Genetic aspects of pre-eclampsia: mutation screening of the low-density lipoprotein receptor, methylenetetrahydrofolate reductase, prothrombin and factor V candidate genes

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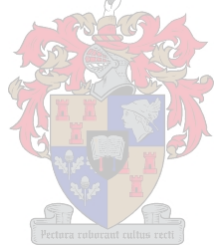

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature **Date**

Summary

Pre-eclampsia is a condition unique to pregnancy and primarily affects the maternal and placental vascular endothelium. It has significant morbidity and mortality consequences for both mother and infant. Despite global research into the aetiology of the condition, the cause for this condition remains unknown. Several factors, including a strong family history of hypertension in pregnancy point to a familial or genetic component in the pathophysiology of this complication.

The purpose of this research project was to investigate candidate genes implicated in endothelial damage. Common methylene-tetra-hydrofolate reductase (MTHFR) gene mutations C677T and A1298C, factor V Leiden mutation R506Q and prothrombin mutation A20210G were investigated in 50 patients with an uncomplicated pregnancy outcome (controls) and 350 patients with various clinical manifestations of pre-eclampsia, including severe, early onset forms and abruptio placentae. Fasting homocystein levels were determined biochemically on all participants.

In addition, 126 consecutive pregnant patients were recruited at booking, fasting lipograms were performed on them as well as mutation screening of 7 common mutations in the low-density lipoprotein receptor gene. This was correlated with eventual pregnancy outcome, and those with an uncomplicated outcome were selected as an additional control group.

A significant association between hyperhomocysteinaemia and early onset severe pre-eclampsia could be demonstrated. Mutant allele T of the C677T mutation could be associated with hyperhomocysteinaemia but not with pre-eclampsia whilst mutant allele C of mutation A1298C demonstrated a significant correlation with diastolic

blood pressure. In addition, combined heterozygosity for these mutations may serve as a marker for abruptio placentae.

Opsomming in Afrikaans

Pre-eklampsie is 'n hipertensiewe toestand uniek aan menslike swangerskap en dit affekteer hoofsaaklik die vaskulêre endoteel. Die toestand hou ernstige morbiditeit en mortaliteit vir beide ma en baba in en na jare se navorsing is die oorsaak van hierdie toestand steeds onbekend. Epidemiologiese studies toon 'n duidelike familiële verband aan wat die vermoede laat ontstaan dat daar 'n onderliggende genetiese aspek tot die ontwikkeling van die siektetoestand is.

Die doel van hierdie navorsingsprojek was om gene te ondersoek wat geïmpliseer word in endoteel skade. Twee algemene mutasies, C677T en A1298C in die MTHFR geen asook faktor V Leiden R506Q en protrombien A20210G mutasies is ontleed in 50 pasiënte met 'n ongekompliseerde swangerskapsverloop en in 350 pasiënte met 'n swangerskap gekompliseer deur verskillende kliniese manifestasies van die siekteproses, insluitende vroeë aankoms erge pre-eklampsie en abruptio placentae. Op alle pasiënte is ook 'n vastende homosistiën vlak biochemies bepaal.

'n Verdere 126 opeenvolgende pasiënte is gewerf tydens hulle eerste besoek aan die voorgeboortekliniek en vastende lipogramme is op almal uitgevoer. Mutasie sifting vir 7 algemene mutasies in die lae-digtheids lipoproteïen reseptor geen is op hierdie groep gedoen en die resultaat is met die uiteindelike swangerskapsuitkoms gekorreleer. Pasiënte met 'n uitkoms ongekompliseer deur hipertensie is gekies om deel te wees van 'n verdere kontrolegroep.

Daar was 'n betekenisvolle verband tussen hiperhomositiënemie en erge, vroeë aankoms pre-eklampsie. Die T alleel van die C677T mutasie is geassosieer met hiperhomosistiënemie maar nie met pre-eklampsie nie. Die C alleel van die A1298C mutasie toon 'n betekenisvolle verband met diastoliese bloeddruk. Gekombineerde

heterosigositeit vir beide MTHFR mutasies kan 'n moontlike merker vir abruptio placentae wees.

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List of abbreviations

µg	Microgram
µl	Microlitre
°C	Degrees Celsius
A	Adenosine
APS	Ammonium persulphate
ASA-PCR	Allele-specific amplification polymerase chain reaction
ATP	Adenosine triphosphate
APCR	Activated protein C resistance
BMI	Body mass index
bp	Basepair
C	Cytosine
CI	Confidence Interval
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
F	Forward
FFA	Free fatty acid
FH	Familial hypercholesterolaemia
FV	Factor V
G	Guanosine
g	Gram
HDL	High-density lipoprotein
HELLP	Hemolysis, elevated liver enzymes, low platelets (syndrome complex in severe pre-eclampsia)
HEX-SSCP	Heteroduplex-single-strand conformation polymorphism method of mutation detection

HLA	Human leukocyte antigen
IUGR	Intra-uterine growth restriction
l	Litre
kg	Kilogram
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
M	Molar
m	Meter
mg	Milligram
mmHg	Millimetres of mercury
mmol	Millimolar
MTHFR	Methylenetetrahydrofolate reductase
n	Number
OR	Odds ratio
PCR	Polymerase chain reaction
pI	Isoelectric point
PIH	Pregnancy-induced hypertension
R	Reverse
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Relative risk
SDS	Sodium dodecyl sulphate
SSCP	Single-strand conformation polymorphism
T	Thymidine
TBE	Tris, Boric acid and EDTA buffer
TEMED	Tetramethylethylenediamine
U	Unit

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Chapter 1

1.1 Introduction

Pre-eclampsia is a condition unique to pregnancy and it primarily affects the maternal and placental vascular endothelium. One of the main problems encountered with pre-eclampsia is the definition of the condition. For the purpose of this thesis, pre-eclampsia will be defined by the criteria for the International Society for Hypertension in pregnancy [Davey and MacGillivray, 1988]. This includes a blood pressure of 140/90 mmHg measured on two occasions, at least four hours apart, arising for the first time after 20 weeks of gestation, coupled with significant proteinuria (300 mg/l in a 24-hour urine collection or 2+ on diagnostic urine sticks). All patients recruited for the different studies in this project were identified according to these diagnostic criteria.

Hypertension is central in the diagnostic criteria of most definitions but is considered only part of the spectrum of the disease that may include intra-uterine growth restriction (IUGR), platelet dysfunction, liver enzyme abnormalities, hemolysis and systemic disturbances of the kidneys, clotting system and endothelial vasculature. Pre-eclampsia is therefore a clinical syndrome recognised by its signs and not a disease, and does not automatically leads to eclampsia, as the name implies.

The hypertensive conditions of pregnancy complicate 5-10% of all pregnancies [Sibai, 1992]. World-wide, between 14% and 20% of primigravidae and 5.7% - 7.3% of multigravidae will have pregnancies complicated by pre-eclampsia [Gabbe et al, 1996]. The only known cure for the condition is delivery of the feto-placental unit. The implication for the often pre-term infant is the complications of prematurity, including neurological damage, respiratory distress syndrome and necrotising enterocolitis. Derangement of multiple organ systems characterise the maternal

symptom complex, including renal failure, liver dysfunction, hepatic rupture, thrombocytopenia, hemolysis, intracranial haemorrhage and diffuse intravascular coagulation. The long-term implication for women with pre-eclampsia is an increased death rate in later life due to ischaemic heart disease [Jonsdottir et al, 1995].

In 1998, hypertension in pregnancy was responsible for 23.2% of direct maternal deaths in South Africa [Moodley and Pattinson, 1998]. Hypertension is the direct cause of 30% of perinatally related wastage at Tygerberg Hospital [Prins et al, 1997] and may indirectly contribute to a further 10-20% of perinatal deaths due to IUGR and (iatrogenic) prematurity with induction of labour for worsening maternal disease [Murphy and Stirrat, 1999]. The perplexing aetiology and pathophysiology of this condition have made it one of the most researched conditions in obstetrics.

There are several theories proposed for the origin of pre-eclampsia. In all likelihood, the common pathophysiological pathway for the development of pre-eclampsia is endothelial cell dysfunction [Dekker et al, 1995]. The classical pathological lesion observed in the placental bed, described as 'acute atherosis', is a nectrotizing arteriopathy consisting of fibrinoid necrosis, the accumulation of lipid-laden macrophages in the decidua and a perivascular infiltrate in the spiral arteries [Sattar and Greer, 1999]. These features are similar to that in arteriosclerotic changes in the non-pregnant population. There is enough evidence from research on hyperlipidemic and cardiovascular patients that elevated serum lipids are associated with endothelial dysfunction [Goode et al, 1995].

In normal pregnancy there is an 8-10 fold increase in prostacyclin production with a less pronounced rise in the vasoconstrictory prostaglandin thromboxane [Wang et al, 1991a]. There is an imbalance in this relationship in patients with pre-eclampsia, with increased levels of thromboxane leading to platelet aggregation on the inner lining of

the spiral arteries which induce fibrin formation. Another endothelium-derived vasodilatory substance, nitric oxide, is probably more important than prostacyclin in maintaining a low vascular tone in the feto-placental unit [Myatt et al, 1992].

A further independent factor for arteriosclerotic disease is hyperhomocysteinaemia. The most common genetic defect that results in mild hyperhomocysteinaemia is a single base-pair substitution in the methylenetetrahydrofolate reductase (MTHFR) enzyme, resulting in decreased plasma folate and impaired homocystein remethylation [Rozen, 1997]. The homocystein levels in women with pre-eclampsia are significantly higher than in nulliparas without pre-eclampsia [Rajkovic et al, 1997]. Hyperhomocysteinaemia is also associated with placental infarctions, a finding common in pre-eclampsia [Goddijn-Wessel et al, 1996].

The presence of a hypercoagulable state in pre-eclampsia is suggested by an increased incidence of heritable causes of thrombosis in pre-eclampsia. A mutation (the Leiden mutation) in exon 10 of the Factor V gene (A1691G), which results in the substitution of arginine with glycine, causes resistance to activated protein C, a naturally occurring anti-clotting factor, by abolishing the site of protein C cleavage [Bertina et al, 1994]. A G/A transition at position 20210 of the prothrombin gene is also associated with an increased incidence of venous and arterial thrombosis. Both of these mutations act as independent risk factors for pre-eclampsia in certain (predominantly Caucasian) populations [Grandone et al, 1999].

Epidemiological studies indicate a strong familial component to pre-eclampsia [Chesley et al, 1961]. Genetic susceptibility to pre-eclampsia has been linked to various chromosomal regions including 7q36 [Guo et al, 1999]. It is likely that there are several different genetic factors associated with maternal susceptibility.

The different factors implicated in the pathophysiology of pre-eclampsia will be investigated in the following sections: 1.2 Genetic aspects of pre-eclampsia 1.3 Homocystein metabolism and mutation, 1.4 Thrombophilia and pre-eclampsia and 1.5 Lipoproteins and pre-eclampsia. Chapter 2 describes the materials and methods used in the studies. The demographic characteristics of the patients involved in the different studies performed are analysed in section 2.2. The results of the different studies are presented in article format in chapter 3.

1.2 Genetic aspects of pre-eclampsia

The problem with collecting affected pedigrees in this disease is that pre-eclampsia affects only women, and then usually only during their first pregnancy. Analysis of the pattern of inheritance is difficult, as the only known marker of a possible defective gene is the development of pre-eclampsia. Half of the population (men) is excluded in any model, so are non-pregnant women and when pregnant, the disease only manifests more than halfway through gestation. With a pregnancy involved, it is difficult to determine whether the syndrome develops from the genotype of the fetus, the mother or a combination of the two.

The susceptibility to pre-eclampsia is highly heritable. Chesley et al [1961] reported the incidence of pre-eclampsia and eclampsia in daughters and sisters of patients with eclampsia to be increased compared with the incidence in the local maternity hospital. The same authors reported an eight-fold increased incidence of pre-eclampsia in daughters of mothers with pre-eclampsia compared with the normal population [Chesley, 1978]. They proposed that pre-eclampsia is caused by a single recessive gene in the affected mother, based on data collected over 49 years [Chesley and Cooper, 1986]. The authors followed 147 sisters, 248 daughters and 74 granddaughters of women who had eclampsia in pregnancy. As controls they used

131 daughters-in-law of these same women. The speculation was that, if the genotype of the fetus was responsible, then the incidence of pre-eclampsia would be the same in all the cohorts, as the babies are all similarly related to the grandmother.

The diagnosis of pre-eclampsia was made in 26% of first viable pregnancies in the daughters and in 6.1% of daughters-in-law. Pre-eclampsia was diagnosed in 16.2% of viable first pregnancies in the granddaughters. The incidence of pre-eclampsia in the sisters of women with eclampsia was 37%. When compared to the incidence of pre-eclampsia in the general population (5-7%) this data clearly demonstrated strong familial incidence. It also implicates susceptibility rather than a shared environment [Morgan and Ward, 1999]. However, this single recessive gene model does not explain the high incidence of pre-eclampsia in twin pregnancies and pregnancies complicated by trophoblastic neoplasia.

Liston and Kilpatrick [1991] examined six possible genetic models for pre-eclampsia with a hypothetical susceptibility gene, with a dominant allele (A) and a recessive allele (a). These are:

1. A maternal recessive gene hypothesis (a,a).
2. A shared recessive gene hypothesis, where both the mother and fetus are (a,a).
3. A fetal recessive gene hypothesis, where only the fetus is (a,a).
4. The maternal dominant gene hypothesis (A,A or A,a).
5. A shared dominant gene hypothesis, where both mother and fetus must be (A,A) or (A,a).
6. The fetal dominant gene hypothesis, where the fetus alone is of genotype (A,A) or (A,a).

They then used a mathematical model to predict an expected proportion of affected pregnancies and compared this with published observations of the proportions of

affected pregnancies in three studies [Kilpatrick et al, 1989; Cooper et al, 1979; Adams and finlayson, 1961] (Table I). They concluded that only the shared recessive gene hypothesis fits all the data and that it is improbable that the other five are correct [Liston and Kilpatrick, 1991].

Early reports demonstrated that pre-eclampsia may be weakly associated with the HLA DR4 genotype [Simon et al, 1988; Kilpatrick et al, 1989]. However, family studies with restriction fragment length polymorphisms did not show close linkage between maternal susceptibility to pre-eclampsia and the HLA-DR4 region [Wilton et al, 1990]. More recently, HLA class II haplotypes DR4 and DQw2 were shown to be associated with low birthweight and low placental weight in 30 pre-eclamptic women [Omu et al, 1998]. This association may represent a direct susceptibility factor.

Other possible candidate genes for pre-eclampsia are those associated with hypertension. So far, no evidence for linkage has been demonstrated between pre-eclampsia and renin, pronatriodilatin, the mineralocorticoid and glucocorticoid receptors, the amiloride-sensitive sodium/potassium antiporter and the alpha-3 and beta-1 sodium/potassium ATPase genes [Hayward et al, 1992].

A genome-wide scan on 343 pre-eclampsia-affected patients and 239 unaffected relatives showed a maternal susceptibility factor for pre-eclampsia on chromosome 2p13 [Arngrimsson et al, 1999]. This data are consistent with the chromosome 2 locus harbouring a highly penetrant variant which is not common in the general population, but is responsible for the cases of pre-eclampsia with a strong familial component.

The possible role of paternal genes in the pathophysiology of the condition is reflected in the high incidence of pre-eclampsia (27%) in women with gestational

trophoblastic neoplasia [Curry et al, 1975]. This intriguing cancer is the result of an abnormal fertilisation process, where an ovum is fertilised by a haploid sperm, which then duplicates its own genetic material. The ovum nucleus is either absent or inactivated. The karyotype of the tumour is usually 46,XX with the chromosomes entirely of paternal origin [Kajii and Ohama, 1977].

Another observation is the linear decrease in the risk for pre-eclampsia with timing of conception within the first year of sexual cohabitation with a male partner. With pregnancy occurring within 4 months of exposure to the male genetic material (sperm), the risk for pre-eclampsia is 40%, a risk which drops to 5% after 12 months of cohabitation [Robillard et al, 1994]. This suggests primipaternity rather than primigravidity as a possible model to explain the high incidence of pre-eclampsia in first pregnancies, especially teenage pregnancies, where pregnancy usually follows rapidly on sexual exposure [Robillard et al, 1999].

If a genetic-immunological model is supposed, it may help explain why the same gene product (for example a recessive gene) could induce pre-eclampsia in the first pregnancy and impose protection against the disease in the next pregnancy.

Table I

Expected proportion of affected pregnancies according to different mathematical models

	Kilpatrick et al, 1989	Cooper et al, 1979	Adams et al, 1961
Observed proportion affected (%)	11.1	18.2	13.8
Expected proportion for each model (%)			
Maternal recessive gene	32.7	38.2	38.2
Shared recessive gene	11.2	18.3	18.3
Fetal recessive gene	5.2	10.1	10.1
Maternal dominant gene	27.0	30.3	30.3
Shared dominant gene	14.3	17.2	17.2
Fetal dominant gene	14.3	17.2	17.2

The correlation between the shared recessive gene model (shaded row) and the actual proportion of the population affected as published in different studies.

1.3 Homocystein metabolism, hyperhomocysteinaemia and placental vasculopathy

The only source of homocystein in Man is that derived from methionine, an essential sulphur-containing amino acid of animal origin. Levels of methionine are four times higher in extra-embryonic coelomic fluid and twice as high in amniotic fluid as in maternal serum during early pregnancy. There is a concomitant lower level of homocystein in these fluids [Steegers-Theunissen et al, 1997]. This suggests a role for methionine metabolism in early pregnancy. Concentrations of homocystein in maternal blood, umbilical vein and umbilical artery taken during parturition reveal a descending gradient, suggesting the likely incorporation of homocystein into the fetal metabolic cycle [Malinow et al, 1998].

Homocystein is metabolised via two pathways, namely re-methylation or trans-sulphuration (Figure I). In the trans-sulphuration pathway, the sulphur atom of homocystein is ultimately transferred to cysteine in a series of reactions. A pyridoxal-5'-phosphate (Vitamin B6) dependent enzyme, cystathione-ß synthetase, catalyses homocystein to condense with serine to form cystathione. In cystathione-ß synthetase deficiency there is accumulation of homocystein and methionine in body fluids with decreased concentrations of cysteine and cystine. Homocystein interferes with normal cross-linking of collagen and plays an important role in the vascular thrombotic complications that arise from this disease [Rosenberg, 1994].

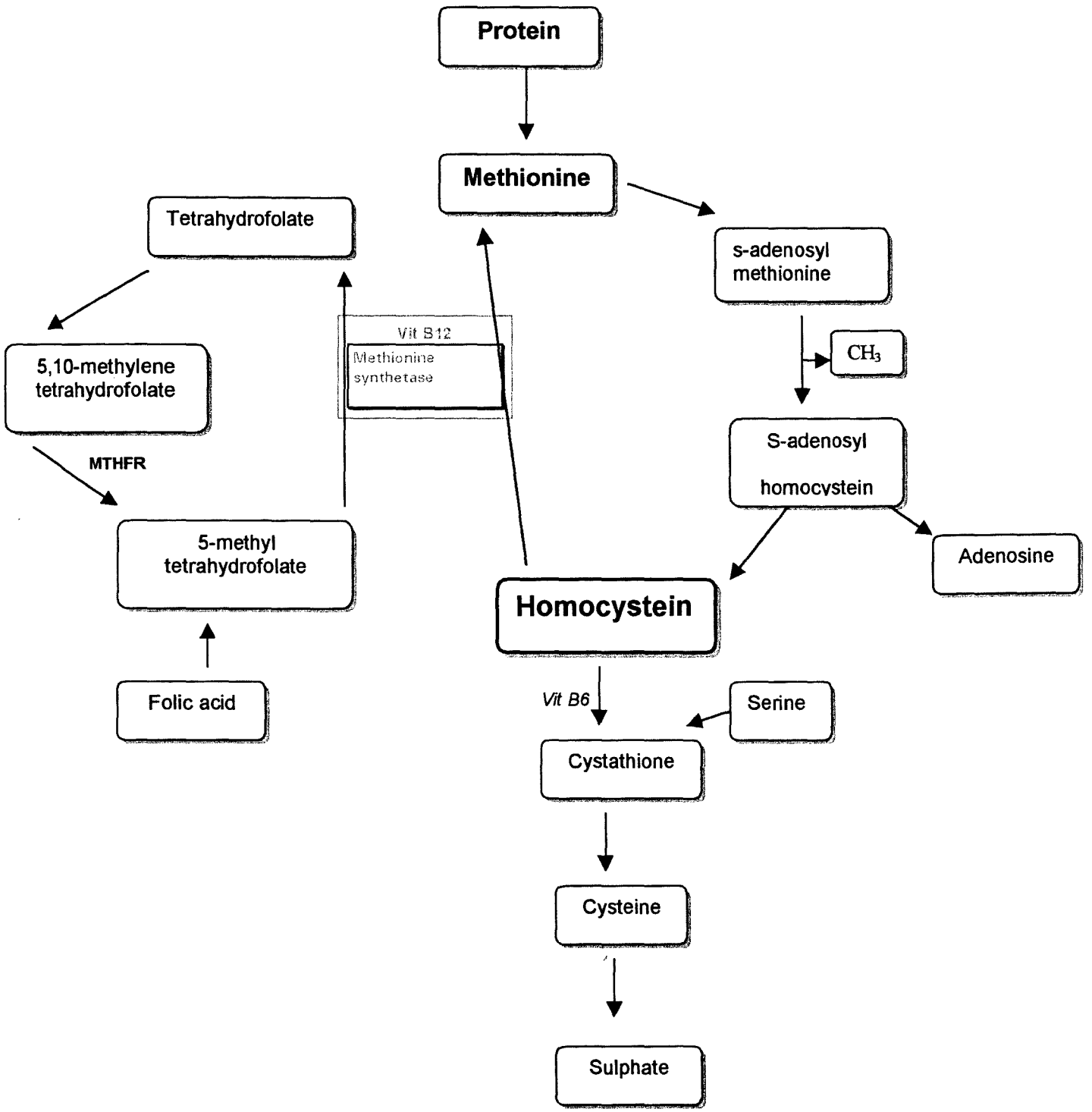


Figure I
Homocystein metabolism

In the re-methylation pathway, a methyl group is added to homocystein to convert it to methionine. The methyl group is derived from the conversion of 5-methyltetrahydrofolate to tetrahydrofolate. This reaction is Vitamin B12 dependent and requires the enzyme methionine synthetase. 5-Methyltetrahydrofolate is synthesised enzymatically from 5,10-methylenetetrahydrofolate by another folate cycle enzyme, 5,10-methylenetetrahydrofolate reductase (MTHFR). This MTHFR enzyme is therefore critical for methionine synthesis and for tetrahydrofolate generation. These reactions are essential for normal DNA and RNA function [Perry, 1999].

Hyperhomocysteinaemia can occur in two forms. The severe form, usually referred to as homocysteinuria, is autosomal recessively inherited and results from deficiencies in the cystathione-ß synthetase or methylenetetrahydrofolate reductase (MTHFR) enzymes. The mild form is a risk factor for vascular disease, but does not cause overt disease [Boushey et al, 1995].

The severe forms of hyperhomocysteinaemia are rare, with only 10 children diagnosed with the reductase deficiency by 1990 and about 50 cases described world-wide. These deficiencies are too rare to account for more than a small proportion of hyperhomocysteinaemic patients with cardiovascular disease [Kluijtmans et al, 1996]. A thermolabile variant of MTHFR was described in 1995 where a point mutation at nucleotide 677 is responsible for the thermolabile phenotype [Frosst et al, 1995]. Although defective, the enzyme is still functional. It has decreased stability and is a plausible candidate for moderate hyperhomocysteinaemia and arteriosclerotic disease.

The human MTHFR locus was mapped to chromosome 1p36.3 by *in-situ* hybridisation [Goyette et al, 1994]. With the single-strand conformation polymorphism

method (SSCP) for mutation detection, a C to T substitution at basepair 677 was identified. This resulted in the substitution of a valine for an alanine residue. Homozygous mutant individuals have significantly higher plasma homocystein levels [Ma et al, 1996]. Plasma folate plays an important role in regulating homocystein in these patients, and hyperhomocysteinaemia is observed primarily when the plasma folate levels are lower [Jacques et al, 1996].

Boushey and colleagues [1995] published a meta-analysis that summarised data on homocystein levels in more than 4000 patients and several thousand individuals. These data were calculated on fasting levels of homocystein alone. The relative risk for persons with elevated homocystein for coronary artery disease was 1.7 (95% CI 1.5-1.9); for cerebrovascular disease 2.5 (95% CI 2.0-3.0) and for peripheral arterial disease 6.8 (95% CI 2.9-15.8). A large multicenter case-control study from 19 centres and 11 European countries, the European Concerted Action Project, determined the risk factor of elevated plasma homocystein in vascular disease in a prospective manner. They found that mild hyperhomocysteinaemia was an independent risk factor for vascular disease, as was smoking, hypertension and hypercholesterolaemia. Interestingly, there were synergistic interactions between hyperhomocysteinaemia and hypertension and to a lesser effect with smoking, indicating a potentiating effect if these factors were jointly present [Boers, 1997].

