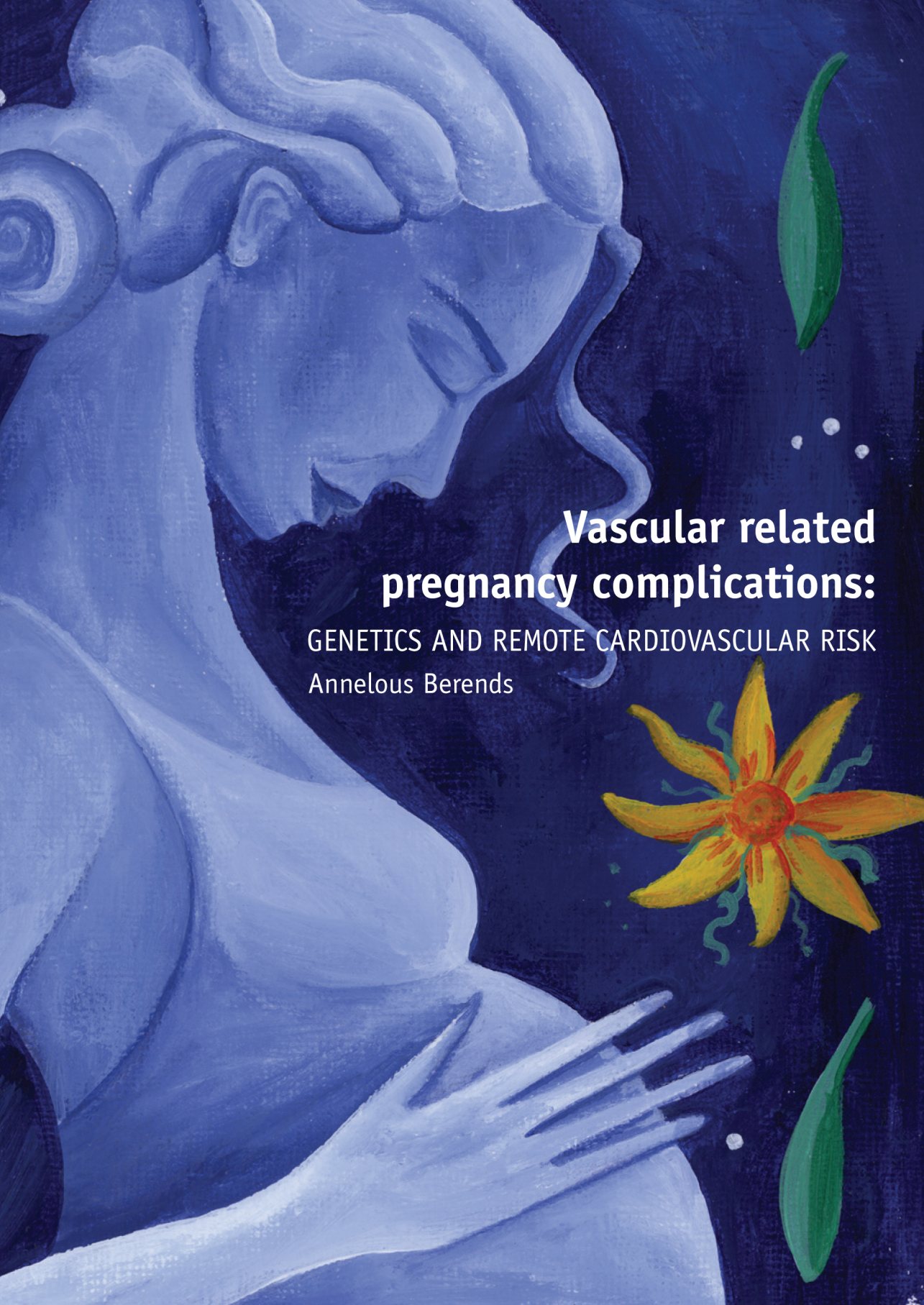

Vascular related pregnancy complications:

GENETICS AND REMOTE CARDIOVASCULAR RISK

Annelous Berends

Vascular related pregnancy complications:
genetics and remote cardiovascular risk

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Chapter 3.1

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Chapter 3.2

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Chapter 3.3

AL Berends, MC Zillikens, CJM de Groot, F Rivadeneira, BA Oostra, CM van Duijn, EAP Steegers. Body composition by dual energy-X-ray absorptiometry in women with previous preeclampsia or small for gestational age offspring. *BJOG*, in press

Chapter 3.4

AL Berends, MC Zillikens, CJM de Groot, FH de Jong, BA Oostra, CM van Duijn, EAP Steegers. Insulin, androgens and bone mineral density in women with previous preeclampsia or pregnancies complicated by intrauterine growth restriction. *In progress*

CONTENTS

1

Introduction

Preeclampsia and intrauterine growth restriction (IUGR) are common vascular related pregnancy syndromes of unknown cause. Both preeclampsia and IUGR are responsible for a significant maternal and perinatal morbidity and mortality worldwide[1]. Preeclampsia affects approximately 2.5-3.0% of women[2]. It is diagnosed by *de novo* hypertension and proteinuria after 20 weeks of gestation and potentially progresses to a multisystemic syndrome characterized by increased vasoconstriction, metabolic changes, endothelial dysfunction, activation of coagulation cascade and inflammatory response[3]. Eclampsia is considered an end stage of the syndrome characterized by generalized seizures. In addition to the maternal clinical symptoms, in approximately one third of the preeclamptic pregnancies fetal growth is impaired suggested as a consequence of impaired placental function[4]. Impaired fetal growth related to placental dysfunction can also occur in absence of a maternal clinical syndrome. In this thesis we focus on the phenotype of impaired intrauterine growth defined by placental dysfunction excluding impaired intrauterine growth caused by e.g. smoking, infection and congenital diseases.

Because of the unknown aetiology, preeclampsia and IUGR cannot be prevented and the only definitive treatment is still delivery. Although delivery is almost always curative for the mother in case of preeclampsia, it might not be best for a premature fetus[5]. Consequently, balancing maternal and fetal risks and benefits is a challenge for clinicians in the management of these pregnancy disorders[6].

Extensive research has not elucidated the aetiology of preeclampsia and IUGR, but it has provided better understanding of their pathophysiology. It is generally agreed upon that a placenta is required for the development of preeclampsia[7]. The current and widely accepted concept is that preeclampsia progresses in two stages; preclinical and clinical[2]. The first stage arises from poor placental development in early pregnancy and its suboptimal maternal blood supply[2] related to reduced endovascular trophoblast invasion and inadequate uteroplacental artery remodeling[7]. Poor placentation is not specific to preeclampsia but is also observed in IUGR[8]. Therefore, it has been hypothesized that preeclampsia and IUGR may be related disorders yet with different clinical manifestations[9]. In women who develop preeclampsia, an oxidatively stressed placenta is thought to release factors into the maternal circulation causing the maternal clinical syndrome (second stage) of which generalized endothelial dysfunction is considered a central pathophysiological feature[2]. The mechanism(s) by which poor placentation occurs and the subsequent progression into the systemic maternal response are intense areas of research[6]. Numerous factors are proposed to be involved among which oxidative stress, trombophilia, immunological factors, angiogenic factors and their endogenous antagonists (summarized by Ilekis et al [6]). Furthermore, genetic factors are likely to contribute since a familial component is well established in both preeclampsia as well as IUGR[10-12].

In the first part of this thesis we describe the search for new susceptibility genes since this strategy helps to elucidate the underlying pathogenetic mechanisms of disease. Although familial clustering of the disease has been long recognized, the genetics of preeclampsia

and IUGR is far from understood. Different modes of inheritance have been proposed over the years varying from single gene models to complex segregation involving both maternal and fetal genotypes[10,11,12]. In addition to Mendelian modes of inheritance alternative genetic mechanisms such as parent-of-origin effects were proposed for both preeclampsia and IUGR[13,14]. Most efforts aiming to identify genes have targeted preeclampsia, while IUGR was studied to a lesser extent. Numerous candidate gene studies have been performed, but have

Table 1. Candidate genes studied in preeclampsia

Gene name	Gene symbol	Gene name	Gene symbol
Thrombophilia		Interleukin 1β	*IL1B*
Factor V Leiden	*F5*	Interleukin 1 receptor antagonist	*IL1RN*
Prothrombin 20210	*F2*	Interleukin 10	*IL10*
Methylene tetrahydrofolate reductase	*MTHFR*	T-lymphocyte-associated protein 4	*CTLA4*
Cystathione β-synthase	*CBS*	TNF-receptor superfamily member 6	*FAS*
Plasminogen activator inhibitor I	*SERPINE1*	**Oxidative stress**	
β-Fibrinogen	*FGB*	Microsomal epoxide hydrolase	*EPHX1*
Platelet glycoprotein IIIa	*ITGB3*	Glutathione S-transferase pi	*GSTP1*
Thrombomodulin	*THBD*	Glutathione S-transferase mu1	*GSTM1*
Factor VII	*F7*	Glutathione S-transferase theta1	*GSTT1*
Platelet collagen receptor α2β1	*ITGA2*	Myeloperoxidase	*MPO*
Factor XIII A-subunit	*F13A1*	Manganese superoxide dismutase	*SOD2*
Haemodynamics		Cytochrome IAI	*CYP1A1*
Angiotensinogen	*AGT*	Haptoglobin	*HP*
Renin	*REN*	p22*phox*	*CYBA*
Angiotensin-converting enzyme	*ACE*	**Lipid metabolism**	
AT1 receptor	*AGTR1*	Lipoprotein lipase	*LPL*
AT2 receptor	*AGTR2*	Apolipoprotein E	*APOE*
Epithelial sodium channel	*SCNN1B*	Peroxisome-proliferator-activated receptor γ	*PPARG*
Endothelial function		Cholesteryl ester transfer protein	*CETP*
eNOS	*NOS3*	β_3-Adrenergic receptor	*ADRB3*
Endothelin 1	*EDN1*	**Endocrine**	
Dimethylarginine dimethylaminohydrolase 1	*DDAH1*	Oestrogen receptor α	*ESR1*
Dimethylarginine dimethylaminohydrolase 2	*DDAH2*	Oestrogen receptor β	*ESR2*
G-protein β3	*GNB3*	Inhibin α	*INHA*
Cytokines		**Angiogenesis**	
TNFα	*TNF*	VEGF	*VEGF*
IGF II	*IGF2*	Matrix metallopeptidase 1	*MMP1*
Interleukin 1α	*IL1A*		

Modified from Chappell and Morgan, Clin Sci (Lond). 2006 Apr;110(4):443-58

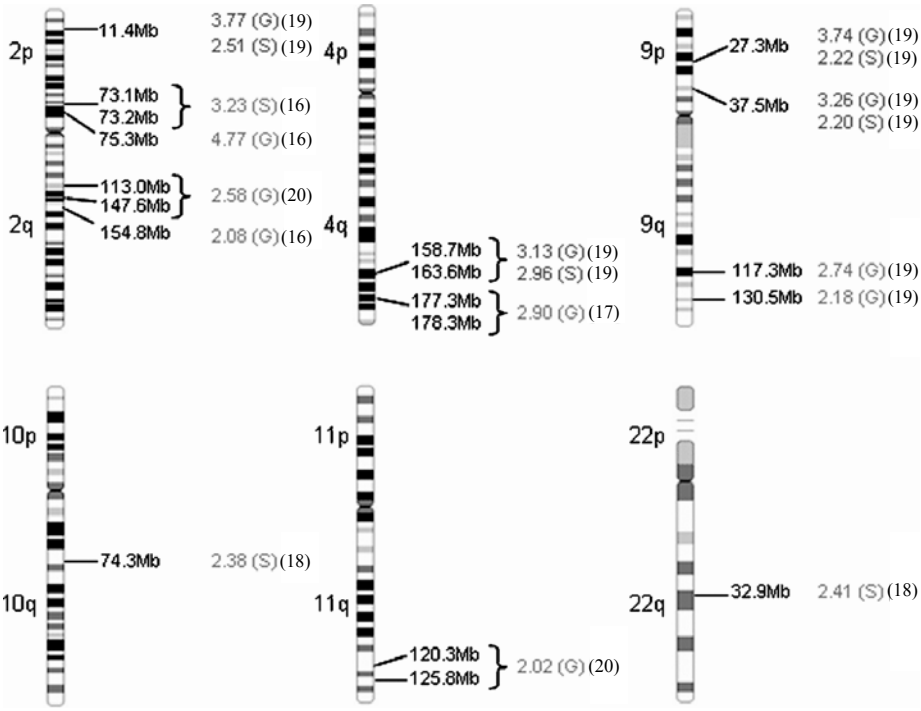

Figure 1 Chromosomal position is given according to NCBI (National Center for Biotechnology Information) Build 35.1. LOD scores or non-parametric linkage scores are shown; the highest scores provide the strongest evidence of linkage. The results of linkage analysis using both strict diagnostic criteria (S, pre-eclampsia/eclampsia only) and general diagnostic criteria (G, includes pregnancy-induced hypertension) are shown. *Modified from Chappell and Morgan, Clin Sci (Lond). 2006 Apr;110(4):443-58*

not yielded consistent results[15] (Table 1). Genome wide linkage studies, have shown evidence for linkage of several loci on chromosome 2p, 2q, 4q, 9p, 10q and 22q with preeclampsia[16-20] (Figure 1). Up to now, follow up of these regions has resulted in identification of one new candidate gene, *STOX1*, which is thought to be imprinted[21].

In the second part of this thesis we focus on the association of preeclampsia and IUGR with cardiovascular disease in later life[22,23]. It is hypothesized that a predisposition to vascular and/or metabolic disease underlies both these pregnancy complications as well as future cardiovascular disease[24]. The physiological stress of pregnancy causes this predisposition to become temporary manifest as preeclampsia or IUGR[25]. Therefore, pregnancy provides a unique opportunity to identify women at increased risk of future disease already at early age, and renders the possibility for prevention[26]. Detailed description of risk profiles in women with prior preeclampsia and IUGR will help to design individual tailored preventive strategies. Additionally, such descriptions allow speculations on aetiology.

The main objectives of this thesis can be summarized as follows:

1. Investigation of genetic factors that are involved in the development of preeclampsia and IUGR, which may enhance our understanding of the aetiological mechanisms.
2. To provide detailed descriptions of the cardiovascular risk profiles of women with previous preeclampsia and IUGR pregnancies to help designing preventive strategies.

SETTING

All but one of the studies described in this thesis were performed within the framework of the research program Genetic Research in Isolated Populations (GRIP), which aims to identify susceptibility genes in complex diseases[27]. Isolated populations are less heterogeneous than outbred populations as, by definition, these isolates originate from a limited number of founders. It has been hypothesized that genetic mechanisms underlying complex diseases in isolated populations might also have reduced heterogeneity, which would facilitate identification of susceptibility genes[28,29]. Moreover, environmental variability is thought to be reduced in these populations. We studied preeclampsia and IUGR in a genetically isolated population in the Southwest of the Netherlands[30]. This population was founded at around 1750 and has been characterized by minimal inward migration and rapid population growth over the last two centuries. Currently, the population consists of over 20,000 individuals. A large genealogical database is available containing information on more than 110,000 individuals from the GRIP region up to 23 generations.

OUTLINE OF THE THESIS

The first part of this thesis focuses on genetic factors of preeclampsia and IUGR. In chapter 2.1 familial aggregation, consanguinity and parent-of-origin effects were investigated. The role of *STOX1* gene, which was previously identified as a putative imprinted gene for preeclampsia following matrilineal inheritance was evaluated in chapter 2.2. In chapter 2.3 a genome wide association analysis of 250.000 single nucleotide polymorphisms (SNPs) was conducted. The second part of this thesis focuses on cardiovascular risk factors in women with a past history of pregnancies complicated by preeclampsia or IUGR. Chapter 3.1 provides detailed descriptions of cardiovascular risk profiles of women with previous complicated pregnancies as compared to those with uncomplicated pregnancies. Additionally, risk profiles of parents of these women were assessed. Lastly, the prevalence of metabolic syndrome was estimated for these women and their mothers. In chapter 3.2 cardiovascular risk profiles were assessed in women after very early onset preeclampsia before 24 weeks of gestation and in the men who fathered these pregnancies. Chapter 3.3 describes maternal body composition and fat

distribution after preeclampsia, IUGR or uncomplicated pregnancies measured by means of anthropometrics and dual energy X-ray absorptiometry. In chapter 3.4 endocrine alterations in these women in relation to fat distribution were studied. Finally, in chapter 4, the main findings of this thesis, aetiological and clinical implications, and suggestions for future research are presented.

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2

Genetics of preeclampsia and intrauterine growth restriction

2.1

Familial aggregation of preeclampsia and intrauterine growth restriction in a genetically isolated population in The Netherlands

ABSTRACT

Objective- Preeclampsia and intrauterine growth restriction are related, pregnancy-specific, disorders with a substantial genetic influence, which may have a joint genetic aetiology. We investigated familial aggregation, consanguinity and parent-of-origin effects for preeclampsia and intrauterine growth restriction.

Methods- Fifty women with previous preeclampsia and 56 with previous pregnancies complicated by intrauterine growth restriction were recruited from a recent genetically isolated population in the Netherlands. Their relationships were estimated by means of a large genealogy database that contains information on more than 110,000 individuals from the isolate over 23 generations. Relationships were quantified using kinship and inbreeding coefficients. Parent-of-origin effects were evaluated by comparing parental kinships.

Results- Eighty-six women (39 preeclampsia and 47 intrauterine growth restriction) could be linked to one common ancestor within 14 generations. The proportion of related women with previous preeclampsia (95.6%) and or pregnancies complicated by intrauterine growth restriction (95.1%) was significantly greater than expected by chance ($p<0.001$). Combined analysis of both disorders did not change the magnitude of familial aggregation. The proportion of women born from consanguineous marriages was increased in women with previous preeclampsia (81.8%) and those with intrauterine growth restriction (78%) compared to a random sample ($p<0.001$). Maternal and paternal kinships were not significantly different in both disorders.

Conclusions- We demonstrate cosegregation of preeclampsia and intrauterine growth restriction, supporting a common genetic aetiology. The high proportion of parental consanguineous marriages suggests the possibility of an underlying recessive mutation. No evidence was found for a parent-of-origin effect either in preeclampsia or in intrauterine growth restriction.

INTRODUCTION

Preeclampsia is a pregnancy-specific disease and leading cause of maternal and fetal morbidity and mortality worldwide[1]. It is defined by de *novo* hypertension and proteinuria. Although maternal symptoms present in the second and third trimester of pregnancy, preeclampsia finds its origin in early placentation. Its exact aetiology remains unknown, but shallow endovascular trophoblast invasion and inadequate uteroplacental artery remodelling resulting in malperfusion of the placenta are key pathologic features of preeclampsia[2]. Similar placental pathology was previously associated with (idiopathic) intrauterine growth restriction (IUGR)[2,3], a condition affecting fetal growth without appreciable maternal disease. The shared placental pathology has led to the hypothesis that preeclampsia and IUGR are related conditions, yet with different clinical manifestations[4].

A familial component, long recognized in the aetiology of preeclampsia[5], supports the hypothesis of a genetic origin. Different modes of inheritance were proposed over the years varying from single gene models[5] to complex segregation involving both maternal and fetal genotypes[5]. Additionally, there is substantial evidence for familial clustering[6, 7] of IUGR, with both maternal and paternal genes contributing to the IUGR phenotype[6]. In addition to Mendelian modes of inheritance alternative genetic mechanisms such as parent-of-origin effects were proposed for both preeclampsia and IUGR[8, 9]. Imprinted genes play a fundamental role in the regulation of human growth and disruption of these genes may cause growth disturbances as is seen in uniparental disomies [9]. Recently, dysregulation of imprinted genes was reported in human placental tissue of IUGR pregnancies[10]. Evidence supporting the role of imprinting in preeclampsia was described in mutant mice models[11]. In humans, the *STOX1* gene was identified as a putative imprinted gene for preeclampsia following matrilineal inheritance.[12] Thus far, however, neither the role of *STOX1* in preeclampsia nor the evidence for imprinting could be confirmed in other populations[13-15].

Whereas familial aggregation is well established for both preeclampsia and IUGR, a question that remains to be answered is whether a joint genetic aetiology underlies the common pathology of both disorders. Further, the evidence for parent-of-origin effects in preeclampsia is still scarce and not replicated. Availability of genealogical information over multiple generations is helpful to assess familial clustering of these disorders for which absolute recurrence risks are low. We investigated familial aggregation of preeclampsia and IUGR in an isolated population for which extensive genealogical data were available. In addition, we studied parent-of-origin effects, which may indicate genomic imprinting and inbreeding effects, suggestive of recessive forms of disease.

METHODS

Population

The study was conducted in a genetically isolated population in the Southwest of the Netherlands,[16] and is part of a larger research program called Genetic Research in Isolated Populations (GRIP), which aims to identify genetic factors in the development of complex disorders[17]. This population was founded around 1750 and has been characterized by minimal inward migration until the period 1960-1970 and rapid population growth over the last two centuries. Currently the population consists of >20,000 individuals[16]. The Erasmus Medical Ethical Committee of the Erasmus Medical Centre Rotterdam approved the study protocol.

Participants

Women with a history of preeclampsia or IUGR pregnancy were selected from the GRIP area. Ascertainment of participants has been described in detail elsewhere[13]. Briefly, 50 women with pregnancies complicated by preeclampsia and 56 with IUGR pregnancies were included in the study. Women were identified from National Birth Registration Records dating from 1983 up to 2004. Diagnoses were confirmed by the research physician after reviewing the medical records. Only women who were living in the isolate at the time of delivery were included in the study.

Preeclampsia was defined as *de novo* hypertension (systolic ≥ 140 and/or diastolic ≥ 90 mm Hg) and proteinuria ≥ 300 mg per 24 hours, or at least 1+ on semi-quantitative analysis after 20 weeks gestation. Superimposed preeclampsia was defined as new onset proteinuria after 20 weeks of gestation in women with chronic hypertension. Preeclampsia was defined as "early-onset" when it was diagnosed before 34 weeks of gestation, and as "late-onset" when diagnosed after 34 weeks. IUGR was defined as a newborn birth weight equal to or below the 5th percentile for gestational age at delivery, according to the Dutch fetal growth charts[18]. If preeclampsia and IUGR co-occurred, women were categorized in the preeclampsia group. Women who gave birth to children with congenital anomalies were excluded from the study group. Only singleton pregnancies were included. Women were invited to participate in the study by their general practitioner or obstetrician. All participants provided written informed consent.

Genealogy

Genealogical data, comprised of the names, dates, and places of birth and death of relatives were obtained from the participants by questionnaires. Municipal and church registers and data from a large genealogy database containing information on more than 110,000 indi-

viduals from the GRIP region were used to extend this pedigree information up to 23 generations.

The relationship between two individuals can be expressed as the pairwise kinship coefficient. This is the probability that a randomly drawn allele from one person is identical by descent with a randomly drawn allele at the same locus of another person. The probability that two alleles in one individual are identical by descent is expressed as the inbreeding coefficient. This coefficient represents the degree of consanguinity between the parents of this individual.

Pairwise kinship and inbreeding coefficients were calculated for a subset of women with previous preeclampsia or IUGR pregnancies. Those with both parents born outside the isolate were excluded from analyses since genealogical information is only available for people born in the isolate.

Parent-of-origin effects

We evaluated parent-of-origin effects in a subset of women who could be linked to a single common ancestor. We tested whether women were more often related through the paternal or maternal lineage by comparing kinship coefficients for the maternal line to those of the paternal line [19]. Under the null hypothesis of no parent-of-origin effect, the average degree of relationship of the fathers should not differ from that of the mothers.

Statistical analysis

General characteristics were compared between groups using Student's t-test for continuous variables and χ^2 statistics for dichotomous variables with SPSS 11.0.1 for Windows. We calculated pairwise kinship and inbreeding coefficients using PEDIG software.[20] This method is based on the same rationale as standard "heritability" estimates methods. We studied whether kinship (K) and inbreeding (I) coefficients in women with previous preeclampsia or IUGR pregnancies deviated from the expected by chance. We made three categories for K: K between ½ and $½^6$ denoting those related within three generation; K<$½^6$ denoting those related more distantly than three generations; and K=0 not related. The frequencies of pairs in each category were calculated. Similarly, we categorized the inbreeding coefficients, I between ½ and $½^6$, I <$½^6$ and I=0, denoting close-, remote- en no inbreeding, respectively. Women with previous preeclampsia and with previous IUGR pregnancies were analysed as separate groups as well as one combined group. To test for evidence for familial aggregation, we performed Monte Carlo analysis. For each patient group, randomly chosen age and sex matched control groups were drawn from the genealogical database of this genetically isolated population. Thousand replicas were used to estimate a null distribution of kinship and inbreeding coefficients reflecting the baseline level in this population. Empirical p-values were estimated from

these distributions. The same procedure was performed to generate the null distribution of the parental kinships.

RESULTS

Genealogy

A total of 106 women were included in the study. Fifty had previous preeclampsia, and 56 had previous IUGR pregnancies. After extensive genealogical analysis, 39 of 50 (78%) women with a history of preeclampsia could be linked to one common ancestor over 14 generations. For IUGR, 47 of 56 (84%) women could be similarly linked. After pooling the two groups of patients, 86 (81%) could be linked to a single ancestor, which was a significantly greater number than was expected by chance (56%, p-value <0.001). Figure 1 depicts the pedigree linking these 86 women. General descriptions for the total groups are presented in Table 1. There were no significant clinical differences between those connected and those not connected to one common ancestor.

Familial aggregation

Seventy-four women (33 with previous preeclampsia and 41 with previous IUGR pregnancies) met the criterion of having at least one parent born in the isolate. We tested whether these

Table 1. Description of women in the total study group, and of those who could be and could not be linked to one common ancestor

Characteristics	PE total	PE with common ancestor	PE, no common ancestor	IUGR total	IUGR with common ancestor	IUGR, no common ancestor
	n=50	n=39	n=11	n=56	n=47	n=9
Age at delivery, years	29.2 (3.8)	29.0 (3.8)	29.9 (3.8)	29.7 (3.6)	29.8 (3.4)	29.5 (4.6)
Gestational age at delivery, weeks	37.0 (3.4)	37.0 (3.3)	37.1 (3.9)	38.6 (2.8)	38.8 (2.8)	38.0 (2.8)
Birthweight of newborns, grams	2559 (886)	2594 (876)	2435 (954)	2223 (547)	2240 (544)	2136 (587)
Early preeclampsia	16 (32)	11 (28.2)	5 (45.5)	NA	NA	NA
Preeclampsia and IUGR co-occurence	8 (16)	6 (15.4)	2 (18.2)	NA	NA	NA

Values are presented as means (SD) or absolute numbers (%)
PE, preeclampsia
NA, not applicable

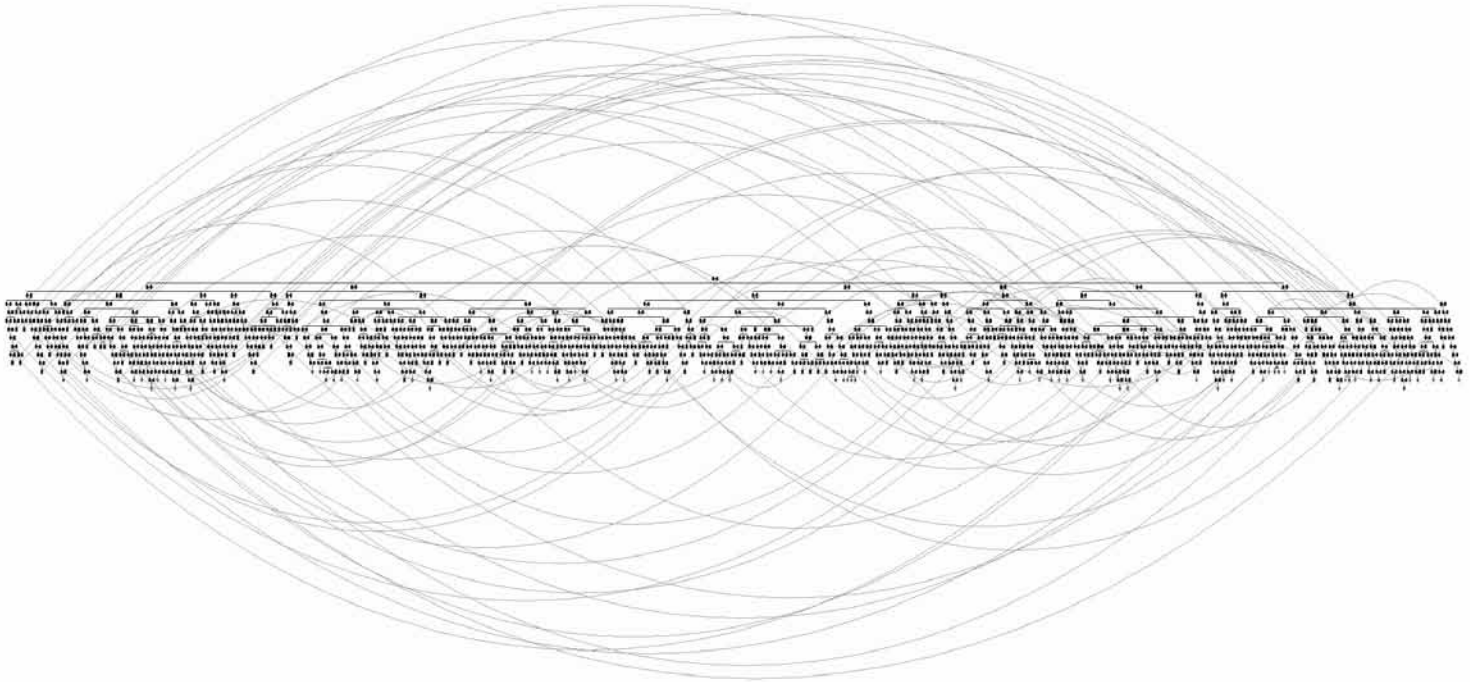

Figure 1

Pedigree of 86 women with previous preeclampsia or IUGR pregnancies (youngest generation) and their relationships to one common ancestor.

