Early-Onset Preeclampsia

Constitutional Factors and Consequences for Future Pregnancy Outcome and Cardiovascular Health

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Early-Onset Preeclampsia

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Vroege Pre-eclampsie

Constitutionele Factoren en Gevolgen voor Toekomstige Zwangerschapsuitkomst en Cardiovasculaire Gezondheid

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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Bas Boudewijn van Rijn

geboren op 23 mei 1977 te Rotterdam

Promotor:	Prof.dr. H.W. Bruinse
Co-promotoren:	Dr. A. Franx
	Dr. H.A.M. Voorbij
	Dr. M. Roest

"... Dr. Rumack: 'You'd better tell the Captain
we've got to land as soon as we can.
This woman has to be gotten to a hospital.'
Stewardess: 'A hospital? What is it?'
Dr. Rumack: 'It's a big building with patients,
but that's not important right now' ..."
– Airplane ! 1980

Contents

1

Introduction

Introduction

Early-onset preeclampsia is a severe complication of pregnancy, commonly defined as *de novo* hypertension and proteinuria before 34 weeks gestational age [1,2]. It is characterized by marked vascular, metabolic and inflammatory changes leading to generalized endothelial dysfunction and end-organ damage due to vascular compromise [3]. Early-onset preeclampsia is a potentially life-threatening disease for both mother and baby [2,4]. Current figures of the World Health Organization estimate that about 1.4 million women are affected by the preeclampsia each year, causing an annual mortality of 65,000 women worldwide [5]. Although extensive efforts have been aimed at unravelling its pathogenesis, the etiology of preeclampsia remains to be elucidated. Several theories have emerged and numerous hypotheses have been postulated [2,3,6,7]. Most likely, preeclampsia is a result of an interplay between maternal constitution, placental factors and inappropriate adaptive changes to pregnancy predominantly involving the cardiovascular and inflammatory system (**Figure 1**) [3,8-10]. Many pathophysiological hallmarks of preeclampsia, however, are not unique to the disease and are also commonly observed in other cardiovascular and inflammatory disorders and in normal pregnancy. Also, in the past two decades, data from large-scale epidemiological studies have shown an association between hypertensive disorders of pregnancy and excess maternal mortality due to coronary heart disease and stroke, consistent with a high risk of developing premature cardiovascular disease after early-onset preeclampsia [11]. These data support the hypothesis that preeclampsia is to be considered as an inappropriate vascular and inflammatory response to the adaptive challenges of pregnancy. Furthermore, women who develop early-onset preeclampsia might exhibit a shared predisposition for pregnancy-related vascular problems and future development of cardiovascular disease.

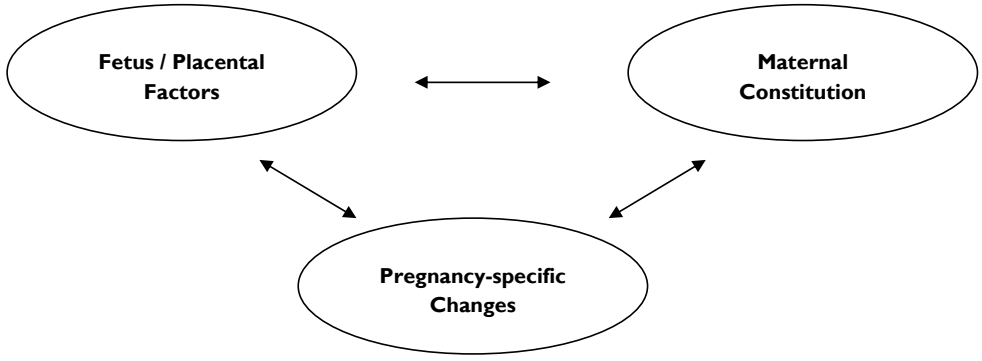


Figure 1 Proposed model for the pathogenesis of early-onset preeclampsia

The following paragraphs of this chapter will outline current thinking on the pathogenesis of early-onset preeclampsia. Next, the concept of the *cardiovascular challenge of pregnancy* will be discussed and its implications for future reproductive outcome and maternal health after early-onset preeclampsia.

1 Pathogenesis

1.1 Endothelial activation

The endothelium is a monolayer of cells lining all blood vessels, that are considered to play a central role in the regulation of vascular homeostasis [12]. Healthy endothelial cells are able to respond to a wide range of factors that regulate vascular tone and permeability, cellular adhesion, smooth muscle cell proliferation, inflammation and coagulation.

Endothelial function is critical for controlling blood pressure and maintaining optimal organ perfusion in response to changes in cardiac output. As first demonstrated by Furchgott and Zawadski and awarded with the Nobel Prize in 1998, this regulatory function of the endothelium mainly depends on its production of endothelium-derived relaxing factor, that was subsequently shown to be nitric oxide [13,14]. Nitric oxide (NO) is synthesized from L-arginine by endothelial nitric oxide synthase (eNOS) in the presence of co-factors such as tetrahydrobiopterin [15]. Upon synthesis, NO induces cGMP-mediated vasodilatation and interacts with other vasomotor substances, e.g. prostacyclin, endothelin, prostanoids, angiotensin I and II in the local regulation of vascular tone and structure [12]. In normal physiological conditions, NO plays a key role to maintain the endothelium in a quiescent state. However, various disturbances in NO-mediated silencing of cellular processes within the endothelium, cause the endothelial cells to become activated. Endothelial activation represents a switch from a quiescent state towards a phenotype that is thought to involve a host defence response [12,16]. Activated endothelial cells express a wide range of chemokines, cytokines, and adhesion molecules capable of signalling leukocytes, platelets and binding various soluble coagulation factors [16]. In contrast to previous thinking of the endothelium as more or less static system, recent evidence has shown a remarkable regenerative capacity of the endothelial lining, dependent on a circulating pool of endothelial progenitor cells (EPCs) [17]. Prolonged endothelial activation can give rise to endothelial injury that leads to detachment of degenerated endothelial cells and subsequent repair by EPCs. Circulating EPCs are a promising marker of endothelial injury, that was recently also evaluated in pregnancy and preeclampsia [18,19].

Normal pregnancy is characterized by endothelial activation[20,21], which has previously been demonstrated by elevated levels of numerous markers association with activated

endothelium, such as von Willebrand factor [22,23], soluble adhesion molecules [24], end products of oxidative stress[25] and inflammation [9]. In comparison to normal pregnancy, preeclampsia is associated with excessive endothelial activation and signs of endothelial injury, to an extent that is comparable to atheroscerosis, sepsis or end-stage renal disease [8,16,21]. Although some controversy exists over its extent and distribution [26], the maternal syndrome of preeclampsia is characterized by generalized endothelial activation, that is believed to underlie most clinical symptoms and severe adverse complications of the disease, due to underperfusion of multiple organs including the kidney, brain, liver and the placenta [27]. The precise mechanism driving this state of endothelial disturbance is unknown. Many plausible candidate factors have been named, including inflammatory molecules [9], anti-angiogenic factors [28], components of the metabolic syndrome [29], syncytiotrophoblast microparticles[30] and reactive oxygen species [31]. However, none of the observed changes in these factors have been proven to precede the onset of clinical disease, or to clearly discriminate between normal pregnancy and preeclampsia. Furthermore, none of these candidate markers of endothelial disturbance seem unique to preeclampsia and mild similar changes are consistently found in normal pregnancy, as well as in other inflammatory and vascular disorders [3,12,16]. Therefore, although an important aspect of its pathophysiology, the endothelial activation of preeclampsia is more likely to represent an extreme maternal host response to common pregnancy-induced inflammatory and vascular stimuli, rather than a specific entity driving its pathogenesis.

1.2 Placenta

Previous investigations into the etiology of preeclampsia have focused on the placenta as the culprit causing the disease [32]. It is still widely believed that preeclampsia is a two-stage disorder, initiated by abnormal placentation with concomitant placental underperfusion and ischemia (first stage), that results in the release of one or more placental factors into the maternal circulation causing the clinical syndrome (second stage). In support of this hypothesis, early observations from histological studies of the placenta and placental bed biopsies in the 1970s revealed abnormal features of placentation and trophoblast invasion in women with preeclampsia [33]. During normal placental development, fetal trophoblast cells of the placental villi hatch to the maternal endometrium and decidual layer and further differentiate to adopt an invasive phenotype characteristic for the so-called 'second wave of trophoblast invasion', that occurs between gestational week 18 to 20 [34]. Intriguingly, invasive trophoblast cells have an important role in expansion and remodelling of the maternal spiral arteries within the myometrium. First, they replace the endothelial lining and express factors characteristic of endothelial cells. Second, they produce an array of cytokines and signalling molecules that act in concert with local maternal decidual and

inflammatory cells, i.e. monocytes and NK cells, to initiate and regulate remodelling of the spiral arteries' intimal and smooth muscle layer [34,35]. If appropriately controlled, this process results in a low resistance placental vascular bed with vessels of large diameter, and unresponsive of vasoconstrictive agents, that provides the placenta with an adequate blood supply required for fetal growth and development [36].

Conversely, abnormal spiral artery remodelling is associated with poor placentation and underperfusion of the placenta. Consequent inadequate blood supply to the fetus is associated with fetal growth restriction, which is a common feature associated with preeclampsia [37]. Moreover, studies of placental bed biopsies in women with preeclampsia confirmed the presence of inadequate trophoblast invasion and arterial remodelling in a majority of cases [33]. In addition, in spiral arteries of a proportion of women with preeclampsia, characteristic lesions were observed, reminiscent of vascular changes typically observed in atherosclerosis, that involved fibrinous deposits, lipid droplets and foam cells, infiltrated mononuclear inflammatory cells and platelet aggregates, named 'acute atherosis' [38]. Further, many studies of placental tissue from preeclamptic patients describe characteristic hallmarks of vascular compromise, such as ischemia [39], thrombosis [40], inflammation [9], oxidative stress [31] and apoptosis [30].

Of interest, and accordant with the maternal endothelial and vascular changes observed during the clinical syndrome, defective spiral artery remodelling is not uniquely observed in preeclampsia. Although most striking in preeclampsia, similar changes are also observed in women with fetal growth restriction [41], in about a third of preterm deliveries without hypertensive disease [42], and even in normal pregnancy [43]. Notably, several comparative studies performed with placental tissue from women with preeclampsia and normal pregnancy show conflicting results [44], and are hampered by methodological difficulties due to inadequate matching for gestational age, effects of labour and inappropriate sampling. Also, abnormal placentation and fetal growth restriction have a rather weak correlation with severity and clinical outcome of the maternal syndrome of preeclampsia [37]. In addition, poor placental function and concomitant intrauterine growth restriction are predominantly associated with early-onset preeclampsia, whereas women with term preeclampsia deliver infants that have birth weights comparable to infants born to normotensive mothers [45,46].

Over the past two decades, many research groups have aimed to find candidate factors released into the maternal circulation, that might explain the link between these placental abnormalities and the subsequent development of maternal disease, which usually occurs many weeks after the completion of placentation [6]. Although the list of candidate factors that the placenta is capable of producing, and that are elevated during preeclampsia, is continually growing and seems unlimited, none of these factors have been shown to

universally correlate with clinical outcome, precede the onset of disease and lack convincing scientific proof to support a plausible pathophysiological mechanism [3,27,37]. In our opinion, most promising results have been gained from the studies of circulating placental syncytiotrophoblast microparticles capable of endothelial injury [30], of inflammatory biomarkers [9], and of the release of anti-angiogenic peptides [28]. However, the two-stage model for preeclampsia clearly does not fit a one-to-one relationship for all women that develop preeclampsia [27]. Thus, defective placental factors may not be considered as the sole basis underlying its pathogenesis, but are likely to interact with several maternal constitutional factors to determine clinical outcome.

In summary, abnormal placentation and subsequent release of circulating factors is an attractive concept to explain the pathogenesis of preeclampsia. However, current evidence of placental pathology in preeclampsia at best supports the notion that defective placentation is a strong risk factor for preeclampsia, but is not a pathognomonic feature present in all affected women (**Figure 1**).

1.3 Metabolic Syndrome

Many features of preeclampsia resemble changes associated with atherosclerosis and cardiovascular risk. Indeed, adaptive changes to normal pregnancy may be described as a shift towards a pro-atherogenic state, that is found to be more severe in women who develop preeclampsia [47]. Furthermore, maternal risk factors for preeclampsia include hypertension, obesity and diabetes, that are all associated with cardiovascular risk [48]. Also, women with preeclampsia more often exhibit dyslipidemia, hypertriglyceridemia and impaired glucose tolerance shortly after pregnancy, reminiscent of the so-called metabolic syndrome [49-51]. Metabolic syndrome, also named insulin resistance syndrome or syndrome X, is a complex of traits commonly found in persons at high risk of cardiovascular events characterized by central obesity, hypertension, hypertriglyceridemia, hyperglycaemia and low levels of high density lipoprotein (HDL)-cholesterol [52].

The prevalence of metabolic syndrome is estimated at 15 to 25 % of western adult individuals [53,54], and this complex of interrelated risk factors is thought to contribute to at least a quarter of all cardiovascular events and up to 40% of incident type 2 diabetes mellitus [55]. In women, the majority of cases of premature atherosclerosis are associated with the metabolic syndrome. Overall, according to population-based data from the United Kingdom and the Framingham Heart Study, women with the metabolic syndrome had a relative risk (RR) of developing any cardiovascular disease of RR=2.25 (95 percent confidence interval [CI], 1.31–3.88) and RR=1.54 (95 percent CI, 0.68–3.53) for coronary heart disease and a RR=6.90 (95 percent CI, 4.34–10.94) for diabetes [56]. In women who develop preeclampsia, components of the metabolic syndrome are more commonly

present before, during and after pregnancy [37,51].
Therefore, some authors speculate that maternal changes leading to metabolic syndrome provide an explanation for the link between placental dysfunction and the maternal syndrome of preeclampsia, as well as a common link between a history of preeclampsia and future risk of cardiovascular disease [51,57-59].

1.4 Inflammation

First proposed by Redman and colleagues, the endothelial dysfunction characteristic of the maternal syndrome of preeclampsia may be considered as a component of a more generalized low-grade systemic inflammatory response to the semi-allogeneic fetus [60]. In support of this hypothesis, many studies have demonstrated altered levels of circulating biomarkers of inflammation during preeclampsia, as compared to healthy pregnancy, as will be reviewed in detail in Chapter 5 of this thesis [9]. In this chapter, we describe recent advances in immunology that have made an important contribution to preeclampsia research.

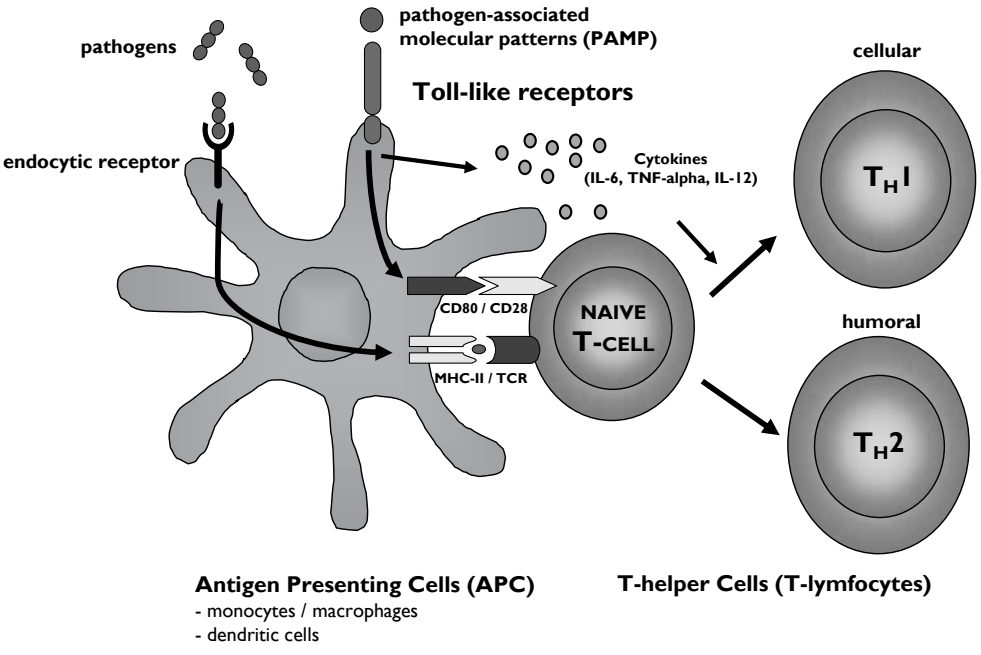


Figure 2

In general, the immune system can be divided in two components: an innate and an adaptive component (**Figure 2**). The innate immune system, which includes a cellular component (monocytes, granulocytes and dendritic cells) and a humoral component (mostly complement factors) provides a rapid and non-specific response system that plays a major role in determining and controlling the type of adaptive immune response [61]. More recently however, it became clear that cells of the innate immune system are far less non-specific than previously assumed. As first proposed by Janeway and colleagues in 1992 [62], and subsequently demonstrated in 1998 [63], human innate immune cells express a series of evolutionary conserved receptors known as pattern-recognition receptors (PRRs). PRRs recognize and bind highly conserved sequences known as pathogen-associated molecular patterns (PAMPs). PAMPs, such as bacterial lipopolysaccharide (LPS), peptidoglycan and viral double stranded ribonucleic acid (dsRNA) are unique to and expressed by microbes [61]. One of the principal families of the PRRs are the Toll-like receptors (TLRs), which are central components of the innate immune system [64,65]. Each TLR is distinct in its specificity. However, following ligation, all TLRs signal through a common adaptor molecule named MyD88, to activate the nuclear factor kappa B (NF-κB) pathway, which results in a cellular immune response characterized by the production of pro-inflammatory cytokines such as interleukins 2 (IL-2) and 6 (IL-6), antimicrobial products, and the upregulation of costimulatory molecules [66].

The adaptive immune system is composed of T and B lymphocytes, which are activated through antigen recognition and clonal expansion, leading to the generation of an antigen specific immune response. CD4-positive T helper-1 (Th1) cells produce Th1 type cytokines, such as IL-2, interferon (IFN) -γ and tumor necrosis factor (TNF) -α, that support natural killer (NK) cell and macrophage activation and the generation of cytotoxic T cells, while CD4-positive T helper-2 (Th2) cells synthesize Th2 type cytokines, such as IL-4, IL-5 and IL-13 that induce B-cell activation and antibody production [67]. Wegmann and colleagues were the first to propose that successful pregnancy induces an immune bias towards Th2 type immune responses and suppressed Th1 type immunity [68]. Women with preeclampsia show an exaggerated inflammatory response, characterized by aberrant cytokine production towards harmful Th1 type immunity [69]. The sources of these increased levels of pro-inflammatory cytokines in the circulation of preeclamptic women have not been fully determined. Some authors indicate excessive synthesis by the placenta [70,71], while others suggest circulating activated leukocytes as the main source of pro-inflammatory cytokine production [21].

In support of an immunological basis for preeclampsia are the observations that the disease predominately occurs in first pregnancy and has a relatively low recurrence rate [2,72]. Although not fully understood, these data suggest a model of immunotolerance

to pregnancy-related, or fetal-paternal antigens. Indeed, epidemiological studies suggest reduced preeclampsia risk after repeated exposure to paternal antigens [73] and an increased risk after a prolonged interval between two subsequent pregnancies [74]. Although disputed by some, recurrence risk of preeclampsia seems higher if a subsequent pregnancy is conceived with a different partner [74-77]. In addition, women who become pregnant with a partner who previously fathered a preeclamptic pregnancy, have a 2-fold higher risk of developing preeclampsia [77]. This so-called 'dangerous father' theory is further supported by intergenerational data from the Norwegian Birth Registry [78].
Previously, many study groups have aimed to unravel the immunology of placentation and induction of tolerance to the allogeneic placental trophoblast cells of the fetus. However, despite extensive research, characterization of the precise mechanisms involved in trophoblast invasion and induction of immunotolerance essential for fetal survival, is still largely mysterious [79]. Although inconclusive, components of the immune system thought to be critical players at the maternal-fetal interface are the major histocompatibility complex (MHC) or the human lymphocytes antigens (HLA), especially HLA-C, -E and -G that are uniquely expressed on invasive trophoblast cells [80], maternal natural killer (NK) cells [81], regulatory cytokines interleukin (IL)-10, IL-12 and transforming growth factor β [9].
Second to the local immune response at the placenta, preeclampsia risk may be dependent on maternal sensitivity to inflammation. It is well recognized that the capacity to elicit and control an inflammatory response has a strong variation between individuals, that cannot be explained by the type and nature of the encountered antigens [82]. Because individuals differ in their sensitivity to low-grade acute-phase stimuli, it has been speculated that women who are "high responders" are more susceptible to pregnancy-induced inflammatory changes. Similar observations have been shown for other inflammatory mediated conditions, such as meningococcol sepsis and coronary heart disease [83,84]. Maternal constitutional factors that involve the inflammatory response might therefore be an important link between preeclampsia and long-term risk of atherosclerosis. In population studies, high baseline plasma levels of inflammatory markers, such as C-reactive protein (CRP) and interleukin-6 (IL-6) are predictive of future myocardial infarction and stroke.[85] CRP is a member of the so-called 'acute-phase' reactants, synthesized in the liver in response to inflammation, infection or tissue damage [86]. It has been shown that women with a history of preeclampsia exhibit higher CRP levels up to 20 years after delivery, in comparison to women with a history of only uneventful pregnancies [87].

In summary, recent advances in immunology provide a promising area of research to unravel the pathophysiology of preeclampsia and subsequent maternal health.

1.5 Genetic constitutional factors

In concordance with other multifactorial vascular disorders, it can be assumed that certain maternal constitutional factors that contribute to preeclampsia have a genetic basis. Women with first degree relatives, i.e. sisters or mothers, with previous hypertensive disorders of pregnancy are at increased risk of preeclampsia [2]. Recently, overall heritability was estimated at about 30% for preeclampsia and 20% for pregnancy-induced hypertension [88]. Although family history contributes to preeclampsia risk, no monogenetic pattern of inheritance can be adopted to preeclampsia. Several genome-wide screening studies have been performed, that have identified substantial linkage with at least four different loci [89-91]. However, these loci only explain a small percentage of the overall cases of preeclampsia. More likely, preeclampsia can be considered as a complex disease of multigenetic origin and as a phenotype that is dependent on multiple gene-environment interactions. In recent years, studies have identified a rapidly growing number of susceptibility genes, many of which interact with the maternal cardiovascular or haemostatic system, or with the regulation of maternal inflammatory responses [92]. In recent years, promising results have been obtained from candidate-gene approaches using allelic variants known as single-nucleotide polymorphisms (SNPs). Similar to observations in other complex traits, several candidate SNPs have been found that alter the function or expression of critical receptors, enzymes and other biologically active proteins involved in the pathogenesis of preeclampsia [92,93]. Genetic association studies have the major advantage that these allelic variants are fixed at birth and thus, unlike acquired or environmental risk factors, provide a measure of life-long exposure, not likely to be subject to confounding due to the timing of investigation [94]. Most of these functional allelic variants are associated with a moderate (2- to 3-fold) increase in overall disease risk. However, many studies of allelic variants associated with risk of preeclampsia (as reviewed in reference [93]) have provided inconclusive results, mostly due to lack of power, considerable selection bias, non robust evaluation of linkage disequilibrium and functionality and population stratification. Candidate genes consistently associated with preeclampsia include allelic variants of eNOS, MTHFR, angiotensinogen, TNF, factor V Leiden and prothrombin [93].

2 Maternal health after preeclampsia

2.1 Subsequent pregnancy and reproductive outcome

After delivery, most clinical signs of early-onset preeclampsia disappear within days to weeks. However, women with previous preeclampsia are at an increased risk of developing future disorders of similar etiology. Although several studies on reproductive outcome

after preeclampsia have reported a significantly reduced risk of hypertensive disorders in subsequent pregnancies, recurrence risk remains considerably higher than in the general population. Recurrence rates of preeclampsia have been reported differently, ranging from 13% up to 65%, depending on definitions for onset and severity of the disease [77,95-97]. Also, reports on risk factors for recurrence of preeclampsia have provided inconsistent data. Although debated, commonly acknowledged risk factors for recurrence include the presence of chronic hypertension, early onset of disease in first pregnancy, hereditary or acquired thrombophilias, paternity change and prolonged interpregnancy interval [2]. At present, few studies have been performed to assess recurrence risk of early-onset onset disease, and risk factors for recurrence are largely unknown. Of interest, women with previous preeclampsia are more prone to develop fetal growth restriction and deliver small-for-gestational age infants in subsequent pregnancies. Most likely, this effect is due to shared constitutional factors that contribute to abnormal placentation.

2.2 Pregnancy as a stress test for cardiovascular health

As mentioned in the previous paragraphs, many features of early-onset preeclampsia are similar to atherosclerosis. Both disorders are characterized by endothelial dysfunction and inflammation. Common risk factors include obesity, dislipidemia, insulin resistance, chronic hypertension, thrombophilias and hyperhomocystinaemia. In addition, evidence from large-scale epidemiological studies have consistently shown that a history of preeclampsia exerts an independent risk for future cardiovascular disease. Recently, these studies have been reviewed in a comprehensive meta-analysis by Bellamy and colleagues. Pooled follow-up data of up to 200,000 women with a history of preeclampsia show significantly increased relative risks for future chronic hypertension, ischaemic heart disease, venous thromboembolism and stroke (**Figure 3**). Overall mortality after 14.5 years was an estimated 1.49-fold higher (95% confidence interval 1.05 – 2.14). Long-term maternal prognosis is especially poor for women who experienced early-onset disease (<37 weeks gestational age), in whom long-term follow-up studies revealed a 7- to 8-fold increased cardiovascular mortality, compared to a 2-fold increase in women with a history of late-onset preeclampsia.

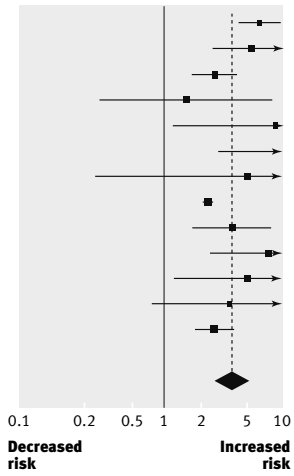
These data have led to the concept of pregnancy as a 'stress test for cardiovascular health'. In this concept, the adaptive challenge of pregnancy can be seen as an indicator of maternal susceptibility to develop future vascular, inflammatory and thrombotic conditions. Women who experience early-onset preeclampsia have 'failed' the challenge test and should be considered high risk. Conversely, women who have uneventful pregnancies, 'pass' the challenge test and might be considered low risk.

Figure 3 Pre-eclampsia and risk of fatal and non-fatal ischaemic heart disease events in later life. *Early and late pre-eclampsia combined (see table 2). † Mild and severe eclampsia combined (see table 2). ‡ All maternal placenta syndromes.

A Preeclampsia and risk of hypertension in later life.

Study	Total No of cases/ women who had pre-eclampsia	Total No of cases/ women who did not have pre-eclampsia	Weight (%)	Relative risk (random) (95% CI)
Adams 1961	40/53	21/185	14.25	5.91 (3.89 to 8.98)
Epstein 1964	37/48	7/114	8.97	5.34 (2.50 to 11.42)
Sibai 1986	60/406	23/409	13.52	2.63 (1.66 to 4.17)
Carleton 1988	3/23	2/23	2.91	1.50 (0.28 to 8.10)
Nisell 1995	9/45	1/44	2.12	8.80 (1.16 to 6.66)
North 1996	20/50	1/50	2.23	20.00 (2.79 to 143.35)
Laivuori 1996	2/22	0/22	1.03	5.00 (0.25 to 99.24)
Hannaford 1997	377/2371	922/14 831	18.59	2.35 (2.08 to 2.65)
Marin 2000	30/94	12/86	8.88	3.70 (1.72 to 7.97)
Shammas 2000	23/47	3/46	5.45	7.50 (2.42 to 23.26)
Hubel 2000	10/30	2/30	3.81	5.00 (1.19 to 20.96)
Sattar 2003	7/40	2/40	3.49	3.50 (0.77 to 15.87)
Wilson 2003	216/443	55/206	14.74	2.62 (1.77 to 3.87)
Total (95% CI)	834/3658	1051/16 086	100.00	3.70 (2.70 to 5.05)

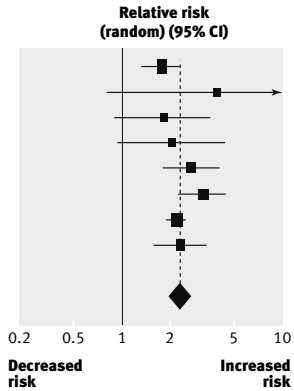
Test for heterogeneity: χ^2=32.05, df=12, P=0.001, I^2=62.6%
Test for overall effect: z=8.20, P<0.001



B Preeclampsia and risk of fatal and non-fatal ischaemic heart disease events in later life.

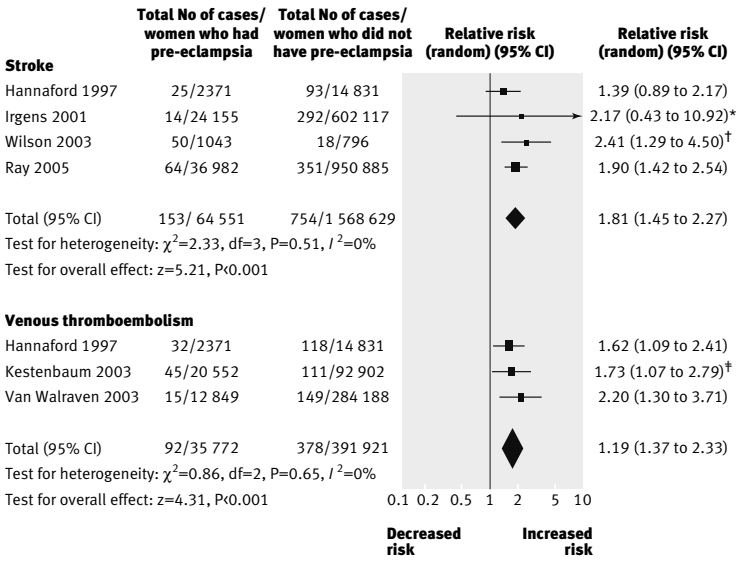
Study	Ischaemic heart disease: Total No of cases/ women who had pre-eclampsia	Ischaemic heart disease: Total No of cases/ women who did not have pre-eclampsia	Relative risk (random) (95% CI)
Hannaford 1997	69/2371	216/14 831	1.65 (1.26 to 2.16)
Irgens 2001	27/24 155	325/602 117	3.61 (0.76 to 17.18)*
Smith 2001	12/22 781	31/106 509	1.70 (0.86 to 3.35)
Wilson 2003	26/1043	10/796	1.95 (0.90 to 4.22)
Kestenbaum 2003	35/20 552	64/92 902	2.55 (1.70 to 3.83)†
Funai 2005	41/1070	269/35 991	3.01 (2.18 to 4.33)
Ray 2005	228/36 982‡	1262/950 885	2.10 (1.82 to 2.42)
Wirkstrom 2005	176/12 533	2306/383 081	2.21 (1.56 to 3.31)†
Total (95% CI)	614/121 487	4483/2 187 112	2.16 (1.86 to 2.52)

Test for heterogeneity: χ^2=9.60, df=7, P=0.21, I^2=27.1%
Test for overall effect: z=10.00, P=0.001



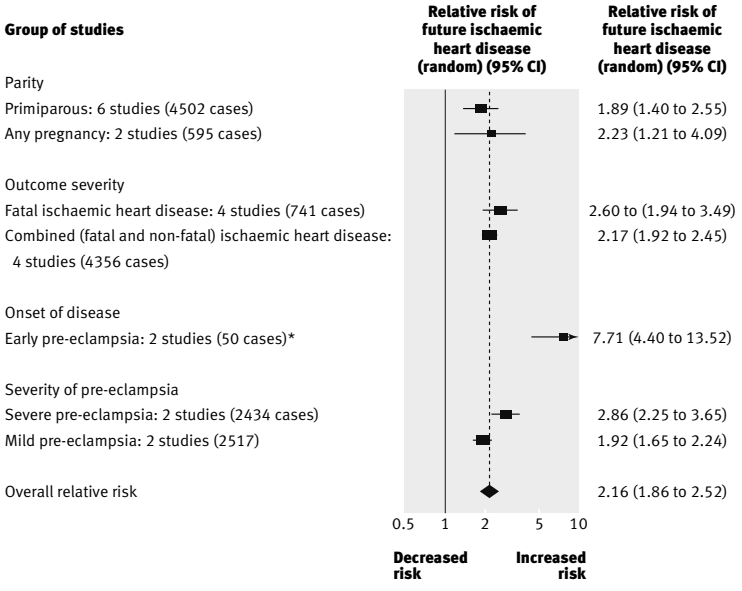
**Early-onset and late-onset preeclampsia combined.*
†Mild and severe preeclampsia combined.
‡All maternal placental syndromes.

C Preeclampsia and risk of fatal and non-fatal stroke and thromboembolism in later life.



**Early-onset and late-onset preeclampsia combined.*
†Fatal and non-fatal stroke combined.
‡Mild and severe preeclampsia combined

D Preeclampsia and risk of ischaemic heart disease in later life by study characteristics.



**Only one study included, as data not available from Smith 2001*

Bellamy, L. et al. BMJ 2007;335:974, copied with permission from the authors.

However, at present, there is limited knowledge on the underlying link between adverse pregnancy outcome and future cardiovascular risk. Although history of early-onset preeclampsia exerts a major risk for future cardiovascular events, structured risk factor assessment programs after delivery are not routinely advocated. Apart from women with known chronic hypertension, women with previous preeclampsia are generally lost to follow-up after delivery and comprehensive data on components of cardiovascular risk are limited. Few studies have measured risk factors predictive of cardiovascular disease after preeclampsia, and these studies have been limited to small numbers of women with mostly late-onset disease, and lack power for an integrated approach to estimate global risk.

Conclusions

In summary, early-onset preeclampsia is a severe condition that continues to contribute to maternal and neonatal morbidity and mortality. Despite extensive research, conclusive evidence on the cause and consequences of preeclampsia remains to be discovered. Recent focus on parallels between preeclampsia and non-pregnancy related vascular conditions have raised important questions concerning its etiology, pathogenesis and the impact on future reproductive and long-term health of those women affected. Advances in the fields of genetics, immunology, epidemiology and cardiovascular research provide opportunities to attempt to unravel several of these issues. In this thesis, we address maternal constitutional factors related to long-term cardiovascular health and subsequent pregnancy outcome in women with early-onset preeclampsia.

Aims of the thesis

Part I

- To evaluate subsequent pregnancy outcome in women with a first pregnancy complicated by early-onset preeclampsia and to study risk factors for recurrence of preeclampsia and preterm delivery (**Chapter 2**)
- To evaluate subsequent pregnancy outcome in women with a first pregnancy complicated by intrauterine growth restriction without maternal hypertensive disease (**Chapter 3**)

Part II

- To characterize classic cardiovascular risk factors predictive of first cardiovascular events in primiparous women with a history of early-onset preeclampsia (**Chapter 4**)
- To estimate global risk of major cardiovascular events in primiparous women with previous early-onset preeclampsia (**Chapter 4**)
- To evaluate a novel circulating marker for ischemia, ischemia modified albumin (IMA), in normal pregnancy and preeclampsia (**Chapter 5**)

Part III

- To provide a comprehensive review of previous studies on inflammatory changes in preeclampsia, with emphasis on current understanding of the maternal innate and adaptive immune response (**Chapter 6**)
- To study the role of allelic variants of the innate immunity receptors Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain 2 (NOD2) and plasma inflammatory biomarkers in women with previous early-onset preeclampsia (**Chapter 7**)
- To evaluate the maternal acute-phase inflammatory response to influenza vaccination, as a model for mild *in vivo* stimulation of the immune system, in women with a history of early-onset preeclampsia (**Chapter 8**)

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2

Outcomes of Subsequent Pregnancy after First Pregnancy with Early-Onset Preeclampsia

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Abstract

Objective

Aim of this study was to report outcome of subsequent pregnancy after early-onset preeclampsia in the first pregnancy, and to evaluate potential risk factors for recurrence of preeclampsia and preterm delivery.

Study design

Reproductive follow-up data were obtained for women with a history of early-onset preeclampsia, resulting in delivery before 34 weeks of gestation at the University Medical Center Utrecht, The Netherlands, between July 1993 and September 2002. The relative contributions of demographic data, outcome variables of first pregnancy, and common thrombophilias to the recurrence risk of preeclampsia and preterm delivery in subsequent pregnancy, were estimated by Cox proportional hazard models.

Results

Subsequent pregnancy outcome data were available for 120 women. Overall, preeclampsia reoccurred in the second pregnancy in 30 women (25%). However, 6 women delivered before 34 weeks of gestation (5%), 20 women between 34 and 37 weeks of gestation (17%), and 94 women after 37 weeks of gestation (78%). Forty-one women (34%) had an uneventful pregnancy. Recurrence rates for preeclampsia or preterm delivery were not related to severity of first pregnancy complications, including delivery before 28 weeks of gestation, occurrence of HELLP syndrome, small-for-gestational age infants, and to hereditary or acquired thrombophilias. Chronic hypertension was related to a higher recurrence risk of preeclampsia in the second pregnancy (HR 2.1, 95%CI 1.0 – 4.4), and smoking was related to a higher recurrence risk of preterm birth (HR 2.4, 95%CI 1.1 – 5.6).

Conclusions

Outcomes of subsequent pregnancy following first pregnancy with early-onset preeclampsia are generally favorable.

Introduction

Preterm preeclampsia is a dangerous and unpredictable maternal and fetal syndrome, usually prompting delivery within days after the onset of disease[1]. First noted by Mauriceau in 1694, severe preeclampsia is primarily observed in first pregnancy[2,3]. Although in most nulliparous women the onset of disease is near term, approximately 10% of cases occur before 34 weeks of gestation[1-3]. Early onset of preeclampsia is related to an increased risk of adverse maternal outcome, and predisposes to potentially lethal complications, such as eclampsia, HELLP syndrome and placental abruption. Furthermore, perinatal outcome of infants born to preeclamptic mothers is closely related to gestational age at delivery[4]. Additionally, early-onset preeclampsia is associated with a higher rate of small-for-gestational age infants. Recurrence rates of preeclampsia have been reported differently, ranging from 13 %[5] up to 65 %[6,7], depending on definitions for onset and severity of the disease. Currently, efforts aimed at secondary prevention of recurrent preeclampsia include screening for common hereditary and acquired thrombophilias, low-dose aspirin, and folic acid supplementation in women with hyperhomocysteinemia[8]. Also, more recent studies focus on the role of anti-oxidants[9], low-molecular-weight heparin[10], life-style intervention programs[11] and psychological support[12] in women with a history of severe preeclampsia. However, secondary preventive measures must only be considered beneficial, when reducing recurrence risk for early-onset disease and preterm delivery in subsequent pregnancy, since maternal and neonatal outcome in multiparous women with near-term preeclampsia is generally favorable[13]. Our aim was to study maternal and fetal outcome of subsequent pregnancies of primiparous women with early-onset preeclampsia in first pregnancy, defined as preeclampsia resulting in delivery before 34 weeks of gestation, and potential risk factors for recurrence of preeclampsia and preterm delivery.