The mechanism by which homocystein promotes atherosclerosis is not well understood. It may exert these effects through a direct action on the endothelium, on clotting factors or on platelets. The platelet life span is normal in hyperhomocysteinaemia. Platelet aggregation is normal and platelet morphology also appears normal [Uhlemann et al, 1976]. It does not appear that the vascular damage is mediated by abnormal platelet function. In high concentrations, homocystein impairs regulation of nitric oxide and other endothelial-derived vaso-active

substances. It may also damage endothelial cells by the generation of free radicals [Perry, 1999].

To investigate the direct effect of homocystein on the endothelium, Chambers and colleagues [1999] studied the brachial artery diameter response with high-resolution ultrasound in 17 healthy volunteers. They measured the response to hyperaemic flow (endothelium dependent) and glyceryl trinitrate (endothelium independent) after methionine and placebo administration. To investigate whether homocystein impairs endothelial function through oxidative stress, they also studied pre-treatment with Vitamin C before the methionine loading. They found that an acute elevation in homocystein concentration is associated with a rapid (within 2 hours) onset of endothelial dysfunction that can be prevented by pre-treatment with an anti-oxidant like Vitamin C.

This also emphasises the importance of nutritional aspects in this condition. Folate supplementation dramatically reduces the incidence of neural tube defects [Centers for Disease Control 1992] and the MTHFR C677T polymorphism frequency is increased two to three times in families of offspring with neural tube defects [Van der Put et al, 1995]. With the relevance of folate, Vitamin B6 and B12 in the metabolic pathways of homocystein, intervention studies with food fortification and vitamin supplementation is necessary [Motulsky, 1996]. Interestingly, plasma concentrations in pregnant women with a controlled (400-800μg daily) intake of folate are still significantly lower than in non-pregnant controls; this may be a physiologic response to pregnancy [Bonnette et al, 1998].

Guttormsen and colleagues [1996] screened 18 043 healthy subjects for elevated total plasma homocystein and identified 67 cases. Homozygosity for the C677T mutation was found in 73.1% as opposed to 10.2% in a control group. In 37 patients

intervention with low dose folate (0.2 mg/day) was started and within 7 weeks plasma homocystein was reduced in 35 of them.

The long-term health impact of this type of intervention has not yet been addressed so far [Bakker and Brandjes, 1997]. A pilot study on 14 patients with a positive methionine-loading test (after a first pregnancy complicated by pre-eclampsia) showed recurrent pre-eclampsia in 50% of them in the following pregnancy. They were each treated with folate and Vitamin B6 in this pregnancy. The median birthweight of the second pregnancy was 2867g compared with 1088 g in the first pregnancy [Leeda et al, 1998].

There is a considerable population specificity in MTHFR locus allele frequencies. Fletcher and Kessling [1998] summarised data from 37 studies published in the English language up to 1998. The frequency of the C677T mutation in healthy individuals varies from 16.3% in an Italian population, to 11% (Japanese), 5.4% (Dutch) and was absent in Black Americans.

Homocystein levels are increased in a subset of women with severe pre-eclampsia. Postpartum levels were measured as part of an investigation into underlying disorders associated with severe early-onset pre-eclampsia [Dekker et al, 1995]. A positive methionine loading status was present in 17.7% of 79 patients tested. Homocystein levels were also measured by Rajkovic and colleagues [1979] to determine whether it is elevated in pregnancy complicated by pre-eclampsia. They found plasma homocystein levels to be significantly higher in 20 nulligravid patients with pre-eclampsia when compared with 20 healthy nulligravidas. However, folic acid and vitamin B12 levels were not significantly different between the two groups.

Hyperhomocysteinaemia is also a risk factor for placental abruption and infarctions [Goddijn-Wessel et al, 1996]. A study group of 84 women with prior placental abruptio or infarctions were subjected to a methionine loading test in the non-pregnant state; the incidence of abnormal homocystein concentration was 31%, in contrast to 9% in a control group of patients with uncomplicated pregnancy ($p < 0.05$).

A second mutation in the MTHFR gene (A1298C) was subsequently reported to occur with a high carrier frequency in the general population [Weissberg et al, 1998]. This mutation is also associated with decreased enzyme activity, but an isolated mutation (heterozygous state) does not result in hyperhomocysteinaemia [Van der Put et al, 1998]. Combined heterozygosity for both C677T and A1298C mutations does lead to hyperhomocysteinaemia [Van der Put et al, 1998] and may also be associated with abruptio placentae [Gebhardt et al, 2000]. The role of this and other novel mutations in placental vasculopathy and other complications of pregnancy remain to be elucidated.

1.4 Thrombophilia and pre-eclampsia

During normal pregnancy there are dramatic changes in the coagulation and fibrinolytic systems. There is deposition of fibrin in the uteroplacental walls and fibrinolysis is suppressed. There is an increase in the levels of clotting factors VII, VIII and X and a doubling in the levels of fibrinogen. The end result is the well-described hypercoagulability of pregnancy, protecting the mother against blood loss at delivery, but also predisposing to thrombotic complications. Pulmonary embolism is the leading cause of maternal deaths, alongside hypertensive disease, in developed countries.

When a blood vessel is damaged, a complex interaction of clotting factors, the coagulation cascade, is initiated by the activation of Factor XII by collagen and the activation of Factor VII by thromboplastin release. The end result is the formation of an insoluble fibrin clot from the soluble precursor fibrinogen. Thromboplastin, a specific lipoprotein, occurs in large concentrations in the placenta. Protection against generalised thrombosis is supplied by naturally-occurring anticoagulants, of which antithrombin III and the protein C-thrombomodulin-protein S complex are the most important. Protein C inactivates Factors V and VIII in conjunction with its cofactors protein S and thrombomodulin. Abnormal forms of Factor V resist such inactivation and can lead to thrombosis.

The association between placental infarcts and adverse pregnancy outcome, especially midtrimester pregnancy loss and the development of pre-eclampsia, is well described in the antiphospholipid syndrome. In this condition, anticardiolipin antibodies are active against phospholipids in arterial and venous cell walls. The higher the titre of the lupus anticoagulant and cardiolipin antibodies, the greater the risk to the fetus [Branch et al, 1985; 1988]. If one accepts this pathogenesis, a logical hypothesis would be that other forms thrombophilia should also be associated with

adverse pregnancy outcome [Nelson-Piercy, 1999]. The term thrombophilia refers to acquired (antiphospholipid syndrome) or inherited abnormalities that alter the haemostatic balance in favour of fibrin formation.

One such abnormality, described by Dahlback and colleagues in 1993, is activated protein C resistance (APCR) Coagulation factor V acts as cofactor for activated Factor X to activate prothrombin in the coagulation cascade. Factor V is inactivated by cleavage by activated protein C. The human Factor V gene contains 25 exons that range from 72 bp to 2820bp in length and spans more than 80 kb of genomic DNA [Simioni, 1999]. Bertina and colleagues [1994] described a mutation in exon 10 of the Factor V gene (A1691G) which results in the substitution of an arginine with a glycine residue and abolishes the recognition site of protein C cleavage. This variant was named the FV Leiden mutation, after the city in which it was discovered. In the United States 5% of Caucasians and 1% of Blacks are heterozygous for this mutation [Rouse et al, 1997].

The relationship between activated protein C resistance and adverse pregnancy outcome was first reported in 1996. At a specialist recurrent miscarriage clinic, the prevalence of APCR was significantly higher among women with a history of second-trimester miscarriage (20%) when compared with a control group (4.3%; $p<0.02$) [Rai et al, 1996]. In the same year results of the European Prospective Cohort on Thrombophilia (EPCOT) were published [Preston et al, 1996]. The study included 1384 women; of 843 women with thrombophilia, 571 had 1524 pregnancies between them. In the control group of 541 women, 395 had 1019 pregnancies. They studied combined effects of FV Leiden mutation, antithrombin III deficiency and protein C and S deficiencies. The highest odds ratio for stillbirth was in women with combined defects (OR 14.3, 95% CI 2.4-86). After adjustment for all possible confounding

factors, FV Leiden mutation was not recognised as a risk factor for miscarriage. Unfortunately the incidence of hypertensive disease in this cohort was not reported.

The association between FV Leiden mutation and pre-eclampsia was first reported in 1995 in a small patient cohort with severe early-onset pre-eclampsia [Dekker et al, 1995]. In a larger study of 158 women with severe pre-eclampsia, the incidence of FV Leiden mutation was 8.9% and 4.2% in a control group (χ^2 4.686, p=0.03). All patients were heterozygous for the mutation [Dizon-Townson et al, 1996]. Since then, several reports from different parts of the world confirmed or disputed this association (Table II). The prevalence of the mutation in Africa is reported to be below 1% [Rees et al, 1995].

In 1996, a guanine to adenine transition at position 20210 in the 3' untranslated region of the prothrombin gene, was described [Poort et al, 1996]. This mutation (G20210A) was associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis. An independent association with pre-eclampsia was reported in 1999 from Italy [Grandone et al, 1999]. In 140 Caucasian patients with pre-eclampsia, compared to 216 healthy normotensive women, the prothrombin A20210G mutation was strongly associated with proteinuric hypertension (OR 3.31; 95% CI 1.12-6.56). In 110 Israeli women (48 who are from the Ashkenazi ethnic group) with serious pregnancy complications (abruptio placentae, severe pre-eclampsia, IUGR) the incidence of the A20210G mutation in the prothrombin gene was 10% compared to 3% in a control group (p<0.03) [Kupferminc et al, 1999]. An association with IUGR could not be established in a small study of 35 Caucasian patients with IUGR [Wisotzkey et al, 1999].

Table II

Distribution of Factor V Leiden mutation world-wide

Country/ region	Condition	n	Incidence	Reference
Israel	Recurrent fetal loss	39	48%	Brenner et al, 1997
Israel	Poor obstetric history	7	100%	Rotmensch et al, 1997
Hungary	Severe pre-eclampsia	pregnant = 71 non-pregnant = 58 pre-eclampsia = 69	7% 5.2% 18.8%	Nagy et al, 1998
New South Wales	Severe pre-eclampsia	pregnant = 150 pre-eclampsia = 50	0.07% 8%	Mimuro et al, 1998
	HELLP	21	19%	Krauss et al, 1998
	pre-eclampsia	116 FV positive women	25%	Hastings et al, 1998
Italy	pre-eclampsia	controls = 4 pre-eclampsia = 11	1.8% 7.9%	Grandone et al, 1999
East Anglia (UK)	pre-eclampsia	control = 200 pre-eclampsia = 283	5.3% 5.5%	O'Shaughnessy et al, 1999
New York	IUGR	General population IUGR = 35	7.9% 0%	Wisotzkey JD et al, 1999
Israel	pre-eclampsia	110	20%	Kupferminc et al, 1999

1.5 Lipoproteins and pre-eclampsia

Fatty acids are an important precursor for prostaglandins and membrane lipids and are also a critical source of metabolic energy. Unsaturated non-esterified fatty acids (free fatty acids, FFA) are intrinsically toxic and have the ability to form free radicals.

During normal pregnancy, there are substantial changes in lipid metabolism. There is a consistent increase in plasma triglycerides, cholesterol and phospholipids with proportional enrichment of triglycerides in the lipoprotein fractions. Plasma cholesterol concentration rises by approximately 25% and the triglyceride concentration rises 2-3 fold [Potter et al, 1979]. FFAs are mobilised from maternal adipose tissue and this increased lipid transport supports growth and development of the feto-placental unit. The greatest change occurs in the very low-density lipoprotein (VLDL) triglycerides [Montelongo et al, 1992]. The main factor responsible for this rise is an increased liver production of VLDL triglycerides and a decreased elimination from the maternal circulation. The importance of these raised levels for growth in the fetus is demonstrated by the marked abnormalities in fetal development occurring in conditions with altered maternal lipid metabolism, such as overt diabetes mellitus or hypothyroidism [Herrera et al, 1997].

VLDL is composed of triglyceride (50%), phospholipid (18%), cholesterol ester (16%), protein (7%) (which consists of approximately 50% Apo B, 45% Apo C and a trace Apo A), cholesterol (6%) and FFA (3%) [Arbogast et al, 1996]. The primary function of VLDL is the transport of endogenous and dietary triglycerides, the major source of stored energy. The abundance of VLDL triglycerides in the maternal plasma may contribute to the accumulation of triglycerides in the low-density (LDL) and high-density lipoproteins (HDL).

In familial hypercholesterolaemia (FH), increased levels of LDL lead to accelerated endothelial damage in the form of atherosclerosis. Endothelial cells line the vascular system and modulate vascular tone by the secretion of modulating factors such as prostacyclin and nitric oxide (vasodilatory) and thromboxane and endothelin (vasoconstricting). Endothelial injury is an early event in pre-eclampsia. This is demonstrated with fibronectin, a marker for endothelial cell damage, where elevated levels are found long before clinical manifestation of pre-eclampsia in patients destined to develop this condition [Gebhardt, 1998]. By similar analogy, the increased levels of lipoproteins observed in pregnancy may cause endothelial damage, precipitating the cascade of events that eventually lead to pre-eclampsia.

In rat models, aortic endothelial cell cultures show injury within one hour of exposure to diabetic rat serum. The toxic component in the diabetic serum was identified as VLDL [Arbogast and Taylor, 1996a]. The toxic activity of VLDL occurred in the presence of all other serum components and the toxicity of the serum disappeared with the removal of VLDL. *In vitro* serum toxicity of VLDL was also shown during pregnancy in rats. This toxicity increased throughout gestation and disappeared after birth [Chan and Pollard, 1978]. All the toxicity is associated with the triglyceride subfraction rather than with the lipoprotein fractions. Other components carried by VLDL (prostaglandin F2α, 17β-estradiol, progesterone, 25 hydroxycholesterol, monopalmitolein, elaidyl alcohol, α-linolenyl alcohol or free fatty acids) were tested for toxicity, but the toxicity appeared to be due to the VLDL fraction itself [Chan and Pollard, 1981].

With these results, it was hypothesised that hypertriglyceridaemic human serum might also be toxic to endothelial cells *in vitro*. However, this was not observed [Arbogast, 1988]. Instead, a factor present in human sera masked this toxicity. This

factor, named toxicity-preventing factor, was identified as human plasma albumin at isoelectric point (pI) 5.6 [Arbogast and Taylor, 1996b]. The other major isoelectric form of human plasma albumin is isoelectric point 4.8. The pI 4.8 form contains more FFA and conversion from pI 4.8 to pI 5.6 suggests a loss of FFA. The pI 4.8 form is responsible for transport of most of the FFA and remains constant throughout pregnancy. The levels of the toxicity-preventing factor, plasma albumin pI 5.6, decreases throughout normal pregnancy and eventually a point must be reached where the toxicity of VLDL is expressed.

Pre-eclampsia is characterised by endothelial damage, leading to permeability of the vascular endothelium, with leakage of plasma proteins, including albumin, to the tissue. This results in the oedema commonly observed. With protracted loss of fluid in the third space, maternal ascitis and pulmonary oedema develop. An increased VLDL and lower toxicity-preventing factor early in pregnancy correctly identified patients who subsequently developed pre-eclampsia [Arbogast et al, 1996].

A lower level of albumin early in pregnancy can be due to nutritional deficiencies. It may also be that a genetic susceptibility to endothelial damage in the form of hypercholesterolaemia predisposes to the development of this disease. The concentration of free fatty acids is elevated months before the development of pre-eclampsia [Lorentzen et al, 1995]. There is also a significant association between first trimester total serum cholesterol and the risk of pre-eclampsia. Van den Elzen and colleagues [1996] measured serum total cholesterol and HDL in 393 women over the age of 36 years in the first trimester of pregnancy. Serial levels were determined throughout pregnancy and correlated with the eventual outcome. They found that the risk for pre-eclampsia increased 2.2 fold for every 1 mmol rise in baseline serum cholesterol (95% Confidence Interval 1.2-4.2). The relative risk for

pre-eclampsia exceeded 5 with a serum total cholesterol above 6 mmol/l when compared to a level below 5 mmol/l (RR = 5.2; 95% CI 1.2 - 22.5).

Postmenopausal women with a history of recurrent hypertension during their pregnancies have significantly increased diastolic blood pressure and atherogenic profiles when compared to matched controls without a prior history of hypertension in pregnancy [Hubel et al, 2000]. Also, the risk of dying from ischemic heart disease in later life is increased in women who had hypertensive disease in pregnancy (RR 2.61, 95% CI 1.11- 6.12) [Jonsdottir et al, 1995]. It is likely that a combination of genetic predisposition and environmental factors for atherosclerosis in later life is unmasked earlier in life, by pregnancy, as pre-eclampsia. Further evidence for this potential metabolic syndrome is an elevated body mass index (BMI; kg/m^2) in women with pre-eclampsia, regardless of parity, before, during and after pregnancy; compared with women with normotensive pregnancies [Barden et al, 1999].

There is very little information available on the reason for the exaggerated response of plasma lipids during and preceding pre-eclampsia. There is a spectrum of mutations identified in the promoter and coding region of the low density lipoprotein receptor (LDLR) gene associated with FH [Day et al, 1997; Varret et al, 1998], incorporated in two databases, http://www.ucl.ac.uk/fh and http://www.umd.necker.fr/. This disease is common in the South African population and three founder mutations in the LDLR gene account for 90% of the FH cases in the Afrikaner group {Kotze et al, 1989; Leitersdorf et al, 1989]. These mutations also contribute to the hypercholesterolaemia phenotype in the indigenous South African population of mixed ancestry [Loubser et al, 1999], which has a high incidence of pre-eclampsia as well. African women are also particularly prone to hyperlipidaemia during normal pregnancy [Ahaneku et al, 1999].

Apart from these three mutations, four other are of particular interest in the study population. A six basepair deletion in exon 2 of the LDLR gene predominates in Africans with familial hypercholesterolaemia [Thiart et al, 2000]. A recently described -175G/T variant in the footprinting 2 (FP2) *cis*-acting regulatory element of the LDLR gene may play an important role in hypertension by virtue of a possible effect on calcium metabolism [Scholtz et al, submitted]. Two other common mutations in exon 4, a 3 bp deletion (651 del GGT) [Meiner et al, 1991] and an A to G substitution at position 662 (D200G) can also be detected using the same primer set as for the other mutations in exon 4 [Hobbs et al, 1992]. The role of these seven mutations has not been examined in the pathophysiology of pre-eclampsia.

Another lipoprotein subfraction that increases significantly in pre-eclampsia is small, dense low-density lipoprotein (LDL-III) [Sattar et al, 1997]. These small LDL subfractions are more atherogenic than larger LDL species [Witzum, 1993]. They are also more susceptible to oxidation [Wang et al, 1991a] and oxidised LDL inhibits prostacyclin and nitric oxide synthesis [Chin et al, 1992]. The result is platelet activation, thromboxane release and vasospasm.

If increased levels of lipoprotein fractions contribute to the pathogenesis of pre-eclampsia through direct endothelial damage, resolution of the disease post-partum should be accompanied by a decrease in lipoprotein fractions. This was investigated by Hubel et al in 1996. They collected venous blood samples pre-delivery and 24 hours and 48 hours post-delivery in eight women with pre-eclampsia and in nine healthy pregnant patients. Circulating triglycerides and FFAs were dramatically elevated in the pre-eclampsia group and this decreased within 48 hours post partum.

Apolipoprotein E (apoE) plays an important part in lipid metabolism and an increased frequency of the ε2 allele has been reported in women with severe pre-eclampsia

[Nagy et al, 1998; Williams et al, 1996]. In a South African study involving a Western Cape population group of mainly Black and Coloured patients, an increased frequency of the ε2 allele was found in the pre-eclampsia group (19.9%) as well as in the control group (19%) [Burton et al, submitted]. In this population the ε2 allele is not a risk factor for pre-eclampsia, but the high frequency thereof in the general population may have cardiovascular implications in later life.

1.6 Hypothesis

The hypertensive conditions of pregnancy constitute a major social health issue and exert a huge strain on the already-stressed health budget of the country. The clinical presentation is highly variable due to the multi-systemic nature of the condition. After more than a hundred years of dedicated research, no definite underlying cause for the condition has been identified. Most research is currently directed towards the management of the condition once it is diagnosed, with different conservative management options involving anti-hypertensive or other drugs. A careful balance must be maintained between early delivery, with its implications for fetal mortality and morbidity, and conservative management with its possible adverse complications for the mother.

Other areas of extensive research has included investigation of fluctuations in various markers for endothelial damage and in possible preventative measures with aspirin and other drugs which exert their influence on the endothelium.

DNA technology has advanced at a rapid pace and the entire human genome has recently been sequenced. The tools are now available to dissect pre-eclampsia on a molecular level. In most complex, multifactorial diseases like multiple sclerosis,

ischemic heart disease or diabetes mellitus, there are complex gene-gene interactions and interaction between genes and environmental factors present.

In this study, an approach was adopted in an attempt to understand the pathophysiology of pre-eclampsia. The aim was to investigate candidate genes implicated in endothelial vascular damage:

- To elucidate the role of mutations C677T and A1298C in the MTHFR gene in pre-eclampsia and abruptio placentae.
- To confirm or dispute a previous finding of combined heterozygosity for these mutations as a possible marker for abruptio placentae.
- To determine the contribution of inherited thrombophilia (the prothrombin mutation A20210G and the factor V Leiden mutation) in the development of pre-eclampsia and abruptio placentae
- To determine whether there is a correlation between mutations in the MTHFR gene and clinical significant hyperhomocysteinaemia in the South African population
- To investigate the role of hyperhomocysteinaemia in the pathophysiology of placental vasculopathy
- To investigate the possible genetic contribution of mutations in the low-density lipoprotein receptor in the development of pre-eclampsia. The hypothesis in this regard is that common mutations in the LDLR gene associated with hypercholesterolaemia also predispose to the development of pre-eclampsia.

Once the genetic aspect of pre-eclampsia is unravelled it may help to make informative conclusions on pre-conceptual supplementation with folic acid and various combinations of vitamins in the prevention or postponement of pre-eclampsia.

Chapter 2

Materials and methods

2.1 Patient selection

The study population consists of several groups and two control groups. The first group (Group A) consists of 50 multigravidae that had pregnancies uncomplicated by any of the hypertensive conditions (in the index pregnancy or any previous pregnancy). They were selected after delivery at term from consecutive uncomplicated pregnancies in the labour ward at Tygerberg Hospital. Patients with infants with a birth weight below the tenth centile for gestation were excluded to prevent inclusion of a possible case of intra-uterine growth restriction. The second control group (Group L) (n=126) were selected at their first antenatal visit to Tygerberg Hospital to represent the hospital population and they were followed prospectively. This selection was done in 3 months, including all consecutive new patients. At booking, fasting lipograms were performed on these patients for the lipid evaluation part of the study.

The study groups were selected as follows:

B. Study group of 50 primigravidae with onset of pre-eclampsia before 34 weeks.
C. Study group of 50 primigravidae with onset of pre-eclampsia after 34 weeks.
D. Study group of 50 multigravidae with severe pre-eclampsia before 34 weeks.
E. Study group of 50 multigravidae with onset of pre-eclampsia after 34 weeks.
F. Study group of 50 primigravidae with pregnancy-induced hypertension.
G. Study group of 50 multigravidae with pregnancy-induced hypertension.
H. Study group of 50 patients who developed abruptio placentae during the pregnancy, regardless whether it was accompanied by pre-eclampsia or not.

Hypertension and pre-eclampsia are defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy (see 1.1, introduction). Abruptio placentae is defined as an abnormal early detachment of the placenta, followed by intra-uterine or vaginal bleeding. This is deemed significant when more than 15% of the placenta is covered by a blood clot following delivery.

This is a non-experimental cohort-analytical study that ran over a 2-year period. Informed consent was obtained from all patients using a specially designed form approved by the Ethics Committee of the University of Stellenbosch (Appendix). The project, including all ethical aspects, was approved by the Ethics Committee on 8 March 1999 (project number 99/025).

2.2 Demographic characteristics

The demographic characteristics of the patients in the different groups are shown in Table III. Information on the taking of folate and iron supplementation was obtained from each patient and verified from the antenatal charts. More than 83% of patients in all the groups were taking prophylactic supplements.

The racial distribution for the 4059 deliveries at Tygerberg Hospital in 1999 was as follows:

- Coloured (Mixed Ancestry Group) 3362 (82.8%)
- Black 636 (15.6%)
- Caucasian 61 (1.6%)

The racial distribution between the groups is shown in Figure II. There were significantly more Black patients with early onset severe pre-eclampsia (primigravidae: $p = 0.0033$; multigravidae: $p = 0.0315$).

Forty percent of patients in the control group admitted to smoking at any time during their pregnancy. There is a significant lower incidence of smokers in all the hypertensive groups (groups B-G), confirming published studies on the puzzling apparent protective effect of cigarette smoking against the development of pre-eclampsia [Zhang et al, 1999]. There were significantly more smokers in the abruptio group ($p = 0.0012$) than in the hypertensive groups combined (Figure III).

There was a strong history of hypertension in pregnancy in the mothers of daughters admitted to the project in all the groups except the control group (Figure IV) and the abruptio group. The numbers are too small to make any significant epidemiological conclusion, but it is conspicuous that abruptio placentae does not seem to have an apparently strong genetic link.

