OXIDATIVE STRESS AND ENDOTHELIAL FUNCTION IN NORMAL PREGNANCY AND PREECLAMPSIA

OXIDATIEVE STRESS EN ENDOTHEELFUNCTIE TIJDENS NORMALE ZWANGERSCHAP EN PREECLAMPSIE

Proefschrift voorgelegd tot het behalen van de graad van doctor in de medische wetenschappen aan de Universiteit Antwerpen door:

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Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

MARIE CURIE (1867-1934)

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Table of contents

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Preeclampsia: the disease of theories

Preeclampsia (PE) is a pregnancy specific disorder, defined as hypertension and proteinuria initiating after the twentieth pregnancy week. Although mild cases occur, PE often derails into a severe life-threatening condition with high maternal mortality. In sharp contrast to a decline of sepsis and haemorrhage as maternal mortality causes in the developed world, unfortunately PE remains high on this list. [1] This implies that despite enduring and vast efforts, modern medicine is still unable to properly treat this dangerous pregnancy complication. PE has a worldwide incidence of 10 million cases per year, resulting in 76.000 maternal deaths. [1] Until now, the only effective treatment for PE is delivery, leading to high rates of premature deliveries, therefore causing 500.000 foetal and neonatal deaths each year. In the western world, 2-7% of all pregnancies suffer from PE and 0.1% of these progress to the convulsive state known as eclampsia. [2] Extensive research over the last decade revealed another crucial aspect of PE, namely the long-term sequelae of this hypertensive disorder. PE is known to be an important risk factor for cardiovascular disease in later life, both for mother and child. [3] Women with a history of PE are exposed to a 3-fold higher risk of hypertension and a 2-fold higher risk of ischemic heart disease and stroke. [4-7] Furthermore, a greater risk for the development of chronic kidney disease has been proven. [8, 9]

As alluded in the title, the exact aetiology of PE remains an enigma. Nevertheless, research concerning the pathophysiologic mechanism of PE has made substantial progress and it is abundantly demonstrated that abnormal placentation plays a key role. However, it remains unclear whether PE is primary a placental problem or whether this placentation failure is secondary to a maladaptation of the maternal cardiovascular system during pregnancy. [10] During the first trimester of normal pregnancy, vascular remodelling of the maternal uterine spiral arteries occurs. Trophoblast cells invade the spiral arterioles and convert the muscular vessels into wide bore, low resistance, large capacity vessels. This process is normally completed by 20 weeks gestation. [11] In PE, this placentation process is disturbed and the spiral arteries remain in a high resistance, vasoconstricted state. [11] This leads to impaired blood supply of the foeto-placental unit and results in placental ischemia-reperfusion damage. Consequently, toxic substances such as free radicals are formed, causing abundant placental

oxidative stress (OS). The ischemic placenta becomes abnormally activated and produces inflammatory, anti-angiogenic (e.g. soluble fms-like tyrosine kinase 1 (sFlt-1)) and oxidative substances, resulting in systemic inflammation, systemic OS and systemic endothelial activation. [12, 13] When this endothelial activation and dysfunction occurs at the level of peripheral organs such as liver, kidney or brain, the clinical presentation of PE arises. [14]

Benefit and drawback of oxidative stress in pregnancy

Oxidative stress is defined as an imbalance between a pro-oxidant and antioxidant state, where the balance is shifted towards the former. [15] In normal pregnancy, a certain amount of placental OS is present during all three trimesters and this is necessary to maintain normal cell function, including activation of redox-sensitive transcription factors and activation of protein kinases. [16-20] Although OS is a necessary feature of normal pregnancy, elevated and persistent OS is a causative factor in the pathogenesis of PE. [18, 20-22] OS arises when produced free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) exceed the antioxidative capacity of the system. The most abundant ROS in the human body is superoxide (O2•-) [23], while nitric oxide (•NO) represents the majority of RNS.

Although an excess in free radicals has a pernicious effect, not all free radicals cause disturbances in the organism and •NO is an example thereof. •NO is released by the trophoblast cells that alter the muscular wall of the spiral arteries and thus exerts a crucial role in the healthy placentation process. [24] Endothelial •NO synthase (eNOS) is the enzyme responsible for •NO formation out of the amino acid L-arginine. eNOS is activated by the binding of angiogenic factors vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) to their respective receptor (fms-like tyrosine kinase-1 (Flt-1), also known as VEGF receptor 1 (VEGFR-1)) on the cytotrophoblast. [25] Although VEGF receptor 2 (VEGFR-2) or KDR (kinase insert domain receptor) is the major mediator of the broad signalling cascades that regulate endothelial cell functions in the human body, it is poorly represented in placental tissue. It can be found in placental endothelial cells, but not in cytotrophoblast cells. [26] In PE, the placenta is known to produce a soluble form of the Flt-1 receptor (sFlt-1), that interferes with •NO production by capturing VEGF and PlGF. [13, 27] (Figure 1) Therefore, it is hypothesized that preeclamptic placental cells produce lower concentrations of •NO. Nevertheless, findings in literature have been heterogenous. [27] In the setting of defective

placentation, oxidative substances become excreted in the maternal circulation and lead to endothelial dysfunction. In a high systemic OS state, •NO and superoxide will react, forming a molecule named peroxynitrite (ONOO-). ONOO- is a very potent deleterious molecule that leads to DNA damage and cell death by inhibiting all mitochondrial processes. [28] Concerning the identity of the causative substances responsible for this systemic OS as well as their normal circulating levels during healthy pregnancy, literature is scarce and confusing. In addition, data on placental •NO concentrations in healthy and PE pregnancies tend to differ, depending on the used technique.

In this doctoral thesis, electron paramagnetic resonance (EPR) was used as a direct method to detect placental •NO and maternal circulating superoxide levels. The basic concept of EPR is comparable to nuclear magnetic resonance (NMR), but instead of detecting the spins of atomic nuclei, in EPR unpaired electron spins of free radicals are excited. EPR has proven to be the most direct and reliable method for the detection of free radicals. [29]

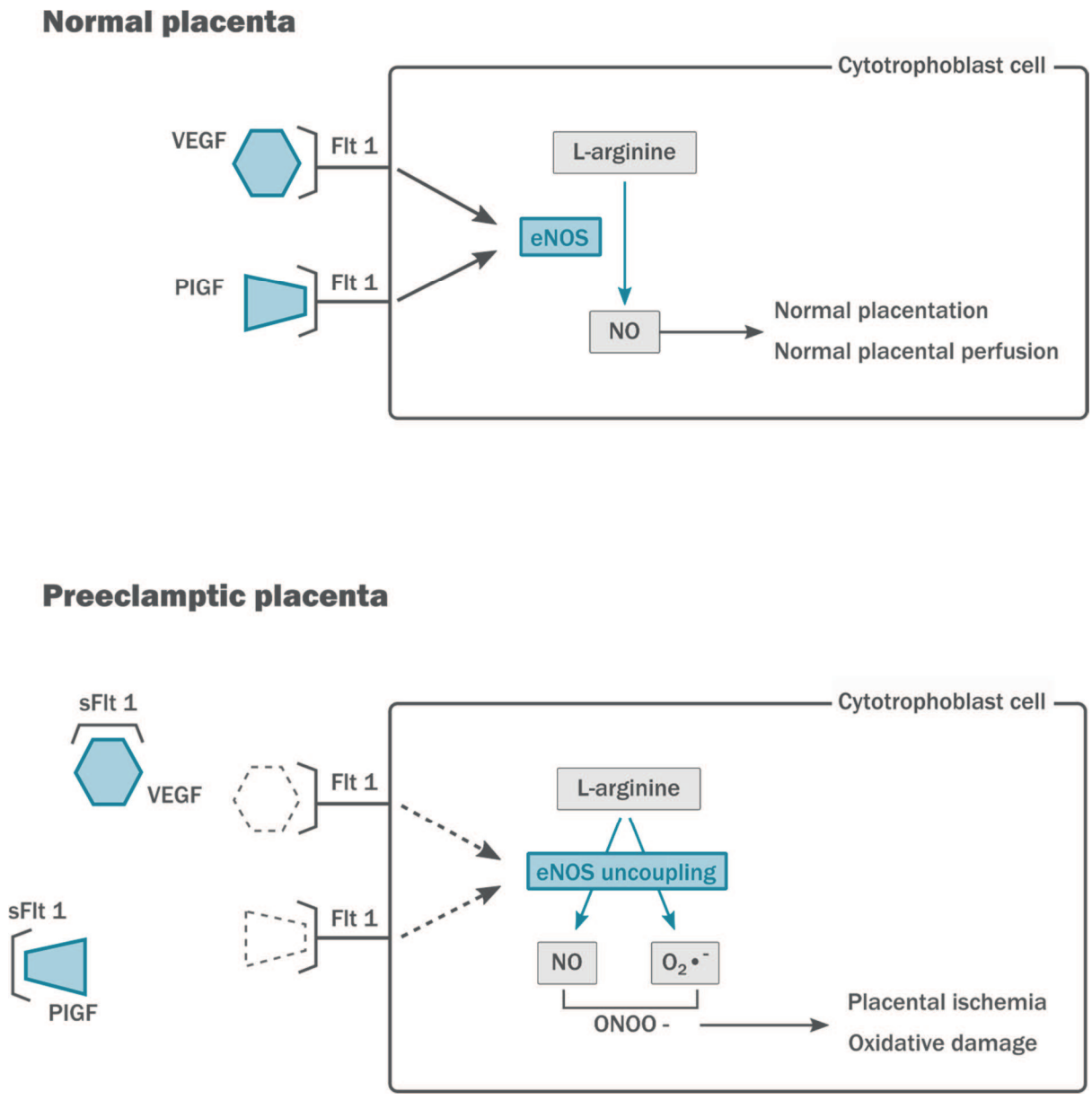

Figure 1: Binding of VEGF and PlGF to their receptor Flt1 stimulates •NO formation via eNOS. In preeclampsia the soluble receptor sFLT1 captures VEGF and PlGF inhibiting •NO formation. In high oxidative stress environments, eNOS undergoes uncoupling, resulting in the formation of superoxide besides •NO. •NO and superoxide react to from ONOO-, resulting in even more oxidative damage and placental ischemia. (VEGF: vascular endothelial growth factor, PlGF: placental growth factor; Flt 1: fms-like tyrosine kinase-1, sFLT 1: soluble fms-like tyrosine kinase-1;•NO: nitric oxide, eNOS: endothelial NO synthase; O2•-: superoxide; ONOO-: peroxynitrite)

Haematological factors representing systemic inflammation

Healthy pregnancy is characterized by physiologic haematological changes. Neutrophils are increased due to augmented physical stress and impaired neutrophilic apoptosis, while lymphocytes decrease [30, 31], resulting in a higher neutrophil to lymphocyte ratio (NLR) in healthy pregnancy. [32] In PE, activation of neutrophils is even more prominent due to exposure to oxidized factors secreted by the placenta when they pass the intervillous space. [33-35] In healthy pregnancy, thrombocytes become activated and perished, precipitating a condition referred to as 'gestational thrombocytopenia'. Endothelial dysfunction, the keystone of PE, results in the activation of thrombocytes, therefore enhancing vascular dysfunction by producing the vasoconstrictor thromboxane A2 (TxA2). This thrombocyte activation can be evaluated by measuring mean platelet volume (MPV) as an indicator of thrombocyte size, synthesis and function [36-38]. In this doctoral thesis, NLR and MPV are both retrospectively and prospectively measured in healthy versus PE pregnancy in order to evaluate their potential as predictive factors for the development of PE.

The importance of optimal arterial and endothelial adaptation to pregnancy

Pregnancy is known to cause massive cardiovascular changes, already starting in the first trimester. This hyperdynamic circulation, characterized by an enormous increase in cardiac output, intravascular volume and systemic compliance, is necessary to meet the metabolic requirements of pregnancy. [10] Since intravascular volume is expanded with approximately 1500ml, it is needless to point out that systemic vasodilation is crucial to maintain maternal blood pressure within normal range. [10] It is the endothelium that plays a critical role in the accommodation of this increased arterial flow. [39] The endothelial lining is situated at the interface of bloodstream and blood vessel wall and has an important function through the production of vasodilators (e.g. •NO and prostaglandins) and vasoconstrictors (e.g. endothelin-1 [ET-1]) in response to changes in shear stress. [32] These substances stabilize platelets preventing fibrin formation, control the influx of inflammatory cells and, most importantly, regulate blood vessel tone by coordinating the vascular smooth muscle cells in the tunica media.

Whereas endothelial dysfunction and vascular maladaptation are the grounding of the house on which PE is build, hypertension is only the roof, visible from all corners. [39] Endothelial dysfunction is characterized by a decreased •NO production and/or diminished response of the smooth muscle cells to •NO. In the abundantly described two stage disease model of PE, defective placentation (stage 1) leads to the production of OS and anti-angiogenic factors, which affect the endothelium. This systemic endothelial dysfunction (stage 2) causes reduced perfusion of target organs such as kidneys, liver and brain, ultimately resulting in the clinical manifestation of PE. [27] However, it is proposed that women who develop PE are characterized by pre-existing cardiovascular impairment prior to pregnancy, which makes them more prone to this vascular and endothelial dysfunction. [10] In this way, we propose an "adapted" two stage mechanism (Figure 2), starting at a pre-existing fragile endothelial situation, leading to defective placentation, and resulting in exacerbated and generalised endothelial dysfunction.

•NO is the protagonist of the endothelium and measurement of brachial artery flow mediated dilatation (FMD), the gold standard technique for non-invasive assessment of endothelial function, is well correlated to •NO bio-availability in the endothelial cells. [40] Peripheral arterial tonometry (PAT) is an additional measurement of microvascular endothelial function and is known to be less operator- and less •NO-dependent. [41] In contrast to FMD regulation by •NO, less is known about the influence of increased levels of ET-1 on endothelial function. [42] Low-flow mediated constriction (L-FMC) has been recently introduced as a promising measure of ET-1 dependent endothelial function, but is only scarcely investigated in pregnancy. [43, 44] Further, applanation tonometry (AT) is a vascular test measuring central and peripheral arterial stiffness. Although it does not measure endothelial function directly, it reliably represents vascular wall stiffness. [45, 46] FMD and AT are both non-invasive cardiovascular measurement techniques recently recommended for the assessment of hemodynamic function in pregnancy. [47]

Since an optimal vascular adaptation to pregnancy is of utmost importance for the establishment of a healthy pregnancy, this doctoral thesis aims to compile a profound assessment of changes in vascular function during and after normal versus PE pregnancy. A combination of recommended and innovative vascular parameters was used to establish a thorough hemodynamic profile in both healthy and PE pregnancy.

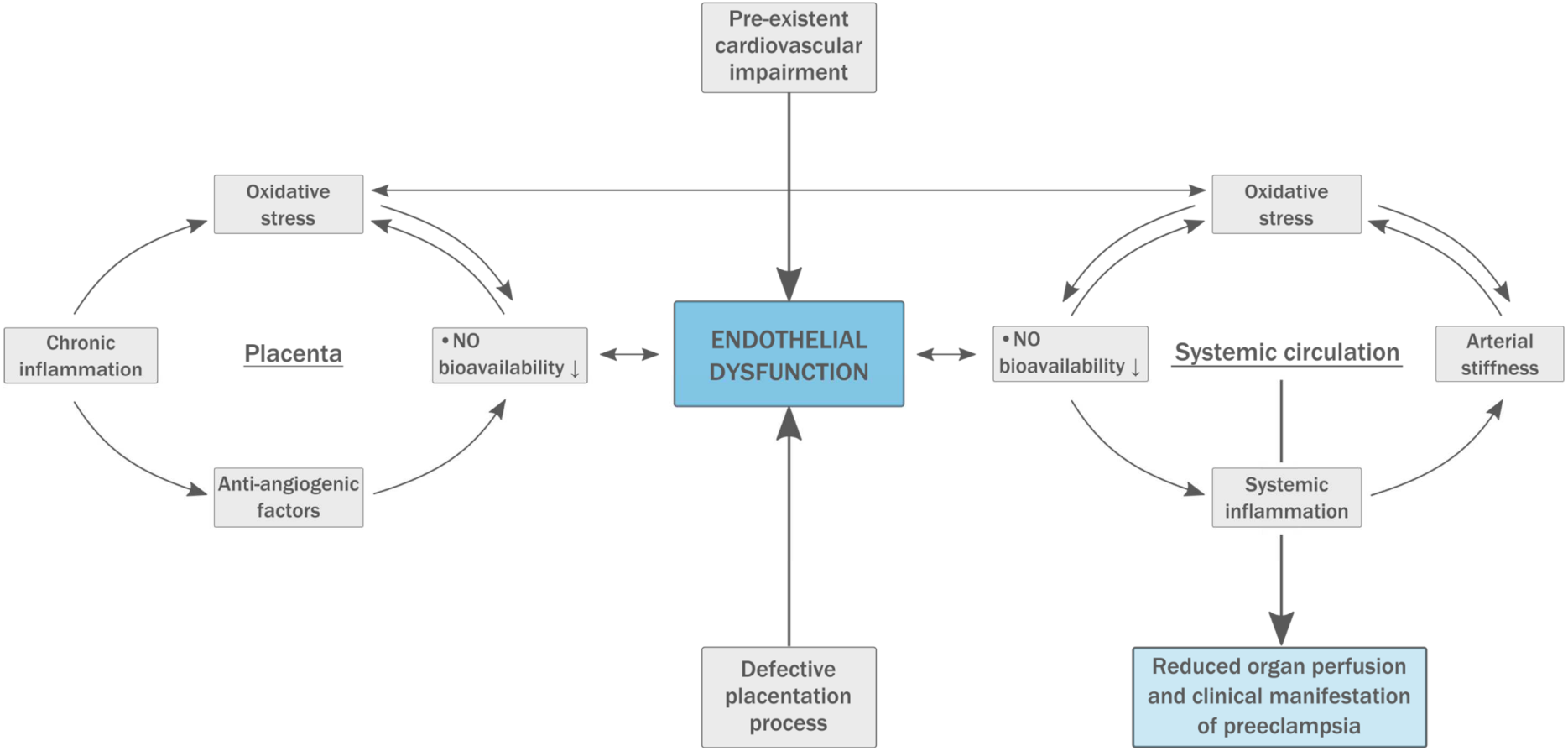

Figure 2: The "adapted" two stage model of preeclampsia.

Aim of the thesis

The overarching objective of this doctoral thesis was to expand our knowledge of the contribution of oxidative stress (OS) and systemic inflammation to arterial and endothelial function in healthy pregnancy and preeclampsia.

The *first aim* was to evaluate and compare the longitudinal course of OS and systemic inflammation during healthy versus PE pregnancy by measuring superoxide, NLR and MPV in maternal blood.

The *second aim* was to make a comprehensive evaluation of the longitudinal course of vascular (arterial and endothelial) function during healthy versus PE pregnancy. A combination of recommended and innovative vascular parameters was used to evaluate their clinical relevance and usefulness in the evaluation of hemodynamic changes during and after pregnancy.

The *third aim* was to determine placental •NO concentrations to elucidate the contribution of altered placental •NO levels in the pathophysiology of PE.

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CHAPTER TWO

STUDY PROTOCOL OF THE ENDOPREG STUDY

STUDY PROTOCOL: OXIDATIVE STRESS AND ENDOTHELIAL FUNCTION IN NORMAL PREGNANCY VERSUS PREECLAMPSIA, A COMBINED LONGITUDINAL AND CASE CONTROL STUDY

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BMC Pregnancy Childbirth. 2018;18(1):60.

Abstract

Background

Preeclampsia (PE) is related to an impaired endothelial function. Endothelial dysfunction accounts for altered vascular reactivity, activation of the coagulation cascade and loss of vascular integrity. Impaired endothelial function originates from production of inflammatory and cytotoxic factors by the ischemic placenta and results in systemic oxidative stress (OS) and an altered bioavailability of nitric oxide (•NO). The free radical •NO, is an endogenous endothelium-derived relaxing factor influencing endothelial function. In placental circulation, endothelial release of •NO dilates the fetal placental vascular bed, ensuring feto-maternal exchange. The Endopreg study was designed to evaluate in vivo endothelial function and to quantify in vitro OS in normal and preeclamptic pregnancies.

Methods/design

The study is divided into two arms, a prospective longitudinal study and a matched case control study. In the longitudinal study, pregnant patients ≥ 18 years old with a singleton pregnancy will be followed throughout pregnancy and until 6 months post-partum. In the case control study, cases with PE will be compared to matched normotensive pregnant women. Maternal blood concentration of superoxide (O2•-) and placental concentration of •NO will be determined using EPR (electron paramagnetic resonance). Endothelial function and arterial stiffness will be evaluated using respectively Peripheral Arterial Tonometry (PAT), Flow mediated dilatation (FMD) and applanation tonometry. Placental expression of eNOS (endothelial NOS) will be determined using immune-histochemical staining. Target recruitment will be 110 patients for the longitudinal study and 90 patients in the case-control study.

Discussion

The results of Endopreg will provide longitudinal information on in vivo endothelial function and in vitro OS during normal pregnancy and PE. Adoption of these vascular tests in clinical practice potentially predicts patients at risk to develop cardiovascular events later in life after PE pregnancies. •NO, O2•- and eNOS measurements provide further inside in the pathophysiology of PE.

Trial registration: This trial has been registered on clinicaltrials.gov. ClinicalTrials.gov Identifier: NCT02603913. Registered October 2015.

Introduction

Preeclampsia (PE) is a potentially life-threatening pregnancy related vasculopathy characterized by hypertension and proteinuria. PE results in high morbi-mortality for both mother and her unborn child. Between five and ten percent of pregnancies are complicated by hypertensive disorders and worldwide the incidence of PE has increased by 25% in the past two decades. [1]

In normal pregnancy vascular remodelling of the maternal uterine spiral arteries occurs. Trophoblast cells invade the spiral arterioles within the first 12 weeks of pregnancy and replace the muscular wall of the vessels converting them into wide bore, low resistance, large capacity vessels, a process normally completed by 20 weeks gestation. [2] The free radical nitric oxide (•NO) is an important mediator of the placentation process. •NO is an endogenous endothelium-derived relaxing factor influencing endothelial function. Under physiologic conditions, endothelial release of •NO in the placental circulation dilates the fetal placental vascular bed, ensuring feto-maternal exchange. [3] •NO is formed out of L-Arginine by NOS (Nitric Oxide Synthase). This reaction is regulated by VEGF (Vascular endothelial growth factor), an endothelial mitogen that has an important function in the proliferation of endothelial cells and in angiogenesis. VEGF stimulates eNOS (endothelial NOS) and induces therefore •NO production. [4] In an oxidative environment, the lack of NOS-stabilizing factors results in NOS-uncoupling. NOS-coupling causes a shift from •NO production to superoxide (O2•-) production which maintains an oxidative setting.

The pathogenesis of generalized endothelial dysfunction is well known in PE and is subdivided into two phases. The first phase exists of a poor trophoblast invasion of the spiral arteries during the placentation process, causing failure to transform the placental bed arteries from high to low resistance vessels. This results in local ischemia, reperfusion damage and oxidative stress (OS). The local damage activates the second phase where disturbed production of angiogenic and anti-angiogenic factors (placental growth factor (PlGF) and soluble fms-like tyrosine kinase 1 (sFlt-1), respectively) results in systemic inflammation, endothelial activation, systemic OS and altered endothelial •NO production. [5, 6] When this vascular endothelial activation and dysfunction occurs at the level of liver, kidney, brain and placenta, the clinical presentation of PE arises. [7]

In the past, PE has been divided in two different entities; angiogenic or placental PE (formerly called early onset, before 34 weeks) and non-angiogenic or maternal PE (formerly called late onset, after 34 weeks). [8] Impaired placentation and endothelial dysfunction have been described in placental PE, whereas pre-existing maternal cardiovascular risk factors (essential hypertension, high BMI (body mass index), diabetes, renal disease,..) usually precede maternal PE. This description however oversimplifies and overstates recent existing findings. Maternal risk factors can precede early onset PE as well as abnormal concentrations of placental angiogenic factors are found in late onset PE. [6] Fetal growth restriction and endothelial dysfunction caused by systemic inflammation are usually described in placental PE, nevertheless they are common in late onset PE. It is therefore more accurate to state that both maternal and placental factors contribute to PE and research should focus on classifications based on pathophysiologic processes, for instance endothelial and vascular dysfunction and amount of systemic inflammation and OS. [9, 10]

In normal pregnancy, placental OS is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and activation of protein kinases. [11-15] Although OS is a common necessary feature of normal pregnancy, persistent OS gives rise to different disease-states, such as PE. [13, 15-17] Although considerable research has been devoted to OS in PE [13-15], less attention has been paid to the evolution of OS during the course of normal pregnancy. Little research has described an increase in •NO concentration with gestational age, suggesting an important role for •NO in the cardiovascular changes of normal pregnancy. [3]

Recent literature has elucidated that PE is an important risk factor for cardiovascular disease in later life. Bellamy et al. and McDonald et al. describe a 3-fold risk for hypertension and a 2-fold risk of ischemic heart disease and stroke in women with a history of PE. [18-21] Women with hypertensive disorders during pregnancy also have a greater risk of chronic kidney disease and end-stage renal disease. [22, 23] With a view to detecting those women at risk, objectifying endothelial function and vascular function after healthy pregnancy and PE can help to establish reference values for disturbed post-pregnancy vascular function.

Methods

Study hypothesis

Endothelial and vascular function improve during healthy pregnancy to answer the higher hemodynamic demands. Due to the deficient placentation in PE, disturbed production of (anti) angiogenic and inflammatory factors results in arterial stiffness and endothelial dysfunction. After PE, this vascular dysfunction continues in patients at risk for developing cardiovascular events later in life.

A certain amount of OS is necessary in healthy pregnancy. The deficient placental oxygenation in PE causes excessive local formation of reactive oxygen and nitrogen species (O2•- and •NO respectively). When the balance between pro-oxidant species and the antioxidants is disturbed, OS arises. We hypothesize that in PE, there is a higher amount of O2•- in the maternal circulation and a lower concentration of •NO and eNOS measurable in the placenta.

Endothelial and vascular dysfunction is correlated to the amount of OS present in the circulation.

Objectives

<u>Primary study objective</u>:

To evaluate OS during pregnancy.

Measurement of •NO in placental tissue and O2•- in maternal blood using EPR.

Measurement of eNOS in placental tissue using immuno-histochemical staining.

Prospective longitudinal study: To evaluate the OS profile in normal pregnancies.

Matched case-control study: To compare the OS profile in normal versus PE pregnancies.

<u>Secondary study objectives</u>:

To evaluate endothelial function during pregnancy (using peripheral arterial tonometry (PAT) and flow mediated dilatation (FMD) techniques) and to relate endothelial function to •NO, O2•- and eNOS concentration.

Prospective longitudinal study: To evaluate endothelial function in normal pregnancies.

Matched case-control study: To compare endothelial function in normal versus complicated pregnancies.

To evaluate arterial stiffness during pregnancy (Pulse wave velocity, pulse wave analysis using Sphygmocor ®) and to relate arterial stiffness to •NO, O2•- and eNOS concentration.

Prospective longitudinal study: To evaluate arterial stiffness in normal pregnancies.

Matched case-control study: To compare arterial stiffness in normal versus complicated pregnancies.

Methodology

Study design

Single centre prospective longitudinal study

The first part of the study will have a prospective longitudinal design. Pregnant women in their first trimester of pregnancy will be eligible and will be followed throughout pregnancy and until 6 months postpartum.

Multicentre matched case-control study

The second part is a case control study where patients with pregnancies complicated by PE will be compared to normotensive controls, matched for maternal and gestational age, parity, smoking behaviour, BMI and ethnic group. Patients will be followed throughout (the rest of their) pregnancy and until 6 months postpartum. Matching will reduce the risk of bias.

Study population

Inclusion criteria

1. Prospective longitudinal study

    Pregnant women ≥ 18 years old with a singleton pregnancy

2. Matched case-control study

    Pregnant women ≥ 18 years old with a singleton pregnancy and > 20 weeks of pregnancy

Cases:

Cases will meet the criteria for preeclampsia following the revised ISSHP definition (2014) [24]: Hypertension (>140 mmHg systolic or >90 mmHg diastolic) developing after 20 weeks gestation and the coexistence of one or more of the following new onset conditions:

1. Proteinuria (spot urine protein/creatinine >30 mg/mmol [0.3 mg/mg] or >300 mg/day or at least 1 g/L [‘2 + ’] on dipstick testing)

2. Other maternal organ dysfunction

- renal insufficiency (creatinine >90 µmol/L)
- liver involvement (elevated transaminases – at least twice upper limit of normal - and/or severe right upper quadrant or epigastric pain)
- neurological complications (examples include eclampsia, altered mental status, blindness, stroke, or more commonly hyperreflexia when accompanied by clonus, severe headaches when accompanied by hyperreflexia, persistent visual scotomata)
- haematological complications (thrombocytopenia: platelet count below 150,000/dL, DIC (diffuse intravascular coagulation), haemolysis)

3. Uteroplacental dysfunction: fetal growth restriction

Hypertension greater than or equal to 140 mmHg systolic or 90 mmHg diastolic, must be confirmed at least 4 hours apart. Hypertension greater than or equal to 160 mmHg systolic or 110 mmHg diastolic can be confirmed after a short interval (minutes) to facilitate antihypertensive treatment. [25]

Exclusion criteria

Exclusion criteria are (gestational) diabetes, multiple pregnancies, fetal abnormalities, hypercholesterolemia, renal disease, auto-immune disorders and connective tissue disease. Intake of low-dose aspirin or vitamin C supplements (>500mg/day) is an exclusion criterion. Since in Belgium folic acid is an advised pre- and periconceptional therapy, this will not act as an exclusion criterion.

The use of other medication / supplements will be listed in the patients record to find eventual confounders afterwards.

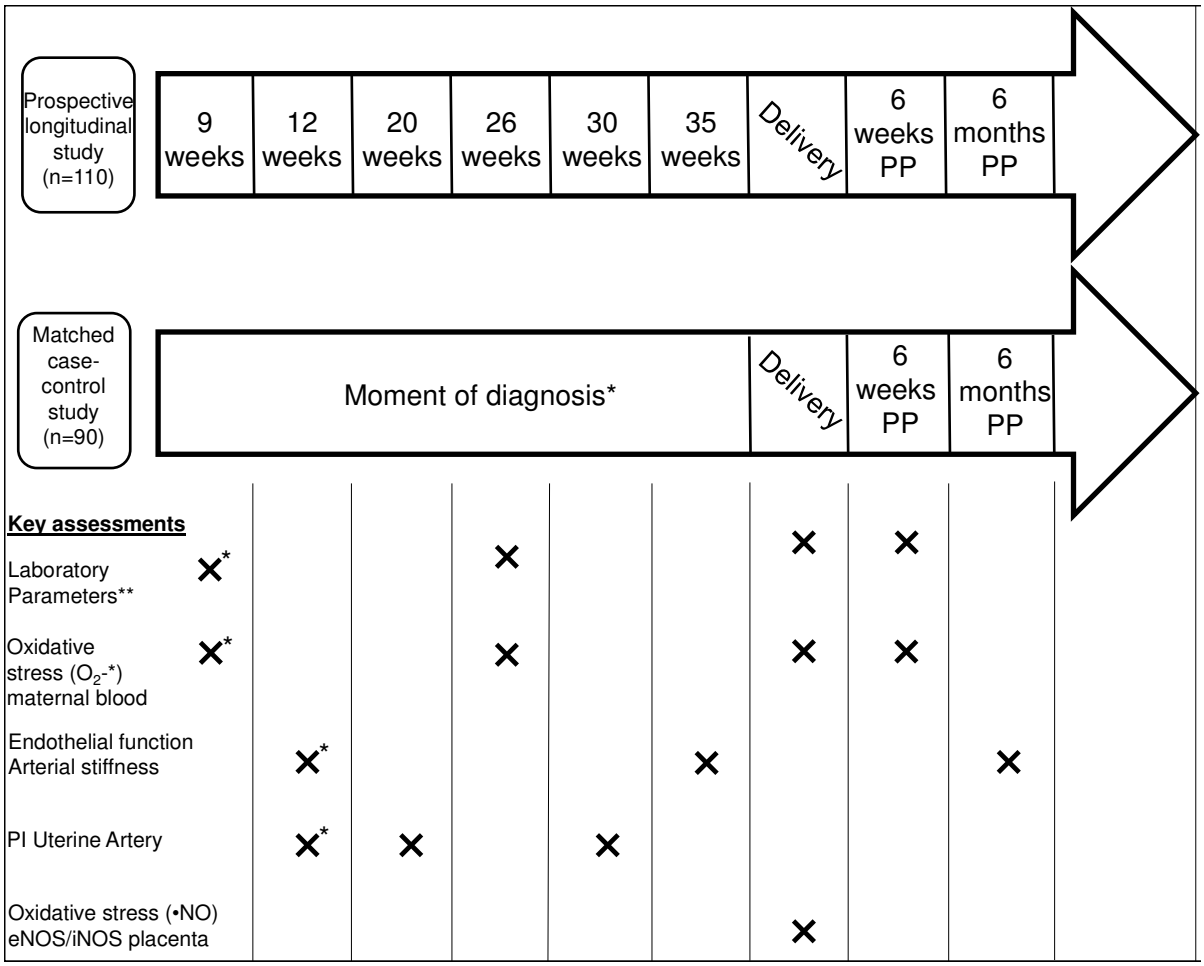

Figure 1: Study design and key assessments.
*PI = pulsatility index; PP = postpartum; * Assessments at moment of diagnosis;*
*** Laboratory parameters tested include NLR, MPV (mean platelet volume), platelet count, Fe, transferrin and ferritin.*

Endothelial function (FMD)

The gold standard for non-invasive assessment of endothelial function is FMD, measuring •NO-dependent vasodilatation of the brachial artery in response to reactive hyperaemia [26]. An ultrasound diagnostic instrument (Prosound alfa6, Hitachi Aloka Medical ®) equipped with vascular software for 2D-imaging, colour Doppler imaging and ECG-triggering, are used with a high frequency linear array transducer (UST-5413, 5-13 MHz, Hitachi Aloka Medical ®). Patients are in a resting, supine position with the arm in a comfortable position for imaging the brachial artery. A blood pressure cuff is placed on the forearm with the upper border of the cuff at a distance of 5 to 10 cm distal from the elbow (lateral epicondyle). The brachial artery is imaged above the antecubital fossa in a longitudinal plane with a clear delineation of both anterior and posterior intima-media interfaces. A special probe-holding device is used to ensure consistency

of images during the measurement. The baseline artery diameter is automatically tracked and the waveform of diameter changes over the cardiac cycle is displayed in real time using an automated edge detection system. (eTracking system, Aloka ®). Arterial occlusion is created by cuff inflation to supra-systolic pressure at least 50 mmHg above systolic pressure (minimum value of 200 mmHg) for 5 minutes. Low–flow mediated constriction (L-FMC) is calculated as the percent decrease in arterial diameter in the last 30 seconds of cuff occlusion as compared with resting diameter. [27] After 5 minutes of occlusion, the cuff is deflated. Brachial diameter is recorded continuously (eTracking) from the time point of cuff inflation to 5 minutes after cuff deflation. FMD (in % from baseline value) is expressed as (post-ischemic maximal diastolic diameter change - baseline diastolic diameter)/ baseline diastolic diameter. [26] During the measurements, brachial artery blood flows will be measured at 3 different time-points; at rest, 15 seconds before cuff deflation, and immediately upon cuff deflation using pulsed-wave Doppler. [27] All recordings are performed by two experienced investigators (IG, TS).

FMD and PAT measurements will be performed simultaneously and patients will be asked 24h prior to examination not to eat high-fat substances nor drink caffeine or alcohol and to refrain from smoking at least 6h prior to examination. Fingernails had to be short and no nail polish applied. Patients were studied in a quiet, temperature-controlled room (21-24°C) and stressful situations for the patient were avoided (people entering the room unexpectedly, telephone ringtones, etc.) Patients will not be measured in active labour.

Endothelial function (RHI)

PAT is recorded using the Endo-PAT2000® (Itamar Medical, software version 3.2.4) and the disposable fingertip probes (Itamar Medical) in accordance with the manufacturer's recommendations. PAT is a less operator-dependent and more reproducible technique. The system uses pneumatic finger probes which assess digital volume changes accompanying pulse waves. Reactive hyperaemia is induced as described for FMD and measurements are performed simultaneously to FMD. The ratio of the average amplitude of the PAT signal over a one minute period starting one minute after cuff deflation (maximum pulse amplitude) divided by the average amplitude of the PAT signal over a 3.5minute period before cuff inflation (baseline pulse amplitude) is calculated. The control arm is used to correct for confounding factors (room temperature, systemic changes). The result is expressed as the reactive hyperaemia index (RHI).

(Figure 2) All recordings are performed simultaneously with the FMD and by the same two experienced investigators (DM, EF).

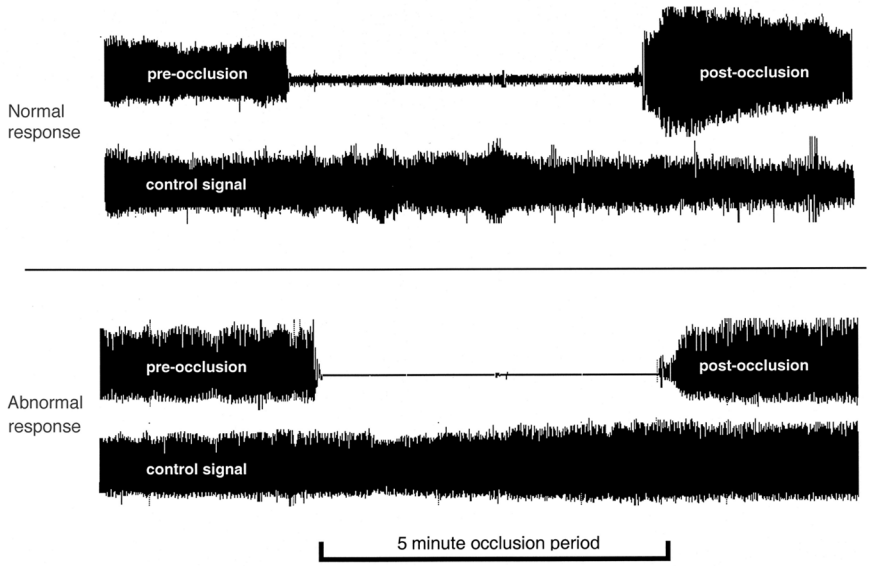

Figure 2: Representative reactive hyperaemia peripheral arterial tonometry recordings of subjects with normal and abnormal reactive hyperaemic response.
Normal response is characterized by a distinct increase in the signal amplitude after cuff release compared with baseline.

Arterial stiffness: PWV, AP, AIx and AIx75

Pulse wave velocity (PWV) and pulse wave analysis (PWA) will be calculated using the Sphygmocor system ® (Atcor Medical, West Ryde, Australia). To calculate PWV, two pressure waveforms must be measured at a known distance apart and the distance between measurement sites is divided by the propagation time. Aortic PWV is measured by carotid-femoral PWV (CF-PWV) as it is the 'gold standard' measurement of the stiffness of the aorta. Measurements of CF-PWV will be performed using a pressure tonometer to transcutaneously record the pressure pulse waveform in the underlying artery. The tonometer contains a micromanometer that provides a very accurate recording of the pressure within the artery. The carotid and femoral PWV will be assessed by gently compressing respectively the carotid artery and the femoral artery with the tip of the tonometer at the site of maximal pulsation. The Sphygmocor device will automatically calculate the CF-PWV.

Pulse wave analysis will calculate AIx (augmentation index) by placing the tonometer at the radial artery (site of maximum pulsation). A generalized transfer function will derive the aortic

pressure waveform from the radial artery waveform. From the aortic pressure waveform, the augmentation pressure (AP) and AIx can be calculated. The AP (ΔP) is defined as the height of the late systolic peak above the inflection point on the waveform. The AIx is defined as AP expressed as a percentage of the aortic PP. As AIx is affected by heartrate, it will be standardized to a heart rate of 75 bmp (AIx-75).

Pulsatility index uterine artery and fetal biometry

Uterine artery (UA) Doppler examinations will be performed using trans-abdominal colour directed pulsed wave Doppler (Voluson, GE Healthcare Technologies, USA). Pulsatility index of both uterine arteries will be obtained on either side of the cervix before 14 weeks' gestation and at the apparent crossover with the external iliac arteries after 14 weeks. [28, 29]

Three similar consecutive waveforms must be obtained before calculating the PI. The mean PI of the two vessels was calculated. Observation on the presence or absence of a bilateral early protodiastolic notch will be performed. A notch is defined as a persistent decrease in blood flow velocity below the diastolic peak velocity, in early diastole.

At the same moment basic fetal biometry parameters will be measured: bi-parietal diameter, head circumference, abdominal circumference, femur length and expected fetal weight using Hadlocks formula. [30]

Markers of systemic inflammation

Performing a complete blood count (CBC), the neutrophil/lymphocyte ratio (NLR), mean platelet volume (MPV) and platelet count will be obtained using a ADVIA 120 Hematology System (Siemens healthcare, Germany). [31-33]

Automated blood pressure measurement

SBP (systolic blood pressure), DBP (diastolic blood pressure) and MAP (mean arterial pressure) after 10 minutes rest in a sitting position, will be measured using a Mindray VS 900 monitor (Mindray ®, China).

