INHERITED FACTORS IN PRE-ECLAMPSIA: MOLECULAR GENETIC AND EPIDEMIOLOGICAL STUDIES IN A SRI LANKAN POPULATION

By

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On one sunny evening in August 1999, when I made my way to an oration in Colombo on 'Pre-eclampsia in the 21st Century' by Prof. Malcolm Symonds of the University of Nottingham, I never thought that that would be my first steps towards reading for a Ph.D. at the University of Nottingham. A post oration meeting with Prof. Symonds was soon followed by an introduction to my supervisors. Following year I was packing my bags to come to Nottingham. A serendipitous meeting in Serendib had changed my life forever.

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ABSTRACT

Pre-eclampsia is a disorder of unknown aetiology that affects about 5% of Sri Lankan women during their pregnancy. It is most likely a multifactorial disorder that is caused by the interaction of genetic and environmental factors. Recent advances in genetics have resulted in a surge of investigations into genetic factors underlying pre-eclampsia. These studies have been conducted mainly in the white Caucasians in the West and the Japanese in the East.

The investigations described in this thesis therefore were undertaken in a genetically distinct South Asian Sinhalese population in Sri Lanka and replicated in a white Caucasian population in Nottingham, UK. Four candidate genes; Epidermal Growth Factor (*EGF*) and Transforming Growth Factor Alpha (*TGFA*), which may play a role in placentation; Angiotensinogen (*ANG*), which is involved in blood pressure regulation; and 5,10-Methylenetetrahydrofolate reductase (*MTHFR*), which is an enzyme involved in folate metabolism, were examined.

These investigations consisted of the following: Recruitment of 80 population volunteers each from the Sinhala, Sri Lankan Tamil and Moor racial groups in Sri Lanka, establishing the allele/haplotype frequency for the candidate genes in those volunteers and in 80 white Caucasian population volunteers, and comparing their allele/haplotype frequencies; recruitment of 180 Sinhalese women with pre-eclampsia and 180 normotensive pregnant Sinhalese women,

establishing the allele/haplotype frequencies of the candidate genes in these women and in 74 white Caucasian women with pre-eclampsia and 81 normotensive pregnant white Caucasian women, comparing the pre-eclampsia phenotype of the Sinhalese with that of the white Caucasians, examining the association of the candidate genes with pre-eclampsia, and examining the association of the candidate genes with quantitative traits such as birth weight and blood pressure in normotensive pregnant women; and examining the functional effects of polymorphisms in the angiotensinogen gene on gene expression.

The phenotyping results of the Sinhalese women reflect the severe morbidity associated with pre-eclampsia elsewhere, and highlight the severe perinatal mortality associated with pre-eclampsia in the Sinhalese. The population genetic results show considerable similarity between allele/haplotype frequencies of the Sri Lankan racial groups and considerable variation between them and the white Caucasians. The *EGF* gene was associated with pre-eclampsia in the Sinhalese and with the weight of babies at birth in both the Sinhalese and the white Caucasians. The *TGFA, ANG* and *MTHFR* genes were not associated with either pre-eclampsia or any quantitative trait. The angiotensinogen reporter gene expression studies revealed the possible existence of a repressor element in the 3' untranslated region of the angiotensinogen gene, but this finding needs confirmation by further investigations.

PUBLICATION AND ABSTRACTS

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1. INTRODUCTION

The development, in the later half of pregnancy, of new-onset hypertension and proteinuria that resolves postpartum – pre-eclampsia – is a potentially life threatening condition. It appears to be a disease unique to human pregnancies. It is an enigmatic condition because in spite of rigorous investigation, the underlying aetiology remains obscure. This thesis describes new investigations into the aetiology of pre-eclampsia using molecular genetic and epidemiological methods in the Sinhalese in the Sri Lankan population and attempts at replicating the results in the white Caucasians in Nottingham, UK.

This introductory chapter is intended to serve several purposes. It begins by highlighting the significant health related implications of pre-eclampsia and the challenges faced by clinicians in diagnosing the condition. In doing so reference will be made to relevant Sri Lankan scientific literature that may not be widely available. Copies of these are provided in a separate folder bound to the back cover of this thesis. The second section of this introduction is on the investigation of genetic factors involved in pre-eclampsia. It examines the evidence for a genetic basis for pre-eclampsia, the evidence for the possible location of pre-eclampsia susceptibility gene(s), and the approaches and the future directions such investigations may take. The third section is on the pathophysiology of the disease. It briefly surveys the evidence for the widely accepted two-stage hypothesis on the pathogenesis of pre-eclampsia and concludes by identifying two novel candidate genes (*EGF* and *TGFA*) that

have not been examined before in any population and two more candidate genes from among those that have already been studied that require further evaluation in the light of this hypothesis. The next four sections are devoted to each candidate gene. In each section a survey will be made of the available data on the genomic structure, the regulation of the gene as far as it is relevant to these investigations, and the functional effects of the protein encoded by the gene. Each section concludes by identifying genetic markers within or near the candidate gene that have been selected for the investigations described in this thesis. This chapter concludes with a statement of the hypothesis and the objectives of this thesis.

The second chapter is on methods. It describes the validation of the urine protein heat coagulation test (HCT) that was central to the establishment of the diagnosis of pre-eclampsia in Sri Lanka; recruitment of subjects and phenotyping; generic molecular biology methods; specific genotyping and functional assays used for these investigations and statistical methods. Results are reported in the third chapter. The fourth chapter is a discussion of the results. The final chapter summarises the conclusions of this thesis.

1.1. The burden of hypertensive disorders of pregnancy

Pre-eclampsia is a major medical problem in pregnancy throughout the world. To appreciate the burden of this problem, however, it has to be considered in the wider context of the spectrum of hypertensive disorders of pregnancy: Gestational Hypertension (GH), Pre-Eclampsia (PE), a majority of cases of Eclampsia (E) and HELLP Syndrome (Haemolysis, Elevated Liver enzymes, Low Platelet count in association with hypertension). Gestational hypertension is the occurrence of *de novo* hypertension in pregnancy. Pre-eclampsia is the occurrence of significant proteinuria in addition to *de novo* hypertension (see section 1.2.1). Eclampsia is the occurrence of generalised convulsions during pregnancy, labour, or within seven days of delivery which is not caused by epilepsy or other convulsive disorders. The HELLP syndrome, the definition of which may vary slightly from country to country, is usually indicated by the presence of an abnormal peripheral blood smear, with schistocytes and/or burr cells; serum lactate dehydrogenase, bilirubin, and transaminase more than twice the upper limit of normal, and platelet count less than $100x10^9$/L.

1.1.1. Incidence of hypertensive disorders of pregnancy

There are many reports on the occurrence of hypertensive disorders of pregnancy in different parts of the world. Very few of these, however, could be used to estimate their incidence with any degree of reliability and an even smaller number could be used for comparison. The reported incidence shows great variation. This may be attributable to differences in definition,

population composition, demographic and obstetric characteristics, actual disease incidence, or access to and availability of antenatal care services (World Health Organisation, 1987). A population based international collaborative study designed to control for these factors found that clinically recognised hypertension during pregnancy varied by a factor of 25 (Incidence range 1.2% to 31.0%) between countries. Even using a strict definition of proteinuric hypertension, the incidence varied by a factor of five (Incidence range 1.5% to 8.3%) (World Health Organisation, 1988).

In the only Sri Lankan study of its kind hypertensive disorders of pregnancy have been reported to occur in 4.9% of pregnancies delivering in a tertiary care hospital (Jayawardana and Fernando, 1995). This study and another from the same hospital (Jayawardana, 1994), report the proportion of hypertensive women having pre-eclampsia (43.4% and 46.5% respectively) as being not much less than that of gestational hypertension (51.1% and 53.4% respectively). In another Sri Lankan study proteinuria defined as >2 g/L was present in 37.2% of patients with hypertension in pregnancy (Jegasothy, Thayalasekaran and Sivasuriya, 1983). This definition of proteinuria is much more rigorous than the currently accepted definition for significant proteinuria in pregnancy of ≥0.5g/L. Even so, in the West, proteinuria is usually reported to occur in only one-fifth or less of women with *de novo* hypertension in pregnancy (Zhang, Meikle and Trumble, 2003). It is possible therefore, that Sri Lankan women who develop *de novo* hypertension in pregnancy may go

on to develop the more severe form of the disease spectrum – pre-eclampsia – than do women in the West. This may perhaps be explained if Sri Lankan women were to have lower blood pressure levels prior to pregnancy than do women in the West, because then they may be expected to have developed severe disease by the time their blood pressure reached the diagnostic threshold, which is of course a Western definition. This possibility is examined in the analysis of the pre-eclampsia phenotype in section 3.1.3.

Similar observations can be made with respect to eclampsia. Eclampsia has been reported to occur in 0.28% of pregnancies in Peradeniya in Central Sri Lanka (Jayawardana *et al.*, 1995), 0.38% of pregnancies in Galle in Southern Sri Lanka (Goonewardene and Sirisena, 1984-85), and 0.66% of pregnancies in Jaffna in Northern Sri Lanka (Jegasothy *et al.*, 1983). This is many times more than that in the United Kingdom (0.049%: Douglas and Redman, 1994), United States of America (0.056%: Saftlas, Olson, Franks, Atrash and Pokras, 1990, 0.1%: Zhang *et al.*, 2003), and Finland (0.024%: Ekholm, Salmi and Erkkola, 1999).

1.1.2. Morbidity and mortality associated with hypertensive disorders of pregnancy

In the developed world, the syndrome of pre-eclampsia and eclampsia is one of the two most common causes of maternal mortality (The National Institute for Clinical Excellence, The Scottish Executive Health Department and The

Department of Health Social Services and Public Safety Northern Ireland, 2001). However, it is the developing world, where pre-eclampsia is the third commonest cause of maternal death behind haemorrhage and infection (World Health Organisation, 1999), that accounts for the overwhelming majority of the estimated 50 000 annual maternal deaths from hypertensive disorders of pregnancy. In Africa, Latin America, and the Caribbean, pre-eclampsia and eclampsia is estimated to account for 20-25% of maternal deaths (Duley, 1992).

Sri Lanka stands out in the developing world as a country that has made vast strides in improving maternal health. The overall maternal mortality rate in Sri Lanka in 1995 was 2.4 per 10 000 deliveries compared with 15 per 10 000 deliveries in 1970 (Seneviratne and Rajapaksa, 2000). Hypertensive disorders of pregnancy accounted for 10.3% of maternal deaths in 1997 (Family Health Bureau, 1998). This, however, does not mean that severe maternal morbidity associated with hypertensive disorders of pregnancy is any less than has been reported in other parts of the world (Zhang *et al.*, 2003) as evidenced by the analysis of the pre-eclampsia phenotype in Sri Lankan women recruited for the investigations described in this thesis (see section 3.1).

Since the 'cure' for pre-eclampsia is delivery there is also a considerably increased morbidity and mortality from iatrogenic prematurity. In one Sri Lankan study as many as 34% of infants born of pregnancies complicated by

pre-eclampsia required special care (Jayawardana, 1994). Perinatal mortality rates (PNMR) in pregnancies complicated by pre-eclampsia are low in the West where it is reported to be less than 5% (Baldwin, Leighton, Kilby, Wyldes, Churchill *et al.*, 2001, Zhang *et al.*, 2003). In comparison, perinatal mortality rates of 17% (Jegasothy *et al.*, 1983), 14.8% (Goonewardene and Kularatna, 1992), 23.2% (Jayawardana, 1994), and 24.1% (Jayawardana *et al.*, 1995) have been reported in Sri Lankan studies.

1.1.3. Recurrence of pre-eclampsia in subsequent pregnancies

The repeated occurrence of pre-eclampsia or gestational hypertension in several pregnancies of the same woman is not a rare event. The recurrence of pre-eclampsia or gestational hypertension in subsequent pregnancies in some studies has been as high as 50% (Chesley, 1980, Campbell, MacGillivray and Carr-Hill, 1985, Sibai, el-Nazer and Gonzalez-Ruiz, 1986, Sibai, Mercer and Sarinoglu, 1991, Makkonen, Heinonen and Kirkinen, 2000, Zhang, Troendle and Levine, 2001). Some retrospective studies estimating the recurrence risks of pre-eclampsia, however, may not give the true picture, as they have not been able to verify the diagnosis by examining case notes since strict criteria may not have been applied to diagnose the condition in the past. Indeed in some studies, including that of long term studies by Chesley and colleagues (Chesley, Annitto and Cosgrove, 1976, Chesley, 1980), 'eclampsia' has been used as a surrogate for the diagnosis of pre-eclampsia as the former was likely to be more accurate.

A previous normal pregnancy, and to some extent even a previous abortion, is associated with a markedly lowered incidence of pre-eclampsia (Dekker, Robillard and Hulsey, 1998). The protective effect of multiparity is lost, however, with a change of partner (Robillard, Hulsey, Alexander, Keenan, de Caunes *et al.*, 1993, Trupin, Simon and Eskenazi, 1996, Dekker, Tubbergen, Valk, Althuisius and Lachmeijer, 1998). Robillard and colleagues coined the word primipaternity to describe this phenomenon, suggesting that pre-eclampsia might be a problem of primipaternity rather than primigravidity because the patterns of changing paternity were significantly correlated with pre-eclampsia in multiparae (Robillard, Dekker and Hulsey, 1999).

In contrast to these findings, two groups who analysed the data from the Medical Birth Registry of Norway covering all births in that country from 1967 to 1998 independent of each other concluded that a change of partner may in fact decrease the risk of pre-eclampsia in a subsequent pregnancy (Trogstad, Eskild, Magnus, Samuelsen and Nesheim, 2001, Skjaerven, Wilcox and Lie, 2002). This casts a strong element of doubt on the entire concept of primipaternity. In both these studies the risk of pre-eclampsia was shown to increase as the interval between pregnancies became longer. Skjaerven *et al.* (2002) report that this increased risk applies to all women, but Trogstad *et al.* (2001) report that it applies only to women who were normotensive in their first pregnancy and observed that the risk of pre-eclampsia for women who have had pre-eclampsia in their first pregnancies reduced with longer inter

pregnancy intervals. However, these registry based epidemiological studies are prone to error. Firstly the paternity recorded in such registers may not be accurate since it is well known that there is a significant rate (5-20%) of false claimed paternities in stable couples in developed countries (Macintyre and Sooman, 1991, Lucassen and Parker, 2001). Secondly early terminations of pregnancies may not be recorded in Medical Birth Registers and as such pregnancies taken as first and second may not actually be the first and second pregnancies of a woman. Finally there may not be adequate data to confirm that the diagnosis of pre-eclampsia was based on uniform criteria in all cases over the years.

1.1.4. Long term sequelae of pre-eclampsia

Chesley, Annitto and Cosgrove (1976) who followed up a group of women with eclampsia delivering in the period 1931 through 1951, upto1973-74 were the first to provide definitive data on the possible long term sequelae of eclampsia. They did not find an excess rate of hypertension or an increase in cardiovascular morbidity or mortality in general in women who had eclampsia only in their first pregnancy. Women who had eclampsia as multiparae, however, had a greater incidence of hypertension as well as higher death rates from all causes, specifically cardiovascular disease later in life. Fisher and colleagues (1981) reported similar results in a group of women with pre-eclampsia. They also showed that an age and race matched group of women who were normotensive during pregnancy were at a significantly lower risk of

developing hypertension later in life than the population at large. These findings suggested that pre-eclampsia/eclampsia in second or subsequent pregnancy may unmask hypertension in women who are destined to have chronic hypertension later in life and that they are at greater risk of these complications than women in the population at large.

Some of these early findings, however, are in conflict with more recent data (Sibai *et al.*, 1986, Marin, Gorostidi, Portal, Sanchez, Sanchez *et al.*, 2000). There seems to be agreement on the finding that women who have normotensive pregnancies are at a lower risk of long-term hypertension than the general female population of a similar age. Women who develop pre-eclampsia in their first pregnancy, however, seem to be at a higher risk of hypertension later in life and women who have pre-eclampsia/eclampsia in more than one pregnancy are at still greater risk (Sibai *et al.*, 1986, Marin *et al.*, 2000). Although Marin *et al.* (2000) reported, in agreement with Chesley *et al.* (1976), that eclampsia is not associated with a greater risk of long-term hypertension, they had studied only 14 women with eclampsia. The long-term neurological effects of women with eclampsia have also been subjected to investigation. It appears that acute manifestations are transient and that long term effects are rare (Sibai, Spinnato, Watson, Lewis and Anderson, 1985). There are no studies on the long-term sequelae of Sri Lankan women who develop pre-eclampsia.

In contrast to the controversy surrounding long-term risk of hypertension, data on the long-term risk of ischaemic heart disease (IHD) are more consistent. It has been found that women who have eclampsia or pre-eclampsia are at a greater risk of death from IHD than are women with normal pregnancies or women in the reference population (Jonsdottir, Arngrimsson, Geirsson, Sigvaldason and Sigfusson, 1995, Hannaford, Ferry and Hirsch, 1997). In a Norwegian cohort study women with pre-eclampsia who delivered pre-term were eight times more likely to die from IHD than women who had gestational hypertension and whose pregnancy went to term (Irgens, Reisaeter, Irgens and Lie, 2001). In another study done in Scotland, women with pre-eclampsia who delivered a small for gestational age infant were seven times more likely to have a hospital admission for IHD or die of IHD (Smith, Pell and Walsh, 2001). Finally in a study done in the USA, severe pre-eclampsia was associated with a three fold increase in risk of cardiovascular disease (Kestenbaum, Seliger, Easterling, Gillen, Critchlow *et al.*, 2003). This tendency for IHD later in life may antedate the pre-eclamptic pregnancy rather than be exacerbated by it as evidenced, for example, by the sharing of common risk factors such as increased body mass index in both conditions.

The emerging picture of pre-eclampsia therefore, is of a life long illness (Brown, 2003) that carries a high degree of immediate as well as long term sequelae not only from hypertension and IHD, but also from stroke (Wilson, Watson, Prescott, Sunderland, Campbell *et al.*, 2003) and venous thrombo-

embolism (van Walraven, Mamdani, Cohn, Katib, Walker *et al.*, 2003). As such, greater understanding of the aetiology of pre-eclampsia is vital to develop meaningful interventions to care for women who develop the condition. It can only be achieved by conducting research on volunteers affected with pre-eclampsia selected after carefully phenotyping the condition on the basis of strict research diagnostic criteria.

1.2. The diagnosis of pre-eclampsia

Pre-eclampsia/eclampsia has its share of history and antiquity. The following short account of which is based on reviews by Thiagarajha (1943) and Chesley (1984).

The pre-Hippocratic Kahun Papyrus from Egypt dating back almost 3000 years alluded to eclampsia. Ancient Greeks also recognized pre-eclampsia: "In pregnancy, the onset of drowsy headaches with heaviness is bad; such cases are perhaps liable to some sort of fits at the same time". There seems to be no mention of the condition, however, in any of the authenticated writings of Hippocrates, but Galen's commentaries on Hippocrates' aphorisms suggest that he knew of the violent manifestations in pregnancy. Similarly there is no mention of it in the writings of Susrutha the great Indian physician in the sixth century BC. In the tenth century AD, a famous monastic physician St. Gall had a trick played on him. It seems that he was about to examine the Duke of Bavaria and, as a trick the worthy nobleman substituted for his urine that of a woman who was pregnant. The priest after making his examination made the solemn announcement that the Duke would give birth to a child. This incident is perhaps the earliest record of recognition of proteinuria in pregnancy, albeit somewhat indirect.

Scientific writing on pre-eclampsia/eclampsia really begins in the seventeenth century. Mauriceau, Barton, and Blundell all recognized the symptoms of pre-

eclampsia. In 1840, Rayer observed proteinuria in three oedematous pregnant women. It was Lever and Simpson, however, who discovered proteinuria in eclampsia independently of each other in 1843. The hard bounding pulse of women with eclampsia had suggested arterial hypertension to the old time clinicians. Vinay, in 1884, used a primitive sphygmomanometer and found blood pressures ranging from 160 to 200 mmHg in pregnant proteinuric women; pressures up to 160 mmHg were said to be normal as estimated by his instrument. The discovery of eclamptic hypertension is generally credited to Vaquez and Nobecourt in 1897, but they remarked that they had confirmed Vinay's observation.

1.2.1. The definition

The definition of pre-eclampsia has been the subject of much debate. Greater appreciation of the underlying pathophysiological changes of the disease, however, has enabled different expert groups working independently of each other to arrive at almost uniform criteria for its definition reflecting the known pathophysiology of the disorder (Brown, Hague, Higgins, Lowe, McCowan *et al.*, 2000, National High Blood Pressure Education Program Working Group, 2000).

The International Society for the Study of Hypertension in Pregnancy (ISSHP) defines pre-eclampsia for research purposes as *de novo* hypertension (systolic blood pressure (SBP) ≥140 mmHg and diastolic blood pressure (DBP) ≥90

mmHg) after 20 weeks of gestation in a previously normotensive pregnant woman, which returns to normal by the end of the third month postpartum, together with properly documented proteinuria (≥300 mg in a 24 hour collection or ≥500mg/L on a spot urine collection or ≥30 mg protein/mmol creatinine in a spot urine collection or, failing other measurement, ≥1+ on dipstick testing) not associated with urinary tract infection or ruptured membranes (Brown, Lindheimer, de Swiet, Van Assche and Moutquin, 2001). The presence of oedema, which was used in the past as a necessary feature of the diagnosis of pre-eclampsia, has now been dropped from both clinical and research definitions because it is not specific to the condition.

1.2.2. The differential diagnosis of pre-eclampsia

The appearance of hypertension and proteinuria for the first time in pregnancy clearly presents a diagnostic dilemma. It may be pre-eclampsia *per se*; it may be a long term problem antedating pregnancy which may or may not have been aggravated during pregnancy; rarely, it may be a previously undiagnosed medical problem such as phaeochromocytoma (Schenker and Chowers, 1971) by chance coinciding with pregnancy; or much more rarely it may be a pregnancy specific condition such as hydatidiform mole (Page, 1939, Newman and Eddy, 1988) or Ballantyne syndrome (Carbillon, Oury, Guerin, Azancot and Blot, 1997) presenting as pre-eclampsia.

1.2.2.1. Hypertension

Hypertension is a *sine qua non* of pre-eclampsia. It is also a common medical problem independent of pregnancy. Women with chronic hypertension and/or renal disease are three to seven times more likely to develop superimposed further hypertension and proteinuria in pregnancy (Chesley, 1980). In a population study of over a million people in America, 4.9% of women under the age of 40 years were hypertensive and among them the condition was previously undiagnosed in 39.7% (Stamler, Stamler, Riedlinger, Algera and Roberts, 1976). In England and Wales the prevalence of treated hypertension in women under the age of 44 years was 2.38% (Ryan and Majeed, 1999). In a Sri Lankan study, 13% of 2047 women aged between 20 to 40 years had a diastolic blood pressure of >90mmHg (Mendis, Ranasinghe and Dharmasena, 1988). Data on variation of the prevalence of hypertension over time is scarce. In a small study of blood pressure and other cardiovascular parameters in 806 adult women of Northern Sweden over the period 1986 to 1999, the prevalence of hypertension in those between the ages of 20 to 44 years ranged from 4.3% to 2.3% (Jansson, Boman and Messner, 2003). The number of women in this age group, however, is too small to draw broad conclusions from this data.

Often the obstetrician is the only physician consulted by women during their reproductive years. As such, records of blood pressure measurements prior to pregnancy, a sure way of excluding chronic hypertension, are often not

available. Records of normal blood pressure during early pregnancy may mask chronic hypertension due to the effect of the normal physiological reduction in blood pressure in the first half of pregnancy (MacGillivray, Rose and Rowe, 1969, Ayala, Hermida, Mojon, Fernandez, Silva *et al.*, 1997). In addition many women with chronic hypertension have an even greater physiological fall in blood pressure during the first half of pregnancy than do normotensive women (Sibai, Abdella and Anderson, 1983). Women with chronic hypertension therefore, may appear normotensive when they start antenatal care and then may show an increase in blood pressure in the third trimester resembling pre-eclampsia. Hypertension occurring before 20 weeks of pregnancy, however, is almost certainly chronic hypertension. It is usually essential hypertension with no identified underlying pathology (Sibai, 2002). Pre-eclampsia *per se* before 20 weeks of gestation has been described only in a handful of cases (Hazra, Waugh and Bosio, 2003).

The only sure way of excluding chronic hypertension, in the absence of pre-pregnancy blood pressure records, is to confirm its resolution postpartum. Davey and MacGillivray (1988) recommended the use of the traditional cut-off of resolution of blood pressure within six weeks to confirm the diagnosis of pre-eclampsia. This recommendation was probably based on practicality rather than on scientific evidence since six weeks is the conventional time for the postnatal check-up in the UK. In more recent recommendations this period has been extended to three months (Brown *et al.*, 2001). There is concern,

however, as to whether insisting on postpartum blood pressure normalisation would misclassify women with pre-eclampsia who may have developed chronic hypertension secondary to irreversible vascular damage caused by the pre-eclamptic state. If this were so, women who develop pre-eclampsia early in pregnancy, and are as a result more likely to have a prolonged illness with greater vascular damage; and women who have recurrent pre-eclampsia, resulting in repeated vascular damage, would be expected to take longer to normalise their blood pressure postpartum and/or develop chronic hypertension later in life. Several groups have reported such observations (Chesley *et al.*, 1976, Sibai *et al.*, 1986, Ferrazzani, De Carolis, Pomini, Testa, Mastromarino *et al.*, 1994). It is difficult to determine how much time is adequate for normalisation of blood pressure due to the scarcity of systematic data on blood pressure in the immediate postpartum period. It is most likely, however, that the earlier recommendation that blood pressure should resolve by six weeks postpartum to confirm the diagnosis was not appropriate (Ferrazzani *et al.*, 1994). It is also likely that there will also be a subgroup that will still be 'unnecessarily' on antihypertensive therapy at this time.

Blood pressure measurement is routine in antenatal care, but it is necessary to ensure that a standardised method is practised in pregnancy (Brown *et al.*, 2001). One area of controversy is the measurement of diastolic blood pressure (Higgins and de Swiet, 2001). The current ISSHP recommendation is to use Korotkoff phase V (K5) (sound disappearance) to record the diastolic blood

pressure, and to use Korotkoff phase IV (K4) (muffling) only when the sound does not disappear (Brown *et al.*, 2001) because K5 is detected more reliably than K4 during pregnancy (Blank, Helseth, Pickering, West and August, 1994, Shennan, Gupta, Halligan, Taylor and de Swiet, 1996), because K5 reflects the true diastolic pressure in pregnancy than K4 (Brown, Reiter, Smith, Buddle, Morris *et al.*, 1994), and because the change from K4 to K5 in hypertensive pregnant women does not alter the figures for the incidence of morbidity for the mother or the baby (Brown, Buddle, Farrell, Davis and Jones, 1998).

1.2.2.2. Proteinuria

De novo proteinuria is the second feature of the diagnosis of pre-eclampsia. A distinct renal lesion, glomerular endotheliosis, has been described and widely accepted as pathognomonic of pre-eclampsia (Spargo, Mc Cartney and Winemiller, 1959). It resolves postpartum. The severity of proteinuria in pre-eclampsia correlates positively with the extent and the severity of the endothelial changes seen in the glomerulus (Fisher *et al.*, 1981). Glomerular changes similar to that of pre-eclampsia, however, have also been reported in women with pregnancies complicated by hypertension alone. Moreover, in an ethically highly controversial study, control renal biopsies of normal pregnant women also show such changes, albeit of a milder degree (Strevens, Wide-Swensson, Hansen, Horn, Ingemarsson *et al.*, 2003). These observations suggest that it is possible that the severe glomerular changes seen in pre-eclampsia may be one extreme of the adaptation process in pregnancy rather

than an abnormal condition specific to pre-eclampsia.

In one of the early studies, glomerular endotheliosis was present in renal biopsies of 70% of primiparous women and 14% of multiparous women with pre-eclampsia diagnosed on the basis of *de novo* hypertension and proteinuria (McCartney, 1964). In another study renal biopsies of 84% of primiparous women with pre-eclampsia and 24% of multiparous women had glomerular endotheliosis (Fisher *et al.*, 1981). These observations together with the observations on the prognostic significance of parity to the development of hypertension later in life (Chesley *et al.*, 1976); and the reported impact of parity on the clinical presentation and on fetal growth and development (Gleicher, Boler, Norusis and Del Granado, 1986) are the basis for the widely accepted view that genetic studies of pre-eclampsia should be confined to primigravid women (Higgins *et al.*, 2001). However, women in their second pregnancy who have experienced an early first trimester abortion in their first pregnancy could also be included in such studies as their uterine morphology essentially resembles that of primigravid women. In fact the risk of developing pre-eclampsia in nulliparous women with a previous abortion who conceive again with the same partner is half that of nulliparous women who do not have such a history (Saftlas, Levine, Klebanoff, Martz, Ewell *et al.*, 2003).

Renal protein excretion increases in normal pregnancy, and may reach levels of up to 300mg day in the third trimester of a normal pregnancy (Davison,

1985, Higby, Suiter, Phelps, Siler-Khodr and Langer, 1994). Proteinuria in excess of 300mg/day is extremely rare in uncomplicated pregnancies (Higby *et al.*, 1994, Waugh, Bell, Kilby, Lambert, Blackwell *et al.*, 2003) and is usually associated with pre-eclampsia or renal disease. Rapid and reliable detection of significant proteinuria has always posed a challenge in clinical settings. Quantitative tests for proteinuria, although available in routine clinical practice in the West, are not widely available even in the best of centres in the developing world. What is available is the urine protein heat coagulation test (HCT) (Guidotti and Jobson, 1992). It is most often the only test available. The cut-off point for detecting significant proteinuria with the HCT, however, seems not to have been formally established.

Pre-eclampsia is the most common cause of gross proteinuria in pregnancy. Other renal diseases that may give rise to a similar clinical picture, the diagnosis of which rely on a renal biopsy, include proliferative or membranoproliferative glomerulonephritis, minimal change nephrosis, lupus nephropathy, hereditary nephritis, diabetic nephropathy, renal vein thrombosis, and amyloidosis (Davison and Baylis, 1998). Non-glomerular renal disease such as reflux nephropathy and polycystic kidney disease can also give rise to significant proteinuria (Ihle, Long and Oats, 1987). A renal biopsy, however, is almost never indicated to resolve the differential diagnosis of *de novo* proteinuria in pregnancy (Lindheimer and Davison, 1987).

1.3. Investigating the genetics of pre-eclampsia

1.3.1. Evidence for a genetic basis for pre-eclampsia

Pre-eclampsia most certainly has a genetic basis because it occurs across the globe in all environmental conditions and because it 'runs' in families (Adams and Finlayson, 1961, Chesley, Cosgrove and Annitto, 1961, 1962, Chesley, Annitto and Cosgrove, 1968, Sutherland, Cooper, Howie, Liston and MacGillivray, 1981, Arngrimsson, Bjornsson, Geirsson, Bjornsson, Walker *et al.*, 1990, Lie, Rasmussen, Brunborg, Gjessing, Lie-Nielsen *et al.*, 1998, Mogren, Hogberg, Winkvist and Stenlund, 1999, Esplin, Fausett, Fraser, Kerber, Mineau *et al.*, 2001, Dawson, Parfrey, Hefferton, Dicks, Cooper *et al.*, 2002). Pre-eclampsia is nearly two and a half times more common in daughters of women with pre-eclampsia than in their daughters in law (Arngrimsson *et al.*, 1990); twice as frequent in pregnancies fathered by men who are themselves the product of pregnancies complicated by pre-eclampsia compared to pregnancies fathered by men who were born of normotensive pregnancies (Esplin *et al.*, 2001); and more frequent in pregnancies fathered by men who have previously fathered a pregnancy complicated by pre-eclampsia in another woman compared to pregnancies in women whose partner does not have such a history (Lie *et al.*, 1998).

Several twin studies have examined the occurrence of pre-eclampsia in pregnancies of twins. The early studies were disappointing as they reported discordance for pre-eclampsia in pregnancies of monozygotic twins (MZ)

(Thornton and Onwude, 1991, Thornton and Macdonald, 1999, Treloar, Cooper, Brennecke, Grehan and Martin, 2001) These studies, however, lacked true population representation because they were based on volunteer twin registers and their sample size did not have sufficient power to arrive at definite conclusions. In contrast, in a recent population based study of 2116 (917 MZ and 1119 dizygotic (DZ)) twin pairs in Sweden, pair wise concordance rates of 0.25 and 0.06 for pre-eclampsia were found in MZ and DZ twin pairs respectively. Furthermore, the estimates for heritability (influence of maternal genes) and non-shared environmental effect (including influence of fetal/paternal genes) were 0.54 and 0.46 respectively (Salone Ros, Lichtenstein, Lipworth and Cnattingius, 2000).

1.3.2. Inheritance of pre-eclampsia

The analysis of the mode of inheritance of pre-eclampsia, which does not show a clear pattern of inheritance, is made difficult by the nature of the condition itself – it is sex-limited and it occurs only during pregnancy. In addition susceptibility to pre-eclampsia could be controlled by the maternal genotype alone, or the fetal genotype alone, or by an interaction of the genotypes of the mother and her fetus. Some of the fetal genes of course would be paternally derived.

In one of the first attempts to answer this question, Cooper and Liston (1979) re-examined the data collected and previously reported by Chesley and

colleagues (Chesley *et al.*, 1962, Chesley *et al.*, 1968). The incidence of eclampsia in the sisters, daughters, and sisters in law of women with eclampsia proved to fit closely with homozygosity for a maternal recessive gene. When they extended their analysis to a further set of data from Aberdeen, however, they found instead evidence for the fetal-genotype hypothesis, as there was a significant deficit of eclampsia among sisters of affected women. In the absence of information about the patients' in-laws in the latter group it was not possible for them to come to a final conclusion. The case for a single recessive gene acting on the mother was further strengthened by an analysis of data which compared the frequency of pre-eclampsia in mothers versus mothers in law of pre-eclamptic index cases (Sutherland *et al.*, 1981) and in daughters versus daughters in law of eclamptic index cases (Chesley and Cooper, 1986) which showed that the maternal genotype alone is mainly responsible for susceptibility to pre-eclampsia. Both studies, however, could not rule out the possibility of multifactorial inheritance. Later the latter group modified their hypothesis to include the fetal genotype, and argued that the primary mode of action of the gene(s) involved is to affect the interaction between uterine and placental tissue (Cooper, Hill, Chesley and Bryans, 1988). They were supported by Liston and Kilpatrick (1991) who examined six simple Mendelian models of inheritance and rejected all except the one in which both the mother and the fetus expressed the same recessive gene to confer susceptibility. Arngrimsson and colleagues also supported the single maternal recessive gene hypothesis, however, their data could also fit a model of

dominant inheritance with incomplete penetrance (Arngrimsson *et al.*, 1990). They analysed their data further on four models of Mendelian inheritance and concluded that a major maternal dominant gene model with reduced penetrance, or multifactorial inheritance (including the possible influence of fetal/paternal genes) could fit their data best (Arngrimsson, Bjornsson and Geirsson, 1995) .

In practice, in the absence of any molecular or biochemical methods for carrier detection, it would be very difficult to 'prove' that pre-eclampsia shows autosomal recessive inheritance. A high degree of parental consanguinity in association with pre-eclampsia, however, would provide strong supportive evidence for autosomal recessive inheritance. The available data on consanguinity and pre-eclampsia does not support such an association (Stevenson, Say, Ustaoglu and Durmus, 1976). Interestingly consanguineous marriages are favoured by at least 20% of the world population. It is widely practised in many parts of Asia, Middle East, Northern Africa, and South America. Prevalence in other parts of the world is believed to be low. Worldwide, it is estimated that at least 8.4% of children have related parents (World Health Organisation, 1996). Two Sri Lanka studies report consanguinity rates of 16% (Corea, 1982) and 10% (De Silva, 1998). While investigating the association of pre-eclampsia with consanguinity may be difficult in most parts of the world due to its rarity, proving such an association in parts of the world where consanguinity is common is made

difficult by the fact that some degree of consanguinity is the norm in many marriages.

Apart from Mendelian modes of inheritance, it has also been proposed that pre-eclampsia could be caused by a mutated mitochondrial gene transmitted by the mother (mitochondrial inheritance) (Torbergsen, Oian, Mathiesen and Borud, 1989) or a mutation in a paternally imprinted, maternally active gene expressed by the fetus (genetic imprinting) (Graves, 1998). Li and colleagues, however, ruled out the possibility of mitochondrial inheritance as a common cause of pre-eclampsia because they found a similar risk for pre-eclampsia among maternal and paternal half sisters, when in fact they should have found a higher risk in the former group if pre-eclampsia were caused by a mutated mitochondrial gene (Lie *et al.*, 1998).

Pre-eclampsia therefore, like most other common disorders that are not inherited in a Mendelian fashion, is almost certainly a complex disorder involving both genetic and non-genetic/environmental factors.

1.3.3. 'Pre-eclampsia genes'

1.3.3.1. Problems in identifying 'pre-eclampsia genes'

Research aimed at identifying genes contributing towards the risk of complex disorders are complicated by several major problems that are centred on phenotyping, genetic heterogeneity, reduced penetrance, and phenocopy.

Problems of phenotyping in pre-eclampsia, where the diagnostic signs of *de novo* hypertension and proteinuria depend on arbitrary cut off points on a continuous distribution of values, have already been discussed in previous sections. Furthermore, the severity of the disorder as well as the involvement of various organs is highly variable and the expression of the original phenotype is masked by medical care. Genetic heterogeneity refers to a situation where alleles at more than one locus can individually trigger the disease. Reduced penetrance results in an individual with a predisposing genotype not expressing the phenotype. In contrast, phenocopy refers to a situation where the disease is triggered by environmental factors in the absence of a predisposing genotype, as may be the case with pre-eclampsia occurring in women with chronic hypertension who develop gestational hypertension and on top of it proteinuria due to a urinary tract infection. Thus we may expect to encounter situations where a predisposing genotype does not result in the disease and, conversely, where the disease occurs without the predisposing genotype. This would make the correlation between genotype and the disease phenotype more difficult to establish. Furthermore, genetic heterogeneity also means that a gene implicated in the disease may be correctly identified in one population, but it may not be replicated in another (Lander and Schork, 1994).

1.3.3.2. Approaches to identifying 'pre-eclampsia genes' and the current state of knowledge

Linkage and association analysis, complementary methods of gene detection, are the two main approaches used to detect specific chromosome regions (loci) and genes that are involved in complex disorders such as pre-eclampsia (Risch and Merikangas, 1996). A third approach, which could be used in the not so distant future, would involve testing for association across the genome using a very dense map of markers (linkage disequilibrium genome screen).

Linkage Analysis

Many early linkage studies into pre-eclampsia that concentrated on candidate loci did not find linkage to any of the loci tested (Wilton, Cooper, Brennecke, Bishop and Marshall, 1990, Wilton, Barendse, Donald, Marshall, Trudinger *et al.*, 1991, Arngrimsson, Geirsson, Cooke, Connor, Bjornsson *et al.*, 1994, Wilton, Kaye, Guo, Brennecke and Cooper, 1995). Arngrimsson and colleagues found linkage to both the angiotensinogen gene on chromosome 1q42-43 (Arngrimsson, Purandare, Connor, Walker, Bjornsson *et al.*, 1993) and to the NOS3 gene on chromosome 7q36 encoding endothelial nitric oxide synthase (Arngrimsson, Hayward, Nadaud, Baldursdottir, Walker *et al.*, 1997). Others, however, could not confirm the latter finding (Guo, Lade, Wilton, Moses, Grehan *et al.*, 1999, Lade, Moses, Guo, Wilton, Grehan *et al.*, 1999, Lewis, Lachmeijer, Downing, Dekker, Glazebrook *et al.*, 1999).

Hayward and colleagues investigated linkage across a small fraction of the

genome and constructed an exclusion map which indicated that the pre-eclampsia gene may lie on one of chromosomes 1, 3, or 9; chromosome 18 was not typed (Hayward, Livingstone, Holloway, Liston and Brock, 1992). Similarly, using a small number of marker loci, Harrison and colleagues, found evidence of linkage to chromosome 4q (Harrison, Humphrey, Jones, Badenhop, Guo *et al.*, 1997). As technology advanced, other groups were able to search for linkage across the whole genome using large numbers of marker loci distributed on all chromosomes. These genome wide scans, when interpreted using the criteria suggested by Lander and Kruglyak (1995), revealed locations with significant linkage on chromosome 2p13 (Arngrimsson, Sigurardottir, Frigge, Bjarnadottir, Jonsson *et al.*, 1999), 2p25 and 9p13 (Laivuori, Lahermo, Ollikainen, Widen, Haiva-Mallinen *et al.*, 2003); and locations with suggestive linkage on chromosome 2q, 3p and 15q (Arngrimsson *et al.*, 1999); 2q23 and 11q23-24 (Moses, Lade, Guo, Wilton, Grehan *et al.*, 2000); 3p,12q,15q, 10q,11,and 22q (Lachmeijer, Arngrimsson, Bastiaans, Frigge, Pals *et al.*, 2001); and 4q32 (Laivuori *et al.*, 2003). It has been suggested, taking into account the poor precision of estimates of map location of disease-predisposing loci for complex disorders, that the two loci identified by Arngrimsson *et al*. (1999) and Moses *et al.* (2000) represent the same location on chromosome 2p13. More support for the presence of a pre-eclampsia susceptibility locus in this region comes from another report of the association between microsatellite marker D2S286, the same significant marker reported by Arngrimsson *et al.* (1999), on 2p13 with pre-eclampsia

(Laasanen, Hiltunen, Romppanen, Punnonen, Mannermaa *et al.*, 2003). Laivuori *et al.* (2003), however, found what is definitely a separate pre-eclampsia susceptibility locus on chromosome 2p25. Interestingly the statistical significance of the genome-wide scan by Arngrimsson *et al.* (1999) was attributed to two large multicase families. When the data was analysed excluding these families there was no linkage to chromosome 2. This observation and the poor overlap between these studies, in addition to highlighting the statistical contribution of large multicase families to such studies, also highlight the possibility of the existence of some families where pre-eclampsia may be caused by a major maternal susceptibility gene on chromosome 2, while a more complex mode of inheritance may be operative in other families.

Association studies

In contrast to family based linkage studies, association studies are based on populations and they search for associations between markers near or within known genes thought to be reasonable candidates for disease susceptibility. In these studies marker frequencies in unrelated cases and controls are compared.

Selection of a suitable marker for analysis is an important consideration in association studies. Over the past few years Single Nucleotide Polymorphisms (SNPs) have emerged as the favoured genetic marker for association analysis (Gray, Campbell and Spurr, 2000, Schork, Fallin and Lanchbury, 2000). SNPs

that are known to be functionally significant based on previous work and/or SNPs in which the minor allele occurs relatively commonly (generally at a frequency of 5-10% or more), so that a study of adequate power could be carried out with a reasonable sample size of several hundred cases and controls, are usually chosen as markers for association analysis in common diseases such as pre-eclampsia. When available resources only permit the assay of a limited number of SNPs, as it usually happens, it seems appropriate to give the highest priority to SNPs in coding regions that lead to non-synonymous and non-conservative variations, as they are most likely to be associated with functional effects and phenotypic outcomes; to SNPs in 5' untranslated regions (UTRs); and to SNPs creating or deleting a splice site (Risch, 2000). SNPs selected for association analysis could either be directly contributory to the disease phenotype, or may be in linkage disequilibrium (LD) with a variation that is contributory. Theoretically, because recombination is less likely to occur between SNPs separated by short chromosomal distances, SNPs in LD with other SNPs contributory to a disease phenotype are expected to be close to each other. In actual fact, however, although generally LD decreases as the chromosomal distances increases (Salisbury, Pungliya, Choi, Jiang, Sun *et al.*, 2003), LD can extend over much larger genomic regions than expected, and the patterns of LD differ markedly among populations (Reich, Cargill, Bolk, Ireland, Sabeti *et al.*, 2001).