Table III Demographic characteristics

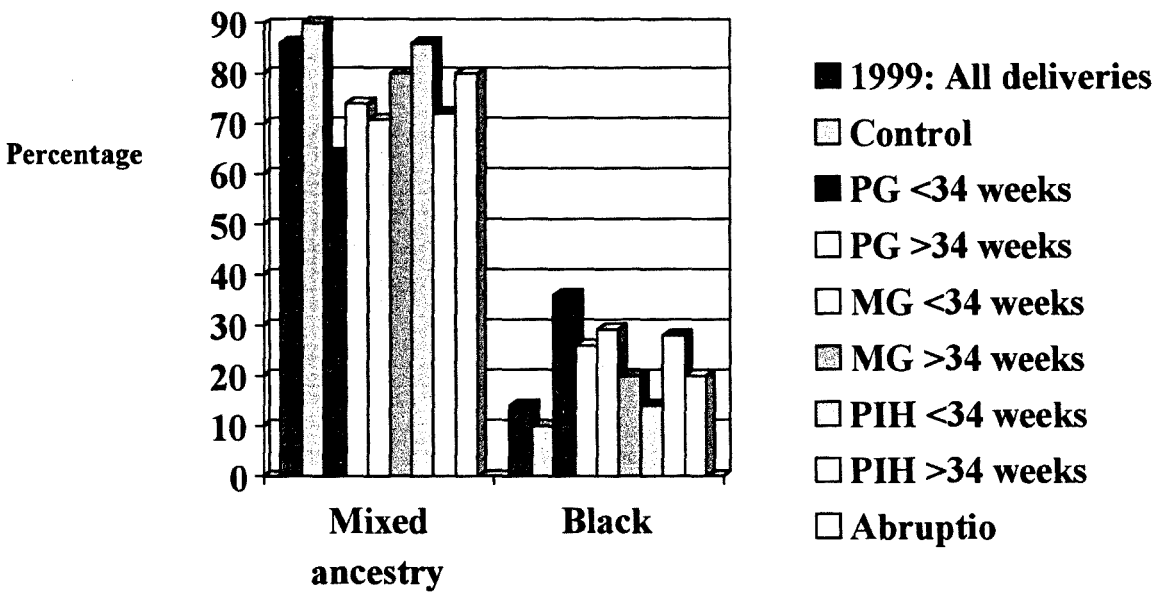
	A (n=50)	B (n=55)	C (n=50)	D (n=56)	E (n=51)	F (n=50)	G (n=53)	H (n=50)
Age	29 (18-43)	21 (14-37) A vs B*	19.5 (15-37) A vs C*	30 (18-42) B vs D*	29 (17-46) C vs E*	22 (14-37) A vs F*	32 (20-43)	28 (16-42)
Gravidity	3 (2-8)	1	1	3 (2-7)	2 (2-8)	1	3 (2-6)	2 (1-6)
Parity	2 (1-7)	0	0	2 (0-6)	1 (0-5)	0	2 (0-5)	1 (0-5)
Race: (%) Black	10%	36%	26%	29%	20%	14%	28%	20%
Race: (%) Coloured	90%	64% A vs B*	74%	71% A vs D*	80%	86%	72% A vs G*	80%
Blood pressure: systolic	120 (100-140)	160 (130-220) A vs B*	160 (130-200) A vs C*	160 (120-220) A vs D*	160 (130-260) A vs E*	140 (120-90) A vs F*	140 (120-210) A vs G*	140 (80-200) A vs H*
Blood pressure: diastolic	80 (60-90)	105 (80-150) A vs B*	110 (90-140) A vs C*	110 (90-160) A vs D*	108 (90-160) A vs E*	100 (90-120) A vs F*	100 (90-130) A vs G*	90 (60-130) A vs H*
Smoking (%)	40%	20% A vs B*	20% A vs C*	16% A vs D*	20% A vs E*	28%	20.8% A vs G*	38%
Iron supplementation (%)	100%	86%	86%	83%	94%	98%	94%	90%
Folic acid (%)	100%	86%	86%	83%	94%	98%	94%	90%
Gestational age at diagnosis	-	29 (19-33)	36 (34-42)	29 (20-37)	36 (34-44)	38 (21-42)	36 (21-41) F vs G*	33 (24-40)
Gestational age at delivery	39 (37-44)	30 (20-39) A vs B*	36 (34-42) A vs C*	31 (24-38) A vs D*	36 (31-44) A vs E*	39 (28-42) A vs F*	38 (26-42) A vs G*	33 (27-40) A vs H*
Birth weight	3256 (2460-4576)	1314 (592-2964) A vs B*	2603 (1358-4052) A vs C*	1218 (1218-3430) A vs D*	2678 (1314-4498) A vs E*	3044 (910-4060) A vs F*	2972 (768-4796) A vs G*	1751 (762-3702) A vs H*

All values are given in the median, unless otherwise stated. The range is given in parenthesis.
*Statistically significant difference between groups as indicated at $p < 0.05$

A Control group
B Primigravidae < 34 weeks
C Primigravidae > 34 weeks
D Multigravidae < 34 weeks
E Multigravidae >34 weeks
F Primigravidae with PIH
G Multigravidae with PIH
H Abruptio group

Figure II

Racial distribution of the different groups



□ vs ■ p=0.0032

PG Primigravida

MG Multigravida

PIH Pregnancy-induced hypertension

Figure III

Smoking during pregnancy: control group vs. abruptio group and hypertensive groups (B-G) combined

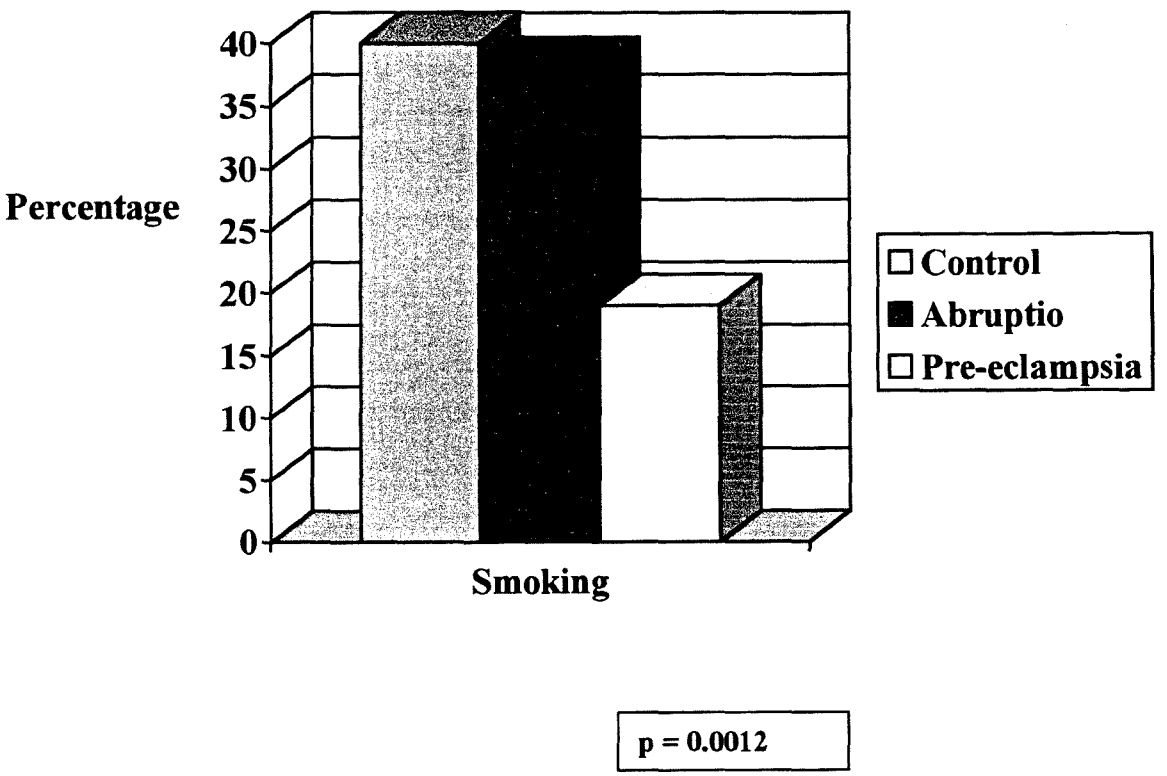
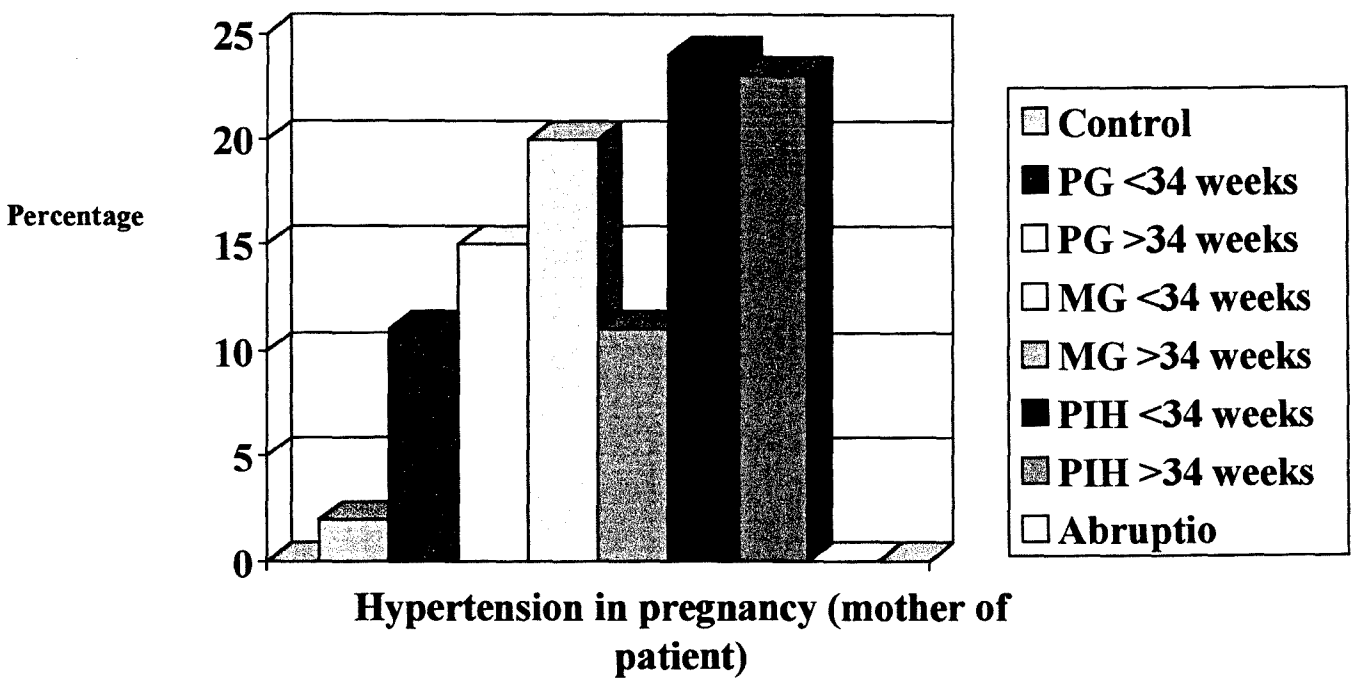


Figure IV

History of hypertension during pregnancy (mothers vs. daughters)



PG Primigravida
MG Multigravida
PIH Pregnancy-induced hypertension

There is a significant relationship of hypertension in pregnancy between mothers and their daughters. Only 2% of patients with an uncomplicated pregnancy outcome had mothers with hypertension during their (the mothers') pregnancies.

($p = 0.0029$)

2.3 Collection of plasma for homocystein determination

Homocystein is synthesised by erythrocytes and leukocytes and production continues after collection of blood. This production is minimised by storing samples on ice. A protein rich meal within 8 hours of sampling can also affect blood levels of homocystein. After separation of plasma, homocystein levels are stable and can remain so for years when frozen at –20°C or less [Perry, 1999].

For this study, blood for homocystein determination was always collected after an overnight fast. An EDTA and clotted blood sample were obtained for plasma and serum samples respectively and were stored on ice until centrifuged. The centrifuged plasma was immediately frozen at -78°C until dispatched for analysis. A methionine-loading test was traditionally used to detect heterozygosity for deficiency in the cystathione-ß synthetase enzyme. This test maybe a valuable adjunct as fasting values sometimes fail to identify a patient at risk for vascular complications [Cattaneo et al, 1996]. A method to measure total homocystein in blood was introduced in the late 1980s; this method determines the free homocystein fraction as well as the protein-bound homocystein, making loading tests unnecessary [Ueland et al, 1992].

For the purpose of this study, hyperhomocysteinaemia resulting from defects in the MTHFR enzyme were deemed more important. Also, the safety of methionine loading in pregnancy and the puerperium is not established as a sudden increase in homocystein can precipitate a vascular crisis like abruptio placentae. Elevated fasting levels constitute an equally strong excessive risk for arteriosclerotic disease as elevated postload concentrations [Boers, 1997]. Homocystein levels were determined at Pretoria University using the method described by Ubbink and co-workers in 1991.

The fasting serum samples were stored for lipogram and vitamin B12 and folate analysis.

2.4 Collection of blood for DNA extraction

Maternal blood for DNA extraction was obtained from a clean vein puncture in the cubital fossa after informed consent was given. Two samples of 5 ml each in a tube containing EDTA (1/10 volume 0.5M sodium EDTA) were frozen; one sample in a conventional freezer until DNA extraction could be performed and the other sample at -78°C as a back-up. When the paternal genetic contributor (male partner) consented, a blood sample was obtained in a similar manner. Fetal blood was obtained after delivery of the fetus and before delivery of the placenta. Taking care not to contaminate the sample with maternal genetic material, two samples of 5 ml each were obtained directly from the umbilical cord on the placental side, in EDTA, and manipulated the same way as the maternal blood. If no blood was obtained at the time of delivery of a live baby, a few drops of blood were taken from a heel prick. In cases of intra-uterine death, blood was obtained after delivery from the fetus by direct cardiac puncture.

2.5 DNA Extraction

DNA extraction was performed using an adaptation of the original salting out procedure originally described by Miller et al [1988]. EDTA stored blood (5 ml) was mixed with 50 ml cold lysis buffer (Appendix) in a polypropylene tube to produce lysis of red cells. The sample was shaken periodically and placed on ice to enhance lysis. Following destruction of red cells, the mixture was centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was discarded without disturbing the centrifuged pellet.

Fetal haemoglobin (HgbF) is composed of two α-chains (identical to adult haemoglobin, HgbA) and two γ chains instead of the ß-chains of HgbA. This

difference amounts to 39 of the 149 amino acid residues in the haemoglobin tetramere and results in a higher oxygen affinity of HgbF [Bissonnette, 1996]. It also makes HgbF relatively more resistant to denaturation by alkali. This is the basis of the Apt test, where easy denaturation of a blood sample of antepartum vaginal bleeding with sodium hydroxide discloses its maternal origin. To extract fetal DNA, the lysis buffer step was repeated until obvious lysis of red cells could be observed.

The pellet was then carefully washed with phosphate-buffered saline (PBS) and spun down again at 1500 rpm for 10 minutes. The cell lysates were incubated overnight at 55°C with 3 ml nucleic lysis buffer (Appendix), 30 µl proteinase K (10 mg/ml) and 300 µl 10% SDS. Subsequently, 1 ml of saturated 6M sodium chloride solution was added and the sample shaken vigorously. It was then centrifuged at 2500 rpm for 15 minutes.

The DNA-containing supernatant was carefully decanted into a clean polypropylene tube and 20-30 ml of ice-cold absolute ethanol was added to precipitate the DNA. The DNA strands were carefully lifted from the solution with a plastic pipette and sprayed with 70% ethanol to remove excess salt. The sample was then carefully blotted against sterile blotting paper to remove excess fluid and transferred to a labelled micro-centrifuge tube, where it was left at room temperature to airdry. Approximately 600 µl of sterile distilled water was added to dissolve the pellet and the mixture placed on a shaker for a few hours before storage at 4°C.

2.5.1 Cleaning of contaminated samples

DNA samples contaminated by protein or other impurities, especially fetal DNA samples, were purified in the following manner. From the contaminated sample, 200 µl was placed in a clean polypropylene tube that was clearly labelled. To this, 200 µl

of phenol chloroform (a mixture of phenol:chloroform:absolute ethanol 25:24:1) was added. The solution was mixed thoroughly on a vortex and then centrifuged at 1500 rpm.

The aqueous layer (more or less 180 µl) was gently removed with a pipette and placed in a new clean, labelled polypropylene tube. An equal volume (180 µl) of chloroform and isoamyl alcohol (24:1 mixture) was added and the solution again vortexed and centrifuged. The aqueous layer (180 µl) was removed and placed in a labelled, clean polypropylene tube. To this, 20 µl of 3M sodium acetate was added and mixed well. Two volumes of cold, absolute ethanol were added to precipitate the DNA. The solution was then centrifuged for 30 seconds and the fluid carefully discarded to retain the DNA-containing pellet. The pellet was washed with 70% ethanol, centrifuged again and the resulting pellet was left at room temperature to airdry, after which it was dissolved in 200 µl of sterile water for use in PCR reactions.

2.6 PCR

2.6.1 Factor V Leiden and Prothrombin mutations

A multiplex allele-specific amplification polymerase chain reaction (ASA-PCR) was performed using primers described by Hezard et al (1998). Allele specific oligonucleotides were synthesised to anneal directly to the wild-type and mutated sequences respectively. In an individual homozygous for the mutation annealing will only occur with the allele specific probe containing the mutation; in a heterozygous individual annealing will occur with both the mutated and the wild-type probe (see Figure XIV).

The mutation in the prothrombin gene was detected with the reverse primer (wild-type allele) 5' cactgggagcattgaggatc 3', the mutated allele 5' cactgggagcattgaggatt 3'

and the forward (consensus) primer 5' tctagaaacagttgcctggc 3'. This part of the reaction yielded a DNA fragment of 340bp. For the Factor V Leiden mutation, the forward primer (wild-type allele) was 5' cagatccctggacagacg 3', the mutated allele 5' cagatccctggacagaca 3', and the consensus primer (reverse) 5' tgttatcacactggtgcttaa 3'. This yielded a 174bp DNA fragment. Polymerase chain reaction (PCR) amplification was carried out in a 50 µl reaction volume with 1 µl of genomic DNA, 1 µl (10pmol/l) of each primer, 5µl (5mM) of each of the four deoxynucleotide triphosphates and 1µl of *Taq* polymerase (5U/µl).

Thermal cycling was performed at 95°C for 1 minute, followed by 30 cycles of 95°C, 1 minute; 56°C, 1 minute; 72°C, 1 minute followed by a final extension step of 72°C, 5 minutes. PCR products were resolved on a 1% Agarose gel, stained with ethidium bromide and visualised under ultraviolet light (A_{260} nm).

2.6.2 MTHFR C677T mutation

Genotyping was performed by PCR amplification and *Hinf* I digestion as originally described by Frosst et al [1995]. The primers used were 5' tgaaggagaaggtgtctgcggga 3' (forward) and 5' aggacggtgcggtgagagtg 3' (reverse). This resulted in a DNA fragment of 198 bp. The PCR reaction was performed in a 50µl reaction mix containing 0.8 µl (10 pmol/l) of each primer, 4 µl mix of the deoxynucleotide triphosphates (dNTPs) (5 mM), 5 µl of 10x PCR buffer and 1 µl of *Taq* polymerase (5U/µl). Thermal cycling was started at 95°C for 1 minute, followed by 10 cycles (95°C, 10 seconds; 60°C' 45 seconds; 72°C, 45 seconds) and a further 20 cycles (95°C, 10 seconds; 57°C, 45 seconds; 72°C, 45 seconds) and a final extension step for 1 minute at 72°C.

2.6.3 MTHFR A1298C mutation

Genotyping was performed by PCR amplification and *Mbo* II digestion. The forward primer is 5' atgtgggggaggagctgac 3' and the reverse 5' gtctcccaacttacccttctccc 3'. The resulting DNA fragment is 241 bp in size. The PCR reaction was performed in a 50 µl reaction mix containing 1.5 µl (10 pmol/l) of each primer, 5 µl mix of the dNTPs (5mM), 5µl of 10x PCR buffer and 1µl of *Taq* polymerase (5U/µl). Thermal cycling was started at 94°C for 1 minute 30 seconds, followed by 30 cycles (94°C, 30 seconds; 55°C, 45 seconds; 72°C, 1 minute) and a final extension step for 3 minutes at 72°C.

2.6.4 LDLR promoter

To determine the recently described -175G/T variant in the regulatory region of the LDLR promoter, the primers used were 5' aggcagagaggacaatggc 3' (15pmol; 0.55 µl, forward primer) and 5' cacgacctgctgtgtccaagcttgaaaccc 3' (15 pmol; 0.25 µl, reverse primer) in a 50 µl reaction mix. PCR buffer 10x, 5 µl, dNTPs (5mM) 10 µl and *Taq* polymerase (5U/µl) 0.15 µl were amplified with 1 µl of genomic DNA. Thermal cycling was started at 95°C for 1 minute, followed by 10 cycles (95°C for 10 seconds, 60°C for 45 seconds, 72°C for 45 seconds) and another 30 cycles (95°C for 10 seconds, 58°C for 45 seconds, 72°C for 45 seconds).

2.6.5 LDLR: D154N, D200G, 652 del GGT and D206E mutations in exon 4

For this reaction, the primers 5' cccccagctgtgggcctgcg 3' (forward, 0.2 µl of a 20 pmol solution) and 5' cgccccccaccctgccccgcc 3' (reverse, 0.2 µl of a 20 pmol solution) were used. A reaction mix of 50 µl was used with 10 µl dNTPs (5mM), 5 µl 10x PCR buffer mix, 0.1 µl *Taq* polymerase (5U/µl)and 1 µl of genomic DNA as template.

Thermal cycling was started at 95°C for 2 minutes and 30 seconds, followed by 35 cycles of 94°C for 1 minute, 71°C for 1 minute and 72°C for 2 minutes. The final extension was done at 72°C for 10 seconds. A DNA fragment of 237 basepairs was produced. *Mbo* II restriction digestion analysis was performed to detect the D154N mutation.

2.6.6 LDLR: V408M mutation in exon 9

Primers H9 (5' cccctgacctcgctccccgg 3', forward primer, 0.33 μl of a 20 pmol solution) and J9 (5' gctgcaggcaggggcgacgc 3', 0.2 μl of a 20 pmol solution, reverse primer) was used in a 50 μl total volume containing 10 μl of the four dNTPs (10mM), 5 μl 10X PCR buffer and 0.1 μl *Taq* polymerase (5U/μl). One microlitre of genomic DNA was used as template.

The initial denaturation step of the PCR reaction was started at 95°C for 1 minute, followed by 10 cycles (95°C for 5 seconds, 63°C for 30 seconds, 72°C for 30 seconds) and another 30 cycles (95°C for 5 seconds, 62°C for 30 seconds, 72°C for 30 seconds and a final extension step for 5 minutes at 72°C).

2.7 Restriction enzyme digestion

2.7.1 C677T Digest (*Hinf* I)

The C to T substitution at nucleotide position 677 creates a *Hinf I* recognition site for enzymatic cleavage. The recognition sequence is 5'...G ♦ ANT C...3'

3'...C TNA ♦G...5'

Hinf I (10U/μl) supplied by Promega Corporation was used. The restriction enzyme buffer (10x) supplied with the enzyme (60mM Tris-HCL, 500mM NaCl, 60mM $MgCl_2$,) and acetylated bovine serum albumin (10 mg/ml) were used in the following quantities: buffer 2.0μl, bovine serum albumin 0.2μl, distilled water 6.8μl and *Hinf* I

1.0µl and 10µl of amplified DNA product in a total volume of 20µl. Digestion was performed in a water bath at 37°C for at least four hours. The 198 bp fragment (C/C, undigested) is cut into 175 bp and 23 bp fragments if the mutation is present. Bovine serum albumin was added as it enhances the activity of the restriction enzyme.

2.7.2 A1298C Digest (*Mbo* II)

The supplier of *Mbo* II (10U/µl) was Amersham Pharmacia Biotech. The A to C substitution abolishes a cleavage site for *Mbo* II in the wild type genome. The fragment is cut into fragments of 204 and 37 basepairs. For the digest, the buffer (10mM Tris-HCl, 7mM MgCl2, 10mM KCL, 7mM 2-mercaptoethanol, 0.01% BSA) supplied with the restriction enzyme is used in the following quantities: 10x buffer 2µl, distilled water 7.5µl, *Mbo* II 0.5µl and PCR product 10µl. Products were digested overnight at 37°C.

2.8 Gel electrophoreses

Non-denaturing polyacrylamide gel solutions were used for restriction enzyme analysis. A polyacrylamide gel was used, supplemented with 10% urea for the V408M mutation analysis and supplemented with 10% glycerol for the combined heteroduplex and single-strand conformation polymorphism (HEX-SSCP) analysis of mutations D206E and D154N [Kotze et al, 1995]. The FV Leiden and prothrombin mutations were resolved on 1% agarose gels. A urea HEX-SSCP gel was used for the detection of the -175G/T LDLR promoter variant. For the restriction enzyme gels, 20µl of the digested product and 5 µl loading dye (appendix) were electrophoresed for 2 hours at 250 volts at room temperature. Polyacrylamide gels were stained with ethidium bromide before visualisation under ultra-violet light. Agarose gels were prepared with ethidium bromide added to the gel to enhance visualisation of products under ultra-violet light. A ficoll orange loading dye was used for agarose gels.