Material and methods

Study population

Between June 1993 and September 2002, all primiparous women with singleton pregnancies complicated by early-onset preeclampsia, admitted to our tertiary referral center (UMC Utrecht, the Netherlands), entered the follow-up database. Early-onset preeclampsia was defined as preeclampsia resulting in delivery before 34 completed weeks of gestation. At three to twelve months after the first pregnancy, demographic, general medical, family history and obstetric data were recorded, and blood samples were obtained for detection of thrombophilic, metabolic, inflammatory and lipid risk factors. A detailed description

of these tests and cut-off values for abnormality was previously published elsewhere[14]. With a minimum follow-up time of two years after their first delivery, reproductive follow-up data were obtained and second pregnancy outcome was recorded and verified from the medical records. All women had received low dose aspirin (80 mg / day) from 12 to 36 weeks of the second pregnancy. Women diagnosed with hyperhomocysteinemia, were treated with 5 mg folic acid and 100 mg vitamin B6 daily. Preeclampsia was defined, according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP)[15]. HELLP-syndrome was defined according to previously described criteria, as lactate dehydrogenase > 600 U / L and/or haptoglobin <= 0.3 g/L, serum aspartate aminotransferase and/or serum alanine aminotransferase > 50 U / L, and platelet count < 100 x 10^9 / L[16]. Small-for-gestational-age was defined as birth weight below the 10^{th} centile for gestational age at delivery, based on the Dutch population charts[17]. Chronic hypertension was defined as hypertension, treated with antihypertensive medication prior to the index and subsequent pregnancy and at follow-up.

Statistics

Demographic and outcome variables of first pregnancy and thrombophilic risk factors were individually tested for their association with recurrent preeclampsia and with preterm delivery < 37 weeks of gestation in second pregnancy. Hazard ratios were calculated using the Cox proportional hazards model, with days of gestation in second pregnancy as timescale. We then calculated adjusted hazard ratios by multilevel Cox proportional hazard models, including chronic hypertension, commonly regarded as confounding follow-up data, and including variables that significantly contributed to recurrence risk at univariate analysis.

Results

At a mean follow-up time of 6.3 years after inclusion, complete reproductive follow-up data were obtained for 185 (93 %) women. Pregnancy rate at follow-up time was 67.8 %, which included 7.5 % early pregnancy loss and 60.3 % deliveries after 16 weeks of gestation. Thus, complete follow-up data were available for 120 women. Clinical maternal and fetal outcome characteristics of index and subsequent pregnancies are summarized in **Table 1** and **2**. Mean (SD) maternal age at first delivery was 29.0 (4) years, and 31.8 (4) years at second delivery, with a mean interval of 2.8 (1) years, or a mean interpregnancy interval of 2.1 (1) years after adjustment for gestational age at second delivery. Chronic hypertension

was found in 27 % at baseline and 34 % at follow-up. Overall, preeclampsia reoccurred in 30 pregnancies (25%), including 18 women with deliveries after 37 weeks (15 %), 9 women with deliveries between 34 and 37 weeks (7.5 %), and 3 women with deliveries before 34 weeks of gestation (2.5 %). Recurrence of HELLP syndrome was found in only 3 cases (2.5 %). Twelve infants were born small-for-gestational-age (10%), thus not exceeding the population frequency. Gestational hypertension without proteinuria occurred in 27 women (22.5 %), and 12 women (10%) developed other non-hypertensive pregnancy complications, including preterm labor and placental abruption, leaving 41 women (34 %) with an uneventful pregnancy. Risk of recurrent preeclampsia was not influenced by maternal age, gestational age at delivery, the interval between first and second delivery, occurrence of HELLP syndrome or delivery of small-for-gestational age infants in the first pregnancy (**Table 2**).

Table 1 Baseline characteristics of 120 women with a history of early-onset preeclampsia at < 34 weeks of gestation and outcome of screening for common hereditary and acquired thrombophilic risk factors

Age* (y)	29.0 ± 4.0
Interpregnancy interval (y)	2.8 ± 1.4
Body-mass index (kg/m^2)	26.2 ± 4.6
Smoking (No.)	36 (31%)
Chronic hypertension (No.)	41 (34%)
Thrombophilia screening† ≥ 1 test positive (No.)	29 (24%)
Protein S deficiency (No.)	2 (1.7%)
Protein C deficiency (No.)	3 (2.5%)
Anti-thrombin III deficiency (No.)	1 (0.8%)
Increased APC resistance‡ (No.)	17 (14%)
Factor V Leiden mutation (No.)	13 (11%)
Lupus anticoagulant positive (No.)	1 (0.8%)
Anticardiolipin IgM positive (No.)	5 (4%)
Anticardiolipiin IgG positive (No.)	8 (7%)
Hyperhomocysteinemia (No.)	20 (17%)

** maternal age at 1st delivery*

† positive test results at baseline include protein S deficiency, protein C deficiency, anti-thrombin III deficiency, factor V Leiden mutation, lupus anticoagulant, anticardiolipin antibodies or hyperhomocysteinemia

‡ genetic analysis revealed 4 women without factor V Leiden mutation

Of 41 women with chronic hypertension, 14 women developed preeclampsia (48 %), as compared to 16 out of 79 normotensive women (20%). Chronic hypertension contributed significantly to recurrence of preeclampsia with a hazard ratio of 2.1 (95 % CI 1.0 – 4.4). In the multivariate analysis hazard ratios for other potential risk factors were not influenced after adjustment for chronic hypertension (**Table 1**).

Table 2 Characteristics of first pregnancy (FP) and subsequent pregnancy (SP) of 120 women with early preeclampsia < 34 weeks of gestation

	FP	SP
Gestational age at delivery (wk)	29.4 ± 2.5	38.0 ± 3.7
Delivery at < 28 wks (No.)	30 (25.0%)	3 (2.5%)
Delivery at > 28 wks < 34 wks (No.)	90 (75%)	3 (2.5%)
Delivery at > 34 wks < 37 wks (No.)	-	20 (17%)
Delivery at > 37 wks (No.)	-	94 (78%)
Preeclampsia (No.)	120 (100%)	30 (25%)
HELLP syndrome (No.)	63 (52.5%)	3 (2.5%)
Abruptio placentae (No.)	7 (5.8%)	4 (3.3)
Mean birth weight (g)	1001 ± 439	2961 ± 800
Small-for-gestational-age infant <p10 (No.)	68 (57%)	12 (10%)
Cesarean section (No.)	97 (81%)	43 (36%)
Perinatal deaths (No.)	41 (35%)	2 (1.7%)

Table 3 Relationship between known risk factors for adverse pregnancy outcome and recurrence of preeclampsia in women with a history of early-onset preeclampsia < 34 weeks, using the Cox proportional hazards model

	Unadjusted Hazard Ratio (95% CI)	Adjusted Hazard Ratio* (95 % CI)
Age	0.9 (0.9 – 1.1)	1.0 (0.9 – 1.1)
Interpregnancy interval	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
Body-mass index	1.0 (0.9 – 1.1)	0.9 (0.8 – 1.0)
Smoking	1.0 (0.4 – 2.5)	1.0 (0.4 – 2.4)
Chronic hypertension	2.1 (1.0 – 4.4)	
Gestational age at 1st delivery	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
Gestational age at 1st delivery < 28 wks	1.1 (0.5 – 2.5)	0.9 (0.4 – 2.1)
HELLP syndrome in 1st pregnancy	1.0 (0.5 – 2.0)	1.0 (0.5 – 2.0)
Small-for-gestational-age infants < p10	1.4 (0.7 – 3.0)	1.4 (0.7 – 3.0)
Thrombophilia screening† ≥ 1 test positive	1.5 (0.7 – 3.3)	1.5 (0.7 – 3.3)
Thrombophilia screening†† ≥ 2 tests positive	2.4 (0.8 – 6.8)	1.9 (0.6 – 5.5)
Hyperhomocysteinemia	1.6 (0.7 – 3.6)	1.4 (0.6 – 3.2)
Factor V Leiden mutation	1.2 (0.4 – 3.6)	1.0 (0.4 – 3.0)
Anticardiolipin antibodies positive‡	1.5 (0.5 – 5.1)	2.1 (0.6 – 7.7)

** adjusted for chronic hypertension*

† positive test results at baseline for protein S deficiency, protein C deficiency, anti-thrombin III deficiency, factor V Leiden mutation, lupus anticoagulant, anticardiolipin antibodies or hyperhomocysteinemia

†† idem for women with two or more positive test results (n=7)

‡ positive test results for either IgM or IgG antibodies

Table 4 Relationship between known risk factors for adverse pregnancy outcome in women with a history of early preeclampsia < 34 weeks, and preterm delivery < 37 weeks in subsequent pregnancy, using the Cox proportional hazards model

	Unadjusted Hazard Ratio (95% CI)	Adjusted Hazard Ratio* (95 % CI)
Age	1.0 (0.9 – 1.1)	1.0 (0.9 – 1.1)
Interpregnancy interval	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
Body-mass index	1.0 (0.9 – 1.0)	1.0 (0.9 – 1.1)
Smoking	2.4 (1.1 – 5.6)	2.4 (1.1 – 5.6)
Chronic hypertension	1.7 (0.8 – 3.8)	1.4 (0.6 – 3.4)
Gestational age at 1st delivery	1.0 (1.0 – 1.0)	1.0 (1.0 – 1.0)
Gestational age at 1st delivery < 28 wks	0.7 (0.3 – 1.8)	0.6 (0.2 – 1.7)
HELLP syndrome in 1st pregnancy	0.7 (0.3 – 1.6)	0.6 (0.3 – 1.5)
Small-for-gestational-age infants < p10	1.5 (0.7 – 3.3)	1.2 (0.5 – 2.9)
Thrombophilia screening† ≥ 1 test positive	1.0 (0.4 – 2.4)	0.7 (0.2 – 2.0)
Thrombophilia screening† ≥ 2 tests positive	0.1 (0.0 – 44.2)	N/A
Hyperhomocysteinemia	1.1 (0.4 – 2.9)	1.2 (0.4 – 3.2)
Factor V Leiden mutation	1.6 (0.6 – 4.7)	0.7 (0.2 – 3.0)
Anticardiolipin antibodies positive‡	1.3 (0.3 – 5.5)	1.5 (0.3 – 7.0)

** adjusted for chronic hypertension and smoking*

† positive test results at baseline for protein S deficiency, protein C deficiency, anti-thrombin III deficiency, factor V Leiden mutation, lupus anticoagulant, anticardiolipin antibodies or hyperhomocysteinemia

‡ positive test results for either IgM or IgG antibodies

Preterm delivery before 37 weeks occurred in 26 subsequent pregnancies (22.5 %), whereas preterm delivery before 34 weeks of gestation occurred in 6 cases (5 %), 3 of which occurred before 28 weeks (2.5 %). Mean (SD) gestational age at delivery was 29.2 (2) weeks for the index pregnancy, compared to 38.0 (4) weeks for the subsequent pregnancy ($p<0.0001$). First pregnancy perinatal mortality was 34.5 %, whereas in second pregnancy two perinatal deaths occurred (1.7%), both due to placental abruption. Furthermore, lower cesarean section rates (80.8 % compared to 35.8 %), and higher mean birth weight (mean difference 1961 (841) g) were observed in subsequent as compared to first pregnancies (**Table 2**). We found no relationship between severity or outcome variables (including gestational age at first delivery, the presence of HELLP syndrome and small-for-gestational-age infants) of the first pregnancy and the reoccurrence of preterm delivery (at < 37 weeks) in the next pregnancy (**Table 3**). Risk of recurrent preterm delivery was not influenced by maternal age, interpregnancy interval, body-mass index or the occurrence of chronic hypertension. However, smoking significantly increased the risk

for recurrent preterm delivery (hazard ratio 2.4 (95% CI 1.1 – 5.6)), independently of the presence of chronic hypertension.

Laboratory tests at baseline revealed that 29 of 120 women (24.2 %) had one or more hereditary or acquired thrombophilias. (**Table 1**). However, these thrombophilias did not contribute to recurrence risk of preeclampsia (**Table 3**) or preterm delivery (**Table 4**), or to incidence of HELLP syndrome, small-for-gestational age infants or preterm delivery (data not shown) in the subsequent pregnancy (**Table 3** and **4**).

Comment

Our study summarizes reproductive follow-up data of a tertiary referral cohort of 120 women with a history of early-onset preeclampsia with delivery before 34 weeks of gestation in their first pregnancy. Our study indicates that maternal and fetal outcome of a second pregnancy after early-onset preeclampsia is generally without serious adverse events. Although in a quarter of women preeclampsia reoccurred, approximately 4 out of 5 develop near-term disease, usually without serious complications for the mother or her infant. Additionally, because severe maternal complications of preeclampsia (including HELLP syndrome) are closely related to an early onset of disease, they were rarely seen in the next pregnancy. Subsequently, cesarean section rate was a 2-fold lower at 43 % of second deliveries.

Previous studies report widely different recurrence rates for preeclampsia[5,6,18], because of non standardized diagnostic criteria for the disorder and its subtypes[3,13]. Fifteen years ago, Sibai and colleagues found that the onset of disease is an important predictor of recurrence risk. They reported that 2 out of 3 women with preeclampsia in the second trimester have recurrent preeclampsia their in subsequent pregnancy, half of which again develops in the second trimester[7]. By contrast, in our population recurrence of preeclampsia in the second pregnancy was not different for women with very early-onset (<28 weeks) of the syndrome in the first pregnancy. More recently, a large Norwegian population based study of 1.7 million women revealed a recurrence rate of 13.1 % for all forms of preeclampsia[5]. Although highly powered, their data do not specify differences between early- and late-onset disease. Our results show that the recurrence rate of early-onset preeclampsia resulting in delivery before 34 weeks of gestation in the second pregnancy, is a 19-fold lower than recurrence of late-onset disease. In contrast to previous reports[19,20], we found no relationship between the occurrence of HELLP syndrome in first pregnancy, and the risk of adverse pregnancy outcome in subsequent pregnancy. Our findings might be explained by the relatively low background risk of preeclampsia in

primiparous and multiparous women.

Generally, severe preeclampsia is regarded as a syndrome of first pregnancy. Why preeclampsia is more common in nulliparous women is not yet fully understood, but is probably due to an immune phenomenon, involving the interaction between innate immune cells, NK cells and trophoblast antigens[1,3]. Apparently, immunological adaptation remains effective long after first delivery and protects the mother from recurrence of preeclampsia in the second pregnancy.

In our study, some cases of early-onset preeclampsia in the second pregnancy, might have been prevented by administration of low-dose aspirin[21], routinely prescribed to all women from the 12th to 36th week of pregnancy. Other more speculative actions that might have influenced the observed recurrence risk of early-onset preeclampsia, include periconceptional control of chronic hypertension and administration of vitamin B6 and folic acid in women with hyperhomocysteinemia.

Common thrombophilias, including factor V Leiden, anticardiolipin antibodies, protein C and S deficiency, antithrombin III deficiency, Lupus anticoagulant, and hyperhomocysteimemia, were observed in approximately 1 of 4 women. Highest rates were found for hyperhomocysteinemia, factor V Leiden and positive anticardiolipin IgG antibodies, which is in concordance with most previous studies in women with preeclampsia at ≤ 34 weeks of gestation[10,22]. In contrast to the study by Van Pampus and colleagues, common thrombophilias were not related to very early onset (≤ 28 weeks of gestation) in our population (data not shown)[23]. Although compared to the general population, an estimated 2-fold of women in our study population exhibited one or more thrombophilic risk factors, none of them were related to adverse pregnancy outcome or preterm delivery in subsequent pregnancy. Likely explanations for this result include a relatively low frequency of serious adverse events in the next pregnancy, false-positive screening for hyperhomocysteinemia and anticardiolipin antibodies related to interval between delivery and testing[24], and the possible effects of low-dose aspirin, folic acid and vitamin B6 supplementation.

In line with previous reports[25], preeclampsia reoccurred significantly more often in chronic hypertensive women, as compared to normotensive women, however almost all near term. Alternately, chronic hypertension was not related to subsequent preterm delivery, or to other adverse events. Smoking was related to a higher rate of recurrent preterm delivery, but not to a higher rate of preeclampsia. This could be explained by known deleterious effects of smoking regarding almost all adverse pregnancy outcomes except preeclampsia, for which others have even found a protective effect[1,3].

In summary, maternal and perinatal outcome of subsequent pregnancy after preeclampsia with early onset before 34 weeks of gestation, is generally favorable. Major complications including HELLP syndrome and preterm delivery reoccur only in a minority of cases. We found no relationship between recurrence of preeclampsia or preterm delivery in the second pregnancy and severity of the disease in first pregnancy, including onset and the occurrence of HELLP syndrome or small-for-gestational age infants, or the presence of hereditary or acquired thrombophilias. Our findings are important for counseling of women with a history of early-onset preeclampsia with respect to future pregnancies. Furthermore, future secondary preventive strategies, including proposed anti-oxidant and heparin treatment, will largely depend on estimated recurrence rates. Our findings show that serious adverse maternal or fetal outcomes in the second pregnancy after early-onset preeclampsia are rare, thus secondary prevention of preeclampsia is probably not feasible due to the high numbers needed to treat.

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3

Subsequent Pregnancy Outcome after First Pregnancy with Normotensive Severe Early-Onset Intrauterine Growth Restriction at < 34 Weeks of Gestation

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Submitted

Abstract

Objective

Aim of this study was to evaluate maternal and fetal outcome of the subsequent pregnancy of primiparous women with a history of normotensive intrauterine growth restriction (IUGR), prompting delivery before 34 weeks of gestation.

Study Design

Reproductive follow-up data were recorded for 22 women with a normotensive first pregnancy complicated by early-onset severe IUGR before 34 weeks, referred to the University Medical Center Utrecht, the Netherlands, between 1993 and 2005.

Results

Mean gestational age at delivery was 29.4 weeks for the index pregnancy compared to 36.4 weeks for the next pregnancy. IUGR recurred in 6 pregnancies (27.3%). Four subsequent pregnancies were complicated by hypertensive disorders. Perinatal mortality was 72.7% in the index pregnancy, compared to 13.6% in the second pregnancy. Overall, eleven women (50%) had an uneventful pregnancy.

Conclusion

Women with first pregnancy early-onset IUGR, without concomitant maternal hypertensive disease, frequently develop severe perinatal complications in their subsequent pregnancy.

Introduction

Intrauterine growth restriction (IUGR), as defined by reduced fetal growth due to compromised uteroplacental perfusion, is an important contributor to perinatal morbidity and mortality [1-3] . Although the onset of IUGR is mostly near term, approximately 20% of cases result in preterm delivery of a small-for-gestational-age (SGA) infant [4]. SGA infants born before 34 weeks of gestation are at increased risk of neonatal mortality, compared to matching preterm infants with appropriate fetal growth.[3,4] In addition, severe preterm growth restriction is associated with stillbirth [5] and serious neonatal complications, e.g. necrotizing enterocolitis and respiratory distress syndrome [1,6,7]. Although at term excess morbidity due to suboptimal growth is limited to those infants with very low birth weight (< 2.5th percentile), in preterm infants the incidence of respiratory distress is directly related to both birth weight percentile and gestational age [1,2,7].

A strong association exists between reduced fetal growth and concomitant maternal hypertensive disorders, that is inversely related to gestational age at onset [8,9]. This association is most striking in women with early-onset preeclampsia, of whom an estimated 53% deliver an infant that is small-for-gestational age [10]. However, severe IUGR that results in preterm delivery at < 34 weeks of gestation, is also observed in women without maternal signs of hypertensive disease. Normotensive early-onset IUGR, without the presence of maternal hypertension, apparent constitutional factors or congenital abnormalities, is an uncommon condition. Although results are inconclusive [11], previous studies suggest a relatively poor perinatal prognosis in normotensive early-onset IUGR when compared to early-onset IUGR in hypertensive women [9]. At present, for this subgroup of women with severe placental disfunction, that results in preterm IUGR without concomitant maternal hypertensive disease, no data exist on recurrence rates, maternal and perinatal outcome in subsequent pregnancies.

In this study, we evaluated maternal and fetal outcome of a subsequent pregnancy of women with normotensive early-onset severe IUGR in first pregnancy, defined as IUGR < 34 weeks of gestation with birthweight < 10^{th} centile in the absence of maternal hypertensive complications, known preexistent disorders and congenital abnormalities.

Materials and Methods

All primiparous women with a singleton pregnancy, complicated by normotensive IUGR, and referred for tertiary care to the University Medical Center Utrecht, the Netherlands, between July 1993 and December 2005, were eligible for inclusion. Early-onset IUGR was

defined as birth weight below the 10th percentile for gestational age at delivery based on Dutch population charts [12], that resulted in delivery before 34 completed weeks of gestation, excluding infants with chromosomal abnormalities and major fetal anomalies. Women with hypertensive disorders, including pregnancy-induced hypertension and preeclampsia in their index pregnancy, or having preexistent chronic hypertension, renal disease, cardiac disease, systemic lupus erythematosus, and diabetes mellitus were excluded. Participants entered follow-up at three to twelve months after delivery, and baseline demographic, general medical, family history and obstetric data were recorded. In addition, fasting blood samples were obtained for detection of thrombophilic, metabolic and inflammatory factors. Details on study design, recruitment of participants, laboratory procedures and cut-off values for abnormality were previously published elsewhere [13]. All women were advised treatment with low-dose aspirin (80 mg per day) from 12 to 36 weeks during their second pregnancy, and women diagnosed with hyperhomocysteinemia were treated with daily supplementation of 5 mg folic acid and 100 mg vitamin B6, according to current standard guidelines for clinical care in the Netherlands. Main outcome measures were recurrent IUGR, perinatal mortality, preterm delivery, preeclampsia, pregnancy-induced hypertension and other major obstetric complications, e.g. placental abruption. Perinatal mortality was defined as the sum of neonatal mortality and the number of stillbirths, with neonatal mortality defined as neonatal deaths within the first week of life and stillbirths as fetal deaths after 22 weeks of gestation. Preterm delivery was defined as delivery before 37 weeks of gestation. Pregnancy-induced hypertension, preeclampsia, HELLP-syndrome were defined according the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP), as previously described in detail elsewhere [13-15].

Results

Within our 12-year inclusion period, 34 healthy nulliparous women were admitted with a normotensive pregnancy complicated by early-onset severe IUGR, that resulted in delivery before 34 weeks of gestation. At a mean follow-up time of 7.4 years after their first delivery, complete reproductive follow-up data were obtained for 31 (91.2%) women. Twenty-two women (71%) had completed a subsequent pregnancy. At inclusion after the index pregnancy, baseline laboratory analyses showed positive test results for hereditary and acquired thrombophilias in 2 women (9.1%), i.e. one carrying the heterozygous Factor V Leiden mutation and another woman positive for anticardiolipin IgG antibodies. During the subsequent pregnancy, 18 women (82%) had used low-dose aspirin from 12 to 36 weeks of gestation or delivery.

Table 1 Characteristics of first pregnancy and subsequent pregnancy of 22 women with normotensive early-onset IUGR at <34 weeks gestational age.

	First Pregnancy	**Subsequent Pregnancy**
Age (y)	28.8 ± 3.9	31.4 ± 4.2
Gestational age at delivery (wk)	29.4 ± 2.9	36.4 ± 4.7
Delivery < 28 wks	4 (18.2%)	2 (9.1%)
Delivery ≥ 28 wks or < 34 wks	18 (81.8%)	2 (9.1%)
Delivery ≥ 34 wks or < 37 wks	-	4 (18.2%)
Delivery ≥ 37 wks	-	14 (63.6%)
Birth weight (g)	723 ± 297	2626 ± 1078
Birth weight percentile < 2.3	12 (54.5%)	2 (9.1%)
Birth weight percentile ≥ 2.3 or < 5	6 (27.3%)	2 (9.1%)
Birth weight percentile ≥ 5 or < 10	4 (18.2%)	2 (9.1%)
Birth weight percentile ≥ 10 or < 25	-	8 (36.4%)
Birth weight percentile ≥ 25 or < 50	-	2 (9.1%)
Birth weight percentile ≥ 50	-	6 (27.3%)
Perinatal mortality	16 (72.7%)	3 (13.6%)
Stillbirth	15 (68.2%)	1 (4.5%)
Postnatal death	1 (4.5%)	2 (9.1%)

Mean (SD) maternal age at first delivery was 28.8 (3.9) years, compared to 31.4 (4.2) years at second delivery. Mean interval (range) between the index and subsequent pregnancy was 1.9 (0.4 – 6.0) years after adjustment for gestational age at second delivery. Data on birth weight percentiles, gestational age at delivery and perinatal mortality, as compared between first and subsequent pregnancy, are shown in **Table 1**. Mean birth weight in first pregnancy was 723 grams, compared to 2626 grams in the second pregnancy. In the first pregnancy complicated by early-onset IUGR, 54% infants had very low birth weight below the 2.3rd percentile. In the subsequent pregnancy, fetal growth restriction with a birth weight below the 10th percentile recurred in 6 infants (27.3%), with two infants (9.1%) having birth weights below the 2.3rd centile (**Figure 1**). Mean gestational age at delivery was 29.4 weeks for the first pregnancy compared to 36.4 weeks for the subsequent pregnancy (**Table 1**). Eight women were delivered preterm (at <37 weeks of gestation), of whom 4 were delivered before 34 weeks of gestation, leaving 14 infants delivered (63.6%) at term. Perinatal mortality in first pregnancy was 72.7% (16 out of 22), that included 15

stillbirths. Stillbirths comprised 6 patients referred after intrauterine demise and 9 patients that, after extensive counseling, chose against surgical intervention because of infaust fetal prognosis. Neonatal death occurred in one case, due to severe necrotizing enterocolitis. In the subsequent pregnancy group, three perinatal deaths (13.6%) occurred, all due to severe growth restriction. Detailed clinical characteristics and perinatal outcome of the 6 women, who developed recurrent IUGR, are shown in **Table 2**. Hypertensive disorders, including pregnancy-induced hypertension and preeclampsia occurred in 4 subsequent pregnancies. Overall, 11 women (50%) had an uneventful pregnancy.

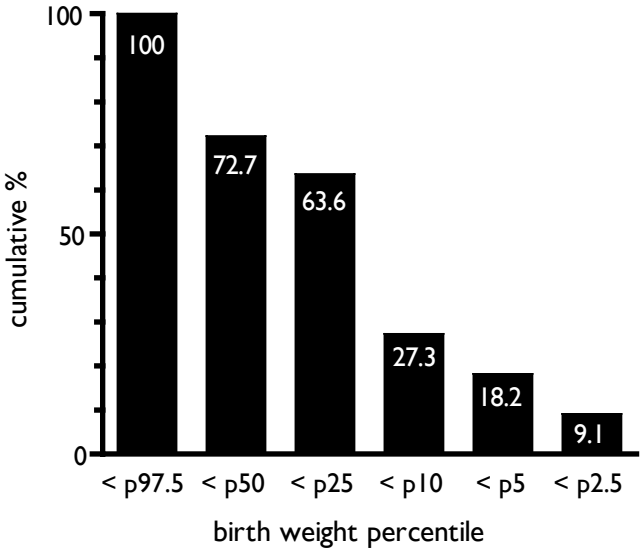

Figure 1 Birth weight percentiles in subsequent pregancy of 22 women with first pregnancy normotensive intrauterine growth restriction at < 34 weeks of gestation

Table 2 Characteristics of the first pregnancy and subsequent pregnancy of 6 women with recurrence of IUGR in the subsequent pregnancy.

	First Pregnancy				Subsequent Pregnancy			
	GA	**Delivery**	**Birth weight**	**Fetal Outcome**	**GA**	**Delivery**	**Birth Weight**	**Fetal Outcome**
1	23	Induction because of IUD	2.3rd-5th centile	† IUD	28	CS for fetal distress	< 2.3rd centile	† respiratory distress
2	25	Induction because of IUD	< 2.3rd centile	† IUD	40	Induction because of PE	5th-10th centile	Alive
3	28	Induction because of IUD	< 2.3rd centile	† IUD	25	Induction because of IUD	< 2.3rd centile	† IUD
4	29	CS for fetal distress	< 2.3rd centile	† NEC	37	CS for fetal distress PIH	5th-10th centile	Alive
5	31	CS for fetal distress	5th -10th centile	Alive	26	Medical termination of pregnancy because of severe PE/HELLP	2.3rd- 5th centile	† IUD
6	33	Induction because of IUD	< 2.3rd centile	† IUD	38	CS for fetal distress during labour	2.3rd- 5th centile	Alive

GA= Gestation age at time of delivery, IUD= Intrauterine demise, NEC= Necroting enterocolitis, CS= caesarean section

Comment

Normotensive early-onset IUGR, without the presence of maternal hypertension, apparent constitutional factors or congenital abnormalities, is an uncommon but severe condition [3,7]. In this study, we present comprehensive follow-up data of a tertiary referral cohort of women with a history of normotensive early-onset IUGR with delivery before 34 weeks of gestation in their first pregnancy. Our results indicate that women with normotensive early-onset IUGR in their first pregnancy, without concomitant maternal hypertensive disease, frequently develop severe perinatal complications in their subsequent pregnancy. In a second pregnancy after normotensive early-onset IUGR, fetal growth restriction recurred in approximately 1 of 4 women, and 1 of 3 women were delivered preterm. Although perinatal mortality was an estimated 5.3-fold lower, in 22 subsequent pregnancies 1 stillbirth and 2 neonatal deaths due to recurrence of severe IUGR and prematurity were observed. Overall, only half of these women had an uneventful subsequent pregnancy.

Current studies on recurrence rates of preterm IUGR have mostly included women with coexistent hypertensive disease [3,10,13,16,17], i.e. pregnancy-induced hypertension and preeclampsia. Indeed, fetal growth restriction has a strong assocation with preeclampsia, that is dependent on gestational age [10]. In a large cohort study, including reproductive follow-up of 154,810 women, pregnancies complicated by fetal growth restriction, preeclampsia, or placental abruption, had an overall increased risk of recurrence of these conditions in their subsequent pregnancy [16]. However, their data did not permit analysis on the association of fetal growth restriction with or without maternal hypertensive disease. More specifically, recent epidemiological data of 307 patients selected from the Norwegian Medical Birth Registry, estimated that early-onset preeclampsia at <34 weeks of gestation is complicated by IUGR in >50% of women, compared to rates of <10% in women with late-onset or near term disease [10]. Our previous follow-up study of 120 primiparous women with severe early-onset preeclampsia at <34 weeks of gestation, revealed similar rates of IUGR in the first pregnancy (57%), compared to only 10% small-for-gestational age infants (< 10th percentile) in the subsequent pregnancy [13]. Thus, we observed a 2.7-fold higher recurrence rate (27%) in our present study of early-onset IUGR in normotensive women, as compared to our previous study of women with both preeclampsia and IUGR. In addition, when compared to women with normotensive early-onset IUGR, our previous data on early-onset IUGR and coexistent preeclampsia showed a lower number of perinatal deaths in both the first (34% versus 73%) and second pregnancy (1.7% versus 13.6%). Taken together, our results support the hypothesis that early-onset IUGR in normotensive women results in poorer perinatal outcome in first and subsequent pregnancy, than in women with early-onset IUGR in the presence of maternal

hypertensive disease.

Interestingly, 4 previously normotensive women (18%) developed hypertensive complications in their second pregnancy. Although fetal growth restriction and hypertensive disorders of pregnancy differ in their association with maternal disease, both are commonly characterized by similar placental pathology. In our opinion, IUGR and preeclampsia and can be considered as closely related disorders. Most likely, after abnormal placentation that results in fetal growth restriction, the development of hypertensive disease will mainly depend on maternal constitutional factors related to cardiovascular fitness [18]. The appearance of preeclampsia after a first pregnancy with normotensive IUGR further supports this hypothesis of shared pathogenesis.

Our study has some of strengths and limitations that deserve attention. To our knowledge, this is the first study on reproductive follow-up of early-onset IUGR in a strictly defined subgroup of women with normotensive early-onset IUGR in their first pregnancy, i.e. in the absence of chronic or pregnancy-induced hypertension, preeclampsia, known pre-existent maternal disorders and congenital abnormalities. However, due to the low prevalence of this condition, are study has limited power to specifically identify subgroups or risk factors for recurrence. Although participants were advised to use low-dose aspirin during their subsequent pregnancy, 4 subjects did not receive treatment. Some, but not all, studies have shown a beneficial effect of aspirin supplementation in reducing recurrence the risk of IUGR [19,20]. Thus, in our study, low-dose aspirin therapy might have lowered the recurrence rate of early-onset IUGR in the next pregnancy. Other potential confounders that might have influenced the estimated recurrence risk are data on paternity change, which were unavailable, and selection due to referral bias in study population eligible for tertiary care. Furthermore, our study only included women early-onset disease at < 34 weeks of gestation and therefore may not be extrapolated to women with fetal growth restriction occuring near term.

In summary, women with first pregnancy early-onset IUGR, without concomitant maternal hypertensive disease, frequently develop severe perinatal complications in their second pregnancy. Women with a history of normotensive early-onset IUGR are at increased risk of recurrent fetal growth restriction, perinatal mortality and hypertensive disorders in subsequent pregnancy. About half of these women have an uneventful subsequent pregnancy. Although of limited sample size, our data are of value for counseling of these women with respect to their reproductive future. Nonetheless, additional studies to evaluate maternal and neonatal follow-up, as well as to elucidate the shared and disparate pathophysiologies of reduced fetal growth and maternal hypertensive disease, merit further investigation.

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4

Classic Risk Factors Predictive of First Cardiovascular Events in Women with a History of Early-Onset Preeclampsia: Opportunitities for Primary Prevention

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Submitted

Abstract

Objective

Women with a history of early-onset preeclampsia are at increased risk of developing major cardiovascular disease (CVD) related events, that have a detrimental effect on their long-term health and life expectancy. In this follow-up study, we measured established risk factors predictive of first CVD events after early-onset preeclampsia.

Study design

Over a 12-year interval, 243 primiparous women with a history of early-onset preeclampsia (delivery <34 weeks gestation) were included and tested for major cardiovascular risk factors at least six months after delivery, in addition to a population-based control group of 374 healthy non-pregnant women. Women with chronic hypertension were excluded.

Results

Mean age was 30.5 years for cases compared to 28.3 years for controls (P<.001). After adjustment for age, we observed significantly increased mean values for weight (P<.001), body-mass index (P<.001), systolic blood pressure (P<.001), diastolic blood pressure (P<.001), total cholesterol (P<.001), LDL-cholesterol (P<.001), total cholesterol to HDL-cholesterol ratio (P<.001), triglycerides (P=.009), fasting blood glucose (P<.001), and lower HDL cholesterol (P<.001) in women with previous early-onset preeclampsia. No differences were found for height, smoking, diabetes, and ethnicity. Estimated 10-year risk of first CVD events by Framingham Risk Scores remained <10% for all women (low-risk). Nonetheless, at mean (SD) 0.7 (1.0) years after early-onset preeclampsia, 14.2% of women met the criteria for metabolic syndrome, 54% of women exhibited ≥2 and 18% of women ≥3 major CVD risk factors.

Conclusion

The majority of women with a history of early-onset preeclampsia exhibit at least one modifiable risk factor for future CVD. Although most of these women are classified as low-risk according to the current AHA guidelines, this is mainly due to their young age masking other, mostly modifiable, major risk factors. Our data thus support routine assessment of risk factors and life-style intervention programs aimed at primary prevention of CVD in women with a history of early-onset preeclampsia.

Introduction

Cardiovascular diseases are the number one cause of death in women, exceeding all other causes and mortality rates in men [1,2]. Evidence continues to grow that most cardiovascular disease is preventable and largely dependent on modifiable risk factors [3,4]. In addition, long-term prospective studies consistently show that women with low levels of risk factors have a life long protection against cardiovascular events [5]. Because of the still-high rate of fatal or severely disabling first cardiovascular events, there is a strong imperative to prevent the first episode of coronary heart disease or stroke by early identification of women at high risk [4]. Importantly, optimal benefit of primary prevention may be gained from matching the intensity of interventions to the hazard for cardiovascular events [6]. To achieve this goal, screening and intervention programs should first aim at further stratifying apparently healthy women into subgroups with known higher rates of cardiovascular mortality [4,6]. Because most major cardiovascular events in women do not occur until after menopause [1], attention is increasingly focused towards clinical events in younger women that predict cardiovascular health, such as adverse pregnancy outcome [7,8].

Hypertensive disorders of pregnancy affect over 4.2 million women worldwide and cause 65,000 maternal deaths each year [9]. Early-onset preeclampsia is its most serious presentation, defined by *de novo* hypertension and proteinuria in the second half of pregnancy, that requires delivery before 34 weeks gestational age [10-12]. Although of unknown origin, the clinical syndrome of preeclampsia is characterized by widespread endothelial damage with marked vascular compromise and metabolic changes, that are potentially lethal to both mother and baby [10,13]. Many features of preeclampsia resemble changes closely related to atherosclerosis, including dyslipidemia, insulin resistance [14], hypercoagulability and inflammation [15]. Although major clinical signs usually cease within days after delivery, mothers with previous preeclampsia are at an increased risk of coronary heart disease and stroke later in life [16]. Long-term maternal prognosis is especially poor for women who experienced early-onset disease (<37 weeks gestational age), in whom long-term follow-up studies revealed a 7- to 8-fold increased cardiovascular mortality, compared to a 2-fold increase in women with a history of late-onset preeclampsia [16]. This has led to some controversy over subclassification of preeclampsia with late and early onset, which some regard as a separate condition from preeclampsia occuring at term [12].

At present, there is limited knowledge on the underlying link between adverse pregnancy outcome and future cardiovascular risk. In addition, although history of early-onset preeclampsia exerts a major risk for future cardiovascular events, structured risk factor assessment programs after delivery are not routinely advocated. Apart from women with

known chronic hypertension, women with previous preeclampsia are generally lost to follow-up after delivery and comprehensive data on components of cardiovascular risk are limited. Few studies have measured risk factors predictive of cardiovascular disease after preeclampsia, and these studies have been limited to small numbers of women with mostly late-onset disease, and lack power for an integrated approach to estimate global risk [17-22].

To provide more information, we assessed major cardiovascular risk factors (age, systolic and diastolic blood pressure, body-mass index, smoking and diabetes) and measured levels of traditional cardiovascular biomarkers that have independent associations with first cardiovascular events[23] (total cholesterol, high- and low-density lipoprotein [HDL and LDL], fasting glucose and triglycerides) in a 12-year cohort of primiparous women with early-onset preeclampsia referred for tertiary care, at least 6 months after delivery, compared to an unselected population-based control group of apparently healthy women of similar age.

The aim of this study was to characterize major risk factors known to be predictive of first cardiovascular events in women with a history of early-onset preeclampsia. We sought to provide insight into shared maternal predisposition to both conditions, as well as to identify women at high risk of developing cardiovascular disease and to specify risk markers which might be used for primary prevention programs.

Methods

Study Population

Between November 1994 and January 2007, all women with a first pregnancy complicated by early-onset preeclampsia referred for tertiary care to the University Medical Center Utrecht, The Netherlands, were eligible to participate in the follow-up study, with the first visit planned at least six months after delivery. Preeclampsia was defined as the presence of gestational hypertension and concomitant proteinuria in the second half of pregnancy, based the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) [24]. According to ISSHP criteria, gestational hypertension was defined as diastolic blood pressure above 90 mm Hg and/or systolic blood pressure above 140 mm Hg, measured on two or more separate occasions at least 4 hours apart; proteinuria was diagnosed when above 300 mg per 24 hour or above 2+ at dipstick urinalysis. Early-onset preeclampsia was defined as preeclampsia, that required delivery before 34 completed weeks of gestational age. Coexistent hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome was defined according to previously described criteria [25], as hemolysis

(defined as serum lactate dehydrogenase (LDH) >600 U/L and/or haptoglobin ≤0.3 g/L), elevated liver enzymes (serum aspartate aminotransferase (AST) >70 U/L and/or serum alanine aminotransferase (ALT) >70 U/L), and a low platelet count (<100 × 10^9/L). Infants were considered small-for-gestational age (SGA), if birth weight was below the 5th centile, based on standardized Dutch population charts [26]. For the purpose of this study, we excluded women with preexistent chronic hypertension as defined by hypertension that required the use of antihypertensive medication before pregnancy. The population-based control group consisted of apparently healthy women recruited for the Atherosclerosis Risk in Young Adults (ARYA) study, that comprises an unselected population-based cohort of similar age, demographic and geographical background. Details of inclusion criteria, recruitment procedures, data and sample handling were previously described elsewhere [27]. At enrolment, demographic, general medical and obstetric data were recorded, and fasting blood samples were obtained for detection of metabolic, inflammatory and lipid risk factors. Risk factor assessment was performed at least six months after delivery and at least six weeks after discontinuation of breast feeding, and women were required to adhere to a minimum of six weeks without taking any vitamin or folic acid supplements. The study was approved by the institutional review board of the University Medical Center Utrecht and participants provided written informed consent.