Oxidative stress , Electron paramagnetic resonance

EPR (electron paramagnetic resonance) is derived from magnetic resonance spectroscopy and uses microwave radiation to detect molecules with an unpaired electron number, like radicals. When an magnetic field is created by the EPR spectrometer, all radicals will align. The EPR spectrometer sends out a radio frequent microwave, causing the electrons to jump from a low to a high energy state. This energy absorption can be measured and is directly correlated to the amount of free radicals in the sample. A 'spin trap' will be added to scavenge the very reactive radicals and to prolong their half live. EPR spectra will be obtained using the Bruker EMX 1273 spectrometer equipped with an ER 4119HS high-sensitivity resonator (=cavity) and 12-kW power supply operating at X band frequencies. The Bruker WINEPR -post processing system software (Germany, 1996) will be used to analyse the spectra. [34]

Placental concentration of •NO

Placental tissue will be obtained within two minutes after (vaginal or caesarean) delivery. At a standardized central location, a viable sample of 1cm³ placental tissue will be taken avoiding placental infarcts. The sample will be rinsed with saline (NaCl) and immediately added to the spin trap 750µl FeSO4+750 µl DETC (iron (II)diethyldithiocarbamate solution). After one hour of incubation at 37°C, the sample will be snap frozen and stored in -80°C until analysis with EPR. [34]

Since labour causes ischemia-reperfusion injury at the site of the placenta, which will influence placental NO concentrations, samples will be subdivided in labour (vaginal delivery, secondary caesarean section) versus no labour (elective caesarean section). [35]

Maternal blood concentration of O2•-

Maternal blood will be obtained at 12weeks and 24-28 weeks in a heparin tube (BD vacutainer) and transported on ice. After 15 minutes, 30µl of spin trap CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) will be added to 30µl blood. The whole will be incubated on ice and transferred into a capillary after 5 minutes. The sample will then be snap frozen and stored in -80°C until analysis. [34]

Immuno-histochemical staining for eNOS and iNOS

Placental tissue will be obtained shortly after delivery and stained for eNOS and iNOS as previously described by Du et al. [36]

Statistical methods

Sample size calculations

As sample size determining factor, we took the RHI since this is an important variable in our study, there is normal distribution and the population standard deviation is known.

Prospective longitudinal study

For the physiologic study of the RHI in pregnancy we calculate that for a 95% confidence interval, a population standard deviation of 0.5 (as described in other populations for RHI) and a tolerable standard error of the mean (SEM) value of 0.1, 97 women have to be followed. [37] Taking at least a 10% dropout into account the starting sample size will be 110 women.

Matched case-control study

In a pilot study by Yinon [38] the RHI in normotensive pregnancies was 1.8, and in PE 1.5; in most populations standard deviation is 0.5. [37] For 80% power and a two sided $\alpha = 0.05$ and considering a 0.3 difference clinically relevant, the sample size for each group would be 44 (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html); which we consider the sample size for our cross-sectional study comparing preeclamptic patients with normotensive controls . The sample size of this study will be 90 patients, 45 in each group.

Descriptive statistics and data analysis

Prospective longitudinal study

For the physiologic study of the RHI in pregnancy we will calculate the reference values and 95% confidence interval. Longitudinal data will be plotted and a linear mixed-effects model with random intercept will be fitted. Percentiles for RHI, FMD and PWV/PWA will be calculated based on this model. Correlation coefficients between baseline RHI, FMD, PWV/PWA, UA Doppler PI, fetal biometry, NLR, MPV, MAP, birth-weight percentile, eNOS, •NO and O2•- will be analysed.

Matched case-control study

RHI, FMD, PWV/PWA, UA Doppler PI, fetal biometry, NLR, MPV, MAP, birth-weight, eNOS, •NO and O2•- concentration and other continuous variables in PE versus healthy pregnancies will be tested for normality using the Shapiro Wilk Test. If there is normality, they will be expressed as mean, standard deviations and 95% confidence intervals and compared using two sided T

test. If not, they will be expressed as median and interquartile ranges and compared using Mann Whitney U Test.

Discussion

PE is responsible for 11.5% of maternal deaths in Flanders. Ten percent of early neonatal deaths are caused by maternal hypertensive disorders. [39] This project will contribute to the knowledge of PE with the ultimate goal of reducing maternal morbidity and mortality.

As stated before, the gold standard for non-invasive assessment of endothelial function is FMD. [26] Previous literature suggests that during normal pregnancy, there is a steady increase in FMD until week 32, with a stabilization or even decline at week 36. [40] In PE, a significant reduction in FMD is suggested. [41] Data using PAT during pregnancy and PE are limited, based on small studies and they show opposing results. [38, 42] Due to measurement of peripheral microcirculatory function, PAT is less •NO-dependent than FMD. As such, FMD and PAT assess different aspects of vascular function. [43-45] Arterial stiffness has been evaluated in pregnancy, using applanation tonometry (AT). [2] During normal pregnancy AIx falls during mid pregnancy and rises at the end of pregnancy. In PE AIx is significantly increased and a significant role of first trimester AIx in the early screening of PE has been proposed. [2] Arterial stiffness is independently associated with cardiovascular risk and may, therefore, provide a potential marker to select women who will develop cardiovascular events later in life after PE. [2, 46] Concerning endothelial function and arterial stiffness in normal pregnancy and PE there is still no consent in literature and further research is warranted.
Both in normal pregnancy and PE are inflammatory effects present, which can be objectified by higher neutrophil to lymphocyte ratio (NLR) and higher mean platelet volume (MPV). [47] Increase in NLR and MPV are described to be more prominent in PE, and these factors have been proposed as predictive biomarkers for PE. [31, 48] Increased systemic low grade inflammation possibly contributes to alterations in endothelial function.

Previous research has demonstrated that markers of OS, like O2•-, hydrogen peroxide (H2O2), hydroxyl radical (•OH), nitric oxide (•NO), and peroxynitrite (ONOO-) are involved in the

pathophysiology of placental pregnancy disorders. OS at the site of the placenta causes placental damage and this ischemic placenta releases cytotoxic, anti-angiogenic and inflammatory markers in the circulation resulting in systemic endothelial dysfunction and peripheral organ damage. [3] O2•-, the most abundant free radical, encloses an important role in the beneficial effects of OS. Studies measuring O2•- concentration longitudinally in normal pregnancy and during PE are lacking, creating a gap in the knowledge of OS in pregnancy and PE. Evidence for OS at the placenta and in the maternal circulation in PE has led to the suggestion that antioxidant therapy can improve or even prevent PE. Vitamins E and C, L-arginine (precursor of •NO) and •NO donors have been proposed to limit both endothelial injury and for the prevention of PE with conflicting results. [49-55] Substantial knowledge of the evolution of OS in healthy and PE pregnancies might influence introduction of these therapies in regular medical practice.

ENDOPREG is the first clinical study comparing in vivo measurements of endothelial function with in vitro markers of endothelial dysfunction in a longitudinal and case-control setting. To our knowledge, serial changes in maternal endothelial function have not been evaluated previously in pregnancy using two different methods, i.e. PAT/FMD and EPR, simultaneously. The main strength of our study is the longitudinal design. Little studies determined maternal •NO or reactive oxygen species concentration at the moment of diagnosis [56] and only a few performed a longitudinal approach. Studying endothelial function and OS profile in and after normal pregnancies and PE, will give a better insight in the pathophysiology of this pregnancy complication and will help with the detection of patients a risk of developing cardiovascular events later in life. Within the ENDOPREG study, research groups from biomedical and pharmaceutical sciences will collaborate to unravel yet another step in the pathophysiology of the disease of many theories, PE.

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CHAPTER THREE

ARTERIAL AND ENDOTHELIAL FUNCTION IN HEALTHY PREGNANCY AND PREECLAMPSIA

A. LOW-FLOW MEDIATED CONSTRICTION AS A MARKER OF ENDOTHELIAL FUNCTION IN HEALTHY PREGNANCY AND PREECLAMPSIA

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Pregnancy Hypertension. (submitted)

Abstract

Objectives

Overwhelming clinical evidence exists on disturbed vascular and endothelial function in the pathophysiology of preeclampsia (PE). In a non-pregnant (NP) population, L-FMC (low-flow mediated constriction) provides insight in the 'resting' endothelial capacity in contrast to the gold standard of flow mediated dilatation (FMD), reflecting endothelial nitric oxide bioavailability.

Study design

Longitudinal follow-up of 100 healthy pregnant (HP) women, 33 PE women and 16 NP controls with non-invasive vascular assessments. HP women were evaluated at 12 and 35 weeks of gestation and at 6 months postpartum. PE patients were assessed at diagnosis (mean 30 weeks) and 6 months postpartum.

Main outcome measures

Endothelial function (L-FMC, FMD, peripheral arterial tonometry (PAT)) and arterial stiffness (pulse wave velocity (PWV) and analysis (PWA)) were measured at the different visits and compared between groups.

Results

Overall endothelial dysfunction is present in PE (FMD HP 9.09±4.20 vs PE 5.21±4.47, p=0.0004; L-FMC HP -1.90±2.66 vs PE -0.40±2.09, p=0.03). L-FMC gradually elevates during the course of a HP (1^{st} trim -0.31±1.75 vs 3^{rd} trim -1.97±3.02, p<0.0001) and is present in 85% of women in the third trimester. In NP, only 27% of women has L-FMC. In PE, L-FMC is present in 50% of cases. Arterial stiffness is increased in PE (all p<0.0001). There is no correlation between L-FMC and other markers of vascular function (p>0.05).

Conclusion

PE is characterized by dysfunction of both resting and recruitable endothelial capacity. This study offers new insights in different aspects of endothelial function in pregnancy, since L-FMC reflects an adaptation in HP that is absent in PE.

Introduction

The pathophysiology of preeclampsia (PE) has not been completely unravelled, yet evidence on endothelial dysfunction (ED) and arterial stiffness is abundant. [1-5] The endothelium plays a critical role in controlling vasomotor tone. Lowering the vasomotor tone is essential to allow haemodynamic adaptation to pregnancy, therefore quantitative measures of endothelial function have gained increasing attention over the last decade. [1, 2, 4, 6, 7]

The gold standard for non-invasive assessment of endothelial function is flow mediated vasodilatation (FMD), measuring nitric oxide (NO)-dependent vasodilatation of the brachial artery in response to reactive hyperaemia and increased shear stress.[8] While FMD reflects the 'recruitable' capacity of the endothelium, it does not provide information concerning basal endothelial function.[9, 10] In order to fill this gap, low-flow mediated vasoconstriction (L-FMC), mainly an endothelin-1 dependent phenomenon, has been recently introduced as an additional measure, however its relationship with changes in blood flow, cardiovascular risk factors and FMD has been less well demonstrated. [9, 10] Research on L-FMC is scarce and discrepancies between studied populations have been described. [11, 12] Controversy exist whether the presence of L-FMC is a beneficial condition or rather an inability of the endothelium to maintain tone during low-flow conditions. [13] The evolution of L-FMC during the course of a healthy pregnancy (HP) is unknown, and L-FMC has never been assessed in early PE.

Applanation tonometry (pulse wave velocity (PWV) and pulse wave analysis (PWA)) represents a reliable measure of vascular wall stiffness associated with endothelial function. [14, 15] Arterial stiffness is an independent predictor of cardiovascular mortality and morbidity [16, 17] and may play a role in the prediction of PE [18]. Recently, normograms for gestational changes in arterial stiffness have been described. [19]

When discussing vascular function in pregnancy and PE, it is critical to apprehend its different aspects (endothelial function and arterial stiffness). In this study, a comprehensive assessment of non-invasive vascular function was performed in a longitudinal follow-up study of healthy pregnancies and PE, with a special focus on the value of L-FMC as potential marker of PE.

We hypothesize that during the course of a HP, vascular function improves to meet the higher hemodynamic needs of pregnancy. Resting endothelial function is important in this

cardiovascular adaptation. We presume that during PE these vascular adaptations are impaired, resulting in lower FMD, higher arterial stiffness and less L-FMC, since increased arterial stiffness will inhibit the vessels to further constrict to a low-flow stimulus.

Methods

Study population

One hundred women with a healthy pregnancy (HP), 33 PE women (gestational age (GA) 25+0 weeks - 36+5 weeks (mean 30 weeks)) admitted to the maternal intensive care unit and 16 non-pregnant controls (NP) were included between January 2016 and December 2017. We defined PE according to the revised ISSHP definition.[20] Exclusion criteria were (gestational) diabetes, multiple pregnancies, foetal malformations, hypercholesterolemia, kidney disease, auto-immune disorders, connective tissue diseases or use of acetylsalicylic acid. Since the Antwerp University Hospital serves as a tertiary referral centre, most women were already initiated on anti-hypertensive medication, low molecular weight heparin (LMWH) and MgSO4 at the moment of referral and inclusion (Table 1). HP were included in the study during their first trimester and were longitudinally followed throughout the whole pregnancy. They were free from medication and did not have a history of PE, pregnancy-induced hypertension, hypertension, cardiovascular disease or other chronic conditions. NP subjects (n=16) matched for age, BMI and parity, served as an additional control group. At 6 months PP, 17 HP women and 19 PE women were re-assessed.

The Research and Ethics committee of the Antwerp University Hospital approved the study protocol of the ENDOPREG study (Belgian number: B300201524783), and written informed consent was obtained from all subjects.

Table 1: Antihypertensive medication and doses given to the PE women

Medication	*n*	*Duration*
No antihypertensive medication	5 women	/
Labetalol 100mg 3x/d	7 women	1-5 days
Labetalol 200mg 3x/d	5 women	1-5 days
Labetolol (IV) 6-10 ml/h	9 women	<24 hours
Labetalol 100mg 3x/d + Felodipine 10mg 2x/d	3 women	1-3 days
Felodipine 5mg 2x/d	2 women	1-5 days
Methyldopa 500mg 3x/d + Nifedipine 30mg 3x/d	2 women	1-2 days
MgSO4 (1gr/h)	27 women	<24 hours
Low Molecular Weight Heparin (Enoxaparin 4000 IE) 1x/d	33 women	<24 hours

Vascular function measurements

Women were asked not to eat high-fat substances nor drink caffeine or alcohol 24h prior to examination and to refrain from smoking at least 6h prior to examination. [21] Fingernails had to be short and no nail polish applied. Women were studied in a quiet, temperature-controlled room (21-24°C) and stressful situations were avoided (people entering the room unexpectedly, telephone ringtones, etc.) The examinations were performed in a supine lying position with the arm in a comfortable position for imaging the brachial artery. In all subjects, one blood pressure (BP) measurement was taken after 5 minutes of rest using an automated BP device (OMRON® Intellisense, Healthcare Japan) in a supine position. The systolic BP was used to determine occlusion pressure for the FMD/L-FMC/peripheral arterial tonometry (PAT) measurements. FMD/L-FMC and PAT measurements were performed simultaneously. After the endothelial function measurements, arterial stiffness was recorded. Repeat measurements in HP and PE groups were performed at the same arm and at approximately the same time of day. [6] All recordings were performed by two experienced investigators (IG, TS).

Brachial artery low-flow mediated constriction and flow mediated dilatation

L-FMC/FMD were assessed by measuring changes in brachial artery diameter in response to a respectively decrease and increase in blood flow and endothelial shear stress, elicited by inflating a cuff at the forearm for 5 minutes. [8, 10] An ultrasound diagnostic instrument (Prosound alfa6, Hitachi Aloka Medical®) equipped with vascular software for 2D-imaging, colour Doppler imaging and ECG-triggering, was used with a high frequency linear array

transducer (UST-5413, 5-13 MHz, Hitachi Aloka Medical ®) to perform the FMD/L-FMC measurements as previously described. [6] L-FMC was calculated as the decrease in brachial arterial diameter in the last 30 seconds of cuff occlusion as compared to the resting diameter. [10] FMD was expressed as % increase in brachial arterial diameter after cuff release (post-occlusion maximal diastolic diameter - baseline diastolic diameter)/ baseline diastolic diameter.[8, 22] Modified FMD (mFMD) is calculated as maximum percentage change in vessel diameter from end-occlusion diameter following cuff release. [22] Figure 1 illustrates the principles for the L-FMC and FMD measurements.

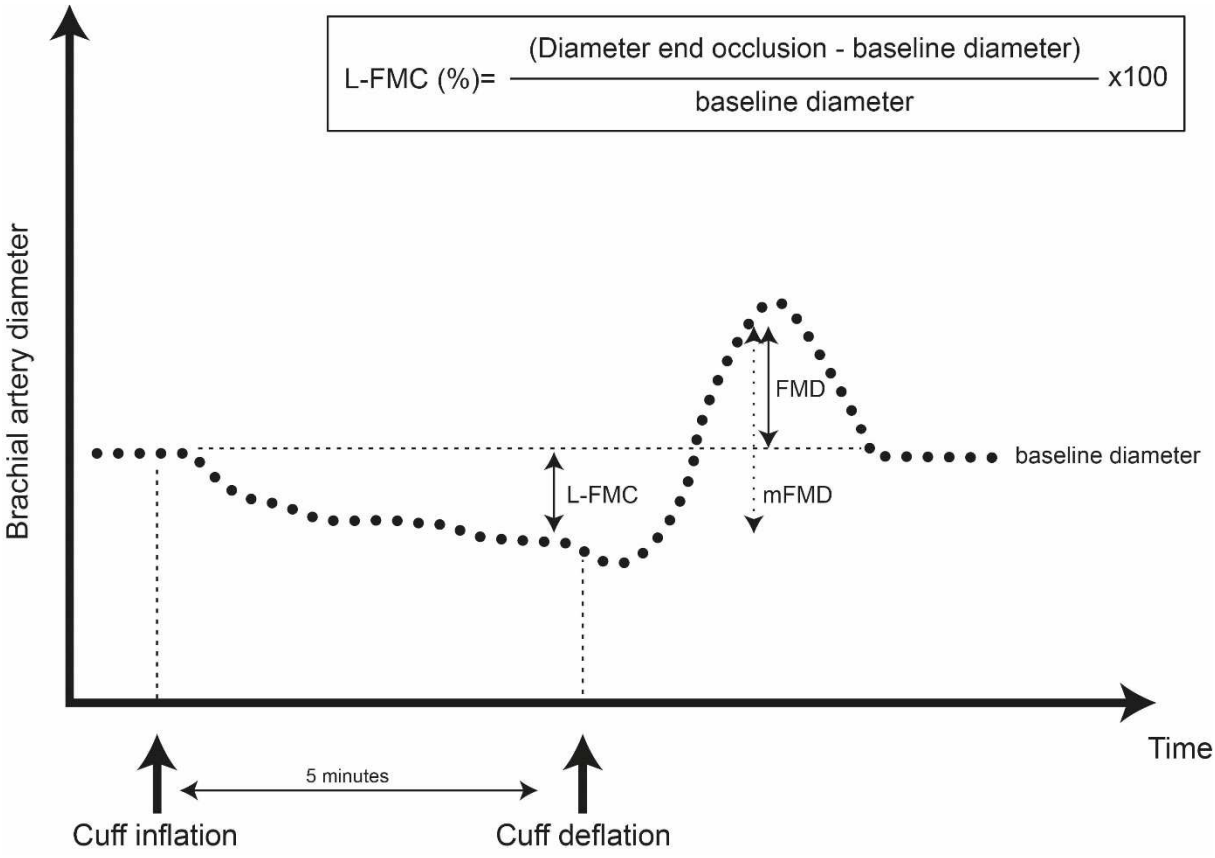

Figure 1: Principles for the L-FMC and FMD measurements.
The brachial artery is visualized with ultrasound for 11 minutes (1 min rest, 5 min cuff inflation and 5 min cuff deflation). L-FMC is acquired during the last 30 seconds of cuff inflation. FMD is calculated as maximum percentage change in vessel diameter from baseline following cuff release. mFMD is calculated as maximum percentage change in vessel diameter from end-occlusion diameter following cuff release. (L-FMC: low-flow mediated constriction; FMD: flow mediated dilatation; mFMD: modified FMD)

Peripheral arterial tonometry (PAT)

PAT was recorded using the Endo-PAT2000® (Itamar Medical, software version 3.2.4) using disposable fingertip probes (Itamar Medical) in accordance with the manufacturer's recommendations and as previously described. [6] PAT is a less operator-dependent and more reproducible technique and measures microvascular endothelial function. The system uses pneumatic finger probes which assess digital volume changes as a response to reactive hyperaemia. The result is expressed as the reactive hyperaemia index (RHI).

Arterial stiffness

Systemic arterial stiffness was evaluated using pulse wave analysis (PWA) and pulse wave velocity (PWV) using the Sphygmocor system® (Atcor Medical, West Ryde, Australia) as previously described. [6, 23, 24] For PWA, a tonometer was placed at the radial artery from which the aortic pressure waveform was derived. [23] From this aortic pressure waveform, the augmentation pressure (AP) and augmentation index (AIx) were calculated. The AP is defined as the height of the late systolic peak above the inflection point on the waveform. The AIx is defined as AP expressed as a percentage of the aortic pulse pressure. As AIx is affected by heart rate, it was standardized to a heart rate of 75 bpm (AIx-75). [24] For PWV three measurements at the level of the carotid artery and subsequently the femoral artery were obtained. As the arterial wall stiffens, the velocity of the travelling waves in the lumen increase. The aortic PWV (measured by carotid-femoral pulse wave velocity (CF-PWV)) is the gold standard method for evaluating arterial stiffness. [25-27] CF-PWV was multiplied by 0.8, a correction factor for body surface distance measurements.[27] All recordings were performed by the same two experienced investigators (DM, EF).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0, SAS 9.4 and GraphPad Prism version 7. Data are expressed as mean ± standard deviation (SD). Normality of continuous variables was evaluated using Kolmogorov-Smirnov test. Unpaired data were compared using analysis of variance (ANOVA) with Tukey's multiple comparisons and Kruskal-Wallis with Dunn's multiple comparisons post-hoc tests as appropriate. Correlation between GA and vascular measurements was studied in the case-control study using Pearson and Spearman correlation analysis as appropriate. In the presence of correlation, analysis of covariance (ANCOVA) was used to correct for the influence of differences in GA. As CF-PWV is influenced by BP and heartrate [27, 28], correlation was investigated and if present, corrected for by ANCOVA in the case-control group and linear mixed models in the longitudinal study. Fisher-exact test was used for comparison of categorical variables. A two-tailed $p < 0.05$ was considered significant.

Results

Patients characteristics

Characteristics of the three groups (HP, PE and NP) are summarized in Table 2. Pregnancy groups were comparable regarding age, BMI and cardiovascular risk. BP and birthweight were significantly different between groups. The differences in birthweight were due to differences in GA at birth. Women with co-existing IUGR were excluded from the study, since most of them were started on transdermal nitro-glycerine before measurements. In our PE population, 82% suffered from “early” PE (diagnosis <34 weeks), while the other 18% suffered from severe “late” (34-37weeks) PE, characterised by severe hypertension and/or disturbed laboratory parameters.

Table 2: Patients characteristics

	PE pregnancy (n=33)	Healthy pregnancy (n=100)	Non-pregnant controls (n=16)	P		
				PE vs HP	PE vs NP	HP vs NP
Age (years)	29.6 ± 4.1	30.4 ± 4.2	28.8 ± 3.4	0.24**		
BMI 3rd trimester (kg/m^2)	28.9 ± 4.3	28.0 ± 4.1	22.9 ± 2.7	<0.0001*		
				0.99	<0.0001	<0.0001°
SBP 3rd trimester (mmHg)	159.0 ± 15.7	125.9 ± 11.9	123.8 ± 9.3	<0.0001*		
				<0.0001	<0.0001	>0.99
DBP 3rd trimester (mmHg)	95.9 ± 11.4	74.0 ± 8.1	75.3 ± 8.0	<0.0001*		
				<0.0001	<0.0001	>0.99
MAP 3rd trimester	116.9 ± 11.8	91.3 ± 8.4	90.2 ± 7.2	<0.0001**		
				<0.0001	<0.0001	0.92
Heartrate (bpm)	77.2 ± 11.7	82.3 ± 13.3	73.1 ± 10.8	0.01*		
				0.34	0.53	0.02
Nulliparous (n)	26	52	15	0.004*		
				0.02	>0.99	0.07
Gestation at measurements 3rd trimester (weeks)	30.6 ± 3.4	34.8 ± 0.9	na	<0.0001	na	na
Gestation at delivery (weeks)	32.6 ± 4.1	38.8 ± 1.8	na	<0.0001	na	na
Birthweight (g)	1462 ± 580.7	3389 ± 539.0	na	<0.0001	na	na
Smoking (n)	2	3	0	0.33	0.54	0.99

*Data are expressed as mean ± SD, as median (range) or as number of total (n). Not applicable (na). BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure. ° There was no significant difference in BMI between NP and HP at 12 weeks (p=0.49). * Statistical analysis was performed using Kruskal-Wallis ** Statistical analysis was performed using ANOVA.*

Vascular function in preeclamptic versus normotensive pregnancy

Results of the vascular measurements of 33 PE women and 100 HP controls are shown in Table 3. FMD and mFMD are significantly decreased in PE compared to HP, confirming ED. L-FMC is significantly attenuated in PE compared to HP. In PE we found vasoconstriction during the occlusion phase (L-FMC) in 50% of patients, while in the third trimester of HP L-FMC was present in 85% of women. (Figure 2) Regarding microvascular endothelial function, RHI was

increased in PE women (p<0.0001). Arterial stiffness is significantly higher in PE women, as seen by increased CF-PWV, AP and AIx75.

CF-PWV, AIx75 and FMD all correlated with GA. After adjustment for GA, all three remained significantly increased in PE (respectively p<0.0001, p<0.0001 and p=0.013). CF-PWV was correlated with MAP, but not with heart rate. After correction for MAP, CF-PWV remained significantly increased in PE (p=0.025).

Table 3: Vascular function in preeclamptic pregnancy, healthy pregnancy and non-pregnant controls.

	PE pregnancy (n=33)	Healthy pregnancy (n=100)	Non-pregnant controls (n=16)	P		
				PE vs HP	PE vs NP	HP vs NP
Endothelial function						
FMD (%)	5.21 ± 4.47	9.09 ± 4.20	8.95 ± 3.76	0.0005**		
				0.0004	0.02	>0.99
mFMD (%)	7.90 ± 6.34	11.41 ± 4.85	8.25 ± 4.40	0.002*		
				0.005	>0.99	0.08
L-FMC (%)	-0.40 ± 2.09	-1.90 ± 2.66	0.60 ±1.22	0.0005**		
				0.03	0.42	0.001
Time to peak diameter (s)	55.1 ± 35.4	52.6 ± 23.5	39.4 ± 13.1	0.1*		
Baseline diameter (mm)	3.72 ± 0.66	3.31 ± 0.34	2.98 ± 0.31	<0.0001**		
				0.0003	<0.0001	0.02
Minimal diameter prior to cuff release (mm)	3.68 ± 0.67	3.25 ± 0.34	2.99 ± 0.32	<0.0001**		
				0.0002	<0.0001	0.11
Maximal diameter post-occlusion (mm)	3.95 ± 0.65	3.60 ± 0.33	3.23 ± 0.32	<0.0001*		
				0.003	<0.0001	0.01
Brachial artery flow, rest (cm/s)	70.9 ± 12.3	72.1 ± 17.0	/	0.441	/	/
Endopat RHI	2.1 ± 0.43	1.49 ± 0.31	1.71 ± 0.41	<0.0001*		
				<0.0001	0.03	0.12

Arterial stiffness						
CF-PWV (m/s)	7.58 ± 0.91	6.01 ± 0.97	6.36 ± 1.11	<0.0001*		
				<0.0001	0.002	0.64
AIx75 (%)	23.77 ± 9.37	3.61 ± 11.36	5.77 ± 10.06	<0.0001**		
				<0.0001	<0.0001	0.74
AP (mmHg)	11.51 ± 7.54	-0.04 ± 4	3.23 ± 3.74	<0.0001**		
				<0.0001	<0.0001	0.048

*Vascular function in PE pregnancy vs healthy pregnancy (35weeks) vs non-pregnant controls. Values are mean ± SD. CF-PWV, carotid femoral pulse wave velocity; AP, augmentation pressure; AIx75, augmentation index; FMD, flow-mediated dilation; mFMD, modified FMD; L-FMC, low-flow mediated constriction; RHI, reactive hyperaemia index. * Statistical analysis was performed using Kruskal-Wallis ** Statistical analysis was performed using ANOVA.*

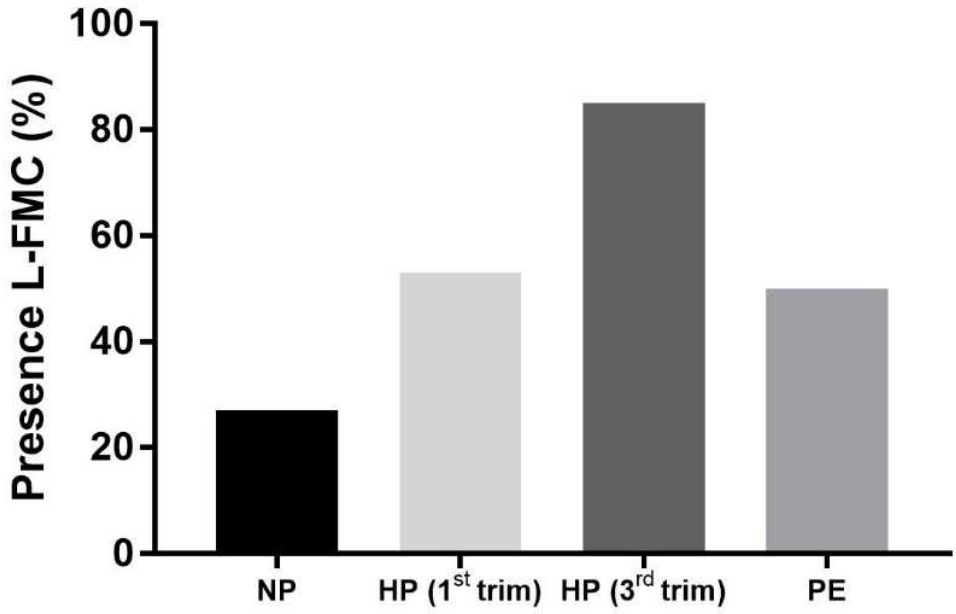

Figure 2: Presence of low-flow mediated constriction (L-FMC) in the non-pregnant (NP), healthy pregnant (HP, first pregnancy trimester), healthy pregnant (HP, third pregnancy trimester) preeclamptic (PE) and preeclamptic (PE) population.

Evolution of vascular function during normal pregnancy

One hundred HP women were included and underwent an extensive vascular assessment at the first (12.0±0.6w, n=100) and third (34.8±0.9w, n=82) trimester of pregnancy and 6 months PP (n=17). At the third trimester, only 82 patients were measured due to drop-outs (n=3), development of pregnancy complications (gestational diabetes (n=3) and of PE at 38-39weeks

(n=2)) and inferior vena cava syndrome during FMD/L-FMC/PAT measurements (n=10). Due to logistic reasons, only 85% of all HP women underwent FMD/L-FMC measurements.
FMD was comparable between the first and third trimester (Figure 3). L-FMC on the other hand became more prominent with advancing pregnancy. In the third trimester, 85% of HP had a decrease in brachial artery diameter during occlusion, compared to 53% in the first trimester (p=0.0003) (Figure 2). Six months after HP, FMD/L-FMC return to NP levels (Figure 3), except for microvascular endothelial function which seems to be improved after a HP compared no NP controls. In contrast to FMD, RHI was significantly lower at the third trimester compared to the first trimester. Figure 3 illustrates that with progression of pregnancy, there is a decrease in aortic stiffness (CF-PWV, corrected for MAP) and AP, but no difference in overall arterial resistance (AIx75). Six months after HP, CF-PWV was again normalized, but arterial resistance (AIx75) was increased compared to 1st trimester values, however, not different from NP.

A negative correlation was seen between baseline diameter of the brachial artery and FMD (1st trim p=0.03, 3rd trim p=0.001) in HP, but this relation was absent in PE (p=0.19). No correlation was found between baseline diameter and L-FMC (1st trim p=0.84, 3rd trim p=0.44, PE p=0.15).

Evolution of vascular function in preeclampsia *(Figure 3)*

L-FMC values during PE were comparable to the values of a NP population (p=0.42). While FMD improved again 6 months after a PE pregnancy, L-FMC, on the other hand, did not significantly change PP. (Figure 3) Regarding arterial stiffness, CF-PWV, AIx75 and AP improved 6 months PP. When comparing vascular measurements after a HP versus after a PE pregnancy, one single parameter remained significantly higher (CF-PWV PE PP 6.88±0.64, HP PP 6.36±0.64, p=0.03), however, after correction for MAP, this was no longer significant (p=0.12). (Figure 3) Microvascular endothelial function (PAT) is not different after a PE pregnancy compared to HP.

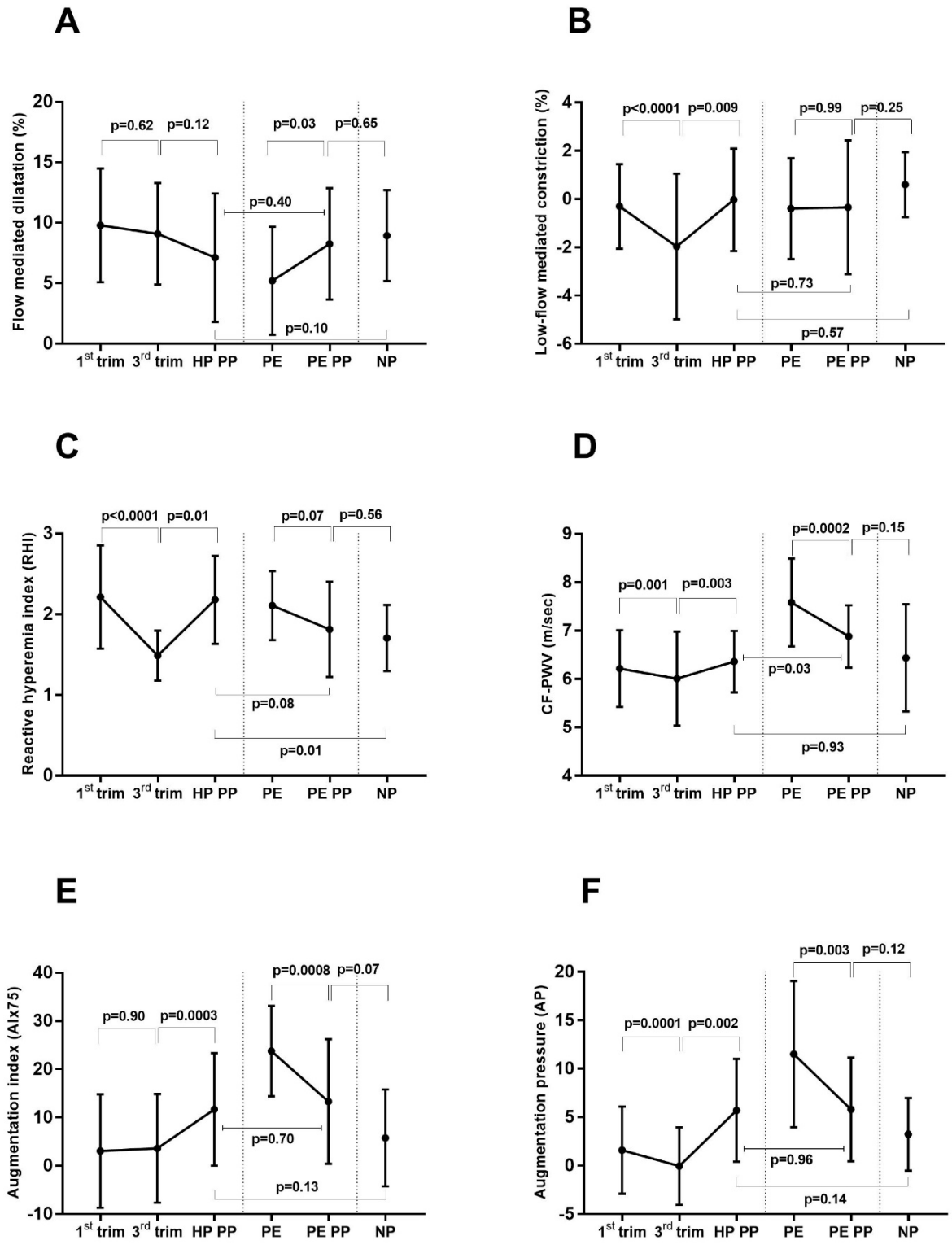

Figure 3: Vascular function in healthy pregnancy (HP) (n=100; first trimester, third trimester and post-partum (PP)), preeclampsia (PE) (n=33, at diagnosis and post-partum (PP)) versus non-pregnant (NP) controls (n=16). (Endothelial function (Flow mediated dilatation (FMD, Fig 3A), Low-flow mediated constriction (L-FMC, 3B), Reactive hyperaemia index (RHI, Fig 3C)) and arterial stiffness (Carotid-femoral pulse wave velocity (CF-PWV, 3D), Augmentation index (AIx75, Fig 3E), Augmentation pressure (AP, Fig 3F))).

Relation between markers of endothelial function

We found no significant correlation between FMD and L-FMC in neither of the groups, nor between FMD and RHI, nor between L-FMC and RHI (all $p>0.05$). No correlation was found between L-FMC and markers of arterial stiffness (all $p>0.05$). In the case-control group, FMD was significantly correlated with CF-PWV ($p=0.03$, $r= -0.3$). Throughout the whole study, CF-PWV was correlated with MAP ($p<0.0001$, $r=0.6$) and was corrected for as appropriate.

Discussion

In this study, an innovative aspect of endothelial function was objectified in early PE and longitudinally during the course of a HP. L-FMC was compared to other markers of endothelial function and arterial stiffness. The key findings presented here are that the extent of L-FMC increases during HP, without a concurrent change in FMD, but that in PE pregnancies, both L-FMC and FMD are attenuated versus HP and arterial stiffness is increased. Six months PP, overall vascular function in PE women recovered to levels comparable to HP and NP.

Brachial artery diameter is significantly higher at the end of pregnancy and a known limitation of FMD is its invers relation to the baseline diameter of the measured artery. [11, 15, 29] Normalising FMD values for baseline diameter (FMD%index) [30] does not seem to correct for this influence. [6] The proposed parameter, L-FMC, attempts at addressing this limitation. [9] Regarding this 'resting' endothelial function we found a substantial L-FMC increase with advancing pregnancy which has never been described. Literature on L-FMC in pregnancy is very scarce and in the NP population discussion remains whether an increase in L-FMC is a compensation rather than a physiologic improvement of endothelial function. [9, 10] Most studies on L-FMC are performed on the radial artery and studies on the brachial artery suggest that the presence of brachial L-FMC varies considerably among a diverse population.[11, 12] Brachial artery FMD, however, is the gold standard for non-invasive assessment of endothelial function and simultaneously measuring L-FMC has large advantages.[8] Weissgerber et al. described a significant L-FMC in a small subgroup of active pregnant women (n=15). In non-active pregnant women and NP women, there was no L-FMC observed in the brachial artery. Similarly, in our small NP population, L-FMC was absent in 73% of cases, while this was only the case in 15% of third trimester HP.

This study adds to the existing studies of vascular function in PE by investigating L-FMC as a novel parameter of endothelial function in a substantial group of early and severe PE women. L-FMC has previously been investigated in 8 cases of mild and late PE, without finding significant vasoconstriction during the occlusion phase.[31] Our results indicate that L-FMC is significantly attenuated in PE, which renders an inability to vasoconstrict to a low-flow stimulus. While an enhancement in L-FMC seems to be a manifestation of HP, L-FMC values in PE are comparable to the NP population, evidencing the hypothesis that PE is a maladaptation to pregnancy. This hypothesis becomes more and more supported by recent research. [3, 32] Another explanation might be that the absence of L-FMC is due to the increased vascular stiffness in PE and acts as a protection mechanism to avoid severe hypertension. Since correlation between FMD and L-FMC is clearly absent, L-FMC describes a completely different aspect of endothelial function, which might fill the missing gap in PE.

PE is undoubtedly associated with a deterioration in vascular function. Compared to the third trimester of HP, PE women express an overall decrease in vascular and endothelial function.[5, 6, 33-35] Surprisingly, a larger baseline brachial artery diameter was found in PE, probably due to anti-hypertensive medication. However, the correlation between baseline diameter and FMD is absent in this population, rejecting the hypothesis that FMD is worse due to this increase in baseline diameter. Literature on the effect of antihypertensive medication on FMD in NP population, claims that FMD is interpretable despite recent intake of anti-hypertensives.[36] The majority of PE women in this study were on beta-blockers at the time of measurements. While calcium channel blockers (felodipine, nifedipine) and third generation beta-blockers (labetalol) are known to improve endothelial function [37, 38], most studies are performed after chronic (>1 month) medication intake, whilst this was not the case in our PE population. In literature, no information is available on the effect of MgSO4 on endothelial function. Concerning LMWH, a recent study describes an acute improvement in FMD three hours after intravenous plus subcutaneous administration of enoxaparin. Our PE population received a significant lower dose of enoxaparin daily, however an effect on our FMD results is likely to be present.[39]

Few prior studies were able to implement PP follow-up. Interestingly, and in contrast with our study hypothesis, differences between healthy and PE PP women were small. Interestingly,

while FMD improved 6 months after a PE pregnancy, L-FMC, on the other hand, did not significantly change PP. This finding questions the influence of anti-hypertensive medication on L-FMC in PE.