Chapter 3

Results and discussion

In this chapter, the most significant data are presented in tables, figures and graphs. A representative photograph of each mutation is included. The data is then presented in article format with articles already accepted or in preparation for publication.

Table IV

Mutation detection: Results Group A (Control)

Number	Race	Age	Homocystein	C677T	A1298C	FV Leiden	A20210G	1 Min Apgar
A01	4	35	1	1	2	1	1	1
A02	4	24	1	1	1	1	1	1
A03	4	36	2	1	2	1	1	1
A04	4	26	1	2	2	1	1	1
A05	8	36	1	1	1	1	1	2
A06	4	28	1	1	2	1	1	1
A07	4	35	1	2	1	1	1	2
A08	4	28	1	1	1	1	1	1
A09	4	35	1	1	1	1	1	1
A11	4	36	2	1		1	1	1
A12	4	19	1	1	2	1	1	1
A13	4	28	2	1	2	1	1	1
A14	4	26	1	1	3	1	1	1
A15	4	28	1	1	2	1	1	1
A16	4	28	2	1	2	1	1	2
A17	8	22	1	1	2	1	1	1
A18	4	23	2	1	1	1	1	1
A19	4	28	1	1	1	1	1	1
A20	4	29	2	1	1	1	1	1
A21	4	23	2	1	2	1	1	1
A22	4	37		1	2	1	1	1
A23	4	29	1	2	1	1	1	1
A24	4	25	2	2	2	1	1	1
A25	4	34	1	1	3	1	1	1
A26	4	35	1	1	1	1	1	1
A27	4	43	1	1	2	1	1	1
A28	4	36	2	1	1	1		1
A29	4	31	1	2	1	1	1	1
A30	4	34	1	1	1	1	1	1
A31	4	34	1	1	2	1		1
A32	4	29	1	1	1	1		1
A33	4	37	1	1	1	1		1
A34	8	19	1	1	1	1		1
A35	4	40	2	1		1		1
A36	4	27	1	2	2	1	1	1
A37	4	20	2	1	2	1	1	1
A38	4	18	1	1	2		1	1
A39	4	30	1	1				1
A40	8	26	1	1				1
A41	4	34	1	1				1
A42	4	39	1	1				1
A43	4	22	2	2		1	1	1
A44	4	28	1	1	2	1	1	1
A45	4	34	1	1	2	1	1	1
A46	4	33	2	3	1	1		1
A47	4	25	1	1	1	1	1	1
A48	8	29	1	1		1	1	1
A49	4	36	1	1	2	1	1	1
A50	4	22		1	1	1	1	1

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table V

Mutation detection: Group B (Primigravidae <34 weeks)

Number	Race	Age	Homocystein	C677	A1298C	FV Leiden	A20210G	1 Min Apgar
B51	2	31	1	2	1	1	1	1
B52	4	16	1	1	2	1	1	0
B53	8	26	1	1	3	1	1	0
B54	4	37	2	1	1	1	1	0
B55	4	20	1	1	1	1	1	0
B56	8	26	1	1	1	1	1	0
B57	4	17	1	1	2	1	2	2
B58	4	21	1	1	1	1	1	1
B59	8	19	1	1	1	1	2	1
B60	8	16	2	2	1	1	1	2
B61	8	19	1	1	1	1	1	1
B62	8	24	1	1	1	1	1	1
B63	4	24	1	1	1	1	1	1
B64	8	20	1	1	3	1	1	0
B65	8	24	1	1	2	1	1	
B66	4	21	1	1	1	1	1	2
B67	8	21	2	1	1	1	1	2
B68	2	26	2	1	1	1	1	1
B69	4	26	1	1	3	1	1	1
B70	4	16	2	1	3	1	1	1
B71	8	15	2	1	3	1	1	2
B72	4	22	1	1	2	1	1	1
B73	4	21	2	2	1	1	1	0
B74	8	22	1	1	1	1	1	1
B75	4	19	2	1	3	1	1	2
B76	8	29	1	1	1	1	1	1
B77	4	27	2	1	1	1	1	1
B78	4	21	1	1	3	1	1	2
B79	8	17	2	1	1	1	1	2
B80	4	19	1	1	1	1	1	0
B81	8	16	2	1	3	1	1	
B82	4	24	1	2	1	1	1	2
B83	4	16	1	2				1
B84	4	25	1	1	1	1	1	1
B85	4	20	2	2	1	1	1	2
B86	4	17	2	1	1	1	1	1
B87	8	26	2	1	1	1	1	0
B88	8	23	2	1	2	1	1	1
B89	4	20	1	2	1	1	1	0
B90	4	14	1	1	2	1	1	1
B91	4	21	2	2	1	1	1	2
B92	8	22	2	1	2	1		0
B93	8	20	2	2	2	1	1	1
B94	8	25	1	1		1		2
B95	4	18	2	1	1	1		1
B96	4	20	1					1
B97	4	18	2	1	1	1	1	1
B98	4	23	2	1	2	1	1	0
B99	4	28	1	1	2	1	1	1
B100	8	22	1	1				2

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table VI

Mutation detection: Group C (Primigravidae > 34 weeks)

Number	Race	Age	Homocystein	C677T	A1298C	FV Leiden	A20210G	1 Min Apgar
C101	8	20	1	1	2	1	1	2
C102	4	18	1	1	2	1	1	1
C103	4	17	2	1	2	1	1	1
C104	8	19	2	1	1	1	1	1
C105	8	20	1	1	2	1	1	0
C106	4	17	2	1	1	1	1	1
C107	4	29	1	1	2	1	1	1
C108	4	18	1	1	1	1	1	1
C110	4	28	1	2	2	1	1	1
C111	4	25	1	2	1	1	1	1
C112	8	20	1	1	1	1	1	1
C113	4	22	1	1	1	1	1	1
C114	4	19	2	1	2	1	1	1
C115	4	19	1	1	2	1	1	1
C116	4	18	1	1	1	1	1	2
C117	4	19	1	1	2	1	1	1
C118	4	21	1	1	2	1	1	1
C120	8	26	1		3	1	1	1
C121	8	20	1	1	1	1	1	2
C122	4	37	1	1	3	1	1	1
C123	4	19	1	1	1	1	1	2
C124	4	25	1	1	1	1	1	1
C125	4	25	1	2	2	1		1
C126	4	19	1	1	2	1	1	1
C127	4	25	1	1	1	1	1	1
C128	4	23	1	1	1	1	1	1
C129	8	16	2	1	1	1	1	1
C130	8	15	1	1	2	1	1	1
C131	4	21	1	2	1	1	1	1
C132	4	16	1	2	1	1	1	1
C133	4	19	1	1	1		1	1
C134	4	15	1	1	1	1	1	1
C135	4	25	1	1	1	1	1	1
C136	4	17	2	1	2	1	1	1
C137	4	19	2	1	2	1	1	1
C138	8	26	1	1	1	1	1	1
C139	4	20	1	1	2	1		1
C140	8	15	1	1	1	1	1	2
C141	4	25	1	1	1	1	1	1
C142	4	16	1	1	2	1	1	1
C143	4	24	1	2	1	1	1	1
C144	4	20	1	1	1	1	1	1
C145	4	19	1	1	1	1	1	1
C146	4	19	1	1	2	1	1	1
C147	4	22	1	1	2	1	1	1
C148	8	31	1	1	1	1	1	1
C149	4	19	1	2	1	1		1
C150	8	19	2					1

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table VII

Mutation detection: Group D (Multigravidae <34 weeks)

Number	Race	Age	Homocystein	C677	A1298C	FV Leiden	A20210G	1 Min Apgar
D151	8	42	2	2	2	1	1	0
D152	8	31						2
D153	4	27	1	1	2	1	1	0
D154	4	25	1	2	1	1	1	1
D155	4	25	1	1	1	1	1	1
D156	4	34	2	2	1	1	1	1
D157	4	31	1	1	1	1	1	0
D158	4	40	1	1	1	1	1	1
D159	4	35	1	1	2	1	1	1
D160	4	31	1	1	2	1	1	0
D161	4	27	1	2	1	1	1	2
D162	4	33	1	1	1	1	1	1
D163	4	32	1	1	2	1	1	2
D164	8	29	2	1	1	1	1	0
D165	4	21	1	1	2	1	1	0
D166	4	32	2					1
D167	4	33		1	1	1	1	1
D168	8	31	1					1
D169	4	25	1	1	1	1	1	1
D170	8	33	2	1	1	1	1	2
D171	4	25	1	1	2	1	1	1
D172	8	26		1	2	1	1	0
D173	4	38	2	1	3	1	1	
D174	4	25	1	1	1	1	1	0
D175	4	37	1	1	3	1	1	0
D176	4	23	1	2	2	1	1	1
D177	4	30	2	1	1	1	1	1
D178	8	27	1	1	1	1	1	1
D179	4	19	1	1	1	1	1	1
D180	4	25	2	1	2	1	1	0
D181	8	35	2	1	1	1	1	0
D182	4	25	1	1	1	1		2
D183	8	36	2	2	1	1	1	1
D184	8	29	2	2	1	1	1	1
D185	4	26	1	2	1	1	1	2
D186	4	27	1	1	2	1	1	2
D187	8	36	2	2	1	1	1	1
D188	8	31	1	1	1	1	1	1
D189	4	33	1	1	2	1	1	0
D190	8	27	2	2	1	1	1	2
D191	4	24	1	1	3	1	1	1
D192	4	41	1	1	1	1	1	1
D193	8	29	1	1	1	1	1	
D194	4	25	1	1	3	1	1	0
D195	4	18	1	1	2	1	1	1
D196	8	26	1	1	1	1	1	0
D197	4	36	1	1	2	1	1	0
D198	4	30	1	1	1	1	1	0
D199	4	31	1	1	2	1	1	2
D200	4	28	1	1	1	1	1	1

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table VIII

Mutation detection: Group E (Multigravidae >34 weeks)

Number	Race	Age	Homocystein	C677	A1298C	FV Leiden	A20210G	1 Min Apgar
E201	4	29	1	1	3	1	1	1
E202	8	46	1	1	1	1	1	2
E203	4	18	1	1	3	1	1	1
E204	4	26	1	1	1	1		1
E205	4	37	1	1	2	1	1	1
E206	4	33	1	1	1	1	1	1
E207	4	33	1	2	1	1	1	1
E208	8	30	1	2	1	1	1	1
E209	4	36	1	1	3	2	1	1
E210	4	37	1	1	3	1	1	1
E211	4	30	1	1	3	1	1	2
E212	4	30	1	1	2	1	1	1
E213	4	20	1	1	2	1	1	1
E214	4	41	1					1
E215	4	21	1	2	1	1	1	1
E216	4	17	1	1	1	1		1
E217	4	26	1	1	2	2	1	1
E218	4	36	1	1	1	1	1	1
E219	4	26	1	1	1	1		1
E220	4	37	1	2	1	1	1	1
E221	8	29	1	1	3	1	1	1
E222	4	33	1	1	2	1	1	1
E223	8	39	1	1	2	1	1	1
E224	4	19	1	1	3	1	1	1
E225	4	34	1	1	2	1	1	1
E226	4	34	1	1	2	1	1	1
E227	4	40	1	1	1	1	1	1
E228	4	22	1	2	1	1	1	1
E229	4	20	1	1	1	1	1	1
E230	4	20	1	1	3	1	1	1
E231	4	32	1	1	1	1	1	1
E232	8	25	1	1	1	1	1	1
E233	4	32	1	1	3	1	1	2
E234	4	25	1	2	1	1	1	1
E235	8	23	1	1	1	1	1	1
E236	4	27	1	1	1		1	2
E237	8	37	1	1	2	1	1	1
E238	4	25	1	1	1	1		1
E239	4	22	1	1	3	1		2
E240	4	26	1	2	2	1	1	1
E241	8	31	1	1	1	1	1	1
E242	4	19	1					1
E243	8	26	1					1
E244	4	28	1	1	1	1		1
E245	8	25	1	1	1	1		1
E246	4	26	1					1
E247	4	20	1	1	2	1		1
E248	4	29	1	1		1		1
E249	4	35	1		1		1	1
E250	4	29	1	1	1	1		1

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table IX

Mutation detection: Group F (Primigravidae PIH)

Number	Race	Age	Homocystein	C677	A1298	FV Leiden	A20210G	1 Min Apgar
F251	4	22	1	1	2	1	1	1
F252	4	23	1	2	2	1	1	1
F253	4	19	2	1	2	1	1	1
F254	4	17	1	1	1	1	1	1
F255	4	32	1	1	3	1	1	1
F257	4	23	1	1	1	1	1	1
F258	4	16	1	1	3	1	1	1
F259	8	24	1	1	3	1	1	1
F260	4	15	1	1	3	1	1	1
F261	4	20	1	1	1	1	1	1
F262	8	24	1	1	2	1	1	1
F263	4	32	1	1	2	1	1	1
F264	8	29	1	1	1	1	1	2
F265	4	15	1	1	1	1	1	1
F266	4	21	1	1	2	1	1	1
F267	4	15	1	1	1	1	1	1
F268	4	17	1	1	1			1
F269	4	26	1	1	1	1	1	1
F270	4	18	1	1	3	1	1	1
F271	4	19	1	1	1	1	1	1
F272	4	21	1	1	2	1	1	1
F273	4	30	1	1	2	1	1	1
F274	4	22	2	2	2	1	1	1
F275	4	23	1	1	1	1	1	1
F276	4	20	1	1	2	1	1	1
F277	4	19	1	1	1	1	1	1
F278	8	19	1	1	1	1	1	1
F279	4	15	1	1	2	1	1	1
F280	4	28	1	1	2	1	1	1
F281	4	15	1	1	2	1	1	1
F282	4	15	1	1	2	1	1	1
F283	4	26	1	1	3	1	1	1
F284	4	14	1	1	1	1		1
F285	8	25	1	1	1	1	1	1
F286	4	24	1	2	2	1	1	1
F287	4	23	1	1	2	1	1	2
F288	4	37	1	1	2	1	1	1
F289	4	20	1	1	1	1	1	1
F290	4	17	1	1	2	1	1	1
F291	4	23	1	1	3	1	1	1
F292	8	28	1	1	2	1	1	1
F293	4	28	1	1	2	2	1	1
F294	8	24	1	1	1	1	1	1
F295	4	19	1	1	1	1		1
F296	4	30	1	1				1
F297	4	24		1				2
F298	4	24	1	2	1	1		1
F299	4	27	1	2				1
F300	4	20	1					1

Homocystein 1 = normal, 2 = >11 μ mol/l

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table X

Mutation detection: Group G (Multigravidae PIH)

Number	Race	Age	C677T	A1298C	FV Leiden	A20210G	1 Min Apgar
G301	8	24	1	1	1	1	1
G302	4	25	1	2	1	1	1
G303	4	37	1	2	1	1	1
G304	8	34	1	2	1	1	1
G305	8	36	1	1	1	1	1
G306	4	35	1	1	1	1	1
G307	8	23	1	2	1	1	2
G308	4	42	1	2	1	1	1
G309	4	43	1	2	1	1	1
G310	4	35	1	1	1	1	1
G311	4	37	1	1	1	1	1
G312	8	26	1		1	1	1
G313	4	26	1	2	1	1	2
G314	4	36	1	3	1	1	1
G315	8	30	1	2	1	1	1
G316	4	28	1	1	1	1	1
G317	4	40	2	1	1	1	1
G318	4	29	1	2	1	1	1
G319	8	26	1	3	1	1	1
G320	8	32	1	1		1	2
G321	4	35	2		1	1	1
G322	4	33	1				1
G323	4	29	1	1	1	1	1
G324	4	33	1	3	1	1	1
G325	4	38	1	2	1	1	1
G326	4	32	2	2	1	1	1
G327	4	33	1	2	1	1	1
G328	8	34	1	2	1	1	1
G329	4	30	1	3	1	1	2
G330	8	29	1	2	1	1	1
G331	8	35	1	3	1	1	1
G332	8	30	1	1	1	1	1
G333	4	31	1	2	1	1	1
G334	4	34	1	1	1	1	1
G335	8	26	1	1	1	1	1
G336	4	28	1	1	1		1
G337	4	25		1	1	1	1
G338	8	32	1	1	1	1	1
G339	4	29	1	2	1	1	1
G340	4	34					0
G341	4	20					1
G342	4	37	2	1	1	1	1
G343	8	25	1	2	1	1	0
G344	4	39	2	2	1	1	1
G345	4	35	1	3	1	1	1
G346	4	27	1	2	1	1	1
G347	4	25	1	2	1	1	1
G348	4	36	1	2	1	1	1
G349	4	37	1	1	1	1	1
G350	4	38	1	2	1	1	1

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table XI

Mutation detection: Group H (Abruptio placentae)

Number	Race	Age	C677T	A1298C	FV Leiden	A20210G	1 Min Apgar
H351	4	36	1	1	1	1	0
H352	4	34	1	1	1	1	1
H353	4	33	1	2	1	1	0
H354	4	27	1	2	1	1	0
H355	8	36	1	1	1	1	0
H356	8	40	1	3	1	1	0
H357	4	38	1	1	1	1	1
H358	4	24	1	1	1	1	1
H359	4	31	1	1	1	1	0
H360	8	25	1	1	1	1	0
H361	4	36	1	2	1	1	2
H362	8	35	2	1	1	1	0
H363	4	35	1	1	1	1	1
H364	8	29	2	1	1		0
H365	4	25	1	1	1	1	2
H366	4	26	1	1	1	1	0
H367	4	19	3		1	1	0
H368	4	35	2	1	1	1	1
H369	4	18	2	1	1		0
H370	8	22	1	2	1	1	0
H371	4	28	2	2	1	1	0
H372	8	28	1	1	1	1	0
H373	4	19	1	1	1	1	1
H374	4	28	1	2	1	1	0
H375	8	22	1	1	1	1	1
H376	4	29	1	1	1	1	0
H377	4	32	1	1	1	1	0
H378	4	42	1	3	1	1	0
H379	4	35	1		1		2
H380	4	38	1	2		1	2
H381	4	19	1	1	1	1	2
H382	4	32	2	2	1	1	0
H383	4	23	2	2	1	1	1
H384	4	27	1	1	1	1	0
H385	4	31	1	2	1	1	0
H386	8	32	1	1	1	1	0
H387	4	30	1	2	2		1
H388	4	28	1	1	1	1	0
H389	2	18	1	2	1	1	0
H390	8	21	1	1	1	1	0
H391	4	22	2	1	1	1	0
H392	4	19	1	1	1	1	0
H393	4	20	1	1	1	1	1
H394	4	20	1	1	1	1	1
H395	4	16	1	1	1	1	0
H396	4	17	1	2	1	1	0
H397	4	20	1	2	2	1	0
H398	4	37	1	3	1	1	0
H399	4	21	2	1			2
H400	4	33	1	3	1	1	0

Mutations: 1 = wild-type (-/-), 2 = heterozygous state (+/-), 3 = homozygous mutant (+/+)

1 minute Apgar: 1 = score above 5, 2 = score 5 or less, 0 = fetal death

Race: 2 = Caucasian, 4 = Coloured, 8 = Black

Table XII

Mutation detection: Results Group L

Nr	R	G	P	A	BMI	S-C	677	1298	LDLR	FH1	FH3	FH2	X2	PL	SFG	PH	MO
L1	4	6	5	1	46	1	2	1	1	1	1	1		2	1	1	3
L2	4	4	2	1	33	1	1	1	1	1	1	1		1	1	6	1
L4	4	1	0	2	23	3	1	2	1	1	1			1	1	1	2
L5	8	1	0	1	24	1	1	3	1					1	1	1	4
L6	8	3	2	0	23	1	2	1	1	1	1		1	2	1	1	4
L7	4	7	5	1	29	1	1	1	2	1	1			1	1	2	2
L8	4	2	1	1	40	1	3	1	1		1			1	1	1	2
L9	4	2	1	1	23	1	1	1	1	1	1			2	1	5	1
L10	4	2	1	1	34	1	1	3	1	1	1	1		1	1	1	1
L13	4	3	2	1	29	1	1	1	1		1			1	1	1	1
L15	4	3	0	1	30	1	1	1	1	1	1			1	1	6	1
L17	4	5	3	1	33	2	1	2	1	1	1			1	1	1	2
L18	4	5		1	24	1	1	2	1	1	1			2	1	2	1
L19	4	4	3	1	37	1	1	1	2					1	1	1	1
L20	8	4	3	1	34	3	1	2	1	1	1			1	1	1	1
L21	4	6	3	1		1	1	2	1	1	1			1	1	6	1
L22	4	2	1	1	41	2	1	1	1	1	1			1	1	1	2
L23	4	3	1	1	20	3	1	2	1	1	1			1	1	1	1
L24	4	3	2	1	32	1	1	2		1	1			1	1	3	2
L26	4	3	2	1	17	1	2	1	1	1	1			2	1	6	1
L29	4	4	3	0	0	1	2	1	1		1			3	3	1	1
L30	4	6	5	1	36	1	2	1	1	1	1			1	1	1	1
L33	4	7	1	0	27	1	1	1	1	1				3	3	6	1
L34	4	2	1	1	28	3	1	1	1	1	1			1	1	1	1
L35	4	1	0	1	23	3	2	1	1	1	1			1	1	1	1
L36	4	1	0	1	22	1	1	2	2	1	1			1	1	1	1
L12	4	2	0	1	29	1	1	1	1			1		1	1	6	1
L16	8	10	7	1	34	1	1	1	1	1	1		1	1	1	1	1
L28	4	2	1	1	18	2	1	2	1					1	1	1	1
L31	4	3	2	1	21		1	2	1					1	1	2	1
L39	4	6		1	36	1	2	1	1	1	1			1	1	3	4
L40	4	4	3	1	31	1	2	1	2	1	1			1	1	1	1
L41	4	1	0	1	19	1	2	1	1	1	1			1	1	1	1
L42	4	5		1	28	1	1	1	1	1	1			2	1	1	1
L44	4	4	3	1	23		1	2	1	1	1			1	1	3	1
L45	8	4	3	1	19	1	2	1	1	1	1		1	2	2	2	1
L46	4	2	1	1	51	1	1	2	1	1	1			1	1	3	2
L47	4	4	0	0	18	1	1	3	1	1	1			3	3	6	1
L48	4	4	3	1	25		2	1	1	1	1			1	1	2	1
L49	8	3	1	1	29	1	1	1	1		1			1	1	2	1
L50	4	5		1	30	1	1	1	1	1	1			1	1	2	1
L51	4	4	0	0	35	1	1	1	1	1	1			3	3	6	1
L53	4	1	0	1	21	1	2	2	2	1	1			1	1	1	1
L54	4	6	4	1	48		1	1	1	1	1			1	1	2	2
L56	4	4	1	0	27	1	1	2	1	1	1			3	3	1	1
L58	4	2	1	1		2	1	1	1	1	1			1	1	1	1
L59	4	2	1	1	28	1	1	2	1					1	1	1	1
L60	4	2	1	1	24	1	2	1	1	1	1			2	1	1	1
L61	4	3	1	0	27	1	2	1	1	1	1			3	3	2	1
L62	4	2	0	1	32	1	1	2	1	1	1			1	1	1	1
L63	4	3	2	1	25	1	1	2	1		1			1	1	2	1
L64	4	3	2	1	20	1	2	1	1	1	1			1	1	1	1
L105	8	2	0	1	23	2	1							1	1	1	1
L65	4	2	1	1	0	3	2	2	1	1	1	1		2	1	1	1
L66	4	3	2	0	25	1	2	1	1	1	1			1	2	1	1
L67	4	2	1	1	33	1	1	1	1	1	1			2	1	1	3
L70	4	1	0	1	29	1	1	1	1	1	1			1	1	1	1