When a SNP is in LD with a polymorphism contributing to the disease

phenotype, the power to detect an association decreases as the LD between the SNP and the disease-predisposing variation becomes weaker. This could be overcome by constructing haplotypes using SNPs on the same chromosome and testing association with haplotypes. Since haplotype based methods incorporate LD information, they are more powerful than methods based on single SNPs (Akey, Jin and Xiong, 2001, Zaykin, Westfall, Young, Karnoub, Wagner *et al.*, 2002). Haplotype based association analysis is also more powerful than SNP based methods when multiple disease susceptibility variants occur within a gene (Joosten, Toepoel, Mariman and Van Zoelen, 2001, Morris and Kaplan, 2002). It is best to use the maximally informative set of common SNPs (tagSNPs) that contribute to the maximum haplotype diversity in the gene or genomic region of interest when carrying out haplotype analysis of candidate genes (Huang, Fu and Boerwinkle, 2003). To do so information on the LD that exists between polymorphic sites in the region is required.

LD is commonly measured using Lewontin's disequilibrium coefficient (D') and the correlation coefficient (r^2; also referred to as Δ^2) (Lewontin, 1964, Devlin and Risch, 1995). 'Complete LD' is said to exist when $D'=1$. This occurs when neither site has experienced recurrent mutation or gene conversion and if there has been no recombination between sites. 'Perfect LD' is said to exist when $r^2=1$. This occurs when there is perfect correlation between the polymorphic sites, i.e. when both sites have the same minor allele

frequency. In case-control disease association analysis using a polymorphic marker, the statistical power to detect unassayed disease-associated polymorphisms depends on the correlation (r^2) between the unassayed sites and the assayed site. Such LD information can be made use of to select the tagSNPs for candidate gene disease association studies (Carlson, Eberle, Rieder, Yi, Kruglyak *et al.*, 2004).

Another factor that could undermine SNP base association analysis is genotyping error. It is necessary to take steps to minimise genotyping errors in any association study. It is especially important when polymorphisms with very low minor allele frequencies are genotyped, because otherwise infinitely large samples are required to account for genotyping errors and to maintain acceptable levels of power and significance (Gordon, Finch, Nothnagel and Ott, 2002, Kang, Gordon and Finch, 2004). Haplotype analysis is another way of minimising the impact of genotyping errors, as rare, possibly false, haplotypes generated due to their presence can be excluded from analysis.

It is not always possible to determine haplotypes present in an individual unequivocally using genotype data unless genotype data is available for parents and/or other siblings in a family. Technically too, haplotyping is a laborious process (Eitan and Kashi, 2002). The alternative approach that has been widely employed therefore is to use the expectation-maximization (EM) algorithm (Dempster, Laird and Rubin, 1977) to infer maximum likelihood

haplotype frequencies from genotype data, in which the gametic phase is unknown in a proportion of subjects, under the assumption of Hardy-Weinberg equilibrium (HWE) (Excoffier and Slatkin, 1995, Hawley and Kidd, 1995, Long, Williams and Urbanek, 1995). The accuracy of the EM-based maximum likelihood method in estimating haplotype frequencies has been validated in a variety of simulated conditions (Schipper, D'Amaro, de Lange, Schreuder, van Rood *et al.*, 1998, Fallin and Schork, 2000). These studies have shown that its accuracy is better with large sample size (>100 chromosomes), more loci (>5 loci), and greater dispersion of haplotype frequency values (with some very common haplotypes and many rare haplotypes) (Fallin *et al.*, 2000).

Associations between disease and genetic markers, however, can also be due to factors other than LD such as unsuspected stratification of case and control groups (Cardon and Palmer, 2003). Stratification is a problem if the marker alleles have different frequencies in admixed racial groups and if one of them has a higher incidence of disease resulting in its over representation in cases and under representation in controls. There are clear differences in the pattern of variation in SNPs and haplotypes of genes in different populations (Salisbury *et al.*, 2003). In association studies therefore, to avoid spurious genetic associations, it is essential that sufficient measures be taken to establish the correct racial origin of each individual in both case and control groups and to match the two groups for race. Taking extra care to ensure that

stratification is eliminated or minimised is particularly important when subjects are recruited for association analysis in racially diverse countries such as Sri Lanka. Sri Lanka has several major racial groups – Sinhalese (74%), Sri Lankan Tamils (11%), Indian Tamils (6%), Moors (7%), Malays (<1%) and Burghers (<1%) (Department of Census and Statistics, 2001).The Sinhalese are probably the descendents of the original inhabitants of the Island, but their origin is also traced to the Bengali region in Northern India. The Sri Lankan Tamils are the descendents of Tamils from Southern India who came to the Island and settled down at various times in the past when Sri Lanka was invaded by South Indian rulers. The Moors and the Malays are the descendents of Arab and Malay traders respectively. They came to the country in search of trade and settled down. Burghers are a hybrid population of Dutch and other Western Europeans who married mainly the Sinhalese during the colonial rule in the 16^{th} to 20^{th} centuries.

Most genetic studies of pre-eclampsia to date have been case-control candidate gene pre-eclampsia association studies that examine the association between SNPs in candidate genes and the disease phenotype (see review by Lachmeijer, Dekker, Pals, Aarnoudse, ten Kate *et al.*, (2002)). The results of these studies have not been consistent (see tables 1.3 and 1.4). There are many reasons why this may be so. The most obvious reason is differences in study design especially the sample size that makes many studies under powered to detect actual genetic effects leading to false negative results (Lalouel and

Rohrwasser, 2002). In addition, pre-eclampsia is almost certainly a polygenic disorder, and as such although one gene may be responsible for causing the disorder in one population, it may not necessarily be so in another. Another reason is spurious association in admixed populations as discussed above. It is also possible that there are true differences between these populations such as the existence of modifier genes that may mask the effects of the gene being studied (epistatic gene-gene interactions) or environmental modifiers specific to the population. Additionally, it is also possible that what is important above is a specific haplotype, rather than any of the single loci that define the haplotype. In fact there are very few studies that have carried out haplotype analysis in candidate genes in pre-eclampsia (e.g. Kaiser, Brennecke and Moses, 2000, Plummer, 2001, Levesque, Moutquin, Lindsay, Roy and Rousseau, 2003).

An issue that none of the association studies of pre-eclampsia have addressed adequately is the issue of correction for multiple testing to adjust the level of significance to determine a positive association. It is dealt with in the discussion (see section 4.4.1). Another concern is the selection of the control group. It is widely accepted that confining genetic studies of pre-eclampsia to nulliparous women would reduce the chance of those with underlying disorders, especially renal disease, being included among cases (Higgins *et al.*, 2001). However, there seems to be no such widely accepted consensus on what should constitute a suitable control group. Some of the control groups

that have been used in these studies include population-based controls, multiparous women in whom all pregnancies were normal, and nulliparous normotensive pregnant women (see tables 1.3 and 1.4). In addition there is also the question as to what factors they should be matched for. Matching for parity and age seems appropriate, but should they also be matched for body mass index (BMI) bearing in mind that there is a strong association with BMI and pre-eclampsia? These concerns have to be addressed when designing case-control candidate gene pre-eclampsia association studies.

Properly designed case-control candidate gene disease association studies are the most practical and powerful approach to identify genetic factors involved in complex diseases (Risch *et al.*, 1996). In spite of this, however, the most persuasive test of association involves the use of family-based controls such as is the case in the transmission disequilibrium test (TDT) (Spielman, McGinnis and Ewens, 1993). In the TDT, if a given allele contributes to disease, then the probability that an affected person has inherited the allele from the heterozygous parent should vary from the expected probability under Mendelian segregation of 0.5; the association of a neutral polymorphism due to admixture displays no such deviation. Some authors believe that any association not confirmed by the TDT should be regarded as provisional pending proof of causality (Altshuler, Kruglyak and Lander, 1998). In addition the TDT has the added advantage that it is not subject to confounding due to racial stratification, and can distinguish between maternal and fetal

susceptibility genes (Zusterzeel, Visser, Peters, Merkus, Nelen *et al.*, 2000, Zusterzeel, te Morsche, Raijmakers, Roes, Peters *et al.*, 2002).

1.3.3.3. Future direction of genetic studies of pre-eclampsia

A major impediment to attempts at understanding the mode of inheritance and to establishing linkage in pre-eclampsia research has been the absence of any large-scale study in non-European populations other than in the Japanese population. It would be most appropriate therefore, to carry out a study to fill that void in a South Asian country such as Sri Lanka, an Island nation in the Indian Ocean with considerable genetic diversity between its five main racial groups that have by and large remained genetically heterogeneous, possibly due to cultural differences (Papiha, Mastana and Jayasekara, 1996a).

It is evident from the study of Arngrimsson and colleagues (1999), where support for the susceptibility locus on chromosome 2p13 came mainly from two large multicase families, that the success in pinpointing pre-eclampsia susceptibility loci by linkage analysis would require the investigation of a large number of multicase families than has been possible so far. This will become increasingly difficult in view of the social trend towards smaller family size. In addition, there is ample epidemiological evidence for a fetal/paternal genetic contribution to pre-eclampsia (Lie *et al.*, 1998, Esplin *et al.*, 2001) that needs to be addressed adequately in future studies as most candidate gene studies and all genome-wide scans so far have focused mainly on maternal genetic factors. Such studies of course would require the

mobilisation of huge amounts of financial and other resources and collaboration among teams of researchers as in the case of the UK Genetics of Pre-eclampsia Collaborative (GOPEC) study (http://www.gopec.org). In many populations including that of the Sri Lankan population therefore, candidate gene pre-eclampsia association studies will play an important role. Selection of candidate genes for such studies will depend on chromosome locations indicated by genome wide scans, the pathophysiological processes operative in pre-eclampsia, and the tissue expression pattern of the proposed candidate genes.

1.4. Some pathophysiological aspects of pre-eclampsia

Pre-eclampsia has been termed the 'disease of theories' due to the confusion surrounding its aetiology (Ness and Roberts, 1996, Dekker and Sibai, 1998, Redman, Sacks and Sargent, 1999). It is possible that the hypertension and proteinuria that are relied on to define the condition may simply be the final common pathway through which more than one type of pathology is being expressed. Pre-eclampsia is now widely accepted to be a two-stage disorder: reduced placental perfusion usually secondary to abnormal placentation and a consequent maternal disorder characterized by endothelial dysfunction and a systemic maternal disease (Roberts and Redman, 1993, Roberts, 2000).

1.4.1. Stage 1: Reduced placental perfusion

Pre-eclampsia only occurs in the presence of a placenta and its resolution begins with the removal of the placenta. More than 70 years ago Page proposed that decreased placental perfusion results in pre-eclampsia (Page, 1939). This has subsequently been confirmed by direct measurements of intervillous blood flow (Kaar, Jouppila, Kuikka, Luotola, Toivanen *et al.*, 1980) and by animal experiments where it has been shown that reducing blood flow to the pregnant uterus and placenta results in a condition remarkably similar to pre-eclampsia, including glomerular endotheliosis (Aladjem, Lueck and Brewer, 1983, Combs, Katz, Kitzmiller and Brescia, 1993).

In many cases reduced perfusion is secondary to abnormal placentation. In addition conditions that are associated with microvascular disease such as chronic hypertension (Dekker, de Vries, Doelitzsch, Huijgens, von Blomberg *et al.*, 1995, Sibai, Ewell, Levine, Klebanoff, Esterlitz *et al.*, 1997) and diabetes (Garner, D'Alton, Dudley, Huard and Hardie, 1990, Lao and Tam, 2001, Xiong, Saunders, Wang and Demianczuk, 2001, Bryson, Ioannou, Rulyak and Critchlow, 2003, Ostlund, Haglund and Hanson, 2004); and conditions that are thrombophilic such as anticardiolipin antibody syndrome (Branch, Andres, Digre, Rote and Scott, 1989) may also decrease blood supply to the placenta and increase the pre-eclampsia risk. Moreover, obstetric conditions that increase placental mass such as multiple pregnancies (Coonrod, Hickok, Zhu, Easterling and Daling, 1995, Mastrobattista, Skupski, Monga, Blanco and August, 1997) and hydatidiform mole (Newman *et al.*, 1988, Soto-Wright, Bernstein, Goldstein and Berkowitz, 1995) can have the same effect, possibly due to a 'relative' decrease of placental blood flow.

1.4.1.1. Placentation: Morphological changes

The process of human placentation is an invasive phenomenon in which embryo-derived cytotrophoblastic cells progressively integrate into maternal tissue. After implantation, specialised epithelial cells of the placenta that attach the fetus to the mother, cytotrophoblasts, differentiate along one of two pathways. In the first, cytotrophoblasts fuse to form the multinucleate syncytium that covers the floating chorionic villi. These villi, which are in

direct contact with maternal blood in the intervillous space, perform nutrient and gas exchange functions for the fetus. In the second pathway, extra villous cytotrophoblasts in the anchoring villi proliferate to form a shell lining the uterine cavity, and invade the uterine wall (interstitial invasion) and its blood vessels (endovascular invasion).

Cytotrophoblasts that invade the uterine wall (interstitial cytotrophoblasts) reach the superficial myometrium by the eighth week of gestation (Pijnenborg, Bland, Robertson, Dixon and Brosens, 1981). Later, they invade deeper into the myometrium especially at the centre of the placental bed (Pijnenborg, Bland, Robertson and Brosens, 1983). When they reach the end of their invasion path they fuse to form multinuclear giant cells (Pijnenborg, Bland, Robertson and Brosens, 1996). Endovascular cytotrophoblasts invade the spiral arteries and migrate along the arteries to reach myometrial spiral arteries by the tenth week of gestation. This process, however, may continue for several more weeks (Pijnenborg *et al.*, 1983). It has been hypothesised that endovascular invasion occurs in two waves – a first wave at the beginning of pregnancy following fecundation and a second deeper wave at the end of the first trimester around 14 to 16 weeks of gestation that involves the inner third of the myometrium (Robertson, Khong, Brosens, De Wolf, Sheppard *et al.*, 1986). There is also data, however, that suggest a progressive endovascular migration into myometrial arteries rather than it occurring in two stages (Robson, Ball, Lyall, Simpson, Ayis *et al.*, 2001).

The process of trophoblast invasion, in addition to physically anchoring the fetal placenta to the uterus, transforms the small diameter muscular spiral arteries into large-diameter low resistance flaccid vessels that carry blood to the intervillous space (Pijnenborg *et al.*, 1983). It is clear that this process, named physiological change, has to take place in both decidual and myometrial segments of spiral arteries as an essential prerequisite for the provision of an adequate blood supply to the rapidly growing fetus and placenta. The changes in myometrial arteries never return to a 'non-pregnant' state, so that second and subsequent pregnancies begin with an advantage in terms of flow (Brosens, Robertson and Dixon, 1967, Khong, Adema and Erwich, 2003).

Interstitial trophoblast invasion is deficient in pre-eclampsia, but endovascular invasion is greatly impaired and physiological changes do not take place in both decidual and myometrial spiral arteries. They remain low calibre high resistance vessels that are unable to provide an adequate blood supply to the intervillous space (Khong, De Wolf, Robertson and Brosens, 1986, Meekins, Pijnenborg, Hanssens, McFadyen and van Asshe, 1994, Naicker, Khedun, Moodley and Pijnenborg, 2003). Spiral artery remodelling in normal pregnancies and pregnancies complicated by pre-eclampsia is summarised in figure 1.1.

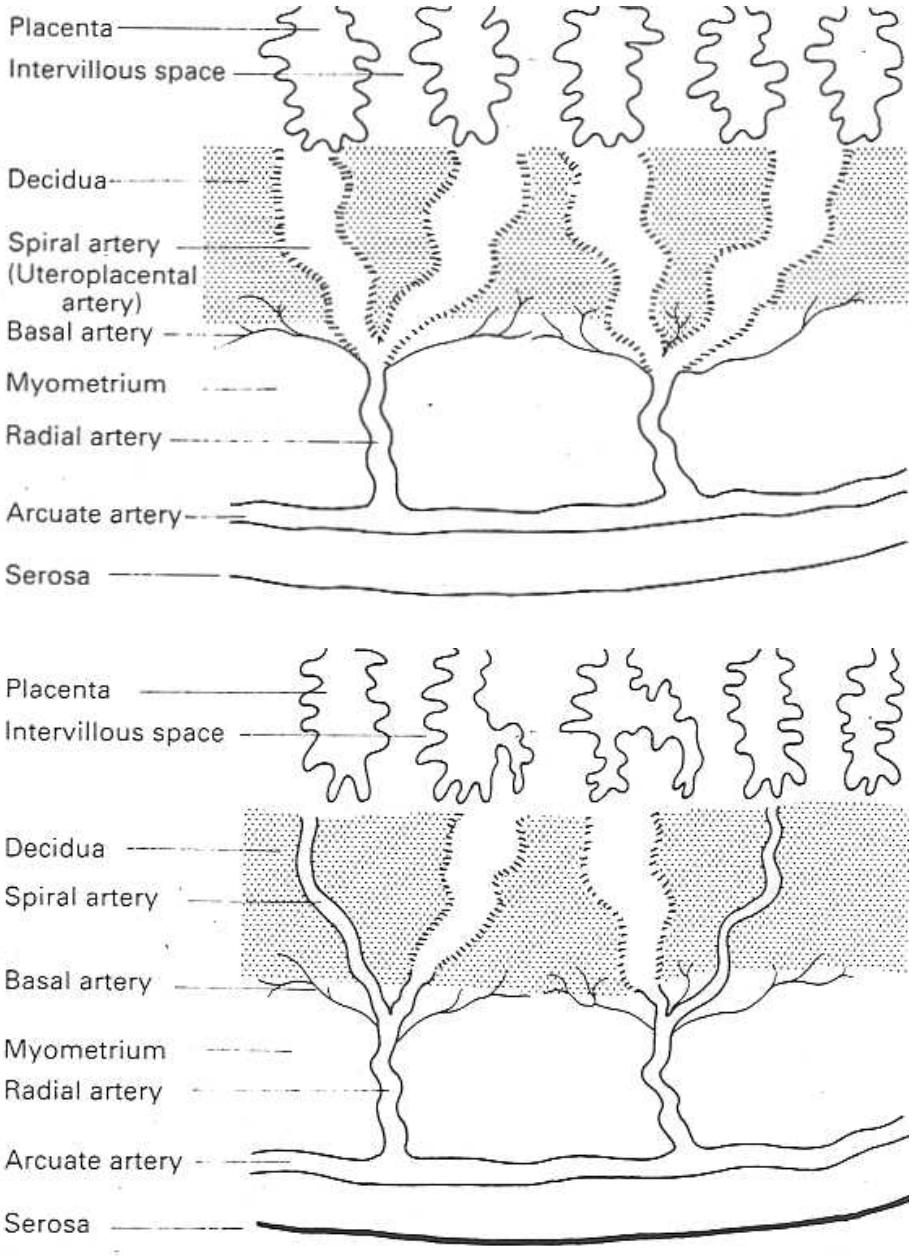


Figure 1.1. Spiral artery remodelling in normal pregnancies and pregnancies complicated by pre-eclampsia. The upper diagram shows normal adaptation of spiral arteries to pregnancy, whereas the lower one depicts this process in pre-eclampsia (adapted from Khong *et al.* (1986)).

1.4.1.2. Placentation: Molecular mechanisms

At a molecular level, trophoblast invasion is a complex process. It has been studied using *in vitro* systems consisting of enzyme-dispersed cells, extravillous cytotrophoblast (EVT) growing out of first trimester explants, or EVT cell lines derived from first trimester explants maintained on matrices similar to either decidual extracellular matrix or basal lamina (Matrigel^{TM}). These studies point to a complex interaction between EVT and its environment in regulating invasion. The downside to these elegant *in vitro* studies is that it is impossible to obtain trophoblast cells that have not been exposed to normal environmental levels of O_2 in harvesting and preparation from placental tissue. It is not clear therefore whether such cells actually mimic cells *in vivo.*

A comprehensive review of the large body of scientific literature in this field is beyond the scope of this introduction. A brief survey therefore is made below of the molecular mechanisms that are most relevant to the investigations undertaken in this study.

The cytotrophoblast, which lies at the feto-maternal interface, is influenced by circulating and local factors from both compartments. Among them is Epidermal Growth Factor (*EGF*) (see section 1.5.1). The *EGF* gene, maps to chromosome 4q25-27 (Morton, Byers, Nakai, Bell and Shows, 1986) and is approximately 57cM away from the closest markers bordering the suggestive

locus for pre-eclampsia detected in the genome-wide scan of Finnish families by Laivuori *et al*. (2003). At four to five weeks of gestation EGF might act to induce proliferation, whereas, at six to 12 weeks and later, it induces differentiation (Maruo, Matsuo, Murata and Mochizuki, 1992). EGF has been shown to promote invasion of first trimester cytotrophoblasts on Matrigel™ assays and produce morphologic changes such as extension of pseudopodia and ruffling of the cell membrane, but not syncytium formation (Bass, Morrish, Roth, Bhardwaj, Taylor *et al.*, 1994). This suggests that EGF may induce phenotype differentiation along the invasive pathway. In addition EGF has been shown to inhibit apoptosis in first trimester (Perkins, St John and Ahmed, 2002) and term cytotrophoblasts (Levy, Smith, Chandler, Sadovsky and Nelson, 2000). This can contribute to cytotrophoblast survival during placentation and throughout the pregnancy. Interestingly apoptosis occurs at a high rate in pre-eclampsia whereas little or no apoptosis is observed in placentae of normal pregnancies (DiFederico, Genbacev and Fisher, 1999, Allaire, Ballenger, Wells, McMahon and Lessey, 2000). What is not clear, however, is whether apoptosis occurs as a primary or secondary phenomenon.

EGF receptors (EGFR) are found in most reproductive tissues. The placenta, however, has many times more EGFR than the uterus, endometrium or decidua (Hofmann, Rao, Barrows, Schultz and Sanfilippo, 1984, Hofmann, Rao, Carman and Siddiqi, 1988). In addition EGF can upregulate EGFR in the cytotrophoblast (DePalo and Das, 1988). EGF in the feto-maternal interface is

most likely to be of maternal origin (Haining, Schofield, Jones, Rajput-Williams and Smith, 1991, Hofmann, Scott, Bergh and Deligdisch, 1991, Bass *et al.*, 1994). There is limited data on systemic levels of EGF in the maternal circulation, but EGF levels in maternal urine in the first trimester and maternal plasma at term is reduced in women who deliver small for gestation age (SGA) babies (Shigeta, Hiramatsu, Eguchi and Sekiba, 1992, Lindqvist, Grennert and Marsal, 1999). It is possible therefore, that genetic polymorphisms in the maternal *EGF* gene (see section 1.5.1.3) affecting its functional activity can cause defective placentation. The *EGF* gene therefore is a candidate gene for pre-eclampsia.

The EGFR is the prototypal member of the super family of receptors with intrinsic protein tyrosine kinase activity. EGFR serves as the sole or overwhelmingly predominant receptor for multiple distinct ligands including EGF, Transforming Growth Factor Alpha (TGFA), Amphiregulin (AR), Heparin Binding EGF (HB-EGF), Betacellulin (BTC), Epiregulin, and Epigen (Harris, Chung and Coffey, 2003). Of these, TGFA (see section 1.5.2) is of interest to pre-eclampsia because the gene encoding for *TGFA* maps to chromosome 2p13 (Tricoli, Nakai, Byers, Rall, Bell *et al.*, 1986) within the boundary of the most likely location for a pre-eclampsia susceptibility gene identified in genome-wide scans (Arngrimsson *et al.*, 1999, Moses *et al.*, 2000). TGFA can be localised in the endometrium during the proliferative and the secretory phase of the menstrual cycle, but its expression is particularly

high in decidual cells (Horowitz, Scott, Drews, Navot and Hofmann, 1993, Lysiak, Han and Lala, 1993). TGFA is reported to be present in all forms of trophoblast cells (Filla, Zhang and Kaul, 1993, Hofmann, Horowitz, Scott and Navot, 1993). This is probably an artefact brought on by the massive expression of EGFR in the cytotrophoblast because Bass *et al.* (1994) could not find TGFA mRNA in cytotrophoblasts. TGFA in the feto-maternal interface like EGF is most likely to be of maternal origin (Haining *et al.*, 1991, Bissonnette, Cook, Geoghegan, Steffen, Henry *et al.*, 1992, Bass *et al.*, 1994). Interestingly TGFA treatment of primary human cytotrophoblasts stimulates their proliferation in culture (Lysiak *et al.*, 1993). The *TGFA* gene therefore is also a candidate gene for pre-eclampsia.

In addition to molecules that bind directly to EGFR, there are others that can activate it without binding to it directly. Among them is angiotensin II (AngII) (Eguchi, Numaguchi, Iwasaki, Matsumoto, Yamakawa *et al.*, 1998, Wang, Yu, Cohen and Brecher, 2000). AngII is a component of the renin-angiotensin system (RAS) that also includes prorenin, renin, angiotensinogen (AGT), angiotensin I (AngI), angiotensin-converting enzyme, and angiotensin receptors (AT1R and AT2R). All these are expressed in and around the remodelling spiral arteries forming a local RAS (Morgan, Craven and Ward, 1998, Cooper, Robinson, Vinson, Cheung and Broughton Pipkin, 1999). AngII has two possible actions – acting via AT1R it is capable of promoting angiogenesis and cell growth; and acting via AT2R it is capable of exerting

anti-proliferative effects (Le Noble, Hekking, Van Straaten, Slaaf and Struyker Boudier, 1991, Yamada, Horiuchi and Dzau, 1996). Only the AT1R, however, has been proven to be present in the human placenta (Cooper *et al.*, 1999). The important role that this local RAS, which has only been recently recognised, may play in remodelling of spiral arteries has been overshadowed by the long standing knowledge of the changes in the circulating RAS during pregnancy.

The RAS in the circulating system, which is of critical importance in the regulation of blood pressure, is activated in normal pregnancy as evidenced by an increase in the concentration of plasma renin and angiotensinogen (Kalenga, de Gasparo, de Hertogh, Whitebread, Vankrieken *et al.*, 1991). The resulting increase in AngII does not normally result in hypertension as normotensive pregnant women are resistant to the pressor effects of infused AngII (Abdul-Karim and Assalin, 1961). This appears to be due to the down-regulation of the AT1R that mediate vasoconstriction (Baker, Broughton Pipkin and Symonds, 1992a) and increased secretion of vasodilator substances, among them placental and probably renal prostacyclin (Pedersen, Aalkjaer, Christensen, Christensen, Danielsen *et al.*, 1984, Fitzgerald, Entman, Mulloy and Fitzgerald, 1987). AngII is one of the substances that stimulate placental prostacyclin production (Glance, Elder and Myatt, 1985). The changes observed in this system in women with established pre-eclampsia, however, differ markedly from that observed in normal pregnancies. Their plasma renin concentrations are significantly lower than in normotensive

pregnancies and it is often associated with low plasma AngII concentrations (Wier, Brown, Fraser, Lever, Logan *et al.*, 1975). In addition, the AT1R density on platelets is increased (Baker, Broughton Pipkin and Symonds, 1991), and pressor sensitivity to infused AngII is increased towards pre-pregnancy values (Baker, Broughton Pipkin and Symonds, 1992b). The latter change is present even prior to the onset of clinically evident hypertension (Gant, Daley, Chand, Whalley and MacDonald, 1973). Other observations of note in women with pre-eclampsia, in relation to the RAS, include reduced placental secretion of prostacyclin (Fitzgerald *et al.*, 1987) and an increase in the presence in the plasma of a high molecular weight form of AGT, which is believed to be of placental origin (Tewksbury and Dart, 1982). A recent finding sheds light on the possibility that agonistic autoantibodies directed against the AT1R in the plasma of women with pre-eclampsia could, through activation of Nicotinamide Adenine Dinucleotidephosphate (NADPH) oxidase, contribute to the production of reactive oxygen species and the inflammatory responses in pre-eclampsia (Dechend, Viedt, Muller, Ugele, Brandes *et al.*, 2003). In addition to the above evidence for the role of angiotensinogen in blood pressure regulation, there is also evidence for a possible role for maternal AGT in determining fetal growth. In one study significant inverse correlations were found between the maternal plasma AGT level at 18 weeks of gestation and the birth weight and the head circumference of their babies (Broughton Pipkin, Sharif and Lal, 1995). This evidence supports the possibility that genes encoding components of the RAS are

candidates for pre-eclampsia; of them AGT (see section 1.5.3), being the precursor of AngII and the rate limiting factor in conversion of AGT to AngI in pregnancy, deserves particular consideration.

1.4.2. Stage 2: The systemic disease

The normal endothelium buffers response to circulating pressors, prevents activation of platelets, activates circulating anticoagulants, and maintains fluids in the intravascular compartment (Rubanyi, 1993). Injury to the endothelium sets in motion a dysfunctional cascade of vasoconstriction, coagulation, and intravascular fluid redistribution that is at the centre of the systemic disease of the clinical syndrome of pre-eclampsia (Roberts, Taylor, Musci, Rodgers, Hubel *et al.*, 1989).

There is morphologic evidence of endothelial cell injury in the kidney – glomerular endotheliosis (see section 1.2.2.2), and in the placental bed and adjacent uterine vessels (Shanklin and Sibai, 1989) of women with pre-eclampsia. In addition biochemical markers of endothelial cell activation appear in the circulation. Among these are: increased von Willebrand Factor (Redman, Denson, Beilin, Bolton and Stirrat, 1977), growth factor activity (Taylor, Heilbron and Roberts, 1990), cellular fibronectin (Friedman, de Groot, Taylor, Golditch and Roberts, 1994, Halligan, Bonnar, Sheppard, Darling and Walshe, 1994), plasminogen activation inhibitor -1 (Halligan *et al.*, 1994), anticardiolipin antibodies (Scott, 1987, Branch *et al.*, 1989),

endothelin (Taylor, Varma, Teng and Roberts, 1990, Florijn, Derkx, Visser, Hofman, Rosmalen *et al.*, 1991), thromboxane (Kraayenbrink, Dekker, van Kamp and van Geijn, 1993), tissue plasminogen activator (Friedman, Schiff, Emeis, Dekker and Sibai, 1995), and vascular cell adhesion molecule concentration (Lyall, Greer, Boswell, Macara, Walker *et al.*, 1994); and reduced prostacyclin metabolite excretion (Fitzgerald *et al.*, 1987). Some of these changes have been demonstrated prior to clinically evident pre-eclampsia supporting the central role of altered endothelial function in the pathophysiology of the disease.

One of the features of an activated endothelium is a disturbance of the balance that is normally dominated by vasodilator autocoids, such as prostacyclin and nitric oxide, to a vasoconstrictor phenotype with the production of more endothelin, thromboxane, and adhesion molecules. The pathological changes present in various organs of women dying of eclampsia which show the widespread presence of haemorrhage and necrosis is also more consistent with reduced perfusion possibly secondary to vasoconstriction rather than mechanical disruption of blood vessels (Roberts *et al.*, 1993).

An activated endothelium can also activate the haemostatic system via platelets and the coagulation cascade. Activation of the coagulation cascade that creates a maternal hypercoagulable state is likely to further reduce organ perfusion by the formation of microthrombi. The maternal hypercoagulable

state in pre-eclampsia is characterised by increased platelet consumption and reduced platelet lifespan (Redman, Bonnar and Beilin, 1978), increased concentration of pro-coagulant factors like tissue plasminogen activator, plasminogen activator inhibitor –1, von-Willebrand factor, anticardiolipin antibodies, endothelin and fibronectin; and reduced expression of endothelial cell associated anticoagulant proteins including antithrombin III (Weenink, Borm, Ten Cate and Treffers, 1983) and protein C (Gilabert, Fernandez, Espana, Aznar and Estelles, 1988). Finally, fluid is lost from the vascular compartment secondary to vasoconstriction and to an endothelial leak (Brown, Zammit and Lowe, 1989). These changes further reduce organ perfusion. Here too the reduction in plasma volume antedates the clinical diagnosis of pre-eclampsia. The significance of this last observation, however, is arguable because 'dry' pre-eclampsia, without the movement of plasma from the vascular tree into the tissues, may sometimes be more dangerous than the oedematous form (Vosburgh, 1976, National High Blood Pressure Education Program Working Group, 2000).

It has been proposed that a hypercoagulable state created by a hereditary predisposition to thrombophilia can increase the risk of pre-eclampsia (Kupferminc, Eldor, Steinman, Many, Bar-Am *et al.*, 1999, van Pampus, Dekker, Wolf, Huijgens, Koopman *et al.*, 1999, Kupferminc, Fait, Many, Gordon, Eldor *et al.*, 2000). The 5,10-methylenetetrahydrofolate reductase (*MTHFR*), factor V, and prothrombin genes have been shown to have

polymorphisms that increase the thrombophilic tendency. Among these the *MTHFR* gene is the one that has received most attention (see section 1.5.4) because its *677C>T* polymorphism is the commonest genetic cause of hyperhomocysteinaemia, which is an independent risk factor for vascular disease (Clarke, Daly, Robinson, Naughten, Cahalane *et al.*, 1991, Boers, 2000). Homocysteine is a metabolic intermediary. It is formed during the metabolism of methionine, a sulphur-containing essential amino acid, in the diet. Once formed homocysteine is either transulfurated by cystathionine β synthase into cystathionine or remethylated into methionine in the presence of 5-methyltetrahydrofolate, which is formed from 5,10-methylene tetrahydrofolate by the action of *MTHFR* (see section 1.5.4.1). This process is impaired in 677TT homozygotes because their *MTHFR* gene encodes an enzyme that has reduced enzymatic activity.

The mechanisms that underlie the relationship between homocysteine and vascular damage have been investigated extensively and various mechanisms involving platelets, endothelial cells and clotting factors have been proposed. Shortened platelet survival was suggested as a possible mechanism in a study on baboons (Harker, Harlan and Ross, 1983), but that has not been confirmed (Uhlemann, TenPas, Lucky, Schulman, Mudd *et al.*, 1976, Hill-Zobel, Pyeritz, Scheffel, Malpica, Engin *et al.*, 1982). Increased platelet adhesion (McDonald, Bray, Field, Love and Davies, 1964), inhibition of the expression of thrombomodulin and protein C activation on endothelial cells (Rodgers and

Conn, 1990, Lentz and Sadler, 1991), and an increase in factor V activity (Rodgers and Kane, 1986) have all been reported in different studies. All these could shift the haemostatic balance to coagulation. A direct toxic effect on endothelial cells has also been suggested (Harker, Slichter, Scott and Ross, 1974, Harker, Ross, Slichter and Scott, 1976). In addition oxidized homocysteine could lead to an increased generation of hydrogen peroxide that could damage the endothelium, reduce prostacyclin synthesis, and increase atherogenic oxidised Low Density Lipoprotein (ox-LDL) in plasma (Starkebaum and Harlan, 1986). These studies, however, have been widely criticised as unrepresentative of the mechanisms *in vivo* because they have been conducted using extremely high concentrations of homocysteine (1 to 10 mM). Interestingly in a group of patients with premature atherosclerosis, plasma levels of endothelium derived proteins, von Willebrand factor and thrombomodulin, that are markers of endothelial activation, decreased following homocysteine lowering treatment suggesting a reversible toxic effect of hyperhomocysteinaemia on the endothelium at concentrations found *in vivo* (Van den Berg, Boers, Franken, Blom, Van Kamp *et al.*, 1995).

Plasma homocysteine levels in normal pregnant women are almost 50% lower than in non-pregnant women (Andersson, Hultberg, Brattstrom and Isaksson, 1992). In women who develop pre-eclampsia, however, mean plasma homocysteine levels are elevated in the first (Cotter, Molloy, Scott and Daly, 2003) and second trimesters (Sorensen, Malinow, Williams, King and Luthy,

1999) of pregnancy prior to clinically evident disease, during the acute phase of the illness (Rajkovic, Catalano and Malinow, 1997, Rajkovic, Mahomed, Malinow, Sorenson, Woelk *et al.*, 1999, Sanchez, Zhang, Rene Malinow, Ware-Jauregui, Larrabure *et al.*, 2001) and postpartum (Dekker *et al.*, 1995, van Pampus *et al.*, 1999). Hyperhomocysteinaemia, as evidenced by the above-mentioned studies, is neither a feature of all pregnancies complicated by pre-eclampsia nor is its presence in pregnancy always associated with pre-eclampsia, but plasma homocysteine levels, when elevated in pre-eclampsia, positively correlate with that of cellular fibronectin (Powers, Evans, Majors, Ojimba, Ness *et al.*, 1998). This indicates that homocysteine may play a role in promoting endothelial dysfunction in susceptible women. The *MTHFR* gene therefore is a candidate gene for pre-eclampsia.

1.4.3. The link between the two stages

It has been hypothesised that women affected by pre-eclampsia have a circulating factor released by the hypoxic placenta that damages the endothelium. A serum factor that has selective affinity for endothelial cells found in the serum of women with pre-eclampsia has been shown to damage human endothelial cells (Rodgers, Taylor and Roberts, 1988, Tsukimori, Maeda, Shingu, Koyanagi, Nobunaga *et al.*, 1992, 1994). Others have failed to confirm this (Kupferminc, Mullen, Russell and Silver, 1996), and instead found the plasma and not the serum of women with pre-eclampsia to be toxic to endothelial cells (Smarason, Sargent and Redman, 1996). This discrepancy

could be accounted for by the physiological differences between plasma and serum and the fact that different aspects of endothelial cell function were assayed by the two groups, i.e. endothelial cell proliferation compared to endothelial cell injury.

It has been proposed that the possible explanation for the link between the two stages of the disease lies in observations made in relation to the pathogenesis of atherosclerosis and parallels to it that are seen in pre-eclampsia. In normal human pregnancy, plasma cholesterol and triglyceride concentrations rise by up to 50% to 300% respectively, with all major lipoproteins reflecting these changes (Potter and Nestel, 1979). These changes are accentuated in pre-eclampsia resulting in pre-eclampsia sharing a common dyslipidaemia with atherosclerosis, which is characterised by increased triglyceride and fatty acid levels (Hubel, McLaughlin, Evans, Hauth, Sims *et al.*, 1996), decreased high-density lipoproteins (HDL) (Rosing, Samsioe, Olund, Johansson and Kallner, 1989), and increased small dense low-density lipoproteins (LDL) (Sattar, Bendomir, Berry, Shepherd, Greer *et al.*, 1997). In fact triglyceride and fatty acid levels in women with pre-eclampsia are elevated from early pregnancy (Lorentzen, Endresen, Clausen and Genriksen, 1994). This may be a feature of pregnancies that are destined to develop early-onset, but not-late onset pre-eclampsia (Clausen, Djurovic and Henriksen, 2001). It is not known, however, whether these changes antedate pregnancy.

The small dense LDLs, which are a part of the atherogenic dyslipidaemia, are proposed to have preferential access to the subendothelial space, where they bind to proteoglycans and reside longer than other LDLs. Small dense LDLs are inherently more easily oxidisable. Protected from circulating antioxidants in the subendothelial space they form very reactive oxidized-LDL (ox-LDL). They damage endothelial cells as they are cytotoxic to them and attract monocytes, as they are also a potent chemo-attractant to circulating monocytes. Monocytes take up ox-LDL to form foam cells and eventually the fatty streak characteristic of atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo and Witztum, 1989). This process that leads to an excess of free radicals and reactive oxygen species over antioxidant scavengers, termed 'oxidative stress', is thought to be responsible for the endothelial changes in atherosclerosis, and has been proposed as the link between decreased placental perfusion and the maternal syndrome in pre-eclampsia (Roberts and Hubel, 1999).

Changes in circulating lipids alone, however, are unlikely to be enough to bring about the widespread damage to endothelial cells that occur during a relatively short period of time during a pregnancy complicated by pre-eclampsia. The answer to the link therefore, may perhaps lie in recent discoveries involving circulating factors that interfere with angiogenesis (Maynard, Min, Merchan, Lim, Li *et al.*, 2003), engage AngII signalling (Dechend *et al.*, 2003), and directly impair endothelial function (Savvidou,

Hingorani, Tsikas, Frolich, Vallance *et al.*, 2003). Among them the study by Maynard *et al.* (2003) deserves special attention. They studied the gene expression profile of placental tissue in normotensive women and women with pre-eclampsia using microarray chips and discovered that the production of placental soluble fms-like tyrosine kinase 1 (sFlt1), an antagonist of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), is upregulated in pre-eclampsia, which could lead to increased systemic levels of sFlt1 that fall after delivery. They also found that the increased level of circulating sFlt1 in patients with pre-eclampsia is associated with decreased circulating levels of free VEGF and PlGF, which are necessary to maintain the integrity of the vascular endothelium. They were able to show that depleted levels of VEGF and PlGF result in endothelial dysfunction in vitro that can be rescued by exogenous VEGF and PlGF, and that the administration of sFlt1 to pregnant rats induces the classical features of pre-eclampsia – hypertension, proteinuria, and glomerular endotheliosis. The same group studied the gestational patterns of circulating sFlt1, free PlGF and, free VEGF in normotensive and pre-eclamptic pregnancies and concluded that increased levels of sFlt1 and reduced levels of PlGF, beginning five weeks before the clinical detection of the condition, predict the subsequent development of pre-eclampsia (Levine, Maynard, Qian, Lim, England *et al.*, 2004). These observations suggest strongly that sFlt1 may be a pre-eclampsia factor, but what upregulates sFlt1 is not known.

1.5. The candidate genes

The pathophysiological evidence discussed above has suggested numerous candidate genes for pre-eclampsia. From the list of possible candidates *EGF*, *TGFA*, *AGT*, and *MTHFR* genes have been selected for the investigations described in this thesis.

1.5.1. Epidermal Growth Factor

1.5.1.1. Introduction

EGF is a growth factor that has many biological functions, including proliferation of epidermal tissue (Groenen, Nice and Burgess, 1994). It is a globular protein of 6.4kDa consisting of 53 amino acids that is characterised by a consensus sequence consisting of six spatially conserved cysteine residues that form three intramolecular disulphide bonds. This consensus sequence, which is known as the EGF motif, present in all mammalian ligands that bind the EGFR, is crucial for EGFR binding. EGF proteins are closely conserved during evolution as indicated by approximately 70% similarity between the amino acid sequence of human and mouse *EGF* genes, and the conservation of the relative position of the cysteine residues. EGF is synthesized as a prepro-protein of 1207 amino acids. It exists as a membrane bound molecule from which EGF is released by proteolytic cleavage (Bell, Fong, Stempien, Wormsted, Caput *et al.*, 1986).

There have been numerous studies examining the properties of the EGFR, its

ligands, and its signalling pathway. The following brief description is based on recent reviews by Wells (1999) and Harris *et al.* (2003): EGFR also referred to as HER (human EGF receptor) is the prototypal member of a family of transmembrane receptors with intrinsic protein tyrosine kinase activity. EGFR binding by its ligands, including EGF and TGFA, activates EGFR kinase and initiates numerous signalling pathways. The biological outcome of many of these pathways has not yet been determined. There are, however, two strongly activated pathways that interact with each other to bring about enhanced cell motility and cell proliferation. These pathways act via phospholipase C-γ (PLCγ) and *ras*. The PLCγ pathway enhances cell motility by hydrolysis of PIP_2 (Phosphatidylinositol (4,5) bisphosphate) and mobilization/activation of actin modifying proteins (AMP) such as gelsolin, profilin, and cofilin, and possibly via protein kinase C (PKC) and calcium mediated events. Motility is blocked, however, if MEK (MAP kinase kinase) signalling is abrogated. The activation of the ras-MAP kinase (extracellular signal-regulated kinase, mitogen-activated protein kinase) pathway results in cell proliferation and motility. The phosphorylation of EGFR at threonine 654 by PKC, however, preferentially disrupts activation of the ras-MAP pathway, which is postulated to result in intermittent *erk* activity that favours cell motility over proliferation. These pathways are summarised in figure 1.2.