Table 2. Distribution of kinship coefficients for women with previous preeclampsia or IUGR pregnancies and controls

Kinship coefficient	PE	Controls	IUGR	Controls	PE+IUGR	Controls
½ - ½ ^{6}	0.9 (5)	0.6	1.0 (8)	0.6	1.1 (29)	0.6
< ½ ^{6}	94.7 (500)*	62.1	94.1 (772)*	56.6	94.2 (2544)*	58.8
0	4.4 (23)*	37.3	4.9 (40)*	42.8	4.7 (128)*	40.6
Total no. of pairs	100 (528)	100	100 (820)	100	100 (2701)	100

All values are percentages with absolute numbers between parentheses

* p <0.001compared with controls

PE, preeclampsia

women were more closely related than expected by chance, adjusting for age and sex (Table 2). The proportion of women that was related (K>0) in the preeclampsia group (95.6%) was higher than expected (62.7%; p<0.001). In the IUGR group this proportion was 95.1%, which was also significantly increased (57.2%; p<0.001). Given the possible confounding effect of smoking on birth weight, we analysed K also after exclusion of those who reported smoking during pregnancy. The proportion of related women did not significantly change (96.7%; p=0.5, Table 3). When preeclampsia and IUGR were analysed as a combined group, 95.3% were related as compared to the expected 59.4% (p<0.001). In addition, significantly more women with previous preeclampsia and IUGR pregnancies could be linked to one common ancestor (97%) than expected by chance (66%, p<0.001). We further compared kinship coefficients between women with early- and late onset preeclampsia. No significant differences were found.

Consanguinity and parent-of-origin effects

Next, we calculated the proportion of women born from consanguineous marriages (I>0) (Table 4). For preeclampsia this proportion was 81.8%, which was significantly greater than expected by chance (38.8%; p<0.001). In the IUGR group (78% versus 35.4%) and combined groups (79.7% versus 36.5%) these proportions were also significantly increased (p< 0.001).

Table 3. Distribution of kinship and inbreeding coefficients for women with previous IUGR pregnancies who did not smoke during pregnancy

	Kinship coefficients*	Inbreeding coefficients†
Coefficient	IUGR non-smokers	IUGR non-smokers
½ - ½ ^{6}	0.5 (1)	0 (0)
< ½ ^{6}	96.2 (202)	76.2 (16)
0	3.3 (7)	23.8 (5)
Total no. of pairs/ absolute no.	100 (210)	100 (21)

All values are percentages with absolute numbers between parentheses

*kinship coefficients were not significantly different from the complete group IUGR

† inbreeding coefficients were not significantly different from the complete group IUGR

Table 4. Distribution of inbreeding coefficients for women with previous preeclampsia or IUGR pregnancies and controls

Inbreeding coefficient	PE	Controls	IUGR	Controls	PE+IUGR	Controls
Close inbreeding	3.0 (1)	3.2	2.4 (1)	3.2	2.7 (2)	3.2
Remote inbreeding	78.8 (26)*	35.6	75.6 (31)*	32.2	77.0 (57)*	33.3
No inbreeding	18.2 (6)*	61.2	22.0 (9)*	64.6	20.3 (15)*	63.5
Total no.	100 (33)	100	100 (41)	100	100 (74)	100

All values are percentages with absolute numbers between parentheses

*p <0.001 compared with controls

PE, preeclampsia

Additional analyses in those women of the IUGR group who did not smoke during pregnancy showed similar proportions (76.2%; p=0.6, Table 3). To evaluate the evidence of parent-of-origin effects we compared the mean kinship of the mothers and fathers of related women. For women with previous preeclampsia, the ratio of mean maternal kinship to paternal kinship was 0.91. This ratio was not significantly different from the ratio expected by chance (p=0.50), implying no evidence of parent-of-origin effect. For IUGR we found a ratio of 1.01, which was not significantly different either (p=0.30). Finally, we evaluated consanguinity and parent-of-origin effects in subsets of women with early-and late-onset preeclampsia. In these groups the ratio of mean maternal kinship and paternal kinship was not significantly different from controls.

DISCUSSION

As expected, we found significant evidence for familial aggregation for women with previous preeclampsia and IUGR pregnancies. The analysis of preeclampsia and IUGR separately or pooled, yielded no significant differences in magnitude of familial aggregation. Pooling two disorders with different genetic origins should result in a smaller proportion of related pairs if the two traits are independent. As the proportion of related pairs in the pooled group was similar to that of the separate patient groups, our data suggest that preeclampsia and IUGR cosegregate in families and probably have a common genetic origin. This finding corroborates the hypothesis of a, at least to some extent, joint aetiology of preeclampsia and IUGR[4].

To our knowledge, no other studies have investigated familial aggregation of preeclampsia and IUGR in a similar way. It was, however, previously reported that women being born small for gestational age have an increased risk to develop preeclampsia[21], which is also compatible with a joint genetic origin. Already in 1977, a common pathophysiology[2] was suggested as morphologic examinations of placental bed biopsies indicated similar pathological changes in preeclampsia and IUGR[3]. Additional evidence for this hypothesis comes from the observation that both disorders share common risk factors[4] and, as has become evident over the last

years, are both associated with similar long-term disease such as an increased risk of cardiovascular disease[22,23]. In this cohort we have reported earlier that more than 40%, and almost 30% of the women with previous pregnancies complicated by preeclampsia or IUGR respectively, were diagnosed with chronic hypertension[24]. This association could not be explained by admixture.

An increased number of women with previous preeclampsia and IUGR pregnancies were born from consanguineous marriages. This proportion suggests a recessive mode of inheritance, at least in a subset of these women. This is in agreement with several previous studies suggesting recessive inheritance for preeclampsia[5]. Given the high risk of chronic hypertension later in life[24], this finding is in line with the higher prevalence of consanguinity in parents of patients with chronic hypertension[25]. However, two earlier studies that compared the incidence of consanguinity in women with preeclampsia and non-hypertensive pregnancies, could not confirm any impact of consanguinity on the occurrence of preeclampsia[26,27]. An important difference between these studies and ours is that consanguinity was based on self-reports instead of on extensive genealogical data, which did not allow for analyses of distant relationships. Regarding IUGR, one large study proposed a recessive inheritance pattern in a subset of their families, yet the majority of the families would best fit a dominant model[7]. A recent large scale study conducted in Lebanon indicated that consanguinity is associated with decreased birth weight[28]. Alternatively, the increased number of women born from consanguineous marriages is also consistent with a polygenic mode of inheritance, particularly in an inbred population.

We also used our genealogic database to evaluate the evidence for parent-of-origin effects in the transmission of preeclampsia or IUGR in a subset of women that could be linked to one common ancestor. No evidence for a parent-of-origin effect was found either for women with a history of preeclampsia or in those with IUGR pregnancies. These findings are in accordance with our previous findings that no maternal preferential transmission of *STOX1* gene in women with preeclampsia and IUGR was observed[13]. However, our observations are at odds with prior findings of a maternal parent-of-origin effect in Dutch preeclampsia patients[8]. Differences between the study of Oudejans et al.[8] and our study may be explained by the selection of patients. Oudejans et al. included affected sib pairs of whom a majority were born to mothers who experienced preeclampsia or pregnancy induced hypertension[8]. In this way, they aimed to target a familial form of preeclampsia with early onset disease. This highly selected group represents only a small proportion of the overall group of preeclamptic women. As we included an unbiased series of women with preeclampsia, our study may have lacked statistical power to pick up the effect of such a small subgroup. Analysing a subgroup of our patients with early and late onset preeclampsia similarly, yielded no evidence for a parent-of-origin effect. Moreover, we found no differences in the degree of relationship between early- and late onset preeclampsia, although it was previously suggested that familial aggregation

is stronger for the clinically more severe type of preeclampsia that often manifests early in pregnancy[29].

The strength of our study is the availability of an extensive genealogy database. Most of the previous studies on familial aggregation and parent-of-origin effects are based on two or three generations [5,7,8,29]. The availability of the genealogy revealed familial aggregation in seemingly unrelated cases. The extended pedigree containing a large proportion of the women with previous preeclampsia and IUGR, may prove to be helpful in future in discovering genes involved in the pathogenesis of both disorders.

Our study also has limitations. The IUGR phenotype was defined as small-for-gestational-age babies. We based our definition on birth weight, because ultrasound examination was not widely used at the time of delivery of the women that we studied. This may have resulted in the inclusion of newborns that were constitutionally small, but not growth restricted. However, by using the stringent criterion of birth weight equal or below the fifth percentile we aimed to minimize misclassification. Familial aggregation of IUGR due to environmental factors such as severe nutritional deficiencies or smoking cannot be excluded with certainty, but the impact of malnutrition on birth weight in studies within developed countries is not likely to be large. Regarding smoking, reanalysis of kinship and inbreeding coefficients after exclusion of women who reported smoking during pregnancy, did not change our results.

Finally, studying patients in an isolated population may raise the question whether the findings can be generalized to the population at large. However, because our population is of more recent isolation the genetic makeup may more closely resemble that of the general population[30]. Further, our simulation studies based on the genealogy have shown that this potential problem concerns primarily rare variants[30]. For common genetic variants, our simulation studies show that no substantial differences between the GRIP isolate and the general population are expected[30]. This is in line with the finding that common variants identified in isolates such as Iceland and Sardinia, are found with similar frequencies and effect in outbred populations.

In summary, we found evidence for familial aggregation in women with previous preeclampsia and IUGR pregnancies in a genetically isolated population. The increased frequency of parental consanguineous marriages suggests recessive mutations play a role. Further, we observed cosegregation of preeclampsia and IUGR, supporting the hypothesis of a common genetic pathogenesis. No evidence was found for a parent-of-origin effect either in preeclampsia or in IUGR.

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2.2

STOX1 gene in preeclampsia and intrauterine growth restriction

ABSTRACT

Objective- Evaluation of the role of *STOX1* gene, which was previously identified as a putative imprinted gene for preeclampsia following matrilineal inheritance, in preeclampsia and intrauterine growth restriction (IUGR).

Methods- Allele frequencies of *STOX1*- Y153H were compared between women with a history of preeclampsia (n=157) and controls (n=157) in the general population. Next, segregation of *STOX1*-Y153H was studied in families of women with pregnancies complicated by preeclampsia (50 families) or IUGR (56 families) in an isolated Dutch population.

Results- *STOX1*-Y153H frequencies were similar in women with preeclampsia (65%) and controls (64%). In families with pregnancies complicated by preeclampsia or IUGR no distortion could be demonstrated in the transmission of *STOX1*-Y153H from heterozygous mothers to offspring which would be suggestive for imprinting.

Conclusions- Our findings do not confirm previous suggestions that *STOX1* plays a major role in Dutch preeclamptic patients.

INTRODUCTION

Preeclampsia is a pregnancy-specific disorder defined by *de novo* hypertension and proteinuria and is a major cause of maternal and fetal morbidity and mortality. Although maternal symptoms present in the second and third trimester of pregnancy, preeclampsia finds its origin in early placentation. Intra uterine growth restriction (IUGR) shares a common pathogenesis with preeclampsia. Abnormal placentation including shallow trophoblast invasion combined with maternal endothelial cell dysfunction are key features in both diseases[1].

A strong familial component in the aetiology of preeclampsia has been described in several studies[2]. Different modes of inheritance have been proposed over the years varying from single gene models to complex segregation involving both maternal and fetal genotypes[2].

Familial aggregation of IUGR supports the role of a genetic component in its aetiology[3], with both maternal and paternal genes contributing to the IUGR phenotype[3]. Epigenetic phenomena such as genomic imprinting are described in the regulation of fetal growth[4].

Oudejans et al.[5] reported evidence for epigenetic phenomena in preeclampsia in Dutch women. In 2005, Van Dijk et al.[6] combined linkage results with expression data of candidate genes in first trimester placentas and identified *STOX1* as a new imprinted gene for preeclampsia. *STOX1* is expressed in early placenta and is subject to imprinting with preferential expression of the maternal allele. *STOX1* encodes a putative DNA binding protein, which is involved in differentiation of the trophoblast. Van Dijk et al.[6] hypothesized that maternal transmission of the variant allele to the fetus induces premature trophoblast differentiation and results in shallow trophoblast invasion, resulting in preeclampsia in such cases[6]. As the paternal imprinted allele is silenced, the maternal allele acts dominantly. Van Dijk et al.[6] showed maternal transmission of *STOX1*-Y153H variant in all preeclamptic patients they studied.

In this study we aim to replicate the finding that *STOX1*-Y153H is involved in the aetiology of preeclampsia. Considering the shared pathogenesis of preeclampsia and IUGR on the one hand and the role of *STOX1* in defective placentation on the other hand, we hypothesized that *STOX1* is involved in the aetiology of IUGR as well.

First, we investigated allele frequency differences of *STOX1*- Y153H variation in the general population between women with a history of preeclampsia and controls. Secondly, we studied the segregation of *STOX1*-Y153H variation in families of women with pregnancies complicated by preeclampsia or IUGR in an isolated Dutch population.

MATERIAL AND METHODS

Subjects

Hospital-based study

Patients (n=157) and controls (n=157) were recruited from the obstetric services of the Leiden University Medical Centre and the St. Joseph Hospital Veldhoven as previously described[7]. Women with a history of preeclampsia had been diagnosed by strictly adhering to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP). All women were in their first pregnancy. The data were analysed anonymously. The study protocol was approved by the local Ethics Review Board.

Family-based study

Women with a history of preeclampsia or a pregnancy complicated by IUGR were selected from a genetically isolated community in the Southwest of the Netherlands. This study is part of a larger research program named Genetic Research in Isolated Populations (GRIP)[8].

The scientific protocol of GRIP has been approved by the Medical Ethics Committee of the Erasmus Medical Centre. All participants provided informed consent. Preeclampsia was defined as *de novo* hypertension (≥ 140/ 90 mm Hg) and proteinuria ≥ 300 mg per 24 hour or at least 1+ on semi quantitative analysis. IUGR was defined as birth weight of newborns equal to or below the 5th percentile, according to the Dutch fetal growth charts of Kloosterman. Mothers of children with congenital anomalies were excluded. Only singleton pregnancies were included. Women with a history of preeclampsia or IUGR were identified from National Birth Registration Records dating from 1983 up to 2004. The records reported 93 women with preeclamptic pregnancies and 104 with IUGR living in this community at the time of delivery. Medical records, if available, were obtained and reviewed by the research physician to confirm diagnosis. Diagnoses were confirmed in 140 women (61 women with preeclampsia and 79 women with IUGR). In the remaining 57 cases either patient's identity could not be traced or medical records were absent. One hundred and six women (50 women with preeclampsia and 56 women with IUGR) were willing to participate in the study (response rate of 75.7%). Thirteen women did not respond to repeated inquiries and 21 declined to participate. Inclusion of mothers, who were asked to fill in a questionnaire about their obstetric history (n=68), and fathers (n=50) of the affected women, as well as the children (n=91) born from the preeclamptic pregnancies (if alive) and their spouses (n=85), allowed for segregation study and reconstruction of haplotypes (Figure 1).

Laboratory analyses

Genomic DNA was extracted from peripheral blood (salting out method) using the Puregene DNA purification kit from Gentra Systems (Minneapolis, MN, USA). DNA from children was obtained using buccal swab samples (MasterAmp kit from Epicentre Biotechnologies, Madison, WI, USA) and from saliva samples using the Oragene DNA self-collection kit from DNA Genotek Inc (Ottawa, Ontario, Canada).

Genotypes corresponding to the single nucleotide polymorphism (SNP) rs1341667 of the *STOX1* gene were determined in all available individuals. The SNP is located at position 19193015 from the human genomic contig NT_008583.16 (NCBI build 36.1 and Van Dijk et al.[6]) and consist of a T/C change (in the reverse strand) leading to a change of amino acid Tyrosine to Histidine at position 153 of the *STOX1* protein (Y153H). The TaqMan validated SNP genotyping assay method (allele T was labelled with VIC and allele C with FAM) on an Applied Biosystems 7300 Real Time PCR System was used.

Eight samples were selected based on their genotype (homozygous CC, TT and heterozygous CT) for direct sequencing and confirmation of the genotypes previously determined with the TaqMan assay.

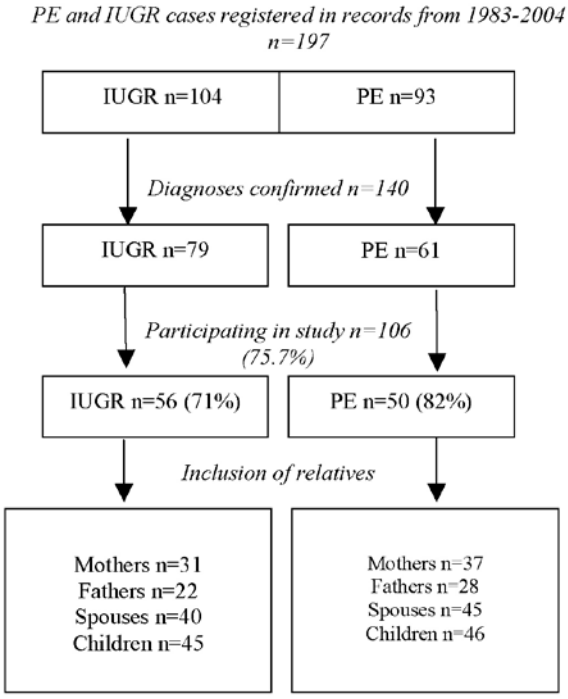

Figure 1. Recruitment of women with preeclampsia or IUGR and their relatives.
PE, preeclampsia

Two additional polymorphic markers at 10q21.3, D10S1678 at 70.35 Mb (according to NCBI build 36.1) and D10S210 (69.71 Mb), flanking *STOX1* (70.25 Mb) were used to reconstruct haplotypes for segregation analysis. Fluorescent PCR products were load on an ABI 3100 automated sequencer from Applied Biosystems. Data were analysed using Genemapper 2.1 software (Applied Biosystems, Foster City, CA, USA).

Statistical analyses

Chi-square statistics were used for testing Hardy Weinberg equilibrium and to evaluate distortion in transmission of the alleles.

RESULTS

In the population-based study, genotyping was successful in 149 women (preeclampsia) and 154 controls. The genotype frequencies and allele proportions did not deviate from Hardy Weinberg Equilibrium. The frequency of the C-allele in controls was 65% (genotype distribution: CC 43.5%, CT 43.5%, TT 13%), which was not significantly different from the allele frequency in cases (C-allele frequency 64% with genotype distribution: CC 43%, CT 43%, TT 14%).

In the isolated population, similar frequencies were found (p=0.5). Of the 106 cases, 104 were successfully genotyped. In women with a history of preeclampsia the C-allele frequency was 71% (genotype distribution: CC 53.1%, CT 36.7%, TT 10.2%), which did not differ significantly from the frequency in women with IUGR pregnancies (C-allele frequency: 60%, genotype distribution CC 40% CT 40% TT 20%). In spouses of women with pregnancies complicated by preeclampsia or IUGR we found a C-allele frequency of 68% (genotype distribution: CC 45.6% CT 44.3% TT 10.1%). Genotype frequencies and allele proportions of all groups were in Hardy Weinberg Equilibrium.

Segregation of *STOX1*-Y153H variation was studied separately in families with preeclampsia and IUGR. In 42 of the 50 preeclamptic families sufficient genotype information was available to study the maternal transmission of the C-allele to children born from the complicated pregnancies. In 34 (81%) of these families we observed maternal transmission of the C-allele from mother to child. When excluding the CC homozygous mothers and only considering the informative heterozygous mothers (n=10), the C: T transmission ratio was 7:3 (p=0.21). The observed allele transmission ratios were tested against the expected Mendelian proportions (i.e. 0.5 for both alleles).

Similarly we studied the 56 families with pregnancies complicated by IUGR. In 47 families maternal transmission of the C-allele could be evaluated. In 31 (66%) families transmission

Table 1. C/T allele transmissions from cases to children born from affected pregnancies

	Number of heterozygous subjects	Number of C-allele transmissions	Number of T-allele transmissions	p-value
Mothers with a history of PE	10	7	3	0.21
Mothers with a history of IUGR	13	9	4	0.17
Grandmothers with a history of vascular related pregnancy complication	12	8	4	0.25
Pooled groups				
Mothers with a history of PE + IUGR	23	16	7	0.06
Mothers with a history of PE + IUGR and grandmothers	35	24	11	0.03

PE, preeclampsia

of the C-allele from mother to child was observed. When only considering the informative heterozygous transmissions (n=13) the C-allele was transmitted 9 times (p=0.17).

We extended the pedigrees with grandmothers (mothers of preeclampsia or IUGR cases) who reported a history of preeclampsia, pregnancy induced hypertension or IUGR (n=30). Twelve heterozygous grandmothers transmitted the C-allele 8 times (p=0.25). The allele transmissions and corresponding P-values are depicted in Table 1. We found no significant evidence for a distortion in transmission of maternal alleles. Finally, we evaluated the allele transmissions after pooling of the data. First, mothers with preeclampsia and IUGR were combined; second, affected grandmothers and mothers with preeclampsia and IUGR were pooled. The P-values were 0.06 and 0.03 respectively (Table 1.).

DISCUSSION

No significant differences were found in allele frequencies of *STOX1*-Y153H variation between women with preeclampsia and controls. Surprisingly, the disease-associated allele (C-allele) of *STOX1* gene appeared to be the predominant allele in the populations studied. The C-allele frequencies we observed were higher than the 50% described by Van Dijk et al.[6]. They based the allele frequency on the genotyping of 32 controls in contrast with 154 controls in our study [6]. Our findings are in accordance with the frequencies described in the NCBI SNP database. Similar frequencies were found in women with a history of preeclampsia or IUGR in an isolated Dutch population. Van Dijk et al.[6] reported a preferential transmission of the C-allele of *STOX1* from mothers with preeclampsia to offspring. We examined the allele transmissions

from heterozygous women with preeclampsia or IUGR to their offspring. Heterozygotes are expected to transmit alleles equally to their offspring when no preferential transmission is assumed. A distortion in transmission of alleles would be suggestive for a parent-of origin effect. We, however, found no evidence for such a distortion in transmission in our population.

It is important to note that, although the study of Van Dijk and our study used the same criteria to diagnose preeclampsia, the studies selected women differently. Van Dijk et al.[6] included affected sib pairs only with significant linkage with 10q, resulting in a selection of preeclampsia patients with a presumably larger genetic component. Oudejans et al.[9] point out that Van Dijk et al.[6] in this way target the early familial form of preeclampsia associated with IUGR, designating this condition as placental preeclampsia[9]. However, Van Dijk does not explicitly state that they included women with early preeclampsia only. When we performed a subanalysis on the women with early and late onset preeclampsia recruited from the general population, we found no significant (p=0.71) difference in genotype distribution or allele frequencies between early (<34 weeks of gestation, n= 83) and late onset preeclampsia (≥ 34 weeks of gestation, n=66).

We did not differentiate between subtypes of preeclampsia in the analysis of the patients recruited from the isolated population, which may imply that our patients are more heterogeneous. On the other hand, by studying patients from an isolated population we reduced heterogeneity.

We observed an excess of maternal transmission of the C-allele compared with transmission of the T-allele. However, this deviation from the expected Mendelian proportions did not reach a significant level when the phenotypes were analysed separately. When we pool the data of preeclampsia and IUGR based on the assumption that *STOX1*-Y153H variant is involved in placental pathology underlying both phenotypes a P-value of 0.06 is found. When including data of the affected grandmothers in the analyses a significant P-value of 0.03 is reached.

This indicates a distortion in transmission in favour of the C allele supporting maternal preferential transmission in women with abnormal pregnancy outcome with shared aetiological placental pathology. However, we must be cautious when pooling the data of the grandmothers as diagnoses were based on self-reported disease. Nevertheless, pooling the data points out that we cannot reject the hypothesis that *STOX1* adds to the risk of preeclampsia in a subset of women.

Two recent studies, however, performed in Dutch and Finnish populations, could not confirm the involvement of *STOX1* gene in preeclampsia[10,11]. No differences were detected in expression of *STOX1* mRNA between placentas form preeclamptic and uncomplicated pregnancies. Additionally, Iglesias-Platas et al.[10] demonstrated biallelic expression in fetal tissues and placentas of preeclamptic and uncomplicated pregnancies, in both human and mouse, contradicting the imprinting status of *STOX1*.

Despite the fact that the imprinting status of *STOX1* gene is questioned, there is substantial support for epigenetic phenomena such as genomic imprinting in placental pathology.

Altered expression of imprinted genes has been described in association with IUGR[4]. The involvement of imprinted genes in preeclampsia was already suggested years ago by Graves[12] as a solution for the genetic conflict between maternal genes that limit growth and paternal genes that promote growth. Kanayama et al. reported evidence for genomic imprinting in preeclampsia in animal models. Heterozygous mice with respect to the maternally expressed imprinted gene *Cdkn1c* develop preeclampsia only when the maternal mutant allele is transmitted to the offspring[13].

In conclusion, we studied the role of *STOX1*-Y153H variation in the pathogenesis of preeclampsia and IUGR in an isolated Dutch population. We found no significant evidence for preferential transmission of *STOX1*-Y153H mutation from women with preeclampsia or IUGR to their offspring. Our findings, therefore, do not confirm the hypothesis that *STOX1*-Y153H plays a major role in Dutch women with preeclampsia. The relevance of *STOX1*-Y153H variation in women with preeclampsia in other populations remains to be studied.

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2.3

Genome wide association analysis of preeclampsia and intrauterine growth restriction

ABSTRACT

Objective- Preeclampsia and intrauterine growth restriction (IUGR) are related, pregnancy specific disorders, which are presumed to have a partly genetic origin. A genome wide association study was conducted to identify genes involved in preeclampsia and IUGR.

Methods- From a genetically isolated Dutch population, women with previous preeclampsia (n=44) or IUGR pregnancies (n=52) were selected. Controls (n=97) were recruited from the same isolated population. Participants were genotyped with the Affymetrix 250K Nsp array from the GeneChip® Human Mapping 500K array Set. Genome wide association analyses were performed using GenABEL. Subsequently, the associations of single nucleotide polymorphisms (SNPs) located in previously reported linkage regions were evaluated.

Results- None of the associations reached an established genome wide significance level (p value 5 $x10^{-8}$). Twenty-two SNPs were associated with preeclampsia with a p-value < 10^{-4}, of which four were intragenic. Six of those SNPs were located within a 100 Kb region on chromosome 2, one located within the EHBP1 gene and 5 nearby. Two of the top SNPs associated with preeclampsia were located within previous reported linkage regions on chromosome 2 and 10. Eighteen SNPs were associated with IUGR with a p value < 10^{-4}, of which 13 were located within 8 different genes. Four of these top SNPs were located in a previously reported linkage region for preeclampsia on chromosome 10.

Conclusions- Our genome wide association study did not yield genome wide statistical evidence for a new locus for preeclampsia or IUGR. Suggestive associations were found for two SNPs associated with preeclampsia on chromosome 2 and 10, and for four SNPs associated with IUGR on chromosome 10, which were supported by previous linkage studies. Additionally, a new gene, EHBP1, associated with preeclampsia on chromosome 2 was identified. Our findings require verification by replication in other cohorts.

INTRODUCTION

Preeclampsia is a pregnancy specific disorder characterized by *de novo* hypertension and proteinuria. It is a leading cause of maternal and fetal morbidity and mortality worldwide[1]. Despite the fact that maternal symptoms develop in the latter half of pregnancy, preeclampsia finds its origin in early placentation. Shallow endovascular trophoblast invasion and inadequate uteroplacental artery remodelling are key pathologic features of preeclampsia[2], yet the exact aetiology remains unknown. Similar placental pathology has been suggested to precede intrauterine growth restriction (IUGR)[2,3], a condition affecting fetal growth without substantial clinical impact on the mother. Hence, it has been hypothesized that preeclampsia and IUGR are related conditions sharing a common cause but differing in clinical manifestation[4]. Additional support for a common pathogenesis comes from the observation that preeclampsia and IUGR share common risk factors as well as long term consequences, such as an increased risk of cardiovascular disease[5-9].

Familial clustering has been recognized for both preeclampsia (reviewed by Lachmeijer et al.[10]) and IUGR[11,12], supporting a genetic contribution to their development. Also we observed familial aggregation of preeclampsia and IUGR in the same families in a Dutch genetically isolated population, supportive of a (partly) joint genetic etiology[13]. Accordingly, women with previous growth-restricted babies (without preeclampsia) have an increased risk of preeclampsia in the subsequent pregnancies [14,15].

Most efforts aiming to identify genes have targeted preeclampsia, while IUGR was studied to a lesser extent. The genetics of preeclampsia has been studied with varying degrees of success (reviewed by Lachmeijer et al.[10,16]). Candidate gene approaches have not yielded consistent results[16]. Genome wide linkage studies have shown evidence for several loci on chromosome 2p, 2q, 4q, 9p, 10q and 22q for preeclampsia.[17-20] The only gene identified up to date as a follow up of these linkage studies is *STOX1*, a gene which appears to be imprinted[21]. However, we as well as others, failed to find convincing evidence for a major role of the *STOX1* gene in preeclampsia or IUGR[22-24], nor did we find convincing evidence for imprinting based on our genealogical data for this gene or other ones[13]. Recent advances in genotyping technologies with high-throughput platforms now allow for rapid screening of common genetic variations across the human genome for association with disease. In the current study we conducted a genome wide association analysis on preeclampsia and IUGR in a genetically isolated population in the Netherlands.