Nr	R	G	P	A	BMI	S-C	677	1298	LDLR	FH1	FH3	FH2	X2	PL	SFG	PH	MO
L71	4	6	3	1	34	2	1	1	1	1	1	1		2	1	1	1
L73	4	5	3	1	34	1	2	1	1	1	1			1	1	2	1
L74	4	3	0	0	21	1	2	2		1	1			3	3	6	1
L75	4	3	2	1	21	1	2	1	1	1	1			1	1	2	1
L76	4	1	0	1	0	1	2	1	1	1				1	1	1	2
L77	4	1	0	1	0	1	1	2	1					2	2	1	3
L124	8	3	2	1	43	1	1	2	1	1	1			1	1	1	4
L80	4	1	0	1	25	2	1	2	1	1	1			1	1	1	1
L81	4	4	3	1	36	2	2	1	1	1	1			1	1	1	2
L82	4	1	0	1	26	1	1	2	1	1	1			2	2	1	1
L83	4	3	2	1	22	1	1	1	1	1	1			1	1	1	2
L84	4	5	2	1	26	1	1	2	1	1				2	2	6	1
L85	4	3	2	1	0	1	1	1	1		1			2	1	2	1
L88	4	5	3	2	38	1	1	2	1	1	1			2	2	1	2
L89	4	3	1	1	23	3	2	1	1					1	1	1	1
L90	4	3	0	1	23	1	1	1	1	1	1			1	1	3	1
L91	4	3	2	2	33		1	1	1	1	1			1	1	3	2
L92	4	1	0	1	23	1	1	3	1	1	1			2	2	1	1
L93	4	4	1	0	26	1	2	2	1	1	1			3	3	1	1
L94	4	1	0	1	26	1	1	1	1	1	1			1	1	1	1
L95	4	3	2	1	35	1	1	2	1	1	1			1	1	1	2
L96	8	3	2	1	22	1	1	1	1	1	1		1	1	2	1	1
L97	4	5	3	1	30		1	2	1	1	1			1	1	1	4
L98	4	2	1	1	33	1	1	1	1	1	1			1	1	3	1
L99	8	1	0	1	24	3	1	1	1	1	1		1	1	1	1	1
L100	4	3	2	1	20	1	1	1	1	1				1	1	2	1
L101	4	3	0	1	32	1	1	1	2					1	1	6	1
L103	4	2	1	1	21	1	1	3	1	1	1			1	1	2	1
L104	4	1	0	1	25	1	1	2	1	1	1			1	1	1	1
L106	2	1	0	1	27	2	1	2	1	1	1			1	1	1	2
L107	4	1	0	1	20	1	1	2	1		1			1	1	1	3
L108	4	4	3	1	27	1	1	1	1	1				1	1	1	1
L109	8	1	0	1	39	1	1	1	1	1			1	1	1	1	2
L110	4	1	0	1	29	2	1	1	1	1	1			1	2	1	2
L111	4	2		1	32	1	1	2	1	1				2	1	6	1
L112	4	2	0	1	39	1	1	2	2	1				1	1	6	1
L113	4	3	2	1	21	1	1	1	1	1				1	1	2	2
L114	4	2	1		20	1	1	1	1	1	1			3	3	1	1
L115	4	3	2	2	28	1	2	1	1	1	1			1	1	1	1
L116	8	5	1	1		1	1	1	1		1			1	1	6	1
L117	4	3	2	1	38		1	2	1	1				1	1	1	1
L118	4	4	3	0	32	1	1	2	1	1	1			3	3	2	1
L119	4	2	1	1	0	2	2	2		1	1			1	2	1	1
L120	8	7	5	1	39		1	2	2	1			1	1	1	2	1
L122	8	5	4	1	31		2	1	1	1	1		1	1	1	1	1
L123	8	5	0	0	33	1	1	1	1	1	1		1	3	3	6	1
L125	4	1	0	1	25	1	3	1	1		1			1	1	1	1
L126	2	1	0	1	22	3	1	1	1	1	1			1	1	1	1
L128	4	6	4	1	0	1	2	2	1	1	1			1	1	1	1
L129	4	2	1	2	31	1	1	2		1	1			1	1	1	1
L131	4	5		1	26	1	1	2	1		1			1	1	1	1
L132	4	5	4	1	33	1	1	2	1	1	1			1	1	1	1
L133	4	5	4	0	32	1	2	1	2	1	1			1	1	2	1
L134	4	2	1	1	25	1	1	1	1	1	1			1	1	1	2
L135	4	4	3	1	22	1	1	2	1	1	1			1	1	2	1
L136	4	3	3	1	36	1	1	1	1	1	1			1	1	1	1
L137	4	7	2	0		1	1	2	1	1				3	3	1	1
L3	4	2	1		35	1	1	1	1	1	1					1	1
L11	4					2	1	3	1	1	1					1	1
L14	2	2	1			1	1	1	1	1	1					1	1
L32	4	3			22	1	1	1	1					1		3	1
L37	4					1	1	2	1							1	1

Nr	R	G	P	A	BMI	S-C	677	1298	LDLR	FH1	FH3	FH2	X2	PL	SFG	PH	MO
L43	4	2				1	2	1	1							1	
L57	4					3	1	2	1	1	1					1	4
L72	8					2	1	1	1	1	1		1			1	1
L86	4	3	2		25	2	1	2	1	1						1	1
L87	8	5		1		1	1	2	1	1			1	1	1	1	1
L127	4	4	3			1	1	2	1	1						1	1
L130	4	4				1	3		1	1	1					1	1

Key to legend: Group L

Nr Number

R Race: 2 = Caucasian, 4 = Coloured, 8 = Black

G Gravidity

P Parity

A Apgar score: score >5 = 1; 5 or less = 2, fetal death = 0

BMI Body mass index (height in kg/ length in m squared)

S-C Serum cholesterol: 1 = normal, 2 = probable familial hypercholesterolaemia, 3 = possible familial hypercholesterolaemia

677 MTHFR C677T mutation: 1 = wild-type (-/-); 2 = heterozygote (+/-); 3 = homozygote (+/+)

1298 MTHFR A1298C mutation: 1 = wild-type (-/-); 2 = heterozygote (+/-); 3 = homozygote (+/+)

LDLR LDLR promoter mutation -175G/T: 1 = mutation absent, 2 = mutation present

FH1 D206E LDLR mutation: 1 = absent

FH2 V408M LDLR mutation: 1 = absent

FH3 D154N LDLR mutation: 1 = absent

X2 6bp deletion in Exon 2 of LDLR mutation: 1 = absent

PL Preterm labour: 1 = none, 2 = preterm labour, 3 = miscarriage

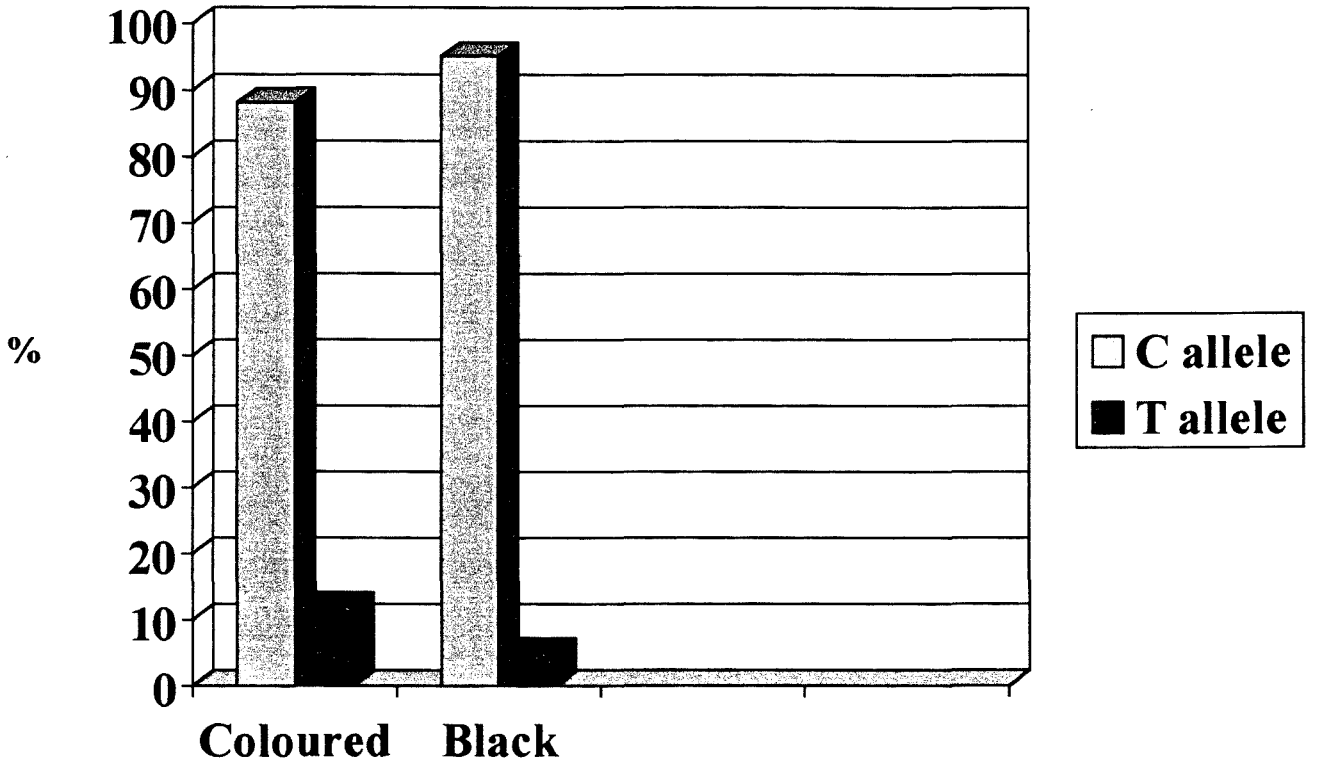
SFG Fetus small for gestational age 1 = normal, 2 = SFG, 3 = miscarriage

PH Previous history: 1 = none, 2 = previous intra-uterine death, 3 = previous hypertension, 4 = previous pre-eclampsia, 5 = previous preterm labour, 6 = previous miscarriage

MO Maternal outcome: 1 = normal, 2 = hypertension, 3 = develops pre-eclampsia, 4 = preterm labour

Figure V

Allele frequencies of the C and T alleles of the C677T MTHFR mutation: racial distribution

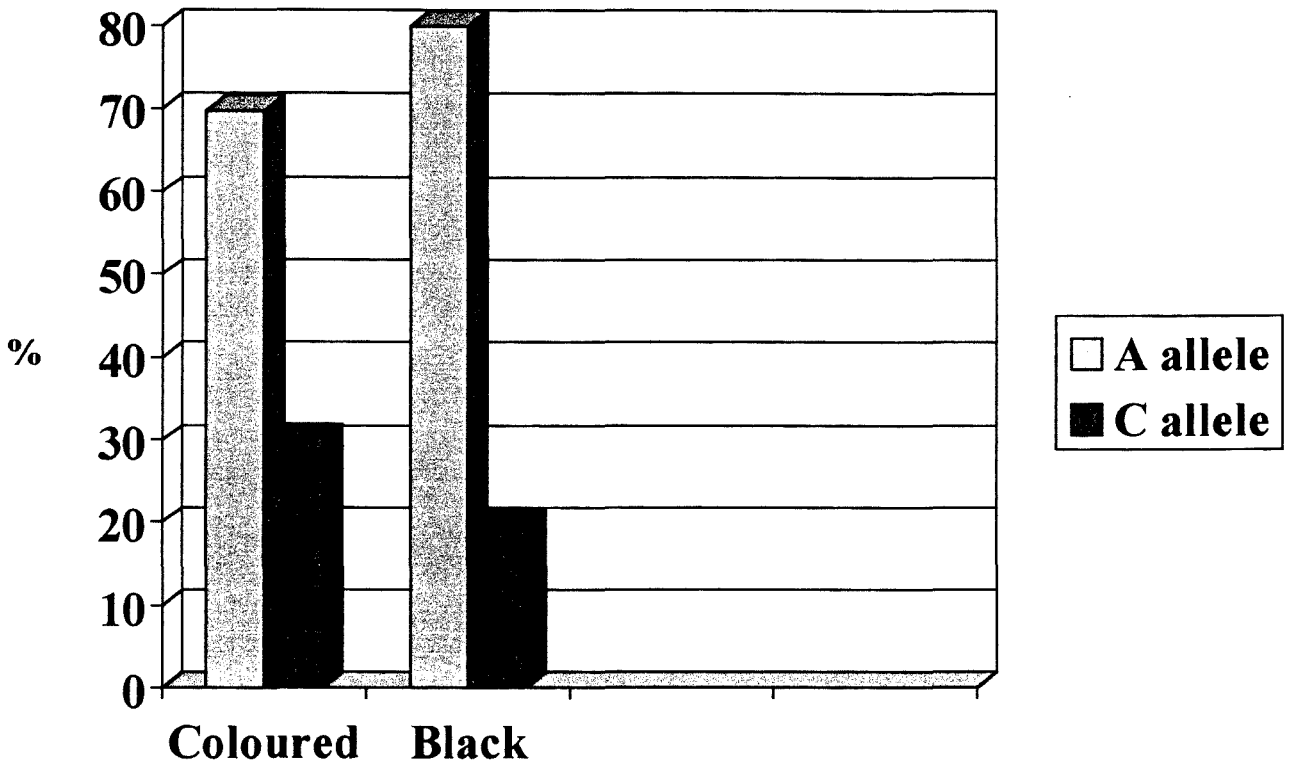


p=0.0246, OR 2.08 (95%CI 1.05-4.21)

The mutated T allele of the C677T mutation occurs significantly more frequently in the Coloured (mixed ancestry) population than in the Black population.

Figure VI

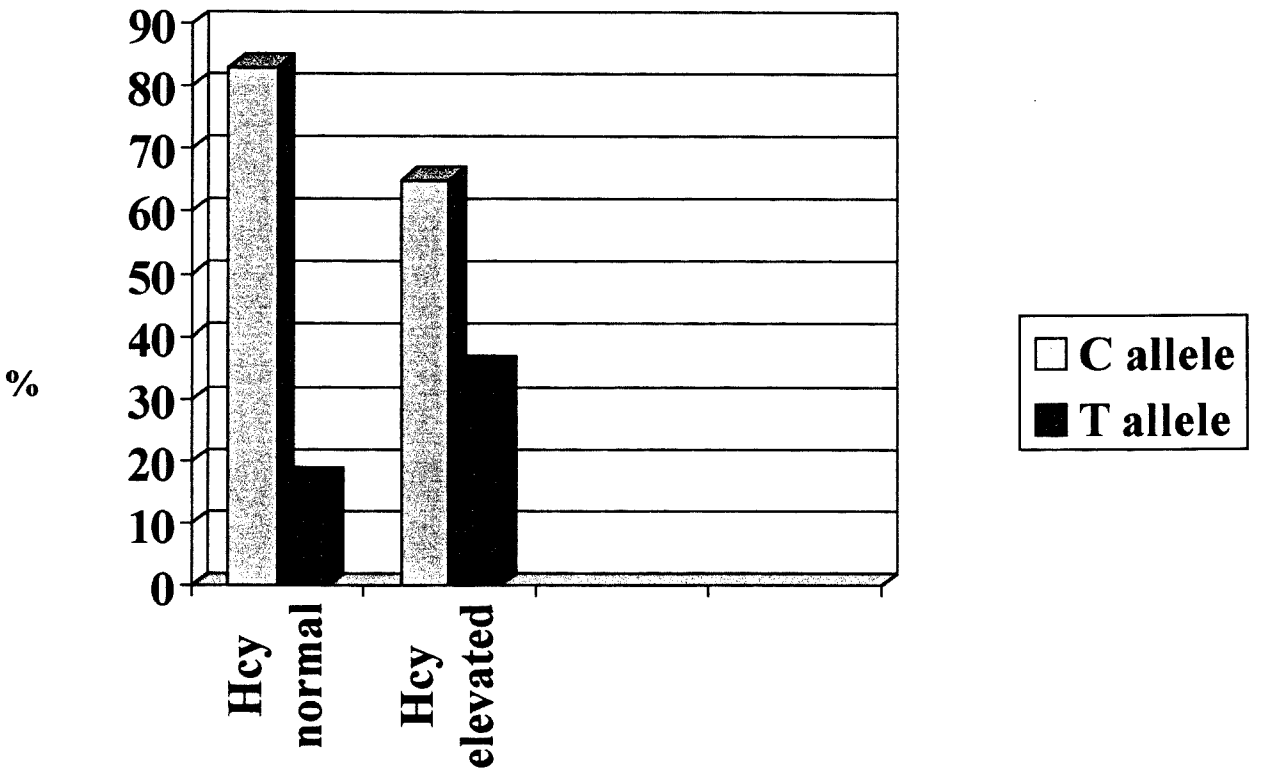
Allele frequency of the A and C alleles of the A1298C MTHFR mutation: racial distribution



p = 0.0407, OR 1.80 (95% CI 1.14-2.83) The mutated C allele occurs significantly more frequently in the Coloured population than in the Black population.

Figure VII

Genotype frequency of the C677T MTHFR mutation: association with hyperhomocysteinaemia

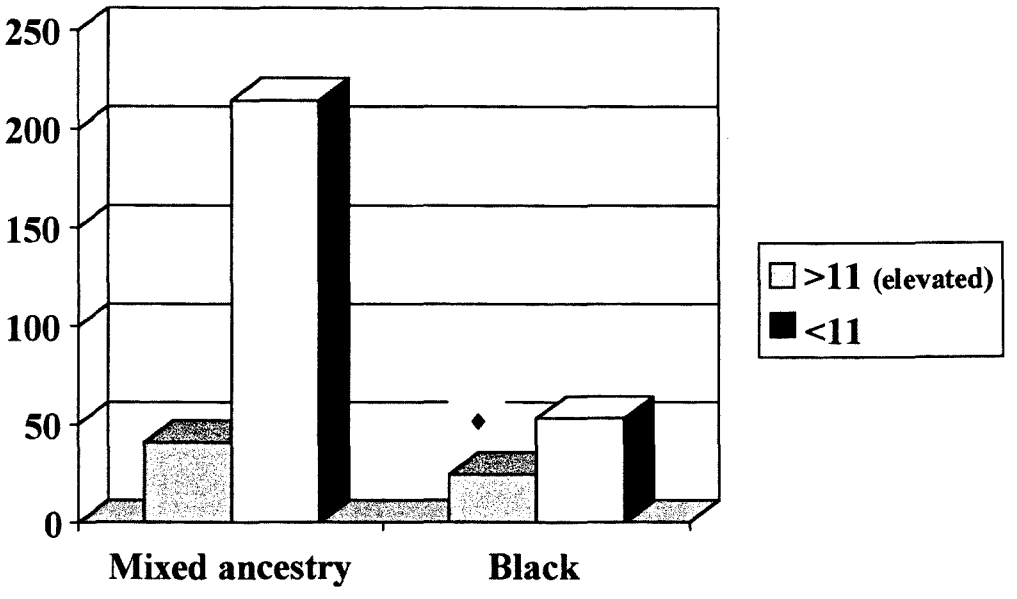


p = 0.0095 OR 2.27 (95% CI 1.15-4.46)

The mutated T allele is significantly associated with hyperhomocysteinaemia

Figure VIII

The incidence of elevated homocystein levels: racial distribution

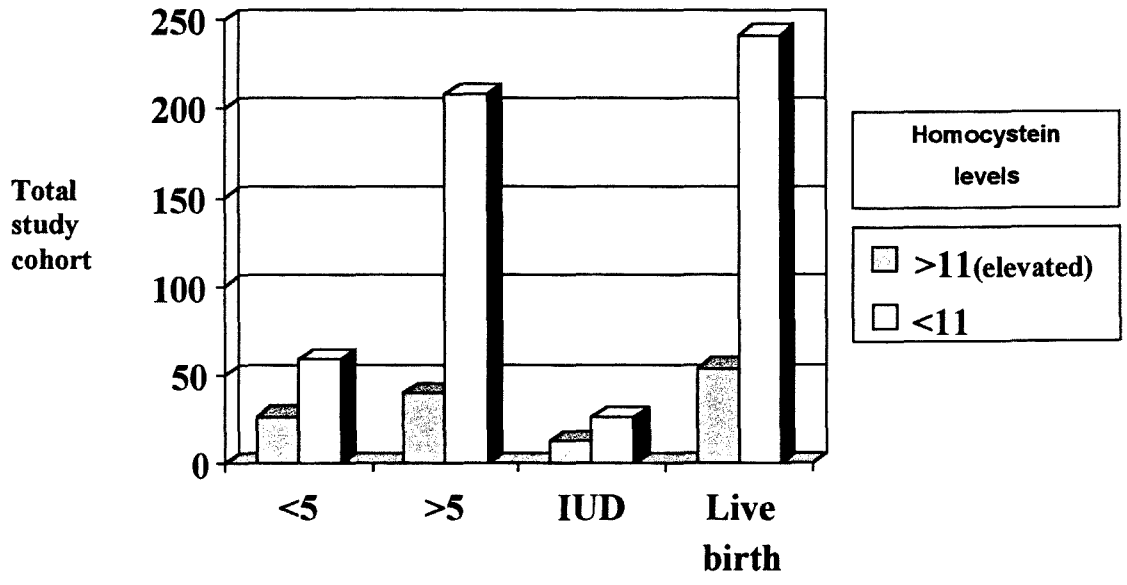


♦ p = 0.0033

There were significantly more Black patients with abnormal homocystein levels (all patient groups combined).

Figure IX

The relationship between elevated homocystein levels and neonatal outcome (1 minute Apgar score)

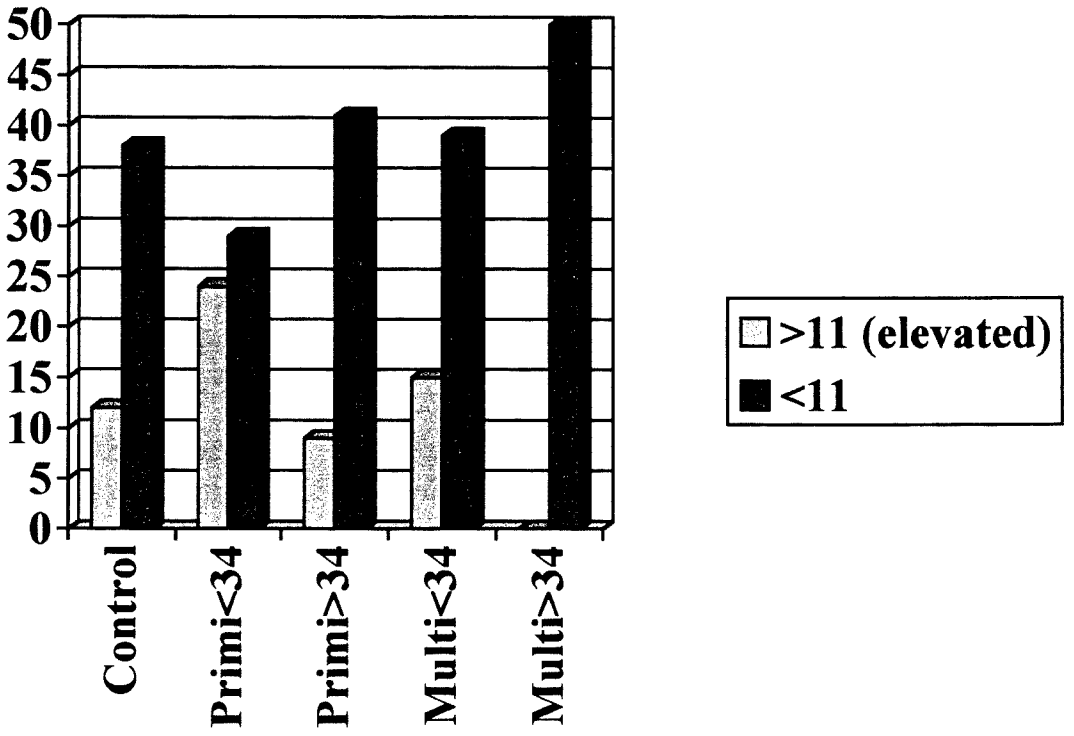


There is a significant association between elevated homocystein level and

- a one minute Apgar Score of 5 or less (p = 0.043)
- intra-uterine death (p = 0.046)

Figure X

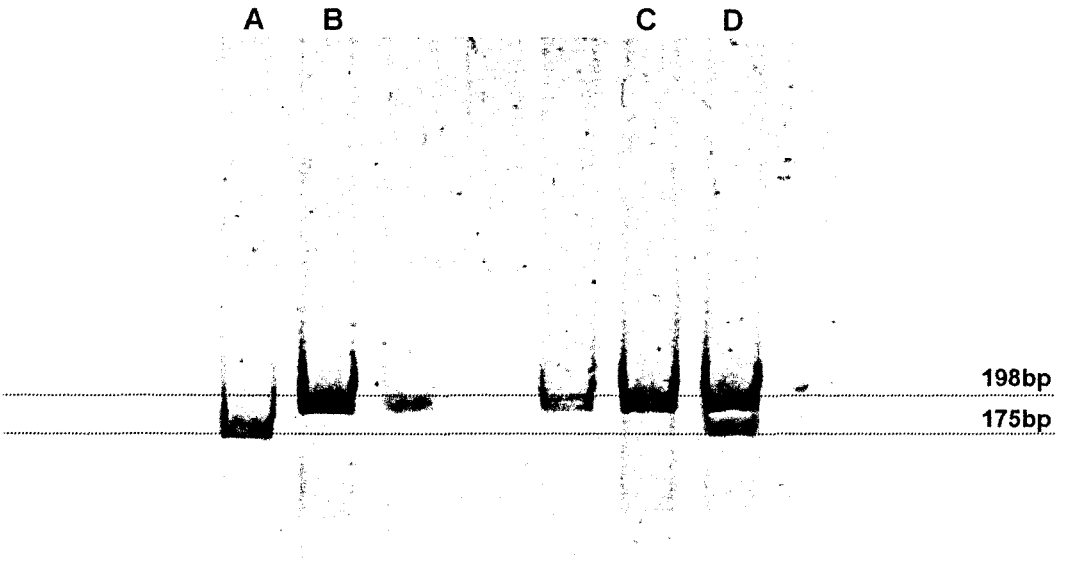
Abnormal homocystein levels: distribution between patient groups



A significant number (43%) of primigravidas with early onset, severe pre-eclampsia had hyperhomocysteinaemia when compared with the other groups (p = 0.0396).

