodeled spiral arteries in the placental basal plate at term by Labarrere et al. [5] would point towards a more severe preeclampsia phenotype. However, the clinical data for the PE group and information of co-morbidities are not included in the Labarrere paper [5]. A previous study by Tziotis et al., did not find any significant difference in level of ICAM-1 and other markers of endothelial activation between preeclampsia and normotensive pregnancies in samples from the placental bed [27].

Our finding of lack of a smooth muscle cell layer in AA vessels is in line with a previous report by Katabuchi et al. [19]. The processes causing the lack of smooth muscle cells in these vessels cannot be established from our work, but smooth muscle cells necrosis or apoptosis is likely a major cause, as suggested by Katabuchi et al. We cannot exclude that the smooth muscle cell layer was present at the time of lesion initiation. In line with existing literature [26], we observe a lack of intramural trophoblasts in arteries with AA lesions [1]. The lack of intramural trophoblasts and trophoblast-derived fibrionid indicate that the vessels were not remodeled prior to AA formation.

It is noteworthy that we observe AA arteries (lacking intramural trophoblasts) and completely transformed arteries (with intramural trophoblasts) in close proximity to one another. In completely transformed arteries, bright purple PAS-positive fibrinoid is observed in the vessel wall, which is most likely trophoblast-derived [8,22]. In contrast, AA arteries lack the purple PAS-positive fibrinoid, instead fibrinoid necrosis (grey-pink PAS-stain) is observed, which also stains bright red with MSB staining. In a few cases, we observed a mix of both features, with part of the vessel displaying full physiological transformation with intramural trophoblasts, and the other part having foam cells and red MSB stain in-between the endothelium and a normal outer vessel wall. This suggests that local factors cause AA since neighboring arteries display a normal physiological remodeling status, and in some cases only part of the vessel is affected.

Our data from vacuum suction samples of decidua basalis complements earlier studies of placenta bed biopsies, placenta basal plates and decidua parietalis tissue. The major strength of this study is our vacuum suction method, which yields a higher number of spiral artery sections compared to placenta bed biopsies and placenta surface biopsies [3]. Our systematic immunohistochemical and histological staining enables a clear visualization of the histological and cell specific composition of AA lesions and neighboring arteries. Of necessity, our study is of a relatively low number of patients, owing to the extensive staining and evaluation protocol.

In summary, our observations indicate that AA is a focal lesion, associated with an altered endothelial phenotype and lack of intramural trophoblasts in the affected arteries only. We are not able to establish whether an altered endothelial phenotype precedes and potentially inhibits endovascular trophoblast invasion, or if lack of trophoblast invasion would lead to endothelial alteration and AA formation. As AA is also observed in spiral arteries of decidua parietalis, where no trophoblast invasion takes place, it is tempting to speculate that the endothelial alterations may be the initiating events. We were not able to detect ICAM-1 positive staining of the endothelium in a majority of AA arteries, dissimilar from what is observed for atherosclerosis.

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.04.006.

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Supplemental Material

Acute atherosis of decidua basalis; characterization of spiral arteries, endothelial status and activation

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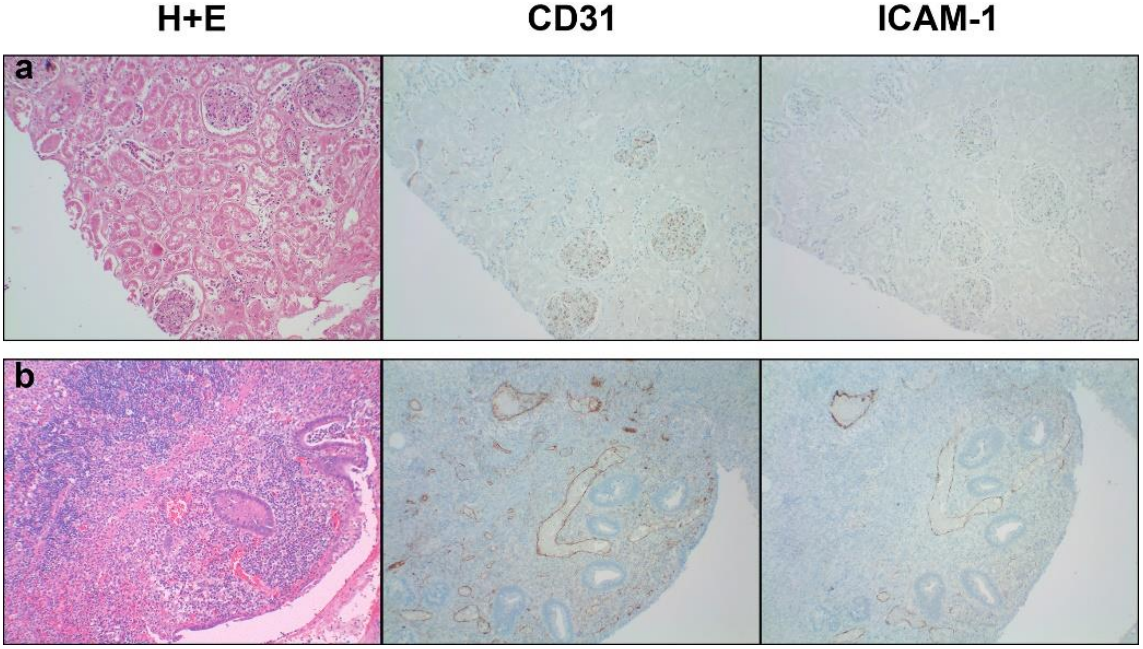
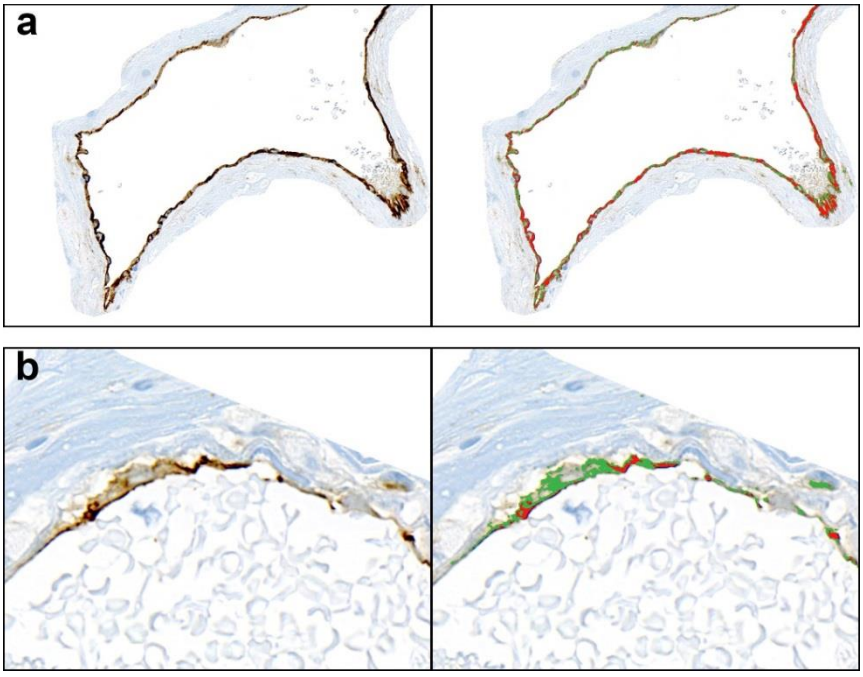
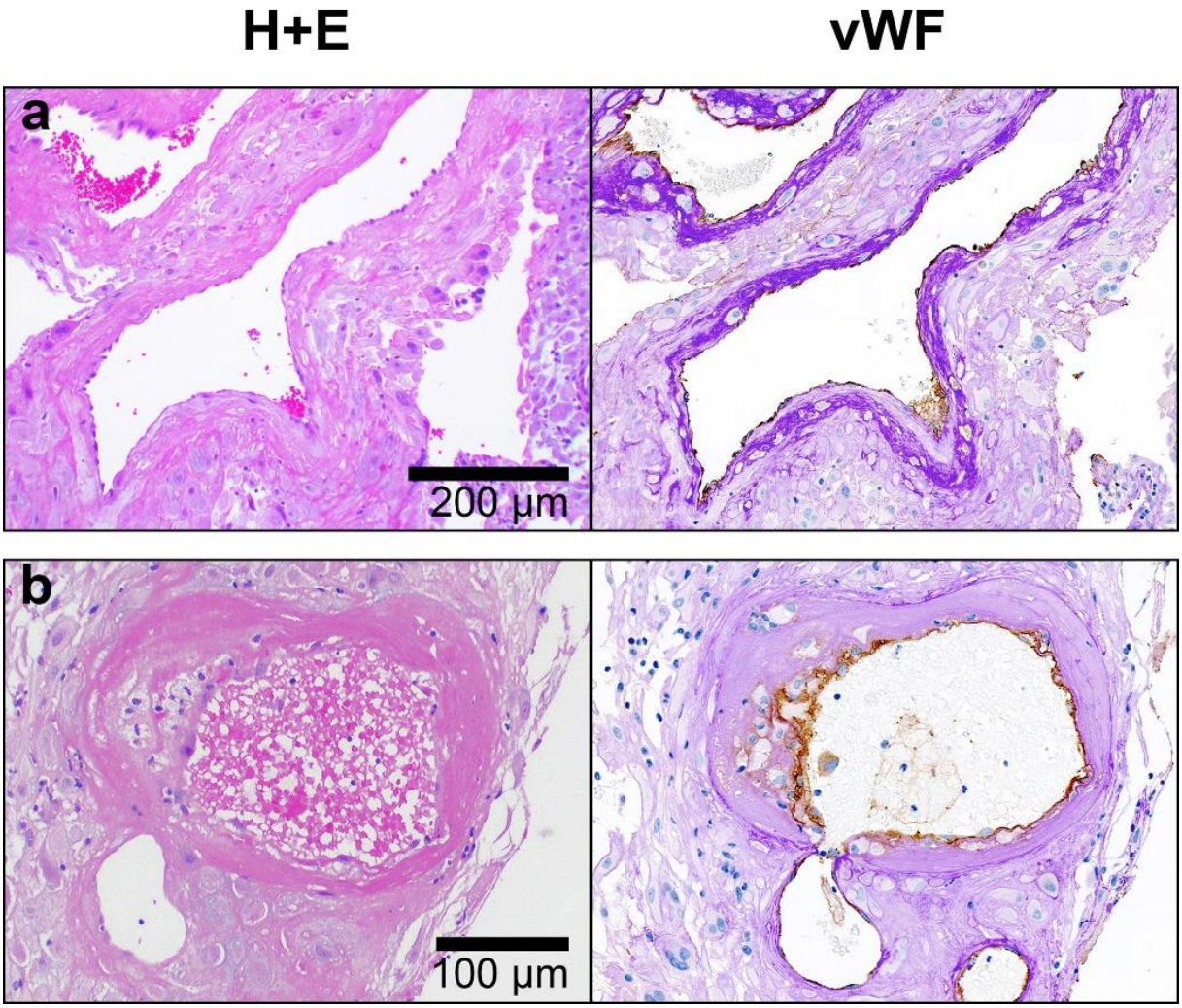
Supplementary figure 1. Positive and negative control tissue. Slides are stained with (from left to right) Hematoxylin+Eosin, CD31, and ICAM-1. Images of **a)** kidney tissue, CD31 positive endothelium visible, but no endothelial activation (ICAM-1 negative), **b)** appendicitis, vessels with endothelium visible (CD31 positive) with endothelial activation (ICAM-1 positive).



Supplementary figure 2. Image analysis of CD31 intensity. The Image analysis module of the Zen Blue software was used to identify the areas of High (red) and low (green) CD31 intensity for each vessel. The software determined the area of each category (high/low intensity), and a ratio of low intensity to total area of endothelium was calculated. Images of **a)** spiral artery with predominantly high intensity of stain, **b)** spiral artery with predominantly low intensity of stain.



Supplementary figure 3. Staining of serial FFPE sections of decidua basalis tissue to evaluate endothelium. Slides are stained with (from left to right) Hematoxylin+Eosin (H+E), von Willebrand factor (vWF) counterstained with periodic acid-Schiff, to identify endothelium in combination with CD31. Images of **a)** nonAA-artery with normal endothelium, **b)** AA-artery with abnormal endothelium.



Supplementary Table 1: Clinical characteristics of the women participating in the study. Gestational age at delivery was defined by routine ultrasound screening at gestational week 17-20. Sex-specific newborn weight percentiles were calculated according to Norwegian fetal growth curves [1]. Small for gestational age was defined as a birth percentile below the 10th percentile. Fetal growth restriction (FGR) was defined as abnormal Doppler values [2] and/or a sex-specific newborn weight below the 3rd percentile, excluding pregnancies with fetal structural/chromosomal abnormalities.

	Normotensive controls (NC)			***Preeclampsia (PE)***			***P-value***		
	NC n=16	***NCAA- n=11***	***NCAA+ n=6***	***PE n=16***	***PEAA- n=9***	***PEAA+ n=7***	***NC vs PE***	***NCAA- vs NCAA+***	***PEAA- vs PEAA+***
Gestational age at delivery (weeks)	39	39	38.9	34.3	34.1	34.9	<.001*	.510	.606
Preterm delivery <37 weeks	6.3% (1/16)	0 %	20% (1/5)	87.5%	88.9%	85.7%	<.001*	0.313	>.999
Early onset PE <34 weeks				37.5%	33.3%	42.8%	-	-	>.999
New born weight (g)	3433	3472	3240	2124	2163	2085	<.001*	.145	.536
New born weight percentile	51.3	68.4	48.7	2.2	2.1	2.3	<.001*	.510	.837
Small for gestational age	0 %	0 %	0 %	75 %	66.7%	85.7%	<.001*	>.999	.585
Fetal growth restriction	0 %	0 %	0 %	68.8%	66.7%	71.4%	<.001*	-	>.999
Mean systolic BP at delivery (mmHg)	121	118	125	166	167	165	<.001*	.441	.758
Mean diastolic BP at delivery (mmHg)	70	70	70	100	100	100	<.001*	.827	.606
Maternal age at delivery (years)	33.5	34	31	35,5	39	34	.239	.743	.681
BMI, pre-pregnancy (kg/m2)	22.1	21.7	23.2	22.6	22.3	24.8	.724	.913	.351
Mean systolic BP <20 weeks (mmHg)	111.5	113	110	115.5	115	116	.341	.377	.837
Mean diastolic BP <20 weeks (mmHg)	69.5	69	72	72	75	69	.305	.221	.536
First pregnancy	50%	45.5%	60 %	37.5%	33.3%	42.9%	.722	>.999	>.999
First delivery	56.3%	45.5%	80 %	56.3%	55.6%	57.1%	>.999	.308	>.999
Previous PE	13.3%	10 %	20 %	28.6%	22.2%	40 %	.390	>.999	.580

Statistical differences were calculated for the total group of normotensive controls (NC) against preeclampsia (PE), and within the controls and PE group for women without acute atherosis (AA-) against women with acute atherosis (AA+). We compared medians of continuous variables by non-parametric Mann-Whitney U test, and frequencies of categorical variables by Fishers exact test. Differences were considered to be statistically significant when $p<0.05$.