Assessment of Classic Cardiovascular Risk Factors

The presence of diabetes and chronic hypertension was recorded and body-mass index was calculated, using self-reported height and weight at inclusion. Blood pressure was measured with non-automated validated sphygmomanometer by a trained research nurse, as the mean value of two separate measurements. Fasting blood samples were collected, immediately centrifuged and directly analysed for lipid markers, glucose and triglyceride levels by standard procedures at the routine Clinical Chemistry Laboratory of our hospital. A detailed description of measurements and laboratory procedures was previously published elsewhere [27-29]. Briefly, fasting total cholesterol, HDL-cholesterol, triglycerides and glucose were determined using a Vitros950 dry-chemistry analyzer (Johnson & Johnson, Rochester, NY). LDL cholesterol was calculated using the Friedewald formula. Within-run variation coefficients were 1.7% for total cholesterol, 2.3% for HDL-cholesterol, 1.9% for triglycerides and 4.3% for fasting glucose levels. All analyses were performed by technicians blinded for outcome.

Statistical Methods

Statistical analyses were performed using SPSS version 12.0.1 (SPSS Inc.). Baseline variables were expressed as means and standard deviation (SD), or number and percentage where

appropriate, and comparisons were made between women with a history of early-onset preeclampsia (cases) and population-based controls by the independent samples T-test and chi-squared test, respectively. Next, plasma inflammatory markers were summarized as medians and interquartile range and comparisons between cases and controls were made by the non-parametric Mann-Whitney *U* test. To account for missing data (<5% for all variables), a common and previously described imputational method using regression lines was used [30]. Subsequently, stratified analyses were performed for body-mass index (BMI), comparing the number of cases and controls in increasing quintiles, with cut-off points based on the distribution of values within the control population; odds ratios and corresponding 95 percent confidence intervals (CIs) were calculated for each quintile compared to the lowest quintile as a set reference group. Further data analysis included comparisons of multiple risk factors and estimation of 10-year absolute cardiovascular risk by a validated global risk factor algorithm based on the Framingham Heart Study [23], that includes weighted risk scores for age, smoking status, systolic and diastolic blood pressure, diabetes, total cholesterol and HDL-cholesterol. Next, we estimated the global risk score by modeling the 10-year risk extrapolated to the age of 60 years, as is recommended in the European guidelines (Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Practice) [31]. Where appropriate, logistic regression models were age-adjusted and controlled for potential confounders.

Results

Baseline Characteristics, First Pregnancy Outcome and Determinants of Cardiovascular Risk

Baseline clinical and outcome characteristics of the study group and the population-based controls are summarized in **Table 1**. Compared to women with previous early-onset preeclampsia, controls had a small but significant difference in mean (SD) age at inclusion of –2.2 (.24) years, that only marginally affected all of the other baseline variables and for which was adjusted in the subsequent logistic regression models. In our 12-year tertiary referral cohort, first pregnancies with early-onset preeclampsia had a mean age at delivery of less than 30 completed gestational weeks, and were characterized by high rates of concurrent maternal and fetal complications that included 62% of women who met the criteria for HELLP syndrome, 11% placental abruptions and more than half of infants born small-for-gestational (birth weight <5th centile) due to severe intrauterine growth restriction. At a mean (SD) interval of .78 (.95) years after delivery, primiparous women with previous early-onset preeclampsia had higher systolic and diastolic blood pressure, weight, and BMI.

Table 1 Baseline characteristics, first pregnancy outcome and determinants of cardiovascular risk

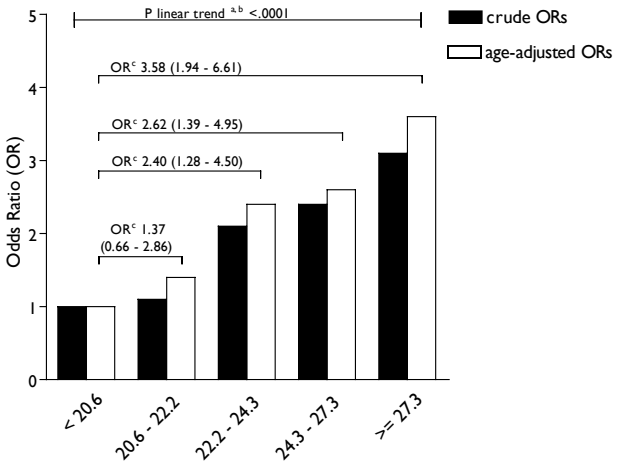
	Early-Onset Preeclampsia (N=243)	Controls (N=374)	P Value
Age, mean (SD), yr	30.5 (4.5)	28.3 (0.9)	<.001
Height, mean (SD), cm	170 (6.6)	170 (6.4)	.302
Weight, mean (SD), kg	75 (16)	70 (14)	<.001
Body-mass index, mean (SD), kg/m^2	26.1 (5.5)	24.3 (4.6)	<.001
White race, no. (%)	242 (99.6)	370 (98.9)	.373
Gravidity, mean (SD)	1 (1)	1 (1)	-
Parity, mean (SD)	1 (0)	0 (1)	-
Characteristics of first pregnancy (cases only) †			
Gestational age at delivery, mean (SD), wk	29.8 (2.4)	-	-
Infant's birth weight, mean (SD), g	1083 (452)	-	-
Hellp-syndrome, no. (%)	150 (62)	-	-
Placental abruption, no. (%)	27 (11)	-	-
Small-for-gestational-age infant (<5th centile), no. (%)	148 (61)	-	-
Blood pressure, mean (SD), mm Hg			
Systolic	126 (13)	120 (12)	<.001
Diastolic	79 (10)	70 (8)	<.001
Plasma lipid profile			
Total cholesterol, mean (SD), mg/dL	198 (41)	186 (32)	<.001
HDL-cholesterol, mean (SD), mg/dL	55 (14)	61 (14)	<.001
LDL-cholesterol, mean (SD), mg/dL	119 (36)	104 (31)	<.001
Triglycerides, mean (SD), mg/dL	121 (76)	108 (49)	.009
Total cholesterol to HDL-cholesterol ratio, mean (SD), mg/dL	3.81 (1.22)	3.21 (0.96)	<.001
Fasting blood glucose, mean (SD), mmol/L	5.1 (0.6)	4.8 (0.4)	<.001
Diabetes, no. (%)	3 (1.2)	2 (0.5)	.085
Current smoker, no. (%)	76 (25)	105 (26)	.774
The metabolic syndrome*, no. (%)	34 (14.2)	15 (4.0)	<.001
Body-mass index > 30 kg/m^2	39 (16.3)	35 (9.4)	.020
Triglycerides >=150 mg/dL	52 (21.7)	57 (15.3)	.137
HDL-cholesterol < 50 mg/dL	95 (39.6)	72 (19.4)	<.001
Diastolic blood pressure >= 85 mm Hg, systolic blood pressure >=130 mm Hg	121 (50.4)	76 (20.4)	<.001
Fasting blood glucose >= 6.11 mmol/L	3 (1.3)	1 (0.3)	.706

Data are expressed as means ± SD, or as number (%) and compared by the age-adjusted logistic regression analysis.
† Data represent outcomes of first pregnancy.
** Defined as having 3 or more risk factors described in the subcategories, excluding women with known diabetes mellitus.*

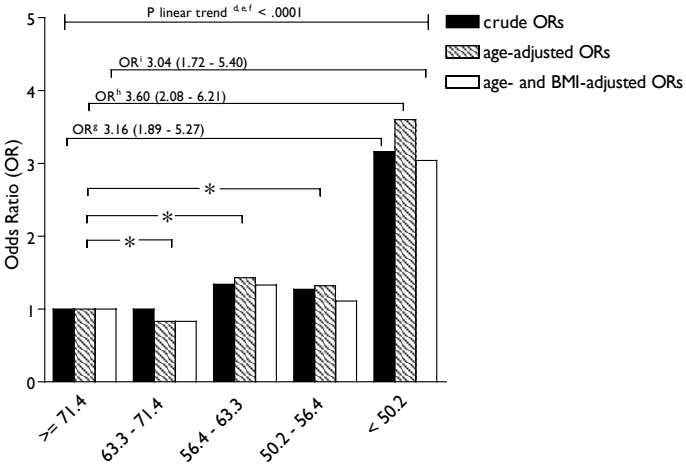
In comparison to controls, concentrations of total cholesterol, LDL cholesterol, total cholesterol to HDL-cholesterol ratio, triglycerides and glucose were also higher after early-onset preeclampsia. Of women with previous early-onset preeclampsia 14.2% met the criteria for metabolic syndrome (**Table 1**), compared to 4% of controls (age-adjusted odds ratio 3.6 [95% CI 1.8 – 7.2]). No significant differences were observed in height, ethnicity, diabetes and current smoking.

Figure 1 Association between quintiles of body-mass index, plasma lipid profile, high blood pressure and a history of early-onset preeclampsia

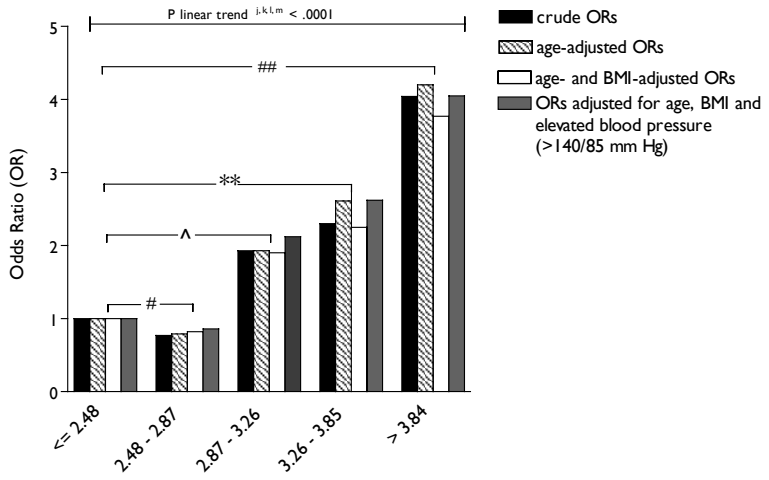
A Body-mass index quintiles



B HDL-cholesterol quintiles



C Total cholesterol to HDL-cholesterol ratio, quintiles



Odds ratios for women stratified by quintiles of body-mass index (BMI), calculated from the distribution of BMI levels in the population-based control group, by univariate and multivariate logistic regression analysis, with the lowest quintile as reference. Similar analyses were performed for quintiles of HDL-cholesterol, and total cholesterol to HDL-cholesterol ratio, each with the lowest quintile as reference group. Closed black bars represent crude odds ratios for early-onset preeclampsia, compared to controls, according increasing quintiles of either BMI, HDL-cholesterol, or total cholesterol to HDL-cholesterol ratio. Accordingly, dashed bars represent odds ratios for early-onset preeclampsia adjusted for age at inclusion, open bars represent odds ratios adjusted for age at inclusion and BMI, and grey bars represent odds ratios adjusted for age at inclusion, BMI and elevated blood pressure, i.e. systolic blood pressure > 140 mm Hg, or diastolic blood pressure > 85 mm Hg.

[a]Linear trend among increasing quintiles of BMI

[b]Linear trend among increasing quintiles of BMI, adjusted for age

[c]Age-adjusted odds ratios and 95% confidence intervals for each quintile of BMI

[d]Linear trend among increasing quintiles of HDL-cholesterol

[e]Linear trend among increasing quintiles of HDL-cholesterol, adjusted for age

[f]Linear trend among increasing quintiles of HDL-cholesterol, adjusted for age and BMI

[g]Crude odds ratio and 95% confidence interval (CI) for the lowest quintile of HDL-cholesterol (<50.2 mg/dL), compared to the highest quintile for HDL-cholesterol (≥71.4 mg/dL)

[h]Age-adjusted odds ratio and 95% CI for the lowest quintile of HDL-cholesterol (similar to [g]), compared to the highest quintile for HDL-cholesterol

[i]Odds ratio and 95% CI for the lowest quintile of HDL-cholesterol, compared to the highest quintile for HDL-cholesterol, adjusted for age and BMI

[j]Linear trend among increasing quintiles of total cholesterol to HDL-cholesterol ratio

[k]Linear trend among increasing quintiles of total cholesterol to HDL-cholesterol ratio, adjusted for age,

[l]Linear trend among increasing quintiles of total cholesterol to HDL-cholesterol ratio, adjusted for age and BMI,

**No significant assocation (P>.05) was observed between each of these lower HDL-cholesterol quintiles and previous early-onset preeclampsia, compared to the highest quintile of HDL-cholesterol as reference*

#No significant assocation (P>.05) was observed between each of these higher total cholesterol to HDL-cholesterol ratio quintiles and previous early-onset preeclampsia, compared to the lowest quintile of total cholesterol to HDL-cholesterol ratio as reference

^Odds ratio for early-onset preeclampsia according the third higher quintile of total cholesterol to HDL-cholesterol ratio, as compared to the lowest quintile; crude odds ratio 1.93 (95% CI 1.07 to 3.47; P=.028); odds ratio adjusted for age 1.93 (95% CI 1.01 to 3.69; P=.046); odds ratio adjusted for age and BMI 1.90 (95% CI 0.99 to 3.66; P=.054); odds ratio adjusted for age, BMI and elevated blood pressure 2.12 (95% CI 1.07 to 4.18; P=.030)

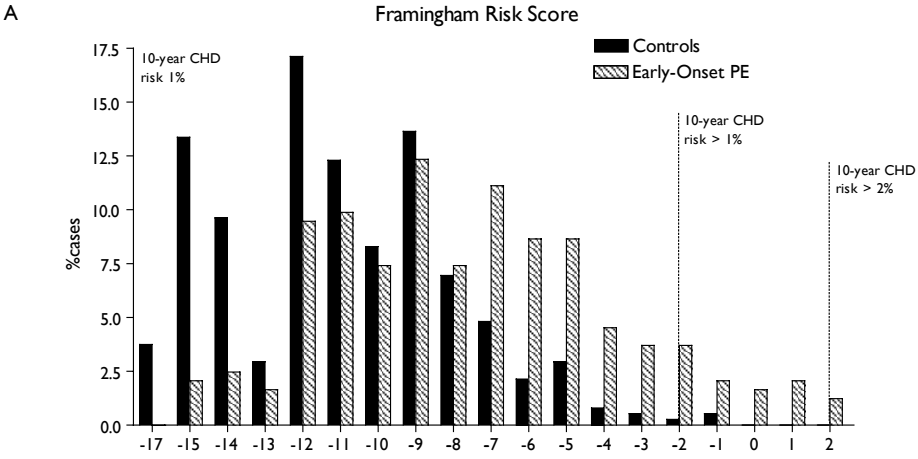
***Odds ratio for early-onset preeclampsia according the fourth higher quintile of total cholesterol to HDL-cholesterol ratio, as compared to the lowest quintile; crude odds ratio 2.30 (95% CI 1.29 to 4.10; P=.004); odds ratio adjusted for age 2.61 (95% CI 1.38 to 4.93; P=.003); odds ratio adjusted for age and BMI 2.25 (95% CI 1.17 to 4.34; P=.016); odds ratio adjusted for age, BMI and elevated blood pressure 2.62 (95% CI 1.32 to 5.20; P=.006)*

##Odds ratio for early-onset preeclampsia according the highest quintile of total cholesterol to HDL-cholesterol ratio, as compared to the lowest quintile; crude odds ratio 4.04 (95% CI 2.33 to 7.01; P<.001); odds ratio adjusted for age 4.20 (95% CI 2.35 to 7.51; P<.001); odds ratio adjusted for age and BMI 3.77 (95% CI 2.06 to 6.92; P<.001); odds ratio adjusted for age, BMI and elevated blood pressure 4.05 (95% CI 2.18 to 7.53; P<.001)

We observed a linear association of BMI with previous early-onset preeclampsia (**Figure 1**). When we examined different cut-off levels for BMI based on the normal distribution within the control group, higher quintiles of BMI were associated with higher odds ratios for previous preeclampsia. For example, after adjustment for age, the highest quintile of BMI, compared with the lowest, was associated with an odds ratio of 3.6 [95% CI 1.9 – 6.6]) for previous preeclampsia. When applying clinical criteria for overweight (BMI>25 and <30) and obesity (BMI>30), women with previous early-onset preeclampsia were overweight in 34.2% and obese in 16.9%, with an overall age-adjusted odds ratio of 2.0 (95% CI 1.4 – 2.8) for having a BMI>25. Similar associations were observed with lower quintiles of HDL-cholesterol (**Figure 1**). The lowest quintile of HDL-cholesterol, compared with the highest, was associated with an age-adjusted odds ratio of 3.6 (95% CI 2.1 – 6.2), that was only moderately attenuated after additional adjustment for BMI (odds ratio 3.0 [95% CI 1.7 – 5.4]).

Multiple classic cardiovascular risk factors, as defined by the AHA/ACC criteria (**Table 2**) [32], were present in 54% of women with previous early-onset preeclampsia, compared to 23% of controls (P<.001), including 18% of formerly preeclamptic women with ≥ 3 classic independent risk factors for cardiovascular disease, compared to 3% of controls (P<.001).

Figure 2 Framingham risk scores of women with previous early-onset preeclampsia and controls at least six months after delivery and extrapolated to the age of 60 years



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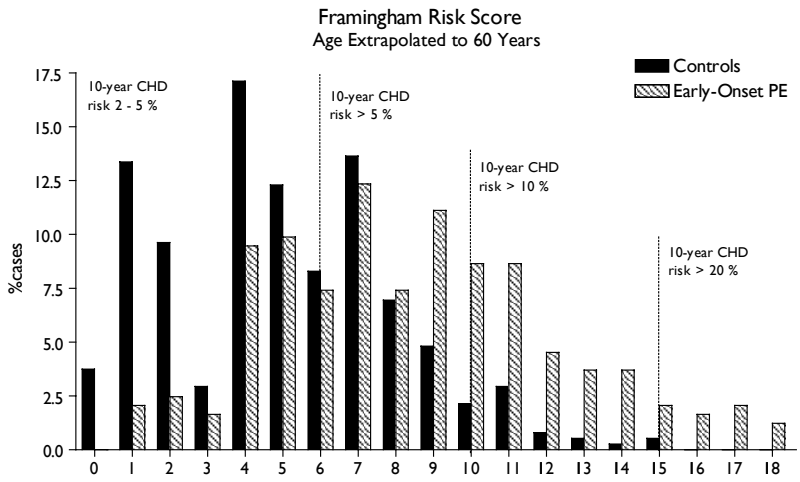

Distribution of values for estimated global cardiovascular risk score by the Framingham algorithm, based on major independent cardiovascular risk factors, i.e. age, diabetes, smoking, systolic and diastolic blood pressure, total cholesterol and HDL-cholesterol levels, for women with a history of early-onset preeclampsia (dashed bars) and population-based controls (closed bars).

Table 2 Association Between Classic Risk Factors Predictive of Cardiovascular Events[a] and Previous Early-Onset Preeclampsia

	Unadjusted OR (95% CI)	Age-adjusted OR (95% CI)	P[d] Value
Blood pressure ≥ 140/90 mm Hg[a]	5.12 (3.15 – 8.61)	3.49 (2.04 – 5.99)	<.001
Total cholesterol ≥ 200 mg/dL[a]	1.85 (1.33 – 2.59)	1.53 (1.07 – 2.19)	.019
HDL-cholesterol < 40 mg/dL[a]	2.85 (1.52 – 5.30)	3.82 (1.95 – 7.47)	<.001
Current Smoking, %[a]	0.89 (0.62 – 1.29)	1.07 (0.72 – 1.58)	.748
Diabetes[b], %[a]	2.33 (0.39 – 14.02)	1.47 (0.20 – 10.7)	.701
Blood pressure ≥ 140/90 mm Hg and total cholesterol ≥ 200 mg/dL	6.29 (3.09 – 12.79)	4.43 (2.10 – 9.33)	<.001
Blood pressure ≥ 140/90 mm Hg and HDL-cholesterol < 40 mg/dL	9.48 (2.02 – 44.39)	10.50 (2.01 – 54.83)	.005
Blood pressure ≥ 140/90 mm Hg, total cholesterol ≥ 200 mg/dL and HDL-cholesterol < 40 mg/dL	11.36 (1.25 – 103.16)	11.10 (1.15 – 107.06)	.037
≥ 1 Classic Risk Factor[c]	2.14 (1.76 – 3.60)	2.14 (1.47 – 3.12)	<.001
≥ 2 Classic Risk Factors[c]	3.91 (2.44 – 6.25)	3.18 (1.93 – 5.24)	<.001
≥ 3 Classic Risk Factors[c]	6.13 (2.23 – 16.87)	4.86 (1.65 – 14.32)	.004

[a]Major independent risk factors according to the American Heart Association and American College of Cardiology consensus statement using prediction algorithms from the Framingham Heart Study [Grundy SM et al, JACC 1999]
[b]Diabetes was only present in 3 cases and 2 controls
[c]The presence of 1 or more major independent risk factors as mentioned[a], compared to subjects without any of the major risk factors as controls

Estimated 10-year Absolute Risk of a First Cardiovascular Event

Results of global cardiovascular risk assessment, using the five independent classic risk factors predictive of a first cardiovascular event (age, diabetes, smoking, systolic and diastolic blood pressure, total and HDL-cholesterol), as estimated by the Framingham Risk Score (FRS) algorithm [23], are shown in **Figure 2**. In comparison with controls, the distribution of FRS values in women with previous early-onset preeclampsia showed a relative shift towards higher estimated global risk. However, due to young age, absolute 10-year risk of a first cardiovascular event is low (< 5%) for all women. Nonetheless, based a the observed classic risk factor profile, estimated long-term risk is high for the majority of women with previous early-onset preeclampsia (**Figure 2**). For example, if extrapolated to the age of 60 years, low-risk (< 5%) was present in only 7.0% of women with previous preeclampsia, compared to 29.7% of controls (OR 0.18 [95% 0.10 – 0.31]); high risk (> 10% and ≤ 20%) was present in 35.8% of cases compared to 11.2% of controls (OR 13.52 [95% 7.21 – 25.38], compared to low-risk [< 5%] women as reference); and very high risk (> 20%) was present in 2.8% of cases compared to 0.8 % of controls (OR 15.24 [95% 3.59 – 64.67], compared to low-risk [< 5%] women).

Comment

In this follow-up study of a 12-year population of 243 apparently healthy primiparous women with a history of early-onset preeclampsia, we found high rates of multiple major classic risk factors predictive of cardiovascular events, when compared to a population based age-matched control group, at least six months after delivery. Women with previous early-onset preeclampsia more often show marked dyslipidemia, high blood pressure, high body-mass index, and other components of the metabolic syndrome, including higher fasting glucose and triglyceride levels. Our results demonstrate that more than half of women with previous early-onset preeclampsia exhibit two or more major modifiable risk factors for future CVD. Based on these independent cardiovascular risk factors, global risk estimation with the Framingham multiple risk score at a mean age of 30.5 years, showed higher overall predicted risk of cardiovascular events in women with a history of early-onset preeclampsia, compared to population-based controls. Although most of these women are classified as low-risk (absolute 10-year risk < 5 %) according to the current AHA/ACC guidelines [4], this is mainly due to their young age masking other, mostly modifiable, major risk factors.

Large-scale epidemiological studies have consistently shown a link between preeclampsia and future cardiovascular disease. After preeclampsia, women have an increased risk of fatal

and non-fatal coronary heart disease, stroke, hypertension and venous thromboembolism in later life [16]. Early-onset preeclampsia (< 34 weeks of gestation) is associated with an even greater relative risk of ischemic heart disease, which was estimated at 7.71, 95 percent confidence interval 4.40 to 13.52, in a recent meta-analysis [16]. The underlying link between early-onset preeclampsia and long-term cardiovascular disease is unclear. Findings from our study strongly support the involvement of traditional independent risk factors, that include pro-atherogenic lipid profile, high blood pressure and markers of insulin resistance. Indeed, of mothers with previous first pregnancy early-onset preeclampsia, 1 out of 7 women met the criteria for metabolic syndrome, that is generally considered to be a precursor of type 2 diabetes mellitus and atherosclerosis [33].

Although preeclampsia is widely believed to be a state of generalized maternal endothelial activation in response to abnormal placental development,[10,13] it is likely that early-onset preeclampsia and cardiovascular diseases have a common pathogenesis inititiated by similar risk factors. Thus, early-onset preeclampsia may be considered as an early cardiovascular risk marker. Indeed, our findings match those from previous of women with a history of late-onset preeclampsia (> 34 weeks gestational age), although of relatively small sample size, that report signs of dyslipidemia, insulin resistance and high blood pressure remote from delivery [17-22]. Although some contoversy exists [12], our study supports the notion that women with early-onset preeclampsia exhibit a constitutional risk profile after delivery, that matches women with previous late-onset disease, albeit more pronounced. Early- and late-onset preeclampsia are therefore likely to represent clinical extremes of the same condition, the onset of disease being dependent on the presence of multiple maternal constitutional factors, also common to cardiovascular disease in later life [10,12].

The observed associations between previous preeclampsia and markers of cardiovascular risk cannot be entirely explained by differences in maternal weight and body-mass index. In contrast to recent population data from the United States, that estimated 49% of non-Hispanic white women age 20 to 40 years to be overweight or obese and 25% of these to be obese in 2002 [34], in The Netherlands overall prevalence of adiposity and obesity was lower with 33% of women being overweight and 9.9% obese [35]. We observed comparable adiposity (34%) and obesity (9.6%) rates in our population-based control group, and in this study approximately half of women with previous early-onset preeclampsia were overweight or obese, that included no more than 17% obese women. We found a linear association with body-mass index and a history of early-onset preeclampsia. Surprisingly, this only had a moderate effect on fasting levels of HDL-cholesterol and total cholesterol to HDL-cholesterol ratio. Our findings are consistent with previous studies that showed higher body-mass index and higher weight circumference after preeclampsia, which is thought

to contribute to development of the metabolic syndrome [18,19,36]. In addition, in older women, high body-mass index is strongly related to adverse cardiovascular biomarker levels and provides an important target for primary prevention of cardiovascular disease by diet and physical activity [37,38].

Importantly, we studied apparently healthy women without known cardiovascular disorders at the time of inclusion, and without preexistent chronic hypertension. Nonetheless, mean systolic and diastolic blood pressure were higher in women with previous early-onset preeclampsia and subclinical mild hypertension (i.e. systolic blood pressure > 130 mm Hg, and/or diastolic blood pressure > 85 mm Hg), was present in 1 out of 2 women, compared to 1 out of 5 age-matched population-based controls. This is an important finding, because mild blood pressure elevation may precede the onset of chronic hypertension and contributes to overall cardiovascular risk [39]. However, clustering of multiple risk factors was not exclusive to hypertensive women. Indeed, when stratifying risk factors according to blood pressure, in women with low blood pressure (i.e. systolic blood pressure ≤ 130 mm Hg and diastolic blood pressure ≤ 85 mm Hg), history of early-onset preeclampsia showed similar associations with increased total cholesterol to HDL-cholesterol ratio (age-adjusted P<.001) and higher fasting glucose levels (age-adjusted P<.001). Furthermore, for women with low blood pressure, the assocations persisted after additional adjustment for BMI (age-adjusted P<.001 for previous early-onset preeclampsia and increased total cholesterol to HDL-cholesterol ratio, and age-adjusted values P<.001 for increased fasting glucose levels, in comparison to population-based controls as reference). Therefore, it is unlikely that the observed abnormal lipid profile after preeclampsia can be attributed to the presence of subclinical hypertension or high body-mass index alone.

Our findings have several important implications for clinical practice. At present, despite the considerably higher predicted long-term risk of first cardiovascular events, routine assessment of cardiovascular risk factors is not routinely performed in women with previous early-onset preeclampsia. However, in recent years, it has become clear that most cardiovascular disease is preventable and related to exposure to modifiable risk factors. Therefore, in our study, the presence of multiple traditional cardiovascular risk factors provides an opportunity for primary prevention of cardiovascular events in women with previous early-onset preeclampsia. Preventive strategies are best matched to those individuals considered at high risk (i.e. absolute cardiovascular risk > 5%, in the next 10 years or if extrapolated to the age of 60 years), based on the current AHA/ACC guidelines, as well as guidelines from the European Society of Cardiology (ESC). Most likely, women with previous early-onset preeclampsia will benefit most from an overall healthy lifestyle, that includes regular excercise, consuming a healthy diet, maintaining a desirable body weight and discontinuing smoking. Nonetheless, although absolute

10-year global cardiovascular risk was < 5% for all women in our study, a proportion of women may require more aggressive risk factor reduction that includes antihypertensive treatment and pharmacological control of dyslipidemia. For example, in our study 22.2% of women with previous early-onset preeclampsia met the AHA/ACC criteria for LDL-lowering drug therapy, as defined by the presence of ≥1 major risk factor and blood LDL-cholesterol ≥ 160 mg/dL, or the presence of ≥ 2 risk factors and blood LDL-cholesterol ≥ 130 mg/dL. In addition, ESC guidelines recommend that in young women, the desired goal of lipid lowering therapy should be lowered to < 100 mg/dL for LDL-cholesterol and <175 mg/dL for total cholesterol, for those women with an estimated absolute cardiovascular risk of > 5% if extrapolated to the age of 60 years (82% of women with a history of early-onset preeclampsia). However, adherence to and effectiveness of risk factor reduction programs in women with previous early-onset preeclampsia are presently unclear and await the results of appropriate intervention trials.

There are several limitations of this study. The study was cross-sectional in design and women were included after a first pregnancy complicated by early-onset preeclampsia. Therefore a causal relationship between cardiovascular risk factors and the development early-onset preeclampsia cannot be inferred. Obviously, from retrospective analysis of cardiovascular risk factors after early-onset preeclampsia, it is not possible to determine whether these changes were present before the indicated pregnancy. Although some prospective studies support evidence for preexistent constitutional risk factors in women destined to develop preeclampsia [11,40], data from these studies are inconclusive for early-onset disease. It cannot be ruled out that maternal adaptation to pregnancy itself leads to long-term metabolic, cardiovascular and inflammatory changes, and that early-onset preeclampsia leaves a permanent 'scar'. In an attempt to exclude any temporary effects, we enrolled women at least six months after delivery. Furthermore, levels of cardiovascular risk factors were not influenced by the interval between delivery and assessment, suggesting that biomarker levels returned to baseline at the time of inclusion. However, to provide a more conclusive answer to this question, prospective data from a large cohort of pregnant women with pre-pregnancy risk factor assessment would be required. In the current study, we examined only data on traditional risk factors for cardiovascular disease, based on their independent contribution to global cardiovascular risk [23]. Recent studies, however, have demonstrated that for women up to 20% of coronary events occur in the absence of these major risk factors [41]. Therefore, it is likely that additional non-traditional risk factors contribute to the link between early-onset preeclampsia and future cardiovascular disease. Possible candidate factors that are associated with a history of preeclampsia, and are predictive of long-term cardiovascular risk, include C-reactive protein [42,43], interleukin-6 [44,45], fibrinogen [44,45], family

history of premature cardiovascular disease [43,46], dietary patterns and physical activity [3,47]. Furthermore, our study was designed to include only primiparous women with previous early-onset preeclampsia, who delivered before 34 weeks of gestation. Therefore, our data may not be extrapolated to multiparae and women with a history of late-onset or near term preeclampsia. Although similarities are evident, pathophysiologic features of the disorder leading to onset after 34 weeks' gestation might be different from those observed in early-onset disease [12].

Strengths of the present study include a large number of apparently healthy women with previous early-onset preeclampsia included over a 12-year interval, with comprehensive measurements of traditional cardiovascular biomarkers. In addition, our study summarizes the first data on predicted global cardiovascular risk after early-onset preeclampsia. Therefore, our results may be of clinical importance, since individual strategies to prevent cardiovascular sequelae after early-onset preeclampsia are best aimed at multiple modifiable risk factors and estimated long-term absolute risk [4,31]. Finally, few studies have examined risk factors after preeclampsia, in comparison to a population-based control group. Rather, most studies have compared post partum findings in women with previous preeclampsia to a control group of women who experienced uneventful pregnancies. This is important, because normotensive pregnancy predicts a high probability of being free from long-term hypertensive and cardiovascular sequalae [48]. Therefore, the use of a population-based control group provides more appropriate risk estimates useful for clinical counseling.

In summary, we found evidence that first pregnancy early-onset preeclampsia is associated with high rates of multiple modifiable classic risk factors predictive of cardiovascular disease, at least six months after delivery. The results of our study have implications for follow-up and counseling women after early-onset preeclampsia and justify raised awareness that women with previous early-onset preeclampsia require appropriate recordings of blood pressure, diabetes, smoking status, measurement plasma total cholesterol, LDL-cholesterol or HDL-cholesterol in the first year after delivery, aimed at primary prevention of cardiovascular disease.

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5

Ischemia Modified Albumin in Normal Pregnancy and Preeclampsia

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Abstract

Objective

Ischemia-modified albumin (IMA) has emerged as a new biomarker of myocardial ischemia. Currently, no information is available on maternal IMA levels during normal and complicated pregnancy. Preeclampsia is associated with ischemia and increased formation of free radicals in the placenta. We therefore hypothesized that production of IMA may occur in preeclamptic pregnancy.

Methods

Serum IMA and albumin concentrations were assessed in 12 patients with preeclampsia, 12 normal pregnant controls, and 12 non-pregnant controls. IMA levels were compared between groups and corrected for albumin by multivariate regression analysis.

Results

Mean IMA levels were elevated in normal pregnant controls (107.3 U/mL, 95% CI 102.5 to 112.01), compared to non-pregnant controls (94.5 U/mL, CI 89.4 to 99.6; p=0.015). In patients with preeclampsia, IMA levels were similar to normal pregnant controls (109.7 U/mL, CI 102.2 to 117.2; p=0.65). Also, no difference in IMA levels was observed between preeclamptic women who delivered small-for-gestational-age (SGA) infants (99.0 U/mL, CI 87.9 to 110.1; p=0.13) and preeclamptic women without SGA.

Conclusion

Serum IMA, which has been advocated as a clinical marker of cardiac ischemia, appears to be elevated during normal pregnancy. We found no significant relationship between IMA levels and preeclampsia, with or without SGA.

Introduction

Ischemia-modified albumin (IMA), as measured by the albumin cobalt binding test [1] has been advocated as a new biological marker for myocardial ischemia [2]. Under physiological conditions, transition metals are able to bind tightly to the N-terminus of albumin. In the presence of ischemia however, reactive oxygen species are thought to cause structural changes in the N-terminus of the protein, which reduce this binding capacity [3]. IMA levels rise in the early subclinical stages of myocardial infarction, before the appearance of other biomarkers, such as cardiac troponins, CK-MB, and electrocardiographic abnormalities [2]. Recent reports show IMA changes during conditions other than cardiac ischemia, raising concerns about its tissue-specificity, and suggesting a possible role as a biomarker for other oxidative stress or ischemia related diseases [4].

Currently, no information is available on maternal IMA levels during normal and complicated pregnancy. Preeclampsia is characterized by generalized dysfunction of the endothelium, which is widely regarded as a consequence of placental ischemia that also explains concurrent fetal growth restriction [5]. We have shown before that preeclampsia is associated with increased formation of free radicals in the placenta, which is most likely due to ongoing ischemia [6]. We therefore hypothesized that excessive production of IMA may occur in preeclamptic pregnancy.

Methods

Serum IMA and serum albumin concentrations were assessed in 12 patients with preeclampsia, 12 gestational-age matched normal pregnant controls, and 12 age matched non-pregnant controls. Patients and controls were recruited from the obstetric population of the University Medical Center Utrecht, The Netherlands. The study was approved by the Institutional Review Board and informed consent was obtained from all participants. Preeclampsia was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) as *de novo* hypertension and proteinuria (≥300 mg/24 h) [7]. The syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP syndrome) was defined as a platelet count $<100\times10^9$/L, an amino aspartate transferase concentration >70 IU/L and a lactate dehydrogenase concentration >600 IU/L [8]. Small-for-gestational age (SGA) was defined as birth weight below the 5th centile for the Dutch population [9]. Serum samples were obtained after withdrawing 10 mL venous blood by antecubital venipuncture using Vacutainer® tubes without any additive, and immediately stored at –80 °C.

Serum IMA was measured by the Albumin Cobalt Binding (ACB) test (Ischemia Technologies, Inc., Colorado, USA), according to the modified manufacturers protocol on a Hitachi 911 colorimetric analyzer, as described previously [2]. All samples were measured in a single run. The intra-assay CV was 6%, which is in accordance with the manufacturer's references. Serum albumin levels were assessed by Bromocresol Green on a Vitros colorimetric analyzer in our routine clinical chemistry laboratory. Mean serum levels were compared between groups by univariate logistic regression analysis and corrected for serum albumin levels by multivariate logistic regression analysis.

Results

Baseline and outcome characteristics of the study population are shown in the Table. No significant differences were found in maternal age, gestational age at sampling, gravidity, parity and smoking rate between the study groups. As expected, preeclamptic women delivered at an earlier gestational age, and had infants with a significantly lower birth weight. Results of serum albumin and IMA measurements are shown in **Figure 1**. Mean IMA levels were elevated in normal pregnant controls (107.3 U/mL, 95% confidence interval (CI) 102.5 to 112.01) as compared to non-pregnant controls (94.5 U/mL, 95% CI 89.4 to 99.6; p=0.015 after correction for albumin). In patients with preeclampsia, IMA levels were similar to normal pregnant controls (109.7 U/mL, 95% CI 102.2 to 117.2; p=0.65). No significant difference in mean IMA level was observed between preeclamptic women who delivered SGA infants (N=4, 99.0 U/mL, 95% CI 87.9 to 110.1; p=0.13) and preeclamptic women without SGA, or normal pregnant controls. Also, mean IMA levels of women with preeclampsia and HELLP syndrome (N=4, 103.0 U/mL, 95% CI 89.4 to 116.6; p=0.88) were comparable to women with preeclampsia only. As shown in **Figure 2**, we found a statistically significant negative correlation between serum albumin and IMA levels (Pearson r = -0.44, p= 0.007). Moreover, there was a weak negative correlation between corrected IMA levels and infant's birth weight (r = -0.34, p= 0.04).

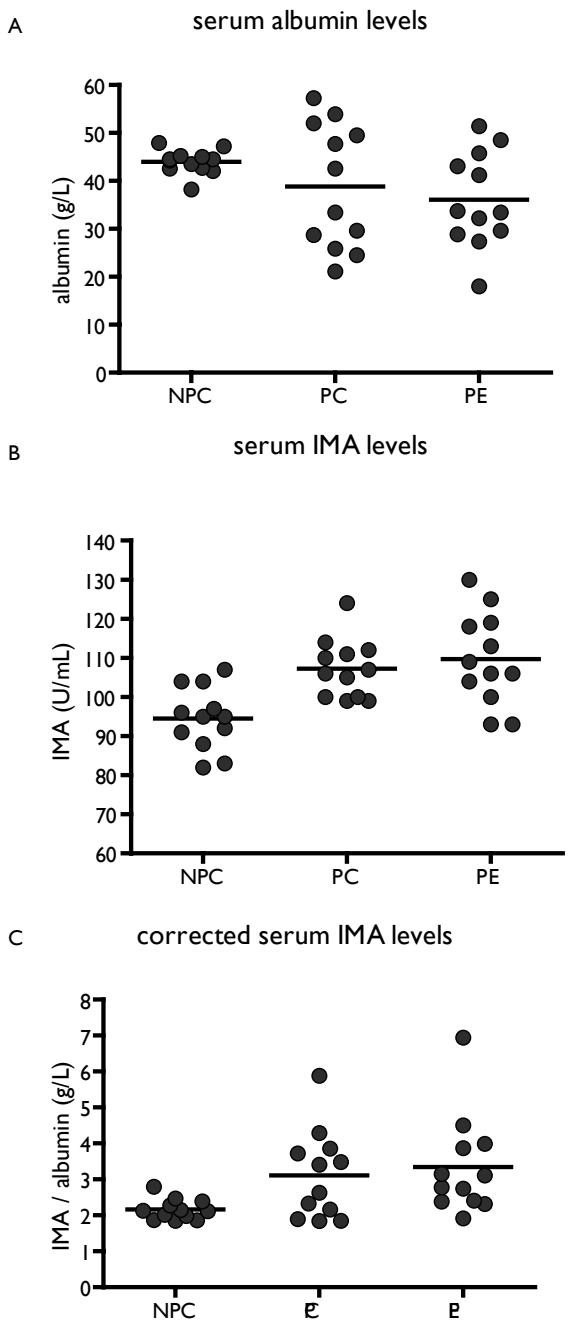

Figure 1 Serum IMA, albumin and IMA/albumin-ratio levels, as measured among women with preeclampsia (PE), gestational-age matched pregnant controls (PC) and age-matched non-pregnant controls (NPC). Horizontal reference lines represent the mean value for each study group.