Figure XI

C677T mutation detection

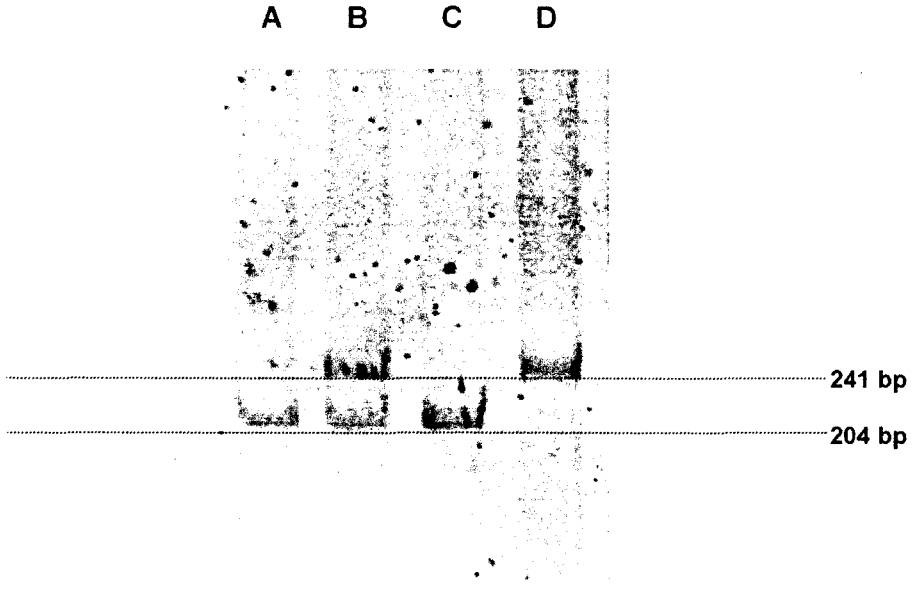


A representative photograph of a wild type individual (C/C), lane B and C. A single, undigested 198 bp band is observed.

In lane A, homozygosity (T/T) for the mutation is demonstrated by a 175 bp fragment. In lane D, heterozygosity is shown by the presence of both bands (mutant allele digested, wild-type allele undigested).

Figure XII

MTHFR Mutation A1298C



Representative photograph of mutation A1298 analysis. The mutation abolishes a *Mbo* II cleaving site present on the wild-type allele.

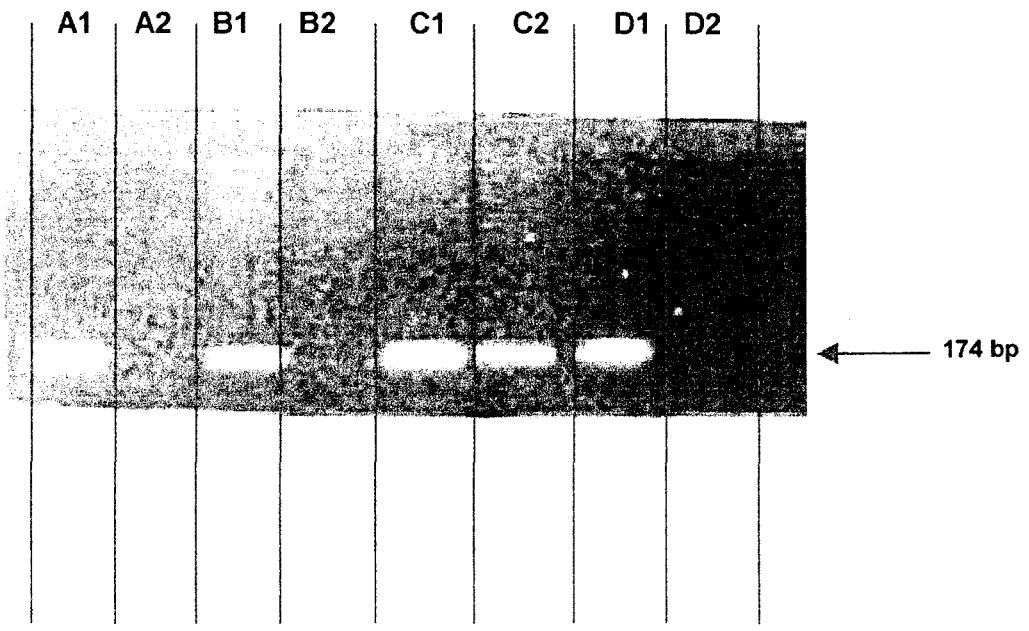
Lane A: Digested 204 bp fragment in a normal (wild-type) individual

Lane B: Two fragments at 241 bp and 204 bp indicating heterozygosity for the mutation.

Lane D: Undigested 241 bp fragment in a homozygous mutant individual.

Figure XIII

Factor V Leiden mutation



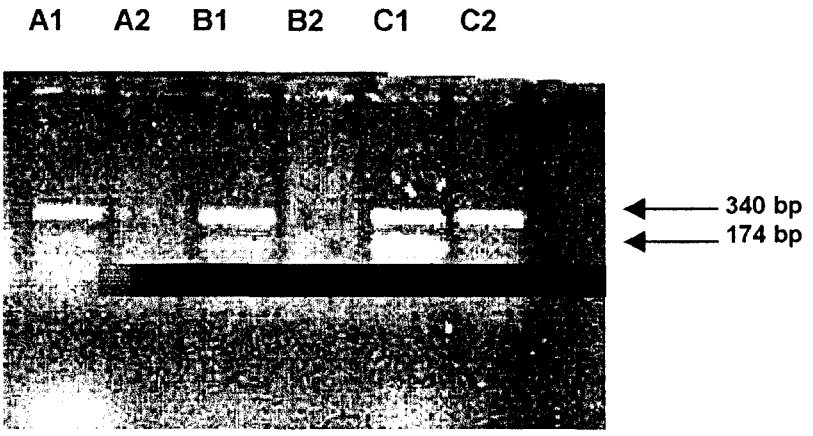
Allele specific PCR analysis for the factor V Leiden mutation:

Lane A: Wild-type individual (A1 normal primer, A2 mutant annealing primer; no annealing)

Lane C: DNA from heterozygous individual annealing with both the wild-type primer (C1) and the mutant primer (C2).

Figure XIV

Simultaneous prothrombin A20210G and factor V Leiden mutation detection



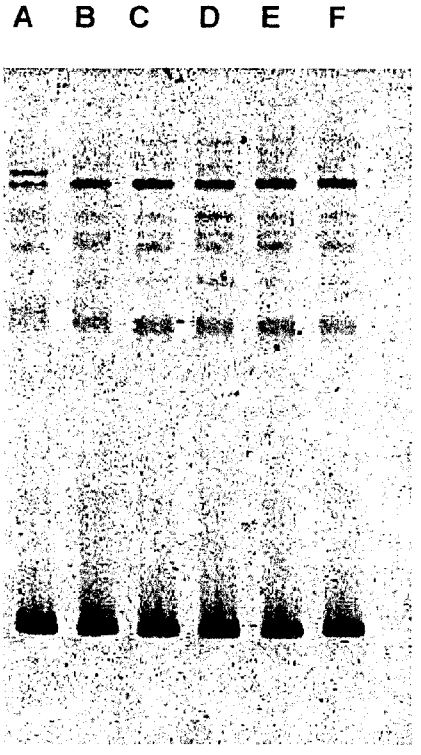
Multiplex allele specific PCR for factor V and Prothrombin A20210G mutation.

Lane C1 and C2: annealing of the wild-type and mutant primers, respectively. At 340 bp, two fragments representing heterozygosity at this locus (prothrombin).

174 bp: fragment in wild-type lane only, representing wild-type homozygosity for factor V Leiden mutation.

Figure XV

LDLR exon 4B (mutations D206E and D154N)



A D206E mutation

B D154N mutation

C-F Individuals negative for both mutations

3.1 Combined heterozygosity for methylenetetrahydrofolate reductase (MTHFR) mutations C677T and A1298C is associated with abruptio placentae but not with intrauterine growth restriction.

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Abstract

Objective. This study was undertaken to investigate the involvement of MTHFR gene mutations C677T and A1298C implicated in vascular disease, in patients with abruptio placentae and intrauterine growth restriction (IUGR).

Study design: DNA was extracted from blood samples of 54 patients with placental vasculopathy (18 patients with abruptio placentae and 36 with IUGR) and 114 control patients and amplified by the polymerase chain reaction. The resulting fragments were subjected to restriction enzyme analysis and resolved by gel electrophoresis.

Results: A significant association could be demonstrated between mutation A1298C and both abruptio placentae and IUGR. Combined heterozygosity for mutations C677T and A1298C was detected in 22.2% of abruptio placentae cases.

Conclusions: Combined heterozygosity for MTHFR mutations C677T and A1298C may represent a genetic marker for abruptio placentae.

Keywords: MTHFR combined heterozygosity, abruptio placentae

1. Introduction

Abruptio placentae is the most common cause of intrauterine death at our institution and the second most frequent cause of perinatal death after preterm labour (1). It is the primary cause of fetal distress in patients where early onset severe pre-eclampsia is treated expectantly (2). Within this population, at least 36% of patients with severe pre-eclampsia also present with IUGR (3).

Abruptio placentae was associated with biochemical hyperhomocysteinaemia in the population served by our institution (4). Elevated homocystein levels have been shown to damage the vascular endothelium (5) and result in placental vasculopathy (6). There are several documented mutations in the gene encoding methylenetetrahydrofolate reductase (MTHFR) which is involved in the remethylation of homocystein to methionine. Homozygosity for a mutated MTHFR gene was found to be a risk factor for placental vasculopathy (7) while the A1298C mutation has been associated with decreased MTHFR activity and neural tube defects (8).

This study was designed to determine the prevalence of the MTHFR enzyme defects in the local population and to investigate their role in abruptio placentae and IUGR.

2. Subjects and methods

2.1. Clinical

The first study group (n=18) comprised patients who delivered at our institution in the period 1 January to 31 December 1996 with the pregnancy complicated by abruptio placentae. Only patients from the immediate catchment area were approached and requested to participate. Abruptio placentae were diagnosed clinically and subsequently confirmed when more than 15% of the placental surface was covered with blood clots. After informed consent was obtained, five millilitres of blood was collected in an EDTA-tube and sent to the laboratory for subsequent analysis.

The second study group (n=36) comprised mothers with IUGR identified at the fetal evaluation clinic where all cases with possible poor fetal growth (symphysis-fundus measurements below the tenth centile for gestational age) are evaluated (9). Doppler flow velocity waveforms of the umbilical artery, determined by a continuous wave Sonicaid Vasoflow machine with a 100Mhz filter, were used to differentiate between IUGR and a constitutionally small fetus. Intrauterine growth restriction is defined as a resistance index (RI) above the 95th centile (10). The study was explained to these patients and informed consent obtained. Blood was collected in EDTA tubes and sent to the laboratory for further analysis.

The control group was recruited from pregnant patients attending their first antenatal clinic. In the period 15 July 1999 - 15 September 1999, 114 consecutive new patients were invited to participate in the study. Venous blood was collected in EDTA tubes for DNA extraction following informed consent.

All patients at this institution receive prophylactic iron and folate supplements during the antenatal period. The majority (91%) of individuals included in this study is from

the mixed ancestry (Coloured) indigenous population of South Africa (study group 50/54 [93%]; control group 103/114 [90%]). The Coloured population of South Africa originated in the seventeenth century after union between European males and females of two indigenous populations, the Khoi and the San (11).

2.2. Mutation detection

Genomic DNA was extracted from whole blood (12) and amplified by the polymerase chain reaction (PCR) using previously described oligonucleotides (13). Amplified products (198 and 163 basepairs) were digested with *Hinf* I and *Mbo* II restriction enzymes for detection of the C677T and A1298C mutations at the MTHFR locus, respectively. Restricted products were resolved on 12% and 20% polyacrylamide gels respectively, and visualised under UV light following ethidium bromide staining.

The frequency of the MTHFR mutations in the two study groups was compared with that of the control group. Chi-square, Fisher's exact tests (where appropriate) and odds ratios were used to compare categorical data. Statistical analysis was performed by an independent statistician using Epi-info version 6.04, the statistical package from the Centers for Disease Control.

3. Results

The frequency of the mutant 677T allele did not differ significantly between the mothers with abruptio placentae (13.9%) or IUGR (11.1%) and the control group (14.9%) [Table I]. No homozygotes for the C677T mutation was detected in either of the study groups but could be identified in two (1.75%) of the mothers in the control group. In contrast, the A1298C mutation occurred significantly more frequently in mothers who developed abruptio placentae (p=0.0157) and in mothers who had IUGR (p=0.0192) when the frequency of the C allele is compared with that of the control group [Table II].

Combined heterozygosity for mutations C677T and A1298C was identified in 4 (22.2%) of mothers who developed abruptio placentae, 2 (5.6%) of mothers who had IUGR and in 4 (3.5%) of the control group. The abruptio group differed significantly from the control group [Table III].

4. Discussion

In this study, MTHFR mutation C677T did not occur more frequently in patients who had abruptio placentae or IUGR than the control group. This compares favourably with the recent study by Chikosi *et al,*. who could not identify more C677T mutations in a cohort of Zulu mothers who developed pre-eclampsia (14). This would suggest that the 677T mutant allele, *per se*, does not represent a major susceptibility locus/factor for abruptio placentae or IUGR in these South African ethnic groups, in whom gestational vascular disease is common.

Homozygosity for the mutant T allele of mutation C677T (combined with wildtype AA genotype of mutation A1298C) was identified exclusively in two individuals from the control group. This status is usually associated with reduced MTHFR activity, elevated plasma homocystein and reduced folate levels and these individuals would usually be distinguishable biochemically. In contrast, homozygosity (and heterozygosity to a lesser extent) for mutant allele 1298C is characterised by reduced MTHFR activity but plasma homocystein and folate levels within the normal range (15). Consequently individuals with this genotype would normally escape detection by conventional plasma screening, reinforcing the value of DNA testing. Plasma homocystein levels and serum and plasma folate and vitamin B12 status were not determined in this study.

Strikingly, when combinations of the mutations were examined in this study, combined heterozygosity for the two mutations was found to occur in almost a quarter of the abruptio cases [odds ratio 5.14 (95% CI 1.05-24.47)]. Could this represent a susceptibility/predisposition factor for abruptio placentae? The apparent combined effect of the two mutations would suggest that variant A1298C represents

an abruptio placentae susceptibility factor which can be triggered/influenced by a modifier factor, in this case, variant C677T.

In this study, the A1298C mutation occurred significantly more commonly in the abruptio and IUGR group than in the control cohort implicating this allele as a minor susceptibility marker for placental vasculopathy. Interestingly, a role for this sequence variant as a possible contributing factor to neural tube defects (NTDs) has previously been proposed (8). Further studies are warranted to elucidate any molecular links between placental vasculopathy and NTDs, both of which may result from low levels of folate and/or raised homocystein levels.

A previous study from our institution demonstrated significantly higher serum homocystein levels in patients who had abruptio placentae when compared with a control group (4). Red blood cell count, serum folate, vitamin B_6 and B_{12} levels did not differ significantly although folate levels were lower in the abruption group. In a case control study at the same institution, no significant differences could be demonstrated in the nutritional status of mothers who had had abruptio placentae when compared with a control group from the same residential area (16). It is therefore likely that genetic factors play a more important role than nutritional conditions in our population. Folic acid appears to be the most important supplement; as little as 0.65 mg per day (3.25x the recommended daily allowance) has been shown to normalise homocystein levels (17). Chronic alcohol misuse is associated with raised homocystein levels and administration of folic acid, vitamin B_6 and B_{12} corrected this (18). Quitting cigarette smoking is another way of reducing elevated homocystein levels (19).

Raised homocystein levels can be normalised by supplementation with folic acid, vitamin B_6 (17) and vitamin C (20). Folic acid and vitamin B supplementation in

particular is effective in lowering homocystein levels in patients with TT and CT genotypes for the 677CT MTHFR mutation (21). Future studies will focus on whether i) a correlation exists between MTHFR mutation status and the clinical presentation of placental vasculopathy and ii) intervention with folic acid and vitamin B supplementation in mutation positive mothers is likely to be beneficial in reducing the incidence and recurrence of abruptio placentae.

Acknowledgements

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Table I

Genotype distribution of MTHFR mutations C677T and A1298C in abruptio placentae, intrauterine growth restriction and a control group. Percentages are shown in brackets.

	Abruptio placentae	IUGR	Control
	n = 18	**n = 36**	**n = 114**
Mutation C677T	**a**	**b**	**c**
Genotype			
CC	13 (72%)	28 (78%)	82 (72%)
CT	5 (28%)	8 (22%)	30 (26%)
TT	0	0	2 (2%)
Mutation A1298C	**d**	**e**	**f**
Genotype			
AA	6 (33%)	16 (44%)	70 (61%)
AC	9 (50%)	14 (39%)	39 (34%)
CC	3 (17%)	6 (17%)	5 (4%)

a vs. c: odds ratio 0.99; 95% CI 0.28 - 3.30; p = 0.7974

b vs. c: odds ratio 0.73; 95% CI 0.27 - 1.91; p = 0.6343

d vs. f: odds ratio 3.18; 95% CI 1.01 - 10.37; p = 0.0473

e vs. f: odds ratio 1.99; 95% CI 0.07 - 4.55; p = 0.1095

Table II

Allele frequencies of MTHFR mutations C677T and A1298C in abruptio placentae, intrauterine growth restriction and a control group. Percentages are shown in brackets.

	Abruptio placentae n = 18	IUGR n = 36	Control n = 114
Mutation C677T	**a**	**b**	**c**
C	31 (86%)	64 (89%)	194 (85%)
T	5 (14%)	8 (11%)	34 (15%)
Mutation A1298C	**d**	**e**	**f**
A	21 (58%)	46 (64%)	179 (79%)
C	15 (42%)	26 (34%)	49 (21%)

a vs. c: odds ratio 0.92; 95% CI 0.29-2.72; p = 0.926

b vs. c: odds ratio 0.71; 95% CI 0.29-1.71; p = 0.538

d vs. f: odds ratio 2.61; 95% CI 1.18-5.77; p = 0.015*

e vs. f: odds ratio 2.06; 95% CI 1.12-3.82; p = 0.019*

*Statistically significant at $p<0.05$

Table III

Combined genotype distribution of MTHFR mutations C677T and A1298C in patients with abruptio placentae, intrauterine growth restriction and a control group.

Mutation C677T	Mutation A1298C	Abruptio placentae (n=18)	IUGR (>95%)† (n=36)	Controls (n=114)
CC	*AA*	5	10	42
CC	AC	5	12	35
CC	CC	**3**	**6**	**5**
CT	AA	1	6	26
CT	AC	4	2	4
CT	CC	0	0	0
TT	AA	0	0	2
TT	AC	0	0	0
TT	CC	0	0	0
Combined heterozygosity		**a** **4 (22%)**	**b** **2 (5.6%)**	**c** **4 (3.5%)**

a vs. c: odds ratio 5.14; 95% CI 1.05 – 24.47; p = 0.0306*

b vs. c: odds ratio 1.06; 95% CI 0.14-6.22; p = 0.6135

† 95th centile refers to Doppler flow velocity waveforms in the umbilical artery

* Statistically significant at p<0.05

3.2 Lipid disturbances in pre-eclampsia: analysis of the low-density lipoprotein receptor gene

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(Presented at the 44th Academic Yearday of the University of Stellenbosch, 24 August 2000. Paper in preparation)

Abstract

Objective

To investigate the hypothesis that pre-eclampsia is associated with atherogenic lipid profiles and corresponding disease-causing mutations in the low-density lipoprotein receptor (LDLR) gene.

Design

One hundred and twenty six consecutive patients were recruited at their first antenatal visit. Blood was obtained for DNA extraction and fasting serum lipograms. The eventual pregnancy outcome was correlated with seven LDLR mutations that are relatively common in South African patients with familial hypercholesterolaemia.

Methods

Maternal DNA was genotyped for mutations V408M, D206E, D200G, D154N, 561 del GGT, -175G/T promoter polymorphism and a 6 bp deletion in exon 2 of the LDLR gene using polymerase chain reaction amplification, gel electrophoresis and restriction enzyme analysis.

Results

None of the mutations screened for in the coding region of the LDLR gene were detected in the study population, including 10 patients with abnormal lipid profiles. Nine patients were positive for the –175G/T promoter polymorphism, but no association with hypertension could be demonstrated. There was a positive correlation between body mass index (BMI) and the deelopment of pre-eclampsia ($p=0.0002$), although this association was not observed in subjects with the T-allele at nucleotide position –175 (BMI > 25 in 7/9 cases).

Conclusion

This study has found no evidence that LDLR gene mutations may contribute to elevated lipid levels in pre-eclampsia. Further investigation is warranted to explain the apparent absence of pre-eclampsia in subjects with a relatively high body mass index in the presence of mutation –175G/T.

Introduction

Fatty acids are an important precursor for prostaglandins and membrane lipids and are also a critical source of metabolic energy. Unsaturated non-esterified fatty acids (free fatty acids, FFA) are intrinsically toxic and have the ability to form free radicals. The concentration of these fatty acids is elevated months before the development of pre-eclampsia [Lorentzen et al, 1995]. There is also a significant association between first trimester total serum cholesterol and the risk of pre-eclampsia [Van den Elzen et al, 1996]. This dyslipidaemia of pre-eclampsia is an exaggerated response of the typical increase in circulating free fatty acids that take place in the late second trimester during normal pregnancy.

The fasting serum triglyceride concentration also increases in pre-eclampsia and levels of triglycerides and free fatty acids decrease within 48 hours postpartum [Hubel et al, 1996]. It is postulated that the link between elevated lipids and the endothelial dysfunction of pre-eclampsia could be oxidative stress [Gratacos, 2000]. Free radical reaction products, such as malondialdehyde, a metabolite of lipid peroxidase, is positively correlated with pre-eclampsia [Hubel et al, 1996].

Postmenopausal women with a history of recurrent hypertension during their pregnancies have significantly increased diastolic blood pressure and atherogenic profiles when compared to matched controls without a prior history of hypertension in pregnancy [Hubel et al, 2000]. Also, the risk of dying from ischemic heart disease in later life is increased in women who have had hypertensive disease in pregnancy (Relative Risk 2.61, 95% Confidence Interval 1.11- 6.12) [Jonsdottir et al, 1995]. It is likely that a combination of genetic predisposition and environmental factors for atherosclerosis in later life is unmasked earlier in life, by pregnancy, as pre-eclampsia. Further evidence for this potential metabolic syndrome is an elevated body mass index (BMI; kg/m^2) in women with pre-eclampsia, regardless of parity,

before, during and after pregnancy; compared with women with normotensive pregnancies [Barden et al, 1999].

There is very little data available on the reason for the exaggerated response of plasma lipids during and preceding pre-eclampsia. Mutations in the low-density lipoprotein receptor (LDLR) gene cause familial hypercholesterolaemia (FH). This disease is common in the South African population and founder mutations in the LDLR gene contribute significantly to the hypercholesterolaemia phenotype in the indigenous South African Afrikaner and mixed-ancestry populations [Loubser et al, 1999]. There is also a high incidence of pre-eclampsia in this same population. African women are also more prone to hyperlipidaemia during normal pregnancy [Ahaneku et al, 1996].

Three founder-related mutations in exons 4 and 9 of the LDLR gene (D206E, V408M and D154N) account for most cases of FH in the Afrikaner [Kotze et al, 1989; 1991, Leitersdorf et al, 1989] and Coloured population of South Africa [Loubser et al, 1999]. A six basepair deletion in exon 2 of the LDLR gene predominates in FH Africans [Thiart et al, 2000]. A recently described -175G/T variant in the footprinting 2 (FP2) *cis*-acting regulatory element of the LDLR gene may play an important role in hypertension by virtue of a possible effect on calcium metabolism [Scholtz et al, submitted]. Two other common mutations in exon 4, a 3 bp deletion (651 del GGT) [Meiner et al, 1991] and an A to G substitution at position 662 (D200G) can also be detected using the same primer set as for the other mutations in exon 4 [Hobbs et al, 1992]. The role of these seven mutations has not been examined in the pathophysiology of pre-eclampsia.

Our hypothesis is that common mutations in the LDLR gene implicated in dyslipidaemia and endothelial damage may also be associated with pre-eclampsia.

The purpose of this study was to obtain a fasting lipogram at booking and to perform mutation analysis of the seven common mutations for correlation with the eventual pregnancy outcome.

Materials and methods

Patient selection

One hundred and thirty seven patients were recruited at the time of their first antenatal visit at a tertiary institution in this non-experimental cohort analytical study. Most patients book at this clinic because of prior pregnancy complications, especially hypertensive conditions or recurrent miscarriages. However, the clinic also serves as a primary care facility for the immediate community and a fair number of low risk patients visit the clinic.

Eleven patients were excluded from the study due to failure to obtain required genotypic and biochemical information. The study was approved by the Ethics Committee of the University of Stellenbosch as part of the ongoing Genetic Aspects of Pre-eclampsia investigation (GAP study) and all patients provided informed consent.

At the time of booking, a blood sample for DNA extraction was obtained (in 1/10 volume ethylenediaminetetraacetic acid (EDTA)) and frozen at -78°C until DNA extraction could be performed. The patients were asked to return the following morning for a fasting lipogram, unless they were fasting at the time of booking. They were then followed up routinely and clinical information was obtained from the clinician's notes following delivery. DNA extraction and mutation analysis were only performed after delivery so as not to influence the management by the attending physician. Results were only correlated with the clinical outcome after all information was collected.