Supplementary table 2. Distribution of the spiral artery categories across the endothelial cell subtypes. Data are presented for both normotensive controls (NC) and preeclampsia (PE) and for both diagnosis categories merged (NC+PE). The samples are categorized as *nonAA sample* (no acute atherosis (AA) arteries in sample) or *AA samples* (≥1 AA artery present in sample). The spiral arteries identified in each sample are classified as arteries with complete spiral artery remodeling (Fully), partial spiral artery remodeling (Partially) or AA arteries. The Fully and Partially categories are also merged as a *NonAA artery* category. Data are shown as the number of spiral arteries in each category and % of total arteries in the category.

Clinical diagnosis	*AA vessels present in sample (yes/no)*	*Spiral artery subtype*	*Endothelial status (#, % of total)*			*Total arteries*
			Normal	*Abnormal*	*Destroyed*	
NC	**nonAA sample**	Fully	18 (54.5%)	13 (39.4%)	2 (6.1%)	33
		Partially	5 (55.6%)	3 (33.3%)	1 (11.1%)	9
		nonAA artery (Fully+Partially)	**23 (54.8%)**	**16 (38.1%)**	**3 (7.1%)**	**42**
	AA sample	Fully	4 (44.4%)	5 (55.6%)	0	9
		Partially	3 (42.9%)	4 (57.1%)	0	7
		nonAA artery (Fully+Partially)	**7 (43.8%)**	**9 (56.3%)**	**0 (0%)**	**16**
		AA artery	**0** [a)]	**4 (50.0%)**	**4 (50.0%)** [b) c)]	**8**
PE	**nonAA sample**	Fully	6 (30.0%)	12 (60.0%)	2 (10.0%)	20
		Partially	2 (28.6%)	5 (71.4%)	0	7
		nonAA artery (Fully+Partially)	**8 (29.6%)**	**17 (63%)**	**2 (7.4%)**	**27**
	AA sample	Fully	8 (72.7%)	2 (18.2%)	1 (9.1%)	11
		Partially	4 (100.0%)	0	0	4
		nonAA artery (Fully+Partially)	**12 (80%)**	**2 (13.3%)**	**1 (6.7%)**	**15**
		AA artery	**4 (25.0%)**	**8 (50.0%)**	**4 (25.0%)**	**16**
						124
NC+PE	**nonAA sample**	Fully	24 (45.3%)	25 (47.2%)	4 (7.5%)	53
		Partially	7 (43.8%)	8 (50%)	1 (6.3%)	16
		nonAA artery (Fully+Partially)	**31 (44.9%)**	**33 (47.8%)**	**5 (7.2%)**	**69**
	AA sample	Fully	12 (60.0%)	7 (35%)	1 (5%)	20
		Partially	7 (63.6%)	4 (36.4%)	0	11
		nonAA artery (Fully+Partially)	**19 (61.3%)**	**11 (35.5%)**	**1 (3.2%)**	**31**
		AA artery	**4 (16.7%)**	**12 (50.0%)**	**8 (33.3%)**	**24**
						124

[a)] Significantly different (p=0.005) when comparing *AA artery* to *NonAA artery in nonAA sample* for the "Normal" endothelial phenotype within the normotensive controls. [b)]Significantly different (p=0.004) when comparing *AA artery* to *NonAA artery in nonAA sample* and [c)] *NonAA artery in AA sample* (p=0.007) for the "Destroyed" endothelial phenotype within the normotensive controls. Fishers exact test.

Supplementary table 3. Distribution of vessels with endothelial activation (>3 ICAM+ cells) in the different spiral artery categories across the endothelial cell subtype categories. Data are presented for both normotensive controls (NC) and preeclampsia (PE) as well as both diagnosis groups merged (NC+PE). The samples are categorized as *nonAA sample* (nonAA arteries in sample) or *AA samples* (≥1 AA artery present in sample). The spiral arteries identified in each sample are classified as vessels without AA (*nonAA artery*) (combination of spiral arteries with complete or partial spiral artery remodeling) or *AA arteries*. Data are shown as the number of spiral artery in each category with endothelial activation and % of total spiral arteries in the category.

Clinical diagnosis	***AA status sample***	***Spiral artery subtype***	***Endothelial activation (#, % activated)***			***Total***
			Normal	***Abnormal***	***Destroyed***	
NC	**nonAA sample**	**nonAA artery**	6/23 (26.1%)	1/16 (6.3%)	0/3 (0%)	7/42 (16.7%)
	AA sample	**nonAA artery**	1/7 (16.7%)	2/9 (22.2%)	0/0 (-)	3/16 (18.8%)
	AA sample	**AA artery**	0/0 (-)	3/4 (75%)	0/4 (0%)	3/8 (37.5%)
						13/66 (19.7%)
PE	**nonAA sample**	**nonAA artery**	3/8 (37.5%)	5/17 (29.4%)	0/2 (0%)	8/27 (29.6%)
	AA sample	**nonAA artery**	3/12 (25%)	1/2 (50%)	0/1 (0%)	4/15 (26.7%)
	AA sample	**AA artery**	3/4 (75%)	1/8 (12.5%)	0/4 (0%)	4/16 (25%)
						16/58(27.6%)
NC+PE	**nonAA sample**	**nonAA artery**	9/31 (29.0%)	6/33 (18.2%)	0/5 (0%)	15/69 (21.7%)
	AA sample	**nonAA artery**	4/19 (21.1%)	3/11 (27.3%)	0/1 (0%)	7/31 (22.6%)
	AA sample	**AA artery**	3/4 (75%)	4/12 (33.3%)	0/8 (0%)	7/24 (29.1%)

Supplemental References

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Decidua basalis and acute atherosis: Expression of atherosclerotic foam cell associated proteins

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ABSTRACT

Introduction: Uteroplacental acute atherosis is frequently observed in preeclampsia, and shares features with early atherosclerotic lesions, including artery wall foam cells. The lipid-associated proteins FABP4 (fatty acid binding protein 4), perilipin-2, and LOX-1 (lectin-like oxidized LDL-receptor 1) are involved in atherosclerotic foam cell formation. Increased levels of these proteins have been associated with preeclampsia systemically and in placental tissue. Their role in acute atherosis is yet unidentified. Our aim was to describe the presence of these proteins in acute atherosis, and compare our findings to what is known in early atherosclerotic lesions.

Methods: Serial sections of decidua basalis tissue from 12 normotensive (4 with acute atherosis) and 23 pre-eclamptic pregnancies (16 with acute atherosis) were stained with HE and immunostained for CK7, CD68, FABP4, perilipin-2, and LOX-1. Artery wall and perivascular protein expression was assessed in 190 spiral artery sections; 55 with acute atherosis.

Results: Acute atherosis foam cells were commonly positive for perilipin-2 (55%), less often for FABP4 (13%), and never for LOX-1. LOX-1 was frequently observed in intramural trophoblasts of normal spiral arteries. Perivascularly, LOX-1 positivity of decidual stromal cells surrounding arteries with acute atherosis was significantly increased as compared to arteries lacking acute atherosis (38% vs. 15%, $p < 0.001$).

Discussion: We found that perilipin-2 and FABP4 are expressed by acute atherosis foam cells, similar to atherosclerosis, supporting possible shared pathways for foam cell generation. Unlike atherosclerosis, LOX-1 is not present in acute atherosis, possibly explained by pregnancy-specific routes to decidua basalis foam cell generation.

1. Introduction

Acute atherosis (AA) is a lesion of the uteroplacental spiral arteries, which occurs frequently in preeclamptic pregnancies, but is also seen in normotensive pregnancies [1,2]. AA lesions were classically defined by intramural foam cells and fibrinoid necrosis, and a mononuclear perivascular infiltrate [3]. The qualitative and quantitative details of these morphological entities are however lacking in relation to acute atherosis. Fibrinoid necrosis has been defined as "a grayish-bluish color" upon Periodic acid-Schiff stain [4] or, in relation to acute atherosis, as "a complete or partial fibrin ring" [5]. Perivascular infiltrate in decidual acute atherosis has by us been defined as "a focal perivascular accumulation of lymphocytes (within 50 μm to the outer margin of the spiral artery), more dense than in the surrounding tissue" [1]. Our previously published criterion for acute atherosis using presence of ≥2 adjacent vacuolated CD68-positive foam cells has proven reliable and useful when comparing the rates of acute atherosis across studies, pregnancy outcomes, and different tissue sources [1,6].

The causes and consequences of acute atherosis in preeclampsia and other obstetric syndromes mediated by placental dysfunction are incompletely understood [7], although inflammation is likely a key factor in the development of the lesion [8]. As revised by us recently [7], acute atherosis and graft vascular disease share morphological similarities that have been highlighted for several decades [8–10], although subendothelial foam cell lesions are less prominent in graft vascular disease.

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Women with a history of preeclampsia have an increased risk of cardiovascular disease later in life [11]. Our recent work has shown that AA is associated with dyslipidemia (i.e. increased Low Density Lipoprotein and Apolipoprotein B), a classical cardiovascular risk factor, in older pregnant women [12]. AA occurs more frequently in preeclamptic pregnancies, and we have previously suggested that acute atherosis detection could represent a supplementary biomarker predicting future risk for maternal cardiovascular disease [13,14].

Atherosclerosis is the main histopathological lesion of major cardiovascular diseases [15]. AA shares some morphological features with early stages of atherosclerosis ("fatty streaks") [16], including presence of artery wall foam cells. Both lesions are characterized by inflammation, oxidative stress, and dyslipidemia [8]. There are several differences between the lesions, including differences in the size of affected arteries [8] and molecular composition [7,17–20], suggesting possible differing mechanisms of lesion development. Foam cells of atherosclerotic lesions accumulate cholesterol esters, triglycerides, and phospholipids in cytoplasmic lipid droplets [21]. These lipid droplets express several proteins, with perilipin-2, also named adipophilin and adipose differentiation-related protein, being the most abundantly expressed [22]. Perilipin-2 expression increases during foam cell formation [23], leading to increased lipid accumulation while blocking cholesterol efflux [22,24]. Absence of perilipin-2 has been found to be atheroprotective in mouse models [25]. Atherosclerotic lesions also express the lipid-handling proteins FABP4 (fatty acid binding protein 4) and LOX-1 (lectin-like oxidized LDL-receptor 1) [26–29]. FABP4 is involved in intracellular lipid transport and is associated with atherosclerosis both locally (in macrophages and endothelial cells [27,29]) and systemically [27,29,30]. Increased FABP4 expression in advanced atherosclerotic lesions has been linked to plaque instability [27,31]. LOX-1 is a receptor-bound membrane glycoprotein which binds and internalizes oxidized LDL (oxLDL) [32], promoting lipid accumulation and foam cell formation by both smooth muscle cells and macrophages [33,34]. In atherosclerotic lesions, LOX-1 is located to endothelial cells (particularly in early atherosclerotic lesions), macrophages, and intimal smooth muscle cells [26,28]. Like FABP4, increased LOX-1 promotes plaque instability [35].

In decidua basalis, the major maternal-fetal tissue interface, the levels of phospholipids and cholesterol [36] are increased in preeclampsia compared to normotensive pregnancies. Furthermore, Brosens et al. [37] recently demonstrated by Oil Red O staining that increased amounts of intra- and extracellular lipids in decidua basalis is associated with chronic hypertension and preeclampsia. FABP4, perilipin-2, and LOX-1 expression is also increased in placentas of preeclamptic women [38–40], and a recent study found increased levels of circulating FABP4, measured in early pregnancy, to represent an independent predictor of preeclampsia [41]. The presence and possible role of these proteins in decidua basalis acute atherosis is not known. While the mechanisms of atherosclerotic lesion development are well studied, including the roles of FABP4, perilipin-2, and LOX-1 [22,26–30,33], the mechanisms of AA development remain unclear [7]. The aim of this study was thus to assess whether decidua basalis acute atherosis lesions express lipid associated protein markers known to be of importance in the development of atherosclerotic foam cell lesions of larger arteries.

2. Materials and methods

2.1. Recruitment of pregnant women and decidua basalis tissue collection

Pregnant women (n = 35) were recruited prior to delivery to Oslo Pregnancy Biobank at Oslo University Hospital, as described previously [1]. All gave informed written consent. The study was approved by the Regional committee for Medical and Health Research Ethics in South-Eastern Norway, and conducted in accordance with the principles of the Helsinki Declaration.

Preeclampsia was defined as new onset hypertension (blood pressure ≥140/90 mmHg) and proteinuria (≥1+ on dipstick or ≥30 total protein/creatinine ratio) at ≥20 weeks' gestation [42]. Women with preeclampsia (n = 21) and chronic hypertension with superimposed preeclampsia (n = 2) were combined to one preeclampsia group (n = 23) for statistical analyses. None of the included women had chronic diseases, except for the two women in the preeclampsia group with chronic hypertension.

Gestational age at delivery was defined by routine ultrasound screening at gestational week 17–20. Sex-specific newborn weight percentiles were calculated according to Norwegian fetal growth curves [43]. Small for gestational age was defined as a birth percentile below the 10th percentile.