METHODS

Population

The study was conducted in a genetically isolated population in the Southwest of the Netherlands[25] and is part of a larger research program called Genetic Research in Isolated Populations (GRIP), which aims to identify genetic factors in the development of complex disorders[26]. This population was founded around 1750 by a limited number of individuals (<400) and has been characterized by minimal inward migration and rapid population growth over the last two centuries. Currently the population consists >20,000 individuals[25]. The Erasmus Medical Ethical Committee of the Erasmus Medical Centre Rotterdam approved the study protocol.

Participants

Women with a history of preeclampsia or IUGR pregnancies were selected from the GRIP area. The recruitment of cases has been described in detail elsewhere[22]. In brief, women with pregnancies complicated by preeclampsia and IUGR were identified from National Birth Registration Records dating from 1983 up to 2004. Only women who were living in the isolated area at time of delivery were included in the study. Preeclampsia was defined as *de novo* hypertension (systolic ≥ 140/ diastolic ≥ 90 mm Hg) and proteinuria ≥ 300 mg per 24 hours or at least 1+ on semi quantitative analysis[27]. Superimposed preeclampsia was defined as new onset proteinuria after 20 weeks of gestation in women with chronic hypertension[27]. IUGR was defined as birth weight of newborns equal to or less than the 5th percentile for gestational age at delivery, according to the Dutch fetal growth charts of Kloosterman[28]. If preeclampsia and IUGR co-occurred (n=8), women were categorized in the preeclampsia group. Women, who gave birth to children with congenital anomalies, were excluded from the study group. Only singleton pregnancies were included. Women were invited to participate in the study by their general practitioner or obstetrician. In total, 106 women with a history of preeclampsia or IUGR (47 with preeclampsia, 3 with superimposed preeclampsia, and 56 with IUGR) agreed to participate. For the purpose of this study women with chronic hypertension prior to pregnancy (3 women with superimposed preeclampsia and 3 women with IUGR pregnancies) were excluded from analyses, as these might represent a distinct phenotype. Three individuals were excluded as they were close relatives of other study participants (2 with PE and 1 with IUGR) and in one woman venous blood sampling failed, resulting in a total of 44 women with prior preeclampsia and 52 with IUGR pregnancies for further analysis. Women who participated in the Erasmus Rucphen Family study (ERF), a study that is also embedded in the GRIP population, served as controls. A total of 1616 women participated in ERF of whom 811 were premenopausal. Genome wide SNP data were available for 97 of those who were previously selected

for a study on eye colour and height.[29] None of these women reported a history of pregnancy complicated by hypertension. All participants provided written informed consent.

Genotyping

Genomic DNA was extracted from whole blood samples, using the salting out method[30].The 250K Nsp array from the GeneChip® Human Mapping 500K Array Set (Affymetrix) was utilized to determine genome-wide genotypes. The chips were run and analysed according to the manufacturer's protocols.

Statistical analysis

Prior to running the analyses genotypes for autosomal single nucleotide polymorphisms (SNPs), monomorphic SNPs (n=25017) were excluded as well as SNPs with call rate <95% and SNPs with a p-value < 1×10^{-8} (n=31765) for an exact test of Hardy Weinberg Equilibrium, using GenABEL package (1.3-7) for R[31]. In total, 205,388 SNPs were considered for further analyses. General characteristics were compared between groups using student T-test or Mann Whitney test with SPSS 11.0.1. The SNPs were tested for association with preeclampsia and IUGR separately, using genomic control to adjust for unknown relationships and population mixture based on the EIGENSTRAT method[32] as implemented in egscore function of the GenABEL package[33]. These corrections for residual inflation of the association test statistics are necessary as they often occur in genetically isolated populations as a result of relations between study subjects. Alleles were coded as 0,1,2, rendering nominal p-values with 1 degree of freedom. The p-values derived from the egscore function will be further reported in this paper. Subsequently, we evaluated the association of SNPs located under previously reported linkage peaks[17-20, 34]. The -2 LOD score was used to define the regions. We searched the Ensembl Project (www.ensembl.org) and the National Centre for Biotechnology Information (NCBI) Human Genome Resource databases (www.ncbi.nlm.nih.gov) for known genes surrounding or containing the SNPs associated with preeclampsia and IUGR in this study.

RESULTS

General characteristics of our study population are presented in Table 1. The mean age was higher in women with previous preeclampsia and previous IUGR pregnancies as compared to controls. Women with previous preeclampsia had a significantly higher body mass index and systolic and diastolic blood pressure than controls. No significant differences were found for BMI or blood pressure between women with IUGR pregnancies and controls. Height was not significantly different between the groups.

Table 1. General characteristics of participants

	Preeclampsia n=44	IUGR n=52	Controls n=97
Mean age, y	35.1± 5.3*	38.6±5.2*	30.3±6.9
Mean body mass index, kg/m^2	27.9± 6.6*	24.6 ±4.4	24.3± 4.6
Mean systolic blood pressure, mmHg	132.2±16.1*	122.3 ±12.1	122.6± 12.7
Mean diastolic blood pressure, mmHg	81.9±10.4*	76.6± 9.2	74.9± 9.3
Median height, cm	165(161-169)	162 (157-167)	165 (156-175)

Values are presented as means with ± SD or medians (interquartile ranges)

* significantly different from controls, p<0.001

The results of the genome wide association analyses (GWA) are displayed in Figures 1a (preeclampsia) and 1b (IUGR). In these figures, -log$_{10}$ p-values are plotted on the Y axis, and the physical chromosomal position of the SNP in the genome on the x-axis. None of the SNPs reached an established level for genome wide significance level (p=5 x10^{-8}). The smallest p values were found for rs4942158 on chromosome 13 (p value of 0.2 x 10^{-5}) for preeclampsia and rs7757142 on chromosome 6 (p value of 0.3 x 10^{-5}) for IUGR. In table 2a and 2b, SNPs associated with preeclampsia and IUGR, with a nominal p– value ≤10^{-4} are depicted. For these SNPs, genotype distribution and allele frequency were in Hardy Weinberg Equilibrium. The tables also show the chromosome, the position on the chromosome, and the gene in which the SNP is located or the nearest gene in the region. For preeclampsia we found 22 SNPs associated with a p value < 10 $^{-4}$, of which four were intragenic (Table 2a). Remarkably, six of these 22 SNPs were positioned in the same region of chromosome 2, one located within the EH domain binding protein 1(EHBP1) gene and 5 nearby. Four additional regions with 2 hits were found on chromosomes 3, 6, 9 and 11. The region on chromosome 3 was near synaptoporin (SYNPR), on chromosome 6 near HUS1 checkpoint homolog b (HUS1B), on chromosome 9 within the

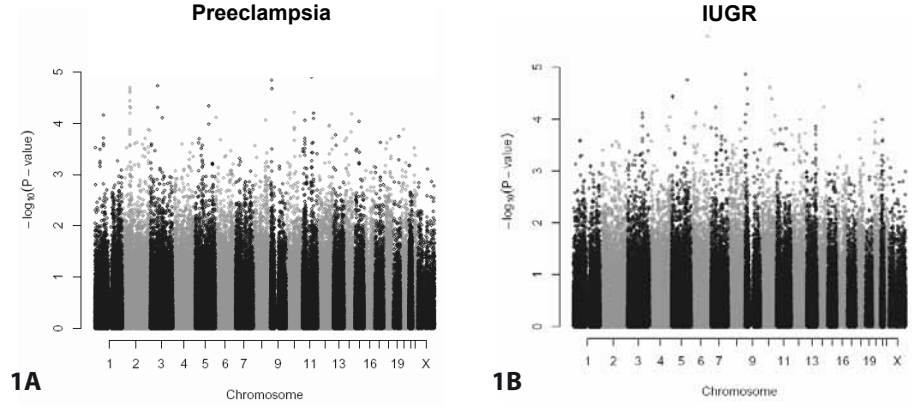

Figure 1a. Genome wide association for preeclampsia. Nominal p-values.

Figure 1b. Genome wide association for IUGR. Nominal p-values.

Table 2a. Single nucleotide polymorphisms significantly ($p<10^{-4}$) associated with preeclampsia*

rs number	Nominal p-value (1df) x 10^{-5}	Chromosome	Position	Gene	Nearest Gene
rs10493536	8,40	1	77528815	AK5	
rs6752010	2,50	2	62712546		EHBP1
rs2204656	4,50	2	62737372		EHBP1
rs1534420	2,80	2	62737557		EHBP1
rs6545969	5,90	2	62744978		EHBP1
rs6757128	7,80	2	62745256		EHBP1
rs360801	3,10	2	62808891	EHBP1	
rs7578047	6,10	2	68433435		PLEK
rs1901304	2,30	3	63365781		SYNPR
rs9311875	6,00	3	63367449		SYNPR
rs17835169	9,50	3	104138957		ZPLD1
rs2972345	5,60	5	121291607		SRFBP1
rs2804737	1,30	6	660196		HUS1B
rs2804744	9,30	6	678163		HUS1B
rs7874540	1,80	9	8990476	PTPRD	
rs10809068	2,60	9	10319917		PTPRD
rs10996467	7,50	10	66921953		CTNNA3
rs17142789	1,60	11	81100270		RPS28
rs666825	9,80	11	99079899		CNTN5
rs614096	7,80	11	99082600		CNTN5
rs4942158	0,20	13	42254313	C13orf30	
rs41508545	8,00	14	82052605		SEL1L

*sorted on chromosome and position

protein tyrosine phosphatase, receptor type, D (PTPRD) region and chromosome 11 near contactin 5 (CNTN5). For IUGR, 18 SNPs were associated with a p value < 10^{-4}, of which 13 were located within 8 different genes (Table 2b). There were two regions on chromosome 10 in two different genes, potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (KCNMA1) and zinc finger protein 518 (ZNF518). Furthermore, two regions were found on chromosome 9, one within the ADAMTS-like 1(ADAMTSL1) gene and one near to SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SMARCA1) gene. Two additional regions with 2 hits were found on chromosome 5 within the catenin delta 2 (CTNND2) gene and on chromosome 6 within the glucagon-like peptide 1 receptor (GLP1R) gene. We observed no overlap in the SNPs associated with preeclampsia and IUGR.

Subsequently, we evaluated for preeclampsia the associations with SNPs that were positioned in previous reported linkage regions (Table 3). These regions had been located on chromosomes 2, 4, 9,1 0, and 22 [17-20, 34]. Considering the (partly) common pathophysiology

Table 2b. Single nucleotide polymorphisms significantly ($p<10^{-4}$) associated with IUGR*

rs number	Nominal p-value (1df) x 10-5	Chromosome	Position	Gene	Nearest Gene
rs1450049	9,20	3	134394111	TMEM108	
rs852621	4,40	5	11065130	CTNND2	
rs852622	4,40	5	11065202	CTNND2	
rs432793	2,20	5	145219312	NP_001073985.1	
rs9296283	9,10	6	39142974	GLP1R	
rs7766275	9,10	6	39143814	GLP1R	
rs7757142	0,30	6	143762160		ADAT2
rs2908282	7,10	7	44215353	YKT6	
rs7045276	9,60	9	1916444		SMARCA2
rs10123583	1,70	9	1918115		SMARCA2
rs1991881	6,20	9	18728908	ADAMTSL1	
rs7031387	3,20	9	18733676	ADAMTSL1	
rs660465	3,00	10	78855117	KCNMA1	
rs584141	3,00	10	78860360	KCNMA1	
rs17385450	4,90	10	97880590	ZNF518	
rs17476713	4,90	10	97884446	ZNF518	
rs8020585	7,10	14	74849642		O95431-HUMAN
rs12373195	2,90	18	27983601		RNF138

*sorted on chromosome and position

of preeclampsia and IUGR we evaluated these regions for IUGR also. The smallest p values for SNPs associated with preeclampsia were found in the linkage region on chromosome 2 reported by Arngrimsson et al.[17] (rs7578047) and on chromosome 10 reported by Lachmeijer et al.[18] (rs10996467), with p values of 6.1×10^{-5} and 7.5×10^{-5}, respectively (Table 3). Both SNPs were intergenic with pleckstrin (PLEK) being the nearest gene on chromosome 2 and catenin alpha 3 (CTNNA3) on chromosome 10. These genes were also detected as the top SNPs in our GWA analysis. The SNPs in the remaining regions were less significantly associated with preeclampsia ($>10^{-4}$). For IUGR the smallest p values were found in the linkage region on chromosome 10 (table 3), located within the KCNMA1 (rs660465, p value=$3,0 \times 10^{-5}$, rs584141, p value=$3,0 \times 10^{-5}$) and ZNF518 gene (rs17385450 and rs17476713, both with p value of 4.9×10^{-5}). These SNPs were also found among the top SNPs of our GWA. The remaining associations had p-values of $>10^{-4}$.

DISCUSSION

In this genome wide association study of preeclampsia and IUGR we found that six SNPs associated with preeclampsia with p values $< 10^{-4}$ were located within a 100Kb region on chro-

Table 3. Top 5 SNPs with smallest nominal P-value in previous reported linkage regions*

	Preeclampsia			IUGR		
Chromosome	SNP	Position	nominal p value	SNP	Position	nominal P value
Region with significant linkage reported by Arngrimmson et al.(1999)17						
2	rs1575027	66248910	$1{,}20 \times 10^{-3}$	rs10165209	79305109	$3{,}04 \times 10^{-3}$
2	rs13383903	66254338	$6{,}63 \times 10^{-4}$	rs1864550	79553090	$5{,}70 \times 10^{-3}$
2	rs7578047	68433435	$6{,}10 \times 10^{-5}$	rs10520295	81074599	$1{,}59 \times 10^{-3}$
2	rs12713802	74078772	$8{,}32 \times 10^{-4}$	rs1427648	82432439	$6{,}05 \times 10^{-3}$
2	rs3771738	74394425	$9{,}28 \times 10^{-4}$	rs7563050	82458101	$5{,}72 \times 10^{-3}$
Region with suggestive linkage reported by Moses et al.(2000)19						
2	rs430253	104762930	$2{,}96 \times 10^{-4}$	rs403491	104762723	$3{,}67 \times 10^{-4}$
2	rs961303	124748687	$2{,}79 \times 10^{-4}$	rs430253	104762930	$3{,}71 \times 10^{-4}$
2	rs12994131	138135345	$7{,}26 \times 10^{-4}$	rs4954821	140609145	$3{,}17 \times 10^{-4}$
2	rs2163960	174024297	$3{,}07 \times 10^{-4}$	rs10192208	165826396	$3{,}58 \times 10^{-4}$
2	rs6739583	178261815	$5{,}05 \times 10^{-4}$	rs1990606	171208870	$4{,}09 \times 10^{-4}$
Region with suggestive linkage reported by Lachmeijer et al.(2001)18						
10	rs16921228	66751654	$2{,}55 \times 10^{-4}$	rs660465	78855117	$3{,}00 \times 10^{-5}$
10	rs10996467	66921953	$7{,}50 \times 10^{-5}$	rs584141	78860360	$3{,}00 \times 10^{-5}$
10	rs1544237	66953884	$1{,}59 \times 10^{-4}$	rs10509681	96788739	$1{,}90 \times 10^{-4}$
10	rs10822584	66958252	$1{,}48 \times 10^{-4}$	rs17385450	97880590	$4{,}90 \times 10^{-5}$
10	rs7071067	66962534	$1{,}48 \times 10^{-4}$	rs17476713	97884446	$4{,}90 \times 10^{-5}$
Region with suggestive linkage reported by Lachmeijer et al.(2001)18						
22	rs9917605	26062798	$1{,}18 \times 10^{-3}$	rs9613287	25607262	$5{,}59 \times 10^{-3}$
22	rs35196735	26066108	$3{,}55 \times 10^{-4}$	rs35196735	26066108	$3{,}69 \times 10^{-3}$
22	rs4822938	26494050	$1{,}63 \times 10^{-3}$	rs4821628	36052247	$2{,}06 \times 10^{-3}$
22	rs8135828	28259239	$2{,}30 \times 10^{-3}$	rs4821705	36482875	$3{,}90 \times 10^{-3}$
22	rs243243	32727453	$4{,}94 \times 10^{-4}$	rs139316	37829709	$4{,}03 \times 10^{-3}$
Region with significant linkage reported by Laivuori et al.(2003)20						
2	rs4669229	8018726	$8{,}58 \times 10^{-3}$	rs7593098	16581786	$1{,}31 \times 10^{-3}$
2	rs16858275	12155807	$8{,}19 \times 10^{-3}$	rs4666475	19357020	$8{,}14 \times 10^{-4}$
2	rs2278551	12164266	$6{,}43 \times 10^{-3}$	rs703317	19364082	$4{,}67 \times 10^{-4}$
2	rs12618369	13544455	$6{,}91 \times 10^{-3}$	rs1373781	19364386	$9{,}11 \times 10^{-4}$
2	rs41328347	19749078	$1{,}33 \times 10^{-3}$	rs1373780	19364510	$8{,}14 \times 10^{-4}$
Region with significant linkage reported by Laivuori et al.(2003)20						
9	rs2297694	27548918	$5{,}78 \times 10^{-3}$	rs866630	28769376	$1{,}13 \times 10^{-2}$
9	rs10812622	27610642	$2{,}59 \times 10^{-3}$	rs10758226	33749363	$8{,}89 \times 10^{-3}$
9	rs7019647	33883073	$1{,}99 \times 10^{-3}$	rs10758227	33752135	$1{,}01 \times 10^{-2}$
9	rs1543605	34030774	$2{,}94 \times 10^{-3}$	rs10738949	35826259	$3{,}17 \times 10^{-3}$
9	rs10738949	35826259	$5{,}49 \times 10^{-3}$	rs4394481	38446840	$9{,}87 \times 10^{-3}$

Table 3. Continued

		Preeclampsia		IUGR		
Chromosome	SNP	Position	nominal p value	SNP	Position	nominal P value
Region with suggestive linkage reported by Laivuori et al.(2003)20						
4	rs6842837	143040527	$8,15 \times 10^{-4}$	rs6818900	152285134	$1,62 \times 10^{-4}$
4	rs894510	143040785	$5,86 \times 10^{-4}$	rs7688165	168245326	$2,47 \times 10^{-3}$
4	rs17484678	149487596	$4,34 \times 10^{-4}$	rs10002479	168372854	$1,59 \times 10^{-3}$
4	rs17581884	149518521	$8,57 \times 10^{-4}$	rs999959	169934946	$1,92 \times 10^{-4}$
4	rs17525479	169089786	$2,95 \times 10^{-4}$	rs17544750	170409932	$1,75 \times 10^{-3}$

*regions based on -2 LOD score

mosome 2 within the EHBP1 gene region. Furthermore, we found 2 associated SNPs located within previous reported linkage regions on chromosome 2 and 10[17,18]. These SNPs were intergenic with PLEK and CTNNA3 as nearest genes in the region. Also 4 SNPs associated with IUGR with p values $<10^{-4}$ were located within a previously reported linkage region for preeclampsia on chromosome 10[18]. These SNPs were located within the KCNMA1 and ZNF518 gene.

To our knowledge this is the first genome wide association study on preeclampsia and IUGR. We found six SNPs associated within a distinct 100 kb region on chromosome 2 which may represent a new region harbouring susceptibility genes for preeclampsia. One SNP was located within EHBP1 gene and the others in the surrounding region. EH domain binding protein 1 is involved in insulin regulated endocytic trafficking (www.genecard.org). Women with previous preeclampsia and IUGR pregnancies are more often diagnosed with hypertension and insulin resistance than those with uncomplicated pregnancies[6,8,35], making the association of EHBP1 to preeclampsia of interest. In this light, the associations found for IUGR with two SNPs in the GLP1R gene on chromosome 6 are also of special interest. This gene, glucagon-like peptide 1 receptor, is described to play a role in several endocrine disorders including insulinomas, hyperinsulinemia and type 2 diabetes (www.genecard.org).

Earlier, several linkages studies on preeclampsia have been conducted[17-20]. Three studies out of 5 linkage studies found evidence for linkage of preeclampsia to chromosome 2[17, 19, 20], of which two showed overlapping regions[17,19]. In one of these previously reported regions, described by Arngrimmson et al[17], we found a SNP associated with a p value $6,1 \times 10^{-5}$ with preeclampsia, providing additional support for the involvement in preeclampsia. The SNP was intergenic and located nearest to the gene encoding pleckstrin. Pleckstrin is a major substrate for protein kinase C in blood platelets. Its phosphorylation triggers responses that ultimately lead to platelet activation and blood clot formation, but its exact function is not known (www.ncbi.nlm.nih.gov). The activation of pleckstrin has also been associated to the development of complications in diabetes including retinopathy, atherosclerosis and nephropathy[36,37], which are of interest with respect to preeclampsia because they are all vasculopathies.

Previously, suggestive linkage with chromosome 10 was reported by Lachmeijer et al[18] in Dutch women with prior preeclampsia. These patients all had early onset preeclampsia with fetal growth restriction suggesting that this region may also play a role in IUGR[38]. We found one SNP associated with preeclampsia within this region. This SNP was located nearest to the gene encoding the catenin, alpha 3. The protein has a general function in cell signalling (KEGG-04520). Remarkably, for IUGR we found 4 SNPs associated within this region (p values $<10^{-4}$) in the KCNMA1 gene and ZNF518 gene. These SNPs were not associated to preeclampsia ($p>0.1$). KCNMA1 codes for calcium-activated potassium channel subunit alpha-1, which is fundamental to the control of smooth muscle tone and neuronal excitability (www.genecard.org). Involvement in smooth muscle control may be of interest in light of defective placentation which is characterized by inadequate uterine artery remodelling. The process of successful maternal uterine vessel adaptation depends on a delicate interaction between trophoblast and maternal tissue including smooth muscle cells[39,40].

The finding of four SNPs associated with IUGR in a previously reported linkage region for preeclampsia is in favour of the hypothesis of a joint genetic aetiology of preeclampsia and IUGR. However, this was only observed for a subset of women with preeclampsia, i.e. those with early onset preeclampsia with fetal growth restriction. On the contrary, no overlap was observed in the SNPs we found associated with preeclampsia and IUGR which seems to contradict the hypothesis of a joint genetic aetiology.

A limitation of this study is the small sample size and the lack of a replication cohort. Given the multiple testing, the proportion of false positive findings will be considerable. Conversely, true associations may not be detected. Our findings therefore ask for replication in independent samples.

In summary, our genome wide association study did not yield genome wide statistical evidence for a new locus for preeclampsia or IUGR. However, we did find suggestive association which were supported by previous linkage studies, increasing the likelihood of a true positive finding. Our study suggests that a mutation in the PLEK region explains the previously described linkage of preeclampsia to chromosome 2[17] and a mutation in CTNNA3 to chromosome 10[18], while the KCNMA1 and ZNF518 may explain the linkage of early onset preeclampsia with fetal growth restriction to chromosome 10[18]. We identified 2 new genes of special interest, the first (EHBP1) being associated to preeclampsia and involved in insulin regulated endocytic trafficking and the second (GLP1R) being associated to IUGR and involved in the insulin pathway. The latter results require verification by replication in other cohorts.

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3

Preeclampsia and intrauterine growth restriction and remote cardiovascular disease

3.1

Shared constitutional risks for maternal vascular related pregnancy complications and future cardiovascular disease

ABSTRACT

Objective- Maternal predisposition to vascular and metabolic disease may underlie both vascular-related pregnancy complications such as preeclampsia and intrauterine growth restriction (IUGR) as well as future maternal cardiovascular disease. We aimed to substantiate this hypothesis with biochemical and anthropometrical evidence.

Methods- An intergenerational case-control study in an isolated Dutch population was conducted including 106 women after preeclampsia or IUGR (median follow up: 7.1 years) and their fathers (n=43) and mothers (n=64) as well as 106 controls after uncomplicated pregnancies with their fathers (n=51) and mothers (n=68). Cardiovascular risk profiles were assessed including fasting glucose, lipids, anthropometrics, blood pressure, intima media thickness and metabolic syndrome.

Results- We found significantly higher fasting glucose levels, larger waist circumferences and a 5-fold increased prevalence of hypertension in women with a history of preeclampsia as compared to controls ($p<0.001$). Likewise, their parents had higher glucose levels than control parents ($p<0.05$). Their mothers had larger waist circumferences and higher blood pressures ($p<0.05$). Also, women after pregnancies complicated by IUGR had higher glucose levels and increased prevalence of hypertension ($p<0.01$). Their fathers showed higher glucose levels as well ($p<0.05$). Mean carotid intima media thickness was increased in a subset of women after preeclampsia diagnosed with chronic hypertension as compared to those without hypertension ($p<0.01$). Metabolic syndrome was more prevalent both in women with a history of preeclampsia and their mothers ($p<0.05$).

Conclusions- We demonstrated intergenerational similarities in cardiovascular risk profiles between women after preeclampsia or IUGR and their parents. These findings suggest shared constitutional risks for vascular related pregnancy complications and future cardiovascular disease.

INTRODUCTION

Preeclampsia and intrauterine growth restriction (IUGR) are common vascular related pregnancy disorders. Preeclampsia is characterized by *de novo* hypertension and proteinuria and is a major cause of maternal and fetal morbidity and mortality worldwide [1]. It has been suggested that preeclampsia and IUGR share a common pathogenesis involving shallow trophoblast invasion with subsequent maternal endothelial cell dysfunction[2, 3], although this is questioned by others.[4]

Epidemiological studies suggest an association between pregnancies complicated by preeclampsia and IUGR and an increased risk of future cardiovascular disease[5-8]. Common risk factors such as obesity, hyperlipidemia, hypertension and insulin resistance are shared by both these pregnancy disorders as well as cardiovascular disease[9-12]. Moreover, a positive family history of cardiovascular disease in women with preeclampsia has been reported [13] . In addition, endothelial dysfunction provides a link between the pathogenesis of these pregnancy disorders and future cardiovascular disease in that it predisposes to both placental dysfunction and atherosclerosis[14].

The similarities in risk factors, familial predisposition and pathogenesis between preeclampsia, IUGR and cardiovascular disease have led to the hypothesis that it is maternal constitution, i.e. a predisposition to vascular and metabolic disease, that underlies both preeclampsia and IUGR as well as future cardiovascular disease,[15] rather than that preeclampsia or IUGR are cause of cardiovascular disease. The ability to identify young women, through pregnancy complications, at increased risk for future cardiovascular disease may enable unique programs of secondary prevention.

This study aims to provide biochemical and anthropometrical evidence to substantiate the hypothesis that cardiovascular risk factors are constitutional in women with a history of pregnancies complicated by preeclampsia or IUGR. For that purpose we chose a novel approach in that an intergenerational study was conducted, assessing cardiovascular risk profiles of women with a history of pregnancies complicated by preeclampsia, IUGR or uncomplicated pregnancies as well as of their parents. Assuming that constitution is at least partly genetically determined, we hypothesized that intergenerational similarities in risk profiles substantiate the constitutional origin of vascular risk factors in women with a history of pregnancies complicated by preeclampsia or IUGR.

METHODS

Population

The study was conducted in a genetically isolated population in the Southwest of the Netherlands and is part of a larger research program called Genetic Research in Isolated Populations (GRIP), which aims to identify genetic factors in the development of complex disorders[16]. All of the participants were of Caucasian origin.

Participants

Women with a history of preeclampsia or a pregnancy complicated by IUGR and their parents were selected from the GRIP population. Women with a history of pregnancies complicated by preeclampsia or IUGR will be referred to as the "index generation" and their parents as "mothers or fathers of the index generation". The scientific protocol of GRIP was approved by the Medical Ethics Committee of the University Medical Centre Rotterdam. All of the participants provided written informed consent. Preeclampsia was defined as *de novo* hypertension (systolic ≥ 140/ diastolic ≥ 90 mm Hg) and proteinuria ≥ 300 mg per 24 hours or at least 1+ on semi quantitative analysis[17]. The guidelines recommend 24-hour urinary collection, but when dipstick is the only test available, 1+ is accepted as it is associated with ≥ 300 mg/24h proteinuria. In our cohort only 4 women were diagnosed with preeclampsia by using dipstick analysis. For all remaining women 24-hour urine collection data were available. Superimposed preeclampsia was defined as new onset proteinuria after 20 weeks of gestation in women with chronic hypertension[17]. Preeclampsia was considered as "early-onset" when it was diagnosed before 34 weeks of gestation, and as "late-onset" when diagnosed after a gestational age of 34 weeks. IUGR was defined as birth weight of newborns equal to or below the 5th percentile, according to the Dutch fetal growth charts of Kloosterman[18]. If preeclampsia and IUGR co-occurred (n=8), women were categorized in the preeclampsia group. Women of the index generation, who gave birth to children with congenital anomalies, were excluded from the study group. Only singleton pregnancies were included. Women with a history of preeclampsia or IUGR were identified from National Birth Registration Records dating from 1983 up to 2004. The records reported 93 women with preeclamptic pregnancies and 104 with IUGR complicated pregnancies, living in this community at time of delivery (Figure 1). In 57 cases either the patients' identities were unknown or medical records could not be retrieved. Of the remaining 140 cases the diagnoses were confirmed by the research physician after reviewing the medical records. These women were invited to participate in the study by their general practitioner or obstetrician. A total of 106 women with a history of preeclampsia or IUGR (47 with preeclampsia, 3 with superimposed preeclampsia and 56 with IUGR) agreed on participation. Subsequently, these women were asked to invite their parents for participation. Thirty-five

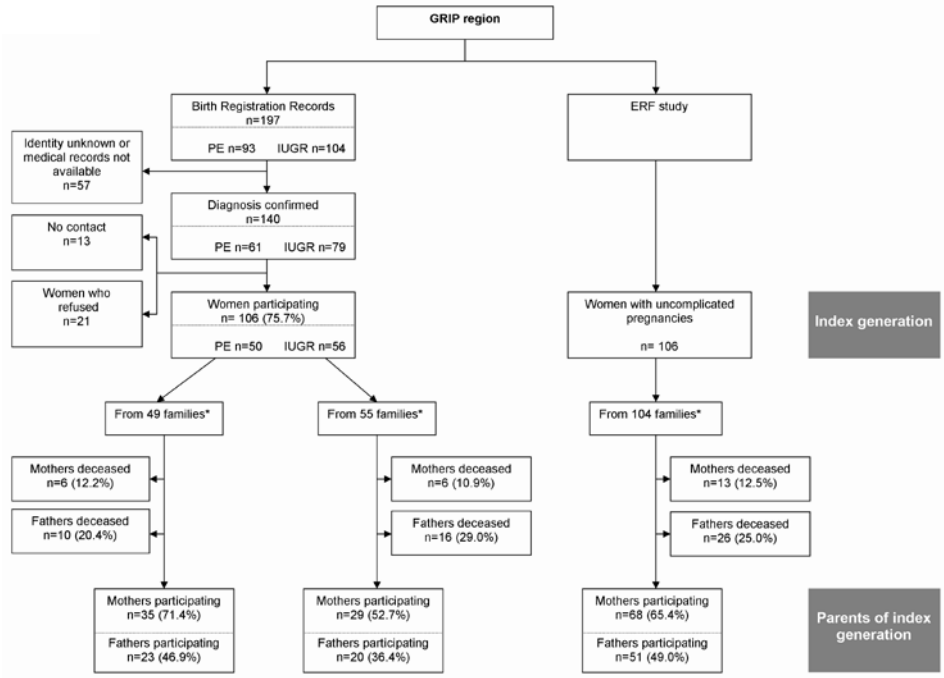

Figure 1. Flow diagram for recruitment of participants.
PE, preeclampsia
IUGR, intrauterine growth restriction
* Differences between number of families and number of participating women in index generation are explained by the fact that some of the women in the index generation are sisters.

mothers and 23 fathers of women with a history of preeclampsia and 29 mothers and 20 fathers of women with pregnancies complicated by IUGR agreed on participation. Parents refrained from participation because of various reasons; long travel distance, no contact with family, or bad health.