Despite these novel findings, our study has some limitations. First, there is a small but significant difference in parity between HP and PE. Second, only a subgroup of our study population was measured PP. Last, PE women were already on medication during their vascular assessment, possibly influencing our results.

The main strength of our study is the longitudinal design in HP. This way we were able to investigate and understand the physiological changes in resting and recruitable endothelial function related to pregnancy itself before comparing them to preeclamptic pregnancies. To our knowledge, L-FMC has not been evaluated previously longitudinally in normal pregnancy, nor in a large group of early PE women. Furthermore, we have studied the association between L-FMC and other vascular function tests, proving that L-FMC clearly reflects an adaptation in HP which is absent in PE.

Highlights

- L-FMC reveals a different aspect of endothelial function in pregnancy
- L-FMC clearly reflects an adaptation in healthy pregnancy which is absent in PE
- L-FMC and FMD (gold standard) are both disturbed in PE and not related
- L-FMC and FMD contribute to our knowledge on the mechanisms underlying PE

Acknowledgements

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B. FLOW MEDIATED DILATATION AND PERIPHERAL ARTERIAL TONOMETRY ARE DISTURBED IN PREECLAMPSIA AND REFLECT DIFFERENT ASPECTS OF ENDOTHELIAL FUNCTION

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Abstract

Background

Endothelial function and arterial stiffness are known to be altered in preeclamptic pregnancies. Previous studies have shown conflicting results regarding the best technique for assessing vascular function in pregnancy. In this study, we made a comprehensive evaluation of in vivo vascular function (including flow mediated dilatation (FMD), peripheral arterial tonometry (PAT), and arterial stiffness) in preeclamptic patients and compared them to normal pregnancies. In addition, we assessed the relation between vascular function and systemic inflammation.

Methods

Fourteen patients with preeclampsia (PE) and 14 healthy pregnant controls were included. Endothelial function was determined by FMD and PAT, arterial stiffness by carotid-femoral pulse wave velocity (CF-PWV) and augmentation index (AIx). Systemic inflammation was assessed using mean platelet volume (MPV) and neutrophil-leucocyte ratio (NLR).

Results

The reactive hyperaemia index (RHI), assessed using PAT is decreased at the third trimester in comparison to the first trimester in a normal uncomplicated pregnancy ($p=0.001$). Arterial stiffness is significantly higher in PE versus normal pregnancy ($p<0.001$). Endothelial function obtained by FMD is deteriorated in PE versus normal pregnancy ($p=0.015$), while endothelial function assessment by PAT is improved in PE versus normal pregnancy ($p=0.001$). Systemic inflammation (MPV and NLR) increases during normal pregnancy.

Conclusions

Flow mediated dilatation and peripheral arterial tonometry are disturbed in preeclampsia. Endothelial function assessed by FMD and PAT show distinct results. This may indicate that measurements with FMD and PAT reflect different aspects of endothelial function and that PAT should not be used as a substitute for FMD as a measure of endothelial function in pregnancy.

Introduction

The physiological changes that occur during pregnancy are necessary to meet the higher cardiovascular demand and to maintain sufficient feto-maternal perfusion. Regulation of vascular tone is an important mechanism involved in the maternal physiological adaptation to pregnancy, with the endothelium as key player. Normal endothelial function is critically dependent on the bioavailability of nitric oxide (•NO). Recently, •NO has been attributed an important role in the endovascular trophoblast invasion at the uterine spiral arteries during early placentation [1, 2]. In case of a deficient placentation, the uterine vessels in the placental bed maintain their high resistance [3]. This causes a suboptimal maternal circulatory adaptation to pregnancy and results in placental ischemia, reperfusion damage and oxidative stress. This local damage initiates the production of placental 'toxins', such as trophoblast debris, inflammatory cytokines and anti-angiogenic factors. These factors have a systemic impact and lead to general inflammation and peripheral endothelial dysfunction. When this vascular endothelial activation and dysfunction occurs at the level of liver, kidney, brain and placenta, the clinical presentation of preeclampsia (PE) arises with liver and kidney failure, thrombocytopenia and seizures [3].

Preeclampsia affects 3-10 % of pregnancies and is associated with increased maternal and perinatal morbidity and mortality worldwide [1]. PE has been linked to general endothelial dysfunction and maternal systemic inflammation, but it remains the "disease of theories", since extensive research has not yet been able to unravel the complete aetiology and pathophysiology of this common and threatening pregnancy disorder [4]. It is however important to investigate and understand the physiological changes in endothelial function and systemic inflammation related to normal pregnancy before evaluating them in preeclamptic pregnancies.

The gold standard for non-invasive assessment of endothelial function is flow mediated vasodilatation (FMD), measuring •NO-dependent vasodilation of the brachial artery in response to reactive hyperaemia [5]. During normal pregnancy, there is a steady increase in FMD until week 32, with a stabilization or even decline at week 36 [6]. Preeclamptic women are characterized by a significant reduction in FMD at time of diagnosis [7]. An alternative, user-independent technique in the assessment of endothelial function is peripheral arterial tonometry (PAT), measuring changes in digital pulse volume following reactive hyperaemia. The

resulting reactive hyperaemia index (RHI) has prognostic value in the general and cardiovascular population, comparable to FMD [8]. Data using PAT during pregnancy and PE are limited, based on small studies and showing conflicting results. Yinon et al. described a reduced RHI in women with PE compared to controls [9]. A study comparing RHI measurements at 16 and 28 gestational weeks in normal and PE pregnancies, showed a lower RHI at gestational week 28 compared to week 16 in both groups, but no difference between PE and healthy pregnancies [10]. However, due to measurement of peripheral microcirculatory function, PAT is less •NO-dependent than FMD. As such, FMD and PAT assess different aspects of vascular function [11, 12].

Arterial stiffness has been evaluated in pregnancy, using applanation tonometry (AP). When arterial wall stiffness is increased, the arterial pulse wave travels more rapidly away from the heart and the reflected wave returns more rapidly, resulting in a significant augmentation of the systolic peak. This can be measured as a raised augmentation index (AIx) [13]. During normal pregnancy AIx falls during mid pregnancy and rises at the end of pregnancy. In PE AIx is significantly increased and a significant role of first trimester Aix in the early screening of PE has been proposed. Arterial stiffness is independently associated with cardiovascular risk and may, therefore, provide a potential marker to select women who will develop cardiovascular events later in life after PE [13, 14].

Normal pregnancy and PE are both known to exert inflammatory effects, apparent by higher neutrophil to lymphocyte ratio (NLR) and higher mean platelet volume (MPV) [15]. Increase in NLR and MPV are described to be more prominent in PE, and these factors have been proposed as predictive biomarkers for PE [16, 17]. This increased systemic low grade inflammation in pregnancy, possibly contributes to alterations in endothelial function.

In this study, we made a comprehensive evaluation of in vivo vascular function (including FMD, PAT and arterial stiffness) in PE patients and compared them to normal pregnancies. In addition, we assessed the relation between vascular function and inflammation (NLR and MPV). We hypothesize that PE is associated with a disturbed endothelial function (as determined by FMD and PAT) and increased arterial stiffness probably even more so due to an exaggerated inflammatory response. During the course of normal pregnancy, we expect a mild deterioration of endothelial function due to an augmented systemic inflammation at the end of pregnancy.

Methods

Study population

Fourteen preeclamptic patients (gestational age 29+0w - 36+5w, mean 31w) admitted to the maternal intensive care unit at the Antwerp University Hospital were included between January 2016 and September 2016. We defined PE according to the revised ISSHP definition from 2018 [18]. Exclusion criteria were (gestational) diabetes, multiple pregnancies, foetal malformations, hypercholesterolemia, kidney disease, auto-immune disorders, connective tissue diseases or use of acetylsalicylic acid. Since the Antwerp University Hospital serves as a referral centre, patients were already started on anti-hypertensive medication (Table 1) and MgSO4 at the moment of referral and inclusion. Fourteen age-, BMI- and parity-matched healthy pregnant women served as controls. They were included in the study during their first trimester and were longitudinally followed throughout the whole pregnancy. They were free from medication and did not have a history of PE, pregnancy-induced hypertension, hypertension, cardiovascular disease or other chronic conditions. The Research and Ethics committee of the Antwerp University Hospital approved the study protocol (Belgian number: B300201524783), and written informed consent was obtained from all subjects.

Table 1 : Antihypertensive medication and doses given to the PE patients

No antihypertensive medication	4 patients
Labetalol 100mg 3x/d	4 patients
Labetalol (IV) 6-8 ml/h	4 patients
Labetalol 100mg 3x/d + Felodipine 10mg 2x/d	1 patient
Felodipine 5mg 2x/d	1 patient

Vascular function measurements

Patients were asked 24h prior to examination not to eat high-fat substances nor drink caffeine or alcohol and to refrain from smoking at least 6h prior to examination. Fingernails had to be short and no nail polish applied. Patients were studied in a quiet, temperature-controlled room (21-24°C) and stressful situations for the patient were avoided (people entering the room unexpectedly, telephone ringtones, etc.) The examinations were performed in a supine lying position with the arm in a comfortable position for imaging the brachial artery. After 5 minutes of rest, one blood pressure measurement was taken using an automated blood pressure device

(OMRON ® Intellisense, Healthcare Japan). This systolic blood pressure value was used to determine occlusion pressure for the FMD/RHI measurements.

FMD and RHI measurements were performed simultaneously. Repeat measurements in the control group were performed at the same arm and at approximately the same time of day.

Brachial artery flow mediated dilatation

FMD was assessed by measuring changes in brachial artery diameter in response to an increased shear-stress during reactive hyperaemia [5]. An ultrasound diagnostic instrument (Prosound alfa6, Hitachi Aloka Medical ®) equipped with vascular software for 2D-imaging, colour Doppler imaging and ECG-triggering, was used with a high frequency linear array transducer (UST-5413, 5-13 MHz, Hitachi Aloka Medical ®). Patients were in a resting, supine position with the arm in a comfortable position for imaging the brachial artery. A blood pressure cuff was placed on the forearm with the upper border of the cuff at a distance of 5 to 10 cm distal from the elbow (lateral epicondyle). The brachial artery was imaged above the antecubital fossa in a longitudinal plane with a clear delineation of both anterior and posterior intima-media interfaces. A special probe-holding device was used to ensure consistency of images during the measurement. The baseline artery diameter was automatically tracked and the waveform of diameter changes over the cardiac cycle was displayed in real time using an automated edge detection system. (eTracking system, Aloka ®). Arterial occlusion was created by cuff inflation to suprasystolic pressure at least 50 mmHg above systolic pressure (minimum value of 200 mmHg). After 5 minutes of occlusion, the cuff was deflated. Brachial diameter was recorded continuously (eTracking) from the time point of cuff inflation to 5 minutes after cuff deflation. FMD (in % from baseline value) was expressed as (post-ischemic maximal diastolic diameter change - baseline diastolic diameter)/ baseline diastolic diameter. All recordings were performed by two experienced investigators (IG, TS).

Peripheral arterial tonometry (PAT)

PAT was recorded using the Endo-PAT2000® (Itamar Medical, software version 3.2.4) and the disposable fingertip probes (Itamar Medical) in accordance with the manufacturer's recommendations. PAT is a less operator-dependent and more reproducible technique. The system uses pneumatic finger probes which assess digital volume changes accompanying pulse waves. Reactive hyperaemia was induced as described for FMD and measurements were performed simultaneously to FMD. The ratio of the average amplitude of the PAT signal over a

one minute period starting one minute after cuff deflation (maximum pulse amplitude) divided by the average amplitude of the PAT signal over a 3.5minute period before cuff inflation (baseline pulse amplitude) was calculated. The control arm was used to correct for confounding factors (room temperature, systemic changes). The result is expressed as the reactive hyperaemia index (RHI). All recordings were performed by the same two experienced investigators. (DM, EF).

Arterial stiffness

Systemic arterial stiffness was evaluated using pulse wave analysis (PWA) and pulse wave velocity (PWV) using the Sphygmocor system® (Atcor Medical, West Ryde, Australia). For PWA, three measurements at the level of the radial artery were obtained with a Quality Operator Index of at least 80%. The tonometer was placed at the area of interest and its position was adjusted until a strong, accurate and reproducible waveform was obtained. The augmentation index (AIx) was calculated by analysis of the pressure waveform, expressed as the ratio of augmented pressure (attributed to wave refection) to pulse pressure. Pulse pressure (PP) is defined as systolic pressure minus diastolic pressure. A rise in arterial stiffness causes earlier reflection (augmentation) of the pulse wave that reaches the heart in late systole and thus increases the cardiac workload. As AIx is affected by heart rate, it was standardized to a heart rate of 75 bpm (AIx-75). For PWV the distance between the site of maximal pulsation of the carotid artery and the femoral artery was measured using a tape measure in a straight line. Three measurements at the level of the carotid artery and subsequently the femoral artery were obtained with a standard deviation below 10%. As the arterial wall stiffens, the velocity of the travelling waves in the lumen increase. In assessing the PWV, the aortic PWV (measured by carotid-femoral pulse wave velocity (CF-PWV)) is the gold standard [19]. All recordings were performed by the same two experienced investigators (DM, EF).

Maternal venous blood

Maternal venous blood samples were taken for the quantification of mean platelet volume (MPV) and neutrophil-lymphocyte ratio (NLR). Peripheral blood was collected by venepuncture at 9-11 weeks, 24-28 weeks and at term using vacuette tube. Ethylenediaminetetraacetic acid (EDTA) samples were analysed using an ADVIA 120 Hematology System (Siemens healthcare ®, Germany).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0. Data are expressed as mean ± standard deviation (SD). Normality of continuous variables was evaluated using Kolmogorov-Smirnov test. Groups were compared using independent T-test and paired sample T-test as appropriate. Spearman correlation coefficient was used for univariate correlation analysis. A two-tailed $p < 0.05$ was considered significant.

Results

Patient characteristics

Characteristics of preeclamptic and normotensive patients are summarized in Table 2. Groups were comparable regarding age, parity, BMI and cardiovascular risk. Blood pressure and birthweight ware significantly different between groups. The differences in birthweight were due to differences in gestational age at birth. Patients with co-existing IUGR were excluded from the study.

Table 2: Patient characteristics

	Preeclampsia (n=14)	Normotensive (n=14)	p value
Age (years)	29 (22-37)	31 (25-36)	0.15
BMI 3rd trimester (kg/m2)	26.7 ± 3.4	28.0 ± 3.6	0.37
SBP 3rd trimester (mmHg)	155.7 ± 15.1	128.8 ± 11,2	0.00
DBP 3rd trimester (mmHg)	92.5 ± 8.5	74.6 ± 7.6	0.00
MAP 3rd trimester	113.6 ± 10.0	92.7 ± 7.9	0.00
Nulliparous (n)	11	10	na
Gestation at delivery (weeks)	30.7 (25-37)	38.9 (37-40)	0.00
Birthweight (g)	1435 ± 699	3457 ± 418	0.00
Smoking (n)	1	0	na

Data are expressed as mean ± SD, as median (range) or as number of total (n). Not applicable (na).

Evolution of vascular function during normal pregnancy

Fourteen healthy pregnant controls underwent vascular assessment at the first (11+6w – 13+2w, mean 12+4w) and third (34+1w – 36+3w, mean 35+0w) trimester of pregnancy. FMD was comparable between the first and third trimester (8.95±4.67% vs 8.53±4.09%, p=0.78) (Figure 1A). The time to reach the peak diameter in the third trimester did not change compared to the first trimester (54.0±17.5s vs 47.1±18.9s, p=0.31), but the change in artery diameter between baseline and end of occlusion was significantly higher in the third trimester (0.094±0.112mm vs 0.003±0.036mm, p=0.03). Baseline diameters were as well significantly different between the first and third trimester (2.9±0.26mm vs 3.3±0.45mm, p=0,002). To account for the influence of this augmented baseline diameter we re-calculated the FMD results of both the first and third trimester by dividing them with the baseline diastolic diameter of the 1st trimester. As a result, FMD measurements were even more comparable between the first and third trimester of pregnancy (3.14±1.71 vs 2.97±1.48, p=0.76).

In contrast to FMD, RHI was significantly lower at gestational week 35 compared to week 12 (1.53±0.33 vs. 2.30±0.56, p=0.001). This evolution was observed in all patients and suggests a deterioration in endothelial microvascular endothelial function with advanced pregnancy (Figure 1B).

There was no significant difference in PWA (5.14±9.47 vs 9.00±9.74, p=0.21) nor in CF-PWV (6.18±0.67 vs. 6.03±0.78, p=0.31) between the first and third trimester of pregnancy. (Figure 1C and 1D)

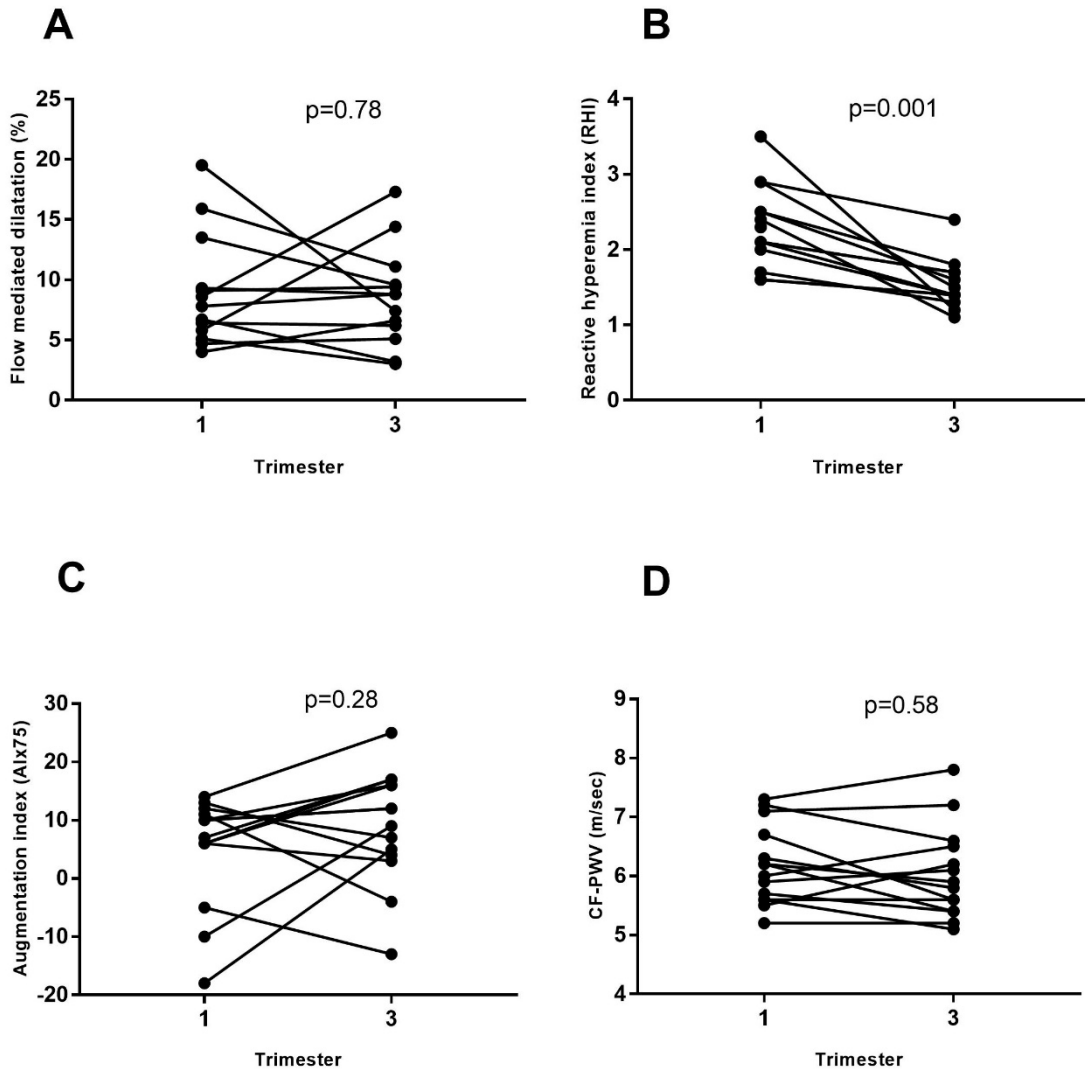

Figure 1: Vascular function during the 1st and 3rd trimester of normal pregnancy (n=14), as assessed by flow mediated dilatation (FMD, Fig 1A), reactive hyperaemia index (RHI, Fig 1B), Augmentation index (AIx, Fig 1C) and carotid-femoral pulse wave velocity (CF-PWV, Fig 1D). Paired sample T-test.

Vascular function in preeclamptic versus normotensive pregnancy

Results of the vascular function assessments that were performed simultaneously in 14 preeclamptic patients (mean gestational week: 30.6 ±3.67) and in 14 age-matched control patients (mean gestational week: 34.6±0.75) are shown in Table 3 and Figure 2. Flow mediated dilatation measurements and RHI measurements were performed simultaneously to reduce diurnal and biological variation. FMD was severely reduced in preeclamptic patients compared to control patients (p=0.014). Totally opposite to this observation, there was a higher RHI in preeclamptic versus control patients (p=0.0008). Arterial stiffness, as measured by

augmentation index, PWA and CF-PWV were significantly higher in preeclamptic patients compared to normotensive patients (all p<0.001).

Table 3: Vascular function in preeclamptic versus normotensive pregnancy

	Preeclampsia	Normotensive pregnancy	P-value
FMD (%)	4.83±3.15	8.53±4.09	p=0.014
Time to peak diameter (s)	61.3±44.3	54.0±17.5	p=0.58
Change in artery diameter between baseline and end of occlusion (mm)	0.049±0.166	0.094±0.0112	p=0.45
RHI	2.08±0.38	1.53±0.33	p=0.0008
AIx75	23.00±8.80	9.00±9.74	p<0.0001
CF-PWV	7.66±0.93	6.03±0.78	p=0.0005

Values are mean ± SD. P-value represents between-group comparison (T-test) FMD= flow mediated dilatation; RHI= reactive hyperaemia index; Aix= augmentation index; CF-PWV= carotid femoral pulse wave velocity.

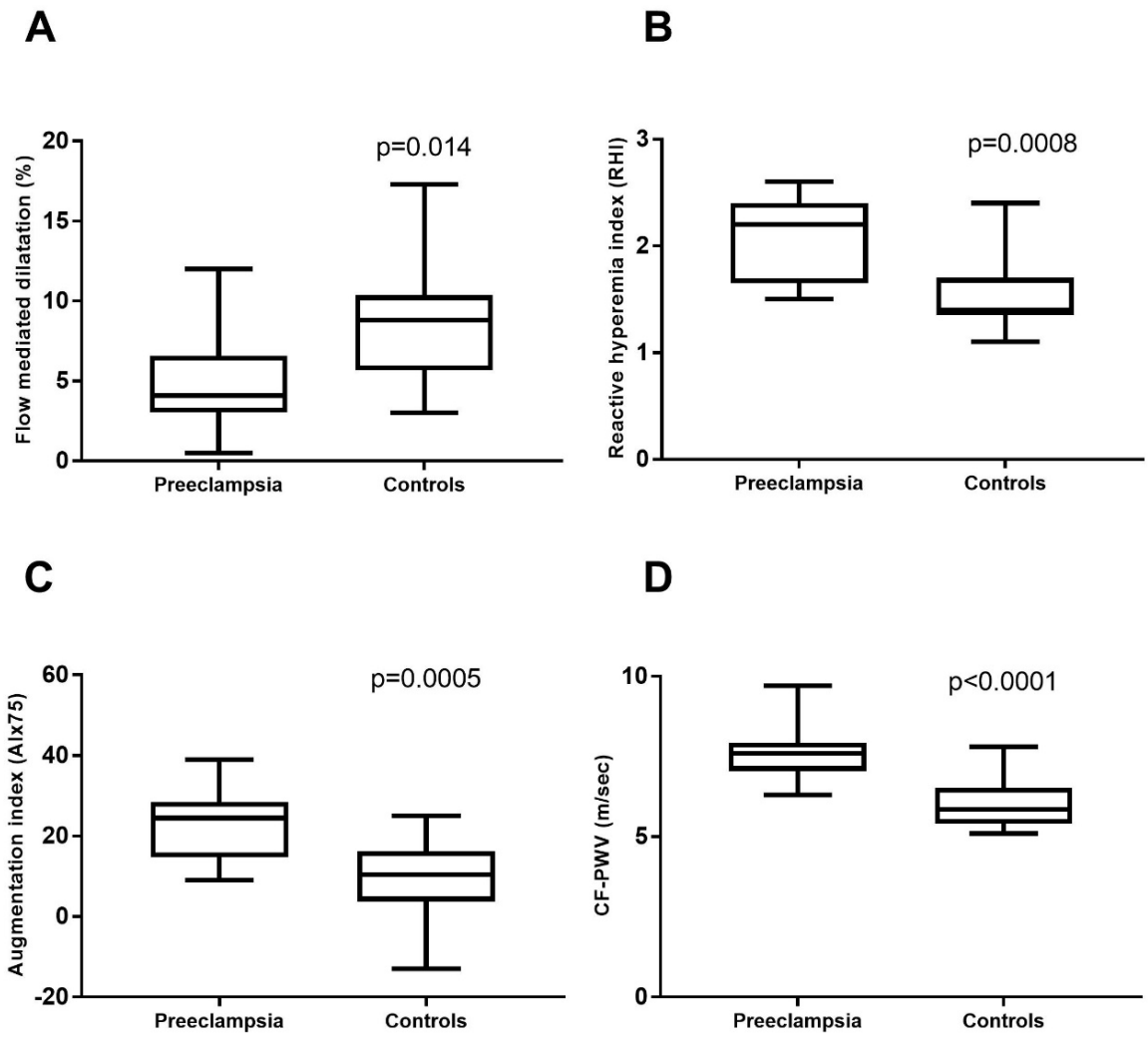

Figure 2: Vascular function in preeclampsia (n=14) versus normal pregnancy (n=14), as assessed by flow mediated dilatation (FMD, Fig 2A), reactive hyperaemia index (RHI, Fig 2B), Augmentation index (AIx, Fig 2C) and carotid-femoral pulse wave velocity (CF-PWV, Fig 2D). Independent T-test.

MPV and NLR measurements

Gestational-age-specific longitudinal changes for MPV and NLR are presented in Figure 3. There is a significant increase in MPV during pregnancy (overall significance, P=0.0002) (First trim 7.94±1.05; Second trim 8.70±1.05; Third trim 9.42±1.50). NLR was significantly increased between the first trimester (3.62±0.99) and the second trimester (5.63±2.10), but decreased significantly between the second and third trimester (4.58±1.47). We found no significant difference in MPV nor NLR between PE patients (MPV 8.96±1.35; NLR 6.67±3.62) and healthy pregnant controls (respectively p=0.42 and p=0.06). Neutrophils were significantly higher in the PE group compared to normal pregnancy (10.86±4.25 vs 8.14±2.45, p=0.05) (Figure 4).

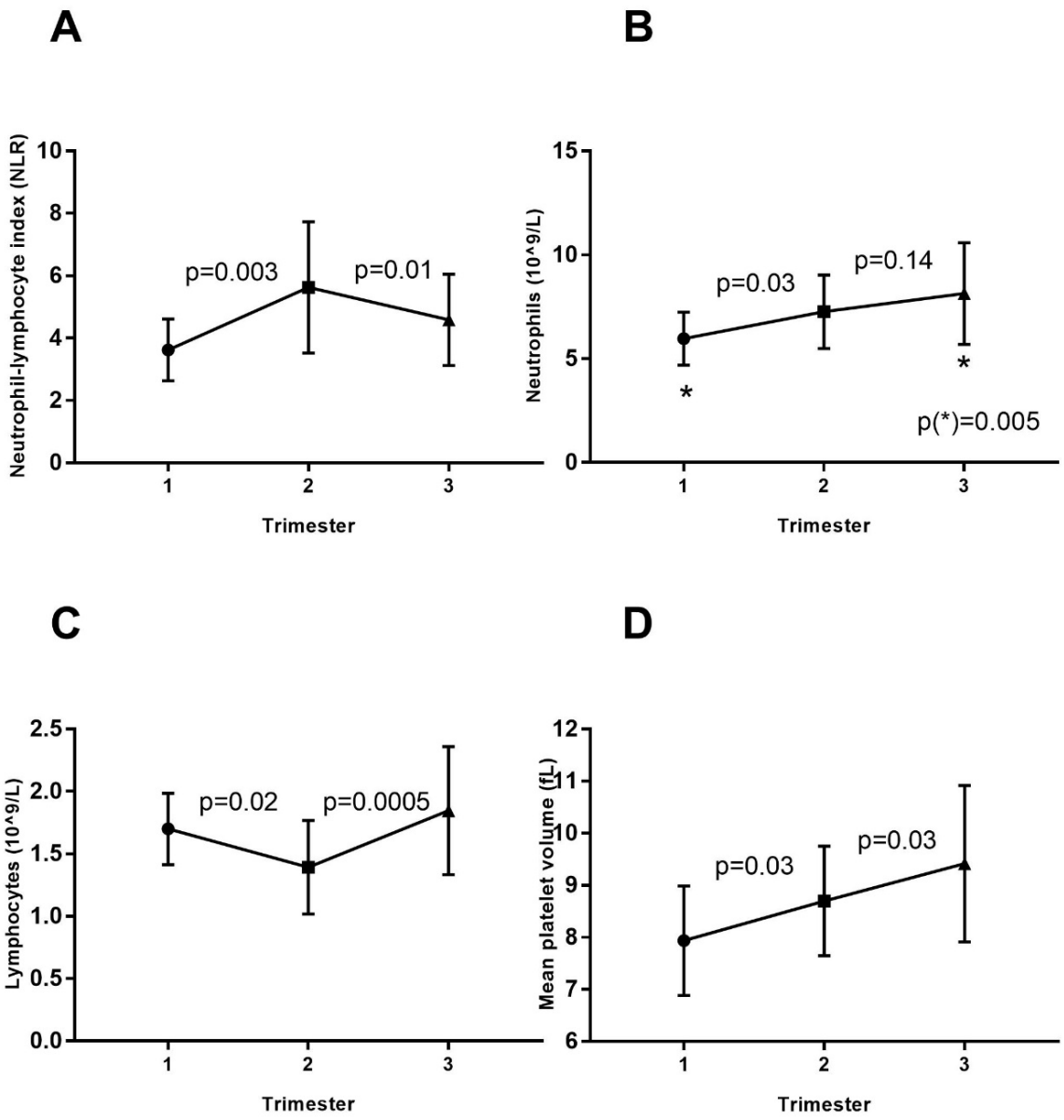

Figure 3: Evolution or NLR, neutrophils, lymphocytes and MPV during the 1st, 2nd and 3rd trimester of normal pregnancy (n=14). Paired sample T-test.

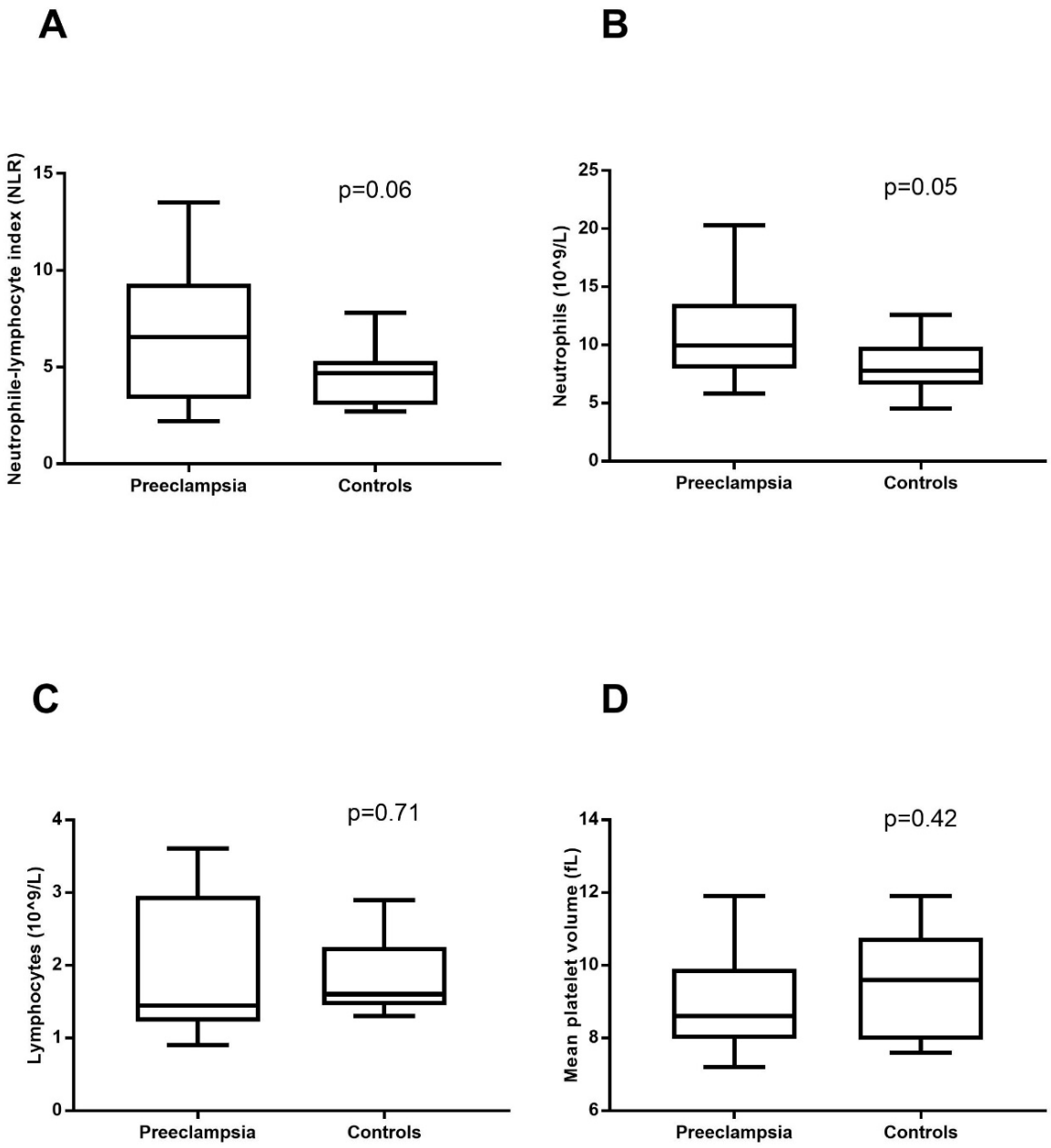

Figure 4: NLR, neutrophils, lymphocytes and MPV in preeclampsia (n=14) compared to normal controls (n=14). Independent T-test.

Correlation between vascular function and inflammatory markers

In the case-control study, we found no correlation between RHI and FMD (p=0.42), nor between endothelial function and inflammatory parameters (RHI vs MPV (p=0.54), FMD vs MPV (p=0.72), FMD vs NLR (p=0.34) and RHI vs NLR (p=0.16)).

Discussion

Vascular dysfunction plays a key role in the pathogenesis of PE. However, differences in assessment techniques have generated apparently paradoxical results when it comes to the presence of endothelial dysfunction and arterial stiffness in PE. This is the first study to compare simultaneously two methods for assessing endothelial function, namely FMD and RHI and to perform a thorough assessment of different aspects of in vivo vascular function in PE and in normotensive pregnant women.

We found that RHI is decreased at the end of pregnancy in comparison to the first trimester in a normal uncomplicated pregnancy, whereas no changes are seen when FMD is measured.
In literature, FMD is known to increase during normal pregnancy until 32 weeks, and to stabilize or to decline at 36 weeks [6]. Most papers that describe a higher FMD at the end of gestation, take their last measurement between 30-32 weeks [20]. In contrast with our hypothesis, we were not able to detect a deterioration in FMD between the first and last trimester of pregnancy. There was, however, a significant increase in baseline diameter of the brachial artery during normal pregnancy, which makes it difficult to interpret our results. Recalculation of the FMD measurements to minimise for the influence of an augmented baseline diameter, showed FMD measurements that were even more comparable between the first and third trimester of pregnancy. Since vascular stiffness is directly proportional to vascular diameter, a possible explanation can be found in the inability to vasodilate further from baseline in advanced pregnancy, due to the overt vasodilatation at the end of pregnancy. Regarding the execution of FMD measurements in particular, the methodological approach can critically impact the magnitude of the FMD response, therefore the paradoxical results between the present study and previous studies could also be explained by differences in technical aspects, such as cuff placement. [5]
The RHI, on the contrary, is decreased at the end of pregnancy in comparison to the first trimester in our study population, as we stated in our hypothesis. In the third trimester of normal pregnancy, microvascular endothelial dysfunction seems to arise. It is uncertain whether this is caused by a higher level of systemic inflammation (higher neutrophils), a mild form of disseminated intravascular coagulation (DIC) (higher MPV) or a chronic volume overload at the end of normal pregnancy. We found an increase in NRL and MPV during

pregnancy, supporting this theory of increased inflammatory response at the end of pregnancy. A recent paper by Melchiorre et al. describes an increase in total vascular resistance and end-systolic wall stress at term as a consequence of the persistent overload of volume during pregnancy [21]. During normal pregnancy, there is an overt systemic vasodilatation at all levels of the vascular tree, resulting in a higher shear-stress, possibly compromising microvascular endothelial function. An alternative explanation is that the lower RHI results are not a sign of endothelial dysfunction, but rather a consequence of normal pregnancy. Since the RHI is the ratio of the post-occlusion pulse amplitude over the baseline pulse amplitude, the RHI decreases when the vessel is in vasodilated state [10]. This can explain why the PAT results are lower at the end of normal pregnancy, without the presence of endothelial dysfunction.

As stated in our hypothesis, we found an increased arterial stiffness in PE. This can be explained by the inflammation and oxidative stress caused by circulating toxic factors produced by the ischemic placenta, attacking the vessel resulting in widespread endothelial dysfunction [2]. Our results are in line with published literature on arterial stiffness in PE. Arterial stiffness is independently associated with cardiovascular risk and may, therefore, provide a potential marker to select women who will develop cardiovascular events later in life after PE [13, 14]. We found no difference in arterial stiffness between the first and last trimester of pregnancy, suggesting normal pregnancy has no influence on the stiffness of maternal vessels.

Supporting our initial stated hypothesis, FMD is decreased in preeclamptic pregnancies compared to normal controls in our study population. This is in line with literature, suggesting endothelial dysfunction in PE [22]. Women with PE show a significant reduction in brachial artery diameter as compared to normotensive women.

When we compare RHI among preeclamptic patients and healthy controls, we surprisingly find a higher RHI in PE. This finding is not in accord with our hypothesis stating that overall endothelial dysfunction is present in PE. In the literature, less research exists concerning the use of PAT during preeclamptic pregnancy (in comparison to FMD) and small studies show conflicting results. The control group in a study by Yinon et al., had an average gestational age of 29 weeks, while the preeclamptic patients were on average 32 weeks pregnant [9]. Control patients in our study were on average 35 weeks pregnant, which makes it less reliable to

compare results. Our finding suggests that PAT and FMD are not interchangeable and that PAT should not be used as a substitute for FMD in order to measure endothelial function, according recent literature [11, 12].

We hypothesize that measurements with FMD and PAT reflect different aspects of endothelial function. This can be explained by the different measuring targets in the arterial tree. FMD measures endothelial regulation of vascular reactivity at the conduit arteries (brachial artery), while PAT measures the transient increase in blood flow that occurs following a brief period of ischemia in resistance arteries of the finger. There are important differences between the microvasculature in the finger and the brachial artery, including vessel size, number of capillaries and arteriovenous anastomoses, and they may all show a different response to ischemia. During PE, there is a vasoconstriction at the level of the arteries and arterioles, this in combination with an increased blood volume during pregnancy, results in augmented shear stress (at the level of the arteries). The constricted arterioles protect the microvascular system, which could explain the increased RHI. Another explanation can be found in a paper by Beinder et al. [23]. They describe changes in microcirculatory reactivity in patients with PE and found that in PE there is a higher vasodilatory reserve during reactive hyperaemia compared to healthy pregnancy, indicating an increased resting vasomotor tone in the microcirculation. They used local cooling as a vasoconstrictor stimulus and found that vascular reactivity was significantly greater in preeclamptic patients than in controls. These findings are in line with our findings, suggesting vasoconstriction in the microcirculation during PE with the ability to vasodilate after stimulation. We therefore hypothesize that not only FMD and PAT assess different aspects of endothelial function in pregnancy and cannot be used interchangeably, we also suggest that the arterial vasculature and microcirculation undergo distinct changes during preeclamptic pregnancies.

In PE the mechanism that causes endothelial dysfunction is not yet fully established. Toxic factors that are released from the ischemic placenta injure the endothelial wall (glycocalyx), compromising the endothelial function and in particular •NO release. Endothelial cells release •NO, and the main stimulus for this is shear-stress caused by increased blood flow. Evidence exists that PAT is a less •NO-dependent technique in relation to FMD [11]. Similar to •NO, prostacyclin (PGI2) is secreted by endothelial cells and acts as a potent vasodilator. In healthy arteries, •NO has an inhibitory effect on PGI2 secretion. It is postulated that in contrast to

normotensive young subjects, hypertensive patients produce significant amounts of vasoactive PGI2. Since PAT is a less •NO-dependent technique, it is possible that it encloses a larger role for PGI2, and this may be another explanation for the better RHI results in preeclamptic pregnancies [24]. An alternative explanation can be that vasodilatation in resistance arteries is less •NO dependent than in the conduit arteries, possibly because of a higher amount of vascular smooth muscle in the conduit arteries, sensitive to •NO.