None of the women had regular uterine contractions or signs of infection, and fetal membranes were intact at time of delivery. Decidual tissue was collected by vacuum suction of the placental bed following caesarean delivery, as described previously [44]. Collected tissue was formalin fixed and paraffin embedded (FFPE). One decidua basalis biopsy was evaluated per pregnancy, as presented and evaluated previously for spiral artery detection rates [6].

Table 1 displays the clinical characteristics of the clinical groups. The clinical characteristics were similar, except for the expected group differences, including significantly higher BMI and blood pressures, as well as lower gestational age at delivery, lower fetal birthweight and birthweight percentile for the preeclampsia group as compared to the normotensive group (all $p < 0.05$).

2.2. Decidua basalis standard staining, immunohistochemistry, and evaluation

For all women, serial 3 μm FFPE decidua basalis sections were stained with Hematoxylin-Eosin (HE) using standard histological technique, cytokeratin 7 (CK7, M7018, Dako, USA, 1:300, counterstained with Periodic acid-Schiff [PAS]), CD68 (M081401, Dako, USA, 1:1500, counterstained with PAS), FABP4 (HPA002188, Sigma-Aldrich, USA, 1:1000), perilipin-2 (651102, Progen, Germany, 1:300), and LOX-1 (MABS186, Millipore, USA, 1:750). The following positive controls were used to verify antibody specificity; CK7: placenta, CD68: appendicitis, FABP4: placenta, perilipin-2: liver, LOX-1: placenta. As negative control, non-immune mouse ascites served as primary antibody.

Decidua basalis FFPE slides were scanned with Axio Scan slide scanner and evaluated using Zen Blue 2.6 (Zeiss) for presence of spiral arteries and acute atherosis, as previously described [1]; spiral arteries with ≥2 intramural adjacent CD68-positive vacuolated cells (foam cells). Presence of decidual stromal cells and CK7-positive trophoblasts confirmed decidua basalis tissue. Vessels <140 μm in diameter were not analyzed so as to ensure the exclusion of smaller basal arteries. Artery sections with AA were categorized as *AA arteries*, while artery sections without AA were categorized as *non-AA arteries*. Samples with ≥1 *AA artery* were categorized as *AA samples*. Samples without *AA arteries* were categorized as *non-AA samples*.

We included decidua basalis tissue samples from 12 normotensive pregnancies (of which 4 *AA samples*), 23 preeclamptic pregnancies (of which 16 *AA samples*). We chose to over-represent samples with AA compared to the prevalence of the lesions for our pregnancy groups [1,2] as the focus of this study was foam cell characterization. Artery wall foam cells were evaluated on adjacent serial sections for FABP4, perilipin-2, and LOX-1 staining.

In addition to describing the presence of the lipid-associated proteins in the foam cell lesions of the spiral artery wall, we evaluated their presence in the perivascular area of the same arteries (designated by us as a perimeter around the vessel, expanding 100 μm beyond the outer margin of the vessel wall, described previously [20]). We also evaluated the vessel wall and perivascular areas of *non-AA arteries*. The perivascular staining patterns of FABP4, perilipin-2, and LOX-1 were classified as "no stain" (no immunopositive cells), "scattered" (single positive cells seemingly without relation to one another), "focal" (≥5

Table 1
Clinical characteristics of the study groups (n = 35 women).

	Normotensive			Preeclampsia		
	All n=12	*Non-AA-samples n=8*	*AA-samples n=4*	*All n=23*	*Non-AA-samples n=7*	*AA-samples n=16*
Maternal age at delivery (years)	32.6	32.6	35.5	32.7	32.7	33.0
BMI, pre-pregnancy (kg/m^2)	22.5	21.9	23.0	26.0	30.4	24.8
Mean systolic BP < 20 weeks (mmHg)	110	110	107	116	109	120
Mean diastolic BP < 20 weeks (mmHg)	65	65	69	70	70	70
First pregnancy	33%	38%	25%	48%	57%	44%
First delivery	50%	50%	50%	61%	71%	56%
Previous preeclampsia[a)]	17%	0%	50%	56%	50%	57%
Mean systolic BP at delivery (mmHg)	120	121	120	160	150	172
Mean diastolic BP at delivery (mmHg)	70	73	70	100	95	105
Gestational age at delivery (days)	272	270	273	232	238	232
Preterm delivery (<37 weeks)	17%	13%	25%	96%	86%	100%
Early onset preeclampsia (delivery <34 weeks)	–	–	–	61%	43%	69%
Newborn weight (g)	3437	3455	3338	1689	1777	1680
Newborn weight percentile	66	69	57	1	2	1
Small for gestational age	8%	13%	0%	70%	57%	75%

[a)] Primiparous women were excluded from analyzing rate of previous preeclampsia.

positive cells clustered together), or "generalized" (larger portions of the perivascular area).

2.3. Additional immunohistochemistry of aorta atherosclerosis lesions

For methodological comparison of the subcellular location of the immunohistochemical stains, we tested FABP4, perilipin-2, and LOX-1 on one aorta tissue sample with early ("fatty streak") and one aorta sample with advanced atherosclerotic lesions, using the same antibodies and dilutions as described for decidua basalis sections in the Methods section. Foam cells expressed CD68, FABP4, and perilipin-2 (data not shown), although not all CD68-positive foam cells in the atherosclerotic lesions were positive for FABP4 and/or perilipin-2. FABP4 was located to cytoplasmic granules and perilipin-2 to lipid droplets. LOX-1 was observed in a few foam cells of the advanced atherosclerotic sample, but in a diffuse intracellular pattern and not as the expected plasma membrane stain [40].

3. Statistics

Statistical analyses were performed using SPSS Statistics 25 (IBM). Non-parametric Mann-Whitney *U* test was used for continuous

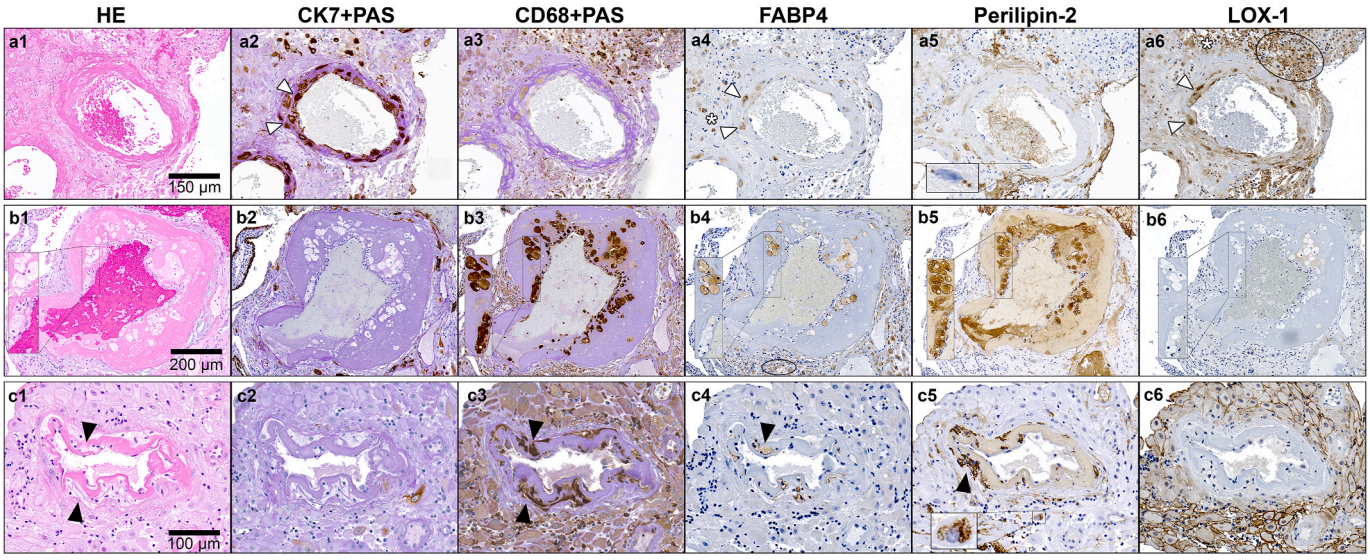

Fig. 1. Serial FFPE sections of decidua basalis tissue: identification of spiral arteries, acute atherosis, and lipid-associated proteins. Slides are stained with (from left to right, 1–6) Hematoxylin-Eosin (HE), CK7+PAS, CD68+PAS, FABP4, perilipin-2, and LOX-1. Representative images of:
a1-a6: Spiral artery without acute atherosis from a normotensive pregnancy, characterized by presence of CK7-positive trophoblasts (a2, white arrowhead) and intramural fibrinoid (a2, bright purple upon PAS staining) in the artery wall. Intramural trophoblasts are positive for FABP4 (a4, white arrowhead). Scattered perivascular FABP4 stain of stromal cells (asterisk). Small intracellular perilipin-2 positive lipid droplets in one trophoblast (a5, inset). LOX-1 shows positive stain in trophoblasts (a6, white arrowhead) and a combination of areas with scattered (a6, asterisk) and focal (a6, circled) positive staining patterns perivascularly. Note also varying degrees of unspecific LOX-1 stain in spiral artery fibrinoid, a frequent observation.
b1-b6: Spiral artery with acute atherosis from a preeclamptic pregnancy. ≥2 intramural CD68-positive vacuolated cells are present (inset), confirming acute atherosis. Fibrinoid necrosis is visible as a pale grey-pink material upon PAS staining in the artery wall. Numerous foam cells are positive for perilipin-2, fewer are positive for FABP4, and none for LOX-1 (b4-6, inset). Focal staining pattern of perivascular stromal cells (b4, encircled) with FABP4, as well as scattered positive stromal cells. Unspecific cross-reactivity between fibrin and perilipin-2 was observed frequently, and can be seen in a5) and b5).
c1-c6: Spiral artery with acute atherosis from a preeclamptic pregnancy, confirmed by ≥ 2 intramural CD68-positive vacuolated cells (foam cells, black arrowhead). Inset; intracellular perilipin-2 positive lipid droplets in decidual stromal cell. LOX-1 demonstrates generalized staining pattern of perivascular stromal cells.

variables. Fisher's exact test was used for categorical variables, expected cell count <5. A p-value of <0.05 was considered statistically significant.

4. Results

4.1. Spiral artery identification and acute atherosis diagnosis

We identified and evaluated 190 spiral artery sections (*non-AA arteries* and *AA arteries*) across the 35 included pregnancies. Distribution of spiral arteries across the clinical groups is presented in Supplemental Table 1. As described in the Methods section, we observed *non-AA arteries* in both *AA samples* and *non-AA samples*. We observed *non-AA arteries* in 28/35 (80%) pregnancy samples. Representative images of *non-AA arteries* and *AA arteries* are presented in Fig. 1. There was no significant difference in median number of spiral arteries detected between the preeclampsia and normotensive groups (Supplemental Table 1). Due to our over-selection of *AA samples*, 33% of the normotensive women and 71% of the women with preeclampsia had one or more *AA arteries* (Supplemental Table 1). In *AA samples*, the rate of *AA arteries* was somewhat higher in the preeclampsia group than in the normotensive group, but the difference was not significant (57% in preeclampsia vs. 46% in normotensive, p = 0.419).

4.2. Immunohistochemistry staining of lipid associated proteins in spiral artery walls with acute atherosis (AA arteries)

In our sample selection of AA pregnancies, there was no significant difference between the normotensive and preeclampsia groups regarding total number of foam cells and median number of foam cells (normotensives: n = 350 foam cells, median = 4 per section. Preeclampsia: n = 1423 foam cells, median = 16 per section, p = 0.6 for both comparisons) per pregnancy sample.

Perilipin-2 expression was located to cytoplasmic lipid droplets (Fig. 1, b5 and c5). Almost all (93%) AA lesions had ≥1 perilipin-2 positive foam cell. More than half (55%) of all evaluated AA foam cells were perilipin-2 positive (Fig. 2). FABP4 expression was located to the cytoplasm of foam cells (Fig. 1, b4 and c4), and was present in 36% of AA lesions, and in 13% of AA foam cells (Fig. 2). Perilipin-2 was significantly more often positive in foam cells as compared to FABP4 (p < 0.001).

LOX-1 was only detected in 3% of *AA artery* walls and only in 0.4% (7/1773) of all foam cells. Similarly to our aorta atherosclerotic sample, the LOX-1 stain was not located to the cell membrane as expected from previous publications [40], but to the cytoplasm. We considered this as an unspecific LOX-1 staining of the foam cells and thus excluded these numbers from further analysis.

FABP4 (p = 0.366) and perilipin-2 (p = 0.755) expression in foam cells was not significantly different between normotensive and preeclamptic pregnancies (Fig. 2) when comparing the frequency of positive foam cells for each *AA artery*. However, when all observed foam cells were "pooled" and evaluated according to clinical group, we found that foam cells in normotensive pregnancies were significantly more often positive for FABP4 (24%, 83/350, for normotensive and 10%, 140/1423, for preeclampsia, p < 0.0001) and perilipin-2 (62%, 217/350 for normotensive and 52%, 746/1423 for preeclampsia, p < 0.0015) than in preeclamptic pregnancies.

We rarely observed positive perilipin-2, FABP4, or LOX-1 staining of other cell types than foam cells in the artery wall of *AA arteries*.

4.3. Immunohistochemistry staining of lipid associated proteins in spiral artery walls without acute atherosis (non-AA arteries)

FABP4, perilipin-2, and LOX-1 protein expression was also detected in the vessel wall of *non-AA arteries* in normotensive and preeclampsia groups, but in different cell types than in *AA arteries*. FABP4 was the most prevalent protein expressed in *non-AA artery* walls. FABP4 was observed in 53% (Fig. 1, a4), perilipin-2 in 19% (Fig. 1, a5), and LOX-1 in 12% (Fig. 1, a6) of *non-AA artery* walls. There were no significant differences in frequency of positive staining for FABP4, perilipin-2, or LOX-1 between *non-AA arteries* in *non-AA samples* in the normotensive and preeclampsia groups, nor between *non-AA arteries* in *non-AA samples* and *non-AA arteries* in *AA samples*, irrespective of clinical diagnosis. All three markers primarily located to trophoblasts, but were also seen sporadically in macrophages. FABP4 and LOX-1 were also sporadically seen in endothelial cells, whereas perilipin-2 was never seen in spiral artery endothelial cells.