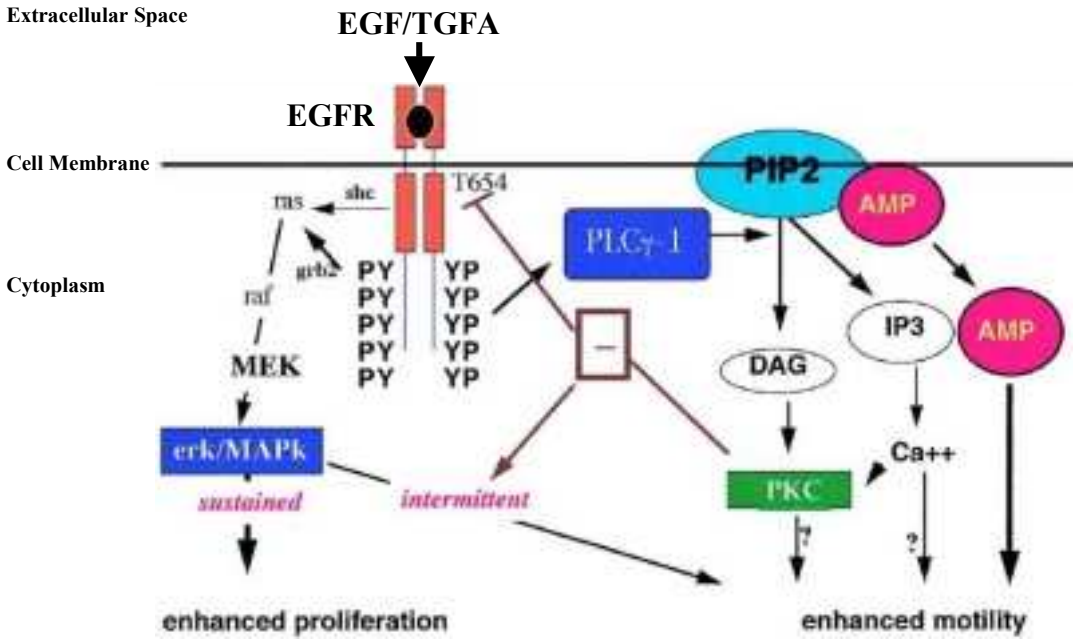


Figure 1.2. A general model of the cross-talk between downstream pathways activated by EGFR in different types of cells. See text for description. EGF: Epidermal Growth Factor, TGFA: transforming growth factor, EGFR: Epidermal growth factor receptor, MAPk: mitogen activated protein kinases, MEK: MAP kinase kinase, PLCγ: phospholipase C-γ, PIP_2: Phosphatidylinositol (4,5) bisphosphate, AMP: Actin Modifying Protein, DAG: Diacylglycerol, IP3: Inositol-1,4,5-triphosphate, PKC: Protein Kinase C (Adapted from Wells (1999)).

1.5.1.2. Genomic structure

The gene encoding the EGF precursor maps to chromosome 4q22-25 (Brissenden, Ullrich and Francke, 1984, Morton *et al.*, 1986). It is located approximately 47.6cM and 67.3cM away from the suggestive locus for pre-eclampsia identified by Laivuori *et al*. (2002) and Harrison *et al.* (1997) respectively (Figure 1.3). The genomic structure of the *EGF* gene was described by Bell *et al.* (1986). It has a length of approximately 110kb and consists of 24 exons. The introns interrupt the coding sequence of the gene such that many of the domains of the protein are the products of individual exons. Moreover, most of the protein (amino acids 43-952) is apparently the result of the tandem duplication of a block of eight to nine exons. This gene has been described as a mosaic because 15 of its 24 exons encode sequences that are homologous to exon-encoded regions in other proteins (eight of these exons encode the cysteine-rich EGF-like repeat motif). It is also a member of three gene families: one, which includes proteins that have EGF-like repeat motifs; a growth factor family which includes the TGFA precursor; and a receptor family which includes the LDL receptor (Figure 1.4).

Bell *et al.* (1986) described some of the regulatory regions of the gene. There is a consensus 'cap site' and 'TATA box' 23 and 29 base pairs respectively, upstream of the transcription start site. The first 66 base pairs of the gene that encode the first 22 amino acids most likely represent the signal peptide. Mullhaupt, Feren, Jones and Fodor (2000) characterised the *EGF* promoter

further and showed that it is active during cell proliferation and negatively regulated in contact inhibited and quiescent cell cultures. They hypothesised, because of the link between *EGF* gene activity and growth induction, that the *EGF* promoter comprises a yet unidentified growth responsive regulatory domain. Other regulatory regions of this gene have not yet been characterised.

1.5.1.3. Polymorphisms/haplotypes

There are two studies that report on screening for *EGF* polymorphisms. Semina, Datson, Leysens, Zabel, Carey *et al.*(1996) screened all exons of the *EGF* gene and identified four SNPs in exons 7, 14, 15 and 19. They established the frequency of these polymorphisms in 50 white Caucasians (Table 1.1). Subsequently Shahbazi, Pravica, Nasreen, Fakhoury, Fryer *et al*. (2002) detected another SNP at position 61 in the 5' UTR (Table 1.1). They carried out *in vitro* studies to examine EGF production in peripheral blood mononuclear cells (PBMC) and found that cells of individuals homozygous for the 61A allele produced significantly less EGF than cells of heterozygous individuals and individuals homozygous for the 61G allele. The mechanism by which this polymorphism affects EGF production however, is not known. They also found that the 61G allele was associated with a significant risk of developing malignant melanoma (odds ratio (OR)=2.7, 95% Confidence Interval (CI), 1.9 to 4.0; $P<0.0001$). This finding, however, could not be replicated in another group of white Caucasians (McCarron, Bateman, Theaker and Howell, 2003).

In the two association studies mentioned above, in white Caucasian controls, the frequency of the 61A allele was 56.1% and 60.2% respectively. In the study by Semina *et al.* (1996) the frequency of the 67149A allele was 50%, and the variant allele of all other polymorphisms occurred in <10% of chromosomes. Thus the 61G>A and 67149G>A polymorphisms occur frequently enough in the population to justify their selection as markers for association analysis as it would be possible to carryout a study that has 80% statistical power at a significance level of 0.05 to detect a doubling of the risk of pre-eclampsia with the sample size of this study. In addition the 61G>A is the only *EGF* polymorphism that has been shown to have functional effects and to be implicated in the aetiology of a disease. The 67149G>A polymorphism that occurs in exon 14 of the gene results in a substitution of the amino acid methionine by isoleucine in the LDL receptor homology domain of the gene. These two polymorphisms were therefore selected for this study.

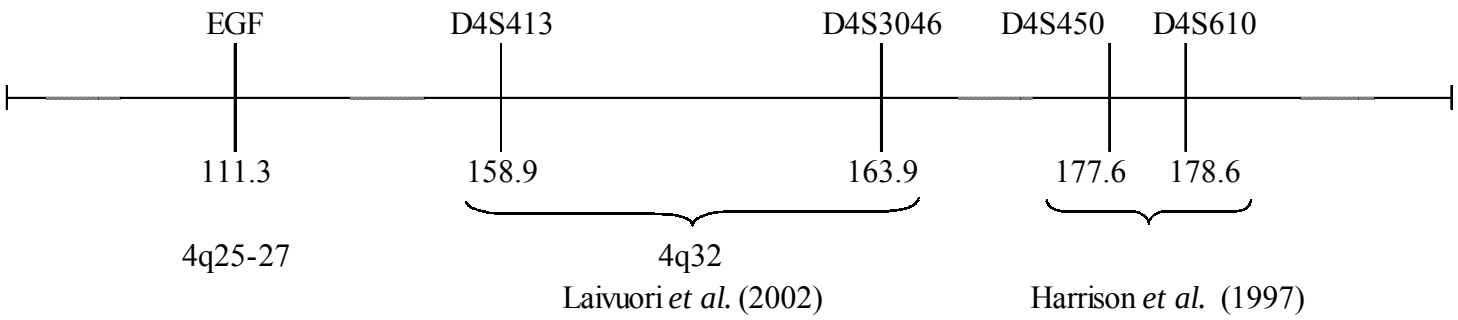


Figure 1.3. A diagrammatic representation of the relative position of the *EGF* gene on a Sequence Tagged Site (STS) map of chromosome 4 in relation to markers that were associated with suggestive loci for pre-eclampsia in genome wide studies by Laivuori *et al.* (2002) and Harrison *et al.* (1997). Position of *EGF* and markers are from the STS map of chromosome 4 at the NCBI (see section 2.10).

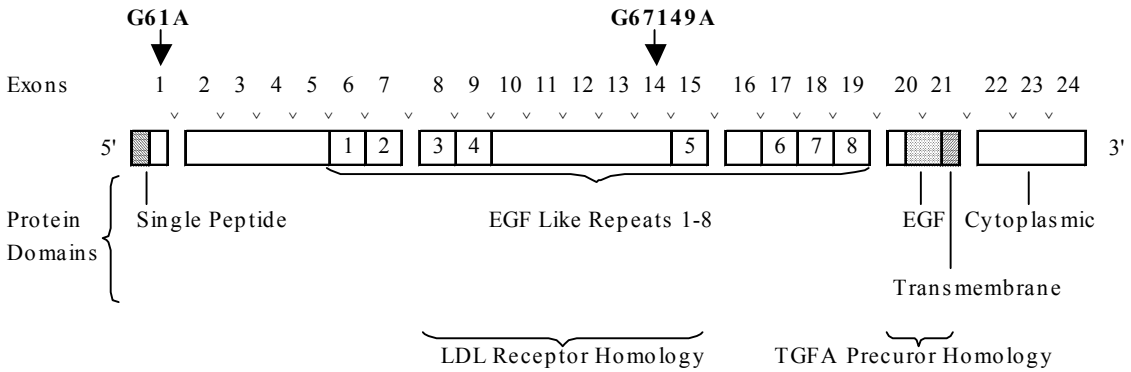


Figure 1.4. A diagrammatic representation of the exon-intron organisation and the protein domains of the *EGF* gene and the location of SNPs used for association analysis in this study. Arrowheads indicate the positions at which introns interrupt the coding region. Exon numbers are shown between the arrowheads. The locations of the two SNPs are indicated. Modified and adapted from Bell *et al.*(1986)

Nucleotide[1]	Location	Allele at SNP	Amino Acid	Frequency
61	Exon 1	G	-	0.44 [2]
		A	-	0.56
48002	Exon 7	T	His	0.99 [3]
		C	His	0.01
67149	Exon 14	G	Met	0.49 [3]
		A	Ile	0.50
68062	Exon 15	T	Leu	0.07 [3]
		A	Met	0.93
80378	Exon 19	T	Val	0.94 [3]
		A	Glu	0.06

Table 1.1. SNPs reported in the *EGF* gene. [1] Nucleotides are numbered with respect to the transcription start site (see section 2.10 below). [2]Shahbazi *et al.* (2002): frequencies are for 99 white Caucasians. [3]Semina *et al*. (1996): frequencies are for 50 white Caucasians.

1.5.2. Transforming Growth Factor Alpha

1.5.2.1. Introduction

TGFA is a 50 amino acid protein that is synthesized as a prepro-protein of 160 amino acids. It is a structural (shows 40% similarity in sequence) and functional homologue of EGF. It competes with EGF for binding to the EGFR. It too is believed to exist as a membrane bound molecule from which TGFA is released by proteolytic cleavage.

1.5.2.2. Genomic structure

The gene encoding TGFA precursor maps to chromosome 2p11-2p13 (Brissenden, Derynck and Francke, 1985, Tricoli *et al.*, 1986). It lies between the markers at 70.8cM and 79.4cM within the most likely location for a pre-eclampsia susceptibility gene according to the genome wide scan of Arngrimsson *et al.* (1999) (Figure 1.5). The gene is approximately 100kb long and consists of six exons, of which exon 1 encodes the 5' UTR and signal peptide, exon 2 is the N' terminal precursor, exon 3 is the mature peptide through the first two disulphide loops of EGF motif, exon 4 is the third disulphide loop and transmembrane domain, exon 5 is the cytoplasmic domain, and exon 6 is the 3'UTR (Figure 1.6) (Collin, Marshall, Naggert and Nishina, 1999, Machida, Yoshiura, Funkhauser, Natsume, Kawai *et al.*, 1999). This exon organisation is similar to the genes of other EGFR ligands such as HB-EGF, AR, and BTC (Harris *et al.*, 2003).

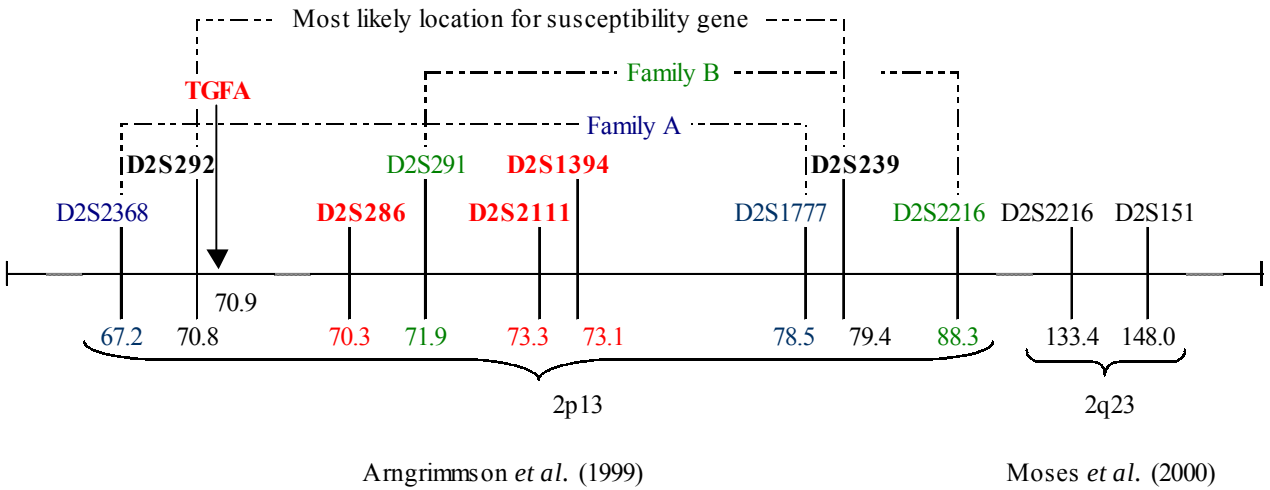


Figure 1.5. A diagramtic representation of the relative position of the *TGFA* gene on a STS map of chromosome 2 in relation to markers that were associated with a suggestive locus for pre-eclampsia in genome wide studies by Arangrimmson *et al.* (1999) and Moses *et al.* (2000). The position of *TGFA* and microsatellite markers were obtained from the STS map of chromosome 2 at NCBI. The positions assigned to markers in this map differed from positions assigned to them on the radiation hybrid Marshfield map that were referred to in the report by Arangrimmson *et al.* (1999), but the order of markers on the two maps are the same. The most significant linkage under general criteria that included both women with gestational hypertension and pre-eclampsia was to marker D2S286, and under strict criteria including only women with pre-eclampsia and eclampsia it was between markers D2S211 and D2S1394. The markers bordering the area of distribution of the haplotypes shared by the members of the two large families (A&B) that contributed to the significant linkage to this region is also indicated.

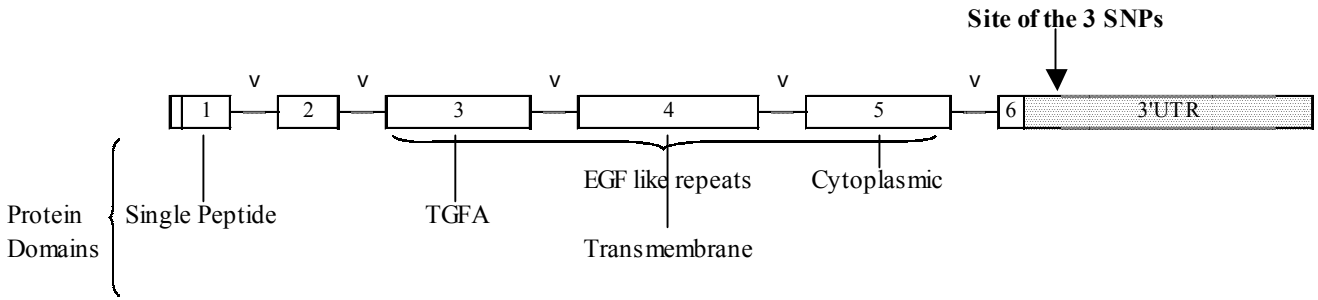


Figure 1.6. A diagrammatic representation of the exon-intron organisation and the protein domains of the *TGFA* gene and the location of SNPs used for association analysis in this study. Arrowheads indicate the positions at which introns interrupt the coding region. Exon numbers are shown. The location of the SNPs in the 3'UTR is indicated.

1.5.2.3. Polymorphisms/haplotypes

There are several well-characterised polymorphisms in the *TGFA* gene that have been used in association analysis. A 4bp deletion/insertion polymorphism (DIP) in intron 5 of the gene, designated *Taq*I restriction length polymorphism (Hayward, Nancarrow and Bell, 1987), was among the first to be identified and has been found in less than 10% of chromosomes tested (Basart, Qian, May and Murray, 1994, Tanabe, Taketani, Endo-Ichikawa, Tokunaga, Ogawa *et al.*, 2000). A second 4-bp DIP in the 3'UTR of the gene is more common and it has been has been reported to be present in about 13% of white Caucasian chromosomes (Shiang, Lidral, Ardinger, Buetow, Romitti *et al.*, 1993) and 24% of Japanese chromosomes (Tanabe *et al.*, 2000). There is also a cluster of SNPs at nucleotides 3822, 3827, and 3851 in the 3'UTR of the gene. These three SNPs are in complete linkage disequilibrium and define four haplotypes (Table 1.2). They have been found to be associated with the development of two disorders involving tissue remodelling, viz. non syndromic cleft lip and palate (Ardinger, Buetow, Bell, Bardach, VanDemark *et al.*, 1989) and cleft palate only (Shiang *et al.*, 1993) in some populations, but not in others (Machida *et al.*, 1999). Polymorphisms in 3'UTR conserved regions of genes can play a role in mRNA stability or tissue specific targeting (Siomi and Dreyfuss, 1997). These three polymorphisms were therefore selected for this study.

Haplotype			Frequency	
3822G>A	**3827T>C**	**3851T>C**	**White Caucasian[1]**	**Japanese[2]**
G	T	T	0.29	0.21
G	C	T	0.50	0.50
G	C	C	0.07	0.08
A	C	T	0.14	0.20

Table 1.2. Haplotypes defined by the three SNPs in the 3'UTR of the *TGFA* gene and the reported frequency of the haplotypes in two distinct populations [1]Shiang *et al.* (1993) [2]Tanabe *et al.* (2000)

1.5.3. Angiotensinogen

1.5.3.1. Introduction

Angiotensinogen (AGT) is the precursor of AngII, which is a potent vasoconstrictor, a major determinant of salt and water homeostasis and a growth factor. AGT is mainly synthesised in the liver, but it is expressed in a wide range of tissues, including the placenta.

1.5.3.2. Genomic structure and gene regulation

The *AGT* gene maps to chromosome 1q42-43 (Isa, Boyd, Morrison, Harrap, Clauser *et al.*, 1990). It is approximately 12kb in length and consists of five exons and four introns of which exon 1 encodes the 5' UTR, exon 2 encodes the signal peptide and AngI, and exons 3 to 5 encode the remainder of the coding sequence. A part of exon 5 also contains the 3'UTR (Figure 1.7) (Gaillard, Clauser and Corvol, 1989, Fukamizu, Takahashi, Seo, Tada, Tanimoto *et al.*, 1990).

There are many studies that have examined the regulation of angiotensinogen gene expression. A brief survey is made here of the studies most relevant to the investigations undertaken in this thesis. Fukamizu *et al.* (1990) characterised the minimal promoter required for expression in HepG2 cells and other putative regulatory motifs in the 5' flanking region, including the TATA box, the oestrogen response element, the glucocorticoid responsive element, the acute phase response element, the cAMP response element, and

the heat shock response element. Later the same group identified other elements responsible for maintaining promoter activity. Among them is angiotensinogen core promoter element 1 (AGCE1), located between the TATA box and transcription initiation site, that interacts with an unidentified nuclear factor AGCF1 (AGCE-binding factor 1) (Yanai, Nibu, Murakami and Fukamizu, 1996), and AGCE2 and 5'AGCE2, that interact with another unidentified nuclear factor AGCF2 (Yanai, Matsuyama, Murakami and Fukamizu, 1997). AGCF1 seems to play the major role in mediating the enhancer effects of the downstream enhancers of the *AGT* gene on gene expression. Two such enhancer elements spanning 80-bp (Nibu, Tanimoto, Takahashi, Ono, Murakami *et al.*, 1994b) and 23-bp (Nibu, Takahashi, Tanimoto, Murakami and Fukamizu, 1994a) have been identified in exon 5 and the 3' flanking region of the gene. Both these regions that bind HepG2 specific nuclear factors are responsible for activating the *AGT* promoter in a cell type dependent manner, and are both necessary for full enhancer activity. Polymorphisms in the *AGT* minimal promoter have been shown to affect promoter activity (see section 1.5.3.3), but it is not known whether polymorphisms in the *AGT* 3'UTR have any effects on enhancer activity.

The regulation of the *AGT* gene is affected by many substances, among them AngII. AngII has been reported to stimulate the synthesis and secretion of AGT in primary cultures and on hepatocytes in suspension in rats (Ruiz, Jimenez, Montiel, Narvaez, Diego *et al.*, 1987, Klett, Muller, Gierschik and

Hackenthal, 1990). Further work on rat hepatocytes show that this increase in AGT mRNA following stimulation with AngII is due to stabilisation of AGT mRNA by a 12-kD intracellular hepatic protein that selectively binds to the 3'UTR of the AGT mRNA rather than due to increased transcription (Hackenthal and Klett, 1993, Klett, Bader, Ganten and Hackenthal, 1994). No attempt to replicate these results in human cells has been reported.

1.5.3.3. Polymorphisms/haplotypes

Members of our pre-eclampsia research group and others have extensively investigated polymorphisms and the haplotype structure of the *AGT* gene and their prevalence in white Caucasian and Japanese populations. These studies show wide variation in both the prevalence of SNPs and the haplotype structure in these populations (Morgan, Broughton Pipkin and Kalsheker, 1996, Jeunemaitre, Inoue, Williams, Charru, Tichet *et al.*, 1997, Nakajima, Jorde, Ishigami, Umemura, Emi *et al.*, 2002, Plummer, Morgan and Kalsheker, 2002).

Of the many polymorphisms in the *AGT* gene Ward and colleagues investigated the 235Met>Thr polymorphism in exon 2 for its possible role in pre-eclampsia. It was selected because it had been found to be associated with essential hypertension (Jeunemaitre, Soubrier, Kotelevtsev, Lifton, Williams *et al.*, 1992). They found that in their population pre-eclampsia was associated with the 235Thr allele (Ward, Hata, Jeunemaitre, Helin, Nelson *et al.*, 1993).

Then they extended their investigations to functional studies and found that 235Met/Thr heterozygotes exhibited significantly elevated expression of the 235Thr allele compared to the 235Met allele, suggesting that the elevated expression of the 235Thr allele in decidual spiral arteries may cause first trimester atherotic changes leading to pre-eclampsia (Morgan, Craven, Nelson, Lalouel and Ward, 1997). Subsequently they found evidence in support of their hypothesis when 235Thr/Thr homozygous women were found to have a greater media area/external diameter ratio than 235Met/Met homozygous women (Morgan, Craven, Lalouel and Ward, 1999b). Results of studies that have examined the association of the 235Met>Thr polymorphism with pre-eclampsia are summarised in table 1.3. The initial positive result has not been replicated in all populations. In one recent study, the 174Met allele of the 174Thr>Met polymorphism and the 1035A/174Met/235Thr haplotype of the *AGT* gene are reported to confer a two-fold increased risk of pre-eclampsia in a French Canadian population (Levesque *et al.*, 2003). The same study failed to find any significant increased risk associated with the 235Thr allele alone.

The 235Thr allele is in linkage disequilibrium with the –20C, –6A and 174Met alleles of the –20A>C, –6G>A and 174Thr>Met polymorphisms of the *AGT* gene (Morgan *et al.*, 1996, Jeunemaitre *et al.*, 1997). Reporter gene constructs containing the *AGT* minimal promoter with the nucleotide A at –6 and C at –20 have increased promoter activity upon transient transfection in human liver derived HepG2 cells compared with reporter constructs containing

nucleotide G at –6 and A at –20 respectively (Inoue, Nakajima, Williams, Quackenbush, Puryear *et al.*, 1997, Zhao, Zhou, Narayanan, Cui and Kumar, 1999). This may account for the reported association of high levels of plasma AGT with the 235Thr allele (Jeunemaitre *et al.*, 1992). In contrast, in normotensive pregnant women the 235Thr allele seems to be associated with lower plasma angiotensinogen concentrations (Morgan, Crawshaw, Baker, Broughton Pipkin and Kalsheker, 1999a). It is possible therefore, that complex mechanisms are operative in regulating angiotensinogen expression in pregnancy.

The above-mentioned SNPs are situated 5' to the 235Met>Thr polymorphism. Recently members of our group screened the 3'UTR/flanking region of the *AGT* gene, and described the presence of six novel SNPs in this region (11535C>A, 11608C>A, 12058G>A, 12194A>C, 12429C>T, 12822T>C) that together with the 235Met>Thr polymorphism define five common *AGT* haplotypes that are found in 91% of white Caucasian chromosomes (Plummer *et al.*, 2002). Interestingly two of these SNPs, 11535C>A and 11608C>T, are in linkage disequilibrium with each other, and split the common haplotype bearing 235Met; all other haplotypes have been described in association with the 235Thr allele (Plummer *et al.*, 2002). The 174Thr>Met, 235Met>Thr and 11535C>A polymorphism of the *AGT* gene were therefore selected for this study.

All the 3'UTR/flanking region SNPs are located outside the two regions that have been showed to be responsible for the 3' enhancer activity under basal conditions in HepG2 cells (Nibu *et al.*, 1994a). What effect, if any, that the SNPs have on enhancer activity and enhancer activity in HepG2 cells under stimulated conditions has not been studied so far. A further study was undertaken therefore, to investigate the functional significance of the 3'UTR/flanking region SNPS on *AGT* gene expression using reporter gene constructs containing enhancer elements harbouring the three common haplotypes defined by the 3'UTR/flanking region SNPs (see table 2.12) under basal and AngII stimulated conditions in HepG2 cells.

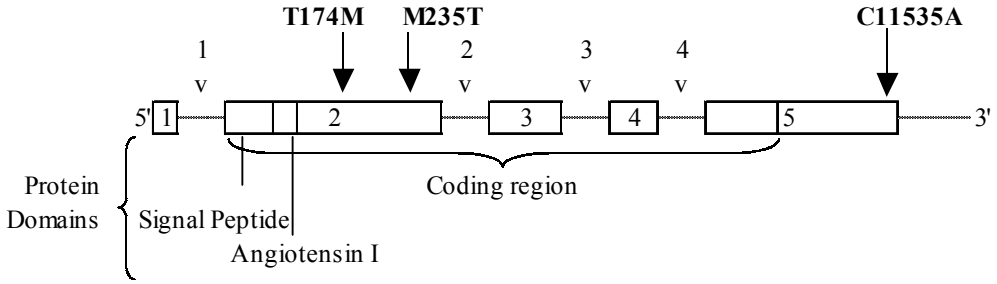


Figure 1.7. Genomic structure of the *AGT* gene. The exons and introns are numbered. The protein domains are indicated. The positions of the three polymorphisms used in these investigations are indicated.

Population	Cases	Controls	Primi/ Multi	Exclusion criteria	T allele frequency		
					Cases	Controls	*P*
White Caucasian,USA[1]	45 PE	571 normal pregnant women	Both	CHT, RD, MP	0.60	0.41	<0.001
Japanese[1]	18 PE	80 normal pregnant women	Both	DM, CHT, RD, MP.	0.90	0.71	0.041
Indonesian[2]	20 E	100 non eclamptic women	?	?	0.65	0.62	NS
White Caucasian,Australia[3]	106 PE/E	81 normal pregnant women	?	?	0.47	0.38	NS
Chinese[3]	72 PE/E	48 normal pregnant women	?	?	0.78	0.75	NS
Japanese[4]	33 GH/PE	280 normal pregnant women	Both	CHT, DM, RD, MP	0.79	0.80	NS
White Caucasian, UK[5,6]	43 PE	84 normal pregnant women	Both	CHT	0.48	0.48	NS
Japanese[7]	115 GH/PE/E	381 normal pregnant women	Both	CHT, DM, RD, FA, HELLP	0.91	0.76	<0.001
Japanese[8]	58 PE	164 normal pregnant women	Primi	CHT, DM, RD, MP, FA	0.78*	0.59*	0.01
Hispanic[9]	87 PE	53 women with at least 2 normal pregnancies	Both	CHT, DM, RD, PlatD, AID	0.72	0.70	NS
Romanian[10]	11PE	6 normal pregnant women	?	?	0.42	0.33	NS
Hellenic (north west Greece)[11]	41 PE	102 normal with at least 2 normal pregnancies	Both	MP	0.59	0.40	0.005
French Canadian[12]	203 PE	357 normal pregnant women	Primi	MP, CHT	0.44	0.40	NS

Table 1.3. A summary of the case-control *AGT* 235Met>Thr pre-eclampsia association studies with some studies reporting an association. *The frequency of the *AGT* 235Thr/Thr genotype.

Abbreviations:
?-Information not available
AID-Auto Immune Disorders
CHT-Chronic Hypertension
DM-Diabetes Mellitus
FA-Fetal and amniotic fluid volume abnormalities
GH-Gestational Hypertension
HELLP-HELLP Syndrome
MP-Multiple Pregnancies
PE-Pre-eclampsia
PlatD-Platelet Disorders
RD-Renal Disease
Primi-Primiparous
Multi-Multiparous

References:

1. (Ward *et al.*, 1993)
2. (Romi, Patria and Matsuo, 1997)
3. (Guo, Wilton, Fu, Qiu, Brennecke *et al.*, 1997)
4. (Suzuki, Tanemura, Murakami and Suzumori, 1999)
5. (Morgan, Baker, Broughton Pipkin and Kalsheker, 1995)
6. (Morgan *et al.*, 1999a)
7. (Kobashi, Hata, Shido, Kato, Yamada *et al.*, 1999)
8. (Kobashi, Shido, Hata, Yamada, Kato *et al.*, 2001)
9. (Bashford, Hefler, Vertrees, Roa and Gregg, 2001)
10. (Procopciuc, Jebeleanu, Surcel and Puscas, 2002)
11. (Bouba, Makrydimas, Kalaitzidis, Lolis, Siamopoulos *et al.*, 2003)
12. (Levesque *et al.*, 2003)

1.5.4. 5,10-Methylenetetrahydrofolate Reductase

1.5.4.1. Introduction

MTHFR (Enzyme Commission 1.5.1.20) is the enzyme that catalyses the conversion of 5,10-methylenetetrahydrofolate to 5- Methyl tetrahydrofolate, the major form of folate in plasma, that is a cosubstrate for remethylation of homocysteine to methionine (Figure 1.8). Thus normal biological activity of MTHFR is important for the regulation of methionine and homocysteine concentrations in the circulation.

Severe forms of MTHFR deficiency (residual enzyme activity that correspond to 0-20% of control values) caused by rare mutations in the *MTHFR* gene result in homocysteinuria as well as severe neurological and vascular complications (Sibani, Christensen, O'Ferrall, Saadi, Hiou-Tim *et al.*, 2000). Milder forms of MTHFR deficiency (residual enzyme activity 35-50% of control values) caused by other common polymorphisms in the *MTHFR* gene result in mild hyperhomocysteinaemia (Frosst, Blom, Milos, Goyette, Sheppard *et al.*, 1995, van der Put, Gabreels, Stevens, Smeitink, Trijbels *et al.*, 1998, Weisberg, Tran, Christensen, Sibani and Rozen, 1998). These common polymorphisms have been associated with complications in pregnancy such as recurrent pregnancy loss (Nelen, Steegers, Eskes and Blom, 1997) and pre-eclampsia. The latter association, however, has not been replicated in most of the studies (Table 1.4).

1.5.4.2. Genomic structure

The *MTHFR* gene maps to chromosome 1p36.3 (Goyette, Sumner, Milos, Duncan, Rosenblatt *et al.*, 1994). It consists of 11 exons with lengths ranging from 102-bp to 432-bp and introns with lengths ranging from 250-bp to 1.5-kb, except for one large intron of 4.2-kb (Figure 1.9). There is approximately 90% similarity between the nucleotide sequences of the human and mouse *MTHFR* genes. Both genes have 11 exons of similar sizes and similar exon-intron boundaries (Goyette, Pai, Milos, Frosst, Tran *et al.*, 1998).

The promoter region of the *MTHFR* gene contains numerous consensus sequences for transcription factor binding sites, but lack a TATA box. This points to complex regulation of the *MTHFR* gene at transcriptional level. Three MTHFR transcript variants, predicted to give rise to 74.5, 78.9 and 76.8 kDa molecular weight MTHFR polypeptides, have been identified in cultured human fibroblasts (Homberger, Linnebank, Winter, Willenbring, Marquardt *et al.*, 2000). However, the major MTHFR isoform that has been identified in most human tissues so far appears to have a molecular weight of 77 kDa. In addition a smaller isoform with a molecular weight of 70 kDa has also been found in the human fetal liver (Frosst *et al.*, 1995). Interestingly the human and mouse *MTHFR* genes are in head to head orientation in close proximity to the *CLCN6* (a putative chloride channel) gene with only 3.1-kb and 2.5-kb respectively in each species between the translation initiation codons of each gene (Gaughan, Barbaux, Kluijtmans and Whitehead, 2000).

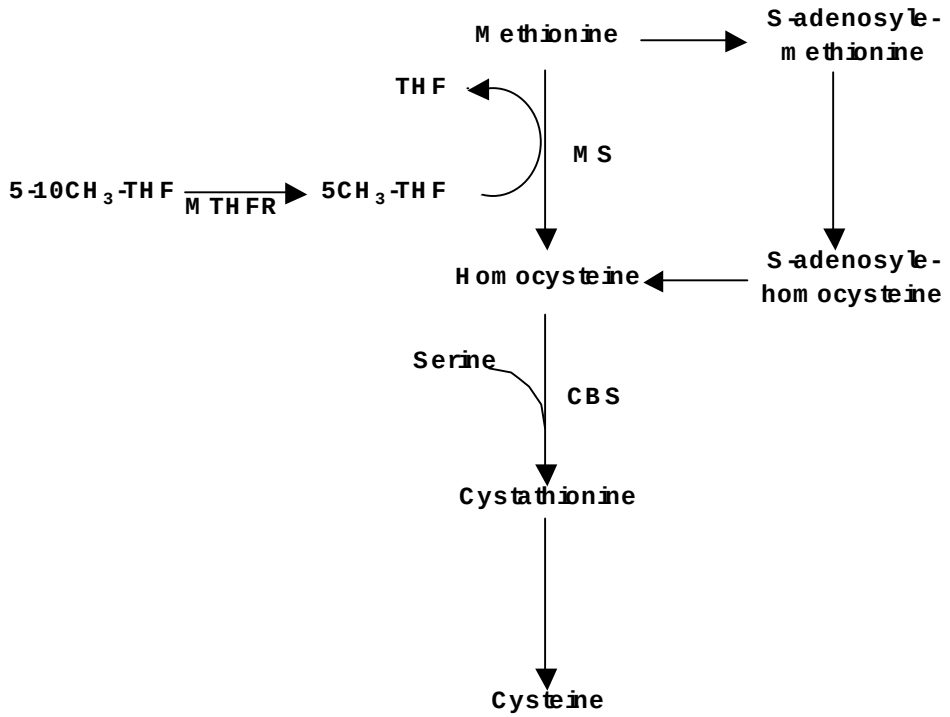


Figure 1.8. Pathways of folate metabolism relating to MTHFR. MS – Methionine Synthase, CBS – Cystathionine β Synthase, THF – Tetrahydrofolate.

1.5.4.3. Polymorphisms/haplotypes

There are many variations described in the *MTHFR* gene. Most of them are rare mutations found in patients with homocysteinuria (Goyette, Frosst, Rosenblatt and Rozen, 1995, Goyette, Christensen, Rosenblatt and Rozen, 1996, Sibani *et al.*, 2000). It also has several common polymorphisms. The 677C>T and 1298A>C polymorphisms are the most well characterised of the common *MTHFR* polymorphisms (Frosst *et al.*, 1995, van der Put *et al.*, 1998, Weisberg *et al.*, 1998). In addition to these, other polymorphisms have also been described, but their functional relevance has not yet been investigated. They are the 1317C>T polymorphism that does not result in a change in the amino acid sequence that has been reported in a frequency of 5% in a small group of African-American women (Weisberg *et al.*, 1998), and the 1793G>A polymorphism that results in an arginine to glutamine substitution at codon 594 that has been reported in frequencies of less than 10% in different racial groups from among a clinic population in Texas and New York (Rady, Szucs, Grady, Hudnall, Kellner *et al.*, 2002).

The 677C>T polymorphism is characterized by a point mutation at nucleotide 677 in exon 4 of the gene. It results in the substitution of the amino acid alanine by valine in the folate-binding site of the N-terminal catalytic domain of the enzyme in an evolutionary conserved region (Frosst *et al.*, 1995). The MTHFR variant found in 677TT homozygous individuals that has reduced enzyme activity had been characterised biochemically as a ‘thermolabile’

variant of MTHFR before its genetic characterisation (Kang, Zhou, Wong, Kowalisyn and Strokosch, 1988). The enzyme activity of MTHFR in 677TT homozygotes is 50-60% lower at 37^{o}C and approximately 65% lower at 46^{o}C than in 677CC homozygotes under similar conditions. 677CT heterozygotes are in the intermediate range. Reduced activity of the mutated allele has been demonstrated in both lymphocytes obtained from peripheral blood (Frosst *et al.*, 1995) and placental tissue (Daly, Molloy, Mills, Lee, Conley *et al.*, 1999) These variations in MTHFR activity appear to correlate with biochemical abnormalities. 677TT homozygotes have higher total levels of homocysteine in their plasma than 677CC homozygotes. The homocysteine levels, however, depend in part on folate levels, in that homocysteine is increased among those who have an inadequate intake of folate (indicated by low serum folate levels) while it is normal in others who have an adequate intake of folate (Jacques, Bostom, Williams, Ellison, Eckfeldt *et al.*, 1996, Ma, Stampfer, Hennekens, Frosst, Selhub *et al.*, 1996). Thus it should be possible to overcome hyperhomocysteinaemia caused by the 677T allele by folate supplementation (Litynski, Loehrer, Linder, Todesco and Fowler, 2002).

There are many studies examining the prevalence of the 677T allele in many parts of the world. They show a wide variation of the frequency of the allele between geographical areas and between different racial groups within geographical areas. These variations may reflect the ancestral origin of these populations. In these studies the prevalence was highest among Hispanic

groups in Italy, Spain and Mexico; and lowest among Africans. White Western Europeans and Chinese had frequencies intermediate between these two groups. The prevalence among Asian populations varied widely (Pepe, Camacho Vanegas, Giusti, Brunelli, Marcucci *et al.*, 1998, Schneider, Rees, Liu and Clegg, 1998, Wilcken, Bamforth, Li, Zhu, Ritvanen *et al.*, 2003). The prevalence of the 677T allele among the white Caucasians in Britain is reported to range from 33 to 38% (Botto and Yang, 2000) and among the Sri Lankans it is reported to range from 4.5% (Schneider *et al.*, 1998) to 10% (Alagratnam, Wierzbicki, Swaminathan, Turner and Wickramasinghe, 2000).

There are two hypotheses to explain the high frequency of the 677T allele in some populations, which attribute it to a selection advantage in the past (Blom, 1998). First, in times of famine, reduced MTHFR activity would have lead to decreased remethylation of homocysteine and thus preserved the available one-carbon moieties of the tetrahydrofolate metabolism for the vital synthesis of purines and thymidine. Secondly, those who carry the mutated allele have a lower risk of colon cancer. As a consequence the frequency of the 677T allele would have increased gradually. The unexpectedly high prevalence of the 677T allele in Mexico, northern China, and southern Italy has suggested the possibility of heterozygote advantage with respect to the risk of neural tube defects (NTD) because of the reported excess of 677CT heterozygotes among first degree relatives of patients with NTD (Weitkamp, Tackels, Hunter, Holmes and Schwartz, 1998). These hypotheses, however, are unconfirmed.

The 677C>T polymorphism is in partial linkage disequilibrium with the 1298A>C polymorphism that is characterized by a point mutation at nucleotide 1298 in exon 7 of the gene. It results in an amino acid substitution (glutamine to alanine) within the c-terminal regulatory domain of the enzyme, and is believed to affect the regulation of the enzyme by S-adenosylmethionine, an allosteric inhibitor of MTHFR that is known to bind in the c-terminal region (Weisberg *et al.*, 1998).

In all studies thus far, almost all subjects with the 677TT genotype and most subjects with the 677CC genotype have had the 1298AA genotype and the haplotype with both substitutions, 677T/1298C, have been extremely rare (<1% or absent). These findings suggest that each substitution would have evolved on a separate wild-type allele. (Adjalla, Amouzou, Sanni, Abdelmouttaleb, Chabi *et al.*, 2003, Ogino and Wilson, 2003). The activity of the enzyme encoded by the 677CC/1298CC haplotype is approximately 60% of that of the enzyme encoded by the 677CC/1298AA haplotype. This is not as low as the activity of the enzyme encoded by the 677TT genotype. In addition individuals with the 677CC/1298CC haplotype do not appear to have higher serum homocysteine levels than controls. Subjects who harbour the 667TT/1298AA haplotype, however, have reduced enzyme activity and elevated homocysteine levels similar to that seen in individuals with the 677TT genotype (van der Put *et al.*, 1998, Weisberg *et al.*, 1998, Adjalla *et al.*, 2003).

It would appear therefore, given the pathophysiological basis for the selection of the *MTHFR* gene as a candidate gene for pre-eclampsia, that it would be sufficient to carry out association analysis using the 677C>T polymorphism, as it is the polymorphism that has the predominant functional effect. The 677C>T polymorphism was therefore selected for this study.

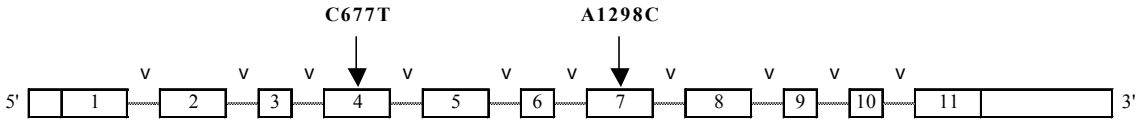


Figure 1.9. Genomic structure of the *MTHFR* gene. The exons are numbered. Arrowheads indicate the position of the introns. The positions of the two common polymorphisms are indicated.

Population	Cases	Controls	Primi/ Multi	Exclusion criteria	T allele frequency		
					Cases	Controls	*P*
Japanese[1]	67 PE	98 healthy pregnant delivering >22 weeks & 260 healthy adults	?	?	0.48	0.37	<0.02
Italian[2]	45PE/51GH	129 healthy parous women	Both	CHT, RD, DM, PD	0.43	0.52	0.057
Black South African[3]	150 PE	110 healthy pregnant, >37 Weeks	Both	ED, MD.	0.10	0.06	NS
Jewish[4]	34 PE	110 healthy pregnant	Both	?	0.22*	0.08*	0.005
White northern European[5]	283 PE	100 healthy pregnant women at delivery & 100 healthy non pregnant women with no history of PE	Both	CHT, RD, DM, MP	0.31	0.31	NS
White[6]	99 PE	114 healthy pregnant women	Primi	CHT, RD, MD	0.34	0.40	NS
Black Zimbabwian[7]	171 PE	185 healthy pregnant at delivery	Both	CHT	0.09	0.09	NS
Japanese[8]	73 PE	215 healthy pregnant at 1 month postpartum	Both	HELLP, CHT, RD, DM, MP, FA	0.38	0.38	NS
Jewish[9]	63 PE	126 pregnant women with at least 1 normal pregnancy immediately postpartum	Both	CHT, MP, TE	0.24*	0.10*	0.008
Hungarian[10]	120 PE	101 healthy pregnant women	Both	CHT, DM, RD, MP	0.34	0.32	NS
Japanese[11]	133 PE	224 healthy pregnant women	?	CHT, RD	0.48	0.37	0.007
White[12]	281 PE	360 women who have had 2 normal pregnancies	Both	?	0.33	0.33	NS
White & African American[13]	100 PE	97 health pregnant women immediately postpartum	Both	CHT, DM, RD, MP, TE, FA	0.22	0.25	NS
Italian[14]	54 PE	74 health pregnant women	?	CHT, DM, RD	0.10*	0.12*	NS
Mexican[15]	13 PE/E	15 healthy pregnant women	?	CHT, DM, RD	0.58	0.33	0.059
German[16]	15 PE	34 healthy pregnant at delivery	Both	HELLP, CHT, DM, RD, VD, HD	0.30	0.43	NS
Croat[16]	25 PE	28 healthy pregnant at delivery	Both		0.32	0.33	NS
Indonesian[16]	41 PE	27 healthy pregnant at delivery	Both		0.10	0.09	NS
French Canadian[17]	203 PE	357 healthy pregnant women	Primi	MP, CHT	0.16*	0.13*	NS

Table 1.4. Continued in next page.