In contradiction with our initial hypothesis, we found no significant difference in MPV and NLR comparing normal pregnant patients in their third trimester of pregnancy, to preeclamptic patients. Since the majority of patients in the control group were in the first stage of labour when blood samples were collected, this could have influenced our results. [25] Still, there is a trend towards significance regarding the NLR. NLR in normal pregnancy was 4.58±1.47 versus NLR in PE 6.67±3.62 (p=0.06). Also, neutrophils were significantly higher in the PE group compared to normal pregnancy (10.86±4.25 vs 8.14±2.45, p=0.05). A larger sample size is mandatory in order to examine whether NLR, and thus systemic inflammation, is indeed augmented in preeclamptic pregnancies.

Despite these novel findings, our study has limitations. First, in the normal pregnancy group, there is a large gap between the vascular measurements (12 weeks – 35 weeks). In this manner we do not know how vascular stiffness, FMD and PAT fluctuate during the course of normal pregnancy. Second, we only studied vascular function in PE at the moment of diagnosis, we cannot compare these results with previous measurements in the same patient. Third, the PE patients were already on medication started at moment of their vascular assessment and blood sample, which could have influenced our results. Last, our blood samples in the control group were taken during the first stage of labour, possibly influencing our markers of inflammation.

The main strength of our study is the longitudinal design in normal pregnancy. This way we were able to investigate and understand the physiological changes in systemic inflammation and endothelial function related to pregnancy itself before comparing them to preeclamptic pregnancies. To our knowledge, serial changes in maternal endothelial function have not been evaluated previously in normal pregnancy using two different methods, i.e. FMD and RHI, simultaneously. Nor have FMD and RHI been investigated in preeclamptic pregnancies

simultaneously. Furthermore, we have studied the association between endothelial function and arterial stiffness on the one hand, and between endothelial function and maternal inflammatory response on the other hand, in the same population.

Perspectives and significance

The results of this study allow us to conclude that there is higher arterial stiffness and lower flow mediated dilatation in preeclamptic pregnancies compared to healthy pregnant controls. Controversially, the RHI is higher in PE compared to normal pregnancy. Together these findings support the assertion that measurements with FMD and PAT reflect different aspects of endothelial function and that PAT should not be used as a substitute for FMD as a measure of endothelial function in pregnancy. We hypothesize however, that arterial vasculature and microcirculation undergo distinct changes during preeclamptic pregnancies. Future research is imperative to improve our understanding of the longitudinal evolution of vascular function during and after preeclamptic pregnancies to better apprehend the pathophysiology of this severe pregnancy complication.

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CHAPTER FOUR

OXIDATIVE STRESS IN HEALTHY PREGNANCY AND PREECLAMPSIA MEASURED BY ELECTRON PARAMAGNETIC RESONANCE

A. OXIDATIVE STRESS DETERMINATION IN PREGNANCY AND PREGNANCY COMPLICATIONS, A SYSTEMATIC REVIEW ON THE APPLICATIONS OF ELECTRON PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY

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(resubmitted after revisions)

Abstract

Significance

In normal pregnancy, oxidative stress (OS) is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and activation of protein kinases. Although OS is an important feature of pregnancy, persistent OS gives rise to different disease-states, such as preeclampsia (PE).

Recent advances

The central facet of OS in the pathophysiology of PE, a potentially life-threatening hypertensive pregnancy disorder, has received much attention in literature lately. Different methods have been used to evaluate and adequately measure radical concentrations. This systematic review aims to report on what is known about the determination of OS in pregnancy and pregnancy complications using electron paramagnetic resonance (EPR) and concentrates on the broad spectrum of used materials and techniques.

Critical issues

Determination of OS in pregnancy is of wide interest to specialists in perinatal medicine, but the best technique to access OS has been matter of debate. Research focusses on OS in PE, while large longitudinal studies assessing the contribution of specific radicals in OS during pregnancy are lacking. Therefore, a good understanding of the EPR technique and resume of its advantages and disadvantages will open new research avenues on OS determination in pregnancy.

Future directions

In order to unravel the relation between OS and the appearance of PE, it is of utmost importance to further investigate the longitudinal changes of radical concentrations in healthy pregnancy and to compare them to PE pregnancies using the most direct and reliable method for detecting free radicals, EPR.

Introduction

Oxidative stress (OS) is defined as a disturbance in the pro-oxidant antioxidant balance, where the balance is shifted towards the former. This imbalance leads to potential damage, disrupting cell function and cellular signalling, leading to various pathophysiological events and disease states. [1] In normal pregnancy, placental OS is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and activation of protein kinases. [2-6] Although OS is a common necessary feature of normal pregnancy, augmented OS gives rise to different disease-states, such as preeclampsia (PE). [4, 6-8] (Figure 1)

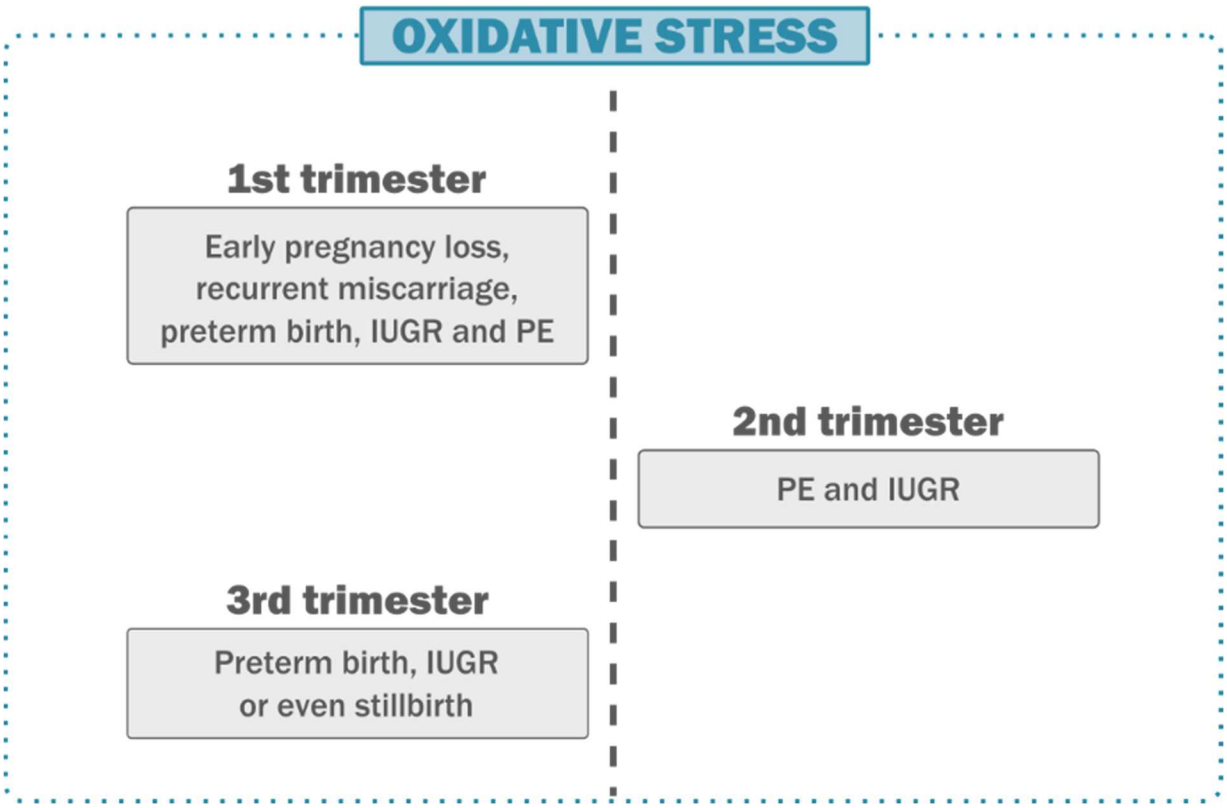

Figure 1: The effect of augmented oxidative stress during the different trimesters of pregnancy. (PE: preeclampsia; IUGR: intra-uterine growth restriction)

PE is a potentially life-threatening pregnancy complication clinically detected after 20 weeks of pregnancy and characterized by the appearance of hypertension and proteinuria. The pathophysiology of PE however, starts in the first trimester of pregnancy, caused by failure of the placentation process resulting in placental ischemia and the formation of OS. [3, 7] In PE, hypertension arises to compensate this decreased placental blood flow. Placental insufficiency prevents the organ meeting the needs of the foetus, and as a consequence, the growth and viability of the foetus become compromised. Severe PE is life-threatening and is associated with HELLP syndrome, characterised by **h**aemolysis, **e**levated **l**iver enzymes and **l**ow **p**latelets. PE occurs in 5-7% of pregnancies and is associated with a high foetal and maternal morbi-mortality. In the developed world, PE is responsible for 20% of the yearly 15 million preterm births, making it an important health problem. [3] Due to the severity and global importance of the disease, extensive research has been performed to unravel its origin and pathophysiology. Whether PE is primary a placental problem or whether the maternal cardiovascular system is unable to adapt to pregnancy, remains an important point of discussion. [9] However, the deficient placental oxygenation in PE causes local formation of reactive oxygen and nitrogen species (ROS and RNS respectively). Previous research has demonstrated that ROS and RNS, like superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$) are involved in the pathophysiology of placental pregnancy disorders. [2] (Figure 2) OS at the site of the placenta causes placental damage and this ischemic placenta releases cytotoxic, anti-angiogenic and inflammatory markers in the circulation, such as soluble fms (Feline McDonough Sarcoma)-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), resulting in systemic endothelial dysfunction and peripheral organ damage. [10, 11] Typical for PE, compared to other pregnancy-related disease-states, is the extension of disease into the maternal vasculature.

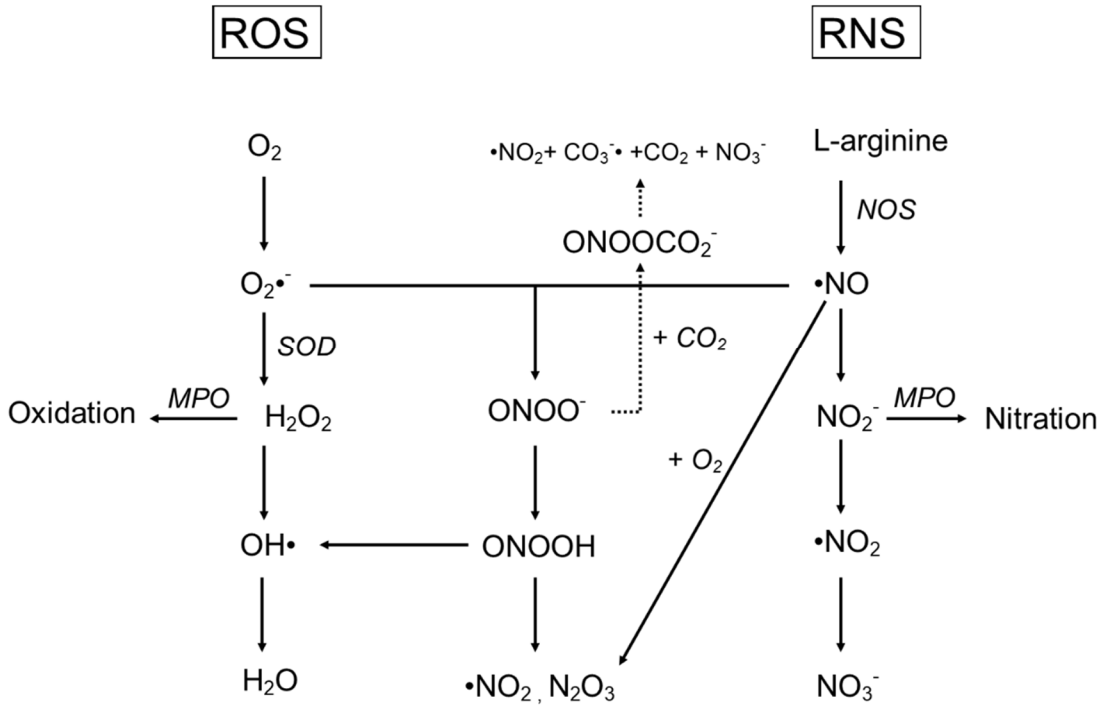

Figure 2: Scheme of ROS (reactive oxygen species) and RNS (reactive nitrogen species) formation.
(O2 : oxygen; O2•-: superoxide; SOD: superoxide dismutase; H2O2: hydrogen peroxide; MPO: myeloperoxidase; •OH: hydroxyl radical; H2O: aqua; •NO2: nitrogen dioxide; CO3-•: carbonate radical; CO2: carbon dioxide; NO3-: nitrate; ONOOCO2-: nitrosoperoxycarbonate; ONOO-: peroxynitrite; ONOOH: peroxynitrous acid; N2O3: dinitrogen trioxide; NOS: nitric oxide synthase; •NO: nitric oxide; NO2-: nitrite.

Although considerable research has been devoted to OS in PE [4-6], less attention has been paid to the evolution of OS during the course of normal pregnancy. In normal pregnancy, •NO is known to promote endovascular invasion by the cytotrophoblast during the first trimester and •NO is an important mediator of vasomotor tone and endothelial function throughout pregnancy. Little research has described an increase in •NO concentration with gestational age, suggesting an important role for •NO in the cardiovascular changes of normal pregnancy. [12] (Figure 3)

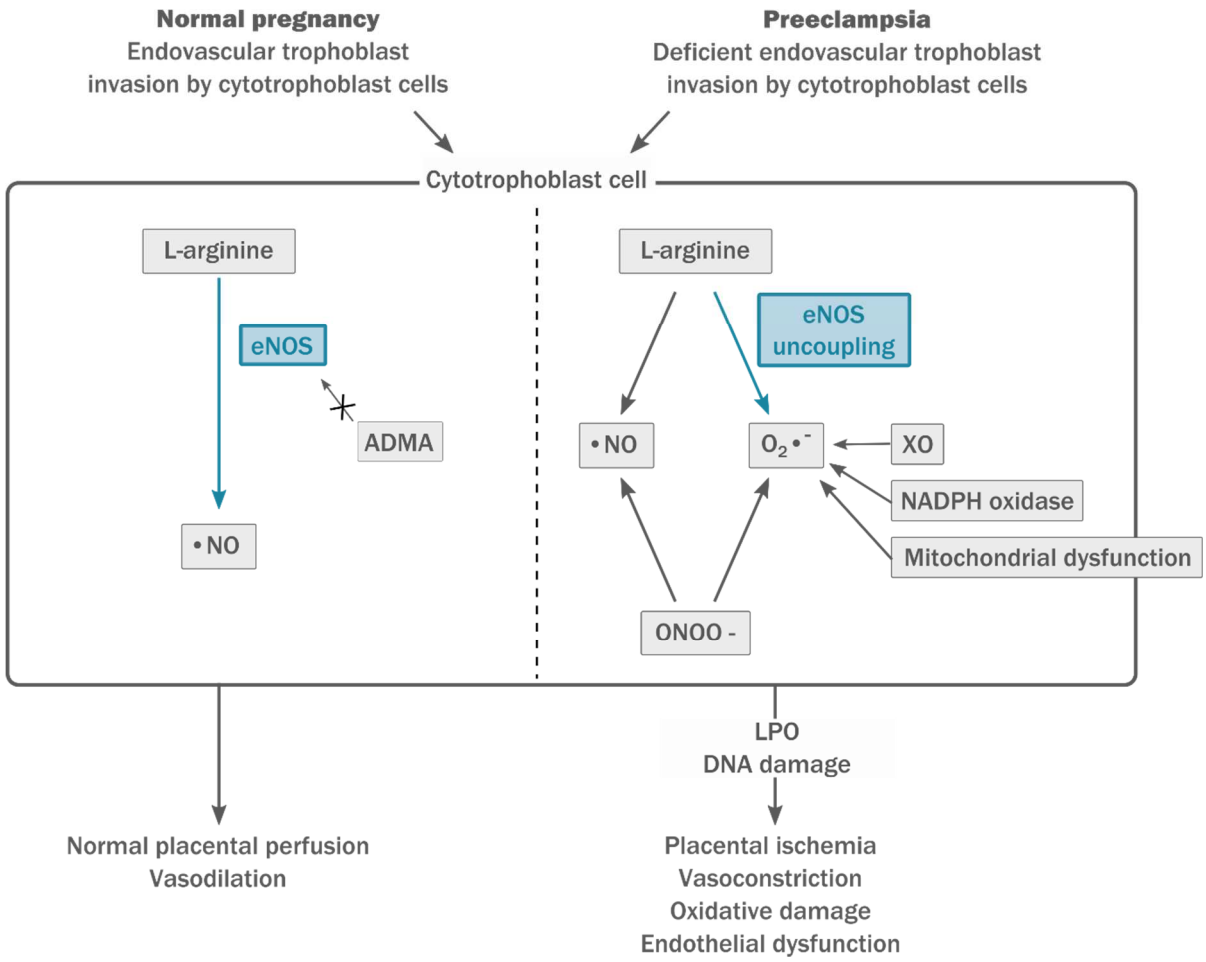

Figure 3: The role of nitric oxide, superoxide and eNOS in the placentation process.
(eNOS: endothelial nitric oxide synthase; •NO: nitric oxide; O2•-: superoxide; XO: xanthine oxidase; NADPH: nicotinamide adenine dinucleotide phosphate; ONOO-: peroxynitrite; LPO: lipid peroxidation)

Determination of antioxidant (AO) enzymes suggests that there is an increase in SOD (superoxide dismutase) and catalase activity in healthy pregnancy, possibly acting as a compensatory mechanism for the formation of free radicals. [13] During physiologic labour, the utero-placental unit undergoes an extensive amount of ischemia-reperfusion (I/R) injury, resulting in OS. [14] Intracellular ROS, mostly O2•-, can be produced by a number of pathways after I/R. These include mitochondrial electron transfer processes, and a variety of enzymes such as NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and xanthine dehydrogenase/oxidase (XDH/XO). During the period of hypoxia there is also a net catabolism of ATP (adenosine triphosphate), resulting in an increased concentration of hypoxanthine. Consequently, when oxygen is reintroduced XO will catalyse the accumulated hypoxanthine to produce O2•- and H2O2 will arise from spontaneous disproportionation of O2•-. [15]

Determination of ROS and RNS in PE has been studied extensively in recent years and is of wide interest to specialists in perinatal medicine, but the best technique to access OS has been matter of debate. A direct method to detect ROS/RNS is electron paramagnetic/spin resonance (EPR/ESR) spectroscopy. The basic concept of EPR is comparable to nuclear magnetic resonance (NMR), but instead of the spins of atomic nuclei that are detected, in EPR electron spins are excited. Despite being the most direct and reliable method for detecting free radicals, EPR remains a largely unknown and underused technique.

Since the presence of OS is abundantly proven in the pathophysiology of hypertensive pregnancy disorders, most literature on the use of EPR in pregnancy complications is about PE. In this review, we make a comprehensive resume of the use of EPR in pregnancy and highlight the possibilities and restrictions of this technique in relation to detecting OS and its consequences in normal pregnancy and PE. We explore the broad application of EPR spectroscopy for ROS/RNS detection including the different approaches used in wide-ranging topics of research to spread the use of this technique in the field of pregnancy related conditions The aim of this review is to make EPR a more notorious and accessible technique in the field of perinatal medicine.

Results

EPR spectroscopy for ROS/RNS detection (Figure 4)

In order to determine OS, many techniques have been investigated, e.g. the use of fluorescent probes and detection of oxidation products by spectrophotometry such as protein carbonyl assay. [16] Although these are used in many studies due to their easiness to apply in the laboratory, their main disadvantage is that they lack specificity to assess the specific type of ROS that is detected. [17, 18] EPR is a magnetic resonance technique that identifies and quantifies paramagnetic species with one or more unpaired electrons. These include simple molecules like •NO and O2•-, but also larger, paramagnetic molecules. Small molecular complexes with a transition metal ion, proteins with transition metal ions as cofactors (such as iron in transferrin (TF) or copper (Cu) in ceruloplasmin (CP)) and free radicals also possess unpaired electrons and can therefore be detected by EPR. [19, 20] EPR is based on the magnetic moment arising from the electron spin that aligns in an externally applied magnetic field, resulting in two non-degenerate spin energy states. (Figure 4) The low, more stable spin

magnetic moment is associated with the spin magnetic moment aligned with the magnetic field, while the high, less stable energy state is associated with a spin magnetic moment aligned opposite to the magnetic field. Transition between the two different energy states takes place by absorption of radiation in the microwave frequency and this absorption can be detected to determine the concentration of unpaired electron spins present in the sample. In an EPR spectrum, the amplitude of the second derivative of the EPR spectrum directly correlates with the number of unpaired electrons present in the sample and is therefore used for quantification. The local magnetic moment caused by some nuclei (for example 1H, 13C, 14N) in close vicinity of the unpaired electrons can interact with the magnetic field and results in a hyperfine splitting of the EPR spectrum. [20, 21] This distinctive 'fingerprint' spectrum characterised by the hyperfine coupling constants, in combination with the g-factor, helps to determine the identity of paramagnetic species. While EPR is able to identify and quantify long-lived radicals (such as the ascorbyl free radical (AFR)) directly, for short-lived radicals stabilization is mandatory. This stabilisation can be achieved by forming stable spin adducts with diamagnetic properties (spin trapping) or by scavenging the unpaired electron to form a new more stable radical (spin probing). Spin traps are usually small organic diamagnetic molecules, either nitroso (N=O) or nitrone (C=N) compounds (e.g. DMPO: 5,5-dimethyl-1-pyrroline-N-oxide) [21] or iron chelated spin traps which can stabilize •NO (e.g. diethyldithiocarbamate (DETC)2Fe(II) complex). [22] Spin probes are mostly small redox-sensitive molecules that capture unpaired electrons to form a long-lived radical itself. The spin traps and spin probes have an unique spectrum, but unfortunately the source of the unpaired electron or radical species cannot directly be identified. [23] Specifically spin probes can quantify radical formation, however direct identification of the original free radical is only possible by using a specific inhibitor (for example SOD in the case of O2•-) to retrospectively detect the source of the free radical. [24] Apart from the detection and quantification of free radicals, EPR can also be used to define membrane characteristics. In this case, lipophilic spin probes doxyl stearates (DS) are used that readily insert into the cell membrane, where DSs show restricted motion resulting in spectra broadening. This feature is used to calculate the order parameter which is reciprocally proportional to membrane fluidity. By varying the depth of the inserted doxyl group in the membrane, membrane local polarity and fluidity can be established. [20] Another advantage of EPR is that it can be applied on many biological samples

and enables the identification and quantify free radicals in different tissues, e.g. whole blood, plasma, serum, placenta and sperm. [20, 22, 25]

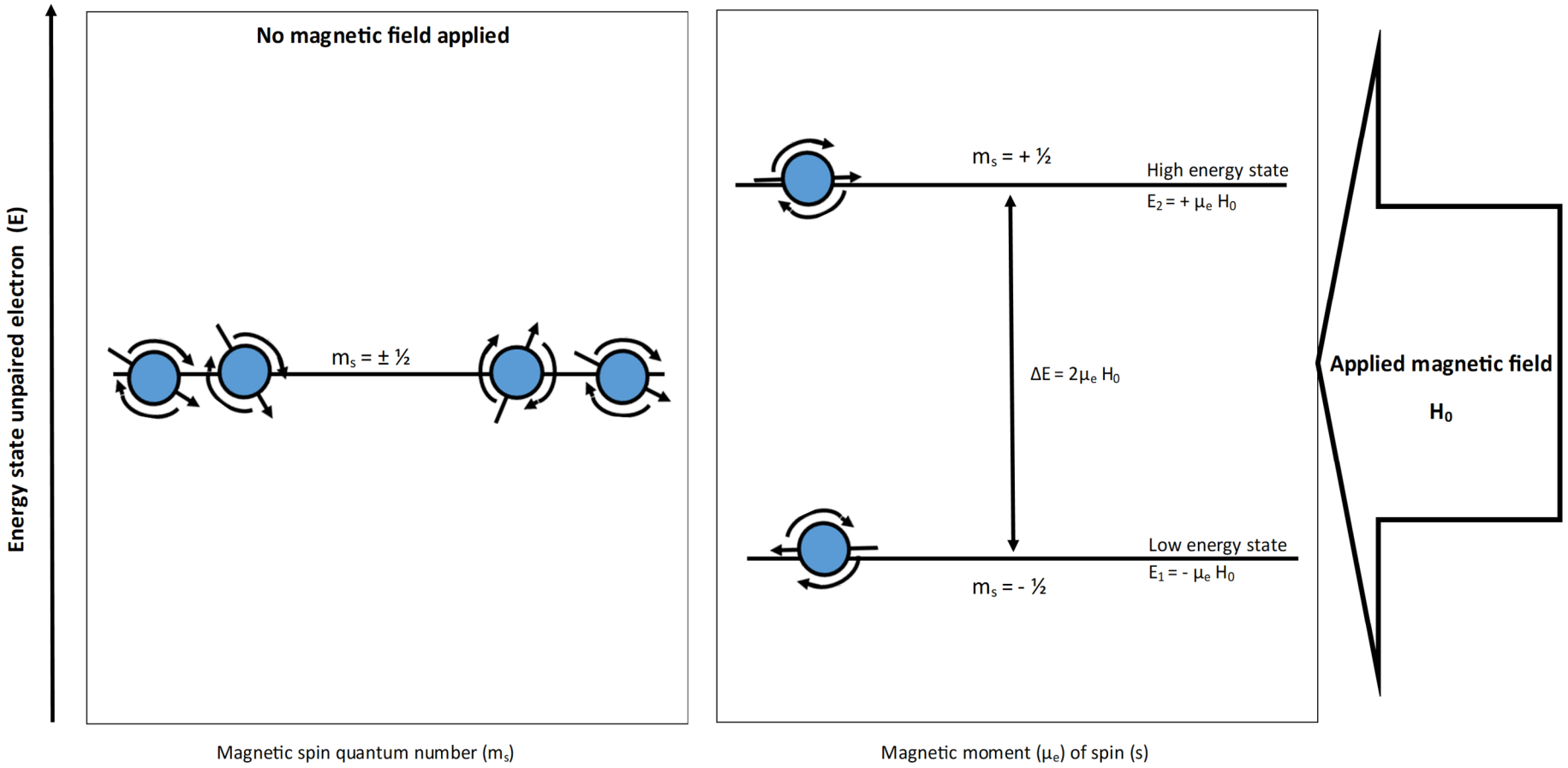

Figure 4: The physical principles of the EPR method. (ms : Magnetic spin quantum number; μe: Magnetic moment of spin (s))

Detection of OS in pregnancy

Oxidative stress in physiologic pregnancy and labour

ROS/RNS identification and quantification

During normal pregnancy, the placenta is a site of active oxygen metabolism that continuously generates OS. The major source of cellular ROS/RNS is the mitochondrial respiratory chain and in physiologic conditions approximately 1-2% of electrons are estimated to leak out. [26] In the placenta, ROS/RNS play a dual role by inducing beneficial regulatory effects, for example the induction of AO, but if they are overwhelmed in situations of OS, become harmful due to their high reactivity. OS is necessary in the normal metabolism of the placenta, especially in regulating apoptosis, immune tolerance, angiogenesis, vascular reactivity, invasion and anti-microbial function. [27] Pro-oxidant enzymes such as NOS (nitric oxide synthase), NADPH oxidase and XO are an important source of ROS/RNS, but their radical products exert harmful effects if they become abnormally activated. [2, 27] •NO is synthesized out of L-arginine in the placenta via the NOS enzymes including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Apart from being part of the ROS/RNS family, •NO is the most important mediator of endothelial function due to its potent vasodilator effects and plays a crucial role in lowering vascular resistance of the uterus and placenta unit throughout pregnancy. A paper by Khetsuriani compared free •NO content in the blood of non-pregnant, pregnant and preeclamptic women using EPR and found •NO concentration to be unchanged during physiologic pregnancy compared to the non-pregnant state. In this paper DETC was used as a spin trap for •NO detection and the blood-DETC mixture was incubated for ten minutes at room temperature. Samples were snap frozen after incubation. [28] Since haemoglobin can be used as an endogenous spin trap for •NO, [29] care should be given in the interpretation of these results, since a large part of the present •NO concentration will be trapped by haemoglobin. Labour is characterized by effective contractile activity and the formation of OS as stated above. Uterine inertia is a pathological state in which there is absence of effective uterine contractions during labour. Zyrianov et al. [30] evaluated the amount of free radicals in myometrium of the subplacental area and in uterine tissue using EPR. Samples were immediately snap frozen after collection and the freeze-trapping technique was used to detect an unidentified radical signal with g=2,0036. Undoubtedly, freeze-thaw processing can influence the integrity of the tissue and can enhance oxidation of nitrones, which will result in the generation of artefactual EPR

signals, lowering the sensitivity and specificity of free radical detection. [31] Based on this approach, physiologic labour was compared with uterine inertia and they found that low radical formation was associated with disturbances in the contractile activity of the myometrium. Furthermore, concentration of free radicals was considerably lower in the body of uterus compared to the subplacental myometrium, confirming that the main site of radical formation is the placenta. [30] Studies on OS during normal pregnancy have mainly focussed on its formation during labour. In contrast, little is known about the evolution of OS during a physiologic pregnancy.

Antioxidant capacity

In order to maintain normal intercellular integrity and function in processes susceptible to OS during pregnancy, the AO system plays an important role. The AO system acts in response to changes of OS during pregnancy. [32] Therefore, quantification of the antioxidant reserve capacity gives an idea of the amount of OS in the body. In placental I/R, XO transforms xanthine or hypoxanthine to uric acid and simultaneously catalyses reduction of oxygen to O2•-. The formation of OS during labour is a physiologic phenomenon, however, OS in utero is an important determinant of neonatal morbidity and mortality. During labour, contractions result in continuous I/R, both in the placenta and the foetus. Umbilical artery (UmA) and vene (UmV) cord blood gas analysis is often performed to assess foetal acidosis. Another approach to determine the amount of distress a foetus underwent during labour is measurement of OS in foetal blood. Due to the working of the mitochondrial transport chain, O2•- is the most widely produced free radical in times of stress. [33] Ascorbate (vitamin C) is the most important small molecular, water soluble AO in plasma and reacts with O2•- to form AFR. During its antioxidant reaction, ascorbate undergoes two consecutive one electron oxidations to dehydroascorbic acid with intermediate formation of the AFR. Only the AFR is measurable using EPR, ascorbate and dehydroascorbate are EPR silent. When interpreting the AFR signal obtained by EPR, the amplitude is directly proportional to the overall rate of ascorbate oxidation, whereas the signal duration is inversely proportional. [34] Cord blood ascorbate levels are four times higher than those in maternal blood and are responsible for a large part of the antioxidant capacity (AOC) of cord blood. During OS, O2•- is produced in excess and AO ascorbate serves as scavenger to form AFR. A study by Nishida et al. [14] compared AFR/DMSO (dimethyl sulfoxide) levels in cord blood of foetuses born after spontaneous vaginal birth, elective caesarean section (CS) and

emergency CS due to non-reassuring foetal status, using EPR. While blood gas analysis showed no different amount of foetal distress between the three groups, UmA-AFR/DMSO level of the elective CS group was significantly lower compared to the spontaneous delivery group and the emergency CS group showed significantly lower levels of UmA-AFR/DMSO levels compared to the elective CS group. The umbilical artery contains foetal blood flowing from the foetus to the placenta, thus blood deprived from the AO ascorbate in situations of foetal stress. In this paper, EPR is successfully used to asses foetal stress in a supplementary manner compared to standard blood gas analysis and in addition it is proven that emergency and elective CS cause higher foetal stress compared to spontaneous vaginal delivery. [14] Furakawa et al. [32] measured oxygen radical absorbance capacity (ORAC) and superoxide radical-eliminating ability (SREA) in maternal serum using EPR. Instead of measuring the level of an individual AO, which will not always reflect overall antioxidant ability, ORAC and SREA are measures of the eliminating ability for ROS. In brief, the ORAC method uses an azo-radical initiator, AAPH (2,2-azobis(2-amidinopropane) dihydrochloride), which is used as a thermal free radical source in combination with the spin trap CYPMPO (5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline N-oxide). After irradiation (at 200W for 5 sec), the competitive reaction between the spin trapping of the AAPH-derived free radical and its elimination by serum (antioxidant ability of the serum) is measured. [35] For the SREA method, blood was added to a mixture of riboflavin, DTPA (diethylenetriamine pentaacetic acid chelate, 1mM) and CYPMPO spin trap. After irradiation (at 250W for 10s), the obtained EPR spectra depend on the spin trapping of $O_2^{\bullet-}$ by CYPMPO versus the AO in the serum. While the ORAC responds to numerous AO and stands for total antioxidant ability, the SREA on the other hand, stands for superoxide radical specific antioxidant ability. In the first trimester, a low concentration of both ORAC and SREA is seen compared to non-pregnant controls. It is well documented that OS peaks in the first trimester when maternal circulation is established in the placenta, thus lowering antioxidant ability. Both ORAC and SREA increase during the course of pregnancy, with the highest concentration in the late third trimester. Previous studies also demonstrated a progressive increase in plasma total AOC during normal pregnancy. [36] Apart from healthy pregnancy, in one paper EPR was used to evaluate the relative level of AFR in amniotic fluid in patients with inherited thrombophilia. They snap froze aliquots with amniotic fluid but measured the samples at room temperature. It was found that the oxidative status of the amniotic fluid remains unchanged in thrombophilia compared to healthy controls. [37]

Lipid peroxidation & membrane characteristics

Pregnancy is known to be associated with considerable metabolic changes in the body, in particular activation of lipid metabolism. During pregnancy, the demand for metabolic fuels for foetal growth and development of its associated structures is increased and hormonal changes in the body may lead to changes in lipid profile during different trimesters of the pregnancy. [38] Higher lipid content in combination with OS results in lipid peroxidation (LPO). Lipids are the main constituents of different membranes and organelles and there composition determines membrane state and properties, including their osmotic stability and permeability. Cell deformability is of utmost importance for erythrocytes. Erythrocytes are essential for adequate oxygen transport and their deformability is critical for optimal microcirculatory function. Erythrocytes with a diameter of 8 µm are able to pass through capillaries with a diameter of 3-4 µm, allowing oxygen uptake and release. In case of LPO, erythrocyte deformability is reduced, compromising microcirculation. Impaired microcirculation is involved in the development of serious prenatal and postnatal complications such as intra-uterine growth restriction (IUGR), PE and necrotizing enterocolitis. Eguchi et al. [39] examined erythrocyte deformability in healthy pregnant and non-pregnant women using EPR with the fatty acid spin label 5-doxyl stearic acid. This spin label has a nitroxide-containing ring at the carboxyl end and is easily incorporated into the red cell membrane. [40] The unpaired electron of the spin label is located in the 2pn orbital of the nitroxide and the spin label is known to undergo rapid anisotropic rotation about the chain axis when incorporated in the cell membrane. The resonance parameters of the obtained EPR signal (g-factor and hyperfine coupling constant) depend upon the direction of the applied magnetic field with respect to the 2pn orbital. The average deformability of the red cells was determined by calculating the difference between the spectrum with red cells in rest-state versus the spectrum under flow condition. They found erythrocytes in pregnancy to be significantly less deformable compared to the non-pregnant state. Given this, it may be deduced that the higher amount of OS in pregnancy impairs microcirculation in a mild manner. The microvillus membrane in the placenta is another type of membrane strongly influenced by the consistency of the lipid bilayer and a similar method was used in the study by Cester et al. [41], where three different spin labels were added to incorporate in the mitochondrial, microsomal and microvillus membranes. Two spin labels were derivatives from stearic acid, namely 5-NS (5(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic acid) and 16-NS (16(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic

acid) and the third was N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MSL). Spin labels were incorporated with the ratio spin label/phospholipids being 1:150. The obtained amplitudes of the EPR spectra reflect the incorporation and mobility of the spin labels, thus representing the rigidity of the membranes. Their results suggest a very unique, stable and rigid character of the microvillus membrane.

Despite the widespread belief in increased OS in pregnancy, large longitudinal studies assessing the contribution of specific radicals in OS during pregnancy, and especially their role in normal labour, are lacking. There is a stronger tendency to investigate the amount of OS in disease-states. Since OS is abundantly investigated in PE, we will further elaborate on this in the next section.

Oxidative stress in preeclampsia

PE is characterized by an inflammatory response after ischemia and reperfusion at the site of the placenta. Placental reperfusion injury results in a damaging cascade that is responsible for increased placental production of ROS/RNS resulting in systemic OS. Accumulation of several biomarkers of OS and depletion of AO takes places in plasma during PE. As a result, OS in PE can be objectified in placental tissue as well as in maternal blood.

The following paragraphs describe the use of EPR in determination of ROS/RNS concentrations, AOC and membrane characteristics respectively.

ROS/RNS identification and quantification

Research has been performed on the quantification of different ROS/RNS and enzymes responsible for ROS/RNS generation in PE. One of the most abundant RNS present in the human body is •NO. As stated before, •NO is important for countless physiologic functions and plays an important role in cytotrophoblast invasion and endothelial function throughout pregnancy. Once formed, •NO has the tendency to rapidly react with other molecules to form other, more dangerous RNS (ONOO-, N2O3, and others) and these RNS can induce protein nitration. Myeloperoxidase (MPO), an enzyme reducing the bioavailability of •NO, is noted to be elevated in the plasma of PE patients. [42] Free •NO content in placental tissue seems to be decreased during PE, confirming the theory of impaired placentation. Tortladze et al. [43] used a DETC spin trap to measure •NO in placental tissue. In the same placental tissue, they also quantified lipoperoxyl radicals (LOO·), HbNO (nitrosyl haemoglobin), FeSNO, FeS (iron sulphide) and cytochrome-p450 using EPR. •NO concentration was significantly lower in PE placentae, in

contrast to the presence of complexes of •NO with hemic and nonhemic iron (respectively FeSNO and HbNO). LOO· are generated after the reaction of lipid hydroperoxides (LOOH) with various haeme compounds such as myoglobin and cytochrome c and they were detected with an α-phenyl-tert butylnitron (PBN) spin trap. In PE, a higher concentration of LOO· was found in the placenta. Placental tissue is an important source of steroidogenesis during pregnancy and the electron transport chain responsible for this carries electrons from FeS-centres of adrenodoxine to cytochrome-p450. In PE, insufficient placental blood flow results in decreased oxygen supply, disturbing the mitochondrial electron transport chain. Cytochrome-p450 is a paramagnetic molecule itself but becomes less detectable by EPR when it is in high state of steroidogenesis. The decreased concentration of adrenotoxin FeS-centres and increased intensity of cytochrome-p450 reflects the disturbed steroidogenesis in PE. An explanation for this disturbance can be found in the nitrosilation of mitochondrial electron transport chain proteins (FeSNO and HbNO), which significantly alters their activity, disturbing normal mitochondrial electron transport. [43] Iron plays an important role in the formation of OS, since it catalyses the reaction of O2•- with H2O2 and •OH is formed. This reaction is called the Fenton and Weis-Haber reaction. [44] Besides •NO, O2•- is an important ROS essential for immunoregulation, but when both are present in excess, ONOO- is formed. ONOO- is an oxidant and nitrating agent and because of its oxidizing properties, ONOO- can damage a wide array of molecules, including DNA and proteins. A study by Sikkema et al. [45] demonstrates that the concentration of O2•- is significantly increased in the placental tissue of PE women. In this study, O2•- has been quantified in placental tissue with a PBN spin trap and half of the samples were pre-incubated with DETC. After incubation and denaturation, the sample was centrifuged and the chloroform layer containing the lipophilic PBN-spin adducts was stored at -80° until analysis. O2•- adducts present in the sample resulted in three doublets. However, some spectra were contaminated with another PBN adduct with a larger proton hyperfine coupling. The source of this adduct was not further investigated in the study and the authors attributed it to PBN adducts from carbon-based radicals resulting from LPO. Unfortunately, the authors did not state whether this adduct was more frequently found in the PE group, as would be expected since the higher level of LPO in PE. [46] In samples pre-incubated with DETC to inhibit Cu-Zn-SOD activity, another spin adduct was detected with g=2.01 which was attributed to the paramagnetic Cu-(DETC)2 complex formed upon chelation of Cu by DETC. These contaminating EPR signals were not further investigated in the paper. Upon inhibition of Cu-

Zn-SOD, the relative increase of O2•- was significantly smaller in placentae of PE patients. These findings suggest a decreased basal Cu-Zn SOD activity in PE placentae, accordingly in the past it has been proven that Cu-Zn SOD is indeed inactivated under conditions of high OS. Therefore, the reduction in its activity in PE may be a consequence of prolonged exposure to high levels of O2•-. [45] The higher concentration of O2•- can also be partly attributed to a phenomenon called 'eNOS uncoupling'. In conditions of ischemia and OS, eNOS can undergo uncoupling, a state in which eNOS produces O2•- besides •NO. [2] In PE, eNOS expression is increased in the placental tissue, which could be an adaptive response to the increased resistance and poor trophoblastic invasion. [47]

The endothelium is an important organ in maintaining adjusted vascular tone throughout pregnancy. To regulate vascular tone, two indispensable factors are produced, namely •NO and endothelin-1 (ET-1), vasodilator and vasoconstrictor respectively. In a study by Khetsuriani et al. [28] the concentration of these two antagonistic vaso-active substances were determined in maternal blood of healthy and PE patients. Free •NO was quantified using EPR with a (DETC) 2Fe(II) complex spin trap and was found to be 10% decreased in comparison to healthy pregnancy. ET-1 content was 71% increased and this was stated to be compensatory to low placental perfusion. The low free •NO concentration was considered due to a low capacity of eNOS and a redox dependent transformation of •NO in ONOO-. Since high ET-1 is cytotoxic, it causes intensification of OS and contributes to the endothelial dysfunction in PE. [28] Another approach to measuring •NO content in plasma, is by determination of •NO deposition in haemoglobin (HbNO). In normal physiologic conditions, HbNO is undetectable. In hypoxic conditions, such as PE, •NO production is increased as compensatory mechanism. Haemoglobin is more sensitive for •NO as for O2 and binds more easily. Zhorzholadze et al. [48] states that although compensatory mechanisms apply in the pathophysiology of PE, the higher •NO production cannot reach the increasing demands and vasoconstriction and hypertension arises.