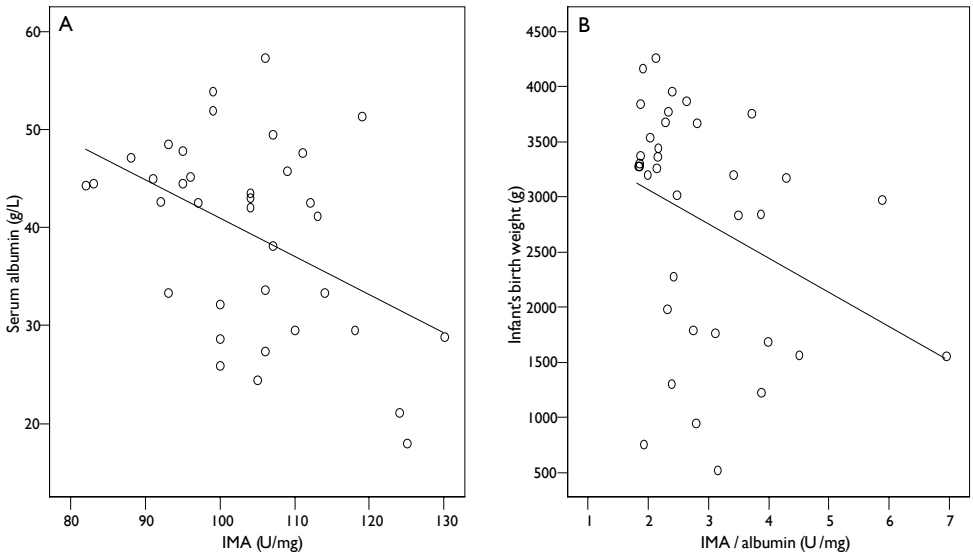

Figure 2 Correlation between serum albumin levels (g/L) and serum IMA levels (U/mL) (above) and between corrected serum IMA levels (U/mg) and infant's birthweight (g) (below).

Table 1 Baseline and outcome characteristics.*

Characteristic	Preeclampsia (N=12)	Pregnant Controls (N=12)	Non-pregnant Controls (N=12)	P Value
Maternal age – yrs	32 ± 3	34 ± 3	32 ± 4	
Gestational age at sampling - wks	31 ± 3	30 ± 3	-	
Gestational age at delivery - wks	32 ± 3	39 ± 1	-	< 0.0001
Birth weight - g	1451 ± 516	3464 ± 466	-	< 0.0001
Gravidity – mean no.	2 ± 1	2 ± 2	-	
Parity – mean no.	0 ± 1	1 ± 2	1 ± 0	
Current smoker – no.	4	3	1	
HELLP syndrome – no.	4	-	-	
Small-for-gestational-age infant (<5th centile) – no.	4	-	-	

**Data are expressed as mean ± SD or number. P values are given only for significant differences between the preeclampsia and normal pregnant control group.*

Discussion

Serum IMA, which has recently been developed as a clinical marker of ongoing myocardial ischemia, appears to be elevated during normal pregnancy. Many previous reports have documented ongoing ischemia and formation of oxygen free radicals during physiological pregnancy [10]. Normal pregnancy is characterized by a marked maternal adaptive response which includes activation of inflammation, endothelial cell activity and coagulation of the mother, as well as production of many pro-oxidant and vasoactive substances by the placenta. Preeclampsia is characterized by inappropriate generalized activation and dysfunction of the maternal inflammatory system and vascular endothelium [5].

Most circulating biomarkers for the measurement of ongoing ischemia and oxidative stress have been proven unreliable during pregnancy. Caution is needed when applying these methods, because of a relatively low specificity for organ-specific tissue damage and wide biological and inter-assay variability. Also, biokinetic and biodynamic changes, as well as upregulation of many metabolic and vascular systems occurs during the normal pregnant state [10,11]. Serum IMA has been proposed as a promising new marker to monitor ongoing ischemia. Although early reports indicated a high sensitivity and specificity for myocardial ischemia [2,4,12], others have questioned its tissue-specificity since elevated IMA levels have been observed during a number of other clinical conditions, e.g. systemic sclerosis [13] and end-stage renal disease [14]. IMA levels change significantly during and after strenuous physical exercise, i.e. after marathon racing or in professional athletes [15,16]. Also, peroperative IMA levels increase during tourniquet surgery [17] or arterial clamping during revascularization procedures [18], suggesting transient changes due to skeletal muscle ischemia, rather than myocardial damage.

Modification of the N-terminus of albumin could well occur during any condition characterized by ongoing oxidative stress, reflecting a normal antioxidant defense mechanism against excessive production of oxygen free radicals. Although evidence on mechanisms underlying IMA formation is still lacking, recent *in vitro* experiments by Roy and others suggest direct damage to albumin by reactive oxygen species, especially in the presence of the highly reactive hydroxyl ($OH\cdot$) radical [19]. Sources of increased free radical production during pregnancy include the endothelium, inflammation and the placenta. Also, many antioxidant defense mechanisms are upregulated during normal pregnancy and may be important in protecting the mother against pregnancy specific conditions such as preeclampsia, congenital malformations and intra-uterine growth restriction [6,10]. Therefore, increased IMA formation during pregnancy and preeclampsia is likely to reflect a defense strategy of the mother against circulating prooxidant triggers. Although in our data IMA levels of preeclamptic women did not exceed those of normal pregnant controls,

these results might be influenced by the relatively small sample size.
Intriguingly, we also found a weak but significant inverse correlation between infant's birth weight and IMA levels. Although this study is too small to draw any firm conclusions, one could argue that changes in IMA levels partly represent differences in placental perfusion. Impaired placental circulation is clearly related to restricted fetal growth, leading to small-for-gestational age infants. The clinical syndrome of preeclampsia is believed to arise from a placental damage due to hypoxia and ischemia, and is frequently complicated by marked fetal growth restriction [5]. IMA levels might therefore be useful to monitor placental ischemia or oxidative stress. Although we can only report on N=4 women who delivered SGA infants, our results do not support this hypothesis, as we found no difference between preeclamptic women with and without SGA. Obviously, larger numbers are needed to test this hypothesis thoroughly.
Previous reports have demonstrated that IMA levels are inversely related to serum albumin concentration [17,20,21]. Although our findings show a similar correlation, we found a higher and even more consistent rise in IMA levels in pregnancy after correction for albumin levels by multivariate regression analysis. As proposed in the recent authorative review by Apple and others, IMA test results should be interpreted with caution when serum albumin levels are < 20 g/L or > 50 g/L [4]. As shown in our data, the impact of serum albumin concentration on IMA levels is still considerable within the normal range (serum albumin between 20 and 50 g/L). Correction for albumin levels is therefore especially important in populations with broad differences in albumin concentrations, such as pregnant women in the third trimester. However, this also raises serious concern for the interpretation of uncorrected IMA data in all other population studies. Other potential confounders include lactate, which is thought to interfere with the ACB test [20]. Although data on serum lactate levels were unavailable for our study population, previous reports show no change in lactate concentrations in normal pregnancy [22]. Lactate metabolism in preeclampsia has not been previously addressed, however metabolic abnormalities including lower pyruvate and lactate synthesis in placentas of preeclamptic mothers have been described [23]. Interference of the ACB test in preeclampsia by high lactate levels thus seems unlikely to have occurred.

In conclusion, we found elevated serum IMA concentrations in normal pregnancy, as compared to age matched non pregnant controls. In women with preeclampsia, IMA levels were similar to normal pregnant controls. Our results suggest that IMA values are influenced by many other mechanisms than myocardial damage, most likely involving ongoing ischemia and oxidative stress in other vascular beds, such as the placenta. Additional studies are required to further evaluate the sensitivity and specificity of the

ACB test for detecting cardiac ischemia during pregnancy. Also, as serum IMA appears to be inversely related to birth weight, it might be of interest in monitoring ongoing ischemia or free radical production in placental disorders. However, future studies are warranted to test this hypothesis thoroughly.

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6

Inflammatory Changes in Preeclampsia: Current Understanding of the Maternal Innate and Adaptive Immune Response

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Abstract

Preeclampsia is characterized by generalized endothelial dysfunction as a result of an inappropriate maternal immune response against the fetus. It has been postulated that the adaptive immune system plays a key role in the etiology of preeclampsia by generating a pro-inflammatory Th1 type immune reaction. In this review, recent studies on Th1 and Th2 type cytokine mapping in preeclampsia are reviewed, as well as on the possible sources of pro-inflammatory cytokines and the role of regulatory cytokines and chemokines. In addition, we discuss the possible role of Toll-like receptors of the innate immune system in the pathophysiology of preeclampsia.

Abbreviations

CD	Cluster of differentiation
DARC	Duffy blood group antigen receptor of chemokine
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MIF	Macrophage migration inhibitory factor
MyD88	Myeloid differentiation primary response gene 88 (adaptor molecule)
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
PBMC	Peripheral blood mononuclear cells
PAMP	Pathogen-associated molecular pattern
PHA	Phytohemagglutinin
PRR	Pattern recognition receptor
RT-PCR	Reverse transcription polymerase chain reaction
sEng	Soluble endoglin
TGF	Transforming growth factor
TLR	Toll-like receptor
Th	T helper cell (CD4 positive)
TNF	Tumor necrosis factor

Introduction

Preeclampsia is a common and potentially dangerous disorder of human pregnancy. It affects 2-3% of all pregnancies and is a leading cause of maternal morbidity and mortality in the Western world [1]. Despite extensive research which has elucidated many of its pathophysiological features, preeclampsia remains a highly variable and unpredictable disease of unknown origin [2,3]. However, generalized maternal endothelial cell dysfunction has been proposed as the underlying pathophysiological process [4]. First proposed by Redman and colleagues, evidence accumulates that the endothelial dysfunction of preeclampsia is to be attributed to an inappropriate maternal immune response against the semi-allogeneic fetus, leading to activation of inflammatory cells such as monocytes and granulocytes [5-7]. Over the past decade, multiple analyses of cytokine biomarkers have provided substantial insight into the key players of the maternal immune system involved in preeclampsia. However, major advances in the field of innate and adaptive immunology, including the recently discovered role of Toll-like receptors [8,9], are currently changing the way we think about the pathogenesis of inflammatory diseases in general [10-12]. Understanding the nature of the complex immunoregulatory processes that constitute the maternal inflammatory response could thus be of crucial importance to unravel the pathophysiology of preeclampsia.

Traditionally, two compoments are discerned in the immune system : an innate and an adaptive component. The innate immune system, which includes a cellular component (monocytes, granulocytes and dendritic cells) and a humoral component (mostly complement factors) provides a rapid and non-specific response system that plays a major role in determining and controlling the type of adaptive immune response [13]. More recently however, it became clear that cells of the innate immune system are far less non-specific than previously assumed, as they were shown to express a series of receptors known as pattern-recognition receptors (PRRs). PRRs recognize and bind highly conserved sequences known as pathogen-associated molecular patterns (PAMPs). PAMPs, such as bacterial lipopolysaccharide (LPS), peptidoglycan and viral double stranded ribonucleic acid (dsRNA) are unique to and expressed by microbes [13]. One of the main families of the PRRs are the Toll-like receptors (TLRs), which are central components of the innate immune system [14,15]. Each TLR is distinct in its specificity. However, following ligation, all TLRs signal through a common adaptor molecule named MyD88, to activate the nuclear factor kappa B (NF-κB) pathway, which results in a cellular immune response characterized by the production of pro-inflammatory cytokines such as interleukins 2 (IL-2) and 6 (IL-6), antimicrobial products, and the upregulation of costimulatory molecules [8,14].

The adaptive immune system is composed of T and B lymphocytes, which are activated

through antigen recognition and clonal expansion, leading to the generation of an antigen specific immune response. CD4-positive T helper-1 (Th1) cells produce Th1 type cytokines, such as IL-2, interferon (IFN) -γ and tumor necrosis factor (TNF) -α, that support natural killer (NK) cell and macrophage activation and the generation of cytotoxic T cells, while CD4-positive T helper-2 (Th2) cells synthesize Th2 type cytokines, such as IL-4, IL-5 and IL-13 that induce B-cell activation and antibody production [16]. Wegmann and colleagues were the first to propose that successful pregnancy induces an immune bias towards Th2 type immune responses and suppressed Th1 type immunity [17]. It has been postulated that women with preeclampsia show an exaggerated inflammatory response, characterized by aberrant cytokine production towards harmful Th1 type immunity [17-19]. The sources of the increased levels of pro-inflammatory cytokines in the circulation of preeclamptic women have not been fully determined. Some authors indicate excessive synthesis by the placenta [20,21], while others suggest circulating activated leukocytes as the main source of pro-inflammatory cytokines [22,23]. Here, we review the current knowledge on cytokine mapping in preeclampsia and provide a conceptual framework for future research into innate and adaptive immunity as a key feature of preeclampsia.

The adaptive immune system in preeclampsia

The first strong support for the deleterious effects of Th1 type cytokines in pregnancy was reported by Wegmann and colleagues, who showed that injection of Th1 type cytokines (IL-2, IFN-γ and TNF-α) into pregnant mice results in termination of pregnancy [17]. Thereafter, many others have studied Th1/Th2 cytokine balance in human pregnancy. **Table 1** summarizes recent studies on maternal cytokine production in preeclampsia and shows whether cytokine levels were found to be increased, decreased or not different from normal pregnancy. In addition, **Table 2** gives an overview of the results of recent studies on cytokine production by fetal and placental cells in preeclampsia. **Table 3** shows the results of recent studies on maternal cytokines in normal pregnant women, compared to non-pregnant women.

Pro-inflammatory cytokines

Luppi and colleagues showed that the spontaneous synthesis of the pro-inflammatory cytokines IL-1β and IL-6 by monocytes of preeclamptic women is higher than in normal pregnant and non-pregnant women [24]. In contrast, Jonsson and colleagues were unable to detect a significant difference in IL-1β levels in sera from normal pregnant and preeclamptic women [25]. In a study by Rein and colleagues, release of IL-1β or IL-6 by trophoblast cells was similar for preeclamptic and normal pregnant women [26]. Rinehart

and colleagues studied cytokine expression in placentas from women with preeclampsia, and found an increased IL-1β expression [21], whereas Benyo and colleagues did not observe a significant difference [27]. However, both study populations were too small to allow definitive conclusions.

IL-6 is a major pro-inflammatory cytokine, however with pleiotropic effects, also including inhibition of IL-1 and TNF-α, stimulation of antibody production by B-cells and activation of T-cells [28]. Studies on IL-6 levels in maternal serum or plasma generally show a significantly higher level in preeclamptic women [25,29-32]. Due to its versatile nature, it is hard to speculate on the clinical effects of elevated IL-6 levels in preeclampsia [28]. Studies on placental production of IL-6 showed no significant difference between normal pregnancy and preeclampsia [21,26,27,33]. However, IL-6 levels in decidual lymphocytes and umbilical artery serum were found decreased in preeclamptic women [34,35]. This would suggest that maternal circulating leukocytes rather than fetoplacental inflammatory cells are the main source of IL-6 production in preeclampsia.

TNF-α is another powerful pro-inflammatory cytokine and is known to be present in human placental and uterine cells at the early and late stages of gestation [36]. Several studies have reported elevated maternal circulating TNF-α concentrations in preeclampsia, suggesting that TNF-α is involved its pathogenesis [22,27,32,37,38]. However, others contradict these findings, reporting no significant difference in maternal TNF-α levels in preeclampsia as compared to normal pregnancy [25,31,39,40]. So far, few studies have been performed on in vitro TNF-α production. Luppi and colleagues found no difference in intracellular TNF-α levels between normal pregnancy and preeclampsia [24], while Azizieh and colleagues found that TNF-α production was elevated in preeclampsia at phytohaemagglutine (PHA) stimulation [22]. Studies on TNF-α expression in placental tissue and in trophoblast cultures generally do not show altered levels in preeclampsia [26,27,37]. Only Rinehart and colleagues demonstrated increased placental production of TNF-α in preeclampsia [21]. However, their control placentas of healthy pregnant women did not express TNF-α at all, which is conflicting with previous data in the literature.

Th1 type cytokines

As shown in **Table 1**, all studies demonstrating increased IL-2 levels in preeclampsia were performed using cultured peripheral blood mononuclear cells (PBMCs). Both Darmochwal-Kolarz and colleagues and Rein and colleagues showed that PBMCs in preeclamptic women express higher intracellular IL-2 concentrations [23,41]. Furthermore, in a second study, Darmochwal-Kolarz and colleagues showed increased IL-2 production by PHA stimulated PBMCs in preeclampsia [42]. Other studies on IL-2 production by PBMCs or in maternal serum or plasma have failed to detect significant differences between preeclamptic women and controls [22,25,39,43]. Also, both cultured trophoblast cells and choriodecidual cells

of preeclamptic versus normal pregnant women did not show any differences in IL-2 production [26,43].

IFN-γ is a major cytokine in the induction of a Th1 type cytotoxic immune response and specifically inhibits B cell activation [28]. Darmochwal-Kolarz and colleagues found a consistent increase in *in vitro* IFN-γ production both by unstimulated and PHA stimulated PBMCs during preeclampsia [41,42]. However, several reports of similar experiments failed to reproduce these findings [22,23,43,44]. Furthermore, IFN-γ levels in maternal serum or plasma were not significantly different in preeclampsia, compared to normal pregnancy [25,43]. Thus far, only few studies have focused on the role of IFN-γ produced by fetoplacental cells in preeclampsia. Both Wilczynski and colleagues and Arriago-Pizano and colleagues found increased IFN-γ production in decidual lymphocytes and choriodecidual cells respectively [34,43].

Table 1 Maternal cytokines in preeclampsia, detected in serum or plasma, and in cultured peripheral blood mononuclear cells (PBMCs).

	Serum / plasma levels				Cultured PBMCs			
Cytokine	Increased	Decreased	NS	*cases / controls (N)*	Increased	Decreased	NS	*cases / controls (N)*
Pro-inflammatory								
IL-1β			• [25]	*15/15*	• [24]			*15/31*
IL-6	••••• [25,29-32]		• [33]	*152/146*	• [24]	• [22]	• [46]	*62/100*
TNF-α	•••• [27,32,37,38]		•••• [25,31,39,40]	*152/155*	• [22]		• [24]	*47/85*
Th1 type								
IL-2			••• [25,39,43]	*54/62*	••• [23,41,42]		•• [22,43]	*111/125*
IFN-γ			••	*32/30*	•• [41,42]		•••• [22,23,43,44]	*126/140*
Th2 type								
IL-4		•• [40,43]	• [25]	*60/58*		• [22]	••• [23,41,44]	*91/105*
IL-5			• [25]	*15/15*		•• [22,46]		*47/69*
IL-13			• [25]	*15/15*			• [46]	*15/15*
Regulatory								
IL-10	•• [29,51]		•••• [25,31,39,43]	*149/143*		•••• [22,41,42,46]	•• [43,44]	*117/135*
IL-12			• [25]	*15/15*	•• [52,54]		•• [44,46]	*81/86*
IL-18	• [55]	• [56]		*57/59*			• [54]	*24/21*
TGF-β1	•••• [29,51,61,62]		•• [63,64]	*285/233*				-
Chemokines								
IL-8	••• [25,31,38]			*53/42*	• [24]			*15/31*
MIF	• [79]		•• [25,80]	*76/116*				-

IL = interleukin, TNF-α = tumor necrosis factor α, IFN-γ = interferon γ, TGF-β1 = transforming growth factor β1, MIF = macrophage migration inhibitory factor. Cytokine levels are either increased, decreased or not significantly (NS) different from normal pregnancy. Every • indicates one study (references in superscript).

Table 2 Fetoplacental cytokines in preeclampsia.

	Fetus / placenta			
Cytokine	Increased	Decreased	NS	*cases / controls (N)*
Pro-inflammatory				
IL-1β	• [21]		•• [26,27]	*23/21*
IL-6		•• [34,35]	•••• [21,26,27,33]	*83/79*
TNF-α	• [21]		••• [26,27,37]	*41/44*
Th1 type				
IL-2			•• [26,43]	*28/26*
IFN-γ	•• [34,43]			*38/26*
Th2 type				
IL-4			• [43]	*17/15*
IL-5				-
IL-13				-
Regulatory				
IL-10	• [21]	•• [26,34]	• [43]	*55/41*
IL-12		• [34]		*21/11*
IL-18	• [55]			*27/28*
TGF-β1			• [64]	*12/14*
Chemokines				
IL-8				-
MIF				-

IL = interleukin, TNF-α = tumor necrosis factor α, IFN-γ = interferon γ, TGF-β1 = transforming growth factor β1, MIF = macrophage migration inhibitory factor. Cytokine levels are either increased, decreased or not significantly (NS) different from normal pregnancy. Every • indicates one study (references in superscript).

Th2 type cytokines

Normal pregnancy seems to induce a bias towards Th2 type immunity, while preeclampsia is consistent with suppressed Th2 type cytokines [45]. Several studies have shown a decrease in circulating IL-4 or levels or decreased production by PBMCs in preeclamptic women, compared to normal pregnant controls [22,40,43]. However, in other studies on spontaneous IL-4 production by maternal PBMCs no significant difference could be detected [23,41,44]. Arriaga-Pizano and colleagues measured IL-4 expression in placentas of preeclamptic women and reported levels similar to normal pregnancy [43].

Jonsson and colleagues found that spontaneous IL-5 secretion by PBMCs was significantly reduced in preeclampsia [46]. However, Azizieh and colleagues were unable to detect any IL-5 production in unstimulated PBMC cultures. After mitogen stimulation however, they also observed lower IL-5 production in PBMCs isolated from women with preeclampsia as compared to normal pregnant controls [22].

Table 3 Maternal cytokines in normal pregnancy, detected in serum or plasma and in cultured peripheral blood mononuclear cells (PBMCs).

	Serum / plasma levels				Cultured PBMCs			
Cytokine	Increased	Decreased	NS	*cases / controls (N)*	Increased	Decreased	NS	*cases / controls (N)*
Pro-inflammatory								
IL-1β				-			• [24]	*31/41*
IL-6	• [32]			*26/21*			• [24]	-
TNF-α	• [32]			*26/21*			•• [24,87]	*43/53*
Th1 type								
IL-2				-				-
IFN-γ				-		• [87]		*6/6*
Th2 type								
IL-4				-			• [87]	*6/6*
IL-5				-				-
IL-13				-				-
Regulatory								
IL-10				-				-
IL-12				-	• [87]	•• [52,54]		*68/64*
IL-18				-	• [54]			*21/17*
TGF-β1		•• [62,63]		*52/63*				-
Chemokines								
IL-8				-			• [24]	*31/41*
MIF	• [80]			*60/20*				-

IL = interleukin, TNF-α = tumor necrosis factor α, IFN-γ = interferon γ, TGF-β1 = transforming growth factor β1, MIF = macrophage migration inhibitory factor. Cytokine levels are either increased, decreased or not significantly (NS) different from non pregnant women. Every • indicates one study (references in superscript).

So far, the role of IL-13 in the generation of a type 2 immune response of preeclampsia has received little attention, except in two studies by Jonsson and colleagues who found no difference in IL-13 secretion by PBMCs between preeclamptic patients and controls [25,46].

In addition to the current knowledge on Th1 type and Th2 type inflammatory responses, it is important to note the newly described Th17 lineage of IL-17 and IL-6 producing CD4+ effector cells [47]. Th17 cells develop through a pathway separate from Th1 and Th2, and are thought to have a central role as one of the most potent pro-inflammatory cells involved in immune regulation, immune pathogenesis, angiogenesis and host defense [47,48]. Recent work by Pongcharoen and colleagues demonstrated production of IL-17 by human extravillous trophoblast [49]. The role of IL-17 producing T cells in preeclampsia has yet to be established.

Regulatory cytokines and chemokines

Regulatory cytokines

Regulatory cytokines such as IL-10, IL-12, IL-18 and transforming growth factor (TGF) -β1 have a major role in controlling the cytokine balance of the inflammatory response. IL-10 exerts potent anti-inflammatory effects, which are believed to be important in the maintenance of pregnancy [50]. The increased pro-inflammatory response observed in preeclampsia could theoretically be due to decreased levels of IL-10. However, evidence in support of a role for IL-10 in preeclampsia is inconsistent. Several studies demonstrated a decrease in maternal IL-10 production during preeclampsia [22,41,42,46], however most studies were unable to detect any significant differences at all [25,31,39,43,44], while some authors even demonstrated elevated IL-10 concentrations [29,51]. Interestingly, all studies which suggest a decrease in IL-10 levels used *in vitro* production by stimulated PBMCs as a read-out, while studies that measured actual IL-10 levels in maternal plasma generally show an increase. It is tempting to speculate that the increase in IL-10 levels observed in preeclamptic patients occurs in response to elevated levels of TNF-α, IL-2 and IL-6, suggesting some sort of cytokine balancing. Studies involving fetoplacental tissue demonstrated increased expressions of IL-10 in the placenta [21], but also decreased expression in cultured trophoblast cells and decidual lymphocytes [26,34].

IL-12 is known to induce the production of IFN-γ and favours the differentiation of Th1 cells in physiological and pathological immune responses [16]. Sakai and colleagues showed in two studies that spontaneous IL-12 production by maternal PBMCs is significantly

higher in preeclamptic patients than in normal pregnant women [52]. They included a non-pregnant healthy control group in their studies and showed that IL-12 levels are decreased in normal pregnancy, compared to healthy non-pregnant women. These data support the theory that normal pregnancy induces a Th2 type predominance. Nonetheless, others found no difference in IL-12 levels between preeclamptic women and normal pregnant controls in both maternal serum and in cultured PBMCs [25,44,46]. IL-12 secretion by decidual lymphocytes was found to be decreased in preeclampsia when compared to normal pregnancy [34].

Elevated serum IL-18 levels have been reported in complicated pregnancies [53]. IL-18 is a pro-inflammatory cytokine of the IL-1β family known to induce IFN-γ production and divert the immune response towards the Th1 type when IL-12 is present. On the other hand, IL-18 alone has the capacity to induce Th2 responses [54]. Results on IL-18 production in preeclamptic women are conflicting. Huang and colleagues found that serum and placental IL-18 are elevated in preeclampsia [55], while others found decreased plasma levels [56]. In the latter study, administration of betamethasone might have been responsible for the lower IL-18 levels. After patients who received corticosteroids were excluded, IL-18 levels were found similar for preeclamptic and normal pregnant women. Furthermore, since IL-18 is known to divert towards Th1 immunity only in the presence of IL-12, these cytokines should always be measured together. One study measured both IL-18 and IL-12 secretion by PBMCs, demonstrating that elevated IL-18 secretion, together with decreased IL-12 secretion, may induce Th2 dominance in normal pregnancy, while elevated secretion of both IL-18 and IL-12 may cause Th1 dominance in severe preeclampsia [54].

TGF-β1 is a multifunctional growth factor known to have powerful immunoregulatory properties [57]. Furthermore, members of the TGF-β superfamily are widely expressed in the endometrium and placenta and are closely related to many reproductive processes [58]. Recent attention for the TGF-β superfamily includes the promising first results on the role of the TGF-β coreceptor soluble endoglin (sEng) in the pathogenesis of preeclampsia [59,60]. Plasma derived TGF- β1 appears to be elevated in preeclampsia [29,51,61,62]. In a case-control study of 100 preeclamptic and 100 pregnant controls, Muy-Rivera and colleagues assessed plasma TGF-β1 concentrations in relation to preeclampsia risk among Peruvian women. They showed an estimated 15-fold higher odds ratio for preeclampsia across successively higher quartiles of TGF-β1 [61]. However, two other studies were unable to detect a significant difference in serum or plasma TGF-β1 in preeclampsia versus normal pregnancy [63,64]. TGF-β1 production by cultured maternal PBMCs has not been studied thus far. Interestingly, enhanced immunostaining and expression for TGF-β1 and TGF-β2 in placental bed biopsies appeared related to abnormal spiral artery invasion of extravilluos trophoblast [58,64,65]. At term however, TGF-β1 immunostaining was found

negative in placentas of both preeclamptic and normal pregnant women, suggesting a temporal role for TGF-β in early placentation rather than in development of late gestation preeclampsia [64].

Chemokines

Although chemotactic cytokines are primarily involved in the recruitment of inflammatory cells, other biological functions that have recently been attributed to chemokines include cell growth and proliferation, microbial pathogenicity, tumor metastasis and inflammation [66,67]. The complex interactions between chemokines and their receptors provide a new and rapidly growing area of research, aimed at understanding leukocyte trafficking as well as fine tuning of the cellular immune response. Currently, there are no data yet on the role of chemokines in preeclampsia, except for IL-8, which is locally produced in response to tissue injury, leading mainly to the recruitment and activation of neutrophils. Because neutrophils are considered important mediators of the endothelial dysfunction of preeclampsia [68], IL-8 may play a pivotal role in its pathogenesis. In support of this hypothesis, several studies demonstrated increased maternal serum IL-8 levels and increased IL-8 production by maternal PBMCs in preeclampsia [24,25,31,38].

The innate immune system in preeclampsia

Normal pregnancy induces a remarkable upregulation in number and activation of granulocytes, suggesting a major role for cells of the innate immune system in controlling the maternal immune response [69]. Although originally thought to be an evolutionary redundant immune pathway with a lower level of complexity than its adaptive counterpart, the innate immune response is not entirely non-specific [10,13]. In fact, innate immune cells are able to discriminate between a self and variety of non-self antigens through a set of highly conserved pattern-recognition receptors (PRRs). The effector responses of PRR activation include release of many pro-inflammatory and regulatory cytokines, linking the innate immune system to the Th1 and Th2 cytokines of the adaptive immune system, as well as to chemotaxis and apoptosis [15]. The role of the innate immune response in normal pregnancy and preeclampsia remains to be established, but is a provocative topic for future investigations. Here, we discuss the current knowledge on innate immunity relevant to preeclampsia.

TLRs

In 1994, Faas and colleagues developed an animal model for preeclampsia by infusing low doses of endotoxin (also known as lipopolysaccharide or LPS) into pregnant rats [70]. This suggests that administration of LPS during pregnancy, which exerts its pro-inflammatory effects through activation of TLR-4 , induces a preeclampsia-like syndrome. More recently, two studies have investigated the immunohistochemical distribution of TLR-2 and TLR-4 in the human placenta. Holmlund and others showed strong immunoreactivity for TLR-2 and TLR-4 in the villous and the intermediate trophoblasts [71], while Kumazaki and others localized TLR-4 in the extravillous trophoblasts and the intermediate trophoblasts [72]. Even though their results show different localizations for TLRs in the placenta, they both confirm the presence of TLRs on trophoblast cells. Furthermore, recent experiments with cultured trophoblast cells have shown that ligation of a TLR with its specific ligand LPS, leads to induction of chemokine secretion [71,73,74]. In addition, Abrahams and colleagues showed that production of these chemokines causes increased monocyte and neutrophil chemotaxis [73]. So far, only one study has been conducted to determine whether changes in TLR-2 and TLR-4 can be detected in the trophoblasts at the placental bed of women with and without preeclampsia [75]. In this study, Kim and colleagues showed that TLR-4 protein expression is increased in interstitial trophoblasts of patients with preeclampsia. They hypothesized that "danger signals" (host or microbial in nature) at the feto-maternal interface are recognized by trophoblasts through TLR-4 and may play a role in creating a local abnormal cytokine milieu leading to the development of preeclampsia. However, the actual existence of an endogenous TLR-4 ligand which would act as a danger signal, is still controversial [76].

MIF

Recently it has been shown that the chemotactic cytokine macrophage migration inhibitory factor (MIF) has a central role in the regulation of inflammatory immune responses. It exerts direct pro-inflammatory action by promoting the expression of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-2, IL-6 and IL-8 [77]. Also, Roger and colleagues implicated that MIF is an essential regulator of innate immune responses through modulation of TLR-4 [78]. This suggests that MIF is important in the recognition of LPS and Gram-negative bacteria by cells of the innate immune system. A recent study on cytokine production in preeclampsia has shown that MIF serum levels are more than doubled in preeclamptic patients compared with normal pregnant women [79], whereas other studies were unable to reproduce these findings [25,80]. Hristoskova and colleagues found MIF levels to be significantly elevated in pregnancy, but not further increased in preeclampsia [80].

Discussion

Although several studies lend support to the original theory of increased Th1 type and decreased Th2 type immunity in preeclampsia, at least as many reports demonstrate lack of reproducibility, conflicting results and considerable overlap in cytokine levels between normal pregnancy and preeclampsia. These discrepancies may be due to different experimental approaches or technical considerations, such as differences in ELISA, RT-PCR, immunostaining, flow cytometry or cell culture techniques. For instance, investigators have noted that TGF-β1 is released from platelets during clotting, thus detection of TGF-β1 in serum does not correlate to plasma concentrations. In addition, the possible transient episodic release and the short half life of cytokines in the human body are serious impediments to the detection of cytokine production *in vivo* [6]. Analysis of cytokines secreted by cultured PBMCs using flow cytometry would circumvent this problem, but requires direct measurements in fresh material and, more importantly, may not always be extrapolated to the *in vivo* situation.

In some studies, patients received glucocorticoid treatment prior to the time of blood sample collection. As demonstrated by Adams and colleagues, this could influence cytokine levels [56]. It cannot be ruled out that other medications administered prior to blood sampling may have influenced cytokine analysis in preeclampsia as well. Also, normal pregnancy already requires considerable and ongoing immunological adaptations of the mother to the placenta and the fetus. As a consequence, cytokine levels are likely to fluctuate during different stages of gestation. Hence, blood samples from healthy pregnant women should be carefully matched to preeclamptic patients for gestational age. Many authors did not acknowledge this and applied broader criteria for obtaining blood samples during the third trimester. Other potential confounders that may influence cytokine levels include maternal age, body-mass index (BMI) and smoking.

It is also important to note that conflicting results might reflect differences in study design and inclusion criteria, allowing for the selection of mild rather than severe cases. Most studies define preeclampsia either according to the definition of the American College of Obstetricians and Gynaecologists (ACOG), or according to the World Health Organization (WHO). These definitions are comparable, in the use of both systolic and diastolic blood pressure criteria. Some authors diagnosed preeclampsia on diastolic blood pressure alone, according to the criteria defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP) [30]. Others did not provide a definition of preeclampsia at all [61,63], or specifically included a more severe form of preeclampsia, using predefined stricter criteria [41,79]. These discrepancies might have resulted in different preeclampsia study populations, making it more difficult to compare results.

Most studies have adopted a two-group study design comparing preeclamptic patients to a control group of healthy pregnant women. Only few studies included a third group to the study population, consisting of healthy non-pregnant women. However, for most cytokines, changes in circulating levels throughout normal pregnancy have not been clarified. Among others, Sacks and colleagues convincingly demonstrated by their flow cytometry studies that normal pregnancy itself gives rise to intense inflammatory changes comparable to those observed in sepsis [81]. Thus, more research on cytokine levels during normal pregnancy is needed to fully appreciate the significance of the alterations in cytokine levels found in preeclampsia.

In addition, several studies may have been limited by small sample size, having too little statistical power to detect true differences between cases and controls. Also, ethnicity affects cytokine expression patterns and handling by effector cells, as suggested by Velzing-Aarts and others [38]. For instance, Caucasians use the Duffy blood group antigen receptor of chemokine (DARC) to bind excessive IL-8 in the circulation, thus impeding the activation of effector cells. Most West-Africans however do not express DARC on their red blood cells. Velzing-Aarts and colleagues studied IL-8 levels in Afro-American women with preeclampsia and proposed that higher levels of IL-8 in preeclampsia may be due to the DARC negative phenotype. In the studies reviewed here, participants of many different ethnic backgrounds were included. Fortunately, North-American, South-American, European and Asian ethnicities generally do not seem to influence cytokine levels.

Future directions

Previous research has shown that there is a strong genetic component in determining cytokine production and control of inflammation. Westendorp and colleagues assessed genetic influence on cytokine production and its contribution to fatal outcomes by determining the capacity to produce TNF and IL-10 in families of patients who had meningococcal disease [82]. They found that families characterized by low TNF production and high IL-10 production had an increased risk of fatal outcome. This suggests that the innate capacity to produce cytokines contributes to familial susceptibility for inflammatory diseases and also has a profound effect on severity and outcome. It has indeed been documented that certain cytokine gene polymorphisms may account for the abnormal cytokine profiles observed in preeclampsia [83-85]. Maternal predisposition, rather than pregnancy specific changes, could therefore be of crucial importance for the pathogenesis of preeclampsia. Studying cytokine levels in women, both during pregnancy and in the non-pregnant state will lead to a better understanding of maternal predisposition for preeclampsia.

Recently discovered PRRs are essential for the induction of innate immune responses and there is growing evidence that TLRs at the feto-maternal interface may play a key role in creating a locally abnormal cytokine milieu. PRR-mediated trophoblast responses provide a relatively new but interesting topic for future preeclampsia research, as many TLRs and other PRRs, such as the intracellular nucleotide-binding oligomerization domain (NOD) receptors, are likely to have critical roles in health and disease.

In conclusion, although many immunological aspects of preeclampsia have been studied, the precise mechanisms of immune dysfunction remain to be elucidated. For a better insight, more information is needed on cytokine levels during normal pregnancy and on the influence of possible maternal predisposition on cytokine levels. It is probably not sufficient to analyse the Th1/Th2 dysbalance alone, as interactions between Th1, Th2, regulatory cytokines and chemokines may be responsible for directing the immune response towards the harmful pro-inflammatory immune reaction observed in preeclampsia. Modern techniques, such as multiple bead array systems allow simultaneous and integral detection of the molecules of interest [86]. This is of particular interest when regarding the evidence that most cytokines act in concert with each other, leading to combined rather that separate effects. Also, future studies should focus on the role of regulatory cytokines and chemokines in the bias towards cytotoxic immunity in preeclampsia. Although recent studies show the pivotal role of PRRs and other components of the innate immune system in controlling the inflammatory response, as well as the presence of TLRs on trophoblasts and inflammatory cells in the human placenta, there are no data yet on the role of TLRs in the etiology of preeclampsia. Future studies into the nature of the mother's immunological adaptation to pregnancy will continue to improve our understanding of the pathways leading to the clinical syndrome of preeclampsia. Hopefully, this will reveal new approaches to identify patients at risk and provide new perspectives for the treatment of preeclampsia.

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7

Maternal TLR4 and NOD2 Gene Variants, Pro-Inflammatory Phenotype and Susceptibility to Early-Onset Preeclampsia and HELLP syndrome

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Abstract

Background

Altered maternal inflammatory responses play a role in the development of preeclampsia and the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. We examined whether allelic variants of the innate immune receptors Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain 2 (NOD2), that impair the inflammatory response to endotoxin, are related to preeclampsia and HELLP syndrome.