Population	Cases	Controls	Primi/ Multi	Exclusion criteria	T allele frequency		
					Cases	Controls	*P*
Chinese[18]	57 PE	120 normal women	?	?	0.58	0.39	0.001
Finnish[19]	113 PE	103 healthy pregnant women postpartum	Both	CHT, RD, AID	0.23	0.26	NS
Dutch[20]	176 PE	403 population based controls	Both	CHT, RD, HD, MP	0.33	0.29	NS
Dutch[21]	174 PE	120 healthy blood donors	?	?	0.30	0.34	NS
Australian (White Anglo-Saxon)[22] include cases in[23]	156 PE	79 healthy pregnant women	Primi	CHT, RD, MD	0.33	0.34	NS
Scottish[24]	164 PE	103 non pregnant women who have had a uncomplicated first singleton pregnancy	Primi	MP	0.28	0.30	NS
	303 GH				0.37	0.30	NS
Black South African[25]	204 PE	338 normotensive pregnant women	Both	?	0.07	0.06	NS

Table 1.4. A summary of case-control *MTHFR* 677C>T pre-eclampsia association studies with most studies failing to show an association. *The frequency of the *MTHFR* 677TT genotype.

Abbreviations:

?-Information not available
CHT-Chronic Hypertension
DM-Diabetes Mellitus
ED-Endocrine Disease
FA-Fetal Anomaly
GH-Gestational Hypertension
HELLP-HELLP Syndrome
HD-Hepatic Disease
MD-Metabolic Disease
MP-Multiple Pregnancies
PD-Pulmonary Disease
PE-Pre-eclampsia
RD-Renal Disease
TE-Thromboembolism
VD-Vascular Disease
Primi-Primiparous
Multi-Multiparous

References:

1. (Sohda, Arinami, Hamada, Yamada, Hamaguchi *et al.*, 1997)
2. (Grandone, Margaglione, Colaizzo, Cappucci, Paladini *et al.*, 1997)
3. (Chikosi, Moodley, Pegoraro, Lanning and Rom, 1999)
4. (Kupferminc *et al.*, 1999)
5. (O'Shaughnessy, Fu, Ferraro, Lewis, Downing *et al.*, 1999)
6. (Powers, Minich, Lykins, Ness, Crombleholme *et al.*, 1999)
7. (Rajkovic, Mahomed, Rozen, Malinow, King *et al.*, 2000)
8. (Kobashi, Yamada, Asano, Nagano, Hata *et al.*, 2000)
9. (Kupferminc *et al.*, 2000)
10. (Rigo, Nagy, Fintor, Tanyi, Beke *et al.*, 2000)
11. (Watanabe, Hamada, Yamakawa-Kobayashi, Yoshikawa and Arinami, 2001)
12. (Kim, Williamson, Murray, Andrews, Pietscher *et al.*, 2001)
13. (Livingston, Barton, Park, Haddad, Phillips *et al.*, 2001)
14. (D'Elia, Driul, Giacomello, Colaone, Fabbro *et al.*, 2002)
15. (Perales Davila, Martinez de Villarreal, Triana Saldana, Saldivar Rodriguez, Barrera Saldana *et al.*, 2001)
16. (Prasmusinto, Skrablin, Hofstaetter, Fimmers and van der Ven, 2002)
17. (Levesque *et al.*, 2003)
18. (Li, Zheng, Xue, Sun, Chen *et al.*, 2000)
19. (Laivuori, Kaaja, Ylikorkala, Hiltunen and Kontula, 2000)
20. (Zusterzeel, Visser, Blom, Peters, Heil *et al.*, 2000)
21. (Lachmeijer, Arngrimsson, Bastiaans, Pals, ten Kate *et al.*, 2001)
22. (Kaiser, Brennecke and Moses, 2001)
23. (Kaiser *et al.*, 2000)
24. (Morrison, Miedzybrodzka, Campbell, Haites, Wilson *et al.*, 2002)
25. (Pegoraro, Chikosi, Rom, Roberts and Moodley, 2004)

1.6. Objectives

As elaborated above *EGF* 61G>A, *EGF* 67149G>A, *TGFA* 3822G>A, *TGFA* 3827T>C, *TGFA* 3851T>C, *AGT* 174Thr>Met, *AGT* 235Met>Thr, *AGT* 11535C>A and *MTHFR* 677C>T polymorphisms and the haplotypes defined by them were selected as markers for pre-eclampsia to test the hypothesis that *EGF, TGFA, AGT* and *MTHFR* are candidate genes for pre-eclampsia in a racially distinct South Asian Sinhalese population in Sri Lanka and in a white Caucasian population in Nottingham, UK using the case-control candidate gene disease association model. The objective of the cases-control candidate gene pre-eclampsia association study was:

1. to compare allele/haplotype frequencies stated above in normotensive pregnant women and in women with pre-eclampsia to determine whether they are associated with the susceptibility to pre-eclampsia among Sinhalese women in the Sri Lankan population; and in white Caucasian women in Nottingham, UK.

Since results of such studies would have to be interpreted in relation to the population frequencies of these alleles/haplotypes, it was necessary to supplement these investigations by examining the allele/haplotype frequencies in the Sri Lankan and UK populations. The objective of the population genetic study was:

2. to compare allele/haplotype frequencies stated above in the Sinhalese, the Sri Lankan Tamil and the Moor racial groups in the Sri Lankan population

and the white Caucasians in Nottingham, UK.

As evidenced by the pathophysiological roles played by them, described in previous sections of this chapter, the above mentioned candidate genes may also play a role in determining the phenotypic expression of quantitative traits. EGF; because of its similarity with EGF, TGFA; and AGT may all play a role in determining the birth weight of babies. In addition AGT may determine the blood pressure of pregnant women. It was decided therefore, to investigate them by examining candidate gene quantitative trait associations in normotensive pregnant women and their babies using the above-mentioned polymorphic markers. The objectives of the candidate gene quantitative trait association study were:

3. to determine whether haplotypes of the *EGF, TGFA* and *AGT* genes in normotensive pregnant women are associated with the birth weight of their babies among Sinhalese women in the Sri Lankan population; and in white Caucasian women in Nottingham, UK.
4. to determine whether haplotypes of the *AGT* gene in normotensive pregnant women are associated with their antenatal booking blood pressure among Sinhalese women in the Sri Lankan population; and in white Caucasian women in Nottingham, UK.

As mentioned above in 1.5.3.3, the *AGT* gene has been a long-standing interest of our pre-eclampsia research group and it was hoped that it would be possible

to further understand the regulation of the *AGT* gene by characterising the functional effects of the three common haplotypes in the 3'UTR/flanking region of the gene defined by the *AGT* 3'UTR/flanking region SNPs on gene expression using reporter gene constructs under basal and AngII stimulated conditions. The objectives of the reporter gene expression study was:

5. to study the effects of the three common haplotypes of the 3'UTR/flanking region of the *AGT* gene on reporter gene expression in HepG2 cells under basal and AngII stimulated conditions.

2. METHODS

All the methods used in these investigations are described in this chapter. Since there were no pre-existing DNA resources for Sri Lankans, subjects had to be recruited in Sri Lanka afresh. Obtaining ethical clearance to conduct these investigations was therefore, the first consideration and it is described at the outset. The validation of the heat coagulation test (HCT), which was central to establishing the diagnosis of pre-eclampsia in Sri Lanka, is described in the second part of the chapter. It is followed by a description of the recruitment of subjects for population genetic and case-control candidate gene disease association studies and their phenotyping. The generic molecular genetic methods are in the fourth part of the chapter. It is followed by a detailed description of specific genotyping and functional assays that were used for these investigations. The constitution of the buffers and culture media prepared in house is in appendix 2. The constitution of the buffers that were purchased from outside suppliers is given when it is first mentioned in the text if the manufacturer had disclosed it. A list of suppliers is in appendix 3. The chapter concludes with a description of the statistical methods, databases and software tools used for these investigations and a special note on polymorphism nomenclature.

2.1. Ethical considerations

The Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka, approved these studies. All Sri Lankan volunteers were recruited after obtaining written informed consent using consent forms in Sinhala and Tamil languages. Some Moors are literate in Sinhala and Tamil while others are literate in Tamil only. In addition the Sri Lankan studies were also approved by the Department of Health Services, Sri Lanka.

The recruitment of subjects for the white Caucasian DNA resources at the University of Nottingham was done with the approval of the Hospital Ethics Committee of the Queen's Medical Centre, Nottingham, UK. All these subjects had been recruited after obtaining written informed consent.

2.2. Validation of the urine protein heat coagulation test

It was necessary to establish that women classified as having pre-eclampsia by the clinicians had actually satisfied the research definition for pre-eclampsia before recruiting them for the case-control candidate gene disease association study. How this was done is described in section 2.4.1. An important consideration in the research definition of pre-eclampsia was the use of a stringent cut off for the detection of significant proteinuria as has been done in the GOPEC study to ensure recruitment of women with strictly defined pre-eclampsia. In the GOPEC study the following were used as being indicative of significant proteinuria: proteinuria of ≥500 mg/day on a 24 hour collection, ≥300mg/L on a 24 hour collection, ≥50 mg protein/mmol creatinine in a spot urine collection, ≥2+ on dipstick testing.

In the two recruiting hospitals in Sri Lanka none of these tests were available routinely. What was available was the HCT that was performed in the wards by midwives and nurses (Guidotti *et al.*, 1992). The sulphosalycilic acid test (SAT) for urinary protein (Gyure, 1977) and in exceptional circumstances 24-hour urine protein estimations were also available on request from the laboratory when false negative HCT results were suspected on clinical grounds, but it was the HCT that was usually relied upon by the clinicians to detect significant proteinuria. ≥2+ on the SAT had been recommended as the cut-off for significant proteinuria in a previous version of the ISSHP recommendations (Davey *et al.*, 1988), but it does not appear in the current

one (Brown *et al.*, 2001). The threshold for significant proteinuria using the HCT, however, seems not to have been determined before.

In a Sri Lankan study, ≥1+ on the HCT had been found to correspond on average to >300 mg of protein on a 24 hour collection (Kudalugoda Arachchi, Malalasekara, Premaratne, Lanerolle and Sheriff, 1998). This article, however, did not report the sensitivity, the specificity, the positive predictive value (PPV), or the negative predictive value (NPV) of the test. Although the authors were contacted with the view to obtaining the raw data to carry out this analysis, they were unable to trace the original data. Another drawback in that study was that a research assistant had performed the HCT once the sample reached the laboratory. In actual practice, however, it is a midwife or nurse who performs it in a busy ward setting.

It was decided to adopt the cut-off of ≥1+ on the HCT as the definition for significant proteinuria for the purpose of recruiting women with pre-eclampsia in Sri Lanka, but at the same time the need to validate the use of this cut-off to detect significant proteinuria was recognised. To do so, a study was carried out with the aim of determining whether ≥1+ on a standardised HCT reliably detects significant proteinuria defined as ≥500 mg/day on a 24-hour urine collection and to compare it with the Roche Combur^{10} Test® dipstick test for protein (Roche Diagnostics Ltd.).

2.2.1. Recruitment of subjects and the study protocol

The recruitment was conducted in the De Soysa Hospital for Women (DSHW) and the Castle Street Hospital for Women (CSHW), the two main teaching hospitals of the University of Colombo, Sri Lanka, between August 2002 and April 2003. The aim of recruitment was to recruit as many women as possible during this period. 107 hospitalised pregnant women of >20 weeks of gestation whose proteinuria status was known based on a HCT result within the preceding 24 hours as a part of routine inpatient clinical care were recruited after obtaining written informed consent. At recruitment they passed a sample of urine and immediately thereafter began a 24-hour urine collection under supervision. A midwife or nurse performed a HCT and a dipstick test on separate aliquots of the initial sample. Midwives and nurses were trained on performing the HCT according to a standard protocol (see appendix 4) and the dipstick test according to manufacturer's instructions. This dipstick test is based on the principle of the protein error of pH indicators. The 24-hour urine protein estimations were made in the laboratory using the pyrogallol red method (Randox Laboratories Ltd, UK) by a technician who was blinded to the results of the tests done in the ward. A third aliquot of the initial sample was sent to the laboratory for a SAT. The SAT is used routinely in Sri Lanka as a backup test if the HCT is negative when there is strong clinical suspicion of significant proteinuria. Briefly the SAT involved acidifying urine by adding 10% acetic acid, centrifugation of the sample for 5 minutes at 3000 rpm, placing 2ml of urine in a test tube, adding 2ml of 5-sulphosalicylic acid,

and making a qualitative assessment of turbidity to report the result. The coefficient of variance for 24-hour proteinuria estimations in this lab between different runs was 3%.

2.2.2. Results

Five women were excluded because their 24-hour urine collections were not completed. The dipstick test results of 31 women were not available because the midwife or nurse performing the HCT had either not performed the dipstick test or having performed it, had not recorded the result. SAT results were available for 88 women.

The results of the women for whom results of both the HCT and dipstick test were available are summarised in Table 2.1. ≥1+ on the HCT was comparable with ≥2+ on the dipstick test in detecting proteinuria of ≥300mg/day or ≥500mg/day. ≥1+ on the dipstick test had the highest sensitivity for detecting proteinuria of ≥300mg/day, but lacked specificity at ≥500mg/day.

2.2.3. Discussion

≥1+ on the HCT is as good as ≥2+ on the dipstick test in detecting proteinuria of ≥500mg/day. The HCT, however, is less sensitive than ≥1+ on the dipstick test in detecting lesser degrees of proteinuria. In the West, where dipstick testing is routine in clinical practice, its sensitivity is perceived to be much higher than evidence from this and other studies would suggest (Kuo,

Koumantakis and Gallery, 1992, Brown and Buddle, 1995, Bell, Halligan, Martin, Ashmore, Shennan *et al.*, 1999). Induced diuresis is often cited as a reason for false negative dipstick results. This may be true of the HCT as well. The practice in Sri Lanka, to overcome the problem of false negative HCT results, is to test another aliquot of the urine sample using a more sensitive laboratory test such as the SAT when significant proteinuria is clinically suspected. In this study, of the false negatives on the HCT, 70% with proteinuria of ≥500mg/day and 50% with ≥300mg/day were picked up by the SAT. The sensitivity of the combined HCT and SAT based on the results of the 88 women for whom results of both tests were available was 0.90 for proteinuria of ≥500mg/day and 0.73 for proteinuria of ≥300mg/day. It is clear therefore that having a backup test for HCT such as the SAT would be useful in clinical settings where access to 24-hour urine protein estimations is not readily available. The prevalence of significant proteinuria in this hospitalised series of women was high. It was 0.59 for significant proteinuria of ≥300mg/day and 0.37 for significant proteinuria of ≥500mg/day. The positive and negative predictive values may be distorted because of this high prevalence. These values were tested therefore across a prevalence range from 0.05 to 0.60. These results showed that the results of the HCT and the dipstick test are comparable across this range (Table 2.2).

In conclusion therefore, ≥1+ on the HCT is a suitable cut off point for detecting proteinuria of ≥500mg/day.

24hr Urine Protein Estimation	Heat Coagulation Test			Dipstick Test						Sulphosalycilic Acid Test		
	Negative	≥1+	Total	Negative	≥1+	Total	Negative/1+	≥2+	Total	Negative	≥1+	Total
<300 mg/day	25	1	26	24	2	26	25	1	26	24	2	26
≥300mg/day	15	30	45	9	36	45	23	22	45	7	29	36
Total	40	31	71	33	38	71	48	23	71	31	31	62*
Sensitivity			0.67			0.80			0.49			0.81
Specificity			0.96			0.92			0.96			0.92
PPV			0.97			0.95			0.96			0.94
NPV			0.63			0.73			0.52			0.77
<500 mg/day	59	6	65	28	12	40	37	3	40	30	8	38
≥500mg/day	11	26	37	5	26	31	11	20	31	1	23	24
Total	70	32	102	33	38	71	48	23	71	31	31	62*
Sensitivity			0.70			0.84			0.65			0.96
Specificity			0.91			0.70			0.93			0.79
PPV			0.81			0.68			0.87			0.74
NPV			0.84			0.85			0.77			0.97

Table 2.1. Comparison of the results of the HCT, dipstick test and SAT. Results are from data of women who had both the HCT and dipstick test result. *Nine women did not have the SAT result. All figures, except the sensitivity, specificity, PPV, and NPV, refer to number of women. PPV: Positive Predictive Value. NPV: Negative Predictive Value.

ence	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50
	0.52	0.70	0.79	0.84	0.87	0.90	0.92	0.93	0.94	0.95
	0.97	0.95	0.92	0.89	0.86	0.82	0.79	0.75	0.71	0.66
	0.35	0.54	0.65	0.72	0.78	0.82	0.85	0.87	0.89	0.91
	0.99	0.98	0.96	0.95	0.93	0.92	0.90	0.87	0.85	0.82
	0.40	0.59	0.69	0.76	0.81	0.84	0.87	0.89	0.91	0.93
	0.97	0.94	0.91	0.88	0.85	0.81	0.78	0.74	0.70	0.65

gative (NPV) predictive values of the heat coagulation test and the dipstick test across a proteinuria prevalence range from 0.05 to 0.60. Based on PPV and NPV of all 102 wom

2.3. Recruitment of population volunteers

2.3.1. Sri Lankan subjects

The genetic epidemiological component of this study was designed to identify gross differences in allele frequencies between racial groups. 80 Sinhalese, 80 Sri Lankan Tamil, and 81 Moor subjects were recruited for this study in Colombo, Sri Lanka (50% male). They were recruited from among women attending antenatal clinics at the DSHW, staff and students of the University of Colombo, and accompanying persons of patients attending General Practice clinics. With a sample of 80 subjects it was possible to detect a minimum difference of –0.07 or +0.11 in the frequency of an allele, between two populations, when that allele occurs in a frequency of ≥0.10 in one of them, at a significance level of P=0.05 with 80% power (Table 2.3). All subjects were assigned to the correct racial group by inquiring from them about the race and country of origin (to identify and exclude Indian Tamils) of their parents and all four grandparents. Those of mixed race were excluded. The medical history of all volunteers was recorded. 1.5 ml of venous blood was obtained from each volunteer.

2.3.2. British subjects

The white Caucasian population in Nottingham, UK was studied using an already existing DNA resource at the University of Nottingham (Morgan *et al.*, 1996, Plummer *et al.*, 2002). Samples in this resource had been collected from volunteers attending a blood donor clinic in Nottingham. 80 subjects (50% male) were selected randomly from this resource.

Allele frequency in population 1	Minimum difference in allele frequency detectable in population 2
0.10	– 0.07 , + 0.11
0.25	– 0.12 , + 0.14
0.50	– 0.15 , + 0.15

Table 2.3. Population frequencies of polymorphic variants detectable at significance level *P*=0.05, with Power = 80% with a sample size of 80. Power calculation was done with Sample Size Software, SPSS Inc., USA.

2.4. Recruitment of women with pre-eclampsia and normotensive pregnant women

2.4.1. Sri Lankan subjects

Recruitment of women with pre-eclampsia and normotensive pregnant women in the Sri Lankan population was done from the DSHW and the CSHW from August 2001 to January 2003. The nine consultant obstetrics units in these two hospitals account for approximately 25 000 deliveries per year.

Pre-eclampsia, for the purpose of recruitment, was defined as hypertension of ≥140/90 mmHg on two occasions six hours apart occurring after 20 weeks of gestation in a previously normotensive woman, together with proteinuria of ≥1+ on the HCT (see section 2.2) not associated with urinary tract infection or ruptured membranes.

Potential recruits were identified by doctors, midwives, and nurses in the referring units and referred for recruitment. A total of 324 gravida 1 para 0 (G1P0) and 32 gravida 2 para 0 (G2P0) women were referred for recruitment. All multigravid women had conceived again with the same partner. On the basis of the referral 228 (64%) women were deemed to fulfil recruitment criteria. Four of them had left the hospital prior to being approached for recruitment. The rest were approached for recruitment; of them eight declined. The balance, 216 women, were recruited after obtaining written informed consent; of them six were subsequently withdrawn from the study either because they were deemed

not to have actually fulfilled the recruitment criteria or because they were subsequently found to have one or more exclusion criteria information about which had come to light after recruitment. Therefore only 210 (59%) of the 356 women were recruited. A much higher exclusion rate is reported in the GOPEC study where similar rigorous inclusion criteria were applied (O'Malley, Personal Communication). It is interesting to note that in both studies the main reason for non-participation was the failure to fulfil the criteria for significant proteinuria (Table 2.4).

Almost all women with pre-eclampsia were recruited before delivery. Two Sinhalese women who developed postpartum eclampsia following delivery at term were found to have blood pressure and proteinuria that fulfilled the diagnostic criteria and were recruited. Women were not approached for recruitment or were withdrawn from the study following recruitment for the following reasons: mixed race or current pregnancy fathered by a man of a race other than that of the woman; renal disease, chronic hypertension, or persistent proteinuria (defined as ≥1+ on the HCT in the first three urine samples tested in pregnancy with or without urinary tract infection); ischaemic heart disease, cerebrovascular accidents, or insulin or non-insulin dependent diabetes mellitus; BMI ≥30kg/m^2 based on height and weight measured at the antenatal booking visit or, in case of unbooked pregnancies, postpartum; hydatidiform mole, multiple fetuses, or gestational diabetes in the current pregnancy; and pregnancy conceived as a result of in-vitro fertilisation where such information was

volunteered by the woman. Race was established as in section 2.3.1. When recording the family history, information regarding consanguinity was also recorded. However, consanguinity was reported only in one family.

A summary of recruitment is given in table 2.4. It also includes an analysis of the reasons for non-participation and/or withdrawal. An analysis of the race of the recruited women is given in table 2.5. 10ml of venous blood was obtained at the time of recruitment from all recruits.

Recruitment of normotensive pregnant women was conducted from May 2002 to January 2003. The normotensive pregnant women were matched for race, parity, age, and BMI with the women with pre-eclampsia. In June 2002, a preliminary analysis of the racial breakdown of women with pre-eclampsia recruited up to then indicated that by the end of the recruitment period it would not be possible to recruit sufficient numbers of Tamil and Moor women with pre-eclampsia to carry out any meaningful genetic analysis. It was decided therefore, to confine the genetic studies to Sinhalese women. As such recruitment of normotensive controls was confined to primigravid Sinhalese women. In addition to ensuring that they were normotensive and non-proteinuric throughout the pregnancy, all other exclusion criteria applied to women with pre-eclampsia were also applied to normotensive pregnant women. Moreover, normotensive pregnant women delivering before the 37^{th} week of gestation and women delivering babies weighing <2.0 kg were not

recruited. This was an arbitrary cut-off; however, the mean weight of Sinhalese babies delivering at term in Sri Lanka is reported to be 2824g (standard deviation (SD) 413g; Male 2911g (SD 427g), Female 2820g (SD 440g)) (Nanayakkara, 1998).These women were excluded because it is possible that pre-eclampsia and low birth weight may have common aetiological factors as evidenced by the changes seen in the placenta in both conditions (Khong *et al.*, 1986). Normotensive pregnant women were identified by midwives and nurses on the first postpartum day while in postnatal wards and referred for recruitment. A total of 180 Sinhalese normotensive pregnant women were recruited as controls. During the recruitment period weekly comparisons were made between case and control groups to determine the inter group variability in age and BMI, and based on that, appropriate controls were recruited to match the two groups as closely as possible.

The phenotyping booklet for women with pre-eclampsia of the GOPEC study was modified and adopted to collect the phenotyping data of the Sri Lankan women. Data were stored in a SPSS database. Data relating to severity of disease, delivery, and the baby of one Sinhalese woman with pre-eclampsia who was transferred to a district hospital for delivery were not available. It was possible, however, to confirm that the mother and baby were safe and well after delivery, and that she had made a complete recovery postpartum.

	No
Total Referred	356
Could not be approached for recruitment	4
Consent not given	8
Excluded or withdrawn	
Failed proteinuria criteria	64
Chronic hypertension	18
Twin pregnancy	12
Diabetes mellitus/Gestational diabetes	11
Failed blood pressure criteria	10
Body Mass Index ≥30 kg/m^2	8
Mixed race marriage	7
G1 abortion at a period of gestation > 12 weeks	3
Blood sampling could not be done	1
Total Recruited	210

Table 2.4. Summary of recruitment of Sri Lankan women with pre-eclampsia.

Race	Total		Population %[1]
	No	%	
Sinhalese	180	86%	76%
Sri Lankan Tamil	17	8%	11%
Indian Tamil	1	0%	1%
Moor	12	6%	9%
Total	210	100%	

Table 2.5. The racial breakdown of the Sri Lankan women with pre-eclampsia. [1]As a % of the total population of each race in the district of Colombo (Department of Census and Statistics, 2001).

2.4.2. British subjects

The white Caucasian women with pre-eclampsia and normotensive pregnant women in Nottingham, UK were studied using an already existing DNA resource in the University of Nottingham. Samples in this resource were collected from volunteers delivering in obstetric units at the Queen's Medical Centre and the City Hospital in Nottingham between 1988 and 1999. All subjects had given written informed consent to participate. This collection had been made by Morgan and colleagues for preliminary investigations into genetics of pre-eclampsia (Morgan *et al.*, 1995, Morgan, Crawshaw, Baker, Edwards, Broughton Pipkin *et al.*, 1997, Morgan, Crawshaw, Baker, Brookfield, Broughton Pipkin *et al.*, 1998, Morgan *et al.*, 1999a). It contained both primiparous and multiparous samples. 74 primiparous pre-eclamptic and 81 primiparous normotensive samples from this collection were selected for the study. Some samples in this collection were not selected because there was insufficient DNA left for analysis. A SPSS phenotyping database of these women had been maintained. It was however, not as comprehensive or complete as the phenotyping database of the Sri Lankan women. At the time this collection was made, since the emphasis was on genetic studies, the samples were coded as pre-eclamptic or normotensive and anonymised. It was not possible therefore, to identify the subjects for whom data was missing to retrieve their case notes to complete the missing data.

2.5. Generic molecular genetic methods

2.5.1. DNA extraction

Venous blood obtained from Sri Lankan population volunteers, women with pre-eclampsia and normotensive pregnant women were collected into EDTA containing tubes and stored at –20^{o}C prior to DNA extraction. DNA extraction was done using QIAamp® DNA mini kits (Qiagen Ltd., UK) according to the manufacturers protocol. This protocol involves lysis of leukocytes, adsorption of DNA on to a silica membrane, washing of membrane to ensure the removal of proteins and other possible contaminants, and the elution of DNA from the column.

The QIAamp® DNA Blood Mini Kit DNA extraction procedure was carried out as follows: 20µl of protease (or proteinase K), 200µl of venous blood, and 200µl of lysis buffer AL were added to a 1.5 ml microcentrifuge tube in that order and mixed by pulse vortexing for 15 seconds. Then the reaction was incubated at 56^{o}C for 10 minutes. Following incubation the tube was centrifuged briefly to remove drops from the inside of the lid. Then 200µl of ethanol was added to the reaction, mixed by pulse vortexing for 15 seconds, and the tube briefly centrifuged again to remove drops from the inside of the lid. This reaction mix was then applied to a QIAamp spin column that was placed on a 2ml collection tube and the column was centrifuged at 8000 rpm for 1 minute. Next 500µl of wash buffer AW1 was applied to the spin column. This was followed by centrifugation at 8000 rpm for 1 minute. Lastly 500µl of

wash buffer AW2 was applied to the spin column. This was followed by centrifugation at 14 000 rpm for 1 minute. After each centrifugation step the flow-through was discarded. Finally the spin column was placed in a 1.5ml collection tube and 200µl of elution buffer AE was added to the spin column. After incubating the column for 5 minutes at room temperature, DNA was eluted from the column by centrifugation at 8000 rpm for 1 minute. The eluted DNA samples were labelled and stored at –20^{o}C.

2.5.2. Polymerase Chain Reaction

Amplification of DNA segments of interest was the starting point for almost all the experiments described in this thesis. This was done using the polymerase chain reaction (PCR). PCR amplification involves simultaneous primer extension on complementary strands of DNA with two oligonucleotide primers, specific to each strand that flank the genomic region to be amplified, using thermostable *Taq* DNA polymerase enzyme in the presence of deoxynucleotides and a reaction buffer containing Mg^{2+} (Saiki, Scharf, Faloona, Mullis, Horn *et al.*, 1985, Mullis and Faloona, 1987, Saiki, Gelfand, Stoffel, Scharf, Higuchi *et al.*, 1988). PCRs were performed on Techne Genius (Techne, UK) or DNA Engine: Tetrad – PTC225 (Genetic Research Instrumentation, UK) programmable thermal cyclers without the need for oil overlay. All PCR buffers and enzymes used in these investigations were from Roche Diagnostics GmbH, Germany and MBI Fermentas, Lithuania (Helena Biosciences, UK).

PCR primers were designed using Primer3 software (see section 2.8). PCR were conducted in a reaction mix containing 100-200ng of template genomic DNA, 200μM deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), PCR reaction buffer (10mM Tris-HCL, 50mM KCl, 1.5 mM $MgCl_2$, pH 8.3,20^{o}C), and 1U *Taq* DNA polymerase. PCR amplification involved repeated cycles of heat denaturation of the genomic DNA at 94^{o}C for 30 seconds, annealing of the primers to their complementary sequences at an annealing temperature depending on the melting temperature of the two primers, which was determined empirically, for 1 minute, and extension of the annealed primers at 72^{o}C for 1 minute. The temperature cycles were preceded by 2 minutes at 94^{o}C and concluded with 10 minutes at 72^{o}C followed by cooling at 4^{o}C. The presence of PCR amplified products was confirmed by electrophoresing a fraction of the reaction mix on an agarose gel (see section 2.5.3)

2.5.2.1. Multiplex PCR

Multiplex PCR is a variant of PCR in which two or more genomic regions are simultaneously amplified in the same reaction using multiple sets of oligonucleotide primers (Chamberlain, Gibbs, Ranier, Nguyen and Caskey, 1988, Henegariu, Heerema, Dlouhy, Vance and Vogt, 1997). It is described in section 2.6.5.

2.5.2.2. Mutagenically Separated PCR

Mutagenically separated PCR (MS-PCR) is a variant of PCR that is used for genotyping biallelic polymorphisms. It enables genotyping by examining PCR products on an agarose gel without the need for further downstream processing. It is described in section 2.5.5.2.

2.5.3. Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate and identify DNA fragments. During electrophoresis negatively charged DNA molecules migrate towards the anode with smaller fragments having greater mobility. The location of DNA within the gel can be determined directly by staining with low concentrations of the dye ethidium bromide that intercalates with DNA as it migrates and fluoresces when examined over ultra violet (UV) light on a UV transilluminator.

Agarose gels were prepared by mixing an appropriate amount of molecular biology grade agarose (Helena Biosciences, UK) in an appropriate volume of 1 × TAE buffer to obtain the correct percentage gel (weight/volume). 0.5µg/ml of ethidium bromide (Sigma-Aldrich, UK) was added to each gel. Gels were poured into gel casting trays fixed with combs with the desired number of wells and allowed to set at room temperature. After that the gels were submerged in 1 × TAE buffer. Next approximately 5µl of each sample of DNA to be resolved mixed with 5µl of gel loading buffer (15% (w/v) Ficol;

Sigma-Aldrich, UK) were placed in separate wells and a 50 volt current was applied. The pattern created by different size DNA fragments was visualised by examining the gel on a UV transilluminator. The size of the DNA fragments can be confirmed by electrophoresing in the presence of a size marker in one of the wells. 100bp DNA ladder and ΦX 174 size markers (Promega, UK) were used as the size marker in the investigations described in this thesis.

2.5.4. Automated sequencing

Automated sequencing was done to confirm the genotype of control samples used in genotyping assays, as well as to confirm the insert sequence of plasmid constructs used in the reporter gene expression investigations. PCR amplified products of genomic regions flanking polymorphic sites were sequenced to confirm the genotype while miniprep plasmid DNA were sequenced to confirm the insert sequence.

PCR mixes contain various impurities such as excess primers and deoxynucleotides in addition to the PCR products. Excess primers can be removed by treatment with Exonuclease I (ExoI). Excess deoxynucleotides can be removed by treatment with Shrimp Alkaline Phosphatase (SAP). This was done by incubating 5µl of a PCR reaction mix in a final volume of 10µl containing 5U ExoI and 1U SAP (Amersham Biosciences, UK) at 37^{o}C for 15 minutes. The reaction was stopped by inactivating the enzymes by further

incubation for 15 minutes at 80^{o}C. The reaction was then cooled on ice and spun down before being subjected to the cycle sequencing reaction.

Cycle sequencing was done using the ABI PRISM® BigDye® Primer v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, UK). This system works on the basis of the Sanger dideoxy chain termination principle (Sanger, Nicklen and Coulson, 1977). It contains four ddNTPs with different fluorescence labels and AmpliTaq® DNA polymerase. At the end of the reaction PCR products of varying sizes all terminating with a fluorescent-labelled dideoxy nucleotide are generated. The sequence is then read out automatically by capillary electrophoresis of cycle sequencing products through an automated sequencing machine.

Cycle sequencing reactions were set up in a final volume of 10µl, containing 3µl enzyme treated PCR products, 4µl big dye ready reaction mix, and approximately 5pmol of the sequencing primer in a final volume of 10µl. All reactions were set up and run in the dark (samples covered with aluminium foil) to prevent degeneration of light sensitive dyes. The reactions were subject to 25 cycles of denaturation at 96^{o}C for 30 seconds, primer annealing at 50^{o}C for 15 seconds and primer extension at 60^{o}C for 4 minutes. The temperature cycles were concluded with 28^{o}C for 1 minute followed by cooling. Following cycling, products were cleaned by loading them on to a Centriflex® gel filtration cartridge (Edge Biosystems, USA) and subjecting it to centrifugation

at 3600 rpm for 2 minutes. The filtrate was evaporated to dryness at 90^{o}C and then stored at –20^{o}C prior to automated sequencing.

2.5.5. Genotyping methods

2.5.5.1. Restriction Fragment Length Polymorphism

Polymorphisms sometimes create or abolish recognition sites for restriction enzymes. This can be made use of for genotyping as follows: Firstly genomic DNA flanking the polymorphic site is amplified by PCR. Then the PCR amplified products are digested with the appropriate restriction enzyme. Finally the genotype is determined by inspecting the pattern created by resolving the restriction-digested PCR fragments by electrophoresis on an agarose gel. This is termed restriction fragment length polymorphism (RFLP).

All restriction enzymes and buffers used in these investigations were from Roche Diagnostics GmbH, Germany or MBI Fermentas, Lithuania (Helena Biosciences, UK). Restriction digestion was carried out in a reaction mix containing 10µl PCR products, 2U appropriate restriction enzyme and 1 × appropriate restriction digestion buffer in a final volume of 15µl. The reaction was incubated overnight at 37^{o}C. The digested products were resolved by electrophoresing a fraction of the reaction mix by agarose gel electrophoresis (see section 2.5.3) to identify the genotype.

It is useful to build in an internal quality control to any RFLP assay to

distinguish truly undigested samples from those in which the restriction enzyme fails to digest a recognition site that is actually present in the sample. One way to do this is to locate a naturally occurring non-polymorphic constant recognition site for the same restriction enzyme close to the polymorphic site and design PCR primers so that the constant restriction site is also incorporated into the amplified PCR product. When such a PCR product is digested, the constant site should be digested in all reaction tubes. If it has failed to digest, then that sample has to be repeated. This method was used for internal quality control in all PCR/RFLP assays described in this thesis. When a constant restriction site is not found near the polymorphism of interest a multiplex PCR could be set up to co-amplify a different genomic region containing a constant restriction site or a PCR primer could be designed incorporating a constant restriction site within the primer sequence. The use of both these strategies in PCR/RFLP genotyping assays, for example, to genotype the *MTHFR* 677C>T polymorphism has been described before (Van Amerongen, Mathonnet, Boucly, Mathieu, Vinatier *et al.*, 1998, Bravo-Osorio and Bydlowski, 2000).

2.5.5.2. Mutagenically Separated PCR

In this method genomic DNA is amplified by PCR using two primers of different lengths specific to each allele of a biallelic polymorphism and a common reverse primer. Then the genotype is determined by inspecting the pattern created by resolving the PCR products by electrophoresis on an

agarose gel.

This method makes use of the inherent amplification refraction property of primers mismatched at their 3' end (Kwok, Kellogg, McKinney, Spasic, Goda *et al.*, 1990) and the inability of *Taq* DNA polymerase to perform 3' to 5' proofreading to correct and amplify such mismatched primers. As a result, only the primer(s) specific for the allele(s) present in a sample of genomic DNA are amplified under appropriate conditions (Rust, Funke and Assmann, 1993). As such, designing primers for a MS-PCR assay required special consideration. The 3' end of the each primer should contain a nucleotide specific for one allele of the polymorphism. One or more nucleotide mismatches should be incorporated near the 3' end to improve the allele specificity of the primer and two mismatches should be incorporated into the longer primer at two positions corresponding to the 5' end of the shorter primer to inhibit filling up of heteroduplex molecules that may be formed between long and short PCR products during the final cycles of the PCR. The effects of the incorporation of such mismatches on primer extension have been studied extensively (Newton, Graham, Heptinstall, Powell, Summers *et al.*, 1989, Kwok *et al.*, 1990, Rust *et al.*, 1993).

2.5.5.3. Competitive Allele Specific Oligonucleotide Hybridisation

Under certain conditions short oligonucleotide probes bind to a single stranded DNA target only when perfectly matched – a difference in even a single base

is adequate to prevent binding. This property is made use of in allele specific oligonucleotide (ASO) hybridisation assays to genotype biallelic polymorphisms. In this method genomic regions of interest are first amplified by PCR. Then PCR products are spotted on duplicate sets of nylon membranes. Next each membrane is incubated with a radiolabelled oligonucleotide probe specific for either the wild type or the variant allele of the polymorphism. Then hybridisation of labelled probes is revealed by autoradiography to determine the genotype.

Oligonucleotide probes can vary from 15 to 25 bases in size and the polymorphic site is usually located towards their centre. This method can be improved by reducing non-specific binding of probes to target DNA by pre-hybridisation of each filter with a 10 fold molar excess of the unlabelled alternative oligonucleotide prior to hybridisation with the labelled probe. This improved competitive method increases the accuracy of ASO hybridisation by enhancing the specificity of probe binding and reducing background noise (Gunneberg, Scobie, Hayes and Kalsheker, 1993).

In preparation for competitive ASO hybridisation, genomic regions flanking the polymorphism of interest were amplified by PCR. Then 2µl of the PCR product were denatured in 200µl of 0.2N NaOH/2 × SSC and spotted onto duplicate nylon membranes (Hybond N+, Amersham Biosciences, UK). In addition, two oligonucleotides, each specific for one of the alleles of the

polymorphism were radiolabelled in a 10µl reaction containing 3pmol oligonucleotide, 5U T4 polynucleotide kinase (PNK), 1 × PNK buffer (70mM Tris-HCl; pH 7.6 at 25ºC, 10mM $MgCl_2$, 5mM DTT) (Promega, UK), and 6pmol γ^{32}P-dATP. The reaction was incubated at 37ºC for 1 hour and terminated by incubation at 80ºC for 10 minutes. Un-incorporated radiolabelled nucleotides were removed by size exclusion chromatography through Sephadex® G-50 (Amersham Biosciences, UK) spin columns.

Competitive ASO hybridisation consisted of two steps. First each membrane was prehybridised at 52°C for 1 hour in 10ml 5 × SSPE/1% SDS containing 30pmol of unlabelled oligonucleotide corresponding to each allele, and then they were hybridised at 52°C for 1 hour in 10ml 5 × SSPE/1% SDS containing 3pmol of the alternative ^{32}P end labelled ASO. Following hybridisation membranes were washed in 2 × SSPE/0.1% SDS for two 30 minute periods at room temperature, then twice for 15 minutes in 5 × SSPE/0.1% SDS at 52°C, and autoradiographed at –80°C overnight. Once the genotypes were determined the probes were removed from the filter by placing them on a tray containing boiled 0.1% SDS on a shaker for 30 minutes. As a result filters could be reused to determine the genotype at multiple sites within the same PCR product.

2.5.5.4. Quality control considerations

Quality control was an important consideration in all genotyping assays. In PCR before RFLP and competitive ASO hybridisation assays, and in MS-PCR assays DNA-free controls were used. If any of these negative controls were found to have PCR amplified products on gel electrophoresis, then the entire PCR batch was repeated. In MS-PCR and in RFLP assays sequenced positive controls were also used. If they gave a wrong genotype the entire batch was repeated. In addition in PCR/RFLP assays as described in section 2.5.5.1 constant restriction sites were used for internal quality control of each restriction digestion reaction. In competitive ASO hybridisation assays, at least one sample of each genotype was sequenced to confirm the genotype.

Two independent observers read all gels and autoradiographs. Samples were re-genotyped when the observers did not agree on the genotype. Genotype frequencies of all polymorphisms were examined to confirm that they were in HWE. If the genotypes of samples of normotensive pregnant women or samples of population volunteers were not in HWE then they were re-genotyped. In the investigations described in this thesis this had to be done once (see section 2.6.2). The final level of quality control was applied statistically when haplotype analysis was done. Rare haplotypes occurring in frequencies of ≤0.05% could represent either actual rare haplotypes or false haplotypes created as a result of genotyping errors. Such haplotypes were therefore excluded from the analysis.

2.5.5.5. Selection of genotyping methods

Nine polymorphisms were genotyped in the investigations described in this thesis. When a genotyping method had already been described for a particular polymorphism that method was used for genotyping; however, modifications were made to such methods to improve quality control when appropriate. New genotyping methods had to be developed to genotype several polymorphisms. Different genotyping strategies were employed for each one of them so that the author gained experience with different methods. The amount of time taken up for genotyping, workload, and cost are important considerations in selecting a genotyping method. In terms of time and the amount of work involved MS-PCR was the quickest and least labour intensive. In terms of cost PCR/RFLP methods were the most expensive because of the additional cost of restriction enzymes, but the relative cost of the genotyping methods employed in the investigations described in this thesis did not differ greatly.

2.5.6. Mammalian cell culture

Cells from a human hepatoma cell line (HepG2) obtained from the European Collection of Cell Cultures (ECACC) were used in the reporter gene expression investigations described in this thesis (Aden, Fogel, Plotkin, Damjanov and Knowles, 1979, Knowles, Howe and Aden, 1980). Cell culture procedures were carried out under sterile conditions in a Class I flow cabinet. All culture media and reagents used in these investigations were from Sigma-Aldrich, UK or Gibco BRL (Invitrogen, UK).

Stocks of HepG2 cells were maintained in a cryopreserved state in liquid nitrogen. They were rapidly thawed and grown in 25ml or 75ml flasks in complete Eagle Minimal Essential Medium (EMEM) at 37ºC in a humidified atmosphere containing 5% carbon dioxide. The complete EMEM contained 84% EMEM, 10% heat-treated fetal bovine serum, 2% l-glutamine, 1% penicillin/streptomycin, 1% amphotericine B, 1% non essential amino acids, and 1% sodium pyruvate.

2.5.6.1. Feeding cells

The culture medium in the flask was replaced every 2-3 days to provide the cells with a continuous supply of nutrition. This entailed the removal of the old culture medium, washing the cells with phosphate buffered saline (PBS), pipetting out all the PBS, and replenishing the flask with complete EMEM.

2.5.6.2. Passaging

Once the cells reached 90-100% confluence they were harvested and subcultured into new flasks. Cell harvesting entailed removal of the old culture medium, washing the cells with PBS, discarding the PBS, trypsinising the cells with trypsin-EDTA and incubation for 5 minutes at 37ºC in a humidified atmosphere containing 5% CO_2. 5ml or 10 ml of trypsin-EDTA was used for 25ml or 75ml flasks respectively. At the end of the incubation period the flask was struck firmly to dislodge any adherent cells and the medium was agitated by pipetting to break down cell clumps. Cell clump formation is a recognised

feature of HepG2 cells. Once cells were resuspended, an equal volume of complete EMEM was added to inactivate trypsin. Thereafter more complete EMEM was added to the flask to make up the volume necessary to seed new flasks. Finally 5ml or 15ml of complete EMEM containing suspended HepG2 cells was transferred into new 25ml or 75ml flasks respectively.