Antioxidant capacity & radical intermediates

In a study by Mikaelyan et al [49] EPR was used to determine the content of ceruloplasmin (CP) and apotransferrin (apoTF), TF not bound to iron. CP and apoTF are copper- and iron-containing AO proteins that bind O2•-, oxidise iron (FE(II)) and inhibit LPO. ApoTF is concerted into diferric TF upon binding iron, TF delivers iron to cells. During normal pregnancy, a high CP and apoTF

was measured, compared to a low CP and apoTF in PE. CP and TF can be measured by EPR without using spin traps or probes, since they respectively contain paramagnetic Cu and iron. As already mentioned, iron concentration is important in the formation of free radicals. In plasma, the concerted iron-binding AOC of TF and CP decreases with increasing transferrin saturation by iron. Hubel et al. [19] used EPR to determine diferric TF, total TF and CP concentrations and to examine interactions of organic hydroperoxides with haemoglobin and TF in PE patients and healthy pregnant controls. EPR demonstrated that in PE, the release of iron from free haemoglobin by lipid hydroperoxides, increases TF saturation. O2•- generated by XO during post-ischemic reperfusion at the placenta, can mediate release of iron from ferritin, similarly leading to an increased TF saturation. Thus, in PE they found an increased TF saturation, an increased serum iron concentration and a decreased total iron-binding capacity, which may occur following to OS but further promotes OS by decreasing serum AO buffering against redox-active iron. Mild intravascular haemolysis or increased extravascular destruction of red blood cells in damaged tissue may be responsible for the increase in iron. [19]

Kagan et al. [50] used EPR in four different experiments, measuring the life span of artificially created AFR with EPR. In the first experiment, the redox-cycling activity of exogenous added Cu to plasma of PE patients (incubated with phosphate buffer, ascorbate, deferoxamine mesylate (DFO) and CuSO4) was measured analysing the amount of formed AFR in 30 minutes. The second experiment determined the redox-cycling activity of endogenous Cu in plasma (incubated with phosphate buffer, ascorbate, DFO and tert-Butyl hydroperoxide (ButOOH)) with or without pre-incubation with a Cu (II) chelator. In the third experiment the redox-cycling activity of exogenous Cu in plasma with or without exogenous human serum albumin (hSA) and oleic acid was measured in function of the obtained AFR spectra. The fourth experiment was performed in phosphate buffer with ascorbate, DFO and CuSO4 wherein the redox cycling activity of Cu was measured in the presence of hSA and/or oleic acid. Enhanced OS is associated with improper Cu binding by plasma albumin, resulting in enhanced Cu redox-cycling activity, mainly in the presence of increased free fatty acids. By determining the decay of the AFR signal in different settings in PE plasma, they concluded that in PE improper Cu binding by plasma albumin is present, contributing to higher amounts of OS. These findings suggest a place for Cu chelators as AO therapy in PE. [50] PE has many characteristics similar to the metabolic syndrome, such as an aberrant lipid metabolism with a higher concentration of free fatty acids (FFA). [51] These FFA bind albumin, resulting in a lower ability of albumin to bind Cu. Higher

concentrations of Cu become available for Cu redox-cycling activity resulting in more OS. EPR can be used to access the redox activity of Cu by ascorbate radical formation and to study the formation of Cu(II)/albumin complexes in the presence or absence of FFA. In PE, the "loosely bound" Cu is responsible for the generation of ROS and disturbance of endothelial function.

Human plasma contains an efficient arsenal of low molecular weight, non-enzymatic AO that serve to protect the vasculature from oxidant damage. [19] Of these, reduced ascorbate is of primary importance in protecting plasma lipoproteins from peroxidation during exposure to a wide spectrum of water- or lipid-soluble free radical generators in vivo. Depletion of AO in PE seems to be associated mainly with water-soluble AO such as ascorbate and thiols, whereas levels of lipid-soluble AO (α-tocopherol (vitamin E), carotenoids) are not significantly decreased in PE. [50] Ascorbate can be used to determine the amount of OS in maternal plasma. A study by Hubel et al. [34] used EPR to determine the ascorbate-oxidizing activity in plasma of PE patients and compared them to healthy controls. They found that ascorbate concentrations were 50% lower in PE, but concentrations of thiols and α-tocopherol (measured using high-pressure liquid chromatography) were no different. Furthermore, they explored the elapsed time prior to half-consumption of plasma ascorbate and this appeared to be three-fold decreased in blood of PE pregnancies, reflecting the increased ascorbate-oxidizing activity in PE. [34]

As discussed, higher lipid content in combination with OS results in LPO. LPO is abundantly present in PE and is one of the most potent consequences of OS. Placental microsomes are highly susceptible to LPO and in PE, placental tissue may be an important source of LPO products. Placental microsomes synthesize 2-hydroxy-estradiol (2-OH-E), the main catabolite of oestrogens. Jankowski et al. [52] performed a small study on the AOC of 2-OH-E in placental microsomes using EPR. Human placental tissue was homogenized and centrifuged after which the sedimented microsomal fraction was re-suspended. The formation of malondialdehyde (MDA) was measured using the thiobarbituric acid test and by adding ButOOH to initiate LPO. The addition of ButOOH resulted in the formation of spin adducts with the spin trap DMPO which were observed with EPR. The DMPO spin trap was used to differentiate the radicals arising from the cytochrome-p450 mediated ButOOH cleavage. 2-OH-E was found to be an effective AO since it inhibited spin adduct and thus MDA formation, concluding that 2-OH-E acts as a natural endogenous AO for preventing LPO in placental tissue.

Membrane characteristics

As mentioned before, the increased lipid content in PE contributes to enhanced free radical LPO. [50] Since lipids are major components of the cell membrane, Mitsui et al studied erythrocyte deformability using a fatty acid spin label (5-doxyl-stearic acid). This spin label becomes incorporated into the outside of the lipid bilayer and measurements are performed under flowing conditions (flowrate 10ml/min) and non-flowing conditions. Using this technique, erythrocyte deformability is influenced by intrinsic factors only. They found that during normal pregnancy, erythrocyte deformability decreases with advancing pregnancy. Haemodilution, a physiologic process in normal pregnancy, compensates for this increase, since haematocrit levels needed for high deformability are lower in pregnancy. In severe pregnancy-induced hypertension, haemoconcentration is present and erythrocytes become more rigid. This can result in microcirculatory disturbances in maternal organs, including the uteroplacental unit. [53] Mikaelyan et al. [49] investigated the rigidity of erythrocyte membranes in PE using these fatty acid spin labels. In the same paper they found a low CP and apoTF concentrations, indicating a decreased AO capacity and a higher amount of LPO. As a result, in PE the higher LPO is responsible for the reduced content of cholesterol and phospholipids causing increased erythrocyte membrane microviscosity and hydrophobicity of the phospholipid bilayer. Consequently, PE is characterized by increased rigidity of the surface and deep layers of erythrocyte membranes. [49]

Discussion

The importance of OS in pregnancy and in the pathophysiology of pregnancy complications like PE, makes OS determination an attractive research area. It is increasingly apparent that OS plays a central role in many signal transduction pathways and it is important to recognise that homeostatic concentrations are present in all tissues. The benefits of well-controlled ROS and RNS production are gradually being recognized. These reactive species are involved in many important cellular signalling pathways and induce the expression of physiologically necessary genes. ROS and RNS can be generated through many pathways within cells, but the mitochondria, endoplasmatic reticulum and enzymes such as NADPH oxidase and XO are the most important sources. These pathways can respond to a variety of stimuli, not arguably the most important ones for pregnancy are perturbations in the maternal blood supply to the

placenta and systemic inflammation. Pregnancy disorders such as recurrent miscarriage, PE and IUGR represent a spectrum of placental OS-related disorders, secondary to deficient trophoblast invasion. [6]

Many attempts have been made to obtain a proper insight in the rate of ROS/RNS production and AO formation on the one hand, and their effects on cell and organ function on the other hand, using a broad spectrum of techniques. In this review we aimed to make a comprehensive overview of free radicals and AO important in pregnancy and listed how they can be measured using EPR. The use of EPR is not restricted to the direct measurement of OS, but can also be implemented to measure AO concentrations, AOC and radical intermediates or to assess membrane-characteristics, all influenced by the amount of OS. EPR is able to directly quantify the amount of OS in maternal blood as well as placental tissue.

Despite being the most direct and reliable method for detecting free radicals, EPR remains a largely underused technique. This is probably due to the scarce availability of EPR-devices, the expensive nature of the spectrometer and the relatively unknown character of the technique. Unfortunately, there are limitations to the EPR technique. First, EPR is a quantitative technique, however quantification of free radicals is not entirely genuine with the use of spin traps. The stability of spin traps and the competition of free radicals binding to spin traps versus other scavenging agents differs between physiological and pathological conditions. For example, EPR performed at room temperature using DETC as a spin trap seems to be a simple and sensitive method for •NO detection in vivo in animals. However, it has been shown that that this in vivo detection is highly influenced by the iron availability in tissues. [54] So the rate of •NO production in an animal disease model that also causes iron overload in tissues, will be overestimated if this is compared to a control that has no iron overload. Stability of dithiocarbamate complexes and reactions like conversion into EPR-silent Binuclear dinitrosyl iron complexes (B-DNIC) also has to be taken into consideration. [55] Second, EPR is, compared to other methods to detect ROS, not the most sensitive technique, specifically not when it is applied in combination with the spin trapping technique. Radicals are detected in concentrations in the micromolair range [56], while fluorescent probes detect these in concentrations 10 to 100 times lower. [57] The capacity of spin traps to cross biological barriers like membranes might also hamper detection of free radical species by EPR. [58] Another limitation is the instability of the spin adducts in biological systems which leads to a fast decay of EPR signals. This decay is highly dependent on the cell type and on the activation state of the

cells [59, 60] and caused by the reduction via intracellular reductants. For example, reactions with cytosolic ascorbate might form EPR silent hydroxylamines, prone to fast elimination of small molecules, yielding the parent nitrone. [61, 62] Glutathione peroxidase in the presence of glutathione (GPx/GSH) has been shown to catalyse the reduction of the O2•- adduct to the •OH adduct. [63] A drawback of using the spin trap DETC/Fe2+ for •NO detection is that high levels of iron complexes might be toxic, so that (auto)oxidation of the complex is likely to result in oxidant formation and the stability of the complexes in water, especially at low pH values, and the poor water solubility can limit their use. [22] Due to its strong Cu-chelating properties, DETC can inhibit, when applied in high concentrations (10 mM), enzymes with Cu in the active site, including Cu-Zn-SOD. [35] The resulting higher O2•- levels could result in an underestimation of •NO levels.

The determination of OS in pregnancy is largely focused on OS in pregnancy complications. Less research exists on the evolution of OS during the course of a normal pregnancy. Previous studies reached the conclusion that OS is measurable in pregnancy and that assessment of OS unravels yet another part of the largely unknown pathophysiology of PE. In PE the placenta produces a large amount of circulating free radicals, lipid peroxides and anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng). These factors induce cell injury and recruit macrophages when they are taken up by endothelial cells. Endothelial dysfunction is the key pathological feature of PE. Endothelia produce vasodilators, such as •NO and vasoconstrictors, such as ET-1. In PE, OS increases ET-1 expression and although eNOS is highly activated, in an oxidative environment the generated •NO undergoes further reaction to form ONOO- and eNOS uncoupling results in more ONOO- production.

Conclusion

Evidence is abundantly present confirming the importance of OS in pregnancy and the pathophysiology of PE. However, several questions remain unanswered at present. It is therefore of utmost importance to further investigate the longitudinal changes of specific ROS/RNS concentrations in healthy pregnancy and to compare them to PE pregnancies. Research should focus on longitudinal changes of O2•- in healthy pregnancy versus pregnancy

complications and on the role of placental NO during the course of pregnancy. In this way, OS could be used in the future to predict adverse pregnancy outcomes in the first trimester. At the end of the first trimester, placentation is complete, thus OS markers must be present in the circulation and the perfect moment to start preventive measures has not yet passed.

Material and methods

Search strategy

Embase, Central and PubMed databases were searched in order to collect all relevant articles on the application of EPR spectroscopy in pregnancy, published from 1985 to May 2017. A string of the following terms and their synonyms was used: 'pregnancy', 'electron paramagnetic resonance', 'electron paramagnetic spectoscopy', 'EPR' and 'ESP'. The search was not restricted to publications in the English language, translations of Russian and Italian articles were obtained by native-speakers. Papers were only included if they concerned human populations. Literature reviews were excluded from the search.

Study selection

Two independent reviewers (D.M and E.F.) screened the titles and abstracts of all retrieved articles for relevance. Studies that did not use EPR were excluded, as were studies that were not performed in pregnancy. As the aim was to describe the application of EPR in pregnancy, there were broad selection criteria concerning the material used (for example blood, plasma, placenta). The full-text versions of 21 articles were obtained and underwent a second trial of selection according to inclusion criteria. After this second selection, another 4 papers were excluded. All disagreements regarding exclusion or inclusion were resolved by discussion between the two reviewers until full agreement was achieved. A total of 17 papers was included in this systematic review. (Figure 5) Table 1 gives a detailed resume of all included papers, listing their targets, samples and spin traps/probes used.

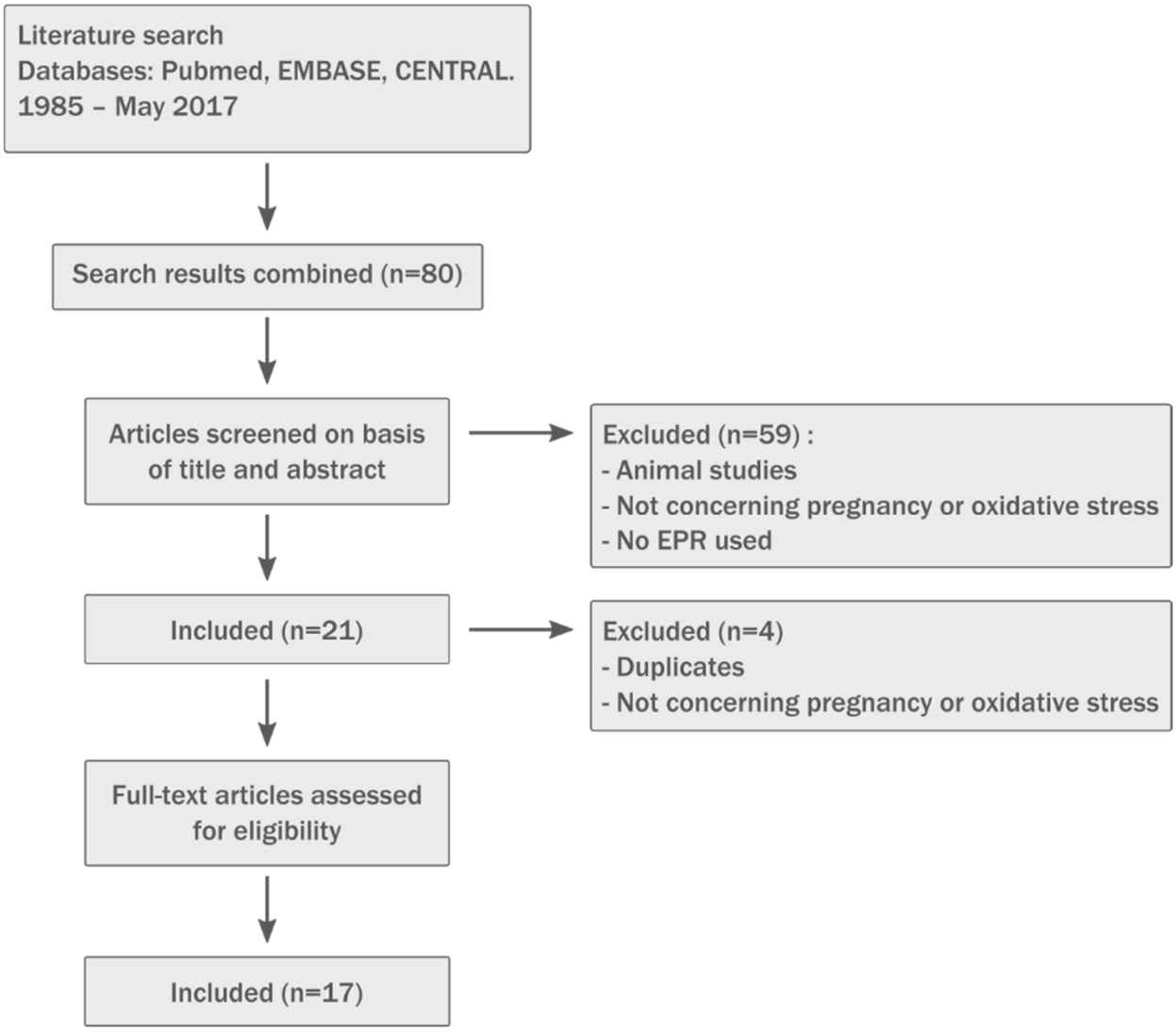

Figure 5: Search strategy and study selection.

Author, year	Study topic	Species detected by EPR	Sample type	Spin trap/spin probe	n
1. Physiologic pregnancy & labour					
Nishida, 2014 [14]	Foetal OS	AFR	Cord blood	None	75
Zyrianov, 2003 [30]	Myometrial contraction	Unidentified radical	Myometrium	None	16
Furukawa, 2015 [32]	AO status: ORAC (1) & SREA (2)	(1) AAPH-derived free radical (2) O_2•-	Maternal blood	(1) CYPMPO, AAPH (2) CYPMPO	33
Eguchi, 1995 [39]	Erythrocyte deformability	Spectral changes related to erythrocyte deformability	Maternal blood	5-doxyl stearic acid	40
Cester, 1985 [41]	Membrane characteristics	Spectral changes related to membrane deformability	Placenta	5-NS/16-NS/Maleimide	5
2. Preeclampsia					
Tortladze, 2012 [43]	Placental •NO metabolism	•NO (1), LOO· (2), HbNO, FeSNO, FeS, cytochrome P-450	Placenta	(1) DETC / $FeSO_4$ (2) α-phenyl-tert butilnitron	80
Khetsuriani, 2006 [28]	•NO concentration in maternal blood	•NO	Maternal blood	DETC / $FeSO_4$	49
Zhorzholadze, 2006 [48]	•NO metabolism in maternal blood	•NO (1) , HbNO	Maternal blood	(1) DETC / $FeSO_4$	59
Kagan, 2001 [50]	Copper redox cycling	AFR, Cu (II)/Albumin complex	Maternal blood	Ascorbate	34
Mikaelyan, 2001 [49]	AO capacity EM microviscosity (2)	CP, TF (1)	Maternal blood	(1) None (2) Doxyl stearates	173
Sikkema, 2001 [45]	Placental superoxide	O_2•-	Placenta	α-phenyl-tert butilnitron	23
Hubel, 1997 [34]	Ascorbate-oxidizing activity	AFR, thiols, α-tocopherol	Maternal blood	Ascorbate, phenoxyl radicals	25

Hubel, 1996 [19]	AOC	CP, TF	Maternal blood	None	36
Mitsui, 1994 [53]	Erythrocyte deformability	Spectral changes related to erythrocyte deformability	Maternal blood	5-doxyl stearic acid	57
Jankowski, 1994 [52]	AOC in placental microsomes	2-hydroxyestradiol AOC	Placenta	t-butylhydroperoxide, DMPO	/
3. Trombophilia					
Pristov, 2009 [37]	OS in inherited trombophilia	AFR	Amniotic fluid	Ascorbate	19

Table 1: Included articles

EPR: electron paramagnetic resonance; n: number of total included patients/samples in the study; OS: oxidative stress; AFR: ascorbyl free radical; AO: antioxidant; ORAC: oxygen radical absorbance capacity; SREA: superoxide radical-eliminating ability; AAPH: 2,2-azobis(2-amidinopropane) dihydrochloride; O2•-: superoxide; CYPMPO: 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline N-oxide; 5-NS: 5(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic acid; 16-NS: 16(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic acid; •NO: nitric oxide; LOO·: lipoperoxyl radicals; DETC: diethyldithiocarbamate; EM: erythrocyte membrane; CP: ceruloplasmin; TF: transferrin; AOC: antioxidant capacity; DMPO: 5,5-dimethyl-1-pyrroline-N-oxide.

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B. OXIDATIVE STRESS IN HEALTHY PREGNANCY AND PREECLAMPSIA IS LINKED TO CHRONIC INFLAMMATION, IRON STATUS AND VASCULAR FUNCTION

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Abstract

Background: During normal pregnancy, placental oxidative stress (OS) is present during all three trimesters and is necessary to obtain normal cell function. However, if OS reaches a certain level, pregnancy complications might arise. In preeclampsia (PE), a dangerous pregnancy specific hypertensive disorder, OS induced in the ischemic placenta causes a systemic inflammatory response and activates maternal endothelial cells. In this study, we aimed to quantify superoxide concentrations (as a measure of systemic OS) using electron paramagnetic resonance (EPR) and correlate them to markers of systemic inflammation, iron status and vascular function.

Methods: Fifty-nine women with a healthy pregnancy (HP), 10 non-pregnant controls (NP) and 28 PE patients (32±3.3weeks) were included. During HP, blood samples for superoxide, neutrophil to lymphocyte ratio (NLR), mean platelet volume (MPV) and iron status were taken at 10, 25 and 39 weeks. Vascular measurements for arterial stiffness (carotid-femoral pulse wave velocity (CF-PWV), augmentation index (AIx), augmentation pressure (AP)) and microvascular endothelial function (reactive hyperemia index (RHI)) were performed at 35 weeks. In PE, all measurements were performed at diagnosis. CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) was used as spin probe for EPR, since the formed CM radical corresponds to the amount of superoxide.

Results: Superoxide concentration remains stable during pregnancy ($p=0.92$), but is significantly higher compared to the NP controls ($p<0.0001$). At 25 weeks, there is a significant positive correlation between superoxide and ferritin concentration. ($p=0.04$) In PE, superoxide, systemic inflammation and iron status are much higher compared to HP (all $p<0.001$). During HP, superoxide concentrations correlate significantly with arterial stiffness (all $p<0.04$), while in PE superoxide is significantly correlated to microvascular endothelial function ($p=0.03$).

Conclusions: During HP there is an increased but stable oxidative environment, which is correlated to ferritin concentration. If superoxide levels increase, there is an augmentation in arterial stiffness. In PE pregnancies, systemic inflammation and superoxide concentrations are higher and result in a deterioration of endothelial function. Together, these findings support the hypothesis that vascular function is directly linked to the amount of OS and that measurement of OS in combination with vascular function tests might be used in the prediction of PE.

Introduction

Pregnancy is a state characterized by many physiological changes, which would be pathological in the non-pregnant state. Haematologically, neutrophils are increased due to augmented physiologic stress and impaired neutrophilic apoptosis during pregnancy, while lymphocytes are known to decrease during normal pregnancy, with a rise during the third trimester. Delivery is a highly stressful situation, resulting in a brisk leucocytosis. [1, 2] Thrombocytes on the other hand, become activated during pregnancy, particularly in the third trimester, and thus decrease, a condition referred to as 'gestational thrombocytopenia'. [2] This systemic inflammatory response in pregnancy results in high amounts of circulating reactive oxygen species (ROS), produced by activated blood cells. [3] The central organ regulating pregnancy, the placenta, is a major source of ROS. During normal pregnancy, placental oxidative stress (OS) is present during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and protein kinases. [3-7] Although OS is a common necessary feature of normal pregnancy, augmented OS could give rise to different disease-states, such as preeclampsia (PE).

PE is a potentially life-threatening complication of pregnancy, clinically detected after 20 weeks gestation. It affects 5% of pregnancies and is characterized by hypertension and proteinuria in mild cases, but can derail into organ damage, seizures and maternal death in severe cases. In the classic two-stage model of PE, OS induced in the ischemic placenta causes release of cytotoxic factors into the maternal circulation, stimulating the inflammatory response and activating maternal endothelial cells. [3] Increased neutrophil to lymphocyte ratio (NLR) and mean platelet volume (MPV) have been suggested as parameters of this chronic low-grade inflammation and enhanced OS. [8-10] Both systemic inflammation and OS result in the formation of ROS and reactive nitrogen species (RNS). ROS and RNS can react with nitric oxide (NO), resulting in a lower bio-availability of NO, the main player in endothelial function. The combination of superoxide and NO forms ONOO- (peroxynitrite), a harmful molecule with cell destructive effects. [11] This disturbance in endothelial homeostasis can lead to endothelial dysfunction, a condition characterized by a vasoconstrictive, pro-inflammatory and prothrombotic tendency. [12-14] In PE, OS, systemic inflammation and vascular dysfunction are obviously linked and capable of forming dangerous positive feed-forward systems. [3]

Under physiological conditions, the most common oxygen free radical in the human body is the superoxide anion radical (O2·-). Superoxide concentration is increased under conditions of hypoxia, when the availability of oxygen to act as final electron acceptor in the mitochondrial respiratory chain is reduced, which results in accumulation of unpaired electrons on oxygen. [3] As a result, iron (Fe) plays a crucial catalysing role in the production of ROS via the formation of hydroxide (OH−) and the very reactive hydroxyl radical (HO·), the main products of the Haber-Weiss and Fenton reactions. [15, 16] Literature suggests that an increased iron and ferritin concentration is linked to a higher risk of PE. [16] The profound hemodynamic changes of pregnancy can be objectified by assessing endothelial function and vascular stiffness with peripheral arterial tonometry (PAT) and applanation tonometry and they are proven to be disturbed in PE pregnancies. [9] Determination of ROS in blood during pregnancy and PE is of wide interest to specialists in perinatal medicine, but the best technique to access OS has been matter of debate. [5, 17]

Therefore, we performed a study by applying electron paramagnetic/spin resonance (EPR/ESR) spectroscopy as a direct method to detect ROS by using the spin-probe CMH (hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) to detect superoxide concentrations in blood. [18-21]

Although endothelial dysfunction and vascular stiffness are proven to be present in PE, no direct correlation with markers of OS and systemic inflammation has been proven. In this study, we aim to elucidate this correlation by measuring superoxide concentrations, haematological parameters of systemic inflammation and iron concentrations in HP and PE and correlate them with vascular function measurements.

Material and methods

Study population

Fifty-nine women with a healthy pregnancy (HP) and 28 PE patients (gestational age 25+6 weeks - 38+1 weeks (mean 32±3.3weeks)) admitted to the maternal intensive care unit were included between January 2016 and September 2017. We defined PE according to the revised ISSHP definition (2014).[22] Exclusion criteria were (gestational) diabetes, multiple pregnancies, foetal malformations, hypercholesterolemia, kidney disease, auto-immune

disorders, connective tissue diseases or use of acetylsalicylic acid. HP were included in the study during their first trimester and were longitudinally followed throughout the whole pregnancy. They were free from medication and did not have a history of PE, pregnancy-induced hypertension, hypertension, cardiovascular disease or other chronic conditions. A small group of non-pregnant (NP) healthy controls (n=10) was included to compare OS concentrations. The Research and Ethics committee of the Antwerp University Hospital approved the study protocol (Belgian number: B300201524783), and written informed consent was obtained from all subjects.

Oxidative stress: superoxide concentrations

EPR measurements were carried out on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high-sensitivity resonator and 12-kW power supply operating at X band frequencies. [23] The EPR analysis setting were as follows: frequency 9.86 GHz, power 50.41 mW, modulation frequency 100 kHz, modulation amplitude 1 G, sweep time 41.94 sec, time constant 40.96 msec, sweep width 50 G, number of scans 1. For the measurements, maternal blood was obtained at 9-11 weeks (mean 10+3 weeks), 24-28 weeks (mean 25+2 weeks) and at 38-41 weeks (mean 39+3weeks) in a heparin tube (BD vacutainer®, Canada) and transported on ice. After 15 minutes, 30µl of spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) was added to 30µl blood. The mixture of CMH and blood was incubated on ice and transferred into a 50 µl glass capillary (Hirschmann®, Germany) after 5 minutes. The sample was snap frozen and stored at -80°C until analysis. [23] The concentrations reported were obtained via double integration of the respective simulated spectra of the formed CM radical and this corresponds to the amount of superoxide that was present in the sample. The simulations were performed using a NIEHS P.E.S.T. WinSIM ver. 0.96 using the hyperfine values obtained from literature. [24] EPR calibration was performed using aqueous solutions of a stable radical 4-hydroxy-TEMPO (97%, Sigma-Aldrich, Germany) in a range of concentrations 2 - 200 µM as previously described. [25]

Haematological parameters of systemic inflammation (NLR/MPV) and iron status

Maternal venous blood samples were taken for the quantification of mean platelet volume (MPV), neutrophil-lymphocyte ratio (NLR), iron, ferritin and transferrin concentrations. Peripheral blood was collected by venepuncture at standard follow-up visits: 9-11 weeks (mean 10+4 weeks), 24-28 weeks (mean 25+3 weeks), and at 38-41 weeks (mean 39+3weeks) using vacuette tubes. Ethylenediaminetetraacetic acid (EDTA) and serum samples (BD Vacutainer®, Canada) were analysed respectively using an ADVIA 120 Hematology System (Siemens healthcare®, Germany) and Dimension Vista 1500 System (Siemens healthcare®, Germany).

Vascular function measurements

Vascular function measurements were taken during the third pregnancy trimester in HP and at the time of diagnosis in the PE group. Blood pressure measurements were taken using an automated blood pressure device (OMRON® Intellisense, Healthcare Japan). Microvascular endothelial function was evaluated with PAT using the Endo-PAT2000® (Itamar Medical, software version 3.2.4) and systemic arterial stiffness was evaluated using applanation tonometry with pulse wave analysis (PWA) and pulse wave velocity (PWV) using the Sphygmocor system® (Atcor Medical, West Ryde, Australia). PWA derives augmentation pressure (AP) and augmentation index (standardized to a heart rate of 75 bpm, AIx75) from the aortic pressure waveform. While PWV measures the carotid-femoral pulse wave velocity (CF-PWV), the gold standard for assessing aortic stiffness. PAT is an operator-independent and highly reproducible technique. [26] The system uses pneumatic finger probes which assess digital volume changes accompanying pulse waves. Relative ischaemia was induced by inflation of a blood-pressure cuff to suprasystolic pressure on the forearm of the patient for 5 minutes, after which the pressure was released and reactive hyperaemia was measured. The increased shear stress induced by reactive hyperaemia, increases endothelial NO production and subsequent vasodilation of the vessel. The ratio of the average amplitude of the PAT signal over a one minute period starting one minute after cuff deflation (maximum pulse amplitude) divided by the average amplitude of the PAT signal over a 3.5 minute period before cuff inflation (baseline pulse amplitude) was calculated. The control arm was used to correct for confounding factors (room temperature, systemic changes). The result is expressed as the reactive hyperaemia index (RHI). Vascular measurements were performed as previously described. [9]

To keep the focus on OS, detailed results of vascular measurements are described separately. (Chapter 3)

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 and GraphPad Prism version 7. Data are expressed as mean ± standard deviation (SD). Normality of continuous variables was evaluated using Kolmogorov-Smirnov test. Groups were compared using analysis of variance (ANOVA) with Tukey's multiple comparisons and Kruskal-Wallis with Dunn's multiple comparisons post-hoc tests as appropriate. Fisher-exact test was used for comparison of categorical variables. Spearman and Pearson correlation coefficient was used for univariate correlation analysis as appropriate. A two-tailed $p < 0.05$ was considered significant.

Results

Patient characteristics

Characteristics of the three groups (HP, PE and NP) are summarized in Table 1. Groups were comparable regarding age, BMI and cardiovascular risk. Blood pressure was significantly different between groups. Due to urgency for delivery, only 17 of the 28 PE patients underwent vascular measurements. There was no significant difference between BMI in the NP population and BMI at 12weeks in HP (NP 23.0 ± 2.7 vs HP 24.0 ±4.2, p=0.5).

Table 1: Patient characteristics

	Preeclamptic pregnancy (n=28)	Healthy pregnancy (n=59)	Non-pregnant (n=10)	p		
				PE vs HP	PE vs NP	HP vs NP
Age (years)	28.5 ± 3.9	30.2 ± 4.7	29.6 ± 4.1	0.27**		
BMI 3rd trimester (kg/m2)	30.2 ± 5.0	28.8 ± 4.2	22.3 ± 2.8	<0.0001*		
				0.99	<0.0001	<0.0001°
SBP 3rd trimester (mmHg)	160.2 ± 14.9	123.0 ± 9.2	122.9 ± 9.3	<0.0001*		
				<0.0001	<0.0001	>0.99
DBP 3rd trimester (mmHg)	96.0 ± 11.8	72.9 ± 8.2	73.8 ± 6.8	<0.0001*		
				<0.0001	<0.0001	>0.99
Nulliparous (n)	23 (82%)	31 (53%)	9 (90%)	0.004*		
				0.008	>0.99	0.07
Gestation at delivery (weeks)	32.0 ± 3.0	38.2 ± 2.2	/	<0.0001	/	/
Smoking (n)	0	0	0	/	/	/

Data are expressed as mean ± SD or as number of total (n). BMI= Body Mass Index, SBP= Systolic Blood Pressure, DBP= Diastolic Blood Pressure. PE= Preeclampsia, HP= Healthy Pregnancy, NP= Non-pregnant.
*° There was no significant difference in BMI between NP and HP at 12 weeks (p=0.50). * Statistical analysis was performed using Kruskal-Wallis ** Statistical analysis was performed using ANOVA.*

Oxidative stress and systemic inflammation in healthy pregnancy

During the course of a HP, NLR significantly increased (10 weeks 3.3±1.4 vs 25 weeks 4.7±1.8, p<0.001) caused by an increase in neutrophils (10 weeks 5.9±1.4 109/L vs 25 weeks 7.2±1.9 109/L, p<0.001) and a decrease in lymphocytes (10 weeks 1.9±0.7 109/L vs 25 weeks 1.7±0.5 109/L, p<0.001), while no difference was noted in MPV (10 weeks 8.4±1.2 fL vs 25 weeks 8.3±0.6 fL, p=0.38). Superoxide concentrations remained stable during HP (10 weeks 197.1±73.0 µM vs 25 weeks 199.2±105.1 µM, p=0.91) but were significantly higher compared to the NP controls (109.1±32.0, p<0.0001). Superoxide concentration at 25 weeks compared to 39 weeks (without labour) was not significantly different (25 weeks 208.3±131.1 µM vs 39 weeks 286.8±140.7 µM, p=0.28), confirming a stable superoxide environment during the whole pregnancy. Iron (10 weeks 101.5±38.0 µg/dL vs 25 weeks 81.7±35.9 µg/dL, p<0.001) and ferritin (10 weeks 42.0±28.2 mg/L vs 25 weeks 15.3±10.9 µg/L, p<0.001) concentrations decreased, while transferrin concentrations (10 weeks 2.9±0.8 g/L vs 25 weeks 4.1±0.7 g/L, p=0.007)

increased with advancing pregnancy. At 25 weeks of pregnancy, there was a significant positive correlation between superoxide concentration and ferritin concentration. (Fig 1)

Regarding the blood samples taken at term, 60% of patients were in labour (a condition characterised by overt ischemia and reperfusion injury), resulting in a significant difference in NLR (labour 6.2±3.9 vs no labour 4.0±1.6, p<0.001) and superoxide concentrations (labour 409.8±105.8 µM vs no labour 303.9±119.8 µM, p=0.03). NLR and superoxide during labour were positively correlated (p=0.03).

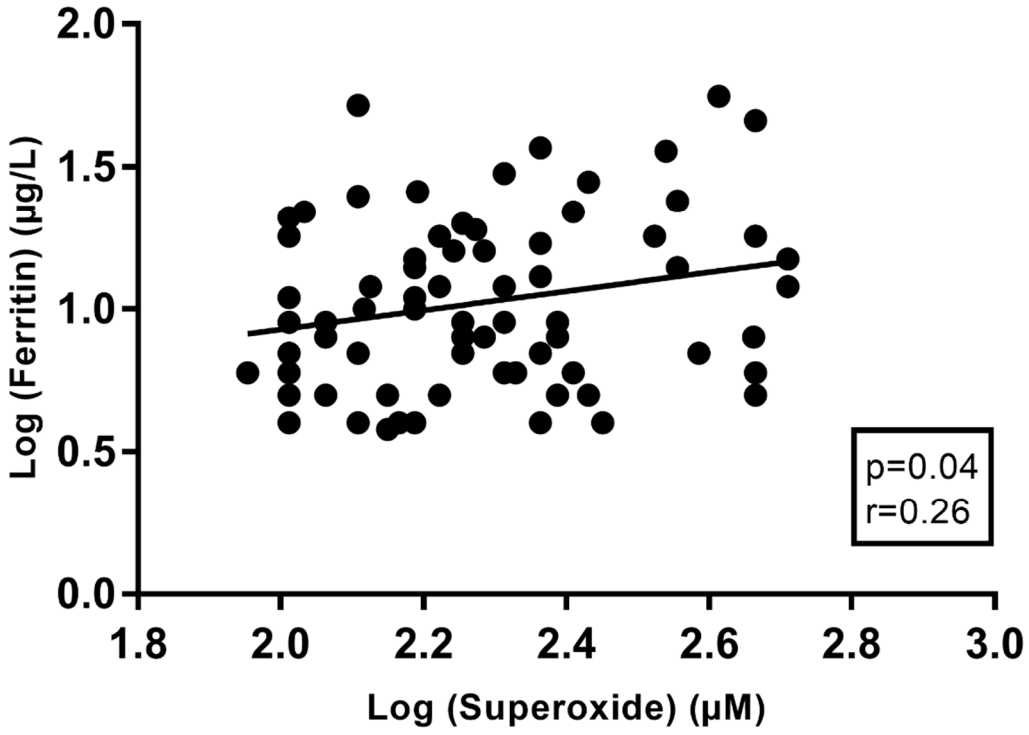

Figure 1: Correlation of superoxide concentrations with ferritin levels at 25 weeks of pregnancy. (r: pearson correlation coefficient)

Oxidative stress and systemic inflammation in preeclampsia

In comparison with HP, OS and markers of systemic inflammation were much higher in PE. (Table 2) Regarding iron status, iron concentration and intracellular iron reserve (ferritin) were significantly higher in PE, while transferrin concentrations were not different. There was no significant correlation between superoxide and ferritin concentration in PE (p=0.14), nor with other haematological parameters.

Table 2: Oxidative stress and systemic inflammation in PE vs HP

	PE	HP	p
Gestational age at blood sample	32+3 weeks	25+3 weeks	>0.05
Superoxide (µM)	307.2 ± 108.7	199.2.0 ± 105.1	<0.0001
NLR	6.5 ± 4.7	4.7 ± 1.8	0.0003
Neutrophils (10^9/L)	10.1 ± 5.1	7.2 ± 1.9	<0.0001
Lymphocytes (10^9/L)	1.9 ± 0.7	1.6 ± 0.5	0.01
MPV (fL)	9.7 ± 1.8	8.3 ± 0.6	<0.0001
Iron (µg/dL)	115.3 ± 70.3	80.9 ± 44.1	0.0003
Ferritin (µg/L)	176.9 ± 325.2	13.5 ± 10.4	<0.0001
Transferrin (g/L)	3.8 ± 3.1	3.9 ± 3.0	0.82

Data are expressed as mean ± SD. NLR= Neutrophil to Lymphocyte ratio, MPV= Mean platelet volume.

Correlation oxidative stress and arterial stiffness in healthy pregnancy

In the HP group, superoxide concentrations at 25 weeks of HP correlated significantly with all markers of arterial stiffness (CF- PWV, AIx75 and AP) measured during the third trimester (mean 34+2weeks) (Fig 2 A, B, C). There was, on the contrary, no significant correlation between superoxide and microvascular endothelial function (RHI) (Fig 2 D).