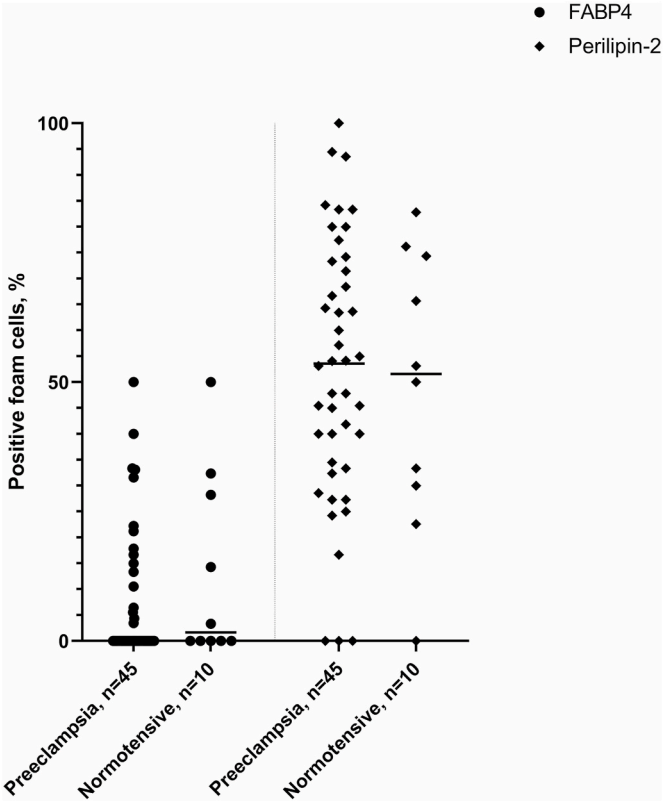

Fig. 2. Distribution of lipid-associated protein expression in decidual acute atherosis foam cells. Percentage of observed CD68-positive, vacuolated (foam) cells also positive for FABP4 (circle) and perilipin-2 (diamond) for each acute atherosis spiral artery across normotensive and preeclamptic pregnancies. Line at median.

5. Immunohistochemistry staining of lipid associated proteins in perivascular decidua basalis tissue

The protein expression of FABP4, perilipin-2, and LOX-1 in the decidua basalis spiral artery perivascular area (up to 100 μm from the outer margin of the vessel wall) varied greatly both within and between women, regardless of clinical group. Overall, FABP4 was the most often detected protein, followed by LOX-1, which in the perivascular area stained the cell membrane as expected (Fig. 1, c6) [40]. Perilipin-2 was the least often detected perivascular protein. We detected positive staining mainly in decidual stromal cells (Fig. 1, a4-6 and c4-6), less often in interstitial (extravillous) trophoblasts, macrophages and lymphocytes.

LOX-1 was most often observed in a "generalized" staining pattern surrounding *AA arteries* (Fig. 1 c6, Supplemental Fig. 1), both for normotensive and preeclampsia groups. LOX-1 was also significantly more often positive in a generalized pattern perivascularly of *AA arteries* as compared to *non-AA arteries* (38% vs. 15%, p < 0.001). Similarly,

perilipin-2 was significantly more frequently observed surrounding *AA arteries* than *non-AA arteries* (59% vs. 32%, $p < 0.001$). Perilipin-2 and LOX-1 co-localized around the same spiral arteries (though not necessarily in the same cells) significantly more often for *AA arteries* compared to *non-AA arteries* (30% vs. 13%, $p = 0.01$). In contrast, FABP4 was significantly more often positive surrounding *non-AA arteries* than *AA arteries* (69.6% vs 52.7%, $p = 0.031$).

For the perivascular decidua basalis tissue, decidual stromal cells stained most often positive for the 3 investigated markers (data not shown). Occasionally, interstitial trophoblasts and macrophages also stained positive.

6. Discussion

Our study showed some clear similarities in lipid binding protein expression between artery wall foam cell lesions of atherosclerosis and acute atherosis. We found that perilipin-2 was abundantly expressed in AA lesions, in line with findings in atherosclerosis [22,24], and more often present in acute atherosis foam cells than the other investigated lipid-associated proteins FABP4 and LOX-1. Perilipin-2 was detected in a majority of foam cells while FABP4 was detected in much fewer foam cells. The localization of positive staining of lipid droplets for perilipin-2 and cytoplasmic staining for FABP4 in foam cells of decidua basalis spiral artery walls was identical to observations reported previously from atherosclerosis [45,46].

Although perilipin-2 and FABP4 were detected in many foam cells, we rarely found *all* foam cells of an AA lesion to be positive for perilipin-2 or FABP4. FABP4 expression in atherosclerosis is linked to plaque instability [27,31] and may represent a marker of more advanced lesion development that is lacking in AA, possibly due to its much shorter duration as compared to atherosclerosis (i.e. pregnancy months compared to several years). Perilipin-2 lacking foam cells could indicate that there has been a disruption of the lipid droplets or that the lipid droplets are larger than in perilipin-2 positive foam cells, as both situations have been shown to reduce perilipin-2 expression in in vitro studies [47]. An alternative interpretation is that the lipid droplets of perilipin-2 negative foam cells may express other members of the perilipin protein family.

Another reason for not all foam cells staining positive for the investigated proteins lies in the nature of serial tissue sections, as not all foam cells identified by HE and CD68 were present on the following parallel slides stained with FABP4, perilipin-2 and LOX-1. This represents a potential (albeit unavoidable) weakness of our method for studying AA, as the lesion is patchy, not affecting the full circumference or the full length of a spiral artery. In some of the AA lesions, we identified artery wall cells that were positive for FABP4 and/or perilipin-2 (but never LOX-1). Although these cells also likely represented foam cells, they were not included in our analyses as they were not present on the HE or CD68 slides and thus could not be confirmed to be foam cells.

LOX-1 was observed in relation to a few foam cells in the spiral artery walls, but this was likely non-specific, as the stain was located diffusely intracellularly. LOX-1 is a transmembrane glycoprotein expressed on the plasma membrane [32], in line with what we found for the perivascular LOX-1 staining. We expect the intracellular LOX-1 staining in the vascular wall foam cells to stem from non-immune, non-specific cross-reactivity with some intracellular element or internalization of the receptor-ligand compound [48].

One important identified difference between our study of decidua basalis acute atherosis and previous atherosclerosis studies is that early atherosclerotic lesions often express LOX-1 in endothelial cells, and LOX-1 is thought to be important in foam cell formation and lipid deposition [26,33,49]. In contrast, we did not observe LOX-1 protein expression in the endothelial cells of acute atherosis arteries, although it is possible that LOX-1 expression is present at earlier gestational ages than in our third trimester decidua basalis samples. This discrepancy, together with our previous finding of lacking endothelial activation of decidual acute atherosis [19], may suggest that endothelial cells play a different role in the development of acute atherosis lesions as compared to atherosclerosis. However, as described by us and others previously [19,50–52], endothelial cells in AA are often disrupted, degenerated, or lacking, in contrast to endothelial cells in early atherosclerosis, which are mainly intact [16]. This difference may have contributed to our observed differences in LOX-1 staining of AA and atherosclerotic endothelium. In addition, LOX-1 has been shown to be of importance in advanced stages of atherosclerosis [26,28].

Foam cells in atherosclerosis of larger arteries derive from macrophages, endothelial cells, and vascular smooth muscle cells [35]. Previous studies of acute atherosis have demonstrated monocyte-macrophages [18] and smooth muscle cells [50] as possible sources for foam cells. The role of endothelial cells or other possible sources for foam cells, such as trophoblasts or fibroblasts, in acute atherosis is unknown. Our group has previously demonstrated that trophoblasts in vitro accumulate neutral lipids and express LOX-1 in an environment of oxidative stress (typically present in preeclampsia [53]) in a trophoblastic cell line, suggesting that trophoblasts may be able to convert into foam cells [54]. Intriguingly, we observed quite frequently positive LOX-1 and sometimes perilipin-2 positive staining in intramural trophoblasts in *non-AA arteries*. We propose that LOX-1-positive trophoblasts of *non-AA arteries* could represent precursors to foam cells and that given more time, the trophoblasts could indeed form foam cells and participate in the formation of decidua basalis acute atherosis lesions. Hypoxia, which is implicated in the preeclamptic placenta [55], has, along with hyperlipidemia, been shown to increase FABP4 and perilipin-2 expression in cultured term trophoblasts [56–58], supporting our observation that trophoblasts and foam cells display some molecular similarities. While the assumption that trophoblasts could be a potential source of foam cells in acute atherosis is not supported by the lack of CK7- and LOX-1-positivity in acute atherosis foam cells, it is possible that CK7- and LOX-1-expression could decrease as the trophoblasts develop into foam cells. Trophoblasts may represent an added pregnancy-specific source of acute atherosis foam cells, in addition to monocyte-macrophages and smooth muscle cells.

The present study is not epidemiologically representative for acute atherosis prevalence, as we purposely chose to over-represent pregnancies with acute atherosis. Interestingly, our study indicates that, when acute atherosis is present, decidua basalis foam cell characteristics are similar, with similar lipid associated protein patterns across preeclampsia and normotensive pregnancies. Acute atherosis lesions are however much more frequent in preeclampsia than normotensive pregnancy, as shown by several studies [59,60]; including ours [1,2,6].

We also observed interesting lipid associated protein staining patterns in the perivascular area of decidua basalis spiral arteries. Specifically, there was slightly more generalized LOX-1 staining surrounding *AA arteries* of preeclampsia as compared to normotensive women, though this difference was not statistically significant. LOX-1 was also significantly more often expressed in the perivascular area of *AA arteries* compared to *non-AA arteries*, indicating higher levels of oxLDL in decidua basalis tissue in samples from women with preeclampsia and acute atherosis.

Our group [53] found increased 8-iso-prostaglandin $F_{2\alpha}$ levels in decidua basalis in preeclampsia, and later confirmed that this compound, a marker of oxidative stress, increases LOX-1 expression in a trophoblastic cell line [54]. While these results do not directly confirm that LOX-1 is upregulated in decidua basalis during preeclampsia, LOX-1 has been shown to be highly expressed in the endothelium of omental arteries [61] and in syncytiotrophoblasts in placentas [40] of preeclamptic pregnancies. Taken together with our present results, this could indicate a role of LOX-1 in acute atherosis, both in preeclampsia and normotensive pregnancies. We propose that the presence of oxLDL in the decidua basalis creates an inflammatory and oxidative environment surrounding the spiral arteries, facilitating LOX-1 expression and foam cell lesion development. A recent study also suggests a role for

cholesterol crystals mediating inflammation in decidua basalis in pre-eclampsia [62], although its role in acute atherosis lesions is not described.

Perilipin-2 was infrequently positive in the perivascular area, compared to LOX-1 and FABP4. Even so, we did find that perilipin-2 was significantly more often positive in perivascular stromal cells surrounding *AA arteries* than *non-AA arteries*. We suggest that this observation reflects the increased lipid contents of *AA arteries* [18,50] and that the increased perilipin-2 observed around *AA arteries* is a direct consequence of locally increased lipid levels. The latter would be in line with our previous study showing increased levels of phospholipids and cholesterol in decidua basalis of preeclamptic pregnancies [36], and in line with our present finding of increased LOX-1 surrounding *AA arteries*, indicating increased oxLDL locally.

Our study is, to our knowledge, the first to assess FABP4, perilipin-2, and LOX-1 immunohistochemically in uteroplacental tissues and acute atherosis lesions of spiral arteries. We found that perilipin-2 and FABP4 are expressed by acute atherosis foam cells, similar to atherosclerotic foam cells of larger arteries. We propose that foam cells of these arterial lesions may share some developmental features. The lack of LOX-1 in acute atherosis lesions differs from that of atherosclerosis, where LOX-1 has a more clearly defined role in lesion development. LOX-1 was however present in many intravascular trophoblasts of *non-AA arteries*, and particularly in decidual stromal cells surrounding both *non-AA* and *AA arteries*. Our findings regarding LOX-1 also suggest possible discrepant routes for foam cell origin and development between these two lesions of different artery calibers.

In conclusion, we propose that acute atherosis may share some development pathways with atherosclerosis, but also that there are important differences between the two lesions. We propose that pregnancy-specific pathways, such as local oxidative stress, immune-mediated factors and possible trophoblast-associated pathways, that may contribute in the development of decidua basalis acute atherosis, could explain these differences. This study contributes to the understanding of acute atherosis development at the maternal-fetal interface and explores molecular features of the lesion, and highlights the role of foam cell associated proteins in the lesion process.

Declaration of competing interest

The authors declare that they have no competing conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2021.03.001.