Controls and their parents were recruited from participants of the Erasmus Rucphen Family (ERF)[19] study, a study that is also embedded in the GRIP program. Women, who reported no history of hypertensive complications during pregnancy, were identified. Their obstetric records were obtained from the midwife's practice within the community and reviewed by the research physician. From all women with a history of uncomplicated pregnancies with term deliveries and children with normal birth weight, 106 controls (equal to the total number of women with a history of preeclampsia or IUGR) were randomly selected. Sixty-eight mothers and 51 fathers of controls participated in the study (Figure1).

Data collection

Participants were invited for examination at our research centre located within the community. Fasting blood samples were drawn for measurements of lipids and glucose levels according to a standardized procedure[20, 21].

All of the participants were interviewed about their medical history, medication use and life style. Participants were classified as non smokers or current smokers (≥ 1 cigarette per day). Alcohol consumption was defined as regular use of alcoholic drinks (≥ 1 U/ week).

Educational level was categorized into low (primary school/ lower vocational training), intermediate (secondary school/ intermediate vocational training) and high education (higher vocational training/ university). Blood pressure measurements and anthropometrics were limited to female participants.

Blood pressure was measured twice in the sitting position at the right upper arm using an automated device (OMRON 711, automatic IS). The mean of these two measurements was used in the analyses. Hypertension was defined as diastolic blood pressure ≥ 90 mmHg and/ or a systolic blood pressure ≥ 140 mmHg and/or use of anti-hypertensive medication (grades 1,2, and 3 of the World Health Organization criteria[22]) for the index generation and a diastolic blood pressure ≥ 100 mmHg and/ or a systolic blood pressure ≥ 160 mmHg and/or use of anti-hypertensive medication (grades 2 and 3 of the World Health Organization criteria[22]) for the mothers of the index generation. Height and weight were measured with the participant dressed in light underclothing. Waist circumference was measured on uncovered skin using a tape measure with the participant in upright position, halfway between the rib cage and the pelvic bone.

Intima-media thickness (IMT) as well as the prevalence of metabolic syndrome were assessed in the index generation and in mothers of the index generation. IMT of the common carotid artery was assessed by duplex scan ultrasonography using a 7.5 MHz linear-array transducer (ATL Ultra-Mark IV). IMT was measured offline from frozen images recorded on videotape[23] over an average distance of 10 mm of the common carotid arteries. The mean was calculated of the maximum IMT of the near and far wall measurements of both left and right arteries. In presence of a plaque at the 10 mm site, IMT was measured in the region adjacent to the plaque. The reproducibility of IMT measurements, performed in our department, has been described previously[24].

Metabolic syndrome was retrospectively defined according to the consensus statement from the International Diabetes Federation[25]. According to this definition, for a woman to be defined as having metabolic syndrome, she must have central obesity (waist circumference ≥80 cm) plus any two of four additional factors; raised triglycerides (≥1.7 mmol/L), reduced high-density lipoprotein (HDL) cholesterol (<1.29mmol/L or treatment for lipid abnormalities), raised blood pressure (systolic blood pressure ≥130 or diastolic blood pressure ≥85 mmHg or

treatment of previously diagnosed hypertension), or raised fasting glucose (≥5.6 mmol/L or previously diagnosed type 2 diabetes).

Population for analysis

For a description of baseline characteristics all of the subjects were included. For subsequent analyses on anthropometrics, lipids, glucose levels, blood pressure measurements and IMT the following exclusion criteria were applied. Pregnant women and postmenopausal women were excluded from the "index generation" in order to create a more homogenous subgroup of premenopausal women only. Analogously, we excluded premenopausal women from the "mothers of the index generation", resulting in a group of postmenopausal women only. Subjects with diabetes mellitus, or using lipid lowering- or antihypertensive medication were excluded from analyses on fasting glucose, lipids, and blood pressure, respectively. In the analyses on metabolic syndrome all of the (non-pregnant) participants were included. Measurement of one blood sample in the preeclampsia group in the index generation failed. IMT measurements were available for a limited number of subjects; 86 controls and 100 cases (n=48 for preeclampsia and n=52 for IUGR) in the index generation and for 107 mothers of the index generation (n=29 for preeclampsia, n=24 for IUGR and n=54 for controls).

Statistical analyses

Comparisons between groups were performed for women of the index generation, mothers and fathers separately. General characteristics were compared using T-test, $\chi 2$ statistics and Fisher's exact test where appropriate. Differences in cardiovascular risk factors were analyzed using univariate analysis of variance. Risk estimates were assessed by cross tabulations. For all statistical analyses we used SPSS for Windows, version 11.0.1.

Variables with skewed distribution were normalized using (natural) logarithm transformation. The analyses on anthropometrics were adjusted for age and time interval between delivery and pregnancy in the index generation and for age only in the parents of the index generation. Additional adjustments were made in the analyses on lipids and glucose levels for body mass index, smoking, use of anti hypertensive medication, educational level, and hormone replacement therapy (only in mothers of index generation). Similar adjustments, with exception of antihypertensive medication, were made in the analyses on blood pressure. Analyses on IMT measurements were adjusted for age and time interval between delivery and study.

RESULTS

General characteristics

General characteristics of cases and control subjects of the index generation and their parents are depicted in Table 1.

Cardiovascular risk factors

Index generation

Women with a history of preeclampsia had a significantly greater body mass index and larger waist circumference as compared with control subjects. After adjustment for body mass index, fasting glucose levels were significantly higher in both formerly preeclamptic women and women with pregnancies complicated by IUGR than in women with uncomplicated pregnancies. Similar levels of total serum cholesterol, low-density lipoprotein (LDL) choles-

Table 1. General characteristics of index generation and parents of index generation

Characteristic	Preeclampsia	IUGR	Controls
Index generation	(n=50)	(n=56)	(n=106)
Index pregnancy			
Age, y	29.2 (± 3.8)*	29.7 (± 3.6)*	26.2 (± 4.3)
Birth weight, g	2559 (± 886)*	2223 (± 547)*	3345 (± 379)
Gestational age, weeks	37 (± 3.4)*	38.6 (± 2.8) †	39.9 (± 1.4)
Early preeclampsia	16 (32)	NA	NA
Current study			
Age, y	36.2 (± 5.8) †	39 (± 5.3)	39.2 (± 5.6)
Time interval delivery- study, y	7 (± 5.6) *	9.3 (± 4.6) *	13.1 (± 5.7)
Educational level			
Low	19 (38)*	39 (69.6)	77 (72.6)
Intermediate	24 (48) †	11 (19.6)	27 (25.5)
High	7 (14) †	6 (10.7) ‡	2 (1.9)
Premenopausal status	48 (96)	52 (94.6)	101 (95.3)
	(n=47§)	(n=56)	(n=106)
Anti hypertensive drugs	9 (19.1)*	8 (14.3) †	1 (0.9)
Lipid lowering drugs	1 (2.1)	1 (1.8)	1 (0.9)
Diabetes mellitus	2 (4.3)	2 (3.6)	0
Current smoking	11 (22) †	31 (55.4)	52 (49.1)
Alcohol consumption	16 (32)	15 (26.8)	33 (31.1)

Mothers of index generation	(n=35)	(n=29)	(n=68)
Age, y	60.6 (±8.9)	61.9 (±6.4)	61.3 (±7.3)
Time interval delivery- study, y	35.2 (± 5.1)	36.8 (± 4.4)	36.9 (± 5.8)
Hypertensive pregnancy complication	13 (37.1) †	6 (20.7)	8 (11.8)
Educational level			
Low	32 (91.4)	27 (93.1)	64 (94.1)
Intermediate	3 (8.6)	2 (6.9)	4 (5.9)
High	0	0	0
Postmenopausal status	32 (91.4)	29 (100)	62 (91.2)
Hormone replacement therapy	2 (5.7)	5 (17.2)	6 (8.8)
Anti hypertensive drugs	15 (42.9)	14 (48.3)	21 (30.9)
Lipid lowering therapy	8 (22.9)	8 (27.6)	17 (25)
Diabetes mellitus	5 (14.3)	2 (6.9)	6 (8.8)
Current smoking	12 (34.3)	11 (37.9)	34 (50)
Alcohol consumption	17 (48.6) ‡	6 (20.7)	16 (23.5)
Fathers of index generation	(n=23)	(n=20)	(n=51)
Age, y	62.7 (±8.8)	63 (±5.0)	62.8 (±6.9)
Educational level			
Low	17 (73.9) †	14 (70) †	50 (98)
Intermediate	6 (26.1) †	6 (30) †	1 (2)
High	0	0	0
Anti hypertensive drugs	9 (39.1)	7 (35)	20 (39.2)
Lipid lowering therapy	4 (17.4)	6 (30)	13 (25.5)
Diabetes mellitus	2 (8.7)	0	5 (9.8)
Current smoking	5 (21.7)	7 (35)	19 (37.3)
Alcohol consumption	18 (78.3)	14 (70)	32 (62.7)

Data are expressed as mean (SD) or as absolute numbers (%).

Each comparison was performed between the case group (preeclampsia, IUGR) and the control group, using T-test, $\chi 2$ or Fisher's exact test.

NA, not applicable

* P value <0.001

† P value <0.01

‡ P value <0.05

§ Pregnant women (n=3) were excluded

terol, HDL-cholesterol and triglycerides were found in all of the groups. Elevated systolic and diastolic blood pressure was observed in women with a history of preeclampsia (Table 2). Diagnosis of hypertension was 5 times more common in formerly preeclamptic women compared with control subjects (46.7% versus 8.9%, p<0.001) and 3 times in women with IUGR complicated pregnancies (26.9% versus 8.9%, p<0.01). Intima media thickness of the common carotid artery was not significantly different between cases (median, interquartile range, for

Table 2. Cardiovascular risk factors of index generation and parents of index generation

Risk factor	**Preeclampsia**	**IUGR**	**Controls**
Index generation	(n=45*)	(n=52*)	(n=101*)
Body mass index, kg/m^2	27.2 (23.9-33.1)†	23.6 (21.6-25.9)	24.4 (21.8-27.5)
Waist circumference, cm	90 (76.8-103.8)†	79.9 (71.5-85)	77.6 (71.5-83)
	n=42‡	n=50‡	n=101‡
Fasting glucose, mmol/L	4.8 (4.4-5.2)†	4.7 (4.2-4.9)†	4.2 (3.8-4.5)
	n=43§	n=51§	n=100§
Cholesterol, mmol/L	4.8 (4.4-5.8)	5.3 (4.7-6.0)	5.4 (4.6-6.2)
LDL-cholesterol, mmol/L	3.0 (2.6-3.8)	3.6 (2.9-4.3)	3.6 (2.9-4.1)
HDL-cholesterol, mmol/L	1.3 (1.1-1.6)	1.3 (1.1-1.5)	1.3 (1.1-1.6)
Ratio cholesterol/HDL	3.8 (3.1-4.6)	4.2 (3.3-4.8)	3.9 (3.3-4.7)
Triglycerides, mmol/L	1.0 (0.7-1.6)	0.9 (0.7-1.3)	1.0 (0.7-1.3)
	n=36וו	n=44וו	n=100וו
Systolic blood pressure, mm Hg	126 (120-144)¶	121 (113-135)	121 (115-130)
Diastolic blood pressure, mm Hg	81 (72-89) #	76 (70-84)	75 (69-79)
Mothers of index generation	n=32**	n=29**	n=62**
Body mass index, kg/m^2	28.1(26.3-32.6)	27.8 (24.6-30.9)	28.2 (25.9-31.4)
Waist circumference, cm	96 (88.9-105.6) #	92 (81.9-102.8)	90.3 (82-97.3)
	n=27‡	n=27‡	n=56‡
Fasting glucose, mmol/L	5.2 (4.6-5.6) #	4.8 (4.4-5.1)	4.6 (4.2-5.0)
	n=25§	n=21§	n=45§
Cholesterol, mmol/L	5.9 (5.0-6.6)	6.0 (5.3-6.4)	6.4 (5.8-7.1)
LDL-cholesterol, mmol/L	3.8 (2.9-4.5)	4.0 (3.5-4.6)	4.4 (3.8-5.0)
HDL-cholesterol, mmol/L	1.3 (1.2-1.7)	1.3 (1.2-1.7)	1.4 (1.2-1.6)
Ratio cholesterol/HDL	4.4 (3.3-5.5)	4.5 (3.8-5.3)	4.6 (3.9-5.5)
Triglycerides, mmol/L	1.3 (1.0-1.9)	1.3 (1.1-1.6)	1.4 (1.0-1.9)
	n=17וו	n=15וו	n=43וו
Systolic blood pressure, mm Hg	152 (140-177)¶	139 (129-163)	140 (129-153)
Diastolic blood pressure, mm Hg	85 (77-93)	81 (74-85)	80 (74-86)
Fathers of index generation	n=23	n=20	n=51
Body mass index, kg/m^2	26.9(24.4-30.3)	26.2(24.5-28.2)	27.1(24.4-29.4)
	n=21‡	n=20‡	n=45‡
Fasting glucose, mmol/L	5.2 (5.0-5.7)¶	5.1(4.8-5.8)¶	4.8 (4.3-5.3)
	n=19§	n=14§	n=38§
Cholesterol, mmol/L	4.7(4.1-5.3) #	5.3 (4.3-5.8)	5.6 (5.1-6.2)
LDL-cholesterol, mmol/L	3.1(2.6-3.6) #	3.4 (2.8-3.8)	3.8 (3.4-4.5)
HDL-cholesterol, mmol/L	1.1(0.7-1.4)	1.1 (0.9-1.2)	1.0 (0.9-1.2)
Ratio cholesterol/HDL	4.3(3.2-5.3)	5.2 (3.9-6.0)	5.6 (4.5-6.5)
Triglycerides, mmol/L	1.2(0.8-1.6)	1.6 (1.0-1.9)	1.6 (1.1-2.4)

Data are presented as median (interquartile range).
Each comparison was performed between the case group (preeclampsia, IUGR) and the control group, using analysis of variance.
* Pregnant women and postmenopausal women were excluded.
† P value <0.001,¶ P value < 0.05 ,# P value <0.01
‡ Subjects with diabetes mellitus were excluded
§ Subjects using lipid lowering medication were excluded
ת Subjects using antihypertensive medication were excluded
**Premenopausal women were excluded

preeclampsia 0.66, 0.58-0.73 mm, for IUGR 0.65, 0.6-0.74 mm) and controls (0.68, 0.62-0.74 mm). However, after stratification for hypertension we found increased IMT in women with hypertension compared with women without the diagnosis of hypertension in the preeclampsia group (0.72, 0.6-0.78 mm versus 0.65, 0.58-0.69 mm, p<0.01) and IUGR group (0.72, 0.67-0.88 mm versus 0.64, 0.59-0.71 mm, p=0.08). No differences were found after stratification in the control group (0.7, 0.63-0.77 versus 0.68, 0.62-0.74, p=0.9).

Early versus late-onset preeclampsia

Diastolic blood pressure was higher in women with early-onset preeclampsia compared to those with late-onset disease (median (IQR), 88 (77-93) mm Hg for early-onset, 81 (72-88) mm Hg for late-onset, p=0.04). Diagnosis of hypertension (grades 1,2, and 3) was more common in the early-onset than late-onset group (66.7% for early-onset and 34.4% for late-onset, p=0.04). No significant differences were found in the remaining risk factors.

Mothers of index generation

Fasting glucose levels, waist circumferences and systolic blood pressures were significantly higher in mothers of preeclamptic women but not in mothers of women with pregnancies complicated by IUGR compared to control mothers (Table 2). Hypertension was more frequent in mothers of women with a history of preeclampsia, although not significantly (59.4% in mothers of preeclamptic women, 63% in mothers of IUGR women and 42.6% in control mothers, p=0.1 and p=0.08 respectively). IMT of the common carotid artery was not significantly different between mothers of case subjects (median, interquartile range, for preeclampsia 0.86, 0.78-1.1 mm, for IUGR 0.93, 0.88-1.0 mm) and mothers of control subjects (0.95, 0.85-1.0 mm). After stratification for hypertension, mothers with the diagnosis of hypertension in the preeclampsia (0.98, 0.8-1.1 mm versus 0.83, 0.76-0.94 mm, p=0.3), IUGR (0.94, 0.87-1.1 mm versus 0.92, 0.87-0.95 mm, p=0.5) as well as control group (0.99, 0.94-1.2 mm versus 0.9, 0.8-0.99 mm, p=0.2) tend to have increased IMT compared to mothers without hypertension, although not significant. Exclusion of mothers, who reported an obstetric history with preeclampsia or pregnancy induced hypertension (n=13 in the group of mothers of preeclampsia

women, n=5 in the mothers of IUGR women and n=8 in the control mothers), did not significantly change the results.

Fathers of index generation

Fasting glucose levels were significantly higher in fathers of formerly preeclamptic women and women with pregnancies complicated by IUGR compared with control subjects. Fathers of preeclamptic women showed lower concentrations of cholesterol and LDL-cholesterol compared to fathers of women with pregnancies complicated by IUGR and control subjects (Table 2).

Metabolic syndrome

The prevalence of metabolic syndrome in the index generation was 3 fold higher in women with a history of preeclampsia compared with control subjects (Figure 2). No significant difference in prevalence of metabolic syndrome was found between women with IUGR and control subjects. A similar pattern was observed with regard to comparisons between mothers (Figure 2). Mothers of women with a history of preeclampsia had a 1.6-increased risk (RR, 95% CI 1.1-2.2) of having metabolic syndrome as estimated by the relative risk. For mothers of women with pregnancies complicated by IUGR this risk was also increased but the association was not significant (RR 1.5, CI 95% 1.0-2.2).

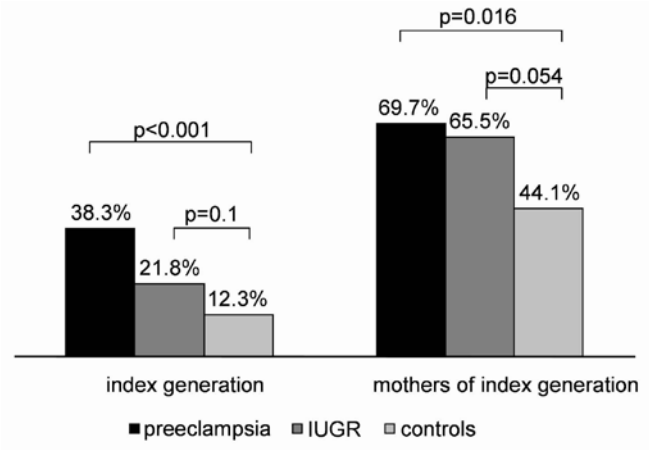

Figure 2. Prevalence of metabolic syndrome in index generation and mothers of index generation. All participants were included except for three pregnant women in the preeclampsia group.

DISCUSSION

In the present study, women with pregnancies complicated by preeclampsia or IUGR and their mothers have unfavourable cardiovascular risk profiles, marked by higher glucose levels, prevalence of hypertension and larger waist circumferences, compared with women with uncomplicated pregnancies and their mothers. Our findings suggest shared constitutional risks for vascular related pregnancy complications and future cardiovascular disease.

This study is the first that we are aware of that assessed biochemical and anthropometrical risk factors of cardiovascular disease, not solely in women with a history of preeclampsia and IUGR but also in their parents. We hypothesized that intergenerational similarities in risk profiles substantiate the hypothesis that a predisposition to cardiovascular disease underlies preeclampsia and IUGR.

The most marked similarity in risk profiles of women with a history of preeclampsia and their mothers and fathers was the finding of higher fasting glucose levels. These findings could not be explained by differences in body mass index. These results are in line with the results of studies describing an association with hyperinsulinemia and higher HbA1C levels in women 15-25 years after preeclampsia [26, 27]. The higher glucose levels, although still in the reference range, are predictive for an increased risk of cardiovascular disease and type 2 diabetes[28]. Women with a history of preeclampsia had previously been suggested to be at increased risk of developing type 2 diabetes[29]. Also in women with IUGR complicated pregnancies and their fathers we found higher fasting glucose levels. This is in accordance with a previous cross-sectional study showing an association between insulin resistance in women at older age and low-birth-weight offspring[30].

Intergenerational similarities were also found for hypertension. Previous preeclamptic women, at relative young age, as well as their mothers have a higher prevalence of chronic hypertension, with hypertension being even more prevalent after early-onset than late-onset preeclampsia. Similarly, women with a history of a pregnancy complicated by IUGR and their mothers were diagnosed with chronic hypertension more often than control subjects, but less frequent than previously preeclamptic women. These findings correspond with previous studies[27, 31] and are consistent with the observation that preeclamptic women more frequently have a family history of hypertension[10, 13]. Carotid IMT, which is used as a marker of subclinical atherosclerosis[23, 32], was increased in this specific subset of women who were diagnosed with chronic hypertension, as compared to those without hypertension.

In addition, we found that women with a history of preeclampsia have larger waist circumferences as well as their mothers, when compared to control subjects. Likewise, women with pregnancies complicated by IUGR as well as their mothers tend to have larger waist circumferences than controls, although not significantly. This points at an unfavourable, that is abdominal, fat distribution in women with preeclampsia which is known to be strongly associated with the metabolic syndrome[25] and thus, with increased risk of future cardiovascular disease.

Indeed, metabolic syndrome was more prevalent in women with a history of preeclampsia compared to control subjects. Consistent with studies describing a familial history of cardiovascular disease in women with preeclampsia[13], we found that metabolic syndrome was also more prevalent in mothers of women with a history of preeclampsia.

Hyperglycaemia, hypertension and (abdominal) obesity[14, 33] are associated with impaired endothelial function. Therefore, our data fit the hypothesis of Ness and Sibai[2] in which they propose that both women with preeclampsia and IUGR complicated pregnancies enter pregnancy with some degree of endothelial dysfunction predisposing to poor placentation. Abnormal placentation occurring in women with concurrent metabolic syndrome would result in preeclampsia whereas in absence of metabolic syndrome IUGR would develop. Accordingly, we found that metabolic syndrome, as compared with control subjects, was more prevalent in women with a history of preeclampsia.

The strength of this study is the intergenerational study design that provides the opportunity to explore the constitutional origin of the risk factors. We consciously use the term "constitutional" to indicate that (subclinical) risk factors were likely to be present in women before pregnancy without making any assumptions on their genetic and/ or environmental origin. The similarities in risk profiles, particularly for fasting glucose levels, are suggestive for familial aggregation of these traits in families with preeclampsia and IUGR. Previous studies demonstrated genetic influence for familial clustering of features of the metabolic syndrome[34, 35] . Whether familial aggregation, in our study is due to shared environmental and/ or genetic factors cannot be concluded. Nonetheless, our data suggest a potential role for the glucose metabolism in the pathogenesis of both pregnancy disorders. Another interpretation of our data could be that higher levels of fasting glucose are one of the first features marking an unfavourable metabolic and/ or vascular phenotype, as described by Haffner et al.[36] who found in a prospective study that elevations of insulin concentrations precede numerous metabolic disorders.

A limitation of this study is the low response rate of the parents, particularly of women with a history of IUGR. However, we have no strong evidence for a selection bias as the prevalence of hypertension in participating and non-participating parents, based on statements of women in the index generation about their parents' health, was not significantly different. Yet, since these comparisons were based on small numbers, selection bias cannot be definitively excluded.

Aside from the aetiological implications, our study has important clinical implications. More than 40%, and almost 30% of women in our study with pregnancies complicated by preeclampsia or IUGR, within a decade after the index pregnancy, are diagnosed with chronic hypertension. For metabolic syndrome these numbers are nearly 40% and 20%, respectively. Identification and follow up of these women in clinical practice is of major importance as long-term consequences may be reduced or avoided by preventive strategies. Especially, the subset of women with hypertension should be recognized and followed up on as we demon-

strated that these women, despite their relative young age, already have subclinical atherosclerosis.

PERSPECTIVES

Women with pregnancies complicated by preeclampsia or IUGR have a constitutionally determined, unfavourable vascular and metabolic profile. Given the high prevalence of cardiovascular disease it is essential that clinicians consider preeclampsia or pregnancies complicated by IUGR as novel, distinct risk indicators of cardiovascular disease.

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3.2

Severe, very early onset preeclampsia: subsequent pregnancies and future parental cardiovascular health

ABSTRACT

Objective- To study subsequent pregnancy outcome in women with previous severe, very early onset preeclampsia (onset before 24 weeks' gestation) and to analyze cardiovascular risk profiles of these women and their partners.

Methods- Twenty women with preeclampsia with an onset before 24 weeks' gestation, admitted between 1 January 1993 and 31 December 2002 at a tertiary university referral centre, were enrolled in the study. Data on subsequent pregnancies were obtained from medical records. Their cardiovascular risk profiles and those of their partners (n=15) were compared with those of 20 control women after uncomplicated pregnancies only, matched for age and parity, and those of their partners (n=13). Body weight, height, waist and hip circumference, blood pressure and intima media thickness (IMT) of the common carotid artery were measured. Fasted blood samples were drawn for detection of metabolic cardiovascular risk factors.

Results- Of the 20 case women 17 women had 24 subsequent pregnancies, of which 12 (50%) were complicated by preeclampsia. Severe preeclampsia developed in 5 (21%) pregnancies. No perinatal deaths occurred. Case women had significantly more often chronic hypertension as compared to controls (55% vs. 10%, p=0.002). IMT of the common carotid artery was increased in a subset of case women using antihypertensive medication (p=0.03). Case women showed increased micro-albuminuria ($p<0.05$). No differences were found in cardiovascular risk profiles between partners of cases and controls.

Conclusions- Women with severe, very early onset preeclampsia have an increased risk of preeclampsia in future pregnancies, yet neonatal outcome is, in general, favourable. Regarding cardiovascular health, women after severe, very early onset preeclampsia exhibit more risk factors compared to controls whereas men who fathered these pregnancies do not.

INTRODUCTION

Severe, very early onset preeclampsia before 24 weeks' gestation is a rare complication in pregnancy. Small series have described high maternal morbidity and very poor perinatal outcome[1,2]. However, we could locate no reports on the outcome of subsequent pregnancies and future health parameters of these women with very early onset preeclampsia. Women with preeclampsia in general, especially complicated by HELLP syndrome, are at increased risk for all forms of hypertension related complications in subsequent pregnancies [3-6].

In addition to offering advice about family planning, these women may have to be counselled in the future regarding their long-term cardiovascular prognosis. Several epidemiological studies have suggested an association of hypertensive disorders in pregnancy and increased risk of cardiovascular disease later in life[7]. These studies suggest a more pronounced effect in women with preeclampsia, premature delivery <37 weeks' gestational age and low birth weight offspring[7-9]. It is hypothesized that common risk factors predispose to both hypertensive pregnancy disorders as well as cardiovascular disease[10,11]. Knowledge of the pathophysiological determinants involved is indispensable when it comes to patient counselling and future preventive strategies in order to reduce the risk of cardiovascular disease in this specific group of women.

Genetic factors that increase the risk of cardiovascular disease may also be involved in the development of preeclampsia. Such genes may also be paternally derived, since it has been suggested that paternal genes, as expressed in the placenta and fetus, contribute to the risk of preeclampsia[12-14]. Subsequently, it can be hypothesized that men who fathered preeclamptic pregnancies have an increased risk of cardiovascular disease themselves.

In the present study we investigated the recurrence risks of hypertension related pregnancy complications and the cardiovascular risk profiles of women, as well as of their partners, who had experienced a pregnancy complicated by severe, very early onset preeclampsia before 24 weeks' gestation, in a case-control study design.