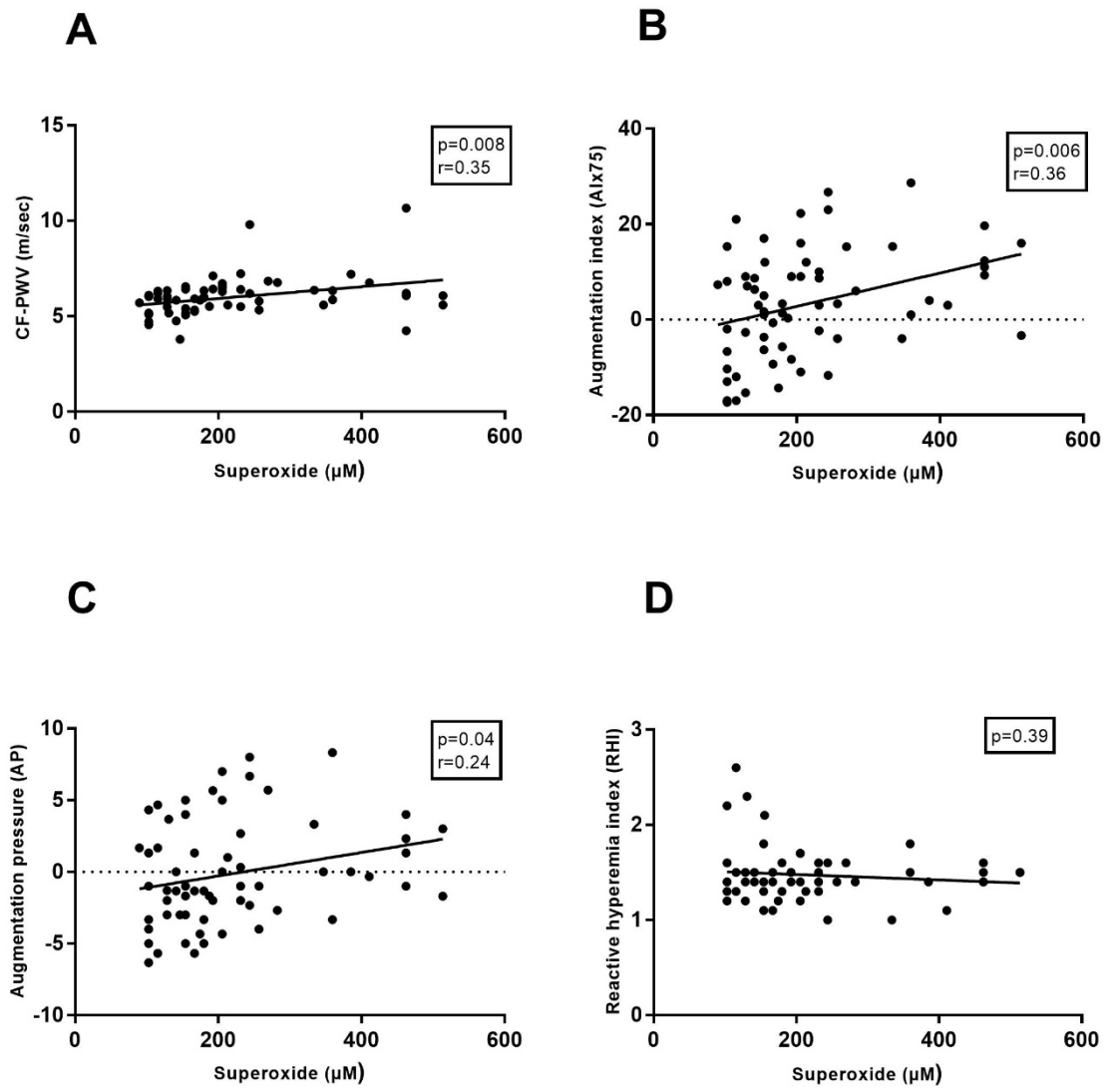

Figure 2: Correlation of superoxide concentrations with vascular function in the third trimester of a healthy pregnancy. Vascular function was assessed by carotid-femoral pulse wave velocity (CF-PWV (6.2±0.8), Fig 2A), augmentation index 75 (AIx75 (4.4±11.7), Fig 2B), augmentation pressure (AP (0.3±4.1), Fig 2c) and reactive hyperaemia index (RHI (1.6±0.4), Fig 2D). (r: pearson correlation coefficient)

Correlation oxidative stress and microvascular endothelial function in preeclampsia

A significant negative correlation was observed between superoxide concentration and microvascular endothelial function (RHI) in PE (Fig 3D), while no correlation was found with arterial stiffness (CF- PWV, AIx75 and AP) (Fig 3 A, B, C).

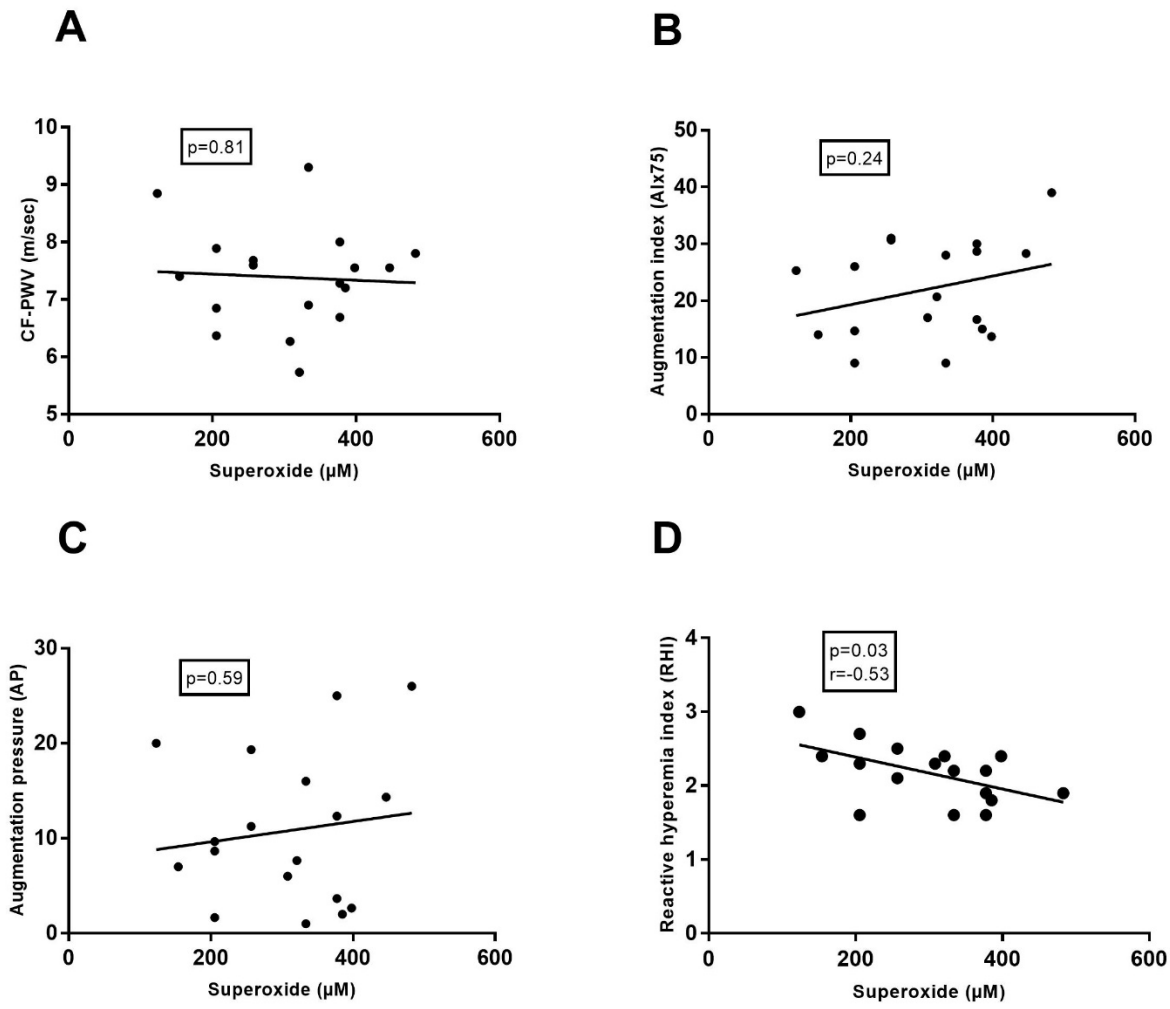

Figure 3: Correlation of superoxide concentrations with vascular function in preeclampsia. Vascular function was assessed by carotid-femoral pulse wave velocity (CF-PWV (7.6±0.9), Fig 2A), augmentation index 75 (AIx75 (24.0±9.6), Fig 2B), augmentation pressure (AP (11.8±7.8), Fig 2c) and reactive hyperaemia index (RHI (2.1±0.4), Fig 2D). (r: pearson correlation coefficient)

Discussion

There are 4 major findings of this study on OS and systemic inflammation and their relationship with endothelial function and arterial stiffness in HP and PE.

First, consistent with previous studies, this study demonstrates that systemic inflammation increases with advancing pregnancy. During labour, a condition associated with brisk leucocytosis, a positive correlation was found between NLR and superoxide concentration, confirming the theory that activated blood cells and ischemia-reperfusion damage at the site of the placenta result in higher OS levels.

Second, OS appears to maintain stable concentrations during pregnancy, but is correlated to ferritin levels. Literature on the longitudinal course of OS during a HP is very scarce. An increased extracellular antioxidant status with advancing pregnancy and an increase in lipid peroxides in the second trimester have been described [27, 28], however our results imply a stable OS environment from the first trimester of pregnancy to term.

Since placental OS is produced throughout pregnancy, is it probable that the antioxidant system acts in response to OS changes in order to maintain normal intercellular integrity and function in processes susceptible to OS in pregnancy. If this balance collapses, pregnancy complications might arise such as pregnancy-loss, intra-uterine growth restriction or PE. [3] Superoxide concentrations in the NP population are much lower compared to HP, proving once more the oxidative environment in a HP. Iron is abundantly present in the placenta and is an important co-factor in the formation of ROS. In the placenta, iron is stored in both ferritin and in transferrin bound to the transferrin receptor. [29] The placenta is a very oxygen-rich organ (starting from the second pregnancy trimester) and its abundant mitochondrial mass favours the production of ROS. Since iron is a necessary cofactor in the production of free radicals, iron excess results in acceleration of the formation of OS. On the contrary, iron deficiency results in defective mitochondrial function and mitochondrial DNA damage, with results in the release and leakage of ROS out of deficient mitochondria. [30, 31] Since both iron deficiency and iron excess result in free radical mitochondrial damage, iron supplementation is now only advisable for pregnant women with proven iron-deficiency anaemia. [30, 32]

In this study, we found a positive correlation between superoxide concentration and ferritin concentration at 25 weeks of pregnancy, which confirms that an excess in iron supplementation can cause harm by augmenting the amount of OS. This finding has previously been described in another pregnancy complication, gestational diabetes mellitus. Research by Hininger et al. describes a high maternal iron status and iron supplementation in the non-anaemic pregnant population as a potential risk factor for the development of gestational diabetes mellitus and suggests that a ferritin level of >70 g/L may be associated with a higher risk of developing gestational diabetes. They proposed association of iron supplementation with vitamin E and antioxidant rich foods to prevent the formation of oxidative radicals associated with iron overload in pregnancy. [33, 34]

Third, PE is characterized by higher levels of superoxide, markers of systemic inflammation and iron status (Table 2) which is in line with previous literature. [3, 35, 36] Studies on OS in PE have mainly focussed on indirect biomarkers of OS or on antioxidants levels, while the methodology of our work is entirely different. In this study we were able to directly measure the amount of CM radical formed which is proven to correspond directly to the amount of superoxide. [18-21]We acknowledge that the concentration of the formed CM radical may not represent the total concentration of the superoxide radical anion which was present in the solution. However, their concentrations are directly related, allowing to obtain trends for the superoxide formation. [18, 19] Superoxide levels were clearly higher in PE and we hypothesize that this is induced by activated blood cells, the ischemic placenta, higher iron and ferritin levels and the inability of the antioxidant system to meet and neutralise the produced ROS. Recently, the ratio of neutrophil to lymphocyte has been proposed as a prognostic and predictive marker in several low-grade inflammation disease states, such as cancer, cardiac diseases and PE. [8, 37-40] Although lymphocyte levels were higher in our PE population, NLR remained significantly higher, endorsing once again the activation of peripheral blood cells in PE.

Last, this study innovatively approaches vascular function by affirming its direct relationship with the amount of OS present in peripheral blood. HP is associated with profound alterations in the maternal cardiovascular system. Due to a decrease in vascular resistance necessary to answer the higher needs of the growing foeto-placental unit, vascular stiffness during HP is lower compared to the NP state. [41, 42] In our HP study population we assessed arterial stiffness with applanation tonometry and although the results were in the normal range for a pregnant population, there was a significant positive relationship between OS concentration and vascular stiffness in the third pregnancy trimester. This finding might imply that when OS rises above a certain 'harmful' level, vascular damage arises resulting in vascular dysfunction and stiffness. [4, 5] On the contrary, in our PE population vascular stiffness was higher, yet no correlation was found between the amount of OS and vascular stiffness. A possible explanation could be that at a certain level of OS, vascular stiffness reaches a maximum, without increasing further with higher OS. Microvascular endothelial function on the other hand, is inversely correlated to superoxide concentration, evidenced by worse RHI results in PE patients with higher superoxide levels.

Despite these novel findings, our study has limitations. There was a significant difference in parity between the HP and PE group, which could influence our results. Furthermore, since blood samples at term were taken during labour in 60% of the cases, the sample size to compare blood parameters at 39 weeks without labour was small. Likewise, vascular measurements were performed in a subgroup of PE patients. Last, we did not record iron supplementation in our population, as a result there are no reliable data on the intake of iron-supplements. From previous published literature, we know that in our population approximately 76% of pregnant women take vitamin supplements, containing approximately 26mg of iron daily. [43] Since iron supplementation might be determinant in the superoxide production, interventional studies are planned in the future and exact monitoring and randomisation of iron intake will take place.

To the best of our knowledge, this is the first paper to compare superoxide concentrations between HP and PE directly measured with EPR and to correlate them with vascular function tests. The strengths of this study were the repeated measures of systemic inflammation and OS during HP and the finding that vascular function was correlated with the amount of OS in both HP and PE.

Conclusions

The results of this study allow us to conclude that during HP there is an increased but stable oxidative environment, which is correlated to ferritin concentration. If superoxide levels increase, there is an augmentation in arterial stiffness. In PE pregnancies, systemic inflammation and superoxide concentrations are higher and result in a deterioration of microvascular endothelial function. Together, these findings support the hypothesis that vascular function is directly linked to the amount of OS, suggesting an important place for antioxidant treatment in the prevention on PE. Future research is imperative to investigate a broader spectrum of ROS in PE and to explore the effect of antioxidant therapy on vascular function.

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CHAPTER FIVE

HAEMATOLOGICAL PARAMETERS OF CHRONIC INFLAMMATION IN PREECLAMPSIA

ARE NEUTROPHIL/LYMPHOCYTE RATIO (NLR), PLATELET/LYMPHOCYTE RATIO (PLR), AND/OR MEAN PLATELET VOLUME (MPV) CLINICALLY USEFUL AS PREDICTIVE PARAMETERS FOR PREECLAMPSIA?

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J Matern Fetal Neonatal Med. 2017:1-8.

Abstract

Objective

Preeclampsia (PE) is a severe pregnancy complication with significant maternal and neonatal morbimortality resulting in high health care costs. Prevention, mainly based on the administration of acetylsalicylic acid, is only possible if timely identification of high-risk patients can be realized in an easy, non-expensive and widely available way. This paper explores the clinical usability of neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and/or mean platelet volume (MPV) in discriminating between women that will and those that will not develop PE.

Study design

Demographic data and laboratory results were retrospectively collected and compared in 2050 pregnant women (164 PE and 1886 controls) between 1 January 2014 and 31 January 2016.

Results

In the PE group, gravidity, parity, gestational age, and birth weight were significantly lower compared to the control group. Before the 20th pregnancy week, MPV was significantly elevated in the PE group compared to the controls ($p = 0.006$), hence analysis revealed an optimal cut-off point of 8.15 (sensitivity 66.7%, specificity 56.3%) for predicting PE. At the end of pregnancy, NLR and MPV appeared to be higher and PLR lower in the PE group compared to the controls, which strengthens the current knowledge on the pathogenesis of PE.

Conclusion

MPV is significantly elevated in the first half of pregnancy in women who later develop PE and might therefor be implemented in combination with other parameters in a PE prediction model.

Introduction

Preeclampsia (PE) constitutes a major pregnancy complication, as it occurs in 2-8% of pregnancies and is associated with significant maternal and neonatal morbimortality resulting in high health-care costs [1-5]. It is a leading cause of maternal death in developing countries, where mortality is attributed to eclampsia, a result of untreated PE [1]. Regarding the mother, PE can lead to caesarean section, renal failure, liver failure, coagulopathy, stroke, adult respiratory distress syndrome, cardiac arrest, and eventually death [1, 6, 7]. There is widespread empirical evidence that PE is a major risk factor for cardiovascular disease later in life [8-10]. Neonatal risks are intra-uterine growth restriction and low birth weight (due to the placental dysfunction), perinatal death and iatrogenic prematurity [1, 3]. The main features of PE are hypertension and proteinuria [3]. PE is caused by placental dysfunction and placental hypoxia [3, 11, 12]. This leads to activation of immunological factors [13, 14], increased neutrophil counts [15, 16], thrombocyte activation [17-19], systemic inflammation, and endothelial dysfunction [20, 21]. To date, there is only symptomatic but no curative therapy for PE. Delivery is the only way to end the disease, but the timing of delivery should be weighed against the fetal risks of premature birth [1, 3, 7, 22]. In women with increased risk of PE, low dose acetylsalicylic acid is recommended as prevention of PE, but has a high number needed to treat [22-24]. According to the WHO (World Health Organisation), acetylsalicylic acid should be started at 75 mg per day before 20 weeks of pregnancy [25].

The significant morbidity and mortality call for a predictive test in early pregnancy concerning the future development of PE, in order to provide close follow-up and preventive measures.

Based on the pathogenesis of the disease, this study was designed to examine differences in serum inflammatory and thrombocyte factors, such as NLR (neutrophil / lymphocyte ratio), PLR (platelet / lymphocyte ratio), and MPV (mean platelet volume), between women who developed PE and healthy pregnant women. The purpose of this study is to implement these easy applicable parameters as low-cost predictive factors for the development of PE.

Methods

Patient population

This retrospective cohort study was conducted at the Antwerp University Hospital (UZA). The study group consisted of all women who gave birth from 1 January 2014 until 31 January 2016. Patients were divided in two groups: a PE group and a healthy control group.

PE was diagnosed in accordance with the American Congress of Obstetricians and Gynecologists (ACOG) as hypertension (a systolic blood pressure of 140 mmHg or higher, or a diastolic blood pressure of 90 mmHg or higher, that occurs after 20 weeks pregnancy in a woman with previously normal blood pressure), and proteinuria (measured as 0.3 gram proteins or more in a 24-hour urine specimen) [7], or signs of other maternal organ dysfunction, such as renal insufficiency (elevated creatinine), liver involvement (elevated transaminases, right upper quadrant or epigastric abdominal pain), neurological complications (headache, hyperreflexia, visual scotoma) or hematological complications (thrombocytopenia, DIC, hemolysis) or signs of utero-placental dysfunction, such as fetal growth restriction [22]. No difference was made between severe or mild PE. Women with HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet counts), were also considered to have PE, since HELLP syndrome is a more serious condition in the same spectrum of this disorder [22].

Data collection

Data were obtained from pregnancy reports and laboratory results. Collected data were age, body mass index (BMI) at the beginning of pregnancy, abuses, and maternal chronic diseases such as kidney diseases, diabetes mellitus, and autoimmune diseases.

Obstetric data were GPA-status (gravidity, parity and abortion), gestational age, single or multiple gestation, personal and familial history of PE, neonatal birth weight, type of delivery (vaginal delivery or primary or secondary caesarean section) and the presence of gestational hypertension, gestational diabetes, gestational cholestasis, PE, HELLP, PPROM (preterm premature rupture of membranes), premature contractions without PPROM, placenta previa, vaginal blood loss, and fetal abnormalities. Proteinuria throughout pregnancy was evaluated using dipstick. Data on blood pressure and blood samples were taken at two occasions: before the 20th pregnancy week and right before primary caesarean section (PCS).

Since the UZA serves as a referral center, first trimester blood results of referred patients were collected through contacting general practitioners and referring gynecologists. Maternal venous blood samples in UZA were taken using vacuette tube. Ethylenediaminetetraacetic acid (EDTA) samples were analyzed using an ADVIA 120 Hematology System (Siemens healthcare®, Germany). Blood results involved complete blood count (CBC) with leukocyte differentiation and biochemical factors such as liver enzymes, creatinine, and glucose.

Exclusion criteria were multiple gestation, previous pregnancy with PE, kidney disease, diabetes mellitus, obesity (BMI ≥ 30kg/m²), gestational diabetes, cholestasis of pregnancy, chronic hypertension, inflammatory bowel diseases, thyroid disorders, auto-immune diseases, cardiovascular diseases, the use of acetylsalicylic acid during pregnancy, hepatitis B or C, human immunodeficiency virus, syphilis, signs of active infection, fetal death, and fetal chromosomal or morphological disorders.

Statistical analysis

Data analysis was executed using SPSS (Statistical Package for the Social Sciences) for Windows, version 24 (SPSS Inc., Chicago, IL).

Normal distribution of variables was tested with the Kolmogorov-Smirnov test. Independent T-test was used to compare mean NLR, PLR, and MPV between the PE and the control group for the first and second lab report for parametric data. Non-parametric data were analyzed using Mann-Whitney U-test. Outliers, defined as values > 2 standard deviations of the mean, were eliminated. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off level for NLR, PLR, and MPV in predicting PE. Binary multiple logistic regression analysis was performed to assess the independent predictors of PE. Statistical significance was defined as $p < 0.05$.

Results

Patient characteristics

A total of 2050 patients were included, of which 164 preeclamptic patients. After applying the exclusion criteria and withholding cases with missing lab reports, 1495 patients remained in the control group and 118 patients in the PE group. (Table 1) Regarding the second blood sample (right before labor), patients undergoing PCS were filtered, since being in labor (vaginal delivery

or secondary caesarean section) may affect lab results. There remained 138 patients in the control group and 59 patients in the PE group with PCS. (Table 2)

Table 1: Patients characteristics

	Preeclampsia (n=118)	Controls (n=1495)	p-value
Maternal age (years)	28.91 ± 4.91	30.20 ± 5.19	*0.006*
Nulliparous, n (%)	96 (81.4%)	697 (46.6%)	*0.00*
Smoking, n (%)	6 (0.08%)	87 (0.08%)	0.95
BMI at beginning pregnancy (kg/m^2)	23.60 ± 3.66	22.69 ± 3.12	0.13
Gestational age at delivery (weeks)	32.94 ± 4.02	38.11 ± 3.29	*0.00*
Neonatal birth weight (g)	2216 ± 3802	3199 ± 1147	*0.006*

Data expressed as mean ± standard deviation, median (minimum-maximum) or number (%).

Table 2: Patients characteristics (PCS)

	Preeclampsia (n=59)	Controls (n=138)	p-value
Maternal age (years)	28.03 ± 5.06	31.96 ± 4.50	*0.00*
Nulliparous, n (%)	48 (81.4%)	51 (37.0%)	*0.00*
Smoking, n (%)	3 (0.09%)	7 (0.07%)	0.74
BMI at beginning pregnancy (kg/m^2)	22.79 ± 3.11	23.13 ± 2.63	0.72
Gestational age at delivery (weeks)	30.98 ± 2.74	38.58 ± 0.80	*0.00*
Neonatal birth weight (g)	1475 ± 522	3298 ± 438	*0.00*

Data expressed as mean ± standard deviation, median (minimum-maximum) or number (%).

Blood pressure, serum inflammatory, and thrombocyte factors before 20th pregnancy week

Systolic and diastolic blood pressure were significantly elevated in the PE group. Leukocytes and thrombocytes were significantly higher in the PE group. No statistical significant difference was found between the PE and control group, in terms of NLR and PLR. MPV was significantly

higher in the PE group, compared to the control group. (Table 3) The optimal cut-off ratio for MPV is determined by ROC-analysis, as shown in figure 1. The area under the curve (AUC) is 0.652 (95% confidence interval 0.515-0.790). The optimal cut-off point is set at 8.15 with a sensitivity of 66.7% and a specificity of 56.3%. Logistic regression with MPV and maternal BMI before the 20th pregnancy week was statistically significant (Hosmer and Lemeshow test with significance 0.21, values > 0.05 are considered significant) in predicting PE. This model has an overall significance of p=0.02 and is able to divide 96.4% of the patients in the correct group (PE versus healthy pregnancy). The Nagelkerke R square however was 0.12, indicating a rather weak model quality.

Table 3: Blood pressure and haematological parameters before the 20th pregnancy week

	Preeclampsia group	Control group	p-value
Systolic BP (mmHg)	125.53 ± 15.40	118.67 ± 12.78	***0.025***
Diastolic BP (mmHg)	74.05 ± 8.46	68.12 ± 9.32	***0.007***
Hemoglobin (g/dL)	12.53 ± 1.19	12.65 ± 1.95	0.693
Hematocrit (%)	37.19 ± 3.21	36.62 ± 3.32	0.171
MCV (fL)	87.15 ± 5.51	85.72 ± 9.94	0.237
MCHC (g/dL)	33.87 ± 1.26	34.27 ± 2.58	0.216
Leukocytes (*10^9/L)	19.67 ± 29.16	8.70 ± 2.31	***0.003***
Neutrophils (*10^9/L)	7.89 ± 10.04	5.63 ± 2.32	0.214
Lymphocytes (*10^9/L)	2.96 ± 4.91	1.83 ± 0.71	0.205
NLR	2.81 ± 0.95	3.08 ± 1.07	0.173
Thrombocytes (*10^9/L)	265.75 ± 67.62	249.07 ± 57.53	***0.022***
MPV (fL)	8.64 ± 1.17	8.06 ± 0.87	***0.006***
PLR	128.86 ± 49.95	132.29 ± 39.74	0.662

Data expressed as mean ± standard deviation.
BP = blood pressure, MCV = mean corpuscular volume, MCHC = mean corpuscular hemoglobin concentration, NLR = neutrophil/lymphocyte ratio, MPV = mean platelet volume, PLR = platelet/lymphocyte ratio.

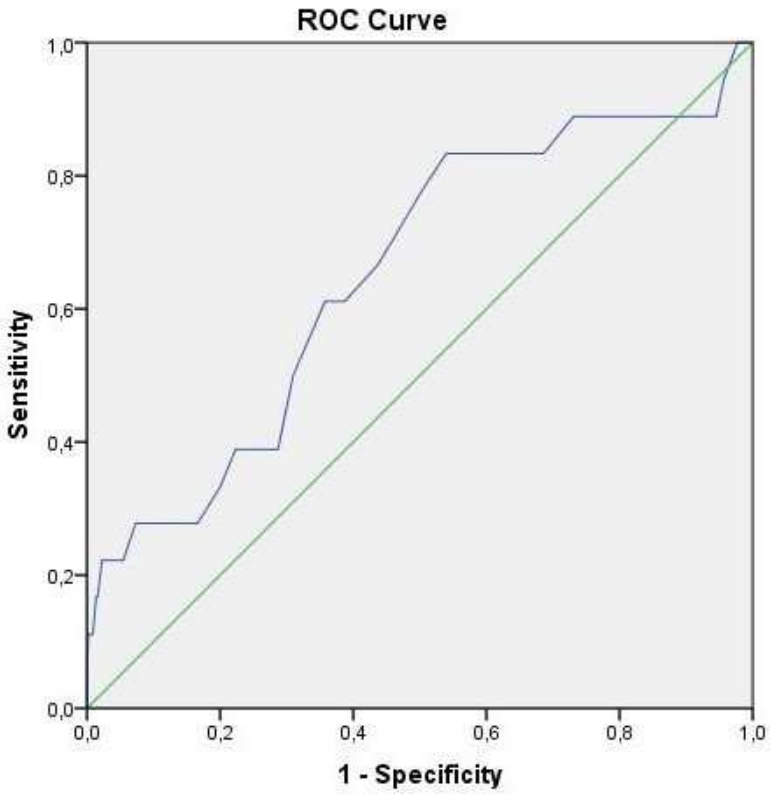

Figure 1: Receiver operating characteristic (ROC) curve for MPV before the 20th pregnancy week

Blood pressure, serum inflammatory, and thrombocyte factors right before PCS

Right before PCS, blood pressure was significantly elevated in the PE group, with hypertensive ranges both in systolic as in diastolic blood pressure, which is a known symptom of PE. Leukocytes were not significantly different between the two groups. Neutrophils were significantly higher, and thrombocytes were significantly lower in the PE group, compared to the control group. There was a significantly higher NLR, a lower PLR and a higher MPV, in the PE group compared to the control group. (Table 4) Corresponding ROC curves are shown in figures 2, 3, and 4. AUC with their optimal cut-off points for NLR, PLR, and MPV are listed in Table 5. Logistic regression analysis with NLR, PLR, and MPV was borderline significant (Hosmer and Lemeshow test with 0.05 significance, values > 0.05 are considered significant). Logistic regression with NLR and PLR as variables is promising, with a significance of 0.791. This model gives a correct prediction of PE in 80% of the cases. (Formula: Logit (p) = -3.911 + 3.520*NLRcutoffpoint + 2.789*PLRcutoffpoint) Transforming the model in ROC curve analysis had an AUC of 0.870 (95% confidence interval 0.790-0.949), as shown in figure 5. The optimal

cut-off for the probability of PE was 0.32, with a sensitivity of 83.9% and a specificity of 70.5%. The positive likelihood ratio for this cut-off point was 2.84 and the negative 0.23.

Table 4: Blood pressure and haematological parameters right before PCS

	Preeclampsia group	Control group	p-value
Systolic blood pressure (mmHg)	161.22 ± 21.56	122.31 ± 12.29	***0.00***
Diastolic blood pressure (mmHg)	101.14 ± 10.91	72.62 ± 8.21	***0.00***
Hemoglobin (g/dL)	12.09 ± 3.48	11.46 ± 1.11	0.186
Hematocrit (%)	33.85 ± 4.73	33.34 ± 2.79	0.468
MCV (fL)	86.10 ± 5.20	82.36 ± 6.13	***0.00***
MCHC (g/dL)	34.52 ± 1.60	34.37 ± 1.15	0.537
Leukocytes (*10^9/L)	13.27 ± 4.54	12.34 ± 15.34	0.656
Neutrophils (*10^9/L)	11.33 ± 4.22	6.49 ± 2.05	***0.00***
Lymphocytes (*10^9/L)	1.70 ± 0.62	1.77 ± 0.49	0.533
NLR	6.79 ± 2.84	3.60 ± 1.17	***0.00***
Thrombocytes (*10^9/L)	151 ± 73.89	232.96 ± 63.40	***0.00***
MPV (fL)	9.51 ± 1.21	8.90 ± 1.17	***0.005***
PLR	91.47 ± 47.48	129.05 ± 40.89	***0.0003***

Data expressed as mean ± standard deviation.
BP = blood pressure, MCV = mean corpuscular volume, MCHC = mean corpuscular haemoglobin concentration, NLR = neutrophil/lymphocyte ratio, MPV = mean platelet volume, PLR = platelet/lymphocyte ratio.

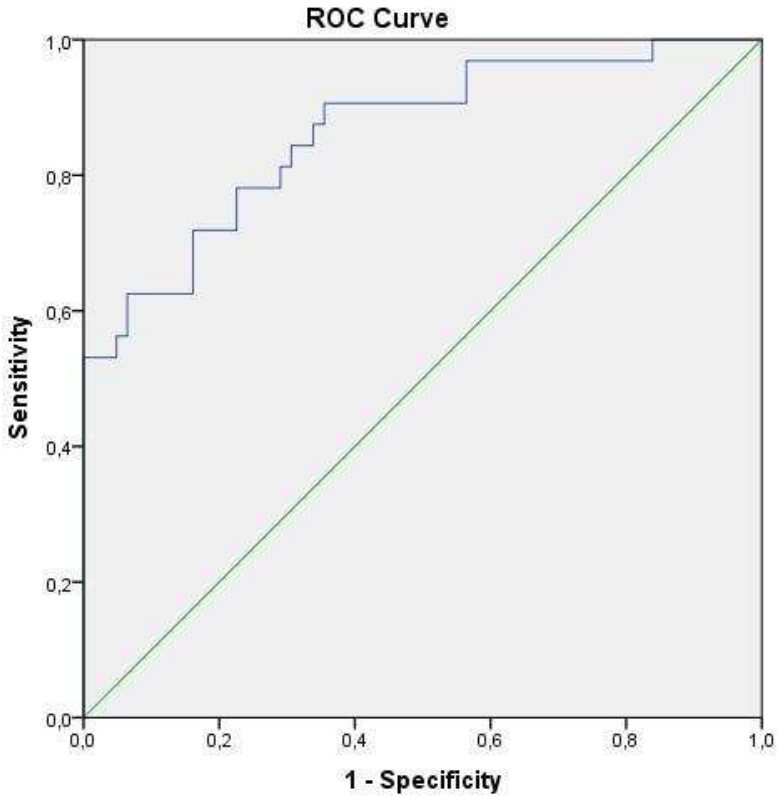

Figure 2: ROC curve for NLR right before PCS

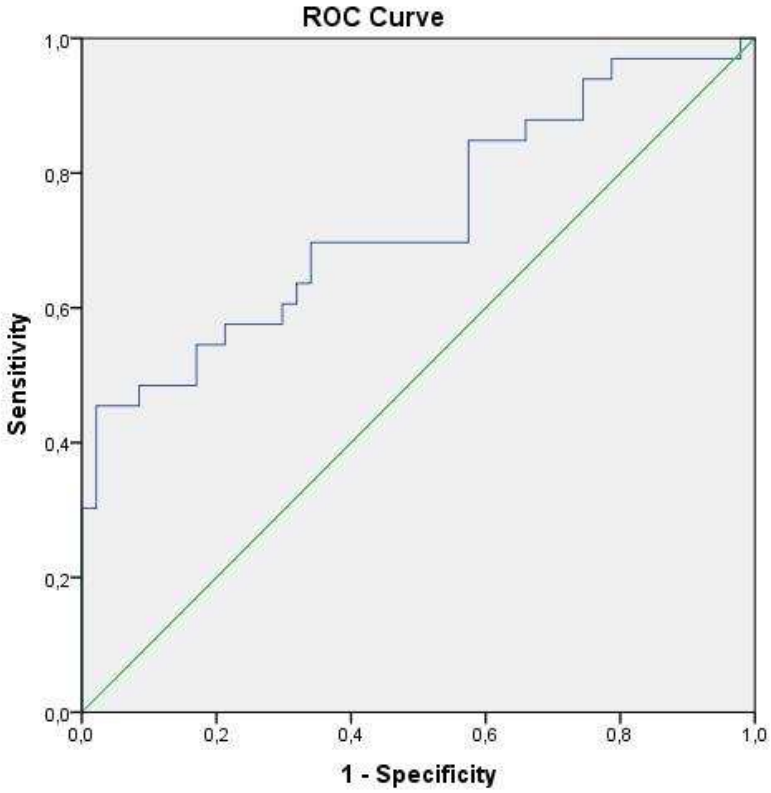

Figure 3: ROC curve for PLR right before PCS

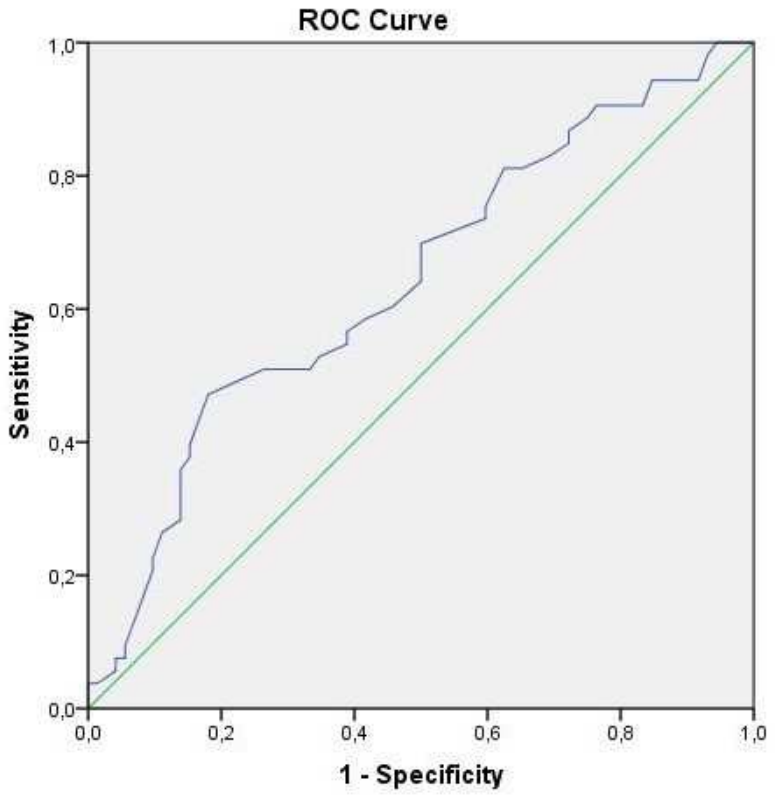

Figure 4: ROC curve for MPV right before PCS

Table 5: AUC with optimal cut-off point for NLR, PLR, and MPV right before PCS

	NLR	PLR	MPV
AUC	0.863	0.732	0.642
95% confidence interval	0.783-0.944	0.616-0.848	0.544-0.741
Optimal cut-off point	3.92	109	8.85
Sensitivity	84.4%	69.7%	69.8%
Specificity	69.4%	66%	50%

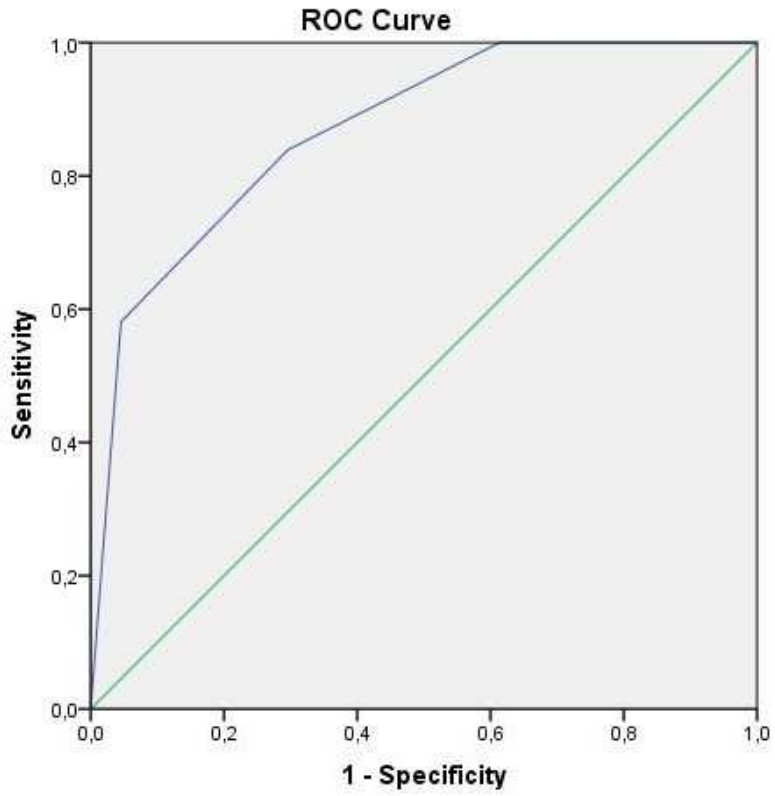

Figure 5: ROC curve for logistic model with NLR and PLR right before PCS.

Discussion

The pathogenesis of PE exists of two consecutive stages. The first stage occurs at the maternofoetal junction where a deficient invasion of cytotrophoblasts in the uterine wall and the spiral arteries leads to reduced utero-placental arterial flow and inadequate perfusion of the placenta [3, 11, 12]. This leads to hypoxia and the release of reactive oxygen species, which further contributes to placental oxidative stress and placental dysfunction [3]. This hypoxic state also induces inflammation through the release of chemokines, pro-inflammatory cytokines, anti-angiogenic factors and the activation of monocytes and neutrophils [13]. The neutrophil plays an important role in the pathogenesis of PE. Activation of neutrophils occurs by exposure to oxidized lipids secreted by the placenta, when they pass the intervillous space [26-28].

The second stage of PE starts when these activated neutrophils infiltrate maternal vascular tissue and is associated with maternal systemic vascular inflammation [13, 26, 29]. This leads to thrombocyte activation, vasoconstriction, hypertension, endothelial dysfunction, and end-

organ ischemia [3, 5, 12]. For this reason, the clinical stadium of PE is characterized by hypertension, proteinuria, edema, headache, scotoma, coagulopathy, and renal and hepatic dysfunction [3, 9, 14]. Systemic inflammation occurs in normal pregnancies. There is a shift towards Th2 (suppressor T-helper) lymphocytes in normal pregnancies, which leads to suppression of Th1 cytokines, which in turn enables maternal immune tolerance to the fetus, whereas in PE there is a shift towards the Th1 response, an immune maladaptation, and a hyper-inflammatory state [12, 14, 30]. Based on this pathogenesis cascade, there have been many studies about predictive factors for PE, but consensus about a significant and useful predictive parameter has not yet been achieved [31]. Some studies described blood pressure [32] or uterine artery Doppler velocimetry (an increased pulsatility index alone or combined with bilateral notching) [31, 33, 34] in the screening for PE. Biochemical parameters that have been tested as predictive factors are mostly based on the hyper-inflammatory state, anti-angiogenesis or platelet activation specific for PE. Recently under research in this area is the increase in soluble FMS-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) and decrease in placental growth factor (PlGF), caused by an imbalance in the VEGF signaling pathway [6].