Methods and findings

We determined five common mutations in TLR4 (D299G and T399I) and NOD2 (R702W, G908R and L1007fs) in 340 primiparous women with a history of early-onset preeclampsia, of whom 177 women developed HELLP syndrome and in 113 women with a history of only uneventful pregnancies as controls. In addition, we assessed plasma levels of pro-inflammatory biomarkers C-reactive protein, interleukin-6, soluble intercellular adhesion molecule-1, fibrinogen and von Willebrand factor in a subset of 214 women included at least six months after delivery. After adjustment for maternal age and chronic hypertension, attenuating allelic variants of TLR4 were more common in women with a history of early-onset preeclampsia than in controls (OR 2.9 [95% CI 1.2– 6.7]). Highest frequencies for TLR4 variants were observed in women who developed HELLP syndrome (adjusted OR 4.1 [95% CI 1.7– 9.8]). In addition, high levels of interleukin-6 and fibrinogen were associated with a history of early-onset preeclampsia. Combined positivity for any of the TLR4 and NOD2 allelic variants and high levels of interleukin-6 was 6.9-fold more common in women with a history of early-onset preeclampsia (95% CI 2.1–23.2) compared to controls.

Conclusions

We observed an association of common TLR4 and NOD2 gene variants, and pro-inflammatory phenotype with a history of early-onset preeclampsia and HELLP syndrome, that suggests involvement of the maternal innate immune system in severe hypertensive disorders of pregnancy.

Introduction

Preeclampsia is a complex multi-system disease of unknown origin, characterized by hypertension and proteinuria in the second half of pregnancy, with potentially life-threatening consequences for both mother and baby [1]. Worldwide an estimated 4.2 million women are affected by preeclampsia each year and an annual 60,000 maternal deaths are attributed to the disorder [2].

The inflammatory system has a pivotal role in the pathogenesis of common placental and hypertensive disorders of pregnancy, including preeclampsia, intra-uterine growth restriction (IUGR) and the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome [1,3,4]. Maternal adaptation to pregnancy requires a prominent and tightly regulated interplay of innate and adaptive immunity to allow normal growth and development of the semi-allogeneic fetus. Inappropriate inflammatory patterns have been related to abnormal trophoblast invasion [5], endothelial damage [3], and renal dysfunction [6]. Furthermore, preeclampsia is characterized by excessive production of pro-inflammatory cytokines and chemokines [7], as well as disturbances in immunosuppressive regulators such as IL-10 and TGF-β [7,8].

Endotoxin or lipopolysaccharide (LPS) is a potent antigen normally present in gram-negative bacteria, known to induce a systemic pro-inflammatory reaction. LPS-mediated inflammation is believed to be of critical importance in many systemic infectious and non-infectious or auto-immune disorders, including sepsis [9], allergic asthma [10] and inflammatory bowel diseases [11]. In an animal model, Faas and colleagues demonstrated that infusion of low-dose LPS induces a preeclampsia-like syndrome, including hypertension, proteinuria and glomerular endotheliosis [12]. LPS leads to release of pro-inflammatory cytokines through activation of two major pattern recognition receptors (PRRs) present on innate immune cells (macrophages, NK cells and dendritic cells), i.e. the extracellular Toll-like receptor 4 (TLR4), and the intracellular nucleotide oligomerisation domain 2 (NOD2) protein, also known as the caspase-activating recruitment domain (CARD15). Upon activation, these receptors signal cells of the adaptive immune system (mainly T cells) via production of nuclear factor kappa B and release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) to constitute an inflammatory response necessary for effective clearance of the harmful pathogen.

Allelic variants of TLR4 and NOD2 have been related to an attenuated immune response to LPS, leading to inappropriate inflammatory patterns which can be both ineffective in dealing with an infectious agent, as well as cause the adverse effects of uncontrolled inflammation [13,14]. Genetic variation in the extracellular (TLR4) and intracellular (NOD2) endotoxin sensing system have been related to sepsis [9], Crohn's disease [11,15], premature delivery

[16] and atherogenesis [17]. In this study we examined the contribution of two common allelic variants of the TLR4 (D299G and T399I) and three common allelic variants of NOD2 (R702W, G908R and L1007fs) to the pathogenesis of early-onset preeclampsia and HELLP syndrome. In addition, we determined plasma levels of biomarkers representative for the acute-phase inflammatory response (C-reactive protein, fibrinogen, interleukin-6, soluble intercellular adhesion molecule-1 and von Willebrand factor) in women with a history of early-onset preeclampsia and controls with only uneventful pregnancies at least six months after delivery. We hypothesized that allelic variants of TLR4 and NOD2, as well as high circulating levels of pro-inflammatory biomarkers after delivery would relate to a history of early-onset preeclampsia and HELLP syndrome.

Methods

Participants

Participants were recruited between August 1995 and September 2005 in four tertiary referral centres (University Medical Center Utrecht; N=280, Leiden University Medical Center; N=45, St Radboud University Medical Center Nijmegen; N=98 and Maxima Medical Center Veldhoven; N=30) in the Netherlands. Inclusion criteria for cases were primiparity, a history of preeclampsia or HELLP syndrome, and delivery before 34 weeks of gestation. Controls were women with a history of at least one uneventful pregnancy, recruited at the University Medical Center Utrecht (N=75) and St Radboud University Medical Center Nijmegen (N=38). At inclusion, demographic data and whole blood samples for DNA isolation were obtained from all participants. For a subgroup of 214 women (all recruited at the University Medical Center Utrecht, at least six months after delivery; N=144 cases), plasma samples were available for analysis of metabolic, lipid and inflammatory markers. Inclusion criteria, sample collection and data acquisition were previously described elsewhere [18-20]. The study was approved by the local Institutional Review Boards and written informed consent was obtained from all participants. Preeclampsia was defined as gestational hypertension and proteinuria according to the criteria of the International Society for the Study of Hypertension in Pregnancy [21], i.e. de novo hypertension above 90 mm Hg at two or more consecutive measurements, at least 4 h apart, and proteinuria above 0.3 g per 24 hours. HELLP syndrome was defined according to previously described criteria [22], as Small-for-gestational age (SGA) was defined as birth weight below the 5th centile, based on the Dutch population charts [23]. Finally, chronic hypertension was defined as hypertension, treated with anti-hypertensive medication initiated before first pregnancy.

Procedures

Genomic DNA was isolated from blood, using standard commercially available kits (Gentra Systems, Minneapolis, MN, USA). Two common missense mutations of the TLR4 gene (GenBank: NM_138554; OMIM: 603030) were detected by a previously described polymerase chain reaction (PCR) [24], based on exonuclease degradation of dual labelled allele-specific oligonucleotides: a substitution of adenosine by guanine at position 896 in the fourth exon, leading to replacement of aspartic acid by glycine at amino acid position 299 in the extracellular domain of TLR4 (D299G; RefSNP ID: rs4986790) and a frequently co-segregating mutation, leading to an amino acid substitution at position 399 of threonine by isoleucine (T399I; RefSNP ID: rs4986791). In addition, three common allelic variants of the NOD2 gene (GenBank: NM_022162; OMIM: 605956) were determined by a similar genotyping method: a substitution of cytosine by thymine at position 2104 leading to amino acid replacement of arginine by tryptophan at position 702 in the fourth exon (R702W; RefSNP ID: rs2066844), a substitution of cytosine by guanine at position 2722 leading to replacement of glycine at arginine at position 908 in the eighth exon (G908R; RefSNP ID: rs2066845) and an insertion of cytosine at position 3020 leading to an amino acid frame shift from leucine at position 1007 (L1007fs; RefSNP ID: rs2066847). Primers and probes were designed by commercially available custom-made development kits (Assay-by-Design; Applied Biosystems, Foster City, CA, USA) and performed on a 384-wells PCR machine (Biometra, Goettingen, Germany) and an automated fluorimeter (Fluostar Galaxy, BMG LABTECH, Offenburg, Germany), as described in detail before [24].

For phenotype analysis, fasting blood samples were collected, immediately centrifuged and plasma was stored at –80°C until further analysis. Plasma concentrations of high sensitivity C-reactive protein (CRP) were determined using a commercially available reagents for nephelometric analysis (Dade Behring, Marburg, Germany). Fibrinogen levels were measured by the Claus' clotting method using the Sta-R automatic coagulation analyzer with STA Fibrinogen reagent (Diagnostic Stago, Taverny, France). Plasma levels of interleukin-6 (IL-6) and soluble intercellular adhesion molecule-1 (sICAM-1) were assessed by means of commercially available enzyme-linked immunosorbent assay development kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Plasma antigen levels of von Willebrand factor (vWf) were measured with an enzyme-linked immunosorbent assay using commercial antibodies (Dako, Glostrup, Denmark), as previously described [25].

Statistics

Baseline and clinical outcome variables were compared by Students t-tests for continuous variables and Chi-squared tests for categorical variables where appropriate. The relative contributions of TLR4 and NOD2 genotypes to the occurrence of early-onset preeclampsia were estimated by logistic regression analysis, and expressed as odds ratios with 95% confidence intervals (CIs). Because time of diagnosis and the presence of pre-existent hypertensive disorders could theoretically influence the diagnosis of preeclampsia, odds ratios were adjusted for age at first delivery and chronic hypertension, by multivariable logistic regression analysis. To account for their skewed distributions, circulating levels of inflammatory biomarkers were presented as medians with interquartile range, and comparisons between cases and controls were based on Mann-Whitney *U* statistics. Correlations between continuous variables were calculated with the use of age-adjusted Spearman partial correlation coefficients. In addition, interactions between inflammatory markers and allelic variants of TLR4 and NOD2 were evaluated by calculating odds ratios for occurrence of preeclampsia or HELLP syndrome, according to each tertile of the plasma level distribution of the control population, also by logistic regression analysis. To evaluate the combined contributions of genotype and tertiles of plasma inflammatory markers, trend analysis was performed by logistic regression analysis among the following groups using a regression equation for early-onset preeclampsia [26]: women negative for any of the minor allelic variants of TLR4 and NOD2 and within the lowest tertile of plasma inflammatory markers, according to the distribution among controls (reference group; score=1), women negative for allelic variants within the highest tertiles and women positive for one or more allelic variants within the lower two tertiles (score=2), and women positive for one or more allelic variants within the highest tertiles (score=3). Subsequently, multivariable logistic regression analysis was used to control for age at inclusion, differences in body-mass index (less than 20, 20 to 25, 25 to 30 and 30 or more), interval between delivery and inclusion, current smoker (yes or no), and chronic hypertension (yes or no).

Results

Women with a history of early-onset preeclampsia had significantly higher body-mass index and slightly lower age at enrolment in the study than controls. Among the 340 women with first pregnancy early-onset preeclampsia, 177 women developed HELLP syndrome, and 98 delivered small-for-gestational age infants (**Table I**).

All genotype frequencies were in Hardy-Weinberg equilibrium with allele frequencies for

TLR4 at 7.4% for the D-allele at position 299 and 7.2% for the I-allele at position 399, and for NOD2 at 4.4% for the W-allele at position 702, 1.0% for the R-allele at position 908 and 1.8% for the frame shift deletion at position 1007. As expected, TLR4 D299G and T399I genotypes showed a high co-segregation rate of 97%.

Table 1 Baseline characteristics, first pregnancy outcome and plasma markers of inflammation and the acute-phase response

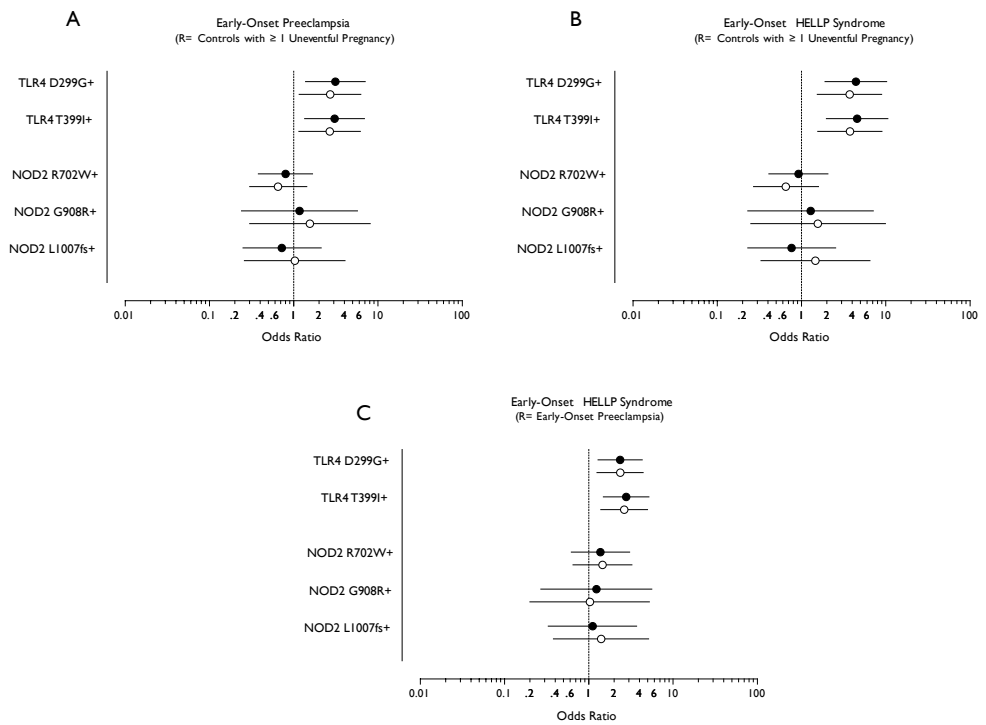
Characteristic	Early-Onset Preeclampsia (N=340)	Controls (N=113)	P Value
Maternal age at inclusion – yr	30.6±4.4	32.8±4.3	<0.001
Maternal age at delivery – yr	29.7±4.4	31.1±4.2	0.005
Height – cm	169±7	169±10	0.73
Weight – kg	73±15	66±11	0.001
Body-mass index	25.7±5.3	22.7±4.0	<0.001
Chronic hypertension – no. (%)	61 (19)	7 (7)	<0.001
Ethnicity – caucasian no. (%)	326 (97)	112 (99)	0.21
Current smoker – no. (%)	43 (17)	19 (21)	0.31
Smoking during pregnancy † – no. (%)	59 (18)	16 (18)	0.92
Gestational age at delivery † – wk	29.9±2.4	40.0±1.3	<0.001
Infant's birth weight † – g	1090±427	3576±469	<0.001
Hellp-syndrome † – no. (%)	177 (51)	-	-
Small-for-gestational-age infant (<5th centile) † – no. (%)	98 (28)	1 (1)	<0.001
C-reactive protein – mg/L	0.9±2.8	0.6±1.0	0.05
Fibrinogen – g/L	2.8±1.2	2.5±1.4	0.02
Interleukin-6 – pg/mL	3.6±2.7	2.8±2.4	0.002
sICAM-1 – ng/mL	121±148	138±121	0.04
von Willebrand factor – µg/mL	8.4±5.0	8.2±3.6	0.52

Data are expressed as means ± SD, or as number (%) and compared by the independent sample T-test. Plasma inflammatory markers were measured in a subset of 214 women at least six months after delivery and are expressed as medians ± interquartile range and compared by the nonparametric Mann-Whitney U test.
† Data represent outcomes of first pregnancy.

Among all women with a history of early-onset preeclampsia, positivity for one or more of the minor allelic variants of TLR4 was observed significantly more often than in controls, with an odds ratio of 3.3 (95% CI 1.5 to 7.5). This association between TLR4 genotype and outcome remained significant (adjusted OR 2.9 [95% CI 1.2 to 6.7]), after adjustment for maternal age and chronic hypertension in our multivariable model (**Figure 1a**). In women with early-onset preeclampsia complicated by HELLP syndrome (N=177), positivity for

one or more allelic variants of TLR4 was observed in 1 out of 4 women, with an crude odds ratio of 4.7 (95% CI 2.0 to 10.9) and adjusted odds ratio of 4.1 (95% CI 1.7 to 9.8), when compared to controls with only uneventful pregnancies (**Figure 1b**). In addition, TLR4 polymorphisms were significantly more common in women who developed HELLP syndrome, when compared to women with preeclampsia only (excluding those with HELLP) with an OR of 2.3 (95% CI 1.3 to 4.3) after adjustment for maternal age and chronic hypertension (**Figure 1c**). Individually, each of the NOD2 allelic variants (R702W, G908R and L1007fs) did not contribute to the development of early onset preeclampsia with or without HELLP syndrome (**Figure 1**).

Figure 1 Association of early-onset preeclampsia and HELLP syndrome with common allelic variants of TLR4 and NOD2 related to impaired innate inflammatory responses to endotoxin



Odds ratios (95% confidence intervals) for carrying either heterozygous or homozygous common functional allelic variants of TLR4 (D299G and T399I) and NOD2 (R702W, G908R and L1007fs) are presented as unadjusted values (closed symbols) and were adjusted for maternal age at first delivery and chronic hypertension (open symbols). Comparisons were made between women with a history of early-onset preeclampsia and controls with a history of only uneventful pregnancies (A), between women with a history of early-onset HELLP syndrome and controls with a history of only uneventful pregnancies (B) and between women with a history of early-onset HELLP syndrome and women with a history of early-onset preeclampsia without HELLP syndrome (C).

Median levels of CRP, fibrinogen, and IL-6 were significantly higher in women with a history of early-onset preeclampsia (**Table 1**). Conversely, median sICAM-1 levels were lower in cases and vWf levels showed no difference between cases and controls. We observed no difference in median plasma levels of inflammatory markers between women with a history of HELLP syndrome, compared to women with early-onset preeclampsia without HELLP syndrome (data not shown).

Table 2 Age-adjusted spearman partial correlation coefficients between plasma inflammatory biomarkers, body-mass index, and interval between delivery and enrolment among 70 controls with a history of only uneventful pregnancies and 144 cases with a history of early-onset preeclampsia *

Controls

	CRP	Fibrinogen	IL-6	sICAM	vWf	BMI	Interval
CRP	–						
Fibrinogen	0.17	–					
IL-6	-0.21	0.10	–				
sICAM	0.06	0.01	0.02	–			
vWf	-0.04	-0.03	-0.06	-0.05	–		
BMI	0.22	0.32†	-0.06	-0.14	0.04	–	
Interval	0.00	-0.22	-0.33§	-0.09	-0.08	-0.07	–

Cases

	CRP	Fibrinogen	IL-6	sICAM	vWf	BMI	Interval
CRP	–						
Fibrinogen	0.38¶	–					
IL-6	0.14	0.12	–				
sICAM	0.22§	0.13	-0.03	–			
vWf	0.20†	0.38¶	0.11	-0.03	–		
BMI	0.30§	0.12	0.05	0.08	-0.05	–	
Interval	-0.04	-0.03	0.03	-0.28‡	0.13	-0.07	–

* CRP denotes C-reactive protein, IL-6 interleukin-6, sICAM soluble intercellular adhesion molecule-1, vWf von Willebrand factor, BMI body-mass index and Interval time between delivery and enrolment.
† P value < 0.05
§ P value < 0.01
‡ P value < 0.001
¶ P value < 0.0001

Correlations between plasma levels of inflammatory markers (CRP, IL-6, sICAM-1), acute-phase reactants (fibrinogen, vWf) and baseline variables are shown in **Table 2**. In women with a history of early-onset preeclampsia significant positive correlations were observed between CRP, sICAM-1, fibrinogen and vWf, ranging from 0.20 for CRP and vWf to 0.38 for fibrinogen and vWf and for CRP and fibrinogen. In addition,

body-mass index related to plasma levels of fibrinogen in cases (0.30) and CRP in controls (0.32). Interestingly, plasma levels of CRP, fibrinogen and vWf were unaffected by the time between delivery and enrolment for both cases and controls. However, IL-6 levels showed a significant negative correlation with interval between delivery and inclusion for controls only (–0.33). This correlation was not observed within women with a history of early-onset preeclampsia, probably due to the significantly higher median IL-6 levels in women with a history of early-onset preeclampsia (3.6±2.7 pg/mL compared to 2.8±2.4 pg/mL; p=0.002). A similar effect of interval between delivery and inclusion upon sICAM-1 levels (–0.28; p<0.001) was observed for cases only.

Table 3 shows the relationship between plasma inflammatory markers measured at least six months after delivery and history of early-onset preeclampsia, according to tertiles based on the distribution of plasma levels within the control group of women with a history of at least one uneventful pregnancy. Higher circulating IL-6 levels within both the mid and highest tertiles were observed more frequently in women with a history of early-onset preeclampsia compared to controls (P for trend <0.001), with an unadjusted OR of 3.4 for the mid tertile (95 % CI 1.5 to 7.7) and 4.9 for the highest tertile (95 % CI 2.2 to 11.0). Adjustment for interval between delivery and enrolment, age at inclusion, body-mass index, chronic hypertension and smoking did not affect this relationship. In addition, a history of early-onset preeclampsia was related to lower plasma levels of sICAM-1. After adjustment for confounding variables, ORs (95% CI) for early-onset preeclampsia were 0.3 (0.1 to 0.9; P for trend 0.02) for both the mid and highest tertiles of sICAM-1.

Median levels of CRP, IL-6, sICAM-1, fibrinogen and sICAM-1 showed no direct relationship with heterozygous, homozygous or combined positivity for any of the TLR4 and NOD2 allelic variants (all P values > 0.05 after group comparison by Kruskal-Wallis testing; data not shown). Nonetheless, increasing tertiles of interleukin-6 and fibrinogen levels were associated with higher odds ratios for early-onset preeclampsia in women carrying one or more TLR4 or NOD2 mutations, compared to women with the wild-type TLR4 or NOD2 genotype (**Table 4**). Overall, highest odds ratios for early-onset preeclampsia were observed for women carrying any of the five allelic variants of TLR4 or NOD2, within the highest tertiles for IL-6 and fibrinogen, with ORs of 6.9 (95% CI 2.1 to 23.2; P for trend 0.03 after adjustment for confounding variables) for IL-6 and 3.8 (95% CI 1.2 to 11.8; adjusted P for trend 0.04) for fibrinogen, respectively.

Table 3 Odds ratios (95 percent confidence intervals) for women with a history of early-onset preeclampsia, compared to controls with only uneventful pregnancies, according to plasma levels of acute-phase inflammatory biomarkers at least six months after delivery

	Unadjusted OR (95% CI)				Adjusted OR (95% CI) Model 1 †				Adjusted OR (95% CI) Model 2 §			
	Low (R)	Mid	High	P for trend *	Low (R)	Mid	High	P for trend *	Low (R)	Mid	High	P for trend *
CRP, mg/L [0.4 – 1.0]	1	0.7 (0.4 – 1.5)	1.6 (0.8 – 3.2)	0.16	1	0.8 (0.4 – 1.6)	1.6 (0.8 – 3.2)	0.17	1	0.4 (0.1 – 1.2)	0.9 (0.3 – 2.3)	0.86
Fibrinogen,g/L [2.1 – 2.7]	1	0.9 (0.4 – 2.0)	2.1 (1.0 – 4.5)	0.03	1	0.8 (0.4 – 1.8)	1.9 (0.9 – 4.1)	0.07	1	1.6 (0.5 – 5.2)	1.2 (0.4 – 3.4)	0.71
IL-6, pg/mL [2.1 – 3.5]	1	3.4 (1.5 – 7.7)	4.9 (2.2 – 11)	<0.001	1	2.9 (1.3 – 6.7)	3.9 (1.7 – 9.1)	0.002	1	3.6 (1.1 – 11)	4.6 (1.4 – 14)	0.01
sICAM-1, ng/mL [93 – 179]	1	0.4 (0.2 – 0.8)	0.6 (0.3 – 1.3)	0.09	1	0.3 (0.2 – 0.7)	0.5 (0.3 – 1.0)	0.06	1	0.3 (0.1 – 0.9)	0.3 (0.1 – 0.9)	0.02
vWf, µg/mL [7.1 – 9.4]	1	0.5 (0.3 – 1.1)	1.0 (0.5 – 1.9)	0.82	1	0.5 (0.2 – 1.1)	1.0 (0.5 – 2.0)	0.91	1	0.4 (0.2 – 1.2)	0.8 (0.3 – 1.9)	0.41

** P values represent trends accross increasing tertiles of plasma levels, by logistic regression analysis.*

† Model 1 includes tertiles of plasma levels and interval between delivery and enrolment.

§ Model 2 includes tertiles of plasma levels, interval between delivery and enrolment, age at inclusion, body-mass index, current smoking and chronic hypertension.

Table 4 Genotype-phenotype interactions for TLR4 and NOD2 genotypes and plasma inflammatory markers, among women with a history of early-onset preeclampsia and controls with only uneventful pregnancies *

	TLR4 negative (n=194)		TLR4 positive (n=36)			
	Low (R)	High	Low	High	*P Value*†	*P_a Value*¶
CRP	1	1.6 (0.8 – 3.4)	3.9 (0.8 – 19.0)	3.0 (0.8 – 11.6)	0.05	0.13
Fibrinogen	1	2.2 (1.0 – 4.8)	3.5 (0.7 – 18.0)	4.7 (1.2 – 18.0)	0.007	0.02
IL-6	1	4.4 (1.9 – 10.3)	9.0 (1.7 – 46.5)	7.5 (1.8 – 30.8)	<0.001	0.006
sICAM	1	0.6 (0.3 – 1.3)	2.4 (0.7 – 9.0)	1.3 (0.2 – 6.7)	0.86	0.34
vWf	1	0.9 (0.5 – 1.9)	2.7 (0.7 – 10.1)	2.0 (0.4 – 10.1)	0.43	0.43
	NOD2 negative (n=200)		NOD2 positive (n=30)			
	Low (R)	High	Low	High	*P Value*†	*P_a Value*¶
CRP	1	1.5 (0.7 – 3.1)	0.9 (0.3 – 2.8)	2.7 (0.5 – 13.4)	0.19	0.39
Fibrinogen	1	1.9 (0.9 – 4.2)	0.8 (0.2 – 2.6)	3.9 (0.8 – 19.3)	0.06	0.14
IL-6	1	4.5 (1.9 – 10.7)	2.1 (0.6 – 7.3)	9.2 (1.8 – 48.2)	<0.001	0.01
sICAM	1	0.6 (0.3 – 1.3)	1.0 (0.3 – 3.6)	0.7 (0.2 – 2.5)	0.34	0.10
vWf	1	0.8 (0.4 – 1.7)	0.7 (0.2 – 2.1)	2.1 (0.4 – 10.2)	0.85	0.93
	TLR4 or NOD2 negative (n=170)		TLR4 or NOD2 positive (n=60)			
	Low (R)	High	Low (R)	High	*P Value*†	*P_a Value*¶
CRP	1	1.6 (0.7 – 3.5)	1.7 (0.6 – 4.5)	2.6 (0.8 – 8.0)	0.07	0.23
Fibrinogen	1	2.2 (1.0 – 5.1)	1.6 (0.5 – 4.5)	3.8 (1.2 – 11.8)	0.02	0.04
IL-6	1	4.3 (1.7 – 10.5)	3.6 (1.2 – 10.7)	6.9 (2.1 – 23.2)	<0.001	0.03
sICAM	1	0.7 (0.3 – 1.5)	1.6 (0.6 – 4.2)	0.8 (0.3 – 2.5)	0.76	0.27
vWf	1	0.9 (0.4 – 1.9)	1.3 (0.5 – 3.2)	1.9 (0.6 – 6.5)	0.43	0.57

** Data represent odds ratios and 95% CIs for early-onset preeclampsia compared to controls with at least one uneventful pregnancy, with respect to the reference group (R) of women negative for the all minor allelic variants of TLR4 and NOD2 and with low values for plasma inflammatory markers. High values are values within the highest tertiles of the distribution of plasma levels of the control group. Low values are values within the lowest tertiles (reference group) and values within the lower two tertiles (TLR4 and NOD2 positive group), respectively; CRP denotes C-reactive protein, IL-6 interleukin-6, sICAM soluble intercellular adhesion molecule-1 and vWf von Willebrand factor.*

† P values for trend between groups, compared to the reference group (R) of women negative for allelic variants of TLR4 and NOD2, and with low levels of plasma inflammatory markers, by univariable logistic regression analysis.

¶ P_a values for trend between groups, compared to the reference group (R), after adjustment for age at inclusion, interval between delivery and enrolment, body-mass index, smoking and chronic hypertension, by multivariable logistic regression analysis.

Discussion

We observed two novel findings from our study. First, that maternal predisposition to early-onset preeclampsia and HELLP syndrome is related to allelic variants of genes that impair the innate immune response, as demonstrated by the association with common TLR4 polymorphisms. Second, that high circulating levels of biomarkers representative of the inflammatory response are related to a history of early-onset preeclampsia and HELLP syndrome in apparently healthy non-pregnant women. Our findings are consistent with a central role for the maternal innate immune system in the development of severe hypertensive disorders of pregnancy. Moreover, our findings provide the first evidence for a potential role of endotoxin responsiveness in the susceptibility to preeclampsia and HELLP in humans.

Endotoxin, also known as lipopolysaccharide (LPS), has been shown to induce pregnancy-specific inflammatory and endothelial cell disturbances in rats, including hypertension and proteinuria and characteristic glomerular endotheliosis lesions, with striking similarities to the clinical syndrome of preeclampsia [12]. Taken together with human data showing elevated circulating biomarkers of soluble and cellular components of the innate immune system before, during and after pregnancies complicated by early-onset preeclampsia [3,7,27], we hypothesized that genetically encoded individual differences in the inflammatory response to LPS could influence maternal predisposition to the disorder. In the present study, we demonstrated that the allelic variants D299G and T399I of the extracellular pattern-recognition receptor TLR4, that impair the inflammatory response to LPS, are related to a history of early-onset preeclampsia. Alternately, we found no association for three attenuating polymorphisms (R702W, G908R and L1007fs) of the innate immune receptor NOD2, which binds the bacterial component muramyl dipeptide (MDP) and is involved in intracellular LPS-signalling [14].

Toll-like receptors (TLR) are considered as the most important class of pattern-recognition receptors (PRRs), involved in host defense against a variety of microbes by induction and regulation of innate immunity. At present 10 members of the TLR family have been discovered in humans, of which TLR4 was first recognized as a key receptor for the LPS component of Gram-negative bacteria. In addition, TLR4 has been shown to recognize chlamydial heat shock protein, respiratory syncytial virus fusion (F) protein and molecular patterns of malarial parasites. Two functional mutations in the extracellular LRR domain of human *TLR4* have been identified at position A896C, leading A/G amino acid transition at position 299, and C1196T, leading to T/I transition at position 399. Although co-segregation of the D299G and T399I genotype occurs approximately 98% of Western populations, both polymorphic variants appeared have been shown to independently

affect responsiveness to inhaled LPS. Recent stoichiometric analysis of the D299G and T399I polymorphic variants of TLR4 confirmed that the presence of each of the D299G and T399I variants results in distinct structural changes in the ligand-binding site of the receptor, which account for increased susceptibility to infectious disease such as Gram-negative sepsis, disseminated candidiasis and malaria.

As TLR4 mutations have been shown to determine susceptibility to a number of exogenous infectious agents, should we consider potential infectious agents responsible for causing preeclampsia or HELLP syndrome? Recently, it has indeed been demonstrated that women with preeclampsia are prone to periodontal infections, mainly caused by gram-negative bacteria [28]. Other associations with preeclampsia have been observed for adeno-associated virus 2 (AAV-2) [29], parvovirus B19 [30] and cytomegalovirus [31]. Of interest, preeclampsia appears more commonly in women who are seropositive for *Chlamydia Pneumoniae* [32], which is a TLR4 dependent intracellular pathogen related to the development of atheromatous lesions. Also, in a recent study of 304 pregnant women infected with *P. Falciparum* from South Ghana, the D299G allelic variant of TLR4 was associated with a 5-fold increased risk of severe maternal anemia, as a result of ineffective clearance of the malaria parasites. Although the interaction of TLR4 with *P. Falciparum* is not fully understood, Krishnegowda and colleagues showed in vitro that TLR4 can be activated by malarial glycosylphosphatidylinositols (GPIs) to induce a pro-inflammatory response. It is, however, not known if the D299G variant has any effect on this interaction. Furthermore, in women with HELLP syndrome, in vitro experiments show striking differences in monocyte handling of LPS and gram-negative bacteria, when compared to women with preeclampsia only [33]. In our study, TLR4 mutations were 2.4 times more common in women with HELLP syndrome than those with preeclampsia only, suggesting that the TLR4 pathway might be involved in the development of HELLP episodes in women with early-onset preeclampsia. The HELLP syndrome is generally considered the clinical extreme of preeclampsia, although its acute onset and frequent spontaneous (albeit temporary) resolution within days, even in those women who do not deliver [34,35], suggests a more rapid deterioration initiated by temporally released, yet unknown triggers. Our data suggest that exogenous (infectious) agents involved in the TLR4 pathway, might be candidate factors triggering HELLP syndrome within the already immuno-compromised pre-eclamptic patient. On the other hand, it has been suggested that TLR4 can be activated by number of endogenous ligands involved in vascular damage, including heat-shock protein 60 and the extracellular domain A (EDA) of fibronectin [36,37]. Although these data are inconclusive because of potential LPS contamination during analytical procedures, they suggest a role for TLR4 in the so-called "danger hypothesis" [38]. This hypothesis states that tissue damage (either by endogenous or exogenous agents) involves

the release of signalling molecules that are capable of eliciting an immediate immune reaction. In preeclampsia, elevated circulating levels of cellular and sub-cellular placental debris, including shedding of syncytiotrophoblast membrane fragments [1,3], cell-free fetal DNA [39] and soluble fms-like tyrosine kinase 1 (sFlt-1) [40], have been shown to induce endothelial damage and precede onset of the clinical syndrome. It would be interesting to investigate the role of TLR4 in the maternal response required to eliminate excess shedding of placental factors.

NOD2 is a member of the Apaf1/Ced4 superfamily of apoptosis regulators, mainly expressed in monocytes, that acts as an intracellular pattern recognition proteins (PRRs), containing LRR domains similar to TLR4. Although NOD proteins have an indirect role in LPS signaling, NOD2 primarily recognizes a muramyl dipeptide (MDP) motif of peptiglycans found in virtually all classes of bacteria, similar to the NOD1 ligand diaminopimelic acid (DAP)-containing muramyl tripeptide (M-TriDAP), found in gram-negative bacteria. In addition to its role as an intracellular PRR, genetic mapping studies have identified NOD2 as a strong susceptibility gene for Crohn's disease. The allelic variants L1007fs, R702W and G908R, each represent three independent haplotypes commonly observed in patients with European ancestry. Individuals homozygous for any of these mutations exhibit an odds ratio of 42.1 for developing Crohn's disease. Crohn's disease is an inflammatory disorder that affects the digestive tract, the underlying cause of which is largely unknown. However, the discovery of NOD2 gene variants related to the disorder, suggests a role for defective bacterial sensing in its etiology. Recent studies have clarified the genetic and molecular basis for this association, as reviewed in ref xx. All three variants affect the LRR-domain and are considered loss-of-function mutations, likely to impair the recognition of microbial components, interfering with appropriate activation of NF-κB in monocytes, as well as disregulating NOD1 function. Also, NOD2-/- mice exhibit a TH-1 type pro-inflammatory phenotype following stimulation with TLR agonists, that suggests a more fundamental role for NOD2 in controlling the innate immune response. Although the role of NOD proteins in pregnancy and placentation has not yet been investigated, protein expression and induction of pro-inflammatory cytokine release after NOD1 and NOD2 activation, was recently observed in first trimester trophoblast cells. In our study, no association was observed for common NOD2 polymorphisms alone and a history of early-onset preeclampsia or HELLP syndrome. However, our data suggest a combined association of NOD2 allelic variants and maternal pro-inflammatory phenotype with pregnancy outcome, which will be further discussed below.

In addition, we observed elevated levels of circulating biomarkers for inflammation and the acute-phase response in women with a history of early-onset preeclampsia, when measured at least six months after delivery, including CRP, fibrinogen and IL-6, as compared

to women who experienced only uneventful pregnancies. Similar to previous reports [27], our data demonstrate that non-pregnant women with a history of early-onset preeclampsia more frequently exhibit a pro-inflammatory phenotype. Biomarkers of the acute-phase inflammatory response, including CRP, fibrinogen, IL-6 and vWf, have all been shown to be predictive of long-term cardiovascular risk, although not independently adding to classic risk factor algorithms [41]. This study suggests that an innate maternal pro-inflammatory phenotype might be a common underlying risk factor for early-onset preeclampsia and cardiovascular disease [42]. Surprisingly, we observed an inverse relationship between sICAM-1 levels and history of early-onset preeclampsia, which is in contrast with previous findings by Freeman and colleagues [27]. However, their study reported on sICAM-1 levels determined 20 years after delivery, whereas our data represent samples collected at a mean interval between delivery and enrolment of 0.7 years (range 0.5 to 8.5 years).

In this study, elevated plasma inflammatory biomarker levels were unaffected by TLR4 and NOD2 genotype, and correlated with increasing odds ratios for early-onset preeclampsia even after careful correction for confounding factors, including age, interval between delivery and enrolment, body-mass index, smoking status and chronic hypertension. When examining the combined association of TLR4 or NOD2 mutations and circulating levels of inflammatory markers, highest odds ratios were observed for women carrying allelic variants of TLR4 or NOD2, who were in the upper tertiles of CRP, fibrinogen and IL-6. Compared to women with plasma levels within the lowest tertiles of the control distribution and negative for any of the TLR4 and NOD2 polymorphisms, the highest tertiles of fibrinogen and IL-6 and the presence of attenuating allelic variants of TLR4 or NOD2 showed both independent and combined associations with a history of early-onset preeclampsia.

Our case-control study has several strengths and limitations. To our knowledge, this study describes genotype and phenotype data of the largest series of women with a history of early-onset preeclampsia and HELLP syndrome yet, recruited over an interval of more than a decade. However, we designed our study to include only primiparous women, who delivered before 34 weeks of gestation. Therefore, our data may not be extrapolated to multiparae and women with a history of late-onset or near term preeclampsia, as the pathophysiology of the disorder leading to onset after 34 weeks' gestation might differ from that which causes early-onset disease [21]. A major strength of our study is in the assessment of genetic single-nucleotide polymorphisms, which unlike acquired or environmental risk factors are fixed at birth and therefore provide a measure of life-long exposure, not likely to be subject to confounding due to the timing of investigation [43]. Despite this considerable advantage, in any genetic association study with a case-control design, selection bias and population stratification should be considered. Conversely, because plasma samples for

analysis of inflammatory biomarkers were collected after pregnancy, we cannot exclude the possibility that pregnancy itself might have led to temporary or permanent changes in inflammatory status. For some (IL-6 and sICAM-1) but not all (CRP, fibrinogen and vWf) of the measured inflammatory markers we indeed observed a significant correlation with the interval between delivery and enrolment, suggestive of at least short-term changes up to several months after pregnancy. Nonetheless, the observed associations between high levels of interleukin-6 and fibrinogen and a positive history of early-onset preeclampsia were only moderately influenced by adjustment for interval between delivery and sampling. At present no prospective data comparing pre- and post-pregnancy levels of inflammatory markers are available to provide a more conclusive answer to this question.

In summary, we are the first to show a relationship between allelic variants of TLR4, that impair the innate inflammatory response to LPS, early-onset preeclampsia and HELLP syndrome. Next, we observed that a history of early-onset preeclampsia is related to higher plasma levels of CRP, fibrinogen and IL-6 at least six months after delivery. Our results support the hypothesis that the innate immune system contributes to maternal susceptibility to early-onset preeclampsia and HELLP syndrome. Therefore, identification of components of the maternal innate inflammatory system that predispose to hypertensive disorders of pregnancy merits further investigation.

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8

In Vivo Acute-Phase Inflammatory Response to Influenza Vaccination in Women with a History of Early-Onset Preeclampsia

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Submitted

Abstract

Objective

Inflammation plays a central role in early-onset preeclampsia. Currently it is unknown whether maternal inflammatory changes are specific to pregnancy or reflect an innate susceptibility to inflammation. C-reactive protein (CRP) and interleukin-6 (IL-6) are markers of the acute-phase inflammatory response and predictive of future cardiovascular events. We compared CRP and IL-6 levels after influenza vaccination, as an *in vivo* model for low-grade inflammation, in non-pregnant women with a history of early-onset preeclampsia and controls with only uneventful pregnancies.