MATERIALS AND METHODS

Participants

All consecutive women (n=26) who had been admitted to the University Medical Centre Rotterdam between 1993 and 2003, with the diagnosis severe, early onset preeclampsia before 24 weeks' gestation were selected for participation in the study. Maternal and perinatal outcome data of this cohort have been published previously[1]. Severe preeclampsia was defined as an absolute diastolic blood pressure of ≥110 mm Hg and proteinuria (≥ 2+ [1 g/l]) on a catheterized specimen on admission, or the occurrence of preeclampsia (blood pressure ≥ 140 mmHg

systolic or ≥ 90 mmHg diastolic measured on at least two occasions in women normotensive before 20 weeks gestation and proteinuria ≥ 300 mg/24h (or ≥ 2+ on dipstick of voided specimen) in combination with eclampsia or HELLP syndrome. HELLP (hemolysis, elevated liver enzymes, and low platelets) was defined as thrombocytes <100x10^9/l, and both ASAT (aspartate aminotransferase) and ALAT (alanine aminotransferase) >70 U/l and lactate dehydrogenase > 600 U/L. Superimposed preeclampsia was defined as a rise of blood pressure ≥30 mm Hg systolic or ≥15 mm Hg diastolic over values in the first 20 weeks and proteinuria ≥300 mg/24 h (or ≥2+ [1 g/l] on a voided specimen or ≥1+ [0.3 g/l] on a catheterized specimen. Gestational hypertension was defined as a blood pressure of >140/90 mmHg after 20 weeks' gestational age in formerly normotensive women, measured on two separate occasions with an interval of at least 4 hours. Twenty women (80%) consented to participation. There was 1 maternal death. Four women refrained from participation because of psycho-emotional distress due to traumatic memories of their hospital admission and poor perinatal outcomes, as all experienced intrauterine fetal death. One patient declined participation because of language difficulties. These five women did not differ from the participating women with regard to parity, presence of chronic hypertension or neonatal survival. There was no difference in occurrence of severe complications (HELLP syndrome, eclampsia, pulmonary oedema) between the refraining and participating group (5/5 versus 18/20). The characteristics of the 20 participating women are described in Table 1. After consent of the woman, partners were approached for participation. Fifteen men (75%) consented to participation, four men declined and one man had died due to a violence offence. All participants provided informed written consent. The study was approved by the Medical Ethics Committee of the University Medical Centre Rotterdam.

Healthy control patients after uncomplicated term pregnancies only, with healthy, appropriate for gestational age weight babies, matched for age, parity, race and year of delivery were selected by the computerized hospital database. Two control patients declined participation and 2 did not respond to our mailing. Each participating case was matched with one control patient (n=20). Thirteen partners (65%) of these control patients consented participation, of which one declined blood sampling.

Data collection

All participants were invited for examination at our hospital. Information on medical and obstetrical history, medication use and smoking habits was obtained by means of interviews by the research physician. Any statement of hypertensive complications of subsequent pregnancies were confirmed by review of their medical records. Participants were classified as non- or current smokers. Current smoking was defined as smoking more than 1 cigarette daily. Family history of hypertension and diabetes was defined by the use of antihypertensive drugs and/or the use of blood glucose lowering drugs by first or second degree relatives.

Table 1. General characteristics of women with a history of severe, early onset preeclampsia and men who fathered these preeclamptic pregnancies compared to controls

	Women		**Men**	
	Preeclampsia (n=20)	Controls (n=20)	Preeclampsia (n=15)	Controls (n=13)
Index pregnancy				
Age, years	32.2 (18.1-40.6)	31.6 (18.8-40.1)	33.5 (22.1-53.2)	34.5 (25.8-50)
Caucasian	12 (60)	12 (60)	10 (66.7)	8 (61.5)
Primiparous	12 (60)	12 (60)	NA	NA
Gestational age, weeks	26 (22-29)*	40 (36-42)	NA	NA
Birth weight, grams	561 (300-775)*	3435 (2303-5200)	NA	NA
HELLP syndrome	10 (50)*	0	NA	NA
Eclampsia	4 (20)	0	NA	NA
Live infant**	4 (18)*	22 (100)	NA	NA
Hypertension†	7 (35)‡	0	NA	NA
Current study				
Age, years	38.8 (22.1-47.7)	37.7 (23.8-41.9)	39.9 (26.2-60.4)	38.8 (31.6-57.8)
Time since index pregnancy, years	5.5 (4-10)	5.9 (4.4-10.9)	NA	NA
Parity	3 (1-4)	2 (1-4)	NA	NA
Anti-hypertensive drugs	7 (35)†	0	1 (6.7)	0
Lipid-lowering drugs	0	0	2 (13.3)	0
Diabetes mellitus	0	0	1 (6.7)	0
Current smoking	2 (10)	3(15)	8 (53.3)	5 (38.5)
Family history				
Family hypertension	15 (75)	12(60)	5 (33.3)	7 (53.8)
Family diabetes	13 (65)	7 (35)	5 (33.3)§	10 (76.9)
Family preeclampsia	3 (15)	2 (10)	1 (6.7)	0

Data are presented as medians (range) or absolute number (%), NA, not applicable

† Hypertension defined as systolic blood pressure ≥140 mm Hg and/or diastolic ≥90 mmHg in the first trimester of pregnancy

* p value <0.001, ‡ p value <0.01, § p value <0.05

** two twin pregnancies included

Subsequently, cardiovascular health was described in cases and controls by means of the following variables. Body mass index (kg/m2) was calculated from height and weight. Waist and hip circumference were measured using a tape measure with the participant in upright position. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. Waist-to-hip ratio was calculated from these measurements. Blood pressure was measured twice with the participant in the sitting position at the right upper arm using a sphygmanometer. The mean of these two measurements was used in the analyses. Hypertension was defined as a systolic blood pressure ≥ 140 mm Hg and/ or a diastolic blood pressure ≥ 90 mmHg and/or

use of anti-hypertensive medication. Intima media thickness (IMT) of the common carotid artery was assessed by duplex scan ultrasonography using a 12-5 MHz linear-array transducer (Philips iU22). IMT was measured offline from frozen images over an average distance of 10 mm of the common carotid arteries[15]. The mean was calculated of the maximum IMT of the near and far wall measurements of both left and right arteries. In presence of a plaque at the 10 mm site, IMT was measured in the region adjacent to the plaque.

Fasting blood samples were drawn for measurements of biochemical parameters. Routine biochemical parameters were performed on a Hitachi 917 chemistry analyzer (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, uric acid, glucose, albumin, and urinary micro-albumin (Roche Diagnostics)). Apolipoprotein A, apolipoprotein B and lipoprotein(a) were measured by immunonephelometry on an Immage 800 nephelometer analyzer. HbA1c was measured using a colorimeter (Menarini HA-8160). Liquid chromatography mass spectrometry was used for measurement of homocystein and flowcytometry (HST 302) was used for measurement of leucocytes.

Statistical analyses

Continuous variables are expressed as medians with ranges. General characteristics were compared between groups using T-test, $\chi 2$ statistics and Fisher's exact test where appropriate. Differences in cardiovascular risk parameters were analyzed using analysis of variance. Variables with skewed distribution were normalized using (natural) logarithm transformation. All analyses on cardiovascular risk factors were adjusted for smoking and body mass index except for anthropometric variables, which were solely adjusted for smoking. A p-value <0.05 was considered statistically significant. For all statistical analyses we used SPSS for Windows, version 11.0.1.

RESULTS

General characteristics of participants are depicted in Table 1. Seven (35%) women had a history of severe, early onset superimposed-preeclampsia since they exhibited hypertension in the first trimester of the index pregnancy, but none of them had been treated with medication prior to pregnancy. At time of study, 5.5 years (range 4 to 10 years) after index pregnancy, five of those seven women used antihypertensive medication. Overall, women with a history of preeclampsia used significantly more often antihypertensive medication as compared to controls ($p<0.01$).

Men who fathered preeclamptic pregnancies did not differ significantly from control men with respect to paternal age and medical history. Diabetes mellitus was significantly more

common in families of men who fathered uncomplicated pregnancies as compared to men who fathered preeclamptic pregnancies ($p<0.05$).

Subsequent pregnancies

Of the 20 women with severe, early onset preeclampsia in their index pregnancies 17 women had in total 24 subsequent pregnancies (17 first subsequent and 7 second subsequent pregnancies). The median birth weight (range) was 2950 (720-3860) grams and gestational age 38 (27-41) weeks. This was significantly different from the median birth weight of 3500 (2960-3980) grams and gestational age of 40 (38-42) weeks in the 15 subsequent pregnancies of the control women ($p=0.001$ and $p=0.01$, respectively). There were no perinatal deaths in any of the 39 subsequent pregnancies. No hypertensive complications or fetal growth restriction occurred in the control group. Figure 1 summarizes the outcome of subsequent pregnancies of women with severe early onset preeclampsia in the index pregnancy. In 92% of the subsequent pregnancies some form of hypertensive complication occurred. Preeclampsia developed in 12 of the 24 subsequent pregnancies (50%), of which 5 developed severe preeclampsia (3 patients with HELLP syndrome and 2 women with uncontrolled severe hypertension). Recurrence of preeclampsia before 36 weeks' gestation was found in 5 pregnancies. Ten pregnancies (42%) were complicated by gestational hypertension. Only 2 (8%) pregnancies were not complicated by hypertensive disease, although two mildly growth retarded fetuses were born with birth weights between the 5th and 10th percentile. The 10 women who had HELLP syndrome in their index pregnancy had 11 subsequent pregnancies of which 3 (27%) were complicated by recurrence of HELLP syndrome.

Next, we compared women with recurrent and non-recurrent preeclampsia with regard to various parameters of the index pregnancy (HELPP syndrome, eclampsia, gestational age and

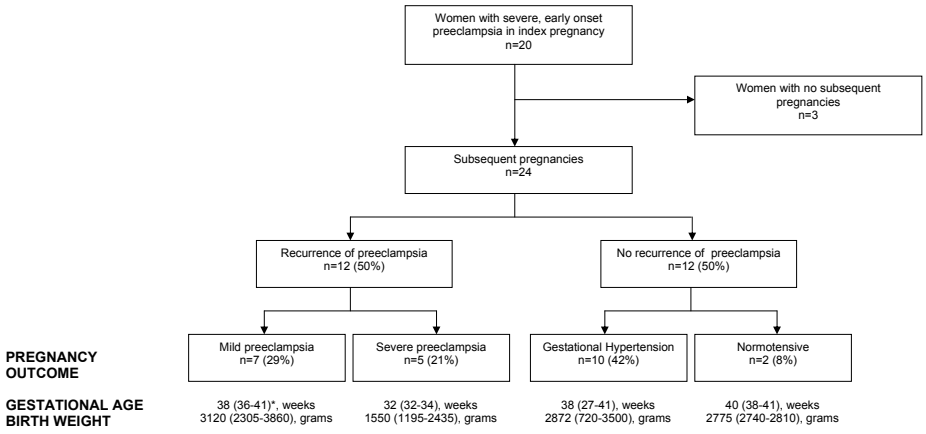

Figure 1. Outcome of subsequent pregnancies after severe, early onset preeclampsia in index pregnancy

birth weight) and their family history of hypertensive disease, yet no significant differences were found.

Cardiovascular health

Cardiovascular risk variables of participants are depicted in Table 2. Anthropometric parameters (body mass index and waist-to-hip ratio) were not significantly different between women with a history of preeclampsia as compared to controls, although there was a tendency to greater body mass index in women with a history of preeclampsia. Regarding the vascular variables, women with a history of preeclampsia were found to have higher diastolic blood pressures than controls. Although the median value for diastolic blood pressure did not pass the threshold of 90 mmHg, which was used in the definition of hypertension, women with preeclampsia were more often diagnosed with chronic hypertension. This could be explained by the fact that diagnosis of chronic hypertension was not solely based on blood pressure measurements but also on the use of antihypertensive medication. No differences were observed in IMT of the common carotid artery between groups. However, when we included only women with a history of preeclampsia using antihypertensive medication, we found that women with a history of preeclampsia with medication had significantly increased IMT (0.71, 0.58-0.80 mm, p=0.03) as compared to controls (0.59, 0.52-0.70 mm). With respect to metabolic variables associated with insulin insensitivity, no significant differences were observed between groups. Fasting lipid concentrations did not differ between cases and controls. Lipoprotein(a) which was not significantly different (p=0.05) in women with previous preeclampsia as compared to controls. Microalbuminuria was significantly higher in women with a history of preeclampsia ($p<0.05$).

Subsequently, we compared the cardiovascular risk parameters between women who had recurrent preeclamptic pregnancies and women with only one preeclamptic pregnancy. No significant differences were observed between the groups. None of the above mentioned variables were significantly different between men who fathered preeclamptic pregnancies as compared to control men (Table 2).

DISCUSSION

Subsequent pregnancies

We found a 50% recurrence rate of preeclampsia in women with a history of severe, early onset preeclampsia before 24 weeks' gestation. Neonatal survival was 100% in contrast to 18% in the index pregnancy consistent with higher gestational age (on average + 12 weeks) and higher birth weight (on average + 2400 grams) compared to the index pregnancy.

Table 2. Cardiovascular risk variables of women with a history of severe, early onset preeclampsia and men who fathered these preeclamptic pregnancies compared to controls

	Women			**Men**		
	Preeclampsia (n=20)	Controls (n=20)	P	Preeclampsia (n=15)	Controls (n=13*)	P
Body mass index, kg/m^2	25.7 (20.2-37.1)	22.7 (17.8-43.1)	0.2	25.4 (18.9-32.2)	25.3 (20.8-29.7)	0.5
Waist-hip ratio	0.80 (0.71-0.90)	0.78 (0.7-0.9)	0.3	0.86 (0.72-0.98)	0.84 (0.8-0.94)	0.7
Systolic blood pressure, mmHg	130 (95-155)	115 (100-170)	0.1	120 (105-170)	120 (105-150)	>0.9
Diastolic blood pressure, mmHg	83 (65-110)	75 (55-110)	0.02	75 (55-90)	80 (65-105)	0.3
IMT common carotid artery, mm	0.6 (0.46-0.92)	0.59(0.46-0.85)	0.4	0.59 (0.52-0.87)	0.64 (0.52-0.88)	0.5
Hypertension	11 (55)	2 (10)	0.002	1 (6.7)	4 (30.8)	0.2
HbA1c, %	5.2 (4.4-5.8)	4.9 (4.4-5.7)	0.4	5.0 (4.7-6.4) †	5.1 (4.4-5.6)	0.8
Fasting glucose, mmol/L	3.7 (2.5-5.6)	4.0 (2.9-4.8)	0.3	3.8 (2.7-5.1) †	4.0 (3.4- 4.7)	0.7
Insulin, mmol/L	37 (14-121)	32 (14-120)	>0.9	29 (14-111) †	31 (14-92)	>0.9
HOMA	159 (50-296)	179 (52-312)	>0.9	210 (49-316) †	193 (62-286)	0.8
Cholesterol, mmol/L	5.0 (4.1-7.1)	4.9 (3.1-5.9)	0.08	4.8 (3.8-6.2)	5.5 (4.3-6.0)	0.08
LDL-cholesterol, mmol/L	2.9 (2.1-5.2)	2.7 (1.5-4.1)	0.09	3.1 (1.3-4.3)	3.4 (2.0-4.5)	0.1
HDL-cholesterol, mmol/L	1.6 (1.3-2.9)	1.6 (1.1-2.9)	0.6	1.3 (0.84-1.8)	1.3 (0.98-2.3)	0.4
Triglycerides, mmol/L	0.86 (0.47-1.7)	0.76 (0.44-6.1)	0.8	1.1 (0.35-11.2)	0.88 (0.71-1.8)	0.4
ApoA, g/L	1.6 (1.3-2.6)	1.5 (1.2-2.8)	0.2	1.4 (1.1-1.6)	1.5 (1.1-2.0)	0.1
ApoB, g/L	0.85 (0.65-1.5)	0.88 (0.46-1.4)	0.1	0.96 (0.7-1.3)	1.0 (0.7- 1.4)	0.2
Lipoprotein (a), g /L	0.27 (0.02-1.2)	0.08 (0.02-1.1)	0.05	0.16 (0.02-0.77)	0.15 (0.02-0.59)	0.9
Leucocytes, x10^9/L	5.7 (1.6-10)	6.3 (3.4-11.1)	0.3	6.1 (3.6-17.9)	5.2 (4.4-7.3)	0.4
CRP, mg/L	2.0 (1.0-11.0)	1.5 (1.0-11.0)	0.3	1.0 (1.0-4.0)	1.0 (1.0-4.0)	0.9
Homocysteine, µmol/L	9.4 (6.3-21.4)	9.8 (6.3-15.4)	>0.9	12.9 (8.8-17.3)	12.7 (8.6-20.2)	0.9
Micro-albuminuria, g/mol	0.008 (0.001-0.2)	0.006 (0.002-0.01)	<0.05	0.005 (0.003-0.03)	0.004 (0.002-0.04)	0.8
Uric acid, mmol/L	0.28 (0.17-0.37)	0.26 (0.13-0.37)	0.2	0.34 (0.24-0.48)	0.37 (0.27-0.45)	0.4

Values are presented as medians (range) or as absolute number (%)

* n=12 for parameters obtained from blood samples

† n=14, one participant with type 1 diabetes was excluded

The described recurrence rate of preeclampsia of 50% is similar to that described by Sibai et al.[3] after second trimester severe preeclampsia. Regarding the recurrence of HELLP syn-

drome we observed a recurrence rate of 27%, whereas previous reports found different rates of 2-5%[16-18]. Sullivan et al [19] found a recurrence rate of 19%, however, gestational age at disease was not studied in relation with risks of recurrence. Differences in recurrence rates might be explained by differences in study population and sample size. In addition, management of the disorder may have been different; women in our study with recurrent preeclampsia at < 32 weeks' gestation were treated with intention to extend their gestational age. The higher incidence of HELLP syndrome may therefore be explained by a longer time interval between diagnosis of preeclampsia and delivery. We were not able to predict which women would experience recurrence of preeclampsia as neither clinical nor biochemical parameters were different between women who experienced recurrence and those who did not.

In view of counselling patients after severe, very early onset preeclampsia these findings should motivate obstetricians to encourage patients to opt for another pregnancy.

Cardiovascular health

The present study was conducted in view of the expectation that women with severe, very early onset disease in co-occurrence with low birth weight off-spring and preterm delivery would exhibit more risk factors or more pronounced cardiovascular risk profiles compared to controls with uncomplicated obstetric outcome[7-9]. We are aware that the small sample size is a limitation of our study, as it is therefore underpowered to detect small differences between groups. It could, however, be calculated that our study had sufficient power (80%) to detect larger differences, for example differences in blood pressure > 7mm Hg or glucose concentrations >0.3 mmol/L.

In the present study women with such history had more often chronic hypertension and increased microalbuminuria but no differences in features of the metabolic syndrome. These findings are interesting for speculations about differences in the origin of early and late onset preeclampsia. Increased prevalence of chronic hypertension (about 20% in most studies) is the common finding of merely all reports evaluating women with a past history of preeclampsia, even though time intervals between pregnancy and study varied from 3-25 years in the different studies[20-23]. Lipoprotein(a) levels were not significantly different between women with a history of severe, early onset preeclampsia and controls in our study. However our data might suggest a trend towards higher lipoprotein(a) levels in these women since the p-value was 0.05. The one other study, we are aware of, that focussed on lipoprotein(a) levels in severe, early preeclampsia reported higher levels in women with preeclampsia as compared to controls[24]. In contrast, Leerink et al. could not detect any differences in lipoprotein(a) concentrations in a population with both early and late onset disease[25]. Possibly higher lipoprotein(a) levels are more specifically associated with early onset disease. With respect to microalbuminuria, increased levels were also reported in previous studies in women with a past history of preeclampsia as compared to women with uncomplicated pregnancies[26,27]. Hypertension[28,29],

lipoprotein(a)[30] and microalbuminuria[31,32] are well established as independent risk factors of atherosclerotic disease. Microalbuminuria is a particularly strong predictor of ischemic heart disease among subjects with hypertension[32]. In addition, lipoprotein(a) levels are reported to be higher in subjects with renal dysfunction[33]. Therefore, our findings may be a reflection of underlying, early onset atherosclerotic disease in women with a history of severe very early onset preeclampsia. Remarkably, in contrast to previous studies with heterogeneous groups of preeclamptic women,[21, 22, 34] no indication for insulin insensitivity was found. This may be explained either by the fact that not all studies controlled for body mass index[21] or by differences in time interval between pregnancy and study[20]. Another explanation could be that metabolic disturbances associated with insulin insensitivity are a more specific feature of late-onset preeclampsia whereas vascular pathology is more specific for early onset preeclampsia, suggesting different pathogeneses. Recent data on HOMA-IR values in the first trimester of pregnancy, showing significantly higher values in late-onset preeclampsia, but not in early onset preeclampsia when compared to controls support this hypothesis[35].

In addition, cardiovascular profiles of men who fathered preeclamptic pregnancies were assessed as possible indirect evidence for involvement of paternal cardiovascular susceptibility genes, in the development of preeclampsia. We could, however, not detect any differences in profiles between men who fathered preeclamptic pregnancies compared to controls. This is in accordance with the findings of a large epidemiological study that did not observe higher mortality in men who fathered preeclamptic pregnancies[9].

In conclusion, very, early onset preeclampsia is related to some kind of hypertensive disease in almost all subsequent pregnancies. Although the recurrence rate of preeclampsia is as high as 50%, of which half of the cases develop severe preeclampsia mostly before 32 weeks' gestation, neonatal outcome in these subsequent pregnancies was favourable.

The cardiovascular risk profiles after 5.5 years of women who experienced severe, early onset preeclampsia showed more often chronic hypertension and increased microalbuminuria. These data suggest a more hypertension related vascular aetiology rather than a metabolic syndrome origin in severe, very early onset preeclampsia, however, specific biochemical markers for counselling women to discriminate those with an increased risk and those with minor complications in future pregnancies are lacking. Specific markers of endothelial dysfunction e.g. brachial artery dilatation following transient forearm ischemia should be evaluated [23].

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3.3

Body composition by dual energy-X-ray absorptiometry in women with a history of preeclampsia or intrauterine growth restriction

ABSTRACT

Objective- To investigate differences in body composition and fat distribution between women with pregnancies complicated by preeclampsia or intrauterine growth restriction (IUGR) pregnancies and those with uncomplicated pregnancies.

Methods- Women with previous preeclampsia (n=45), IUGR (n=53) and uncomplicated pregnancies (n=106) were recruited from a genetically isolated population in the Southwest of the Netherlands. Women were compared for body composition and fat distribution variables, assessed by dual energy-X-ray absorptiometry (DXA) and anthropometrics at a mean follow up time of 10.8 (SD, ±5.9) years after pregnancy. Main outcome measures were total lean- and fat mass, android fat mass, gynoid fat mass, android-to-gynoid fat ratio, waist- and hip circumference, and waist-to-hip ratio.

Results- Women with previous preeclampsia compared to controls had higher mean total fat mass index (11.5±0.6 vs. 9.7±0.4 kg/m^2; p=0.03), lean mass index (15.8±0.3 vs. 14.5±0.2 kg/m^2; p=0.001) and body mass index (28.4±0.8 vs. 25.4±0.5 kg/m^2; p=0.005). Their waist circumferences (90.7±2.0 vs. 78.5±1.3 cm; p<0.001) and waist-to-hip ratios (0.86±0.01 vs. 0.77±0.01; p<0.001) were also higher as well as android fat mass (2.8±0.2 vs. 2.1±0.1 kg; p=0.01) and android-to-gynoid fat ratios (0.45±0.02 vs. 0.39±0.01; p=0.02). Mean total fat-, lean-, and body mass index was not significantly different between women with previous IUGR and controls, yet waist-to-hip ratios (0.83±0.01; p<0.001) were higher. The observed differences in waist- , hip circumference, waist-to-hip ratio and gynoid fat mass could not be attributed to differences in body mass index.

Conclusions- Women with previous preeclampsia or IUGR pregnancies as compared to those with uncomplicated pregnancies have a preferential fat accumulation in the abdominal- over hip region, which may explain, at least partly, their increased cardiovascular risk.

INTRODUCTION

There is growing evidence for an increased risk of future cardiovascular disease in women with a history of pregnancies complicated by preeclampsia or intrauterine growth restriction (IUGR)[1-4]. It is hypothesized that such adverse pregnancy outcomes unmask women with a predisposition to vascular or metabolic disease[5]. Pregnancy, therefore, provides a unique opportunity for early identification of women at increased cardiovascular and metabolic risk and renders the possibility for risk reduction by preventive strategies in this specific group of women. To optimise preventive strategies it is essential to know which cardiovascular risk factors, especially those that are treatable and modifiable, are present in women with a history of preeclampsia or IUGR.

Obesity[6, 7] and in particular abdominal obesity [8-10], is well recognized as major risk factor of cardiovascular disease and type 2 diabetes. It has been suggested that particularly intra-abdominal (visceral) adipose tissue is strongly associated with metabolic disturbances and cardiovascular disease[11], but the exact pathophysiological mechanism underlying this association remains subject of discussion[12].

Body mass index (BMI) is most commonly used as a measure to define obesity. A limitation of BMI, however, is its inability to assess whether excess of body weight is due to excessive adipose tissue or muscle hypertrophy. Anthropometric measurements as waist circumference and waist-to-hip ratio are widely used to estimate abdominal obesity. A drawback of these measurements, however, is that they cannot discriminate between visceral and subcutaneous fat, nor do they discriminate between fat- and lean mass. Moreover, anthropometric measurements are subject to intra- and inter examiner variations. An alternative, more accurate, method that does discriminate between fat and lean mass is dual-energy X-ray absorptiometry (DXA)[13]. Although DXA can also not distinguish between visceral and subcutaneous fat, new DXA measurements that quantify adipose tissue in the lower trunk region correlate strongly with visceral fat[14-16].

Recently, we found in our study population an increased prevalence of metabolic syndrome in women with a past history of preeclampsia or IUGR as compared to women with uncomplicated pregnancies[17]. In that respect we performed waist circumference measurements, which suggested a higher prevalence of abdominal obesity in these women. Abdominal obesity may well be an important contributor to the increased cardiovascular disease risk observed in women with pregnancies complicated by preeclampsia or IUGR[18]. More such detailed information on the characterization of women at future risk of cardiovascular disease is essential for preventive strategies to be implemented by clinicians caring for these women. Therefore, this study aimed to further explore body composition as well as fat distribution by means of DXA, in addition to traditional anthropometric measurements, in women with a history of preeclampsia, IUGR and uncomplicated pregnancies.

MATERIALS AND METHODS

Subjects

Women with a history of preeclampsia or IUGR and women with a history of uncomplicated pregnancies only were recruited from a genetically isolated population in the Southwest of the Netherlands[19]. This population was founded around 1750 by a limited number of individuals (~150) and has been characterized by minimal inward migration and rapid population growth over the last two centuries. Descendants of this population show less genetic diversity than outbred populations[19]. This study is part of a larger research program called Genetic Research in Isolated Populations (GRIP), which aims to identify susceptibility genes for complex disorders[20]. All participants were of Caucasian origin. The scientific protocol of GRIP was approved by the Medical Ethics Committee of the University Medical Centre Rotterdam. All participants provided informed consent. Recruitment of participants has been described in detail elsewhere[17]. In brief, 197 women with pregnancies complicated by preeclampsia or IUGR, living in the isolate at time of delivery, were identified from National Birth Registration Records (anonymous data). Of those 57 could not be traced because of unknown identity or absence of medical records. From the remaining 140 women that were approached for the study, 106 agreed to participate (response rate 76%). Fifty women had a history of preeclampisa of whom 43 (95.6%) were nulliparous at index pregnancy and 56 had a pregnancy complicated by IUGR of whom 35 (66%) were nulliparous at index pregnancy. An equal number of unmatched controls were randomly selected from the midwife's practice, located within the same community. Women who gave birth to children with congenital anomalies were excluded from the study. Only singleton pregnancies were included. Preeclampsia was defined as *de novo* hypertension (systolic ≥ 140/ diastolic ≥ 90 mm Hg) with proteinuria ≥ 300 mg per 24 hour or at least 1+ on semi quantitative analysis after 20 weeks of gestation or as superimposed preeclampsia when new onset proteinuria after 20 weeks of gestation occurred in case of chronic hypertension. IUGR was defined as birth weight of newborns, born to women without severe nutritional deficiency, equal to or below the 5th percentile for gestational age at delivery, according to the Dutch fetal growth charts of Kloosterman[21]. Additionally, we calculated the birth weight percentiles of these IUGR babies according to customised birth weight percentiles (www. gestation.net). These recently developed growth curves adjust for physiological factors that affect fetal growth, such as maternal height, weight in early pregnancy, parity and ethnicity as well as the sex of the baby, hereby limiting misclassification of constitutionally small babies. Maternal prepregnancy weight or weight in early pregnancy was known for 27 of the 56 (48.2%) women with prior IUGR pregnancies. The customised birth weight percentiles in this subgroup were all equal or below the 3rd percentile and therefore met the criteria of IUGR.

Data on body composition were available for 45 women with a history of preeclampsia (3 excluded because of pregnancy and 2 due to technical error) and 53 women after pregnancies complicated by IUGR (3 missing due to technical errors), and for 106 controls.

Data collection

Participants were invited for examination at our research centre located within the community. All participants were interviewed by the research physician about their medical history, medication use, educational level, and smoking habits. Diabetes mellitus was defined as the use of blood glucose-lowering medication. Educational level was categorized into low (primary school/ lower vocational training), intermediate (secondary school/ intermediate vocational training) and high education (higher vocational training/ university). Participants were classified as non- or current smokers (≥ 1 cigarette / day).

Anthropometric measurements

Height and weight were measured with the participant dressed in light underclothing. Waist and hip circumference were measured on uncovered skin using a tape measure with the participant in upright position. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. Waist-to-hip ratio was calculated from these measurements.