Endothelial dysfunction in PE leads to uncontrolled intravascular thrombocyte activation with increased thrombocyte consumption in the maternal peripheral circulation [17-19]. Different studies showed that thrombocytes were significantly decreased [35, 36] and mean platelet volume (MPV) was significantly increased [5, 37-42] in women who developed PE, compared to women who did not develop PE [17, 19, 43-45]. This difference was even demonstrated in the first trimester of pregnancy [17, 39]. MPV is an indicator of thrombocyte size, synthesis, and function [19, 41]. MPV is increased in PE, more than in normal pregnancy, as a result of thrombocyte activation and aggregation [19, 37]. Thrombocyte activation in PE is related to the change in the coagulation process between thrombocytes and the damaged endothelial cells [35, 36, 46].

PE is also associated with an increase in leukocyte count, more than in normal pregnancy, and this increase is mainly due to the increase in neutrophil count [15, 16]. Lymphocytes are decreased in PE [15]. An increase in neutrophils and a decrease in lymphocytes results in an increased NLR in PE, compared to normal pregnancies. Few studies focused on this increased NLR, but there were no homogeneous conclusions for the use of NLR as a predictive factor for PE [4, 5, 26, 30, 47-51]. Regarding PLR, there were also inhomogeneous results [4, 5, 49]. The minority of studies examined blood results in the first trimester of pregnancy [4, 49]. The

majority of previous papers about NLR/PLR and MPV however, were limited to late pregnancy [5, 26, 30, 47, 48, 50, 51]. To the best of our knowledge, this is the first large retrospective cohort study comparing early (<20weeks) and late (3rd trimester) MPV, NLR, and PLR values between normal pregnancies and preeclamptic pregnancies. Consistent with most of the previous studies and the current knowledge on the pathogenesis of PE, our study demonstrates that NLR and MPV are significantly higher and PLR significantly lower in established PE. We thus hypothesize that PE is associated with an enhanced inflammatory response and platelet activation due to systemic inflammation and endothelial dysfunction. The observation that MPV is already augmented in early pregnancy, leads to the conclusion that this systemic inflammation and thrombocyte activation is already present in early pregnancy and MPV can serve as an additional predictor of PE. The weakness of our logistic prediction model with NLR and BMI before 20 weeks, suggests that these factors alone are not capable of correctly predicting PE. However, in combination with other parameters (pulsatility index uterine artery, familial history, anti-angiogenic factors) they might be of additional value in the prediction of PE.

Recent literature proposes NLR as an interesting prognostic factor of cardiovascular disorders since this ratio is significantly increased and since an elevated NLR gives a higher risk for cardiovascular morbidity and mortality [52-54]. For this reason, NLR can be suggested as a prognostic factor in PE for future cardiovascular disease. Future research is required to study whether a higher NLR in PE is associated with a higher prevalence of long-term cardiovascular disease.

Limitations

Limitations to our study are due to the retrospective character of the study, resulting in missing data in the obstetric files. Patients were mostly followed in private practiced hospitals and only transferred to the UZA after the diagnosis of PE. Because of this, their first blood results, before the 20th pregnancy week, were collected from other laboratories. As a result, leukocyte differentiation was often not executed in other laboratories, which caused missing data for NLR and PLR. The blood results before the 20th pregnancy week were thus derived from different laboratories, and were taken at different gestational weeks before the 20th week, which makes it difficult to compare these blood results between patients. Other limitations to our study were

the retrospective model, in which confounding remains possible, and the lack of stratification in the PE group between severe and mild PE.

Conclusions

This study examined NLR, PLR, and MPV as predictive markers for PE. Before the 20th pregnancy week, only MPV was significantly elevated in future PE compared to healthy pregnancy. However, the discriminative power of MPV is, in our opinion, not strong enough to recommend use as a single parameter in clinical practice. There might be a place to implement MPV determination in combination with other parameters in a PE prediction model. At the end of pregnancy (right before PCS), there was a significantly higher NLR, a lower PLR, and a higher MPV, in the PE group compared to the control, which supports the current knowledge of the pathogenesis of PE. Further research is needed on predictive factors for PE, with the purpose of starting preventive treatment in these women, since PE is a pregnancy complication with serious implications for mother and child.

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CHAPTER SIX

PLACENTAL NITRIC OXIDE METABOLISM IN PREECLAMPSIA VERSUS HEALTHY PREGNANCY

PLACENTAL NITRIC OXIDE METABOLISM IN PREECLAMPSIA VERSUS HEALTHY PREGNANCY

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Placenta (submitted)

Abstract

Introduction

Augmented oxidative stress (OS) in pregnancy leads to various pathophysiological events including disturbed cell function and disrupted cellular signalling resulting in different disease-states, such as preeclampsia (PE). Placental nitric oxide (•NO) is produced out of L-arginine by endothelial and inducible NOS (eNOS and iNOS) and is essential for a healthy endothelial function. The best technique to access •NO concentration has been matter of debate and the relative contribution of •NO in the pathogenesis of PE has yet to be fully elucidated.

Methods

Fifteen PE women (mean 32 weeks) and fifteen age-, BMI- and parity-matched healthy pregnant (HP) women (mean 39 weeks) were included. Electron paramagnetic/spin resonance (EPR/ESR) spectroscopy was used as a direct method to detect •NO concentrations by applying an iron-DETC spin-trap (Fe (II) DETC2 (iron (II)diethyldithiocarbamate)) to stabilize the free radical •NO. eNOS and iNOS expression was determined using immunohistochemical staining. The sensitivity of three different EPR devices was compared.

Results

After PE, a 0.5 times significantly lower placental •NO concentration was present compared to after HP (PE 2.9±1.7 vs HP 6.0±4.0, p=0.01). The intensity of eNOS staining was significantly higher in PE compared to HP (p=0.006). iNOS was similarly expressed in PE compared to HP.

Discussion

Our findings suggest a disturbed placental •NO metabolism in PE which might be responsible for the deficient placentation distinctive for PE. Although compensatory eNOS activation is present, eNOS uncoupling and formation of peroxynitrite counteract this mechanism, resulting in lower placental •NO concentrations in PE.

Introduction

Pregnancy is a state of oxidative stress (OS) arising from increased placental mitochondrial activity and placental production of free radicals. In the human body, free radicals are mainly represented by reactive oxygen species (ROS) and reactive nitrogen species (RNS), of which respectively superoxide and nitric oxide (•NO) are the most abundant [1]. In normal pregnancy, placental OS is present during all three trimesters and is necessary to obtain normal cell function [2-6]. However, augmented OS leads to various pathophysiological events including disturbed cell function and disrupted cellular signalling resulting in different disease-states, such as preeclampsia (PE) [4, 6-9]. Although an excess in free radicals has a pernicious effect, not all free radicals cause disturbances in the organism [3] and •NO is an example.

•NO has a wide range of functions in the human body; it is the most important vasodilator, inhibits platelet aggregation, modifies the expression of inflammatory cytokines, inhibits interaction between immune and endothelial cells and is essential for a healthy endothelial function. •NO is produced out of L-arginine by •NO synthases (NOS) of which two isoforms are present in the placenta: endothelial and inducible NOS (eNOS and iNOS) [10]. eNOS is considered the key enzyme in the production of •NO in pregnancy and is inhibited by asymmetric dimethylarginine (ADMA) [11]. eNOS is activated by the binding of the angiogenic factors vascular endothelial growth factor (VEGF) and placental endothelial growth factor (PlGF) to their respective receptor (Flt-1). [12] In PE, the placenta is known to produce a soluble form of this receptor (sFlt-1), that interferes with •NO production by capturing VEGF and PlGF. [13, 14] In addition, in conditions of high OS, eNOS can undergo uncoupling, producing superoxide besides •NO [15], provoking even more OS. (Figure 1)

•NO has essential functions throughout pregnancy. During early placentation, it influences endovascular trophoblast invasion at the uterine spiral arteries [11, 16]. Moreover, apoptosis of trophoblast cells and cytokine production, both important placental functions, are influenced by oxygen and •NO concentration [17]. When the placentation process is deficient, the uterine vessels in the placental bed maintain their high resistance [18]. This causes a suboptimal maternal circulatory adaptation to pregnancy, resulting in placental ischemia-reperfusion damage which in turn results in systemic OS, inflammation and endothelial

dysfunction, all seen in the pregnancy-specific disorder PE [18]. The complex contribution of •NO in the pathogenesis of PE is illustrated in Figure 1. [13] During labour, repeated short periods of ischemia-reperfusion result in acute increased placental OS [1, 15, 19]. As a compensatory mechanism, plasma total antioxidant capacity and placental antioxidants seem to be increased at labour [15, 19]. •NO is known to play a role in the initiation of spontaneous labour, participating in a complex interaction with cytokines, prostaglandins and steroids. [20]. A tocolytic effect of •NO donors has been described [21]. Endothelial produced •NO has a relaxing effect on uterine smooth muscle cells, in this way preventing preterm contractions and delivery, which suggests that a decline in •NO can be considered one of the causative factors in the initiation of parturition.

In literature, controversy exists on placental •NO concentration in normal pregnancy versus PE [11, 22] and on fluctuations of •NO concentration during labour [20, 23]. Most studies measure •NO metabolites as a marker of •NO concentration since •NO is difficult to measure and the best technique to access •NO concentration has been matter of debate. [3, 24] Thus, the relative contribution of •NO in the pathogenesis of PE and in the parturition process has yet to be fully elucidated [11, 22].

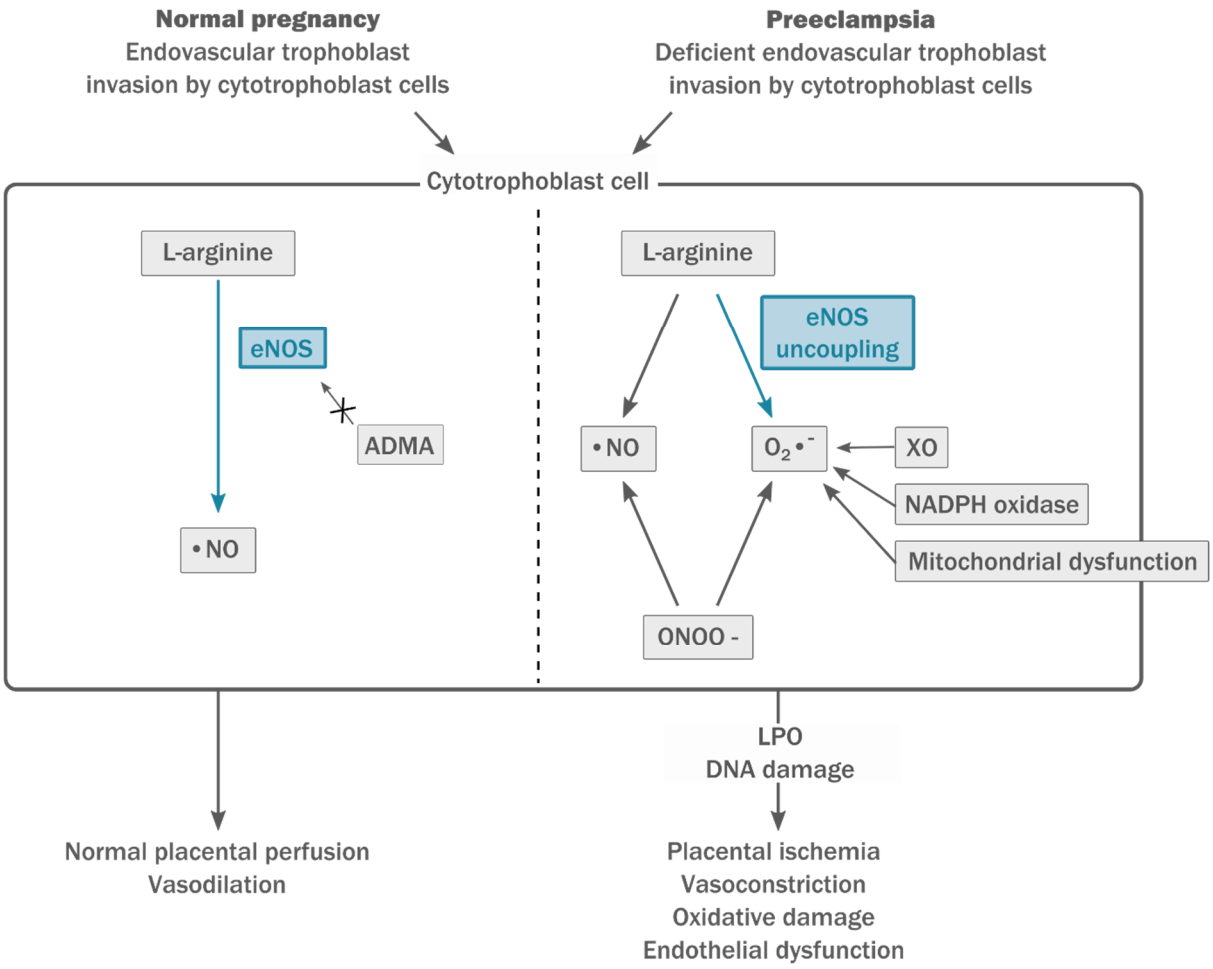

Figure 1: The role of nitric oxide, superoxide and eNOS in the placentation process.
(eNOS: endothelial nitric oxide synthase; •NO: nitric oxide; O2•-: superoxide; XO: xanthine oxidase; NADPH: nicotinamide adenine dinucleotide phosphate; ONOO-: peroxynitrite; LPO: lipid peroxidation, ADMA: asymmetric dimethylarginigne)

In this study, we used electron paramagnetic/spin resonance (EPR/ESR) spectroscopy as a direct method to detect •NO concentrations by applying an iron-DETC spin-trap (Fe (II) DETC2 (iron (II)diethyldithiocarbamate)) to stabilize the free radical •NO. To deduce the origin of •NO, a subgroup of the placentae underwent immunohistochemical (IHC) staining to determine placental eNOS and iNOS expression. We hypothesize that since PE is characterized by a deficient placentation process, placental •NO concentration will be lower compared to healthy pregnancy (HP).

Methods

Study population

Fifteen preeclamptic women (gestational age 28+0 weeks – 35+3 weeks, mean 32 weeks) admitted to the maternal intensive care unit at the Antwerp University Hospital were included between January 2016 and September 2016. We defined PE according to the revised ISSHP definition (2014) [25]. Exclusion criteria were (gestational) diabetes, multiple pregnancies, foetal malformations, hypercholesterolemia, kidney disease, auto-immune disorders, connective tissue diseases or use of acetylsalicylic acid. Since the Antwerp University Hospital serves as a referral centre, women were already started on antihypertensive medication and MgSO4 at the moment of referral and inclusion. (Table 1) Patients with intra-uterine growth restriction on •NO donors were excluded from the study. Fifteen age-, BMI- and parity-matched healthy pregnant women (gestational age 36+6 weeks – 41+4 weeks, mean 39 weeks) served as controls. They were free from medication and did not have a history of PE, pregnancy-induced hypertension, hypertension, cardiovascular disease or other chronic conditions. In the PE group, 11 women were delivered by elective caesarean sections (ECS) and 4 had a vaginal delivery (VD). In HP, all patients delivered vaginally. Patients with secondary caesarean section (after labour) were excluded from the study. eNOS/iNOS expression by IHC staining was performed in 12 PE placentae after ECS and compared to 10 placentae after a HP undergoing ECS. The Research and Ethics committee of the Antwerp University Hospital approved the study protocol (Belgian number: B300201524783), and written informed consent was obtained from all subjects.

Table 1: Antihypertensive medication and doses given to PE patients (n=15)

No antihypertensive medication	5 women
Labetalol 100mg 3x/d	5 women
Labetalol 200mg 3x/d	4 women
Labetalol 100mg 3x/d + Felodipine 10mg 2x/d	1 women
MgSO4 (1gr/h)	11 women

Placental sampling for EPR

Placental tissue was obtained within two minutes after (vaginal or elective caesarean) delivery. At a standardized central location, a viable sample of 1cm³ placental tissue was taken avoiding placental infarcts or necrotic/calcified tissue. As previously described by Sikkema et al. [24], no significant differences in placental OS were found as long as evidently necrotic tissue was avoided. The sample was rinsed with saline (NaCl) and immediately added to the spin trap 750 µL 0.81 mM FeSO4.7H2O and 750 µm 2.1 mM DETC ((iron (II) diethyldithiocarbamate) solution. After one hour of incubation at 37°C, the sample was snap frozen and stored in -80°C until analysis with EPR. [26] The spin trap DETC/FeSO4 was prepared freshly before addition to the sample and stored at -20°C before use.

•NO detection by electron paramagnetic resonance (EPR)

EPR is a magnetic resonance technique that identifies and quantifies paramagnetic species with one or more unpaired electrons. These include simple molecules like •NO and superoxide (O2•-), but also larger, paramagnetic molecules. Small molecular complexes with a transition metal ion, proteins with transition metal ions as cofactors (such as iron in transferrin (TF) or copper (Cu) in ceruloplasmin (CP)) and free radicals also possess unpaired electrons and can therefore be detected by EPR. [27, 28] EPR is based on the magnetic moment arising from the electron spin that aligns in an externally applied magnetic field, resulting in two non-degenerate spin energy states. The low, more stable spin magnetic moment is associated with the spin magnetic moment aligned with the magnetic field, while the high, less stable energy state is associated with a spin magnetic moment aligned opposite to the magnetic field. Transition between the two different energy states takes place by absorption of radiation in the microwave frequency and this absorption can be detected to determine the concentration of unpaired electron spins present in the sample. In an EPR spectrum, the amplitude of the second derivative of the EPR spectrum directly correlates with the number of unpaired electrons present in the sample and is therefore used for quantification. The local magnetic moment caused by some nuclei (for example 1H, 13C, 14N) in close vicinity of the unpaired electrons can interact with the magnetic field and results in a hyperfine splitting of the EPR spectrum. [28, 29] This distinctive 'fingerprint' spectrum characterised by the hyperfine coupling constants, in combination with the g-factor, helps to determine the identity of paramagnetic species. While EPR is able to

identify and quantify long-lived radicals (such as the ascorbyl free radical (AFR)) directly, for short-lived radicals stabilization is mandatory. This stabilisation can be achieved by forming stable spin adducts with diamagnetic properties (spin trapping) or by scavenging the unpaired electron to form a new more stable radical (spin probing). In this study, the DETC spin trap was added to scavenge •NO in order to prolong its half-life time. EPR measurements were carried out on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high-sensitivity resonator (QL= 3000) and 12-kW power supply operating at X band frequencies. [26] The EPR analysis setting were as follows: frequency 9.39 GHz, power 20.07 mW, modulation frequency 100 kHz, modulation amplitude 5 G, sweep time 83.89 sec, time constant 81.92 msec, sweep width 200 G, number of scans 10. The relative •NO concentrations in arbitrary units (A.U.) were obtained by calculating the double integral of the second •NO peak using the WinEPR software (version 2.11.0.0). (Figure 2)

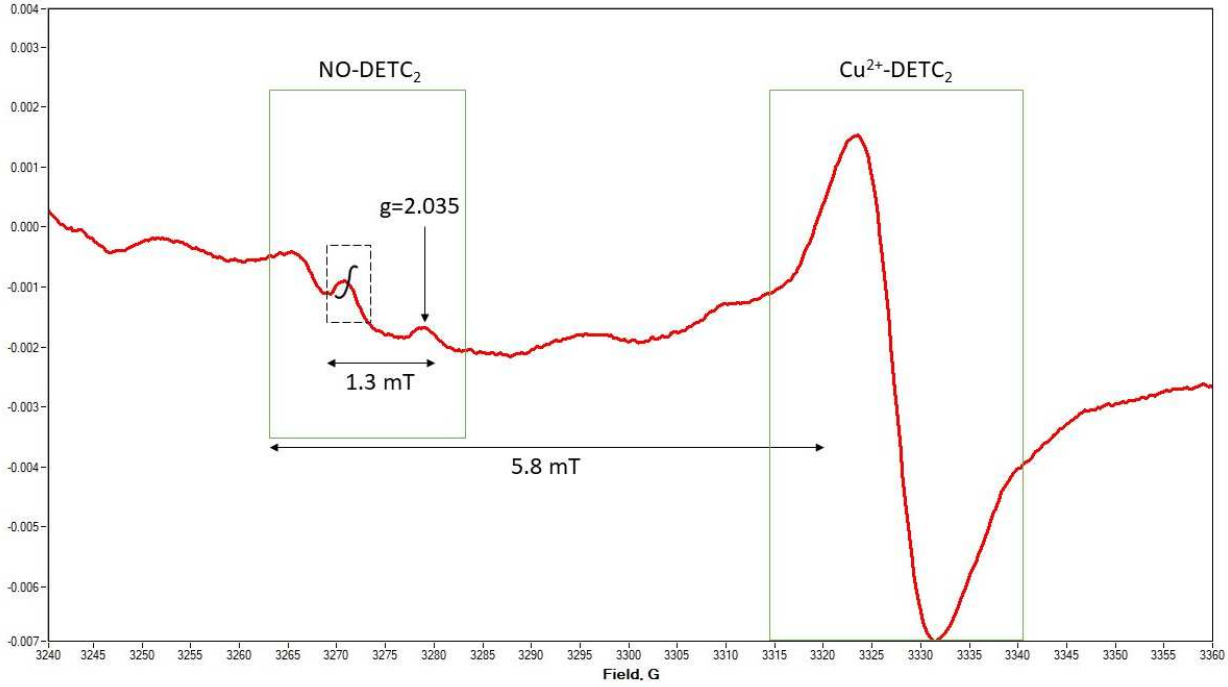

Figure 2: Placental nitric oxide concentration spectrum, obtained using the Bruker EMX 1273 spectrometer at the University of Maastricht. Fe-DETC2 traps NO resulting in a characteristic triplet signal, and copper, creating a Cu2+-DETC peak. The double integral of the second NO peak, used to calculate NO concentrations is plotted on the spectrum.
(NO: nitric oxide; mT: militesla; g= g-factor typical for the free radical NO)

Comparison of the sensitivity of different EPR devices

•NO concentrations are known to be low in human tissue and •NO has a very short half-life of a few seconds. [30] Therefore, direct measurement of •NO concentration remains a difficult task. EPR is one of the best suitable techniques to detect •NO concentrations, especially when used in combination with iron complexes as spin trap. [31] However, EPR spectra are largely dependent on the properties of the EPR device and the storage time of the samples. Placental samples in this study were analysed six to maximal twelve months [32] after sampling on the continuous wave Bruker EMX 1273 spectrometer at the University of Maastricht. Unfortunately this device experienced technical problems whereby it was impossible to analyse the next batch of placental samples on this device. In total, 100 placental samples were collected of which 50 after a HP [29 VD and 21 ECS,) and 50 after a PE pregnancy (12 VD and 38 ECS). Especially the elective caesarean group after HP was a very important group to determine the influence of labour on placental •NO concentration. To quantitatively compare placental •NO concentrations, all samples must be analysed on the same EPR device since differences in power and frequency could influence results. As a consequence, in the second experiment we analysed all one hundred placental samples on a bench-top MiniScope MS2000 EPR spectrometer (Magnettech Ltd., Berlin, Germany) at the University of Antwerp. [26] The EPR analysis settings were as follows: frequency 9.4 GHz, power 17 dBm (50.1 mW), modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep time 30 s, time constant 0.1, center field 329 mT, sweep width 20 mT, number of scans 3. This bench-top device is a high throughput analysis spectrometer, more adapted for integration in the clinical field, but unfortunately it is less sensitive since its magnetic field and microwave radiation are smaller. The signal intensity of the •NO-DETC2 complex is rather small and as a result this device was not able to pick up the •NO-DETC2 signal. To encounter this technical problem, a third experiment was performed using an ELEXSYS E600 spectrometer equipped with a 4102ST /9322 cavity with conversion factor: 1.4 G/√W at QL= 2500 at the University of Antwerp. The EPR analysis setting were as follows: frequency 9.49 GHz, power 20.07 mW, modulation frequency 100 kHz, modulation amplitude 1 G, sweep time 83.89 sec, time constant 20.48 msec, center field 3333G, sweep width 200 G, number of scans 10. This device is more sensitive compared to the bench-top Miniscope, however the obtained spectra (Figure 3) were not as pristine as the ones from the Bruker EMX 1273 spectrometer at the University of Maastricht. In the spectra from Maastricht (Figure 2), the beginning of the •NO-DETC2 triplet was at 5.8 mT from the copper (Cu2+)-DETC

complex. Using the known g-factor (g=2.035) and hyperfine-coupling constant (1.3mT) described by respectively Faassen et al. [32] and Kleschyov et al. [31], the position of the •NO-DETC2 signal was determined. The triplet typical for the •NO-DETC2 signal was not clearly visible so proper quantitative analysis of the samples was not possible. (Figure 3) Therefore, we decided to limit our presented data to the first 30 placental samples measured at the Bruker EMX 1273 spectrometer at the University of Maastricht were we obtained optimal spectra.

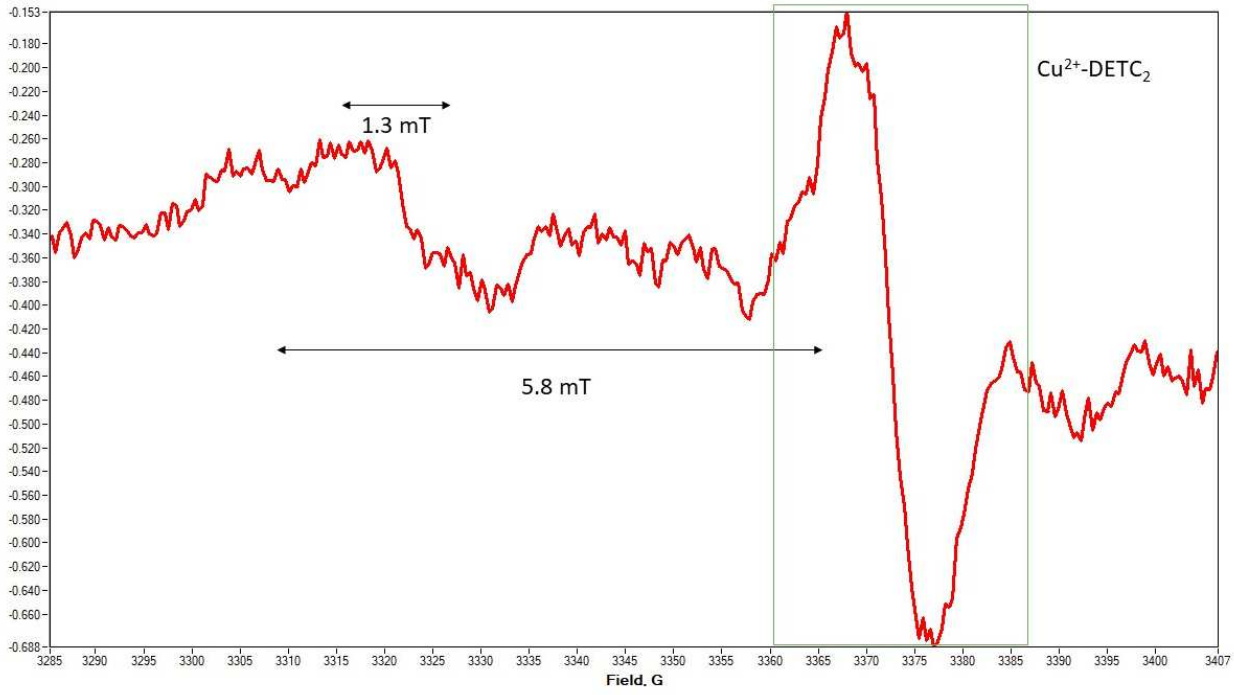

Figure 3: Indefinable placental nitric oxide concentration spectrum, obtained using the ELEXSYS E600 spectrometer at the University of Antwerp.
The characteristic triplet signal of NO is absent. The Cu2+-DETC peak is clearly visible, demonstrating the region where the NO- DETC2 signal must be present.
(NO: nitric oxide; mT: militesla)

Immuno-histochemical staining for eNOS and iNOS expression

Placental tissue specimens were fixed and paraffin embedded on a routine basis. Five µm-thick sections were prepared from FFPE (formalin fixed-paraffin embedded) specimens. Sections were dewaxed and subjected to heat-induced antigen retrieval (HIER) by incubation in a low pH buffer (Envision Flex Target Retrieval Solution low pH, DAKO, Glostrup, Denmark) for 20 min at 97°C. Subsequently, endogenous peroxidase activity was quenched by incubating the slides in peroxidase blocking buffer (DAKO) for 10min. The sections were incubated with normal goat serum (Sigma Aldrich, Saint Louis, USA) for 60 min to prevent non-specific binding. Incubation

with primary rabbit monoclonal anti-eNOS (Clone D8A6N, dilution 1:100, Cell Signalling Technology, Leiden, The Netherlands) and rabbit polyclonal anti-iNOS (dilution 1:600, Abcam, Cambridge, UK) was performed at room temperature for 60 min using the Envision FLEX+ detection kit (DAKO) according to the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated and mounted. Positive controls were included in each staining run and consisted of prostate carcinoma tissue. Scoring was performed by an experienced pathologist. Staining intensity was classified as follows: no staining (0), weak (+), moderate (++) and strong staining (+++). Both eNOS and iNOS were determined in syncytiotrophoblast (ST) and intermediate trophoblast (IT).

Statistical analysis

Statistical analysis was performed using SPSS version 22.0. Data are expressed as mean ± standard deviation (SD). Normality of continuous variables was evaluated using Kolmogorov-Smirnov test. Groups were compared using independent T-test. Chi squared test was used for comparison of categorical variables. A two-tailed $p < 0.05$ was considered significant.

Results

Patient characteristics

Characteristics of the two groups (HP and PE) are summarized in Table 2. Both groups were comparable regarding age, BMI, parity and cigarette abuse (all $p>0.05$). Blood pressure, gestational age at delivery, birthweight and delivery type were significantly different between HP and PE. (Table 2)

Table 2: Patients characteristics

	HP (n=15)	PE (n=15)	p
Elective CS	n=0	n=11 (73%)	**<0.0001**
Vaginal delivery	n=15 (100%)	n=4 (27%)	**<0.0001**
Spontaneous labour	n=8 (53%)	n=0	**<0.0001**
Age (years)	30.4±4.2	29.6±4.1	0.34
Gestation at delivery (weeks)	39.1±1.3	32.1±2.3	**0.001**
BMI 3rd trimester (kg/m2)	28.0±4.1	28.9±4.3	0.89
SBP 3rd trimester (mmHg)	121.9±8.9	148.5±10.2	**<0.0001**
DBP 3rd trimester (mmHg)	73.6±10.8	95.3±8.7	**<0.0001**
MAP 3rd trimester	89.7±9.5	113.0±8.3	**<0.0001**
Nulliparous (n)	n=15 (100%)	n=15 (100%)	0.99
Birthweight (g)	3527±550	1730±470	**0.001**
Smoking (n)	n=0	n=0	na

Data are expressed as mean ± SD or as number of total (n). Not applicable (na). HP, healthy pregnancy; PE, preeclampsia; VD, vaginal delivery; ECS, elective caesarean section; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Nitric oxide concentration in placental tissue

Figure 2 shows a typical •NO absorption spectrum with the double integral plotted on the second •NO peak. Figure 4A demonstrates the mean •NO concentrations in the two groups. After PE, a 0.5 times significantly lower placental •NO concentration was present compared to after HP (p=0.01).

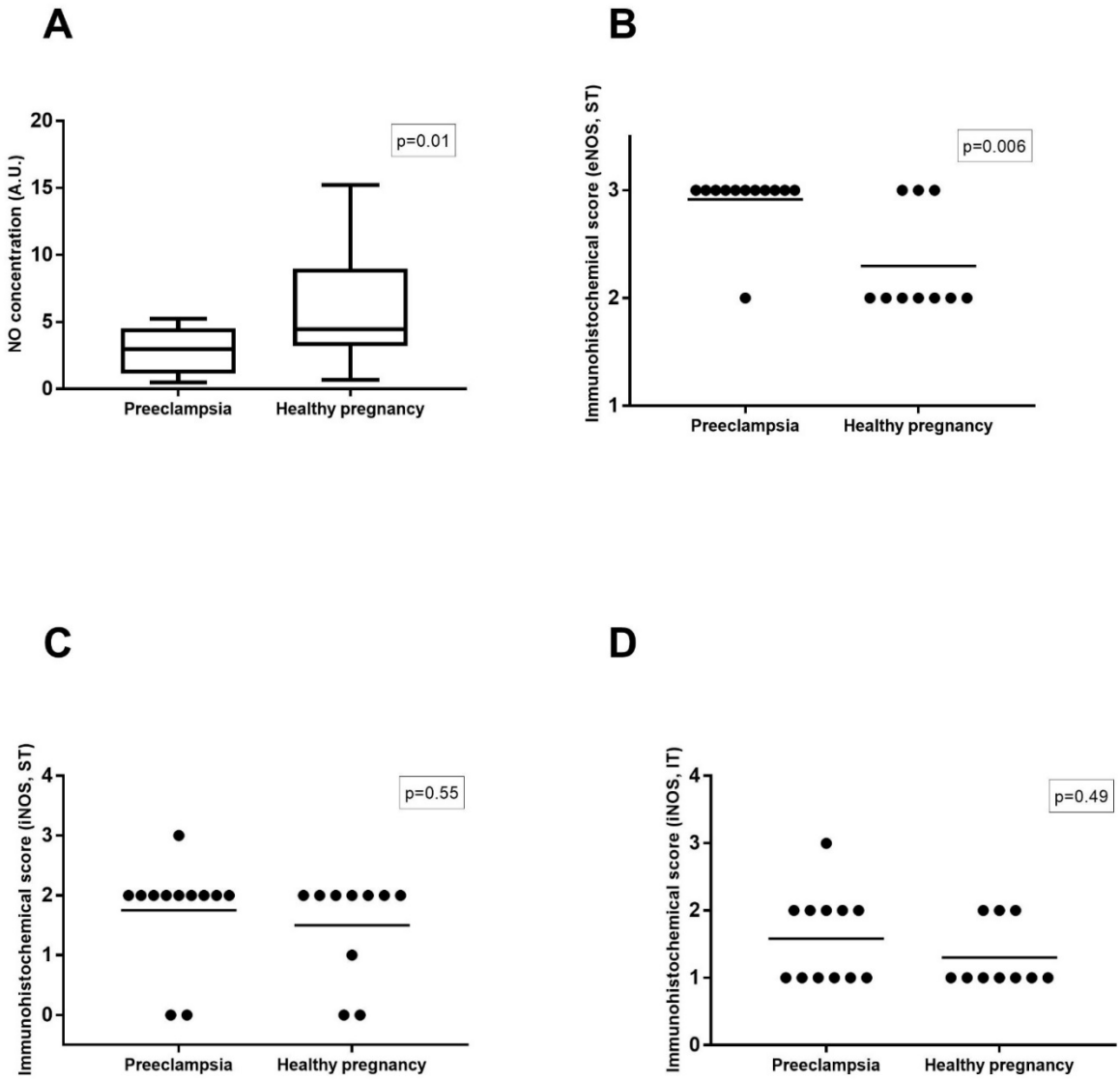

Figure 4: NO, eNOS and iNOS concentration in preeclampsia versus healthy pregnancy.
NO concentration assessed by electron paramagnetic resonance (EPR, Fig 4A), eNOS expression in syncytiotrophoblast (ST) using immunohistochemical staining (IHC, Fig 4B), iNOS expression in syncytiotrophoblast (ST) using immunohistochemical staining (IHC, Fig 4C) and iNOS expression in intermediate trophoblast (IT) using immunohistochemical staining (IHC, Fig 4D). Staining intensity was classified as follows: no staining (0), weak (+), moderate (++) and strong staining (+++).

eNOS/iNOS expression in placental tissue after ECS

Twelve PE placentae and ten HP placentae, all after ECS, were stained for eNOS and iNOS expression. The intensity of eNOS staining in the ST was significantly higher in PE compared to HP (p=0.006). (Figure 4B) eNOS expression could not be determined in IT. iNOS was similarly expressed in PE compared to HP, in both the ST and the IT. (Figure 4C and 4D) Figure 5A and 5B respectively demonstrate 2+ and 3+ staining for eNOS in ST.

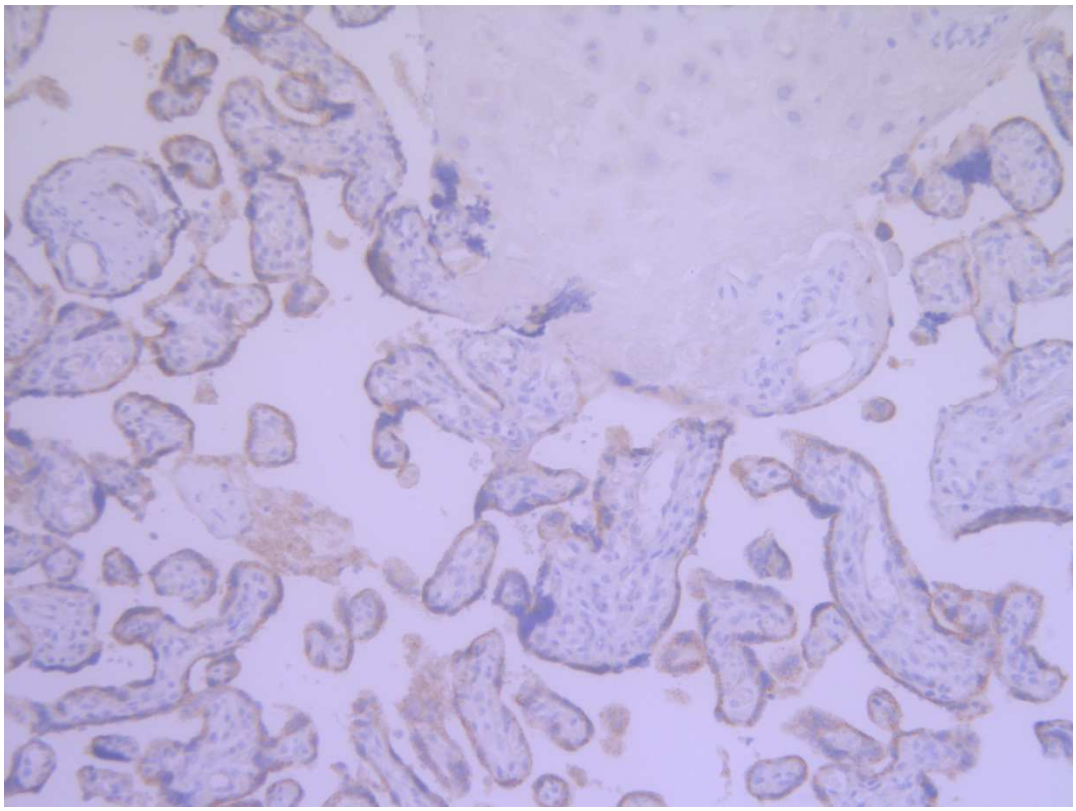

Figure 5A: Placental eNOS expression using immunohistochemical staining: eNOS expression 2+ after healthy pregnancy

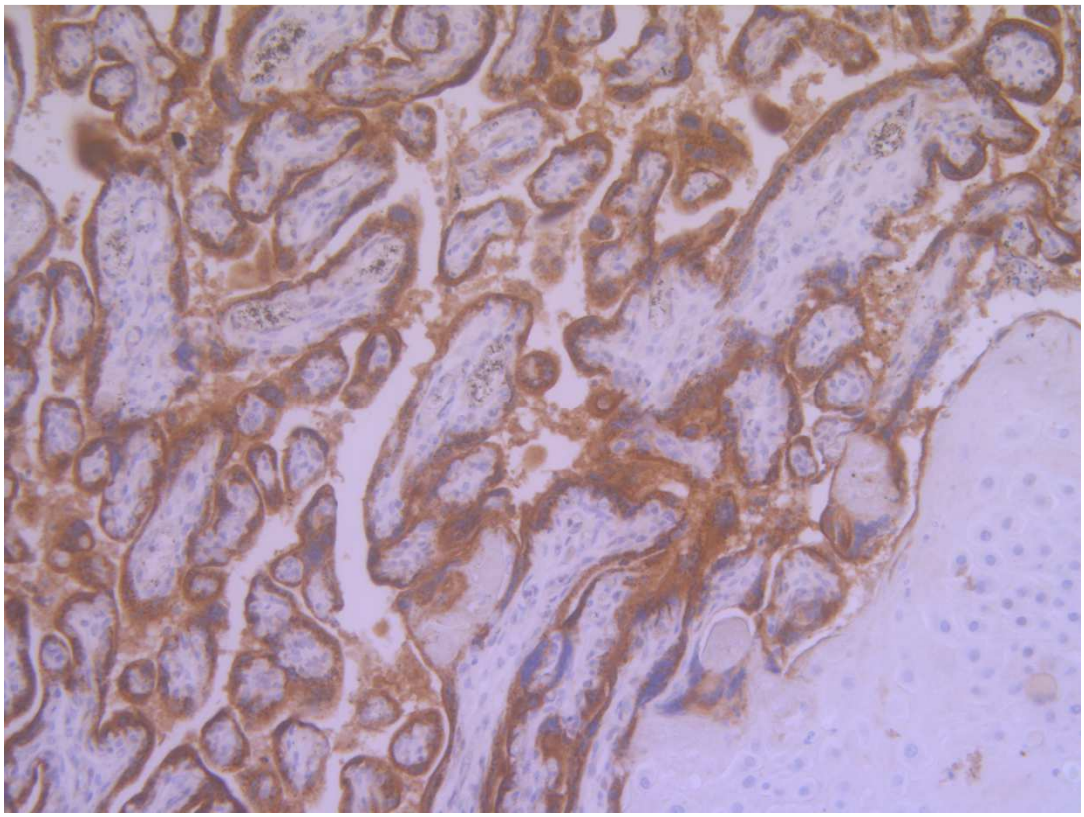

Figure 5B: Placental eNOS expression using immunohistochemical staining: eNOS expression 3+ after preeclampsia.