Serum lipograms were performed at a routine chemical pathology laboratory using standard protocols. Atherogenic lipid profiles were defined as levels above the 80th percentile for gender and age [Rossouw et al, 1985]. This cut-off point should correctly identify more than 90% of cases of FH. Pre-eclampsia was defined by the criteria determined by the International Society for Hypertension in pregnancy. This is a blood pressure of 140/90 mmHg on two occasions, at least four hours apart, arising for the first time after 20 weeks of gestation, coupled with significant proteinuria (300 mg/l in a 24-hour urine collection or 2+ on diagnostic urine sticks) [Davey and MacGillivray, 1988].

DNA extraction

DNA extraction was performed using an adaptation of the salting out procedure as originally described by Miller et al (1988). EDTA stored whole blood (5 ml) was lysed with buffer and the cell lysates incubated overnight at 55 °C with nucleic lysis buffer and proteinase K. The next morning, 1 ml of saturated 6M sodium chloride solution was added and the solution centrifuged. The DNA containing supernatant was carefully decanted into a clean polypropylene tube and 20-30 ml of ice-cold absolute ethanol added to precipitate the DNA. The precipitated DNA was briefly washed in 70% ethanol prior to airdrying and subsequently diluted sterile distilled water for use in polymerase chain reactions (PCR).

LDLR mutation analysis

LDLR promoter amplification

PCR reaction mix (per reaction)

Forward primer: 5' aggcagagaggacaatggc 3'	0.55 µl
Reverse primer: 5' cacgacctgctgtgtccaagcttgaaaccc 3'	0.25 µl
PCR buffer (10x)	5 µl
Taq polymerase (5U/µl)	0.15 µl
Deoxynucleotides (5mM)	10 µl
Genomic DNA	1 µl
Distilled water	33.05 µl
Total volume	50µl

Thermal cycling was started at 95°C for 1 minute, followed by 10 cycles (95°C for 10 seconds, 60°C for 45 seconds, 72°C for 45 seconds) and another 30 cycles (95°C for 10 seconds, 58°C for 45 seconds, 72°C for 45 seconds). The resultant DNA fragment was 430 basepairs in size.

LDLR coding region amplification (exon 4)

PCR reaction mix (per reaction)

Forward primer: 5' cccccagctgtgggcctgcg 3'	0.2 µl
Reverse primer: 5' cgcccccaccctgccccgcc 3'	0.2 µl
PCR buffer (10x)	5 µl
Taq polymerase (5U/µl)	0.1 µl
Deoxynucleotides (5mM)	10 µl
Genomic DNA	1 µl
Distilled water	33.5 µl
Total volume	50µl

Thermal cycling started at 95°C for 2 minutes and 30 seconds, followed by 35 cycles of 94°C for 1 minute, 71°C for 1 minute and 72°C for 2 minutes. The final extension was done at 72°C for 10 seconds. A DNA fragment of 237 basepairs was generated.

LDLR coding region amplification (exon 9)

PCR reaction mix (per reaction)

Forward primer: 5' cccctgacctcgctccccgg 3'	0.33 µl
Reverse primer: 5' gctgcaggcaggggcgacgc 3'	0.2 µl
PCR buffer (10x)	5 µl
Taq polymerase (5U/µl)	0.1 µl
Deoxynucleotides (5mM)	10 µl
Genomic DNA	1 µl
Distilled water	33.37 µl
Total volume	50µl

A denaturation step of the PCR reaction was initiated at 95°C for 1 minute, followed by 10 cycles (95°C for 5 seconds, 63°C for 30 seconds, 72°C for 30 seconds) and another 30 cycles (95°C for 5 seconds, 62°C for 30 seconds, 72°C for 30 seconds). A DNA fragment of 224 basepairs was generated.

Mutation detection by gel electrophoresis

A 10% polyacrylamide gel was used, supplemented with 7.5 % urea for the V408M mutation analysis and supplemented with 5% glycerol for the combined heteroduplex and single-strand conformation polymorphism (HEX-SSCP) analysis of mutations D206E, D154N and the 3 bp deletion (codon 197) [Kotze et al, 1995]. A 10% polyacrylamide gel supplemented with 7.5% urea was used for the HEX-SSCP detection of the -175G/T LDLR promoter variant. When a variant in the SSCP was detected in exon 4, exon 4 PCR products were digested with *Msp* I restriction enzyme to identify mutation D200G. Non-denaturing 10% polyacrylamide gel (3%C) were used to resolve restriction enzyme digested PCR products.

Results

The demographic characteristics of the study population are listed in Table I. The pregnancy of 12 (9.5%) patients ended in a miscarriage. Nineteen (15%) developed pregnancy-induced hypertension and 10 (7.9%) developed pre-eclampsia. Ten patients (7.9%) had significantly elevated blood lipids (>90th centile). An elevated body mass index (BMI) at booking was significantly associated with the development of pregnancy- induced hypertension (p = 0.0002). The median BMI of patients with normal outcome was 26 and in the hypertensive groups 33.

There was no relationship demonstrated between elevated BMI and abnormal cholesterol levels (BMI 25 vs. 27). No patient had any of the mutations screened for in the coding region of the low-density lipoprotein receptor.

In the promoter region, nine patients tested positive for the -175G/T variant. These patients tended to be heavier (BMI 31), but there was no significant association with the development of hypertension in pregnancy or with a history of prior hypertension. Three of these patients (2.4%) had a history of previous abruptio placentae but this did not differ significantly from the other patients (OR 3.44, 95% CI 0.41- 33). The average lipid profile for these patients was normal.

Discussion

This study has found no evidence that mutations in the coding region of the LDLR gene are implicated in elevated serum lipids in pre-eclampsia. The -175G/T polymorphism has been associated with elevated diastolic blood pressure in non-pregnant patients (Scholtz et al, submitted). In the study cohort described above, patients with the polymorphism had a higher BMI, which on its own is associated with the development of pre-eclampsia. However, the incidence of pre-eclampsia did not differ from the rest of the group. It may be that the vasodilatation of normal

pregnancy masks the described association or that it may be more important in the non-pregnant state.

Another possible explanation lies in the induction of LDLR gene transcription by calcium [Makar et al, 1994]. This may be the mechanism by which elevated LDL is associated with hypertension. In normal pregnancy, there is a doubling in the gut absorption of calcium to supply in the fetal demand. Furthermore, calcium supplementation in pregnancy leads to an important reduction in both systolic and diastolic blood pressure and pre-eclampsia [Bucher et al, 1996]. None of these patients was on calcium supplementation. This possible confounding factor needs to be addressed outside of pregnancy.

Table I

Demographic characteristics of the study population

Age (median)	31 (16-44)
Gravidity	3 (1-10)
Parity (median)	1 (0-7)
Gestation at booking	16 weeks (16-38)
Race	Coloured 86.5% Black 13.5%
Gestation at delivery	38 weeks (28-42)
Birthweight (median)	2981 gram (824-4630)

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3.3 The contribution of inherited thrombophilia to placental vasculopathy in the Western Cape, South Africa.

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In preparation

Summary

During normal pregnancy, complex alterations in clotting and fibrinolytic factors tip the scale in favour of coagulation. This is advantageous in the face of blood loss during delivery. However, disease processes causing thrombosis and infarctions in the placentae, as with the antiphospholipid syndrome, eventually lead to intra-uterine growth restriction and the development of pre-eclampsia. By similar analogy, thrombophilic mutations can contribute to the development of placental vasculopathy. This has been observed in Caucasian populations with the factor V Leiden and the A20210G prothrombin mutations, while these mutations are virtually absent in Black patients. In this study, performed on Coloured and Black patients with pre-eclampsia in the Western Cape region of South Africa, the frequency of both mutations was shown to be very low and does most likely not contribute significantly to the observed high incidence of placental vasculopathy.

Introduction

Pre-eclampsia is a condition unique to pregnancy and primarily affects the maternal and placental vascular endothelium. It is a major cause of both morbidity and mortality and the perplexing aetiology and pathophysiology have made it one of the most researched conditions in obstetrics.

During normal pregnancy, there are dramatic changes in the coagulation and fibrinolytic system. There is deposition of fibrin in the uteroplacental walls and fibrinolysis is suppressed. There is an increase in levels of clotting factors VII, VIII and X and a doubling in the levels of fibrinogen. The end result is the well-described hypercoagulability of pregnancy, protecting the mother against blood loss at delivery, but also predisposing her to thrombotic complications. Protection against generalised thrombosis is supplied by naturally-occurring anticoagulants, of which antithrombin III and the protein C-thrombomodulin-protein S complex are the most important. Protein C inactivates Factors V and VIII in conjunction with its cofactors protein S and thrombomodulin. Abnormal forms of Factor V, such as those arising from DNA mutations, resist such inactivation and can lead to thrombosis.

The association between placental infarcts and adverse pregnancy outcome, especially midtrimester pregnancy loss and the development of pre-eclampsia, is well described in the antiphospholipid syndrome. Progressive thrombosis and infarcts in the placenta lead to intra-uterine growth restriction (IUGR), pre-eclampsia and abruptio placentae. If one accepts this pathogenesis, a logical hypothesis would be that other forms thrombophilia should also be associated with adverse pregnancy outcome [Nelson-Piercy, 1999].

One such inherited abnormality, described in 1993, is activated protein C resistance (APCR) [Dahlback et al, 1993]. Coagulation factor V (FV) acts as cofactor for

activated Factor X to activate prothrombin in the coagulation cascade. Factor V is cleaved and inactivated by activated protein C. The human Factor V gene contains 25 exons that range from 72 bp to 2820 bp in length and spans more than 80 kb [Simioni et al, 1999]. Bertina et al (1994) described a mutation in exon 10 of the factor V gene (A1691G) which result in the substitution of an arginine with a glycine residue and abolishes the site of protein C cleavage. This variant was named the FV Leiden mutation, after the city in which it was discovered.

The relationship between activated protein C resistance and adverse pregnancy outcome was first reported in 1996 [Rai et al, 1996]. At a specialist recurrent miscarriage clinic, the prevalence of APCR was significantly higher among women with a history of second-trimester miscarriage (20%) than a control group (4.3%; $p<0.02$). In the same year results of the European Prospective Cohort on Thrombophilia (EPCOT) were published [Preston et al, 1996]. The study included 1384 women; of 843 women with thrombophilia, 571 had 1524 pregnancies. In the control group of 541 women, 395 had 1019 pregnancies. The highest odds ratio for stillbirth was in women with combined defects of coagulation (FV Leiden mutation, antithrombin III deficiency and protein C and S deficiencies) (OR 14.3, 95% CI 2.4-86).

The association between FV Leiden mutation and pre-eclampsia was first reported in 1995 in a small patient cohort with severe early-onset pre-eclampsia [Dekker et al, 1995]. In a larger study of 158 women with severe pre-eclampsia, the incidence of FV Leiden mutation was 8.9% and in a control group 4.2% (χ^2 4.686, $p=0.03$). All patients were heterozygous for the mutation [Dizon-Townson et al, 1996]. Since then, several reports from different parts of the world have confirmed or contradicted this association (Table I).

In 1996, a guanine to adenine transition at position 20210 in the 3' untranslated region of the prothrombin gene, was described [Poort et al, 1996]. This mutation (G20210A) was associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis. An independent association with pre-eclampsia was recently reported in Italy (OR 3.31; 95% CI 1.12-6.56) [Grandone et al, 1999]. In another study of 110 women with serious pregnancy complications (abruptio placentae, severe pre-eclampsia, IUGR) the incidence of the A20210G mutation in the prothrombin gene was 10% compared to 3% in a control group ($p<0.03$) [Kupferminc et al, 1999].

The frequency of factor V Leiden mutation in patients with venous thrombosis in the Western Cape is 26% in Caucasians, 8% in Coloured and absent in Black African patients [Rubinstein et al, 2000], while the frequency of the prothrombin mutation is unknown in this population. With the broad scope in incidence between different populations and with the high incidence of placental vasculopathy in the study population, we investigated the contribution of these two mutations to pre-eclampsia in the pregnant population of the Western Cape province in South Africa.

Patients, material and methods

Patients and controls

All the patients involved in this study were consecutively enrolled between March 1999 and December 2000. The control group (n=50) consists of 50 multigravidae who had pregnancies uncomplicated by any of the hypertensive conditions (Coloured n = 40, Black n = 10). They were selected after term delivery in the labour ward at Tygerberg Hospital. Patients with infants with a birth weight below the tenth centile for gestation were excluded to prevent inclusion of possible undiagnosed placental vasculopathy.

The study group (n=300) consisted of 150 primigravidae and 150 multigravidae with severe pre-eclampsia or pregnancy-induced hypertension (Coloured n = 222; Black n = 78). Hypertension in pregnancy is defined by the criteria determined by the International Society for Hypertension in pregnancy. This is a blood pressure measurement of 140/90 mmHg or more on two occasions, at least four hours apart, arising for the first time after 20 weeks of gestation. If coupled with significant proteinuria (300 mg/l in a 24-hour urine collection or 2+ on diagnostic urine sticks) the diagnosis of pre-eclampsia was made [Davey and MacGillivray, 1988].

The demographic characteristics of the patients in the different groups are listed in Table II.

Informed consent for the study was obtained from all patients and the Ethics Committee of the University of Stellenbosch approved the study (project number 99/025).

Blood collection and DNA extraction

Blood samples for DNA extraction were obtained the morning following delivery from a clean venepuncture in the cubital fossa. Five millilitres of blood were collected in a tube containing EDTA (1/10 volume 0.5M sodium EDTA) and samples were frozen at -78°C until DNA extraction could be performed. DNA extraction was subsequently performed using an adaptation of the original salting out procedure as originally described by Miller et al (1988).

PCR protocol

A multiplex allele specific amplification polymerase chain reaction (ASA PCR) was performed using primers described by Hezard et al (1998).

PCR reaction mix

A reaction mix (total volume 50 μl) consisting of the following was set up for each microliter of template DNA: *Taq* polymerase buffer (10x), 5mM deoxynucelotide mix, 1 U *Taq* polymerase (5U/μl), distilled water and a combination of prothrombin reverse primer (wild-type), prothrombin forward primer (consensus) and factor V forward primer (wild-type) and reverse primer (consensus) for the detection of the wild-type allele. The primer concentration was 10pmol/μl for all the primers used.

Primers prothrombin reverse (mutant) and forward (consensus) and factor V forward (mutant) and reverse (consensus) were utilised for the detection of the mutant prothrombin and factor V Leiden alleles.

PCR conditions

Thermal cycling was performed at 95°C for 1 minute, followed by 30 cycles of 95°C, 1 minute; 56°C, 1 minute; 72°C, 1 minute followed by a final extension step of 72°C for 5 minutes. PCR products were resolved on a 1% Agarose gel, stained with ethidium bromide and visualised under ultraviolet light.

Results

Five patients (1.6%) were positive (heterozygous) for the Factor V Leiden mutation and two (0.6%) tested positive (heterozygous) for the A20210G prothrombin gene mutation (Table III). The five patients in the factor V group all belonged to the Coloured population group (5/222 = 2.2%); none of the Black patients tested positive. Both patients with the prothrombin gene mutation were primigravidae with early onset, severe pre-eclampsia (Coloured 1/222, 0.4%; Black 1/74, 1.3%).

No mutation could be detected in any of the relatively healthy patients in the control group. All mutation positive patients had an uneventful postpartum course and none developed any sign of thrombotic complications.

Conclusion

The public health relevance of mutations associated with disease would largely depend on the mutation frequency in a specific population. The incidence of these thrombophilic mutations is extremely rare in the population studied and is probably not a significant contributing factor to the development of pre-eclampsia. Antenatal screening for inherited thrombophilia in these patients (South African Blacks and Coloureds) is not warranted. It remains to be seen whether these mutations are more common in South African Caucasians with pre-eclampsia. The frequency of these mutations in the general South African population has not been determined yet.

Molecular studies of disease may produce significant findings in only a small number of patients, but once the full genetic tapestry is unravelled, it may reveal more about the mechanism of a disease. The apparently small contribution of inherited thrombophilia to placental infarctions and pre-eclampsia in the South African Coloured and Black population suggests that there may be other as yet unknown underlying hypercoagulability factors that play a role in these conditions. It may also imply that the real cause of placental vasculopathy lies elsewhere, and that a tendency to increased clotting only unmasks this condition earlier in susceptible patients.

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Table I

The distribution of FV Leiden mutation and relationship to adverse pregnancy outcome

Country/ region	Condition	n	Incidence	Reference
Israel	Recurrent fetal loss	39	48%	Brenner et al, 1997
Israel	Poor obstetric history	7	100%	Rotmensch et al, 1997
Hungary	Severe pre-eclampsia	pregnant = 71 non-pregnant = 58 pre-eclampsia = 69	7% 5.2% 18.8%	Nagy et al, 1998
	HELLP	21	19%	Krauss et al, 1998
New South Wales	Severe pre-eclampsia	pregnant = 150 pre-eclampsia = 50	0.07% 8%	Mimuro et al, 1998
	Pre-eclampsia	116 FV positive women	25%	Hastings et al, 1998
Italy	Pre-eclampsia	controls = 4 pre-eclampsia = 11	1.8% 7.9%	Grandone et al, 1999
East Anglia (UK)	Pre-eclampsia	control = 200 pre-eclampsia = 283	5.3% 5.5%	O'Shaughnessy et al, 1999
New York	IUGR	General population IUGR = 35	7.9% 0%	Wisotzkey JD et al, 1999
Israel	Pre-eclampsia	110	20%	Kupferminc et al, 1999

Table II

Demographic characteristics of the study population

	Control group	Study group	p value
n	50	300	
Gravidity	3 (2-8)	2 (1-8)	
Parity	2 (1-7)	2 (1-8)	
Race: % Coloured	90%	74%	
Race: % Black	10%	26%	
Gestational age: delivery	39 (37-44)	33 (20-44)	
Birth weight	3256g (2460g-4576g)	2400g (1220g-4498g)	p<0.05
Smoking in pregnancy	40%	19%	p<0.05
Systolic blood pressure (mmHg)	120 (100-140)	160 (130-220)	p<0.05
Diastolic blood pressure (mmHg)	80 (60-90)	105 (80-105)	p<0.05
Age	29 (18-43)	25 (14-46)	

All values are the median. The range is given in parenthesis

Table III

Patient characteristics

Age	Race	G	P	Mutation	Complication	Smoke	Gestation at delivery	Mass
17	Coloured	1	0	Prothrombin	Severe Pre-eclampsia	Yes	30	1208
19	Black	1	0	Prothrombin	Severe Pre-eclampsia	No	32	1722
36	Coloured	4	2	Factor V	Pre-eclampsia	No	36	3794
26	Coloured	3	2	Factor V	Pre-eclampsia	Yes	40	3260
30	Coloured	2	1	Factor V	Abruptio placentae	No	30	1394
20	Coloured	1	0	Factor V	Abruptio placentae	No	34	1250
28	Coloured	1	0	Factor V	Hypertension	No	41	2306

G = gravidity

P = parity

Prothrombin = A20210G Prothrombin mutation

Factor V = Factor V Leiden mutation

Smoke = smoking during pregnancy

Hypertension = pregnancy-induced hypertension

3.4 Hyperhomocysteinaemia and mutations in the methylenetetrahydrofolate reductase gene- a role in the genetic predisposition to placental vasculopathy?

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In preparation

Abstract

Objective

To investigate the role of hyperhomocysteinaemia and two common mutations (C677T and A1298C) in the methylenetetrahydrofolate reductase (MTHFR) gene in the pathophysiology of pre-eclampsia.

Design

One hundred and ten normotensive and 350 women with pregnancies complicated by pre-eclampsia, hypertension or abruptio placentae participated in the study.

Methods

Fasting levels of homocystein were obtained after delivery. Genotyping for the two mutations was performed on genomic DNA with polymerase chain reaction amplification, restriction enzyme analysis and gel electrophoresis.

Results

The mutant T allele of the C677T mutation was significantly associated with hyperhomocysteinaemia ($p = 0.0095$; OR 2.27, 95% CI 1.15-4.46). There were significantly more Black patients with abnormal homocystein levels ($p = 0.0033$). Most of the patients (44%) with abnormally elevated homocystein levels presented with early-onset severe pre-eclampsia ($p = 0.0396$). The allele frequency of the mutated C allele of mutation A1298C was significantly associated with diastolic blood pressure (OR 1.90, 95% CI 1.20-3.03; $p = 0.0055$).

Conclusion

The high incidence of homozygosity for A1298C, a mutation not usually or apparently associated with homocysteinaemia, in pre-eclampsia, and the elevated homocystein levels in Black patients lacking the mutation screened suggests other possible

contributions of these mutations to the pathophysiology of pre-eclampsia in this patient cohort.

Introduction

Hyperhomocysteinaemia can occur in two forms- the severe form, usually referred to as homocysteinuria, is autosomal recessively inherited and results from deficiencies in the cystathione-ß synthetase or methylenetetrahydrofolate reductase (MTHFR) enzymes. The mild form is a risk factor for vascular disease, but does not cause overt disease [Boushey et al, 1995].

The most common genetic defect that results in mild hyperhomocysteinaemia is a substitution in the methylenetetrahydrofolate reductase (MTHFR) enzyme, resulting in decreased plasma folate levels and impaired homocystein remethylation [Rozen, 1997]. The homocystein levels in women with pre-eclampsia are significantly higher than in nulliparas without pre-eclampsia [Rajkovic et al, 1997]. Hyperhomocysteinaemia is also associated with placental infarctions, a finding common in pre-eclampsia [Goddijn-Wessels et al, 1996].

The human MTHFR locus was mapped to chromosome 1p36.3 by *in-situ* hybridisation [Goyette et al, 1994]. DNA screening of the MTHFR gene, using the single-strand conformation polymorphism (SSCP) method for mutation detection, resulted in the identification of a C to T substitution at basepair 677. This resulted in the substitution of a valine codon for an alanine residue. Although defective, the enzyme is still functional. It has decreased stability and is a plausible candidate for moderate hyperhomocysteinaemia and arteriosclerotic disease. Homozygous mutant individuals have significantly increased plasma homocystein levels [Ma et al, 1996].

A second mutation in the MTHFR gene (A1298C), subsequently identified, was reported to occur with a high carrier frequency in the general population [Weissberg et al, 1998]. This mutation is also associated with decreased enzyme activity, but an isolated mutation (heterozygous state) does not result in hyperhomocysteinaemia.

Combined heterozygosity for both C677T and A1298C mutations does lead to hyperhomocysteinaemia [Van der Put et al, 1998] and also appears to be associated with abruptio placentae [Gebhardt et al, in press].

There is a considerable population specificity of allele frequencies. The frequency of the C677T mutation in the normal population varies from 16.3% in Italy, to 11% (Japan), 5.4% (Holland) and 0% (Black Americans) [Fletcher et al, 1998].

A significantly higher frequency of the C677T mutation in patients with pre-eclampsia has been reported in several different populations [Grandone et al, 1997; Nagy et al, 1998; Grandone et al, 1999; Kupferminc et al, 1999]. The role of this mutation in the development of pre-eclampsia is uncertain, as in other populations no relationship between pre-eclampsia and C677T mutation could be demonstrated. These include patient cohorts from East Anglia [O'Shaughnessy et al, 1999], Japan [Kobashi et al, 2000], South African Blacks [Chikosi et al, 1999] and Australia [Kaiser et al, 2000].

The aim of this study was to determine the frequency of these two mutations in the predominantly Coloured (mixed ancestry) population of the Western Cape and to correlate this with biochemical hyperhomocysteinaemia and the development of pre-eclampsia. The Coloured population of South Africa originated in the sixteenth century after union between European males and females of two indigenous populations, the Khoi and the San [Loubser et al, 1999].

Materials and methods

The control group (n=110) comprised patients with pregnancies uncomplicated by any hypertensive condition (in the index pregnancy or any previous pregnancy). Patients with a birth weight below the tenth percentile for gestational age were excluded to prevent inclusion of possible undiagnosed placental vasculopathy. The

study group (n=350) consisted of 100 successive patients with early-onset severe pre-eclampsia (<34 weeks gestation, group A), 100 successive patients with late-onset pre-eclampsia (>34 weeks, group B), 100 individuals with pregnancy-induced hypertension (group C) and 50 patients who developed abruptio placentae (Group D). More than 83% of patients were taking iron and folate supplementation regularly through their pregnancy.

The hospital is one of two tertiary referral units for the Western Cape Province of South Africa. The catchment population comprises mainly Coloured and Black individuals; in 1999 the racial distribution of all deliveries included Coloured 82.8%; Black 15.6% and Caucasian 1.6%. Participating patients were recruited following delivery. Blood was obtained the morning following a fasting period to determine homocystein levels. A clean venepuncture was performed and two tubes of 5 ml each EDTA blood were collected; one was centrifuged and the plasma removed for homocystein determination and the other was stored at -78°C for DNA extraction.

All patients participated in an interviewer-administered questionnaire and clinical details were obtained form the clinician notes. All patients provided written informed consent and the ethical committee of the University of Stellenbosch approved the study.

Hypertension in pregnancy is defined by the criteria determined by the International Society for Hypertension in pregnancy. This is a blood pressure measurement of 140/90 mmHg or more on two occasions, at least four hours apart, arising for the first time after 20 weeks of gestation. If coupled with significant proteinuria (300 mg/l in a 24-hour urine collection or 2+ on diagnostic urine sticks) a diagnosis of pre-eclampsia was made [Davey and MacGillivray, 1988]. Homocystein levels were determined in a

chemical pathology laboratory using the method described by Ubbink and co-workers [1991].