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Supplemental Material

Decidua basalis and acute atherosis: Expression of atherosclerotic foam cell associated proteins

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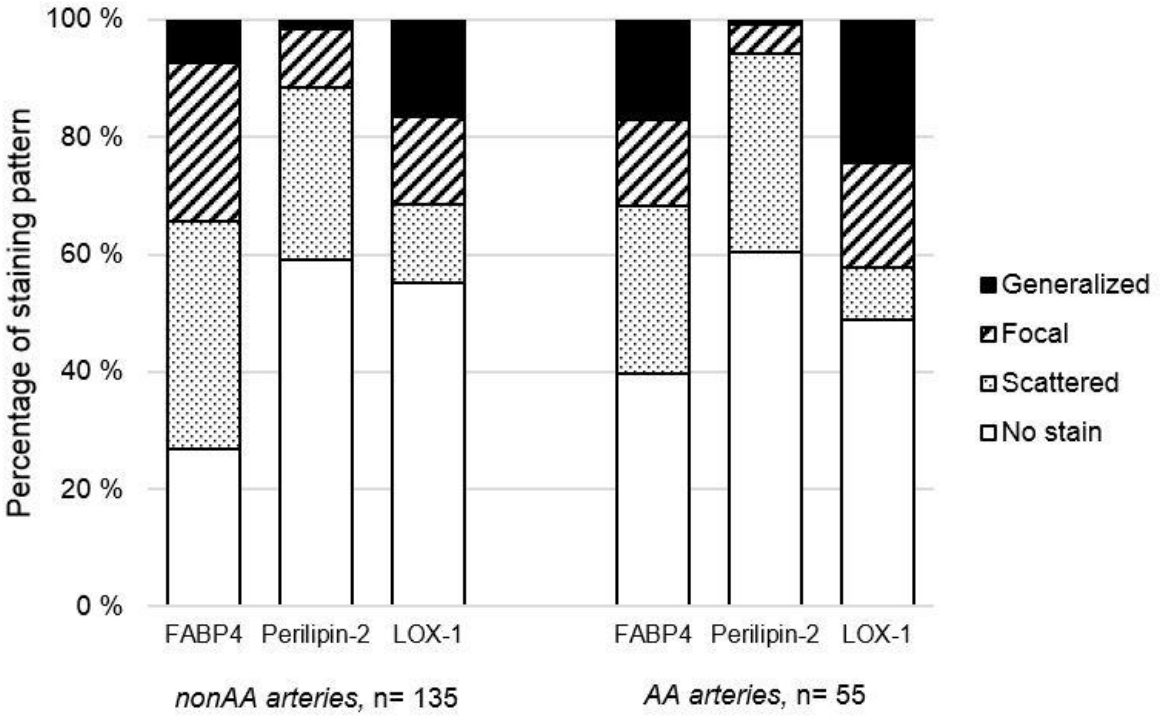
Supplemental Figure 1. Distribution of lipid-associated protein staining patterns in the perivascular area of decidua basalis spiral arteries. Bars show the percentage of arteries in each category displaying each staining pattern for FABP4, perilipin-2, and LOX-1 in the perivascular area, across normotensive women and women with preeclampsia. Observed staining patterns were “no stain” (white), “scattered” (single positive cells seemingly without relation to one another, dots), “focal” (clusters of ≥5 positive cells, diagonal lines, and “generalized” (continuous positive stain of larger portions of the perivascular area, black).



Supplemental Table 1: Distribution of observed spiral arteries across the study groups. The total number of decidua basalis spiral arteries observed within each study group, as well as the number of *non-AA*- and *AA arteries*. The median number (and range) of spiral artery sections observed per pregnancy evaluated for each study group. *Non-AA sample*; sample lacking spiral artery with acute atherosis (AA), *AA sample*; sample containing ≥1 spiral artery with acute atherosis. The distribution (in percentage) of *non-AA*- and *AA arteries* of the total number of spiral arteries observed per pregnancy evaluated is presented as median (and range) across the samples in each category.

	Normotensive		***Preeclampsia***		***Superimposed preeclampsia***	
Number of pregnancies evaluated, n=	***12***		***21***		***2***	
Total numbers of artery sections evaluated, n=	***69***		***114***		***7***	
	Non-AA sample	***AA sample***	***Non-AA sample***	***AA sample***	***Non-AA sample***	***AA sample***
Number of pregnancies per sample category, n=	***8***	***4***	***6***	***15***	***1***	***1***
Total number of artery sections evaluated per study group, n=	37	32	25	89	5	2
Non-AA arteries, n=	37	22	25	46	5	0
AA arteries, n=	-	10	-	43	-	2
Number of spiral artery sections observed per tissue section, median (range)	4 (1-11)	8(1-15)	5 (1-8)	4 (1-16)	5	2
Non-AA arteries, median (range), %	100	54 (0-89)	100	43 (0-81)	100	0
AA arteries, median (range), %	0	46 (11-100)	0	57 (19-100)	0	100

Superimposed preeclampsia: Women with chronic hypertension developing preeclampsia after gestational week 20 (e.g. developing proteinuria. Statistical differences were calculated for the total group of normotensive controls against preeclampsia, and within the normotensive and preeclampsia group for *non-AA sample* against *AA sample*. We compared medians of continuous variables by non-parametric Mann-Whitney U test. Differences were considered to be statistically significant when $p<0.05$.

PAPER II (corrigendum)

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Corrigendum

Corrigendum to "Decidua basalis and acute atherosis: Expression of atherosclerotic foam cell associated proteins" [Placenta 107 (2021) 1–7]

Ingrid Knutsdotter Fosheim [a,b], Guro Mørk Johnsen [a], Patji Alnaes-Katjavivi [a], Gitta Turowski [c], Meryam Sugulle [a,b], Anne Cathrine Staff [a,b,*]

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The authors regret that the clones for the different antibodies used were incorrectly reported in our published paper. The correct clones are as follows: CK7: OV-TL 12/30, CD68: KP1, FABP4: polyclonal, perilipin-2: AP125, LOX-1: 9E12.1. Additionally, antibodies purchased from Dako were incorrectly written as originating from USA, the correct country of origin is Denmark.

Thus, the text in the Materials and methods section, page 2, should have read: "2.2. Decidua basalis standard staining, immunohistochemistry, and evaluation.

For all women, serial 3 μm FFPE decidua basalis sections were stained with Hematoxylin-Eosin (HE) using standard histological technique, cytokeratin 7 (CK7, OV-TL 12/30, Dako, Denmark, 1:300, counterstained with Periodic acid-Schiff [PAS]), CD68 (KP1, Dako, Denmark, 1:1500, counterstained with PAS), FABP4 (polyclonal, Sigma-Aldrich, USA, 1:1000), perilipin-2 (AP125, Progen, Germany, 1:300), and LOX-1 (9E12.1, Millipore, USA, 1:750)."

The authors would like to apologise for any inconvenience caused.

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III

Serum amyloid A1 and pregnancy zone protein in pregnancy complications and correlation with markers of placental dysfunction

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ABSTRACT

BACKGROUND: Hypertensive disorders of pregnancy (preeclampsia, gestational hypertension, and chronic hypertension), diabetes mellitus, and placental dysfunction confer an increased risk of long-term maternal cardiovascular disease. Preeclampsia is also associated with acute atherosis, lesions of uteroplacental spiral arteries resembling early stages of atherosclerosis. Serum amyloid A1 is involved in hypercoagulability and atherosclerosis, and may aggregate into amyloid, aggregations of misfolded proteins. Pregnancy zone protein may inhibit amyloid aggregation. Amyloid is involved in Alzheimer's disease and cardiovascular disease, and has been identified in preeclampsia, but its role in preeclampsia pathophysiology is unclear.

OBJECTIVES: We hypothesized that serum amyloid A1 would be increased and pregnancy zone protein decreased in hypertensive disorders of pregnancy and diabetic pregnancies, and that serum amyloid A1 and pregnancy zone protein would correlate with placental dysfunction markers (fetal growth restriction and dysregulated angiogenic biomarkers) and acute atherosis.

STUDY DESIGN: Serum amyloid A1 is measurable in both serum and plasma. In our study, plasma from 549 pregnancies (normotensive, euglycemic controls: 258; early-onset preeclampsia: 71; late-onset preeclampsia: 98; gestational hypertension: 30; chronic hypertension: 9; diabetes mellitus: 83) was assayed for serum amyloid A1 and pregnancy zone protein. Serum levels of angiogenic biomarkers soluble fms-like tyrosine kinase-1 and placental growth factor were available for 547 pregnancies, and acute atherosis evaluation for 313 pregnancies. Clinical characteristics and circulating biomarkers were compared between the pregnancy groups using Mann-Whitney U, chi-square, or Fisher's exact test as appropriate. Spearman's rho was calculated for assessing correlations.

RESULTS: In early-onset preeclampsia, serum amyloid A1 was increased compared to controls (17.1 vs. 5.1 µg/mL, $p<0.001$), whereas pregnancy zone protein was decreased (590 vs. 892 µg/mL, $p=0.002$). Pregnancy zone protein was also decreased in diabetes compared to controls (683 vs. 892 µg/mL, $p=0.01$). Serum amyloid A1 was associated with placental dysfunction (fetal growth restriction, elevated soluble fms-like tyrosine kinase-1 to placental growth factor ratio). Pregnancy zone protein correlated negatively with soluble fms-like tyrosine kinase-1 to placental growth factor ratio in all study groups. Acute atherosis was not associated with serum amyloid A1 or pregnancy zone protein.

CONCLUSIONS: Proteins involved in atherosclerosis, hypercoagulability, and protein misfolding are dysregulated in early-onset preeclampsia and placental dysfunction, linking them, and potentially contributing to future maternal cardiovascular disease.

Key words: preeclampsia, hypertensive disorders of pregnancy, gestational hypertension, chronic hypertension, diabetes mellitus, gestational diabetes, protein misfolding, hypercoagulability, acute atherosis, biomarker analysis

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Introduction

Preeclampsia is a potentially severe pregnancy complication, defined as new-onset hypertension and at least one other preeclampsia-associated sign of organ dysfunction (e.g. proteinuria, fetal growth restriction, and elevated liver transaminases) in the second half of pregnancy.[1] Hypertensive disorders of pregnancy, including preeclampsia, and other pregnancy complications associated with placental dysfunction (e.g. fetal growth restriction, preterm birth, and gestational diabetes mellitus (GDM)) are associated with a 2-8-fold increased risk of future maternal cardiovascular disease.[2-4] Pregnancy can thus be regarded as a sex-specific stress test for predicting maternal risk of cardiovascular disease. Identifying predictors of future maternal cardiovascular health during pregnancy merits further investigation. Cardiovascular diseases remain a major cause of death for both men and women, but female-specific pathophysiological mechanisms have so far been understudied.[5]

The maternal features of preeclampsia result from excessive systemic vascular inflammation, secondary to increased shedding of pro-inflammatory factors from a malperfused and dysfunctional placenta.[6,7] Placental malperfusion leads to oxidative and endoplasmic reticulum stress, eliciting release of pro-inflammatory and antiangiogenic factors.[8] Placental dysfunction in early-onset preeclampsia is associated with insufficient physiological spiral artery transformation.[6,9] Both early- and late-onset preeclampsia are associated with excessive placental senescence and high rates of uteroplacental acute atherosis.[6,10,11] Acute atherosis is a spiral artery wall lesion, with some morphological similarities to early stages of atherosclerosis.[12,13] It is characterized by subintimal foam cells, artery wall necrosis and inflammation, and is associated with thrombosis and risk of downstream placental infarcts.[14]

Circulating proangiogenic placental growth factor (PlGF) and antiangiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) are mainly of placental origin during pregnancy, and are dysregulated in preeclampsia and other placental dysfunction syndromes such as fetal growth restriction.[15,16] Consequently, we have argued that dysregulated angiogenic biomarkers (e.g. low PlGF, high sFlt-1/PlGF ratio) may be utilized as proxy markers for placental dysfunction and syncytiotrophoblast stress.[7]

Amyloid consists of fibrous aggregates of misfolded proteins, has a slow spontaneous dissociation,[17] and is implicated in several chronic, progressive diseases, including Alzheimer's disease, type 2 DM, and atherosclerosis.[18,19] Amyloid also accumulates in the urine and

GLOSSARY

Acute atherosis: pregnancy-specific foam cell lesions in spiral arteries; may affect any pregnancy, but is most frequent in preeclampsia.

Protein misfolding: process by which proteins lose or fail to maintain normal structure, or fail to fold correctly during protein synthesis, thus losing physiological properties.

Amyloid: fibrillar aggregates of misfolded proteins; very stable compounds due to their cross-β sheet structure, involved in numerous human diseases.

AT A GLANCE

Why was this study conducted?

Preeclampsia and placental dysfunction place women at risk of future cardiovascular disease, but the mechanisms are largely unknown. This study aimed at assessing biomarkers that may contribute to this risk.

What are the key findings?

Circulating serum amyloid A1 and pregnancy zone protein, markers of protein misfolding, are dysregulated in early-onset preeclampsia, and levels correlate with antiangiogenic biomarkers and fetal growth restriction, proxies for placental dysfunction.

What does this study add to what is already known?

Our finding of a correlation between protein misfolding markers and proxies for placental dysfunction is novel. Markers of protein misfolding are implicated in early-onset preeclampsia and placental dysfunction, and may provide a pathway linking these pregnancy complications to the epidemiological increased risk of maternal cardiovascular disease.

placenta of women with preeclampsia.[20,21] Circulating amyloid precursors transthyretin and amyloid-precursor-protein are dysregulated in preeclampsia.[20-22] Urine testing for amyloid has been suggested as a test for preeclampsia severity.[23] Serum amyloid A1 (SAA1) is an acute-phase protein mainly produced in the liver, and one of many proteins able to aggregate into amyloid.[17] Placental expression of SAA1 may play a role in initiating parturition, though its expression throughout pregnancy is uncertain.[24] SAA1 is also implicated in hypercoagulability, by promoting amyloid formation in fibrin(ogen) and driving platelets into a pro-thrombotic state.[25]

Pregnancy zone protein (PZP) is a plasma protein that is highly upregulated during pregnancy,[26] and may play a pro-gestational role through immuno-modulation.[27] Further, low circulating levels have been linked to pregnancy loss.[28] In vitro, PZP stabilizes and inhibits misfolded amyloid-β from aggregating into amyloid fibrils.[29] Whether PZP inhibits SAA1 aggregation into amyloid-A, the amyloid type derived from SAA1, is unknown.

Circulating SAA1 has been shown to be increased in GDM,[30] and has been studied in preeclampsia as well, though results from these predominantly small studies are conflicting.[31-36] Derived PZP levels were decreased in women with preeclampsia in two small studies.[37,38] These previous SAA1 and PZP studies from pregnancy have not assessed biomarker concentrations in relation to placental dysfunction.

As SAA1 and PZP have opposite roles in augmenting and attenuating amyloid aggregation, we hypothesized that circulating SAA1 is increased and PZP decreased in preeclampsia, particularly in early-onset disease, and in pregnancies complicated by diabetes. We further hypothesized that SAA1 and PZP levels correlate with proxies for placental dysfunction (e.g. dysregulated angiogenic biomarkers, fetal growth restriction, or acute atherosis), as an indication of amyloid deposition in placental dysfunction.