Dual-energy X-ray absorptiometry

Total body and regional fat mass, lean mass and bone mineral content were obtained from DXA scans performed using a Prodigy™ total body fan-beam densitometer and analysed with the enCORE™ 2005 software V. 9.30.044 (GE Lunar Corporation Madison, WI). Total body scans were auto analysed by the software, which employs an algorithm that divides body measurements into areas corresponding to head, trunk, arms and legs. The trunk region was limited by an upper horizontal border below the chin (neck cut), vertical borders lateral to the ribs, and a lower border by the iliac crest. The arm region was limited by cuts that cross the arm sockets, as close to the body as possible and separate the arms and hands from the body. The leg region is limited above by the oblique lines passing through the hip joint, and cuts that separate the hands and forearms from the legs and a center leg cut which separates the right and left leg. Two additional regions were defined using the software provided by the manufacturer; the “android” and “gynoid” region (Figure 1). The “android region” has a lower boundary at the pelvis cut and the upper boundary above the pelvis cut by 20% of the distance between the pelvis and neck cuts. The lateral boundaries are the arm cuts. The “gynoid region” has an upper boundary between the upper part of the greater trochanters and a lower boundary

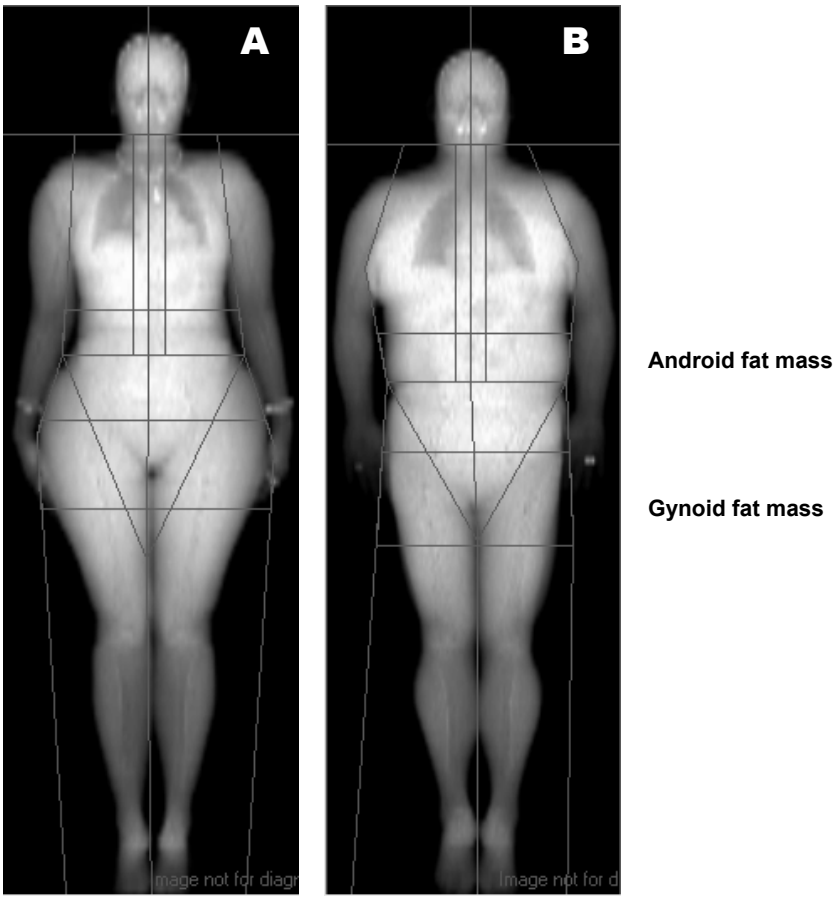

Figure 1.

Two examples of DXA scans showing the special regions of interest corresponding with android fat mass and gynoid fat mass.

A.Gynoid fat deposition

B.Android fat deposition

defined at a distance equal to twice the height of the "android region". The lateral boundaries are the outer leg cuts. The android and gynoid fat mass and android-to-gynoid fat ratio were calculated from these measurements. All analyses were verified by a trained technician who performed adjustments when necessary. Daily quality assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 1%.

Definitions

Total body weight is the sum of total fat- and lean mass and bone mineral content. BMI is total body weight divided by height squared. We calculated the following variables to describe body composition.
Fat mass index = total fat mass (kg)/ height^2 (m^2)
Lean mass index= total lean mass (kg)/ height^2 (m^2)
Total body fat percentage was defined as: (total fat mass x 100%)/ (total fat mass + total lean mass + total bone mineral content).

Statistical analyses

General characteristics were compared between groups using Dunnett's test, $\chi 2$ statistics and Fisher's exact test where appropriate. Differences in body composition and fat distribution were analysed using a general linear model controlling for age, time interval between pregnancy and study, smoking status and educational level. Postmenopausal women were excluded from these analyses given the changes in body composition associated with this condition (1 with prior preeclampsia, 4 with prior IUGR pregnancies and 5 controls). Next, the relation between BMI and fat distribution variables (waist- and hip circumference, waist-to-hip ratio, android fat, gynoid fat, android-to-gynoid fat ratio) was investigated using linear regression analysis with BMI as independent variable and the fat distribution variables as dependent variables. The analyses were adjusted for age, time interval between pregnancy and study, smoking status and educational level. A possible effect of preeclampsia or IUGR (disease status) in the relation between body mass index and fat distribution variables was evaluated by adding an interaction term BMI x disease status to the model. Finally, we repeated our initial analyses on fat distribution with additional adjustment for BMI. For all statistical analyses we used SPSS for Windows, version 11.0.1

RESULTS

General characteristics of participants are depicted in Table 1. At index pregnancy, women with pregnancies complicated by preeclampsia or IUGR were on average 3 years older than controls. At time of study women with a history of preeclampsia were younger than women with IUGR and controls. Accordingly, the time interval between delivery and study was significantly shorter in women with prior preeclampsia and prior IUGR pregnancies as compared with controls. Other significant differences between groups were found for educational level, use of antihypertensive medication and current smoking (Table 1). Hypertension defined as systolic blood pressure ≥140 mm Hg and/or diastolic ≥90 mmHg and/or use of antihyper-

Table 1. General characteristics of women with a history of preeclampsia, IUGR and controls

	Preeclampsia (n=45)	**IUGR** (n=53)	**Controls** (n=106)
Index pregnancy			
Age, years	29.2 (±3.9)*	29.9 (±3.6)*	26.2 (±4.3)
Birth weight newborn, grams	2562 (±902)*	2215 (±555)*	3345 (±379)
Gestational age, weeks	37 (±3.7)*	38.6 (±2.9)†	39.9 (±1.4)
Chronic hypertension	6.7 (3)†	5.7 (3)†	0
Gestational diabetes	4.4 (2)	0	0
Current study			
Age, years	36.1 (±5.5)‡	39.3 (±5.1)	39.2 (±5.6)
Time since pregnancy, years	7.0 (±5.3)*	9.5 (±5.1)*	13.1 (±5.7)
Premenopausal	97.8 (44)	92.5 (49)	95.3 (101)
Educational level			
Low	40 (18)*	71.7 (38)	72.6 (77)
Intermediate	46.7 (21)†	18.9 (10)	25.5 (27)
High	13.3 (6)‡	9.4 (5)†	1.9 (2)
Hypertension#	46.7 (21)*	28.3 (15)‡	8.5 (9)
Anti hypertensive drugs	20 (9)*	13.2 (7)‡	0.9 (1)
Lipid lowering drugs	2.2 (1)	1.9 (1)	0.9 (1)
Diabetes mellitus	4.4 (2)	3.8 (2)	0
Current smoking	22.2 (10)‡	54.7 (29)	49.1 (52)
Alcohol consumption	31.1 (14)	28.3 (15)	31.3 (33)

Data are presented as means (±SD) or percentages (absolute numbers).

Differences between women with a history of preeclampsia or IUGR and controls were examined with Dunnett's test for continuous variables and with χ^2 statistics or Fisher's exact test for dichotomous variables

* $p<0.001$, ‡ $p<0.01$, †$p<0.05$ as compared to controls

#Hypertension defined as systolic blood pressure ≥140 mm Hg and/or diastolic ≥90 mmHg and/or use of antihypertensive medication

tensive medication was significantly more prevalent among women with prior complicated pregnancies as compared to controls. No significant differences were found between groups in use of lipid lowering medication or diabetes mellitus (Type 1 and 2).

Anthropometric measurements

Weight, BMI, waist circumference and waist-to-hip ratio were higher in women with previous preeclampsia compared to controls (Table 2). In contrast, women after pregnancies complicated by IUGR did not differ significantly from controls with respect to body weight, BMI and waist circumferences. However, their hip circumferences were smaller and subsequently their waist-to-hip ratios were significantly higher than in controls (Table 2).

Dual-energy X-ray absorptiometry

Women with a history of preeclampsia had an excess of total lean and fat mass compared to controls. After controlling for height these differences remained significant, reflected by the higher lean and fat mass indices (Table 2). With respect to fat distribution, women with previous preeclampsia had an excess of fat in the android region and subsequently higher android-to-gynoid fat ratios than controls, whereas the total body fat percentage was not significantly different (Table 2). No significant differences were detected in body composition and fat distribution between women with pregnancies complicated by IUGR and controls, except for borderline significant lower total body fat percentage (Table 2).

Fat distribution in relation to BMI

Next, we investigated for each group of women separately, the relation between BMI and the fat distribution variables; waist circumference, hip circumference, waist-to-hip ratio, android fat, gynoid fat, and android-to-gynoid fat ratio. All fat distribution variables were positively

Table 2. Body composition and fat distribution of women with a history of preeclampsia or IUGR compared to controls

	Preeclampsia n=44#	**P***	**IUGR** n=49#	**P ***	**Controls** n=101#
Antropometric measurements					
Height (cm)	164.2 (1.0)	0.9	162.6 (0.9)	0.1	164.4 (0.7)
Weight (kg)	76.7 (2.4)	0.01	65.4 (2.2)	0.2	68.8 (1.6)
Body mass index (kg/m^2)	28.4 (0.8)	0.005	24.8 (0.8)	0.5	25.4 (0.5)
Waist circumference (cm)	90.7 (2.0)	<0.001	81.2 (1.8)	0.2	78.5 (1.3)
Hip circumference (cm)	104.5 (1.8)	0.3	97.7 (1.6)	0.03	102.0 (1.1)
Waist-to-hip ratio	0.86 (0.01)	<0.001	0.83 (0.01)	<0.001	0.77 (0.01)
DXA measurements					
Lean mass (kg)	42.6 (0.9)	0.004	39.0 (0.8)	0.7	39.4 (0.6)
Lean mass index (kg/m^2)	15.8 (0.3)	0.001	14.8 (0.3)	0.4	14.5 (0.2)
Fat mass (kg)	31.0 (1.7)	0.04	23.2 (1.5)	0.1	26.4 (1.1)
Fat mass index (kg/m^2)	11.5 (0.6)	0.03	8.8 (0.6)	0.2	9.7 (0.4)
Android fat (kg)	2.8 (0.2)	0.01	2.1 (0.2)	0.8	2.1 (0.1)
Gynoid fat (kg)	5.8 (0.3)	0.1	4.8 (0.2)	0.1	5.3 (0.2)
Android-to-gynoid fat ratio	0.45 (0.02)	0.02	0.41 (0.02)	0.3	0.39 (0.01)
Total body fat %	39.1 (1.2)	0.3	34.8 (1.1)	0.06	37.5 (0.8)

Data presented as adjusted means (SE)

postmenopausal women were excluded from analyses

*Differences between women with a history of preeclampsia or IUGR and controls were examined in a general linear model adjusted for age, interval between pregnancy - study, smoking and educational level

associated with BMI (all p-values <0.01). Slopes are depicted in Table 3. The associations between hip circumference and gynoid fat mass on the on hand and BMI on the other hand were significantly different in women with prior preeclampsia than in controls, as indicated by the significant interaction terms (Table 3). The association between BMI and gynoid fat was also significantly different in women with a history of IUGR (Table 3).

Given the positive association between BMI and the fat distribution variables we repeated our analyses on fat distribution with additional adjustments for BMI. After controlling for BMI, both women with a history of preeclampsia and IUGR showed narrower hips, larger waist circumferences and waist-to-hip ratios (Table 4). In addition, we found that both women with

Table 3. Association between fat distribution variables and body mass index stratified for previous disease status

Fat distribution variables	Disease status	Slope* (unit/ kg/m2)	Effect of disease status* BMI ‡	P†
Waist circumference	Preeclampsia	2.3	0.26	0.2
	IUGR	2.3	0.29	0.2
	Controls	2.0		
Hip circumference	Preeclampsia	1.7	-0.4	0.01
	IUGR	2.1	-0.04	0.8
	Controls	2.0		
Waist-to-hip ratio	Preeclampsia	0.007	0.004	0.06
	IUGR	0.005	0.002	0.8
	Controls	0.004		
Android fat	Preeclampsia	0.21	-0.02	0.2
	IUGR	0.21	-0.02	0.2
	Controls	0.23		
Gynoid fat	Preeclampsia	0.24	-0.1	<0.001
	IUGR	0.27	-0.09	0.002
	Controls	0.34		
Android-gynoid fat ratio	Preeclampsia	0.017	0.0006	0.8
	IUGR	0.016	0.0005	0.9
	Controls	0.017		

* Values based on linear regression models with fat distribution variables as dependent variables and body mass index as independent variable, adjusted for age, interval between pregnancy - study, smoking and educational level.

‡ Regression coefficient indicating interaction effect of previous disease and BMI on fat distribution variables

† p-value for the comparison with controls

BMI, body mass index

Tabel 4. Fat distribution of women with a history of preeclampsia or IUGR compared to controls adjusted for body mass index

	Preeclampsia n=44#	P*	IUGR n=49#	P *	Controls n=101#
Antropometric measurements					
Waist circumference (cm)	85.4 (0.9)	<0.001	83.6 (0.8)	<0.001	79.6 (0.6)
Hip circumference (cm)	99.7 (0.8)	0.001	99.8 (0.7)	<0.001	103.1 (0.5)
Waist-to-hip ratio	0.85 (0.01)	<0.001	0.84 (0.01)	<0.001	0.77 (0.01)
DXA measurements					
Android fat (kg)	2.2 (0.08)	0.8	2.3 (0.07)	0.5	2.2 (0.05)
Gynoid fat (kg)	5.1 (0.1)	<0.05	5.1 (0.1)	0.03	5.5 (0.09)
Android-to-gynoid fat ratio	0.41 (0.02)	0.5	0.43 (0.02)	0.08	0.39 (0.01)

Data presented as adjusted means (SE)

postmenopausal women were excluded from analyses

*Differences between women with a history of preeclampsia or IUGR and controls were examined in a general linear model adjusted for age, interval between pregnancy - study, smoking, educational level and body mass index

preeclampsia and IUGR complicated pregnancies had less fat deposition in the gynoid region (Table 4).

DISCUSSION

In this study women with pregnancies complicated by preeclampsia or IUGR show an unfavourable fat distribution in comparison with women with uncomplicated pregnancies, marked by an excess of fat deposition in the abdominal region relatively to fat deposition in the hip region. These differences could not be explained by differences in BMI.

As previously described[22, 23] we found that women with a past history of preeclampsia have greater BMI as compared to women with uncomplicated pregnancies. The association between obesity and preeclampsia has been well established[24-26]. Little attention, however, has been paid to the actual composition of body weight in these women, despite the fact that it is excessive fat deposition, rather than body weight per se, that is independently associated to cardiovascular disease[27]. The few studies that did investigate body composition in relation to preeclampsia by means of bio impedance analysis or DXA, were performed during pregnancy or very shortly there after[28-30]. Therefore, these studies do not answer questions on body composition in these women in a non-pregnant state.

We found that women with previous preeclampsia have an excess of total fat mass accompanied by an increase of lean mass, resulting in a higher BMI as compared to controls. Total body fat percentage was slightly, yet not significantly, higher. With regard to fat distribution, both DXA and anthropometric measurements revealed increased abdominal fat deposition in

these women as compared to controls, which is consistent with previously reported anthropometric data[22,23]. Women after pregnancies complicated by IUGR did not differ significantly in total fat- and lean mass from our controls. Unlike previous studies[24,31] we did not observe a significantly lower BMI in these women. However, women with IUGR pregnancies may have lower body fat percentage, although the difference from controls was only significant at p = 0.06. Remarkably, we found that these women, despite their leanness, had an increased abdominal fat deposition reflected by higher waist-to-hip circumferences. We are aware of one other study examining fat distribution in women with low birth weight offspring at a large interval post pregnancy[32]. These women, aged 70-79 years, had larger abdominal circumferences, in comparison with women with normal birth weight offspring, after adjusting for BMI[32].

We evaluated whether the differences observed in fat distribution in both women with a history of preeclampsia or IUGR were attributable to a possible confounding effect of BMI, by adjusting our analyses for BMI. Also then, our data indicated a preferential accumulation of fat in the abdominal- over hip region.

With respect to cardiovascular disease, waist circumference and waist-to-hip ratio are well established as independent predictors[9, 10], whereas such an association has not been studied yet for android fat mass measured by DXA. Therefore, our results strongly suggest that both women with a history of preeclampsia and IUGR are at increased risk of cardiovascular disease. The ultimate confirmation of this increased risk would have been to investigate cardiovascular disease as outcome in these women, yet due to the overall low incidence of cardiovascular disease in young women, and due to limited numbers this was not feasible. However, we previously described in this cohort that 40% and almost 30% of the women with prior preeclampsia and IUGR pregnancies, respectively, were diagnosed with chronic hypertension within a decade after the index pregnancy[17]. Furthermore, metabolic syndrome was diagnosed in nearly 40% and 20% of women with prior preeclampsia and IUGR pregnancies, respectively[17].

Whether the differences in body composition and fat distribution between women with pregnancies complicated by preeclampsia or IUGR and women with uncomplicated pregnancies are causally related to pregnancy complications or are a result from these cannot be concluded from this study, as no preconceptional data are available. However, body composition and fat distribution are largely influenced by genetic factors[33,34] and therefore it is likely that differences were present prior to pregnancy. Differences in body composition and fat distribution may be a possible explanation for the distinct clinical manifestations observed in women with preeclampsia and IUGR during pregnancy. Pregnancy in obese women is associated with endothelial impairment and an exaggerated proinflammatory status when compared to pregnancy in lean women[35].

Several other factors may influence fat distribution like age, hormonal status, and life style and may therefore potentially confound our analyses[34]. Total and abdominal fat mass increase

with age[34,36] and postmenopausal status is associated with abdominal obesity[37]. As we controlled for age in our analyses and as the far majority of women was premenopausal, we expect no confounding effects of these factors. Moreover, women with a history of preeclampsia were younger than controls implicating an underestimation, rather than overestimation of the observed difference. Additionally, we adjusted for smoking status since smoking is associated with lower body mass index and induces abdominal obesity[38]. A limitation of this study is that we could not control for other relevant life style factors such as dietary habits and physical activity, as these data were not available. Another limitation is that we have not controlled for parity, while women with succeeding pregnancies have a tendency to abdominal obesity[39]. However, we speculate that parity is higher in women with a history of uncomplicated pregnancies than in women who encountered problems during pregnancy. Therefore, adjusting for parity may even magnify the observed difference.

Our study was conducted in a genetically isolated population. It has been previously demonstrated for this population that individuals are genetically more homogeneous than individuals of outbred population[19]. Findings in this population may therefore reflect a common underlying genetic predisposition. It can be questioned whether these findings can be entirely generalized to the population at large. However, because our population is of more recent isolation, the genetic makeup may more closely resemble that of the general population[40]. Furthermore, our simulation studies based on the genealogy have shown that this potential problem concerns primarily rare variants. For common genetic variants, our simulation studies show that no substantial differences between isolate and the general population are expected[40]. Finally, our findings are in line with previous studies in outbred populations supporting the generalizability of our findings[22, 32] .

In summary, the present study demonstrates that women after pregnancies complicated by preeclampsia or IUGR have a preferential accumulation of fat in the abdominal- over the hip region. As abdominal obesity is an independent risk factor of cardiovascular disease[10], it may be an important contributor to the increased cardiovascular risk observed in these women[18].

The findings of our study may help to design, individually tailored, optimal preventive strategies with regard to future cardiovascular disease. Especially strategies targeting accumulation of excessive abdominal fat, including diet and physical exercise[41], might be relevant not only for women with previous preeclampsia who are more commonly overweight, but also for women with pregnancies complicated by IUGR, despite their tendency towards lower BMI.

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3.4

Insulin, androgens and bone mineral density in women with previous preeclampsia or pregnancies complicated by intrauterine growth restriction

ABSTRACT

Objective- To investigate the presence of endocrine alterations associated with abdominal obesity and their effect on bone mineral density in women with previous preeclampsia, IUGR pregnancies and uncomplicated pregnancies.

Methods- Women with previous preeclampsia (n=43), IUGR pregnancies (n=50) and uncomplicated pregnancies (n=101) were recruited from a genetically isolated population in the Southwest of the Netherlands. Endocrine parameters, including plasma fasting glucose and insulin, HOMA-IR scores, plasma adiponectin, and sex hormones, as well as bone mineral density assessed by dual energy-X-ray absorptiometry, were compared between groups of women at a mean follow up time of 9.5 (SD, ± 5.1) years after pregnancy.

Results- Women with prior preeclampsia had significantly higher values of fasting glucose ($p<0.001$), insulin ($p=0.002$), HOMA-IR scores ($p<0.001$), non-SHBG-testosterone % ($p=0.03$) and lower levels of adiponectin ($p=0.02$) and SHBG ($p=0.03$) as compared to controls. Women with prior IUGR pregnancies also had higher levels of glucose ($p<0.001$), insulin ($p=0.02$) and HOMA scores ($p=0.002$). After adjustments for body mass index differences remained significant, whereas these disappeared after adjustments for waist-hip-ratio, except for fasting glucose. Bone mineral density was significantly higher in women with previous preeclampsia than in controls ($p=0.003$), and remained significant after adjustments for waist-to-hip ratio ($p=0.04$). Adjustments for body mass index resulted in borderline significant difference ($p=0.07$). No difference in bone mineral density was observed between women with previous IUGR pregnancies and controls.

Conclusions- Women with prior preeclampsia exhibit increased insulin levels with associated endocrine alterations largely attributable to abdominal obesity. Comparable disturbances in insulin levels, also attributable to abdominal obesity, are observed in women with prior IUGR pregnancies. Bone mineral density was increased in women with prior preeclampsia and could not be fully explained by abdominal obesity or obesity per se.

INTRODUCTION

Preeclampsia and intrauterine growth restriction (IUGR) are pregnancy-specific syndromes with common pathophysiological features, but with different clinical manifestations. Shallow endovascular trophoblast invasion and inadequate uteroplacental artery remodelling resulting in malperfusion of the placenta are key pathological features of both syndromes[1], with yet unknown aetiology. Clinically, preeclampsia is characterized by de *novo* hypertension and proteinuria, often accompanied by fetal growth restriction. Fetal growth restriction associated with placental dysfunction can also occur in the absence of the maternal syndrome.

There is substantial epidemiological evidence that both syndromes are associated with an elevated risk of future maternal cardiovascular disease[2, 3], with an even higher risk in women with early onset preeclampsia[2]. Detailed data on traditional cardiovascular risk factors in women with prior preeclampsia have emerged over the last years[4-6], however less is known about risk profiles of women with previous IUGR pregnancies[7]. More knowledge of risk factors in these women is required to develop preventive models regarding their increased cardiovascular risk.

Obesity, as assessed by body mass index (BMI) is a major risk factor for cardiovascular disease[8], and is also a well known risk factor for preeclampsia[9]. Abdominal obesity, as measured by waist circumferences or waist-to-hip ratios, has been independently associated with an increased risk of cardiovascular disease, even in women with normal body weight[10]. Recently we found preferential fat accumulation in the abdominal- over hip region in women with previous preeclampsia as well as in women with previous IUGR pregnancies, while the latter were lean in comparison to those with previous preeclampsia[11]. Excessive fat deposition, and in particular abdominal fat deposition, is associated with numerous endocrine alterations including hyperinsulinemia, hypo-adiponectinemia, changes in sex hormone levels (increased free androgens levels in women) and reduction of sex hormone binding globulin (SHBG) concentrations[12-14]. Apart from the deleterious effect of hyperinsulinemia and hyperandrogenism on cardiovascular health, these conditions are positively associated with bone mineral density[14], implying a potential protective effect against the development of osteoporosis.

The purpose of this study was to investigate levels of fasting glucose and insulin, HOMA-IR, adiponectin, and sex hormone levels including androgen and SHBG concentrations, as well as bone mineral density in women with prior preeclampsia and previous IUGR pregnancies. Furthermore, we aimed to investigate whether differences in these traits were related to obesity per se as assessed by BMI or to abdominal fat distribution as measured by the waist-to-hip ratio. Finally, we examined differences between clinical subgroups of women with previous preeclampsia, namely those with early onset and those with late onset disease.

MATERIALS AND METHODS

Subjects

Women with previous pregnancies complicated by preeclampsia or IUGR and women with uncomplicated pregnancies only were recruited from a genetically isolated population in the Southwest of the Netherlands. The study is part of a larger research program called Genetic Research in Isolated Populations (GRIP), which aims to identify susceptibility genes for complex disorders[15]. All participants were of Caucasian origin. The scientific protocol of GRIP was approved by the Medical Ethics Committee of the University Medical Centre Rotterdam. Selection of participants has been described in detail elsewhere[4]. Briefly, 50 women with pregnancies complicated by preeclampsia and 56 by IUGR were included in the study. Women were identified from National Birth Registration Records and diagnoses were subsequently confirmed by the research physician after reviewing the medical records. An equal number of women with uncomplicated pregnancies with term deliveries of newborns with appropriate birth weight were randomly selected from the midwife's practice, located within the same community. Preeclampsia was defined as *de novo* hypertension (systolic blood pressure ≥ 140 and/or diastolic ≥ 90 mm Hg) with proteinuria ≥ 300 mg per 24 hour or at least 1+ on semi quantitative analysis after 20 weeks of gestation or as superimposed preeclampsia when new onset proteinuria after 20 weeks of gestation occurred in women with chronic hypertension (n=3). IUGR was defined as birth weight of newborns, equal to or below the 5th percentile for gestational age at delivery, according to the Dutch fetal growth charts of Kloosterman[16]. If preeclampsia and IUGR co-occurred (n=8), women were categorized in the preeclampsia group. Women who gave birth to children with congenital anomalies were excluded from the study. Only singleton pregnancies were included. All participants provided informed consent.

Data collection

Participants were invited for examination at our research centre located within the community. Venous blood samples were collected in the morning after an overnight fast, at a random moment in the menstrual cycle. All participants were interviewed by the research physician about their medical history, medication use and smoking habits. Participants were classified as non- or current smokers (≥ 1 cigarette / day). Blood pressure was measured twice in the sitting position at the right upper arm using an automated device (OMRON 711, automatic IS). The mean of these two measurements was used in the analyses. Height and weight were measured with the participant dressed in light underclothing. Body mass index (BMI) was calculated from these data (weight (kg)/ height^2 (m^2)). Waist and hip circumference were measured on uncovered skin using a tape measure with the participant in standing position. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip

circumference was measured at the maximal circumference of the hips. Waist-to-hip ratio was calculated from these measurements, reflecting a measure of abdominal obesity.

Bone mineral density

Total bone mineral density (grams/cm^2) was measured by dual energy X ray absorptiometry using a Prodigy™ total body fan-beam densitometer and analyzed with the enCORE™ 2005 software V. 9.30.044 (GE Lunar Corporation, Madison, WI). Daily quality assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 1%.

Laboratory measurements

Blood glucose was measured according to a standardized procedure [17] and total plasma insulin concentrations were analyzed with the Irma kit of Biosource (Nivelles, Belgium). Insulin sensitivity was assessed by the validated homeostasis model assessment (HOMA-IR) index [18] using the following formula: HOMA-IR=(fasting plasma glucose * fasting plasma insulin)/22.5. Total plasma adiponectin was measured with the Human adiponectin RIA kit of Linco Research (St. Charles, MO). All measurements were performed conform the manufactures protocol. Plasma albumin was measured by photometry (Beckman Lx 20, Fullerton, CA). Serum concentrations of testosterone were estimated using radioimmunoassay (Coat-a-count, Siemens, Los Angeles, CA). Serum levels of dehydroepiandrosterone sulphate (DHEAS), androstenedione and SHBG were estimated using luminescence-based immunoassays (Immulite 2000, Siemens). As measure of biologically available testosterone non-SHBG bound testosterone was calculated according to the method as described earlier[19]. Insulin measurements were missing in 2.6% of the subjects, adiponectin in 2.1%, Testosterone in 1.8%, DHEAS and SHBG in 4.4%, and androstenedione, in 3.5%.

Population for analysis

Pregnant women (3 in the preeclampsia group) and postmenopausal women (2 with previous preeclampsia, 3 after IUGR pregnancies and 5 controls) were excluded from analyses because of the metabolic changes associated with these conditions. Additionally, women diagnosed with diabetes mellitus (defined as the use of blood glucose-lowering medication, 2 with previous preeclampsia and 2 after IUGR pregnancies) were excluded. This resulted in a total number of 43 women with previous preeclampsia, 50 with previous IUGR pregnancies and 101 controls included in the analyses.

Table 1. General characteristics of women with previous preeclampsia, IUGR pregnancies and uncomplicated pregnancies

	Controls n=101	Preeclampsia n=43	IUGR n=50
Index pregnancy			
Age at delivery, years	26.1 (4.3)	29.3 (3.9)*	30.0 (3.2)*
Gestational age at delivery, weeks	40.0 (1.3)	37.1 (3.4)*	38.9 (2.5)*
Birth weight newborn, grams	3359 (371)	2570 (877)*	2283 (496)*
Current study			
Age, years	38.9 (5.5)	36.0 (5.6)*	38.9 (5.0)
Time since index pregnancy, years	12.8 (5.7)	6.8 (5.2)*	8.9 (4.5)*
Body mass index, kg/m^2	25.1 (4.5)	28.9 (6.8)*	24.9 (5.0)
Waist-to-hip ratio	0.77 (0.05)	0.86 (0.09)*	0.83 (0.06)*
Hypertension#	8.9 (9)	46.5 (20)*	30 (15)*
Current smoking	48.5 (49)	20.9 (9)*	58 (29)
Oral contraceptives	45.5 (46)	41.9 (18)	32 (16)

Data are presented as means (SD) or percentages (absolute numbers).