Methods

Forty-four women with a history of early-onset preeclampsia (delivery <34 weeks' gestation) and twenty-nine controls with at least one uneventful pregnancy received an influenza vaccination. We then compared plasma levels of CRP and IL-6 at baseline, 1.3 days and 3.3 days after vaccination.

Results

Median baseline CRP and IL-6 levels of women with a history of early-onset preeclampsia were comparable to controls (1.6 versus 0.8 mg/L; p=0.44 and 5.0 versus 3.4 pg/L; p=0.34, respectively). However, high CRP and IL-6 responses to vaccination were more common in cases (ORs for response >75th, >80th, >85th, >90th and >95th percentile based on the distribution of control values of 2.3, 2.7, 3.1, 4.3 and ∞ for CRP [P for trend 0.11] and of 0.9, 1.4, 1.9, 2.6 and 4.5 for IL-6 [P for trend 0.043], respectively). The relationship between high IL-6 responses and early-onset preeclampsia persisted after adjustment for body-mass index (P for trend 0.048).

Conclusion

Women with a history of early-onset preeclampsia more frequently exhibit an innate pro-inflammatory phenotype not specific to pregnancy.

Introduction

Preeclampsia remains a major cause of maternal and neonatal morbidity and mortality throughout the world, that affects 4.2 million women each year [1]. Despite considerable advances in understanding its disease process [2], preeclampsia has no known underlying cause. Most likely, preeclampsia is a vascular disease of multifactorial origin, resulting from an abnormal interaction between maternal constitution, fetal and placental factors that lead sto damage to the maternal endothelium [2,3]. Many features of preeclampsia are similar to those observed in atherosclerosis [4]. Both disorders are characterized by endothelial dysfunction. Common risk factors include obesity, dyslipidemia, chronic hypertension, insulin resistance, thrombophilias and hyperhomocysteinemia [3,4]. Furthermore, recent findings in population based cohort studies demonstrated an excess long-term risk of premature atherosclerosis [5], coronary heart disease [6], stroke [7], and death due to cardiovascular causes [8] in women with a history of preeclampsia. Importantly, cardiovascular risk seems especially related to women who developed preeclampsia at an early gestational age [6,8]. Early-onset preeclampsia might therefore reveal a shared susceptibility to vascular disease, disclosed in response to pregnancy induced maternal adaptive changes.

The hypothesis has been postulated that the endothelial dysfunction seen in preeclampsia is part of a more generalized excessive maternal inflammatory response to pregnancy [2,9,10]. A mild inflammatory response, as demonstrated by altered cytokine production and activation of circulating leukocytes, is observed in normal pregnancy [9,10]. In preeclampsia, this response is more pronounced, possibly causing the endothelial changes associated with the maternal syndrome. Furthermore, women with a history of preeclampsia exhibit short-term and long-term changes in plasma inflammatory biomarkers representative of the acute-phase inflammatory response, such as C-reactive protein (CRP), interleukin-6 (IL-6) and fibrinogen up to 20 years after the index pregnancy [11]. This implicates that any factor that increases the maternal systemic inflammatory response would theoretically predispose to preeclampsia. Candidate factors that might lower the threshold for developing preeclampsia may thus include pathways not specific for pregnancy, such as susceptibility to infections, chronic inflammatory diseases and a pre-existent pro-inflammatory tendency.

CRP is an acute-phase reactant synthesized in hepatocytes, predominantly under transcriptional control of IL-6, in response to inflammation, infections and tissue damage [12]. Interestingly, subjects in the general population tend to have relatively stable baseline CRP and IL-6 levels characteristic for each individual, apart from occasional spikes related to minor trauma, injury or inflammation. In population-based studies, these baseline CRP

and IL-6 levels are higher in women destined to develop cardiovascular disease [13,14]. Twin studies have shown a significant heritable component in baseline IL-6 levels, and to a lesser extent in CRP levels, that is modified by age and body-mass index [15,16]. By contrast, there is a substantial variation between individuals in the intensity of the acute-phase response to identical inflammatory stimuli, such as vaccinations. In response to the same vaccine, some subjects respond repeatedly with high fever, while others have not vaccination attributable effects at all [17,18]. In addition, monozygotic twins show remarkable similarities in their reaction to *ex vivo* stimulation of the immune system, which suggests a strong heritable component in the intensity of the inflammatory response, independent on the type of stimulus [19]. We have previously shown that individuals who are higher "CRP responders", through genetic or acquired mechanisms, can be identified by measuring the inflammatory response shortly after influenza vaccination, as an experimental model to study mild stimulation of the inflammatory system [18].
In the present study, we hypothesized that the intensity of the acute-phase response to influenza vaccination is associated with the risk of early-onset preeclampsia. To examine this hypothesis, we compared the *in vivo* inflammatory response after administration of a regular influenza vaccination between women with a history of early-onset preeclampsia and women with a history of only uneventful pregnancies, at least twelve weeks after delivery. We measured plasma levels of CRP and IL-6 before vaccination and at two time points within four days after vaccination. In addition, we examined the impact of body-mass index on the acute-phase inflammatory response to vaccination.

Materials and methods

Seventy-three non-pregnant women were recruited at the University Medical Center Utrecht, The Netherlands, at least twelve weeks after delivery, between September and December 2004. Inclusion criteria for cases (n=44) were a history of first pregnancy preeclampsia and delivery before 34 weeks' gestation. Controls (n=29) were women with a history of only uneventful pregnancies. Preeclampsia was defined as *de novo* gestational hypertension and proteinuria, according to the criteria of the International Society for the Study of Hypertension in Pregnancy [20]. The hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome was defined according previously published criteria proposed by Sibai and colleagues [21]. Small-for-gestational age (SGA) was defined as birth weight below the 5th percentile, based on the Dutch population charts [22]. Chronic hypertension was defined as hypertension, requiring anti-hypertensive treatment. The local Institutional Review Board approved of the study and all women gave written informed consent.

At enrolment, demographic data were recorded and a baseline venous blood sample was obtained before vaccination. Next, all women received a standard influenza vaccination (Influvac®, Solvay Pharma, Weesp, The Netherlands). Subsequently, venous blood samples were obtained at 1.3 days and 3.3 days after vaccination. All blood samples were collected in Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) containing EDTA, immediately centrifuged and plasma was stored at –80 °C until further analysis. None of the women reported adverse events after vaccination.

Plasma high sensitivity CRP was determined using commercially available reagents for nephelometric analysis (Dade Behring, Marburg, Germany). IL-6 levels were determined using a commercially available enzyme-linked immunosorbent assay development kit (R&D Systems, Minneapolis, MN, USA). Both assays had a within-run and day-to-day variability of less than 15%.

Baseline characteristics were compared between cases and controls by the independent samples T-test for continuous variables and the chi-squared test for categorical variables, where appropriate. Because plasma levels of CRP and IL-6 showed skewed distributions, comparisons between cases and controls at baseline, 1.3 days and 3.3 days after vaccination, were compared by the non-parametric Mann Whitney *U* test. Inflammatory response to vaccination was determined by calculating the relative change ($t2_{max}$ /t1) in plasma level of CRP and IL-6 from baseline (before vaccination, t1) to the maximum value at either 1.3 or 3.3 days after vaccination ($t2_{max}$). Subsequently, odds ratios for early-onset preeclampsia were calculated by a series of logistic regression analyses, according to predefined cut-off points of the maximum relative change ($t2_{max}$/ t1) in plasma levels of CRP and IL-6 after vaccination. Cut-off points were calculated to divide the study sample according to the 50th, 75th, 80th, 85th, 90th and 95th percentiles based on the distribution of values within the control group. In addition, multivariable logistic regression analysis was applied to adjust for body-mass index. All P values are two-tailed and differences were considered significant at $P<0.05$. Analyses were performed with the SPSS 12.0 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics are shown in **Table 1**. Women with a history of early-onset preeclampsia had significantly higher body-mass index, systolic and diastolic blood pressure

at inclusion. The hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome was diagnosed in 78% of cases, and 45% delivered small-for-gestational age (SGA) infants, compared to none in the control group. In addition, 41% of women with a history of early-onset preeclampsia had chronic hypertension, compared to 3% of controls (P=0.004). No difference between groups was observed for age, smoking, alcohol intake and oral contraceptive use.

Table 1 Characteristics and first pregnancy outcome of women with a history of early-onset preeclampsia and women with a history of only uneventful pregnancies.*

Characteristic	Women with a History of Early-onset Preeclampsia (N=44)	Women with a History of only Uneventful Pregnancies (N=29)	P Value
Age – yr	34.5±5.0	34.8±4.7	
Height – cm	169±7	171±7	
Weight – kg	72.4±15.5	65.3±8.8	0.03
Body-mass index	25.5±5.7	22.4±2.6	0.008
Systolic blood pressure – mm Hg	122±15	110±10	0.001
Diastolic blood pressure – mm Hg	80±10	73±5	0.0007
Primipara – no. (%)	25 (57)	16 (55)	
Interval between last delivery and enrolment – m	30.4±19.2	31.1±31.3	
Interval between first delivery and enrolment – m	40.8±28.0	43.9±41.6	
Gestational age at delivery – wk	29.5±2.9	40.2±1.2	<0.0001
HELLP syndrome – no. (%)	32 (73)	0 (0)	<0.0001
Infant's birth weight – g	1058±474	3480±628	<0.0001
Small-for-gestational age infant (<5th percentile) – no. (%)	20 (45)	0 (0)	<0.0001
Current smoker – no. (%)	11 (27)	3 (10)	
Current alcohol intake > 1 U / wk – no. (%)	21 (51)	21 (72)	
Oral contraceptive use – no. (%)	17 (41)	9 (31)	
Chronic hypertension – no. (%)	13 (32)	1 (3)	0.004
Coronary angioplasty – no. (%)	2 (5)	0 (0)	
Pulmonary embolism – no. (%)	2 (5)	0 (0)	
Diabetes mellitus – no. (%)	1 (2)	0 (0)	
Hyperhomocysteinemia – no. (%)	3 (7)	0 (0)	
Chronic kidney disease – no. (%)	1 (2)	0 (0)	
Hypothyroidism – no. (%)	1 (2)	1 (3)	

**Data are expressed as means ±SD, or as number (%). P values are given only for significant differences, for comparison by the Student's t-test.*

Table 2 shows medians ± interquartile range for plasma CRP and IL-6 levels at baseline, 1.3 days and 3.3 days after influenza vaccination. Comparison between cases and controls showed no significant differences for median CRP and IL-6 levels, except for a trend towards higher median CRP levels at 1.3 days and 3.3 days after vaccination (p=0.09).

Table 2 C-Reactive Protein (CRP) and interleukin-6 (IL-6) plasma levels in women with a history of early-onset preeclampsia and women with a history of only uneventful pregnancies at baseline, at 1.3 days, and at 3.3 days after influenza vaccination.*

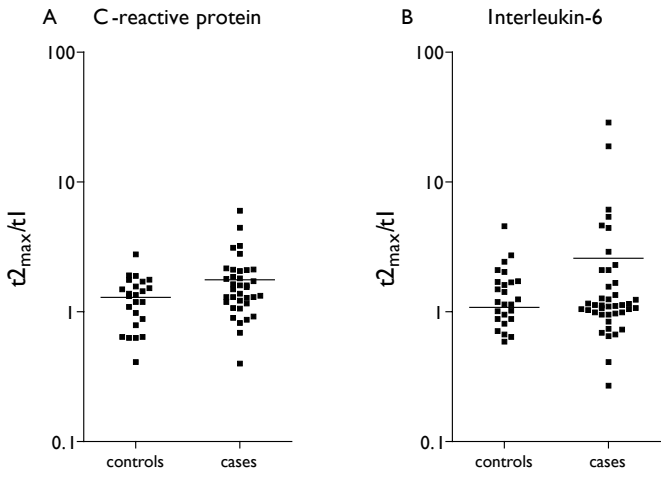
Characteristic	Women with a History of Early-onset Preeclampsia (N=44)	Women with a History of only Uneventful Pregnancies (N=29)	P Value
Median CRP level at baseline – mg/L	1.6±3.4	0.8±2.7	0.44
Median CRP level at 1.3 days after vaccination – mg/L	1.8±4.0	0.9±3.2	0.09
Median CRP level at 3.3 days after vaccination – mg/L	1.8±4.2	0.9±1.8	0.09
Median IL-6 level at baseline – pg/L	5.0±16.8	3.4±14.8	0.34
Median IL-6 level at 1.3 days after vaccination – pg/L	6.2±20.1	4.2±10.7	0.18
Median IL-6 level at 3.3 days after vaccination – pg/L	5.2±16.9	4.7±11.0	0.75

**Data are expressed as medians ±interquartile range, or as number (%). P values are given for statistical comparison by the Mann-Whitney U test.*

The acute-phase inflammatory response to influenza vaccination, as measured by the relative change in CRP and IL-6 levels from baseline to the maximum plasma level at 1.3 and 3.3 days after vaccination, is shown in **Figure 1**. The median increase in levels of inflammatory markers after vaccination was 55% for cases compared to 34% for controls for CRP, and 12% versus 8% for IL-6, respectively. This comparison at group level did not reach significance.

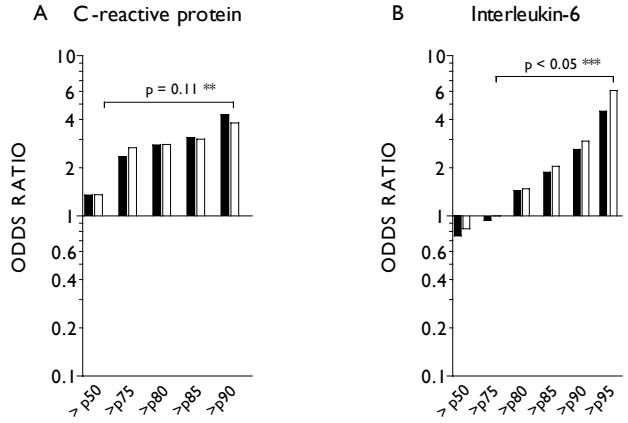
However, odds ratios for early-onset preeclampsia, according to the predefined cut-off points for the relative change in CRP and IL-6 levels after influenza vaccination based on the distribution of responses in the control group, revealed an association with increasing response values for IL-6 (**Figure 2**). High IL-6 responses were related to an increase in odds ratios for early-onset preeclampsia from 1.44 in excess of the 80th percentile to 4.50 in excess of the 95th percentile (P for trend > 75th percentile 0.044). For CRP responses after vaccination, a similar increase in odds ratios for early-onset preeclampsia was observed, ranging from 1.35 above the 50th percentile to 4.28 above the 90th percentile, although this effect did not reach significance (P for trend > 75th percentile 0.11). Both changes in CRP and IL-6 were unaffected by adjustment for body-mass index by multivariable logistic regression analysis (**Figure 2**).

Figure 1 In vivo inflammatory response after influenza vaccination in women with a history of early-onset preeclampsia (cases) and women with a history of only uneventful pregnancies (controls), as measured by the increase in C-reactive protein and interleukin-6 within three days after administration.*



** Data represent the maximum change in plasma levels of inflammatory markers measured at t=32 hours and t=80 hours after vaccination ($t2_{max}$), relative to the baseline value before vaccination (t1), for C-reactive protein in mg/L and for interleukin-6 in pg/L.*

Figure 2 Odds ratios for first pregnancy early-onset preeclampsia, according to the *in vivo* inflammatory response after influenza vaccination, compared to controls with only uneventful pregnancies, as measured by the increase in C-reactive protein and interleukin-6 within four days after administration.*



** Data represent crude odds ratios (closed bars) for first pregnancy early-onset preeclampsia compared to controls with only uneventful pregnancies, according to different cut-off points for the maximum relative change ($t2_{max}$/t1) in plasma level of C-reactive protein and interleukin-6 between baseline (t1) to 1.3 and 3.3 days (t2) after influenza vaccination, by univariable logistic regression analysis; subsequent adjustment for body-mass index (open bars) was performed by multivariable logistic regression analysis. P values for trend were calculated for the upper two quartiles.*

*** P = 0.13 after adjustment for body-mass index.*

**** P = 0.048 after adjustment for body-mass index.*

Discussion

In this experimental case-control study of apparently healthy non-pregnant women, we examined the maternal immune response to influenza vaccination, as an *in vivo* model of low-grade acute inflammation. The principal observation of our study is that women with a history of early-onset preeclampsia more frequently exhibit a high acute-phase inflammatory in response to mild stimulation of the immune system, compared to women with a history of only uneventful pregnancies. At baseline, plasma levels of CRP and IL-6 measured at least six months after delivery, showed values comparable between cases and controls. However, a prominent increase of CRP and IL-6 in response to vaccination was more common in women with a history of early-onset preeclampsia. For both inflammatory biomarkers, vaccination attributable changes in plasma levels were related to a history of early-onset preeclampsia when above the 75th percentile cut-off point, with IL-6 exhibiting an even stronger association in excess of the 90th and 95th cut-off point in a dose-dependent manner. This acute inflammatory response appears to be independent of body-mass index and may be considered as a shared risk factor for preeclampsia and cardiovascular disease.

Epidemiological studies have convincingly demonstrated a greater incidence of coronary heart disease [5,6], stroke [7,23] and mortality due to cardiovascular diseases (CVD) [8] in women with a history of preeclampsia, ranging from a 2-fold relative risk of CVD in women who experienced term preeclampsia to an 8-fold increase in women with early-onset disease (prompting delivery before 34 weeks of gestation) [6,8]. Little is known about the mechanisms leading to this excess CVD risk. Potential predisposing factors include obesity, genetic polymorphisms, hypertension, abnormal lipid profile, insulin resistance and other components of the metabolic syndrome. Previous studies demonstrated the prognostic significance of CRP and IL-6 in the prediction of future cardiovascular event [13,24], which is especially important in women [25]. Chronic low-grade systemic inflammation is known to be proatherogenic [12]. This may reflect a direct causal relationship between CRP and IL-6 production and atherosclerosis, of which women with a history of early-onset preeclampsia more frequently exhibit a subclinical "early phenotype". However, another possibility is that individuals differ in their sensitivity to low-grade acute-phase stimuli, and that women who are "high responders" are more susceptible to pregnancy-induced inflammatory changes, as well as cardiovascular diseases, regardless of a causal effect.

In our study, participants were enrolled approximately 2.5 years after delivery. Although we cannot exclude the possibility that pregnancy itself might have led to temporary or permanent changes in the maternal acute-phase inflammatory response, it is more likely that our results reflect an innate maternal predisposition to inflammation. Acute-phase

production of CRP and IL-6 solely reflects *de novo* synthesis in response to an underlying trigger, and completely returns to baseline, with a plasma half-life of about 19 hours, when the stimulus ceases. The synthesis rate remains unaffected by almost all clinical conditions in health and disease, except by liver failure. In addition, we and others demonstrated that the *in vivo* acute-phase inflammatory response to vaccination is largely independent of the type of vaccine administered [12,18]. Recent vaccination studies in monozygotic and dizygotic twins revealed a strong heritable component in the regulation of experimentally induced inflammatory responses [19]. Thus, unlike baseline values, individual CRP and IL-6 mediated acute-phase responses are remarkably stable and do not reflect the type of stimulus, but an innate sensitivity to inflammation in general.

Obesity is an important risk factor for early-onset preeclampsia [3]. Some authors have argued that the increased inflammatory burden predisposing women to preeclampsia is caused by excess body fat [26]. It has indeed been demonstrated that approximately 25% of IL-6 production originates from adipose tissue [12]. Our study showed no association between body-mass index, baseline values and the relative increase in CRP and IL-6 after influenza vaccination. Furthermore, after adjustment for body-mass index, the association been high CRP and IL-6 responses and a history of early-onset preeclampsia remained unchanged. Our data thus show that obesity is more likely to contribute to preexistent chronic inflammation, rather than to the intensity of the maternal inflammatory response to pregnancy.

In summary, our data suggest that maternal predisposition towards early-onset preeclampsia reflects an innate susceptibility towards inflammation which is not specifically linked to a pregnancy-related stimulus. In addition to the current hypothesis that preeclampsia originates from excess production of specific placental trigger(s) released into the maternal circulation, we propose that preeclampsia is more likely to arise in the presence of preexistent maternal constitutional factors predisposing to inappropriate immunological adaptation to pregnancy. This innate maternal susceptibility to inflammation may prove to be a common risk factor for preeclampsia and long-term cardiovascular disease. If direct involvement of CRP and IL-6 are assumed, this opens perspectives for developing targeted therapeutic and preventive strategies for early-onset preeclampsia, such as CRP blocking agents [27], anti-inflammatory therapy [28,29] and life-style intervention programs [30].

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9

General discussion

In this thesis, follow-up of women with a history of early-onset preeclampsia well after delivery revealed common and previously unknown constitutional factors that related to prior and subsequent pregnancy outcome and might predict future maternal cardiovascular health. Our results have several implications for further research and clinical care. Conclusions and questions that remain from the findings in the previous chapters will be discussed in this final chapter.

1. Next pregnancy outcome after early-onset preeclampsia

1.1 Implications for follow-up and counseling.

Outcome of subsequent pregnancy after early-onset preeclampsia in first pregnancy is generally favorable. As concluded in Chapter 2, approximately 25% of women referred to a tertiary care centre with early-onset preeclampsia in their first pregnancy, develop preeclampsia in their next pregnancy. Although the incidence of recurrent preeclampsia is obviously considerably higher than in women with a prior normotensive pregnancy, most women (78%) with previous early-onset preeclampsia deliver near term (at >37 weeks gestation), and 95% of women deliver at > 34 weeks gestation in their next pregnancy. To the best of our knowledge, we are the first to provide comprehensive data on a reproductive follow-up of women with a history early-onset preeclampsia. In comparison to our results, previous studies have estimated widely different recurrence rates for preeclampsia, that range from 13% to as high as 65% [1-3]. However, these studies did not apply strict criteria for gestational age at onset and included mostly women with near-term preeclampsia, and very small series of women with very early-onset disease. In addition, previous authors have focused on overall recurrence of preeclampsia in subsequent pregnancy, regardless of gestational age at onset. From a clinical point of view, if complications reappear in subsequent pregnancy, maternal and perinatal outcome is most dependent on the gestational age of onset and delivery [4-6]. Furthermore, our data reflect an almost complete 10-year cohort of preeclamptic women referred for tertiary care, a population most representative for clinical practice.

In addition, we performed a comprehensive follow-up study to investigate subsequent pregnancy outcome of women who develop early-onset intrauterine growth restriction in their first pregnancy, that resulted in small-for-gestational age infants (birth weight < 5th percentile), without concomitant maternal hypertensive disease, as described in Chapter 3. Compared to women with early-onset preeclampsia who delivered small-for-gestational

age infants, women with early-onset normotensive intrauterine growth restriction more frequently developed severe perinatal complications in their next pregnancy. Although this study describes a relatively small series (N=22) of patients referred for tertiary care during more than a decade, to our knowledge, no other data exist for this specific subgroup of patients with previous normotensive intrauterine growth restriction at < 34 weeks gestational age. Taken together, we feel that the findings from both studies are an important tool for counseling women who developed early-onset preeclampsia and intrauterine growth restriction in their first pregnancy, with respect to future pregnancies. Of note, counseling should focus on the estimation of recurrence risk for severe adverse pregnancy outcomes, in particular for recurrent preterm delivery.

In our study population, term preeclampsia recurred more often in women with chronic hypertension. Because chronic hypertension could be diagnosed post partum in approximately 1 out of 3 women with a history of early-onset preeclampsia, we recommend routine assessment of blood pressure after pregnancy in these women. Although not as yet demonstrated for women with previous early-onset preeclampsia, this association has been shown in other studies of women with term preeclampsia [7]. In addition, smoking was related to recurrence of preterm delivery, with or without preeclampsia. Although previous reports have shown a reduced likelihood of developing pregnancy-induced hypertension and late-onset preeclampsia [8], this protective effect was not confirmed in early-onset preeclampsia [9]. In our data, described in Chapter 4, no association was observed between smoking and previous early-onset preeclampsia. Nonetheless, others have shown that in preeclamptic pregnancy, smoking is associated with increased perinatal mortality, placental abruptions, fetal growth restriction and preterm delivery [10]. Recent multicentre data from the United Kingdom confirm the association between smoking an a doubled risk of delivery before 34 weeks gestational age in women with early-onset preeclampsia, similar to the findings of our follow-up study [11]. This effect of smoking deserves to be discussed, and encouragement to quit smoking should be part of counseling after early-onset preeclampsia.

1.2 Should we consider secondary prevention of preeclampsia?

Prospective studies on secondary prevention of preeclampsia conducted up to now, vary in their selected end-points, i.e. recurrence of early-onset versus late-onset disease, disease severity and definitions for perinatal outcome [12,13]. Most of these studies have demonstrated no effect of the investigated preventive treatments. One exception is low-dose aspirin, for which prospective data provide evidence for a moderate benefit in preventing recurrent preeclampsia for women at high risk, as recently reviewed by the

Cochrane collaborators [14]. In general, our data show that primiparous women who develop early-onset preeclampsia, even in its most severe form including HELLP syndrome and IUGR, have a low recurrence rate of early-onset hypertensive complications and low rates of preterm delivery in their next pregnancy. When considering secondary prevention programs aimed to reduce recurrence of early-onset disease and improve perinatal outcome, it should be acknowledged that the current practice of standard clinical care with low-dose aspirin supplementation, and blood pressure control of those women with chronic hypertension, is associated with favorable outcome. Therefore, efforts to further reduce severe complications in the next pregnancy by additional secondary preventive measures, such as low-molecular weight heparin or other pharmacological or life-style interventions, are likely to yield only little gain, and would always require high numbers needed to treat. Moreover, risk factors for recurrence of severe early-onset complications and recurrence of preterm delivery are not well characterized, with the exception of smoking status. Thus, apart from directing efforts aimed at cessation of smoking in pregnancy, at present selection of high risk patients eligible for secondary prevention of severe adverse pregnancy outcomes is probably not feasible. Conversely, secondary prevention aimed at recurrence of near-term preeclampsia requires fewer numbers needed to screen and treat. However, maternal and perinatal prognosis of near-term hypertensive disorders of pregnancy is generally good, because the condition can usually be treated by prompt delivery, without serious adverse events for either the mother or her baby [14].

It should be noted that in our study chronic hypertension predicted recurrence of late-onset preeclampsia. In our cohort, women with chronic hypertension were routinely monitored and advised to control blood pressure, usually with methyldopa or labetalol, preferably before or early in their subsequent pregnancy. Although no randomized trials have been performed to evaluate the effect of preconceptional blood pressure regulation and next pregnancy outcome [15], strict adherence to blood pressure management might have prevented recurrence of mild or severe disease in some patients. Other potential targets of secondary prevention might include life-style interventions aimed at reduction of body-mass index and a more favorable cardiovascular risk profile. Currently, no studies have been performed to evaluate the efficacy of these measures in women with previous early-onset preeclampsia.

In conclusion, risk of recurrence of severe or early-onset preeclampsia and associated complications is low under current obstetric care, with low-dose aspirin supplementation and adequate blood pressure control. Additional interventions aimed at secondary prevention of preeclampsia were proven ineffective or have not been thoroughly investigated. Most likely, no single measure will be very effective to further prevent severe adverse outcomes due to high numbers needed to treat.

2. Early-onset preeclampsia and risk of cardiovascular disease in later life

2.1 Opportunities for primary prevention of cardiovascular events

The association of hypertensive disorders of pregnancy with long-term risk of cardiovascular disease has consistently been shown in population-based retrospective epidemiological studies, as recently reviewed in a meta-analysis by Bellamy and colleagues [16]. Our data, that are summarized in Chapters 4, 5, 7 and 8, provide insight into cardiovascular risk factors observed in women who experienced preeclampsia in its most severe form, prompting early delivery before 34 weeks gestation in a tertiary care hospital. Follow-up of these women at least six months after delivery revealed a high prevalence of multiple classic and novel cardiovascular risk factors, including obesity, dyslipidemia, components the metabolic syndrome, a pro-inflammatory profile, a pro-coagulant state and chronic hypertension. Taken together, these data provide a window of opportunity for global risk estimation aimed at primary prevention of cardiovascular disease and stroke [17-19].

2.2 Should women with a history of early-onset preeclampsia be considered for periodic assessment and treatment of cardiovascular risk factors?

Current guidelines for effective treatment of cardiovascular risk factors aimed at primary prevention of cardiovascular events, propose stratifying patients by the level of risk and matching the intensity of interventions to the hazard for cardiovascular diseases events [20]. Prospective evidence consistently shows that women with low levels of risk factors have a lifelong low risk of coronary heart disease and stroke [21]. Contrary, women with multiple major cardiovascular risk factors, or with a high estimated absolute 10-year risk of a cardiovascular event, are likely to benefit most from intensive follow-up and life-style intervention programs [20]. In our study summarized in Chapter 4, 89% of women with a history of early-onset preeclampsia exhibited ≥ 1 major independent cardiovascular risk factor at least six months after delivery, 51% of women exhibited ≥ 2 risk factors and 19% exhibited ≥ 3 risk factors. Global cardiovascular risk, as estimated by the Framingham Risk Score algorithm, revealed higher predicted absolute risk of cardiovascular events in women with a history of early-onset preeclampsia, as compared to population-based controls. Although overall absolute 10-year risk of a major cardiovascular event is low (< 5%) for all women, due to maternal age that compensates for the other increased risk factor levels, 9 out of 10 women had an increased risk (> 5%) when extrapolated to the age of 60 years. Current European guidelines recommend

life-style intervention programs and, where appropriate, lipid lowering therapy, for these relatively young women [19]. In our opinion, routine assessment of major modifiable risk factors is justified for women with a history of early-onset preeclampsia. We recommend risk factor assessment to be performed at six to twelve months after delivery, and to include at least appropriate recordings of blood pressure, smoking status, measurement of plasma total cholesterol, LDL-cholesterol or HDL-cholesterol, and preferably also of fasting glucose levels, triglycerides and high sensitivity C-reactive protein [17,18,20]. In addition, we recommend to develop appropriate follow-up programs for women with previous early-onset preeclampsia, to evaluate the effect of global risk estimation and intervention strategies aimed at primary prevention of long-term cardiovascular events, for this specific group of young women at risk.

3. Pregnancy as a stress test for future maternal health

3.1 Do excess cardiovascular events after early-onset preeclampsia represent a shared predisposition for both disorders, or remote sequelae of a complicated pregnancy?

Findings from the studies described in this thesis all show risk factors of preeclampsia common to other cardiovascular and inflammatory disorders, notably to atherosclerosis [22]. Taken together with consistent epidemiological evidence of a link between previous preeclampsia and future cardiovascular risk [16], the origin of preeclampsia seems to involve a shared maternal predisposition central to its pathogenesis. In addition, and as described in Chapter 4, many changes characteristic of the maternal syndrome of early-onset preeclampsia, such as hypertension, microalbuminuria, dyslipidemia, pro-inflammatory phenotype, insulin resistance and obesity, can be observed in a substantial number of women several months to years after delivery. Obviously, due to the retrospective design of our study, it is not possible to determine whether any of the cardiovascular risk factors were preexistent, i.e. present before the complicated pregnancy. It cannot be ruled out that maternal adaptation to pregnancy leads to long-term metabolic, cardiovascular and inflammatory changes, and that early-onset preeclampsia leaves a permanent 'scar'. To answer this critical question, prospective studies with pre-pregnancy risk factor assessment are needed. However, because of the overall incidence of early-onset preeclampsia, which we estimate to be no higher than 1 in 500 pregnancies, one would require the inclusion of at least 100,000 or more pregnancies to gain sufficient study power. At present, such studies are hardly feasible and are not likely to be attempted in the near future. Nonetheless, retrospective studies may be used to evaluate certain components the cardiovascular system that are unlikely to be influenced by pregnancy-induced changes and are likely to

return to baseline after pregnancy. These include genetic tests, which are fixed at birth, and levels of risk markers with a high heritable component that are relatively stable over time, such as lipoprotein levels [23-25], baseline high sensitivity C-reactive protein [26] and interleukin-6 concentrations [27].

3.2 Reconsidering the placenta hypothesis and "toxemia concept" of preeclampsia.

Although the placenta is likely to contribute to the development of preeclampsia, it seems unlikely that a single factor excreted from the placenta can be held responsible for all pathophysiological features of the disease. Studies on levels of placental factors measured in the maternal circulation have almost exclusively shown substantial overlap between normal pregnancy and preeclampsia [4,28]. Moreover, the degree of placental ischemia, release of placental factors, and even the presence of concomitant fetal growth restriction, has quite a poor correlation with the severity of maternal disease [4,29]. These data do not fit a one-to-one model of placental pathology leading to the release of a single toxic substance (once named 'factor X') in all cases of preeclampsia. In our opinion, considering current knowledge, preeclampsia would be more appropriately considered as a common state, or 'final common pathway', of generalized vascular and inflammatory decompensation in response to various pregnancy-related triggers. As supported by our findings, the triggers that might lead to this state of endothelial and inflammatory compromise are not the same in all women who develop preeclampsia. Most likely, predisposition to early-onset preeclampsia and atherosclerosis in later life results from an added effect of a wide range of abnormalities. Predisposed women are more likely to exceed a threshold for endothelial activation, that is the sum of placental factors, inflammation and vascular reserve (**Figure 1**).

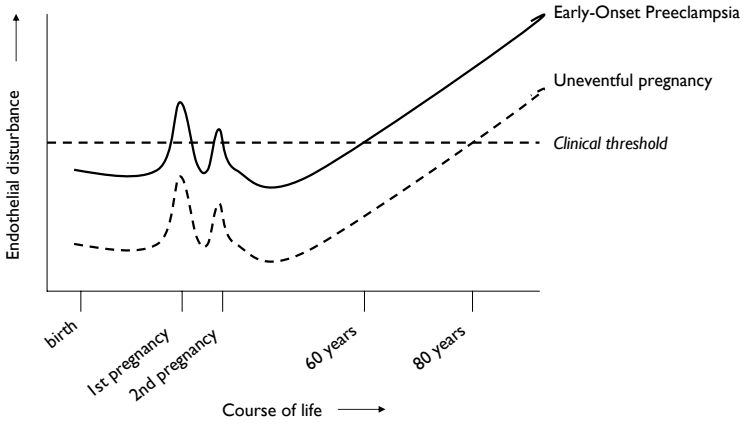

Figure 1 Pregnancy as a stress test for cardiovascular health.

3.3 Is early-onset preeclampsia an inflammatory disorder?

In 1999, Redman and colleagues first proposed that the endothelial damage characteristic of the maternal syndrome of preeclampsia is related to inflammation [30]. By the use of flow cytometric techniques, they showed that third-trimester normal pregnancy is characterized by remarkable activation of peripheral blood leukocytes, akin to changes observed in sepsis patients, which was even further increased in preeclampsia [31]. As summarized in Chapter 6, however, subsequent studies on inflammatory changes during normal pregnancy and preeclampsia have shown conflicting results. In general, evidence of studies of inflammatory markers measured in the maternal circulation, supports the hypothesis that preeclampsia results in a pro-inflammatory phenotype. However, the precise nature of the maternal inflammatory response and the sources which produce substances that control this inflammatory state are not well characterized. Notably, investigations of inflammatory markers produced by the placenta are inconclusive, due to methodological issues, e.g. sample collection, timing of investigation, appropriate controls, and limitated validity of immuno-assays. Another difficulty in comparing these studies, lies in the different criteria used for patient selection. Only few studies have included women with early-onset disease and definitions for mild and severe preeclampsia differ between studies. In addition, present studies have all investigated single or a selected set of immunological markers, instead of using an integrated approach to detect multiple cytokines and chemokines simultaneously. This is important, because most cytokines act in concert with each other, leading to combined rather than separate effects.
Despite these concerns, a body of evidence does support the initial theory by Redman and colleagues, that early-onset preeclampsia is characterized by excessive inflammation. Nonetheless, certain additional questions remain to be addressed.

3.3.1 Is the aggravated inflammatory response to pregnancy, observed in women with early-onset preeclampsia, dependent on a pre-existent pro-inflammatory constitution or phenotype?

In this thesis, we sought to identify inflammatory patterns characteristic of women with a history of early-onset preeclampsia. It is well known that regulation of the immune system has a strong genetic component, which accounts for inter-individual differences in the inflammatory response, after exposure to similar environmental antigens. An innate pro-inflammatory tendency in so-called 'high-responders', has been related to individual susceptibility to a number of inflammatory and vascular disorders [32,33]. In Chapter 7, we showed that women with a history of early-onset preeclampsia more often exhibit

a pro-inflammatory phenotype well after delivery, as demonstrated by higher baseline levels of plasma inflammatory markers C-reactive protein, interleukin-6 and fibrinogen. Subsequently, in Chapter 8, we studied the relative increase of plasma C-reactive protein and interleukin-6 after exposure to a regular influenza vaccination several months after delivery. In these experiments, we demonstrated that women with previous early-onset preeclampsia more often show a high pro-inflammatory response to experimentally induced mild *in vivo* stimulation of the immune system, in comparison to women with a history of only uneventful pregnancies. This observation provides the first evidence that early-onset preeclampsia is more likely to arise in women who are constitutionally predisposed to a more pronounced inflammatory response. Our data are consistent with the hypothesis that 'high-responders', i.e. women with an innate pro-inflammatory phenotype, have a higher risk to develop early-onset preeclampsia. To further confirm this hypothesis, it would be interesting to repeat these experiments in a prospective setting and in women with late-onset or recurrent preeclampsia.

Additional proof that constitutional factors, involving the maternal immune system, are associated with early-onset preeclampsia, is provided in Chapter 7. In this study, we show an association of common low-function allelic variants of the innate immunity receptors Toll-like receptor 4 (TLR4) and nucleotide oligomerization domain 2 (NOD2), with previous early-onset preeclampsia. TLR4 variants were up to a 3-fold more common in women who experienced first pregnancy early-onset preeclampsia and a 4-fold more common in those with concomitant HELLP syndrome, compared to women with uneventful pregnancies. Furthermore, in women with a pro-inflammatory phenotype, as demonstrated by high baseline levels of interleukin-6, the additional presence of TLR4 variants was associated with an odds ratio of 7.5 for previous early-onset preeclampsia. Similar, in women with high baseline interleukin-6 levels and functional NOD2 variants, the odds ratio for early-onset preeclampsia was as high as 9.2.

The results presented in Chapters 7 and 8 provide support for the idea that a maternal pre-existent pro-inflammatory constitution contributes to the risk of developing early-onset preeclampsia. Although the contribution of inflammatory changes to the pathogenesis of early-onset preeclampsia is consistent with the current concept of its etiology, the causality and generalizability of these findings need to be further established. Because our measurements were performed *after* pregnancy, we cannot exclude the possibility that temporary or permanent changes to the immune system are caused by pregnancy itself, and to a greater extent in women who experienced early-onset preeclampsia. For the genetic data, however, this is improbable, because single-nucleotide polymorphism genotypes are obviously fixed at birth and not likely to be altered by pregnancy. Also, we evaluated the effect of the interval between delivery and measurement on levels of

inflammatory markers, as is described in Chapter 7. Although an effect was observed for some of the inflammatory markers at a mean interval of 0.7 years after delivery, notably for interleukin-6, the associations of pro-inflammatory phenotype with previous early-onset preeclampsia were only moderately affected by adjustment for interval in our multivariable models. In addition, there was no effect of interval in the follow-up study presented in Chapter 8, which was conducted at a mean interval of 2.6 years after delivery, suggesting that the effects are temporary. Also, the inflammatory response to influenza vaccination was unaffected by timing of investigation. Furthermore, previous studies of monozygotic and dizygotic twins have shown strong genetic control of the inflammatory response after vaccination, that cannot be attributed to environmental influences [34,35]. In summary, our results identified components of maternal susceptibility to inflammation as novel risk factors for early-onset preeclampsia, that open up perspectives for further studies.

3.3.2 Can the abnormal inflammatory patterns in early-onset preeclampsia be attributed to the innate and/or the adaptive immune system?