Materials and Methods

Patient recruitment and sample collection

Data and biological samples from 549 singleton pregnant women, recruited between 2001 and 2017 to the Oslo Pregnancy Biobank at Oslo University Hospital, were included in this study. All had extensive clinical data from pregnancy and delivery, as well as blood samples available for biomarker analyses. All women gave informed written consent. The study was approved by the Regional committee for Medical and Health Research Ethics in South-Eastern Norway, and conducted in accordance with the principles of the Helsinki Declaration.

Hypertensive disorders of pregnancy (HDP) were defined according to the 2018 Guidelines of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[1] Gestational hypertension (GH) was defined as new-onset hypertension (blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic on ≥2 occasions ≥6 hours apart) at ≥20 weeks' gestation. Preeclampsia was defined as GH accompanied by ≥1 other new-onset preeclampsia-associated feature(s) at ≥20 weeks' gestation. Early-onset preeclampsia (EO-PE) and late-onset preeclampsia (LO-PE) were defined as preeclampsia delivered <34 versus ≥34 weeks' gestation, respectively.[6] Chronic hypertension (CHT) was defined as hypertension occurring pre-pregnancy or diagnosed <20 weeks' gestation. Superimposed preeclampsia was defined as CHT and ≥1 new-onset preeclampsia-associated feature(s) at ≥20 weeks' gestation. Diabetes mellitus (DM) diagnoses (type 1 DM (DM1), type 2 DM (DM2), gestational DM (GDM)) were based on diagnoses identified in the clinical setting by endocrinologists, according to current guidelines.[39,40] Women with both an HDP and DM diagnosis were classified as HDP+DM. Controls were women who remained normotensive and euglycemic throughout pregnancy. None had regular uterine contractions, ruptured fetal membranes, or signs of infection at time of blood sampling. None had known chronic diseases, apart from women with CHT (n=25), pregestational DM (n=79), or hypothyroidism (n=33). Gestational age at inclusion was determined by routine ultrasound screening at gestational week 17-20, except in 25 women where gestational age was determined by embryo transfer date (in vitro fertilization), and 7 women by first day of the last menstrual period before pregnancy. Sex-specific newborn weight percentiles were calculated according to Norwegian fetal growth curves.[41] Fetal growth restriction was defined as sex-specific newborn weight ≤3rd percentile. No newborns had structural or apparent chromosomal abnormalities.

Presence or absence of uteroplacental acute atherosis was assessed in decidua basalis vacuum suction biopsies, collected after delivery of the placenta and evaluated as described previously.[42] Acute atherosis was defined as spiral arteries with ≥2 adjacent CD68-positive vacuolated subintimal cells.[43]

For the majority of women, blood samples were obtained immediately prior to elective caesarean delivery, and

centrifuged within 120 minutes of sampling at 4°C for 10 minutes at 1800*g*. EDTA plasma was stored at -80°C until immunoassay. For a subset of the included women (n=81), blood samples were collected up to 31 days prior to delivery. Blood was sampled from an antecubital vein or intravenous cannula without ongoing infusion, or from an arterial catheter (n=20). Maternal serum samples were also collected at the same time point as EDTA plasma, as described previously.[44]

Biochemistry and immunoassays

SAA1 and PZP concentrations were measured by enzyme-linked immuno-sorbent assay (ELISA) in maternal EDTA-plasma in duplicates. The mean value of the duplicates was used for analysis. All reagents were obtained from R&D Systems, USA, catalogue numbers DY3019-05 (SAA1) and DY8280-05 (PZP). Assays were performed in accordance with the manufacturer's instructions. Optical density was determined at 450 nm and corrected at 540 nm. Coefficients of variation were 6.4% for SAA1 and 2.4% for PZP.

Serum levels of sFlt-1 and PlGF were determined for 547 pregnancies as previously described,[44] using Elecsys® immunoassays (Roche Diagnostics, Switzerland) with a fully automated electrochemiluminescence immunoassay platform (Cobas E analyzer, Roche Diagnostics).

Serum levels of high-sensitivity C-reactive protein (hsCRP) in either fresh (n=219) or thawed (n=161) samples were analyzed as described previously,[45] using a particle-enhanced turbidimetric method (Cobas 8000 c702, Roche Diagnostics).

Statistics

The data were analyzed using SPSS Statistics 26.0 (IBM). Mann-Whitney U test was used for continuous variables. Chi-square or Fisher's exact test were used for categorical variables, as appropriate. Spearman's rho (r_S) was calculated for analyzing correlations between continuous variables, as data were skewed. We did not correct for multiple comparisons. A p-value <0.05 was considered significant.

Results

Table 1 lists the clinical characteristics of the main pregnancy groups (controls, n=258; HDP, n=163; DM, n=83; HDP+DM, n=45), acute atherosis rate, and circulating biomarker concentrations. Pre-pregnancy body mass index (BMI), mean blood pressures in the first half of pregnancy and at inclusion, rates of acute atherosis, hsCRP, and sFlt-1/PlGF ratio were increased, and gestational age and newborn weight percentiles were lower, in HDP and HDP+DM compared to controls.

SAA1 and PZP concentrations in hypertensive and diabetic pregnancies

As shown in Table 1, plasma SAA1 levels were markedly higher in HDP compared to controls (normotensive and euglycemic, $p<0.001$), whereas SAA1 did not differ between DM or HDP+DM compared to controls. PZP concentration was lower in both DM and in HDP+DM compared to controls (both $p=0.01$). In contrast, PZP was similar in HDP and controls.

Table 1. Clinical characteristics and circulating biomarkers, by pregnancy inclusion groups (total cohort n=549)

Variable	Controls (n=258)	HDP (n=163)	P-value	DM (n=83)	P-value	HDP+DM (n=45)	P-value
Maternal age at inclusion (years)	33.5 (20.1-45.5)	33.1 (19.0-52.9)	0.25	33.4 (24.1-45.9)	0.64	33.0 (21.1-44.0)	0.22
Pre-pregnancy body mass index (kg/m^2)	22.4 (17.6-39.5)	24.0 (18.3-41.1)	<0.001	24.5 (19.1-39.4)	<0.001	27.3 (19.6-41.1)	<0.001
Mean SBP <20 weeks (mmHg)	110 (85-140)	120 (90-158)	<0.001	114 (92-138)	<0.001	120 (95-164)	<0.001
Mean DBP <20 weeks (mmHg)	68 (40-88)	75 (55-103)	<0.001	69 (50-84)	0.21	73 (50-90)	<0.001
First pregnancy	24%	45%	<0.001	22%	0.61	42%	0.01
First delivery	39%	62%	<0.001	41%	0.77	53%	0.07
Previous preeclampsia, n (%)[a]	12 (8%)	26 (43%)	<0.001	7 (14%)	0.17	9 (43%)	<0.001
SBP at inclusion (mmHg)	121 (86-156)	151 (117-220)	<0.001	120 (90-155)	0.77	150 (120-189)	<0.001
DBP at inclusion (mmHg)	75 (48-110)	95 (63-130)	<0.001	74 (50-90)	0.94	92 (66-110)	<0.001
Gestational age at inclusion (weeks)	39.0 (33.7-42.1)	34.4 (24.3-41.1)	<0.001	38.0 (28.7-40.4)	<0.001	36.9 (29.0-40.0)	<0.001
Preterm delivery (<37 weeks' gestation)	3%	65%	<0.001	10%	0.01	40%	<0.001
Early-onset preeclampsia (delivery <34 weeks' gestation)	-	48%		-		16%	

New born weight (grams)	3483 (1325-5130)	2087 (500-5040)	<0.001	3802 (988-5857)	<0.001	3574 (620-5424)	0.70
New born weight percentile	62.6 (0-100)	5.0 (0-99.9)	<0.001	86.1 (0-100)	<0.001	90.9 (0-100)	0.01
Fetal growth restriction	2%	45%	<0.001	5%	0.10	4%	0.22
Acute atherosis[b]	9% (14/160)	39% (37/96)	<0.001	7% (2/31)	1.00	27% (7/26)	0.01
Serum-sFlt-1 (pg/mL)[c]	3683 (1081-17185)	11204 (1429-61348)	<0.001	4629 (1260-18857)	0.003	7597 (2003-21430)	<0.001
Serum-PlGF (pg/mL)[c]	172 (23-2074)	58 (6-624)	<0.001	166 (35-1027)	0.82	104 (29-1852)	<0.001
sFlt-1/PlGF ratio[c]	22.3 (1.1-615.1)	208.6 (2.3-3264.3)	<0.001	24.9 (2.7-209.0)	0.24	78.9 (1.4-470.8)	<0.001
Serum-hsCRP (mg/L) [ref.: 0.0-4.0][d]	3.1 (0.6-76.6), n=201	4.4 (0.6-107.0), n=117	0.002	3.9 (0.6-32.7), n=36	0.26	4.8 (0.8-15.9), n=26	0.04
Plasma-SAA1 (µg/mL)	5.08 (0.41-120)	9.90 (0.80-120)	<0.001	4.06 (0.37-120)	0.06	6.07 (0.22-115.95)	0.22
Plasma-PZP (µg/mL)	892 (32-3240)	818 (12-2992)	0.15	683 (40-3406)	0.01	548 (25-3280)	0.01

For continuous variables, median value and range is shown; for categorical, percentages. Statistical differences were calculated by comparing each pregnancy group to the control group (normotensive and euglycemic pregnant women). HDP: hypertensive disorders of pregnancy. DM: diabetes mellitus (pregestational or gestational). HDP+DM: women with both HDP and DM. SBP: systolic blood pressure. DBP: diastolic blood pressure. sFlt-1: soluble fms-like tyrosine kinase-1. PlGF: placental growth factor. hsCRP: high-sensitivity C-reactive protein. SAA1: serum amyloid A1. PZP: pregnancy zone protein.

[a] Nulliparous women were excluded when analyzing rate of previous preeclampsia.

[b] Decidua basalis acute atherosis was assessed in 313 women (all had spiral arteries present).

[c] sFlt-1 and PlGF concentrations were available for all except 2 women with HDP.

[d] hsCRP concentration was available for 380 women.

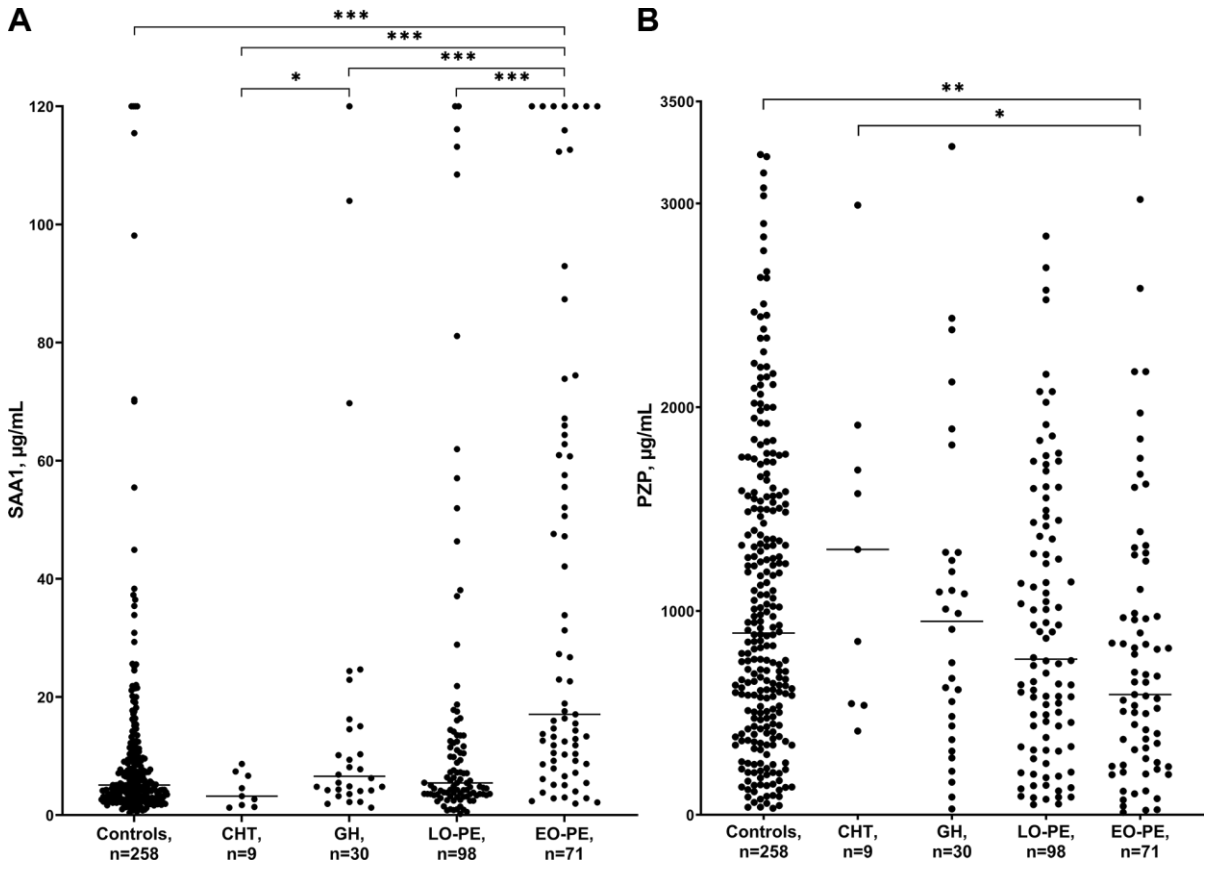
Of the HDP subgroups, the EO-PE group had increased levels of SAA1 compared to controls ($p<0.001$, Supplemental Table 1), and compared to all remaining subgroups of HDP (Figure 1a). Levels of PZP were decreased in EO-PE compared to controls ($p=0.002$, Supplemental Table 1), and compared to CHT (Figure 1b).

SAA1 did not differ between any DM subgroup and controls (normotensive and euglycemic, Supplemental Table 2). PZP, on the other hand, was decreased in women with DM1 and DM2 compared to controls ($p=0.02$ and 0.003, respectively, Supplemental Table 2).