*P-value ≤0.01 compared with controls

#Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or ≥ 90mmHg and/or the use of antihypertensive medication

Statistical analyses

General characteristics are presented as means with standard deviations or as proportions. Groups were compared using T-test, $\chi 2$ statistics and Fisher's exact test where appropriate. Differences in endocrine parameters and bone mineral density were analyzed in a general linear model and presented as adjusted means with standard errors. Variables with skewed distributions were logarithmically transformed and reported as back transformed geometric means. For all statistical analyses we used SPSS for Windows, version 11.0.1.

RESULTS

General characteristics of the study subjects are presented in Table 1. Women with prior preeclampsia as well as those with prior IUGR pregnancies had higher waist-to-hip ratios than controls, whereas body mass index was significantly higher only in women with prior preeclampsia compared to controls. Hypertension was diagnosed more frequently in women with prior complicated pregnancies than in controls. Smoking was less common in women with a history of preeclampsia.

Endocrine parameters and bone mineral density values are depicted in Table 2. Women with prior preeclampsia had significantly higher values of fasting glucose, insulin, and

Table 2. Endocrine parameters and bone mineral density in women with previous uncomplicated pregnancies, preeclampsia, and IUGR pregnancies

	Controls n=101	Preeclampsia n=43	P-value*	IUGR n=50	P-value*
Glucose, mmol/L	4.2 (0.06)	4.8 (0.09)	<0.001	4.6 (0.08)	<0.001
Insulin, pmol/L	67 (1.06)	97 (1.1)	0.002	87 (1.09)	0.02
HOMA-IR	2.1 (1.06)	3.5 (1.1)	<0.001	3.0 (1.1)	0.002
Adiponectin, µg/mL	10.4(1.04)	8.5 (1.07)	0.02	9.4 (1.06)	0.2
Testosterone, nmol/L¶	1.4 (0.7)	1.2 (0.5)	0.2	1.2 (±0.7)	0.08
Non-SHBG-testosterone, nmol/L¶	0.6 (0.4)	0.6 (0.3)	0.9	0.50 (±0.3)	0.2
Non-SHBG-testosterone,%¶	41.8 (12.1)	48.8 (13.3)	0.03	42.3 (±10.2)	0.6
DHEAS, µmol/L¶	3.8 (1.7)	3.1 (1.2)	0.2	3.7 (±1.7)	0.7
Androstenedione, nmol/L¶	8.2 (2.8)	6.9 (2.8)	0.4	8.2 (±3.9)	0.4
SHBG, nmol/L¶	61.3(1.08)	44.4 (1.12)	0.03	57.9(1.1)	0.7
Bone mineral density#, g/cm^2	1.14 (0.07)	1.19 (0.09)	0.003	1.13(±0.07)	0.8

Data are presented as adjusted means (SE)

*P value for differences between women with previous preeclampsia versus controls or IUGR pregnancies versus controls, adjusted for age, time since pregnancy and smoking

additionally adjusted for height

¶ Women using oral estrogens were excluded, resulting in n=56 for controls, n=25 for preeclampsia and n=34 for IUGR

HOMA-IR and lower levels of adiponectin compared to controls. After exclusion of women using oral contraceptives, because of the stimulating effect on SHBG, we observed a significantly increased percentage of non-SHBG bound testosterone and reduced levels of SHBG as compared to controls. In women with prior IUGR pregnancies, we also found higher values of fasting glucose, insulin and HOMA-IR. No differences were found for the other parameters. Bone mineral density was significantly higher in women with prior preeclampsia than in controls. No difference in bone mineral density was observed between women with prior IUGR pregnancies and controls.

Subsequently, we investigated whether the significant differences observed between women with previous preeclampsia or IUGR pregnancies and controls could be explained by differences in BMI or waist-to-hip ratio. After adjusting for BMI, differences in glucose, insulin, HOMA-IR as well as non-SHBG bound testosterone (%) and SHBG levels remained significant for women with previous preeclampsia (Table 3). However, adjusting for waist-to-hip ratio attenuated the differences, except for glucose levels, which remained significantly higher (Table 4). Significant differences in bone mineral density disappeared after adjustment for BMI (Table 3) and decreased but remained significant after adjustment for waist-to-hip ratio (Table 4). In women with prior IUGR pregnancies glucose, insulin and HOMA-IR remained significantly higher than in controls, after adjusting for BMI (Table 3). Only glucose remained significantly higher after adjusting for waist-to-hip ratio instead of BMI (Table 4).

Table 3. Endocrine parameters and bone mineral density additionally adjusted for BMI in women with previous uncomplicated pregnancies, preeclampsia and IUGR pregnancies

	Controls n=101	Preeclampsia n=43	P-value*	IUGR n=50	P-value*
Glucose, mmol/L	4.2 (0.06)	4.8 (0.09)	0.00	4.6 (0.08)	0.00
Insulin, pmol/L	69 (1.05)	84 (1.08)	0.04	94 (1.07)	0.00
HOMA-IR	2.1 (1.05)	3.0(1.09)	0.001	3.2 (1.08)	0.00
Adiponectin, µg/mL	10.3 (1.04)	8.8 (1.07)	0.07		
Non-SHBG-testosterone,%¶	41.6 (1.5)	48.0 (2.3)	0.03		
SHBG, nmol/L¶	60.7 (1.07)	46.5 (1.1)	0.03		
Bone mineral density#, g/cm^2	1.14 (0.007)	1.17 (0.1)	0.07		

Data are presented as adjusted means (SE)

*P value for differences between women with previous preeclampsia versus controls or IUGR pregnancies versus controls, adjusted for age, time since pregnancy, smoking and BMI

additionally adjusted for height

¶Women using oral estrogens were excluded, resulting in n=56 for controls, n=25 for preeclampsia and n=34 for IUGR

Next, we evaluated whether the endocrine parameters and bone mineral density values differed between subgroups of women with previous preeclampsia, i.e. early and late onset disease. Thirteen (30.2%) of all 43 women with prior preeclampsia were diagnosed with early onset disease (onset of disease before 34 weeks of gestation). Their mean gestational age at delivery was 33 (±3.2) weeks with a mean birth weight of newborns of 1561 (±530) grams. The remaining 69.8% delivered at 38.9 (±1.2) weeks with newborn birth weight of 3007 (±586)

Table 4. Endocrine parameters and bone mineral density additionally adjusted for waist-to-hip ratio in women with previous uncomplicated pregnancies, preeclampsia and IUGR pregnancies

	Controls n=101	Preeclampsia n=43	P-value*	IUGR n=50	P-value*
Glucose, mmol/L	4.2 (0.06)	4.8 (0.09)	0.00	4.6 (0.08)	0.00
Insulin, pmol/L	75 (1.06)	81 (1.1)	0.5	81 (1.09)	0.5
HOMA-IR	2.3 (1.07)	2.9 (1.1)	0.09	2.7 (1.09)	0.1
Adiponectin, µg/mL	10.3 (1.04)	8.8 (1.07)	0.8		
Non-SHBG-testosterone,%¶	43.6 (1.8)	45.8 (2.7)	0.5		
SHBG, nmol/L¶	55.6 (1.08)	51.3 (1.1)	0.6		
Bone mineral density#, g/cm^2	1.14 (0.008)	1.18 (0.01)	0.04		

Data are presented as adjusted means (SE)

*P value for differences between women with previous preeclampsia versus controls or IUGR pregnancies versus controls, adjusted for age, time since pregnancy, smoking and waist-to-hip ratio

additionally adjusted for height

¶Women using oral estrogens were excluded, , resulting in n=56 for controls, n=25 for preeclampsia and n=34 for IUGR

grams. BMI and waist-to-hip ratios were not significantly different between women with early or late onset preeclampsia. No significant differences were found in any of the investigated parameters or bone mineral density values between women with early and late onset preeclampsia.

Finally, we calculated the prevalence of insulin resistance based on HOMA-IR scores above 2.9, corresponding with the 75th percentile in controls. The proportions of women with increased HOMA-IR scores were significantly greater (P value ≤0.01) in women with a history of early onset preeclampsia, late onset preeclampsia as well as IUGR pregnancies than in controls (Figure 1). No differences were observed between women with early and late onset preeclampsia.

DISCUSSION

In this study we demonstrated that women with previous preeclampsia, as compared to women with uncomplicated pregnancies, had hyperinsulinemia with associated endocrine alterations including hypoadiponectinemia, hyperandrogenism and reduction of SHBG con-

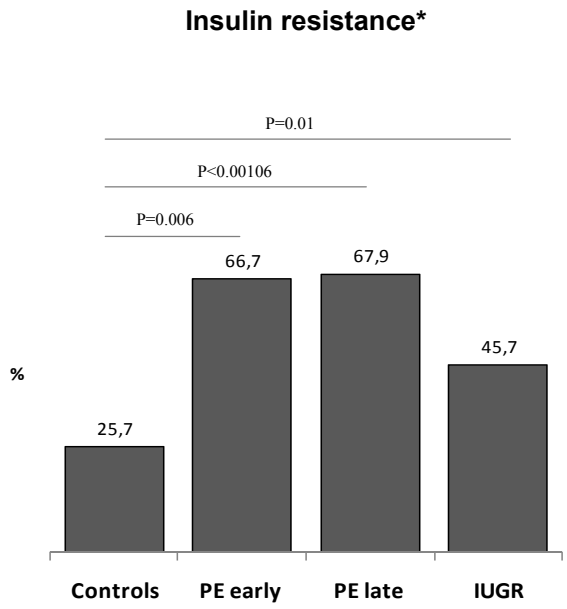

Figure 1.

Prevalence of insulin resistance based on increased HOMA-IR in women with previous complicated and uncomplicated pregnancies.

*percentage of women with HOMA-IR above the 75th percentile of the controls

PE early, early onset preeclampsia

PE late, late onset preeclampsia

centrations which could largely be explained by abdominal obesity. Hyperinsulinemia without alterations in adiponectin levels or sex steroids were observed in women with prior IUGR pregnancies, also largely attributable to abdominal obesity. Furthermore, bone mineral density was increased in women with previous preeclampsia as compared to those with uncomplicated pregnancies.

Hyperinsulinemia was found both in women with previous preeclampsia and with IUGR pregnancies while glucose levels were still within the normal range, although being significantly higher than in controls. Disturbances in glucose metabolism have been previously described in women with prior preeclampsia[5, 20-22] yet the association of hyperinsulinemia with obesity in these women is controversial. Increased insulin levels independent of BMI have been described [20], while others found that differences in insulin levels disappeared after BMI adjustments[22]. An association with abdominal obesity was not specifically studied in most studies[20, 22]. In light of preventive strategies regarding the future cardiovascular risk in these women, it is relevant to know if hyperinsulinemia in these women can be attributed to excessive fat deposition, or more specifically to abdominal fat deposition.

Our data indicate that abdominal obesity is strongly associated with hyperinsulinemia in women with prior preeclampsia and IUGR pregnancies as the differences in insulin levels disappeared after adjustments for waist-to-hip ratio, whereas they did not after adjustments for BMI. This is particularly relevant for women with previous IUGR pregnancies since these women have normal body weight. BMI, used as screening tool to identify individuals with increased cardiovascular risk, fails to identify these women. This phenotype has been previously recognized as metabolically obese normal body weight phenotype which represents a large proportion of the population[23] .

Data on metabolic and cardiovascular risk profiles of women with previous IUGR pregnancies are scarce. However, several studies described hyperinsulinemia in older women who had low birth weight offspring[24, 25]. Additionally, abdominal obesity has been described in such women despite normal BMI[25]. Unfortunately, in most studies birth weight was not corrected for gestational age at delivery, making it impossible to draw conclusions regarding strict IUGR pregnancies. In our study IUGR was strictly defined as birth weight below the 5th percentile according to Dutch growth charts corrected for sex and gestational age[16].

Given the known association of abdominal obesity with hypoadiponectinemia, changes in sex hormone levels and reduction of SHBG concentrations[12-14] we expected to observe such alterations in women with previous preeclampsia and IUGR pregnancies. Indeed lower levels of adiponectin and increased percentage of non-SHBG bound testosterone, and reduced levels of SHBG were found in women with previous preeclampsia, with differences disappearing after adjustments for waist-to-hip ratio. Such endocrine alterations were not observed in women with prior IUGR pregnancies despite increased insulin levels. Furthermore, we hypothesized a positive association of abdominal obesity, mediated by hyperinsulinemia and hyperandrogenism, with bone mineral density. Accordingly, bone mineral density was signifi-

cantly higher in women with previous preeclampsia as compared to controls. When we adjusted our analyses for waist-to-hip ratio the differences decreased, yet remained significant, suggesting that the observed differences could not be fully attributed to abdominal obesity. Moreover, bone mineral density values in women after IUGR pregnancies, despite abdominal obesity and hyperinsulinemia, were similar to those in controls, making a strong effect of abdominal obesity on bone mineral density less likely. Regarding the effect of fat distribution on bone mineral density, a clear association has not been established as data are conflicting[26-28]. However, a positive association between bone mineral density and BMI has been well established[14]. In view of the latter the higher bone mineral density in women with prior preeclampsia is not unexpected, yet after adjusting our analyses for BMI the difference remained borderline significant. No data on bone mineral density in women with previous preeclampsia or IUGR pregnancies are currently available to compare our results with. We are aware of only one study that compared bone mineral density between women with preeclampsia and uncomplicated pregnancies, at time of delivery, revealing no differences[29].

Hyperandrogenism has been previously reported in prior preeclamptic women, with hyperinsulinemia, 17 years post pregnancy[30] and was suggested to be involved in the pathogenesis of preeclampsia. The association with abdominal obesity, however, was not investigated by the authors[30]. Additional support for a pathophysiological role of androgens in the development of preeclampsia came from studies performed during pregnancy, indicating higher androgens levels in women with preeclampsia. Moreover, women with polycystic ovary, a syndrome characterized by hyperandrogenism, are at increased risk of preeclampsia[31]. Alternatively, hyperandrogenism could be a co-phenomenon of hyperinsulinemia which characterizes a common phenotype underlying both preeclampsia and polycystic ovary syndrome.

No differences were observed in endocrine parameters between women with early or late onset preeclampsia as a potential explanation for the increased risk of cardiovascular disease in described in early onset preeclampsia[32]. Important to note is that our numbers in these subgroups were limited. However, previously we described in this cohort a significantly higher prevalence of hypertension after early onset preeclampsia[33].

The increased cardiovascular morbidity and mortality, consistently described in these women [2, 3], calls for action. Pregnancy may help clinicians to identify women with elevated cardiovascular or metabolic risk, yet thus far, no follow up of these women is implemented in clinical practice. Detailed descriptions of risk factors in these women, help to design preventive strategies. The strength of our study is that we examined endocrine alterations in relation to abdominal obesity, indicating that these, with the exception of glucose levels, were largely explained by abdominal obesity and not a specific feature of preeclampsia or IUGR. This implicates that women with abdominal obesity should be a main target of preventive strategies. Regarding the higher glucose levels, follow up at regular intervals is required, as it has been demonstrated that increased glucose levels, even within the normal range puts individuals at

increased risk of developing type 2 diabetes and cardiovascular disease[34], especially in these women in whom we demonstrated hyperinsulinemia.

A limitation of our study is that venous blood samples were drawn randomly during the menstrual cycle. Conclusions with respect to androgens should be interpreted with caution since it has been described that androgens vary during the menstrual cycle[35], although contradicted by others[36]. However, our conclusions with respect to insulin resistance are not likely to be confounded since insulin levels are not significantly affected by menstrual phase.[37]

In summary, women with previous preeclampsia and IUGR pregnancies are more commonly insulin resistant than women with uncomplicated pregnancies which could largely be explained by their abdominal obesity. Since physical exercise has proved to be effective in improving insulin sensitivity[38, 39]and reducing abdominal obesity this should be strongly recommended to these women in order to reduce their cardiovascular risk. Additionally, since glucose levels are elevated in these women, although within the normal ranges, women should be screened for diabetes at regular intervals. Special attention should be paid to women with previous IUGR pregnancies since they are metabolically obese with normal weight and thus will often escape from the attention of medical practitioners as being at risk for future cardiovascular disease.

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4

General discussion

Vascular related pregnancy complications, of which preeclampsia and IUGR are the two most important, are major causes of maternal and perinatal morbidity and mortality worldwide [1].

Despite considerable progress in the understanding of the pathogenesis of preeclampsia and IUGR in the last decades, the aetiology of both disorders remains to be elucidated. Consequently, so far, the only definite cure for preeclampsia is the delivery of the child and the placenta. Further understanding of the pathogenesis of preeclampsia and IUGR is required to be able to predict disease, develop rational therapies and ultimately prevent disease and its remote consequences, i.e. an increased risk of cardiovascular disease.

One of the strategies to elucidate the underlying pathogenetic mechanisms of disease is identification of susceptibility genes. Although a genetic contribution has been long recognized, the genetics of preeclampsia and IUGR is far from understood. Different modes of inheritance have been proposed over the years including both recessive and dominant models as well as alternative genetic mechanisms such as parent-of-origin effects (reviewed by Lachmeijer et al[2] ,Chappell et al[3], and Devriendt et al.[4]). Studies aiming to identify genes involved in preeclampsia, and to a lesser extent, in IUGR have yielded encouraging results but, so far, with lack of consistent reproducibility.

Preeclampsia and IUGR are thought to be multifactorial diseases with genetic and environmental factors, and the interaction of those, contributing to the phenotype. Due to the multifactorial origin and the phenotypic diversity that characterizes clinical practice, research in the field of preeclampsia and IUGR covers a wide range of areas. An impressive number of journal articles have been published (a PubMed search on July 25th 2008, only on "preeclampsia" turns up over 22 000 hits; with a remarkable increase in number of publications over the last 10 years; Figure 1). The latter does not only indicate the impact of the clinical problem but also the major challenge to elucidate the aetiology of these pregnancy disorders.

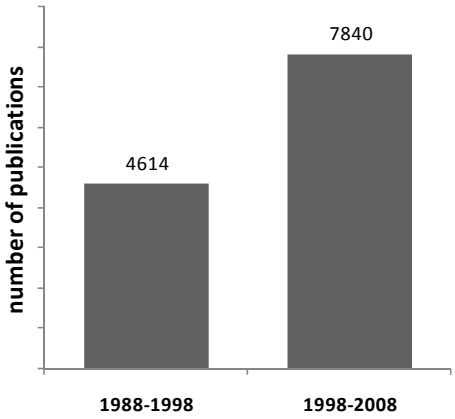

Figure 1. Number of publications in Pubmed on preeclampsia in the last two decades.

The work presented in this thesis aimed at enhancing the understanding of disease mechanisms by studying genetic factors in women with prior preeclampsia and IUGR pregnancies. Additionally, we aimed to provide detailed cardiovascular risk profiles of women with prior preeclampsia and IUGR pregnancies to help designing preventive strategies for future cardiovascular disease in these women.

Of each study the merits and limitations have been described in the previous chapters. The current chapter will focus on the main findings, speculate on aetiological implications and discuss clinical implications, considered in the light of current knowledge and ongoing research. Finally, suggestions for future research are being made.

HYPOTHESES

As with respect to genetics, isolated populations have been suggested to be a powerful tool to study the genetics of complex diseases[5, 6], due to reduced genetic diversity and a decrease in environmental variability. Furthermore, disorders following recessive inheritance are likely to become clear in such populations with substantial inbreeding, due to an excess of homozygotes. Also alternative genetic mechanisms such as imprinting, in which expression of an allele depends upon parental origin, is more likely to become evident. Therefore we studied the genetics of preeclampsia and IUGR in a Dutch genetically isolated population. For this population extensive genealogical data were available which allowed us to study familial aggregation of preeclampsia and IUGR, and the evidence for imprinting based on genealogy without a priori knowledge of the imprinted gene.

Regarding the more clinical hypotheses, it has been suggested, at least for a subset of the cases, that preeclampsia and IUGR are related conditions with different clinical manifestations[7], although this has been disputed by others.[8] Evidence for a shared pathogenesis already became available years ago from pathological studies indicating similarities in placental pathology between preeclampsia and IUGR[9]. Shallow trophoblast invasion and defective uteroplacental artery remodelling are key pathologic features of both syndromes[10]. Further support for a common pathogenesis was provided by epidemiological studies demonstrating that conditions present before pregnancy involving endothelial dysfunction are common to both preeclampsia and IUGR (reviewed by Ness and Sibai [7]). Additionally, preeclampsia and IUGR have similar remote consequences, i.e. an increased risk of cardiovascular morbidity and mortality in later life[11, 12]. These similarities in risk factors and remote disease do not only support a partly common pathogenesis but have also led to the hypothesis that a cardiovascular susceptibility underlies both these pregnancy complications as well as cardiovascular disease in later life[13]. Consequently, the question arises why some women develop preeclampsia only, others IUGR only and many develop both.

Ness and Sibai [7]proposed a unifying pathophysiological model for preeclampsia and IUGR based on the widely accepted concept that preeclampsia is a two stage disorder[14]. Stage 1 is characterized by poor placentation resulting in placental oxidative stress. Subsequently, the placenta is thought to release factors into the maternal circulation causing the maternal clinical syndrome (second stage) of which generalized endothelial dysfunction is considered a central pathophysiological feature. Ness and Sibai[7] posit that preeclampsia and IUGR both arise from a maternal predisposition to endothelial dysfunction which contributes to poor placentation. Preeclampsia subsequently develops when poor placentation interacts with maternal metabolic syndrome whereas IUGR develops in absence of the metabolic syndrome. The main findings of the studies described in this thesis are consistent with this hypothesis.

MAIN FINDINGS

In chapter 2.1 our data supply additional support for a shared pathogenesis by preeclampsia and IUGR. We demonstrated familial aggregation of both preeclampsia and IUGR in the same families, suggestive for a common genetic origin. Our data are in line with previously reported data from the Norwegian Birth registry indicating that women with previous growth-restricted babies (without preeclampsia) have an increased risk of preeclampsia in the subsequent pregnancies[15, 16], which the authors explain by a shared genetic factor involved in both preeclampsia and IUGR[16]. They suggest that the difference in clinical manifestation is explained by a delayed genetic expression of endothelial dysfunction. Alternatively, Roberts and Catov[17] proposed that constitutional factors that render the mother sensitive to develop the maternal syndrome secondly to poor placentation might evolve over time. These constitutional factors would then have not been sufficient to cause the maternal syndrome at time of the first pregnancy which resulted "only" in IUGR. However, by the time of the second pregnancy maternal constitutional factors were sufficient to cause preeclampsia. This reasoning fits with model of Ness and Sibai described above, yet seems to contradict with the observation that the incidence of preeclampsia is by far highest in the first pregnancy and subsequent pregnancies in women with a history of preeclampsia generally have a more favourable outcome, as described in chapter 3.2.

In accordance with the model of Ness and Sibai, we found that women with previous preeclampsia exhibited more cardiovascular risk factors, and therefore more frequently met the criteria of the metabolic syndrome, than women with previous IUGR pregnancies (chapter 3.1). Important to note is that these risk profiles were determined years post pregnancy and therefore are of limited value when discussing causality. However, in the same chapter we demonstrated similarities in risk profiles between these women and their parents, supportive of the constitutional origin of the cardiovascular risk factors in these women. Ideally, consti-

tutional differences in risk factors between women with and without complicated pregnancies would be investigated prior to pregnancy in a prospective study. To date, only one study managed to do this[18]. Linkage of data of Norway's medical birth registry and of a Norwegian population based study of cardiovascular risk markers revealed an association between preeclampsia and prepregnancy larger waist circumferences, systolic and diastolic blood pressures and non fasting cholesterol levels [18]. Using the same database the association between prepregnancy cardiovascular risk factors and offspring's birth weight was investigated. Blood pressure was inversely related to offspring's birth weight whereas unfavourable lipid profile were positively associated with birth weight, suggesting that both low and high birth weight of the offspring may indicate increased cardiovascular risk for the mother [19].

Our observation of familial clustering of cardiovascular risk factors in families with preeclampsia and IUGR is consistent with previous studies reporting a positive family history of cardiovascular disease in women with preeclampsia[20, 21]. Familial clustering of preeclampsia or IUGR could therefore possibly reflect familial clustering of an underlying cardiovascular susceptibility. Clustering of disease in families may be due to shared genetic- or environmental factors or an interaction of those. Our study does not allow to discriminate between these different causes. Previous studies, however, indicated that genetic factors account for more than half of the predisposition for preeclampsia[22, 23]. Additionally, genetic studies on cardiovascular traits demonstrated that familial clustering is largely due to genetic factors[24-26], which supports the hypothesis of shared susceptibility genes in vascular related pregnancy complications and cardiovascular disease. The ultimate evidence would be identification of these genes. From that perspective, numerous candidate gene studies have been conducted based on pathophysiological considerations as reviewed by Lachmeijer et al, and Wilson et al [2, 27]. For example, polymorphisms in genes encoding elements of the renin-angiotensin system, which are associated with chronic hypertension and therefore thought to be involved in cardiovascular disease, were extensively studied in relation to preeclampsia (and to lesser extent to IUGR). Ward and colleagues[28] were the first to describe an association between preeclampsia and a variant in the maternal angiotensinogen gene, which was confirmed shortly after in an Icelandic/Scottish linkage study[29]. However, others could not confirm this association[30, 31]. Similarly, contradictory results were found for polymorphisms in genes of the other elements of the RAS system. Overall, polymorphisms in over 50 candidate genes in various pathways, such as thrombophilia, endothelial function, immunogenetics, oxidative stress and others, have been investigated in relation to preeclampsia with lack of consistent replication, as reviewed by others [2, 3, 27].

Alternative approaches to elucidate the genetic susceptibility of preeclampsia such as genome wide linkage analyses, which in contrast with candidate gene studies do not require a priori hypotheses, have been encouraging but have not yet resulted in major gene discoveries. Several loci have been identified on different chromosomes including chromosomes 2, 4, 9, 10, 11 and 22[32-36]. *STOX1* gene was identified as a putative imprinted gene for preeclampsia

on chromosome 10[37], yet its role in preeclampsia could neither be confirmed by us (chapter 2.2), nor by others[38,39].

A recent development in the research of genetics of complex disease is the genome wide association study (GWA). Modern genotyping platforms permit rapid genotyping of many thousands or even millions of single nucleotide polymorphisms (SNPs). Subsequently these SNPs are tested for association with disease. Genome wide association is an excellent method of identifying genetic variations that may be frequent and have small effect sizes[40,41]. Large sample sizes with adequate statistical power are required to reliably identify susceptibility genes. The downside of GWA is multiple testing, resulting in a great number of false positive findings[42]. The best way to ensure the accuracy of the genome wide findings is through replication in independent samples.

In chapter 2.3 we described the results of a GWA study with 250.000 SNPs. These results are still preliminary and in urgent need for replication, so caution is warranted when interpreting these results. None of our findings reached genome wide significance. However, we did find suggestive association for preeclampsia with two SNPs in previously reported linkage regions on chromosomes 2 and 10[33, 34], which increases the likelihood of a true positive finding. The previously reported linkage region on chromosome 10[34] was found in women with early onset preeclampsia, all with fetal growth restriction[43], suggesting that this region could also be involved in IUGR. In this region we found four SNPs associated with IUGR, located within the KCNMA and ZNF518 genes. These findings may implicate that a subset of women with preeclampsia (those with IUGR) and women with IUGR babies share susceptibility genes, which would support the hypothesis of a joint genetic aetiology, at least in a subset of cases.

Additionally we identified two new genes, the first (EHBP1) being associated with preeclampsia and involved in insulin regulated endocytic trafficking and the second (GLP1R) being associated with IUGR and involved in the insulin pathway. Both are of special interest in view of the increased cardiovascular risk observed in women with previous preeclampsia or IUGR pregnancies. As no GWA studies on preeclampsia or IUGR have been published yet we cannot compare our findings. We would like to emphasize the need for multicentre collaboration to collect large samples and be able to replicate genome wide findings. In that way, maybe, we will be able to disentangle the genetics of vascular related pregnancy complications.

LIMITATIONS IN DIAGNOSTICS

In genetic studies defining stringent phenotype criteria is important, to minimize phenotypic heterogeneity and thus maximize the power of the study. In this respect preeclampsia and IUGR confront researchers with several problems. Preeclampsia has been (internationally) defined as hypertension exceeding 140/90 mmHg with proteinuria over 300mg/day after 20 weeks of gestation[44]. This definition is useful for research purposes but it forces an diagnos-

tic threshold on the continuous distributions of blood pressure and proteinuria, and masks the phenotypic diversity which characterizes clinical practice. Consequently, women with a fulminating disease course, premature delivery and a growth restricted baby and those with slowly progressing disease with term deliveries are considered as one phenotype. Whether this is correct, and clinical diversity thus represents different severities of the same disease or, alternatively, represents different aetiologies, remains unclear.

IUGR is usually defined as "small for gestational age", which often represents placental pathology, but also includes constitutional smallness without pathology. Consequently a proportion of the healthy fetuses will unjustly be assigned as affected. The introduction of customised growth charts, which adjust for this physiological variation due to maternal height, weight and ethnicity, helps to identify those babies that are pathologically small; that is growth restricted[45]. In our study, IUGR was defined as "small for gestational age" according to the growth curves of Kloosterman[46], as data on prepregnancy weight were not available for all women. For those of whom these data were available (27 out of 56), we did calculate the customized birth percentiles, using the calculator on www.gestation.net. All 27 women had offspring with a birth weight equal or below the third percentile (25 below the 1st percentile, 1 between 1st and 2nd percentile and one at the 3rd percentile) and thus met the criteria of fetal growth restriction. These results assured us that the Kloosterman curves were stringent enough to define IUGR.