2.5.6.3. Maintaining cell stocks

Stocks of frozen HepG2 cells were maintained in liquid nitrogen. In preparation for freezing down, cells were harvested as in section 2.5.6.2 and transferred into a 15ml centrifuge tube. This was followed by making the volume in the tube up to 15ml with complete EMEM, centrifuging the tube at 500 rpm at 4^{o}C for 5 minutes, discarding the supernatant and resuspending the cell pellet in 5ml of complete EMEM and an equal volume of freezing medium (2:2:1 complete EMEM: Bovine calf serum: Dimethylsulfoxide (DMSO)). 1.5ml aliquots of the mixture were then transferred into 2ml vials. The vials were frozen at –80^{o}C overnight before being transferred to the liquid nitrogen storage tank.

2.5.7. Study of gene expression and regulation

Genetic reporter systems are used to study eukaryotic gene expression and regulation. They are most frequently used as indicators of transcriptional activity in cells. These systems have an expression vector containing a reporter gene that can be transferred into cells. DNA sequences of interest are cloned

into the expression vector either upstream (promoter elements) or downstream (enhancer elements) of an enzyme reporter gene to create a recombinant molecule. Following transfer, the cells are assayed for the presence of the reporter by measuring the enzymatic activity of the reporter protein. This enables the study of the effects of the insert on reporter gene expression, which serve as an indicator of the effect of the insert on the transcriptional activity of the gene of interest *in vivo*. An ideal reporter gene is not endogenously expressed in the cell type of interest and is amenable to assays that are sensitive, quantitative, rapid, easy, reproducible and safe.

There are many genetic reporters and assay systems designed for analysis of transcriptional regulation in mammalian cells. The pGL3 series of expression vectors (Figure 2.1) containing the firefly luciferase reporter gene (Promega, UK) were used for the investigations described in this thesis. Following co-transfection of the pGL3 reporter gene construct and the control p*Renilla* plasmid into HepG2 cells transcriptional activity was assessed using the Promega dual-luciferase® reporter assay system (Promega, UK). Dual reporter systems are used to improve experimental accuracy. They involve the simultaneous expression and measurement of two individual reporter enzymes within a single system. Normalisation of the effect minimises the experimental variability seen due to differences in cell transfection efficiency, pipetting differences, cell lysis variability, and assay efficiency.

The following account of the Promega dual-luciferase® reporter assay system is based on information provided by the manufacturer. In this system the activity of firefly (*Photinus pyralis)* and sea pansy (*Renilla reniformis)* luciferase are measured sequentially from a single sample containing cell lysates or cell free translation systems. The firefly and *Renilla* luciferase are of distinct evolutionary origins. They have dissimilar enzyme structures and substrate requirements. That makes it possible to selectively discriminate between their respective bioluminescent reactions. Thus, the luminescence from the firefly luciferase reaction (the 'experimental' reporter) may be quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase (the 'control' reporter). In this system the firefly luciferase is quantified by the addition of Luciferase Assay Reagent II (LARII). Then Stop and Glow solution® is added to simultaneously quench the firefly luciferase activity and activate *Renilla* luciferase. Both systems generate full activity within two seconds and remain consistently raised during the 10 second period used for measuring activity.

2.5.7.1. Expression vectors

The pGL3Promoter expression vectors (pGL3P) were the base vectors used to create the recombinant experimental reporter vectors used for the *AGT* reporter gene expression investigations described in this thesis. The pGL3Control (pGL3C) and pGL3P vectors were also used as positive controls in these investigations. The pGL3P vector had the simian virus 40 (SV40)

promoter 5' of the firefly luciferase gene while the pGL3C vector had the SV40 promoter and SV40 enhancer, 5' and 3' of the firefly luciferase gene respectively (Figure 2.1).

2.5.7.2. The construction of recombinant experimental reporter vectors

The traditional method used for constructing recombinant experimental reporter vectors using the pGL3P base vector is restriction digestion of pGL3P and ligation of the insert. In this series of experiments, however, the Gateway® cloning system (Invitrogen, UK) was used for this purpose (see below). To facilitate Gateway® cloning, the pGL3P vectors had the Gateway® modifications at the multiple cloning site downstream of the luciferase reporter gene (pGL3P3'GW).

The following account of the Gateway® cloning system is based on the technical information provided by the manufacturer. This system is based on a site-specific recombination mechanism found in phage λ (Landy, 1989, Hartley, Temple and Brasch, 2000). It uses the key DNA recombination sequences involved in the recombination reactions and the proteins that mediate the recombination reactions in a two-step process to generate recombinant experimental vectors starting with the Gateway® modified base vector and the insert. The DNA recombination sequences are referred to as the *att* sites. In preparation for Gateway® cloning an insert is generated by PCR amplification of genomic DNA. This PCR is carried out with two

oligonucleotide primers that have *att*B1 and *att*B2 sequences at the 5' end of the forward and reverse primer respectively. These two *att*B sequences therefore flank the PCR amplified insert. The first step of the recombination reaction is called the BP recombination reaction. In the BP recombination reaction the *att*B sites of the insert recombines with the *att*P sites of the donor plasmid (pDONR® 201) in a reaction mediated by BP Clonase® enzyme resulting in a recombinant plasmid, referred to as the entry clone that now has *att*L sites flanking the insert. These recombinant plasmids are then chemically transformed into 5DHα competent cells and grown in culture containing kanamycin to obtain a plentiful supply of the entry clone. In the second step, which is called the LR recombination reaction, *att*L sites recombine with the *att*R sites on the destination vector (pGL3P3'GW) in a reaction mediated by LR Clonase® enzyme resulting in a recombinant experimental vector. These recombinant experimental vectors are also chemically transformed into 5DHα competent cells and grown in culture containing ampicillin to obtain a plentiful supply of the expression clone. This is the experimental reporter gene construct that is used to study the effects of the insert on reporter gene expression. The Gateway® cloning system is illustrated in figure 2.2.

When compared with cloning by restriction digestion and ligation, one of the main advantages of the Gateway® cloning system is that the time consuming and sometimes unreliable restriction digestion and ligation steps are not required for cloning. In addition once an entry clone containing an inset of

interest is made, it can be easily sub cloned into a wide variety of destination vectors using the LR Reaction. The system is also designed to make selection of colonies containing either the entry clone or the expression clone easy, because the plasmids that they are derived from, in addition to having an antibiotic resistant gene (donor vector has a kanamycin resistant gene and the destination vector has an ampicillin resistant gene) have a selection marker, the *ccd*B gene, which inhibits growth of *E.Coli.* Transformants selected for antibiotic resistance therefore, will have either the entry clone or the expression clone only. The use of two different antibiotic resistant genes for each step ensures that following the LR recombination reaction transformants selected for ampicillin resistance will only contain the expression clone. A few background colonies may also be seen in culture. They contain inactive or deleted *ccd*B genes.

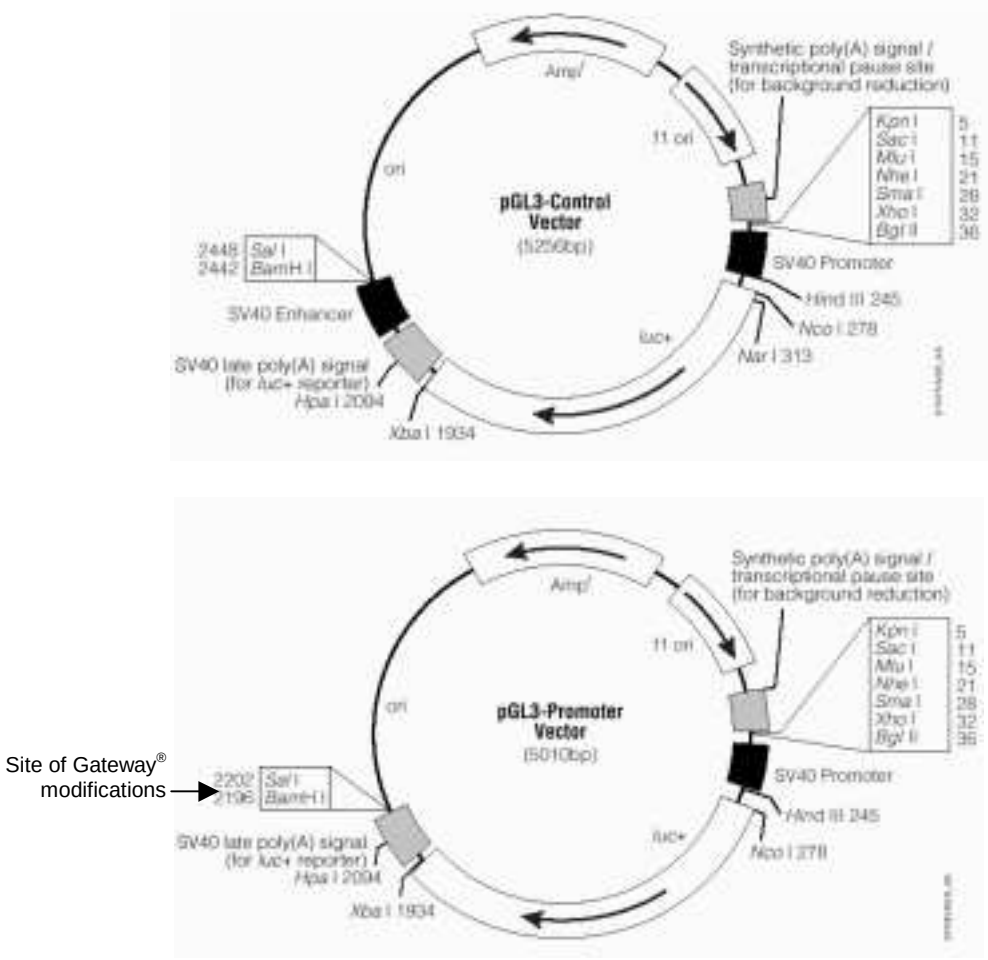
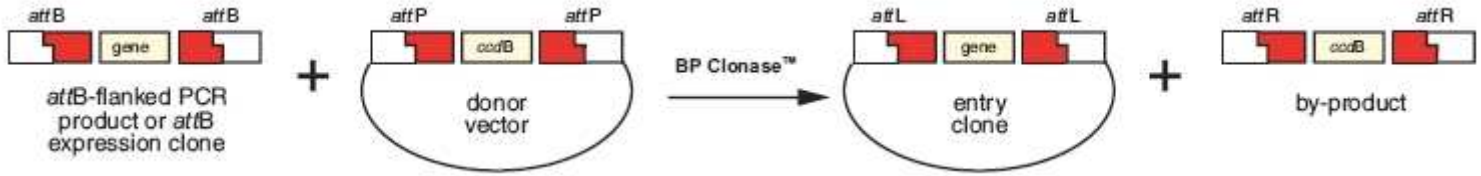


Figure 2.1. Circle maps of pGL3Control and pGL3Promoter vectors. pGL3P3'GW has Gateway® modification at the multiple cloning site downstream of the luciferase gene as indicated in the figure. Source: Promega Protocols and Applications Guide. Catalogue No. P1610.

BP recombination reaction



LR recombination reaction

Figure 2.2. A schematic diagram of the two steps in the Gateway® cloning system. Source: Invitrogen Gateway® Technology Manual. Catalogue Nos:12535-019, 12535-027.

2.5.7.3. Purification of vector DNA from bacterial cells

It is necessary to obtain pure samples of intact vector (plasmid) DNA from transformed 5αDH cell cultures. Two procedures were used for this purpose. Minipreps were performed to obtain plasmid DNA to carry out sequencing and restriction digestion to confirm the presence of the insert sequence. Endotoxin free midipreps were performed to obtain plasmid DNA for transfection. Endotoxins are proficient inducers of the acute phase response process. If present in plasmid preparations they can interfere with reporter gene assays. When endotoxin free preparations are made, endotoxins present in bacterial cells contaminating plasmid DNA are removed from the final preparation. All buffers and kits used for plasmid DNA purification in these investigations were from Qiagen, UK.

Miniprep

The QIAprep® spin miniprep plasmid purification kit was used for minipreps. This protocol involved pelleting of bacterial cells, resuspension of cells, alkaline lysis, cleaning of the bacterial lysate, adsorption of plasmid DNA onto a silica membrane in the presence of salt, washing the membrane and eluting plasmid DNA. The protocol is used to purify of up to 20μg/ml of high copy plasmid DNA from 1-5ml of overnight bacterial culture.

The miniprep procedure was carried out as follows: Transformed bacterial cells were grown overnight in 5ml of Luria-Bertani (LB) medium containing the appropriate selective antibiotic in a 20ml universal container at 37^{o}C with

horizontal shaking at 225 rpm. 1.5 ml of the overnight bacterial culture was applied to a 1.5ml microcentrifuge tube. Cells were then pelleted by centrifugation at 14 000 rpm for 10 minute and the supernatant was discarded. Then the cells were resuspended in 250µl of chilled buffer P1 (50mM Tris-Cl, pH 8.0; 10mM EDTA; 100µg/ml RNase). Next 250µl of the lysis buffer P2 (200mM NaOH; 1% SDS) were added to the resuspended cells. It was mixed by gently inverting 6 times to prevent sheering of plasmid DNA. The lysis reaction was allowed to continue at room temperature for up to but not exceeding 5 minutes by which time a viscous slightly clear mixture was seen. Then 350µl of the neutralisation buffer N3 (3.0M Potassium Acetate, pH5.5) were added to the reaction and mixed immediately by gently inverting the tube 6 times. The reaction was then centrifuged at 14 000 rpm for 10 minutes and the resulting supernatant was applied to a QIAprep spin column. The column was then centrifuged at 14 000 rpm for 1 minute. Next the column was washed by adding 750µl of buffer PE (1M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol) followed by centrifugation at 14 000 rpm for 1 minute. Any residual wash buffer was removed by a further centrifugation at 14 000 rpm for 1 minute. After each of these centrifugation steps the flow-through was discarded. Finally the column was placed in a 1.5ml collection tube and 50µl of buffer EB (10 mM Tris.Cl, pH8.0; 1mM EDTA) was added to the centre of the column membrane. After allowing the column to stand for 1 minute, the plasmid DNA was eluted from the column by centrifugation at 14 000 rpm for 1 minute. The DNA concentration of the eluted sample was measured

spectrophotometrically on a GeneQuant RNA/DNA calculator (Amersham Biosciences, UK) and the sample was stored at –20^{o}C.

Endotoxin free midi preparation

Endotoxin free midipreps were made using a Qiagen Endofree® plasmid maxi kit using the QIAfilter® midiprep protocol. The protocol involves procedures that are similar to that of the miniprep procedures except for an additional step to remove endotoxins and the use of endotoxin free buffers. The protocol is used to purify up to 100µg of high copy plasmid DNA from 25ml overnight bacterial cultures.

Transformed bacterial cells were grown overnight in 25ml of LB medium containing the appropriate selective antibiotic in a 50ml centrifuge tube at 37^{o}C with horizontal shaking at 225 rpm. Bacterial cells in this tube were pelleted by centrifugation at 4600 rpm for 15 minutes and the supernatant was discarded. Then the cells were resuspended in 4ml of chilled buffer P1. Next 4ml of the lysis buffer P2 were added to the resuspended cells. It was mixed by gently inverting 6 times to prevent sheering of plasmid DNA. The lysis reaction was allowed to continue at room temperature for up to but not exceeding 5 minutes by which time a viscous slightly clear mixture was seen. Then 4ml of the chilled neutralisation buffer P3 (3.0M Potassium Acetate; pH 5.5) was added to the reaction and mixed immediately by gently inverting the tube 6 times. The lysate was then poured into a QIAfilter cartridge and filtered

into a 15ml centrifuge tube and the volume noted. Next a volume equal to $1/10^{th}$ the volume noted before, was pipetted from the endotoxin removal buffer ER and added to the tube. The tube was inverted 10 times, and allowed to incubate on ice for 30 minutes. While the reaction was incubating, 5ml of buffer QBT (750mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol, 0.15% Triton®) was added to a QIAGEN-tip100 and the filter in the tip was allowed to equilibrate by gravity. At the end of 30 minutes of incubation, the lysate in the 15 ml tube was applied to the equilibrated tip and it too was allowed to enter the tip aided by gravity. The tip was then washed twice with 10ml of endotoxin free buffer QC (1.0M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol). The flow through was discarded at the end of each step mentioned above. Next the tip was placed on a 50ml centrifuge tube and the DNA was eluted by applying 5ml of endotoxin free buffer QN (1.6M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol) and allowing it to flow through by gravity. After that 3.5ml of isopropanol was added to it to precipitate the DNA. The precipitated DNA was pelleted to the bottom of the tube by centrifuging at 4600 rpm for 90 minutes at 4^oC. At the end of centrifugation the pellet was washed with endotoxin free ethanol and again centrifuged at 4600 rpm for 30 minutes at 4^oC. Finally ethanol was carefully removed and the pellet was air dried before being resuspended in 100µl of endotoxin free TE (10mM Tris, pH8.0; 1mM EDTA). The DNA concentration of the eluted sample was measured spectrophotometrically on a GeneQuant RNA/DNA calculator (Amersham Biosciences, UK) and the sample was stored at $–20^oC$.

2.5.7.4. Strategy for transferring expression vectors into mammalian cells

Transfection is the process of introducing expression vectors into mammalian cells. The Promega Tfx® systems was used for this purpose.

The following brief account of this system is based on the technical information provided by the manufacturer. This system uses artificial liposomes to deliver the plasmid DNA into mammalian cells. The liposome has an overall positive charge at physiological pH and the cationic portion of the liposome associates with the negatively charged plasmid DNA, resulting in the formation of a complex. An overall positive charge tends to improve transfection efficiency, probably due to the combination of the positive complex with the negatively charged cell membrane. Following endocytosis through the membrane the complexes initially appear in endosomes and finally in the nucleus.

2.6. Genotyping the samples of population volunteers, women with pre-eclampsia, and normotensive pregnant women

2.6.1. Epidermal Growth Factor 61G>A genotyping

The substitution of G by A at nucleotide 61 creates a restriction site for the *Alu*I restriction enzyme. This polymorphism therefore, was genotyped using a PCR/RFLP assay described previously (Shahbazi *et al.*, 2002).

The genomic region flanking the polymorphic site was amplified by PCR using primers EGF-61F and EGF-61R (Table 2.6) as described in section 2.5.2. These primers were used to generate PCR products for both genotyping and sequencing. EGF-61F was the sequencing primer for cycle sequencing reactions that were conducted as described in section 2.5.4. Restriction digestion was carried out as described in section 2.5.5.1 with the restriction enzyme *Alu*I and restriction digestion buffer H (5mM Tris-HCl, 10mM NaCl, 1mM $MgCl_2$, 0.1mM dithioerythritol, pH 7.5 at 37^{o}C). Digested PCR fragments were then resolved by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 1 hour at 50 volts on a 3% agarose gel. A summary of the sizes of the digested products is in figure 2.3. The pattern created by them on a 3% agarose gel and the DNA sequence of the genomic region flanking the polymorphic site is figure 2.4.

Prime Name	Sequence (5'→3')	Orientation	Annealing Temperature (^{o}C)	Product Size (bp)
EGF-61F	TGT CAC TAA AGG AAA GGA GGT	Sense	51^{o}C	242
EGF-61R	TTC ACA GAG TTT AAC AGC CC	Antisense		

Table 2.6. The PCR primers used to amplify the genomic region flanking the *EGF* 61G>A polymorphic site for the PCR/RFLP genotyping assay and for sequencing. (Shahbazi *et al.*, 2002).

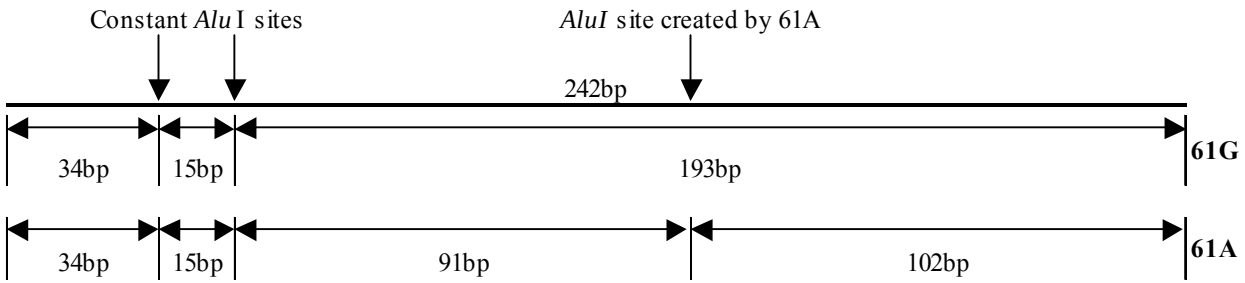


Figure 2.3. A diagrammatic representation of the location of *Alu*I restriction sites on PCR products generated using primers EGF-61F and EGF-61R and the size of the fragments created by restriction digestion of the PCR products with *Alu*I. The expected banding patterns are: 61GG: 193-bp; 61AA: 91-bp and 102-bp; 61GA: 91-bp, 102-bp and 193-bp. 15-bp and 34-bp products were not visible following agarose gel electrophoresis and the 91-bp and 102-bp products appeared together as a single band.

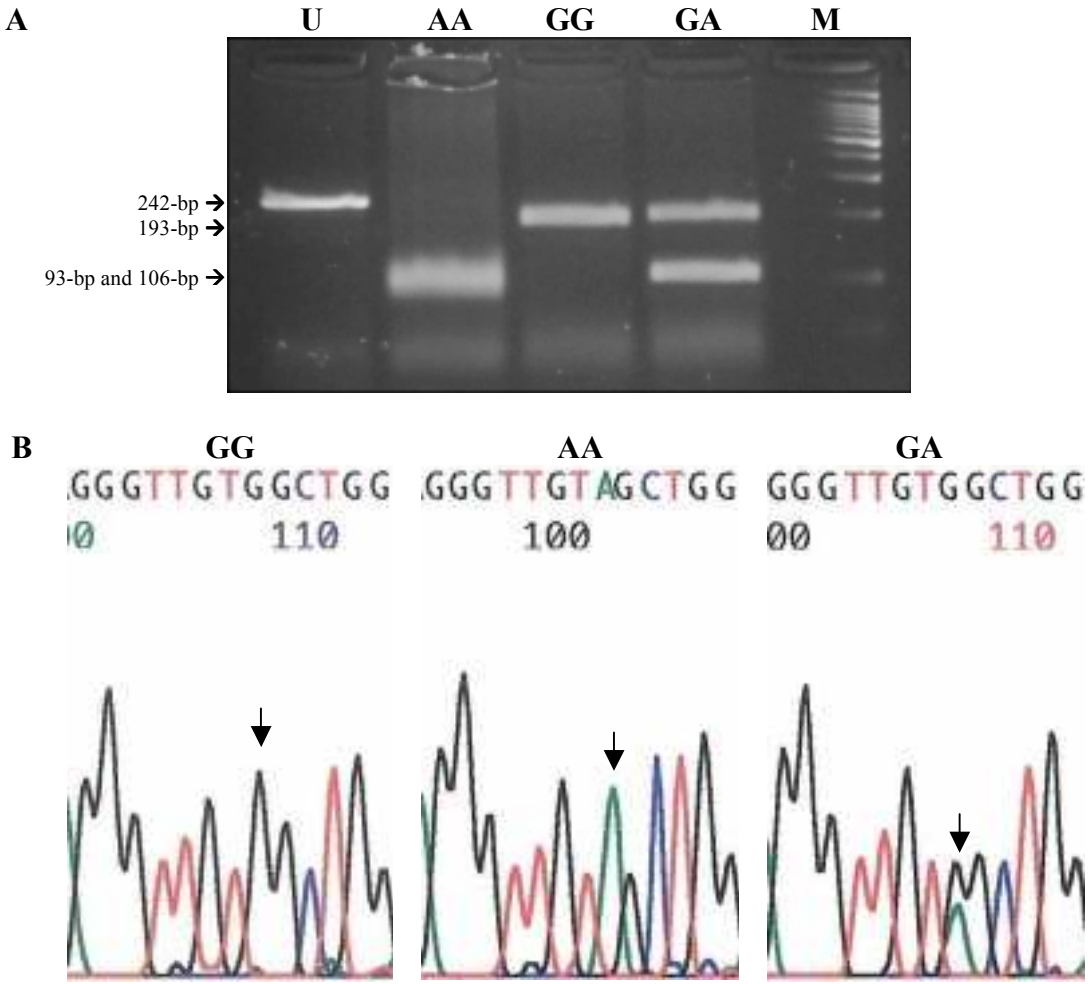


Figure 2.4. *EGF* 61G>A genotyping: **A)** The pattern created by electrophoresing the *Alu*I digested products of samples of the three genotypes on a 3% agarose gel. U: Uncut Products. M:100bp size marker. **B)** The sequence of the three genotypes on the sense strand. Arrowheads indicate the polymorphic site.

2.6.2. Epidermal Growth Factor 67149G>A genotyping

A new MS-PCR assay was developed to genotype this polymorphism. Two allele specific forward primers EGF-G and EGF-A and a common reverse primer EGF-R were designed for this assay (Table 2.7). Principles discussed in section 2.5.5.2 with regard to incorporation of mismatched nucleotides into allele specific primers were made use of in designing EGF-G and EGF-A.

A fourth primer, EGF-F was designed and used together with EGF-R to produce PCR fragments to confirm the genotype by sequencing (Table 2.7). These PCR were conducted as described in section 2.5.2. EGF-R was used as the sequencing primer for cycle sequencing reactions that were conducted as described in section 2.5.4.

In addition to optimising the MS-PCR assay by repeating it at different annealing temperatures, the assay was further optimised by carrying out the reactions varying the number of allele specific primers used in each reaction mix, and by varying the dNTP concentration in the reaction mix. When samples homozygous for the A allele were amplified with only the primer specific for the G allele in the reaction mix, non-specific amplification of the A allele giving a G band on electrophoresis was observed. The same samples were correctly amplified however, when both primers were present in the reaction mix. It is possible that this is a result of competition for templates and other reaction components (Figure 2.5.A). When the same reaction was

repeated at a dNTP concentration of 20μM non-specific amplification of primers did not occur (Figure 2.5.B). Others have also reported similar observations with allele specific primers (Newton *et al.* (1989), Kwok *et al.* (1990), Rust *et al,* (1993)).

The MS-PCR assay was conducted in a final volume of 15μl containing genomic DNA, 0.08μM EGF-G, 0.08μM EGF-A, 0.1μM EGF-R, 20μM of dNTP, PCR buffer; and 1U of Taq Polymerase. The reactions were subjected to 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 60^{o}C for 1 minute, and extension at 72^{o}C for 5 minute. The temperature cycles were preceded by 2 minutes at 94^{o}C and concluded with 5 minutes at 72^{o}C followed by cooling at 4^{o}C.

The 67149G specific primer was 20 nucleotides longer than the 67149A specific primers. Thus when the PCR was carried out a 182-bp product was generated with 67149GG homozygotes, a 162-bp product was generated with 67149AA homozygotes, and both 182-bp and 162-bp products were generated with 67149GA heterozygotes.

MS-PCR products were then resolved by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 1 hour at 50 volts on a 3.5% agarose gel. The pattern created by MS-PCR products and the DNA sequence of the genomic region flanking the polymorphic site is in figure 2.6.

Primer Name	Sequence (5'→3')	Orientation	Annealing Temperature (^{o}C)	Product Size (bp)
EGF-G	TTATGTGTGGTTCTCAGATT**CC**CGCTATGCCATCAGTAA**G**G **CC** **A**	Sense	60	182
EGF-A	GGGCTATGCCATCAG**G**AATA **A**	Sense		162
EGF-F	TGT TTC CAG GTC ACC CAT TT	Sense	51^{o}C	242
EGF-R	CAG ATT CCA GCC AAG GAA AG	Antisense		

Table 2.7. The PCR primers used for the MS-PCR genotyping assay of the *EGF* 67149G>A polymorphism and the PCR primers used to amplify the genomic region flanking the *EGF* 67149G>A polymorphic site for sequencing. The positions where mismatches have been introduced to allele specific primers EGF-G and EGF-A are in bold and the corresponding mismatch on the antisense strand is indicated.

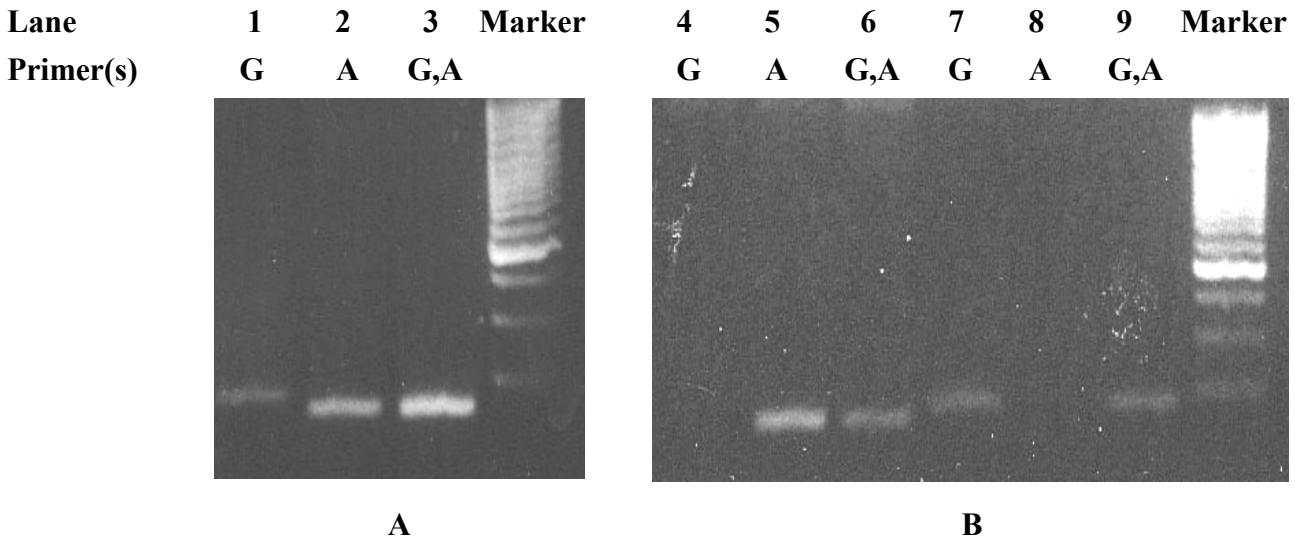


Figure 2.5. A) Non-specific amplification of the 67149A allele by a 67149G specific primer when a 67149AA homozygous sample was amplified only with the 67149G specific primer (lane 1). The same sample amplified with a 67149A specific primer (lane 2) and with both primers (lane 3) show the amplification of the 67149A specific primer only. The dNTP concentration in this reaction was 200µM. **B)** When the same experiment was carried out with the dNTP concentration at 20µM (lanes 4 to 6), non-specific extension of the 67149G specific primer did not occur (lane 4). In a similar experiment with a 67149GG homozygous sample (lane 7-9) the 67149G specific primer was amplified while the 67149A specific primer was not amplified.

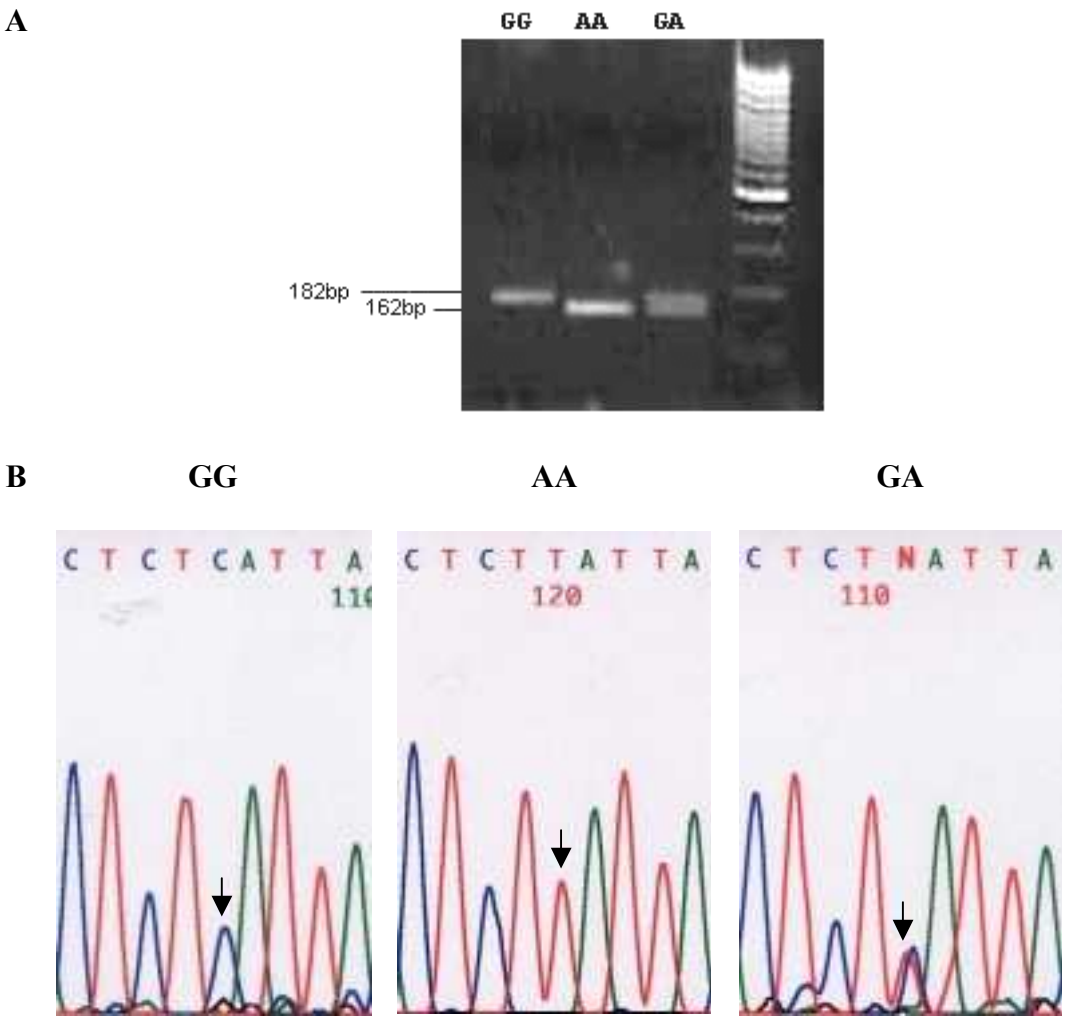


Figure 2.6. *EGF* 67149G>A genotyping. **A)** The pattern created by electrophoresing the MS-PCR products of samples of the three genotypes on a 3.5% agarose gel. **B)** The sequence of the three genotypes on the antisense strand. Arrowheads indicate the polymorphic site.

2.6.3. Transforming Growth Factor Alpha 3827T>C & 3851T>C genotyping

The *TGFA* 3827T>C and 3851T>C polymorphisms were genotyped using a PCR/RFLP assay. The recognition site for *Hinf*I that is present in the wild type 3827T allele is abolished when it is substituted by the 3827C allele. Similarly the recognition site for *NCo*I that is present in the wild type 3851T allele is abolished when it is substituted by the 3851C allele. It was possible therefore, to genotype these two polymorphisms using a PCR/RFLP assay as described before (Shiang *et al.*, 1993). This assay however, lacked an internal control. Two new primers, TGFA-F2 and TGFA-R2 were designed therefore, to amplify a 645-bp fragment flanking the polymorphic region incorporating constant restriction sites for both enzymes (Table 2.8). PCR were conducted as described in section 2.5.2 using primers TGFA-F2 and TGFA-R2. These primers were also used to generate PCR products for sequencing. TGFA-R2 was also used as the sequencing primer in cycle sequencing reactions that were conducted as described in section 2.5.4.

Restriction digestion was carried out as described in section 2.5.5.1 with restriction enzyme *Hinf*I or *NCo*I and 1 × restriction digestion buffer H. Digested PCR fragments were then resolved by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 1 hour at 50 volts on a 3% agarose gel. A summary of the sizes of the digested products is in figure 2.7. The pattern created by them on a 3% agarose gel and the DNA sequence of the

genomic region flanking the polymorphic site is figure 2.8.

2.6.4. Transforming Growth Factor Alpha 3822G>A genotyping

A new competitive ASO hybridisation assay was developed to genotype the 3822G>A polymorphism. It was carried out using oligonucleotide probes TGFA-A, TGFA-G1 and TGFA-G2 (Table 2.8). The 3822G>A polymorphism was just 5-bp away from the 3827T>C polymorphism. If only one probe was used for the 3822G allele with a C corresponding to a 3827C allele, then in the presence of a 3827T allele it would either hybridise inadequately or not at all leading to erroneous genotyping. Two 3822G allele specific probes were used therefore, for hybridisation. One had T at the position corresponding to 3827 and the other had C (Table 2.8). The competitive ASO hybridisation assay was carried out according to the procedure described in section 2.5.5.3. The only modification to that procedure was that nylon filters were blotted in triplicate for each sample to hybridise with the three probes and as such during pre-hybridisation each filter was hybridised with two unlabeled oligonucleotides. For example if hybridisation was with TGFA-A, then pre-hybridisation was with TGFA-G1 and TGFA-G2.

According to previous reports the combination of alleles 3822A and 3827T did not occur together in any chromosome. An oligonucleotide probe for this combination of alleles was not used therefore in the TGFA 3822G>A genotyping assay. However, to further verify that such a combination did not

exist, an ASO hybridisation assay was carried out on 92 samples with a probe containing nucleotides A and T at positions corresponding to 3822 and 3827 (5'-CTA ACC AC**A** AGA C**T**C TCA AC-3') and the TGFA-A probe. No hybridisation was observed with the former probe in any sample.

Primer Name	Sequence (5'→3')	Orientation	Annealing Temperature (^{o}C)	Product Size (bp)
PCR Primers				
TGFA–F2	CGG ACC ACG ATT TCA AGA CT	Sense	55	645
TGFA–R2	GCT TCA ATG GCA CCA TTT CT	Antisense		
ASO Probes				
TGFA–A	CTA ACC AC**A** AGA C**C**C TCA AC	Sense		
TGFA–G1	CTA ACC AC**G** AGA C**C**C TCA AC	Sense		
TGFA–G2	CTA ACC AC**G** AGA C**T**C TCA AC	Sense		

Table 2.8. The PCR primers used to amplify the genomic region flanking the *TGFA* 3'UTR polymorphic site for the PCR/RFLP genotyping assay, for sequencing, and the allele specific oligonucleotides used for the competitive ASO hybridisation assay; and the oligonucleotide probes used for the competitive ASO hybridisation genotyping assay of the 3822G>A polymorphism. The allele specific nucleotides corresponding to the polymorphic sites 3822 and 3827 in the ASO probes are in bold.

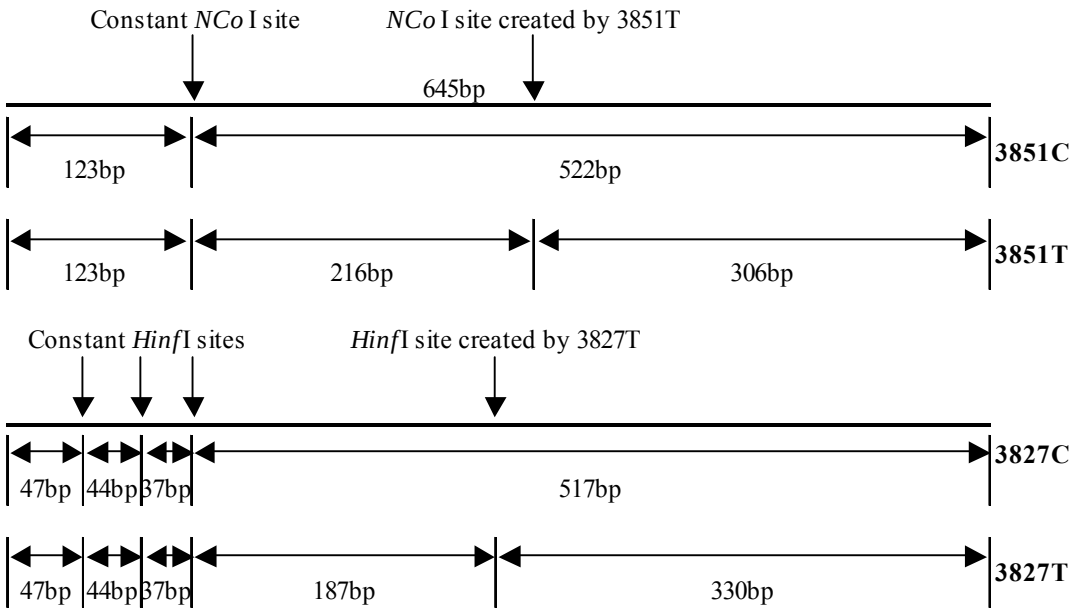


Figure 2.7. A diagrammatic representation of the location of *Hinf*I and *NCo*I restriction sites on PCR products generated using primers TGFA-F2 and TGFA-R2 and the size of fragments created by restriction digestion of the PCR products with *NCo*I and *Hinf*I. The expected banding patterns are: When digested with *NCo*I – 3851TT: 123-bp, 216-bp and 306-bp; 3851CC: 123-bp, and 522-bp; 3851TC: 123-bp, 216-bp, 306-bp, and 522-bp. When digested with *Hinf*I – 3827TT: 187-bp and 330-bp; 3827CC: 517-bp; 3827TC: 187-bp and 330-bp, 517-bp. The 37-bp, 44-bp, and 47-bp products were not visible following agarose gel electrophoresis.

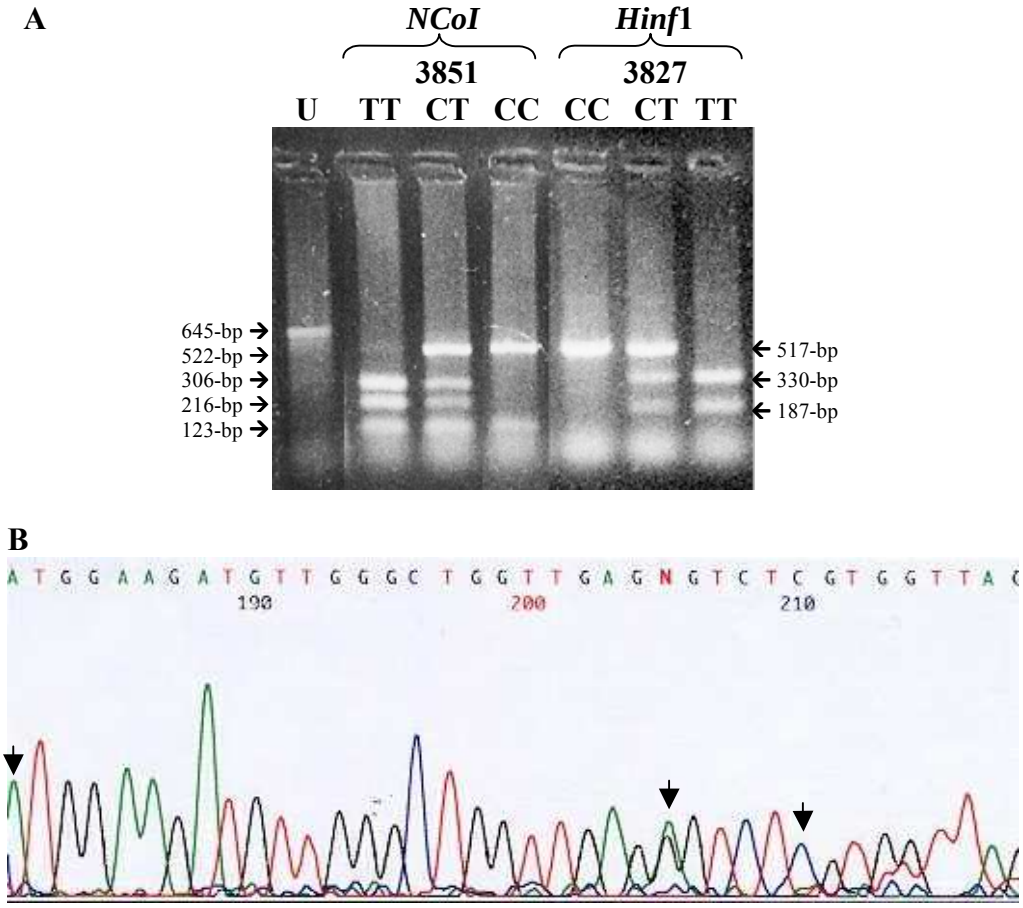


Figure 2.8. *TGFA* 3827T>C and 3851T>C genotyping: **A)** The pattern created by electrophoresing PCR products of samples of the three genotypes at each polymorphic site digested with *NCo*I *and Hinf*I on a 3% agarose gel. Images from two gels have been merged for illustration. U: Undigested PCR products. The sizes of the products digested with *NCo*I and the undigested product are on the left; and the sizes of the products digested with *Hinf*I are of the right. **B)** The sequence of a sample with the genotype 3822GG, 3827TC and 3851TT at the polymorphic sites. Sequence is on the anti-sense strand.