Correlation between SAA1 and HbA1c in diabetic pregnancies

In DM1, SAA1 (at sampling in late pregnancy) correlated negatively with HbA1c measured in the first ($r_S=-0.43$, $p=0.002$), second ($r_S=-0.27$, $p=0.04$), and third ($r_S=-0.33$, $p=0.01$) trimesters. First trimester HbA1c ≥53 mmol/mol was associated with lower SAA1 (2.9 vs. 5.0 µg/mL, $p=0.03$). PZP did not correlate with HbA1c (data not shown).

Figure 1. Scatter plots of circulating biomarkers



Concentrations of circulating biomarkers, by subgroup of hypertensive disorder of pregnancy (irrespective of diabetes mellitus status) and controls (normotensive and euglycemic) (n=466). **A**, serum amyloid A1 (SAA1) concentrations, **B**, pregnancy zone protein (PZP) concentrations. CHT: chronic hypertension, GH: gestational hypertension, LO-PE: late-onset preeclampsia (delivery ≥34 weeks' gestation), EO-PE: early-onset preeclampsia (delivery <34 weeks' gestation). Mann-Whitney U test was used to calculate statistical differences between groups. Line at median. ***) $p<0.001$. **) $p<0.01$. *) $p<0.05$.

SAA1 and PZP concentrations in placental dysfunction

We compared SAA1 and PZP levels to proxies for placental dysfunction. Women with acute atherosis did not differ in SAA1 or PZP levels compared to women without acute atherosis within any of the pregnancy groups (Supplemental Table 3). However, increased SAA1 levels were seen in pregnancies with fetal growth restriction, and correlated positively with the antiangiogenic sFlt-1/PlGF ratio in the total cohort (13.3 vs. 5.1 μg/mL, r_S=0.24, both p<0.001) and in HDP (13.7 vs. 6.1 μg/mL, r_S=0.40, both p<0.001). PZP was not associated with fetal growth restriction, but correlated negatively with sFlt-1/PlGF in the total cohort (r_S=-0.13, p=0.002), HDP (r_S=-0.19, p=0.015), and HDP+DM (r_S=-0.30, p=0.049).

Correlations between SAA1, PZP, and hsCRP

Finally, we compared SAA1 and PZP to the general inflammation marker hsCRP. SAA1 correlated positively with hsCRP in the total cohort (r_S=0.46, p<0.001) as well as in all pregnancy groups; controls (r_S=0.35, p<0.001), HDP (r_S=0.58, p<0.001), DM (r_S=0.35, p=0.04), and HDP+DM (r_S=0.62, p=0.001). Moreover, SAA1 was increased in women with hsCRP above an upper reference limit of 4 mg/L (9.4 vs. 4.3 μg/mL, p<0.001). We found no correlation between PZP and hsCRP (data not shown). We found no correlation between SAA1 and PZP for the total cohort or for any pregnancy groups except for HDP+DM (r_S=-0.37, p=0.01).

SAA1 and PZP concentrations did not consistently correlate with pre-pregnancy BMI, early pregnancy blood pressure, gestational age at sampling, or fetal sex in the total cohort or in any subgroups; the few exceptions are listed in Supplemental Results.

Discussion

Principal Findings

In line with our hypothesis, we found increased SAA1 and decreased PZP in pregnancies complicated by EO-PE. SAA1 and PZP correlated significantly with signs of placental dysfunction, as determined by fetal growth restriction and an antiangiogenic biomarker pattern. In contrast to our hypothesis, we did not find an association between acute atherosis and SAA1 or PZP.

Results

Our finding of increased circulating SAA1 in EO-PE may indicate presence of amyloid-A in these women. Amyloid-A deposits are typically found in chronic inflammatory diseases,[46] as prolonged inflammation (demonstrated by CRP elevation) causing persistently elevated SAA1 may lead to amyloid-A deposits.[17] Other types of amyloid (amyloid-β and transthyretin) have been identified by immunohistochemistry in placentae from preeclamptic pregnancies.[20,21] Whether amyloid-A is deposited in the placenta in response to increased SAA1 is unknown. We speculate that this is the case and that placental amyloid-A might exacerbate existing placental dysfunction, particularly for women with EO-PE, through

disturbing cell-cell interactions and tissue structure.[17]

Previous studies have found increased circulating levels of acute-phase proteins in preeclampsia,[33,36,47] in line with our findings of increased hsCRP in EO-PE (Supplemental Table 1). Also in line with previous observations,[33] SAA1 correlated positively with hsCRP levels in our cohort, confirming a role for excessive inflammation in SAA1 dysregulation.

Preeclampsia is characterized by hypercoagulability and risk of thromboembolism.[1,48] SAA1 increases the thrombotic ability of platelets,[25] and as SAA1 is increased in EO-PE, we speculate that SAA1 participates in mediating hypercoagulability in EO-PE.

Unexpectedly, SAA1 correlated negatively with glycated hemoglobin (HbA1c), a marker of blood glucose control, in women with DM1. Poor glucose control is associated with other inflammatory markers, such as hsCRP, and with increased cardiovascular risk.[30,49,50] A previous study found a positive correlation between SAA and HbA1c in women with GDM.[30] Assays detecting SAA measure both SAA1 and SAA2, nearly identical SAA isoforms. Hence, we expected a positive correlation between SAA1 and HbA1c in our DM1 group as well. BMI likely did not confound our finding, as BMI was not associated with SAA1 in DM1 or other study groups apart from GDM (Supplemental Results). As seen from Supplemental Table 2, median pre-pregnancy BMI of women with DM1 was within the normal range (24.2 kg/m^2). To our knowledge, we are the first to address SAA1 and glycemic control in DM1 during pregnancy. The negative correlation between SAA1 and HbA1c in DM1 may reflect the complex regulation of inflammation in pregnancy, and merits further investigation.

PZP has been shown in vitro to form stable compounds with amyloid-β-peptide, thus inhibiting amyloid-β aggregation.[29] We hypothesize that PZP may similarly inhibit SAA1 misfolding. SAA1 and PZP were both significantly associated with the antiangiogenic sFlt-1/PlGF ratio, but we did not explore any mechanistic interaction between them in this study. We hypothesize that poor placentation leads to dysregulation of sFlt-1/PlGF, SAA1, and PZP, and that low PZP in the setting of increased SAA1 may facilitate amyloid-A formation, as well as other forms of amyloid associated with preeclampsia, such as amyloid-β and transthyretin.

Clinical and Research Implications

Low circulating PZP levels have been linked to pregnancy loss.[28] Women with pregestational DM and those who develop EO-PE may harbor an unfavorable preconception endometrial environment resulting in inadequate placentation and clinical manifestations of preeclampsia during pregnancy.[51] We suggest that low circulating PZP levels in the second half of pregnancy may reflect such placentation issues, and that low PZP may identify women with particularly poor placentation and subsequent high risk of early-onset placental dysfunction.

Amyloid has been identified in atherosclerotic plaques.[52] SAA1 and

SAA2, nearly identical isoforms, may play a role in atherosclerosis both locally and systemically. In a murine atherosclerosis model, SAA expression was increased in both early and late atherosclerosis lesions.[53] SAA possibly acts as a chemoattractant for leukocytes in lesions.[54] Increased circulating SAA levels have been associated with recurrent coronary events and stroke, as have elevated levels of hsCRP.[55] In a mouse model, even a short period of elevated SAA1 increased atherosclerosis lesion size.[56] We speculate that elevated SAA1 in women with preeclampsia may promote accelerated atherosclerosis progression even if the SAA1 elevation were temporary and restricted to some months during pregnancy.

Additional studies are required to elucidate protein-protein interactions between SAA1 and PZP, their possible role in placental (dys)function, and in turn, their impact on circulating angiogenic proteins. Placenta tissue studies may reveal whether increased circulating SAA1, as in EO-PE, leads to placental amyloid-A deposits. Longitudinal studies are needed to identify whether SAA1 shows prolonged elevation following preeclampsia, as hsCRP does,[57,58] placing these women at risk of postpartum amyloid-A deposition and subsequent organ damage.

Strengths and Limitations

This study is the first to examine circulating SAA1 and PZP, potential markers of protein misfolding, in phenotypically well characterized subgroups of women with hypertensive disorders of pregnancy and diabetes mellitus, in relation to different proxies for placental function and uteroplacental acute atherosis. The population size (549 pregnancies) and well-described pregnancy groups are strengths of our study, which establishes that circulating SAA1 is increased in EO-PE and placental dysfunction. Our distinction between EO-PE and LO-PE (delivery <34 or ≥34 weeks' gestation, respectively) could explain why some previous studies report increased SAA1 in preeclampsia[31,33,36] while others do not,[32,34,35] as none have made a similar differentiation. One study quantified circulating SAA1, sFlt-1, and PlGF in preeclampsia, but did not study correlations between SAA1 and sFlt-1/PlGF.[34]

PZP has been indirectly studied (by immunodiffusion gel) in two smaller studies of preeclampsia,[37,38] and directly studied (by ELISA) in one very small study where plasma from 9 women with preeclampsia was pooled before assay.[29] We are, to our knowledge, the first to study circulating levels of PZP by ELISA in individual pregnancy samples from uncomplicated, hypertensive, and diabetic pregnancies.

Pre-pregnancy BMI, early pregnancy blood pressure, gestational age at sampling, and fetal sex did not consistently correlate with SAA1 or PZP levels, thus, we did not perform regression analyses. Gestational age at sampling correlated with SAA1 and PZP, but only in EO-PE, likely reflecting a more severe placental dysfunction with a clinical phenotype requiring earlier delivery. Our results are likely not confounded by our

lack of gestational age matching as gestational age did not correlate with SAA1 or PZP in any other groups.

A limitation to our study is the lack of longitudinal samples to establish whether SAA1 and/ or PZP show prolonged dysregulation during and/ or after pregnancy complications.

Conclusions

Understanding and identifying early risk markers for female cardiovascular disease is essential for achieving targeted primary prevention, optimized health outcomes, and healthier societies. Our finding of increased SAA1 and hsCRP in women with EO-PE, fetal growth restriction, and other signs of placental dysfunction may provide a further explanation as to the known epidemiological link between these pregnancy complications and maternal cardiovascular disease in later life. Dysregulated SAA1 and PZP in EO-PE may point to amyloid-A as a mediator of the observed risk of cardiovascular disease in women with a history of preeclampsia or other forms of placental dysfunction, though longitudinal and mechanistic studies are needed to test this hypothesis.

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Supplemental Material

Serum amyloid A1 and pregnancy zone protein in pregnancy complications and correlation with markers of placental dysfunction

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Supplemental Results

Adjustment strategy for serum amyloid A1 and pregnancy zone protein

Serum amyloid A1 (SAA1) and pregnancy zone protein (PZP) concentrations were not adjusted for pre-pregnancy body mass index (BMI), early pregnancy blood pressure (BP), gestational age at sampling, or fetal sex in our analyses as these exposures in general did not correlate with SAA1 or PZP. The exceptions for lack of correlation were:

Gestational age at sampling correlated negatively with SAA1 and positively with PZP, but only in early-onset preeclampsia (SAA1: r_S=-0.25, p=0.03, PZP: r_S=0.30, p=0.01). BMI correlated positively with SAA1 in gestational diabetes mellitus (r_S=0.35, p=0.02). BMI correlated positively with PZP in controls (r_S=0.19, p=0.002). Early pregnancy BP correlated with SAA1 in gestational hypertension (systolic BP: r_S=-0.36, p=0.049, diastolic BP: r_S=-0.39, p=0.03).

Supplemental Table 1. Clinical characteristics and circulating biomarkers, by subgroup of hypertensive disorder of pregnancy and normotensive, euglycemic controls (n=466)

	Controls (n=258)	EO-PE (n=71)	P-value	LO-PE (n=98)	P-value	GH (n=30)	P-value	CHT (n=9)	P-value
Maternal age at inclusion (years)	33.5 (20.1-45.5)	32.7 (19.0-52.9)	0.12	33.3 (22.0-44.4)	0.25	33.4 (25.6-44.0)	0.93	35.0 (24.3-41.6)	0.68
Pre-pregnancy body mass index (kg/m^2)	22.4 (17.6-39.5)	25.3 (19.0-41.1)	<0.001	23.6 (18.3-39.1)	0.004	27.8 (19.3-41.1)	<0.001	29.4 (20.8-35.9)	0.004
Mean SBP <20 weeks (mmHg)	110 (85-140)	120 (90-158)	<0.001	115 (95-164)	<0.001	123 (105-136)	<0.001	136 (121-155)	<0.001
Mean DBP <20 weeks (mmHg)	68 (40-88)	74 (57-103)	<0.001	73 (50-91)	<0.001	78 (60-87)	<0.001	87 (75-97)	<0.001
First pregnancy	24%	38%	0.02	48%	<0.001	47%	0.009	44%	0.24
First delivery	39%	59%	0.003	61%	<0.001	57%	0.07	67%	0.16
Previous preeclampsia, n (%)[a]	12 (8%)	17 (61%)	<0.001	15 (41%)	<0.001	2 (15%)	0.29	1 (33%)	0.23
Diabetes mellitus	-	7%	-	28%	-	30%	-	44%	-
SBP at inclusion (mmHg)	121 (86-156)	159 (130-220)	<0.001	151 (124-200)	<0.001	145 (117-169)	<0.001	143 (120-189)	<0.001
DBP at inclusion (mmHg)	75 (48-110)	95 (69-120)	<0.001	94 (66-130)	<0.001	91 (63-103)	<0.001	94 (78-103)	<0.001
Gestational age at inclusion (weeks)	39.0 (33.7-42.1)	29.7 (24.3-33.9)	<0.001	36.9 (33.4-40.4)	<0.001	38.4 (32.7-41.1)	<0.001	37.9 (34.4-39.4)	0.008
Preterm delivery (<37 weeks' gestation)	3%	100%	<0.001	46%	<0.001	17%	0.004	33%	0.003
New born weight (grams)	3483 (1325-5130)	1075 (500-2135)	<0.001	2768 (1461-5040)	<0.001	3445 (1820-4754)	0.98	3150 (1830-5424)	0.07