Another difficulty in genetic studies of preeclampsia and IUGR is the fact that disease is only expressed during pregnancy and so there is no recognized counterpart in males or nonparous women.

The lack of consistent replication in genetic studies of preeclampsia may be (partly) explained by the phenotypic diversity, possibly reflecting heterogeneity, which is insufficiently appreciated in the current definitions.

Currently two forms of preeclampsia are proposed in literature; placental and maternal preeclampsia[14]. The following subsets are being designated:

-Placental preeclampsia: the pathophysiological sequelae arise from a placenta under hypoxic conditions with subsequent oxidative stress. Often inadequate and incomplete invasion of the spiral arteries is observed. Placental preeclampsia is associated with fetal growth restriction and early onset of disease.

-Maternal preeclampsia: the problem arises from the interaction between a normal placenta and a maternal constitution that is susceptible to or suffers from microvascular disease, as with chronic hypertension, obesity and diabetes. This form is expected to present later in pregnancy with normal fetal growth.

-Mixed presentations: a combination of maternal and placental contributions.

Appreciation of these subgroups in research, will result in more homogeneous study groups, which may hopefully provide us more insight in the pathogenesis of preeclampsia.

IUGR can be categorized, from the view of the baby, into intrinsic causes such as fetal chromosomal abnormalities and extrinsic causes such as infections, maternal drug abuse, severe nutritional deprivation and uteroplacental insufficiency [47, 48]. The latter group was studied in this thesis, which can be further subdivided into two main groups.
-uteroplacental insufficiency due to abnormal placentation in early pregnancy
-uteroplacental insufficiency due to placental accidents such as infarcts or partial abruptions
-or a combination of both abnormal placentation and placental accidents.
When studying IUGR in relation to preeclampsia, research aims to include IUGR due to uteroplacental insufficiency as a consequence of abnormal placentation. In order to create the most homogeneous study group ultrasound with Doppler examination, using customized fetal growth curves, is indispensable[48,49]. Still, some misclassification is inevitable.

AETIOLOGICAL IMPLICATIONS

Considering the retrospective nature of our studies we can only speculate on aetiological implications of our findings. As discussed in the previous paragraphs the results of the studies described in this thesis support a shared pathogenesis by preeclampsia and IUGR, at least for a subset of cases. Our data provide additional evidence for a cardiovascular susceptibility underlying preeclampsia and IUGR. The cardiovascular risk profiles in chapter 3 suggest that women with a history of either early or late onset preeclampsia or IUGR pregnancies all have endothelial dysfunction, albeit to a different extent. Those with previous early onset preeclampsia are significantly more frequently diagnosed with chronic hypertension which possibly reflect a more generalized endothelial dysfunction which caused them to develop a more severe form of disease. Alternatively, temporizing treatment –prolonging disease duration- for the benefit of the child may have caused irreversible damage to the endothelium. Still, it remains unclear why some women who enter pregnancy with a degree of endothelial dysfunction only develop the maternal syndrome (maternal preeclampsia) without placental pathology and others only the fetal syndrome (IUGR) which implicates placental pathology.

Successful placentation depends on a delicate interaction between maternal tissue and the fetal trophoblast[50]. In this thesis, we mainly focused on maternal factors and "ignored" the fetal influences. It is most likely that the combination of fetal and maternal factors determine the phenotype. The fetal contribution can be inferred from previous studies showing that paternal genes, as expressed in the fetus, contribute to the mother's risk of preeclampsia. The risk of fathering a preeclamptic pregnancy is increased among male whose mothers had preeclampsia[51] and among males who previously fathered a preeclamptic pregnancy with another partner [52]. Additionally, change of paternity is associated with an increased risk of preeclampsia[53,54], which is supportive of an immunological model for preeclampsia. However this is beyond the scope of this thesis.

Given the fact that genetic factors increasing the risk of cardiovascular disease may underlie preeclampsia we hypothesized that such factors could also be paternally derived. Consequently, men who fathered preeclamptic pregnancies would then exhibit unfavourable cardiovascular risk profiles as compared to men who fathered uncomplicated pregnancies. However we could not detect such differences (chapter 3.2). This is in accordance with Irgens et al. who did not find an increased risk of cardiovascular mortality in men who fathered preeclamptic pregnancies[55]. The only paternal gene, we are aware of, that has been related to preeclampsia is glutathione S transferase P1-1, which is involved in detoxification capacity of the trophoblast.[56]

CLINICAL IMPLICATIONS

While our study design only allowed us to speculate on aetiological implications, it does allow us to draw conclusions regarding clinical implications. From our data (chapter 3) it is indisputable that women with prior pregnancies complicated by preeclampsia or IUGR exhibit unfavourable cardiovascular risk profiles, already within a decade post index pregnancy. Some previous studies demonstrated similar profiles for women with prior preeclampsia with disturbances in glucose metabolism and a higher prevalence of chronic hypertension[57,58], however, little was known in this respect for women with IUGR babies. Our findings are in line with the epidemiological studies describing an increased risk of cardiovascular morbidity and mortality in these women[12,55,59,60].

At present, there is substantial evidence that women with prior preeclampsia have an approximately twofold increased risk of fatal and non-fatal ischaemic heart disease[11]. Given the high prevalence of cardiovascular disease and its importance as a major cause of mortality in women, the implications on public health are considerable. So far, however, no special medical care or follow up of these women is implemented in clinical practice. It should be recognized that the absolute risk, in these relatively young women, over the short term is low[11]. However, their risk will evolve over subsequent years and provides an opportunity for prevention. So what should clinicians do? Unfortunately, no evidence is currently available on the effectiveness of preventive measures in this particular group of patients, to guide our decision making. Yet, we propose the following. First, patients should be counselled about their remote cardiovascular risk by their obstetrician. Subsequently, women should be screened for traditional cardiovascular risk factors at regular intervals post partum for which they can be referred to their general practitioner. Screening and management of traditional cardiovascular risk factors can be performed according to existing (inter)national guidelines. Clinicians could consider early treatment of modifiable risk factors, especially in the women with higher risk, for instance those who had early onset preeclampsia[11]. However, the difficulty will be, that given the young age of the women, only few of them will have values above

intervention thresholds. For those with values within the normal range, recommendation of a heart-healthy diet and lifestyle is the first choice[61]. Special attention should be paid to abdominal obesity, especially in those with BMI within the normal or mildly overweight range. BMI in such groups is a poor marker to identify individuals at risk of insulin resistance (chapter 3.4). Waist circumference measurements help identifying those individuals. Cut-off value for women recommended in most guidelines is 88 cm (www.nhlbi.nih.gov/guidelines/obesity or www.hartstichting.nl). Management of abdominal obesity, and improvement of insulin sensitivity, is mainly achieved by physical activity[62]. Additional caloric restriction results in more favourable changes in body composition[63].

Simply advising people to undertake a healthier lifestyle is probably not enough to change their behaviour. However, women who had complicated pregnancies may be more receptive. Studies have demonstrated that long-term patient-provider contact is an important determinant of success[64, 65]. Life-style changes will not only reduce the remote cardiovascular risk in these women but may also reduce the risk of pregnancy complications in subsequent pregnancies. These women, in particular, may benefit from preconception care. The implementation of routine preconception care, in our country, has been recently advised by the Health Council of the Netherlands[66] and the minister of Health. The benefits of these life style interventions should be established in the future.

The abovementioned strategies require education of general practitioners on this topic by obstetricians. A first and easy step towards general awareness could imply that obstetricians mention the increased cardiovascular risk and the need for regular screening in the patient's letter of discharge. All in all, the most important is that physicians become aware of preeclampsia and IUGR as novel risk factors of cardiovascular disease. Knowledge is the initial step towards active risk reduction.

FUTURE RESEARCH

A major challenge in the study of preeclampsia and IUGR is to disentangle the causes of the disease from its consequences. In this respect, genetic studies are particularly valuable, as genetic variation may be the cause of a pathophysiology, but not its consequence. The advantages of high throughput high density genotyping and microarray techniques allowing expression of thousand of genes simultaneously, and techniques facilitating screening of proteins promises interesting results in the years to come.

The question remains whether cardiovascular and metabolic disease is the cause or consequence of vascular related pregnancy complications. Identification of common susceptibility genes for vascular related pregnancy complications and cardiovascular disease would be supportive of the first hypothesis. To date, several genes have been described to be major contributors to type 2 diabetes[67,68]. Identification of genes for cardiovascular disease, how-

ever, has been less successful. Given the higher prevalence of insulin resistance in women with previous preeclampsia or IUGR pregnancies, genotyping of these diabetes genes seems a logical next step in research. Furthermore, epidemiological evidence for a cardiovascular susceptibility underlying preeclampsia and IUGR can be provided by studying the presence of cardiovascular risk factors in women prior to pregnancy in relation to subsequent pregnancy outcome in a prospective manner.

Another issue is that, to date, only few studies have investigated the fetal genotype despite the fact that it is widely appreciated that both fetal and maternal genes contribute to the development of preeclampsia and IUGR. The Genetics of PreEclampsia (GOPEC) consortium genotyped 28 SNPs in 7 previously reported candidate genes and excluded their involvement in preeclampsia by genotyping women as well as their families (including fetal genotype)[69]. In future, genome wide scans of children born from preeclamptic pregnancies as well as their siblings born from uncomplicated pregnancies may prove to be helpful in identifying genes.

Success can only be achieved when phenotypes are strictly defined. Future studies should seriously attempt to describe the phenotypes studied in detail. Distinction should be made between early and late preeclampsia and preeclampsia with and without fetal growth restriction. IUGR should ideally be assessed by repeated ultrasound examination, using customized fetal growth curves[49,70]. If IUGR is assessed post pregnancy customized birth percentiles (www.gestation.net) should be used to minimize misclassification of constitutionally small fetuses. Adequate statistical power can only be achieved if large sample sizes are collected. This requires a multicentre (inter)national collaborative approach. Development of common recruitment protocols which will lead to the establishment of large DNA and tissue resources is needed. In our country such a collaboration has been initiated by the university hospitals; the "String- of –pearls" initiative.

Regarding the remote cardiovascular consequences of preeclampsia and IUGR, studies are needed to investigate the effectiveness of periodic risk assessment and life-style intervention programs. In the short term, improvement in cardiovascular risk factors can be evaluated, and ultimately, reduction of cardiovascular morbidity and mortality later in life. Additionally, effects on subsequent pregnancies can be evaluated by comparing recurrence rates of preeclampsia with historical controls.

In summary, despite extensive research the aetiology of preeclampsia and IUGR remains to be elucidated. Advances in modern molecular genetic techniques promise interesting results for the years to come. Currently, the most important message is that care for women with preeclampsia and IUGR does not end with usual obstetrical care but might extend even to life long screening for diabetes and cardiovascular disease.

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5

Summary/ Samenvatting

SUMMARY

Preeclampsia and IUGR are major obstetric complications leading to substantial maternal and perinatal morbidity and mortality worldwide. Despite considerable progress in the understanding of the pathophysiology of preeclampsia and IUGR in the last decades, including the recognition of a genetic component, the aetiology of both disorders remains to be elucidated. It has been hypothesized that preeclampsia and IUGR are related conditions with a (partly) common pathophysiology, but with different clinical manifestations. At present the only definite cure is the delivery of the child and the placenta. Further understanding of the pathophysiology of preeclampsia and IUGR is required to be able to predict disease, develop rational therapies and ultimately prevent disease and its remote consequences, i.e. an increased risk of cardiovascular disease in later life.

The work presented in this thesis aimed at enhancing the understanding of disease mechanisms by studying genetic factors in women with prior preeclampsia and IUGR pregnancies (chapter 2). Additionally, we aimed to provide detailed cardiovascular risk profiles of women with prior preeclampsia and IUGR pregnancies to help designing preventive strategies for future cardiovascular disease in these women (chapter 3).

Chapter 1 provides a general introduction to this thesis. Chapter 2 continues with studies on genetic factors involved in the development of preeclampsia and IUGR. In chapter 2.1 we investigated familial aggregation, consanguinity and parent-of-origin effects in women with previous preeclampsia or IUGR pregnancies, all originating from an isolated Dutch population. Relationships between women were estimated by means of a large genealogy database that contains information on more than 110,000 individuals from the isolate over 23 generations. We found familial aggregation of preeclampsia and IUGR in the same families, supporting a joint genetic aetiology. Further, a higher proportion of parental consanguineous marriages in these women as compared to women from a random sample of this population suggested the possibility of an underlying recessive mutation. Neither evidence was found for a parent-of-origin effect in preeclampsia nor in intrauterine growth restriction.

In the same isolated population, in chapter 2.2, the role of *STOX1* gene, which was previously identified as a putative imprinted gene for preeclampsia following matrilineal inheritance, was evaluated. Given the partly common pathophysiology of preeclampsia and IUGR we also evaluated the role of *STOX1* in IUGR. A distortion in transmission of *STOX1*- Y153H from heterozygous mothers to offspring, which would have been supportive of a parent-of-origin effect, could not be demonstrated. Additionally, comparison of *STOX1*-Y153H frequencies between women with and without previous preeclampsia originating from an outbred population revealed no differences. Therefore, our findings could neither confirm the role of *STOX1* in preeclampsia nor in IUGR.

Chapter 2.3 describes the results of a genome wide association study of preeclampsia and IUGR. Women with and without complicated pregnancies from the isolated population were

genotyped with the Affymetrix 250K Nsp array from the GeneChip® Human Mapping 500K array Set. None of our findings reached an established level for genome wide significance ($p=5 \times 10^{-8}$). However, we did find suggestive associations which were supported by previous linkage studies increasing the likelihood of a true positive finding, on chromosomes 2 and 10. Additionally, we identified 2 new genes of special interest, the first (EHBP1) being associated to preeclampsia and involved in insulin regulated endocytic trafficking and the second (GLP1R) being associated to IUGR and involved in the insulin pathway. These results require verification by replication in other cohorts.

Chapter 3 deals with remote cardiovascular health in women with previous preeclampsia or IUGR pregnancies. All studies, but one (chapter 3.2), were conducted in the previously described isolated Dutch population. Women were examined at a median follow up of 7.1 years post pregnancy. Chapter 3.1 presents the results of a two-generation study, in which cardiovascular risk profiles were compared between women with previous preeclampsia or IUGR pregnancies and uncomplicated pregnancies as well as their parents. Unfavourable risk profiles were significantly more common among women with previous complicated pregnancies as well as their parents. These intergenerational similarities support the hypothesis that a cardiovascular and/or metabolic predisposition underlies preeclampsia and IUGR.

In chapter 3.2 the focus was on severe, very early onset preeclampsia before 24 weeks of gestation. Participants were recruited from a tertiary university referral centre. Subsequent pregnancy outcome as well as parental cardiovascular health were investigated. Women with previous severe, very early onset preeclampsia had an increased risk of preeclampsia in subsequent pregnancies, yet neonatal outcome was, in general, favourable. Regarding cardiovascular health, these women exhibited more risk factors as compared to women with uncomplicated pregnancies. Particularly, chronic hypertension was significantly more common in these women (55% versus 10%). No differences in cardiovascular risk factors were detected between men who fathered preeclamptic pregnancies and those who fathered uncomplicated pregnancies.

Chapter 3.3 describes the results of a study on body composition and fat distribution measured by means of anthropometrics and Dual Energy X-ray Absorptiometry. As compared to women with previous uncomplicated pregnancies, we found preferential fat accumulation in the abdominal- over hip region in women with previous preeclampsia as well as in women with previous IUGR pregnancies, while the latter were lean in comparison with those with previous preeclampsia. Excessive fat deposition, and in particular abdominal fat deposition, has been associated with numerous endocrine alterations including hyperinsulinemia, hypoadiponectinemia, changes in sex hormone levels and reduction of sex hormone binding globulin (SHBG) concentrations.

In chapter 3.4 we therefore investigated the presence of such endocrine alterations and their relation to abdominal obesity in women with previous preeclampsia or IUGR pregnancies and women with uncomplicated pregnancies. Women with previous preeclampsia, as

compared to women with uncomplicated pregnancies, had hyperinsulinemia with associated endocrine alterations including hypoadiponectinemia, hyperandrogenism and reduction of SHBG concentrations which could largely be explained by abdominal obesity. Comparable disturbances in glucose metabolism, but not in adiponectin levels or sex steroids were observed in women with prior IUGR pregnancies, also largely attributable to abdominal obesity. Apart from the deleterious effect of hyperinsulinemia and hyperandrogenism on cardiovascular health these conditions are positively associated with bone mineral density, implying a protective effect against osteoporosis. Hence we also studied bone mineral density in these women. Bone mineral density was only increased in women with previous preeclampsia as compared to those with uncomplicated pregnancies, yet could not be fully explained by abdominal obesity. Considering the findings described in chapter 3 it is indisputable that women after pregnancies complicated by preeclampsia or IUGR exhibit unfavourable cardiovascular risk profiles, already within a decade post index pregnancy. Our data indicate that abdominal obesity should be a main target of preventive strategies, also in normal weight women with previous IUGR. Due to their normal weight these women will often escape from the attention of medical practitioners as being at risk for future cardiovascular disease.

Chapter 4 provides a general discussion on the main findings and places them in a broader context. In short, regarding genetics of preeclampsia and IUGR our findings support a genetic component in the aetiology of these disorders and suggest, at least in a subgroup of patients, a joint genetic aetiology for preeclampsia and IUGR. Further, our genome wide analysis results support that previously reported loci on chromosome 2 and 10 may harbour susceptibility genes for preeclampsia and IUGR. Regarding the remote cardiovascular risk, our studies provide detailed risk profiles of both women with previous preeclampsia as well as IUGR pregnancies. Hypertension and insulin resistance are far more prevalent in these women than in women with previous uncomplicated pregnancies. These results call for action. Follow-up programs for these women should be designed and implemented in clinical practice. Most importantly physicians must be aware that care for these women does not end with the usual obstetrical care.

SAMENVATTING

Preëclampsie en intrauteriene groei retardatie (IUGR) zijn ernstige zwangerschapscomplicaties die wereldwijd leiden tot substantiële maternale en perinatale morbiditeit en mortaliteit. Ondanks aanzienlijke vooruitgang de afgelopen jaren in de kennis van de pathofysiologie van preëclampsia en IUGR, waaronder het erkennen van een genetische component, dient de oorzaak van deze beide aandoeningen nog opgehelderd te worden. Er is gehypothetiseerd dat preëclampsie en IUGR verwante aandoeningen zijn met een (gedeeltelijk) gemeenschappelijke pathofysiologie, maar met verschillende klinisch beeld. Tot op heden is de enige therapie voor preëclampsie het geboren laten worden van het kind en de placenta. Meer inzicht in de pathofysiologie van preëclampsie en IUGR is noodzakelijk om ziekte te kunnen voorspellen, rationele therapieën te kunnen ontwikkelen en uiteindelijk ziekte en de lange termijn gevolgen daarvan, zoals een verhoogd risico op hart- en vaat ziekten, te kunnen voorkomen.

Het werk dat beschreven wordt in dit proefschrift had als doel het inzicht te vergroten in ziekte mechanismen door het onderzoeken van genetische factoren in vrouwen na een zwangerschap gecompliceerd door preëclampsie of IUGR (hoofdstuk 2). Verder was ons doel om van diezelfde vrouwen gedetailleerde beschrijvingen van cardiovasculaire risicoprofielen te geven die kunnen helpen bij het ontwikkelen van preventieve strategieën ten aanzien van toekomstige hart- en vaat ziekten in deze vrouwen (hoofdstuk 3).

Hoofdstuk 1 geeft een algemene inleiding tot het proefschrift. Hoofdstuk 2 volgt met studies over genetische factoren die betrokken zijn bij de ontwikkeling van preëclampsie of IUGR. In hoofdstuk 2.1 onderzochten we familiaire aggregatie, bloedverwantschap, en "parent-of-origin" effecten in vrouwen die voorheen preëclampsie of een zwangerschap gecompliceerd door IUGR doormaakten, allen afkomstig uit een Nederlandse geïsoleerde populatie. Verwantschap tussen vrouwen werd berekend met behulp van een gegevensbestand dat uitgebreide genealogische informatie bevat van meer dan 110.000 individuen uit deze geïsoleerde populatie over 23 generaties. Wij observeerden familiaire aggregatie van preëclampsie en IUGR in dezelfde families, wat de hypothese van een gemeenschappelijke genetische oorzaak steunt. Verder bleken de ouders van deze vrouwen vaker bloedverwant dan ouders van een willekeurige groep vrouwen uit deze populatie, wat zou kunnen passen bij een onderliggende recessieve genetische mutatie. Noch voor preëclampsie noch voor IUGR kon bewijs worden gevonden voor een "parent-of-origin" effect.

In hoofdstuk 2.2. werd in dezelfde geïsoleerde populatie de rol van het *STOX1* gen geevalueerd. *STOX1* werd eerder geïdentificeerd als een gen voor preëclampsie dat "imprinted" zou zijn en via de moederlijke lijn zou overerven. Gezien de gedeeltelijk gemeenschappelijke pathofysiologie van preëclampsie en IUGR, onderzochten we ook de rol van het *STOX1* gen in IUGR. Een dysbalans in transmissie van *STOX1*-Y153H van heterozygote moeders naar hun kinderen, wat een "parent-of-origin" effect waarschijnlijk zou hebben gemaakt, kon niet worden aangetoond voor preëclamspie of IUGR. Verder liet een vergelijking van *STOX1*-Y153H

frequenties tussen vrouwen met en zonder een preëclampsie in het verleden, afkomstig uit de algemene populatie, geen verschillen zien. Zodoende konden onze data de rol van *STOX1* noch in preëclampsie noch in IUGR bevestigen.

Hoofdstuk 2.3 beschrijft de resultaten van een studie waarin het gehele genoom werd gescreend voor associatie met preëclampsie en IUGR. Met behulp van Affymetrix 250K Nsp array from the GeneChip® Human Mapping 500K array Set werden genotypes bepaald van vrouwen met en zonder gecompliceerde zwangerschappen in het verleden, afkomstig uit de geïsoleerde populatie. Geen van onze resultaten bereikten een significantie niveau dat is vastgesteld voor studies van het hele genoom ($p=5 \times 10^{-8}$). Echter, we vonden wel suggestieve associaties op de chromosomen 2 en 10, die aansloten bij eerdere bevindingen van linkage studies, waardoor de waarschijnlijkheid van een ware associatie groter wordt. Verder, identificeerden we 2 genen die speciaal de interesse wekten, namelijk als eerste, EHBP1, die geassocieerd was met preëclamspie en betrokken is bij insuline gereguleerd endocytisch verkeer en als tweede GLP1R geassocieerd met IUGR en betrokken in de insuline huishouding. Deze resultaten dienen geverifieerd te worden door replicatie in andere cohorten.

Hoofdstuk 3 behandelt de toekomstige gezondheid op het gebied van hart- en vaatziekten in vrouwen na zwangerschappen gecompliceerd door preëclampsie en IUGR. Op één (hoofdstuk 3.2) na alle studies werden uitgevoerd in de eerder beschreven Nederlandse geisoleerde populatie. Vrouwen werden onderzocht na een mediane duur van 7.1 jaar na hun zwangerschap. Hoofdstuk 3.1 presenteert de resultaten van een 2-generatie studie waarin cardiovasculaire profielen werden vergeleken tussen zowel vrouwen die in het verleden een preëclampsie, IUGR zwangerschap of ongecompliceerde zwangerschap doormaakten als tussen hun ouders. Ongunstige risico profielen kwamen significant vaker voor bij vrouwen na een gecompliceerde zwangerschap, evenals bij hun ouders. Deze intergenerationele overeenkomsten steunen de hypothese dat een predispositie voor hart- en vaatziekten en/ of metabole ziekten aan preëclampsie en IUGR ten grondslag liggen.

In hoofdstuk 3.2 werd de aandacht gericht op ernstige, zeer vroege preëclampsie met een begin voor 24 weken zwangerschapsduur. Deelneemsters werden gerekruteerd vanuit het Erasmus MC. Uitkomsten ten aanzien van volgende zwangerschappen alsmede gezondheid op het gebied van hart- en vaatziekten van beide ouders na een preëclamptische zwangerschap werden onderzocht. Vrouwen met zeer ernstige vroege preëclampsie hadden een verhoogd risico op preëclampsie in een volgende zwangerschap maar de neonatale uitkomst was over het algemeen gunstig. Ten aanzien van hart- en vaatziekten, bleek dat deze vrouwen meer risicofactoren voor hart- en vaatziekten hadden dan vrouwen met ongecompliceerde zwangerschappen. Met name chronische hypertensie kwam significant vaker voor (55% versus 10%). Geen verschillen in risicofactoren voor hart- en vaatziekten werden aangetoond tussen mannen van vrouwen met preëclamptische- en ongecompliceerde zwangerschappen.

Hoofdstuk 3.3 beschrijft de resultaten van een studie over lichaamssamenstelling en vet verdeling gemeten met behulp van antropometrie en Dual Energy X-ray Absorptiometry.

We vonden dat, vergeleken met vrouwen na ongecompliceerde zwangerschappen, zowel vrouwen met een preëclampsie als vrouwen na een IUGR zwangerschap, terwijl deze laatste slank waren ten opzichte van de vrouwen na een preëclampsie, een voorkeur hadden tot vet depositie in de abdominale- boven de heup regio. Excessieve vetdepositie, en vooral in de abdominale regio, wordt geassocieerd met talrijke endocriene veranderingen, waaronder hyperinsulinemie, hypo-adiponectinemie, verandering in sex hormonen en verlaging van sex hormoon bindende globuline (SHBG) concentraties.

Zodoende hebben we in hoofdstuk 3.4 de aanwezigheid van bovengenoemde endocriene veranderingen onderzocht in relatie tot abdominale obesitas in vrouwen na zwangerschappen gecompliceerd door preëclamspie en IUGR en ongecompliceerde zwangerschappen. Vrouwen na preëclampsie hadden hyperinsulinemie met geassocieerde endocriene veranderingen waaronder hypoadiponectinemie, hyperandrogenisme and lagere SHBG concentraties, wat grotendeels kon worden toegeschreven aan abdominale obesitas. Vergelijkbare veranderingen in glucose metabolisme, maar niet in adiponectine concentraties of sex steroïden werden gezien in vrouwen na IUGR zwangerschappen, ook grotendeels verklaarbaar door abdominale obesitas. Naast het negatieve effect van hyperinsulinemie en hyperandrogenisme op hart- en vaatziekten zijn deze condities positief geassocieerd met bot mineraal dichtheid, wat een mogelijk beschermend effect tegen osteoporose impliceert. Zodoende onderzochten we bij deze vrouwen ook bot mineraal dichtheid. Deze was verhoogd alleen in vrouwen na preëclampsie ten opzichte van vrouwen na ongecompliceerde zwangerschappen, wat echter niet volledig kon worden verklaard door abdominale obesitas.

Gezien de resultaten beschreven in hoofdstuk 3, is het duidelijk dat vrouwen na zwangerschappen gecompliceerd door preëclampsie of IUGR een ongunstig risicoprofiel ten aanzien van hart- en vaatziekten hebben, al binnen 10 jaar na de zwangerschap. Onze gegevens geven aan dat abdominale obesitas een belangrijk doelwit moet zijn van preventieve strategieën, ook in vrouwen met een normaal lichaamsgewicht na zwangerschappen gecompliceerd door IUGR. Door hun normale gewicht zullen deze vrouwen makkelijk aan de aandacht van artsen ontsnappen als zijnde personen met een verhoogd risico op toekomstige hart- en vaatziekten.

Hoofdstuk 4 levert een algemene discussie over de belangrijkste bevindingen en plaatst deze in een bredere context. Samenvattend, ten aanzien van genetische factoren van preeclampsie en IUGR bevestigen onze resultaten een genetische component in the etiologie van deze aandoeningen and suggereren, tenminste voor een subgroep van patiënten, een gemeenschappelijk genetische etiologie voor preëclampsie en IUGR. Verder ondersteunen onze resultaten van de genoom wijde analyse dat eerder gerapporteerde loci op de chromosomen 2 en 10 mogelijk genen bevatten voor preëclamspie en IUGR. Ten aanzien van het toekomstige risico op hart- en vaatzieken, leveren onze studies gedetailleerde risicoprofielen zowel voor vrouwen na zwangerschap gecompliceerd door preëclampsie als voor vrouwen na IUGR zwangerschappen. Hypertensie en insuline resistentie komen frequenter voor in deze

vrouwen ten opzichte van vrouwen na ongecompliceerde zwangerschappen. Deze resultaten vragen om actie. Vervolg programma's voor deze vrouwen dienen ontwikkeld en ingevoerd te worden in de klinische praktijk. Het allerbelangrijkste is dat artsen realiseren dat de zorg voor deze vrouwen niet ophoudt met de gebruikelijke obstetrische zorg.

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Anne Louise Berends was born on October 19, 1976 in Leiden, the Netherlands. In 1994 she graduated from secondary school at the Stedelijk Gymnasium in Leiden. She attended medical school at the University of Utrecht from 1994-2001. During her studies she participated for a period of four months in a research project at the Department of Diabetes & Endocrinology of King's College Hospital London, United Kingdom. After her medical studies she started working as a resident at the Department of Obstetrics and Gynaecology of the Diakonessenhuis Hospital in Utrecht/Zeist for a period of 6 months. From 2002-2004 she worked as a resident at the Department of Obstetrics and Gynaecology of the Groene Hart Hospital in Gouda. In 2004 she started the research described in this thesis at the Department of Obstetrics and Gynaecology, Division of Obstetrics and Prenatal Medicine, in close collaboration with the Departments of Epidemiology & Biostatistics and Clinical Genetics of the Erasmus MC in Rotterdam. In 2008 she started her training in Obstetrics and Gynaecology at the Sint Franciscus Gasthuis in Rotterdam. In 2008 she married Wouter Metz.