In Chapter 6 we summarized previous investigations of inflammatory markers in normal pregnancy and preeclampsia. Most of these studies involve analysis of biomarkers representative of the adaptive immune system. However, as already recognized by Redman and colleagues, maternal inflammatory changes during pregnancy predominantly involve activation of the non-adaptive effectors of immune reactions, such as neutrophils, monocytes, complement factors and acute-phase reactants, together named the 'innate immune system' [30]. Although their subsequent studies in women with preeclampsia confirm the role of the innate immune system [36], few others have further explored this area of research. Moreover, recent advances in immunology have redefined the role of the innate immune system, by the discovery of its pivotal role in early initiation and regulation of the adaptive immune system, through the action of pattern-recognition receptors (PRRs) [37]. In Chapters 6 and 7, the implications of these findings for preeclampsia research are discussed. Further, we provided the first data that relate common genetic variants of two central players of the innate immune system, i.e. TLR4 and NOD2, to a history of first pregnancy early-onset preeclampsia. In addition, we observed an association with high levels of acute-phase reactants at baseline and after mild *in vivo* stimulation of the immune system, in women with previous early-onset preeclampsia, as described in Chapters 7 and 8. Our findings are consistent with a role for the maternal innate immune system in susceptibility to early-onset preeclampsia. However, these observations certainly do not exclude a role for components of adaptive immunity in the development of early-onset preeclampsia, since the two systems are now thought to be closely interrelated. To further

answer this question, future research should therefore aim at characterizing both systems simultaneously, and include non-pregnant, pregnant and preeclamptic women.

3.4 Can we predict future health by pregnancy outcome?

Several findings described in this thesis fit the previously mentioned conceptual model of pregnancy as a 'stress test for cardiovascular health'. Indeed, pregnancy outcome is likely to predict future cardiovascular health. Pregnancy itself leads to transient inflammatory, metabolic and vascular changes that have the potential to unmask subclinical vascular disease (**Figure 1**). Women who have preexistent vascular compromise and/ or a constitutional sensitivity to inflammation and endothelial activation will develop hypertensive disorders of pregnancy, ranging from mild late-onset pregnancy-induced hypertension to severe early-onset preeclampsia. In the years after delivery, vascular reserve will decline with advancing age, and similar vascular disorders will re-emerge prematurely, while same-age women with normal pregnancies are more likely to remain free of cardiovascular disease. In women with previous early-onset preeclampsia, abnormal levels of plasma lipids, markers of insulin resistance, higher body-mass index, elevated blood pressure and a pro-inflammatory phenotype all have the potential to facilitate the development of cardiovascular disease in later life. It is, however, unknown if these traditional and non-traditional cardiovascular risk factors fully explain the excess cardiovascular morbidity and mortality in women with previous early-onset preeclampsia. Alternately, it is not known whether women who experience only uneventful, normotensive pregnancies have a lower likelihood of developing future cardiovascular disease. Some early data support this assumption [38], but it would be relevant to confirm this hypothesis in a larger population-based cohort study. Although we have focussed on early-onset preeclampsia, it should be noted that a similar model relevant to the potential predictive power of pregnancy outcome, probably also applies to women who develop gestational diabetes, thrombosis and thyroid disease [39]. Also, early manifestations of chronic disease during pregnancy seem to be interrelated. For example, recent evidence has shown that women with a history of preeclampsia have a higher likelihood of developing type 2 diabetes mellitus [40]. In addition, non-hypertensive women with preterm delivery are at an increased risk of developing metabolic syndrome and future cardiovascular disease [41]. In summary, these data open up a promising area of research that links pregnancy outcome to future maternal health and provides new opportunities for preventive strategies relevant to women's health.

4. Future directions

4.1 Evaluation of pregnancy outcome as an independent cardiovascular risk factor

The findings presented in this thesis raise several intriguing issues that deserve to be addressed in future research. Consistent with others, we have found evidence that early-onset preeclampsia shares multiple characteristics with to atherosclerosis and is predictive of long-term cardiovascular health. Nonetheless, the question remains whether the long-term maternal cardiovascular health after early-onset preeclampsia is completely dependent on classic cardiovascular risk factors and components of the metabolic syndrome, or that abnormal pregnancy outcome itself independently contributes to the risk of future cardiovascular disease. To test this hypothesis, future research should attempt to evaluate whether pregnancy outcome independently adds to a predictive model that controls for all major traditional and non-traditional risk factors, such as the algorithm proposed by the Framingham Heart Study, the European SCORE model, or the recently developed Reynolds Risk Score [17-19]. Preferably, prospective, large-scale, population-based cohort studies with adequate follow-up to predict cardiovascular events would be required. Secondary questions would be to determine whether normal pregnancy has a beneficial effect cardiovascular risk profile, to investigate if cardiovascular risk is related to recurrence of preeclampsia and fetal growth restriction, and to evaluate the contribution of novel biomarkers for early detection of atheromatous lesions, such as intima-media thickness and coronary calcification [42,43].

In addition, future studies should clarify the feasibility of cardiovascular follow-up after early-onset preeclampsia, and evaluate the effect of periodic risk factor assessment and life-style intervention to reduce cardiovascular risk. Based on risk factor analysis presented in this thesis, we propose structured follow-up of women with early-onset preeclampsia after delivery. Our findings should raise awareness among health care workers, e.g. gynaecologists, general practicioners, cardiologists and internists, of the association between early-onset preeclampsia and increased cardiovascular risk.

4.2 Preeclampsia as an inflammatory disorder

4.2.1 Advances in immunology, innate immunity and immunotherapy.

Recent breakthrough findings from immunology have opened up a number of perspectives for future studies into the etiology of preeclampsia. Areas of interest include the further exploration of the role of the innate immune system (and notably of Toll-like receptors), and mechanisms relevant to the induction of immunotolerance after preeclampsia. In

addition, the observed association of early-onset preeclampsia and genetic defects in TLR4, which is the receptor for lipopolysaccharide, justifies renewed interest in the low-dose endotoxin infused rat as an animal model for preeclampsia, developed by Faas and colleagues [44]. In view of recent advances in reproductive immunology, this model may again be relevant to evaluate the potential of targeted immunotherapy for the treatment of hypertensive disorders of pregnancy.

4.2.2 Re-investigating the placental bed. Vascular remodeling and innate immunity.

Important recent work in the field of vascular biology and atheroscerosis research has led to improved understanding of processes involved in vascular remodeling and regeneration [45]. Of interest are the recent data on the role of the innate immune system in vascular remodeling, which provides a potential to study the role of the innate immune system in spiral artery remodeling [46]. In addition, future studies may aim to elucidate the defective spiral artery development observed in the placental bed of women with early-onset preeclampsia and severe intauterine growth restriction. Of particular interest are the hallmark 'acute atherosis' lesions observed in early-onset preeclampsia, that resemble early atheromatous plaques, which might relate to maternal cardiovascular risk factors [47].

4.3 Constitutional risk factors for preeclampsia.

4.3.1 Moving away from the factor X hypothesis.

In our opinion, future studies into the etiology of preeclampsia should aim for an integrated approach, that includes interactions between the cardiovascular, coagulative and inflammatory system, to fit the heterogenous components of the final common pathway leading to preeclampsia.

4.3.2 Defining a high risk pregnancy in the molecular age.

Promising new technology in molecular biology have facilitated detailed and efficient characterization of cellular and protein interactions involved in vascular and inflammatory processes. Approaches relevant to future preeclampsia research are the use of multiplex cytokine profiling arrays, such as the recently validated multiple bead array (Luminex) analysis [48], high-throughput genotyping techniques (see also appendix A and B), and protein profiling by proteomics [49]. In addition to the assessment of classic risk factors for preeclampsia, these techniques may reveal previously unknown maternal constitutional factors defining high risk pregnancy. This necessitates the creation of a structured biobank to include blood, plasma an placenta specimens of women with normal and complicated

pregnancies, with the objective to investigate the expression and predictive value of newly discovered genes and proteins. Now that many complex molecular assays have become available, affordable and applicable for testing in routine laboratories, the main concern of future studies into early-onset preeclampsia will be to properly collect specimens and clearly define the clinical disease and its differences in presentation and phenotype.

5. Conclusion

Early-onset preeclampsia remains to be an important cause for maternal and neonatal morbidity and mortality. To date, clinical treatment and prevention of the disorder are challenged by the lack of consistent data to explain its etiology. Despite extensive research, many aspects of its pathogenesis and risk factors underlying the disease remain to be discovered. Important parallels between preeclampsia and other cardiovascular and inflammatory disorders exist and provide a fascinating field for future study.

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Appendix I

Single Step High-Throughput Determination of Toll-Like Receptor 4 Polymorphisms

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Abstract

Background

Toll-like receptors are central components of host defence in humans, responsible for recognition of pathogen-associated molecular patterns and activation of innate immunity. Toll-like receptor 4 (TLR4) is activated by lipopolysaccharide (LPS) and other microbial components, thereby initiating the expression and release of pro-inflammatory cytokines. The common, frequently co-segregating allelic variants Asp299Gly and Thr399Ile have been related to susceptibility to gram-negative infections and sepsis and may be involved in the development of atherosclerosis. Identification of TLR4 Asp299Gly and Thr399Ile genotypes can be important for examination of genotype/phenotype relationships as well as for individual risk assessment of patients.

Methods

TLR4 Asp299Gly and Thr399Ile genotypes were detected by a single tube polymerase chain reaction (PCR), based on exonuclease degradation of dual labelled allele-specific oligonucleotides. The assay results were compared with conventional restriction fragment length polymorphism (RFLP) analysis.

Results

Genotypes of 345 individuals were determined simultaneously in a single PCR assay. Allele frequencies for our population were 6.8% for the TLR4 Asp299Gly polymorphism and 6.4% for the Thr399Ile polymorphism. Validation by RFLP analysis revealed a correct detection of all genotypes.

Conclusions

We have developed a novel method for the detection of the TLR4 Asp299Gly and Thr399Ile mutations, permitting rapid genotyping which should be useful for large-scale population studies as well as applicable for routine clinical testing.

Abbreviations

TLR	Toll-like receptor
LPS	Lipopolysaccharide
Hsp60	Heat shock protein 60
ASO	Allele specific oligonucleotide
NFQ	Nonfluorescent quencher
MGB	Minor groove binder
PCR	Polymerase chain reaction
ARCS	Amplification created restriction site
RFLP	Restriction fragment length polymorphism

Introduction

Toll-like receptors (TLRs) are key components of the innate immune system, responsible for initiating an adequate inflammatory response against microbial pathogens [1]. Individual TLRs recognize distinct structural components of pathogens termed pathogen associated molecular patterns [2]. Toll-like receptor 4 (TLR4) is activated by lipopolysaccharide (LPS) [3], a constituent of the cell wall of Gram-negative bacteria, and a major causative agent of septic shock. LPS stimulates TLR4 in combination with CD14, leading to NF-κB mediated production of pro-inflammatory cytokines [1]. Mutations in the TLR4 gene lead to LPS hyporesponsiveness in mice [4]. Other clinically important microbial ligands of TLR4 include the heat-sensitive, cell-associated factor of Mycobacterium tuberculosis [5] and the F-protein of Respiratory Syncytial Virus (RSV) [6]. In addition, TLR4 is activated by the endogenous ligand heat shock protein 60 (Hsp60) [7], which has been strongly linked to atherosclerosis and coronary artery disease [8]. Two recently described common missense mutations in the TLR4 gene have been shown to affect TLR4 receptor signalling after LPS challenge: a substitution of adenosine by guanine at position 896 in the fourth exon of the TLR4 gene, leading to replacement of aspartic acid by glycine at amino acid position 299 in the extracellular domain of TLR4 (Asp299Gly) and a frequently co-segregating mutation, leading to an amino acid change at position 399 of threonine by isoleucine (Thr399Ile). Both the Asp299Gly and Thr399Ile variants have been associated with hyporesponsiveness to inhaled LPS and lower plasma concentrations of several pro-inflammatory markers [9]. Furthermore, subjects carrying the Asp299Gly polymorphism show a reduced extent and progression of carotid atherosclerosis [10]. In previous studies, the allelic frequency of these mutations has been estimated at 6–11% of the population [10-

12], implicating its potential usefulness in risk-stratifying patients vulnerable to infections, as well as its role in candidate-gene approaches for studying complex traits such as atherosclerosis [13] and the septic shock syndrome [14]. Currently available techniques for genotyping TLR4 polymorphisms are either time-consuming and laborious or require expensive equipment [12,15,16]. In this study, we propose a simple method for reliable high-throughput genotyping, using technology available in most scientific laboratories. The technique is based on the 5′-exonuclease activity of TAQ polymerase to degrade synthetic dual labelled allele specific oligonucleotides (ASOs) that are bound to the single strand DNA templates during a polymerase chain reaction (PCR). The assay contains two ASOs: one specific for the Asp299 or Thr399 allele and another specific for the 299Gly or 399Ile allelic variant. The Asp299 or Thr399 ASO has a FAM label on the 5′-site and a nonfluorescent quencher (NFQ) on the 3′-site, while the 299Gly or 399Ile ASO has a VIC-label on the 5′-site and an NFQ on the 3′-site. FAM and VIC fluorescence are quenched by the NFQ when connected by an ASO. When the ASOs bind to their specific single strand DNA template during the annealing step of the PCR, they will be cleaved by the 5′-exonuclease activity of TAQ polymerase during the amplification step. The presence of the wild-type allele can be determined with specific binding of its ASO to template DNA of a subject carrying the wild-type Asp299 or Thr399 allele, subsequently releasing the FAM fluorogenic probe from its quencher (NFQ). Similarly, the presence of the 299Gly or 399Ile allele can be determined with specific binding of its ASO to template DNA of a subject carrying the mutant 299Gly or 399Ile allele, thereby releasing the VIC fluorogenic probe from the NFQ; free FAM can be detected in a fluorimeter at 492 nm excitation and 520 nm emission, whereas free VIC can be detected at 520 nm excitation and 550 nm emission. A subject is classified as homozygous for the Asp299 or Thr399 variant when the fluorescence of FAM is increased, while the fluorescence of VIC remains low. In contrast, a subject is homozygous for 299Gly or 399Ile when the VIC fluorescence is increased and the FAM fluorescence remains low. Thirdly, a subject is heterozygous when both the FAM and VIC fluorescence are increased.

Materials and methods

DNA samples

Genomic material was obtained from a cohort of 345 women with a history of severe pregnancy complications, recruited at the University Medical Center Utrecht between 1996 and 2002 [17]. Genomic DNA (gDNA) was isolated and purified from buffy coats, using commercially available kits (Puregene genomic DNA purification kit, Gentra Systems, Minneapolis, USA).

Primers and allele specific oligonucleotides (ASOs)

PCR primers were constructed by Operon (QIAGEN, Germany) to amplify a 112 bp fragment containing the TLR4 Asp299Gly mutation site and a 178 bp fragment containing the Thr399Ile mutation site. The following primers were used for the Asp299Gly polymorphism: forward 5'-TGAGTTTCAAAGGTTGCTGTTCTC-3′ and reverse 5′-TGTGGGAAACGTTCCAAATTTACA-3′. Primers for the Thr399Ile polymorphism were: forward 5′-TГАГТТТХАААГТТТГХТГТТХТХ–3′ and reverse 5′-AGGAATACTGAAAACTCACTCATTTGTT-3′. Dual labelled ASOs (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) were designed for the wild-type Asp299 allele and the 299Gly allelic variant with sense sequences: 5′-[6-FAM]-ACCTCGATG*A*TATTAT-[MGB][NFQ]-3′ and 5′-[VIC]-ACCTCGATG*G*TATTAT-[MGB][NFQ]-3′, respectively. Similarly, antisense probes were chosen for the Thr399 and 399Ile alleles. ASO sequences were: 5′-[6-FAM]-TTAGGCTG*G*TTGTCC-[MGB][NFQ]-3′ and 5′-[VIC]-TTAGGCTG*A*TTGTCC-[MGB][NFQ]-3′, respectively. The italicized bases indicate the polymorphic site for each ASO.

Polymerase chain reaction (PCR) conditions

A standard volume of 5 µl gDNA (3–10 ng/l) per reaction was amplified by adding 5 µl of a mastermix, containing final concentrations of 1/10 PCR buffer (Solis BioDyne, Tartu, Estonia), 0.25 mmol/l MgCl2, 50 mmol/l of dNTP mixture (Amersham Biosciences Benelux, Roosendaal, The Netherlands), 10 pmol/µl of each primer, 2 pmol/µl of each hybridization probe and 0.4 U of FIREpol TAQ polymerase (Solis BioDyne). PCR reactions were run in black 384 well fluorescence plates (Nunc Brand Products, Germany) on a Biometra T1 Thermocycler (Göttingen, Germany). Discrimination between wild-type and mutant alleles was observed at annealing temperatures ranging from 55 to 65 ˚C. Optimal PCR conditions were as follows: initial denaturation at 95 ˚C for 10 min, 45 cycles of denaturation for 15 s at 95 ˚C and annealing for 60 s at 60 ˚C, followed by 10 min at 72 ˚C. The ramping rate of the cooling phase between denaturation and annealing was 1˚/s. Fluorescence signals were measured automatically on a microplate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

Data analysis

Genotypes were assigned to each subject, by comparing the FAM signal to its corresponding VIC signal and calculating the -log(FAM/VIC) ratio for each data point. The distribution of -log(FAM/VIC) ratios was displayed in a histogram to arbitrarily determine the cut-off values for each genotype group. Data points with a ratio below the lower cut-off value were classified as homozygous Asp299Asp or Thr399Thr. Similarly, data points with a

ratio between the lower and the higher cut-off value were classified as heterozygous Asp299Gly or Thr399Ile, whereas data points above the higher cut-off value were classified as homozygous Gly299Gly or Ile399Ile.

Validation

The accuracy of this method was validated by restriction fragment length polymorphism (RFLP) analysis, based on a previously described method [15]. In brief, a standard PCR assay was performed by an Amplification Created Restriction Site (ARCS) technique [18], using an altered forward primer sequence to allow generation of a restriction site specific for the mutant TLR4 299Gly or 399Ile allele. In contrast to the approach of Lorenz et al. [15], reverse primers used for the 5′-fluorogenic assay as described above were also used for the ARCS-PCR to amplify a shorter PCR fragment (86 bp for the Asp299Gly site and 167 bp for the Thr399Ile site), which improves optimal discrimination of restriction products on gel electrophoresis. Primer sequences for the Asp299Gly site were: forward 5′-GATTAGCATACTTAGACTACTACCTCCATG-3′ and reverse 5′-TGTGGGAAACGTTCCAAATTTACA-3′. Alternatively, primer sequences for the Thr399Ile site were: forward 5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′ and reverse: 5′-AGGAATACTGAAAACTCACTCATTTGTT-3′. In total, 12 µl of PCR product were digested overnight in 3 µl standard enzyme buffer no. 4 containing 0.5 µl of either NcoI (New England Biolabs, Hitchin, UK) for the Asp299Gly or HinfI (New England Biolabs) for the Thr399Ile fragment. After digestion, gel electrophoresis was performed using 5 µl of sample and 5 µl of 1:500 SYBR Green (Molecular Probes, Leiden, The Netherlands) on a 4% standard multipurpose agarose gel.

Results

For each polymorphism, all 345 individuals were genotyped simultaneously in one PCR microplate with an approximate run-time of 3 hours. In eight samples, fluorescence signals remained below the detection threshold, due to insufficient yield of amplification product after PCR, and these were excluded from the data analysis. Fluorescence signals of the remaining 337 individuals are shown in **Figure 1**. Fluorescent intensities of the FAM probe (X-axis) are plotted against the fluorescent intensities of the VIC probe (Y-axis). Each dot represents the corresponding FAM and VIC signal of one individual. DNA samples of two individuals with a previously determined genotype were added as controls for each genotype group. As shown in **Figure 2**, cut-off points for each genotype group were determined according to the -log (FAM/VIC) ratio of <-0.30 for the homozygous Asp299

genotype, between -0.30 and 0.30 for the heterozygous Asp299/Gly299 genotype and >0.30 for the homozygous 299Gly genotype. Similarly, cut-off levels for each genotype group of the Thr399Ile polymorphism were determined at a -log (FAM/VIC) ratio of <-1.05 for the homozygous Thr399 genotype, between -1.05 and -0.97 for the heterozygous Thr399/Ile399 genotype and >0.97 for the homozygous 399Ile genotype. Of the 337 subjects tested, 292 individuals (86.6%) were classified homozygous Asp299Asp and Thr399Thr, 41 (12.2%) were heterozygous for both Asp299Gly and Thr399Ile, 3 (0.9%) were heterozygous for Asp299Gly only and 1 (0.3%) subject was double homozygous Gly299Gly and Ile399Ile. Corresponding allele frequencies observed in this study population were 6.8% for the 299Gly allele and 6.4% for the 399Ile allele. Co-segregation of the 299Gly and 399Ile allelic variants occurred in 93.5% and genotype distributions were in Hardy–Weinberg equilibrium for both the Asp299Gly (χ^2 = 0.238; 1 df, p = 0.626) and the Thr399Ile (χ^2 = 0.115; 1 df, p = 0.367) polymorphism. These numbers are comparable to previous studies [10-12,15]. Validation of the results by RFLP analysis revealed identical genotyping results for all samples.

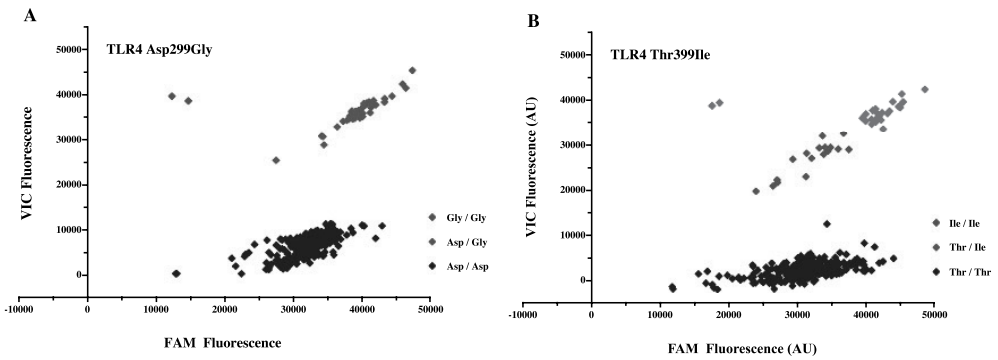

Figure 1 Results of simultaneous genotyping of 345 individuals for the (A) TLR4 Asp299Gly, and (B) TLR4 Thr399Ile genotypes, using dual labelled fluorescent oligonucleotides. For each subject, fluorescent intensities for wild-type (FAM) and mutant (VIC) probes are plotted along the X- and Y-axis, respectively.

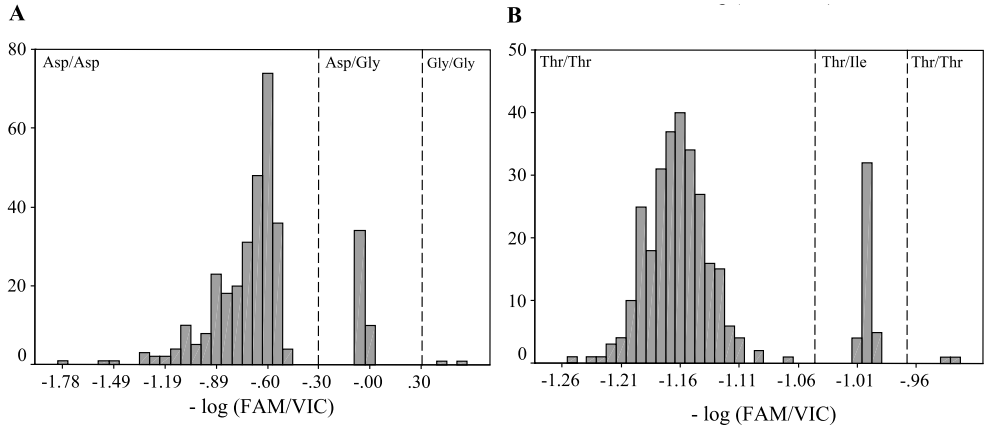

Figure 2 Histogram representing the distribution of -log (FAM/VIC) values within the study population. Reference lines indicate cut-off points for each genotype group. (A) Subjects with a ratio <-0.30 were classified as wild-type for the Asp299/Asp299 alleles, between -0.30 and 0.30 as heterozygous Asp299/299Gly, and >0.30 as homozygous 299Gly/299Gly, respectively. (B) Subjects with a ratio <-1.05 were classified as wild-type for the Thr399/Thr399 alleles, between -1.05 and -0.97 as heterozygous Thr399/399Ile, and >-0.97 as homozygous 399Ile/399Ile, respectively.

Discussion

Susceptibility to infectious diseases is dependent on the nature of the microorganism involved, and the ability of an individual to generate an adequate inflammatory reaction. Induction of this inflammatory response is partly determined by pattern recognition of microbial components [19], such as LPS or heat-shock proteins [2]. TLR4 is a key component of this recognition system in humans, and is able to generate signals leading to activation of genes producing pro-inflammatory cytokines important for host defence [3,7]. Ineffective recognition and activation of TLR4, e.g. in individuals with the Asp299Gly or Thr399Ile genotype, may lead to a relatively weak inflammatory response to various infectious agents [9]. On the other hand, long-term exposure to inflammation is a potentially harmful contributor to cardiovascular diseases, such as atherosclerosis and coronary artery disease [20]. Consequently, subjects carrying polymorphisms that impair TLR4 function seem to benefit from their reduced capacity to generate an inflammatory response to certain stimuli, which is likely to have a protective effect on their cardiovascular risk [10]. Recently, the role of TLR4 mediated inflammation has been implied in the pathogenesis of an emerging number of other clinical conditions, including pregnancy-related disorders, such as preterm delivery and hypertensive disorders of pregnancy [21]. Lorenz et al. [22] showed an increased risk of premature birth in infants carrying the TLR4 Asp299Gly

variant in a Finnish population. However, in another study the risk of preterm premature rupture of membranes (PPROM) was not related to TLR4 genotype in African Americans [23]. Genotyping for this common TLR4 variation could therefore become important in understanding the role of genetically encoded control of innate immunity, as well as early identification of patients at risk of infectious and/or vascular diseases.

We have developed a novel technique for rapid and reliable genotyping of the TLR4 Asp299Gly and Thr399Ile polymorphisms. The technique has several advantages when compared to conventional genotyping by RFLP analysis. Firstly, a maximum of 384 samples can be analysed in one assay, rendering it a powerful tool for large-scale genetic epidemiologic studies. Secondly, the technique permits fast and direct genotyping, requiring no additional post-PCR processing, which is particularly suitable for routine genotyping in a standard clinical setting and minimizes the chances of subsequent contamination by amplicons. Thirdly, it requires no investment in expensive equipment because the genotyping can be performed in a 384 well (or 96 well) PCR thermocycler and a separate fluorescence reader which is capable of handling 384 well microplates. Other groups have recently described a method for TLR4 Asp299Gly genotyping, based on real-time detection [12]. Similar to our approach, this technique is also based on the exonuclease cleavage of allele specific dual labelled probes. The major disadvantage of real-time detection, however, is the expensive LightCycler equipment, normally used for quantitative analysis of PCR yield, e.g. in gene expression studies. Furthermore, the majority of lightcyclers are based on 96-well reactions, thus having a four-fold lower throughput than our 384 well based approach. Another high-throughput method to determine both TLR4 polymorphisms has been described, using Matrix-Assisted Laser Desorption/Ionization Time of Flight mass spectrometry (MALDI-TOF-MS) technology [16]. However, this technique requires highly advanced equipment, which only large genomic centres can afford and is therefore not applicable for the majority of laboratories.

In conclusion, we have developed a rapid one tube assay to determine the TLR4 Asp299Gly and Thr399Ile genotypes. The simplicity and specificity of this assay should facilitate its establishment in molecular research and diagnostic laboratories.

Acknowledgements

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Appendix II

High-Throughput Genotyping with Infrared Fluorescence Allele Specific Hybridization (iFLASH): A Simple, Reliable and Low-Cost Alternative

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Abstract

Objectives

To develop and validate a novel genotyping approach, named infrared Fluorescence Allele Specific Hybridization (iFLASH), which combines the principles of allele specific oligonucleotide (ASO) hybridization with the advanced possibilities of infrared imaging.

Design and methods

As an example, we genotyped the 55L > M and the 192Q > R common genetic variants of the paraoxonase-1 gene in 92 DNA samples using the iFLASH technique, and validated the outcomes with the restriction fragment length polymorphism (RFLP) and TAQman genotyping assays.

Results

There was a 100 percent agreement in genotype outcome among the three methods.

Conclusions

Although we found complete unity in genotype outcome, the iFLASH assay has essential advantages over the RFLP and TAQman genotyping assays. First, the iFLASH technique is capable of handling up to 1536 samples per assay, which makes it a suitable technique for high-throughput genotyping. Secondly, because the costs per assay are lower, high-throughput genotyping with iFLASH is affordable.

Introduction

The candidate-gene approach to investigate the involvement of common genetic variants (also known as single nucleotide polymorphisms or SNPs) in the onset of complex diseases is now commonly used in clinical research and population studies [1]. For the detection of genetic variation in human population studies, different genotyping methods are available. Restriction fragment length polymorphism (RFLP) is the most commonly used technique, but the labourious sample handling and visual inspection of DNA gels for genotyping makes this technique unsuitable for high-throughput screening. Another widely used method is the TAQman assay, utilizing the 5′-exonuclease activity of the TAQ-polymerase to discriminate alleles that differ by a single base substitution [2]. Although this method is quick and accurate, a major disadvantage of the TAQman assay is that it uses relatively expensive dual-labeled allele-specific oligonucleotides (ASOs). This results in substantial costs for genotyping large populations. At the moment, the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has gained interest [3]. However, this technique requires highly advanced equipment, not available for the majority of laboratories. In addition to the techniques described above, there is a wide range of commercial alternatives for genotype determination, less suitable for high-throughput genotyping in the majority of non-commercial laboratories, because of the high cost of equipment and consumables.

The infrared Fluorescence Allele Specific Hybridization (iFLASH) technique described in this study provides a solution to the problem of high assay costs by using inexpensive single-labeled fluorescent oligonucleotides for allelic discrimination. In addition, the iFLASH technique is suitable for handling large amounts of samples. The principle of iFLASH (**Figure 1**) is based on the classical genotyping technique of ASO hybridization [4], in combination with the new possibilities of high sensitive fluorescence imaging. First, the amplified DNA sequence is immobilized on a nylon membrane (step 1-2). Next, the membrane is hybridized with iFLASH oligonucleotides, i.e. single-strand DNA probes complementary to the wild-type or mutant allele of the SNP of interest which are labeled with infrared dyes for detection (step 3). Finally, the membrane is stringently washed to remove nonspecifically bound iFLASH oligonucleotides, followed by signal detection of the iFLASH oligonucleotides on an infrared imaging system. The determination of the genotypes can be done optically, based on differences in colors, or automatically, based on differences in the intensity of the fluorescent signals (step 4).

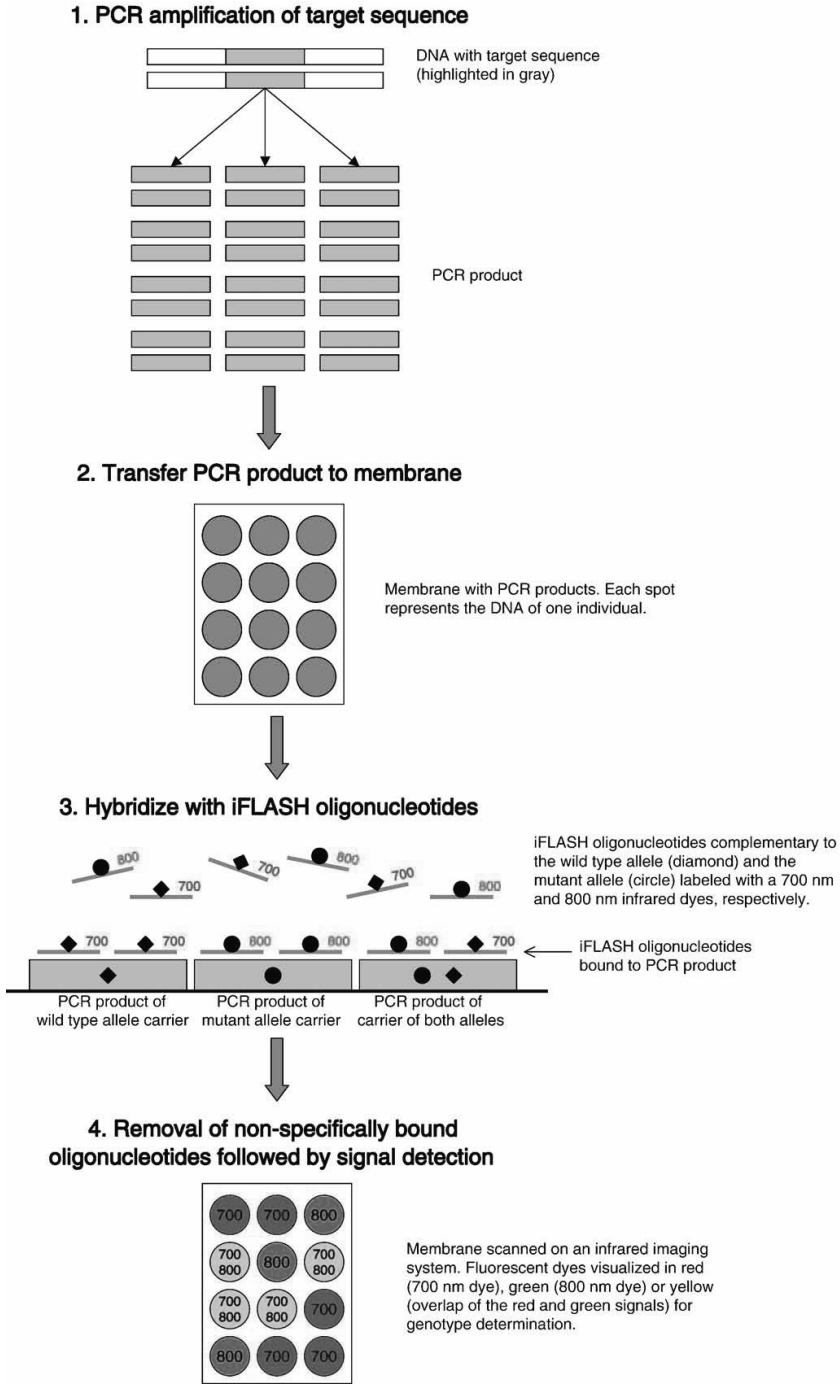

Figure 1 Schematic representation of the infrared Fluorescence Allele Specific Hybridization (iFLASH) genotyping technique.

When considering different techniques for genotyping candidate genes in large-scale population studies, four important aspects should be considered. First, the technique should be reliable and give precise outcomes. Second, the optimization of the genotyping assay should be flexible and easy. Third, the technique should be high throughput, i.e. the technique should be able to handle large numbers of samples, as well as automatically detect and type the genetic variant. Finally, the costs per assay must be kept as low as possible. In the present study, we compared the iFLASH technique with the RFLP and the TAQman genotyping assays in terms of reliability, optimization time, throughput and costs. To this purpose, we determined two common genetic variants in the paraoxonase-1 gene (192Q > R and 55L > M) in 92 DNA samples.

Methods

DNA samples

Genomic DNA was isolated from the blood of 92 healthy volunteers using a commercially available kit (Puregene, Gentra Systems, Minneapolis, USA). All participants gave informed consent.

RFLP 55L > M and 192Q > R genotyping

RFLP primers, restriction enzymes, PCR and restriction conditions are described by Humbert et al. [5]. Digested DNA fragments were separated on a 3% agarose gel and visualized with SYBR Green (Molecular Probes, Leiden, The Netherlands). The 55 L allele corresponded to the presence of a non-digested 170-bp fragment, the 55 M allele to a 44-bp and a 126-bp fragment, the 192 Q allele to a non-digested 99-bp fragment, and the 192 R allele to a 33-bp and a 66-bp fragment.

TAQman 55L > M and 192Q > R genotyping

For the TAQMan assay, PCR primers and FAM and VIC fluorescent dye labeled ASOs were designed by the Assay by-Design service from Applied Biosystems (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), using the following primers and ASOs to genotype the 55L > M polymorphism: forward 5′-ACAACCTGTACTTTCTGTTCTCTTTTCTG-3′ and reverse 5′-CAGAGCTAATGAAAGCCAGTCCAT-3′ in combination with the ASOs 5′-[VIC]-AGTATCTCCAAGTCTTC-[NFQ]-3′ for detection of the 55 L allele and 5′-[FAM]-CAGTATCTCCATGTCTTC-[NFQ]-3′ for detection of the 55 M allele. Similarly for the 192Q > R polymorphism: forward 5′-CTGAGCACTTTTATGGCACAAATGA-3′ and reverse 5′-ACCACGCTAAACCCAAATACATCTC-3′ in combination with the ASOs

5′-[VIC]-CCTACTTACAATCCTG-[NFQ]-3′ for detection of the 192 Q allele and 5′-[FAM]-CCCTACTTACGATCCTG-[NFQ]-3′ for detection of the 192 R allele. The PCR conditions were as follows: initial denaturation at 95 °C for 10 minutes, 40 cycles of denaturation for 15 seconds at 92 °C and annealing for 1 minute at 60 °C, followed by 10 minutes at 72 °C. Fluorescence signals were measured on a microplate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

iFLASH 55L > M and 192Q > R genotyping

For the iFLASH-hybridization technique, two PCR reactions were performed for the amplification of DNA sequences coding for the 55L > M and the 192Q > R polymorphism. The PCR primers and conditions for the amplification were identical to those used for the RFLP assay. PCRs were performed in 384 wells plates, using approximately 12.5 ng template DNA in an end-volume of 10 µL PCR reaction mix. The amplified fragments were dried by heating and resuspended in 5 µL 0.5 M NaOH. For each polymorphism, an individual array was created by transferring the PCR products from the PCR plate to a Hybond N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) by a centrifugation method, as previously described [6]. In brief, a membrane, pretreated in 10× SSC, was placed over the open wells of the PCR plate and covered by all-purpose filter paper. A clamping device kept the membrane and filter paper in place while the PCR products were transferred to the membrane by centrifugation at 1500 rpm in a microplate centrifuge (Mistral 2000, MSE Scientific Instruments, Crawley, UK). For each polymorphism, iFLASH-oligonucleotides were designed for the detection of the wild-type and the mutant alleles. Discrimination between the wild-type and the mutant iFLASH oligonucleotides was achieved by adding an infrared dye excited at either a wavelength of 700 nm (IRD700) or at a wavelength of 800 nm (IRD800). The iFLASH oligonucleotides were commercially obtained from Metabion, Martinsried, Germany. For the genotyping of the 55L > M and 192Q > R polymorphisms we used the following iFLASH-oligonucleotides: 5′-IRD800-CTGAAGACATGGAGAT-3′ (55 M), 5′-IRD700-CTGAAGACTTGGAGA-3′ (55 L), 5′-IRD800-CTACTTACGATCCTGGG-3′ (192 R) and 5′-IRD700-CTACTTACAATCCTGGGA-3′ (192 Q). Membranes were pre-hybridized in 30 mL hybridization buffer (6× SSC, 2.5× Denhahardt's reagent, 0.4% SDS) at 42 °C for 2 hours. After pre-hybridization, 50 pmol of both the wild-type and the mutant iFLASH oligonucleotides were added to the hybridization buffer and the membranes were hybridized for 1 hour at 42 °C. Subsequently, membranes were rinsed in wash buffer (2× SSC, 0.1% SDS) to remove excess iFLASH oligonucleotides, followed by a 30 minute allele-specific temperature wash (at 45°C for 55L > M and at room temperature for 192Q > R). Fluorescent signals were detected on an Odyssey® Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA). The IRD800 dye was detected by the 800 nm channel and was

represented as a green color by the imaging system; the IRD700 dye was detected by the 700 nm channel and was represented as a red color by the imaging system. A yellow color was visible when both signal intensities were present in equal amounts.