2.6.5. Angiotensinogen 174Thr>Met, 235Met>Thr and 11535C>A genotyping

These polymorphisms were genotyped using a competitive ASO hybridisation assay previously developed and used in our laboratory (Morgan *et al.*, 1996).

The genomic region flanking the 174Thr>Met and 235Met>Thr polymorphisms were amplified with primers A21 and A22. The genomic region flanking the 11535C>A polymorphism was amplified with primers H2651 and H2654 (Table 2.9). A multiplex PCR was conducted to generate PCR products of both genomic regions in the same reaction. It was conducted in a reaction mix containing 100-200ng template genomic DNA, 200µM deoxynucleotides, PCR buffer, 0.4µM A21, 0.4µM A22, 0.8µM H2651, 0.8µM H2654 and 1U *Taq* DNA polymerase. PCR amplification involved repeated cycles of heat denaturation of the genomic DNA at 94^{o}C for 1 minute, annealing at 60^{o}C for 1 minute, and extension of the annealed primers at 65^{o}C for 5 minutes. The temperature cycles were preceded by 2 minutes at 94^{o}C and concluded with 3 minutes at 65^{o}C followed by cooling at 4^{0}C. The presence of PCR amplified products was confirmed by agarose gel electrophoresis as in section 2.5.3.

The oligonucleotide probes used for competitive ASO hybridisation are listed in table 2.9. The hybridisation reactions were conducted according to the protocol described in section 2.5.5.3. The only exception was that when

genotyping the 11535C>A polymorphism, the filters had to be pre-hybridised with 300pmols of the competing primer, before hybridisation with 3pmols of the labelled primer, to eliminate a high level of background signal.

Separate PCRs were conducted with primer sets A21 and A22, and H2651 and H2654 to generate PCR products for sequencing as described in section 2.5.2. A21 and H2651 were used as sequencing primers for the cycle sequencing reactions that were conducted as described in section 2.5.4.

Primer Name	Sequence (5' – 3')	Orientation	Polymorphism / Allele	PCR product size
PCR Primers				
A21	AAG GAC AAG AAC TGC ACC TCC CGG CT	Sense	174Thr>Met & 235Met>Thr	365
A22	GCCAGA GCC AGC AGA GAG GTT TGC CT	Antisense		
H2651	AAC CCG CTG AGC ACA GCA TG	Sense	11535C>A	634
H2654	TGG AGG CTT AGT GTG GCA AGA	Antisense		
ASO Probes				
G6745	CTG CTG TCC A**T**G GTG GT	Sense	174Met	
G8041	CTG CTG TCC A**C**G GTG GT	Sense	174Thr	
F016	TCC CTG A**C**G GGA GCC AG	Sense	235Thr	
F017	TCC CTG A**T**G GGA GCC AG	Sense	235Met	
N8804	TGC GGA AC**A** ATA GCT GGT	Sense	11535A	
N8805	TGC GGA AC**C** ATA GCT GGT	Sense	11535C	

Table 2.9. The PCR primers used to amplify the genomic regions flanking the *AGT* polymorphic sites for the competitive ASO hybridisation assay and for sequencing; and the allele specific oligonucleotides used for the competitive ASO hybridisation assay at each polymorphic site. The allele specific nucleotides corresponding to the polymorphic sites in the ASO probes are in bold.

2.6.6. 5,10-Methylenetetrahydrofolate reductase 677C>T genotyping

The *MTHFR* 677C>T polymorphism creates a *Hinf*I recognition site. It was possible therefore, to genotype this polymorphism using an already described genotyping assay (Frosst *et al.*, 1995), which, however, lacked an internal control. A new set of PCR primers incorporating a constant restriction site was therefore designed and used for genotyping the Sri Lankan samples as described below. A MS-PCR assay was also developed to genotype this polymorphism with a view to designing a faster genotyping assay. As the MS-PCR assay was developed prior to the collection of Sri Lankan samples, the white Caucasian women with pre-eclampsia and normotensive pregnant women were genotyped using the MS-PCR assay as described below.

PCR/RFLP assay

The genomic region flanking the polymorphic site was amplified by PCR using primers VAJ-1 and VAJ-2 (Table 2.10). These primers were used to generate PCR products for both genotyping and sequencing. VAJ-2 was also used as the sequencing primer in cycle sequencing reactions that were conducted as described in section 2.5.4. Restriction digestion was carried out as described in section 2.5.5.1 with the restriction enzyme *Hinf*I and 1 × restriction digestion buffer H. Digested PCR fragments were then resolved by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 1 hour at 50 volts on a 3% agarose gel. A summary of the sizes of the digested products is in figure 2.9. The pattern created by them on a 3%

agarose gel and the DNA sequence of the genomic region flanking the polymorphic site is in figure 2.10A and 2.10C respectively.

MS-PCR assay

Two allele specific forward primers and a common reverse primer were used for this assay. Primers described previously for a capillary electrophoresis assay to genotype this polymorphism was modified and used (Ulvik, Ren, Refsum and Ueland, 1998). The only modification made was the addition of a 20-bp CT tail to the 677C allele specific primer (Table 2.10).

MS-PCRs were conducted in a final volume of 25µl containing genomic DNA; 0.18µM MTHFR-C, 0.25µM MTHFR-T, 0.23µM MTHFR-R; 200µM of dNTP; PCR buffer; and 1U of Taq Polymerase. The reactions were subjected to 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 62^{o}C for 1 minute, and extension at 72^{o}C for 1 minute. The temperature cycles were preceded by 2 minutes at 94^{o}C and concluded with 30 minutes at 72^{o}C followed by cooling at 4^{0}C.

The 677C specific primer was 20 nucleotides longer than the 677T specific primer. Thus when the PCR was carried out a 137-bp product was generated with 677CC homozygotes, a 117-bp product was generated with 677TT homozygotes, and both 137-bp and 117-bp products were generated with 677CT heterozygotes.

MS-PCR products were then resolved by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 1 hour at 50 volts on a 3.5% agarose gel. A summary of the pattern created by MS-PCR products is in figures 2.10B.

One drawback in this assay was heteroduplex formation as a result of the failure to introduce nucleotide mismatches to the longer MS-PCR primer to correspond to the 5' end of the shorter primer. It did not however, interfere with genotyping (Figure 2.10.B).

Primer Name	Sequence (5' – 3')	Orientation	Annealing Temperature (^{o}C)	Product Size (bp)
VAJ-1	TAT TGG CAG GTT ACC CCA AA	Sense	55	557
VAJ-2	GGG ACC TGG AGA TCC ACT TT	Antisense		
MTHFR-C	CTCTCTCTCTCTCTCTCTCTGAAGGAGAAGGTGTCTGCGG**T**AGC **G**	Sense	62	137
MTHFR-T	GAAGGAGAAGGTGTCTGCGG**A**AGT **G**	Sense		117
MTHFR-R	ACG ATG GGG CAA GTG ATG	Antisense		

Table 2.10. PCR primers used for the PCR/RFLP and MS-PCR genotyping assays to genotype the *MTHFR* 677C>T polymorphism. The positions where mismatches have been introduced to allele specific primers MTHFR-C and MTHFR-T are indicated in bold and the corresponding mismatch on the antisense strand is indicated.

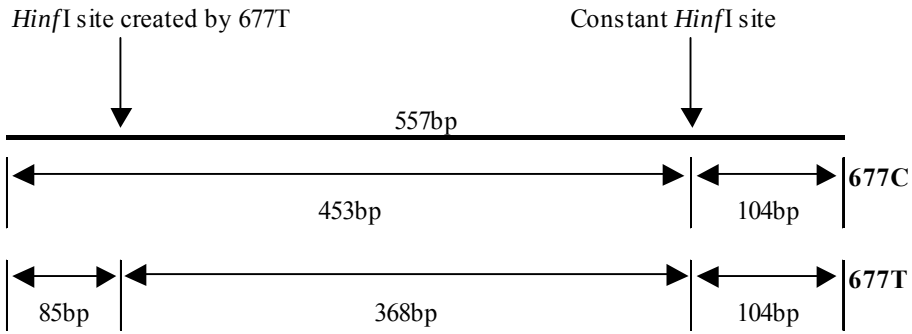


Figure 2.9. A diagrammatic representation of the location of *Hinf*I restriction sites on PCR products generated using primers VAJ1 and VAJ2 and the size of the fragments created by restriction digestion of the PCR products with *Hinf*I. The expected banding patterns are: 677CC: 104-bp and 453-bp; 677TT: 104-bp, 368-bp, and 85-bp; 677CT: 85-bp, 104-bp, 368-bp and 453-bp. The 85-bp and 104-bp products appeared together as a single band on agarose gel electrophoresis.

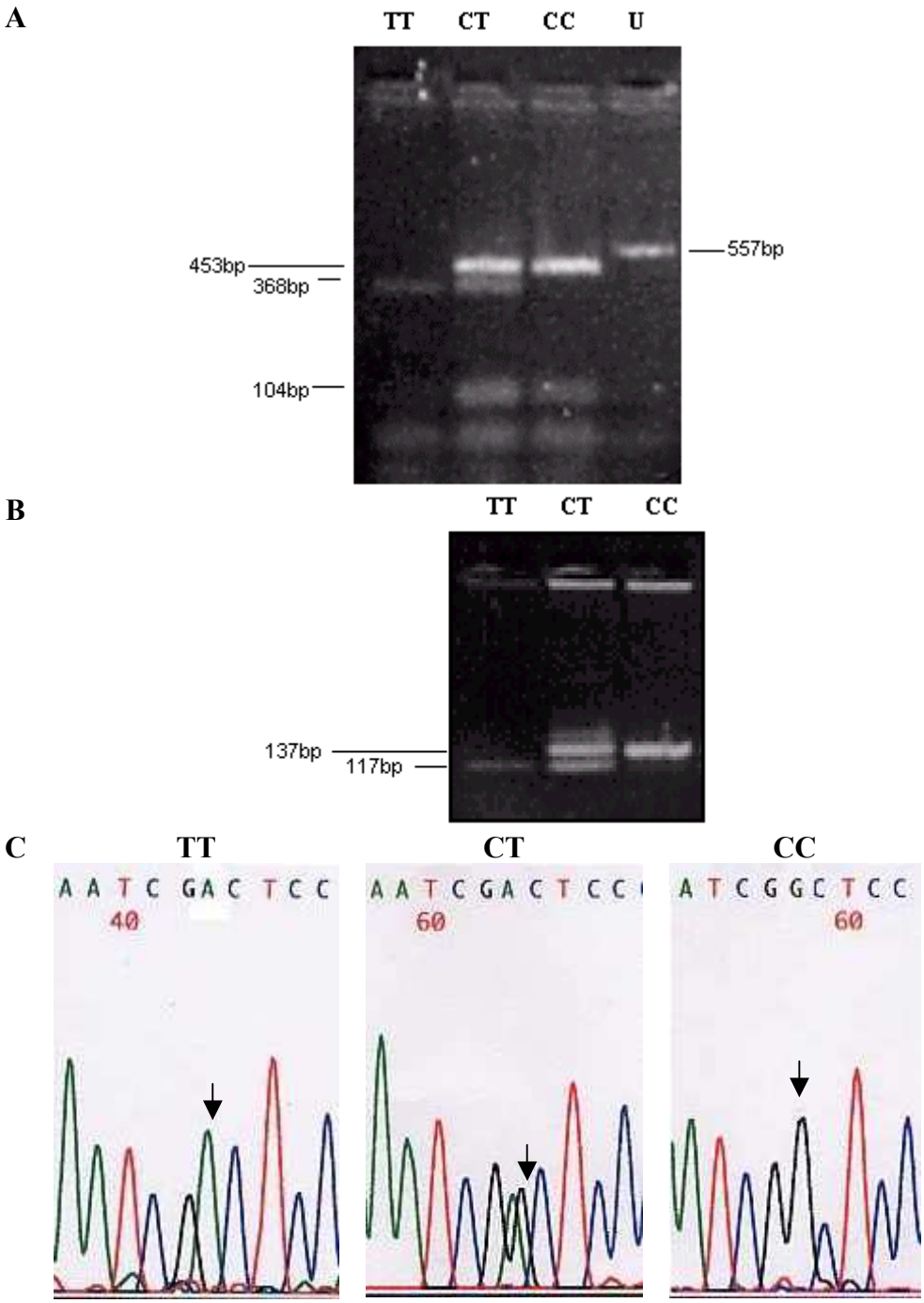


Figure 2.10. *MTHFR* 677C>T genotyping: **A)** The pattern created by electrophoresing the *Hinf*I digested PCR products of samples of the three genotypes on a 3% agarose gel. Visualising the 368-bp and 453-bp fragments was sufficient to assign the genotype. U: Undigested PCR product. **B)** The pattern created by electrophoresing the MS-PCR products on a 3.5% agarose gel. A faint larger heteroduplex band is seen in the CT sample. **C)** The sequence of the three genotypes on the antisense strand. Arrowheads indicate the polymorphic site.

2.7. Angiotensinogen reporter gene expression studies

The three common haplotypes defined by the 3'UTR/flanking region SNPs in the *AGT* gene were examined in this study with relation to their functional effects. The *AGT* 3'UTR/flanking region inserts, which were from a putative enhancer region, were designated E1, E2 and E3. They were cloned into pGL3P3'GW vectors to create the recombinant experimental reporter vectors necessary for these investigations.

2.7.1. Generation of recombinant experimental reporter gene constructs

2.7.1.1. PCR amplification of the *AGT* 3'UTR inserts

The three inserts with *att*B1 and *att*B2 modifications at their 5' and 3' ends respectively were generated by PCR of genomic DNA using primers AGTEF and AGTER (Table 2.11). AGTEF and AGTER had an *att*B1 or *att*B2 sequence at their 5' ends respectively.

Three samples, which had been genotyped for the 3'UTR /flanking region SNPs, were used for these PCRs. Each sample was known to be homozygous for one of the three common haplotypes in the 3'UTR/flanking region. A 1660-bp genomic region from 11259-bp to 12919-bp in the published sequence was amplified. PCR was carried out as described in section 2.5.2. The presence of PCR products was confirmed by agarose gel electrophoresis as described in section 2.5.3 by electrophoresing for 30 minutes at 50 volts on a 1% agarose gel. The haplotype structure of the inserts is listed in table 2.12.

Primer Name	Sequence (5' – 3')	Orientation	Annealing Temperature (C^0)	Product Size (bp)
PCR Primers				
AGTEF	*GGGG ACA AGT TTG TAC AAA AAA GCA GGC T*TT TAG CGC GGG ACT ACT GTT	Sense	58	1660
AGTER	*GGGG AC CAC TTT GTA CAA GAA AGC TGG GT*G AAT GTT CGT ATT TTG TAT CCT TG	Anti-sense		
Sequencing Primers		**Orientation**	**Position on Sequence***	
FRS3	ATT GCC TTC GGT TTC TAT	Sense	11383-11400	
SDEN3	CAT CTG TGT GAT GGA CAG G	Sense	11573-11591	
SSP2	GTT CGG AAT CTT GGG GAG GA	Sense	11781-11800	
SSP1	CAC GGG GAT TAC AAA TCT CG	Sense	12027-12046	
SDEN6	TCA GAA TCT GCA GTT TTA CA	Sense	12218-12237	
H2654	TGG AGG CTT AGT GTG GCA AGA	Antisense	12623-12643	
ASP4	GGA CAG CAT CAC CAT TGC TA	Antisense	12093-12112	
DEN2	CTT CCC TGT CAC CCT CTT	Antisense	11807-11824	
RV4	GAC GAT AGT CAT GCC CCG CG	Antisense	on vector**	

Table 2.11. PCR primers used to amplify the *AGT* exon 5 and 3'UTR/flanking region and the sequencing primers used to confirm the sequence of the enhancer insert in destination vectors. Gateway® modifications in the 5' ends of PCR primers are in *italics*. * Position of primer relative to transcription start site on *AGT* gene. The insert spans from 11259 to 12919. ** downstream of insert.

Construct Name	Polymorphisms					
	11535C>A	**11608C>A**	**12058G>A**	**12194A>C**	**12429C>T**	**12822T>C**
E1	C	C	G	A	C	C
E2	C	C	G	A	C	T
E3	A	T	G	A	C	T

Table 2.12. A summary of the haplotype structure of the three *AGT* 3'UTR/flanking region inserts.

2.7.1.2. BP recombination reaction

The inserts were cloned into pGL3Promoter vectors using the Gateway® cloning system. The BP recombination reaction was set up with 100fmol of PCR products, 2µl of 150ng/µl of the pDONR® 201 vector, and 4µl of the 5 × BP Clonase® reaction buffer, and 1 × TE to make up a final volume of 16µl. BP Clonase® enzyme was removed from –70°C, vortexed briefly and added to the above reaction mix. This reaction mix was vortexed briefly twice and then incubated at 25°C for 1 hour. Then 2µl proteinase K was added to the reaction mix and the reaction was incubated for 37°C for 10 minutes.

2.7.1.3. Transformation of 5αDH competent cells

The entry clone that was generated by the BP recombination reaction was transformed into 5αDH competent cells. To do so 1µl from the BP recombination reaction mix was applied to a 50µl aliquot of 5αDH competent cells and mixed gently. Then it was incubated on ice for 30 minutes and maintained for 30 seconds at 42°C in a water bath before being immediately transferred on to ice. 450µl of SOC medium was then added to the reaction mix and it was incubated at 37°C for 1 hour with horizontal shaking at 225 rpm. 20µl and 100µl aliquots of transformed 5αDH competent cells were then plated on culture plates with LB medium and incubated overnight at 37°C. The plates had kanamycin as the selective antibiotic. The next morning the plates were transferred into a refrigerator. In the afternoon five single colonies were picked up and applied separately to 20ml universal tubes containing 5ml LB

medium with kanamycin. These tubes were incubated overnight at 37^{o}C with horizontal shaking at 225 rpm.

2.7.1.4. Confirmation of the presence of the insert by PCR

The following morning, to confirm the presence of the inset in entry clones in the 5αDH cells growing in each tube, a PCR reaction was set up with 1μ of the culture and AGTEF and AGTER primers as described in section 2.5.2. The initial denaturation of this PCR, however, was conducted for 12 minutes and *Taq* DNA polymerase was added to the reactions at the end of the initial denaturation step. The presence of a 1660-bp PCR products was confirmed by agarose gel electrophoresis as described in section 2.5.2 by electrophoresing for 30 minutes at 50 volts on a 1% agarose. This confirmed the presence of the inset in entry clones growing in the transformed 5αDH cells in culture.

2.7.1.5. Confirmation of the presence of the insert by restriction digestion

2ml of culture medium that had been confirmed to contain 5αDH cells with the insert were next subjected to miniprep as described in section 2.5.7.3. The presence of the insert sequence in the entry clones in this miniprep was confirmed again using a restriction digestion assay. There were two recognition sites for the restriction enzyme *Ssp*I in the entry clone. One was within the insert sequence and the other was in the vector sequence. It was predicted that digestion of miniprep entry clone DNA with *Ssp*I would result in two restriction fragments of 2517-bp and 1664-bp. The results of restriction

digestion confirmed this.

2.7.1.6. LR recombination reaction

The LR recombination reaction was set up with 300ng of entry clone miniprep DNA, 300ng of pGL3P3'GW, 4µl of the 5 × LR Clonase® reaction buffer, and 1 × TE to make up a final volume of 16µl. LR Clonase® enzyme was removed from –70°C, vortexed briefly and added to the above reaction mix. This reaction mix was vortexed briefly twice and then incubated at 25°C for 1 hour. Then 2µl of proteinase K was added to the reaction mix and the reaction was incubated for 37°C for 10 minutes.

The expression clone that resulted from the LR recombination reaction was transformed into to 5αDH competent cells as described in section 2.7.1.3 above. The only exception was that the cells were grown in culture medium containing ampicillin instead of kanamycin. Following this procedure the presence of the insert sequence was confirmed by PCR as described in section 2.7.1.4. Next a miniprep of the expression clone was made as described in section 2.5.7.3 and the entire sequence of the insert was confirmed by sequencing. The sequencing primers are listed in table 2.11.

2.7.1.7. Endotoxin free midi prep of expression vector

5αDH cells containing expression vector plasmids were cultured overnight in 20ml of LB medium containing ampicillin in a 50ml centrifuge tube at 37°C

with horizontal shaking at 225 rpm. An endotoxin free midiprep was made using this culture as described in section 2.5.7.3. The endotoxin free midi preparations of these plasmids were then used for the transfection experiments.

2.7.2. Transfection of experimental plasmid constructs into HepG2 cells and reporter gene assay

2.7.2.1. Transfection assay

In preparation for the assay, HepG2 cells were trypsinised from flasks and then diluted in an appropriate volume of EMEM sufficient to plate on either one or two 12 well plates depending on the requirement. 1.2ml of this HepG2 suspension was then aliquoted into each well. The cells were grown in these plates until they had reached 60-70% confluence.

The transfection mix for each well consisted of 200ng of vector DNA, 20ng p*Renilla, and* 1µl Tfx20 reagent suspended in 400µl of serum free EMEM. The vector DNA was one of either the recombinant experimental vector (pGL3Promoter with 3' *AGT* enhancer insert) or pGL3Promoter containing SV40 promoter or pGL3Control containing both SV40 promoter and enhancer. The latter two were the positive controls of the assay. All assays were performed in triplicate and each experiment was performed at least three times. Serum free EMEM contained all components of complete EMEM listed above except for fetal bovine serum. The sum of the total serum free medium

for each experiment was aliquoted and the required p*Renilla* was added to the total volume. This ensured that the p*Renilla* concentration was uniform in all wells for each experiment. A 1.2ml aliquot of the solution was prepared for each triplicate experiment. To these 1.2 ml aliquots the total vector DNA requirement (600ng of each vector DNA) was added. The transfection reagent (Tfx20) was added to create a 3:1 ratio of DNA to Tfx reagent. This ratio can be varied with most transfection occurring most efficiently at ratios from 2:1 to 4:1. The ratio of 3:1 had been determined as optimum for the HepG2 cell line previously in our laboratory (Marsters, 2000). The mixture was vortexed and incubated at room temperature for 15 minutes. Whilst this incubation proceeded, the cells were washed twice with PBS. After incubation, 400µl of each transfection mix was added to the appropriate well. The transfection reaction was incubated at 37^{o}C with 5% CO_2 for 1 hour. Following this, 800µl of complete EMEM was added, and the cells incubated at 37^{o}C in a humidified incubator with 5% CO_2 for 24 hours.

2.7.2.2. Stimulation

After 24 hours, the cells were either maintained under basal conditions or stimulated with AngII. To maintain cells in a basal state, the overnight culture medium was removed; cells were washed with PBS, and 1.2 ml of serum free EMEM was added to each well. To stimulate cells, the same procedure was followed, but AngII was added to the serum free EMEM. HepG2 cells were stimulation with AngII at concentrations of 10^{-13}, 10^{-11}, and 10^{-9}M. The

rationale for using these concentrations of AngII was that they span the physiological range of AngII, and it has been shown that, at least in the chorionic plate vasculature, such concentrations are physiologically active *in vitro* (Semple, 1977, Odum and Broughton Pipkin, 1987).

2.7.2.3. Harvesting of cells

After 24 hours, both non-stimulated and stimulated cells were washed twice with PBS ensuring that all PBS was removed, and then 200µl of 1 × passive lysis buffer was added to each well. Following this, the cells were incubated for 15 minutes at room temperature. The cells were then scraped off the base of the well. The solution from each well was collected into a separate 1.5ml microcentrifuge tube. The tubes were centrifuged briefly before the reporter gene assay to sediment any cell debris.

2.7.2.4. Reporter gene assay

Expression of the reporter gene in the harvested cells was analysed using the Promega Dual Luciferase® Reporter Gene assay kit on a TD 20/20 luminometer (Turner Designs, USA). To perform the assay, 20µl of the cell lysate supernatant was added to a luminometer tube, and the tube was placed on the luminometer. Then 50µl of 1 × LARII was added to the luminometer tube and the firefly luciferase activity was measured for 10 seconds following a 2 second delay. Next 50µl of 1 × Stop and Glow® solution was added to the

reaction mix in the luminometer tube. Then the luminescent activity of *Renilla* luciferase was measured for 10 seconds following a 2 second delay. This was repeated for the sample from each well.

2.7.3. Study of background luminescence

It is possible that there is background luminescence emanating from HepG2 cells and dual luciferase assay reagents. It is necessary to differentiate that from true luminescence. To investigate background luminescence a 12 well plate was plated with non-transfected HepG2 cells as was done in preparation for a transfection assay as described in paragraph one in section 2.7.2.1. Once it had reached 60-70% confluence, complete EMEM was removed, cells were washed twice with PBS, each well filled with 400µl of serum free EMEM, and incubated at 37^{o}C with 5% CO_2 for 1 hour. Then 800µl of complete EMEM was added to each well and the cells were incubated at 37^{o}C in a humidified incubator with 5% CO_2 for 24 hours. Following that cells were harvested as described in section 2.7.2.3 and a reporter gene assay was conducted as described in section 2.7.2.4.

2.8. Software tools and electronic database information

The software and electronic databases used in these investigations are listed below:

- SPSS for Windows, standard version release 11.0.0, 2001 (SPSS Inc., USA) was used to maintain phenotyping databases and for statistical analysis.
- Sample Size Calculator (SPSS Inc, USA) and QUANTO version 0.5 (Gauderman, 2002) were used for power calculations.
- GenBank at the National Centre for Biotechnology Information (NCBI), USA was searched to obtain DNA sequences and gene mapping information. This is a free database that can be accessed online at URL: http://www.ncbi.nlm.nih.gov
- Primer3 on the WWW for general users and for biologist programmers (Rozen and Skaletsky, 2000) was used to design oligonucleotide primers for PCR. This is a free program that can be accessed online at URL: http://www.broad.mit.edu/genome_software/other/primer3.html
- Webcutter 2.0 was used to analyse restriction enzyme recognition sites on DNA sequences. This is a free program that can be accessed online at URL: http://www.firstmarket.com/cutter
- Group centile calculator software (Gardosi and Francis, 2000) was used to calculate customised birth weight percentiles (Gardosi, Chang, Kalyan, Sahota and Symonds, 1992, Gardosi, Mongelli, Wilcox and Chang, 1995). This is a free programme distributed by the authors over the Internet at

URL: http://www.gestation.net/gest

- The COCAPHASE program of the UNPHASED program suite (Dudbridge, 2002, 2003) was used to estimate haplotype frequencies from genotype data, measure linkage disequilibrium (see section 2.9.2) and to perform hypothesis testing using haplotypes in case-control candidate gene pre-eclampsia association studies. It is available to registered users from the Rosalind Franklin Centre for Genetic Research website at URL: http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased
- The QTPHASE program of the UNPHASED program suite was used to estimate haplotype frequencies from genotype data and to perform hypothesis testing using haplotypes in candidate gene quantitative trait association studies.
- MatInspector Release Professional 6.2.1 (Quandt, Frech, Karas, Wingender and Werner, 1995) was used to examine DNA sequences for transcription factor binding sites. This is a free program that can be accessed online at URL: http://www.genomatix.de

2.9. Statistical Methods

2.9.1. Analysis of phenotyping data of women with pre-eclampsia and normotensive pregnant women

Phenotyping data were entered directly into a SPSS database. Data was verified manually for errors. In the phenotype analysis, summary values are presented as mean (standard deviation (SD)) when data were normally distribution and as median (inter quartile range) when not. The χ^2 or Fisher exact tests were used to determine departures from the hypothesised distribution of categorical data. Student-t test was used to test for differences between groups where the data were normally distributed and appropriate non-parametric tests when the distribution was not normal.

2.9.2. Analysis of population genetic data

χ^2 test was used to test the genotypes at each polymorphic locus for HWE and to compare allele frequency differences between racial groups. The COCAPHASE program of the UNPHASED suite of programs was used to estimate haplotype frequencies and to measure linkage disequilibrium (*D'* and r^2) between genotypes at pairs of polymorphic sites. This programme uses the EM algorithm to obtain maximum likelihood haplotype frequency estimates.

2.9.3. Analysis of case-control candidate gene pre-eclampsia association data

Analysis of HWE and single locus allele frequency comparisons were

performed as described above in section 2.9.2. HWE was tested at each polymorphic locus for case and control groups separately. Haplotype frequency estimation and hypothesis testing was conducted using the COCAPHASE program. This program performs a likelihood ratio test that examines the differences between haplotype frequency profiles between the case and control groups and computes a likelihood ratio statistic for the estimated haplotype frequency likelihoods for the combined case and control groups (Zhao, Curtis and Sham, 2000). Rare haplotypes occurring in frequencies of ≤0.05 were dropped from the analysis in both case and control groups unless otherwise stated.

2.9.4. Analysis of candidate gene quantitative trait association data

The association of the *AGT* gene with systolic and diastolic blood pressures at booking in normotensive pregnant women, and the association of *AGT, EGF* and *TGFA* haplotypes with birth weight of their babies, were analysed using the QTPHASE programme of the UNPHASED software suite. This programme is similar to the COCAPHASE programme, but it uses a generalised linear model for a quantitative trait. The test assumes that the trait is normally distributed with its mean dependent only on the additive effects of haplotypes (Abecasis, Cardon and Cookson, 2000). This program performs a likelihood ratio test for the quantitative trait as described above. ANOVA was used to test the association of individual SNP genotypes with quantitative traits.

2.9.5. Analysis of reporter gene assay data

Average ratios for firefly and *Renilla* luciferase activity were calculated for each triplicate after excluding any outliers (lying outside mean ± 4×SD). The activity of the constructs under basal and stimulated conditions was compared with the activity of the pGL3Promoter vector under basal conditions to calculate the increase/decrease in response. Furthermore, the activity of the constructs under stimulated conditions was compared with the activity of the constructs under basal condition to calculate the increase/decrease in response. Differences in response between the constructs under basal and stimulated conditions were analysed using ANOVA with Tukey HSD *post hoc* after $\log^{-10}$ transformation.

2.10. Polymorphism nomenclature

The nine polymorphisms that were studied in this thesis are referred to by the common names used to refer to them in scientific literature so that there is no confusion when comparisons are made. The only polymorphism that had not been named previously was the *EGF* 67149A polymorphism, which was named by the author based on the position of the polymorphism relative to the transcription start site of the gene on chromosome 4 (GenBank: gi|27477929:440000-550000 homo sapiens chromosome 4 genomic contig)

There is however, a move to standardise nomenclature for description of sequence variations (Antonarakis, 1998, den Dunnen and Antonarakis, 2000). The polymorphisms studied in this thesis together with their corresponding standardised descriptions based on the above-mentioned recommendations are listed in table 2.13.

Polymorphism	Standardised Nomenclature
EGF 61G>A	AJ131611:g.139G>A
EGF 67149G>A	NM_001963:c.2566G>A
TGFA 3822G>A	AF123243:g.599G>A
TGFA 3827T>C	AF123243:g.604T>C
TGFA 3851T>C	AF123243:g.628T>C
AGT 174Thr>Met	NM_000029:c.659C>T
AGT 235Met>Thr	NM_000029:c.842T>C
AGT 11535C>A	M24689:g.834C>A
MTHFR 677C>T	NM_005957:c.716C>T

Table 2.13. A list of the polymorphisms studied in this thesis and a description of the polymorphism using standardised nomenclature (all accession numbers are from GenBank).

3. RESULTS

3.1. The pre-eclampsia phenotype

This section was aimed at analysing the phenotype of the 180 Sinhalese women with pre-eclampsia and comparing it with the 180 normotensive pregnant women from the same population. It was also hoped to compare this data with both previously published data and the phenotyping data of the 74 white Caucasian women with pre-eclampsia and the 81 normotensive pregnant women. It was not possible to compare the Sinhalese data with other published studies as none of the studies reporting morbidity and mortality among women with pre-eclampsia were confined to nullipara. There are several carefully designed clinical trials conducted in nulliparous women that report on maternal and fetal morbidity and mortality (Hauth, Goldenberg, Parker, Philips, Copper *et al.*, 1993, Sibai, Caritis, Thom, Klebanoff, McNellis *et al.*, 1993, Kyle, Buckley, Kissane, de Swiet and Redman, 1995, Levine, Hauth, Curet, Sibai, Catalano *et al.*, 1997), but none of them report this information for women with pre-eclampsia separately, to enable comparison. The Sinhalese phenotype therefore, was compared with that of the white Caucasians. Where phenotyping data for the white Caucasians is incomplete (see section 2.4.2) the number of subjects analysed to derive the result is given. In this section of this thesis the presentation of results is combined with discussion.

3.1.1. Demographic characteristics

The demographic characteristics of the Sinhalese and the white Caucasian women are in table 3.1. The Sinhalese women were matched for age and BMI. The white Caucasians were not matched, and the women with pre-eclampsia had a significantly higher BMI (P=0.001). When the BMI of women with pre-eclampsia was considered, a majority (89.4%) of Sinhalese women had a BMI of <26 kg/m^2. BMI of ≥30kg/m^2 was an exclusion criterion for recruitment of Sinhalese women. However, only eight (2.2%) out of 356 women with 'pre-eclampsia' referred for recruitment were not recruited for this reason (see table 2.4). These results were expected as the prevalence of obesity defined as BMI of >25 kg/m^2 in Sri Lankan women between the ages of 30 to 44 years was reported to be 8.2% (Fernando, Siribaddana, De Silva and Perera, 1994). Among the white Caucasian women, only 44.1% (15/34) had a BMI of <26 kg/m^2. All Sinhalese women were nulliparous, but 20 women with pre-eclampsia (20/180, 11%) were in their second pregnancy. Among the white Caucasians 85% (57 pre-eclamptic (PE) + 58 normotensive (NT); 115/136) were in their first pregnancy, 14% (8 PE + 11 NT; 19/136) in their second, and 1% (1 PE + 1 NT; 2/136) in their third pregnancy. The average age of the Sinhalese and the white Caucasian women with pre-eclampsia as well as those of normotensive pregnant women were comparable. Among the white Caucasians 10.8% (8/66) were smokers and 1.4% (1/66) was an ex-smoker, but none of the Sinhalese women had ever smoked. This was not surprising as smoking is very rare among Sinhalese women in Sri Lanka.

		Sinhalese			White Caucasians		
		Pre-eclamptic (n=180)	**Normotensive (n=180)**	***P***	**Pre-eclamptic (n=74)**	**Normotensive (n=81)**	***P***
Mean Age (Years)	(SD)	27.1 (5.4)	27.2 (5.2)	0.96	27.9 (5.8)	27.7 (4)	0.75
Mean Weight (kg)	(SD)	49.6 (8.8)	48.8 (8)	0.33	68.7 (11.3)	64.5 (9.8)	0.05
Mean Height (cm)	(SD)	153.6 (5.5)	154.0 (6.4)	0.98	162.2 (5.8)	164.7 (7.1)	0.03
Mean Body Mass Index (kg/m^2)	(SD)	21.0 (3.4)	20.7 (3.2)	0.34	26.2 (4.1)	23.7 (3.1)	<0.01
Nulliparous	n(%)	180 (100)	180 (100)		74 (100)	81 (100)	
Ex and Current Smokers	n(%)	0 (0)	0 (0)		9/66 (13.6)	8/70 (11.4)	0.62

Table 3.1. Demographic characteristics of the Sinhalese and the white Caucasian women with pre-eclampsia and normotensive pregnant women. SD: Standard Deviation. Significance (*P)* is for comparison between women with pre-eclampsia and normotensive pregnant women in each population. Note that the Sinhalese women were matched for BMI at recruitment; no such matching was done for the white Caucasian women.

3.1.2. Antenatal booking

Characteristics of both groups of women in each population at the antenatal booking are in table 3.2. There was no difference in the gestation at booking of the Sinhalese and the white Caucasian women with pre-eclampsia and normotensive pregnant women. The blood pressure at booking, however, was significantly higher among the Sinhalese and the white Caucasian women who subsequently developed pre-eclampsia compared to their normotensive counterparts. In the white Caucasians, this difference in blood pressure was greater than in the Sinhalese. The systolic and diastolic blood pressures of the white Caucasian normotensive pregnant women were also higher than that of the Sinhalese normotensive pregnant women (SBP: $P<0.001$, DBP: $P=0.018$).

The differences in blood pressure in the Sinhalese and the white Caucasians are interesting. The white Caucasian women had their antenatal booking later in pregnancy than the Sinhalese women, at a time when a greater physiological reduction in blood pressure was expected. However, their average SBP was nearly 15mmHg higher than that in the Sinhalese. A rise in SBP of ≥30mmHg and/or DBP of ≥15mmHg from blood pressure measurements taken at the pre-conception stage or in the first trimester had at one time been recommended and used by many to establish the diagnosis of pre-eclampsia (National High Blood Pressure Education Program Working Group, 1990, Australasian Society for the Study of Hypertension in Pregnancy, 1993). In the current study, on average, the Sinhalese women had

to have a 25% rise in their SBP from the antenatal booking average of 111.7 mmHg to reach the diagnostic threshold of 140mmHg, compared with 12% for the white Caucasian women. The difference in the rise in DBP that was required was not as marked (19mmHg (27%) in the Sinhalese cf. 20mmHg (22%) in the white Caucasians). If the Sinhalese women were to begin pregnancy at a lower blood pressure than the white Caucasians and the absolute rise in SBP during pregnancy over pre-pregnancy blood pressure levels was more important than reaching a particular diagnostic blood pressure threshold, then that may perhaps explain why many Sinhalese women have severe disease by the time they are diagnosed with hypertension in pregnancy than women in the West (see section 1.1.1).

There were differences in the pulse pressure as well. The average pulse pressure in normotensive women at booking was 40mmHg and 55.7mmHg in the Sinhalese and in the white Caucasians respectively. In Sri Lanka Korotkoff phase IV (K5) is used to record the DBP. When the white Caucasian women were recruited over a decade ago in Nottingham K4 was recommended to record the DBP in pregnancy in UK. If K5 were recorded in these women then the pulse pressure would have been even higher. There are no studies of the pattern of blood pressure of Sinhalese women in pregnancy. The average SBP and DBP of normotensive Sinhalese women at booking in this study is as expected lower than the average SBP (117.5mmHg) and DBP (78.2mmHg) reported in a group of non-pregnant women aged between 20 to 40 years

		Sinhalese			White Caucasians		
		Pre-eclamptic (n=180)	**Normotensive N(=180)**	***P***	**Pre-eclamptic (n=74)**	**Normotensive n=(70)**	***P***
Gestation at booking (weeks)	M(iqr)	13.6 (10.7-16.0)	13 (10.7-15.4)	0.449	17 (14-18)[1]	17 (12-18)[3]	0.578
Blood pressure at booking							
Mean Systolic (mm Hg)	(SD)	111.7 (9.9)	109 (9.3)	0.002	125.2 (14.2)[2]	120.9 (12.9)[4]	0.066
Mean Diastolic (mm Hg)	(SD)	71.1 (7.3)	69 (7.1)	0.002	70.2 (8.9)[2]	65.2 (8)[4]	0.001

Table 3.2. A comparison of antenatal booking data and data at diagnosis of the Sinhalese and the white Caucasian women with pre-eclampsia and normotensive pregnant women. SD: Standard Deviation. M(iqr): median (inter quartile range). The white Caucasian results were derived from data of [1]35, [2]65,[3]69, and [4]70 subjects. Significance (*P)* is for comparison between women with pre-eclampsia and normotensive pregnant women in each population.

in Sri Lanka (Mendis, Athauda, Naser and Takahashi, 1999a). The average pulse pressure of those women (38.2mmHg), however, is similar to that in the current study.

As expected the blood pressure recordings in both countries that were retrieved from case notes show end digit preference. As a result, in many women the recorded blood pressure would have underestimated their true blood pressure.

3.1.3. Diagnosis and management

Characteristics of both groups in each population at diagnosis and delivery are summarised in table 3.3. Most Sinhalese women were diagnosed (124, 69%) and delivered (120, 66%) before 37 weeks of gestation. 106 (58.9%) delivered within 48 hours of the diagnosis being made. Overall 140 (77.8%) women needed a Caesarean section for delivery. The majority of the white Caucasian women (36/66, 54.5%) delivered at term and required a Caesarean section (35/65, 53.8%).

The Sinhalese women with pre-eclampsia on average had lengthy hospital stays. The number of in patient days in hospital from diagnosis to discharge ranged from two to 57 days (Median=9). Only 15 (7.2%) stayed in hospital for less than three days. They were women who had ‘mild’ disease who left the hospital early against medical advice. 160 (76.8%) spent more than five days.

		Sinhalese			White Caucasians		
		Pre-eclamptic n=180	**Normotensive n=180**	***P***	**Pre-eclamptic n=74**	**Normotensive n=81**	***P***
Gestation at presentation (weeks)	M(iqr)	35 (31-38)			35 (32-37)[1]		
<34 Weeks	n(%)	77 (43)			13 (34)		
34-36 Weeks	n(%)	47 (26)			14 (37)		
37-42 Weeks	n(%)	56 (31)			11 (29)		
Gestation at delivery (weeks)	M(iqr)	35 (32-38)	40 (39-40)	<0.001	37 (34-39)[2]	40 (39-41)[4]	<0.001
<34 Weeks	n(%)	69 (38)			13 (20)		
34-36 Weeks	n(%)	51 (28)			17 (26)		
37-42 Weeks	n(%)	60 (33)	180 (100)		36 (55)	69 (100)[4]	
Caesarean deliveries	n(%)	140 (78)			33 (54)[3]		
Diagnosis to delivery (days)	M(iqr)	0 (0-3)					
Delivery to discharge (days)	M(iqr)	7 (5-12)					
Diagnosis to discharge (days)	M(iqr)	9 (5-16)					

Table 3.3. A comparison of data on gestation at diagnosis and delivery, mode of delivery, and the hospital stay of the Sinhalese and the white Caucasian women with pre-eclampsia and the gestation at delivery of the Sinhalese and the white Caucasian normotensive pregnant women. M(iqr): median (inter quartile range). The white Caucasian results were derived from data of [1]38, [2]66, [3]65 and [4]69 subjects.

Three women were managed expectantly for more than four weeks before delivery. Four women were in hospital for more than four weeks postpartum to accompany their babies who required prolonged neonatal care.

3.1.4. Maternal morbidity and mortality

Many Sinhalese and white Caucasian women experienced complications that are associated with adverse maternal and fetal outcomes (Mattar and Sibai, 2000). These included severe blood pressure (SBP≥160mmHg and/or DBP≥110mmHg), severe proteinuria (HCT ≥3+ or Dipstick test ≥3+ or ≥3g/day on 24 hour collection), elevated liver enzymes (AST ≥70IU and/or ALT ≥70IU), placental abruption, eclampsia, and even maternal death. The vast majority of Sinhalese women had severe blood pressure and proteinuria. Two women developed a combination of five of these severe complications. The first woman was diagnosed at 37 weeks of gestation, and her pregnancy was terminated immediately by Caesarean section; both the mother and the baby survived. The second woman was diagnosed at 24 weeks of gestation, her pregnancy too was terminated, but neither mother and nor baby survived.