New born weight percentile	62.6 (0-100)	0.1 (0-75.5)	<0.001	27.4 (0-100)	<0.001	64.2 (4.5-100)	0.59	32.1 (0.1-100)	0.12
Fetal growth restriction	2%	75%	<0.001	20%	<0.001	0%	1	22%	0.01
Acute atherosis[b]	9% (14/160)	52% (27/52)	<0.001	29% (16/56)	<0.001	0% (0/9)	1	20% (1/5)	0.38
Serum-sFlt-1 (pg/mL)[c]	3683 (1081-17185)	14100 (4686-61348)	<0.001	9605 (2651-34240)	<0.001	6227 (1429-12397)	<0.001	5629 (2772-10138)	0.009
Serum-PlGF (pg/mL)[c]	172 (23-2074)	32 (6-231)	<0.001	78 (26-1852)	<0.001	96 (43-624)	<0.001	130 (42-255)	0.02
sFlt-1/PlGF ratio[c]	22.3 (1.1-615.1)	416.9 (78.9-3264.3)	<0.001	123.3 (1.4-555.9)	<0.001	64.3 (2.3-200.9)	<0.001	60.2 (10.9-232.9)	0.006
Serum-hsCRP (mg/L) [ref.: 0.0-4.0][d]	3.1 (0.6-76.6), n=201	6.8 (0.8-107), n=53	<0.001	4.0 (0.6-64.7), n=64	0.24	4.4 (0.9-18.5), n=21	0.08	2.3 (1.0-7.7), n=5	0.68
Plasma-SAA1 (µg/mL)	5.08 (0.41-120)	17.07 (1.95-120)	<0.001	5.46 (0.22-120)	0.37	6.56 (1.27-120)	0.12	3.22 (1.26-8.67)	0.09
Plasma-PZP (µg/mL)	892 (32-3240)	590 (12-3020)	0.002	764 (50-2840)	0.15	950 (29-3280)	0.74	1302 (411-2992)	0.33

For continuous variables, median value and range is shown; for categorical, percentages. Statistical differences were calculated by comparing each pregnancy inclusion group to the control group (normotensive and euglycemic pregnant women). EO-PE: early-onset preeclampsia (delivery <34 weeks' gestation). LO-PE: late-onset preeclampsia (delivery ≥34 weeks' gestation). GH: gestational hypertension. CHT: chronic hypertension. Women with CHT and PE (superimposed PE) were analyzed together with women with de novo PE. SBP: systolic blood pressure. DBP: diastolic blood pressure. sFlt-1: soluble fms-like tyrosine kinase-1. PlGF: placental growth factor. hsCRP: high-sensitivity C-reactive protein. SAA1: serum amyloid A1. PZP: pregnancy zone protein.

[a] Nulliparous women were excluded from analyzing rate of previous preeclampsia.

[b] Decidua basalis acute atherosis was assessed in 282 women (all had spiral arteries present).

[c] sFlt-1 and PlGF concentrations were available for all except 1 woman with EO-PE and 1 woman with LO-PE.

[d] hsCRP concentration was available for 344 women.

Supplemental Table 2. Clinical characteristics and circulating biomarkers, by subgroup of diabetes mellitus in pregnancy and normotensive, euglycemic controls (n=386)

	Controls (n=258)	DM1 (n=61)	P-value	DM2 (n=18)	P-value	GDM (n=49)	P-value
Maternal age at inclusion (years)	33.5 (20.1-45.5)	32.3 (21.1-41.4)	0.03	34.8 (24.3-41.8)	0.62	35.2 (22.0-45.9)	0.11
Pre-pregnancy body mass index (kg/m^2)	22.4 (17.6-39.5)	24.2 (19.1-41.1)	0.003	28.5 (21.5-35.0)	<0.001	26.7 (20.1-39.4)	<0.001
Mean SBP <20 weeks (mmHg)	110 (85-140)	115 (100-138)	<0.001	123 (95-164)	<0.001	115 (92-155)	<0.001
Mean DBP <20 weeks (mmHg)	68 (40-88)	70 (53-85)	0.11	73 (50-90)	0.006	70 (50-90)	0.001
First pregnancy	24%	38%	0.04	44%	0.09	12%	0.06
First delivery	39%	49%	0.15	50%	0.36	39%	0.96
Diet treatment only	-	0%		6%		63%	
Metformin treatment	-	0%		50%		12%	
Insulin treatment	-	100%		89%		33%	
Preeclampsia	-	28%		44%		14%	
Chronic hypertension	-	0%		6%		6%	
Gestational hypertension	-	7%		6%		8%	
Previous preeclampsia[a]	12 (8%)	9 (29%)	0.002	0%	1	7 (23%)	0.02
SBP at inclusion (mmHg)	121 (86-156)	135 (90-183)	<0.001	139 (115-189)	<0.001	127 (99-164)	0.04
DBP at inclusion (mmHg)	75 (48-110)	80 (50-107)	0.007	89 (66-110)	<0.001	80 (57-110)	0.06
Gestational age at inclusion (weeks)	39.0 (33.7-42.1)	37.0 (28.7-40.1)	<0.001	37.5 (29.0-39.0)	<0.001	38.4 (33.0-40.4)	<0.001

Preterm delivery (<37 weeks)	3%	30%	<0.001	17%	0.02	10%	0.03
New born weight (grams)	3483 (1325-5130)	3776 (985-5148)	0.001	4009 (620-5424)	0.01	3590 (1745-5857)	0.13
New born weight percentile	62.6 (0-100)	92.0 (0-100)	<0.001	95.5 (0-100)	0.01	69.9 (0.1-100)	0.05
Fetal growth restriction	2%	7%	0.046	6%	0.29	2%	0.58
Acute atherosis[b]	9% (14/160)	13% (3/24)	0.47	13% (1/8)	0.54	20% (5/25)	0.15
Serum-sFlt-1 (pg/mL)	3683 (1081-17185)	6262 (1744-21430)	<0.001	5394 (2296-15122)	0.01	4715 (1260-13463)	0.002
Serum-PlGF (pg/mL)	172 (23-2074)	136 (35-529)	<0.001	136 (29-1852)	0.81	168 (47-1027)	0.54
sFlt-1/PlGF ratio	22.3 (1.1-615.1)	55.5 (8.7-260.3)	<0.001	29.0 (1.4-470.8)	0.24	29.3 (2.7-175.2)	0.35
Serum-hsCRP (mg/L) [ref.: 0.0-4.0][c]	3.1 (0.6-76.6), n=201	3.9 (0.8-15), n=21	0.75	3.3 (1.2-14.7), n=7	0.71	4.4 (0.6-32.7), n=34	0.01
Plasma-SAA1 (µg/mL)	5.08 (0.41-120)	4.56 (0.41-120)	0.35	5.55 (0.22-115.95)	0.95	4.86 (0.37-116.05)	0.72
Plasma-PZP (µg/mL)	892 (32-3240)	683 (25-3280)	0.02	580 (87-1302)	0.003	640 (45-3406)	0.14

For continuous variables, median value and range is shown; for categorical, percentages. Statistical differences were calculated by comparing each pregnancy inclusion group to the control group (normotensive and euglycemic pregnant women). DM1: type 1 diabetes mellitus. DM2: type 2 diabetes mellitus. GDM: gestational diabetes mellitus. SBP: systolic blood pressure. DBP: diastolic blood pressure. sFlt-1: soluble fms-like tyrosine kinase-1. PlGF: placental growth factor. hsCRP: high-sensitivity C-reactive protein. SAA1: serum amyloid A1. PZP: pregnancy zone protein.

[a] Nulliparous women were excluded from analyzing rate of previous preeclampsia.

[b] Decidua basalis acute atherosis was assessed in 217 women (all had spiral arteries present).

[c] hsCRP concentration was available for 263 women.

Supplemental Table 3. Clinical characteristics and circulating biomarkers, according to presence or absence of decidua basalis acute atherosis by pregnancy inclusion group (n=313)

	Controls (n=160)		P-value	HDP (n=96)		P-value	DM (n=31)		P-value	HDP+DM (n=26)		P-value
	NonAA (n=146)	AA (n=14)		NonAA (n=59)	AA (n=37)		NonAA (n=29)	AA (n=2)		NonAA (n=19)	AA (n=7)	
Maternal age at inclusion (years)	33.5 (22.7-45.5)	32.8 (20.1-40.6)	0.39	33.7 (19.0-42.7)	33.5 (23.4-44.4)	0.59	33.3 (26.8-42.5)	35.6 (30.7-40.6)	0.84	34.4 (24.3-44.0)	32.3 (21.1-36.1)	0.21
Pre-pregnancy body mass index (kg/m^2)	22.2 (17.6-38.1)	22.8 (19.5-30.5)	0.69	22.5 (18.9-41.1)	26.1 (18.6-35.6)	0.04	24.4 (19.8-39.4)	25.1 (24.4-25.7)	0.90	28.3 (19.8-39.1)	26.7 (21.5-36.7)	0.78
Mean SBP <20 weeks (mmHg)	109 (85-135)	118 (90-135)	0.11	119 (95-151)	120 (90-158)	0.87	114 (98-138)	115 (115-115)	0.83	120 (105-164)	121 (95-135)	0.90
Mean DBP <20 weeks (mmHg)	65 (40-85)	68 (60-77)	0.47	75 (55-97)	75 (60-92)	0.93	70 (50-84)	70 (65-75)	0.85	76 (62-90)	75 (50-89)	0.37
First pregnancy	19%	43%	0.04	42%	41%	0.86	14%	0%	1	26%	71%	0.07
First delivery	36%	57%	0.11	63%	51%	0.27	21%	100%	0.06	53%	71%	0.66
Previous preeclampsia, n (%)[a]	8 (9%)	1 (17%)	0.44	5 (24%)	11 (61%)	0.02	3 (13%)	-		4 (44%)	2 (100%)	0.46
SBP at inclusion (mmHg)	120 (90-156)	118 (86-138)	0.22	155 (130-212)	160 (135-220)	0.16	123 (107-155)	132 (124-140)	0.52	154 (130-189)	154 (120-183)	0.81
DBP at inclusion (mmHg)	74 (54-99)	70 (48-83)	0.18	98 (78-120)	99 (80-130)	0.23	73 (60-90)	80 (70-90)	0.50	93 (66-110)	94 (75-107)	0.88

Gestational age at inclusion (weeks)	39.0 (33.7-42.1)	39.0 (34.7-39.7)	0.94	34.4 (25.1-39.3)	31.4 (24.7-40.4)	0.03	38.9 (28.7-40.1)	38.2 (37.9-38.6)	0.62	36.9 (29.0-40.0)	37.0 (32.4-38.7)	0.94
Preterm delivery (<37 weeks' gestation)	3%	7%	0.37	71%	84%	0.16	21%	0%	1	53%	43%	1
Early-onset preeclampsia (delivery <34 weeks' gestation)	-	-		44%	68%	0.03	-	-		15%	33%	0.56
New born weight (grams)	3429 (1690-4760)	3537 (2575-4450)	0.57	2054 (540-4550)	1420 (510-5040)	0.03	4035 (988-4840)	3358 (2960-3756)	0.35	3630 (620-5424)	3150 (1860-4592)	0.23
New born weight percentile	59.7 (0-99.9)	65.3 (5.0-99.4)	0.45	5.0 (0-99.6)	1.2 (0-99.9)	0.07	94.1 (0-100)	57.6 (24.5-90.7)	0.35	98.6 (0-100)	45.5 (7.8-99.9)	0.23
Fetal growth restriction	1%	0%	1	44%	65%	0.047	7%	0%	1	11%	0%	1
Serum-sFlt-1 (pg/mL)[b]	3482 (1081-14085)	4115 (1573-13928)	0.43	11390 (4034-61348)	12256 (4535-44826)	0.63	4629 (1744-14177)	6332 (5846-6817)	0.18	7853 (2651-15619)	7863 (4715-15122)	0.82
Serum-PlGF (pg/mL)[b]	167 (23-2074)	149 (69-314)	0.40	58 (11-279)	39 (6-126)	0.04	158 (35-1027)	126 (102-151)	0.43	130 (29-1852)	81 (47-155)	0.14
sFlt-1/PlGF ratio[b]	20.9 (1.3-615.1)	26.5 (5.1-113.6)	0.22	232.9 (37.3-1884.4)	306.1 (55.0-3141.2)	0.07	31.9 (3.9-139.0)	52.9 (38.7-67.1)	0.31	60.2 (1.4-470.8)	106.3 (34.6-175.2)	0.28

Serum-hsCRP (mg/L) [ref.: 0.0-4.0][c]	3.4 (0.6-76.6), n=121	3.2 (0.9-13), n=13	0.90	4.6 (0.6-55.1), n=47	4.1 (0.8-75.4), n=28	0.88	3.9 (0.7-32.7), n=26	4.8 (3.4-6.2), n=2	0.75	5.2 (0.8-15.9), n=16	6.3 (3.3-14.7), n=7	0.66
Plasma-SAA1 (µg/mL)	5.44 (0.92-120)	4.15 (0.41-18.18)	0.09	10.27 (0.80-120)	12.60 (0.86-120)	0.26	3.11 (0.41-120)	9.18 (1.07-17.29)	1	5.84 (0.22-115.95)	8.90 (3.22-81.12)	0.46
Plasma-PZP (µg/mL)	962 (38-3150)	822 (46-3240)	0.46	820 (50-2840)	700 (12-2685)	0.29	774 (45-3406)	694 (131-1256)	0.67	624 (25-2436)	497 (143-1006)	0.53

For continuous variables, median value and range is shown; for categorical, percentages. Statistical differences were calculated within each pregnancy inclusion group, comparing women without acute atherosis (nonAA) against women with acute atherosis (AA). HDP: hypertensive disorders of pregnancy. DM: diabetes mellitus (pregestational or gestational). HDP+DM: women with both HDP and DM. SBP: systolic blood pressure. DBP: diastolic blood pressure. sFlt-1: soluble fms-like tyrosine kinase-1. PlGF: placental growth factor. hsCRP: high-sensitivity C-reactive protein. SAA1: serum amyloid A1. PZP: pregnancy zone protein.

[a] Nulliparous women were excluded from analyzing rate of previous preeclampsia.

[b] sFlt-1 and PlGF concentrations were available for all except 2 women with HDP.

[c] hsCRP concentration was available for 258 women.