Data analysis

The genotype determination based on the TAQman assay were assigned in a similar fashion as described in detail previously [7]. Briefly, the FAM signal was compared to the VIC signal by calculating the log (FAM/VIC) ratio for each data point. The distribution of the log (FAM/VIC) ratios was displayed in a histogram to arbitrarily determine cut-off values for each genotype group. Genotype assignment for the iFLASH-hybridization assay was similar to the TAQman assay and done by comparing the 800 nm channel signal intensity to the 700 nm channel intensity and calculating the log (800 channel/700 channel) ratios. The iFLASH-hybridization technique was validated by comparing these outcomes to the RFLP and TAQman outcomes. The scatter-plot and the histogram were created with SPSS version 11.5.

Results

Two PCR reactions were performed to amplify the 55L > M and 192Q > R polymorphic regions. PCR products were successfully obtained in 83 and 84 samples for the 55L > M and 192Q > R polymorphism, respectively, and these samples were used for further investigations, shown in **Table 1**.

As depicted in **Figure 2**, the determination of the 55L > M genotype could be performed optically, based on the differences in color: carriers of the 55 L allele displayed a red color during excitation at 700 nm and carriers of the 55 M allele displayed a green color during excitation at 800 nm. In the picture of the merged 700 and 800 channels, the 55 LL homozygotes were red, 55 MM were green and the 55 LM heterozygotes were visualized as yellow. The 192 Q > R polymorphism showed a similar color pattern (data not shown). **Figure 3** shows the scatter plot of the 700 nm and 800 nm channel signal intensities of the iFLASH hybridization assay. PCR samples typed as 55 LL or 192 QQ homozygotes by RFLP had increased signal intensities at 700 nm, while almost no signal could be detected at 800 nm. In contrast, for PCR samples typed as 55 MM or 192 RR by RFLP, almost no signal could be detected at 700 nm, while increased signal intensities were obtained at 800 nm. For the 55 LM and 192 QR heterozygotes, a signal was present at both 700 nm and 800 nm.

Table I Outcomes of the PON1 55L>M and 192Q>R genotyping assay using RFLP, TAQman and the iFLASH techniques.

Sample number	55L > M			192Q > R		
	RFLP	TAQman	iFLASH	RFLP	TAQman	iFLASH
1	*	*	*	*	*	*
2	*	*	*	QQ	QQ	QQ
3	LM	LM	LM	*	*	*
4	LL	LL	LL	RR	RR	RR
5	LM	LM	LM	QQ	QQ	QQ
6	LM	LM	LM	QQ	QQ	QQ
7	LL	LL	LL	RR	RR	RR
8	LM	LM	LM	QQ	QQ	QQ
9	LL	LL	LL	QR	QR	QR
10	LL	LL	LL	QR	QR	QR
11	LM	LM	LM	QQ	QQ	QQ
12	LM	LM	LM	QR	QR	QR
13	LM	LM	LM	QR	QR	QR
14	LM	LM	LM	*	*	*
15	LL	LL	LL	QR	QR	QR
16	MM	MM	MM	QQ	QQ	QQ
17	LM	LM	LM	QR	QR	QR
18	LM	LM	LM	QR	QR	QR
19	LM	LM	LM	QQ	QQ	QQ
20	LM	LM	LM	QR	QR	QR
21	LM	LM	LM	QR	QR	QR
22	LM	LM	LM	QQ	QQ	QQ
23	LL	LL	LL	QQ	QQ	QQ
24	LM	LM	LM	QR	QR	QR
25	LM	LM	LM	QQ	QQ	QQ
26	LL	LL	LL	QR	QR	QR
27	LM	LM	LM	QR	QR	QR
28	LM	LM	LM	QR	QR	QR
29	LL	LL	LL	QQ	QQ	QQ
30	LL	LL	LL	QQ	QQ	QQ
31	LM	LM	LM	QQ	QQ	QQ
32	LM	LM	LM	QR	QR	QR
33	LM	LM	LM	RR	RR	RR
34	LM	LM	LM	QR	QR	QR
35	LM	LM	LM	QQ	QQ	QQ
36	MM	MM	MM	QQ	QQ	QQ
37	LL	LL	LL	*	*	*
38	LM	LM	LM	QR	QR	QR
39	LM	LM	LM	QQ	QQ	QQ
40	LL	LL	LL	RR	RR	RR
41	LM	LM	LM	QQ	QQ	QQ
42	LM	LM	LM	QQ	QQ	QQ
43	LL	LL	LL	QQ	QQ	QQ
44	LM	LM	LM	QR	QR	QR
45	LL	LL	LL	QQ	QQ	QQ

(continued)

Table I continued

46	MM	MM	MM	QQ	QQ	QQ
47	LL	LL	LL	RR	RR	RR
48	LM	LM	LM	*	*	*
49	LL	LL	LL	QR	QR	QR
50	LL	LL	LL	RR	RR	RR
51	LM	LM	LM	QR	QR	QR
52	LL	LL	LL	QQ	QQ	QQ
53	LM	LM	LM	QQ	QQ	QQ
54	LL	LL	LL	RR	RR	RR
55	LL	LL	LL	RR	RR	RR
56	LM	LM	LM	QQ	QQ	QQ
57	LL	LL	LL	*	*	*
58	LL	LL	LL	QQ	QQ	QQ
59	LM	LM	LM	QR	QR	QR
60	LM	LM	LM	QQ	QQ	QQ
61	MM	MM	MM	QQ	QQ	QQ
62	LM	LM	LM	QQ	QQ	QQ
63	MM	MM	MM	QQ	QQ	QQ
64	LM	LM	LM	QQ	QQ	QQ
65	LL	LL	LL	RR	RR	RR
66	LL	LL	LL	QQ	QQ	QQ
67	LM	LM	LM	QQ	QQ	QQ
68	*	*	*	QQ	QQ	QQ
69	LL	LL	LL	QR	QR	QR
70	LM	LM	LM	QQ	QQ	QQ
71	MM	MM	MM	QQ	QQ	QQ
72	LL	LL	LL	QR	QR	QR
73	MM	MM	MM	QQ	QQ	QQ
74	LL	LL	LL	QR	QR	QR
75	LL	LL	LL	RR	RR	RR
76	LL	LL	LL	QR	QR	QR
77	LM	LM	LM	QR	QR	QR
78	LL	LL	LL	QR	QR	QR
79	LM	LM	LM	QQ	QQ	QQ
80	*	*	*	QR	QR	QR
81	*	*	*	*	*	*
82	LL	LL	LL	QQ	QQ	QQ
83	LL	LL	LL	RR	RR	RR
84	LM	LM	LM	QQ	QQ	QQ
85	LL	LL	LL	RR	RR	RR
86	LL	LL	LL	QR	QR	QR
87	*	*	*	QQ	QQ	QQ
88	LL	LL	LL	QQ	QQ	QQ
89	*	*	*	QR	QR	QR
90	*	*	*	QR	QR	QR
91	*	*	*	QR	QR	QR
92	LM	LM	LM	*	*	*

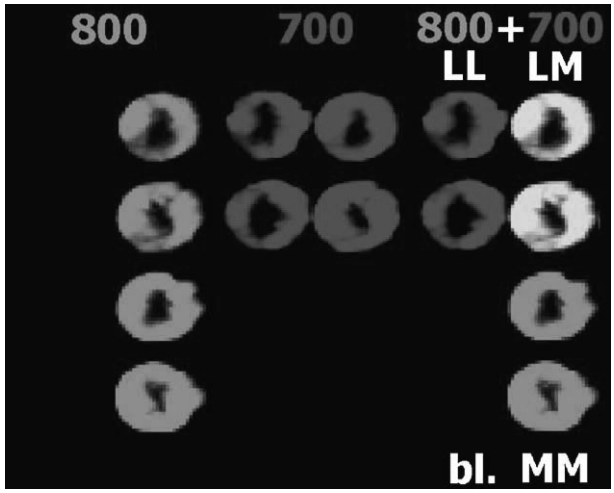

Figure 2 Typical example of the optical representation of the 55L > M polymorphism as detected in the infrared Fluorescence Allele Specific Hybridization (iFLASH) genotyping assay, per 800 nm channel, 700 nm channel and the combination of the 800 nm and 700 nm channel. The genotypes (in duplex) can be read from the combined 800 nm and 700 nm channel, where 55 LL, 55 LM and 55 MM are represented by the red, yellow and green signal, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

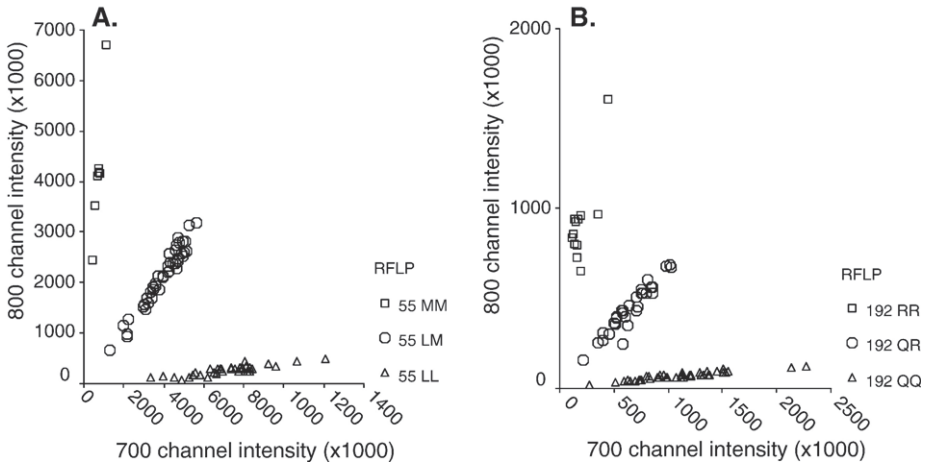

Figure 3 Segregation of the 55L > M (A) and 192Q > R (B) genotypes with the 700 nm and the 800 nm channel intensity plotted along the X- and Y-axis, respectively. Genotypes were determined by restriction fragment length polymorphism (RFLP).

In order to allow automated (by computer) genotype determination, fixed signal-intensity cut-off values were defined. We assigned cut-off values based on the log ratio of the 800 and 700 channel signal intensities **Figure 4**. Log (800 channel/700 channel) signal intensities below –0.75 were classified as 55 LL and 192 QQ homozygotes, those between –0.75 and 0.25 were classified as 55 LM and 192 QR heterozygotes and those above 0.25 were classified as 55 MM and 192 RR mutants.

To validate the iFLASH assay, we compared the results with the RFLP and TAQman assay. There was a 100 percent similarity of genotype-outcomes measured with the three techniques, and all measured genotype distributions were in Hardy-Weinberg equilibrium.

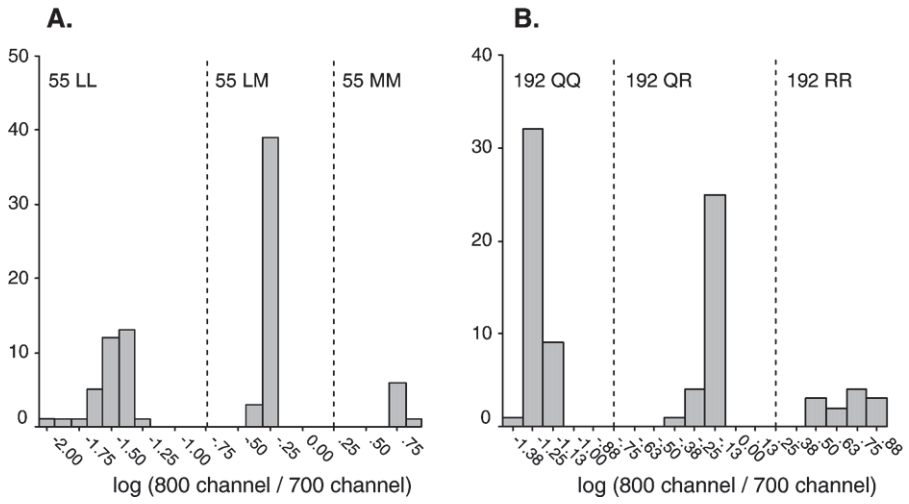

Figure 4 Histograms representing the distribution of the log (800 nm channel intensity/700 nm channel intensity) within the study population for the 55L > M (A) and 192Q > R (B) genotypes. Subjects with signal intensities below –0.75 were classified as 55 LL and 192 QQ homozygotes, those between –0.75 and 0.25 were classified as 55 LM and 192 QR heterozygotes and those above 0.25 were classified as 55 MM and 192 RR mutants. The –0.75 and 0.25 cut-off values were used for full automated determination of the genotypes.

Discussion

We have developed a novel genotyping approach named iFLASH, which combines the advantages of high sensitivity fluorescence imaging with the classic ASO-hybridization technique. Here, we will discuss if the iFLASH assay is an improvement over the RFLP and TAQman techniques with respect to reliability, optimization, throughput and costs.

In this population, we obtained complete consensus on genotype outcome for two common polymorphisms in the PON1 gene (55 L > M and 192Q > R) among the RFLP, the TAQman and iFLASH technique. This 100 percent unity among the three methods suggests that all these techniques are reliable tools for genotyping.

For the optimization, all three methods require PCR primer design to amplify the region of interest followed by 1.) the selection of a restriction enzyme which cuts the polymorphic DNA sequence for the RFLP technique or 2.) the design of ASOs for the TAQman and iFLASH assay. For the RFLP technique, no further optimizing is required. However, the success of the TAQman and the iFLASH assays depends on the selection of suitable hybridization oligonucleotides, which give an allele specific signal. The TAQman assay is a single-tube assay, where the binding of the PCR primers and the hybridization of the TAQman-ASOs to the template DNA take place in the same reaction. This requires that the annealing temperatures of the primers are about the same as the allele specific melting temperatures of the TAQman ASOs. The success of the TAQman assay therefore depends on carefully designed primers and probes. In contrast, for the iFLASH assay, the optimization of the allele specific hybridization temperature can be done separately from the optimization of the PCR conditions. The selection of primers and probes is less critical. This is a benefit of the iFLASH assay over the TAQman assay, especially when the polymorphism is located in a DNA region where a design of primers and ASOs with common annealing and melting temperatures is not possible.

For the genotype-assay comparison in terms of throughput, RFLP can be excluded as a high-throughput method, because the sample handling is labour intensive and determination of genotypes can hardly be automated. TAQman, on the other hand, is a quick method, which has the great advantage that it requires no post-PCR handling. Recently, our group developed a technique based on the TAQman principle using a 384 well PCR apparatus in combination with a fluorescence plate reader [7]. The throughput of the iFLASH hybridization method is equal or higher than the TAQman method. Although we only genotyped 92 samples in this study, the method of centrifugal transfer is capable of creating arrays of 384 or even 1536 samples [6]. A drawback for the throughput of the iFLASH method (when compared to the TAQman assay) is that it requires post-PCR handling. However, because of the high number of samples (up to 1536 per run) in the initial PCR reaction, it is questionable whether this is a serious limitation. In addition, we show that the iFLASH technique gives an excellent signal intensity discrimination among the 55L > M and 192Q > R genotypes, and that clear cut-points can be defined for automated genotype determination using a computer.

Finally, we discuss the cost effectiveness of the genotyping assays. Cost effectiveness depends on the number of samples tested: when genotyping only a few samples, RFLP is

the method of choice. Restriction enzymes are relatively cheap and there is no need for expensive equipment other than a PCR machine and a gel electrophoresis set-up. But, when aiming to determine multiple polymorphisms in populations of considerable sample sizes, we recommend using either the TAQman or the iFLASH assay. Both methods require a one time investment for an expensive DNA amplification and detection system: a real-time PCR machine or a PCR machine in combination with a microplate-reader for the TAQman assay, and a PCR machine and an infrared imaging system for the iFLASH assay. The prices for the machinery will be much the same. However, the essential difference in the costs between the TAQman assay and the iFLASH assay is caused by the type of ASO used. The TAQman assay requires dual labeled oligonucleotides (containing a fluorescent reporter dye and a quencher), which are approximately three times more expensive than the single dye labeled oligonucleotides used in the iFLASH assay. Furthermore, in a 384 well based approach, the TAQman requires approximately 75-fold more labeled oligonucleotides than the iFLASH technique. Therefore, when genotyping numerous polymorphisms in large populations the TAQman assay is considerably more expensive than the iFLASH assay.

In conclusion, we demonstrate that iFLASH-hybridization is a useful technique to genotype the two common polymorphisms in the PON1 gene. Additionally, due to its flexible optimization procedure, it can be used to type virtually any polymorphism desired. Because the iFLASH technique can be used with PCRs performed in 384 (or even 1536) well format and the genotype detection can easily be automated, the iFLASH technique is suitable for high throughput genotyping. Finally, a major advantage of the iFLASH-hybridization technique is that it is cheaper than most techniques available.

Acknowledgments

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Nederlandse samenvatting

Samenvatting in het Nederlands

Pre-eclampsie is een ernstige complicatie van de zwangerschap, in Nederland verantwoordelijk voor ruim eenderde van de moedersterfte en de helft van de babysterfte bij iatrogene vroeggeboorte. Pre-eclampsie wordt gekenmerkt door hypertensie en eiwitverlies in de urine gedurende de tweede helft van de zwangerschap, maar is te beschouwen als een multi-orgaanziekte met een vooralsnog onbekende oorzaak en een zeer variabel beloop. Vroege pre-eclampsie resulterend in een bevalling voor 34 weken zwangerschapsduur komt voor bij 1 op de 500 zwangeren en geldt als de meest ernstige presentatie van het ziektebeeld. Niet zelden geeft vroege pre-eclampsie aanleiding tot levensbedreigende complicaties, waaronder eclampsie, trombocytopenie, leverfunctiestoornissen en hemolyse (samen het HELLP-syndroom), stollingsstoornissen en ernstige hypertensieve crises. Bovendien gaat vroege pre-eclampsie vaak gepaard met intra-uteriene groeirestrictie door bijkomende placenta-insufficiëntie en is daardoor een belangrijke oorzaak van dysmaturiteit en prematuriteit.
Na de bevalling verdwijnen de symptomen van vroege pre-eclampsie meestal binnen een aantal dagen. Recente bevolkingsonderzoeken hebben echter laten zien dat vrouwen met een vroege pre-eclampsie in de voorgeschiedenis een verhoogde kans hebben op hart- en vaatziekten op latere leeftijd.

Het **eerste deel** van het proefschrift beschrijft onderzoek naar de reproductieve toekomst van vrouwen met vroege pre-eclampsie en/of intra-uteriene groeirestrictie in de voorgeschiedenis.

Over complicaties in een volgende zwangerschap van vrouwen die vroege pre-eclampsie hebben doorgemaakt, zijn op dit moment slechts beperkt gegevens beschikbaar. **Hoofdstuk 2** beschrijft de uitkomst van de 2e zwangerschap na een 1e zwangerschap gecompliceerd door vroege pre-eclampsie. Follow-up data werden verkregen van 120 vrouwen met vroege pre-eclampsie en een bevalling voor 34 weken zwangerschapsduur. Alle vrouwen gebruikten van de 12e tot de 36e zwangerschapsweek acetylsalicylzuur in een dosis van 80 milligram per dag. Bij 30 vrouwen (25%) trad opnieuw pre-eclampsie op in de 2e zwangerschap. Bij de meerderheid openbaarde de ziekte zich echter pas laat in de zwangerschap. Bovendien vond bij 78% de bevalling plaats na 37 weken zwangerschapsduur, waardoor ernstige complicaties bij moeder en kind nauwelijks optraden. De herhalingskans voor pre-eclampsie in de 2e zwangerschap werd niet beïnvloed door de ernst van de vroege pre-eclampsie in de 1e zwangerschap, de aanwezigheid van het HELLP-syndroom, intra-uteriene groeirestrictie of erfelijke en verworven trombofiliefactoren. Wel werd

een verhoogd herhalingsrisico voor pre-eclampsie gevonden voor vrouwen met een chronische hypertensie (hazard ratio 2,1; 95% betrouwbaarheidsinterval 1,0 tot 4,4) en een verhoogde kans op vroeggeboorte voor vrouwen die rookten (hazard ratio 2,4; 95% betrouwbaarheidsinterval 1,1 tot 5,6).

In **hoofdstuk 3** worden follow-up data gepresenteerd van vrouwen met een zwangerschap die werd gecompliceerd door intra-uteriene groeirestrictie veroorzaakt door ernstige placenta-insufficiëntie zonder bijkomende bloeddrukstijging of tekenen van pre-eclampsie. Vroege normotensieve intra-uteriene groeirestrictie geldt als een zeldzame aandoening. In de huidige wetenschappelijke literatuur zijn voor deze groep patiënten geen gegevens bekend over de prognose in een volgende zwangerschap. De zwangerschapsuitkomst in een tweede zwangerschap werd onderzocht voor 22 primiparae met vroege normotensieve intra-uteriene groeirestrictie in de een voorgeschiedenis met een kindsgewicht onder de 10e percentiel, bij wie een bevalling voor 34 weken zwangerschapsduur geïndiceerd was. Bij 6 vrouwen (27%) trad opnieuw een intra-uteriene groeirestrictie op in de 2e zwangerschap. Bovendien ontwikkelden 4 vrouwen (18%) zwangerschapshypertensie. De perinatale sterfte was 73% in de 1e zwangerschap, vergeleken met 14% in de 2e zwangerschap. Samengevat verliep de tweede zwangerschap bij slechts 55% van de vrouwen ongecompliceerd.

In het **tweede deel** van het proefschrift worden resultaten gepresenteerd van onderzoek naar het voorkomen van klassieke en moderne risicofactoren voor hart- en vaatziekten bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis.

Hoofdstuk 4 beschrijft het voorkomen van klassieke risicofactoren met een onafhankelijk voorspellende waarde voor de kans op een hartinfarct of beroerte bij 243 vrouwen met vroege pre-eclampsie (voor 34 weken zwangerschapsduur), zoals bepaald tenminste 6 maanden na de bevalling. De resultaten werden vergeleken met een ongeselecteerde controlegroep van 374 gezonde vrouwen binnen dezelfde leeftijdscategorie. Vrouwen met vroege pre-eclampsie in de voorgeschiedenis zonder bekende pre-existente hypertensie bleken gemiddeld vaker belast met één of meer risicofactoren voor hart- en vaatziekten ten opzichte van de controlegroep. Na correctie voor leeftijd hadden vrouwen met vroege pre-eclampsie in de voorgeschiedenis een significant hogere systolische bloeddruk, diastolische bloeddruk, plasma totaal cholesterol-, LDL-cholesterol-, triglyceriden- en nuchtere glucosewaarde, alsmede een lagere HDL-cholesterolwaarde, alle passend bij een verhoogd cardiovasculair risico. Bovendien was een voorgeschiedenis van vroege pre-eclampsie sterk lineair geassocieerd met gewicht en body-mass index. Het vaker

voorkomen van overgewicht en obesitas was echter maar ten dele verantwoordelijk voor de aanwezigheid van de andere risicofactoren. Ook bij vrouwen met een normaal gewicht werden frequent een abnormaal lipidenprofiel, tekenen van insulineresistentie en een verhoogde bloeddruk waargenomen. De resultaten lieten geen associatie zien tussen een voorgeschiedenis van vroege pre-eclampsie en lengte, roken, etniciteit en diabetes mellitus.
Ondanks het veelvuldig voorkomen van één of meer risicofactoren voor hart- en vaatziekten na vroege pre-eclampsie bleek het absolute tienjaarsrisico op een hartinfarct of beroerte, geschat met de Framingham risicoscore, bij geen van de vrouwen in de eerste jaren na de bevalling ernstig verhoogd (>10%). Dit wordt met name verklaard door de lage *a priori* kans op hart- en vaatziekten binnen deze leeftijdscategorie. Niettemin dient op grond van de resultaten rekening gehouden te worden met een belangrijke bijdrage van klassieke cardiovasculaire risicofactoren aan de verhoogde kans op hart- en vaatziekten op latere leeftijd. De uitkomst van deze studie rechtvaardigt dan ook periodieke bepaling van klassieke risicofactoren voor hart- en vaatziekten bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis.

In **hoofdstuk 5** wordt een studie beschreven naar de waarde van een nieuwe merkstof voor cardiale ischemie, genaamd ischemia-modified albumin (IMA), in de normale zwangerschap en tijdens pre-eclampsie en intra-uteriene groeirestrictie. IMA geldt als een veelbelovende test voor het aantonen van de eerste tekenen van ischemie na een hartinfarct. De hypothese van de studie was dat IMA ook wordt gevormd tijdens complicaties van de zwangerschap gekenmerkt door ischemie van de placenta, zoals pre-eclampsie en intra-uteriene groeirestrictie. Hiervoor werden IMA-waarden bepaald bij 12 patiënten met pre-eclampsie, bij 12 gezonde zwangeren in het 3e trimester van de zwangerschap en bij 12 niet-zwangere controlepatiënten. De resultaten van de studie lieten een opvallende stijging zien van de plasma-IMA-spiegels tijdens de normale zwangerschap, vergelijkbaar met de toename van IMA tijdens een hartinfarct. Vergeleken met voor zwangerschapsduur gematchte gezonde zwangeren lieten patiënten met pre-eclampsie geen verdere IMA-stijging zien. Wel bleek IMA omgekeerd evenredig met het geboortegewicht, hetgeen zou kunnen passen bij een relatie tussen ischemie in de placenta en de vorming van IMA. Gezien het relatief kleine aantal proefpersonen in de studie dient deze bevinding uiteraard bevestigd te worden in vervolgonderzoek. Samengevat lijkt IMA geen bruikbare test bij de diagnostiek naar pre-eclampsie, gezien de hoge waarden in de normale zwangerschap en de afwezigheid van een verschil tussen gezonde zwangeren en patiënten met pre-eclampsie. Bovendien lijkt voorzichtigheid geboden bij het gebruik van IMA bij de vroege diagnostiek van het hartinfarct tijdens de zwangerschap.

Het **derde deel** van het proefschrift omvat de resultaten van een drietal studies naar de rol van het immuunsysteem bij de pathogenese van vroege pre-eclampsie.

In **hoofdstuk 6** wordt een overzicht gegeven van beschikbare studies naar de rol van het immuunsysteem van de moeder bij pre-eclampsie. Pre-eclampsie gaat gepaard met een abnormale inflammatoire reactie op zwangerschapsspecifieke veranderingen, mogelijk uitgelokt door de deels lichaamsvreemde foetus. De klassieke hypothese is dat pre-eclampsie wordt gekenmerkt door een verstoord evenwicht in de specifieke afweer, gereguleerd door T-helpercellen, met als gevolg activatie van de type 1 (cellulaire) immuunrespons en onderdrukking van de type 2 (humorale) immuunrespons. Een aantal van de beschikbare studies ondersteunt deze hypothese, onder andere door het aantonen van een toegenomen productie van type 1 cytokinen (zoals TNF-α en IL-6) in plasma en placentaweefsel van patiënten met pre-eclampsie. Minstens evenveel studies bewijzen echter het tegendeel of laten geen duidelijke verschillen zien tussen patiënten met pre-eclampsie en gezonde zwangeren. Bovendien blijkt uit voortschrijdend inzicht in de immunologie dat de regulatie van het immuunsysteem veel complexer is dan voorheen werd gedacht. Naast T-cellen zijn ook andere componenten van de cellulaire en humorale afweer nauw betrokken bij de regulatie van de immuunrespons. De laatste jaren gaat de aandacht daarbij vooral uit naar de centrale rol van de aspecifieke afweer, in het bijzonder naar de eind jaren negentig ontdekte Toll-like receptors (TLRs). Deze receptoren komen vooral voor op macrofagen en neutrofiele granulocyten, maar ook op trofoblastcellen in de placenta. De functie van TLRs in de zwangerschap is nog weinig onderzocht. Wel bestaan aanwijzingen dat TLR4 een rol speelt bij de kans op vroeggeboorte. Ook zijn TLRs betrokken bij sepsis, allergie, het ontstaan van atherosclerose en de kans op hart- en vaatziekten. De rol van TLRs bij het ontstaan van vroege pre-eclampsie lijkt derhalve een veelbelovend onderwerp voor toekomstig onderzoek.

In **hoofdstuk 7** worden de resultaten beschreven van onderzoek naar 5 veelvoorkomende genetische varianten van Toll-like receptor 4 (TLR4) en nucleotide-binding oligomerization domain 2 (NOD2) bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis. TLR4 is een receptor voor lipopolysaccharide, dat ook wel endotoxine wordt genoemd. Endotoxine is een component van de celwand van gramnegatieve bacteriën en heeft sterk pro-inflammatoire eigenschappen. Activatie van TLR4 door endotoxine leidt tot productie van pro-inflammatoire cytokinen en speelt een belangrijke rol bij het aansturen van de inflammatoire respons. Ook NOD2 speelt bij deze interactie een rol en wordt met name geactiveerd door het bacteriële eiwit muramyldipeptide (MDP). Genetische varianten in beide receptoren leiden tot functionele verschillen in de effectiviteit van dit

herkenningssysteem, met als gevolg een minder effectieve afweer. Eerdere studies hebben laten zien dat het voorkomen van de functionele varianten van TLR4, te weten D299G en T399I, alsmede varianten van NOD2, namelijk G908R, R702W en L1007fs, geassocieerd is met het risico op tal van immuungemedieerde aandoeningen, zoals sepsis, astma, de ziekte van Crohn en atherosclerose.

Voor de studie werden 340 vrouwen met vroege pre-eclampsie in de voorgeschiedenis geïncludeerd in 4 derdelijns centra in Nederland, het Universitair Medisch Centrum Utrecht, het Maxima Medisch Centrum Veldhoven, het Leids Universitair Medisch Centrum en het Universitair Medisch Centrum Nijmegen. Ter vergelijking werd een controlegroep samengesteld van 113 vrouwen met in de voorgeschiedenis uitsluitend ongecompliceerde zwangerschappen. Naast genetisch materiaal werd ook plasma verzameld voor bepaling van de ontstekingseiwitten C-reactieve proteïne (CRP), IL-6, fibrinogeen, soluble intercellular adhesion molecule 1 (sICAM-1) en de von Willebrand-factor. De resultaten lieten een sterke associatie zien tussen genetische varianten van TLR4 en een voorgeschiedenis met vroege pre-eclampsie, met een odds ratio van 3,3 (95% betrouwbaarheidsinterval 1,5 tot 6,7) en 2,9 (95% betrouwbaarheidsinterval 1,2 tot 6,7) ten opzichte van de controlegroep, na correctie voor leeftijd en chronische hypertensie. De associatie tussen functionele varianten van TLR4 en vroege pre-eclampsie bleek nog sterker voor patiënten die het HELLP-syndroom ontwikkelden, met een gecorrigeerde odds ratio van 4,1 (95% betrouwbaarheidsinterval 1,7 tot 9,8) ten opzichte van de controlegroep en een odds ratio van 2,3 (95% betrouwbaarheidsinterval 1,3 tot 4,3) ten opzichte van patiënten met pre-eclampsie alleen. Vroege pre-eclampsie was daarentegen niet direct geassocieerd met genetische varianten van NOD2. Ook hadden vrouwen met vroege pre-eclampsie in de voorgeschiedenis minimaal 6 maanden na de bevalling significant hogere plasmaspiegels van de ontstekingsstoffen CRP, IL-6 en fibrinogeen. Een hoge inflammatoire achtergrond, ofwel een 'pro-inflammatoir fenotype', bijvoorbeeld bij vrouwen met een fibrinogeen of IL-6 waarde in het hoogste tertiel van de normaalverdeling, bleek 2 tot 5 keer vaker voor te komen bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis in vergelijking met de controlegroep. Bovendien werd het gezamenlijk voorkomen van TLR4- of NOD2-varianten en een pro-inflammatoir fenotype 4 tot 7 keer vaker geconstateerd na vroege pre-eclampsie. De resultaten bleven gelijk na multivariate correctie voor leeftijd, interval tussen de bevalling en de meting, body-mass index, roken en chronische hypertensie. De resultaten van de studie vormen het eerste bewijs voor de betrokkenheid van de afweer van de moeder en in het bijzonder het aspecifieke immuunsysteem, bij de vatbaarheid voor vroege pre-eclampsie en het HELLP-syndroom.

In **hoofdstuk 8** wordt onderzoek gepresenteerd naar de rol van het acutefasesysteem bij de vatbaarheid voor vroege pre-eclampsie. Hiervoor werden 44 vrouwen met vroege pre-eclampsie in de voorgeschiedenis en een controlegroep van 29 vrouwen met uitsluitend ongecompliceerde zwangerschappen in de voorgeschiedenis geïncludeerd. Aan alle proefpersonen werd tenminste 6 maanden na de bevalling een standaard griepvaccinatie (Influvac®, geproduceerd door Solvay Pharma te Weesp) toegediend. Voor vaccinatie en op de 2e en 4e dag na vaccinatie werd bloed afgenomen ter bepaling van de acutefase-eiwitten CRP en IL-6. De relatieve stijging van CRP en IL-6 na vaccinatie werd als maat gebruikt voor de *in vivo* respons van het immuunsysteem na toediening van een milde inflammatoire stimulus. De resultaten toonden een significant hogere ontstekingsreactie na vaccinatie bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis. Een sterke immuunrespons na vaccinatie, gedefinieerd als een stijging van het CRP of IL-6 boven de 75e percentiel, kwam 2 tot 4 keer vaker voor dan bij de controlegroep. Hoewel vrouwen met vroege pre-eclampsie in de voorgeschiedenis gemiddeld een hogere body-mass index lieten zien, kon dit de bevindingen niet verklaren. Na multivariate correctie voor body-mass index bleven de associaties namelijk vrijwel gelijk. De resultaten ondersteunen de hypothese dat een constitutionele neiging van de moeder tot een versterkte immuunrespons bijdraagt aan het risico op vroege pre-eclampsie.

Conclusies en aanbevelingen

Vroege pre-eclampsie blijft een regelmatig voorkomende ernstige zwangerschapscomplicatie met een grotendeels onbekende oorzaak. Concluderend lijkt de prognose voor een volgende zwangerschap relatief gunstig. Hoewel in een kwart van de gevallen een mildere late vorm van pre-eclampsie optreedt, blijven ernstige complicaties en vroeggeboorte onder het huidige beleid van voorschrijven van aspirine en bloeddrukregulatie bij vrouwen met chronische hypertensie meestal achterwege. Wel is chronische hypertensie geassocieerd met een grotere herhalingskans op pre-eclampsie en roken met een groter risico op vroeggeboorte. Deze gegevens dienen dan ook betrokken te worden bij de counseling van vrouwen met vroege pre-eclampsie in de voorgeschiedenis. Andere aanvullende maatregelen ter secundaire preventie van pre-eclampsie, zoals toediening van laag moleculair heparine of antioxidanten, zijn vooralsnog niet bewezen effectief gebleken. Gezien de resultaten van ons onderzoek lijkt het bovendien niet waarschijnlijk dat dergelijke maatregelen de reeds gunstige prognose voor een volgende zwangerschap na vroege pre-eclampsie verder verbeteren. Wel blijkt uit onze onderzoeksresultaten een duidelijk verhoogd voorkomen van risicofactoren voor hart- en vaatziekten bij vrouwen met een voorgeschiedenis van

vroege pre-eclampsie. Bovendien is uit bevolkingsonderzoek bekend dat deze vrouwen een 8 keer verhoogd risico hebben op sterfte door hart- en vaatziekten op latere leeftijd. Op dit moment worden vrouwen na een vroege pre-eclampsie niet routinematig getest op deze risicofactoren. Het verdient dan ook aanbeveling vrouwen na een vroege pre-eclampsie vanaf het eerste jaar na de bevalling te testen op deze risicofactoren, door bepaling van de bloeddruk, het cholesterolprofiel, gewicht, schatting van het absoluut tienjaarsrisico op een hartinfarct of beroerte (bijvoorbeeld met de Framingham risicoscore) en mogelijk ook bepaling van het CRP-gehalte en indices van het insulineresistentiesyndroom (bijvoorbeeld nuchtere glucosewaarde en HOMA-score). Aangezien het eerste hartinfarct of de eerste beroerte bij de meeste vrouwen (85%) samenhangt met deze beïnvloedbare risicofactoren, biedt dit kansen op primaire preventie van hart- en vaatziekten op latere leeftijd bij vrouwen met vroege pre-eclampsie in de voorgeschiedenis. Hierbij dient wel opgemerkt te worden dat de haalbaarheid en effectiviteit van reductie van hart- en vaatziektenrisico voor deze patiëntengroep nog niet is onderzocht.

De resultaten beschreven in dit proefschrift verschaffen verder nieuwe inzichten in de rol van het immuunsysteem van de moeder bij het ontstaan van vroege pre-eclampsie. In het bijzonder lijkt de vatbaarheid voor vroege pre-eclampsie gerelateerd aan genmutaties in receptoren betrokken bij de aspecifieke afweer. Bovendien vertonen vrouwen met vroege pre-eclampsie in de voorgeschiedenis vele maanden na de bevalling vaker kenmerken van een verscherpte activiteit van het immuunsysteem, zoals verhoogde plasmawaarden van acutefase-eiwitten en cytokinen en een versterkte immuunrespons na vaccinatie. Op grond van de resultaten is het aannemelijk dat de gevoeligheid van het immuunsysteem van de moeder bijdraagt aan het ontstaan van vroege pre-eclampsie en de relatie tussen vroege pre-eclampsie en vatbaarheid voor hart- en vaatziekten op latere leeftijd. De relatie tussen het immuunsysteem, hart- en vaatziekten en zwangerschap bieden een veelbelovend perspectief voor toekomstig onderzoek naar vroege pre-eclampsie.

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Curriculum Vitae

De auteur van dit proefschrift werd op 23 mei 1977 geboren te Rotterdam. Na het doorlopen van de Bloemcampschool en het Rijnlands Lyceum te Wassenaar, behaalde hij in 1995 het gymnasium ß diploma en begon hij de studie Geneeskunde aan de Universiteit Utrecht. Als vierdejaars student volgde hij een wetenschappelijke stage bij de Afdeling Verloskunde van het Universitair Medisch Centrum Utrecht bij toenmalig promovendus Marko Sikkema, onder begeleiding van gynaecologen dr. Arie Franx en prof. dr. Hein Bruinse en grenslaagfysicus dr. Ernst van Faassen van het Debye Instituut, Faculteit Natuur- en Sterrenkunde. Het betrof onderzoek naar de rol van vrije radicalen bij pre-eclampsie, dat werd voortgezet tijdens een wetenschappelijke stage aan The Rayne Institute in Guy's and St. Thomas' Hospital, Kings College te Londen, onder begeleiding van prof. dr. Frank Kelly en prof. dr. Lucilla Poston. Aansluitend werden de co-assistentschappen doorlopen, waarvan het co-assistentschap Gynaecologie en Verloskunde werd gevolgd in het Sint Elisabeth Ziekenhuis te Tilburg en het co-assistentschap Oogheelkunde in Hospital Kuala Lumpur te Maleisië. Met het indienen van de eerste aanmeldingsformulieren voor een ZonMW stipendium ten behoeve van vervolgonderzoek naar pre-eclampsie, in een internetcafé op het Maleise eiland Pulau Perhentian Kecil, werd de keuze voor de specialisatie Gynaecologie en Verloskunde definitief. Na het keuze co-assistentschap bij de afdeling Verloskunde en Gynaecologie van het Meander Medisch Centrum te Amersfoort en behalen van het artsexamen in 2003, begon hij als assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) aan het promotietraject en de opleiding tot gynaecoloog. Het promotie-onderzoek kwam tot stand door een intensieve samenwerking tussen de afdeling Verloskunde en het research laboratorium van de afdeling Speciële Klinische Chemie en werd begeleid door promotor prof. dr. Hein Bruinse en co-promotoren dr. Arie Franx, dr. Ron Voorbij en dr. Mark Roest. Hij begon de klinische opleiding in 2005 in het Sint Elisabeth Ziekenhuis te Tilburg (opleiders dr. Paul Reuwer en dr. Harry Vervest), welke vanaf 2006 en tot op heden wordt voortgezet in het Universitair Medisch Centrum Utrecht (opleider prof. dr. Gerard Visser). Op 25 augustus 2007 trouwde hij met zijn grote liefde Cathelijne.