DNA extraction

DNA extraction was performed on whole EDTA-stored blood using an adaptation of the salting out procedure as originally described by Miller [Miller et al, 1988]. EDTA stored whole blood (5 ml) was lysed with buffer and the cell lysates incubated overnight at 55 °C with nucleic lysis buffer and proteinase K. The following morning, 1 ml of saturated 6M sodium chloride solution was added and the solution centrifuged. The DNA-containing supernatant was carefully decanted into a clean polypropylene tube and 20-30 ml of ice-cold absolute ethanol was added to precipitate the DNA. Precipitated DNA was subsequently briefly washed in 70% ethanol prior to airdrying and dilution in sterile distilled water.

Mutation detection

MTHFR C677T mutation

This genotyping was performed by polymerase chain reaction (PCR) amplification and subsequently *Hinf I* restriction enzyme analysis as originally described by Frosst et al (1995).

Primers sequences:

Forward: 5' tgaaggagaaggtgtctgcggga 3'

Reverse: 5' aggacggtgcggtgagagtg 3'

Each PCR reaction was performed in a 50 µl reaction mix containing 0.8 µl (10 pmol/l) of each primer, 4 µl mix of the four deoxynucleotide triphosphates (5 mM), 5 µl of PCR 10x buffer, 1 µl of *Taq* polymerase (5U/µl) and 1 µl of genomic DNA as template. Thermal cycling was started at 95°C for 1 minute, followed by 10 cycles (95°C, 10 seconds; 60°C' 45 seconds; 72°C, 45 seconds) and a further 20 cycles

(95°C, 10 seconds; 57°C, 45 seconds; 72°C, 45 seconds) and a final extension step for 1 minute at 72°C. This resulted in the generation of a DNA fragment of 198 basepairs (bp).

The C to T substitution at nucleotide position 677 creates a *Hinf* I recognition site for enzymatic cleavage. Restriction was performed on 10 µl of amplified template in a water bath at 37°C for at least four hours. Bovine serum albumin (10 mg/ml) was added to enhance the activity of the restriction enzyme. The resulting fragments were resolved by polyacrylamide gel electrophoresis and ultraviolet illumination. A 198 bp fragment is cut into 175 bp and 23 bp fragments if the recognition site, and hence the mutation is present.

MTHFR A1298C mutation

Genotyping was performed by PCR amplification and subsequently *Mbo* II enzyme restriction. Primer sequences:

Forward primer 5' atgtgggggaggagctgac 3'

Reverse primer 5' gtctcccaacttacccttctccc 3'

The PCR reaction was performed in a 50 µl reaction mix containing 1.5 µl (10 pmol/l) of each primer, 5 µl mix of the four deoxynucleotide triphosphates (5mM), 5µl of PCR 10x buffer, 1µl of *Taq* polymerase (5U/µl) and 1 µl of genomic DNA as template. Thermal cycling was started at 94°C for 1 minute 30 seconds, followed by 30 cycles (94°C, 30 seconds; 55°C, 45 seconds; 72°C, 1 minute) and a final extension step for 3 minutes at 72°C. The resulting DNA fragment is 241 bp in size.

The A to C substitution of the A1298C MTHFR mutation abolishes a cleavage site for *Mbo* II in the wild type genome. PCR amplified products were digested overnight at

37°C with *Mbo* II restriction enzyme and the resulting fragments separated and analysed with polyacrylamide gel electrophoresis. The wild-type fragment is usually cut into fragments of 204 and 37 bp respectively.

Results

From the patient questionnaires, it was apparent that there were significantly more smokers in the abruptio group (D) and in the control group compared with the hypertensive groups combined ($p = 0.0012$). From questions regarding family history, there was also evidence of a significant familial relationship of hypertension during pregnancy between mothers and daughters. Only 2% of control indiviuals (uncomplicated pregnancy) had mothers who smoked in their (the mothers') pregnancy, compared with 10-24% in the other groups with the exception of the abruptio group (2%) ($p = 0.0029$).

The distribution of the mutated T allele of the C677T mutation was shown to be evenly spread in all groups: control group (wild-type C/C 77%, heterozygous state C/T 21%, homozygous state T/T 2%), groups A and B (C/C 80%, C/T 20%), group C (C/C 88%, C/T 10%, T/T 2%) and abruptio group (C/C80%, C/T 18%, T/T 2%). Only three patients were homozygous (T/T) for the C677T mutant allele of whom one was in the control group.

There were four (3.6%) patients homozygous for the mutant C/C allele of mutation A1298C in the control group and 37 (10.6%) in the combined patient groups ($p = 0.0080$; OR 4.18; 95% CI 1.37-14.26). The mutant allele C/C status was evenly spread within the patient groups (12 in group A, $p = 0.0402$; 10 in group B, $p = 0.0357$; 12 in group C, $p = 0.0114$; and 3 in group D ($p = 0.315$)). This represents evidence for a possible association between homozygosity for mutation A1298C and hypertension in pregnancy. The allele frequency of the mutated C allele was

significantly associated with raised diastolic blood pressure (OR 1.90, 95% CI 1.20-3.03; p = 0.0055).

The mutant T allele of the C677T locus occurred significantly more frequently in Coloured than in Black study participants (p = 0.0246; 95% CI 1.05-4.21). Likewise, the mutant C allele of the A1298C locus also occurred more frequently in Coloured participants (p = 0.0407; OR 1.80, 95% CI 1.14-2.83). However, there were significantly more Black patients with early-onset severe pre-eclampsia than in any of the other groups (p = 0.0033). Of the patients with combined heterozygosity for mutations C677T and A1298C, only four demonstrated abnormal homocystein levels.

Homocystein levels were determined on samples from 332 patients of whom 68 had an abnormal elevation in fasting homocystein concentration (>11 μmol/l). The mutant T allele of the C677T locus was significantly associated with hyperhomocysteinaemia (p = 0.0095; OR 2.27, 95% CI 1.15-4.46). When all patient groups were combined, there were significantly more Black patients with abnormal homocystein levels (p = 0.0033). High levels of homocystein could also be associated with a one minute Apgar score of 5 or less in a significant proportion of patients (p = 0.043) as well as with intra-uterine death (p = 0.046). Most of the patients with abnormally elevated homocystein were in the early-onset pre-eclampsia group (44%) (p = 0.0396).

Conclusion

In this study we were unable to determine a direct association between the MTHFR C677T mutation and the development of pre-eclampsia; however, the C677T mutation could be associated with hyperhomocysteinaemia and this condition may contribute to the pathophysiology of pre-eclampsia, with which it is associated. There was a strong correlation demonstrated between homozygosity for the A1298C mutation in the MTHFR gene and the development of hypertensive conditions of

pregnancy in the cohort studied. Since this mutation on its own is not associated with clinical elevated homocystein levels, there may be another mechanism by which it exerts its influence.

One such possibility is interaction or combination with other susceptibility factors. From this study it does not appear that combined heterozygosity for both MTHFR mutations can explain the role of the A1298C mutation in hypertensive conditions of pregnancy, since combined heterozygosity occurred in only 13 patients, including two of the control group; only four had abnormal homocystein levels.

Plasma folate plays an important role in regulating homocystein, and hyperhomocysteinaemia is observed primarily when the plasma folate levels are low or reduced [Jacques et al, 1996]. The simple intervention of folic acid supplementation corrects hyperhomocysteinaemia [Nelson-Piercy, 1999]. Since most (>80%) of the study patients were already on folate supplementation, it may take more than simple supplementation to correct the molecular defects in this population. While homocystein levels may appear normal in biochemical testing there may still be folate deficiency at a cellular/ molecular level due to either or both of these mutations.

There is a significant number of Black patients with early-onset severe pre-eclampsia with hyperhomocysteinaemia while the allele frequencies of the two MTHFR mutations were more common in the Coloured population. This suggests enrichment of a novel mutation in the MTHFR gene in the Black population.

Hypertensive complications of pregnancy tend to recur. It is therefore necessary to examine the relationship between these mutations and homocystein between

pregnancies and to investigate the possible contribution of MTHFR mutation status in the fetus.

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3.5 Analysis of combined heterozygosity for C677T and A1298C MTHFR mutations in patients with abruptio placentae

In the preliminary work (reported in section 3.2), combined heterozygosity for both MTHFR mutations (C677T and A1298C) was significantly associated with abruptio placentae. The control group was randomly selected from group L (lipoprotein study group). To confirm or dispute this association in a larger, more selected study group, and to obtain valid statistical analysis, the abruptio placentae group (Group H) was compared with a control group matched patient by patient with regard to age, race and gravidity.

The control group was recruited from the uncomplicated pregnancy outcome group (group A) and from the lipoprotein study group (group L - all patients with uncomplicated pregnancy outcome). Fifty controls were matched with the abruptio placentae individuals (n = 50).

	Abruptio	Control	p value
Age (median)	28	28	0.91
Gravidity	2	2	1
Parity	1	1	1
Gestational age at delivery (weeks)	33	39	0.000
Birthweight (median) in gram	1751	3021	0.000
Race (Coloured:Black)	40:10	41:9	

There was no difference in the allele frequency of the mutant T allele of the C677T mutation in the MTHFR gene (p = 0.4455). Likewise, the allele frequency of the mutant C allele of the A1298C MTHFR mutation did not differ between the two groups (p = 0.5757). There were three patients with combined heterozygosity for both

mutations in the abruptio placentae group and one in the control group; this difference did not reach statistical significance. This association will be tested again once more patients with abruptio placentae are recruited: it is calculated that at least 180 patients are required in each group for valid statistical analysis.

If the two study groups (original report and this study) and two control groups are analysed together, the association retains its statistical significance.

	Abruptio placentae	**Normal outcome**
Combined heterozygosity	7	5
None	61	159

p = 0.0440

Chapter 4

Conclusion

Pre-eclampsia is likely to be familial in origin and conceivably results from a genetic interaction between maternal and fetal factors. This suggests that genetic screening in the future will probably still require an invasive procedure such as chorionic villus biopsy or amniocentesis. This carries a risk to the pregnancy and until non-invasive methods for obtaining fetal cells are established, non-invasive measures such as blood pressure monitoring will continue to be the most reliable and practical means of diagnosing this condition [Steer, 1999].

The hypertensive complications of pregnancy tend to recur. In this project, most homocystein levels were determined after delivery; they may have been abnormally elevated as part of an acute phase response. It is therefore also necessary to examine the relationship between mutation status and homocystein level between pregnancies and to determine the contribution of MTHFR mutation status in the fetus. It may be that maternal and fetal transmission patterns vary and so influence outcome.

Any mutation or polymorphism that is maintained in a population may be due to heterozygous advantage The factor V Leiden mutation may have spread due to heterozygous advantage; patients with the mutation may have a decreased risk of post-partum haemorrhage. However, the frequency is still very low in our cohort. Whether heterozygous advantage is the reason for the high frequency of MTHFR mutation A1298C remains an open question at this time.

Molecular studies of disease may produce significant findings in only a small proportion of patients, but once the genetic tapestry is unravelled, it may help fully

explain the mechanism underlying the disease. The contribution of inherited thrombophilia to placental infarctions and pre-eclampsia suggests that hypercoagulability may be a pathophysiological route to abnormal placental vasculopathy, but only in a small proportion of mainly European patients. There are likely to be other novel factors underlying hypercoagulability that play a role in placental vasculopathy. It may also imply that the real cause of placental vasculopathy lies elsewhere, and that a tendency to increased clotting only unmasks this condition earlier in susceptible patients.

Future prospects

Future work will include screening for the presence of gene mutations implicated in endothelial damage and hypertension, this will include the angiotensin receptor gene and the nitric oxide gene. The significance of fetal mutation status requires further investigation.

A randomised, controlled trial can be provisionally planned at this stage; the possible ethical dilemma of giving a placebo instead of folate can be managed by routinely giving all patients folate and then randomising groups into various extra vitamin supplementation vs. placebo. More than 80% of patients were taking folate in any case, and it did not seem to make any difference to the development of pre-eclampsia and abruptio placentae.

Finally, for a large scale study an adequate, representative control group is needed, preferably from the representative general population. Planning is currently underway to obtain cord blood from all deliveries at designated, randomly chosen midwife units for a single year; this resultant DNA bank of relative healthy, anonymous individuals can serve as a control group for a variety of molecular studies for years to come.

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Appendix

PASIëNT INLIGTING- EN TOESTEMMINGSVORM

Op soek na genetiese redes vir hipertensie in swangerskap

VERWYSINGSNOMMER:

VERKLARING DEUR PASIëNT

Ek, die ondergetekende, _____ van

_____(adres)

bevestig dat:

1. Ek uitgenooi is om deel te neem aan bogemelde navorsingsprojek wat deur die Departement van Verloskunde, afdeling Mensgenetika van die Universiteit van Stellenbosch onderneem word.

2. Die volgende aan my verduidelik is:

2.1 Doel: Om te kyk of daar 'n genetiese (oorerflike) rede is hoekom sommige swanger vrouens hoë bloeddruk in swangerskap kry.

2.2 Prosedure: Inligting oor my huidige en vorige swangerskap(pe) sal aan my gevra word. Die inhoud is vertroulik en alle inligting sal naamloos gehou word. Tydens die geboorteproses gaan twee ekstra buisies naelstringbloed geneem word vir die bepaling van oorerflike faktore by my baba. Hierdie is addisioneel tot die roetine bloedmonsters wat geneem word. Verder gaan daar twee buisies bloed by my geneem word vroegoggend, voor ek ontbyt gehad het. Die res van die bloedmonsters wat by my geneem gaan word, is standaard in die hantering van my swangerskap en moontlike geassosieerde komplikasies.

3. Ek meegedeel is dat die die inligting wat ingewin word as vertroulik behandel sal word, maar wel aangewend kan word vir referate op kongresse of ander publikasies.

4. Ek meegedeel is dat ek mag weier om deel te neem aan hierdie projek (asook dat ek enige tyd deelname daaraan kan staak) en dat sodanige weiering of staking nie op enige manier my huidige/toekomstige behandeling by hierdie of enige ander inrigting sal benadeel nie.

5. Die inligting wat hierbo weergegee is deur_____ aan my in Afrikaans verduidelik is en dat ek die taal goed magtig is. Ek is 'n geleentheid gegee om vrae te vra en al my vrae is bevredigend beantwoord.

6. Daar is geen dwang op my geplaas om toe te stem tot my deelname aan die projek nie.

7. Deelname aan die projek geen addisionele koste vir my inhou nie.

Ek stem vrywillig in om deel te neem aan bogemelde projek.

Geteken te_____ op _____19_____

_____ _____

Pasiënt se handtekening of regter duimafdruk | Getuie

VERKLARING DEUR OF NAMENS NAVORSER

Ek,_____verklaar dat ek:

1. Die inligting vervat in hierdie dokument aan _____verduidelik het;

2. Haar versoek het om vrae aan my te stel indien daar enige iets onduidelik was;

3. Dat hierdie gesprek in Afrikaans plaasgevind het en dat geen tolk gebruik is nie.

Geteken te_____ op_____19____

_____ _____

Navorser/Navorser se verteenwoordiger | Getuie

PATIENT INFORMATION AND CONSENT FORM

Genetic factors in pre-eclampsia
REFERENCE NUMBER:

DECLARATION BY PATIENT

I, the undersigned, _____
from_____(address)

Confirm that:
1. I was asked to participate in the above-mentioned research project by the department of Obstetrics and Gynaecology, division Human Genetics at the University of Stellenbosch.

2. The following was explained to me:
2.1 Aim: To see if there are genetic reasons why some pregnant patients develop high blood pressure in pregnancy.

2.2 Procedure: I will be asked to give information on my current and previous pregnancies. All information is confidential and will be kept anonymous. At birth, two extra tubes of chord blood will be taken in addition to the routine chord blood taken for blood group determination. This extra blood will be for genetic testing. A further two tubes of blood will be taken from me the next morning before breakfast. The rest of the blood samples taken from me will be the routine blood needed for the management of my pregnancy and any possible complications.

3. That all information gathered will be kept confidential, but the information could be used for presentations at a congress or for publications in medical journals.

4. That I may decline to participate in this project. If I do, this will not influence my current or future management at this or any other institution.

5. This was explained to me by_____ in English and I am fluent in this language. I was given ample time to ask any questions and all questions were answered to my full satisfaction.

6. I was not pressurised to participate in this study.

7. There will be no additional costs involved.

Signed at_____ on _____19_____

_____ _____
Signature or right thumb print witness

DECLARATION BY OR FOR RESEARCHER

I,_____declare that

1. I explained the information in this document to_____;

2. She was given ample time to ask any questions;

3. This conversation was in English and no translator was used.

Signed at _____ on_____19____

_____ _____
Researcher/ representative of researcher witness

Table XIII

Oligonucleotides for amplification and sequencing the different gene exons and flanking introns

Gene Name	Mutation/ variant	Primer name		Sequence (5'-3')	Product size
Prothrombin	G20210A	1A	R: - WT	cactgggagcattgaggatc	
		1B	- MUT	cactgggagcattgaggatt	
		1C	F: - CONSEN	tctagaaacagttgcctggc	340bp
MTHFR	C677T		C677T-F	tgaaggagaaggtgtctgcggga	198bp
			C677T-R	aggacggtgcggtgagagtg	
	A1298C		F	atgtgggggaggagctgac	
			R	gtctcccaacttacccttctccc	241bp
FV Leiden	G1691A	3A	F: - WT	cagatccctggacagacg	
	(ASA_3)				
		3B	- MUT	cagatccctggacagaca	
		3C	R: - CONSEN	tgttatcacactggtgcttaa	174bp
LDL-R prom	-175G/T		F R	aggcagagaggacaatggc cacgacctgctgtgtccaagcttgaaa	430 bp
LDL-R Exon 4	D154N D206E D200G 651del GGT		F H4B R J4B	cccccagctgtgggcctgcg cgcccccaccctgccccgcc	237 bp
LDL-R Exon 9	V408M		F H9 R J9	cccctgacctcgctccccgg gctgcaggcaggggcgacgc	224 bp

LDLR gene mutations

Exon 9 (V408M) amplification

Primer H9 (20pmol)	0.33µl
Primer J9 (20pmol)	0.2µl
dNTPs (5mM)	10.0 µl
10x buffer	5.0 µl
dH_2O	33.37 µl
Taq polymerase (5U/µl)	0.1 µl
DNA	1.0 µl

Total: 50 µl

PCR program

95°C	1 minute	
95°C	5 seconds	x10
63°C	30 seconds	
72°C	30 seconds	
95°C	5 seconds	x30
62°C	30 seconds	
72°C	30 seconds	
30°C	10 seconds	

Resolve on 10% polyacrylamide gel supplemented with 7.5% urea at 150 Volt

Exon 4 (D154N, D206E, D200G, 651 del GGT) amplification

Primer H4B (20pmol)	0.2µl
Primer J4B (20pmol)	0.2µl
dNTPs (5mM)	10.0µl
10x Buffer	5.0µl
Taq polymerase (5U/µl)	0.1µl
dH_2O	35.3µl
DNA	1.0µl

PCR program

95°C	2 minutes 30 seconds	
94°C	1 minute	35 cycles
71°C	1 minute	
72°C	2 minutes	
72°C	10 minutes	
30°C	10 seconds	

Denature DNA for 5 minutes at 95°C, quench on ice before resolving

Resolve on 10% polyacrylamide gel, supplemented with 5% glycerol, 180 Volt, overnight

Digest fragments overnight with *Mbo* II for the D154N mutation

Digest fragments overnight with *MSP* I for the D200G mutation

MTHFR Mutations

1. A1298C mutation

Amplification

10x Buffer	5.0µl
Forward primer (10pmol/µl)	0.8µl
Reverse primer (10pmol/µl)	0.8µl
dNTPs (5mM)	4.0µl
dH_2O	36.4µl
Taq polymerase (5U/µl; 1µl + 5µl dH_2O)	2.0µl
DNA	1.0µl

PCR Program

94°C	1 minute 30 seconds	
94°C	30 seconds	30 cycles
55°C	45 seconds	
72°C	1 minute	
72°C	3 minutes	
20°C	30 seconds	

Restriction enzyme analysis (*Mbo* II)

10x Buffer	2.0µl
dH_2O	7.5µl
Mbo II (12U/µl)	0.5µl
PCR product	10µl
Total volume	20 µl

Resolve on polyacrilamide gel (20µl + 5 µl loading dye) for 2 hours at 250 volt.

2. C677T mutation

Amplification

10x Buffer	5.0µl
Forward primer (10pmol/l)	1.5µl
Reverse primer (10pmol/µl)	1.5ul
dNTPs (5mM)	5.0µl
dH_2O	34.0µl
Taq polymerase (5U/µl; 1µl + 5µl dH_2O)	2.0µl
DNA	1.0µl

PCR program

95°C 1 minute

95°C 10 seconds
60°C 45 seconds
72°C 45 seconds
} x10

95°C 10 seconds
57°C 45 seconds
72°C 45 seconds
} x20

72°C 1 minute

20°C 30 seconds

Restriction enzyme analysis (*Hinf* I)

10x Buffer	2.0 µl
Bovine Serum Albumin	0.2µl
Distilled water	6.8µl
Hinf I (10U/µl)	1.0µl
Total volume	20 µl

Resolve on polyacrilamide gel (20µl + 5 µl loading dye) for 2 hours at 250 volt.

Multiplex PCR for Factor V Leiden and Prothrombin mutation

Amplification

	Normal	**Mutant**
10x Buffer	5.0µl	5.0µl
dNTPs	5.0 µl	5.0 µl
Primers 1a (10pmol)	1.0 µl	-
1b	-	1.0 µl
1c	1.0 µl	1.0 µl
3a (10 pmol)	1.0 µl	-
3b	-	1.0 µl
3c	1.0 µl	1.0 µl
Distilled water	33.0 µl	33.0 µl
Taq polymerase (1µl+5µl dH_2O)	2.0 µl	2.0 µl
DNA	1.0 µl	1.0 µl

PCR Program

95°C	1 minute	
95°C	1 minute	30 cycles
56°C	1 minute	
72°C	1 minute	
72°C	5 minutes	
30°C	10 seconds	

Resolve on 1% Agarose gel

LDLR GENE (promoter)

PCR reaction (50µl)

100 µM of each dNTP

10 x PCR buffer

15 pmol of each primer

0.75U Boehringer Mannheim *Taq* polymerase

Mineral oil (Sigma) overlay

1 µl of genomic DNA

PCR cycling conditions

95°C for 1 minute

95°C for 10 seconds
60°C for 45 seconds
72°C for 45 seconds
} 10 cycles

95°C for 10 seconds
58°C for 45 seconds
72°C for 45 seconds
} 30 cycles

35°C for 5 seconds

HEX-SSCP analysis

10 µl PCR product and 10µl gel loading buffer

Heat denaturisation at 95°C for 5 minutes

Quench on ice prior to loading

Combined 10% polyacrylamide / 7.5% Urea gel

Overnight electrophoresis at 4°C at 25mA in 1.5 x TBE buffer

Gel electrophoresis

10% polyacrylamide gel (supplemented with urea)

4.5 g Urea

18 ml 5x TBE

27 ml dH_2O

15 ml Acrylamide (1% C of a 40% stock)

600 µl ammonium persulphate (APS 10%)

60 µl TEMED

10% polyacrylamide gel (supplemented with glycerol)

3 ml Glycerol (5%)

6 ml 5xTBE

15 ml Acrylamide (1% C of a 40% stock)

36 ml dH_2O

800µl ammonium persulphate (APS 10%)

80µl TEMED

Restriction enzyme gel

5 ml Acrylamide (3.4% C of a 40% stock)

4 ml 5xTBE

10.78 ml dH_2O

160 µl APS

60 µl TEMED

Agarose gel

1%: 1g Agarose gel/ 100 ml 1XTBE buffer

HEX-SSCP gel

4.5 g Urea

15 ml polyacrylamide (1%C, 40% stock)

18 ml 5x TBE

27 ml distilled water

0.8 ml 10% APS

0.08 ml TEMED

Stock solutions

40% Stock (5%C) for restriction enzyme analysis

76 g Acrylamide

4 g Bisacrylamide

Make up to a total volume of 200 ml with distilled water

40% Stock (1%C); for heteroduplex and SSCP analysis

Acrylamide 39.6g

Bisacrylamide 0.4g

Make up to a total volume of 100 ml with distilled water

3.4% C, 40% stock

58.8g Acrylamide

1.2g bis-acrylamide

Fill to 200 ml with distilled water

Lysis buffer (DNA Extraction)	**per 1000 ml**
Ammonium chloride 0.155M	8.3 g
Potassium hydrogen carbonate 0.01 M	1.1 g
EDTA 0.0001M	0.03 g
Adjust pH to 7.4 with concentrated HCl	

Nuclei lysis buffer	**per 1000 ml**
Tris-chloride 0.01 M	1.211 g
Sodium chloride 0.4 M	23.4 g
EDTA 0.002 M	0.6 g
Adjust pH to 8.2 with concentrated HCl	

Gel loading buffer

Consists of a 1:1 ratio of a xylene-bromophenol blue mixture - 0.025% bromophenol blue; 0.025% xylene cyanol; 2.5% ficoll

TBE (5X)

Tris 54 g

Boric Acid 27.5 g

EDTA 4.65 g

Make up to 1 litre with distilled water

10% Ammonium persulphate (APS) solution

1g APS

10 ml distilled water