Since there is clear evidence that early onset pre-eclampsia is a more severe disease than late onset pre-eclampsia in Western populations (von Dadelszen, Magee and Roberts, 2003) data on complications in Sinhalese women is presented in table 3.4 dichotomising it into early (<34 weeks) and late onset groups by gestational age as has been suggested recently (von Dadelszen *et*

al., 2003). Women with early onset pre-eclampsia seem to experience more complications compared to women with late onset pre-eclampsia, although the differences did not reach statistical significance. Eclampsia, however, was slightly higher among women with late-onset pre-eclampsia. This discrepancy may be explained by the $MgSO_4$ therapy given as eclampsia prophylaxis; 46% of women with early onset-pre-eclampsia received $MgSO_4$ while only 29% of women with late-onset pre-eclampsia received it (*P*=0.02). Moreover, those who had early onset pre-eclampsia were more likely to receive closer monitoring in an intensive care unit (ICU) and more aggressive antihypertensive therapy as evidenced by the number of different antihypertensives used. In addition, in 50% of women with eclampsia (5/10) in the late onset group, the presenting symptom was eclampsia. In spite of the severe morbidity in both early onset and late onset groups, overall only 53% of women received care in an ICU because there were only six ICU beds in these two hospitals.

A relatively high proportion (16, 8.9%) of the Sinhalese women with pre-eclampsia also developed eclampsia. There was no difference between the age of the women who developed eclampsia and those who did not (26.1 (6.6) cf. 27.2 (5.4); *P*=0.41), but they had lower BMI at the first antenatal visit (19.3 (1.9) cf. 21.2 (3.4); *P*=0.03). The SBP (107.1 (12.7) cf. 112.0 (9.6); *P*=0.08) and the DBP (68.6 (6.6) cf. 71.3 (7.3); *P*=0.18) at booking, the SBP (160.6 (21.4) cf. 155.7 (14.4); *P*=0.22) and the DBP (103.1 (13.6) cf. 102.1 (9.1);

P=0.22) at diagnosis and the highest recorded SBP (168.8 (18.2) cf. 166.2 (16.1); *P*=0.55) and the highest recorded DBP (115.0 (11.3) cf. 114.1 (10.0); *P*=0.75) were not significantly different in women who had eclampsia and these who did not. Eclampsia occurred before delivery in 14 women and postpartum in two women. Six delivered before 34 weeks, six between 34 and 36 weeks, and four at term. Only one baby was born alive and six (33%) of the babies were small for gestational age. One of these women died.

The white Caucasian women too had severe hypertension and proteinuria. 29.7% (22/74) had SBP≥160mmHg and/or DBP≥110mmHg. At recruitment all white Caucasian women with pre-eclampsia had significant proteinuria of ≥500mg/day on a 24-hour urine protein estimations and/or ≥2+ dipstick testing. Of these women six had proteinuria of ≥3.0g/day and 10 had a dipstick recording of ≥3+. Overall, 21.6% had proteinuria of ≥3.0g/day or ≥3+ on dipstick testing.

In addition to this, 10% (6/66) had thrombocytopaenia, but the liver enzyme levels were not available for them. None of them had eclampsia. In contrast to the Sinhalese women with pre-eclampsia, nearly all of whom (99%) required antihypertensive treatment, only 39% (25/64) of the white Caucasian women with pre-eclampsia received antihypertensive therapy and of them 14 were treated with $MgSO_4$. In addition to them, three others had also received $MgSO_4$ (27%, 17/63). The need to prolong pregnancy by medical intervention

Complication / Treatment	Early Onset (<34 Weeks) n=77 No (%)	Late Onset (≥35 Weeks) n=103 No (%)	*P*
Complications			
SBP ≥160mmHg and/or DBP ≥110mmHg	73 (94.8)	88 (85.4)	0.04
Proteinuria (≥3+ HCT)	65 (84.4)	91 (88.3)	0.44
Renal impairment requiring dialysis	3 (3.9)	1 (0.9)	0.18
Thrombocytopaenia (<100 000/µl)*	14/75 (18.7)	9/101 (8.9)	0.06
Elevated liver enzymes (AST or ALT≥70 IU/L)**	12/65 (18.5)	7/63 (11.1)	0.24
Placental abruption	4 (5.2)	3 (2.9)	0.43
Eclampsia	6 (7.8)	10 (9.7)	0.66
Maternal death	1 (1.3)	0 (0)	0.43
Treatment			
ICU care	54 (70)	41 (40)	<0.01
$MgSO_4$ therapy	35 (46)	30 (29)	0.02
≥2 antihypertensives	72 (94)	69 (67)	<0.01

Table 3.4. Maternal complications in Sinhalese women with pre-eclampsia and the treatment received by them: SBP – Systolic Blood Pressure, DBP – Diastolic Blood Pressure, AST – Aspartate Transaminase, ALT – Alanine Transaminase, HCT – The urine protein heat coagulation test. * Platelet counts were not available for four women. **AST/ALT levels were not available for 52 women.

when the diagnosis is made remote from term increases the likelihood of antihypertensives being used. This could be one explanation for the reason why more Sinhalese women received antihypertensive therapy than did white Caucasian women as only 33% of the Sinhalese women delivered at term compared to 55% of the white Caucasian women. Other factors that would influence the use of antihypertensives are local protocols that vary between hospitals and difference in facilities available for close monitoring of patients in different hospitals.

3.1.5. Perinatal morbidity and mortality

The outcome for the babies of Sinhalese women is summarised in table 3.5. Of them 106 (58.9%) were male. Others have also reported such distortions in the sex ratio of babies born to women with pre-eclampsia. Its significance, however, is not clear because in large studies such as the GOPEC study the sex ratio is almost 1:1 (Broughton Pipkin, F. Personal Communication). Fourteen (7%) pregnancies resulted in stillbirths. The neonatal morbidity and mortality among the Sinhalese babies was much more severe than among the white Caucasian babies. In total, 114 (68.7%) of the 166 babies who were a live at birth received care in a special care baby unit (SCBU). Thirty of them died during the immediate postpartum period, before the mother was discharged from hospital. Of the 136 babies who were taken home, one had died at the age of six days. The condition of 65 others at six weeks postpartum could not be verified, as the mothers could not be contacted for follow up (see

section 3.1.5). Of them, 33 were delivered pre-term and 39 had received SCBU care. Even if we assume that all of them remained alive, overall 45 (25%) babies died during the perinatal period. The smallest surviving baby at six weeks postpartum weighed 750g at birth at 28 weeks of gestation. None of the other babies born following gestation periods shorter than that survived. Of the 77 babies born to mothers developing pre-eclampsia before 34 weeks of gestation, 12 (15.6%) were stillbirths and only 35 (46.7%) survived to go home with the mother. There were only three (2.9%) confirmed perinatal deaths among babies of women who developed late onset pre-eclampsia.

Among the white Caucasians 58% (39/67) of babies were male. All except one (6.6%) of the babies of the 15 women with early onset pre-eclampsia survived, as did all the babies of the 51 women with late onset pre-eclampsia. The PNMR therefore was 1.5% (1/66). Eleven of 13 (84.6%) babies born to women with early onset pre-eclampsia required SCBU care, in comparison to only three of 24 (12.5%) babies born to women with late onset pre-eclampsia.

Customised birth weight centile charts are not available for Sinhalese babies. Indian charts in the customised birth weight centile calculator software (Gardosi *et al.*, 2000) were therefore used as a surrogate to calculate the birth weight centiles of Sinhalese babies. This software takes into consideration the mother's ethnicity, height, weight at antenatal booking, parity, and gestational age at delivery and the baby's sex and birth weight to calculate an adjusted

birth weight that is compared with a standard ultrasound-derived curve for intra uterine weight gain to determine a customised birth weight centile for each baby (Gardosi *et al.*, 1992). Birth weights that were below the 5th centile were considered small for gestational age (SGA). 47.8% (86/180) of babies born to Sinhalese women with pre-eclampsia and 5% (9/180) of babies born to normotensive Sinhalese women were SGA. The latter observation supports the use of Indian charts as a surrogate for this Sri Lankan population. Among the white Caucasians, the percentage of SGA babies in pre-eclamptic and normotensive groups were 20.7% (7/34) and 5.7% (4/70) respectively. In the GOPEC study, which examined 1001 women with pre-eclampsia, 33.8% of all babies born to them were SGA. However, the percentage rose to 62.3% for babies delivered prior to the 34th week of gestation (Broughton Pipkin, F. Personal Communication).

In the current study, overall, the perinatal mortality among babies of Sinhalese women with pre-eclampsia is very high in comparison to babies of the white Caucasian women with pre-eclampsia. However, this is not entirely unexpected because it is evident from the data presented above that the Sinhalese women had much more severe disease than the white Caucasian women. In addition, the scarcity of neonatal care facilities would have also contributed to it. In the SCBU at the CSHW there were only 15 cots with incubators and four cots with ventilators to cater to more than 15 000 babies born there annually. The situation in the other recruiting hospital, DSHW,

where more than 10 000 babies are born annually, was not much better. In Nottingham UK, babies of women with pre-eclampsia occupy about 16% of SCBU cots (Broughton Pipkin, Personal Communication). In the two recruiting hospitals, although such data is not available, a much higher percentage of beds are bound to be occupied by babies of women with pre-eclampsia. National data for perinatal mortality is not available for Sri Lanka, as stillbirths are not registered in all areas. However, the overall PNMR in one of the recruiting hospitals, CSHW, was 1.79% and 1.76% in 2001 and 2002 respectively (Castle Street Hospital for Women, 2003). In comparison it was 25% in babies of Sinhalese women with pre-eclampsia. Among the white Caucasian women, the corresponding rate was 1.5%; this is nearly twice that of the PNMR of 0.83% in England and Wales in 2002 (Office for National Statistics, 2001). In the GOPEC study, the perinatal mortality rate was 2.4%, which is nearly three times that of the overall national rate (Broughton Pipkin, Personal Communication). This data while re-emphasising the severe morbidity in babies of Sinhalese women with pre-eclampsia, also highlight the urgent need to improve perinatal care services in Sri Lanka.

3.1.6. Postnatal resolution of blood pressure and proteinuria

Resolution of hypertension during the postpartum period (before the end of the 13th week) had been confirmed in all white Caucasian women. Postpartum follow up of Sinhalese women, however, was difficult as there was shared care between the recruiting hospital and the primary health care centre near the

woman's place of residence. It was further complicated because Sinhalese women go back to live with their parents following delivery. As a result, the primary health care centre that looked after a woman during the antenatal period may not be the centre that looked after her post partum. Every attempt was made, however, to contact the 179 women at variable times after 13 weeks postpartum to verify whether the condition had resolved. 65 women could not be contacted, but blood pressure and proteinuria had resolved in 26 of them prior to discharge from hospital. A further 110 were confirmed to be normotensive and non-proteinuric postpartum. The condition therefore, was confirmed to have resolved postpartum in 137 women. Four women, however, were on antihypertensives and among them three had had early onset pre-eclampsia. They were excluded from the case-control candidate gene pre-eclampsia association study. A decision, however, had to be made regarding the inclusion of the 39 women, in whom postpartum blood pressure resolution could not be confirmed. It was decided to include them because excluding them would have undermined the power of the study to detect a genetic contribution. It is hoped, that it would be possible to trace at least some of these women once the author returns to Sri Lanka, for a future publication, but it is likely to be time consuming.

3.1.7. Family history

In what is possibly the only study of its kind, Cincotta and Brennecke (1998), who prospectively related the risk of development of pre-eclampsia in a group

Outcome		Sinhalese			White Caucasian		
		Early Onset (n=77)	Late Onset (n=103)	NT (n=180)	Early Onset (n=15)	Late Onset (n=51)	NT (n=81)
Gestation at delivery (weeks)	M(iqr)	30 (28.4-32.1)	37 (35.4-38.7)	40 (38.6-40.4)	31 (29-33)	38 (36-39)	40[2] (39-41)
SGA (<5th percentile)	n(%)	52 (67.5)	34 (33.0)	9 (5.0)	4/9 (44.4)	3/25 (12.0)	4/70 (5.7)
Admission to SCBU	n(%)	62 (100)[1]	52 (50.4)	16 (8.9)	11/13 (84.6)	3/24 (12.5)	0/68 (0.0)
Perinatal losses							
Fetal death	n(%)	12 (15.6)	2 (1.9)	0 (0.0)	0/15 (0.0)	0/51 (0.0)	0/68 (0.0)
Postnatal death (up to six weeks)	n(%)	30 (39.0)	1 (0.9)	0 (0.0)	1/15 (6.7)	0/51 (0.0)	NA

Table 3.5. Perinatal outcome of babies of the Sinhalese and the white Caucasian women with pre-eclampsia and normotensive pregnant women. M(iqr): mode(inter quartile range). [1]Twelve babies were stillborn, and two died soon after deliver before transfer to SCBU, information on one baby whose mother was transferred out of the recruiting hospital to a district hospital for delivery on her request is not available. The white Caucasian data, except gestation at delivery, is presented as number/number of babies for whom the data item was available;[2] Derived from data of 69 women; NA - data not available.

of 368 primigravid women to their self reported family history of the condition, found a four fold increased risk of pre-eclampsia (relative risk (RR)=4.3, 95% CI, 1.6 to 11.5; *P*=0.017) among women who had a mother, sister or both who had had pre-eclampsia. In the current study as a part of phenotyping of Sinhalese women a four-generation pedigree was constructed and the history of the occurrence of hypertensive disorders of pregnancy in female relatives of either side of the family were recorded by questioning the woman. The response to the effect that a female family member developed 'high blood pressure' or 'fits' during pregnancy was taken as being indicative of a positive history of a hypertensive disorder during that woman's pregnancy. No attempt however, was made to trace case notes to verify this information. Similar data had been collected for the white Caucasian women. An analysis of the reported family history of high blood pressure or fits in pregnancy in Sinhalese and white Caucasian women is given in table 3.6. The white Caucasian data, although very limited in size, are in agreement with the data of Cincotta *et al.* (1998), showing that primigravid women with a positive family history of hypertensive disorders of pregnancy in her side of the family carry a three-fold increase risk of pre-eclampsia. It is difficult to explain why this finding could not be replicated in Sinhalese women. It cannot be explained by any social factor such as literacy or non-contact with their extended family that may have influenced awareness of the occurrence of such a devastating condition in a female relative as literacy among Sinhalese women is over 90% and because for the most part Sinhalese have closely knit

extended families. Among the white Caucasians in Nottingham, the increased risk mainly came from a positive history among their mothers and maternal grandmothers (data not shown). Almost equal numbers of sisters of Sinhalese women with pre-eclampsia (176) and normotensive pregnant women (178) had completed at least one pregnancy, but interestingly none of the Sinhalese women gave a positive history in their maternal grandmothers. This may be a result of either a lack of awareness on the part of the women or the severe maternal mortality among women who developed pre-eclampsia/eclampsia in Sri Lanka two generations back (Seneviratne *et al.*, 2000).

	Pre-eclamptic		Normotensive		Odds Ratio	95% CI
FH+ in the woman's family						
Sinhalese	19	(19/176,10.8%)	13	(13/178, 7.5%)	1.5	0.7 to 3.2
White Caucasians	12	(12/26,46.2%)	12	(12/54,22.2%)	3.0	1.1 to 8.2
FH+ in the partner's family						
Sinhalese	9	(9/173, 5.2%)	4	(4/178, 2.2%)	2.4	0.7 to 7.9
White Caucasians	4	(4/25,16.0%)	7	(7/46,15.2%)	1.1	0.3 to 4.0
FH+ in both sides of the family						
Sinhalese	28	(28/176,15.9%)	17	(17/178, 9.6%)	1.8	1.0 to 3.5
White Caucasians	12	(12/26,46.2%)	15	(15/53,28.3%)	2.2	0.8 to 5.8

Table 3.6. Analysis of the reported family history of high blood pressure or fits in Sinhalese and white Caucasian women with pre-eclampsia and normotensive pregnant women. FH+: A positive family history. Percentages were calculated using the formula: number with positive history/number of women for whom family history data was available×100.

3.2. Results of the population genetic analysis

The haplotype frequency estimates of the *EGF*, *TGFA*, and *AGT* genes and the frequency of the alleles of the *MTHFR* 677C>T polymorphism in each population is shown in table 3.7. The frequency of the variant allele of each polymorphism in the four candidate genes is shown in table 3.8. All polymorphisms in each population were in HWE.

Overall there was very little difference in haplotype frequencies between the three Sri Lankan racial groups. The only exception was the difference in the haplotype frequencies of the *TGFA* gene between the Sinhalese and the Moors. Two polymorphisms (3822G>A and 3851T>C), which had significantly different allele frequencies ($P<0.049$ and $P=0.005$ respectively) contributed to this. The only other significant difference between allele frequencies in any polymorphism among the three Sri Lankan racial groups was the difference in the *TGFA* 3851T>C polymorphism between the Sri Lankan Tamils and the Moors ($P=0.025$). The haplotype frequencies in the *EGF*, *TGFA* and *AGT* genes in all three Sri Lankan racial groups were significantly different to that of the white Caucasians ($P<0.01$). Exceptions to this were the *TGFA* gene for the comparison between the Sinhalese and the white Caucasians, and the *EGF* gene for the comparisons between the Sri Lankan Tamils and the white Caucasians, and between the Moors and the white Caucasians.

The haplotype structure of the *EGF*, *TGFA* and *AGT* genes were interesting. In the *EGF* gene, out of a possible four haplotypes, only the 61G/67149A and the 61A/67149G were common and the other two were rare. This was especially so in the three Sri Lankan racial groups. The 61G/67149G haplotype was also found in a frequency of 12% in the white Caucasians. In the *TGFA* gene, out of a possible eight haplotypes as expected, only four were found in all racial groups. In the *AGT* gene, out of a possible eight haplotypes, only six were present. Two of them were very rare. The 174Thr/235Met/11535A and 174Thr/235Thr/11535C haplotypes were common in all four racial groups. The 174Thr/235Met/11535C haplotype was common among the white Caucasians, but was much less frequent in all three Sri Lankan racial groups. This is because of the low frequency of the 235Met allele in all these racial groups compared to that in the white Caucasians ($P<0.001$ in each comparison). Almost all the 235Thr and 11535C alleles seem to occur on the same chromosome in all racial groups with the possible exception of the white Caucasians. The absence of some of the possible haplotype combinations and the differences in the frequency of the different haplotypes of each gene in these four racial groups is a result of linkage disequilibrium. The results of pair-wise linkage disequilibrium analysis between polymorphisms in each gene in each racial group are summarised in table 3.9.

Genes	Haplotypes			Haplotype Frequency				Significance (*P*)					
				S	T	M	W	S v T	S v M	T v M	S v W	T v W	M v W
EGF	***61***	***67149***											
	G	G		0.06	0.06	0.07	0.12						
	G	A		0.53	0.47	0.44	0.37	*	*	*	**0.012**	*	*
	A	G		0.36	0.42	0.45	0.48						
	A	A		0.05	0.04	0.05	0.03						
TGFA	***3822***	***3827***	***3851***										
	G	T	T	0.20	0.24	0.23	0.21						
	G	C	T	0.56	0.48	0.51	0.62	*	**0.007**	*	*	**0.001**	**<0.001**
	G	C	C	0.10	0.08	0.02	0.11						
	A	C	T	0.14	0.20	0.23	0.06						
AGT	***174***	***235***	***11535***										
	T	M	C	0.06	0.11	0.13	0.34						
	T	M	A	0.31	0.25	0.24	0.29						
	T	T	C	0.53	0.51	0.50	0.22	*	*	*	**<0.001**	**<0.001**	**<0.001**
	M	T	C	0.08	0.11	0.11	0.09						
	T	T	A	0.02	0.01	0.01	0.04						
	M	T	A	0.00	0.01	0.00	0.01						
MTHFR	**677**												
	C			0.87	0.91	0.91	0.69	*	*	*	**<0.001**	**<0.001**	**<0.001**
	T			0.13	0.09	0.09	0.31						

Table 3.7. Haplotype frequency estimates of the *EGF*, *TGFA*, and *AGT* genes and the allele frequency of the *MTHFR 677C>T* polymorphism in the Sinhalese (S), the Sri Lankan Tamils (T), the Moors (M), and the white Caucasians (W). **P*>0.05. *P* values for *AGT* haplotypes are based on the four common haplotypes. The *P* value for *MTHFR* is based on allele frequency.

Gene	Polymorphism	Variant Allele	Frequency of the variant allele			
			S	T	M	W
EGF	61G>A	A	0.41	0.46	0.50	0.51
	67149G>A	A	0.58	0.51	0.49	0.40
TGFA	3822G>A	A	0.14	0.20	0.23	0.06
	3827T>C	C	0.80	0.76	0.77	0.79
	3851T>C	C	0.10	0.08	0.02	0.11
AGT	174Thr>Met	M	0.09	0.11	0.11	0.11
	235Met>Thr	T	0.64	0.63	0.63	0.37
	11535C>A	A	0.33	0.27	0.25	0.34
MTHFR	677C>T	T	0.13	0.09	0.09	0.31

Table 3.8. Frequencies of the variant allele of the polymorphisms in the *EGF*, *TGFA*, *AGT* and *MTHFR* genes in the Sinhalese (S), the Sri Lankan Tamils (T), the Moors (M), and the white Caucasians (W).

Gene	Loci Combination		Sinhalese		Tamil		Moor		White Caucasian	
			D'	***r^2***	***D'***	***r^2***	***D'***	***r^2***	***D'***	***r^2***
EGF	61G>A	67149G>A	0.78	0.59	0.83	0.63	0.78	0.58	0.86	0.52
TGFA	3822G>A	3827T>C	1	0.04	1	0.08	1	0.09	1	0.02
	3822G>A	3851T>C	0.38	0.003	0.12	<0.001	0.06	<0.001	0.004	<0.001
	3827T>C	3851T>C	1	0.03	1	0.03	1	0.008	1	0.03
AGT	174Thr>Met	235Met>Thr	1	0.05	1	0.07	1	0.07	1	0.21
	174Thr>Met	11535C>A	1	0.05	1	0.05	1	0.04	0.58	0.02
	235Met>Thr	11535C>A	0.90	0.69	0.92	0.53	0.91	0.48	0.60	0.11

Table 3.9. Pair-wise linkage disequilibrium coefficient (*D'*) and r^2 between pairs of polymorphic loci in *EGF*, *TGFA* and *AGT* genes in the four racial groups.

3.3. Results of the case-control candidate gene pre-eclampsia association analysis

All loci, with the exception of the *EGF* 61G>A polymorphic locus in women with pre-eclampsia in the Nottingham population, were in HWE. The results of haplotype analysis and single locus genotype analysis are shown in tables 3.10 and 3.11 respectively. The only significant association was the association between the *EGF* gene and pre-eclampsia in the Sinhalese women (*P*=0.028). This effect was mainly due to the excess of the 67149G allele in Sinhalese women with pre-eclampsia (*P*=0.025). This association was not replicated in the white Caucasians (P=0.254). In the Sinhalese, using 67149AA as the reference genotype, the OR (95% confidence interval) for pre-eclampsia associated with heterozygosity and homozygosity for the 67149G allele was 1.61 (0.98 to 2.67) and 1.87 (1.05 to 3.31) respectively. The corresponding values for white Caucasians were 0.71 (0.28 to 1.81) and 0.56 (0.23 to 1.38) respectively.

Gene	Haplotype			Sinhalese		White Caucasian	
				PE	NT	PE	NT
EGF	***61***	***67149***					
	G	G		0.12	0.06	0.06	0.06
	G	A		0.43	0.50	0.36	0.28
	A	G		0.41	0.38	0.55	0.63
	A	A		0.04	0.05	0.02	0.03
	Likelihood Ratio Statistic (*P*)			9.07	**(0.025)***	2.74	(0.254)
TGFA	***3822***	***3827***	***3851***				
	G	T	T	0.18	0.21	0.26	0.31
	G	C	T	0.59	0.57	0.39	0.46
	G	C	C	0.08	0.06	0.20	0.15
	A	C	T	0.16	0.16	0.16	0.09
	Likelihood Ratio Statistic (*P*)			1.88	(0.598)	5.46	(0.141)
AGT	***174***	***235***	***11535***				
	T	M	C	0.09	0.09	0.29	0.24
	T	M	A	0.30	0.29	0.30	0.26
	T	T	C	0.50	0.47	0.25	0.32
	M	T	C	0.10	0.13	0.12	0.17
	T	T	A	0.01	0.01	0.00	0.00
	M	M	C	0.01	0.01	0.00	0.00
	Likelihood Ratio Statistic (*P*)			2.36	(0.883)	1.70	(0.638)

Table 3.10. Haplotype frequency estimates of *EGF*, *TGFA*, and *AGT* genes in the Sinhalese and the white Caucasian normotensive pregnant women (NT) and women with pre-eclampsia (PE). Haplotypes with frequencies ≤0.05 were dropped from hypothesis testing. **P* when all haplotypes were included in the analysis was 0.028.

Locus	Sinhalese								White Caucasians							
	No	Genotype frequency						Variant Allele		No	Genotype frequency						Variant Allele	
								f	95%CI for difference in *f*								*f*	95%CI difference in *f*
EGF 61G>A		***GG***		***GA***		***AA***		***A***			***GG***		***GA***		***AA***		***A***	
PE	175	57	(33)	79	(45)	39	(22)	0.45	-0.09 to 0.06	74	20	(27)	23	(31)	31	(42)	0.57	-0.02 to 0.20
NT	180	60	(33)	83	(46)	37	(21)	0.44		81	12	(15)	30	(37)	39	(48)	0.67	
EGF 67149G>A		***GG***		***GA***		***AA***		***A***			***GG***		***GA***		***AA***		***A***	
PE	176	51	(29)	85	(48)	40	(23)	0.47	0.01 to 0.16	74	32	(43)	27	(36)	15	(20)	0.39	-0.18 to 0.03
NT	180	41	(23)	79	(44)	60	(33)	0.55		81	42	(52)	28	(35)	11	(14)	0.31	
TGFA 3822G>A		***GG***		***GA***		***AA***		***A***			***GG***		***GA***		***AA***		***A***	
PE	169	122	(72)	40	(24)	7	(04)	0.16	-0.05 to 0.05	74	52	(70)	21	(28)	1	(01)	0.16	-0.14 to 0.01
NT	178	129	(72)	41	(23)	8	(04)	0.16		79	67	(85)	10	(13)	2	(03)	0.09	
TGFA 3827T>C		***TT***		***TC***		***CC***		***C***			***TT***		***TC***		***CC***		***C***	
PE	176	8	(05)	46	(26)	122	(69)	0.82	-0.09 to 0.03	74	3	(04)	33	(45)	38	(51)	0.74	-0.15 to 0.06
NT	180	4	(02)	67	(37)	109	(61)	0.79		81	5	(06)	40	(49)	36	(44)	0.69	
TGFA 3851T>C		***TT***		***TC***		***CC***		***C***			***TT***		***TC***		***CC***		***C***	
PE	175	150	(86)	24	(14)	1	(01)	0.07	-0.05 to 0.02	74	47	(64)	25	(34)	2	(03)	0.20	-0.13 to 0.04
NT	180	160	(89)	19	(11)	1	(01)	0.06		81	57	(70)	24	(30)	0	(00)	0.15	

Table 3.11. Continued in next page

Locus	Sinhalese								White Caucasians							
	No	Genotype frequency						Variant Allele		No	Genotype frequency						Variant Allele	
								f	95%CI for difference in *f*								*f*	95%CI difference in *f*
AGT T174M		***TT***		***TM***		***MM***		***M***			***TT***		***TM***		***MM***		***M***	
PE	176	143	(81)	30	(17)	3	(02)	0.10	-0.01 to 0.09	29	20	(69)	9	(31)	0	(00)	0.16	-0.10 to 0.13
NT	179	134	(75)	40	(22)	5	(03)	0.14		73	54	(74)	13	(18)	6	(08)	0.17	
AGT M235T		***MM***		***MT***		***TT***		***T***			***MM***		***MT***		***TT***		***T***	
PE	175	32	(18)	75	(43)	68	(39)	0.60	-0.06 to 0.08	73	24	(33)	40	(55)	9	(12)	0.40	-0.01 to 0.21
NT	180	32	(18)	76	(42)	72	(40)	0.61		81	18	(22)	46	(57)	17	(21)	0.49	
AGT 11535C>A		***CC***		***CA***		***AA***		***A***			***CC***		***CA***		***AA***		***A***	
PE	175	84	(48)	73	(42)	18	(10)	0.31	-0.08 to 0.06	72	36	(50)	27	(38)	9	(13)	0.31	-0.13 to 0.07
NT	180	89	(49)	74	(41)	17	(09)	0.30		80	39	(49)	37	(46)	4	(05)	0.28	
MTHFR 677C>T		***CC***		***CT***		***TT***		***T***			***CC***		***CT***		***TT***		***T***	
PE	176	137	(78)	36	(20)	3	(02)	0.12	-0.07 to 0.02	74	34	(46)	36	(49)	4	(05)	0.30	-0.05 to 0.16
NT	180	149	(83)	29	(16)	2	(01)	0.09		81	31	(38)	43	(53)	7	(09)	0.35	

Table 3.11. Genotype and variant allele frequencies (%) and the 95% confidence interval (CI) for the difference between the variant allele frequencies in the Sinhalese and the white Caucasian normotensive pregnant women (NT) and women with pre-eclampsia (PE).

3.4. Results of the candidate gene quantitative trait association analysis

Birth weight

The results of the analysis of the association between the maternal *EGF* haplotypes and birth weight of babies born to women who were normotensive during their pregnancy are shown in table 3.12. The association was significant in the Sinhalese (*P*=0.02) and highly significant (*P*=0.0007) in the white Caucasians. Babies born to mothers with the 61G/67149A haplotype weighed significantly less than the pooled birth weight of babies of mothers who had the other haplotypes (Sinhalese χ^2=5.8, *P*=0.016, white Caucasians χ^2=11.8, *P*=0.0006). The babies of mothers who had the 61A/67149G haplotype weighed significantly more than the pooled birth weight of babies of mothers who had the other haplotypes (Sinhalese χ^2=4.4, *P*=0.037, white Caucasians χ^2=8.5, *P*=0.0035). The actual differences in the mean birth weight of babies born to mothers with these two haplotypes were 113g in the Sinhalese and 398g in the white Caucasians. The birth weight of the Sinhalese and the white Caucasian babies become heavier as the number of *EGF* 61A or *EGF* 67149G alleles that their mother had increased (Table 3.13).

There was no association between the maternal *TGFA* gene or the *AGT* gene and the birth weight of babies born to women who were normotensive during their pregnancy.

AGT and Blood Pressure

There was no association between *AGT* haplotypes and systolic or diastolic blood pressure at booking among women who were normotensive during their pregnancy (Table 3.14).

EGF haplotypes		Sinhalese						White Caucasian					
		Male (n=72)		Female (n=108)		All (n=180)		Male (n=27)		Female (n=42)		All (n=69)	
61	***67149***	***f***	***MBW (g)***	***f***	***MBW (g)***	***f***	***MBW (g)***	***f***	***MBW (g)***	***f***	***MBW (g)***	***f***	***MBW (g)***
All babies (Mean(SD))		2904 (409)		3039 (413)		2985 (421)		3529 (513)		3439 (421)		3474 (464)	
G	G	0.05	2715	0.07	3161	0.06	3023	0.04	3632	0.06	3518	0.05	3551
G	A	0.48	2871	0.51	2968	0.50	2931	0.39	3367	0.26	3177	0.31	3270
A	G	0.43	2979	0.35	3095	0.38	3044	0.55	3631	0.64	3533	0.61	3568
A	A	0.04	2715	0.06	3161	0.05	3023	0.02	3632	0.04	3518	0.03	3551
Likelihood ratio statistic (*P*)		2.2 (0.14)		4.15 **(0.04)**		5.5 **(0.02)**		3.2 (0.074)		10.7 **(0.001)**		11.5 **(0.0007)**	

Table 3.12. Quantitative trait loci analysis of *EGF* haplotypes and birth weight of babies of the Sinhalese and the white Caucasian normotensive pregnant women. Mean (Standard Deviation) given for birth weight of all babies in each group. Equal variance in the birth weight in each group is assumed by QTPHASE. Rare haplotypes with frequencies <0.10 were dropped from hypothesis testing. MBW: Mean Birth Weight.

Population		Genotypes			*P*
		61 AA	**61 GA**	**61 GG**	
Sinhalese	No	41	79	60	
	BWT (kg)	3.1 (0.44)	3.0 (0.43)	2.9 (0.46)	0.06
White Caucasian	No	31	26	12	
	BWT (kg)	3.6 (0.51)	3.5 (0.47)	3.1 (0.33)	0.02
		67149 GG	**67149 GA**	**67149 AA**	
Sinhalese	No	41	79	60	
	BWT (kg)	3.1 (0.41)	3.0 (0.42)	2.9 (0.42)	0.02
White Caucasian	No	33	25	11	
	BWT (kg)	3.6 (0.50)	3.4 (0.47)	3.1 (0.38)	0.01

Table 3.13. Mean (Standard Deviation) birth weight (BWT) of babies of the Sinhalese and the white Caucasian normotensive pregnant women as a function of 61G>A and *EGF* 67149G>A genotypes.

AGT haplotype			Sinhalese (n=180)		White Caucasian (n=81)	
174	***235***	***11535***	***f***	***Mean Blood Pressure (mmHg)***	***f***	***Mean Blood Pressure (mmHg)***
Systolic blood pressure						
All normotensive pregnant women				108.5 (9.2)		121.7 (13.7)
T	M	C	0.09	107.9	0.24	121.7
T	M	A	0.29	108.4	0.26	125.4
T	T	C	0.47	108.1	0.32	127.4
M	T	C	0.13	110.5	0.17	128.2
Likelihood Ratio Statistic (*P*)				2.8 (0.43)		4.0 (0.26)
Diastolic blood pressure						
All normotensive pregnant women				68.6 (7.1)		65.2 (7.9)
T	M	C	0.09	68.5	0.23	66.02
T	M	A	0.29	68.6	0.27	65.88
T	T	C	0.47	68.4	0.32	65.77
M	T	C	0.13	69.4	0.17	62.17
Likelihood Ratio Statistic (*P*)				0.7 (0.86)		4.1 (0.25)

Table 3.14. Quantitative trait loci analysis of *AGT* haplotypes and blood pressure at booking in the Sinhalese and the white Caucasian normotensive pregnant women. Mean (standard deviation) of blood pressure for all women is given. Equal variance in blood pressure in each subgroup is assumed by QTPHASE. Rare haplotypes with frequencies ≤0.05 were dropped from the analysis.

3.5. Results of the reporter gene assay analysis

The dual luciferase reporter gene assay with non-transfected HepG2 cells to determine background luminescence revealed appreciable background signal for *Renilla* luciferase activity (0.144 ± 0.03). The background signal for firefly luciferase activity was much weaker (0.023 ± 0.016). Any reading of <0.204 (mean + 2×SD) for *Renilla* luciferase and <0.056 for firefly luciferase therefore was deemed to be unreliable. Only one reading for *Renilla* luciferase from the transfected HepG2 cells recorded a reading below this cut-off point and was excluded from the analysis.

The results of the reporter gene assay using HepG2 cells transfected with different constructs under basal and AngII stimulated conditions are summarised in tables 3.15 and 3.16 respectively. The pGL3Control vector was the positive vector used to evaluate transfection efficiency. The enhanced response obtained with it was expected, because it has a very potent Simian Virus 40 enhancer. The pGL3Promoter vector was the base vector against which the 'enhancer' effects of the experimental constructs were compared. All three constructs showed significant repression in comparison to the pGL3Promoter vector under both basal and stimulated conditions (P<0.001). The response under basal conditions is illustrated in figure 3.1. There was no significant difference between the repression brought about by the three constructs under both basal and stimulated conditions (Figure 3.2). However, when the effect of stimulation of each construct was compared with the effect

under basal condition, construct E3 demonstrated differential response at various levels of AII. The post-hoc analysis indicated that this was due to a response to AII at 10^{-11}M, which was greater than the response at the other concentrations of AII (for 10^{-11}M vs. 10^{-13}M: P=0.06, 95% CI 0.98 to 3.14-fold; for 10^{-11}M vs. 10^{-9}M: P=0.03 , 95% CI 1.09 to 3.49-fold), but it did not differ significantly from the basal response (P=0.15).

Construct	No of Repeats	Response over promoter		Significance	
		Response	SEM	Construct v Promoter	Between Constructs
pGL3C	8	13.48	5.86		
E1	7	0.17	0.08	<0.001	
E2	7	0.36	0.12	0.006	0.220
E3	7	0.27	0.11	0.001	

Table 3.15. Results of the Dual Luciferase Reporter Gene Assay under basal conditions. SEM: standard error of mean. pGL3C: pGL3Control. E1, E2, E3: the three experimental vectors (see table 2.12).

Construct	Condition (AngII concentration)	The response under AngII stimulated conditions compared with the response under basal conditions			Significance in response between different AngII concentrations
		Mean fold response over basal response	SEM	Significance when compared with basal response	
E1	Basal	1	0.32		
	10^{-9}M	0.75	0.20	0.84	
	10^{-11}M	0.76	0.42	0.61	0.67
	10^{-13}M	0.76	0.16	0.89	
E2	Basal	1	0.18		
	10^{-9}M	1.24	0.22	0.33	
	10^{-11}M	0.94	0.28	0.97	0.14
	10^{-13}M	0.85	0.25	0.90	
E3	Basal	1	0.25		
	10^{-9}M	0.80	0.11	0.57	
	10^{-11}M	1.58	0.27	0.16	0.03
	10^{-13}M	0.89	0.12	0.88	

Table 3.16. Results of the Dual Luciferase Reporter Gene Assay under stimulated conditions compared with results under basal conditions. SEM: standard error of mean. Each experiment was repeated three times. E1, E2 and E3: The three experimental vectors (see table 2.12).

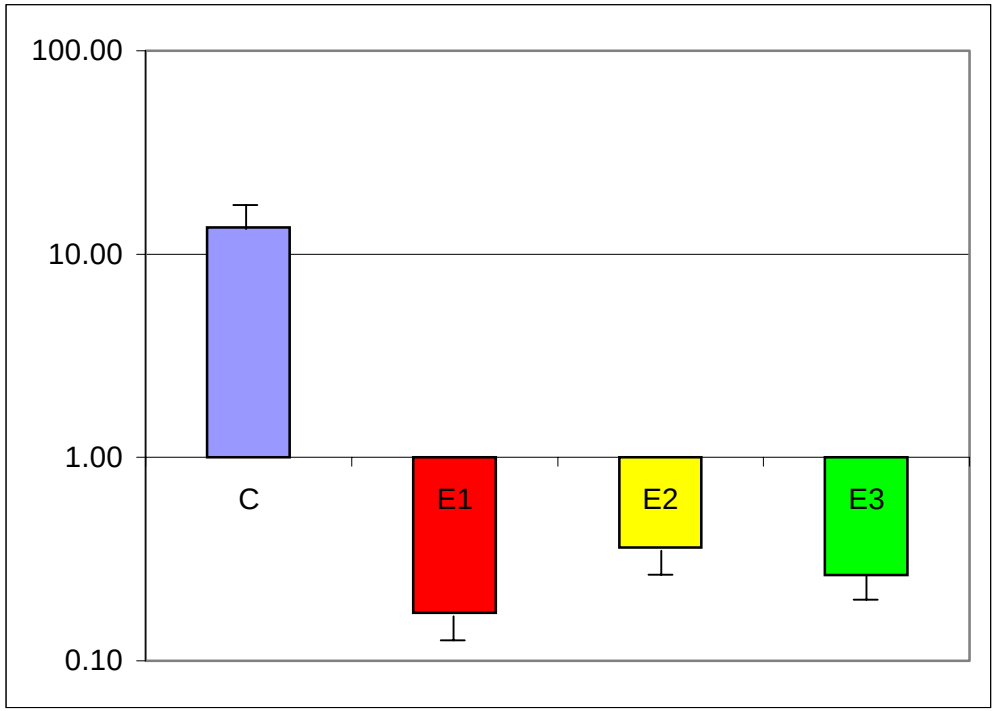


Figure 3.1. A bar chart comparing the enhancer effect of the pGL3Control vector with that of the enhancer effect of the constructs E1, E2, and E3. Y axis is a logarithmic scale. 1=Promoter vector reference. Standard error bars are shown.

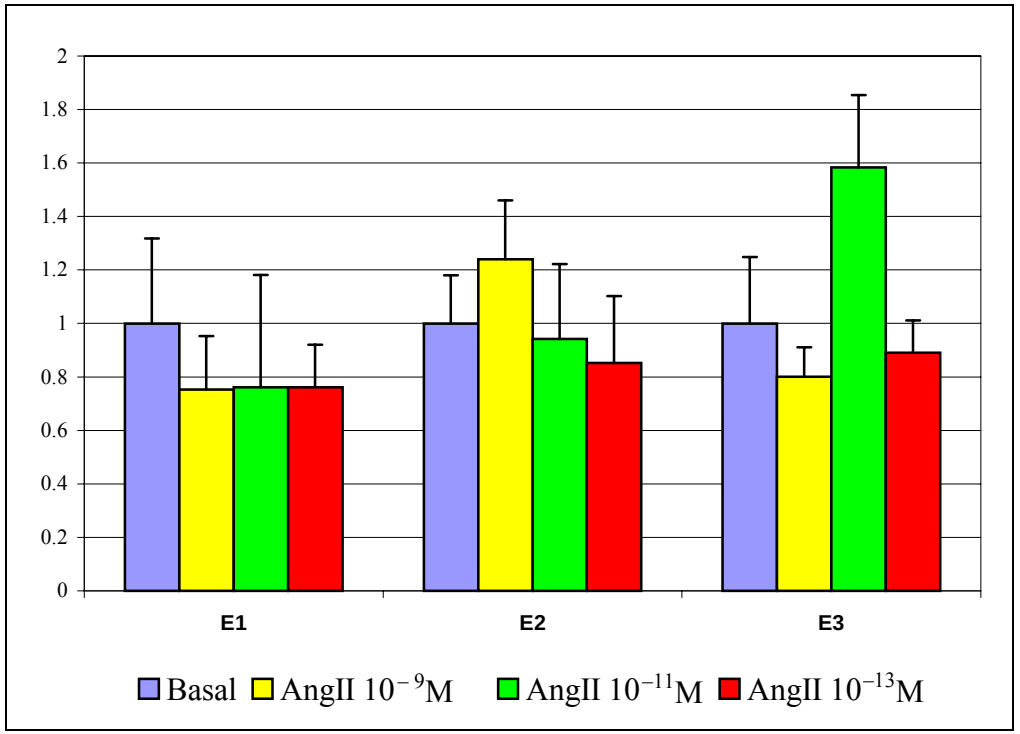


Figure 3.2. A bar chart comparing the response of each construct under AngII stimulated conditions with the response under basal conditions. Y-axis denotes the mean fold response over the response under basal conditions. Standard error bars are shown. Basal response for each construct is normalised to 1 and standard error bars have been adjusted proportionally. The scaled mean (SEM) for each construct under basal conditions is: E1 - 1 (0.32); E2 - 1 (0.18); E3 - 1 (0.25).

4. DISCUSSION

4.1. Population genetic results

4.1.1. Epidermal Growth Factor

This study is the first to report the *EGF* 61G>A and *EGF* 67149G>A polymorphisms frequencies in a non-white Caucasian population. It is also the first to report on haplotype structure in the *EGF* gene in any population although it is inferred from only two polymorphisms. There are significant differences in the allele frequencies at these polymorphic loci, linkage disequilibrium between the alleles at the two loci, and the haplotype structure between the Sinhalese and the white Caucasians. The Moors show a pattern closer to the white Caucasians than the Sinhalese, although they are not significantly different to the latter population. The *EGF* gene is a large gene of approximately 110-kb. It is therefore, not reasonable to assume that two polymorphisms would define the haplotype structure of the *EGF* gene adequately. Many other SNPs in the *EGF* gene have been deposited in the public SNP database at NCBI. Most SNPs in this database have been identified by *in silico* methods that ascertained SNPs from a small number of chromosomes in a limited number of populations. Some of them, however, have been validated in larger samples, and they may be used to select a set of SNPs that can be used to further refine the haplotype structure of the *EGF* gene experimentally.