Discussion

In this study, •NO concentration was determined in placental tissue of patients after PE versus HP. To correlate •NO concentration to the origin of •NO production, eNOS and iNOS expression were assessed in a subgroup of placentae after ECS. Three main findings emerge from this study:

- After a HP, placental •NO concentration is higher compared to after a PE pregnancy.
- Placental eNOS concentration is higher after a PE pregnancy compared to after a HP.
- Placental iNOS expression is not different after PE versus HP.

Our findings suggest a different •NO metabolism in HP compared to PE. As stated before, •NO exerts important functions during the course of pregnancy. It is responsible for endovascular cytotrophoblast invasion in early placentation, regulation of OS production and a decline in •NO is necessary for the initiation of labour [20, 23]. In PE, placentation is deficient and •NO is thought to play a substantial role herein. [33] Aberrant •NO pathways have been described in PE and their relationship with endothelial dysfunction in PE pregnancies has been suggested. [11, 34] However, literature remains inconclusive on the difference in placental and circulating •NO concentration between healthy versus PE pregnancies [11, 35, 36]. Baylis et al. [37] described normal placental levels of •NO and eNOS in PE. Other studies reporting lower placental •NO concentrations in PE [38], postulate that the higher amount of OS in the ischemic placenta causes eNOS uncoupling, production of superoxide and •NO degradation to peroxynitrite (ONOO-), resulting in lower •NO concentrations [13]. ADMA, the endogenous inhibitor of eNOS, has been described to be increased in PE, resulting in lower circulating •NO concentrations [39, 40], however contradictory results exists [41].

In PE, sFlt-1 interferes with NO production by capturing VEGF and PlGF and inhibiting the activity of eNOS. Our results suggest that placental •NO concentration is lower after PE compared to after a HP, while eNOS expression in increased in PE. These at first sight contradictory results can probably be explained by the higher amount of placental hypoxia and OS in PE. Since low placental perfusion, hypoxia and increased blood vessel resistance induce an adaptive activation of eNOS and higher amounts of OS result in eNOS uncoupling, a higher expression of eNOS can be accompanied by lower •NO concentrations. Higher amounts of both

•NO and superoxide, will result in the formation of peroxynitrite, contributing to the lower •NO concentrations. [13] iNOS expression was comparable between both groups, suggesting that iNOS is not involved in the pathophysiology of PE, which is in line with literature. [42]

The discrepancies described for the role of •NO in PE, might be due to the different methodologies applied to measure •NO concentrations. Techniques used in the past range from the Griess reaction, chemiluminescence, gas chromatography-mass spectroscopy and Siever's •NO analyser [3, 43-45]. However, most studies detected the level of nitrate/nitrite (NO3-/ NO2-) from different sources such as plasma, serum, urine, umbilical cord or placenta [3] instead of directly measuring •NO concentrations in placenta tissue. EPR, the method used in this study, is able to directly measure •NO concentrations in combination with the spin trap DETC. EPR is the most direct method to detect free radicals [46]. However, this paper adds to the existing knowledge on the use of EPR that highly sensitive EPR devices are necessary to obtain optimal •NO spectra out of human tissue.

Despite our novel findings, this study has limitations. The first and most important limitation is the difference in delivery type between our two groups. Labour is associated with ischemia-reperfusion damage resulting in OS, causing available •NO to react with formed free radicals. In conditions of high OS, the reaction of superoxide with •NO gives rise to the highly reactive molecule peroxynitrite (ONOO-) and eNOS will undergo uncoupling, both reducing the bioavailability of •NO. [47] Spontaneous labour is known to be associated with lower amounts of •NO. [23] We expect thus •NO concentrations in placentae after labour to be lower compared to elective caesarean section (ECS). In our study population, all placentae after a HP underwent labour and their •NO concentration was still higher compared to the PE group. When we will be able to analyse our placental samples of the ECS HP group, we can further elaborate on this topic. Second, there are large differences in gestational age between the PE and HP population. Since ADMA concentration in known to fluctuate throughout pregnancy [48], both eNOS and •NO concentrations could be dependent on gestational age as well, which might influence our results. It is however difficult to include a healthy preterm ECS group apart from patients with foetal abnormalities. Third, since the idea of determining eNOS/iNOS by immunohistochemistry emerged after the collection of the HP samples, eNOS/iNOS expression was determined in a distinct ECS HP population.

To conclude, our findings suggest a disturbed placental •NO metabolism in PE which might be responsible for the deficient placentation distinctive for PE. Although compensatory eNOS activation is present, eNOS uncoupling and formation of peroxynitrite counteract this mechanism, resulting in lower placental •NO concentrations in PE.

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CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion

Preeclampsia (PE) is a global health issue with significant short and long-term complications. Over the last decade, abundant research has proven that PE is a systemic disease with widespread endothelial damage and an elevated lifelong risk of cardiovascular disease rather than a self-limited occurrence during pregnancy. While the causal factor of PE is still under debate [1], this doctoral thesis enlightens a substantial part of its pathophysiologic steps. In order to understand its pathology, it is of utmost importance to objectify the physiologic processes occurring in healthy pregnancy (HP). We longitudinally evaluated systemic inflammation, oxidative stress (OS) and vascular function in one hundred healthy pregnant women and forty PE women until 6 months post-partum. At delivery, nitric oxide (•NO) metabolism was objectified in placental samples.

As described in the general introduction (Chapter one), it appears that only little is known about the evolution of OS in HP. OS is essential in pregnancy and in contrast to what some might suggest, it not only exerts deleterious effects. The main reason why research concerning this topic is scarce, is the fact that free radicals (such as •NO and superoxide) have a very short half-life and are therefore difficult to accurately measure in the clinical field. The best technique to measure free radicals has been matter of debate, although electron paramagnetic resonance (EPR) as was used in the project, has been proven the most direct method to detect free radicals. [2] The idea that systemic OS and inflammation are responsible for the vascular dysfunction in PE has been fostered by previous research [3]. However, a direct relationship between them has not been objectified previously. The endothelium produces several vasoactive substances, contributing to different aspects of endothelial function. In addition to flow mediated dilatation (FMD), the gold standard for assessing •NO-dependent endothelial function [4], we adopted a novel approach by studying low-flow mediated constriction (L-FMC), which reveals the resting function of the endothelium regulated by endothelin-1 (ET-1). [5] Since prior studies have emphasized the importance of long term cardiovascular observation after PE [6], this study implemented a compound hemodynamic assessment after HP and PE to find a clinically useable and reliable parameter for cardiovascular follow-up. Regarding

placentation, •NO has been appointed to be the essential player. Nevertheless, research concerning exact placental •NO concentrations and its producing enzymes endothelial NO synthase (eNOS) and inducible NOS (iNOS) is lacking. This doctoral thesis aims to fill this gap in knowledge of placental •NO metabolism in HP and PE.

Chapter two describes the methodology used in this project. Regarding EPR, which is proven to be the most direct method to detect free radicals [2], protocols were adopted from previous successful experiments within our research group. [7] Applanation tonometry (AT) and FMD have recently been implemented in the recommendations of vascular function assessment in pregnancy by the International Working Group on Maternal Hemodynamics. [8] PAT (peripheral arterial tonometry) and L-FMC have not yet been validated in pregnancy, yet reflect novel and additional parameters of endothelial function. As stated in the study protocol, PE patients in the ENDOPREG study were not strictly divided into the previously described PE subtypes, namely angiogenic or placental PE (formerly called early onset, before 34 weeks) and non-angiogenic or maternal PE (formerly called late onset, after 34 weeks). [9] These descriptions oversimplify recent existing findings. Maternal risk factors can precede early onset PE as well as abnormal concentrations of placental angiogenic factors are found in late onset PE. [10] Fetal growth restriction and endothelial dysfunction caused by systemic inflammation are usually described in placental PE, nevertheless they are common in late onset PE. It is therefore more accurate to state that both maternal and placental factors contribute to PE and research should focus on classifications based on pathophysiologic processes, for instance endothelial and vascular dysfunction and amount of systemic inflammation and OS. [11-12]

The endothelium is the protagonist of the cardiovascular adaptation in response to the hyperdynamic circulation in pregnancy. It needs to react to the augmented cardiac output and circulating volume by lowering vascular resistance. In HP, increased shear stress provokes vasodilatation. An elevated FMD with a normalisation post-partum, reflects this improved endothelial function in HP. The ability to vasoconstrict to a low-flow stimulus (L-FMC) on the other hand, seems to be a typical aspect of HP since it is absent in the majority of non-pregnant healthy women. A known downside of FMD is its dependence on baseline brachial diameter [5], while in L-FMC this reliance is absent. Chapter three describes that in PE the hemodynamic adaptation to pregnancy is inadequate. In PE, endothelial dysfunction is present, evidenced by

lower brachial FMD and L-FMC values, comparable to the non-pregnant population. Contradictory, microvascular endothelial function (PAT) is increased in PE, suggesting an elevated microvascular vasomotor tone, resulting in a higher vasodilatory reserve during hyperaemia compared to HP. [13] Since there is no correlation between FMD, L-FMC and PAT and taken into account that they thrive on different mediators (•NO, ET-1), these findings indicate that all three parameters reflect different aspects of endothelial function in pregnancy. L-FMC has not been studied previously in a large group of HP and PE women, opening a door to future research on resting endothelial dysfunction and its consequences. Although AT with carotid-femoral pulse wave velocity (CF-PWV) and pulse wave analysis (PWA) are not direct measurements of endothelial function, they represent a reliable marker of arterial stiffness associated with endothelial function. [14, 15] In HP, decreased vascular stiffness is essential in response to the hemodynamic changes. PWA reflects overall vascular stiffness and is already reduced in the first pregnancy trimester, whereas aortic stiffness (CF-PWV) gradually lowers during pregnancy. Both parameters return to pre-pregnancy levels six months after pregnancy. In PE, arterial stiffness (PWA and CF-PWV) is undoubtedly increased, while only aortic stiffness remains increased six months after a HP. Since CF-PWV is predictive for future cardiovascular events [16, 17], this promising parameter might be useful in the long-term follow-up after PE.

We investigated endothelial function in the arterial and capillary system (by FMD and PAT respectively) and related this to peripheral and aortic arterial stiffness. Our findings suggest that endothelial function in PE varies among different vascular beds. However, it needs to be emphasised that only a modest part of total endothelium function has been mapped in this study. Therefore, conclusions on venous, coronary, renovascular and cerebrovascular endothelial function in PE cannot be subtracted. Previous research has described venous dysfunction as a critical factor in severe PE. [18] Nevertheless, literature on venous endothelial function in PE is very scarce. In contrast to the arterial system, where mechanical factors such as shear stress and pulsatile flow induce endothelial dysfunction, venous endothelial function is mainly affected by biochemical factors (•NO). [19] Coronary endothelial dysfunction is expressed by diastolic dysfunction, a condition frequently found during and after PE. [20] Endothelial dysfunction at the glomerulus and the brain, both organs with fenestrated vascular endothelium enabling physiological diffusion of water and solutes, has major clinical implications. Renovascular endothelial dysfunction is characterized by glomerular capillary

endotheliosis, resulting in glomerular enlargement and occlusion of capillary loops. [21] In PE, glomerular filtration rate (GFR) declines and proteinuria is caused by the downregulation of nephrin. When endothelial dysfunction is present at the cerebrovascular system, PE can derail into life-threatening eclampsia.

As described in our comprehensive review on the use of EPR in pregnancy (Chapter four A), this technique encompasses a broad spectrum of applications to determine OS and its consequences. Using EPR, it is possible to, amongst others, determine membrane rigidity, measure antioxidative capacity, or as in this study, directly measure the amount of a single free radical. The physics behind the technique are rather complicated, although clear protocols make it possible for clinicians to directly measure free radical concentration by using EPR.

In the two stage model of PE, the ischemic placenta releases elevated amounts of free radicals into the circulation, thereby affecting the endothelium. Superoxide is the most common oxygen free radical in the human body [22], reflecting the total amount of OS. In order to objectively compare levels of circulating superoxide between HP and PE, the longitudinal course of superoxide concentration in HP was determined (Chapter four B). During HP, circulating superoxide concentrations remain stable, yet are significantly higher compared to the non-pregnant population. This emphasizes again the beneficial and essential role of well-controlled OS in the maintenance of a healthy pregnancy. Iron is a known cofactor in the formation of free radicals and, in HP, superoxide levels were correlated to ferritin concentration. This highlights that iron supplements in pregnancy are only indicated in iron deficiency anaemia, since high iron concentrations result in OS. In PE, a substantially higher concentration of superoxide, systemic inflammation markers and iron were present. Although OS was lower in HP, there was a significant positive correlation between superoxide concentrations and vascular stiffness. Nevertheless, this correlation was absent in PE. These findings suggest that circulating OS affects arterial compliance in a dose-dependent manner, but beyond a certain threshold, this relationship fades out, resulting in overt arterial dysfunction. Superoxide concentration in PE appeared to be correlated to PAT, affecting microvascular endothelial function. During healthy physiologic labour, a known condition of placental ischemia-reperfusion, OS is increased and correlates to the amount of systemic inflammation, represented by neutrophil-lymphocyte ratio (NLR).

NLR and mean platelet volume (MPV) are markers of chronic low grade systemic inflammation. (Chapter five) Low grade inflammation is present in HP, represented by the activation of neutrophils, due to augmented physiologic stress and impaired neutrophilic apoptosis, and the decrease in lymphocytes. [23, 24] In our large retrospective analysis, NLR was confirmed to be higher in PE compared to HP, but only in the third pregnancy trimester, therefore making it unusable for the prediction of PE. MPV on the other hand, a marker of endothelial thrombocyte activation and consumption, was already significantly higher in the first trimester of pregnancies developing in PE later on. This suggest the presence of early endothelial dysfunction in PE. Unfortunately, MPV alone has a low predictive value, but if implemented in combination with others predictive parameters, such as pulsatility index of the uterine artery, familial history and anti-angiogenic factors, it can be valuable in the first trimester prediction of PE.

The combination of endothelial dysfunction, OS and chronic inflammation, has been abundantly recognized as a precursor of cardiovascular disease. [25] Endothelial dysfunction plays an important role in the initiation of atherosclerosis and its presence and severity are directly related to negative outcome in heart failure, ischemic heart disease and chronic kidney disease. [26-28] In heart failure with preserved ejection fraction (HFpEF), a pathophysiologic mechanism very comparable to PE has been described, as circulating inflammatory factors and ROS activate the systemic and coronary endothelium, leading to a vicious circle of worsening endothelial function and progressive heart failure. [28] Findings from cardiac and nephrological research on endothelial function and vascular stiffness enlighten similar pathways as in PE, confirming the important cardiovascular background of this previously falsely assumed specific pregnancy-related disorder.

In the final part of this thesis (Chapter six), the placental metabolism of •NO was explored. •NO is not only the major player in endothelial function, it is also responsible for optimal endovascular trophoblast invasion of the spiral arteries in the first trimester of pregnancy. In placental tissue, •NO is synthesized out of L-arginine by eNOS and iNOS. It is interesting to note that although lower •NO concentrations were found in placental tissue of PE patients, eNOS was undoubtedly more expressed in PE. Soluble fms-like tyrosine kinase-1 (sFlt-1), the soluble receptor for vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)

produced by the placenta, is highly expressed in PE and is an indirect deactivator of eNOS. [21, 29] Moreover, in conditions of high OS, eNOS undergoes uncoupling, resulting in the production of superoxide besides •NO. [14] Although we were able to successfully determine •NO concentrations using EPR, it has to be emphasized that sensitive EPR devices are needed to measure •NO with an iron-DETC spin-trap (Fe (II) DETC2 (iron (II)diethyldithiocarbamate)), considering the low •NO concentrations in human tissue. The encountered difficulties in the •NO measurements gave us the unique possibility to objectively compare different EPR devices.

To conclude, this thesis contributes to the knowledge of the pathophysiology behind the multisystem pregnancy complication, preeclampsia. In PE, augmented OS, systemic inflammation and vascular dysfunction are obviously linked and capable of forming dangerous positive feed-forward systems. Concerning the well discussed question whether disturbed vascular function is caused by systemic inflammation and OS caused by the ischemic placenta, or whether an underlying cardiovascular impairment is the first step in the pathophysiology of PE, our findings suggest that it is probably a combination of both. We suggest that a pre-existing fragile endothelial situation leads to defective placentation, and results in exacerbated and generalised endothelial dysfunction. This study was able to prove a direct relationship between circulating OS and vascular dysfunction. Furthermore, a circulating endothelial dysfunction parameter (MPV) was demonstrated in the first trimester of PE pregnancies. The obtained substantial knowledge on the evolution of OS and vascular function in healthy and PE pregnancies, will hopefully influence and inspire future research in order to develop new screening and treatment strategies for PE.

Future perspectives

Many suggestions for the design and focus of future research have arisen from this doctoral thesis:

Since cardiovascular adaptations seem to start as early as the first weeks of pregnancy, a large prospective study starting in the preconception period would be able to compare cardiovascular adaption between healthy and PE pregnancy.

Since discussion remains on the predictive effect of antioxidants (ascorbic acid, α-tocopherol) and •NO precursors (L-arginine), their direct effects on vascular function should be evaluated.

The clinical value of CF-PWV as predictor of long term cardiovascular disease after PE should be evaluated.

Considering the direct relation between superoxide concentrations and vascular stiffness, and the clinical feasible use of EPR, the added value of superoxide in the prediction of PE should be examined.

Our novel findings on the presence of L-FMC in HP and its shortcoming in PE pregnancies opens the door for future research concerning responsible mediators (ET-1) and mechanisms behind this endothelial function parameter.

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CHAPTER EIGHT

SUMMARY

SAMENVATTING

SUMMARY

Preeclampsia (PE) is a global health issue with significant short and long term health risks. Over the last decade, abundant research has proven that PE is a systemic disease with widespread endothelial damage and high incidence of future cardiovascular disease rather than a self-limited occurrence during pregnancy. While the causal factor of PE is still under debate, this doctoral PhD thesis enlightens a substantial part of its pathophysiologic steps. To understand the pathologic mechanisms behind PE, it is of utmost importance to objectify the physiologic processes occurring in healthy pregnancy (HP). We evaluated systemic inflammation, oxidative stress (OS) and vascular function longitudinally in one hundred healthy pregnant women and forty PE women until 6 months post-partum. At delivery, nitric oxide (•NO) metabolism was objectified in placental samples.

The best technique to measure free radicals has been matter of debate, however electron paramagnetic resonance (EPR) used in this project, has been proven the most direct method to detect free radicals. That systemic OS and inflammation are responsible for the vascular dysfunction present in PE has been fostered by previous research, however a direct relationship between them has not been objectified previously. In this thesis, a direct correlation was proven between the amount of OS and vascular stiffness in HP. Nevertheless, this correlation was absent in PE. These findings suggest that circulating OS affects arterial compliance in a dose-dependent manner, but if a certain threshold has passed, this relationship fades out resulting in overt arterial dysfunction. Moreover, superoxide concentration in PE appeared to be correlated to microvascular endothelial function. The endothelium produces several vasoactive substances, contributing to different aspects of endothelial function. In addition to flow mediated dilatation (FMD), the gold standard for assessing •NO-dependent endothelial function, we adopted a novel approach by studying low-flow mediated constriction (L-FMC) which reveals the resting-function of the endothelium. Increased shear stress provokes vasodilatation (FMD) which is augmented during HP with a normalisation post-partum, reflecting an improved endothelial function in HP. Vasoconstriction to a low-flow stimulus (L-FMC), on the other hand, seems to be a typical aspect of HP since it is absent in the majority of non-pregnant healthy women. We describe that this hemodynamic adaptation to pregnancy is inadequate in PE as reflected by lower brachial FMD and L-FMC values compared to HP. In PE, endothelial dysfunction is present, evidenced by lower brachial FMD and L-FMC values,

comparable to the non-pregnant population. L-FMC has not been studied previously in a large group of HP and PE women, opening a door to future research on resting endothelial dysfunction and its consequences. •NO not only is the major player in endothelial function, it is responsible for optimal endovascular trophoblast invasion of the spiral arteries in the first trimester of pregnancy. In placental tissue, •NO is synthesized out of L-arginine by eNOS and iNOS. It is interesting to note that although lower •NO concentrations were found in placental tissue of PE patients, eNOS was undoubtedly more expressed in PE. Soluble fms-like tyrosine kinase-1 (sFlt-1), the soluble receptor for vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) is highly expressed in PE and is an indirect deactivator of eNOS. Moreover, in conditions of high OS, eNOS undergoes uncoupling, resulting in the production of superoxide besides •NO.

NLR (neutrophil-leucocyte ratio) and mean platelet volume (MPV) are markers of chronic low grade systemic inflammation. In our large retrospective analysis, NLR was confirmed to be higher in PE compared to HP, but only at the third pregnancy trimester, which makes it unusable for the prediction of PE. MPV on the other hand, a marker of thrombocyte activation and consumption by the endothelium, was already significantly higher in the first trimester of pregnancies developing in PE later on. This suggest the presence of early endothelial dysfunction in PE. If implemented in combination with others predictive parameters, MPV can be of additive value in the first trimester prediction of PE.

To conclude, this thesis contributes to the knowledge of the pathophysiology behind the multisystem pregnancy complication, preeclampsia. In PE, augmented OS, systemic inflammation and vascular dysfunction are obviously linked and capable of forming dangerous positive feed-forward systems. Concerning the well discussed question whether disturbed vascular function is caused by systemic inflammation and OS released by the ischemic placenta, or whether an underlying cardiovascular impairment is the first step in pathophysiology of PE, our findings suggest that it is probably a combination of both. We suggest that pre-existing fragile endothelial situation leads to defective placentation, and results in exacerbated and generalised endothelial dysfunction. This study was able to prove a direct relationship between circulating OS and vascular dysfunction besides demonstrating a circulating endothelial dysfunction parameter (MPV) in the first trimester of PE pregnancies. The obtained substantial

knowledge on the evolution of OS and vascular function in healthy and PE pregnancies, will hopefully influence and inspire further research and the development of screening and treatment strategies for and after PE.

SAMENVATTING

Preeclampsie (PE) is een wereldwijd problematisch met belangrijke gezondheidsrisico's op zowel korte als lange termijn. Uitvoerig onderzoek tijdens het voorbije decennium heeft aangetoond dat PE een systeemziekte is met uitgebreide endotheelschade en een verhoogd risico op latere cardiovasculaire ziekte. Dit in tegenstelling tot het eerdere geloof dat PE enkel tijdens de zwangerschap problemen geeft. Hoewel het exacte ontstaansmechanisme van PE grotendeels onbekend blijft, slaagt deze doctoraatsthesis er in om een aanzienlijk deel van zijn pathofysiologisch proces op te helderen. Om het complexe proces van PE te begrijpen, is het essentieel om eerst de fysiologie van een gezonde zwangerschap volledig en nauwgezet in kaart te brengen. Hiervoor hebben we systemische inflammatie, oxidatieve stress (OS) en vasculaire functie longitudinaal gemeten in honderd gezonde zwangere vrouwen en veertig PE patiënten, en hebben dit opgevolgd tot zes maanden postpartum. Tijdens de bevalling werd tevens het stikstofmonoxide (•NO) metabolisme in placentair weefsel bepaald.

In deze thesis werd elektron paramagnetische resonantie (EPR) gebruikt om de concentratie aan vrije radicalen te meten. EPR is hier de meest directe methode voor. Voorafgaand onderzoek heeft aangetoond dat systemische OS en inflammatie verantwoordelijk zijn voor de vasculaire dysfunctie in PE, maar een directe verband tussen hen werd nog nooit aangetoond. Deze thesis bewijst dat er inderdaad een directe correlatie is tussen de hoeveelheid aan OS en arteriële stijfheid in een gezonde zwangerschap. Deze correlatie was echter afwezig in PE. Deze bevinding suggereert dat circulerende OS producten de arteriële compliantie beïnvloeden in een dosisafhankelijke wijze tot een bepaalde drempelwaarde, waarna deze relatie verdwijnt en hoge OS resulteert in diffuse arteriële stijfheid. Bovendien bleek de concentratie aan superoxide gecorreleerd aan microvasculaire endotheeldysfunctie in PE. Het endotheel produceert tal van vasoactieve producten, die allen bijdragen aan de verschillende aspecten van endotheelfunctie. In aanvulling op flow gemedieerde dilatatie (FMD), de gouden standaard voor het beoordelen van •NO-afhankelijke endotheelfunctie, werd in deze thesis een nieuwe parameter bestudeerd, namelijk lage-flow gemedieerde constrictie (L-FMC), dewelke de rustfunctie van het endotheel weergeeft. Flow gemedieerde vasodilatatie neemt toe tijdens de gezonde zwangerschap met een normalisatie postpartum, hetgeen een verbeterde endotheelfunctie in de zwangerschap aantoont. L-FMC blijkt evenzeer een typisch aspect van

een adequate adaptatie tijdens een gezonde zwangerschap te weerspiegelen, aangezien vasoconstrictie tijdens occlusie afwezig is in de grote meerderheid van de niet-zwangere vrouwen doch aanwezig is in meer dan tachtig procent van de zwangere vrouwen in het derde zwangerschapstrimester. In PE blijkt deze hemodynamische aanpassing te ontbreken, aangetoond door lagere FMD en L-FMC waarden in vergelijking tot de gezonde zwangerschap. Dit bewijst opnieuw de veralgemeende endotheeldysfunctie in PE. De nieuwe parameter L-FMC werd voorheen nooit longitudinaal onderzocht in de gezonde zwangerschap en er zijn ook geen data beschikbaar over L-FMC bij PE. Deze bevinding opent deuren naar verder onderzoek omtrent endotheelfunctie in rust en het belang hiervan.

•NO is niet enkel de hoofdrolspeler in endotheelfunctie, •NO is ook verantwoordelijk voor een optimale trofoblastinvasie van de spiraalarteriën in het eerste zwangerschapstrimester. Ter hoogte van de placenta wordt •NO aangemaakt uit L-arginine door eNOS (endotheliaal •NO synthase) en iNOS (induceerbaar •NO synthase). Het is interessant dat lagere •NO concentraties aanwezig waren in placenta's van PE patiënten, terwijl in dit placentaweefsel eNOS duidelijk meer tot expressie kwam. sFlt-1 (oplosbaar fms-like tyrosine kinase-1), de oplosbare receptor voor vasculaire endotheliale groeifactor (VEGF) en voor placentaire groeifactor (PlGF) komt verhoogd tot expressie bij PE en is een indirecte deactivator van eNOS. Bovendien ondergaat eNOS in condities van hoge OS een fenomeen genaamd 'ontkoppeling', hetgeen resulteert in de productie van superoxide naast •NO productie.

NLR (neutrofiel-leukocyt ratio) en MPV (gemiddeld trombocyt volume) zijn beide markers van chronische laaggradige systemische inflammatie. In onze grote retrospectieve analyse was NLR duidelijk hoger in PE in vergelijking tot de normale zwangerschap, maar dit enkel tijdens het derde zwangerschapstrimester. Dit maakt NLR bijgevolg niet geschikt voor de predictie van PE. Dit in tegenstelling tot MPV, een marker voor trombocytactivatie en -verbruik door het endotheel, dewelke reeds significant verhoogd was in het eerste trimester van zwangerschappen die later PE ontwikkelden. Deze bevindingen zijn suggestief voor het vroegtijdig optreden van endotheeldysfunctie in PE. MPV kan dus, in combinatie met andere predictieve parameters, geïmplementeerd worden in de eerste trimester screening voor PE.

Concluderend draagt deze doctoraatsthesis bij tot de kennis omtrent de complexe pathofysiologie achter de ernstige multisystemische zwangerschapscomplicatie preeclampsie. In PE zijn toegenomen OS, systemische inflammatie en vasculaire dysfunctie duidelijk onderling gelinkt en zijn zij in staat tot het vormen van gevaarlijke positieve feed-forward systemen. Aangaande het debat of gestoorde vasculaire functie in PE veroorzaakt wordt door systemische inflammatie en OS geproduceerd door de ischemische placenta, of indien een onderliggend cardiovasculair lijden de eerste stap is in de pathofysiologie van PE, duiden onze bevindingen erop dat het wellicht een combinatie is van beiden. We suggereren dat een preëxistente fragiele endotheliale situatie leidt tot een gestoorde placentatie, hetgeen op zijn beurt resulteert in gegeneraliseerde endotheeldysfunctie. Deze studie heeft een directe relatie tussen circulerende OS producten en vasculaire dysfunctie bewezen, en daarenboven een nieuwe biomarker van endotheeldysfunctie in het eerste trimester van PE zwangerschappen aangetoond. Wij hopen dat de aanzienlijke kennis die met dit doctoraat bekomen werd omtrent de evolutie van OS en vasculaire functie in gezonde en PE zwangerschappen, toekomstig onderzoek zal inspireren en de ontwikkeling van screening en behandelingsstrategieën voor en na PE zal beïnvloeden.

List of abbreviations

•NO	Nitric oxide
•OH	Hydroxyl radical
16-NS	16(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic acid
2-OH-E	2-hydroxy-estradiol
5-NS	5(N-oxyl-4,4'-dimethyloxazolidin-2-yl) stearic acid
AAPH	2,2-azobis(2-amidinopropane)dihydrochloride
ACOG	American Congress of Obstetricians and Gynecologists
ADMA	Asymmetric dimethylarginine
AFR	Ascorbyl free radical
AIx	Augmentation index
AIx-75	AIx standardized to a heart rate of 75 beats per minute
AO	Antioxidant
AOC	Antioxidant capacity
AP	Augmentation pressure
apoTF	Apotransferrin
AT	Applanation tonometry
ATP	Adenosine triphosphate
AUC	Area under the curve
B-DNIC	Binuclear dinitrosyl iron complexes
BMI	Body mass index
ButOOH	tert-Butyl hydroperoxide
CBC	Complete blood count
CF-PWV	Carotid-femoral PWV
CM	3-methoxycarbonyl-proxyl nitroxide
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
CP	Ceruloplasmin
Cu	Copper
CYMPO	5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline N-oxide
DBP	Diastolic blood pressure
DETC	Iron (II)diethyldithiocarbamate

DIC	Diffuse intravascular coagulation
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMSO	Dimethyl sulfoxide
DS	Deoxyl stearates
DTPA	Diethylenetriamine pentaacetic acid chelate
e.g.	Exempli gratia
ECG	Electrocardiogram
ECS	Elective caesarean section(s)
EDTA	Ethylenediaminetetraacetic acid
EF	Endothelial function
eNOS	Endothelial NO synthase
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
eSREA	Superoxide radical eliminating ability
ET-1	Endothelin-1
etc.	Et cetera
Fe	Iron
FeS	Iron sulfide
FeSNO	Adrenodoxin FeS-centres
FFA	Free fatty acids
FFPE	Formalin fixed-paraffin embedded
Flt-1	Fms-like tyrosine kinase 1
FMD	Flow mediated dilatation
FMS	Feline McDonough sarcoma
GFR	Glomerular filtration rate
GPA	Gravidity, parity and abortion
GPx/GSH	Glutathione peroxidase in the presence of glutathione
H2O2	Hydrogen peroxide
HbNO	Nitrosyl hemoglobin
HELLP	Haemolysis, elevated liver enzymes and low platelets
HFpEF	Heart failure with preserved ejection fraction
HIER	Heat-induced antigen retrieval

HP	Healthy pregnancy / Healthy pregnancies / Healthy pregnant women
hSA	Human serum albumin
I/R	Ischemia-reperfusion
IHC	Immunohistochemical
iNOS	Inducible NOS
ISSHP	International Society for the Study of Hypertension in Pregnancy
IT	Intermediate trophoblast
IUGR	Intra-uterine growth restriction
KDR	Kinase insert domain receptor
L-FMC	Low-flow mediated constriction
LMWH	Low molecular weight heparin
LOO•	Lipoperoxyl radicals
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MAP	Mean arterial pressure
MDA	Malondialdehyde
mFMD	Modified FMD
MgSO4	Magnesium sulphate
MPO	Myeloperoxidase
MPV	Mean platelet volume
MSL	N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide
N2O3	Dinitrogen trioxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NLR	Neutrophil to lymphocyte ratio
NMR	Nuclear magnetic resonance
nNOS	Neuronal NOS
NP	Non-pregnant / Non-pregnant women
O2•-	Superoxide
OH-	Hydroxide
ONOO-	Peroxynitrite
ORAX	Oxygen radical absorbance capacity
OS	Oxidative stress

PAT	Peripheral arterial tonometry
PBN	Alpha-phenyl-tert butylnitron
PCS	Primary caesarean section
PE	Preeclampsia
PGI2	Prostacyclin
PI	Pulsatility index
PlGF	Placental growth factor
PLR	Platelet to lymphocyte ratio
PP	Postpartum
PPROM	Preterm premature rupture of membranes
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RHI	Reactive hyperaemia index
RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
sEng	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase 1
SOD	Superoxide dismutase
ST	Syncytiothrophoblast
TF	Transferrin
Th	T-helper
TxA2	Thromboxane A2
UA	Uterine artery
UmA	Umbilical artery
UmV	Umbilical vein
VD	Vaginal delivery
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor 1

VEGFR-2	Vascular endothelial growth factor receptor 2
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

Curriculum vitae

General information

Surname:	Mannaerts
Name:	Dominique Anne Marine
Date of birth:	January 6th, 1988
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Education

Secondary school

Humanities Degree in Latin and Greek, Vita et Pax college, Schoten (Antwerp), 2001.

Humanities Degree in Mathematics and Sciences, St Michielscollege, Schoten (Antwerp), 2005.

Study of Medicine

Academic Degree of Bachelor in Medicine, Antwerp University, 2008, cum laude (73%).

Academic Degree of Master in Medicine, Antwerp University, 2012, magna cum laude (83%).

MRCOG

MRCOG part one exam (2015), Royal college of Obstetrics & Gynaecology, London.

MRCOG part two exam (2017), Royal college of Obstetrics & Gynaecology, London.

2012-2018 Specialization in Obstetrics & Gynaecology

2012-2013: Full-time registrar at the Isala Klinieken, Zwolle, The Netherlands.

2013-2014: Full-time registrar at the University Hospital Antwerp, Belgium.

2014-2018:

- Half-time registrar at the University Hospital Antwerp
- Half-time PhD student at the University of Antwerp

Scientific career

Theses

Master thesis	"Intravenous lidocaine in the treatment of chronic neuropathic pain." Promotor: Prof. Dr. Guy Hans
PhD thesis	"Oxidative stress and endothelial function in normal pregnancy and preeclampsia." Promotors: Prof. Dr. Yves Jacquemyn & Prof. Dr. Marc Spaanderman Copromotors: Prof. Dr. Emeline Van Craenenbroeck, Prof. Dr. Wilfried Gyselaers, Prof. Dr. Paul Cos, Prof. Dr. Jerome Cornette, Dr. Ing. Jacob Briedé

Publications

Mannaerts D, Faes E, Gielis J, Van Craenenbroeck E, Cos P, Spaanderman M, Gyselaers W, Cornette J, Jacquemyn Y.

Oxidative stress and endothelial function in normal pregnancy versus pre-eclampsia, a combined longitudinal and case control study.

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Flow-mediated dilation and peripheral arterial tonometry are disturbed in preeclampsia and reflect different aspects of endothelial function.

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Mortelmans D, Mannaerts D, Van den Broeck S, Jacquemyn Y, Hubens G.
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Use of ticagrelor in human pregnancy, the first experience.
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Ramaekers P, Mannaerts D, and Jacquemyn Y.
Re: Prenatal detection of congenital heart disease--results of a national screening programme.
BJOG, 2015. 122(10): p. 1420-1.

Mannaerts D, Muys J, Blaumeiser B, Jacquemyn Y.
A rare cause of primary amenorrhoea, the XY female with gonadal dysgenesis.
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Mannaerts D, Muys J, Ramaekers P, Jacquemyn Y.
Relapsing fetal bilateral hydrothorax, an isolated expression of a vein of Galen aneurysmal malformation.
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Mannaerts D, Vercruyssen J, Kinget K, Duwel V, Buytaert G.

Malignant Brenner tumor stage pT1a in a 52 year old female.

Gunaikeia 17(2): 27-29., 2012.

Presentations

Oral presentations

Mannaerts D, Faes E, Cos P, Briedé JJ, Gyselaers W, Cornette J, Gorbanev Y, Bogaerts A, Spaanderman M, Van Craenenbroeck E, Jacquemyn Y.

Oxidative stress in healthy pregnancy and preeclampsia is linked to chronic inflammation, iron status and vascular function.

45th Annual meeting of the Fetal and Neonatal Physiological Society, 24-27/6/2018, Maastricht, The Netherlands.

Mannaerts D, Faes E, Cornette J, Gyselaers W, Spaanderman M, Van Craenenbroeck E, Jacquemyn.

Low-flow mediated constriction reveals maternal endothelial dysfunction in preeclampsia.

3rd International Congress on Maternal Hemodynamics, 12-14/4/2018, Cambridge, UK.

Mannaerts D, Faes E, Van Craenenbroeck E, De Bruyn C, Gyselaers W, Spaanderman M , Jacquemyn Y .

Flow mediated dilation and peripheral arterial tonometry are disturbed in pre-eclampsia and reflect different aspects of endothelial function.

27th World Congress on Ultrasound in Obstetrics and Gynecology, 15-19/9/2017, Vienna, Austria.

Mannaerts D, Faes E, Briede JJ, Van Craenenbroeck E, Spaanderman M , Jacquemyn Y .

Determination of nitric oxide concentration in placental tissue of pre-eclamptic patients by direct detection with Electron Paramagnetic Resonance (EPR).

25th Benelux EPR Society Meeting, 1/6/2017, Leiden, The Netherlands.

Mannaerts D, Faes E, Gielis J, Briedé J, Cos P, Van Craenenbroeck E, Gyselaers W, Cornette J, Spaanderman M, Jacquemyn Y.

Oxidative stress in maternal serum as endothelial dysfunction marker in pre-eclampsia, an Electron Paramagnetic Resonance (EPR) pilot study.

Second International Congress on Maternal Hemodynamics, 12-14/5/2016, Rome, Italy.

Mannaerts D, Gielis J, Cos P, Van Schil P, Jacquemyn Y.

Determination of placental nitric oxide concentration in pregnancy complications, an Electron Paramagnetic Resonance (EPR) pilot study.

12th World Congress of Perinatal Medicine, 3-5/11/2015, Madrid, Spain.

Poster presentations

Mannaerts D, Faes E, Cos P, Briedé JJ, Gyselaers W, Cornette J, Gorbanev Y, Bogaerts A, Spaanderman M, Van Craenenbroeck E, Jacquemyn Y.

Oxidative stress in healthy pregnancy and preeclampsia is linked to chronic inflammation, iron status and vascular function.

22nd International Conference on Prenatal Diagnosis and Therapy, 8-11/7/2018, Antwerp, Belgium

Mannaerts D, Gielis J, Van den Eeden L, Cos P, Van Schil P, Jacquemyn Y.

Determination of nitric oxide concentration in placental tissue by direct detection with Electron Paramagnetic Resonance (EPR); a work in progress.

14th World Congress in Fetal Medicine, 21-25/6/2015, Crete, Greece.

Awards

1st Prize Best Oral Presentation

Mannaerts D, Faes E, Cornette J, Gyselaers W, Spaanderman M, Van Craenenbroeck E, Jacquemyn.

Low-flow mediated constriction reveals maternal endothelial dysfunction in preeclampsia.

3rd International Congress on Maternal Hemodynamics, 12-14/4/2018, Cambridge, UK.

Educational activities

Co-supervisor master thesis of master in Medicine

De Cordt C and Heyvaert S

Subject: Neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and mean platelet volume (MPV) as predictive parameters for preeclampsia.

Period: 2014-2018.

Dankwoord

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Professor Cos, bedankt voor uw laboratorium op S7 open te stellen, door u is het oxidatieve stress luik opgenomen in mijn onderzoek. Dokter Cornette, bedankt voor het idee om een cardiologische invalshoek te implementeren in een gynaecologisch doctoraat.

Liefste Ellen, beste collega, geweldige vriendin. Jou kan ik niet genoeg bedanken! Bedankt voor alle hulp bij stalen afnemen, EPR metingen, vaatmetingen en vooral bedankt voor het overnemen van mijn onderzoek toen ik op bevallingsverlof was. Jouw altruïsme is onvoorwaardelijk en zonder onze gezellige ASTARC-momenten, sushi-dates, Maastricht-tripjes en congres-weekends waren de voorbije 4 jaar een stuk minder leuk geweest. Bedankt!

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