4.1.2. Transforming Growth Factor Alpha

This study is the first study to report on the *TGFA* 3822G>A, 3827T>C and 3851T>C polymorphisms in the Sri Lankan population. In contrast to the *EGF* gene, in the *TGFA* gene, the allele and haplotype frequencies in the Sinhalese were similar to that of the white Caucasians, while the Sri Lankan Tamils and the Moors differed significantly from the white Caucasians. In previous studies, the haplotype structure of the *TGFA* gene defined by these polymorphisms in the white Caucasian and the Japanese populations has been similar (Table 1.2). The haplotype frequencies of the white Caucasians in Nottingham differed from that reported previously in the white Caucasians in Iowa, USA (Shiang *et al.*, 1993). Among the white Caucasians in Nottingham, the 3822G/3827C/3851T haplotype was much more common (Nottingham: 0.62 cf. Iowa: 0.50) and the 3822A/3827C/3851T haplotype rare (Nottingham: 0.06 cf. Iowa: 0.20) than in the white Caucasians in Iowa. As for the *EGF* gene, there is a need to further characterise the haplotype structure of the *TGFA* gene using additional SNPs.

4.1.3. Angiotensinogen

The polymorphism at codon 174 deviated from HWE at $P<0.05$ in the white Caucasians. The sample used for the current study was a subset of 153 samples reported previously (Morgan *et al.*, 1996). In that report too the 174Thr>Met genotype distribution of the entire sample collection deviated from HWE. Possible reasons for this have been discussed in that report. Since

that discussion applies to the present results as well it is reproduced here: "There are no obvious explanations for this. This pattern is observed in the presence of two formerly isolated, genetically distinct, sub populations (Wahlund, 1928); however, there are no *a priori* reasons for believing that the Nottingham population would be other than a genetically homogeneous group. Methodical artefacts must be considered. Contamination of polymerase chain reactions with foreign DNA is a well-recognised problem; however, this causes an excess of heterozygotes, not a deficit, and cannot therefore account for the present findings. While ASO hybridisation is a useful technique for genotyping large numbers of samples, lack of specificity can be a problem with conventional methods; this problem has been largely overcome by the use of competitive hybridisation techniques, as employed in the present study. Selective polymerase chain reaction amplification of one allele would result in mistyping of a heterozygote as a homozygote; this would not of course be detected by sequencing of the amplified products, or by running positive control samples. It should be noted, however, that the amplimers used in this investigation for assessment of the 174 genotype were also used for genotyping at codon 235". Other probable reasons for such deviations are the close association of the polymorphism being studied with another selectable markers that increases the amount of homozygosity in the vicinity of the locus for which selection is made and the possibility of bias when the sample size of the study is small.

The frequency of alleles in the 235Met>Thr polymorphism in the three Sri Lankan racial groups is significantly different to that in the white Caucasians and it is closer to that reported in the Japanese (Nakajima *et al.*, 2002). The 174Met allele as reported before seems to occur exclusively in chromosomes carrying the 235Thr allele in all racial groups and it is reflected in the pair-wise linkage disequilibrium data Table 3.9. The 11535C>A is known to split the haplotypes occurring on chromosomes carrying the 174Thr and 235Met alleles. The 11535C allele of this polymorphism occurs in far fewer chromosomes with the above mentioned allele combination in the 174Thr>Met and 235Met>Thr polymorphisms. This together with the differences in the allele frequencies of the 235Met>Thr polymorphism accounts for the significant difference between the haplotype structure of the white Caucasians and all three Sri Lankan racial groups.

4.1.4. 5,10-Methylenetetrahydrofolate Reductase

This study is the first to report on the *MTHFR* 677C>T polymorphism frequency of the three Sri Lankan racial groups separately. Two previous studies that report on the frequency of the alleles of this polymorphism in Sri Lankans do not give the racial background of the population (Schneider *et al.*, 1998, Alagratnam *et al.*, 2000). The low frequency of the 677T allele in the current study is in agreement with the 'pooled' population data of Sri Lankans in the two previous studies. Although statistically not significant, the 677T allele seems to be commoner among the Sinhalese than the Sri Lankan Tamils

or the Moors. The previous studies reported failure to find 677TT homozygotes among Sri Lankans. In contrast two 677TT homozygous Sinhalese subjects were identified in the current study. One factor that could affect the frequency of the 677T allele is the age of the study group because the prevalence of the 677T allele is reportedly lower in older age groups possibly because 677TT homozygous individuals are more prone to cardiovascular disease (Matsushita, Muramatsu, Arai, Matsui and Higuchi, 1997). The Sinhalese (33.0± 8.8) in this study, however, were older than the Sri Lankan Tamils (28.2±6.8; $P<0.001$) and the Moors (27.4±5.6; $P<0.001$). Age therefore is not a factor for the higher prevalence of the 677T allele in the Sinhalese compared to the Sri Lankan Tamils and the Moors in this study. Cardiovascular disease, anyway, is very unlikely to influence the frequencies of the 677T allele at these young ages in any population.

Healthy Sri Lankans are reported to have high levels of homocysteine compared to western populations (Mendis, Athauda and Takashi, 1997). There is no evidence from this study to suggest that the 677C>T polymorphism may contribute to this in a major way. It is possible that low levels of folate in their diet (Mendis, Bulugahapitiya, Ranatunga, Gunawardene and Kandegedera, 1999b) and other genetic and environmental factors may contribute to this.

4.1.5. Overview of population genetic results

These results as expected show that the three Sri Lankan racial groups are more closely related to each other than to the white Caucasians. However, these results can only be used to draw broad conclusions as only a small number of polymorphic loci have been examined. These results are broadly in agreement with previous work on the population structure of the difference racial groups in the Sri Lankan population (Papiha *et al.*, 1996a, Papiha, Mastana, Purandare, Jayasekara and Chakraborty, 1996b).

Although the haplotype structure of the candidate genes in the three Sri Lankan racial groups appear similar, and for most part any differences statistically not significant, the differences that do exist may have an impact on case-control candidate gene pre-eclampsia association studies if any cryptic racial admixture were to be present among either women with pre-eclampsia or normotensive pregnant women (see section 4.2.1). As such, matching the two groups for their racial origin as was done in this study, is essential for case-control candidate gene association studies in Sri Lanka.

The linkage disequilibrium data is also interesting. The r^2 value is an index of the loss of statistical power if one analyses a marker SNP rather than the disease SNP. For a given power and significance threshold, the required increase in sample size to allow for reduced linkage disequilibrium is inversely proportional to r^2. Therefore, for example, in the Sinhalese

population, if 235Met>Thr were a disease locus, but only 174Thr>Met was analysed, one would require a 20-fold ($1/r^2 = 1/0.05$) increase in sample size compared with analysing 235Thr>Met in spite of the fact that these two loci are in strong linkage disequilibrium, with D' = 1. This justifies haplotype analysis in case-control candidate gene disease association studies in preference to analysing single SNPs (Kruglyak, 1999).

4.2. Case-control candidate gene pre-eclampsia association results

4.2.1. Epidermal Growth Factor

This is the first study to report significant association between pre-eclampsia and the *EGF* gene in any population. The results, however, could not be replicated in the white Caucasians. There are several reasons for this. In the Sinhalese, the association was mainly related to the 67149G allele. It was the minor allele of that polymorphism in the Sinhalese, while in the white Caucasians it was the major allele. The allele frequencies were significantly different in the Sinhalese and the white Caucasians (P=0.002). Under these conditions the sample size of the study in the Sinhalese population had 80% power to detect a doubling of risk at a significance level of 0.05, while the sample size in the white Caucasians had only 26% power to detect a doubling of risk at a significance level of 0.05. In addition to this, to find an association, as already mentioned, it is necessary that the polymorphisms tested contribute to the disease phenotype or be in linkage disequilibrium (LD) with a polymorphism that contributes to the disease phenotype. There are differences in the frequency of the alleles of the 61G>A and 67149G>A polymorphisms, haplotypes in the *EGF* gene defined by them, and the LD between them in the Sinhalese and the white Caucasians. If the 67149G>A polymorphism is in LD with another polymorphism that was contributory to the pre-eclampsia phenotype, instead of being the contributory polymorphism by itself, and if the LD between the 67149G allele and the causative polymorphism was weaker in the white Caucasians than in the Sinhalese, that could also have contributed to

the failure to replicate the finding. If this was so, then carrying out association analysis using a more refined haplotype structure using additional SNPs that capture the maximum haplotype diversity within the gene may help to clarify the association further. The other possibility for failure to replicate is the presence of gene-gene interactions. The present study, however, is underpowered to extend the investigation to examine such interactions.

A closer examination of the allele frequency of the *EGF* 67149G>A polymorphism in Sri Lankan population revealed that the 67149G allele, although not statistically significant, is commoner in the Moors than in the Sinhalese or the Sri Lankan Tamils. It is possible therefore, that an admixed Sinhalese population may be over represented among Sinhalese women with pre-eclampsia in this study. This scenario, however, is unlikely in the context of the pattern of inter racial marriages in Sri Lanka, which is more in favour of Sinhalese women marrying into the Moor community and assuming a Moor identity than vice versa, and the care that was taken to verify the racial origin of each recruit for this study

The genotype distribution for the 61G>A polymorphism in white Caucasian women with pre-eclampsia differed significantly from HWE. This was as a result of a relative deficiency of heterozygotes. Such a deficiency of heterozygotes was also noted in Sinhalese women with pre-eclampsia, but it did not reach statistical significance. Given that the *EGF* gene is associated

with pre-eclampsia in the Sinhalese, this may reflect a true departure from HWE. Other probable reasons for such departure from HWE have already been discussed in section 4.1.3 above.

4.2.2. Transforming Growth Factor Alpha

This was the first study to examine the association of pre-eclampsia with the *TGFA* gene. However, there was no association in both the Sinhalese and the white Caucasians. TGFA was selected as a candidate gene because it is a structural and functional homologue of EGF and because the *TGFA* gene is located within the highly suggestive region for a pre-eclampsia susceptibility gene in genome wide studies. There did not appear to be any independent pathophysiological reports linking TGFA to pre-eclampsia.

4.2.3. Angiotensinogen

The angiotensinogen gene was not associated with pre-eclampsia in either population in this study. These results are consistent with most studies that have failed to find an association between pre-eclampsia and the angiotensinogen gene (summarised in table 1.3). It is difficult, however, to draw comparisons between all these studies because of inconsistencies in case and control groups such as the inclusion of multiparous women, inclusion of women with gestational hypertension, and the possible inclusion of women who carry a high risk of pre-eclampsia such as multiple pregnancies and diabetes mellitus in studies where they were not stated as exclusion criteria. In

addition, in some studies the sample size was too small to decide conclusively that the 235Thr allele was not in fact associated with pre-eclampsia. This study in the Sinhalese population had 80% power to detect a doubling of risk associated with the 235Thr allele at a significance level of 0.05. In the Sinhalese, using 235Met/Met as the reference genotype, the OR (95% confidence interval) for pre-eclampsia associated with heterozygosity and homozygosity for the 235Thr allele was 1.01 (0.57 to 1.82) and 1.06 (0.59 to 1.91) respectively. Therefore the contribution of the 235Thr allele to pre-eclampsia, if any, is small.

Recently association of the *AGT* haplotype 1035A/174Met/235Thr haplotype with pre-eclampsia was reported in a French Canadian population (Levesque *et al.*, 2003). The 1035G>A polymorphism which is located at nucleotide –217 upstream of the transcription start site was not examined in the current investigations. It may be useful to genotype these samples for the 1035G>A polymorphism to investigate whether this haplotype is associated with pre-eclampsia in the Sinhalese and/or the white Caucasians.

4.2.4. 5,10-Methylenetetrahydrofolate Reductase

There was no association between the *MTHFR* 677C>T polymorphism and pre-eclampsia in either population. These results are again consistent with the large number of studies with a similar finding (summarised in table 1.4). As was the case with studies examining the association of the *AGT* 235Thr allele

with pre-eclampsia, it is difficult to draw comparisons between these studies for the reasons stated above. In the Sinhalese using 677CC as the reference genotype, the OR (95% confidence interval) for pre-eclampsia associated with combined data for heterozygosity and homozygosity for the 677T allele was 0.73 (0.43 to 1.24). This study in the Sinhalese population had 80% power to detect a doubling of risk at a significance level of 0.05. Therefore the contribution of the 677T allele to pre-eclampsia, if any, is small.

Since non-association with the *MTHFR* 677T allele seems to be the emerging picture from most association studies, irrespective of whether they actually had sufficient power to detect an association or not, it is reasonable to re-examine the original reasons for hypothesising *MTHFR* as a possible candidate. Several recent studies have reported interesting findings. Plasma homocysteine levels are low in normal pregnancies (Raijmakers, Zusterzeel, Steegers and Peters, 2001), and they seem to correlate with the *MTHFR* genotype, with 677TT homozygotes having significantly higher levels than 677CC homozygotes when their folate status is low (Kim, Kim and Chang, 2004). Interestingly, in pregnancies complicated by pre-eclampsia, although homocysteine levels are elevated compared to normal pregnancies, they are comparable to levels in non-pregnant women (Kim *et al.*, 2004) and they do not correlate with the *MTHFR* genotype (Powers, Dunbar, Gallaher and Roberts, 2003). It is possible therefore, that hyperhomocysteinaemia in pre-eclampsia may in fact be an artefact resulting from a relative reduction in

plasma volume. Women with pre-eclampsia, however, have higher levels of homocysteine early in their pregnancies before such changes occur in the circulatory system (Cotter *et al.*, 2003). This is in favour of the possibility that women with the 677TT genotype (with low folate intake, and hyperhomocysteinaemia) begin pregnancy with an already damaged unhealthy endothelium that puts them at a higher risk of pre-eclampsia irrespective of their homocysteine status in pregnancy. Hyperhomocysteinaemia as stated before is reported to be a significant risk factor for cardiovascular disease among Sri Lankans, with normal Sri Lankans having higher levels of homocysteine in their plasma compared to western norms (Mendis *et al.*, 1997). Folate intake among healthy Sri Lankans is also reported to be low (Mendis *et al.*, 1999b). It is not possible from this study to exclude the possibility that hyperhomocysteinaemia due to a mechanism other than the presence of the *MTHFR* 677TT genotype may be contributory to pre-eclampsia in the Sinhalese. It may be premature, however, to discount the possible role of the *MTHFR* gene in the aetiology of pre-eclampsia (Pegoraro *et al.*, 2004).

4.3. Candidate gene quantitative trait association results

In this study the analysis of the association of genes with two quantitative traits was examined only in women with normal pregnancies. The complex disease process in pregnancies complicated by pre-eclampsia, which begins early in pregnancy, is likely to obscure the effects of a single gene on the expression of any quantitative trait, whether it be birth weight or blood pressure. In addition in such pregnancies the gestation at delivery is so variable and that of course would anyway affect the weight of babies at birth making any comparison meaningless. Therefore, although pregnancy data from both women who were normotensive during their pregnancy and women who had pregnancies complicated by pre-eclampsia were available, the quantitative trait association analysis was confined to the former group.

4.3.1. Birth weight

Only the maternal *EGF* gene was associated with the weight of babies at birth. The association was stronger in the white Caucasian population than in the Sinhalese. One possible reason for this is the difference in the haplotype frequency in the Sinhalese and the white Caucasians. The frequency of the 61G/67149A haplotype in the Sinhalese was 0.53 while in the white Caucasians it was 0.37. Another reason is the possible influence of sample size. In both racial groups, the numbers of individuals studied were small (180 Sinhalese and 84 white Caucasian). It is possible that in such small samples the effects of statistically significant quantitative traits may be over

estimated (Barton and Keightley, 2002). Such an effect may account for the more significant result in the white Caucasians than in the Sinhalese. However, the difference in the mean weight between the lightest and the heaviest white Caucasian babies was nearly three and a half times that of the Sinhalese.

Some authors believe that it is not possible to assume that there is no sex effect on any phenotype or trait unless proven otherwise (Yagil and Yagil, 2003). Moreover, it is well known that male babies weigh heavier than female babies. In the current study subgroup analysis by sex of the babies showed that the *EGF* gene was associated with birth weight only in the female babies in both racial groups, but the numbers of male babies were less than the number of female babies in both racial groups. The trend in birth weight, however, remained the same as in the overall group in babies of both sexes in both racial groups. As expected the mean birth weight of the white Caucasian male babies was higher than that of the female babies; but the reverse was seen in the Sinhalese babies.

These results support the association of EGF with the low birth weight of babies reported by Shigeta *et al*. (1992) and Lindqvist, Grennert and Marsal (1999). However, Shahbazi *et al* (2002) found that EGF production was significantly lower in peripheral blood mononuclear cell (PBMC) cultures of *EGF* 61AA individuals, but they were not genotyped at the 67149G>A

polymorphism. This indicates that the underlying pathophysiological mechanisms may be complex. It may also involve interaction with other genes. It is also possible that, since the maternal genotype partially determines the fetal genotype, what is important is not the maternal, but the fetal genotype or the interaction between them. To confirm that, TDT testing would have to be done using data in mother-father-baby trios.

It is interesting to speculate on how *EGF* and other genes involved in placentation may affect birth weight and what implications it may have in the long term. "*The size attained in utero depends on the services which the mother is able to supply. These are mainly food and accommodation.*" (McCance, 1962). Provision of an adequate supply of nutrition to the baby not only requires good maternal nutrition, but also normal placentation. The possible role of EGF in placentation has already been discussed. Variations in the maternal *EGF* gene may result in the production of EGF that is functionally incapable of inducing the desired changes in cytotrophoblast cells to acquire the invasive phenotype, which is necessary for successful placentation. If the nutrition received by babies were to be determined by different grades of success or failure of placentation due to variations in one or more genes involved in placentation, then the effects of such genes may limit the efficacy of maternal nutritional interventions aimed at improving birth weight. It is possible therefore, on the basis of the Barker hypothesis, which states that adult diseases originate in fetal life, as evidenced by, for example,

the increased risk of cardiac disease among men who were small at birth (Barker, Winter, Osmond, Margetts and Simmonds, 1989); and the concept of the 'thrifty phenotype' which is based on the observation that the effect of impaired fetal growth is modified by subsequent growth in infancy and childhood (Hales and Barker, 1992), that such genes may also determine whether or not one is susceptible to adult onset disease.

4.3.2. Blood pressure

There was no association between the *AGT* gene and blood pressure at booking in normotensive pregnant women in this study. Previous studies in non-pregnant subjects have reported both association and non-association between blood pressure variation and the *AGT* gene (Forrester, McFarlane-Anderson, Bennet, Wilks, Puras *et al.*, 1996, Kiema, Kauma, Rantala, Lilja, Reunanen *et al.*, 1996, Bloem, Foroud, Ambrosius, Hanna, Tewksbury *et al.*, 1997, Sethi, Nordestgaard, Agerholm-Larsen, Frandsen, Jensen *et al.*, 2001, Pereira, Mota, Cunha, Herbenhoff, Mill *et al.*, 2003, Sethi, Nordestgaard, Gronholdt, Steffensen, Jensen *et al.*, 2003, Sethi, Nordestgaard and Tybjaerg-Hansen, 2003). In the current study, among the Sinhalese as well as the white Caucasians, women with the 174Thr/235Met/11535C haplotype had the lowest SBP and women with the 174Met/235Thr/11535C haplotype had the highest SBP. It is possible therefore, that the sample size of this study may not have been sufficient to detect subtle differences in the effect of different haplotypes on blood pressure.

4.4. Overview of case-control candidate gene pre-eclampsia association results and quantitative trait analysis results

4.4.1. Correction for multiple testing

The case-control candidate gene pre-eclampsia association studies described in this thesis fall into two broad categories. Association analysis with the *AGT* and *MTHFR* genes were aimed at replicating previously reported associations in the Sinhalese and the white Caucasians in Nottingham. In contrast, association analysis with *EGF* and *TGFA* genes were the first such studies. When such multiple testing is done, a question arises as to whether a more stringent definition of significance should be applied to confirm an association. Although a lower threshold for a significant *P* value would increase the confidence in the association, there is no consensus on whether significance levels should be adjusted for multiple testing or on how such adjustments should be made (Bland and Altman, 1995, Perneger, 1998). Some authors believe that replication of findings in a genetically different population is perhaps more important than correcting for multiple testing (Dekker and van Duijn, 2003). It is in this context that the association of the *EGF* gene with pre-eclampsia in the Sinhalese, that is reported above, has to be considered. It is the first such report, and therefore it is largely exploratory. It could not be replicated in the white Caucasians in this study because the white Caucasian sample collection was underpowered to detect a significant genetic effect. It needs therefore, to be replicated in other populations using a sufficiently powered collection of samples. The *EGF* polymorphism and haplotype

frequency data of the white Caucasian population presented above would be useful to plan out such a study.

4.4.2. The *EGF* gene, pre-eclampsia and birth weight

The most significant findings of these investigations were the association of the *EGF* gene with pre-eclampsia in the Sinhalese and the association of the *EGF* gene with the weight of babies at birth in the Sinhalese as well as in the white Caucasians. In the Sinhalese, the association of the *EGF* gene with pre-eclampsia was mainly due to the high frequency of the 67149G allele in women with pre-eclampsia compared to normotensive pregnant women. However, among the white Caucasians the 67149G allele was found more in normotensive pregnant women than in women with pre-eclampsia. In that racial group it was the 61G allele that was commoner in women with pre-eclampsia. In both the Sinhalese and the white Caucasians, the 61G and 67149A alleles were in association with low birth weight.

These discrepant results can only be explained if two independent polymorphic loci, one each contributing to the determination of birth weight or the development of pre-eclampsia, were present in the *EGF* gene, and the linkage disequilibrium between those loci and the marker polymorphisms studied in the current investigations differed between the Sinhalese and the white Caucasians. Such loci therefore need to be identified by further investigations.

4.5. Reporter gene assay results

The aim of the angiotensinogen reporter gene expression studies was to examine how the *AGT* 3'UTR/flanking region SNPs affect *AGT* gene expression and to examine whether they modify the enhancer activity reported by Nibu and colleagues (Nibu *et al.*, 1994a, Nibu *et al.*, 1994b) in this region. However, instead of enhancer activity, repression was discovered with the 1660-bp *AGT* 3'UTR/flanking region insert used in these investigations.

Gene repression is a relatively new concept in the field of gene regulation. Position-independent elements that direct an active repression mechanism (generally by interfering with the General Transcription Factor (GTF) assembly) are called silencer elements. Position-dependent elements that direct a passive repression mechanism (generally by interfering with upstream elements) are referred to as negative regulatory elements (NRE). Transcription factors associated with NREs are known as repressors (Ogbourne and Antalis, 1998). Such elements have only rarely been identified in the 3'UTRs of genes (Le Cam and Legraverend, 1995, Paul, Simar-Blanchet, Ro and Le Cam, 1998).

The first 451-bp in the 5' end of the insert used in these investigations corresponded with the region showing enhancer activity in Nibu's experiments. The balance 1209-bp of the insert, however, is found in another insert in that study that had been excluded from their investigations because

initial examination had revealed that it had 'no enhancer activity' (Nibu *et al.*, 1994a). It is possible therefore, that repression was present in that region of the 3' flanking region and that it can even override the activity of the 3' enhancer. The logical extension of this work now is to divide the 1660-bp inset into two inserts of 451-bp and 1209-bp each and to attempt to demonstrate, enhancer activity with the former, and repression with the latter. If this hypothesis is supported, then, the 1209-bp insert can be subjected to deletion analysis to define specific DNA elements carrying a repressive function. This could be followed by investigations to identify proteins interacting with those elements. These investigations would include electrophoretic mobility shift assays with nuclear extracts to confirm whether or not any regulatory proteins bind to this region, and should binding be observed, antibody-induced super-shifts to identify novel proteins.

In addition to there being true transcription repression, there are other factors that could have also given similar results. Transfection efficiency with the insert is one concern. This is unlikely as the pGL3Control constructs gave results similar to the ones obtained previously by other researchers in our laboratory. It is also possible that the constructs were lethal, but there did not appear to be any difference in the appearance of the cells following transfection. It is difficult to comment on the viability of cells as a count of the number of cells in each well before transfection and at the time of harvesting was not kept. It is also possible that rather than binding a factor that repressed

transfection; the constructs may have affected the degradation rate of the luciferase gene.

Only construct E3 demonstrated a differential response at various levels of AngII in the stimulation experiments. The insert in construct E3 differed from constructs E1 and E2 at positions 11535 and 11608. E3 had nucleotides A and T at 11535 and 11608 respectively, while both E1 and E2 had nucleotides C and C. In order to examine whether these changes may affect transcription factor binding at these two sites, the sequences of the inserts were examined with MatInspector software for transcription factor binding. At the polymorphic site at nucleotide 11608 there appears to be a consensus sequence for interferon (IFN) regulatory factor 2 (IRF-2) when the 11608C allele is present, but when it is substituted by the 11608T this consensus sequence is abolished. IRF-2 is a transcription factor involved in the regulation of the IFN system (Tanaka, Kawakami and Taniguchi, 1993). The available evidence so far suggest that at least interferon alpha does not stimulate the secretion of angiotensinogen from rat hepatoma cells (Itoh, Matsuda, Ohtani and Okamoto, 1989).

These experiments were conducted in the HepG2 cells because the experimental techniques in this cell line are well established in our laboratory. They could also be extended to other human cell lines expressing angiotensinogen such as the extravillous cytotrophoblast cell lines to examine

whether the regulatory elements behave similarly in those cell lines as well. The stimulation experiments were confined to AngII in these investigations. However, stimulation experiments could have also been performed with other factors, such as EGF, TGFA, corticosteroids, etc., for which HepG2 cells are known to have receptors. These could also be tested in future experiments.

5. CONCLUSIONS

These investigations were undertaken to test the hypothesis that the *EGF, TGFA, AGT* and *MTHFR* genes are susceptibility genes for pre-eclampsia, and that the *EGF, TGFA,* and *AGT* genes contribute to determine the variation in birth weight and/or blood pressure. The hypotheses were tested using molecular genetic techniques in two populations – the Sinhalese in Sri Lanka and the white Caucasians in Nottingham, UK. The first step in these investigations was the recruitment of Sri Lankan research subjects. This afforded an opportunity to validate the urine protein heat coagulation test (HCT) and to collect phenotypic data, unique to Sri Lanka, in a group of 180 Sinhalese women with strictly defined pre-eclampsia.

The main focus of these investigations were the genetic studies, but the establishment of the research cut-offs for the detection of significant proteinuria using the cheap and effective HCT and the demonstration of its use in practice has wide implications for the developing world not just in research, but also in clinical practice. The establishment of a database of the pre-eclampsia phenotype among the Sinhalese, which provides clear evidence of the severe morbidity among women who develop pre-eclampsia and the severe mortality in their babies too is important as it is a call for action on the part of obstetricians, paediatricians and health administrators in Sri Lanka.

Successful subject recruitment was followed by the establishment of a DNA

resource containing both samples from population volunteers of the three main races in Sri Lanka and samples from Sinhalese women with pre-eclampsia and normotensive pregnant Sinhalese women. The only drawback in this part of the study was that resolution of blood pressure postpartum could not be confirmed in some women with pre-eclampsia. An already established DNA resource at the University of Nottingham was used to replicate the studies in a white Caucasian population. Population based DNA resources were successfully used to establish the frequency of polymorphism/haplotype of the candidate genes in the three main Sri Lankan populations. This information, in addition to providing useful information for the current investigation, will also be useful for future genetic research in Sri Lanka. The case-control DNA resources were used to examine the association of the candidate genes with pre-eclampsia; and to examine the association of the candidate genes with quantitative traits in normotensive pregnant women.

The most important finding of these molecular genetic investigations was the discovery of a strong association between polymorphisms/haplotypes of the maternal *EGF* gene and the birth weight of babies in the Sinhalese, which was replicated more strongly in the white Caucasians. Moreover, the *EGF* gene was found to be associated with pre-eclampsia in the Sinhalese. It is not possible, however, with the study design to determine whether the genetic effect was acting in the mother, the fetus or both. These initial findings therefore have to be clarified further not only by more precise haplotyping of

the *EGF* gene in case and control groups, but also by TDT testing in a new sample collection containing mother-father-baby trio's to determine whether the genetic effect is acting on the mother, the fetus or both. A central theme that emerged in the discussion was the need to validate these findings in a larger sample collection that has sufficient power to detect genetic effects. The creation of large white Caucasian pre-eclampsia DNA resources such as has been done in the GOPEC study, would facilitate that in the future. The subsidiary investigation outside the main theme of this thesis was the *AGT* reporter gene expression investigation. It resulted in the discovery of repressor activity in the 3'UTR/flanking region of the *AGT* gene that has hitherto not been described and the preliminary observations made with regard to the possible effects of *AGT* 3'UTR/flanking region polymorphisms on gene expression under AngII stimulated conditions. As elaborated in previous sections, these require further investigation.

In conclusion therefore, it was possible to see almost all the investigations to a successful conclusion and achieve the objectives laid down at the outset. This thesis describes what is possibly the first investigation into genetics of any complex disorder in the Sri Lankan population and as such it has broad implications for genetic research in Sri Lanka in this post genomic era. It is hoped that the publication of these results will encourage researchers in Sri Lanka to take up investigations into the genetics of complex disorders, and there by turn a new leaf in the annals of biomedical research in Sri Lanka.

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APPENDIX 1: LIST OF ABBREVIATIONS

AGT	Angiotensinogen
AMP	Actin Modifying Proteins
AngI	Angiotensin I
AngII	Angiotensin II
AR	Amphiregulin
ASO	Allele Specific Oligonucleotide
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
bp	base pair
BTC	Betacellulin (BTC)
BMI	Body Mass Index
BWT	Birth weight
CBS	Cystathionine β Synthese
CI	95% Confidence Interval
CSHW	Castle Street Hospital for Women
D'	Lewontin's disequilibrium coefficient
DBP	Diastolic Blood Pressure
DIP	Deletion Insertion Polymorphism
DMSO	Dimethylsulfoxide
DSHW	De Soysa Hospital for Women
DZ	Dizygotic
E	Eclampsia
E1	construct containing pGL3P3'GW and insert E1
E2	construct containing pGL3P3'GW and insert E2
E3	construct containing pGL3P3'GW and insert E3
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediamminetetraacetate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

EM	Expectation Maximisation
EMEM	Eagle Minimal Essential Medium
EVT	Extra-villous cytotrophoblast
ExoI	Exonuclease I
GH	Gestational Hypertension
GOPEC	UK Genetics of Pre-eclampsia Collaborative Study
GTF	General Transcription Factor
HB-EGF	Heparin Binding Epidermal Growth Factor
HCT	Urine Protein Heat Coagulation Test
HDL	High Density Lipoprotein
HELLP	HELLP Syndrome
HER	Human Epidermal Growth Factor Receptor
HWE	Hardy-Weinberg equilibrium
iqr	inter quartile range
IFN	Interferon
IHD	Ischaemic Heart Disease
IRF-2	Interferon Regulatory Factor-2
ISSHP	International Society for the Study of Hypertension in pregnancy
K4	Korotkoff phase IV
K5	Korotkoff phase V
kb	kilobase
LARII	Luciferase Assay Reagent II
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
M	Moor
MBW	Mean Birth Weight
MEK	MAP kinase kinase
MS	Methinoine Synthase
MS-PCR	Mutagenically Separated – Polymerase Chain Reaction

MTHFR	5,10-Methylenetetrahydrofolate reductase
MZ	Monozygotic
NADPH	Nicotinamide Adenine Dinucleotidephosphate
NCBI	National Centre for Biotechnology Information, USA.
NPV	Negative Predictive Value
NRE	Negative Regulatory Elements
NT	Normotensive
NTD	Neural Tube Defects
OR	Odds Ratio
ox-LDL	Oxidized Low Density Lipoprotein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Pre-eclampsia/Pre-eclamptic
pGL3C	pGL3Control vector
pGL3P	pGL3Promoter vector
pGL3P3'GW	pGL3Promoter vector with 3' Gateway® modifications
PIP_2	Phosphatidylinositol (4,5) Bisphosphate
PKC	Protein Kinase C
PLCγ	Phospholipase C-γ
PlGF	Placental Growth Factor
PNK	T4 polynucleotide kinase
PNMR	Perinatal mortality rate
PPV	Positive Predictive Value
r^2	Square of correlation coefficient between a pair of loci
RAS	Renin Angiotensin System
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
RR	Relative Risk
S	Sinhalese

SAP	Shrimp Alkaline Phosphatase
SAT	Sulphosalycilic Acid Test
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
sFlt1	Soluble fms-like tyrosine kinase 1
SGA	Small for Gestational Age
SNP	Single Nucleotide Polymorphism
STS	Sequence Tagged Sites
SV40	Simian Virus 40
T	Sri Lankan Tamil
tagSNPs	The maximally informative set of common SNPs
TDT	Transmission Disequilibrium Test
TGFA	Transforming Growth Factor Alpha
THF	Tetrahydrofolate
UTR	Untranslated Region
UV	Ultra violet
VEGF	Vascular Endothelial Growth Factor
W	White Caucasian

APPENDIX 2: BUFFERS AND CULTURE MEDIA

	Amount in 1 Litre
20 × SSC	
Sodium chloride	175.3g
Sodium citrate	88.2g
Adjust the pH to 7.0 with 10N sodium hydroxide	
20 × SSPE	
Sodium chloride	175.3g
Sodium orthophosphate	27.6g
EDTA	7.4g
Adjust the pH to 7.4 with 10N sodium hydroxide	
50 × TAE	242g
Tris base	57.1ml
Glacial acetic acid	100ml
0.5 M EDTA[1] (pH 8.0)	
Luria-Bertani (LB) Medium	
Bacto-tryptone	10g
Bacto-yeast extract	5g
Sodium chloride	10g
Adjust the pH to 7 .0 with 5N sodium hydroxide	
For media containing agar, add 15g bacto-agar to the components listed above	
SOC Medium	
Bacto-tryptone	20g
Bacto-yeast extract	5g
Sodium chloride	0.5g
250mM potassium chloride	10ml
2M $MgCl_2$	5ml
1M Glucose	20ml
Adjust the pH to 7 .0 with 5N sodium hydroxide	

APPENDIX 3: LIST OF SUPPLIERS

Amersham Biosciences UK Limited	Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK.
Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, Cheshire, WA1 7SR, UK.
Edge Biosystems	19208 Orbit Drive, Gaithersburg, MD 20879-4149, USA.
European Collection of Cell Cultures	CAMR, Salisbury, Wiltshire, SP4 0JG, UK.
Genetic Research Instrumentation, Ltd.	Gene House, Queenborough Lane, Rayne, Braintree, Essex, CM77 6TZ, UK.
Invitrogen Ltd.	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF,UK.
MBI Fermentas (Helena Biosciences Europe)	Colima Avenue, Sunderland Enterprise Park, Tyne and Wear, SR5 3XB, UK.
Promega UK Ltd.	Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK.
Qiagen	QIAGEN House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.
Randox Laboratories Ltd.	Diamond Road, Crumlin, Co. Antrim, BT29 4QY, UK.
Roche Diagnostics GmbH	Roche Applied Sciences, Nonnenwald 2, 82372 Penzberg, Germany.
Roche Diagnostics Ltd.	Bell Lane, BN7 1LG, Lewes, E. Sussex, UK.
Sigma-Aldrich Co. Ltd.	Fancy Road, Poole, BH12 4QH, UK.
Techne Ltd.	Duxford, Cambridge, CB2 4PZ, UK.

APPENDIX 4: URINE PROTEIN HEAT COAGULATION TEST

Steps of the standardised HCT

1. Apply 5 ml of the urine sample into a test tube.
2. Add a few drops of dilute acetic acid to the tube to make the sample acidic.
3. Heat the urine column in the tube over a burner without boiling over.
4. Compare the tube against the diagrammatic result interpretation chart (Figure 1) and record the result.

Interpretation of the HCT result

Negative/Trace	No or mild turbidity observed. When the urine column in the tube is placed in front of a typed sheet of paper, printed letters can be clearly read through the tube.
+1	Definite turbidity observed. Printed letters can be clearly read through the tube.
+2	Definite turbidity observed. Printed letters cannot be read clearly through the tube.
+3	Definite turbidity observed. Nothing can be observed through the tube.
+4	Protein clots are seen in the tube.

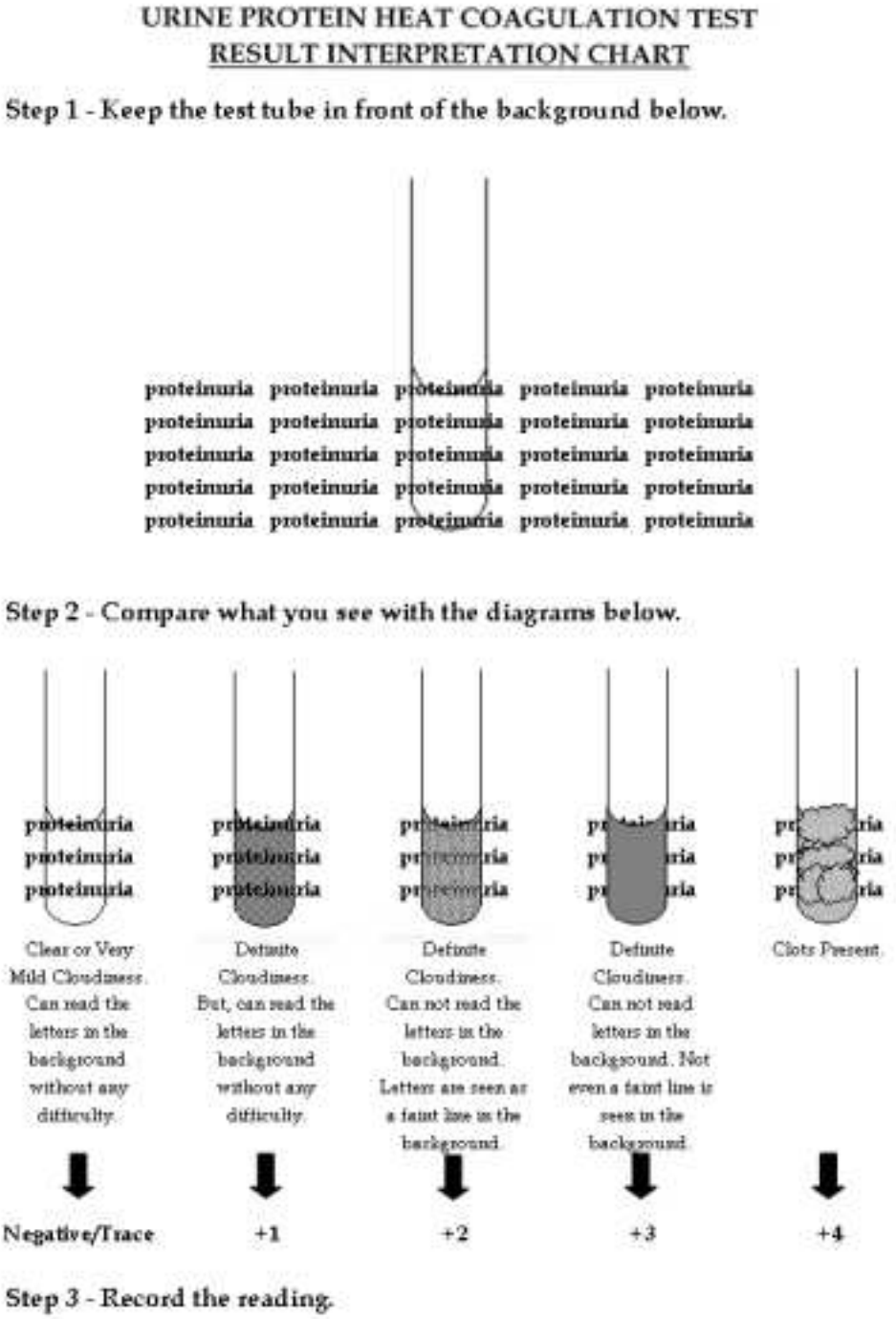

Figure 1. The urine protein heat coagulation test result interpretation chart.

APPENDIX 5: DOCUMENTS USED FOR SUBJECT RECRUITMENT IN SRI LANKA

This appendix contains the English translations of the Sinhala and Tamil language documents listed below, which were used for subject recruitment in Sri Lanka.

- Information leaflet for study participants used for the recruitment of women with pre-eclampsia and the normotensive pregnant women.
- Consent form used for recruitment of women with pre-eclampsia and normotensive pregnant women
- Consent form used for recruitment of volunteers for the population genetic studies
- Consent from used for recruitment of subjects for the urine protein heat coagulation test validation study

Information Leaflet For Study Participants

Invitation to take part in research into the inheritance of Pre-eclampsia (High Blood Pressure in Pregnancy)

You are invited to take part in a research study. Before you decide whether or not you wish to take part it is important for you to understand why the study is being done and what it will involve if you take part. Please read the following information carefully. Discuss it with your friends and relatives if you wish. Ask us if there is anything you do not understand or if you would like more information. You can have as much time as you want to make a decision.

We are asking you and 399 other women (200 women with pre-eclampsia and 200 normal women) and their families to volunteer for this study because without your help we will not be able to carry it out.

Who is organising the study?
This study is organised by the Human Genetics Unit and the Obstetrics and Gynaecology Department of the Faculty of Medicine, University of Colombo. A group of researchers from the University of Nottingham, UK are also helping us to conduct this study.

Why are we doing this study?
We are doing this study because we want to find out why some women develop pre-eclampsia and others don't by studying your genes. Pre-eclampsia is a disease of pregnancy, associated with high blood pressure and loss of protein in the urine. It can endanger both mother and baby. In Sri Lanka, it affects about 1 in every 50 women during pregnancy and results in the death of about 20 women every year. If we know why women develop pre-eclampsia then it may be possible to improve treatment, or prevent its occurrence.

What help do you have to give us?
We would like you to help us by donating 10ml of your blood and by allowing us to examine your medical records. We would also like to study your baby, but it is not necessary to obtain blood samples from the baby. We can use a piece of umbilical cord. We would also like to talk to your sisters and sisters in law if they have been pregnant before to find out whether they have had pre-eclampsia, but they can decided whether they want to talk to us or not. It is not compulsory. They don't have to donate blood.

What benefit do you get?
We cannot guarantee to discover anything that will directly benefit you or your family. Neither you nor us (the researchers involved) will benefit financially from this research. Your participation in this study would be kept

strictly confidential and the results of all the tests done will not be divulged to anyone.

Do you have to take part?
Taking part in this study is voluntary. If you would prefer not to take part you do not have to give a reason. Even if you say 'yes' now and decided to change your mind later, that is fine, you can tell us so. This will not affect your medical treatment.

Whom should you contact with any concerns?
If you have any problems, concerns, complaints or other questions about this study, you should contact Dr. Vajira H. W. Dissanayake by telephone on 695300 ext 349 at the Human Genetics Unit of the Faculty of Medicine in Colombo.

5/Aug/2001

Research Consent Form

PROJECT: Inherited Factors in Preeclampsia

NAME OF INVESTIGATOR: Dr. Vajira H. W. Dissanayake

I …….. give consent for the researchers to examine my medical records to obtain data for this research project.

I know that participation in this study does not bring any direct benefit me. I volunteer because the findings of this study may contribute to the well being of the community at large. I understand that my participation in this study will be kept strictly confidential. It is also my understanding that I can withdraw from this study whenever I wish without giving any reason and that such withdrawal will not result in discontinuation of medical care and treatment given to my daughter / my sister.

…….…… …….…….

Donor's Signature Date

…….……

Witness's Signature

…….……

Principal Investigator's Signature

Research Consent Form

PROJECT: A study of genetic variations in Sri Lankans

NAME OF INVESTIGATOR: Dr. Vajira H. W. Dissanayake

I ……... volunteer to donate 10 ml of venous blood to be used for research purposes.

The blood will be used to study variations in genes. The potential risks of donating blood, none of which are likely to occur, include pain, bruising, fainting or an infection at the puncture site. There will be no direct benefit to me. I understand that my participation in this study will be strictly confidential. It is also my understanding that I can withdraw from this study whenever I wish without giving any reason.

…….…… ……..…….
Donor's Signature Date

…….……
Witness's Signature

…….……
Principal Investigator's Signature

Research Consent Form

PROJECT: Urine protein heat coagulation test validation study

NAME OF INVESTIGATOR: Dr. Vajira H. W. Dissanayake

I ……... volunteer to take part in the above study.

I understand that my participation in this study will be strictly confidential. It is also my understanding that I can withdraw from this study whenever I wish without giving any reason and that withdrawal will not result in discontinuation of medical care and treatment given to me.

…….…… …….…….

Volunteer's Signature Date

…….……

Witness's Signature

…….……

Principal Investigator's Signature